STUDIES ON PHOSPHOGLYCERATE MUTASES

BY

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A thesis submitted for the degree of Doctor of Philosophy

in the University of Stirling , January 1988 .

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CONTENTS.

CHAPTER ONE: INTRODUCTION.

CHAPTER TWO: MATERIALS AND METHODS.

CHAPTER THREE: DENATURATION AND REFOLDING OF BAKERS YEAST
                PHOSPHOGLYCERATE MUTASE.

CHAPTER FOUR: DENATURATION AND REFOLDING OF RABBIT MUSCLE
            PHOSPHOGLYCERATE MUTASE.

CHAPTER FIVE: STRUCTURAL CHARACTERISATION AND THE DENATURATION AND REFOLDING OF *SCHIZOSACCHAROMYCES POMBE* PHOSPHOGLYCERATE MUTASE.

CHAPTER SIX: METAL ION REQUIREMENT AND REFOLDING OF COFACTOR INDEPENDENT PHOSPHOGLYCERATE MUTASES.

V

<u>Dedication and acknowledgements.</u>

This thesis is dedicated to my mother and father in acknowledgement of their continued support during my protracted educational career.

SYNOPSIS.

Phosphoglycerate mutases from a variety of sources have been examined with the aim of defining their denaturation and renaturation (refolding) chracteristics. Both cofactor (2,3-bisphosphoglycerate) dependent and independent enzymes have been examined and examples with differing quaternary configurations are included. The isolation procedures for these enzymes are reported.

The cofactor dependent enzyme from *Schizosaccharomyces pombe* shows a novel monomeric configuration within this group. This configuration has been confirmed here and the amino acid analysis and partial amino acid sequence determined by Prof. J.Fothergill are reported. A moderate degree of homology to the sequence of the cofactor dependent tetrameric enzyme from bakers yeast has been demonstrated. Similarly, the degree of homology between the cofactor dependent dimeric enzyme from rabbit muscle and the bakers yeast enzyme has been examined. These studies indicate moderate relatedness.

The cofactor independent phosphoglycerate mutases exhibit a number of differences from the cofactor dependent enzymes. The divisions between these two groups have been further emphasised in an apparent metal ion requirement and a native Mr of approximately 60,000 observed for the cofactor independent enzymes examined here.

The denaturation of the cofactor dependent enzymes from bakers yeast, rabbit muscle and *S. pombe* by GdnHCl has been examined through loss of catalytic activity and changes in fluorescence and circular dichroism. Only a weak correlation between the denaturation and structural configurations of these enzymes was observed. However, the *S. pombe* enzyme appeared more resistant to structural perturbation in the presence of its cofactor. This difference was also manifest in the enzyme's thermostability and proteinase resistance. The sedimentation velocity of this form of the enzyme was increased implying that the observed changes could have resulted from a more compact structure in the presence of the cofactor.

The renaturation of a range of phosphoglycerate mutases has been examined by measuring the regain of activity; structural aspects of this process have been explored using limited proteolysis as an indicator of bond accessibility.

The bakers yeast enzyme refolds with high efficiency at concentrations between 10 and 50 μg ml^{-1}. Monomeric and dimeric intermediates of refolding exhibiting partial enzymic activity which is sensitive to proteinases have been demonstrated. These partially active species may be similar to a general intermediate formed during the refolding of proteins which has a more "open" structure. Renaturation of the rabbit muscle enzyme is susceptible to the formation of wrong intermolecular aggregates at concentrations above 5

μg ml^{-1}. However, refolding intermediates of the rabbit muscle enzyme also have activity which is sensitive to proteinases. The refolding of the *S. pombe* enzyme is rapid and concentration independent and resembles the refolding of the subunits of the oligomeric bakers yeast and rabbit muscle enzymes.

The cofactor independent phosphoglycerate mutases from *A. nidulans* and wheat germ did not exhibit reactivation following denaturation in GdnHCl. This finding may relate to the possible metal ion requirement of these enzymes or reflect an inability of *in vitro* refolding to mimic accurately *in vivo* folding in which co-translational processes may act to co-ordinate chain folding and domain pairing.

The findings of the renaturation studies are considered in relation to the refolding of other proteins which have been examined and to the current model of protein folding *in vivo*.

CHAPTER ONE : INTRODUCTION

1.1. Phosphoglycerate mutase.

1.1.1. Catalytic reaction and its assay.

Phosphoglycerate mutase is a glycolytic enzyme catalysing the interconversion of 3- and 2-phosphoglycerates. The assay of this reaction is routinely performed by further coupling the formation of 2-phosphoglycerate to phosphoenolpyruvate (which absorbs at 240 nm) using enolase (Rodwell *et al.*, 1957) or to lactate (with the oxidation of NADH monitored at 340 nm) using enolase, pyruvate kinase and lactate dehydrogenase (Leadley *et al.*, 1977) as shown in Fig. 1. Other assay systems have been developed including the direct assay of the phosphate transfered and the measurement of the difference in optical rotation of the molybdate complexes of 3- and 2-phosphoglycerates (Ray and Peck, 1972). However, for routine purposes the enolase coupled assay has been most widely employed (Rodwell *et al.*, 1957, Ray and Peck, 1972, Grisolia and Carreras, 1975). Unfortunately, the absorption of phosphoenolpyruvate at 240 nm is dependent in a complex way on the pH, ionic strength, temperature and Mg^{2+} concentration of the assay (Wold and Ballou, 1957) and therefore comparisons between phosphoglycerate mutase activities obtained under non-identical conditions are difficult.

Phosphoglycerate mutases may be divided into two classes on the basis of their requirement for the cofactor 2,3-bisphosphoglycerate.

1

$$
\begin{array}{ccc}
\text{COO}^- & & \text{COO}^- \\
| & & | \\
\text{CHOH} & \xrightleftharpoons{\;1.\;} & \text{CHOPO}_3^{2-} \\
| & & | \\
\text{CH}_2\text{OPO}_3^{2-} & & \text{CH}_2\text{OH}
\end{array}
$$

3-Phosphoglycerate   2-Phosphoglycerate

$$
\xrightleftharpoons{\;2.\;}
\begin{array}{c}
\text{COO}^- \\
| \\
\text{COPO}_3^{2-} \\
\| \\
\text{CH}_2
\end{array}
$$

Phosphoenolpyruvate

ADP → ATP   3.

NAD$^+$ NADH

$$
\begin{array}{ccc}
\text{COO}^- & & \text{COO}^- \\
| & & | \\
\text{CHOH} & \xrightleftharpoons{\;4.\;} & \text{C=O} \\
| & & | \\
\text{CH}_3 & & \text{CH}_3
\end{array}
$$
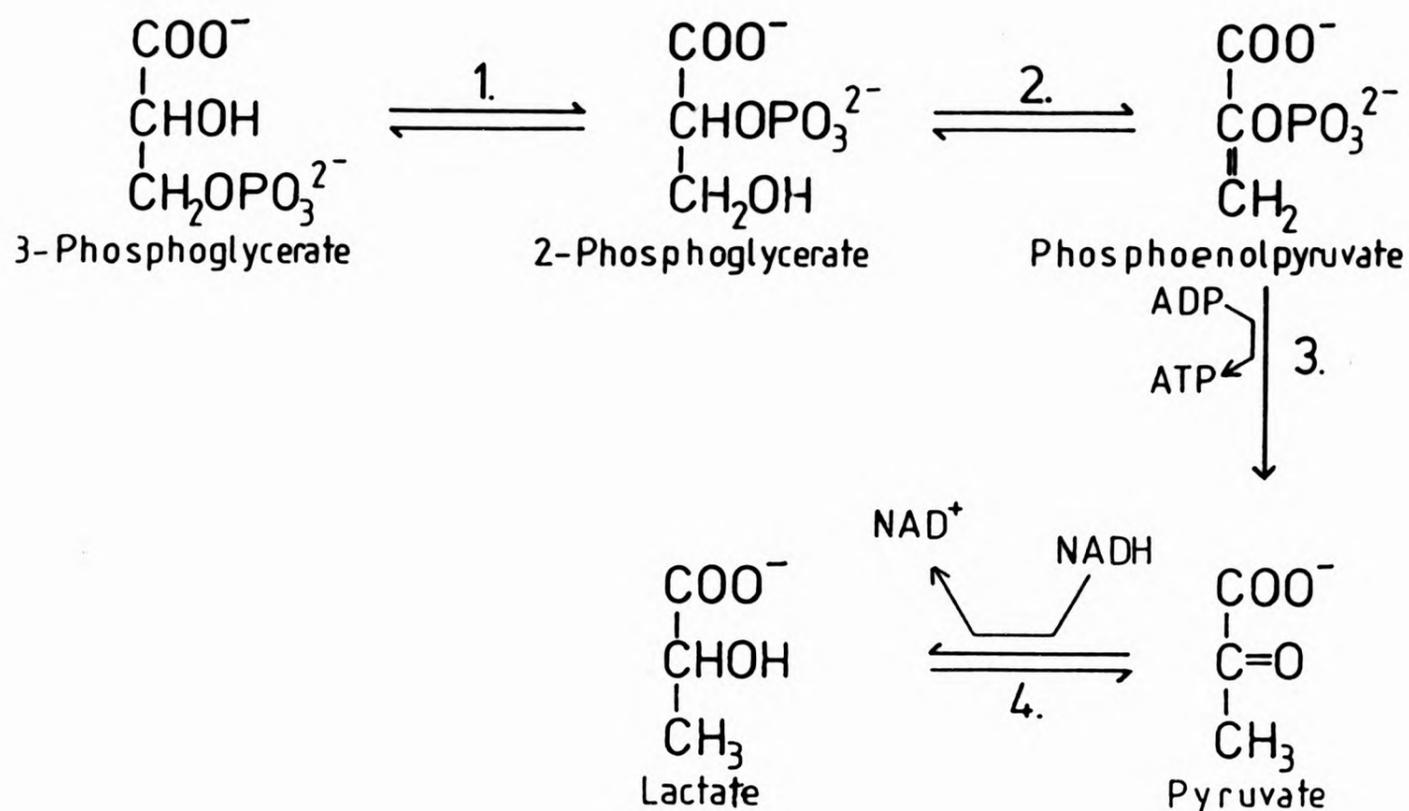
Lactate   Pyruvate

Fig. 1 Catalytic reaction of phosphoglycerate mutase and its assay.

The isomerization of 3-phosphoglycerate to 2-phosphoglycerate is assayed by coupling to phosphoenol pyruvate formation (absorbing at 240 nm) using enolase or to lactate formation using enolase, pyruvate kinase and lactate dehydrogenase with the concomitant oxidation of NADH (declining absorbance at 340 nm).

1. Phosphoglycerate mutase catalysed reaction; may require the cofactor 2,3-bisphosphoglycerate depending on the enzyme source.
2. Enolase catalysed reaction; requires Mg^{2+}.
3. Pyruvate kinase catalysed reaction; requires Mg^{2+} and K^+.
4. Lactate dehydrogenase catalysed reaction.

Cofactor dependent phosphoglycerate mutases have been isolated from a wide range of vertebrate sources, some invertebrates (insects, crustacea, annelids and molluscs), yeasts and in some algae, fungi and bacteria. Cofactor independent phosphoglycerate mutases have been isolated from plant sources, some invertebrates (sponges, coelenterates, arachnids and myriapods) and in some algae, fungi and bacteria (Grisolia and Joyce, 1959, Watabe and Freese, 1979, Singh and Setlow, 1979, Carreras *et al.*, 1982, Price *et al.*, 1983, Price and Stevens, 1983). The evolutionary significance of this phylogenetic distribution is difficult to interpret and does not indicate whether cofactor dependence has evolved from cofactor independent sources or if the independent enzymes have arisen through retrograde processes (i.e. the loss of cofactor).

1.1.2. Mechanism of catalytic reaction.

The mechanism of phosphoglycerate isomerization by the mutase enzymes was first investigated in some detail by Rose (1970, 1971). Rose demonstrated that the cofactor dependent enzymes from rabbit muscle and bakers yeast were phosphorylated on a histidine residue by 2,3-bisphosphoglycerate and that this phosphate could be transfered to the normal substrates or to water in the presence of 2-phosphoglycolate. Subsequent work by Britton *et al.* (1972b) and Britton and Clarke (1972) using an induced transport test system to monitor the fluxes of radio labelled substrates at equilibrium with the enzyme following the addition of non-labelled substrates, suggested a ping-pong phospho-

2

enzyme mechanism for the rabbit muscle enzyme in which each substrate was present at the active site in turn. Britton and co-workers therefore proposed a reaction mechanism involving the intermolecular shuttling of phosphate between the glycerate substrates in which each was present at the active site in turn (ping-pong mechanism). The intermolecular transfer was confirmed by Gatehouse and Knowles (1977) using glycerate skeletons differentially labelled with ^2H and ^{18}O. Using the rabbit muscle enzyme the labels became scrambled as the phosphate (^{18}O) was transfered to the ^2H labelled substrate. Rose *et al.* (1975) isolated and sequenced an active site peptide from chicken muscle phosphoglycerate mutase which also reacted via a phospho-histidine intermediate and Rose and Dube (1976) demonstrated that the rates of phosphorylation and dephosphorylation of this enzyme were consistent with the rates of the enzyme catalysed reaction.

The mechanism of the cofactor independent phosphoglycerate mutase from wheat germ has been studied by Gatehouse and Knowles (1977). These authors showed that no transfer of phosphate occurred between glycerate skeletons differentially labelled using ^2H and ^{18}O and therefore concluded that the phosphoryl transfer was intramolecular. This finding was consistent with the earlier work of Britton *et al.* (1971) using induced transport tests which had implicated an intramolecular phosphoryl transfer in the wheat germ enzyme. The possible involvement of a 2,3-cyclic phosphate ion was excluded by subsequent studies using ^{18}O labelled substrates and $H_2^{18}O$ enriched solutions (Breathnach and Knowles, 1977). The wheat germ enzyme

therefore seemed to catalyse an intramolecular phosphoryl transfer via a phosphoenzyme intermediate. Attempts to isolate such an intermediate were unsuccessful and it was therefore proposed that the phosphoenzyme was probably of high free energy, and thus only transiently populated, and/or bound the substrate extremely tightly (Breathnach and Knowles, 1977). Subsequent work by Blättler and Knowles (1980) defined the stereochemical course of the phosphoryl transfer in the rabbit muscle and wheat germ enzymes using chirally labelled $2-(^{16}O, \ ^{17}O, \ ^{18}O)$ phosphoglycerate. Inversion of the pyramidal configuration of the phosphoryl group is characteristic of single enzyme catalysed transfers (Blättler and Knowles, 1979) and therefore the configuration of the chirally labelled substrates following transfer was used to indicate the mechanism of reaction. In both rabbit muscle and wheat germ phosphoglycerate mutases there was retention of configuration of this group which was consistent with a double displacement process in both cases (Blättler and Knowles, 1980). On the basis of these and earlier observations Blättler and Knowles proposed a reaction mechanism for the rabbit muscle and wheat germ enzymes. These schemes are presented in Figs. 2 and 3 respectively and are currently the generally accepted reaction pathways for the cofactor dependent and independent enzymes which are consistent with the reports reviewed above. The mechanistic differences between the two enzyme groups are emphasized by their enzyme commission numbers with the cofactor dependent phosphoglycerate mutases being considered as tranferases (E.C. 2.7.5.3.) while the cofactor independent mutases considered as true isomerases (E.C. 5.4.2.1.).
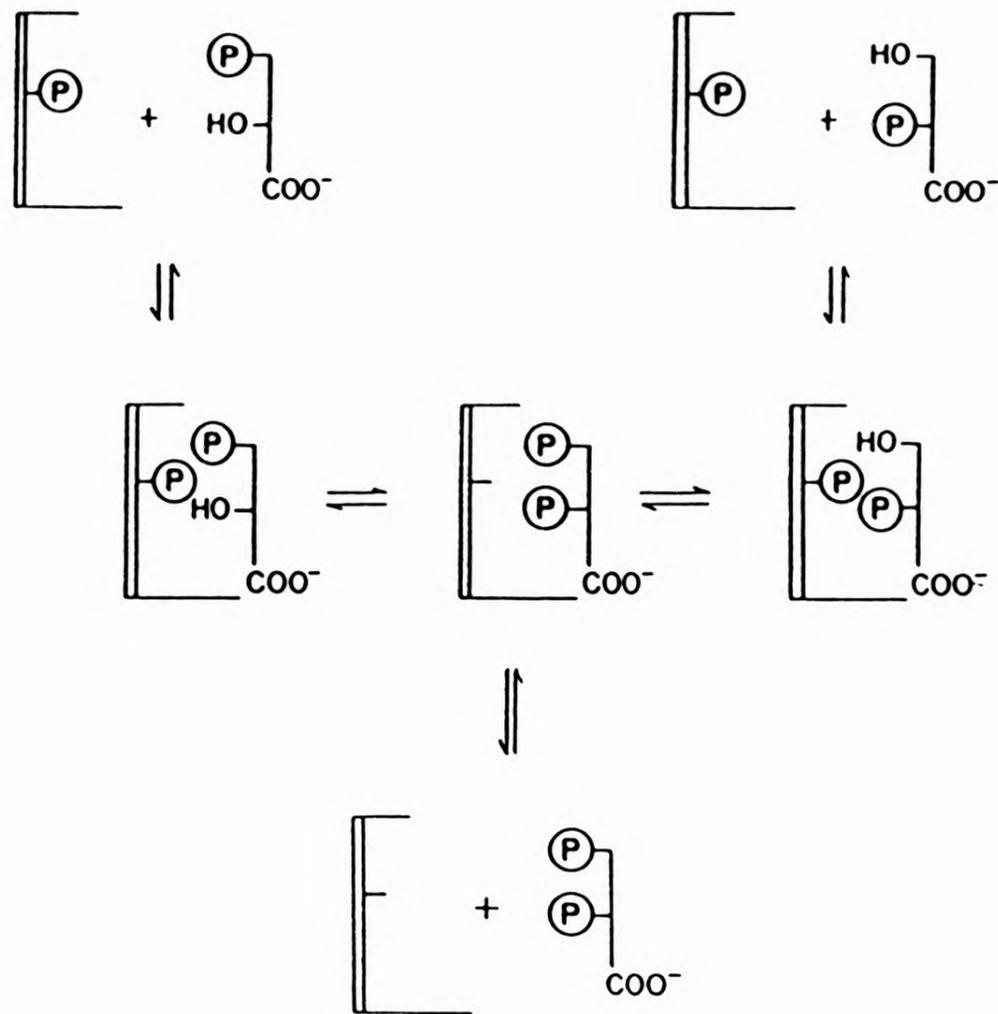
Figure 2.   Proposed mechanism of phosphoryl transfer catalysed by
             cofactor dependent phosphoglycerate mutases (from
             Blättler and Knowles, 1980).

This scheme does not indicate the slow hydrolysis of the phospho-
enzyme (half time 1 to 2 min; Britton et al., 1972a) which accounts
for the observed phosphatase activity of cofactor dependent phospho-
glycerate mutases.
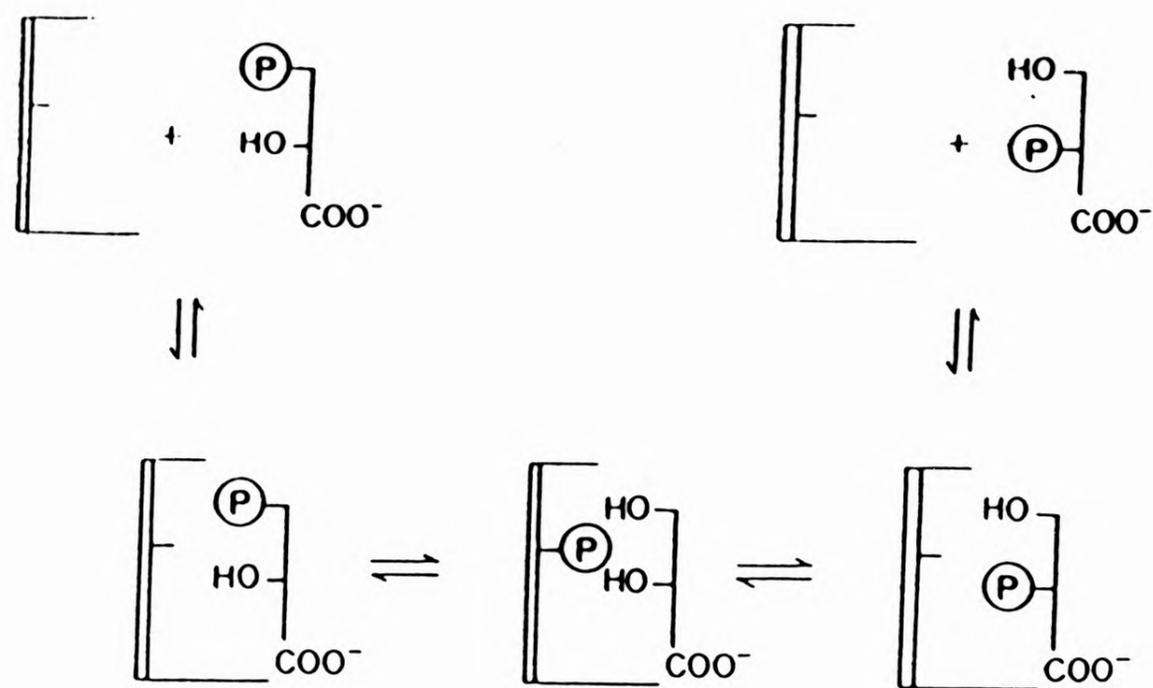
Figure 3. Proposed mechanism of phosphoryl transfer catalysed by
cofactor independent phosphoglycerate mutases (from
Blättler and Knowles, 1980).

In the case of the cofactor dependent phosphoglycerate mutases histidine has been shown to mediate the intermolecular phosphoryl transfer during the catalytic reaction (Rose, 1970, 1971, Rose *et al.*, 1975, Rose and Dube, 1976.). This finding is consistent with the reported pH optima of approximately 7 for this group of enzymes (Grisolia and Carreras, 1975). The pH optima of the cofactor independent enzymes examined appears to be nearer 9 (Ito and Grisolia, 1959, Fernandez and Grisolia, 1960, Carreras *et al.*, 1982) and, although its presence has been implicated, no phosphoenzyme intermediate has been characterised. The differing pH optima is perhaps evidence that the residue mediating the phosphoryl transfer may not be histidine in the cofactor independent enzymes.

1.1.3. Kinetics of catalytic reaction.

A range of values for the kinetic constants Km and Ki for 3-phosphoglycerate, 2-phosphoglycerate and 2,3-bisphosphoglycerate and for the equilibrium constant, Keq, of the isomerization reaction have been documented for both cofactor dependent and independent phosphoglycerate mutases (Ray and Peck, 1972, Grisolia and Carreras, 1975.). Unfortunately, the dependence of the absorption of phosphoenolpyruvate at 240 nm on the various assay conditions employed in such studies makes meaningful comparisons between these values difficult. Furthermore, this thesis contains insufficient kinetic data to warrant a more detailed consideration of this area.

1.2. Cofactor dependent phosphoglycerate mutases.

1.2.1. *Saccharomyces cerevisiae* phosphoglycerate mutase.

The enzyme from *S.cerevisiae* (bakers yeast) has been studied in greater detail than any of the other phosphoglycerate mutases. The bakers yeast enzyme is a tetramer of Mr 110,000 comprised of four identical subunits of Mr 28,000 (Rodwell *et al.*, 1957, Price and Jaenicke, 1982, Hermann *et al.*, 1983). The detailed three-dimensional structure of the enzyme (Campbell *et al.*, 1974) and the complete amino acid sequence (Fothergill and Harkins, 1982) have been determined. The X-ray diffraction structure of the enzyme reveals a symmetrical arrangement of identical subunits. Each subunit is comprised of a core of six strands of β-sheet, the first four of these traced from the N-terminus being parallel and the last two forming an antiparallel pair. This core is flanked by five α-helical regions with a sixth helix not involved in this arrangement (Campbell *et al.*, 1974). This β-core/α-barrel configuration is highly characteristic of a number of nucleotide binding enzymes such as dehydrogenases (Rossman *et al.*, 1975) and kinases (Bryant *et al.*, 1974, Schulz *et al.*, 1974). As noted by Campbell *et al.* (1974) there is considerable structured homology between the phosphoglycerate mutase and lactate dehydrogenase subunit structures as indicated in Fig. 4. Since phosphoglycerate mutases have no known nucleotide requirement the presence of this structural arrangement may reflect the inherent stability of this configuration
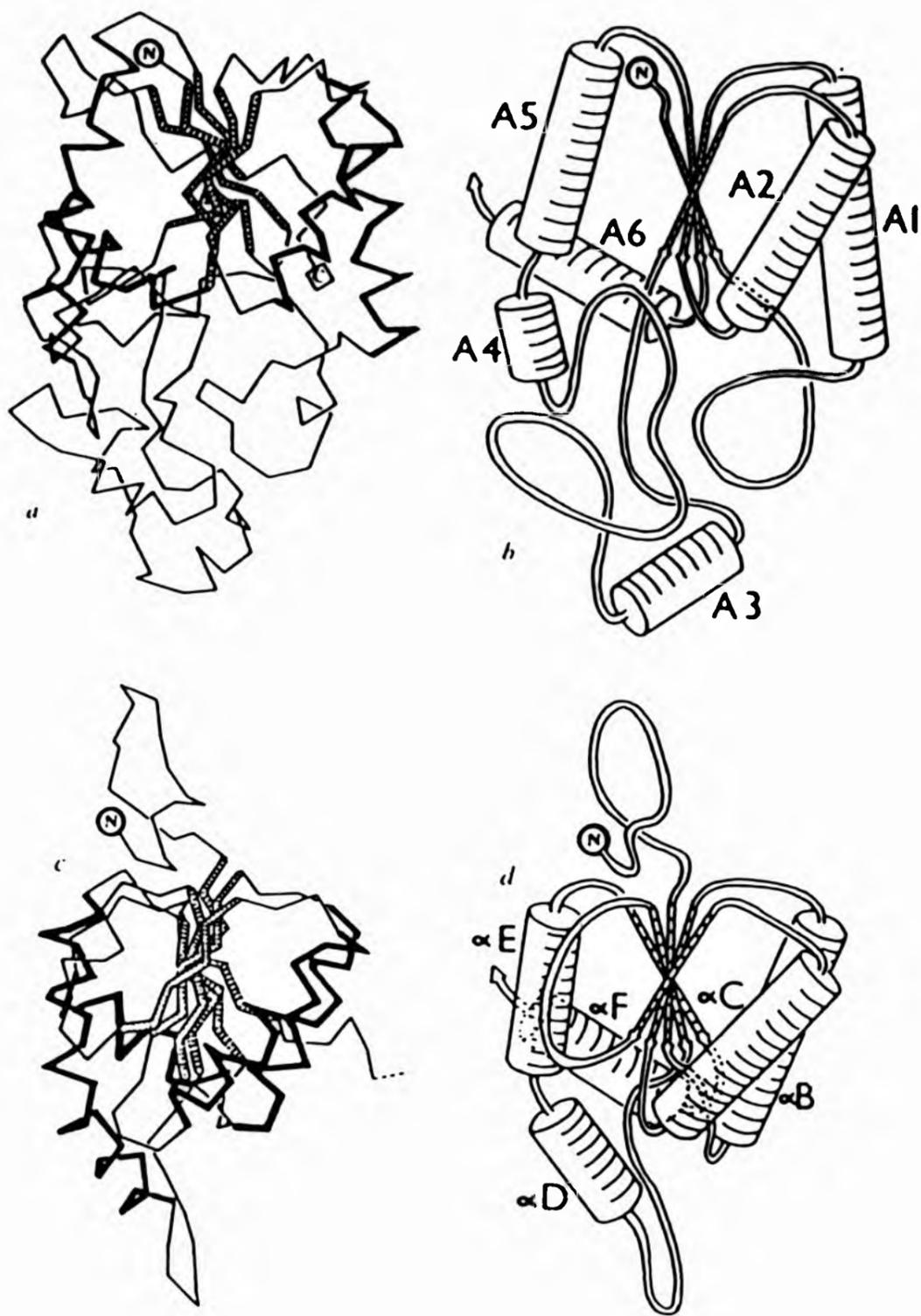
Figure 4.   Structural homology between the subunit structures of
            phosphoglycerate mutase and lactate dehydrogenase (from
            Campbell et al., 1974).

Fig. 4a shows the complete phosphoglycerate mutase subunit in which
β-sheet is indicated by thick hatched lines and α-helix by thick
black lines.   Fig. 4b shows the first 185 residues of the mutase
subunit in a simplified manner with the helices labelled from the
N-terminus according to the scheme of Campbell et al. (1974).   Fig. 4c
and d illustrate the first 165 residues of the lactate dehydrogenase
subunit in a similar manner to Fig. 4a and b for comparison.

as an enzyme core structure or may be evidence of a common evolutionary origin for the glycolytic and related enzymes.

The active site of bakers yeast phosphoglycerate mutase has been located and lies at the base of a deep hollow formed by the residues of one subunit (Winn *et al.*, 1981). The four active sites are well separated in the native enzyme and are freely accessible to solvent. The predicted presence of a histidine residue at the active site (section 1.1.2.) was somewhat complicated by the discovery of two histidines (8 and 179 of the sequence) in a parallel arrangement approximately 0.4 nm apart. In the presence of the substrate histidine 8 is close to the 3-carbon locus and histidine 179 close to the 2-carbon locus. Glutamate residues 15 and 86 are positioned such that their carboxyl groups could act as proton withdrawing groups from the 3- and 2-hydroxyls of the substrate respectively. Similarly, arginine 7 is positioned above and between the histidine 8 and 179 residues and may interact with the carboxyl group of the 1-carbon locus (Winn *et al.*, 1981).

The involvement of histidine 179 in the mechanism of phosphoryl transfer is still a matter of debate. It is always histidine 8 that is isolated with bound phosphate from the active site of the enzyme (Han and Rose, 1979). In addition, the retention of configuration of the phosphoryl group in the rabbit muscle enzyme (Blättler and Knowles, 1980) is consistent with a mechanism in the bakers yeast enzyme proceeding with an even number of transfers, i.e; S-P → His 8-P → S-P

rather than S-P → His 8-P → His 179-P → S-P, where S-P is the phosphorylated substrate. Examination of the active site shows additionally that the histidine 8 → 179 transfer is sterically unfavourable (Winn *et al.*, 1981). The involvement of histidine 179 would therefore require an additional intermediary residue.

Phosphoglycerate mutase from bakers yeast has been reported to exist in a number of electrophoretically distinct forms; components I → V (Chiba and Sugimoto, 1959, Chiba *et al.*, 1960). The specific activity of successive components is approximately one quarter lower than that of component I suggesting combinations of 0 to 4 inactive subunits. Sasaki *et al.* (1966) isolated the enzyme responsible for this inactivation and showed that it cleaved approximately 10 amino acids from the C-terminus of the enzyme subunit. These residues are thought to form a flexible tail which could adopt a structure modulating access to the active site (Winn *et al.*, 1981.). This tail could therefore act to exclude water from the active site such that phosphoryl transfer occurred to the substrate rather than to water. The presence of the tail could in part explain the differences in polypeptide length reported from the X-ray diffraction structure (216 residues) and the amino acid sequence (241 residues) since as a flexible structure it would not appear in the electron density map. The sequence of the C-terminal tail contains two lysine residues which could act as the theoretical intermediary residues in a histidine 8 → 179 transfer. However such a transfer would have to occur extremely

rapidly ($>10^{6}$ s^{-1}) to be consistent with the rate of the enzyme catalysed reaction (Britton *et al.*, 1972a).

As would be predicted from the above structural and mechanistic schemes, bakers yeast phosphoglycerate mutase can be inactivated by histidine and arginine modifying reagents with the substrates, notably 3-phosphoglycerate, providing some protection (Fothergill, 1977, Price *et al.*, 1985b).

1.2.2. Rabbit muscle phosphoglycerate mutase.

The rabbit muscle enzyme is a dimer of Mr 56,000 comprised of two subunits of Mr 28,000 (Pizer, 1960, Hermann *et al.*, 1983). Whilst no structural or comprehensive sequence information are available for this enzyme, two of its histidine containing peptides have been sequenced (Haggarty and Fothergill, 1980) and show almost complete identity to the sequences around histidines 8 and 179 of the bakers yeast enzyme (Fig. 5). No homology was observed to the sequences around the other two histidine residues of the bakers yeast enzyme (88 and 189). The participation of a histidine residue in the phosphoryl transfer catalysed by the rabbit muscle enzyme has been demonstrated by Rose (1970, 1971) and the studies of Britton and Clarke (1972) suggested a ping-pong reaction mechanism similar to that of the bakers yeast enzyme.

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                                    *
1        H₂N-P-K-L-V-L-V-R-H-G-Q-S-E-W-N-E-     (residues 0-15)
2                        V-R-H-G-E-S-T-W


1            -I-A-A-H-G-N-                      (residues 176-181)
2            I-A-A-H-G-N
```

Figure 5 . Partial amino acid sequence of bakers yeast phosphoglycerate
mutase and the aligned rabbit muscle histidine containing
peptides .

1 Bakers yeast phosphoglycerate mutase (Fothergill and Harkins,1982)
2 Rabbit muscle phosphoglycerate mutase (Haggarty and Fothergill,1980)

* ; Histidine mediating phosphoryl transfer

Rabbit muscle phosphoglycerate mutase can be inactivated by histidine, arginine and cysteine modifying reagents with substrates providing some protection (Pizer, 1960, Borders and Wilson, 1976, Hartman and Norton, 1976, Berrocal and Carreras, 1983, Price *et al.*, 1985b). The susceptibility to cysteine modification distinguishes the rabbit muscle enzyme from bakers yeast phosphoglycerate mutase which contains no cysteine residues (Fothergill and Harkins, 1982). Hartman and Norton (1976) showed by titration the presence of approximately 1 mole of cysteine per enzyme subunit under native conditions. A tryptic fragment containing this cysteine residue did not contain histidine. This finding suggested that the cysteine containing sequence of the rabbit muscle enzyme is distinct from the "active site" histidine containing peptides reported by Haggarty and Fothergill (1980). Without further sequence or structural information it is not possible to distinguish whether the cysteine residue is functional in catalysis or if binding of the modifying reagents block access to the active site. However, both possibilities imply its presence at or near the active site.

It is well documented that the phosphoglycerate mutases from mammalian sources such as rabbit muscle occur in three isoenzyme forms that result from the possible combination of two subunit types (M and B). These isoenzymes differ in their thermal stability, electrophoretic mobility and sensitivity to cysteine modifying reagents (Prehu *et al.*, 1984). The BB type isoenzyme is located in the brain, liver, kidney and erythrocytes whereas the MM type isoenzyme is found in muscle.

Both BB and MM types and the MB hybrid are found in heart tissue although there is some evidence that the hybrid isoenzyme also occurs in skeletal muscle (Prehu *et al.*, 1986).

1.2.3. *Schizosaccharomyces pombe* phosphoglycerate mutase.

In contrast to *S.cerevisiae* which divides by budding mechanisms, *S.pombe* divides by medial fission. Further differences in the molecular biology of these two yeasts have been widely reported. For example, there are differences in the respective DNA transcription and initiation regions, the *ras* gene products and aspects of the mitotic cycle such as the presence of a G2 period and processes of chromosome condensation (Russel and Nurse, 1986). The *S.pombe* transcription machinery seems more capable of correctly excising non-coding introns from transcripts of eukaryotic genes and this yeast has therefore been advocated as a suitable vector for their expression (Käufer *et al.*, 1985). Overall these differences are perhaps not surprising given that nucleotide homology indicates that *S.pombe* and *S.cerevisiae* may have diverged in evolutionary terms by some 1,200 million years (Huysmans *et al.*, 1983).

Phosphoglycerate mutase from *S.pombe* has a novel structural configuration amongst the cofactor dependent enzymes in that it is a monomer of Mr 23,000 (Price *et al.*, 1985b). The *S.pombe* enzyme can be inactivated by histidine modifying reagents with substrates, notably 3-phosphoglycerate, providing some protection. The activity of the

11

S.pombe enzyme is not affected by cysteine modifying reagents such as potassium tetrathionate. Furthermore, the potential rôle of arginine in the catalytic mechanism is not clear as the activity of the *S.pombe* enzyme is not stable under the conditions required for the modification of this residue (Price *et al.*, 1985b).

1.2.4. Other cofactor dependent phosphoglycerate mutases.

Cofactor dependent phosphoglycerate mutases have been isolated from a wide range of mammalian sources including chicken muscle (Rose *et al.*, 1975), sheep muscle (James *et al.*, 1971), rabbit liver (Kulbe and Ahrendt, 1973), rabbit muscle (Pizer, 1960), pig kidney (Diederich *et al.*, 1970), pig brain (Tauler and Carreras, 1987) and human erythrocytes (Sheibley and Hass, 1976). The Mr values of these enzymes were reported to be between 55,000 and 65,000 and were examined, the native configuration was dimeric. Cofactor dependent activity has also been isolated from *Escherichia coli* (D'Alessio and Josse, 1971) and from *Hyphomicrobium X* and *Pseudomonas AM1* (Hill and Attwood, 1976). The reported Mr values of these enzymes under native conditions were 56,000, 32,000, and 32,000 respectively. Cofactor dependent activity has also been detected in the yeast *Candida utilis* and a native Mr of 110,000 reported (Price *et al.*, 1983). The quaternary structure of these enzymes have not been determined.

1.2.5. Cofactor dependence: common features.

The cofactor dependent phosphoglycerate mutases isolated from a range of sources seem to fit into a common structural pattern, namely a monomeric, dimeric or tetrameric configuration with a subunit Mr in the range 20,000 to 30,000. Whilst the quaternary structures of some of the enzymes listed above have not been determined there is still no example that cannot be fitted into such a scheme on the basis of their native Mr values. This proposed structural relatedness could be invoked as evidence for a common ancestral source of all the cofactor dependent phosphoglycerate mutases.

A number of the cofactor dependent enzymes can be inactivated by histidine and arginine modifying reagents. In the case of the bakers yeast, rabbit muscle and chicken muscle phosphoglycerate mutases a histidine residue has been shown to mediate the phosphoryl transfer catalysed by these enzymes (Rose, 1970, 1971, Rose *et al.*, 1975). The mammalian enzymes can also be inactivated by cysteine modifying reagents whilst in the yeast enzymes these are absent or not catalytically competent. The pH optima of approximately 7 reported for a range of cofactor dependent phosphoglycerate mutases are consistent with the participation of histidine in their catalytic mechanism and the involvement of other residues in the enzymes interaction with their anionic substrates.

13

A range of cofactor dependent phosphoglycerate mutases have been reported to be inhibited by vanadate (Carreras *et al.*, 1980). This inhibition was pH dependent and could be reversed by dilution or chelation of the vanadate. Vanadate is thought to inhibit enzymes involving a phosphoenzyme intermediate through the structural similarity of the pentavalent vanadium ion to the transition state of the phosphoryl group (Macara, 1980). Such inhibition is therefore evidence of structural and/or mechanistic similarities between the cofactor dependent enzymes.

Cofactor dependent phosphoglycerate mutases have been reported to bind to the triazine dye Cibacron Blue F3GA and to be eluted from the immobilised dye by a pulse of their cofactor, 2,3-bisphosphoglycerate (Price and Stevens, 1983.). Cibacron Blue F3GA was originally thought to be a definitive probe for the "dinucleotide binding fold" in kinases and dehydrogenases (Thompson *et al.*, 1975). However, subsequent work cast doubt on its specificity for this structural element and it is now more widely accepted that a configuration of β-sheets flanked by a α-helices leads to a "funnelling" of the dye into the hydrophobically situated binding sites where further local electrostatic interactions occur (Beissner *et al.*, 1979). Phospho-glycerate mutase has no known dinucleotide requirement but the bakers yeast enzyme seems to possess a structural configuration resembling the dinucleotide fold i.e; 6 β-sheets flanked by α-helices. The structural homology between the bakers yeast enzyme and lactate dehydrogenase has already been considered (section 1.2.1., Fig. 4) and

it is probably this feature that accounts for its binding to Cibacron Blue F3GA. The binding of a range of cofactor dependent phosphoglycerate mutases is therefore evidence that the structural configuration may be common to these enzymes as a group.

1.3. Cofactor independent phosphoglycerate mutases.

1.3.1. Wheat germ phosphoglycerate mutase.

Phosphoglycerate mutase from wheat germ is probably the most extensively studied of the cofactor independent enzymes. A number of purification protocols have been developed for this enzyme (Ito and Grisolia, 1959, Britton *et al.*, 1971, Leadley *et al.*, 1977, Smith and Hass, 1985). These authors have reported a monomeric configuration for this enzyme with a Mr value in the range 50,000 - 60,000. McAleese *et al.* (1985) have also reported a monomeric Mr of 60,000 using their modification of the isolation procedure of Britton *et al.* (1971). Leadley *et al.* (1977) have shown that the wheat germ enzyme contains one cysteine residue. This residue was not accessible to modifying reagents in the native enzyme and was not catalytically competent.

A possible metal ion requirement for activity has been proposed for wheat germ phosphoglycerate mutase. Smith and Hass (1985) and Smith *et al.* (1986) have shown that the enzyme is inhibited by various chelators and apparently requires the presence of Co^{2+} or Mn^{2+} to be

15

reactivated following denaturation in GdnHCl. Leadley *et al.* (1977) also noted that their wheat germ enzyme was inhibited by EDTA.

1.3.2. *Bacillus subtilis* and *Bacillus megaterium* phosphoglycerate mutase.

Interest in the phosphoglycerate mutases from species of *Bacillus* has arisen from the observation that Mn^{2+} is essential for the sporulation of these bacteria and in its absence growth is arrested and 3-phosphoglycerate accumulates in the cells (Oh and Freese, 1976). The phosphoglycerate mutase of *B.subtilis* was shown to have a Mn^{2+} requirement for activity thereby accounting for the elevated levels of 3-phosphoglycerate. The purification of the phosphoglycerate mutase from *B.subtilis* was subsequently developed by Watabe and Freese (1979). These authors reported a monomeric configuration for this enzyme with a Mr of 74,000. The enzyme contained five cysteine and twenty one arginine residues per mole and could be inactivated by modifying reagents specific for these residues. The enzyme activity was inhibited by EDTA and could be reactivated by the addition of 1 mM Mn^{2+}. The apparent Km for Mn^{2+} was 4.5 μM.

The purification of phosphoglycerate mutase from *Bacillus megaterium* has also been reported (Singh and Setlow, 1979). This enzyme is a monomer of Mr 61,000, contains one cysteine per mole and can be inactivated by modifying reagents specific to this residue. The enzyme

16

activity was again inhibited by EDTA and subsequently recovered following addition of 1 mM Mn^{2+}. The apparent Km for Mn^{2+} was 40 μM.

1.3.3. Other cofactor independent phosphoglycerate mutases.

Cofactor independent phosphoglycerate mutases have been isolated from a wide range of plant and fungal sources (Grisolia and Joyce, 1959, Price *et al.*, 1983, Carreras *et al.*, 1982). The enzyme from rice germ has been purified by Fernandez and Grisolia (1960) and the native Mr determined by sedimentation was reported as 30,000. The native Mr of the wheat germ enzyme was also determined using sedimentation (Fernandez and Grisolia, 1960, Ito and Grisolia, 1959). A value of 30,000 was reported. Since the Mr of this enzyme was subsequently determined to be between 50,000 and 60,000 it seems possible that the value quoted for the rice germ enzyme may also be inaccurate. Price *et al.* (1983) have examined phosphoglycerate mutase activity in a range of fungi and determined native Mr values of between 40,000 and 60,000 from these sources.

1.3.4. Cofactor independence: common features.

From the limited information available it seems that the cofactor independent phosphoglycerate mutases may possess a common monomeric configuration and Mr of approximately 60,000. There is also a possible pattern of sensitivity to chelators and a Mn^{2+} or Co^{2+} requirement for activity. The observations of Smith *et al.* (1986) suggest that the

metal ion(s) may be closely associated with the wheat germ enzyme since they appear to play a rôle in structural regain following denaturation in GdnHCl.

The pH optima of the cofactor independent phosphoglycerate mutases are 9; some two pH units higher than that of the cofactor dependent enzymes (Grisolia and Carreras, 1975). Furthermore, the cofactor independent enzymes are not inhibited by vanadate (Carreras *et al.*, 1980) and do not bind to Cibacron Blue F3GA (Price and Stevens, 1983). These observations, in combination with the mechanistic differences discussed in section 1.1.2. indicate that whilst the cofactor dependent and independent phosphoglycerate mutases both catalyse the same overall reaction there are extensive mechanistic and structural differences between the two groups and as such they can only be considered as analogous systems; i.e; different enzymes with similarity of function.

1.4. Bisphosphoglycerate mutases.

Bisphosphoglycerate mutase (E.C. 2.7.5.4.) catalyses the inter-conversion of 1,3- and 2,3-bisphosphoglycerates. In mammals the levels of 2,3-bisphosphoglycerate in erythrocytes profoundly influences the oxygen affinity of deoxyhaemoglobin and thereby modulates the control of oxygen delivery to tissues (Bunn and Forget, 1986). It is now apparent that bisphosphoglycerate mutase has three enzymic activities, namely the interconversion of 1,3- and 2,3-bisphosphoglycerate, the

18

dephosphorylation of 2,3-bisphosphoglycerate and the interconversion of 3- and 2-phosphoglycerates. These three activities are also exhibited by cofactor dependent phosphoglycerate mutase although at different relative rates (Rose and Whalen, 1973; Sasaki *et al.*, 1975; Kappel and Hass, 1976). Bisphosphoglycerate mutase activity and levels of 2,3-bisphosphoglycerate are high in erythrocytes but low in all other tissues examined. Nevertheless, the majority of monophosphoglycerate mutase activity in erythrocytes still seems to be expressed by phosphoglycerate mutase (Fothergill-Gilmore, 1986).

Bisphosphoglycerate mutases and cofactor dependent phosphoglycerate mutases have a number of other common features. For example, the enzymes from human erythrocytes are both dimers of Mr 60,000 and possess similar amino acid compositions (Sheibley and Hass, 1976). Both enzymes have phosphorylated histidine intermediates (Han and Rose, 1979) which have kinetic properties consistent with their participation in the reactions catalysed (Rose and Dube, 1976). Comparison between the amino acid sequences of bakers yeast phosphoglycerate mutase (Fothergill and Harkins, 1982) and human erythrocyte bisphosphoglycerate mutase (Joulin *et al.*, 1986) indicates some 49% homology. Furthermore, the active site histidine sequence of the bakers yeast and the aligned active site peptide of the rabbit muscle phosphoglycerate mutases show considerable homology with the N-terminal sequences of the human, rabbit and horse bisphosphoglycerate mutase enzymes as indicated in Fig. 6.

19

```
                              *
1       H2N-M-S-K-Y-K-L-I-M-L-R-H-G-E-G-A-W-N-K-E-N-
2       H2N-M-S-K-Y-K-L-I-M-L-R-H-G-E-G-A-W-N-K-E-N-
3                               H-G-Q-G-A-W-N-K
4       H2N-P-K-L-V-L-V-R-H-G-Q-S-E-W-N-E-
5                           V-R-H-G-E-S-T-W
```

Figure 6 . Amino acid sequences of bis- and monophosphoglycerate
           mutases around their active site histidine residues .

1 Human bisphosphoglycerate mutase (Joulin et al. ,1986)
2 Rabbit bisphosphoglycerate mutase (Yanagawa et al. ,1986)
3 Horse bisphosphoglycerate mutase (Han and Rose,1979)
4 Bakers yeast phosphoglycerate mutase (Fothergill and Harkins,1982)
5 Rabbit muscle phosphoglycerate mutase (Haggarty and Fothergill,1980)

* ; Histidine mediating phosphoryl transfer

The strong structural similarities between the bisphosphoglycerate mutase and cofactor dependent phosphoglycerate mutase enzymes are consistent with their abilities to catalyse the same reactions. The homology between these two groups is far greater than that between the cofactor dependent and independent phosphoglycerate mutases. Such homology is perhaps evidence of a common evolutionary origin for these two groups or that the bisphosphoglycerate mutases have evolved from the more ubiquitously distributed phosphoglycerate mutases.

1.5. Protein folding and refolding: theoretical aspects.

The mechanism by which polypeptides acquire their unique functional conformation is an area of molecular biology which still remains to be thoroughly defined. This transition from one dimensional to three dimensional conformation, either as a post- or co-translational event, is commonly refered to as protein folding. The study of protein folding using *in vivo* or *in vitro* systems is not a practicable proposition due to the complications arising from the presence of the cellular synthetic machinery and the low levels of folded product obtained.

The pioneering studies of Wu (1931), Northrop (1932) and Anson and Mirsky (1934a, 1934b) in the 1930's demonstrated the reversibility of the protein denaturation renaturation processes. These authors demonstrated that proteins could refold to their native conformation following removal of denaturing forces. Subsequent work by Anfinsen

(see for example, Anfinsen, 1966) showed that all the information required for protein folding was contained within the amino acid sequence of a polypeptide. Anfinsen related these observations to a fundamental theory regarding protein folding, namely that the folded state is more stable than the unfolded state under physiological conditions. These early studies and their implications prompted the extensive use of protein refolding as a suitable model of *in vivo* protein folding. At least two criteria suggest that comparisons between these systems are valid. Firstly, the time scale of refolding is similar to that of *in vivo* folding and secondly, the product of refolding has structural and functional characteristics which are similar, if not identical, to those of the original protein (Jaenicke and Rudolph, 1986).

Since the 1930's protein refolding has been widely employed in an attempt to elucidate the mechanisms of *in vivo* folding. The refolding of pure material under carefully controlled *in vitro* conditions has been defined for a number of proteins. However, it should be noted that refolding of proteins occurs from a denatured but complete polypeptide whereas *in vivo* protein folding could be envisaged as occurring in stages as a co-translational process. There is some evidence that *in vivo* folding does   occur in this way (Bergman and Kuehl, 1979) and this may account for the inability of some larger polypeptides to "fold up" efficiently during refolding studies (Teschner *et al.*, 1987).

In the case of oligomeric proteins it is clear that the processes of polypeptide folding must be closely co-ordinated with the association of subunits into the required quaternary structure. This increased level of complexity requires that the conditions of denaturation and renaturation be carefully controlled during refolding studies. Without such attention the formation of "wrong aggregates" through inter- and intramolecular interactions becomes a major competing reaction to the correct processes of subunit folding and association (Teipel and Koshland, 1971, Zettlmeissel *et al.*, 1979). The refolding of proteins, and in particular that of oligomeric systems, must therefore be examined under optimal conditions in order to mimic accurately the processes of *in vivo* folding.

The time scale of protein refolding is generally rapid (seconds to minutes) and in such periods there is not sufficient time for a polypeptide to examine randomly all potential structures in search of its correct functional conformation (Levinthal, 1968). As a result a number of refolding pathway models have been proposed in order to limit the number of possible conformations into which a protein may fold. Most theories propose an initial collapse from the denatured random coil conformation as a result of hydrophobic interactions with the exclusion of water or through the formation of some secondary structural elements. These elements represent the "kernels" for further collapse to a globular conformation with elements of non-regular and/or ordered secondary structure. This conformation has been termed the "molten globule state" (Dolgikh *et al.*, 1982, Ohgushi and

22

Wada, 1983) and appears to be functionally inactive and lacking certain structural characteristics of the native protein. The molten globule state may be distinguished from the native conformation in terms of certain spectral differences and its marginally more "open" structure (Ptitsyn, 1987). The formation of this semi-ordered state limits the number of folding reactions accessible to a protein and thereby "channels" and accelerates the protein folding towards the native configuration. The subsequent structural alterations generally form the rate limiting steps in refolding and involve further folding processes and the making and breaking of disulphide bonds and non-covalent interactions (Pain, 1987).

This model of refolding represents the simplest situation and it is possible that a number of different kernels or molten globule states, and the pathways interconnecting them, may exist during refolding. However, it is the principle of progressively directing these intermediates towards the native structure by limiting accessibility to all conformations that is the basis of the proposed refolding pathway.

The native conformation of a protein may represent a structure of global free energy minima obtained through a pathway driven by thermodynamic processes. There is some support for such a mechanism of structure formation in the agreement between observed and calculated (on the basis of energy minimisation) protein structures (Sternberg, 1986). However, refolding pathways may act to limit progress to a

23

global energy minimum such that the native conformation only represents the energy minima of kinetically accessible structures. The biosynthesis of the class I histocompatibility antigens provides evidence for such a scheme since they seem to be trapped in native conformations that do not represent the global energy minima (Owen *et al.*, 1980). An experimental method of distinguishing between the "thermodynamic" and "kinetic" models as determinants of native conformation is difficult to envisage and the true situation may include examples from both schemes and some of an intermediate nature.

A number of proteins are subjected to co- or post-translational modification and as these processes are on the pathway between translation of mRNA and expression of protein function or activity they have been considered by some authors within the scheme of protein folding. Modifications include hydroxylation and carboxylation of residues, glycosylation, phosphorylation and limited proteolysis and these have been well reviewed by Freedman and Hawkins (1980). These modifications will not be reviewed in detail here, however, it is of interest that recent evidence indicates that glycosylation of ribonuclease, which appears to be a co-translational process *in vivo* (Pless and Lennarz, 1977), does not affect its *in vitro* refolding (Grafl *et al.*, 1987).

1.6. Protein refolding: experimental approaches.

Proteins are readily denatured by a number of treatments including extremes of pH, heating, organic solvents, detergents (e.g; SDS), chaotropic agents, urea and guanidine hydrochloride. The use of the latter two agents is most common since the degree of unfolding produced is greater and denaturation therefore approaches a two state mechanism (Pace and Vanderberg, 1979). Guanidine hydrochloride (GdnHCl) is generally 1.5 to 2.5 times more effective on a molar basis than urea (Greene and Pace, 1974). Urea also decomposes to yield cyanate (Pace and Vanderberg, 1979). The exact mechanism by which GdnHCl and urea denature proteins is not known but they seem to increase the solubility of non-polar residues and act to disrupt non-covalent interactions (Nozaki and Tanford, 1963).

The refolding of denatured proteins is initiated by the removal of the appropriate denaturing force. In the case of GdnHCl and urea this may be conveniently achieved by dialysis, desalting or dilution. The time scale of protein refolding is generally very short and as such the definition of individual molecular events occurring during this process is difficult. In the transition between the unfolded (U) and native (N) forms of a protein it is generally only U and N that are significantly populated at equilibrium. Intermediate partly folded forms which could be used to characterise the transition pathway do not appear under normal conditions at levels that would permit their characterisation by detailed structural techniques such as X-ray

25

crystallography and NMR. There is some evidence that refolding performed in aqueous-organic cryosolvents at sub-zero temperatures may provide conditions under which these intermediates are significantly populated. Under these conditions the hydrophobic and thermodynamic driving forces towards the native form are reduced thereby allowing partly folded species to accumulate (Fink, 1986). However, a number of other techniques have been successfully adapted to allow an examination of refolding pathways at ambient temperatures. These are listed in Table 1 with appropriate references cited for their use in following protein refolding.

Recent advances in DNA technology and site directed mutagenesis now allow the introduction of specified changes into the sequence of a polypeptide. Clearly any of the techniques in Table 1 could be applied to mutant proteins derived by these methods such that the rôle of specific residues or sequences of amino acids on the mechanisms of refolding might be elucidated (King and Yu, 1986, Ramdas and Nall, 1986, Matthews and Hurle, 1987). The vast potential arising from the use of these mutant proteins will hopefully produce more rapid progress towards an understanding of the processes of protein folding.

1.7. Protein refolding: experimental observations.

The refolding of a number of proteins has been examined over the last twenty years. Initial studies focussed on simple monomeric proteins such as ribonuclease and bovine pancreatic trypsin inhibitor. With the

CATEGORY	TECHNIQUE	REFERENCE
FUNCTIONAL PROBES	Regain of catalytic activity	Chan et al., 1973 Hermann et al., 1985
CONFORMATIONAL PROBES		
	1. SPECTROSCOPIC:	
	a. Absorption	Ikeguchi et al., 1986
	b. Light scattering	West and Price, 1988
	c. Fluorescence	Teipel and Koshland, 1971
	d. Circular dichroism	Craig et al., 1987 Galat et al., 1985 Labhardt, 1986
	2. PHYSICAL:	
	a. Ligand binding	Girg et al., 1983
	b. Limited proteolysis	Girg et al., 1981
	c. Tritium exchange	Kim, 1986
INTERMEDIATE TRAPPING		
	a. Disulphide blocking	Creighton, 1986
	b. Cross-linking	Hermann et al., 1981
	c. Hybridization	Bothwell and Schachman, 1980a,b

Table 1.   Techniques used to examine protein refolding.

refinement of techniques more recent work has examined the refolding
of larger monomeric and oligomeric proteins in which the problems of
obtaining accurate refolding are increased. In this account it is
proposed to examine examples from each of these areas and to relate
the patterns of refolding observed to the general "pathway" model
considered in section 1.5.

1.7.1. Refolding of single domain monomeric proteins.

The refolding of bovine pancreatic ribonuclease A following
denaturation in GdnHCl has been extensively studied by two groups led
by Baldwin (Baldwin, 1980) and Schmid (Schmid, 1986). Evidence was
originally presented for the existence of two unfolded states of this
protein on the basis of the observed biphasic kinetics of reactivation
(Garel and Baldwin, 1973). A fast (U_F) and slow (U_S) refolding species
were detected and a plausible explanation for the two forms relating
to the isomerization of proline residues was first proposed by Brandts
et al. (1975). This theory suggested that following the rapid
unfolding of the native protein (N) to U_F the proline residues
isomerize to the incorrect *trans* configuration giving U_S. The U_S
species would therefore have to isomerize to the *cis* configuration
prior to refolding according to the scheme;

$$U_S \rightleftharpoons U_F \rightleftharpoons N$$

These conclusions were supported by the observation that the activation energy of the $U_S \rightleftharpoons U_F$ reaction (85 kJ mol^{-1}, Schmid and Baldwin, 1978) was similar to that of a *cis-trans* proline isomerization (84 kJ mol^{-1}, Brandts *et al.*, 1975). The typical equilibrium of U_S and U_F in the above scheme is 80% and 20% of the total unfolded species respectively (Garel and Baldwin, 1973).

Subsequent work has indicated that two slow refolding species (U_S^I and U_S^{II}) are populated from U_F. At equilibrium U_S^{II}, the faster of the two slow refolding species, represents 65-70% of the total unfolded species with U_S^I comprising 10-15%. U_S^{II} is thought to contain only one incorrect proline isomer (Pro 93) whereas U_S^I contains additional proline residue(s) in the *trans* configuration (Schmid *et al.*, 1986). The refolding of the U_S^{II} species is fairly well understood and seems to be dependent on the folding conditions. Under strongly native conditions (e.g. in the presence of $(NH_4)_2SO_4$) U_S^{II} refolds through two intermediates, I_I and I_N, before the isomerization of the Pro 93 to the native configuration. I_I is a structure of open configuration but with local H-bonded secondary structure formed by rapid refolding of U_S^{II}. I_N is a native-like intermediate, formed following subsequent refolding of I_I, which has a hydrophobic interior, nucleotide binding capacity and catalytic activity. It differs from N only in some spectral properties and the configuration of Pro 93 (Schmid, 1983, Schmid *et al.*, 1986, Schmid, 1986). The refolding of U_S^I is less well documented primarily due to its low level in the pool of U species.

The refolding of ribonuclease A, and in particular the slightly artificial refolding of the U_s^{II} species under strongly native conditions, fits into the general pattern of the folding pathway considered in section 1.5. Rapid collapse of structure during refolding, presumably via kernel formation, produces the molten globule state (I_I and I_N) which refold further to establish I_N and undergo subsequent covalent modification as the rate limiting reaction in the formation of N.

The refolding of another small monomeric protein, bovine pancreatic trypsin inhibitor has been extensively examined by Creighton and co-workers using a disulphide trapping technique (Creighton, 1980). From a number of studies these workers have proposed a refolding pathway for this protein as indicated in Fig. 7. This scheme involves a rapid initial collapse to a "molten globular" structure containing one of a set of disulphide bonds (5-30, 30-51 or 5-55). These residue pairings characterise the subsequent pathway which involves the rearrangement of covalent and non-covalent bonds to yield the native configuration (N; containing disulphides 30-51, 5-55 and 14-38) as indicated.

The initial structural collapse to the molten globule structure has not been well characterised in ribonuclease or bovine pancreatic trypsin inhibitor. In small monomeric proteins this collapse is so rapid that even with advanced stopped-flow techniques the structural changes during collapse are completed within the dead time of measurement, generally 10-20 ms (Kuwajima et al., 1987).
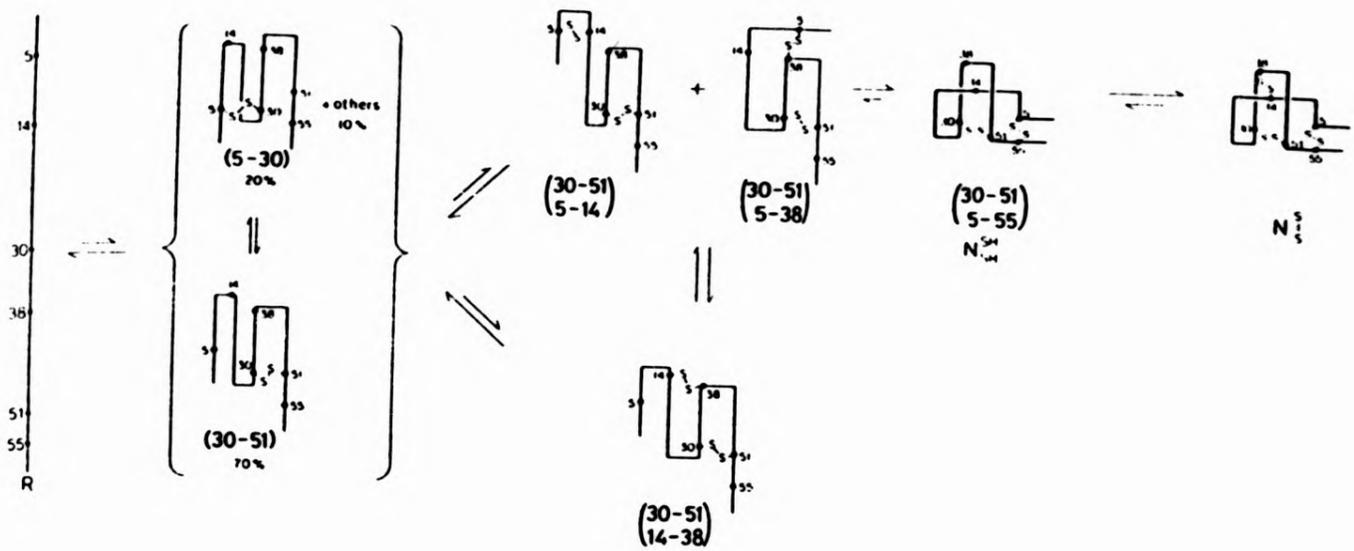
Figure 7.   Refolding pathway of bovine pancreatic trypsin inhibitor
             (from Creighton, 1980).

The solid line represents the polypeptide backbone with the positions
of the six cysteine residues indicated.   The brackets around the
single disulphide species indicate that they are in rapid equilibrium;
only the two most common intermediates are indicated along with their
relative accumulation under normal conditions.   The conformation of
N_{SH}^{SH} and N_{S}^{S} approximate the native configuration of bovine pancreatic
trypsin inhibitor but only N_{S}^{S} represents the true protein configuration.

1.7.2. Refolding of multi-domain monomeric proteins.

Large proteins often contain domains which are thought to act as independent folding units (Adams *et al.*, 1980). In the native protein domains represent compact regions of structure of Mr 10-15,000. Where these domains are separated from local structures by intervening sequences of non-ordered polypeptide they could be envisaged as folding independently with the subsequent association of the folded units comprising the rate limiting reaction in the formation of activity (Goldberg and Zetina, 1980). This scheme of "folding by parts" would have the advantage of accelerating the overall process of folding and allowing structure acquisition to occur as a co-translational process thereby reducing the potential for proteolysis and incorrect folding or aggregation reactions.

The refolding of the monomeric multi-domain protein octopine dehydrogenase has been studied by Zettlmeissl *et al.* (1984) and Teschner *et al.* (1987). The enzyme from *Pecten jacobaeus* has a Mr of 45,000. Reactivation of this enzyme following denaturation in 6 M GdnHCl is characterised by a slow first order process with t_u approximately 30 min. Using spectroscopic techniques an intermediate (I_1) has been defined with fluorescence and circular dichroism properties identical to the native enzyme. I_1 is formed rapidly within 15 s of refolding and seems to represent a highly folded but inactive conformation, i.e; the molten globule state. A second intermediate (I_2) is formed from this state in a slower phase and differs from I_1

in its resistance to the proteinases trypsin and thermolysin. The rate of I_2 formation and the activation energy of the $I_1 \rightarrow I_2$ transition is consistent with a process of proline isomerization. I_2 is subsequently converted to the native enzyme (N) in the slowest process of all. The appearance of N is detected as the reactivation of the enzyme and the conversion $I_2 \rightarrow N$ seems to be affected by solvent viscosity. The conclusion of Teschner *et al.* (1987) from these observations is that this conversion represents processes during which folded units are moving relative to one another such as during domain association. There is some evidence that a direct route of N formation from I_1 exists in that no lag in reactivation is observed corresponding to the $I_1 \rightarrow I_2 \rightarrow N$ pathway. This route presumably reflects I_1 species containing the proline configuration of the native enzyme.

Approximately 30% of denatured octopine dehydrogenase forms an incorrectly folded inactive species (X) which does not arise through intermolecular aggregation. The fluorescence characteristics of X suggest its formation from the denatured enzyme rather than I_1. The origin of the incorrect refolding to X is unknown but does not seem to be irreversible since X may be separated from N, denatured and refolded to yield 70% N and 30% X. The scheme for the refolding pathway of octopine dehydrogenase is thus;
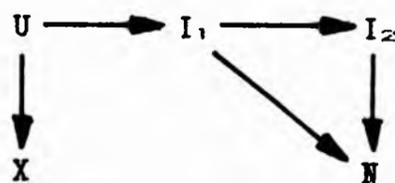
$U \rightarrow I_1$ might be envisaged as the collapse to a molten globule state with $I_1 \rightarrow N$ and $I_1 \rightarrow I_2 \rightarrow N$ representing subsequent structural arrangements confirming the final native conformation. $U \rightarrow X$ would seem to represent a collapse and/or refolding to an inactive trapped configuration. The formation of X during refolding *in vitro* perhaps reflects the contribution of the vectorial processes of *in vivo* protein synthesis to the correct folding of this enzyme. Octopine dehydrogenase may therefore fold by parts as a co-translational process *in vivo*.

1.7.3. Refolding of oligomeric proteins.

The refolding of monomeric proteins considered above is dependent on sequential or parallel first order reactions as rate limiting reactions. In oligomeric proteins second order processes of subunit association may become the rate limiting steps of refolding.

The refolding of lactate dehydrogenase from porcine muscle has been extensively studied by Jaenicke and co-workers. Lactate dehydrogenase is comprised of four identical subunits arranged in a tetrahedral configuration. Intersubunit contact areas show pairwise interactions and the enzyme therefore represents a dimer of dimers (Holbrook *et al.*, 1975). Initial refolding studies defined the effects of temperature, pressure, state of dissociation and coenzymes on the kinetics of reactivation (Rudolph and Jaenicke, 1976, Rudolph *et al.*, 1977a, Rudolph *et al.*, 1977b, Schade *et al.*, 1980). The levels of

reactivation were variable with the formation of non-covalent aggregates the major competing reaction to correct refolding. Aggregate formation has been extensively studied by Zettlmeissel *et al.*, (1979) and shown to be related to the extent of denaturation and the concentration of enzyme during refolding as indicated in Fig. 8. Aggregate formation occurs through processes with reaction order greater than two in competition with the first order subunit refolding reactions on the pathway to the active configuration.

The kinetics of subunit reassociation under optimal conditions of reactivation have been determined using the glutaraldehyde cross-linking technique (Hermann *et al.*, 1981). The reassociation process can be adequately described by a first order association equilibrium between monomers (M) and dimers (D) followed by a second order irreversible association of dimers to tetramers (T). Tetramerization parallels the reactivation kinetics and therefore T is the sole active species. The scheme for the pathway of lactate dehydrogenase refolding is thus;

$$U \longrightarrow 4 \text{ M} \underset{}{\overset{K}{\rightleftharpoons}} 2 \text{ D} \overset{k}{\longrightarrow} T$$

$$\downarrow$$

WRONG AGGREGATES
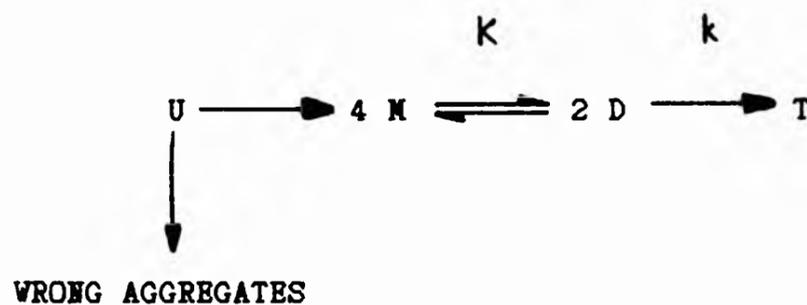
with $K = 3 \times 10^6$ M^{-1} and $k = 3.15 \times 10^4$ M^{-1} s^{-1} at 20°C.
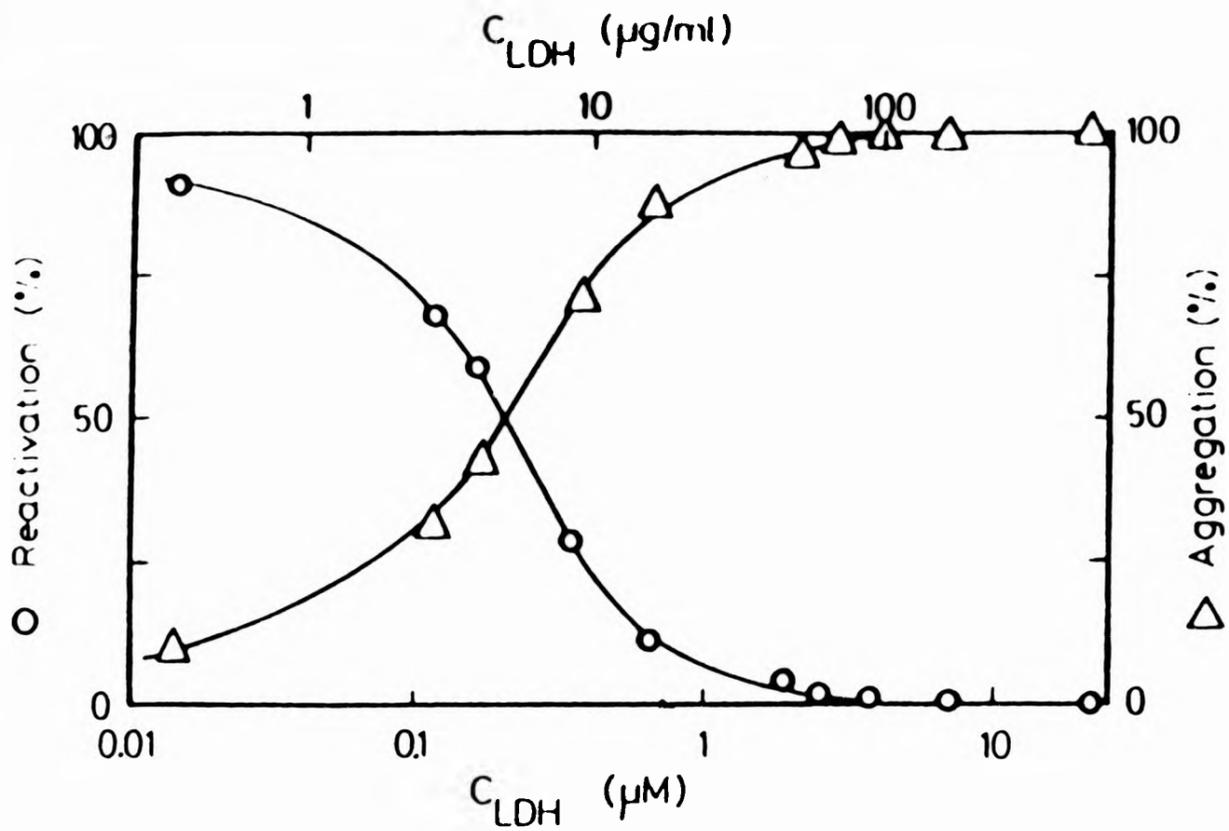
Figure 8.   Effect of enzyme concentration on the extent of reacti-
vation and aggregation during the refolding of lactate
dehydrogenase (from Zettlmeissl et al., 1979).

Subsequent work by Girg *et al.* (1981) showed that the dimeric species could be trapped in a stable configuration by the addition of thermolysin one minute after the initiation of refolding by which time the M → D transition was essentially complete. Endgroup analysis of these dimers showed that the N-terminal acetylation present in the native enzyme was missing. The implication was therefore that a number of amino acids had been cleaved from the N-terminal region and that these residues were required for stabilisation of the native tetrameric configuration. As conformation of this, the X-ray crystallographic structure (Holbrook *et al.*, 1975) did indeed show that the dimer of dimers was held together by an N-terminal arm of approximately 20 amino acids. The sites of cleavage in this arm are only accessible during refolding and not in the native configuration, since a similar thermolysin treatment has no effect on the native enzyme.

Girg *et al.* (1983) examined the characteristics of the proteolytic dimers and the dimers formed during reassociation. Both had circular dichroism similar to the native enzyme and approximately 50% of the native fluorescence. This significant structural component was reflected in the ability of the dimers to bind the affinity dye procion green. Triazine dyes such as procion green are known to interact with the nucleotide fold of dehydrogenases (Biellmann *et al.*, 1979) implying that this structural element is present in the dimers. The dimers also exhibited approximately 40% of the native catalytic activity in the presence of "structure-making" ions such as $(NH_4)_2SO_4$.

Most recently, Opitz *et al.* (1987) have shown that the proteolytic dimers are heterogenous and comprised of species with cleavage sites additional to the N-terminal arm. These internally "nicked" dimers are active and are able to reassociate following denaturation in GdnHCl indicating that separate domains or fragments of a protein may be able to fold and associate correctly even without inter-connecting stretches of polypeptide. These findings therefore further support the possibility that large proteins may fold by parts with the association of domains as a consecutive process (sections 1.5. and 1.7.2.)

Clearly the dimeric (and probably the monomeric) intermediates of lactate dehydrogenase reassociation could be envisaged as representing the molten globule state. The dimers contain considerable native structure and the presence of a nucleotide binding site, although under normal conditions these species are inactive. In the presence of structure-making ions tightening of the globular structure of the dimeric intermediates produces some catalytic activity. A similar process of structural tightening in the molten globule state would presumably lead to the formation of the native enzyme (T) and full activity in the normal pathway of refolding.

1.8. Catalysis of protein folding *in vivo.*

The structural processing that occurs during the transition between the molten globule state and the native conformation are the rate limiting processes of refolding in the examples considered above. The

transition seems to involve a general "tightening" of structure in association with changes in disulphide bonding and non-covalent interactions. Recent evidence has emerged that these processes may be enzyme catalysed during *in vivo* folding.

The enzyme peptidyl prolyl isomerase (PPI) has been isolated from pig kidney by Fischer and Bang (1985). Lang *et al*. (1987) have shown that this enzyme catalyses the refolding of a number of proteins that have U_s refolding species. Evans *et al*. (1987) have also shown that this enzyme may have a rôle in catalysing proline isomerization in the native configuration of Staphylococcal nuclease. Similarly, the enzyme protein disulphide isomerase (PDI) has been shown to catalyse the assembly of procollagen from its constituent polypeptides via disulphide bond formation (Myllylä and Koivu, 1987) and has been found in association with antibody polypeptides *in vivo* (Roth and Pierce, 1987). However, despite this evidence the exact catalytic rôle of these enzymes during *in vivo* folding is not yet known.

1.9. Protein refolding: applications.

Protein refolding is the only technique currently available which allows an examination of the mechanisms of *in vivo* protein folding. The elucidation of these mechanisms is of considerable scientific interest as these complex processes comprise an essential event on the pathway between stored genetic information and its expression in the form of functional protein. Refolding studies are thought to act as a

suitable model for *in vivo* protein folding providing that the refolding conditions are carefully controlled. However, in any comparisons between these two processes consideration must also be made to the contributions of the vectorial nature of protein synthesis (allowing co-translational folding) and the possible enzymic catalysis of certain folding reactions *in vivo* (sections 1.5., 1.7.2., 1.7.3., 1.8.).

A detailed understanding of the processes of protein refolding could have extensive applications in the area of "protein engineering". This term has recently been coined to describe the techniques whereby existing proteins could be genetically manipulated, or new proteins designed and synthesised *ab initio*, to perform a pre-defined biological or non-biological function (Oxender and Fox, 1987). Clearly, a detailed knowledge of protein folding would allow the effects of mutations on the ability of proteins to fold correctly to be predicted. Similarly, it could be envisaged that a complete definition of the mechanisms of folding could allow the design of proteins which would fold to a specified functional configuration.

The study of protein refolding has further applications that are not dependent on its use as a model for folding *in vivo*. These result from problems encountered when eukaryotic genes are cloned into and expressed in prokaryotic vectors such as *Escherichia coli*. In such cases the gene product is often produced as insoluble aggregated material which form dense inclusion bodies (Marston, 1986). This

phenomenon may partly result from the inability of the prokaryotic environment to process successfully the foreign polypeptides. The aggregation process is useful for the purposes of purification since the inclusion bodies will sediment readily with low speed centrifugation. However, to obtain active product the protein must be denatured and refolded into its correct configuration. Aggregated material is denatured in the presence of a reductant which breaks any intermolecular disulphide bonds. In this state the removal of denaturant and provision of an oxidising environment allows the protein to refold to its active configuration. Clearly the definition of the refolding conditions producing optimum yield of correctly folded product could be obtained from preliminary protein refolding studies such as those reviewed in section 1.7.

1.10. Denaturation and refolding studies on phosphoglycerate mutases.

Phosphoglycerate mutases provide an ideal system with which certain aspects of the processes of protein refolding can be studied. The interesting division of cofactor requirement in these enzymes is associated with further mechanistic and structural differences between the two groups (sections 1.1., 1.2. and 1.3.). The cofactor dependent and independent enzymes can therefore provide interesting comparisons between the refolding of analogous proteins. In the case of the cofactor dependent phosphoglycerate mutases considerable structural and mechanistic information is available which can be related to the observed processes of refolding. The X-ray structure and amino acid

38

sequence of the bakers yeast enzyme are known (section 1.2.1.) and the kinetics of reassociation and reactivation during refolding have been extensively studied (section 3.1.).

Phosphoglycerate mutases also provide a range of structural complexities in which protein refolding could be usefully studied. The *S. pombe* enzyme represents a fairly small monomeric protein, the cofactor independent enzymes are larger monomers and therefore probably multi-domain proteins while the oligomeric rabbit muscle and bakers yeast enzymes have a dimeric and tetrameric configuration respectively. The latter two enzymes therefore require both folding and association processes during refolding. These three levels of complexity are comparable to the examples of protein refolding considered in section 1.7.

The aims of this thesis were to examine the denaturation and renaturation (refolding) characteristics of phosphoglycerate mutase enzymes as a group. The refolding of these enzymes could then be compared with the model of "folding pathways" considered in section 1.5. The bakers yeast, rabbit muscle, *S. pombe* and cofactor independent enzymes from wheat germ and *Aspergillus nidulans* were selected as representative examples including different levels of structural complexity and requirement for cofactor. The denaturation transitions were monitored through loss of catalytic activity and changes in protein fluorescence and circular dichroism. Refolding and reassociation following denaturation in GdnHCl were monitored through

reactivation of the enzymes and this process was probed using limited proteolysis as an indicator of bond accessibility. In such studies it was hoped to produce proteinase resistant fragments which might represent compact folded structures or domains. The formation of these structural elements during refolding could be defined and related to published sequence and structural information where available. It was also proposed to investigate the possible metal ion requirement of the cofactor independent enzymes both for catalytic activity and structural regain during refolding. A number of cofactor independent enzymes were examined for a metal ion requirement and structural similarities whilst the refolding of the wheat germ and *A.nidulans* enzymes was examined in more detail.

In addition to developing and/or refining the isolation and purification procedures for the enzymes required in the above studies, it was hoped to produce sufficient quantities of pure *S.pombe* phosphoglycerate mutase to allow its further characterisation through collaborative studies with the Universities of Aberdeen and Regensburg.

CHAPTER TWO : MATERIALS

AND METHODS

2.1. MATERIALS.

2.1.1. Microorganisms and other biological materials.

Fresh bakers yeast (*Saccharomyces cerevisiae*) was the kind gift of the Distillers Company Ltd., Menstrie, Scotland. *Schizosaccharomyces pombe* (CMI 39917) and *Candida utilis* (CMI 23311) were obtained from the Commonwealth Mycological Institute, Kew, England. *Aspergillus nidulans* BVB 272 was supplied by Dr.L.Stevens, University of Stirling, Scotland. *Escherichia coli* and *Klebsiella aerogenes* cultures were supplied by Dr.M.North, University of Stirling, Scotland. Wheat germ, potatoes and mung beans were obtained from local retail suppliers.

2.1.2. Proteins, substrates and co-enzymes.

Purified phosphoglycerate mutase from bakers yeast, *S.pombe*, *A.nidulans* and wheat germ were prepared as described in sections 3.2., 5.2. and 6.2. Purified phosphoglycerate mutase from rabbit muscle was obtained from Boehringer Mannheim as a 5 mg ml^{-1} $(NH_4)_2SO_4$ suspension. Coupling enzymes used including enolase from rabbit muscle (10 mg ml^{-1}) and pyruvate kinase/lactate dehydrogenase mixture from rabbit muscle (4 mg ml^{-1}) were supplied by Boehringer as $(NH_4)_2SO_4$ suspensions. Boehringer also supplied proteinase K from *Tritirachium album* Limber and aldolase from rabbit muscle. Sigma Chemical Co. supplied the following proteins; bovine serum albumin, ovalbumin, trypsinogen, cytochrome C, lysozyme, Dalton Mark VII SDS-PAGE Mr marker set, thermolysin, α-

41

chymotrypsin (tosyllsylchloromethane (TLCK) treated), subtilisin BPN', elastase type IV, V-8 proteinase from *Staphylococcus aureus* and turkey egg-white proteinase-inhibitor protein (type II-T). Trypsin (tosylphenylalanylchloromethane (TPCK) treated) three times recrystallized was obtained from Worthington Corp. and aprotinin (trasylol) was obtained from Bayer. Boehringer supplied the following substrates and co-enzymes; 3-phosphoglycerate (grades 1 and 2), 2-phosphoglycerate, AMP, ADP, ATP, NAD^+ and NADH. Sigma supplied the 2,3-bisphosphoglycerate used.

2.1.3. Chemicals.

GdnHCl (Aristar grade), acrylamide and N,N-methylene-bisacrylamide (both Electran grade) were supplied by BDH. $(NH_4)_2SO_4$ (specially low in heavy metals) was supplied by Fisons. All other chemicals used were of A.R. grade.

2.1.4. Chromatography media.

Cibacron Blue F3GA-sepharose (Reactive Blue 2), QAE-cellulose, Sephadex G-150 and Sephadex G-25 were obtained from Sigma. DEAE-cellulose was obtained from Whatman and Sephacryl S-300 was from Pharmacia.

2.1.5. Distilled water.

Double distilled water obtained from a Fisons glass bi-distillation
unit was used at all times.

2.2. METHODS.

2.2.1. Assessment of protein homogeneity and relative molecular mass.

2.2.1.1. SDS-PAGE.

Polyacrylamide gel electrophoresis in the presence of SDS was performed by the method of Laemmli (1970). Details of buffers, solutions and the preparation of slab gels are given in Appendix I. Polyacrylamide slab gels were routinely prepared in advance and stored for a maximum of 5 days at 4°C until required.

For sample preparation protein solutions were mixed with an equal volume of double strength sample buffer containing 10% v/v 2-mercaptoethanol and boiled for 3 min. After cooling a mixture of 2-mercaptoethanol and 1% w/v bromophenol blue (50:50) was added to a final concentration of 5% v/v and the samples loaded onto gels using a Hamilton syringe. Sample volume after preparation was 10-200 μl and contained 1-20 μg protein. Gels were run at a constant current of 12 mA for 6 h or 4 mA overnight.

Gels were stained for protein in Coomassie Brilliant Blue R-250 solution and destained in 10% v/v acetic acid. When required gels were silver stained by the method of Wray *et al.* (1981). Details of the preparation of solutions and the basic silver staining procedure are given in Appendix I. After staining and destaining, gels were scanned

44

densitometrically using a Shimadzu CS-930 TLC scanner and/or photographed and subsequently dried using a Bio-Rad slab gel dryer.

Protein Mr values were determined from relative migrations during SDS-PAGE with reference to the migration of the standard proteins of the Dalton Mark VII Mr marker set. This proprietry marker set contains bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (36,000), bovine carbonic anhydrase (29,000), trypsinogen (24,000), soybean trypsin inhibitor (20,100) and α-lactalbumin (14,200). A typical calibration for these proteins on a 12% acrylamide separating gel is shown in Fig. 9. Aprotinin (6,500) was used to supplement the Dalton Mark VII Mr marker set when higher percentage acrylamide separating gels were employed in studies of protein fragmentation.

2.2.1.2. Gel filtration.

Gel filtration was performed in 50 mM sodium phosphate buffer, pH 7.5. Both Sephadex G-150 and Sephacryl S-300 gels were used and manufacturers recommendations were followed for the swelling, pouring and storage of columns. Columns were run at an approximate flow rate of 1 ml min^{-1} and were calibrated using the following proteins of known relative molecular mass; bovine serum albumin (66,000), ovalbumin (45,000), trypsinogen (24,000) and cytochrome C (12,400). The elution of standard proteins was detected by absorbance at 280 nm. The column void volume was determined using Blue Dextran (Mr; 2 x 10^6)
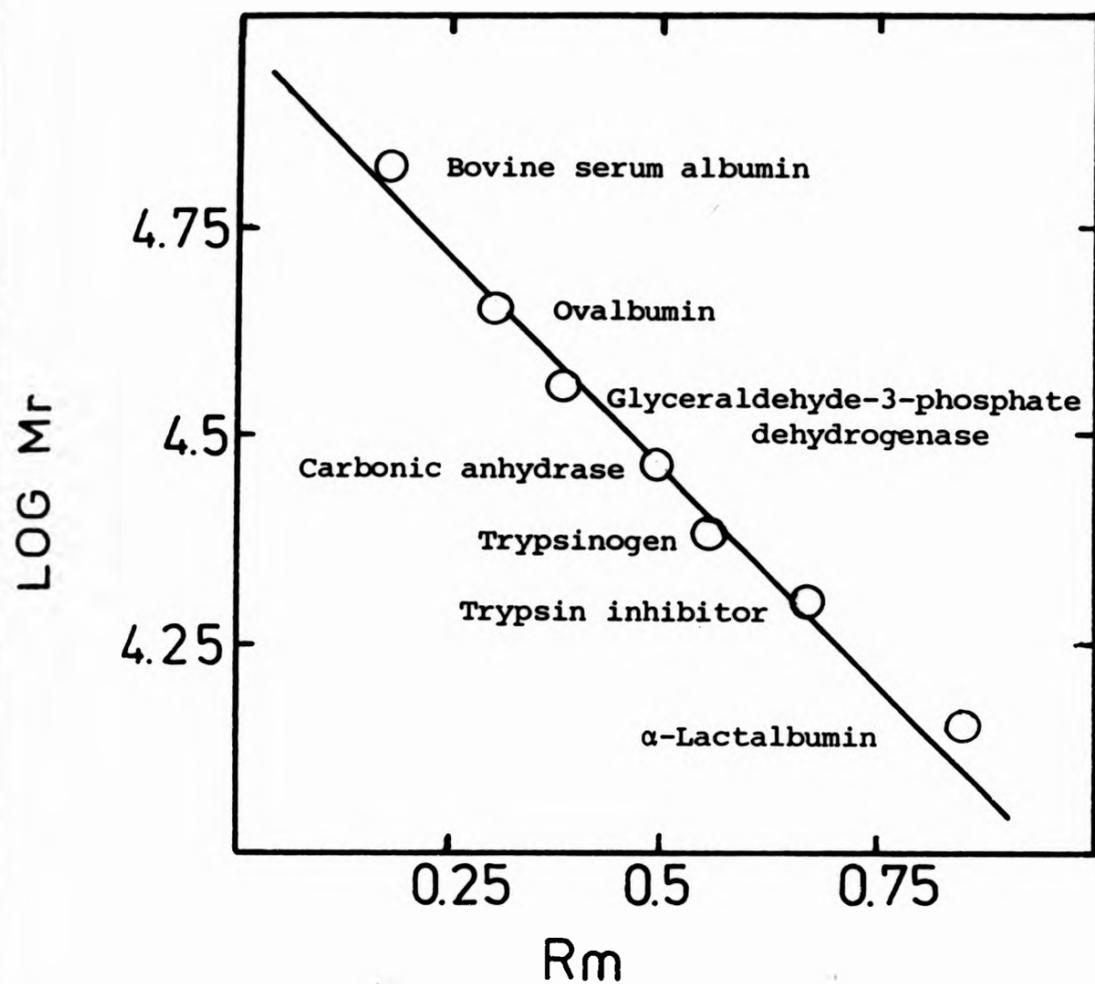
Figure 9. Migration of standard proteins relative to their Log Mr
          following SDS-PAGE on a 12% polyacrylamide separating
          gel.

Rm values express the migration of proteins relative to the bromo-
phenol blue present in each sample.

detected in fractions by absorbance at 600 nm. The calibrations of the Sephacryl S-300 column (38 cm x 4.9 cm²) and Sephadex G-150 column (22 cm x 3.8 cm²) are shown in Figs. 10 and 11 respectively.

2.2.2. Determination of protein concentration.

Protein concentration was routinely determined using the method of Sedmak and Grossberg (1977). To a protein sample made up to a final volume of 1 ml with distilled water was added 1 ml of Coomassie reagent comprising 0.06% w/v Coomassie Brilliant Blue G-250 in 3% v/v perchloric acid. The absorbance of the resulting solution was measured at 620 nm and 465 nm against an absorbance blank of distilled water. The ratio of these absorbances (620/465) for each protein sample were calculated and the absorbance ratio of protein blank (1 ml distilled water and 1 ml Coomassie reagent) subtracted from each value. The corrected values were used to determine the protein concentration from a standard curve prepared using known concentrations of bovine serum albumin between 0 and 50 μg ml^{-1}. A new standard curve was prepared for each new batch of Coomassie reagent. In addition, a single bovine serum albumin standard (20 μg ml^{-1}) was included in each set of protein determinations as a further control.

For purified phosphoglycerate mutase the protein concentration was determined from the absorbance at 280 nm using the published absorption coefficients ($A_{280}^{0.1\%}$) of 1.45 and 1.05 for the enzymes from

46
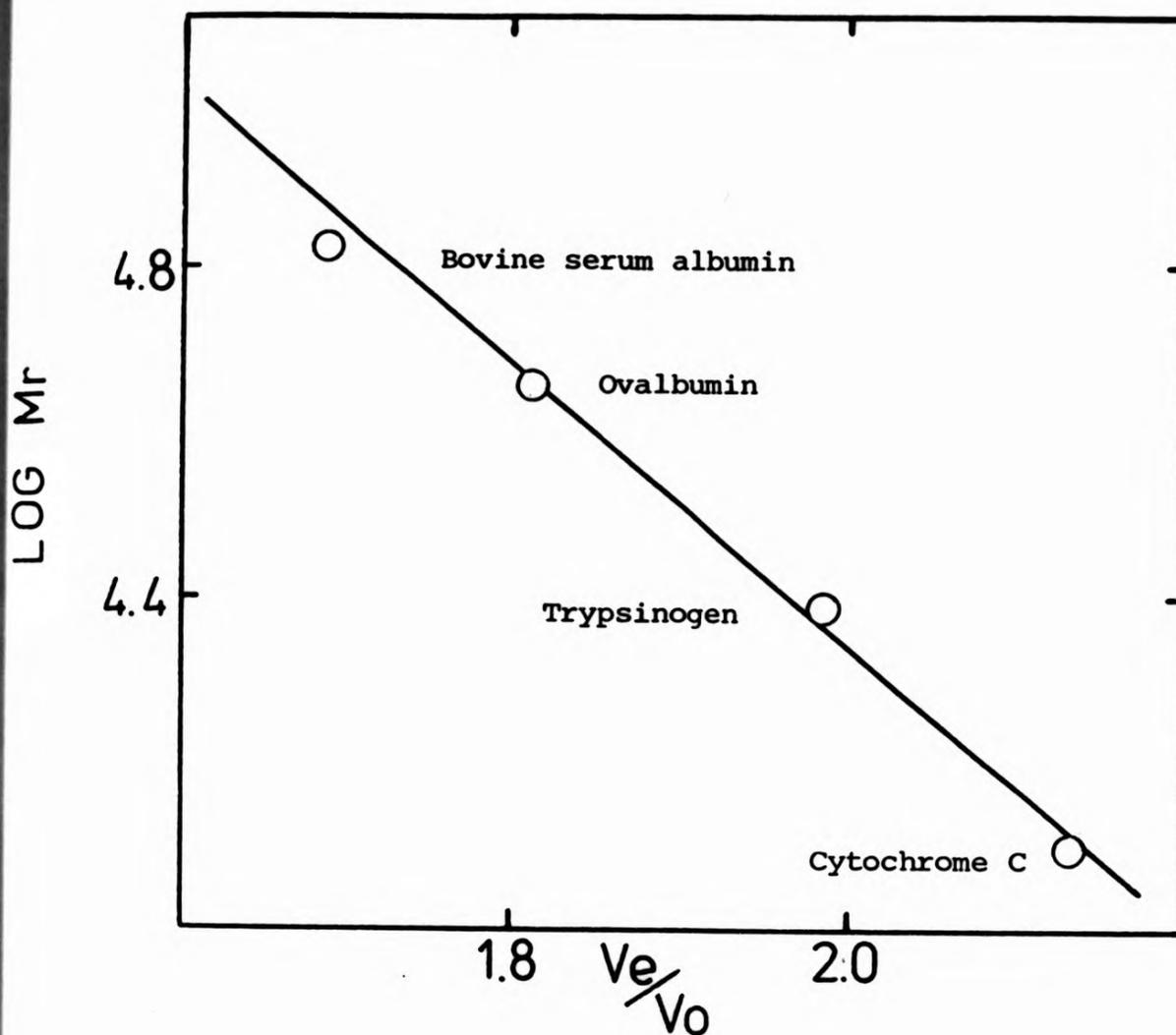
Figure 10. Elution of standard proteins relative to their Log Mr using the Sephacryl S-300 column described in section 2.2.1.2.

Elution volume (Ve) is expressed as a fraction of the column void volumn (Vo) determined from the elution of Blue Dextran.
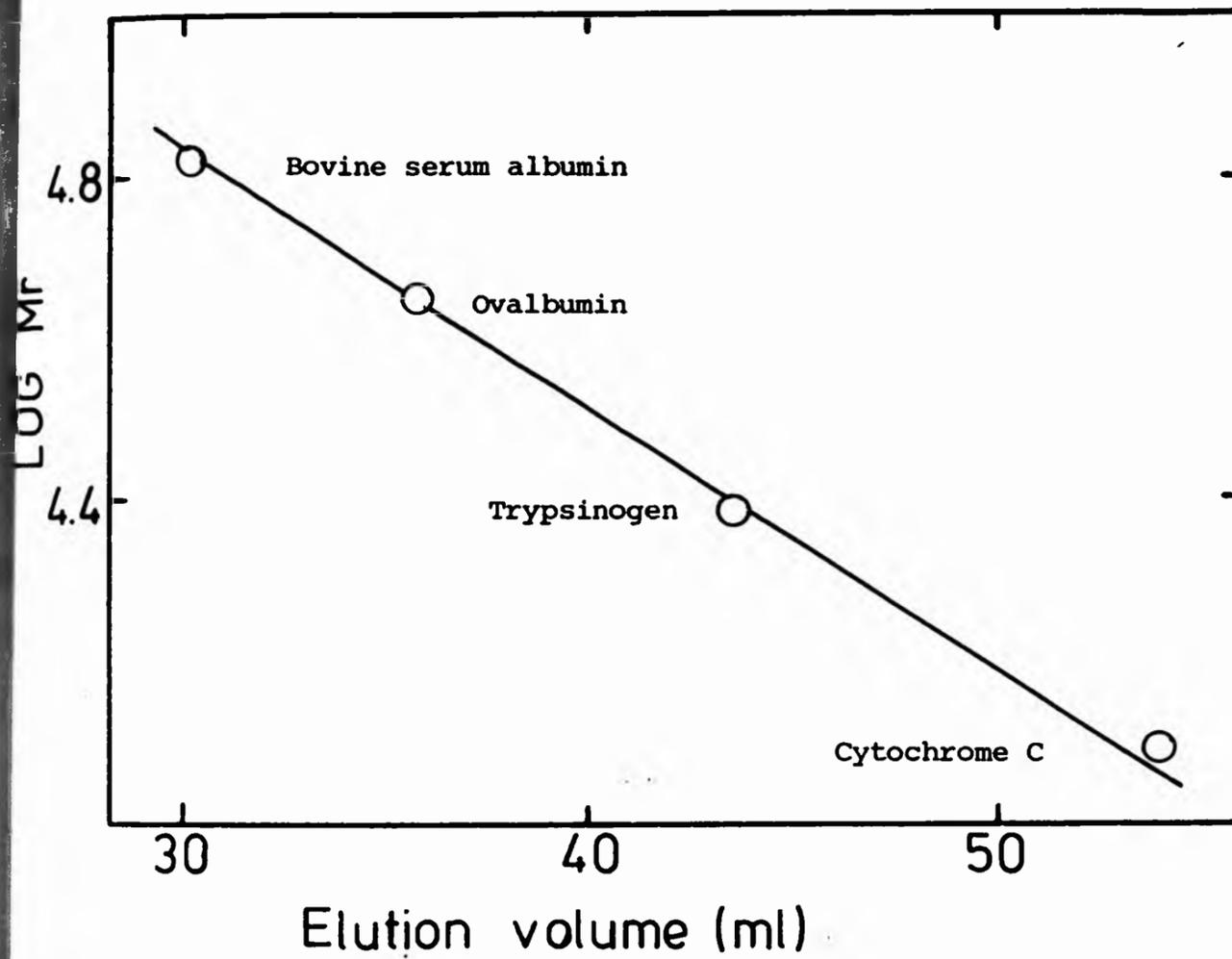
Figure 11. Elution of standard proteins relative to their Log Mr using the Sephadex G-150 column described in section 2.2.1.2.

bakers yeast and rabbit muscle respectively (Edelhoch *et al.*, 1957).
Comparison of the two methods of protein determination using purified
enzyme indicated good agreement (±5%). However, the direct
spectrophotometric method provided a more rapid and non-destructive
technique and was therefore preferentially used for purified enzyme
samples.

2.2.3. Assay of phosphoglycerate mutase activity.

Phosphoglycerate mutase activity was assayed in the forward direction
(3-phosphoglycerate → 2-phosphoglycerate) using two methods.

2.2.3.1. Continuous enolase coupled assay.

The enolase coupled assay of Rodwell *et al.* (1957) has been widely
used for the assay of phosphoglycerate mutase activity. In this assay
the isomerization of 3-phosphoglycerate to 2-phosphoglycerate is
coupled using enolase to the formation of phosphoenolpyruvate (section
1.1.1., Fig. 1). Phosphoenolpyruvate absorbs at 240 nm and the
reaction is therefore followed at this wavelength. The standard assay
system contained in a final volume of 1 ml; 10 mM 3-phosphoglycerate,
0.3 mM 2,3-bisphosphoglycerate (cofactor dependent enzymes only), 3 mM
$MgSO_4$ as a cofactor for enolase and 10μg (0.4 units) of rabbit muscle
enolase in 30 mM Tris-HCl buffer, pH 7.0. The reaction was initiated
by addition of phosphoglycerate mutase (50 μl maximum) and the rate of
increase in A_{240} at 30°C monitored. One unit of phosphoglycerate

47

mutase activity was defined as producing an increase in A_{240} of 0.1 min^{-1} and is equivalent to 0.115 μmol of 3-phosphoglycerate metabolised min^{-1} (Grisolia, 1962). To ensure that the enolase coupling system did not become rate limiting the amount of phosphoglycerate mutase added was adjusted to give a Δ A_{240} of less than 0.15 min^{-1} (i.e.; less than 1.5 units were added).

Phosphoglycerate mutase cofactor dependence was assessed using 10 mM grade 1 3-phosphoglycerate in the standard assay. This substrate is essentially free of contaminating 2,3-bisphosphoglycerate and therefore allows enzyme activities to be determined in the presence and absence of cofactor.

2.2.3.2. Stopped triple coupled assay.

The stopped triple coupled assay used was based on the assay system developed by Leadley *et al.* (1977). In the stopped assay the 2-phosphoglycerate formed by isomerization of 3-phosphoglycerate in a separate reaction is assayed by a coupling system containing enolase, pyruvate kinase and lactate dehydrogenase (section 1.1.1., Fig. 1). The oxidation of NADH as a result of lactate formation is followed as a decrease in absorbance at 340 nm. The initial reaction mixture contained, in a final volume of 1 ml; 10mM 3-phosphoglycerate and 0.3 mM 2,3-bisphosphoglycerate (cofactor dependent enzymes only) in 30 mM Tris-HCl buffer, pH 7.0; at 30°C. Phosphoglycerate mutase (50 μl maximum) was added to this system to initiate the reaction. After

48

defined intervals of incubation, aliquots of the initial assay system were added to the coupling assay system which contained, in a final volume of 1 ml; 20 mM KCl, 5 mM MgSO$_4$, 0.2 mM ADP, 0.15 mM NADH, 20 μg (0.8 units) of enolase, 18 μg (7.5 units) of pyruvate kinase and 6 μg (7 units) of lactate dehydrogenase in 30 mM Tris-HCl buffer, pH 7.0, at 30°C. The immediate drop in A$_{340}$, extrapolated back to the time of addition, was a product of 2-phosphoglycerate formed in the initial assay being converted to lactate. By comparison of the decrease in A$_{340}$ at two time intervals the rate of 2-phosphoglycerate production in the initial system, and thus phosphoglycerate mutase activity, could be determined. Control measurements indicated the decrease in A$_{340}$ produced in the coupling assay system was proportional to the amount of added 2-phosphoglycerate for the range of observed changes in A$_{340}$.

The stopped triple coupled assay, although considerably more complex, has a number of advantages over the continuous enolase coupled assay. For example, the former assay allows phosphoglycerate mutase activity to be determined in the presence of denaturants or inhibitors at concentrations which would normally affect the coupling enzymes since the carry over concentration of denaturant or inhibitor into the coupling assay system is low. Furthermore, the absorbance of phosphoenolpyruvate at 240 nm, monitored in the continuous enolase coupled assay, is dependent on the assay conditions (section 1.1.1.) and therefore the enolase assay is unsuitable for detailed kinetic studies.

49

In the context of the work reported here, the stopped triple coupled assay was most widely used to assay phosphoglycerate mutase activity in the presence of GdnHCl at concentrations up to 2 M. In these experiments 100 μl of the initial reaction mixture was added to the coupling assay system and therefore the maximum carry over concentration of GdnHCl was 0.2 M. This concentration had no effect on the determination of 2-phosphoglycerate by the coupling assay. Appropriate control measurements also indicated that in the case of each enzyme examined the rate of conversion of the 3-phosphoglycerate in the initial system was linear over the time interval during which samples were withdrawn and that this rate was proportional to the amount of phosphoglycerate mutase added over the required range of enzyme concentrations.

2.2.4. Denaturation of phosphoglycerate mutase by GdnHCl.

The denaturation of phosphoglycerate mutase by GdnHCl was monitored using three methods.

2.2.4.1. Loss of catalytic activity.

The activity of phosphoglycerate mutase in the presence of GdnHCl was studied using the stopped triple coupled assay system. GdnHCl was included in the initial assay mixture at concentrations up to 2 M and the rate of 2-phosphoglycerate formation determined using the coupling assay system. The initial assay was initiated by the addition of

phosphoglycerate mutase and so there was no period of pre-incubation
of enzyme in GdnHCl. 2-phosphoglycerate formation was determined 0.5
and 3 min after the addition of phosphoglycerate mutase to the initial
assay mixture.

2.2.4.2. Changes in fluorescence.

Fluorescence measurements were made at 20°C in a Perkin-Elmer MPF-3L
fluorimeter using semi-micro quartz cuvettes of 1 ml capacity.
Fluorescence excitation was at 290 nm and emission was monitored
between 300 and 400 nm. Phosphoglycerate mutase fluorescence emission
was determined in the presence and absence of GdnHCl. Enzyme and
GdnHCl were pre-incubated for 15 min at 4°C prior to the measurement
of spectra.

All measurements were made using 50 mM sodium phosphate buffer, pH
7.5, and appropriate buffer or buffer and GdnHCl blanks were included.
All fluorescence measurements presented are uncorrected for the
wavelength dependence of photomultiplier output but include correction
for the contributions of GdnHCl and/or buffer at the appropriate
wavelengths.

2.2.4.3. Changes in circular dichroism.

Circular dichroism spectra were recorded at 20°C in a Jobin-Yvon
Dichrographe IV using 0.1 mm path length quartz cells. The ellipticity

of phosphoglycerate mutase was monitored between 205 and 250 nm in the presence and absence of GdnHCl. Enzyme and GdnHCl were pre-incubated for 15 min at 4°C prior to the measurement of spectra. All measurements were made using 50 mM sodium phosphate buffer, pH 7.5, and were corrected for the contribution of GdnHCl and/or buffer using appropriate blanks.

Circular dichroism spectra were normalised to zero ellipticity at 250 nm and mean residual ellipticities calculated at 5 nm intervals using the formula;

mean residual ellipticity (θ_{mrw}) = $\dfrac{mrw \times recorded\ ellipticity \times 3300}{protein\ concentration \times cell\ path\ length}$

where mrw is the mean residue weight of an amino acid (110), recorded ellipticity is measured in degrees taking into account the sensitivity setting of the instrument, protein concentration is in mg ml^{-1} and cell path length is in cm. The units of θ_{mrw} are thus deg. cm^2 dmol^{-1}.

2.2.5. Quenching of protein fluorescence using acrylamide and succinimide.

Quenching of protein fluorescence was performed using the methods described by Eftink and Ghiron (1984). Fluorescence conditions and measurement were as described in section 2.2.4.2. except that excitation was at 295 nm and emission was monitored at a fixed

wavelength of 325 nm. The acrylamide and succinimide used in these studies were recrystallised from ethyl acetate and ethanol respectively prior to their use.

Aliquots of 2.5 M stock acrylamide or succinimide were added to 1 ml of phosphoglycerate mutase in the presence and absence of 0.3 mM 2,3-bisphosphoglycerate. Serial additions were made to a final concentration of 0.5 M quencher. Protein fluorescence emission was determined after each addition and recorded values were corrected for protein dilution and the "inner filter effect" described by Ward (1985).

The correction factor (c) for the inner filter effect was calculated as;

$$c = \frac{A_t}{A_o} \cdot \frac{(1-10^{-A_o})}{(1-10^{-A_t})}$$

where A_o is the absorbance of the protein at 295 nm corrected for dilution and A_t is the combined absorbance of the protein and quencher at 295 nm. Initial studies indicated that the absorbance of protein and quencher at 295 nm were linearly related to concentration and therefore absorbance values were extrapolated for intermediate concentrations produced during the serial addition of quencher.

Corrected fluorescence quenching data were expressed in the form of Stern-Volmer plots as the quencher concentration plotted against the ratio of the initial fluorescence emission of the quenched fluorescence (F_o/F). The slopes of these plots give K_A and K_S values for acrylamide and succinimide respectively.

Succinimide is a less effective quencher of protein fluorescence since its physical size in relation to acrylamide (approximately 20% larger; Edward, 1970) reduces its ability to penetrate a protein's matrix and quench buried aromatic residues. In addition, succinimide quenching is relatively inefficient in aprotic solvents (Eftink and Ghiron, 1984) and therefore deeply buried aromatic residues may present a microenvironment in which quenching is reduced. The ratio of quenching efficiencies (K_S/K_A) determined from the Stern-Volmer plots is therefore regarded as an indicator of the relative exposure of fluorophores in a protein. A higher K_S/K_A ratio indicates greater solvent exposure of fluorophores.

Eftink and Ghiron (1984) examined a range of multi-tryptophan containing proteins and reported K_S/K_A values of between 0.1 for aldolase and 0.64 for lysozyme. The quenching of fluorescence in these proteins was re-examined here as a suitable control to the studies on phosphoglycerate mutases.

2.2.6. Renaturation of bakers yeast, rabbit muscle and *S.pombe* phosphoglycerate mutases following denaturation in GdnHCl.

Renaturation of phosphoglycerate mutases following denaturation in GdnHCl was performed essentially as described by Hermann *et al.* (1983). Enzymes were denatured at 4°C in 50 mM sodium phosphate buffer, pH 7.5, containing 1 mM dithiothretiol (DTT). Denaturing conditions were 15 min in 4 M GdnHCl for the bakers yeast and rabbit muscle enzymes and 40 min in 2 M GdnHCl for the *S.pombe* enzyme. Renaturation at 20°C was initiated by rapid dilution of the denatured enzyme with 50 mM sodium phosphate buffer, pH 7.5, containing 1 mM DTT. Rapid conversion from denaturing to renaturing conditions (obtained by dilution) is known to be important in limiting incorrect aggregate formation during refolding (Zettlmeissl *et al.*, 1979). In these studies the dilution factors employed were 40 fold for the bakers yeast and rabbit muscle enzymes and 10 fold in the case of the *S.pombe* enzyme. The residual concentrations of GdnHCl during renaturation were therefore 0.1 M and 0.2 M respectively.

At intervals after the initiation of renaturation samples of enzyme were withdrawn and diluted to a standard concentration of 10 μg ml^{-1} with 50 mM sodium phosphate buffer, pH 7.5, in the case of untreated samples, or into buffer containing proteinase at a final concentration of 5 or 20 μg ml^{-1}. Proteolysis was therefore performed at 1:0.5 or 1:2 w/w (phosphoglycerate mutase: proteinase) respectively. After incubation for 1 min at 20°C, a suitable aliquot was removed and

assayed by the continuous enolase coupled system. As a separate control to these experiments, native enzyme, at the appropriate renaturing concentration and in the presence of the residual concentration of GdnHCl, was sampled and treated in the same manner as the renaturing enzyme (i.e. untreated and proteinase treatments). In all cases the activity of the native enzyme was unaffected by the proteinase treatment and/or the presence of the residual concentration of GdnHCl.

The resistance of the bakers yeast and rabbit muscle phosphoglycerate mutase subunits to proteolysis during the renaturation of these enzymes was examined using SDS-PAGE. Following the 1 min proteinase "pulse" in the scheme described above, proteinases were removed and/or inhibited using the methods described in section 2.2.7. Samples were then prepared for SDS-PAGE analysis as described in section 2.2.1.1. Control samples of native enzyme in the presence of the residual concentration of GdnHCl were processed using identical procedures. In all cases there was no evidence of any proteolytic degradation of the native enzyme subunit in comparison with untreated controls.

2.2.7. Removal and inhibition of proteinases in samples for SDS-PAGE analysis.

2.2.7.1. Removal of trypsin and chymotrypsin.

Trypsin and chymotrypsin were removed from samples for SDS-PAGE analysis as the M_r values of their constituent chains would complicate

the banding pattern resulting from proteolysis of the bakers yeast and rabbit muscle enzymes.

Turkey egg white proteinase-inhibitor protein was coupled to Sepharose 4B using the cynogen bromide activation procedure of March *et al.* (1974) by Dr.N.C.Price. The amount of inhibitor bound to the gel was determined as 2.0 mg ml^{-1} of packed gel. Following incubation of the renaturing enzyme with trypsin or chymotrypsin for 1 min, samples were mixed with the immobilized inhibitor in a ratio 12:1 v/v (sample: inhibitor) and centrifuged at 1,000 g for 1 min to pellet inhibitor and bound proteinase. The supernatant was retained and processed for SDS-PAGE analysis as described in section 2.2.1.1. Control experiments using synthetic peptide substrates to assay trypsin and chymotrypsin showed that at least 97% of proteinase activity was removed from samples using this procedure.

2.2.7.2. Inhibition of thermolysin.

Thermolysin was inhibited in samples for SDS-PAGE analysis by the addition of EDTA to a final concentration of 2 mM. EDTA rapidly chelates Zn^{2+} ions essential for the activity of thermolysin and is thus an effective inhibitor (Girg *et al.*, 1981). Samples were processed for SDS-PAGE analysis as described in section 2.2.1.1. It was not considered necessary to remove thermolysin from such samples since with a Mr of 36,000 this monomeric protein would not interfere

57

with the fragment pattern resulting from the proteolysis of the bakers
yeast and rabbit muscle enzymes (subunit Mr 28,000).

2.2.7.3. Sample preparation for SDS-PAGE.

In addition to the removal or inhibition of the proteinases by the
methods described above, the process of sample preparation for SDS-
PAGE analysis (i.e. rapid boiling in the presence of SDS and 2-
mercaptoethanol) is an effective method of inhibiting proteinase
activity (Price and Stevens, 1982). This process was performed
immediately after the procedures described above to inhibit any
residual proteinase activity.

CHAPTER THREE : DENATURATION

AND REFOLDING OF BAKERS YEAST

PHOSPHOGLYCERATE MUTASE

3.1. INTRODUCTION.

Phosphoglycerate mutase from bakers yeast is a tetrameric enzyme of M_r 110,000 comprised of four identical subunits of M_r 28,000 (Rodwell *et al.*, 1957, Price and Jaenicke, 1982, Hermann *et al.*, 1983). The detailed three dimensional structure (Campbell *et al.*, 1974) and the complete amino acid sequence (Fothergill and Harkins, 1982) are available and the relationship of this information to the enzymes mechanism of catalytic reaction have been considered in sections 1.1.2. and 1.2.1. A detailed and reproducible purification procedure has been developed for this enzyme which seems to produce homogeneous material of high specific activity (Price and Jaenicke, 1982).

The refolding of the bakers yeast enzyme has been examined in some detail by Hermann *et al.* (1983, 1985). These authors used the glutaraldehyde cross-linking technique to examine the kinetics of subunit reassociation following denaturation in GdnHCl. The kinetics of reassociation could be successfully fitted to a model involving an equilibrium between monomers (M) and dimers (D) followed by a bimolecular association of dimers to tetramers (T) as indicated;

$$4M \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 2D \overset{k_2}{\longrightarrow} T$$
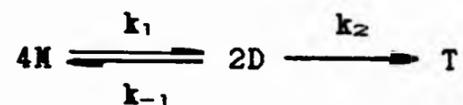
with $k_1 = 6.25 \times 10^3 \ M^{-1} \ s^{-1}$, $k_2 = 2.75 \times 10^4 \ M^{-1} \ s^{-1}$, and $k_{-1} = 6 \times 10^{-3} \ s^{-1}$ in 50 mM sodium phosphate buffer, pH 7.5, at 20°C. The

tetrameric bakers yeast enzyme does not dissociate at concentrations as low as 5 μg ml^{-1} (Price and Jaenicke, 1982) and so the 2D $\rightarrow$ T association appears to be irreversible. The reactivation of the bakers yeast enzyme was also examined under conditions where the refolding enzyme was subjected to a "proteinase pulse" with trypsin (5 min at 20 μg ml^{-1}) prior to assay. This treatment had no effect on the activity of the native enzyme. Using the proteinase pulse procedure the reactivation kinetics of the enzyme concurred with the appearance of the tetrameric species of reassociation. However, omission of the trypsin pulse resulted in a more rapid regain of activity than regain of tetrameric structure. It therefore appeared that folding intermediates of the bakers yeast enzyme possessed some catalytic activity which was sensitive to trypsin. Analysis of the reactivation kinetics in the absence of trypsin indicated that they could be fitted to the kinetic model of reassociation where the monomers and dimers of refolding were assumed to exhibit 35% of the native enzymes activity (Hermann et al., 1983, 1985). The presence of substrates (3-phosphoglycerate and 2,3-bisphosphoglycerate) during refolding increased the rate constants of reassociation, in particular shifting the equilibrium between monomer and dimer towards the dimer. The kinetics of reactivation were also affected and could only be successfully fitted if the dimeric species were assumed to retain their 35% partial activity in the presence of trypsin (Hermann et al., 1985).

Structural changes occurring during the refolding of the bakers yeast enzyme have been examined using circular dichroism and fluorescence (Hermann *et al.*, 1983). The rapid formation of the folded monomers was characterised by the appearance of some 90% of native circular dichroism with the remaining changes occurring in a slower reaction of half time approximately 3 min. Changes in fluorescence were more complex with only 20% of native fluorescence being recovered in the folded monomer. The full native fluorescence was regained in parallel with the appearance of dimeric and tetrameric species. The difference in fluorescence between the monomeric and dimeric/tetrameric species could be related to changes in the environment of aromatic residues occurring during subunit association.

The work of Hermann *et al.* (1983, 1985) provides evidence for the formation of a molten globule state (section 1.5.) during the refolding of the bakers yeast enzyme. The partial activity of the monomeric and dimeric species is sensitive to a trypsin treatment which does not affect the native or refolded tetramer. The structure of these intermediates must therefore be more "open" allowing proteolysis at normally inaccessible sites. Addition of substrates confers resistance to trypsin on the dimeric species, possibly through a process of structural tightening. Spectral analysis of the monomeric and dimeric intermediates also shows that these species do not possess the complete native structure.

As with other oligomeric enzymes such as lactate dehydrogenase the rate limiting reactions during the refolding of the bakers yeast enzyme are the concentration dependent association steps which follow the rapid formation of a folded monomer (section 1.7.3.). However, unlike lactate dehydrogenase, the bakers yeast enzyme does not seem to be susceptible to the formation of wrong aggregates at the refolding concentrations studied. Hermann *et al.* (1983, 1985) showed almost complete reactivation at concentrations of 40 μg ml^{-1}. Incomplete reactivation at lower concentrations was related to the instability of refolding intermediates over the long periods required for association rather than the formation of aggregates.

In order to extend the studies reviewed above the denaturation and refolding of the bakers yeast enzyme were examined in more detail. The denaturation by GdnHCl was monitored as loss of catalytic activity and through changes in protein fluorescence and circular dichroism. Reactivation of the enzyme during refolding was examined in the presence and absence of a proteinase pulse prior to assay. Proteinases used in these studies were selected from preliminary inactivation experiments on the native enzyme. Through the use of a number of different proteinases during the pulse it was hoped that compact proteinase-resistant fragments of structure might be isolated at various stages during refolding. These fragments might represent compact folding domains similar to those generated by thermolysin treatment of lactate dehydrogenase during refolding (section 1.7.3.). Isolation of these fragments might allow the study of reassociation

reactions to reform monomeric and dimeric intermediates of renaturation. In addition, even limited sequence analysis of these fragments would allow their location, size and structure within the native enzyme to be determined. Such information could therefore indicate specific interactions between polypeptide sequences during the refolding of the enzyme.

3.2. METHODS.

3.2.1. Isolation of phosphoglycerate mutase from *S. cervisae.*

Phosphoglycerate mutase was isolated from fresh bakers yeast essentially by Price and Jaenicke's (1982) modification of the method of de la Morena *et al.* (1968). Unless otherwise stated all operations were performed at 4°C and a standard 50 mM sodium phosphate buffer, pH 7.5, was used throughout.

Fresh bakers yeast was lysed by mixing with 1 M NH_4OH (270 ml 1 M NH_4OH/455 g wet weight yeast) and stirring slowly at room temperature for 18-20 h. To the above quantity of lysate was added 250 ml distilled water, 180 g $(NH_4)_2SO_4$ and 16 ml 0.5 M Na_4EDTA. After these salts had dissolved the extract was heated to 70 ± 1°C for 5 min with continued stirring and subsequently cooled on ice to < 30°C. Denatured protein and cell debris were precipitated by centrifugation at 22,000 g for 20 min and discarded. To each 100 ml of supernatant fluid (Fraction I) was added 30 g of $(NH_4)_2SO_4$. After stirring for 30 min Fraction I was centrifuged at 27,000 g for 20 min and the supernatant discarded. The precipitate was re-dissolved in 100 ml of buffer to give Fraction II. Fraction II was cooled in a dry ice/ethanol bath to -20°C and rapidly mixed with an equal volume of acetone cooled to -20°C. After standing for a few minutes the bulk of the supernatant was discarded and the remaining mixture centrifuged at 27,000g for 20 min. The resultant supernatant was discarded and the precipitate

tho roughly re-suspended in 40 ml of buffer using a Potter homogeniser. After centrifuging the re-suspended material at 27,000g for 20 min the supernatant containing re-extracted protein was retained. The precipitate was re-suspended in a further 40 ml of buffer and a supernatant again recovered by centrifugation at 27,000g for 20 min. When pooled, the two re-extractions yielded Fraction III which was dialysed against 4 litres of buffer overnight.

To dialysed Fraction III solid $(NH_4)_2SO_4$ was added to produce 58% saturation. After stirring for 30 min and centrifugation at 27,000g for 20 min the resultant supernatant was retained. Solid $(NH_4)_2SO_4$ was added to produce 70% saturation and after stirring and centrifugation the protein precipitate was retained. This precipitate was then "re-extracted" into a series of 6 ml aliquots of decreasing $(NH_4)_2SO_4$ saturations. The solutions employed were of 66.7, 63.3, 60, 56.7, 53.3, and 50% saturation yielding fractions A-F respectively. The 66.7% solution was added to the retained precipitate, mixed and allowed to stand for 30 min. Centrifugation at 30,000g for 20 min then yielded a supernatant (Fraction A) and a precipitate. The next lower percentage saturation solution was added to this precipitate and the process repeated. Fractions A-F were assayed for activity and protein and the fraction containing the greatest number of units and/or the highest specific activity was dialysed against 10 mM Tris-HCl buffer, pH 8.0, overnight.

Two procedures were employed in the further purification of the re-extracted enzyme preparation. In the first procedure the dialysed fraction was applied to a DEAE-cellulose column (12 cm x 3.8 cm²) equilibrated against 10 mM Tris-HCl buffer, pH 8.0. After washing with this buffer to remove unbound protein, phosphoglycerate mutase was eluted using a linear gradient formed between 10 mM Tris-HCl buffer, pH 8.0, and the same buffer containing 0.2 M NaCl. Phosphoglycerate mutase activity emerged as a single peak and fractions containing more than 10% of the units applied were pooled. The enzyme was concentrated by (NH₄)₂SO₄ precipitation to 95% saturation. The resultant precipitate was redissolved in a small volume of sodium phosphate buffer and dialysed against the same buffer overnight. The dialysed enzyme was then applied to a Sephacryl S-300 column and the activity eluted with sodium phosphate buffer. Fractions were pooled and the enzyme concentrated as with the DEAE-cellulose step.

After some problems in the reproducibility of the ion exchange step a second protocol was devised using a Cibacron Blue F3GA-sepharose column. The dialysed fraction following re-extraction was applied to a Cibacron Blue F3GA-sepharose column (5 cm x 4.9 cm²) equilibrated against 10 mM Tris-HCl buffer, pH 8.0. After washing with this buffer to remove unbound protein a 30 ml wash of 1 mM AMP was applied to elute proteins requiring this cofactor (probably kinases or dehydrogenases). After another 30 ml wash with buffer a 30 ml pulse of 1 mM 2,3-bisphosphoglycerate was applied to elute bound phospho-glycerate mutase. The AMP and 2,3-bisphosphoglycerate solutions were

prepared in 10 mM Tris-HCl buffer, pH 8.0, and their pH re-adjusted to 8.0 with dilute HCl. Phosphoglycerate mutase activity emerged as a single peak and fractions containing more than 10% of the units applied were pooled and the enzyme concentrated as with the DEAE-cellulose step.

The phosphoglycerate mutase prepared by both methods was stored at 4°C in 50 mM sodium phosphate buffer, pH 7.5, to which solid $(NH_4)_2SO_4$ was added to 95% saturation. Prior to use the enzyme preparation was dialysed against ≥ 500 volumes of appropriate buffer.

3.2.2. Denaturation of bakers yeast phosphoglycerate mutase by GdnHCl.

Loss of catalytic activity, changes in fluorescence and changes in circular dichroism observed in the presence of GdnHCl were monitored as described in section 2.2.4.

3.2.3. Susceptibility of native bakers yeast phosphoglycerate mutase to proteinases.

Inactivation and proteolytic degradation of the native bakers yeast enzyme was examined using six proteinases. Native enzyme, at a concentration of 400 μg ml^{-1}, was incubated in 50 mM sodium phosphate buffer, pH 7.5, at 20°C in the presence of the following proteinases; trypsin (40 μg ml^{-1}), chymotrypsin (40 μg ml^{-1}), thermolysin (40 μg ml^{-1}), elastase (20 μg ml^{-1}), subtilisin (10 μg ml^{-1}) and proteinase K

(10 μg ml^{-1}). Samples taken after up to 300 min incubation were assayed for activity and analysed for proteolytic degradation by SDS-PAGE.

Preliminary investigation with all proteinases at 40 μg ml^{-1} indicated a wide range of susceptibilities and the above concentrations were selected as a result of these initial studies such that a general graduated response was achieved over the incubation period.

3.2.4. Renaturation of bakers yeast phosphoglycerate mutase.

Renaturation of the bakers yeast enzyme was examined as described in section 2.2.6. Reactivation was assessed in the presence and absence of proteinases. Resistance of the enzyme subunit structure during renaturation to the proteinases trypsin, chymotrypsin and thermolysin was assessed using SDS-PAGE. These proteinases were selected on the basis of their effects on the activity of the native enzyme determined as described in section 3.2.3.

3.3. RESULTS AND DISCUSSION.

3.3.1. Isolation and characterisation of bakers yeast phosphoglycerate mutase.

Details of a typical purification of phosphoglycerate mutase from 250 g of fresh bakers yeast by the methods described in section 3.2.1. are given in Table 2. The specific activity of the purified enzyme was generally 7-8000 units mg^{-1} equating to 805-920 μmole 3-phosphoglycerate metabolised min^{-1} mg $protein^{-1}$ (section 2.2.3.1). This value is slightly lower than that reported by Price and Jaenicke (1982). However, analysis of the enzyme preparation by SDS-PAGE indicated > 90% homogeneity of staining with Cooma. sse blue R250. The Mr of the enzyme subunit was 28,000 ± 1400 and that of the only contaminant protein 30,000 ± 1500 (Plate 1). Similar results in terms of specific activity and SDS-PAGE profile were obtained following either the DEAE-cellulose, Sephacryl S-300 stages or the Cibacron Blue F3GA-sepharose affinity step in the purification protocol (section 3.2.1.). The co-purification of the contaminant protein under native conditions following Sephacryl S-300 gel filtration indicates that this protein may be oligomeric since its Mr is similar to that reported for native phosphoglycerate mutase (110,000; Price and Jaenicke, 1982). Further extensive investigation of purification conditions, including the use of additional NAD^+, ATP, and NaCl washes during the Cibacron Blue affinity stage, did not remove or reduce the level of this protein from the enzyme preparation. In view of the low

FRACTION	UNITS	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (units mg^{-1})	YIELD (%)	PURIFICATION (fold)
I[*]	1,314,500	2115	622	100	1
II	897,000	1339	670	68	1.1
III	634,300	774	820	48	1.3
A[†]	300	2.7	110		
B	11,400	52	220		
C	48,000	60	800		
D	100,500	87	1155		
E	339,500	191	1780	26	2.9
F	85,500	126	680		
POOLED BLUE[‡] SEPHAROSE FRACTIONS	164,500	21	7830	12.5	12.6

Table 2.   Purification of phosphoglycerate mutase from 250 g of fresh baker's yeast.

* Fraction I represents partially purified material following heat treatment in the presence of $(NH_4)_2SO_4$ and Na_4EDTA.

† Fractions A-F were re-extracted from a 58-70% $(NH_4)_2SO_4$ precipitate of fraction III.

‡ Following fractionation of fraction E by Cibacron blue F3GA sepharose affinity chromatography.

1 2 3 4

66 —

45 —

36 —

29 —
24 —

20.1 —

14.2 —

(Mr x10^{-3})
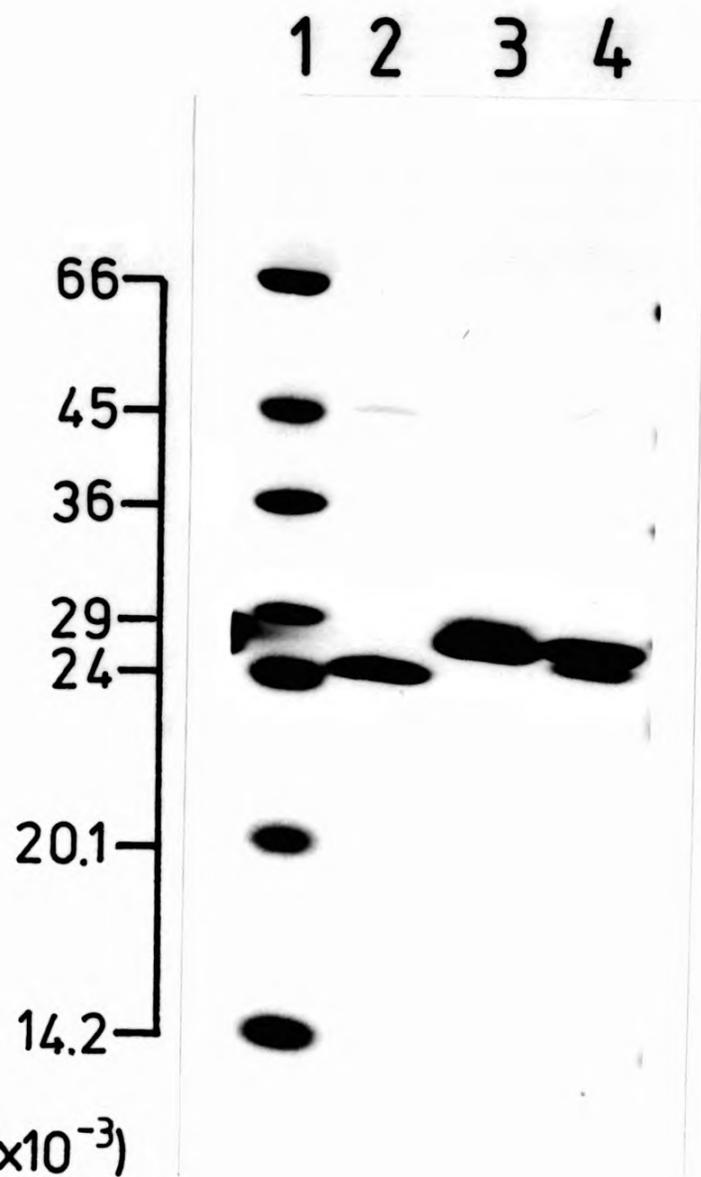
Plate 1.   SDS-PAGE analysis of purified phosphoglycerate mutases.

5 μg of purified phosphoglycerate mutase from: lane 2, S. pombe;
lane 3, baker's yeast;   lane 4, rabbit muscle were analysed by
SDS-PAGE.   Lane 1 contains 5 μg of the Dalton Mark VII Mr marker
set and the Mr values of its constituent proteins are as indicated.
The gel was stained with Coomassie blue R-250 (section 2.2.1.1.).

level of the 30,000 Mr protein (< 10%) it was considered an acceptable contaminant to the phosphoglycerate mutase enzyme preparation.

The Mr of the native enzyme was determined by gel filtration using Sephacryl S-300 as 110,000 ± 11,000. Details of the operation and calibration of this system are described in section 2.2.1.2. This value, and that for the subunit Mr following SDS-PAGE, are comparable with those reported previously (Edelhoch *et al.*, 1957, Ray and Peck, 1972, Price and Jaenicke, 1982, Hermann *et al.*, 1983) and confirm that the phosphoglycerate mutase from bakers yeast is a tetramer of Mr approximately 110,000.

The Km of the enzyme in 30 mM Tris-HCl buffer, pH 7.0, and in the presence of 10 mM grade 1 3-phosphoglycerate for the cofactor 2,3-bisphosphoglycerate was determined at 30°C as 14 ± 1 μM. This value lies within the range (0.8-300 μM) reported for the enzyme under varying conditions of buffer and substrate concentration (Grisolia and Carreras, 1975). In the absence of added 2,3-bisphosphoglycerate the enzyme still exhibited approximately 2% of maximal activity. This level is explicable in terms of the trace amounts of co-factor present in the grade 1 3-phosphoglycerate used (Boehringer quote the contamination as < 0.005% which equates to < 0.5 μM from 10 mM 3-phosphoglycerate). This residual concentration of co-factor was included in the determination and calculation of the Km for the bakers yeast enzyme and other mutases examined.

3.3.2. Denaturation of bakers yeast phosphoglycerate mutase by GdnHCl.

3.3.2.1. Loss of catalytic activity.

The activity of the bakers yeast enzyme in the presence of GdnHCl is shown in Fig. 12. Evidently, there is a progressive decline in activity over the concentration range of approximately 0-1.5 M GdnHCl with 50% inactivation occurring at 0.65 M.

3.3.2.2. Changes in fluorescence.

The fluorescence emission spectra of the bakers yeast enzyme in the presence and absence of 4 M GdnHCl are shown in Fig. 13. The λ max of the native enzyme is 328 nm. In the presence of 4 M GdnHCl there is a strong red shift to a λ max of 348 nm with the fluorescence intensity at 328 nm reduced to one third. There was no difference in the spectrum of the bakers yeast enzyme in the presence of 2 or 4 M GdnHCl indicating that the GdnHCl induced changes in fluorescence were complete following incubation at the lower concentration.

The shape and λ max of the fluorescence spectra in Fig. 13 are in close agreement with those reported by Hermann *et al*. (1983) for the bakers yeast enzyme in the presence and absence of 4 M GdnHCl. The λ max of the spectrum in the presence of 4M GdnHCl is characteristic of highly solvent exposed aromatic residues such as occur in denatured proteins (Teipel and Koshland, 1971). Hermann *et al*. (1983) have
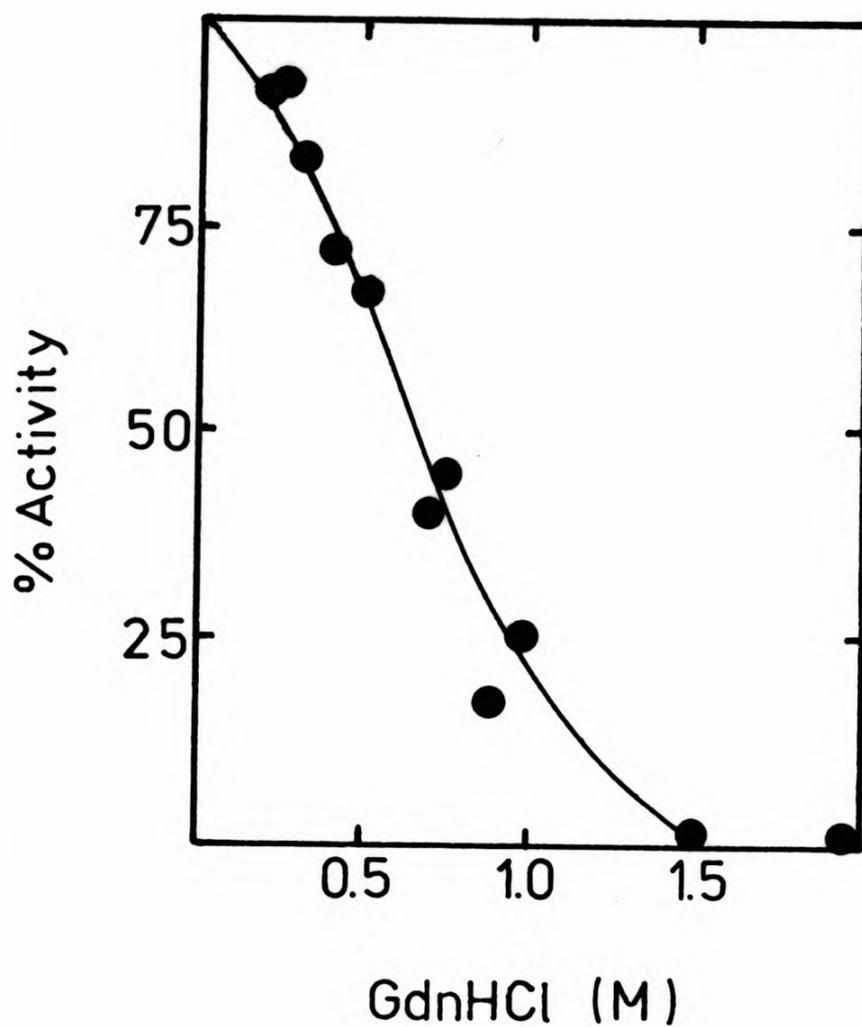
Fig 12 Activity of baker's yeast phosphoglycerate mutase in the presence of GdnHCl.

Enzyme (1 unit) was added to substrate (10 mM 3-phosphoglycerate), cofactor (0.3 mM 2,3-bisphosphoglycerate) and the indicated concentration of GdnHCl in a final volume of 1 ml.   2-phosphoglycerate formation was measured over a 3 min period and activity expressed relative to a control containing no GdnHCl (sections 2.2.4.1. and 5.3.3.1.).
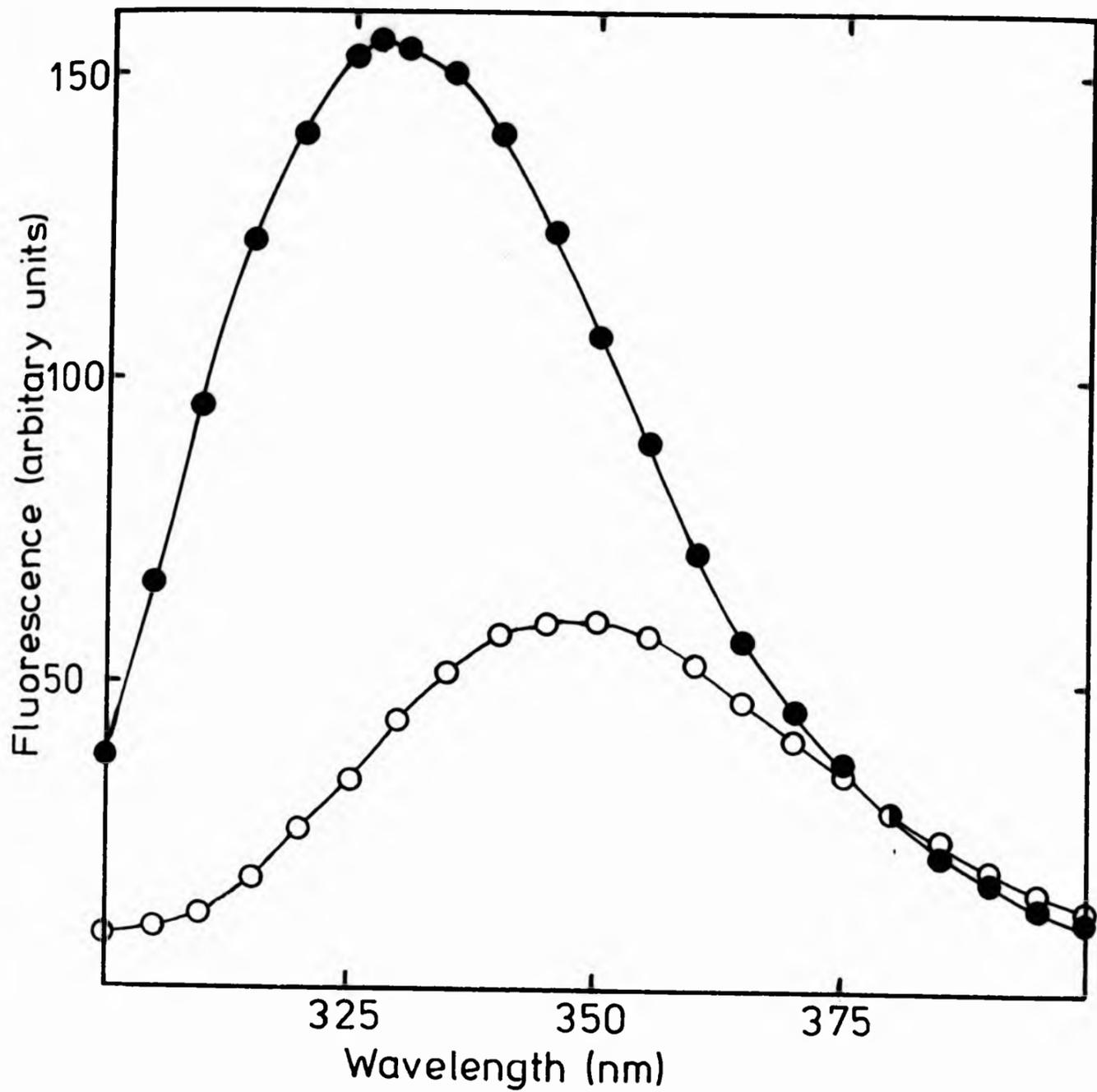
Figure 13.   Fluorescence spectra of baker's yeast phosphoglycerate
mutase in the presence and absence of 4 M GdnHCl.

Baker's yeast phosphoglycerate mutase (10 µg ml^{-1}) at 20°C.   The
fluorescence excitation was at 290 nm and the emission monitored
between 300 and 400 nm.   Instrument   sensitivity was X 10.
●   , enzyme in 50 mM sodium phosphate buffer, pH 7.5;
O   , enzyme in the presence of 4 M GdnHCl.

reported that the bakers yeast enzyme is completely dissociated into subunits in the presence of 4 M GdnHCl. The fluorescence spectra of the enzyme under these conditions (Fig. 13 and Hermann *et al.*, 1983) thus indicate that the dissociation of the subunits is accompanied by greatly increased exposure of aromatic residues presumably as a result of subunit dissociation and unfolding.

The fluorescence emission of the bakers yeast enzyme at 325 nm as a function of GdnHCl at intermediate concentrations between 0 and 2 M is shown in Fig. 14. There is an initial small increase in fluorescence intensity at concentrations up to 1 M GdnHCl followed by a rapid decline between 1 and 1.2 M to a value approximately one quarter that of the native enzyme. The mid-point of this transition is 1.1 M GdnHCl. There was no further change in fluorescence intensity when the GdnHCl concentration was increased to 2 or 4 M. Changes in the λ max of the enzyme occurred at similar concentrations to the reduction in intensity with the red shift from 328 to 348 nm produced between 1 and 1.5 M GdnHCl. The reasons for the initial increase in fluorescence intensity, which is accompanied by a small shift of λ max from 328 to 330nm, are unclear. However, this increase must reflect some change in the environment of the enzyme's aromatic amino acid residues as a result of structural perturbation. Acidic groups are known to quench tryptophan fluorescence and it is thus possible that removal of such a group from the environment of a tryptophan residue could produce an increase in fluorescence intensity.
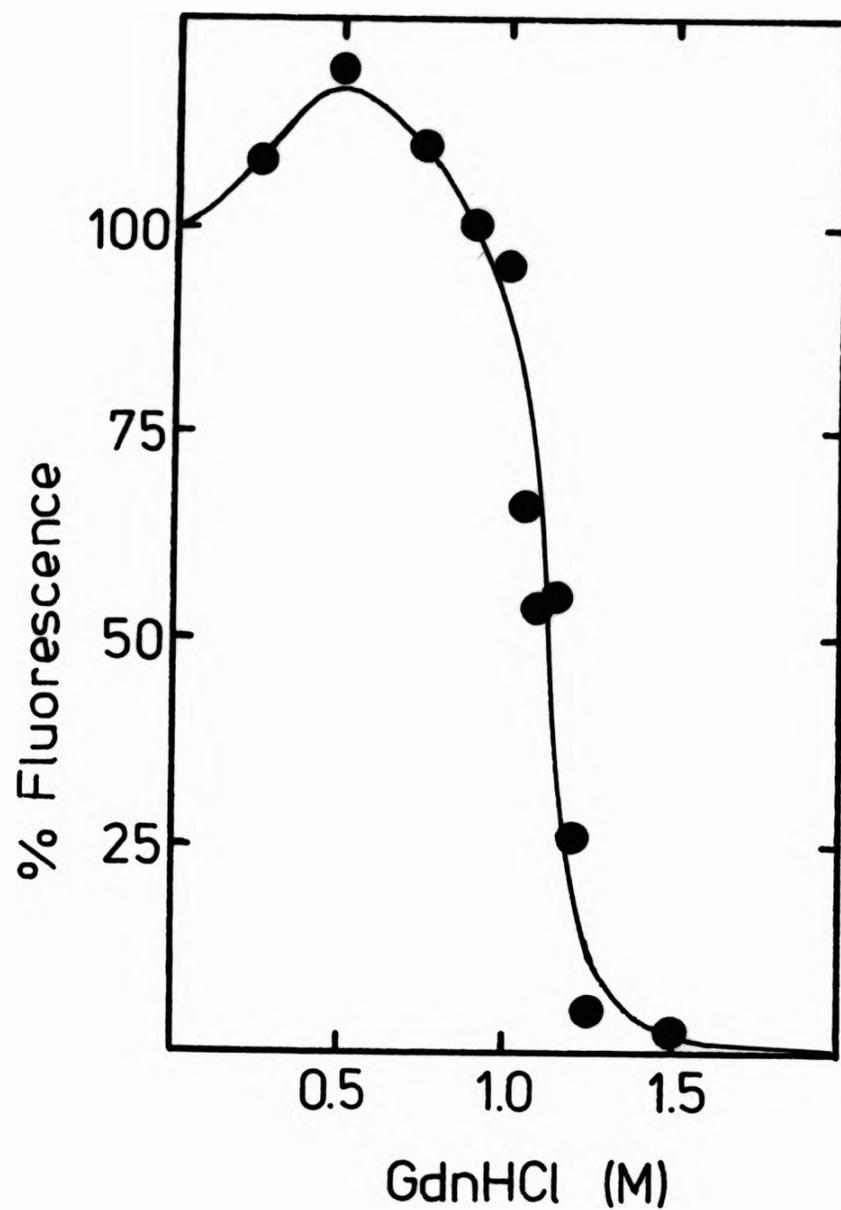
Figure 14.   Fluorescence of baker's yeast phosphoglycerate mutase in
the presence of GdnHCl.

Fluorescence was recorded in 50 mM sodium phosphate buffer pH 7.5, at
20°C.   The excitation and emission wavelengths were 290 and 325 nm
respectively and the change in fluorescence emission is expressed
relative to the total change between 0 and 2 M GdnHCl.   Fluorescence
emission in the presence of 2 M GdnHCl was approximately one quarter
that of the native enzyme (Fig. 13).

3.3.2.3. Changes in circular dichroism.

The circular dichroism spectra of the bakers yeast enzyme in the presence and absence of 4 M GdnHCl are shown in Fig. 15. The spectrum of the native enzyme shows a negative ellipticity over the wavelength range 205-250 nm characteristic of a α-helical structure in proteins. Comparison of this spectrum with that reported by Hermann *et al.* (1983) using the same buffer and conditions indicates close agreement between the two spectra. Any difference between the values of ellipticity may be explicable in Hermann *et al.*'s use of 115 as mean residue weight compared with 110 used in the calculation of the data shown in Fig. 15.

Using the reference values for the ellipticity of pure helix at 208 and 225 nm quoted by Chen *et al.* (1974) the helical content of the native bakers yeast enzyme may be calculated as 21 ± 4%. This estimate is in close agreement with that quoted by Hermann *et al.* (1983) (a value of 20 ± 5%) and is accordance with earlier optical rotatory dispersion measurements (Sugimoto *et al.*, 1966) in indicating that the bakers yeast enzyme has a relatively low helical content. Comparison of these values with that which may be calculated from the X-ray structure of the enzyme (28.6% from the model of Campbell *et al.*, 1974) indicates that the spectral methods are consistently underestimating the percentage helical component. The reason for this discrepancy could lie in the positive contribution of β-turns to the ellipticity of proteins over the wavelength range 200-240 nm (Chang *et*
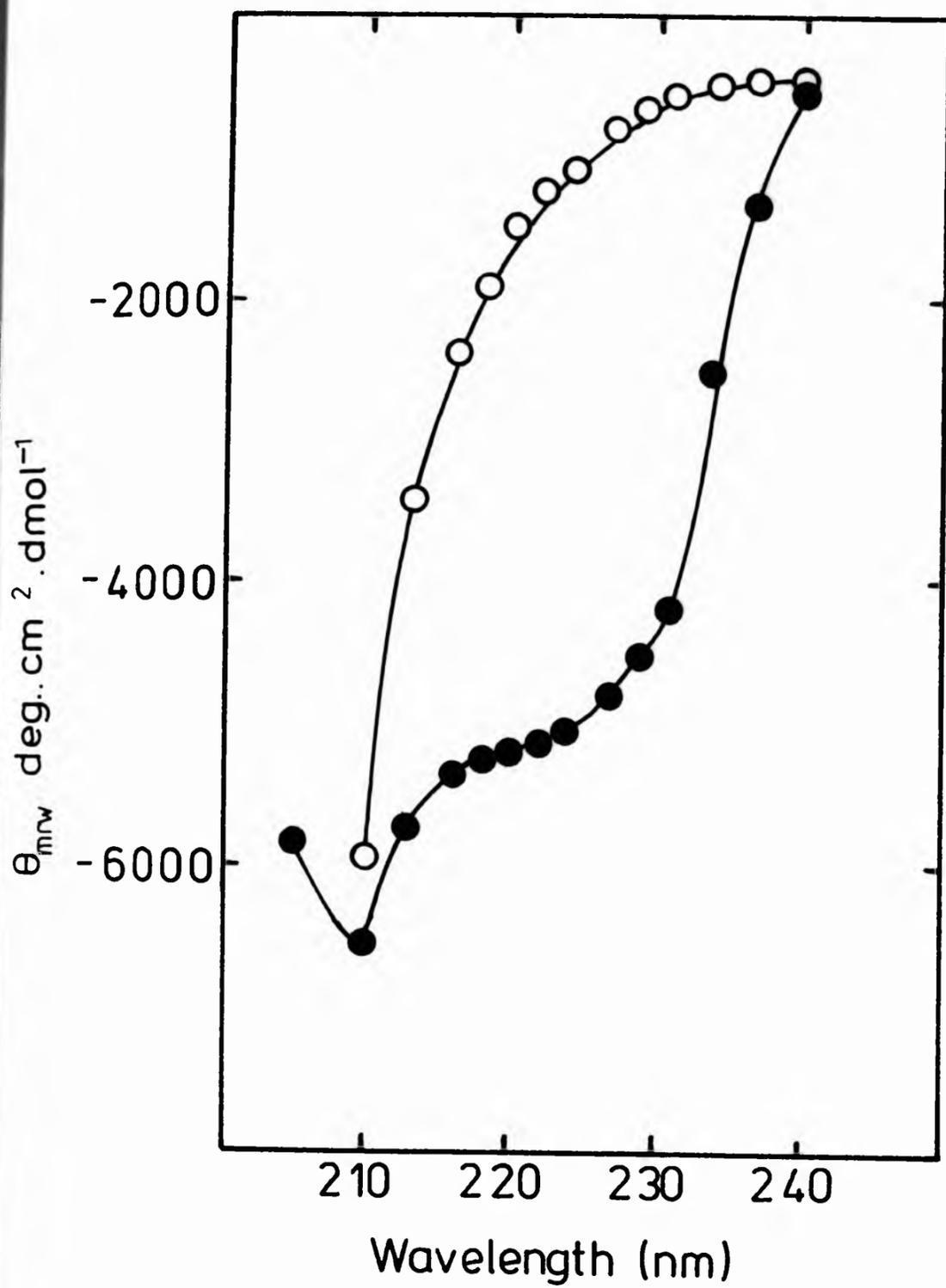
Figure 15. Circular dichroism spectra of baker's yeast phosphoglycerate mutase in the presence and absence of 4 M GdnHCl.

Enzyme in 50 mM sodium phosphate buffer, pH 7.5, at 20°C in the presence (O) and absence (●) of 4 M GdnHCl.

al., 1978). Since a circular dichroism spectrum represents the sum of the spectra of the proteins structural elements then the presence of high nu*m*bers of β-turns could reduce the observed negative ellipticity contributed by the α-helical component. In addition, any comparison between circular dichroism and X-ray structure data is based on the assumption that a proteins structure is identical in crystals and in solution.

The spectrum of the bakers yeast enzyme in the presence of 4 M GdnHCl shows a reduction in ellipticity (i.e; becoming less negative) over the wavelength range studied. This spectrum is similar to that reported by Hermann *et al.* (1983) for their enzyme in the presence of 4 M GdnHCl. Both spectra are similar to that of un-ordered or random structure in proteins (Chang *et al.*, 1978). In the presence of 2 M GdnHCl there appeared to be some helical structure remaining in the bakers yeast enzyme (spectrum not shown). Under these conditions the change in θ_{225} between 0 and 4 M GdnHCl is only 85% complete. This fact reflects the relative resistance of α-helical structure to perturbation and it is presumably the disruption of this component which is one of the final events in the denaturation of the bakers yeast enzyme.

3.3.3. Susceptibility of native bakers yeast phosphoglycerate mutase to proteinases.

The effects of trypsin, chymotrypsin, thermolysin, elastase, subtilisin, and proteinase K on the activity of the native bakers yeast enzyme are shown in Fig. 16. All six proteinases caused inactivation of the enzyme although at markedly differing rates. The native enzyme was relatively resistant to trypsin, chymotrypsin and thermolysin with ≤ 10% activity lost after 5 min incubation. In contrast, elastase produced 50% inactivation and subtilisin and proteinase K produced 65% inactivation over the same time period. In addition, the latter two proteinases were present at only one quarter the concentration of trypsin, chymotrypsin and thermolysin. Progressively greater inactivation was observed with increasing periods of incubation with very similar effects being produced by trypsin and chymotrypsin and by subtilisin and proteinase K. In the case of thermolysin and elastase the initial rapid inactivation over the first 30 min to approximately 30% of control activity was followed by much slower changes with approximately 25% activity remaining after 300 min. The reasons for the apparent biphasic effect of these proteinases on the activity of the bakers yeast enzyme are unclear. However, this data does suggest that a proportion of the enzyme activity and/or a proportion of the enzyme species is/are more resistant to the effects of elastase and thermolysin.
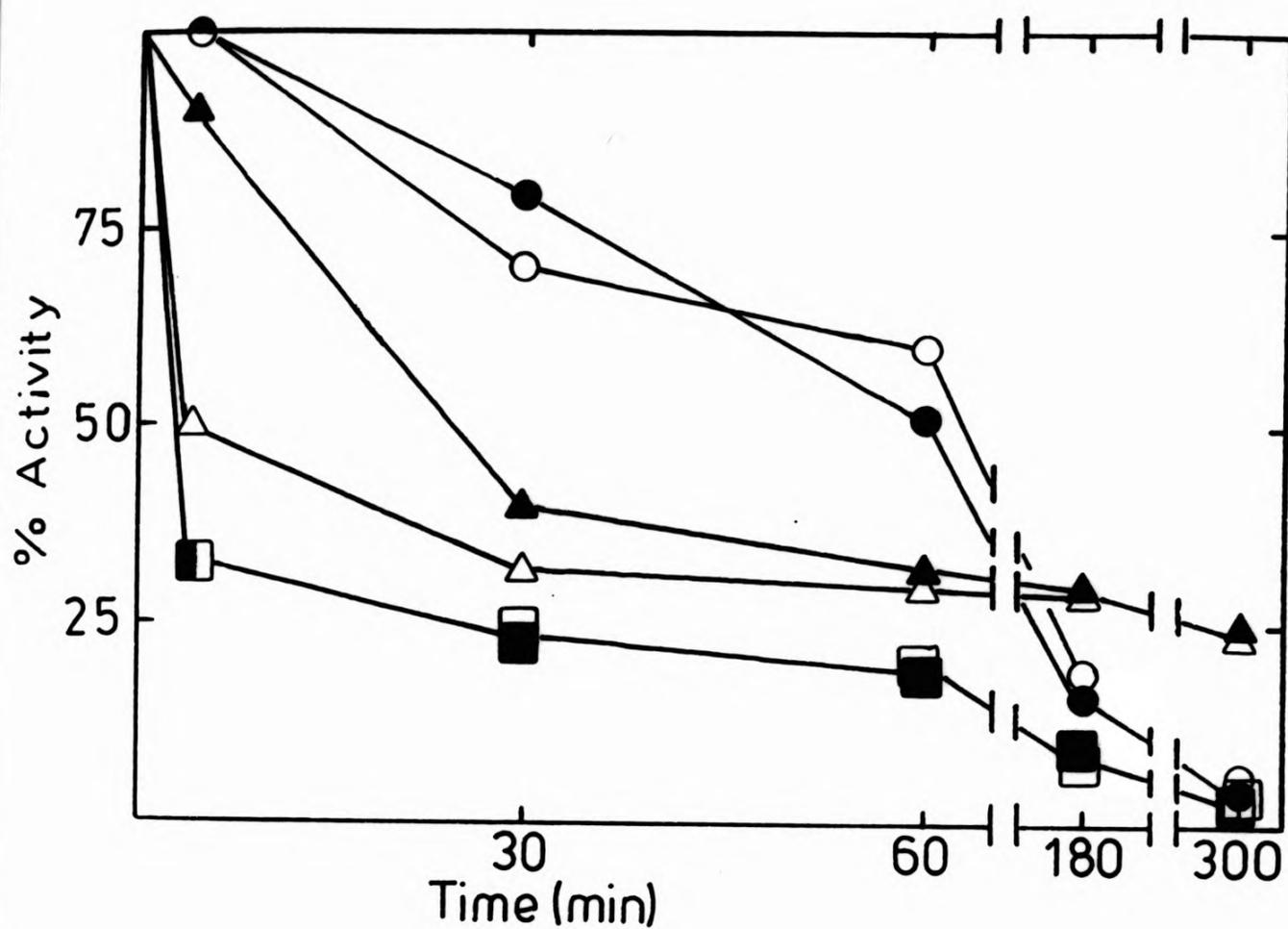
Figure 16.   Effects of proteinases on the activity of native baker's
             yeast phosphoglycerate mutase.

Native enzyme (400 µg ml^{-1}) was incubated in 50 mM sodium phosphate
buffer, pH 7.5, at 20°C in the presence of: ● , trypsin (40 µg ml^{-1});
○ , chymotrypsin (40 µg ml^{-1}); ▲ , thermolysin (40 µg ml^{-1});
△ , elastase (20 µg ml^{-1}); ■ , subtilisin (10 µg ml^{-1}) and
□ , proteinase K (10 µg ml^{-1}).

The effect of the proteinases on the structural integrity of the bakers yeast enzyme was determined using SDS-PAGE. With the proteinases trypsin, chymotrypsin, subtilisin and proteinase K the rates of loss of intact enzyme subunit were generally correlated with the losses in activity shown in Fig. 16. In contrast, treatment with thermolysin and elastase resulted in minimal apparent degradation of the enzyme subunit. However, there was evidence in these cases of conversion of the subunit to a form of slightly lower Mr concomitant with the observed inactivation of the enzyme with these proteinases. These findings could indicate that inactivation of the enzyme occurs following the cleavage of a small number of amino acids from one end of the polypeptide chain (9 amino acids would produce the observed decrease in Mr of approximately 1000). A highly suitable site for this proposed cleavage exists in the C-terminal tail of the bakers yeast enzyme (Winn *et al*., 1981).This tail is susceptible to removal by endogenous proteinases and is essential for activity (Sasaki *et al*., 1966). Furthermore, since this tail does not appear in the electron density map of the enzyme (Winn *et al*., 1981) the residues of this flexible region would be particularly exposed to proteolytic attack. The tail sequence is reported to be :-

Gly-Ala-Ala-Val-Ala-Asn-Gln-Lys-Lys-Gly-COOH

and thus contains suitable sites for cleavage by both thermolysin and elastase.

A similar pattern of inactivation without subunit degradation has been reported for rabbit muscle phosphoglycerate mutase using thermolysin (Price *et al.*, 1985a). These authors have shown that inactivation is accompanied by the loss of approximately 1000 in subunit Mr but that the native enzyme conformation, as assessed by circular dichroism and affinity ligand binding, is maintained. These observations could also be explained by the cleavage of a small tail essential for activity from one end of the rabbit muscle enzyme amino acid chain. However, without the sequence and X-ray diffraction structure of this enzyme the presence of such a tail is a matter of speculation.

3.3.4. Renaturation of bakers yeast phosphoglycerate mutase.

3.3.4.1. Reactivation in the absence of proteinases.

The reactivation of the bakers yeast enzyme at concentrations of 10, 30, and 50 μg ml^{-1} in the absence of proteinases is shown in Fig. 17. At intervals after initiation of renaturation the enzyme was diluted to a standard concentration of 10 μg ml^{-1} and incubated for 1 min at 20°C prior to assay. This process allowed the inclusion of a 1 min proteinase pulse in subsequent studies. The full lines in Fig. 17 were calculated according to the kinetic model of reassociation determined by Hermann *et al.* (1983, 1985) from their extensive examination of this process using the glutaraldehyde cross-linking technique (section 3.1.). In calculation of these reactivation profiles the monomeric and dimeric intermediates of reassociation were
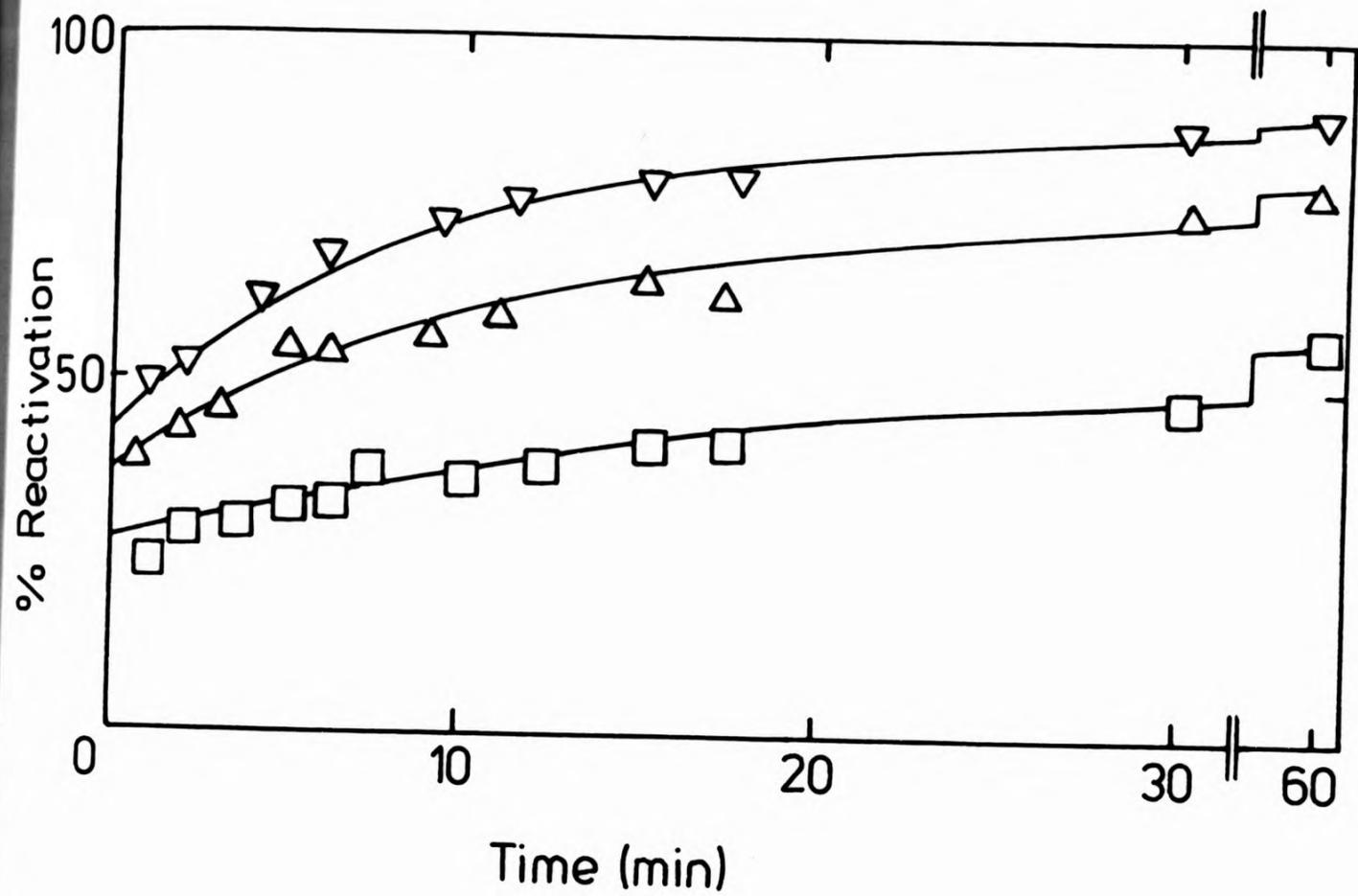
Figure 17.   Reactivation of baker's yeast phosphoglycerate mutase in
the absence of proteinases.

Baker's yeast phosphoglycerate mutase renaturing at; □ ,
10 µg ml^{-1}; △ , 30 µg ml^{-1} and ▽ , 50 µg ml^{-1} was
diluted to a standard concentration of 10 µg ml^{-1} in 50 mM sodium
phosphate buffer, pH 7.5, and incubated for 1 min at 20°C prior to
assay.

assumed to exhibit 35% of the native enzyme activity. This level of partial activity for these species has been reported by Hermann *et al*. (1983, 1985).

Clearly there is close agreement between the observed reactivation kinetics of the bakers yeast enzyme and the model of Hermann *et al*.. This confirms these authors proposal that reactivation of the bakers yeast enzyme preceeds tetramerization (i.e. the attainment of native configuration) since the monomeric and dimeric intermediates of renaturation possess 35% of the native enzymes activity. Fig. 17 also demonstrates the concentration dependence of the rate and yield of reactivation in the bakers yeast enzyme. Increasing concentration during renaturation leads to an increased rate of reactivation as would be predicted from the second order rate constants of subunit association in the model of Hermann *et al*. (1983, 1985, section 3.1.). The high levels of activity which are rapidly regained at 50 μg ml^{-1} (85% of control activity within 60 min) illustrates the reversibility of the denaturation process in the bakers yeast enzyme. Increased periods of reactivation above 60 min would be expected to yield almost complete reactivation. For example, Hermann *et al*. (1985) have reported > 95% reactivation of the bakers yeast enzyme following 24 h renaturation at 40 μg ml^{-1}.

3.3.4.2. Reactivation in the presence of proteinases.

As intermediates produced during renaturation of proteins are generally more susceptible to proteinases (Grig, 1981), trypsin, chymotrypsin and thermolysin were selected from the six proteinases examined in section 3.3.3. as having the least effect on the native enzyme during short periods of incubation (up to 5 min). A one minute pulse of these proteinases at 20°C was included prior to assay during the reactivation of the bakers yeast enzyme. The proteinases were used at 20 and 5 μg ml^{-1} with the bakers yeast enzyme at the standard concentration of 10 μg ml^{-1}.

The reactivation profiles following a 20 μg ml^{-1} pulse of proteinases are shown in Fig. 18a-c for the bakers yeast enzyme renaturing at 10, 30 and 50 μg ml^{-1} respectively. The reactivation data for the enzyme at each concentration in the absence of proteinase (i.e; the data of Fig. 17) is included for comparison. The full lines in Fig. 18a-c were again calculated from the model of Hermann *et al.* (1983, 1985) with the monomeric and dimeric intermediates of reassociation assumed to have 35% and 0% activity of the native enzyme in lines (a) and (b) respectively. Line (a) was thus calculated as for Fig. 17 whilst line (b) represents the formation of tetramer during reassociation.

Clearly Fig. 18a-c show there is a marked decrease in activity regained following the proteinase pulse (particularly at early time points during renaturation) in comparison with the reactivation in the

79

Figure 18a. Reactivation of baker's yeast phosphoglycerate mutase
at 10 µg ml^{-1} assessed following a one minute proteinase
pulse of 20 µg ml^{-1}.

Fig 18a-c. Renaturing baker's yeast phosphoglycerate mutase was
diluted to a standard concentration of 10 µg ml^{-1} at
the indicated times and incubated for 1 min at 20°C prior
to assay in the presence of the following proteinases at
20 µg ml^{-1}; ●, trypsin; ○, chymotrypsin;
□, thermolysin. The full lines were calculated
according to the kinetic model of Hermann et al. (1983,
1985) and assuming both the monomeric and dimeric inter-
mediates of reassociation to have 35% (line (a)) and 0%
(line (b)) of the native enzyme activity. The reacti-
vation data in the absence of proteinases is included at
each concentration for comparison (△).

Figure 18b.   Reactivation of baker's yeast phosphoglycerate mutase at 30 μg ml^{-1} assessed following a one minute proteinase pulse of 20 μg ml^{-1}.

Figure 18c. Reactivation of baker's yeast phosphoglycerate mutase at 50 µg ml^{-1} assessed following a one minute proteinase pulse of 20 µg ml^{-1}.

absence of proteinases. This effect relates only to renaturing activity since the activity of the native enzyme, in the presence or absence of the residual concentration of GdnHCl (0.1 M) during renaturation, was unaffected by the proteinase pulse. Therefore, the observed reduction in reactivation and the accordance of this data with line (b) indicates that the partial activity of the monomeric and dimeric intermediates of reassociation is highly sensitive to the three proteinases examined. The activity of the tetramer formed during reassociation (and the native enzyme) are not affected by the proteinases.

Hermann *et al*. (1983, 1985) have reported a similar observation using a 5 min 20 μg ml^{-1} trypsin pulse during reactivation of the bakers yeast enzyme. These authors examined the period of their trypsin pulse and detected the same effect using between 1 and 20 min. The data in Fig. 18a-c, where 1 min was used, concur with their finding at the shortest time period.

The confirmation of Hermann *et al*.'s observations and their extension to include other proteinases of differing bond specificity indicates that the partially active intermediates of reassociation must have a relatively open structure with large numbers of bonds accessible to attack. Subsequent association of the monomeric and/or dimeric intermediates in combination with structural rearrangements of subunit structure must occur such that these sites are thereby rendered less accessible. Finally, in the tetramer the association and tightening of

80

structure confer the native enzymes complete resistance to the proteinases. Circular dichroism measurements on renaturing bakers yeast phosphoglycerate mutase have shown that the folded monomer possesses approximately 85% of the ellipticity of the native enzyme (Hermann *et al.*, 1983). This species is formed within 30 s of renaturation. However, the final regain of native ellipticity occurs in a slower phase of half time approximately 3 min at a renaturing concentration of 40 μg ml^{-1}. These slower changes could represent the structural rearrangements that confer proteinase resistance.

The reactivation profiles following a 5 μg ml^{-1} proteinase pulse are shown in Fig. 19a-c. Although the effects considered above are less marked at the lower proteinase concentration there is still evidence for a reduction in activity regained relative to untreated controls. The relative speed with which the structural changes occur during renaturation may prevent all accessible sites from being attacked at the lower proteinase concentration and thereby allow some activity of monomeric and dimeric intermediates to remain.

It would be of interest to extend these proteinase pulse studies to include other proteinases of further differing bond specificities to emphasize the "openess" of the intermediate structures. It might also be possible to select a proteinase that had no effect on the activity of the intermediates of renaturation. By comparison of this proteinase's bond specificity with the amino acid sequence (Fothergill and Harkins, 1982) it might be possible to predict areas of the enzyme
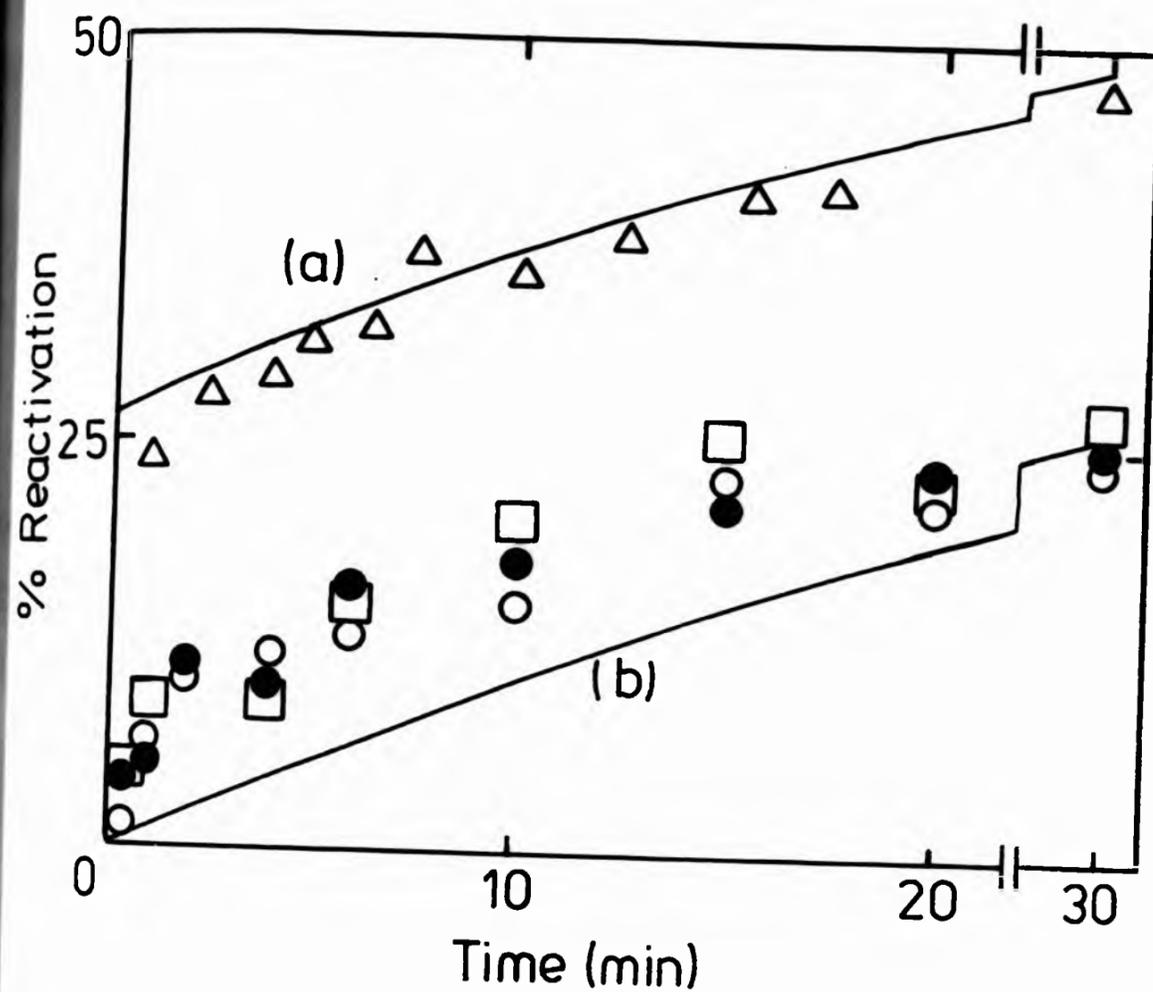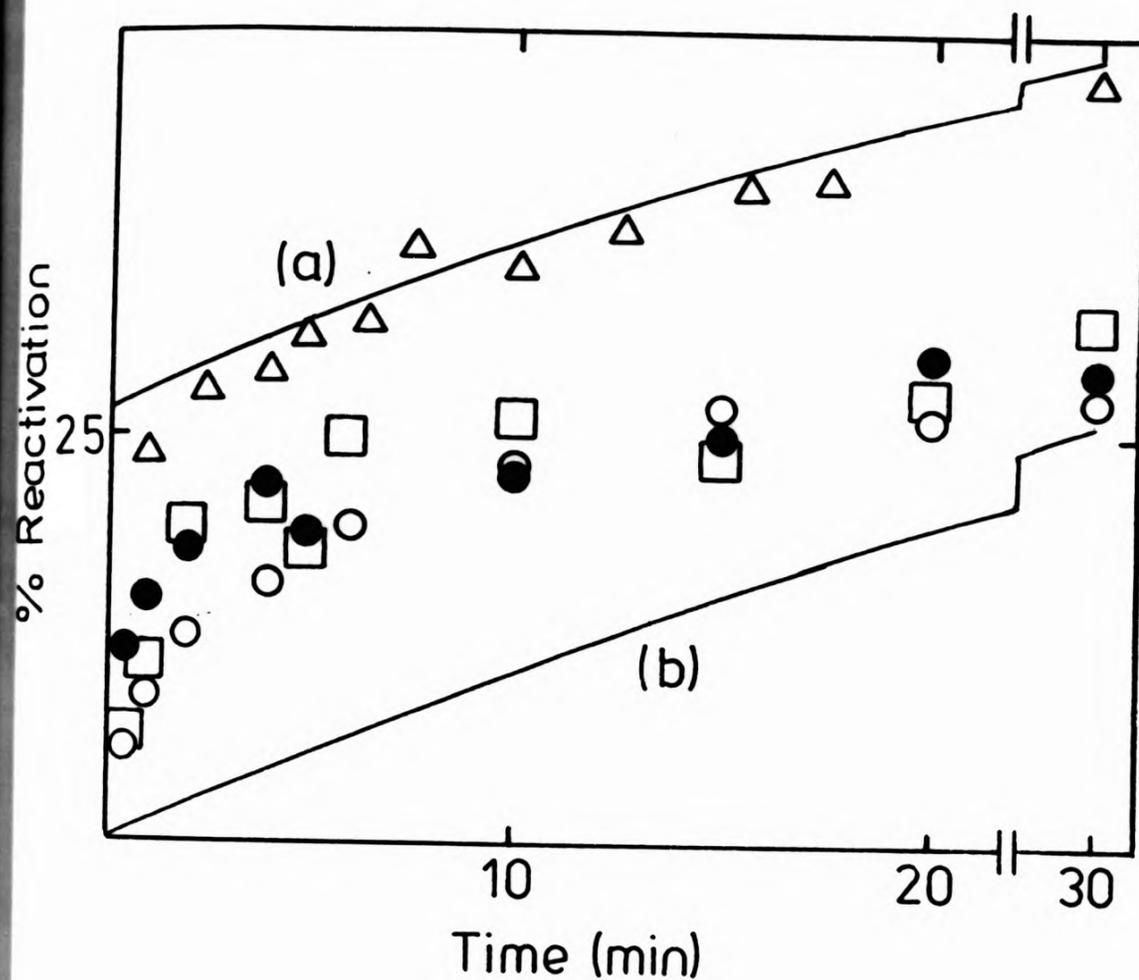
Figure 19a.   Reactivation of baker's yeast phosphoglycerate mutase
at 10 µg ml^{-1} assessed following a one minute proteinase
pulse of 5 µg ml^{-1}.

Fig 19a-c.   Renaturing baker's yeast phosphoglycerate mutase was
diluted to a standard concentration of 10 µg ml^{-1} at
the indicated times and incubated for 1 min at 20°C
prior to assay in the presence of the following proteinases
at 5 µg ml^{-1}; ● , trypsin; ○ , chymotrypsin;
□ , thermolysin.   The full lines were calculated
according to the kinetic model of Hermann et al. (1983,
1985) and assuming both the monomeric and dimeric inter-
mediates of reassociation to have 35% (line (a)) and 0%
(line (b)) of the native enzyme activity.   The reactivation
data in the absence of proteinases are included at each
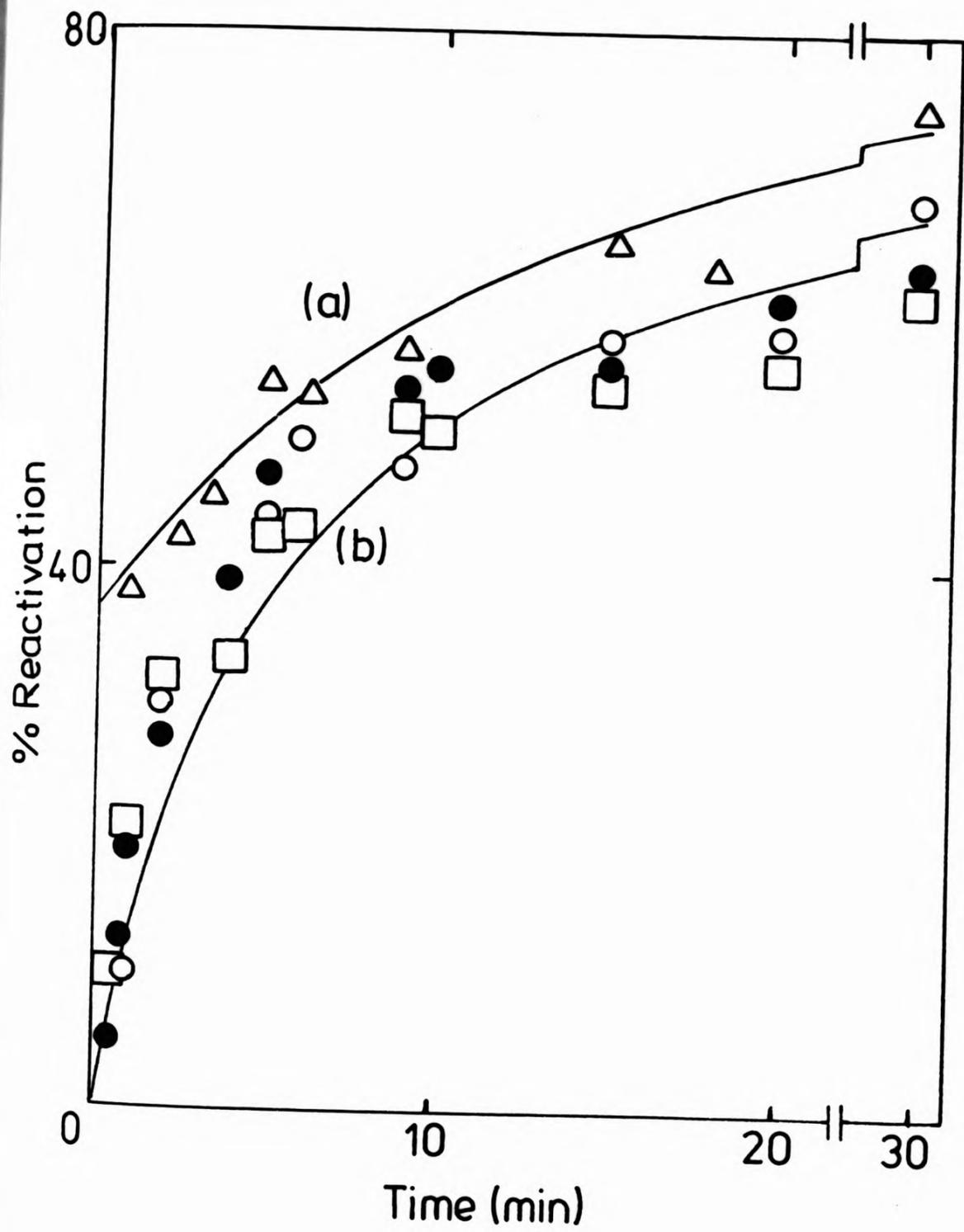concentration for comparison (△   ).
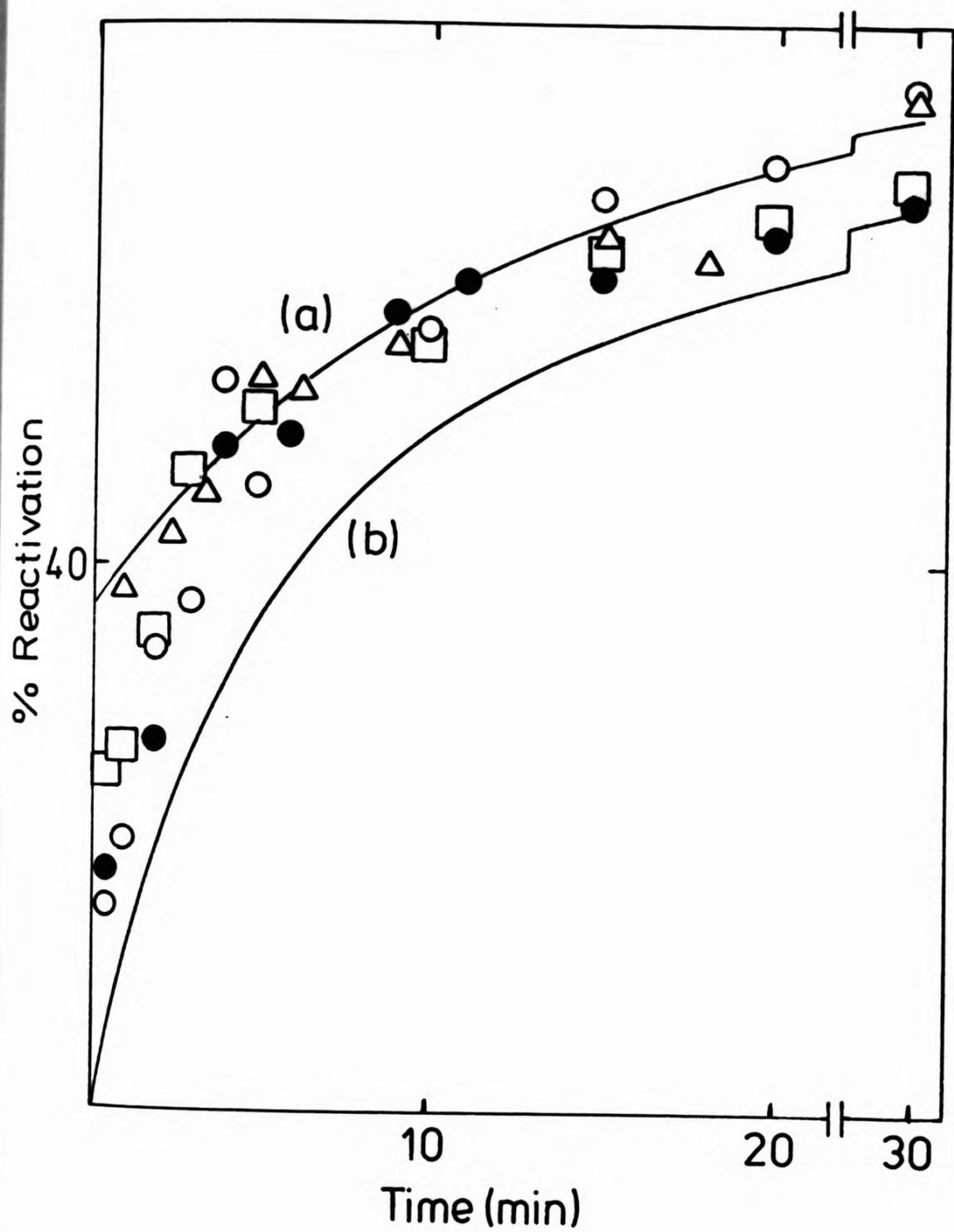
Figure 19b. Reactivation of baker's yeast phospho-glycerate mutase at 30 µg ml^{-1} assessed following a one minute proteinase pulse of 5 µg ml^{-1}.
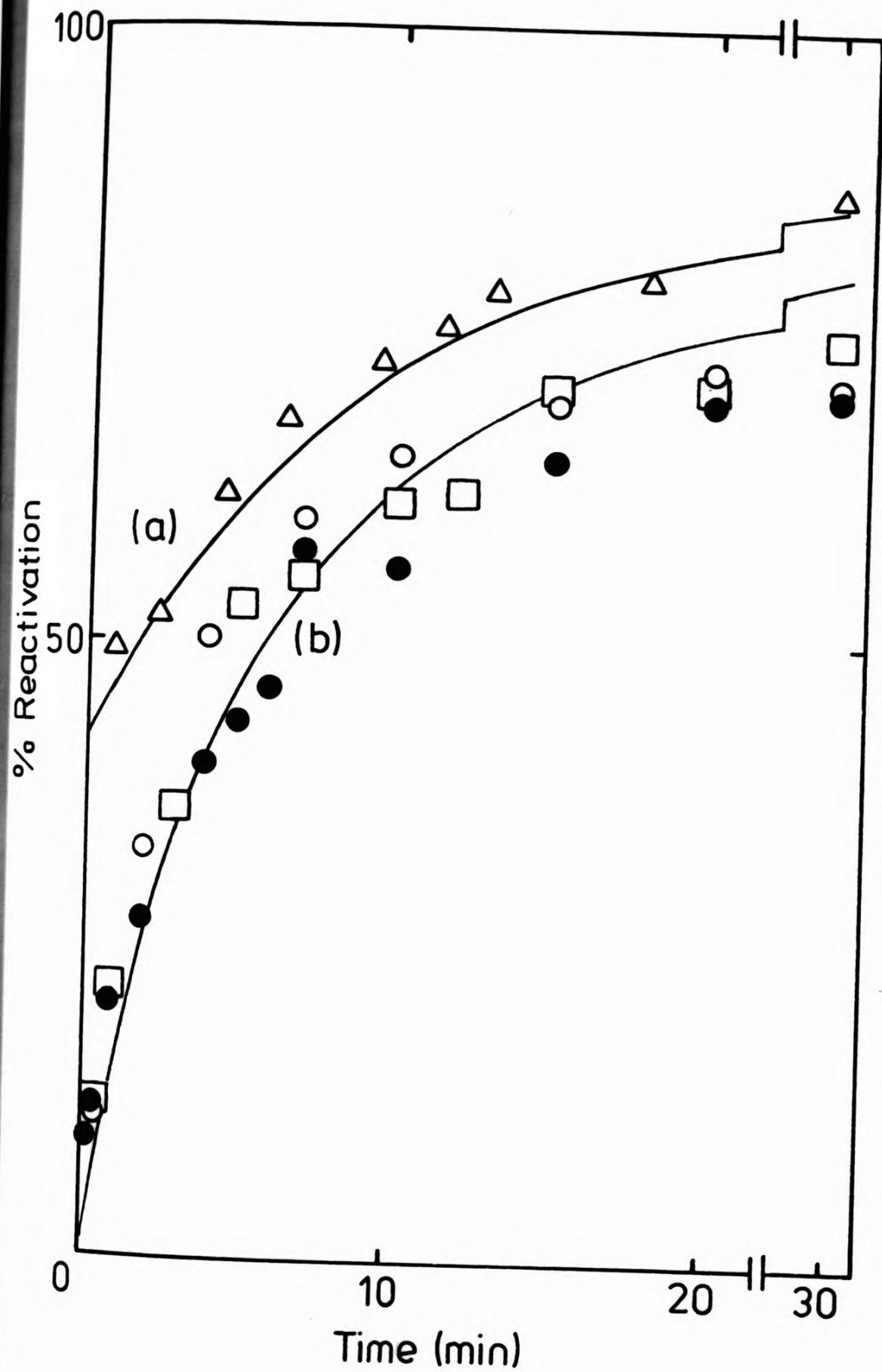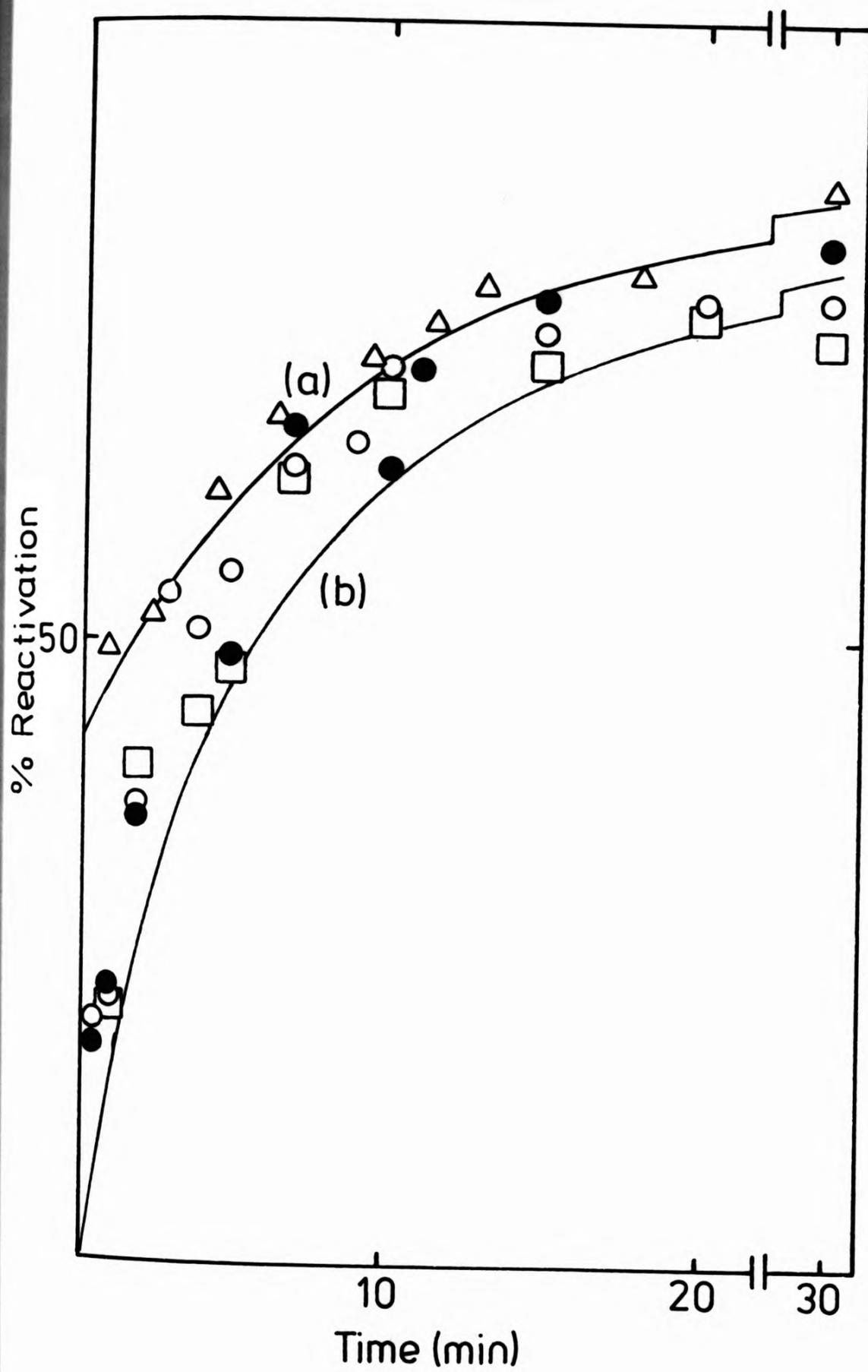
Figure 19c. Reactivation of baker's yeast phosphoglycerate mutase at 50 μg ml^{-1} assessed following a one minute proteinase pulse of 5 μg ml^{-1}.

which rapidly become inaccessible to proteolytic attack at the stage of formation of the folded monomer.

The inclusion of trypsin in the assay of renaturing enzymes was first reported by Chan *et al.* (1973) in their studies on the oligomeric enzyme aldolase. In this case trypsin was included with the intention of preventing enzyme folding and association during the time course of the activity assay. This technique is valid where the formation of native quaternary structure is required for the expression of catalytic activity. However, in cases where intermediate species formed during renaturation may be fully or partially active, the use of proteinases prior to or during assay produces results that require careful interpretation. Although, it is the general rule in oligomeric enzymes that only the native structure is active e.g. alcohol, malate, lactate and glyceraldehyde-3-phosphate dehydrogenases, triose phosphate isomerase, fumarase (Yamoto and Murachi, 1979, Jaenicke, 1984) there is evidence in creatine kinase (Grossman *et al.*, 1981) aldolase (Rudolph *et al.*, 1977c) and bakers yeast phosphoglycerate mutase (Hermann *et al.*, 1985) that the intermediates of renaturation may be at least partially active. The use of trypsin and other proteinases reported here have confirmed this observation for the bakers yeast enzyme.

3.3.4.3. Resistance of subunit structure to proteinases during renaturation

Proteinase pulse techniques can be used to reveal areas of highly folded and compact proteinase resistant structure formed during renaturation. The "fragments" of native structure remaining after proteolysis of unfolded material may correspond to folding domains (Opitz *et al.*, 1987). In the case of bakers yeast phosphoglycerate mutase isolation of such fragments in sufficient quantity for limited sequence analysis would allow the location of these domains within the amino acid sequence and three dimensional structure of the enzyme. Using this technique it was hoped that specific events in the renaturation of the bakers yeast enzyme could be determined and related to the folding of the monomer and subsequent stages of subunit association and tightening of structure defined here and by Hermann *et al.* (1983, 1985).

Proteinases were inhibited and/or removed from the assay mix following the proteinase pulse as described in section 2.2.7. The formation of intact subunit was determined by SDS-PAGE followed by densitometric analysis and compared with control native enzyme treated as in the renaturing samples. The percentage of intact subunit relative to this control is shown in Fig. 20. The full line was calculated according to the model of Hermann *et al.* (1983, 1985) and by assuming the monomeric and dimeric intermediates of renaturation to exhibit no activity in the presence of the three proteinases (section 3.3.4.2.). Fig. 20
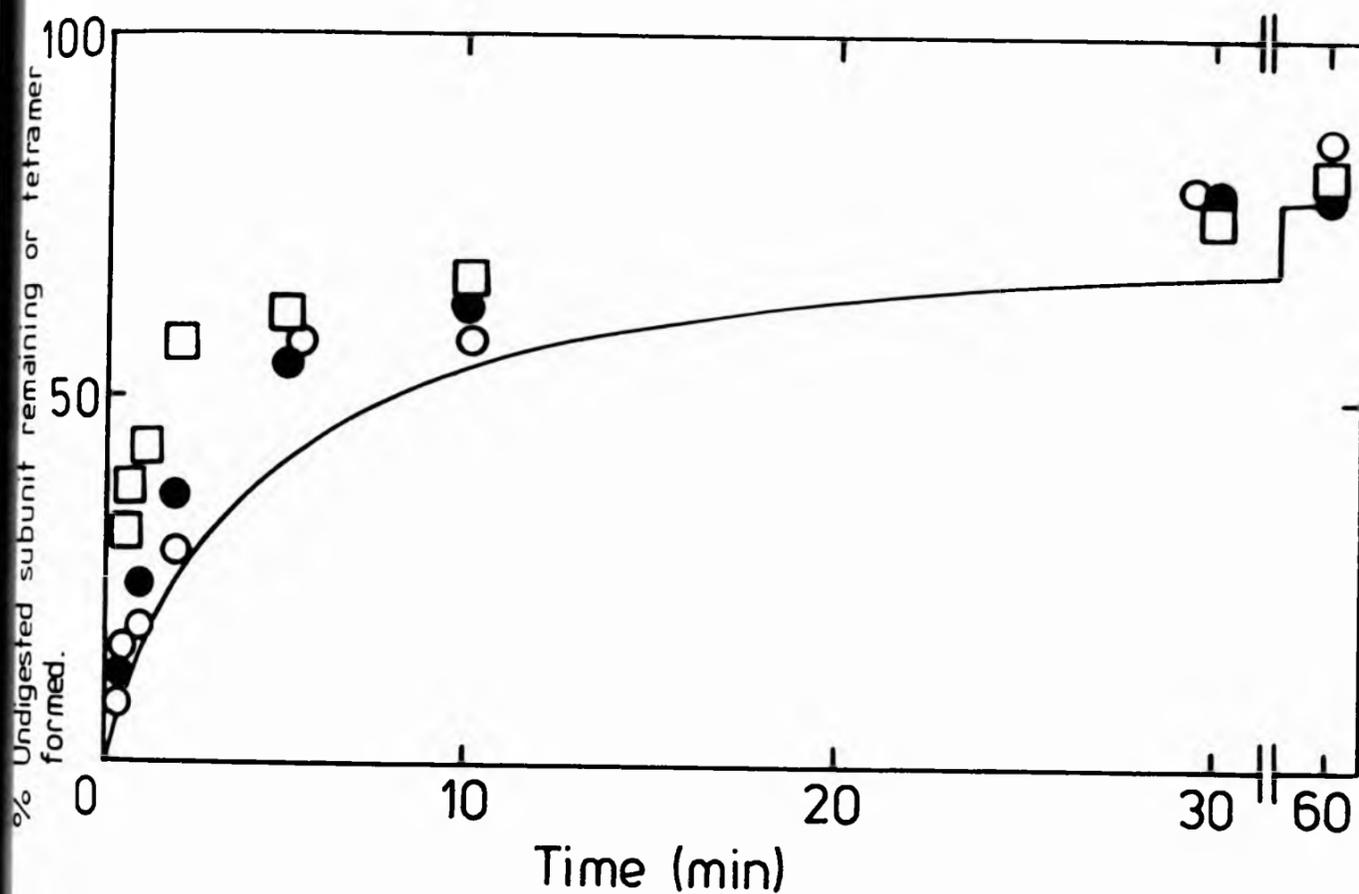
Figure 20. Resistance of baker's yeast phosphoglycerate mutase
subunit structure to proteolysis during renaturation
at 30 µg ml^{-1}.

The percentage of intact subunit remaining was assessed following
SDS-PAGE and densitometric analysis and by comparison with proteinase
treated native controls. Proteolysis for 1 min at 20°C was
performed with renaturing phosphoglycerate mutase diluted to a
concentration of 10 µg ml^{-1} and the following proteinases at a
concentration of 20 µg ml^{-1}. ● , trypsin; ○ ,
chymotrypsin; □ , thermolysin. The full line represents
the predicted tetramer formation calculated according to the
model of Hermann et al. (1983, 1985).

shows that there is extensive degradation of the subunit particularly at early time points during renaturation. However, there is still more undigested subunit remaining than predicted from the formation of proteinase resistant tetramer (represented by the full line). This difference could reflect a greater degree of experimental error in these measurements in comparison with the reassociation and reactivation data. However, it is also possible that inactivation of the monomeric, dimeric and tetrameric species occurs as a result of the cleavage of a small number of amino acids from one end of the amino acid chain. The resultant inactive species would not necessarily be resolved from native subunit under SDS-PAGE conditions. A similar effect has been proposed for the inactivation of the native bakers yeast enzyme by thermolysin and elastase (section 3.3.3.) and has been reported for rabbit muscle phosphoglycerate mutase when treated with thermolysin (Price *et al.*, 1985a). The presence of the C-terminal tail of the bakers yeast enzyme as a candidate for this cleavage has been discussed in section 3.3.3.

The difference between the observed and predicted subunit remaining following proteolysis could be reduced by increasing the concentration of proteinase during the pulse. For example, increasing the thermolysin concentration to 80 μg ml^{-1} reduced the percentage intact subunit remaining to 20% and 65% after 0.25 and 15 min of renaturation respectively. These values were 32% and 78% following the 20 μg ml^{-1} pulse. Concentrations of thermolysin between 80 and 20 μg ml^{-1} produced an intermediate effect between these two sets of values.

84

All the gels in the above series of experiments were examined carefully for the presence of any subunit fragments of Mr > 6500. In the case of trypsin and chymotrypsin there was no evidence of such fragments either under Coomassie blue R250 or silver staining. However, in the case of thermolysin there was evidence of fragments with Mr values between 12 and 15,000. The quantity of these species decreased with increasing periods of renaturation and in parallel with the increasing quantity of undigested subunit remaining. The 12-15,000 Mr species were absent from proteinase only, native enzyme only and native enzyme treated with thermolysin control samples and were thus presumably the result of partial digestion of the intermediates of renaturation (Plate 2). Unfortunately, the staining of these fragments under Coomassie blue R250 indicated quantities of < 5% that of the native enzyme control. Fig. 20 indicates that if the production of these fragments were the sole proteolytic event then quantities of > 50% of the native enzyme control would be predicted. It seems likely therefore that the majority of these species were further digested to fragments of Mr less than 6500.

As a result of these observations a range of lower thermolysin concentrations between 20 and 0.25 μg ml^{-1} were used during the proteinase pulse. However, the effect of this reduction was to increase the amount of undigested subunit remaining (80% after 0.25 min renaturation when treated at 0.25 μg ml^{-1}) without increasing the yield of intact 12-15,000 Mr fragments. Further variations of the thermolysin pulse concentration and/or lowering the temperature of

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |

$$\begin{array}{l} -60 \\ -40 \\ -20 \\ -10 \end{array}$$

$Mr (x10^{-3}$
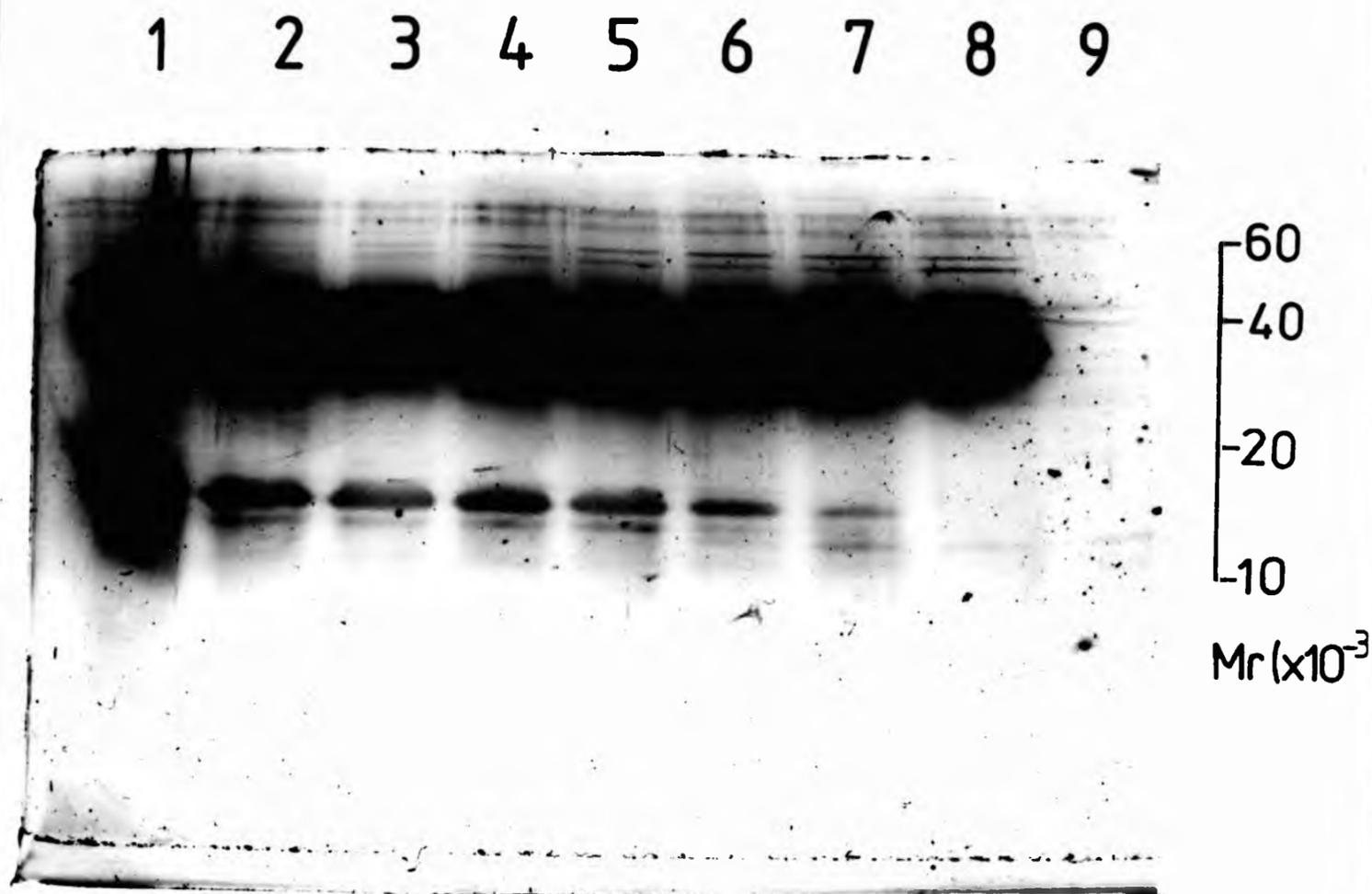
Plate 2. Resistance of baker's yeast phosphoglycerate mutase sub-
unit structure to thermolysin during renaturation at
30 µg ml^{-1}.

Proteolysis for 1 min at 20°C was performed with samples of
renaturing enzyme diluted to a concentration of 10 µg ml^{-1} with
thermolysin at 20 µg ml^{-1}.   Lanes 1-7 represent samples taken
after 0.25, 0.5, 1, 2, 5, 30 and 60 min renaturation respectively.
Lane 8 represents native enzyme incubated with thermolysin under
the defined conditions.   Lane 9 represents thermolysin.   The Mr
scale determined from standard proteins is shown on the right
(section 2.2.1.1.).   The gel was stained with silver according
to the method of Wray et al. (1981) and the possible reasons for
apparent negative staining of some proteins (e.g. thermolysin)
have been discussed by Morrissey (1981).

renaturation and proteolysis (to slow down the relevant reassociation and proteolytic events) might have produced conditions that favoured the production of these fragments. However, in view of the low yields observed above and the constraints of time available, these possibilities were not pursued.

The production of the 12-15,000 Mr fragments by thermolysin suggested that the subunit was being cleaved at or near the centre of its length. The amino acid sequence of the bakers yeast enzyme contains a suitable tetraproline cleavage site at the midpoint (119-122) of the chain of 241 amino acids (Fothergill and Harkins, 1982). Since the initial folding events of polypeptide chains are thought to involve the formation of structural elements such as α-helices (Jaenicke, 1982) which cannot accommodate proline residues (Chou and Fasman, 1978), it seems possible that this tetraproline sequence and its adjacent residues might remain exposed for relatively long periods during renaturation. The separate folding of the two halves of the subunit chain around this proline sequence would produce compact and folded units which, on cleavage near the proline site, would produce fragments of Mr approximately 14,000. These folded units, under suitable conditions, might be sufficiently resistant to further degradation by thermolysin and would therefore appear as the putative fragments in Plate 2.

A similar pattern of subunit cleavage during renaturation has been reported for porcine muscle lactate dehydrogenase (section 1.7.3.).

86

Treatment of this enzyme with thermolysin after 1 min of renaturation produces proteolytic dimers which are incapable of association to the native tetrameric configuration. Analysis of these dimers indicate that the subunit polypeptide chains are "nicked" and three subunit fragments of M_r 33,500, 21,400 and 13,500 are detectable. These fragments represent compact folded units which are capable of reassociation and reactivation to the partial activity of the proteolytic dimers following denaturation in GdnHCl (Opitz *et al.*, 1987). It has been proposed that these fragments represent domains which fold and associate independently. Such processes are therefore considered evidence for *in vivo* cotranslational folding by parts.

Although the cleavage pattern of the bakers yeast enzyme is similar, the folded units produced by thermolysin treatment differ from the lactate dehydrogenase fragments because they are not resistant to further degradation by the proteinase. These folded units must therefore be relatively structured but probably not compact enough to be referred to as true domains.

CHAPTER FOUR : DENATURATION

AND REFOLDING OF RABBIT MUSCLE

PHOSPHOGLYCERATE MUTASE

4.1 INTRODUCTION.

Phosphoglycerate mutase from rabbit muscle is a dimeric enzyme of Mr
56,000 comprised of two subunits of Mr 28,000 (Pizer, 1960, Hermann *et
al.*, 1983). The purified enzyme is available from a number of
commercial sources and therefore represents a convenient enzyme to
include in studies of phosphoglycerate mutases. The rabbit muscle
enzyme seems to have a similar catalytic mechanism to bakers yeast
phosphoglycerate mutase. The isomerization reaction in both enzymes
proceeds via a phosphohistidine intermediate and the sequences around
the two active site histidines of the bakers yeast enzyme have
considerable homology to two histidine containing peptides in the
rabbit muscle enzyme (Haggarty and Fothergill, 1980). In view of this
documented structural and mechanistic similarity it was considered of
interest to further examine the relationships between the bakers yeast
and rabbit muscle enzymes. The relatedness of the two enzymes was
assessed using peptide mapping techniques and through comparisons
between their amino acid compositions.

Preliminary studies by Hermann *et al.* (1983) on the refolding of
rabbit muscle phosphoglycerate mutase had encountered some problems.
These authors were unable to study the reassociation kinetics of this
enzyme using the glutaraldehyde cross-linking technique since they
were unable to obtain quantitative fixation in the dimeric state. In
addition, reactivation of the rabbit muscle enzyme at concentrations
above 5 μg ml^{-1} was complicated by the formation of inactive

aggregates. At enzyme refolding concentrations of approximately 30 μg ml^{-1} only some fifty percent of activity was regained following 24 h refolding. Since this concentration represents approximately 0.5 μM then it is clear that the rabbit muscle enzyme is less susceptible to the formation of aggregates during refolding than lactate dehydrogenase (Fig. 5, section 1.7.3.).

The denaturation and refolding of the rabbit muscle enzyme were also examined here. Denaturation in GdnHCl was monitored by loss of catalytic activity and changes in protein fluorescence and circular dichroism. Reactivation of the enzyme during refolding was examined in the presence and absence of a proteinase pulse prior to assay. Through the use of the proteinase pulse it was hoped to generate proteinase resistant fragments of structure for subsequent reassociation and sequencing studies (section 3.1.). In addition, the denaturation and refolding characteristics of the rabbit muscle enzyme would provide another basis on which comparisons could be made with bakers yeast phosphoglycerate mutase.

4.2. METHODS.

4.2.1. Rabbit muscle phosphoglycerate mutase.

Rabbit muscle phosphoglycerate mutase was obtained from Boehringer as an $(NH_4)_2SO_4$ suspension of 5 mg ml^{-1} concentration. The enzyme was stored at 4°C and was dialysed against $\geqslant$ 500 volumes of appropriate buffer prior to use.

4.2.2. Denaturation of rabbit muscle phosphoglycerate mutase by GdnHCl.

Loss of catalytic activity, changes in fluorescence and changes in circular dichroism observed in the presence of GdnHCl were monitored as described in section 2.2.4. Quenching of fluorescence by acrylamide and succinimide was determined as described in section 2.2.5.

4.2.3. Renaturation of rabbit muscle phosphoglycerate mutase.

Renaturation of the rabbit muscle enzyme was performed under the same conditions and by the same procedures used for the bakers yeast enzyme (section 3.2.4.).

4.2.4. Comparative studies between rabbit muscle and bakers yeast phosphoglycerate mutases.

4.2.4.1. Cleveland mapping.

Cleveland mapping assesses the relatedness between proteins from peptide cleavage patterns produced following partial proteolysis in the presence of SDS (Cleveland *et al*., 1977).

Rabbit muscle and bakers yeast enzyme, at a concentration of 1 mg ml^{-1}, were mixed with an equal volume of digestion buffer comprising 1% w/v SDS, 20% v/v glycerol, 10^{-4}% w/v bromophenol blue in 0.25M Tris-HCl buffer , pH 6.8. The enzymes were boiled for 2 min in the digestion buffer. After cooling, proteinase was added to a final concentration of 33 or 3 μg ml^{-1} and the digestion mixture incubated at 37°C.

At intervals up to one hour samples were withdrawn and processed for SDS-PAGE analysis by addition of SDS (to 2% w/v), 2-mercaptoethanol (to 10% v/v) and boiling for 2 min. After cooling a mixture of 2-mercaptoethanol and 1% w/v bromophenol blue (50:50) was added to each sample to a final concentration of 5% v/v. Samples were subsequently analysed by SDS-PAGE on 15% acrylamide gels.

The proteinases used and their concentration during digestion were chymotrypsin (used at concentrations of 3 μg ml^{-1} and 33 μg ml^{-1} for

the bakers yeast and rabbit muscle enzymes respectively), thermolysin (33 μg ml^{-1}), proteinase-K (3 μg ml^{-1}) and *S.aureus* V-8 proteinase (33 μg ml^{-1}). The optimum concentration of proteinase and the time scale of sampling were determined by performing a number of preliminary experiments.

4.2.4.2. Spectrophotometric determination of tryptophan content.

The tryptophan content of both enzymes was determined by the spectrophotometric method of Edelhoch (1967). Each enzyme was incubated at a concentration of 0.25 mg ml^{-1} in 20 mM sodium phosphate buffer, pH 6.5, and in the presence of 6 M GdnHCl. After 15 min. equilibration the absorbances at 280, 288 and 315 nm were determined. The value at 315 nm is a measure of light scattering by the sample and was used to correct (by subtraction) the values obtained at the other two wavelengths. From the corrected data the enzyme molar absorptions were calculated. The molar absorptions at 280 and 288 nm are 5690 and 4815 for tryptophan and 1280 and 385 for tyrosine. Therefore;

A_{280} (Enzyme molar absorption at 280 nm) = N_{TRP} .5690 + N_{TYR} .1280

A_{288} (Enzyme molar absorption at 288 nm) = N_{TRP} .4815 + N_{TYR} .385

Where N_{TRP} and N_{TYR} are the number of tryptophan and tyrosine residues per mole of enzyme.

Solving the above for;

$$N_{TRP} = (A_{288}/3103) - (A_{280}/10318) \qquad \qquad (a.)$$

$$N_{TYR} = 6.897 (A_{280}/5690 - A_{288}/4815) \qquad \qquad (b.)$$

The number of moles of tryptophan per mole of enzyme were calculated using equation (a.). For tyrosine the errors involved in this method of determination are larger than for tryptophan since its molar absorptions at the two wavelengths are lower. Furthermore tyrosine can be resolved and quantitated during conventional amino acid analysis. In view of these factors the tyrosine content of each enzyme was not calculated using equation (b.) but was obtained by conventional amino acid analysis as described below.

4.2.4.3. Titration of cysteine residues.

The number and accessibility of cysteine residues in the rabbit muscle enzyme was determined using the titrant 5,5'-dithiolbis (2-nitrobenzoic acid) (NbS_2). The bakers yeast enzyme has no cysteine residues (Fothergill and Harkins, 1982).

The rabbit muscle enzyme at a concentration of 0.27 mg ml^{-1} in 100 mM sodium phosphate buffer, pH 8.0, was reacted with 0.25 mM NbS_2 in the presence and absence of 0.1% w/v SDS. The absorbance at 412 nm was used to follow the production of the free 5-thio, 2-nitrobenzoic acid

derivative. The concentration of the thionitrophenolate anion released was calculated from its molar absorption of 13,600 at 412 nm. Using the molar concentration of enzyme the number of reacting cysteine residues under native and denaturing (in the presence of SDS) conditions were calculated.

4.2.4.4. Amino acid analysis.

Amino acid analysis of the rabbit muscle and bakers yeast enzymes after performic acid oxidation was performed by Prof. J. Coggins at the University of Glasgow, Scotland. Comparison of this data with that published for the bakers yeast enzyme (Fothergill and Harkins, 1982) was undertaken by the method of Cornish-Bowden (1983). Using this method protein relatedness is assessed from a difference index (SΔn) which is defined as;

$$S\Delta n = \frac{1}{2} \Sigma (N_{iA} - N_{iB})^2 - 0.035(N_A - N_B)^2 + 0.535(N_A - N_B) \qquad (c.)$$

Where N_A is the number of residues in chain A, N_B the number of residues in chain B, N_{iA} the number of residues type i in chain A and N_{iB} the number of residues type i in chain B. For proteins of equal size the latter two corrections in equation (c.) are not used as $N_A = N_B$. For proteins that differ in chain length by < 18 residues this correction is within the range ± 2.

Values of $S \Delta n < 0.42N$ are considered to indicate strong relatedness between proteins where as values $> 0.93N$ indicate no relatedness. Where the proteins being compared differ in size, the smaller of the two is used in calculating the fraction of N as this tends to slightly under estimate relatedness (Cornish-Bowden, 1983).

4.3. RESULTS AND DISCUSSION.

4.3.1. Rabbit muscle phosphoglycerate mutase.

The specific activity of the rabbit muscle phosphoglycerate mutase supplied by Boerhinger was generally 2500-3000 units mg^{-1}. This equates to 288-345 μmole 3-phosphoglycerate metabolised min^{-1} mg $protein^{-1}$ (section 2.2.3.1.).

Analysis of the enzyme preparation by SDS-PAGE indicated > 90% homogeneity of staining with Coomassie blue R250. The Mr of the enzyme subunit was 28000 ± 1400 and that of the minor contaminant 26000 ± 1300 (Plate 1). Under native conditions the Mr of the enzyme was determined as 56000 ± 5600 using gel filtration on Sephacryl S-300 as described in section 2.2.1.2. These values confirm the earlier reports of Pizer (1960) and Hermann *et al*. (1983) that the phosphoglycerate mutase from rabbit muscle is a dimer of Mr approximately 56000.

The Km of the rabbit muscle enzyme in 30 mM Tris-HCl buffer, pH 7.0, and in the presence of 10 mM 3-phosphoglycerate for its cofactor 2,3-bisphosphoglycerate was determined at 30ºC as 7 ± 0.7 μM. A cofactor K_m of 125 μm has been reported for this enzyme under similar conditions but in the presence of 400 mM KCl during assay (Grisolia and Carreras, 1975). In the absence of added cofactor the rabbit muscle enzyme still exhibited approximately 2% of maximal activity. A

similar observation was made using the bakers yeast enzyme and the possible reasons for this residual activity in terms of trace contamination of grade 1 3-phosphoglycerate have been considered in section 3.3.1.

There have been some reports of a proposed cofactor independent phosphoglycerate mutase activity exhibited by the rabbit muscle enzyme. Grisolia and Joyce (1959) reported some 1% of maximal activity in the absence of cofactor whilst more recently Stankiewicz and Hass (1986) have proposed a bimodality of catalytic activity with 3% of maximal activity remaining in the absence of added 2,3-bisphosphoglycerate. In view of the low Km of the rabbit muscle enzyme for its cofactor and the residual activity observed in its absence which have been reported here, it seems possible that the observations of the above authors may have resulted from a combination of incomplete dialysis of the enzyme to remove endogenous or bound cofactor and/or the presence of trace levels of 2,3-bisphosphoglycerate in "pure" 3-phosphoglycerate. However, it is of interest that Stankiewicz and Hass (1986) reported differing pH optima for the enzyme activity in the presence (maximal activity; pH 7.0) and absence (3% residual activity; pH 6.0) of cofactor. This difference does provide some support to their proposed cofactor independent activity in the rabbit muscle enzyme and therefore the situation with respect to this activity is not firmly established.

4.3.2. Denaturation of rabbit muscle phosphoglycerate mutase by GdnHCl.

4.3.2.1. Loss of catalytic activity.

The activity of the rabbit muscle enzyme in the presence of GdnHCl is shown in Fig. 21. There was a progressive decline in activity between approximately 0 and 1.5 M GdnHCl with 50% inactivation occurring at 0.55 M. Overall, the profile of inactivation for the rabbit muscle enzyme was very similar to that observed for bakers yeast phosphoglycerate mutase (Fig. 12) where 50% inactivation occurred at 0.65 M GdnHCl.

4.3.2.2. Changes in Fluorescence.

The fluorescence emission spectra of the rabbit muscle enzyme in the presence and absence of 4 M GdnHCl are shown in Fig. 22. The λ max of the native enzyme is 340 nm. In the presence of 4 M GdnHCl there is a small red shift to a λ max of 348 nm with the fluorescence intensity at 340 nm reduced to 80% of its native value. The spectrum of the enzyme in the presence of 2M GdnHCl was very similar to that obtained in 4 M (λ max 345 nm, intensity at 340 nm 83% of native) indicating that the gross changes in fluorescence had occurred following incubation at the lower concentration.
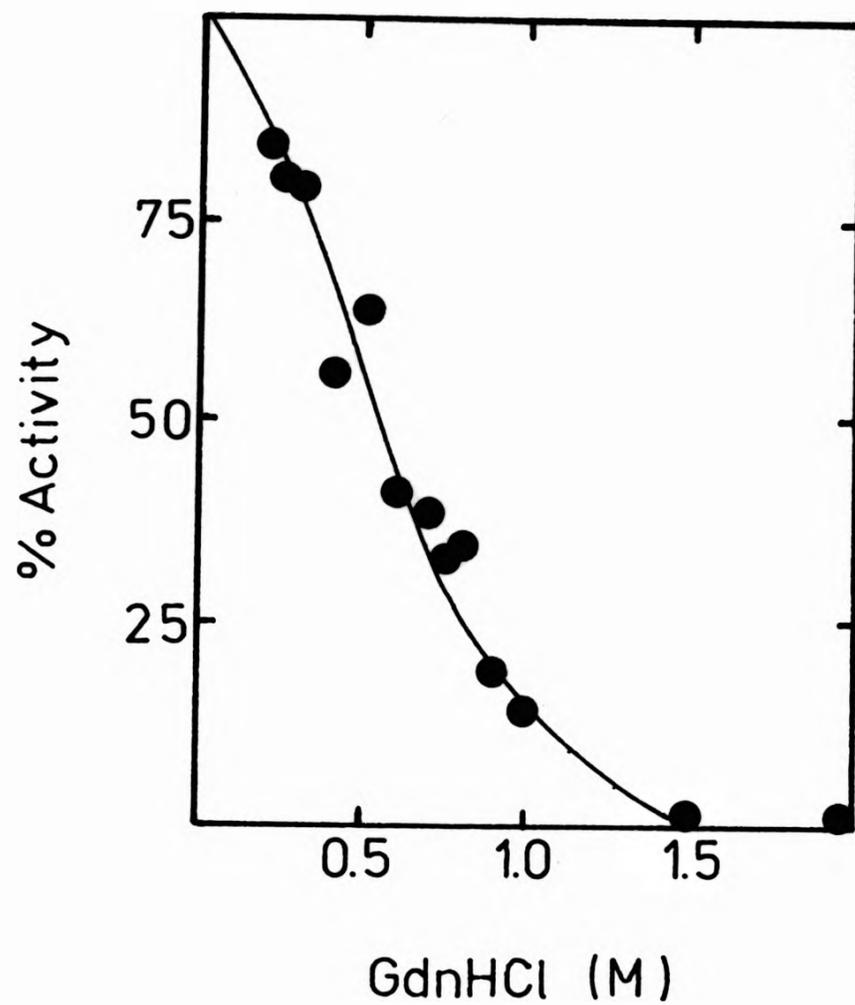
Fig 21 Activity of rabbit muscle phosphoglycerate mutase in the
        presence of GdnHCl.

Enzyme (1 unit) was added to substrate (10 mM 3-phosphoglycerate),
cofactor (0.3 mM 2,3-bisphosphoglycerate) and the indicated
concentration of GdnHCl in a final volume of 1 ml.   2-phospho-
glycerate formation was measured over a 3 min period and activity
expressed relative to a control containing no GdnHCl (sections
2.2.4.1. and 5.3.3.1.).
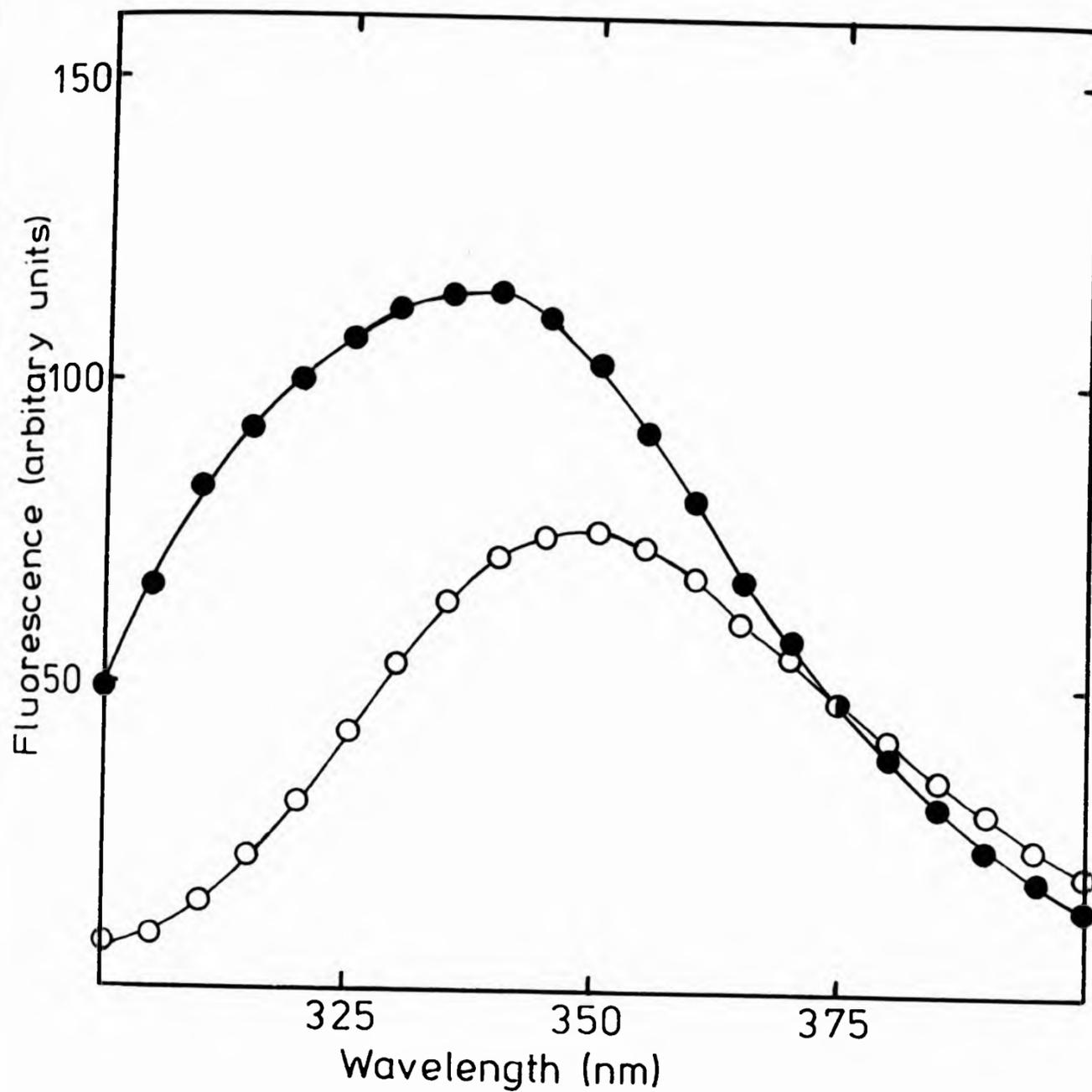
Figure 22. Fluorescence spectra of rabbit muscle phosphoglycerate
mutase in the presence and absence of 4 M GdnHCl.

Rabbit muscle phosphoglycerate mutase (20 µg ml^{-1}) at 20°C.   The
fluorescence excitation was at 290 nm and the emission monitored
between 300 and 400 nm.   Instrument sensitivity was X 10.
●   , enzyme in 50 mM sodium phosphate buffer, pH 7.5;
O   , enzyme in the presence of 4 M GdnHCl.

Teipel and Koshland (1971) have examined the fluorescence spectra for a range of proteins denatured in 6 M GdnHCl. The spectra of these proteins were similar and all showed a common λ max of approximately 350 nm. A mixture of aromatic amino acids (tryptophan, tyrosine and phenylalanine) in the presence of 6 M GdnHCl produced a similar spectrum indicating that these residues were highly solvent exposed in the denatured proteins (Teipel and Koshland, 1971).

Ultracentrifugation studies in the presence of 4 M GdnHCl have indicated that the dimeric rabbit muscle phosphoglycerate mutase is completely dissociated under these conditions (Hermann *et al.*, 1983). The λ max of the fluorescence spectrum of this enzyme in the presence of 4 M GdnHCl (Fig. 22) indicates that this dissociation is accompanied by extensive solvent exposure of aromatic residues presumably as a result of subunit dissociation and unfolding.

The profile and λ max of the fluorescence spectra in the presence of 4 M GdnHCl for the rabbit muscle and bakers yeast enzymes are very similar (Figs. 22 and 13). The intensity of the denatured bakers yeast spectrum at the λ max is approximately double that of the denatured rabbit muscle enzyme when calculated on a per mole basis. These molar intensities correlate well with the differing tryptophan content of the two enzymes. Both enzyme subunits have 5 tryptophans (Fothergill and Harkins, 1982 and section 4.3.4.) thus equating to 20 mol^{-1} and 10 mol^{-1} for the bakers yeast and rabbit muscle enzymes respectively.

In contrast to the spectra in the presence of 4 M GdnHCl, the profiles and λ max of the native enzymes show some marked differences. The relatively high λ max of the rabbit muscle enzyme (340 nm compared with 328 nm for the bakers yeast enzyme) indicates that the aromatic residues are more solvent exposed in this enzyme. In an attempt to verify this observation the quenching of native enzyme fluorescence was examined using acrylamide and succinimide as quenchers (section 2.2.5.).

The Stern-Volmer plots for the rabbit muscle and bakers yeast enzymes (in the presence and absence of 0.3 mM 2,3-bisphosphoglycerate) are shown in Fig. 23 a and b respectively. Examining the fluorescence quenching in the two enzymes it is evident that the aromatic residues in the rabbit muscle enzyme are more readily quenched. For example, at 0.35 M acrylamide fluorescence is reduced by 52% and 39% in the rabbit muscle and bakers yeast enzymes respectively. Succinimide quenches to a lesser extent in both enzymes. The reduced efficacy of succinimide is thought to result from its increased size in relation to acrylamide (approximately 20% larger; Edward, 1970). This increase reduces the ability of succinimide to penetrate the protein matrix and quench buried aromatic residues. In addition, succinimide quenching is relatively inefficient in aprotic solvents (Eftink and Ghiron, 1984) and therefore deeply buried aromatic residues may present a microenvironment in which quenching is reduced. The ratio of quenching efficiencies determined from the slopes of the Stern-Volmer plots (K_s/K_a) is therefore regarded as a sensitive indicator of the relative
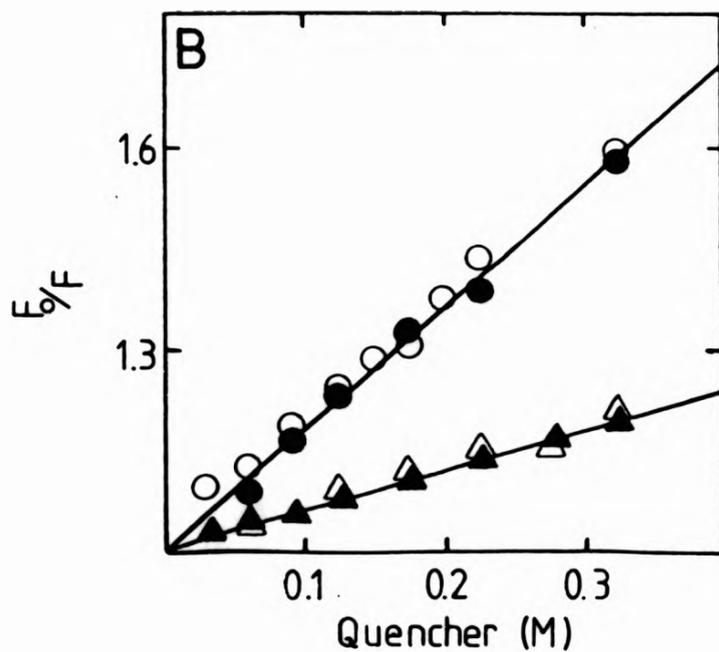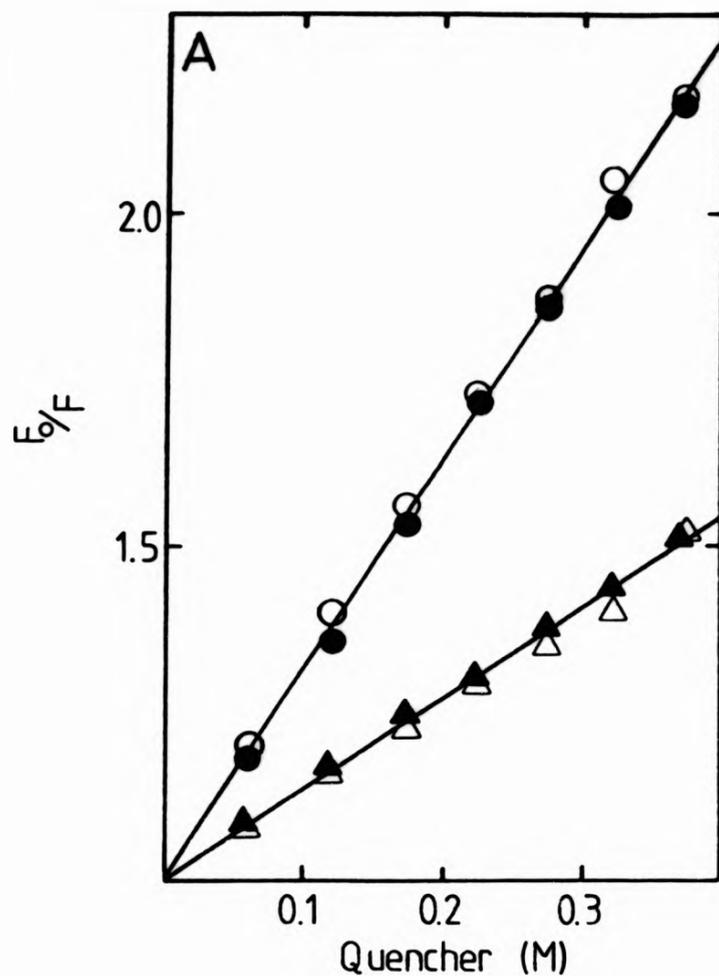
Figure 23. Stern-Volmer plot for quenching of fluorescence in
baker's yeast and rabbit muscle phosphoglycerate
mutases.

23A rabbit muscle enzyme, initial concentration 27 µg ml^{-1}.   23B
baker's yeast enzyme, initial concentration 10 µg ml^{-1}.   On the
ordinates F and Fo refer to the fluorescence in the presence and
absence of quencher respectively. ● ○   , quenching by acryl-
amide; ▲ △   , quenching by succinimide; ● ▲   , dialysed
enzymes; ○ △   , dialysed enzymes in the presence of 0.3 mM
2,3-bisphosphoglycerate.

exposure of the aromatic residues in a protein. This ratio is 0.43 and 0.33 in the case of the rabbit muscle and bakers yeast enzymes respectively. The higher ratio in the former enzyme thus supports the conclusion that its aromatic residues are more highly solvent exposed.

The presence of cofactor had no detectable effect on the quenching of fluorescence in either enzyme (Figs. 23 a and b). This finding is surprising in view of the fact that both enzymes possess a tryptophan residue within their active site peptides (Haggarty and Fothergill, 1980, Fothergill and Harkins, 1982). Furthermore, tryptophan 22 appears to form part of the active site pocket of the bakers yeast enzyme (Winn *et al.*, 1981). It would appear however that the presence of cofactor has no "protective" effect on either of these residues in terms of preventing the quenching of their fluorescence.

Eftink and Ghiron (1984) have reported K_s/K_a ratios of between 0.1 (aldolase) and 0.64 (lysozyme) for a range multi-tryptophan containing proteins. The values obtained for the phosphoglycerate mutases examined are therefore in the middle of this range. As a suitable control to the above studies the quenching of aldolase and lysozyme were re-examined and the comparable K_s/K_a values of 0.1 and 0.61 were obtained (data not shown).

The fluorescence emission of the rabbit muscle enzyme at 325 nm as a function of GdnHCl at intermediate concentrations between 0 and 2 M is shown in Fig. 24. There is a small decrease in fluorescence intensity
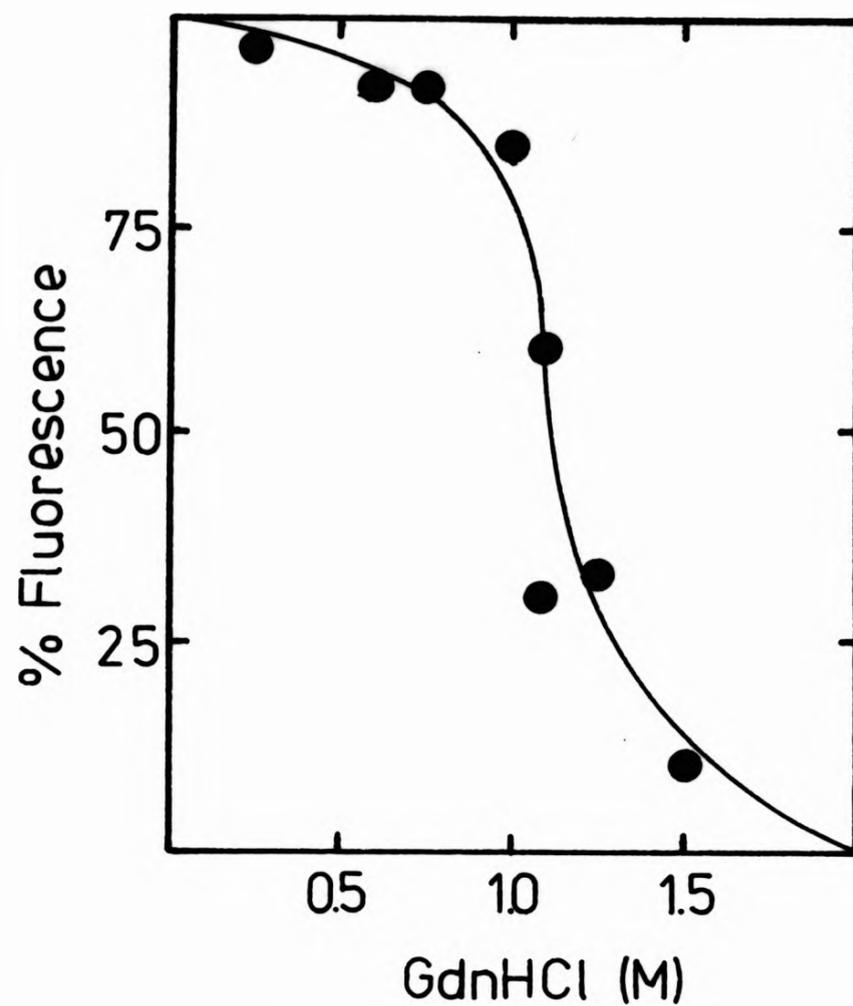
Figure 24.   Fluorescence of rabbit muscle phosphoglycerate mutase
              in the presence of GdnHCl.

Fluorescence was recorded in 50 mM sodium phosphate buffer pH 7.5,
at 20°C.   The excitation and emission wavelengths were 290 and
325 nm respectively and the change in fluorescence emission is
expressed relative to the total change between 0 and 2 M GdnHCl.
Fluorescence emission in the presence of 2 M GdnHCl was approxi-
mately half that of the native enzyme (Fig. 22).

at concentrations up to 1 M GdnHCl followed by a rapid decline between 1 and 1.5 M to a value approximately half that of the native enzyme. A further small decrease in fluorescence intensity occurred on increasing the GdnHCl concentration to 2 M. However, there was no significant change in fluorescence intensity from this value when the GdnHCl concentration was increased to 4 M. The mid-point of the fluorescence transition is 1.1 M. An identical value for this mid-point was obtained for bakers yeast phosphoglycerate mutase although in the case of this enzyme an initial small increase in fluorescence intensity was observed at GdnHCl concentrations < 1 M and the final intensity in 2 M GdnHCl was one quarter that of the native enzyme (Fig. 14). Changes in the fluorescence λ max of the rabbit muscle enzyme occurred at similar concentrations to the reduction in intensity with the red shift in λ max from 340 to 345 nm produced between 0.5 and 2 M GdnHCl.

4.3.2.3. Changes in circular dichroism.

The circular dichroism spectra of the rabbit muscle enzyme in the presence and absence of 2 M GdnHCl are shown in Fig. 25. Using the reference values for the ellipticity of pure helix at 210 and 225 nm quoted by Chen *et al.* (1974) the helical content of the rabbit muscle may be calculated as 12 ± 1% in the absence of GdnHCl. The marked shoulder in the circular dichroism spectrum between 215 and 230 nm in the presence of 2 M GdnHCl is evidence of some helical structure remaining at this concentration. Bakers yeast phosphoglycerate mutase
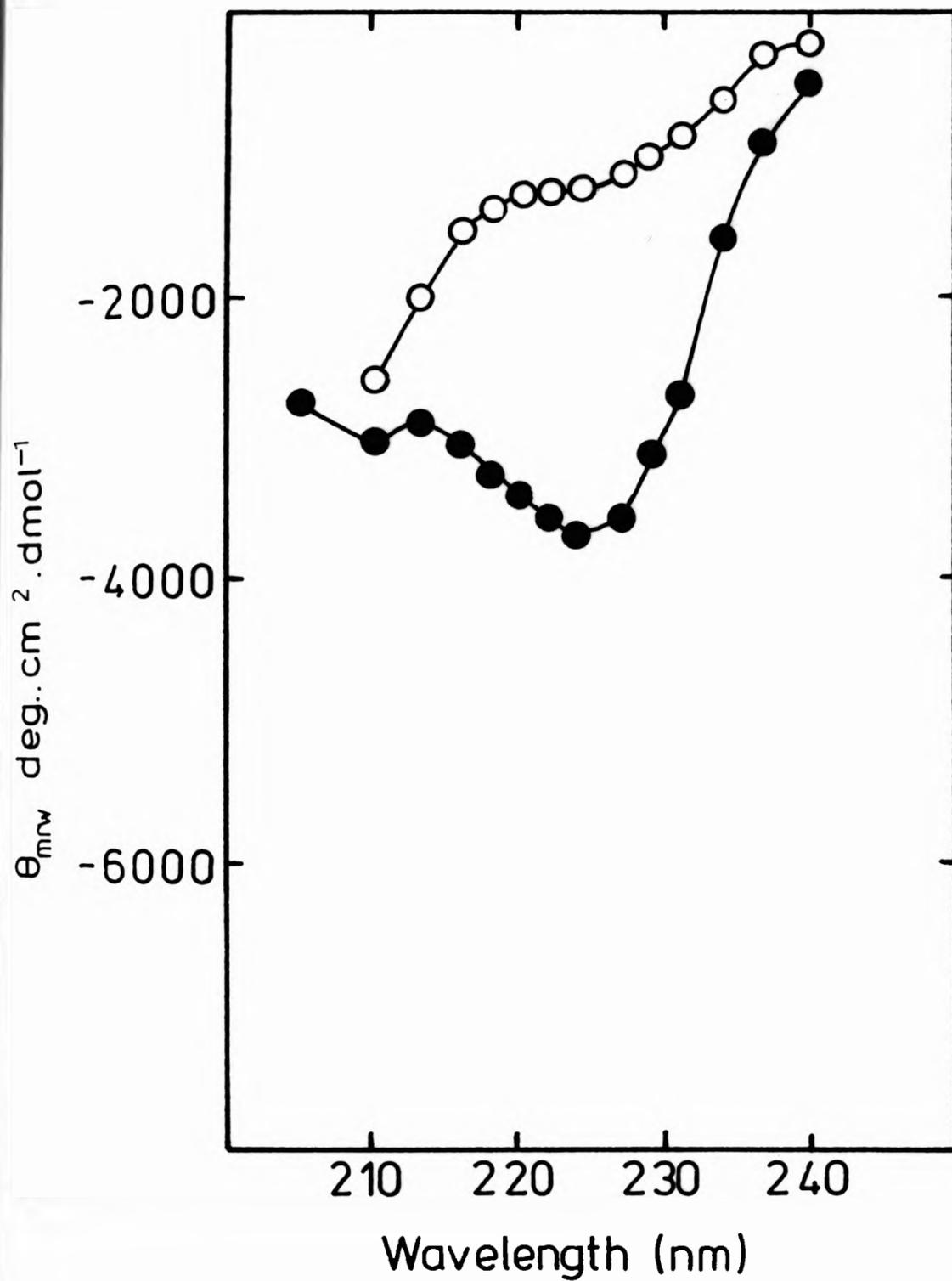
Figure 25. Circular dichroism spectra of rabbit muscle phosphoglycerate mutase in the presence and absence of 2 M GdnHCl.

Enzyme in 50 mM sodium phosphate buffer, pH 7.5, at 20°C in the presence (○) and absence (●) of 2 M GdnHCl.

also exhibited residual helical structure in the presence of 2 M GdnHCl and in the case of this enzyme the ellipticity between 210 and 240 nm was reduced by approximately 10% of the native value on increasing the GdnHCl concentration to 4 M. Unfortunately, the time constraints on the usage of the circular dichroism instrument meant that the spectrum of the rabbit muscle enzyme in the presence of the 4 M GdnHCl was not recorded. However, it seems likely that a spectra under these conditions would exhibit a further decrease in ellipticity between 210 and 240 nm and probably of a similar magnitude to that observed for the bakers yeast enzyme.

Comparison of the circular dichroism spectra of the native rabbit muscle and bakers yeast enzymes (Figs. 25 and 15 respectively) shows that the latter protein has greater (more negative) ellipticity over the wavelength range studied. The bakers yeast enzyme thus appears to have a higher helical content when estimated from this data. This difference must be considered in the context of the assumptions made about the contributions of other structural elements to the ellipticity of proteins (section 3.3.2.3.). Furthermore, the calculation of ellipticities are dependent on reproducible and accurate determinations of protein concentrations. There is thus some potential for experimental error in their calculation.

Whilst the circular dichroism spectra indicate some differences between the bakers yeast and rabbit muscle enzymes, there is some evidence that they may possess a common arrangement of secondary

structural elements. Price and Stevens (1983) have reported that a range of 2,3-bisphosphoglycerate dependent phosphoglycerate mutases bind to the immobilised dye Cibacron blue F3GA and can be displaced from it by a pulse of their cofactor. A number of cofactor independent phosphoglycerate mutases examined by these authors were not retained by this dye. Binding of Cibacron blue F3GA is thought to be characteristic of proteins possessing a regular arrangement of β-sheet and α-helical structure (section 1.2.5.) and therefore binding of the cofactor dependent phosphoglycerate mutases is evidence that they have a common configuration of secondary structural elements.

4.3.3. Renaturation of rabbit muscle phosphoglycerate mutase.

4.3.3.1. Reactivation in the absence of proteinases.

The reactivation profiles of the rabbit muscle enzyme at 20°C and at a renaturing concentration of 10 and 30 μg ml^{-1} are shown in Fig. 26. As with the bakers yeast enzyme, reactivation was assessed following dilution of the renaturing enzyme to a standard concentration of 10 μg ml^{-1} and incubation at 20°C for 1 min. The full lines in Fig. 26 are calculated according to the kinetic model of reactivation of rabbit muscle phosphoglycerate mutase proposed by Hermann *et al.* (1983) involving two parallel first order reactions with rate constants 3.4 x 10^{-4} s^{-1} and 5.0 x 10^{-3} s^{-1} (for the slower and faster reactions respectively). The faster reaction comprises two-thirds of the total reactivation amplitude under this scheme. It should be noted that this
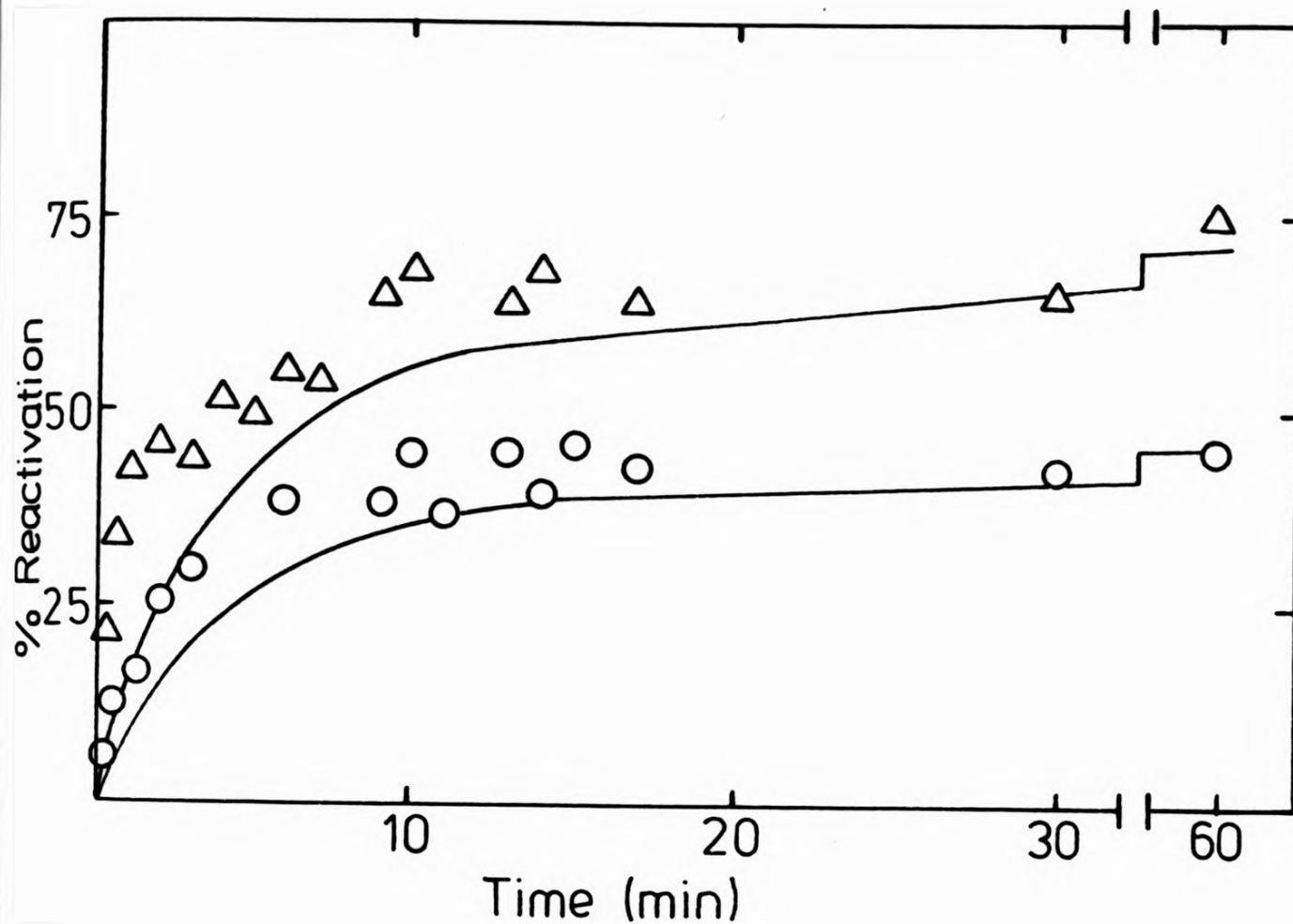
Figure 26. Reactivation of rabbit muscle phosphoglycerate mutase in the absence of proteinases.

Rabbit muscle phosphoglycerate mutase renaturing at; $\triangle$, 10 μg ml^{-1} and $\bigcirc$, 30 μg ml^{-1} was diluted to a standard concentration of 10 μg ml^{-1} in 50 mM sodium phosphate buffer and incubated for 1 min at 20°C prior to assay.   The full lines were calculated according to the kinetic model of reactivation reported by Hermann et al. (1983).

kinetic model was not defined in terms of changes in quaternary structure during reactivation since Hermann *et al.* were unable to quantitatively fix the rabbit muscle enzyme using the glutaraldehyde cross-linking technique. As such the structure of the active species during renaturation are unknown.

The reactivation of the rabbit muscle enzyme at both concentrations runs significantly ahead of the profile predicted by the kinetic model particularly over the first 15 min of renaturation. However, the reactivation data used by Hermann *et al.* (1983) to which their kinetic model was fitted was obtained using a 5 min trypsin treatment at 5 μg ml^{-1} prior to assay. The data in Fig. 26 therefore indicates that some intermediates formed during the renaturation of the rabbit muscle enzyme have activity which is more sensitive to trypsin than the native form (since in the absence of a proteinase "pulse" the reactivation rate is increased). A similar property has been noted for the bakers yeast enzyme (Hermann *et al.*, 1983, section 3.3.4) where the monomeric and dimeric intermediates of renaturation have a partial activity which is proteinase sensitive.

Reactivation of the rabbit muscle enzyme would be expected to be concentration independent from the above first order reaction kinetics. However, reactivation is only completely reversible at renaturing concentrations of < 5 μg ml^{-1}. At higher concentrations the yield of reactivation is reduced although the rate constant is unchanged (Hermann *et al.*, 1983). Fig. 26 shows a similar pattern with

a lower yield of reactivation observed at the higher renaturing concentration examined. The reduction in activity regained occurs as a result of the formation of inactive "wrong" aggregates (Hermann *et al.*, 1983). Aggregate formation has also been reported during the renaturation of lactate dehydrogenase with the level of this competing process dependent on the extent of denaturation and the concentration of this enzyme during renaturation (Zettlmeissel *et al.*, 1979, section 1.7.3.).

4.3.3.2. Reactivation in the presence of proteinases.

The three proteinases employed in the bakers yeast phosphoglycerate mutase reactivation studies, namely trypsin, chymotrypsin and thermolysin, were also used to further examine the proteinase sensitivity of intermediates formed during reactivation of the rabbit muscle enzyme. As with the bakers yeast enzyme a proteinase "pulse" of 1 min. at 20°C was used prior to assessing reactivation. This proteinase treatment had no effect on the activity of the native rabbit muscle enzyme in the presence or absence of the residual concentration of GdnHCl (0.1 M) present during renaturation. The reactivation profiles for the rabbit muscle enzyme renaturing at 10 and 30 μg ml^{-1} are shown in Figs. 27 and 28 respectively. The data obtained in the absence of proteinases (plotted in Fig. 26) is included for comparison and the full lines were calculated as before.
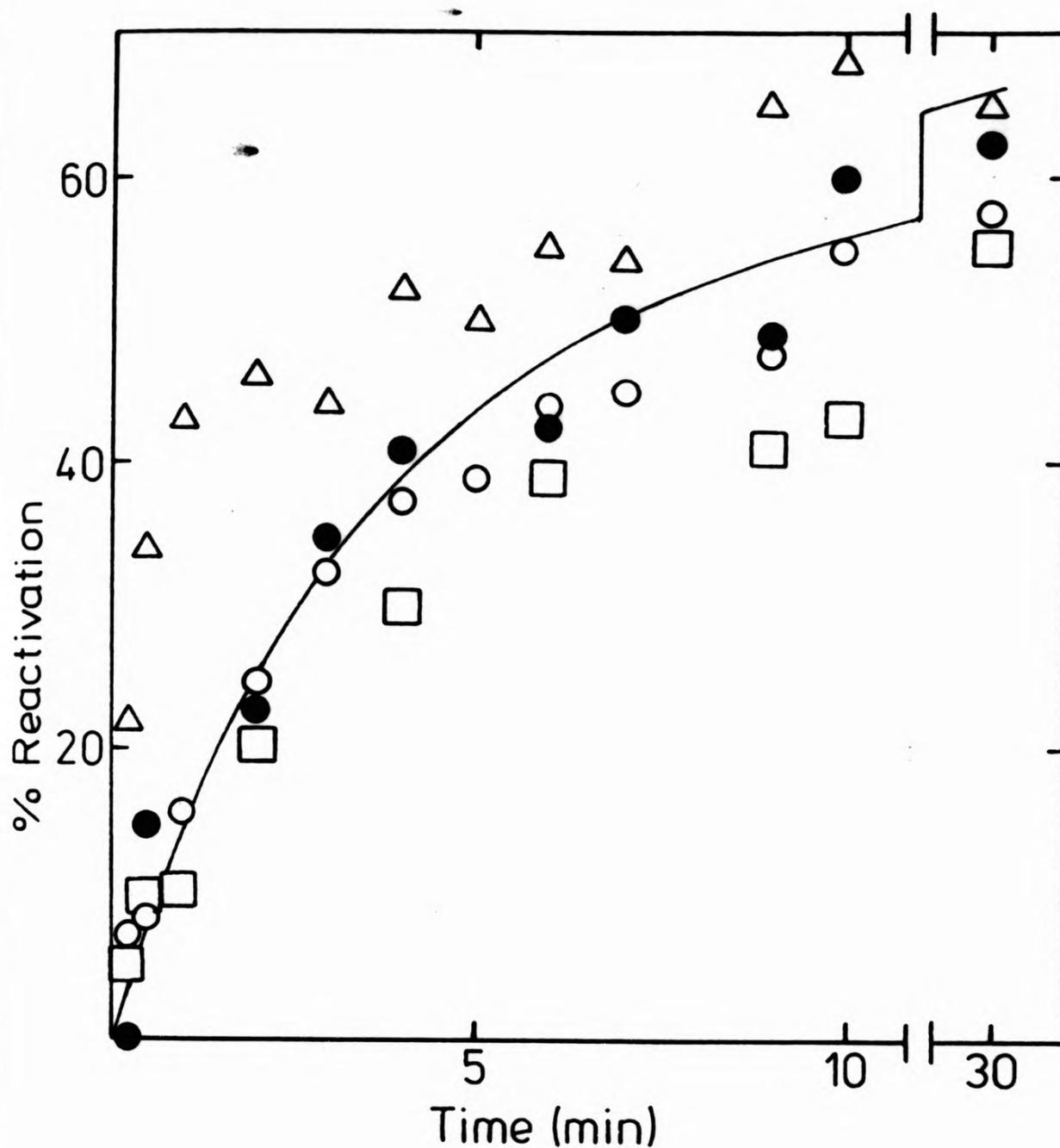
Figure 27.   Reactivation of rabbit muscle phosphoglycerate mutase at
              10 µg ml^{-1} assessed following a one minute proteinase
              pulse of 20 µg ml^{-1}.

Figures 27 and 28.   Renaturing rabbit muscle phosphoglycerate mutase
was diluted to a standard concentration of 10 µg ml^{-1} at the indicated
times and incubated for 1 min at 20°C prior to assay in the presence
of the following proteinases at 20 µg ml^{-1};   ●   , trypsin;
○   , chymotrypsin;   □   , thermolysin.   The full line
was calculated according to the kinetic model of reactivation reported
by Hermann et al. (1983).   Reactivation data in the absence of
proteinases are included for comparison (△   ).
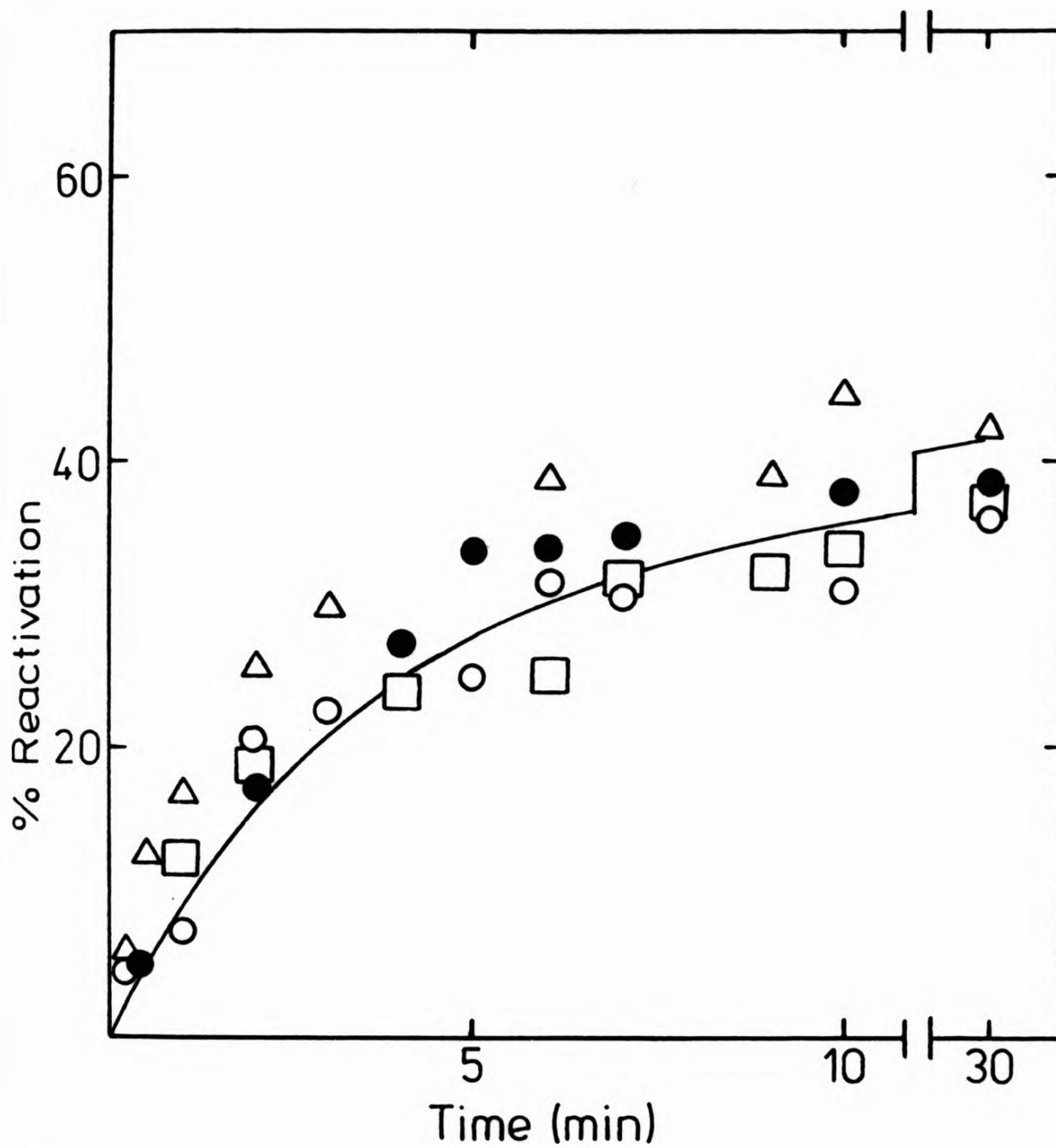
Figure 28. Reactivation of rabbit muscle phosphoglycerate mutase at 30 μg ml^{-1} assessed following a one minute proteinase pulse of 20 μg ml^{-1}.

Figs. 27 and 28 show that the use of a proteinase pulse reduces the yield of reactivation during the first 10 min of renaturation. The reactivation data in the presence of proteinases is now a closer fit to the kinetic model of Hermann *et al.* (1983). There is therefore evidence that intermediates formed during renaturation have activity that is more sensitive to trypsin, chymotrypsin and thermolysin than the native enzyme. The effects at 30 μg ml^{-1} (Fig. 28) are less marked than at 10 μg ml^{-1} (Fig. 27) and it is possible that at the higher renaturing concentration the greater formation of inactive aggregates or their precursors has some effect on the proteolysis of the active enzyme (or its precursors). However, since at this concentration the yield of reactivation is relatively small the experimental error involved in assessing reactivation would be greater relative to the expected difference between the "control" and proteinase pulse treatments.

The observation that the activity of intermediates formed during renaturation are proteinase sensitive implies that these forms must have a relatively "open" structure in comparison with the native enzyme. Changes occuring during subsequent stages must render certain sites inaccessible to proteolytic attack such that the activity of the native enzyme is totally refractory to the 1 min. "pulse". Examples of other enzymes having active or partially active intermediates of renaturation have been considered in section 3.3.4.2.

107

4.3.3.3. Resistance of subunit to proteinases during renaturation.

The integrity of the rabbit muscle enzyme subunit during renaturation following the 1 min. 20 μg ml^{-1} proteinase pulse was assessed using SDS-PAGE as described in section 2.2.6. The percentage of intact subunit with increasing time of renaturation at 30 μg ml^{-1} following proteolysis with trypsin and thermolysin is shown in Fig. 29. The full line was calculated as before representing predicted reactivation of the rabbit muscle enzyme in the presence of proteinase (Hermann *et al.*, 1983). Clearly, Fig. 29 shows that there is extensive degradation of the subunit at early (<2 min) time points during renaturation. With increasing time of renaturation the percentage of intact subunit reaches some 90% indicating that the renatured material and the native enzyme have equivalent resistance to proteolytic degradation. However, at all stages during renaturation the subunit resistance runs considerably ahead of the reactivation profile indicating that inactive but proteinase resistant monomeric and/or dimeric enzyme forms or aggregated material is being produced. This "apparent" formation of subunit may represent the folding of the enzyme to inactive but compact and proteinase resistant aggregates. As discussed above this process is a major competing reaction at higher renaturing concentrations and results in the low yield of reactivation observed. These aggregates would not be resolved from "native" subunit following SDS-PAGE. Alternatively, the possibility that proteolytic inactivation during renaturation results from the cleavage of only a small number of amino acids cannot be excluded. Such an inactive subunit would

108
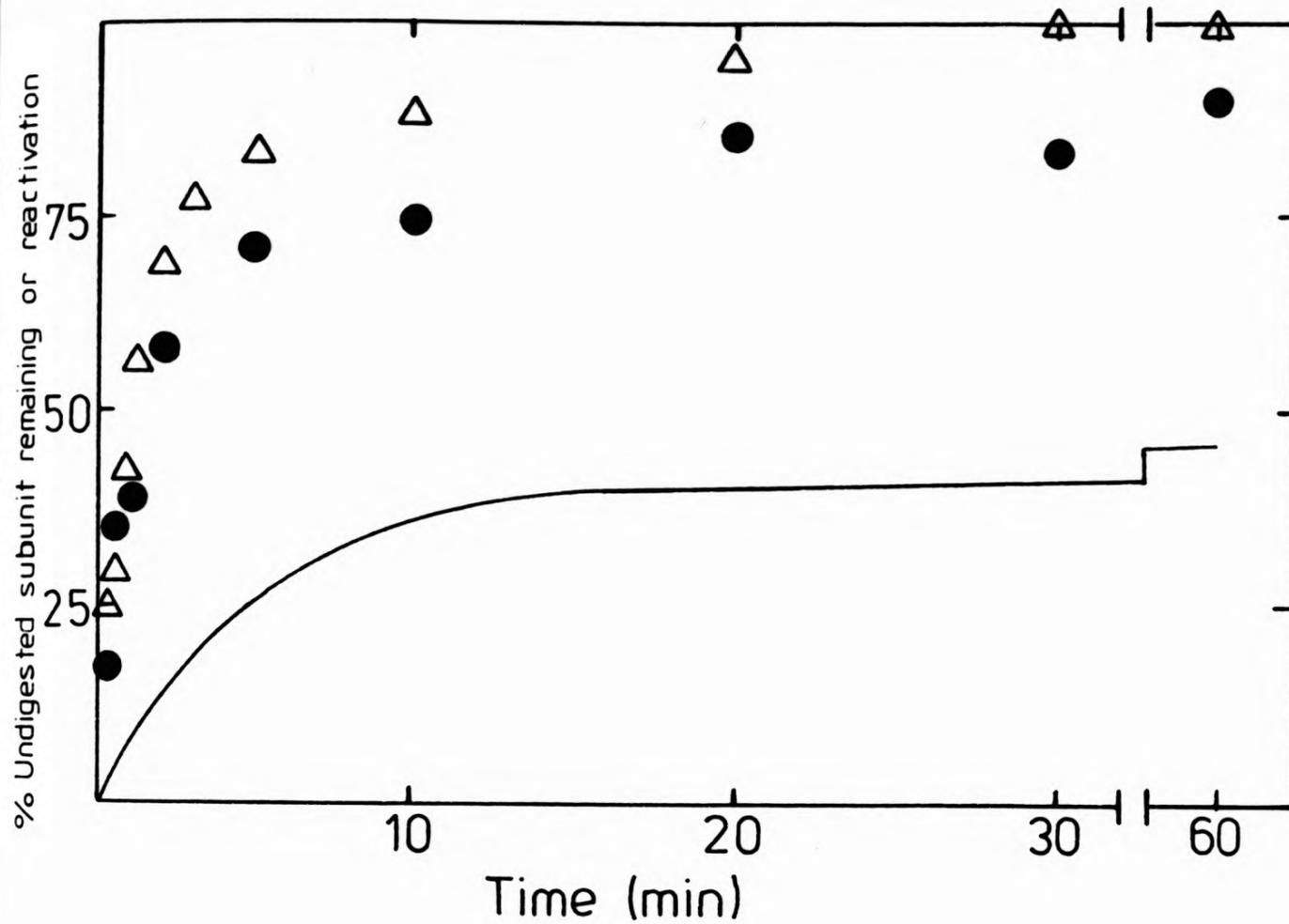
Figure 29. Resistance of rabbit muscle phosphoglycerate mutase
            subunit structure to proteolysis during renaturation
            at 30 μg ml^{-1}.

The percentage of intact subunit remaining was assessed following
SDS-PAGE and densitometric analysis and by comparison with proteinase
treated native controls. Proteolysis for 1 min at 20°C was
performed with renaturing phosphoglycerate mutase diluted to a
concentration of 10 μg ml^{-1} and the following proteinases at a
concentration of 20 μg ml^{-1};   ●   , trypsin;   △   ,
thermolysin.

The full line represents the predicted reactivation in the presence
of proteinases according to the kinetic model of Hermann et al.
(1983).

again not be resolved from active "native" subunit under SDS-PAGE analysis. Price *et al.* (1985a) have reported that inactivation of the native rabbit muscle enzyme by thermolysin occurs with only a minimal decrease in subunit Mr as assessed by SDS-PAGE and with retention of the gross structural features of the native enzyme. Inactivation of the intermediates of renaturation and/or proteolysis of inactive aggregates by this process could explain the "enhanced" integrity of subunit following proteolysis in comparison with the equivalent reactivation data.

Some preliminary experiments were performed using a 20 μg ml^{-1} chymotrypsin pulse and these revealed that no intact subunit remained following proteolysis even after 60 min renaturation. Furthermore, there was no evidence for intact subunit in the proteinase treated native enzyme sample despite the fact that this treatment had no apparent effect on activity. A possible explanation for these observations may be that chymotrypsin cleaves the native or renaturing rabbit muscle enzyme at one or more points but does not produce sufficient disruption of the structure to cause inactivation. Under SDS-PAGE conditions these "nicked" subunits dissociate to smaller fragments $\measuredangle$ 6500 which are not detected. Clearly, these conclusions could benefit from further verification and examination of the above observations.

4.3.4. Comparisons between rabbit muscle and bakers yeast phosphoglycerate mutases.

The rabbit muscle and bakers yeast enzymes are both cofactor dependent phosphoglycerate mutases. Both enzymes have a reaction mechanism of the "ping-pong" type proceeding via a phosphorylated histidine intermediate (section 1.1.2.). The amino acid sequence around this histidine residue is similar and both enzymes are inactivated by the histidine and arginine modifying reagents, (sections 1.2.1. and 1.2.2.). The quaternary structures of the enzymes differ, the bakers yeast being a tetramer and the rabbit muscle being a dimer. However, the subunit Mr of the two enzymes is similar (28,000) and there is evidence of a common structured pattern in the two enzymes reflected in their ability to bind to Cibacron Blue F3GA and to be displaced by a pulse of 2,3-bisphosphoglycerate (Price and Stevens, 1983).

As reported above and in section 3.3.4. the two enzymes can be renatured following denaturation in GdnHCl, although the concentrations at which optimal reactivation is obtained are different. The rabbit muscle enzyme is susceptible to the formation of inactive aggregates at concentrations above 5 μg ml^{-1} (Hermann *et al*., 1983). In contrast the reactivation yield of the bakers yeast enzyme is greater at 50 μg ml^{-1} than at lower concentrations (section 3.3.4.1.).

Despite the above similarities between the two enzymes there are a number of other areas in which they differ. For example, the

110

fluorescence emission spectrum of the two enzymes and its quenching by acrylamide and succinimide have indicated that aromatic residues are considerably more solvent exposed in the rabbit muscle enzyme. The circular dichroism spectra have also indicated that the rabbit muscle enzyme probably has a lower helical content.

The Cleveland mapping technique assesses protein relatedness from cleavage patterns following limited proteolysis in the presence of SDS. Application of this technique to the bakers yeast and rabbit muscle enzymes using the proteinases *S.aureus* V8 proteinase, thermolysin, chymotrypsin and proteinase K as described in section 4.2.4.1. revealed very few common peptides . Only in the case of chymotrypsin (Mr 16500) and proteinase K (Mr 17,000 and 18,000) were any common bands observed.

In view of the range of evidence for homology between the two enzymes it was considered of value to determine the amino acid composition of the rabbit muscle enzyme and compare this with the published data for the bakers yeast enzyme (Fothergill and Harkins, 1982) using the method of Cornish-Bowden (1983).

The tryptophan content of the two enzymes was determined by the spectrophotometric method of Edelhoch (1967) as described in section 4.2.4.2. Values of 4.9 and 4.8 mol^{-1} of subunit were obtained for the rabbit muscle and bakers yeast enzymes respectively. The cysteine content of the rabbit muscle enzyme was determined by titration with NbS_2 as described in section 4.2.4.3. Under native

111

conditions there were two titratable cysteine residues mol^{-1} of enzyme and under denaturing conditions this value was increased to 4 mol^{-1}. There are thus two cysteine residues per subunit of rabbit muscle enzyme with only one being exposed in the native form. The bakers yeast enzyme contains no cysteine (Fothergill and Harkins, 1982). The remainder of the amino acid analysis of the two enzymes was kindly performed by Prof. J. Coggins at the University of Glasgow.

The amino acid compositions of the two enzymes are given in Table 1A of Appendix II. Calculation of the difference index (SΔn) of Cornish-Bowden (1983) described in section 4.2.4.2. gives values of 14.8 (0.061N) between the two sets of data for the yeast enzyme and 147.5 (0.61N) between the rabbit muscle enzyme and Prof. Coggins data for the bakers yeast enzyme. Comparison of the rabbit muscle enzyme with the published data for the bakers yeast enzyme gives a SΔn of 162 (0.67N).

Values of less than 0.42N (SΔn = 94) are considered to indicate strong relatedness between proteins whereas values greater than 0.93N (SΔn = 224) indicate no relationship. From the two rabbit muscle/bakers yeast comparisons it is clear that the Cornish-Bowden index infers only weak relatedness between the two enzymes. Any further conclusions about the homology between the two enzymes must therefore await the determination of the full amino acid sequence and three dimensional structure of the rabbit muscle enzyme.

CHAPTER FIVE : STRUCTURAL

CHARACTERISATION AND THE

DENATURATION AND REFOLDING

OF Schizosaccharomyces pombe

PHOSPHOGLYCERATE MUTASE

5.1. INTRODUCTION.

Phosphoglycerate mutase from *S. pombe* is a monomeric enzyme of Mr 23,000 (Price *et al*., 1985b). Only a limited examination of this enzyme has been performed and this mainly relates to the amino acid residues essential for its activity (Price *et al*., 1985b). The *S. pombe* enzyme is particularly interesting from a structural point of view in that the cofactor dependent phosphoglycerate mutases from the yeasts *Saccharomyces cerevisiae* and *Candida utilis* have Mr values of 110,000; the former enzyme being a tetramer of 28,000 Mr subunits (Hermann *et al*., 1983, Price *et al*., 1983). The cofactor dependent phosphoglycerate mutases from mammalian sources seem to be dimers of Mr approximately 60,000 (sections 1.2.2. and 1.2.4.). It was therefore considered of value to confirm the Mr and monomeric configuration of the *S. pombe* enzyme by ultracentrifugation; these studies were carried out by Prof.R.Jaenicke at the University of Regensburg, West Germany. Sufficient pure enzyme was produced to allow amino acid analysis and partial sequencing to be performed by Prof.J.Fothergill at the University of Aberdeen, Scotland. A comparison of the amino acid composition and sequence data obtained with that of other cofactor dependent phosphoglycerate mutases might indicate why the *S. pombe* enzyme has a monomeric configuration. Similarly, such comparisons might indicate important aspects of oligomer formation in the other enzymes.

In addition to this structural analysis of the *S. pombe* enzyme, the denaturation and refolding of this enzyme were also examined within the overall aims of this thesis. The enzyme was markedly more stable in the presence of 2,3-bisphosphoglycerate and so these studies were performed in the presence and absence of this cofactor whenever possible. Denaturation in GdnHCl was monitored by the loss of catalytic activity and changes in protein fluorescence and circular dichroism. Reactivation of the enzyme during refolding was examined in the presence and absence of a proteinase pulse prior to assay.

5.2. METHODS.

5.2.1. Isolation of phosphoglycerate mutase from
Schizosaccharomyces pombe.

Phosphoglycerate mutase was isolated from *S.pombe* essentially by the
method of Price *et al.* (1985b). All procedures were performed at 4°C
unless otherwise stated.

S.pombe was grown in a liquid culture medium containing per litre,
20 g yeast extract, 25 g KH_2PO_4, 2 g $(NH_4)_2SO_4$ and 20 g glucose for
60 h at 25°C in an orbital incubator. The cells were harvested by
centrifugation at 22,000 g for 20 min (routinely yielding 16 g wet
weight per litre liquid culture) and were subsequently ground for
5 min in a pre-cooled mortar with half their weight of acid washed
sand. Extraction buffer comprised 10 mM Tris-HCl buffer, pH 8.0, 1 mM
EDTA, 0.1% v/v Triton X-100 and 1 mM phenylmethylsulphonyl fluoride
(freshly prepared and added). An equal volume of this buffer was added
to the ground cells. After standing 5 min the crude extract was
recovered by centrifugation at 26,000 g for 30 min and benzamidine
added to a final concentration of 1 mM. The crude extract was then
applied directly to a Cibacron Blue F3GA-sepharose column (5 cm x
4.9 cm²) equilibrated against 10 mM Tris-HCl buffer, pH 8.0. After
washing with this buffer to remove unbound proteins phosphoglycerate
mutase was eluted using a linear gradient formed between 25 ml of Tris
buffer and 25 ml of the same buffer containing 4 mM 2,3-

115

bisphosphoglycerate. The pH of the cofactor containing gradient component was readjusted to pH 8.0 with dilute HCl. Phosphoglycerate mutase emerged as a single peak and fractions containing > 50 units were concentrated six-fold by freeze drying and redissolving in H_2O. Each fraction was analysed for purity by SDS-PAGE and those showing > 90% homogeneity of staining with Coomassie blue R250 were pooled. The pooled enzyme preparation was stored at -18°C and dialysed against > 500 volumes of appropriate buffer before use.

5.2.2. Sedimentation analysis of *S.pombe* phosphoglycerate mutse.

Ultracentrifugation of *S.pombe* phosphoglycerate mutase was performed by Prof.R.Jaenicke at the University of Regensburg, W.Germany. Sedimentation velocity and equilibrium measurements were made using a Beckman Spinco Model E analytical ultracentrifuge as described in detail by Price and Jaenicke (1982).

5.2.3. Denaturation of *S.pombe* phosphoglycerate mutase by GdnHCl.

Loss of catalytic activity, changes in fluorescence and changes in circular dichroism observed in the presence of GdnHCl were monitored as described in section 2.2.4.

5.2.4. Susceptibility of native *S. pombe* phosphoglycerate mutase to proteinases.

Inactivation and proteolytic degradation of the native *S. pombe* enzyme was examined using four proteinases. Native enzyme, at a concentration of 75 μg ml^{-1}, was incubated in 50 mM sodium phosphate buffer, pH 7.5, at 20°C in the presence of the following proteinases; trypsin, chymotrypsin, thermolysin and proteinase K (all at 75 μg ml^{-1}). Samples taken after up to 120 min incubation were assayed for activity and analysed for proteolytic degradation by SDS-PAGE.

5.2.5. Renaturation of *S. pombe* phosphoglycerate mutase.

Reactivation of *S. pombe* phosphoglycerate mutase in the presence and absence of proteinases was examined as described in section 2.2.6. In the absence of proteinase treatment prior to assay samples of renaturing *S. pombe* enzyme were assayed directly from the renaturation mixture.

5.2.6. Amino acid analysis and sequencing of *S. pombe* phosphoglycerate mutase.

The tryptophan content of the *S. pombe* enzyme was determined by the spectrophotometric method of Edelhoch (1967) as described in section 4.2.4.2.   The enzyme concentration was 74 μg ml^{-1} and the GdnHCl concentration 4.2 M. The number and accessibility of cysteine residues

117

was determined by titration with Ellmans reagent NbS_2 as described in section 4.2.4.3.   The enzyme concentration was 74 µg ml^{-1} and the NbS_2 0.25 mM.

The remaining amino acid composition and a proportion of the sequence of the *S.pombe* enzyme were determined by Prof.J.Fothergill at the University of Aberdeen, Scotland. Amino acid analysis was performed following acid hydrolysis of the non-oxidised native enzyme. For sequencing studies the native enzyme was cleaved using the proteinases clostripain and chymotrypsin. The resultant peptides were separated on a C-18 reverse phase HPLC system and sequenced using an Applied Biosystems gas phase sequencer. Detailed methodology of the amino acid analysis and sequencing was essentially as described in Russell *et al.* (1986).

5.3. RESULTS AND DISCUSSION.

5.3.1. Isolation and characterisation of the *S.pombe* phosphoglycerate mutase.

The specific activity of the *S.pombe* enzyme preparation was typically 2000 units mg^{-1} equating to 230 μmole 3-phosphoglycerate metabolized min^{-1} mg protein^{-1}. This specific activity compares well with the value of 250 μmole 3-phosphoglycerate metabolized min^{-1} mg protein^{-1} reported by Price *et al.* (1985b). The minor modification to these authors isolation protocol involving the use of a 0 to 4 mM 2,3-bisphosphoglycerate gradient rather than a 1 mM pulse resulted in a greater yield of activity based on that in the crude extract (70% by gradient, 50% by pulse) with only marginal loss in specific activity.

Analysis of the enzyme preparation by SDS-PAGE indicated > 95% homogeneity of staining with Coomassie blue R250. The Mr of the enzyme was 25,000 ± 1,250 (PLATE 1). The Mr of the native enzyme was determined by gel filtration using Sephacryl S-300 as 23,000 ± 2,300 and using Sephadex G-150 as 23,400 ± 2,400 as described in section 2.2.1.2. These results clearly indicate that the *S.pombe* phosphoglycerate mutase is a monomer of Mr approximately 23,000. This conclusion and the observed enzyme Mr are in close agreement with the findings of Price *et al.* (1985b). These authors reported the Mr of the enzyme to be approximately 25,000 under denaturing conditions using

119

SDS-PAGE and 21,000 ± 2,000 and 23,000 ± 2,000 under native conditions using gel filtration on Sephadex G-150 and gel permeation HPLC respectively.

The K_m of the *S. pombe* enzyme in 30 mM Tris-HCl buffer, pH 7.0, and in the presence of 10 mM grade 1 3-phosphoglycerate for the cofactor 2,3-bisphosphoglycerate was determined at 30°C as 17 ± 1.3 μM. As with the rabbit muscle and bakers yeast phosphoglycerate mutases the *S. pombe* enzyme exhibited approximately 2% of maximal activity in the absence of added cofactor (sections 4.3.1. and 3.3.1. respectively). This activity is again explicable in terms of the trace contamination of the grade 1 3-phosphoglycerate with cofactor and this residual concentration was included in the determination and calculation of the K_m. Price *et al.* (1985b) have reported a K_m of 0.5 mM for 3-phosphoglycerate in the presence of 0.3 mM 2,3-bisphosphoglycerate. However, these authors did not examine the kinetics of the cofactor.

Initially the dialysed enzyme preparation was stored at -18°C. However, it rapidly became evident that repeated freezing and thawing of the enzyme led to considerable loss of activity. Approximately 90% of activity was lost following 5 freeze/thaw cycles performed either over a number of days or repeatedly within a number of hours. The effect thus appeared to be the result of the freezing and thawing process rather than the period of storage. In contrast to this effect the activity of the undialysed enzyme was completely stable to the freeze/thaw effect (Fig. 30 a and b). Furthermore, addition of 2,3-
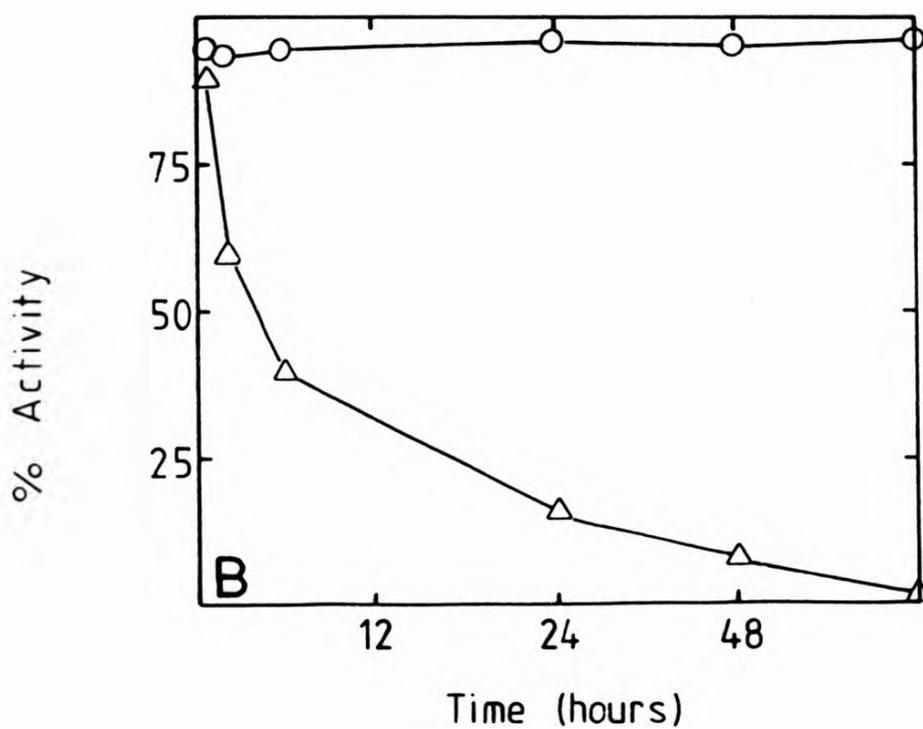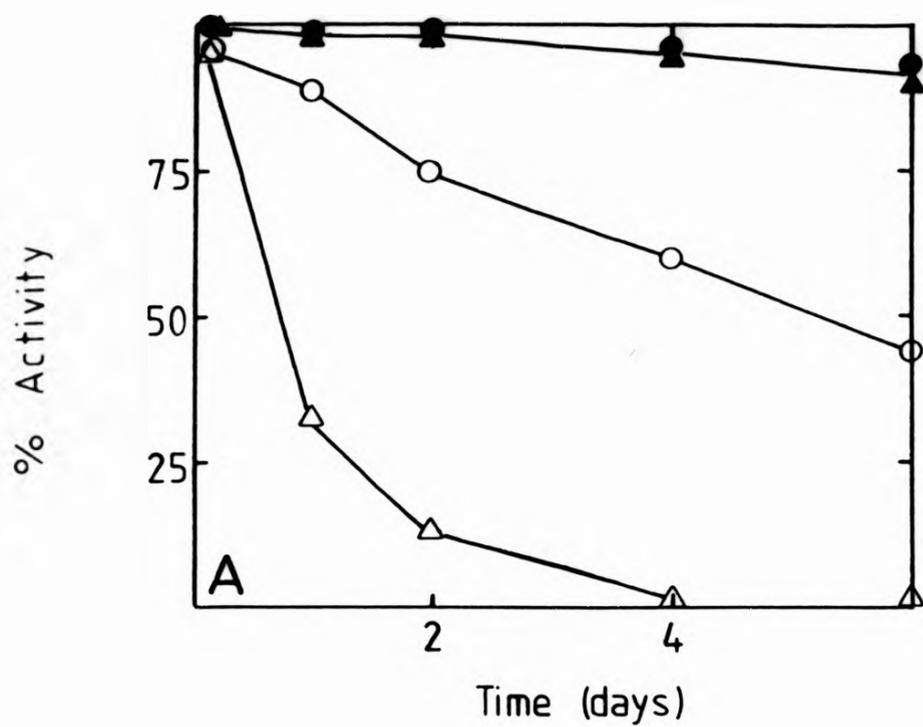
Figure 30. Stability of S pombe phosphoglycerate mutase in 50 mM
           sodium phosphate buffer, pH 7.5.

30A stability at ; ● ○ , 4°C and ▲ △ , following
successive freezing (-18°C) and thawing. ○ △ , dialysed (apo-)
enzyme; ● ▲ , undialysed (phospho-)enzyme.

30B stability of dialysed (apo-)enzyme towards freezing (-18°C) and
thawing. △ , successive freeze/thaw cycles; ○ ,
stored as individual aliquots and thawed once for assay.

bisphosphoglycerate at a concentration of 2 mM to the dialysed enzyme completely restored its resistance to the effect (data not shown). The sensitivity of the enzyme to inactivation by this method is therefore reversible and appears to be dependent on its state of phosphorylation, since dialysis and addition of 2,3-bisphospho-glycerate (which acts to phosporylate the enzyme (section 1.1.2., Fig. 2)) appears to interconvert the resistant and susceptible states.

Enzyme inactivation by processes of freezing and thawing is a fairly common phenomenon particularly at protein concentrations below 2mg ml^{-1} (Scopes, 1982). In the case of phosphoglycerate mutases certain reports have indicated that the cofactor dependent enzymes from rabbit muscle and bakers yeast may be susceptible to freezing and thawing (Cowgill and Pizer, 1956, Grisolia and Carreras, 1975). Substances such as glycerol and sucrose are routinely used to stabilize proteins in solution through their action in reducing water activity (Scopes, 1982). During freezing, solutions containing these substances do not freeze or form a thin slurry of ice surrounding a core of glycerol solute mixture.

Inactivation of enzymes following freezing occurs through a number of possible processes. For example, as a solution freezes the least soluble solute component of a buffer may precipitate first and therefore considerable pH shifts may be produced (Orii and Morita, 1977). Such pH shifts can lead to dissociation of malate dehydrogenase into subunits such that on thawing inactivation of this enzyme is

121

accompanied by the formation of high Mr wrong aggregates (Domenech *et al.*, 1987). The reported effects on malate dehydrogenase were most pronounced in sodium phosphate buffer systems. The use of 50 mM sodium phosphate buffer, pH 7.5, during the stability studies on the *S. pombe* enzyme may therefore have exacerbated the effects of freezing and thawing on the dialysed enzyme.

It also seems possible that freezing of proteins containing internal water molecules may cause some form of structural disruption. Evidence of internal molecules has been reported in a number of proteins where atom packing is less than perfect (Creighton, 1978, Bode and Schwager, 1975, Quiocho and Lipscomb, 1971). The presence of such included solvent molecules in the dialysed *S. pombe* enzyme could be facilitated by a less compact structure (section 5.3.2.). In addition, it has been proposed that bakers yeast phosphoglycerate mutase possesses a C-terminal flexible tail that modulates access of water to the enzyme active site (Winn *et al.*, 1981). The sequence of this tail contains two basic amino acid residues (section 3.3.3.) which could be attracted to phosphate groups of the substrate or cofactor thereby "shutting" the tail. Whether the *S. pombe* enzyme has such a tail is not known as the C-terminal of the enzyme has not been sequenced (section 5.3.7.). However, the effect of cofactor on such a tail in excluding water from the active site could contribute towards its protective effect on the dialysed enzyme towards freezing.

In view of the stability problems reported above the *S.pombe* enzyme preparation was stored at -18ºC either in the phospho- (undialysed) form or in the apo- (dialysed) form as small aliquots which were then thawed once for use. Subsequent characterisation of *S.pombe* phosphoglycerate mutase was performed using the apoenzyme in the presence and absence of 2 mM 2,3-bisphosphoglycerate to determine whether the observed differences in stability of the two forms were reflected in any other properties of the enzyme.

5.3.2. Sedimentation analysis of *S.pombe* phosphoglycerate mutase.

Ultracentrifugation of the *S.pombe* enzyme was performed by Prof. Jaenicke in the presence and absence of 2 mM 2,3-bisphosphoglycerate as described in section 5.2.2. Sedimentation velocity measurements allowed the calculation of the corrected sedimentation coefficients ($S^{o}_{w,20}$) of 2.78 S and 2.3 S in the presence and absence of 2,3-bisphosphoglycerate respectively. These sedimentation coefficients are in agreement with tabulated values for proteins of Mr approximately 20,000. The difference between the two sedimentation coefficients suggests that upon phosphorylation the equivalent hydrodynamic volume of the enzyme changes to a more compact spherical shape thereby allowing more rapid sedimentation per unit centrifugal field. The more compact structure of the phosphoenzyme could explain its enhanced stability to periodic freezing and thawing and might also be expected to affect subsequent comparisons between the two forms of the enzyme.

123

Equilibrium sedimentation of the *S.pombe* enzyme in the presence and absence of 2,3-bisphosphoglycerate resulted in the equilibrium distributions given in Appendix II as Figs. 1A and 2A respectively. From the slopes of the ln absorbance against r^2 plots the Mr of the *S.pombe* enzyme was calculated as 19,900 and 22,450 in the presence and absence of cofactor using the equation described in Appendix II. In the calculation of the Mr a partial specific volume (v_2) of 0.736 cm³ g⁻¹ and a density (ρ) of 1.0021 g cm⁻³ were used. These figures were determined from the amino acid composition data reported in section 5.3.6.

The presence of 2,3-bisphosphoglycerate had not been expected to alter significantly the calculated Mr of the enzyme as was observed in Figs. 1A and 2A. Even if bound to the active site of the enzyme the Mr would not have been expected to increase by more than the Mr of the cofactor (Mr ≈ 360). However, careful examination of the equilibrium distributions in Figs. 1A and 2A shows that the migration range over which the distribution was monitored differ in the two figures. In the presence of cofactor (Fig. 1A) this range is larger and therefore includes any lower Mr impurities and/or proteolytic fragments. Such contaminants may have been present in the enzyme preparation but could also have arisen during transport of the enzyme and the lengthy process of equilibrium sedimentation performed at 25°C. The inclusion of these species in the distribution may have produced the lower calculated Mr in the presence of 2,3-bisphosphoglycerate.

124

The Mr of the native *S. pombe* enzyme from the equilibrium sedimentation data is thus approximately 22,500. Importantly there was no evidence of any higher Mr species in the equilibrium distribution which would indicate oligomer formation. The *S. pombe* enzyme is therefore monomeric as previously reported (section 5.3.1. and Price *et al.*, 1985b). The confirmation of this structural configuration has particular relevance to the studies reported here. The *S. pombe* enzyme is the only documented example of a monomeric cofactor dependent phosphoglycerate mutase. The enzyme from bakers yeast is a tetramer of Mr 110,000 (Hermann *et al.*, 1983) whereas those from mammalian sources seem to be dimers of Mr approximately 60,000 (section 1.2.5.). Other cofactor dependent phosphoglycerate mutases have not been extensively characterised in terms of their structural configurations. The monomeric *S. pombe* enzyme therefore allows interesting comparisons to be made with the oligomeric enzymes. These comparisons are considered, where relevant, in the studies reported within this chapter.

5.3.3. Denaturation of *S. pombe* phosphoglycerate mutase by GdnHCl.

5.3.3.1. Loss of catalytic activity.

The activity of the *S. pombe* enzyme in the presence of GdnHCl was determined using the protocol described in section 2.2.4.1. and as applied to the assessment of the bakers yeast and rabbit muscle enzyme activities in GdnHCl (section 3.3.2.1. and 4.3.2.1.

respectively). This method involved the addition of the enzyme to substrate and cofactor in the presence of GdnHCl and subsequent measurement of 2-phosphoglycerate formation over a 3 min period. Therefore using this procedure it was not possible to examine the inactivation of the apoenzyme since on mixing with the substrate/cofactor/GdnHCl assay it would rapidly become phosphorylated. The data reported below was obtained, as with the other mutases examined, using additions of the dialysed (apo-) enzyme which was presumably converted to the phospho-form during assay.

The activity of the *S. pombe* enzyme in the presence of GdnHCl is shown in Fig. 31. There is a small increase in activity at concentrations up to 0.5 M GdnHCl which is followed by a rapid decline over the range 0.5-1.5 M. The midpoint of the inactivation transition is 0.85 M GdnHCl. In comparison with the bakers yeast and rabbit muscle enzymes, where 50% inactivation occurred at 0.65 and 0.55 M respectively, it is clear that the *S. pombe* enzyme is more resistant to perturbation by GdnHCl. This increased resistance could result from different secondary structure composition, for example, a higher α-helical content in the *S. pombe* enzyme. In addition, the different quaternary structures of the enzymes could also affect their inactivation by GdnHCl. For the tetrameric bakers yeast and dimeric rabbit muscle enzymes dissociation into subunits at concentrations < 1 M GdnHCl would result in considerable loss of activity. There is some evidence that these subunits may exhibit a partial activity relative to the native enzyme (Hermann *et al.*, 1983, 1985, sections 3.3.4. and 4.3.3.)
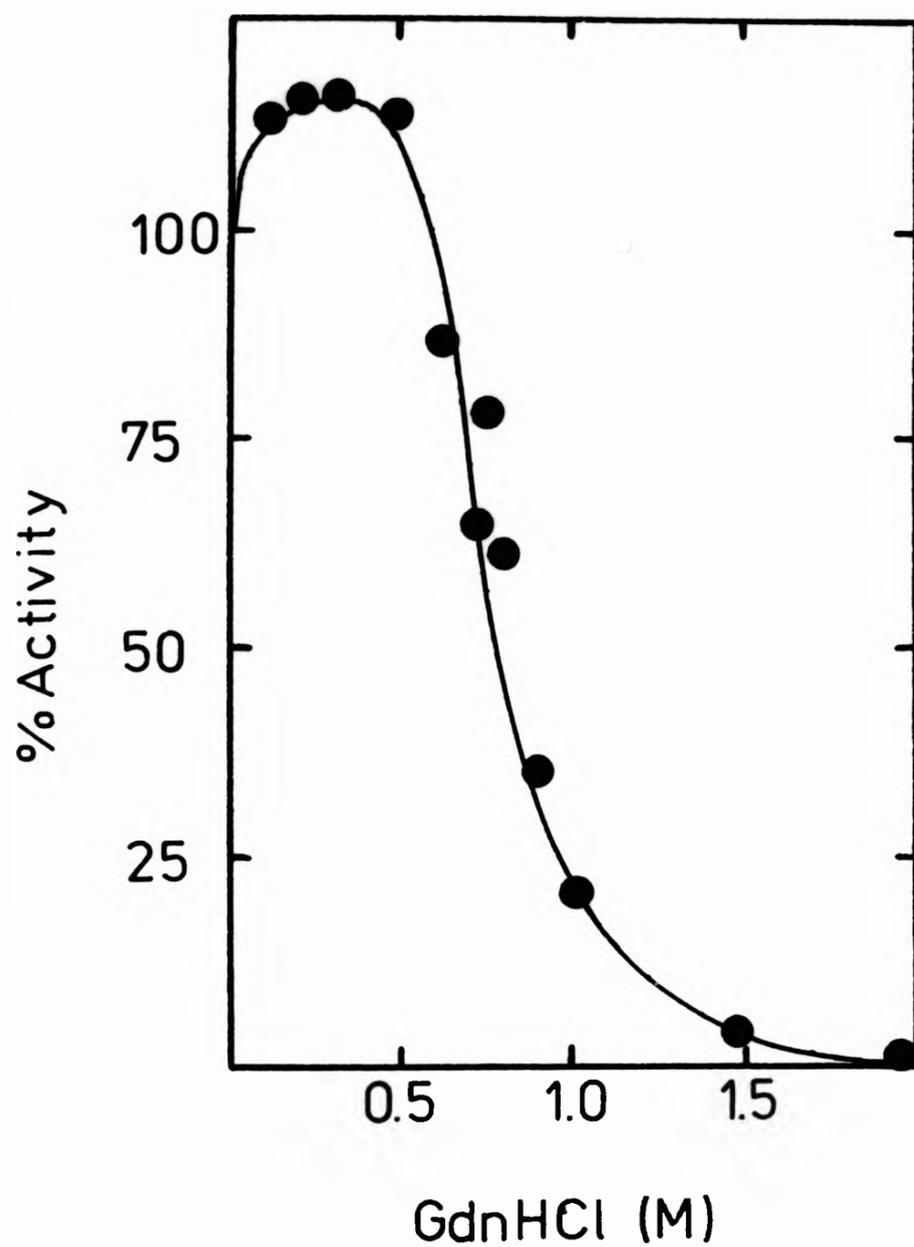
Figure 31. Activity of S. pombe phosphoglycerate mutase in the presence of GdnHCl

Enzyme (1 unit) was added to substrate (10 mM 3-phosphoglycerate), cofactor (0.3 mM 2,3-bisphosphoglycerate) and the indicated concentration of GdnHCl in a final volume of 1 ml.   2-Phosphoglycerate formation was measured over a 3 min period and activity expressed relative to a control containing no GdnHCl (sections 2.2.4.1. and 5.3.3.1.).

and it may be the loss of this activity that subsequently occurs over the higher GdnHCl concentration range (0.75-1.5 M). These possibilities could be examined by following the dissociation of the bakers yeast and rabbit muscle enzymes in the presence of GdnHCl using techniques such as cross-linking (Hermann *et al.*, 1981) or light scattering (Tashiro *et al.*, 1982). However it should be noted that in the case of creatine kinase, an enzyme also showing activity of subunit species (Grossman *et al.*, 1981), inactivation in 0.5 M GdnHCl occurs with the enzyme remaining in its native dimeric state (Tsou, 1986).

The initial increase in activity of the *S. pombe* enzyme observed at GdnHCl concentrations < 0.5 M is particularly interesting (Fig. 31). This effect could be the result of small changes in enzyme conformation which produce enhanced access of the substrate and/or cofactor to the active site. In support of this conclusion Tsou (1986) has presented evidence that the active sites of enzymes may be situated in limited regions of higher structural flexibility. These areas would presumably be more susceptible to structural perturbation by denaturants such as GdnHCl. Strambini and Gonnelli (1986) have also reported an increased "fluidity" of interior regions of equine liver alcohol dehydrogenase at pre-denaturational concentrations of GdnHCl. It thus seems possible that conformational changes such as these could result in the observed activation of the *S. pombe* enzyme between 0 and 0.5 M GdnHCl.

5.3.3.2. Changes in fluorescence.

The fluorescence emission spectra of the *S.pombe* apoenzyme in the presence and absence of 4 M GdnHCl are shown in Fig. 32. The λ max of the native enzyme is 330 nm. In the presence of 4 M GdnHCl there is a red shift to a λ max of 350 nm with the fluorescence intensity at 330 nm reduced to 43% of the native value. The fluorescence spectrum of the enzyme in the presence of 2 M GdnHCl was essentially identical to that obtained in 4 M indicating that the GdnHCl induced changes in fluorescence were completed following incubation at the lower concentration. The spectrum of the native *S.pombe* enzyme is similar in profile to that of the bakers yeast enzyme (λ max of 330 and 328 nm respectively). In the presence of 4 M GdnHCl the spectrum of the *S.pombe* enzyme is similar to those of the bakers yeast and rabbit muscle enzymes and, as discussed previously (sections 3.3.2.2. and 4.3.2.2.), this profile is indicative of solvent exposed aromatic residues in denatured proteins (Teipel and Koshland, 1971). In the monomeric *S.pombe* enzyme the increased solvent exposure of these residues must result from extensive unfolding of native structure. In the presence of 4 M GdnHCl the fluorescence intensity at the λ max could be correlated to the molar tryptophan content of the *S.pombe* enzyme (5.1 mol^{-1}, section 5.3.6.) on the basis of the enzyme molar fluorescence intensities observed for the bakers yeast and rabbit muscle enzymes under the same conditions (section 4.3.2.2.).
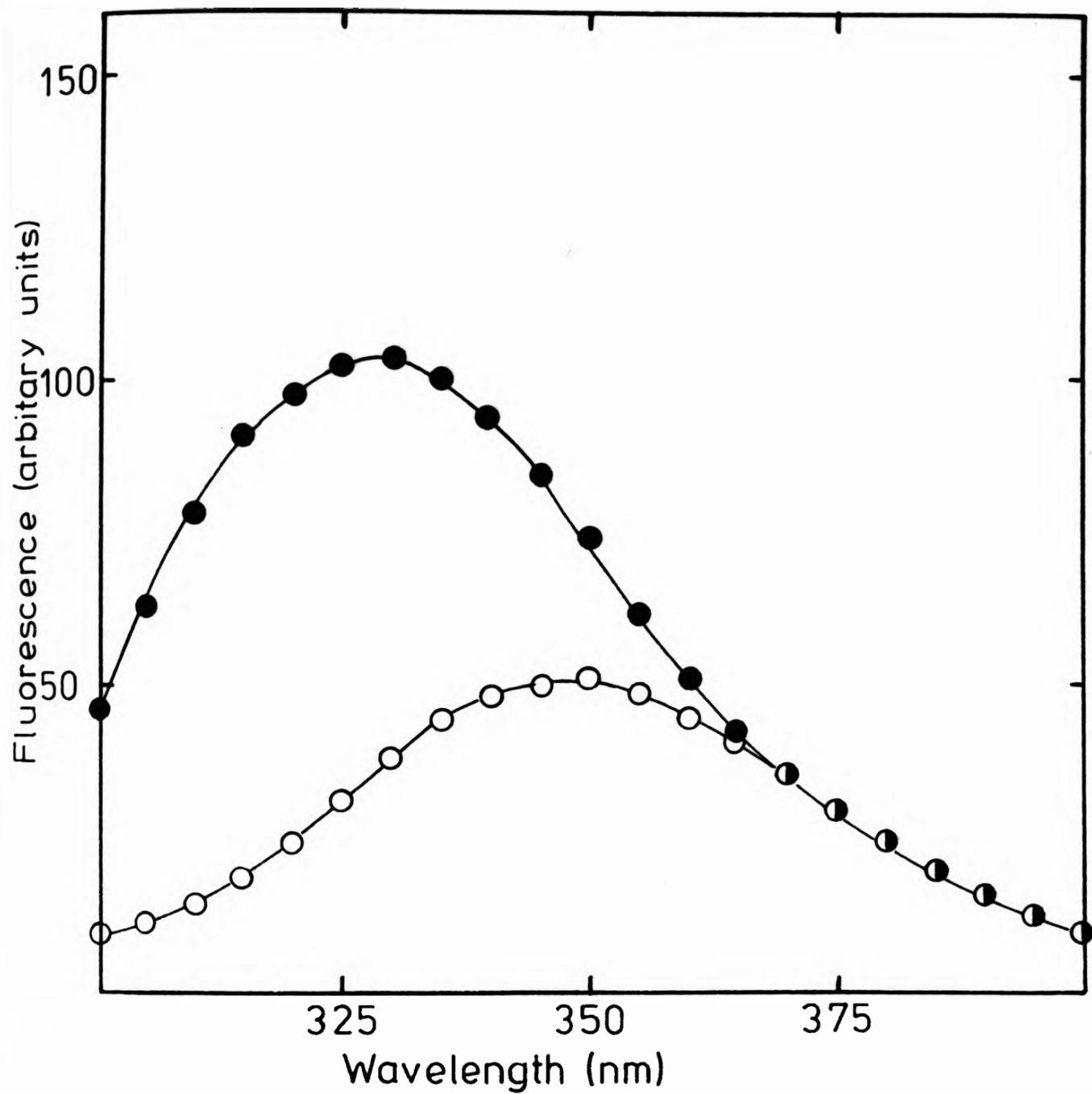
Figure 32.   Fluorescence spectra of <u>S. pombe</u> phosphoglycerate mutase
(apoenzyme) in the presence and absence of 4 M GdnHCl.

<u>S. pombe</u> phosphoglycerate mutase (10.5 μg ml^{-1}) at 20°C.   The
fluorescence excitation was at 290 nm and the emission monitored
between 300 and 400 nm.   Instrument sensitivity was X 15.
●   , enzyme in 50 mM sodium phosphate buffer, pH 7.5;
○   , enzyme in the presence of 4 M GdnHCl.   Spectra of the
<u>S. pombe</u> phosphoenzyme were essentially identical.

There was no detectable difference between the spectra of the apoenzyme in the presence or absence of 2 mM 2,3-bisphosphoglycerate at any of the three GdnHCl concentrations examined. This finding is somewhat surprising in view of the structural differences between the apo- and phospho- forms which have been implied by the sedimentation velocity data (section 5.3.2.). The more compact spherical structure of the native phosphoenzyme might have been expected to alter the relative solvent exposure of some of the enzyme's aromatic residues. It is possible that the aromatic residues in the enzyme which are accessible to solvent are superficially situated and that despite some structural tightening in the phosphoenzyme their overall exposure is still high. The structural changes in the enzyme might also produce decreased exposure of some residues but increased exposure of others such that the overall levels remain unchanged. Alternatively the structural changes between the two forms may produce changes in their fluorescence spectra of a magnitude which is undetectable by the equipment used in obtaining these data.

The fluorescence emmission at 325 nm of the *S. pombe* apoenzyme in the presence and absence of 2 mM 2,3-bisphosphoglycerate as a function of GdnHCl concentration between 0 and 2 M are shown in Fig. 33. For both forms of the enzyme there is a sharp decline in emission between 0.5 and 1.5 M GdnHCl with the midpoints of the transition being 0.95 and 0.8 M for the phospho- and apoenzyme respectively. Thus while the native and denatured spectra of the two forms show no detectable differences, there is evidence of a greater resistance to transition
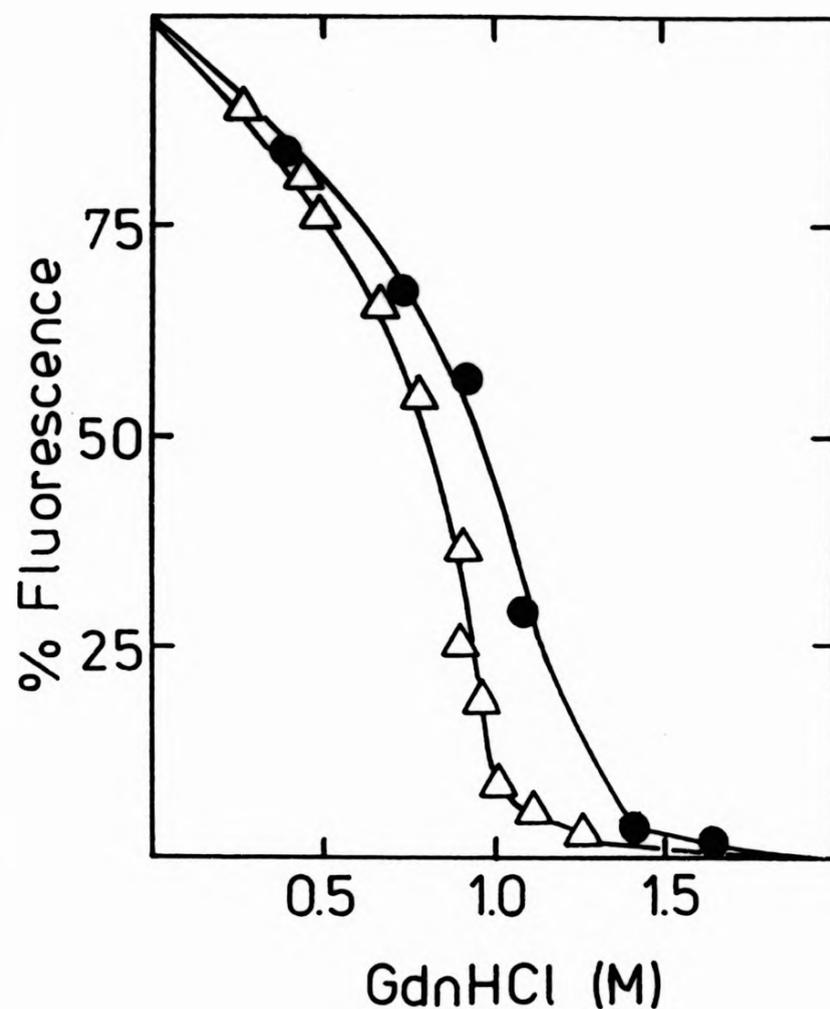
Figure 33. Fluorescence of S. pombe phosphoglycerate mutase in the presence of GdnHCl.

Fluorescence was recorded in 50 mM sodium phosphate buffer, pH 7.5, at 20°C.   The excitation and emission wavelengths were 290 and 325 nm respectively and the change in fluorescence emission is expressed relative to the total change between 0 and 2 M GdnHCl. Fluorescence emission in the presence of 2 M GdnHCl was approximately one third that of the native enzyme (Fig. 32).   △   , S. pombe apoenzyme;   ●   , S. pombe phosphoenzyme.

between these two states in the phosphoenzyme. This increased resistance to perturbation by GdnHCl presumably results from the more compact structure of this form of the enzyme. Comparison of the transitions for the *S. pombe* enzyme in Fig. 33 with similar data obtained for the bakers yeast and rabbit muscle enzymes (Figs. 14 and 24 respectively) indicate some differences. The bakers yeast enzyme exhibits an initial increase in fluorescence intensity followed by a rapid decline in emission with a midpoint of 1.1 M GdnHCl. The rabbit muscle enzyme shows little change in fluorescence emission up to 0.75 M followed once again by a decline with midpoint of 1.1 M GdnHCl. Thus although the activity of the *S. pombe* enzyme appears to be more resistant than the other enzymes to the effects of GdnHCl (section 5.3.3.1.) the structural changes in this enzyme, as judged by changes in fluorescence, occur at lower GdnHCl concentrations than the bakers yeast and rabbit muscle enzymes.

The quenching of the fluorescence of the *S. pombe* apoenzyme in the presence and absence of 0.3 mM 2,3-bisphosphoglycerate was examined using acrylamide and succinimide as described in section 2.2.5. Succinimide is a less effective quencher than acrylamide for deeply buried aromatic residues since its physical size restricts its access to the interior of the protein matrix (Edward, 1970). In addition, succinimide quenching is reduced in aprotic environments and therefore buried residues may provide a micro-environment in which the quenching effect is decreased (Eftink and Ghiron, 1984). The ratio of the quenching efficiencies of succinimide (K_S) and acrylamide (K_A) is

therefore considered a sensitive measure of the overall solvent exposure of the aromatic residues of a protein. The Stern-Volmer plot of fluorescence quenching in the *S. pombe* enzyme is shown in Fig. 34. The K_S/K_A ratio calculated from the slopes of the succinimide and acrylamide quenching is 0.48. The ratios for the bakers yeast and rabbit muscle enzymes are 0.33 and 0.43 respectively (section 4.3.2.2.). Eftink and Ghiron (1984) have reported K_S/K_A ratios between 0.1 for aldolase and 0.64 for lysozyme in a range of multi-tryptophan containing proteins examined using the same protocol as employed here. Clearly, these values indicate that the aromatic residues in the *S. pombe* enzyme are more solvent exposed than those of the bakers yeast and rabbit muscle enzymes. In addition, the exposure of these residues is towards the upper end of the range reported by Eftink and Ghiron (1984).

The presence of 0.3 mM 2,3-bisphosphoglycerate had no effect on the quenching of fluorescence in the *S. pombe* enzyme (Fig. 34). It therefore appears that the structural tightening in the phosphoenzyme, manifest as differences in its sedimentation velocity and thermal stability, does not affect the relative accessibility of the native enzyme's aromatic residues to either of the quenchers examined. This observation is consistent with the identity observed between the fluorescence spectra of the apo- and phosphoenzymes (Fig. 32).

131
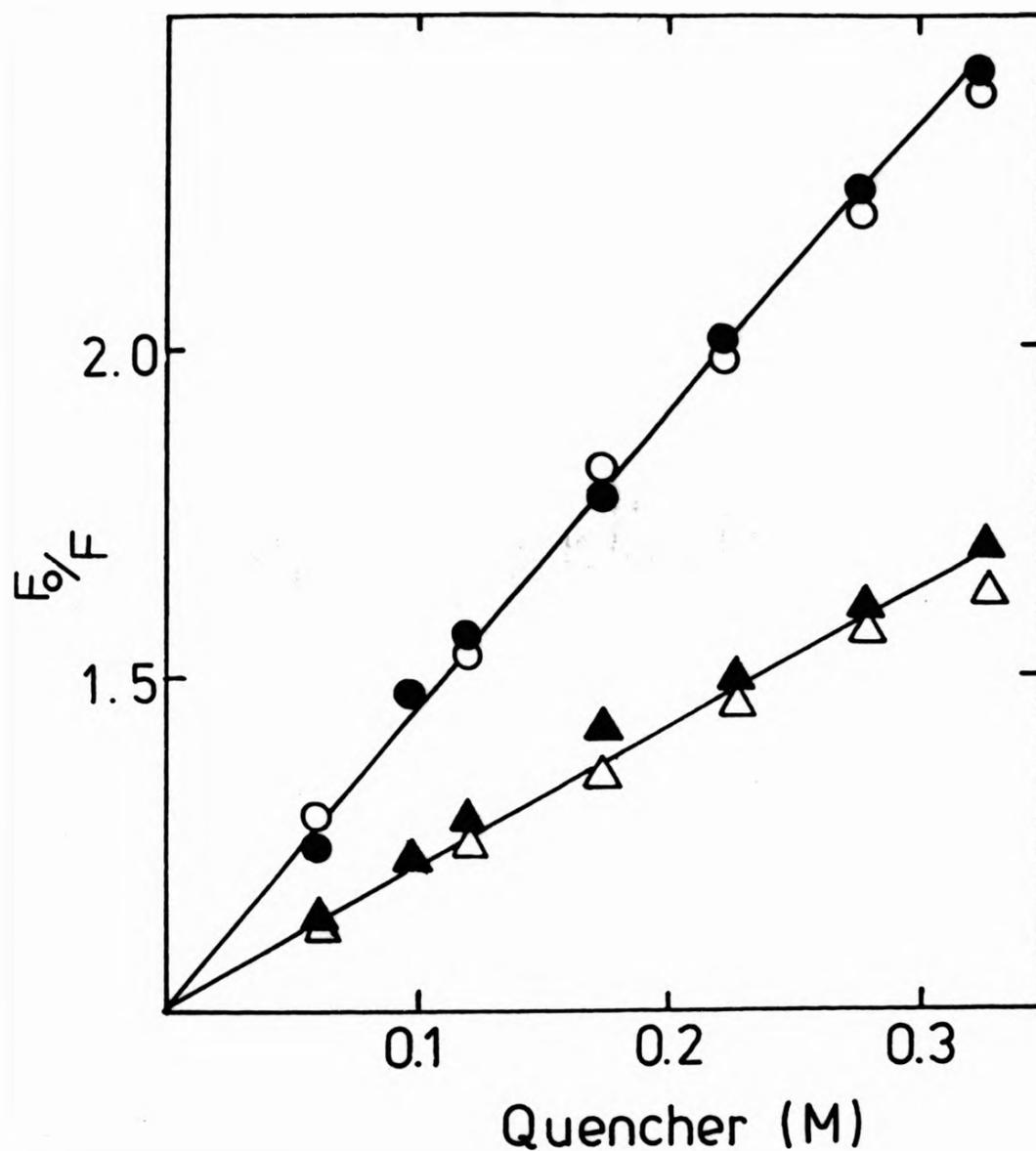
Figure 34. Stern-Volmer plot for quenching of fluorescence in
            S. pombe phosphoglycerate mutase.

On the ordinate F and F_O refer to the fluorescence in the presence
and absence of quencher respectively.   The initial enzyme concen-
tration was 17 μg ml^{-1}. ● ○   , quenching by acrylamide;
▲ △   , quenching by succinimide; ● ▲   , apoenzyme;
○ △   , apoenzyme in the presence of O.3 mM 2,3-bisphospho-
glycerate.

5.3.3.3. Changes in circular dichroism.

The circular dichroism spectra of the *S. pombe* apoenzyme in the presence and absence of 2 mM 2,3-bisphosphoglycerate are shown in Fig. 35. Clearly the spectra of the two enzyme forms differ both in profile and in ellipticity over the wavelength range studied. Using the reference value of pure helix at 210 and 225 nm (Chen *et al.*, 1974) the helical component of the apoenzyme may be estimated as 32 ± 6% and that of the phosphoenzyme 43 ± 12%. An unpublished spectrum of the *S. pombe* enzyme obtained by Price is very similar to that of the phosphoenzyme in Fig. 35 and produces a calculated helical component of 42 ± 2.5% using the method of Siegel *et al.* (1980). Price has indicated that the enzyme from which this spectrum resulted was not extensively dialysed and was therefore likely to be phosphorylated (Price, personal communication). The native *S. pombe* enzyme therefore appears to have approximately 30% helical structure in the apo- form and approximately 40% in the phospho- form. This range of values is considerably higher than the estimates for the bakers yeast and rabbit muscle enzymes of 21 ± 4% and 12 ± 1% respectively. This high helical component may in part explain the increased resistance of the *S. pombe* enzyme to inactivation by GdnHCl (section 5.3.3.1.).

It is difficult to envisage that phosphorylation of the *S. pombe* enzyme could result in such a large increase in helical structure implied by the spectra in Fig. 35. These spectra were obtained on separate occasions and their calculation was therefore dependent on separate

132
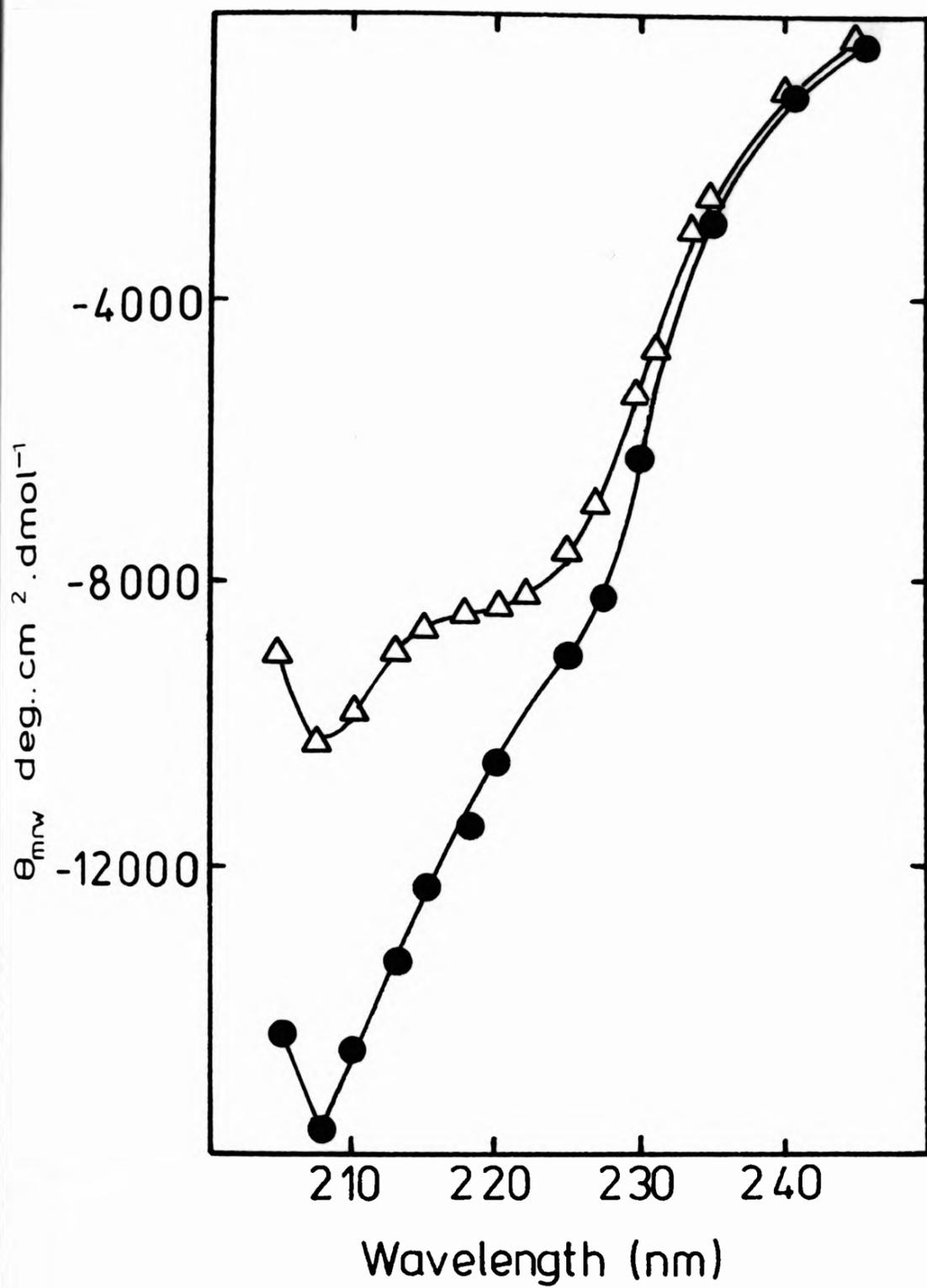
Figure 35.   Circular dichroism spectra of <u>S</u>. <u>pombe</u> phosphoglycerate
mutase.

Enzyme in 50 mM sodium phosphate buffer, pH 7.5, at 20°C.   <u>S</u>. <u>pombe</u>
phosphoenzyme (●   ) and apoenzyme (△   ).

protein determinations. Any inaccuracies in such determinations could in part explain the numerical differences between the two spectra. Nevertheless, the profile of these spectra do differ markedly and there is evidence from other sources that the structure of the two enzyme forms are not identical. It is therefore possible that the observed differences between the two spectra are a true reflection of structural changes such as α-helix or β-turn formation occuring on addition of 2,3-bisphosphoglycerate and therefore phosphorylation of the enzyme.

The spectra of the *S. pombe* apo- and phosphoenzyme in the presence of 4 M GdnHCl are shown in Fig. 36. Both spectra have a profile that is similar to that of unordered structure in proteins (Chang *et al.*, 1978). The ellipticity values differ in the two enzyme forms. However, possible error in protein determination discussed above could in part explain these differences.

The ellipticity at 225 nm of the *S. pombe* apo- and phosphoenzymes as a function of GdnHCl concentrations between 0 and 2 M is shown in Fig. 37. This data is normalised to the total change in ellipticity observed between 0 and 2 M GdnHCl thereby removing the possible contributions of protein concentration. For comparison similar normalised data for the bakers yeast and rabbit muscle enzymes is shown in Fig 38. The midpoints of the transition between 0 and 2 M GdnHCl are 0.95 M for the *S. pombe* apoenzyme, 1.15 M for the *S. pombe* phosphoenzyme, 1.1 M for the rabbit muscle enzyme and 1.2 M for the

133
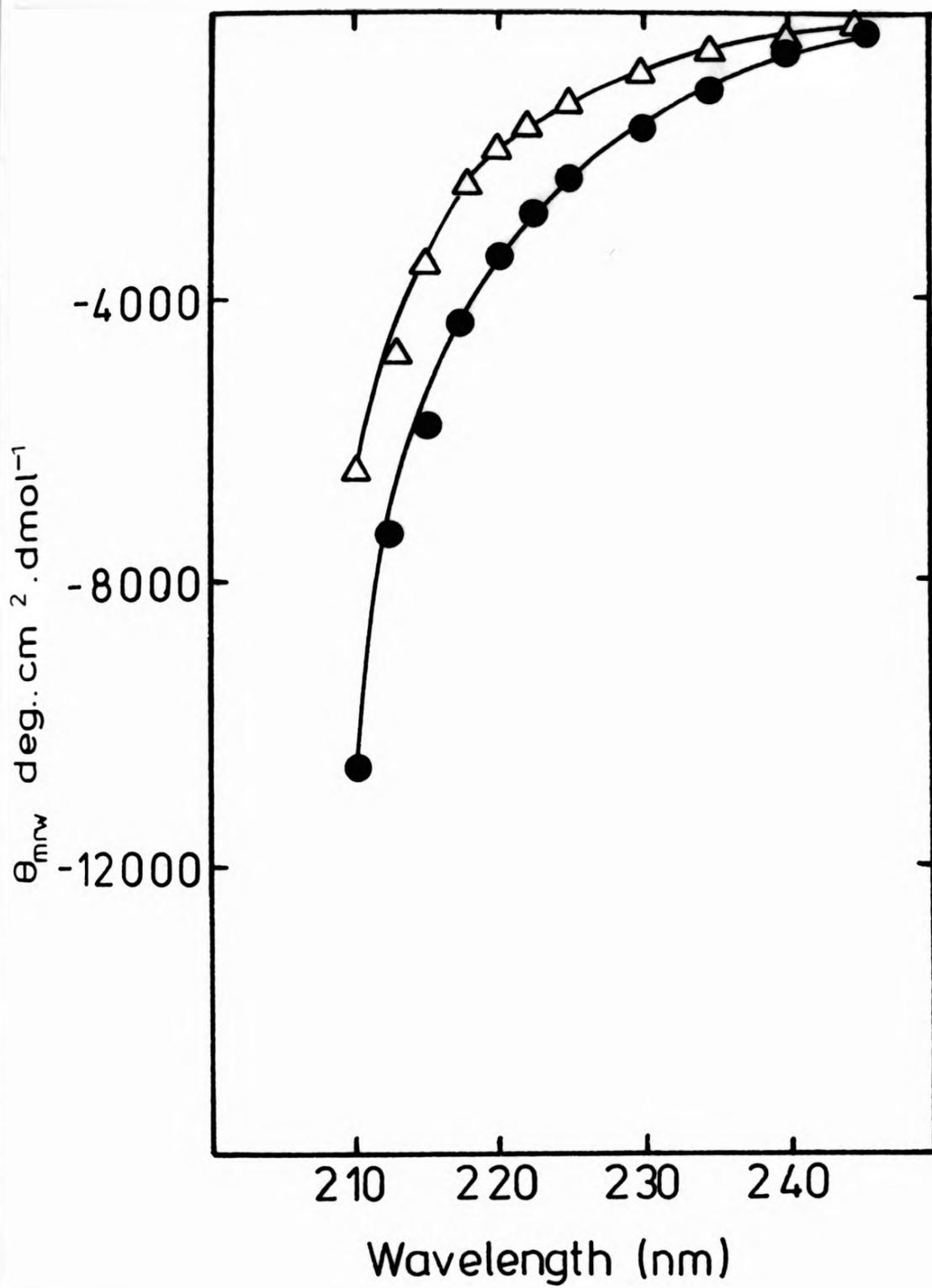
Figure 36. Circular dichroism spectra of S. pombe phosphoglycerate
mutase in the presence of 4 M GdnHCl.

Enzyme in 50 mM sodium phosphate buffer, pH 7.5, at 20°C in the
presence of 4 M GdnHCl.   S. pombe phosphoenzyme (●   )
and apoenzyme (△   ).
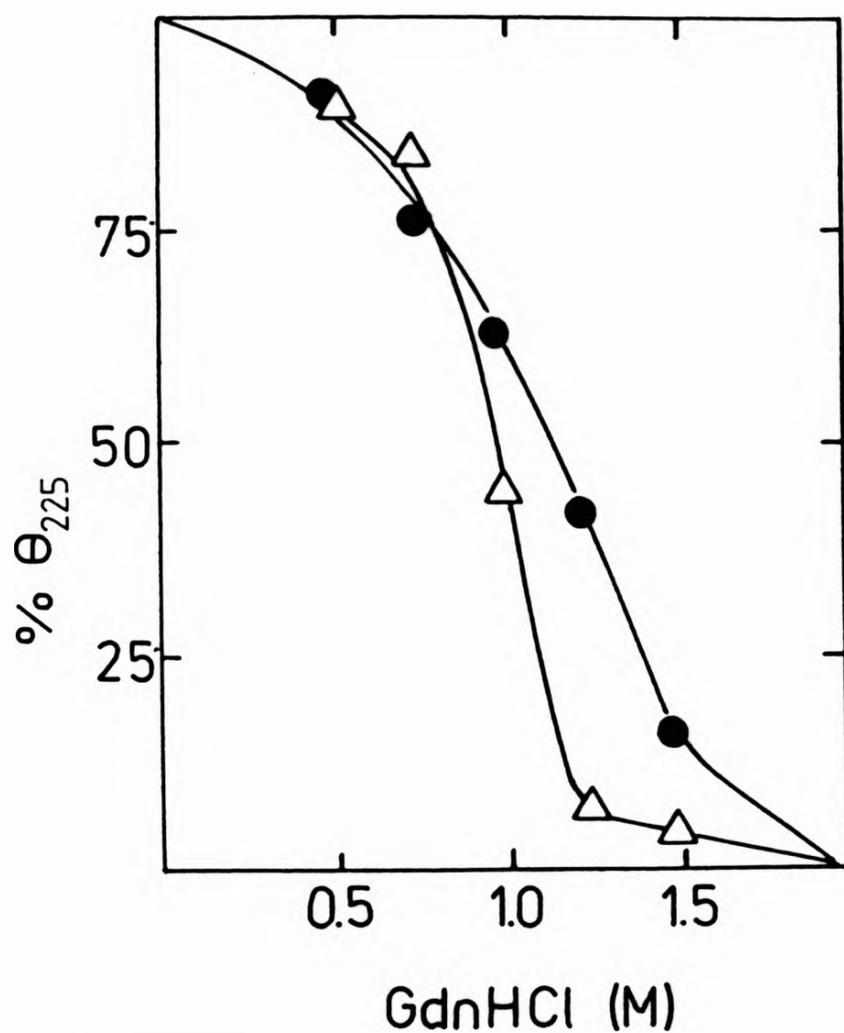
Figure 37. Ellipticity at 225 nm of S. pombe phosphoglycerate
mutase in the presence of GdnHCl.

Circular dichroism was recorded in 50 mM sodium phosphate buffer,
pH 7.5, at 20°C.   Ellipticity at 225 nm is expressed relative to
the total change between 0 and 2 M GdnHCl.   △   , S. pombe
apoenzyme;   ● , S. pombe phosphoenzyme.
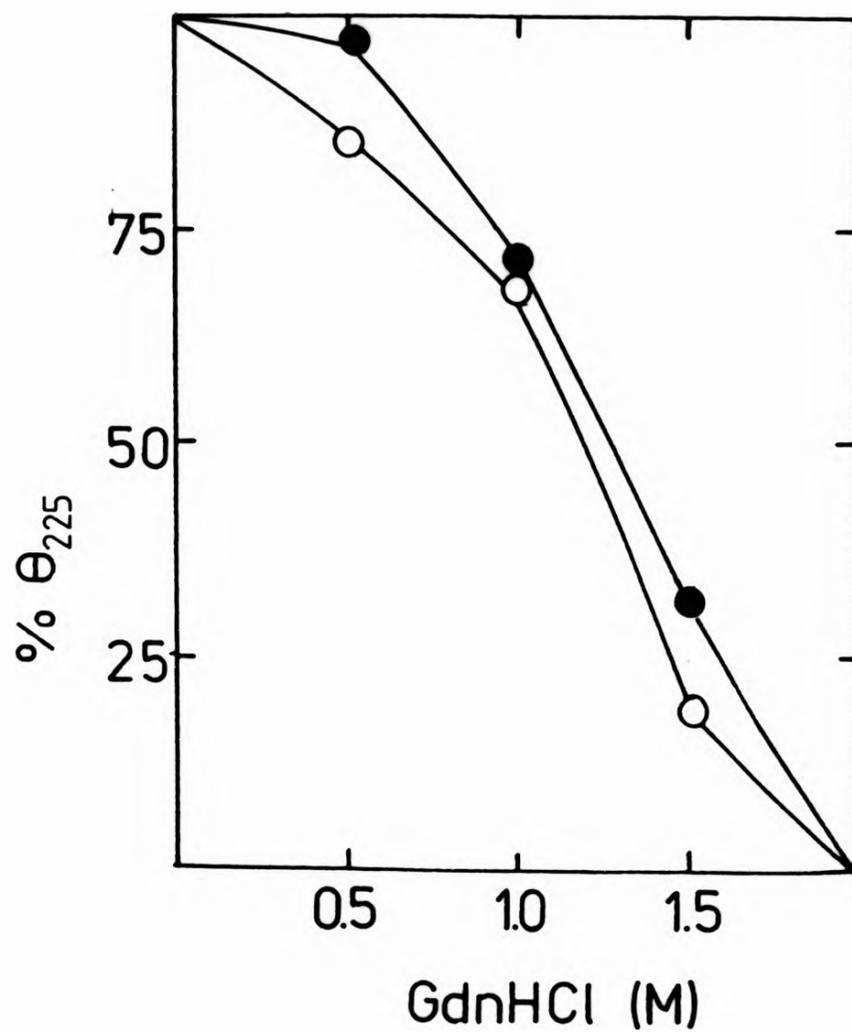
Figure 38. Ellipticity at 225 nm of baker's yeast and rabbit
            muscle phosphoglycerate mutases in the presence of
            GdnHCl.

Circular dichroism was recorded in 50 mM sodium phosphate buffer,
pH 7.5, at 20°C.   Ellipticity at 225 nm is expressed relative to
the total change between 0 and 2 M GdnHCl.   ● , baker's
yeast enzyme;   ○   , rabbit muscle enzyme.

bakers yeast enzyme. The midpoints of all three 2,3-bisphosphoglycerate dependent enzymes thus occur within a similar range. This finding is not necessarily surprising because ellipticity at 225 nm is considered a sensitive index of helical structure. Since helical structure is relatively resistant to denaturation then its pertubation in GdnHCl may occur without influence from other structural elements that are present in the native protein; these elements having been disrupted at lower GdnHCl concentrations. As such it could thus be envisaged that the loss of ellipticity at 225 nm, as a function of its total change between native and denatured values, might occur over a similar GdnHCl concentration range in a variety of proteins.

Once again there is evidence of structural differences between the apo- and phospho- forms of the *S. pombe* enzyme. The lower transition midpoint in the apoenzyme is indicative of a structure more susceptible to perturbation by GdnHCl (Fig. 37). A similar shift to a lower GdnHCl concentration during the fluorescence transition was observed for the apoenzyme and it may be that its more open structure contributes to these effects.

5.3.4. Susceptibility of native *S. pombe* phosphoglycerate mutase to proteinases.

The effects of the four proteinases, trypsin, chymotrypsin, thermolysin and proteinase K on the activity of the native *S. pombe*

phospho- and apoenzymes are shown Figs. 39 and 40 respectively. The phosphorylated enzyme is clearly extremely resistant to the four proteinases with > 75% activity remaining after 120 min incubation (Fig. 39). In contrast the apoenzyme is considerably more sensitive to inactivation with approximately 25% activity remaining after 120 min incubation with thermolysin and < 5% in the case of the other three proteinases (Fig. 40). The marked difference in proteinase sensitivity between these two forms suggests that the phosphoenzyme has a more compact structure with less sites accessible to proteolytic attack. This conclusion is supported by the sedimentation velocity measurements on the *S. pombe* enzyme which have indicated that the phosphoenzyme has a more compact spherical shape. It is also possible that the presence of the phosphoryl group at the active site of the enzyme has some direct protective rôle towards local susceptible sites.

Price *et al.* (1985a) have also reported that rabbit muscle phosphoglycerate mutase is less susceptible to proteolysis by thermolysin when in the phospho- form.

Fig. 40 shows that addition of 2,3-bisphosphoglycerate to the apoenzyme completely restores its resistance to proteolytic inactivation. The minimal inactivation observed in the presence of cofactor is similar to that of the undialysed phosphoenzyme (Fig. 39). The protective effect conferred by the added cofactor is similar to its rôle in restoring the apoenzymes resistance to freezing and
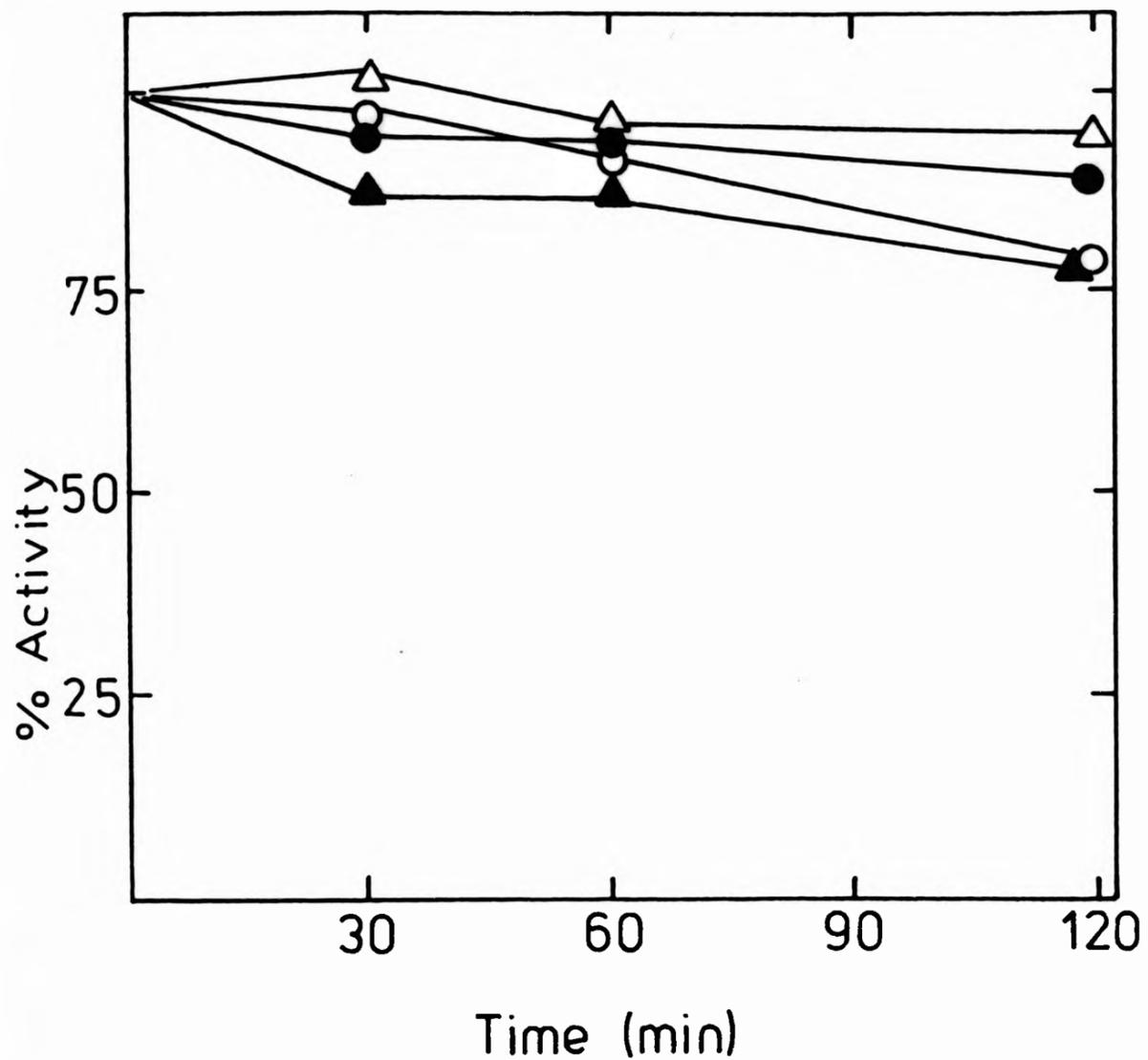
Figure 39. Effect of proteinases on the activity of S. pombe phospho-
glycerate mutase phosphoenzyme.

Native enzyme 75 µg ml^{-1} was incubated in 50 mM sodium phosphate buffer,
pH 7.5, at 20°C in the presence of the following proteinases all at
75 µg ml^{-1}; ●   , trypsin; ▲   , chymotrypsin; ○   ,
thermolysin and △   , proteinase K.
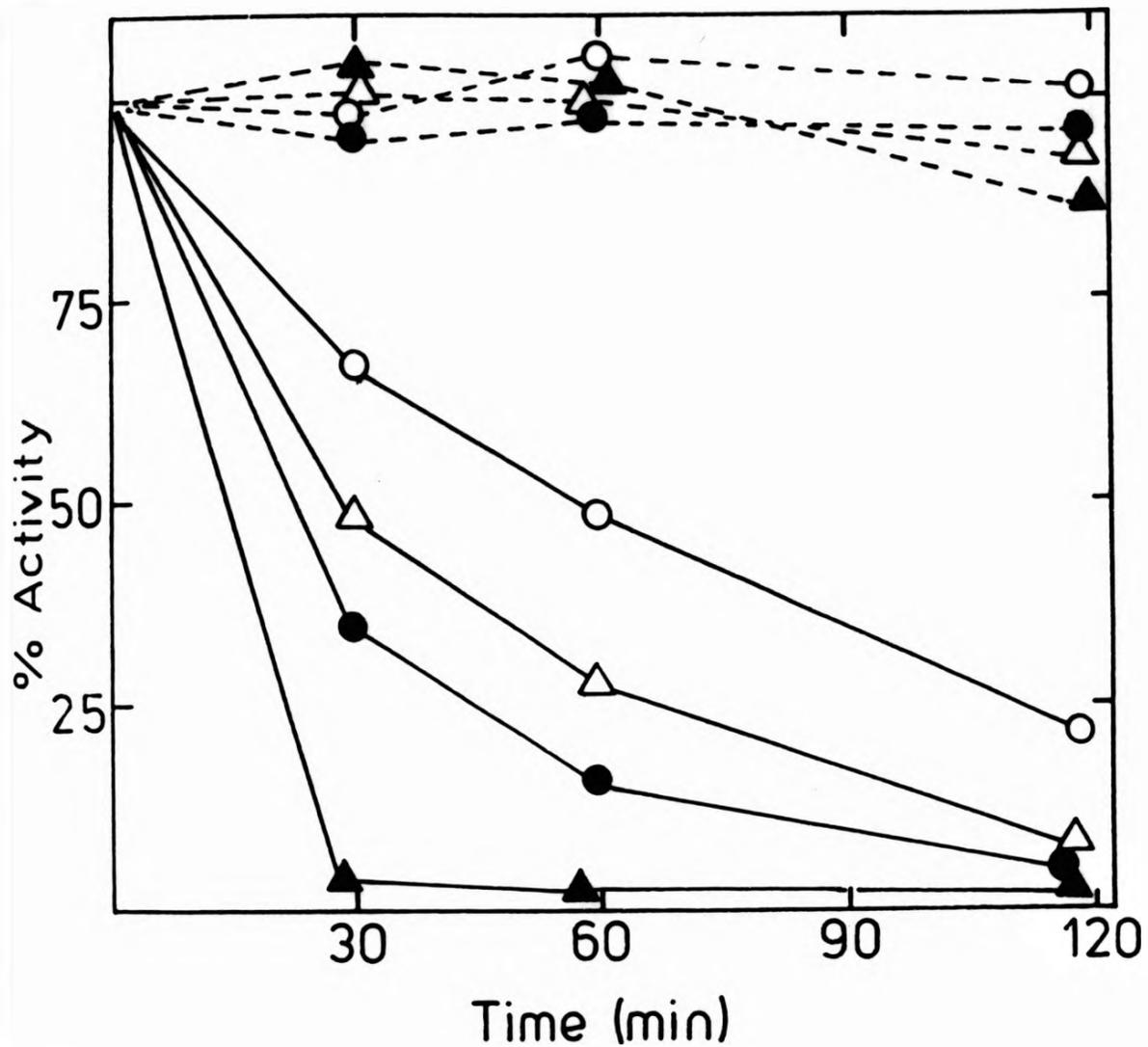
Figure 40.   Effect of proteinases on the activity of S. pombe phospho-
              glycerate mutase apoenzyme.

Native enzyme 75 μg ml^{-1} was incubated in 50 mM sodium phosphate buffer,
pH 7.5, at 20°C in the presence of the following proteinases all at
75 μg ml^{-1};   ● , trypsin;   ▲   , chymotrypsin;   ○   ,
thermolysin and   △   , proteinase K.

Enzyme in the absence (———————) and presence (– – – – – –) of 2 mM
2,3-bisphosphoglycerate.

thawing. In combination these findings suggest that the structural changes between the two forms are readily reversible upon addition or removal of the cofactor.

The proteolytic inactivation of the dialysed (apo-) enzyme shown in Fig. 40 may be compared with that the bakers yeast enzyme (Fig. 16). Similar rates of inactivation are observed in both enzymes. However, in the case of the *S. pombe* enzyme the relative concentration of proteinase to enzyme was 100% w/w (i.e. both 75 µg ml^{-1}) whereas in the case of the bakers yeast enzyme the exposure was at ‹ 10% w/w (i.e. proteinase ‹ 40 µg ml^{-1}, enzyme 400 µg ml^{-1}). The *S. pombe* enzyme is thus approximately 10 fold more resistant to proteolytic inactivation on w/w exposure basis. It is likely that the *S. pombe* enzymes monomeric confirmation and its relatively high helical content contribute significantly to this resistance.

In general the loss of activity in the apoenzyme (Fig. 40) was associated with extensive degradation of the polypeptide chain as judged by SDS-PAGE analysis. In no case was there any evidence of proteolytic fragments of Mr › 6500. The phosphoenzyme exhibited little if any degradation in accordance with the small losses in activity observed (Fig. 39).

5.3.5. Renaturation of *S. pombe* phosphoglycerate mutase.

Renaturation of *S. pombe* phosphoglycerate mutase was initially studied using the apoenzyme. There was some evidence that renaturation of the apoenzyme in the presence of 2,3-bisphosphoglycerate produced higher levels of reactivation. Unfortunately there was not sufficient time to examine fully the potentially interesting effects of the cofactor and/or substrate on the reactivation of the *S. pombe* enzyme. The data considered below therefore refers to the apoenzyme only.

5.3.5.1. Reactivation in the absence of proteinases.

Reactivation of the *S. pombe* enzyme was studied following denaturation in 2 M GdnHCl for 40 min. Reactivation was initiated by a 10 fold dilution resulting in a residual refolding concentration of 0.2 M GdnHCl. These conditions differed from those used in the examination of the bakers yeast and rabbit muscle enzymes where denaturation in 4 M GdnHCl for 15 min followed by 40 fold dilution to 0.1 M was employed. The requirement for the use of these modified conditions resulted from the smaller amounts and lower concentrations of the *S. pombe* enzyme available. The enzyme preparation produced as described in section 5.2.1. was generally between 200 and 400 μg ml^{-1}. Therefore to study refolding of the *S. pombe* enzyme at concentrations of approximately 30 μg ml^{-1} required a maximum 10 fold dilution. From 4 M GdnHCl this would produce a residual concentration of 0.4 M which was considered unacceptable. On the basis of these considerations the 2 to

0.2 M GdnHCl transition was used. Fluorescence measurements had indicated that the spectra of the *S. pombe* enzyme in the presence of 4 and 2 M GdnHCl showed identity following 15 min incubation (section 5.3.3.2.). Furthermore, activity measurements indicated that the enzyme was totally inactive in 2 M GdnHCl (section 5.3.3.1.). Therefore, these modified conditions for denaturation and reactivation were considered suitable to favour the production of the denatured and native enzyme forms.

Preliminary attempts at reactivation of the *S. pombe* apoenzyme produced rather variable and low yields of activity (60 ± 20% of control activity). The reactivation occurred rapidly within 2 min of the initiation of renaturation. Yields of reactivation increased significantly following the inclusion of 1 mM dithiothreitol during the denaturation and reactivation processes. This reductant had been included during earlier studies on the bakers yeast and rabbit muscle enzymes. The improved reactivation of the *S. pombe* apoenzyme at concentrations between 1 and 25 μg ml^{-1} during renaturation is shown in Fig. 41. The rapid reactivation of the *S. pombe* apoenzyme to approximately 85% of control activity within 4 min of reactivation is not significantly affected by the renaturing concentration. This finding is in contrast to the kinetics of reactivation of the other cofactor dependent enzymes examined. In the bakers yeast enzyme increasing renaturing concentration between 10 and 50 μg ml^{-1} leads to increasing rate and yield of reactivation (section 3.3.4.1.). In the rabbit muscle enzyme increasing renaturing concentration above 10 μg

138
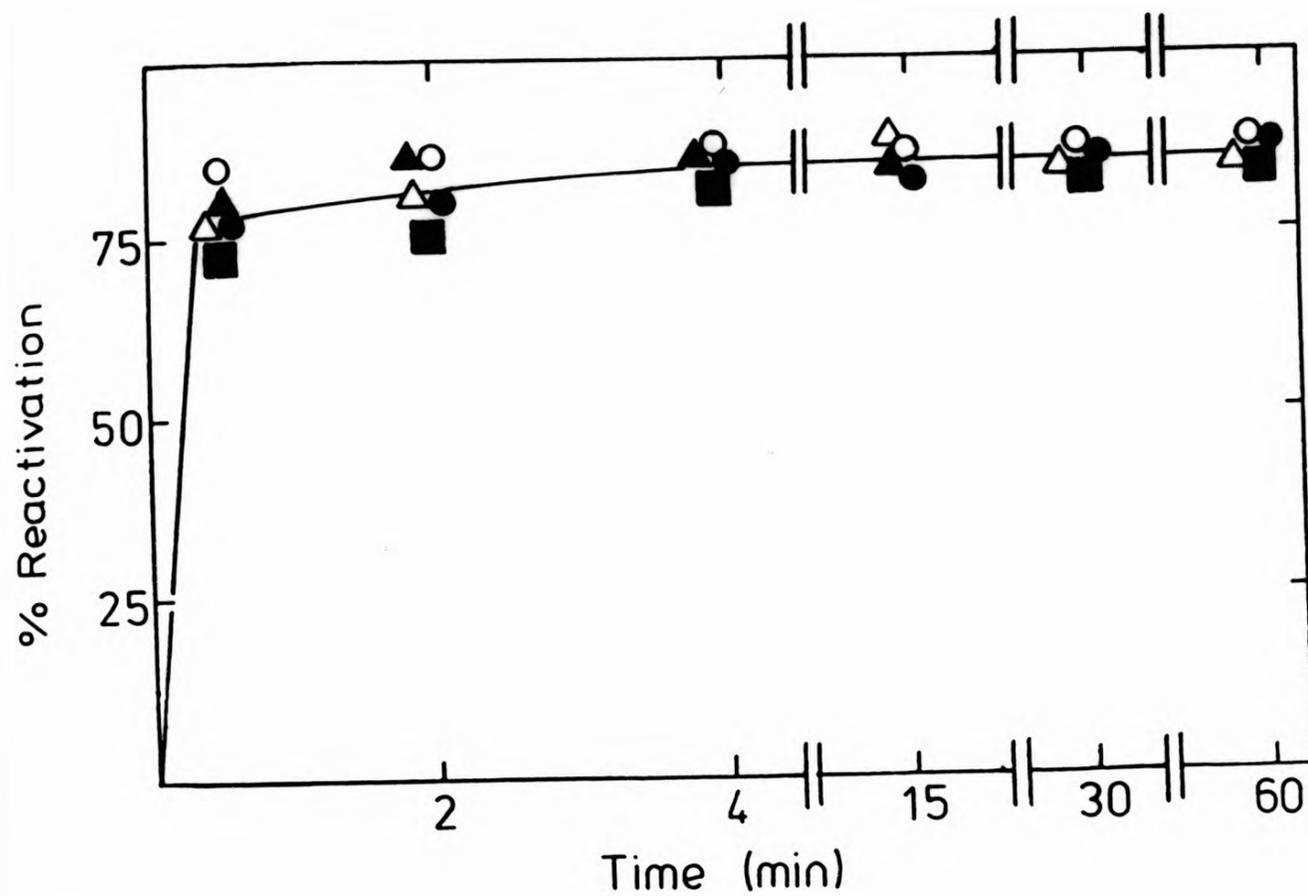
Figure 41. Reactivation of <u>S</u>. <u>pombe</u> phosphoglycerate mutase.

Renaturation of the apoenzyme at; ■ , 25 μg ml^{-1}; ● ,
12 μg ml^{-1}; ○ , 4 μg ml^{-1}; ▲ , 2 μg ml^{-1} and △ ,
1 μg ml^{-1} in 50 mM sodium phosphate buffer, pH 7.5, at 20°C.

ml^{-1} leads to enhanced formation of inactive intermolecular aggregates
(section 4.3.3.1.). In contrast to these systems, the reactivation of
the *S. pombe* enzyme appears to be a first order process with no
dependence on high order reactions of subunit association or
aggregation exhibited in the oligomeric systems.

On the basis of a first order reaction process of reactivation between
0 and 85% reactivation a minimum value for the rate constant may be
calculated. From Fig. 41 it is evident that within 30 s of
reactivation approximately 75% reactivation has occured which
represents 88% of the 0 - 85% reactivation transition. Therefore, at
least three $t_{1/2}$ periods of the first order reaction have elapsed and $t_{1/2}$
is thus < 10 s. From the standard first order equation the rate
constant k is therefore > 0.07 s^{-1}.

The incomplete reactivation of the *S. pombe* apoenzyme may be indicative
of the formation of an inactive enzyme species. The concentration
independence of the yield of reactivation is strong evidence against a
process of intermolecular aggregate formation. A competing process
such as this would have higher order kinetics and therefore would be
reduced at lower enzyme concentrations. It is possible that the
conditions of renaturation, i.e; temperature, buffer composition,
ionic strength, pH, presence of dithiothretiol, might contribute to
the formation of an inactive enzyme species. Indeed, the increased
yields of reactivation of the *S. pombe* apoenzyme following the
inclusion of dithiothretiol suggests that the enzyme could possess one

or more thiol groups which could be "damaged" during the denaturation/renaturation process. As reported in section 5.3.6. the *S.pombe* enzyme does have one titratable cysteine per mole of enzyme although this residue does not appear to be essential for activity in the native form (Price *et al.*, 1985b). Preliminary evidence also indicated that renaturation of the *S.pombe* apoenzyme in the presence of cofactor produced higher levels of reactivation. The enzyme cofactor and/or substrates may thus have some function as a "template" during renaturation thereby enhancing the formation of active species. A further effect on reactivation may result from the residual concentration of GdnHCl (0.2 M) present during renaturation. This concentration has been shown to increase the enzymes activity some 15% above that of the "native" level (section 5.3.3.1.). Both the renaturing enzyme and the native control system (on which the percentage reactivation was based) contained this concentration of GdnHCl. However, if the effect of the residual GdnHCl concentration were different in the native (undenatured) and renatured enzymes then the apparent reactivation might be affected. Clearly a more detailed examination of the reactivation of the *S.pombe* enzyme and the effects of the presence and absence of cofactor and/or substrates might define conditions under which higher levels, if not complete, reactivation would be obtained.

A similar pattern of incomplete reactivation has been reported during studies into the refolding of the monomeric enzyme octopine dehydrogenase (Teschner *et al.*, 1987). As considered in section 1.7.2.

140

this enzyme refolds to yield 70% of the total species in a native and active configuration. The remaining 30% comprises inactive species which are not aggregates but seem to represent incorrectly folded monomers that cannot proceed further to the native configuration. The formation of these species as a competing reaction to the correct pathway of *in vitro* refolding may indicate a possible rôle for co-translational processes controlling the folding of large monomeric proteins *in vivo*. Whilst the *S. pombe* enzyme is a smaller protein than octopine dehydrogenase and may therefore possess fewer folding domains, a similar inability of *in vitro* refolding to mimic the conditions of *in vivo* folding may also contribute to the incomplete reactivation observed for the *S. pombe* enzyme.

5.3.5.2. Reactivation in the presence of proteinases.

The reactivation of the *S. pombe* apoenzyme at 12 μg ml^{-1} was also examined following a 1 min proteinase pulse of 20 μg ml^{-1} trypsin, chymotrypsin, thermolysin and proteinase K. The renaturing enzyme was diluted to the standard concentration of 10 μg ml^{-1} during the proteinase pulse. The proteinase treatments had no effect on the native *S. pombe* apoenzyme activity either in the presence or absence of the residual concentration of GdnHCl during renaturation (0.2 M).

The reactivation of the *S. pombe* apoenzyme following the proteinase pulse is shown in Fig. 42. The proteinases trypsin and thermolysin had no effect on the reactivation of the enzyme in comparison with the

141
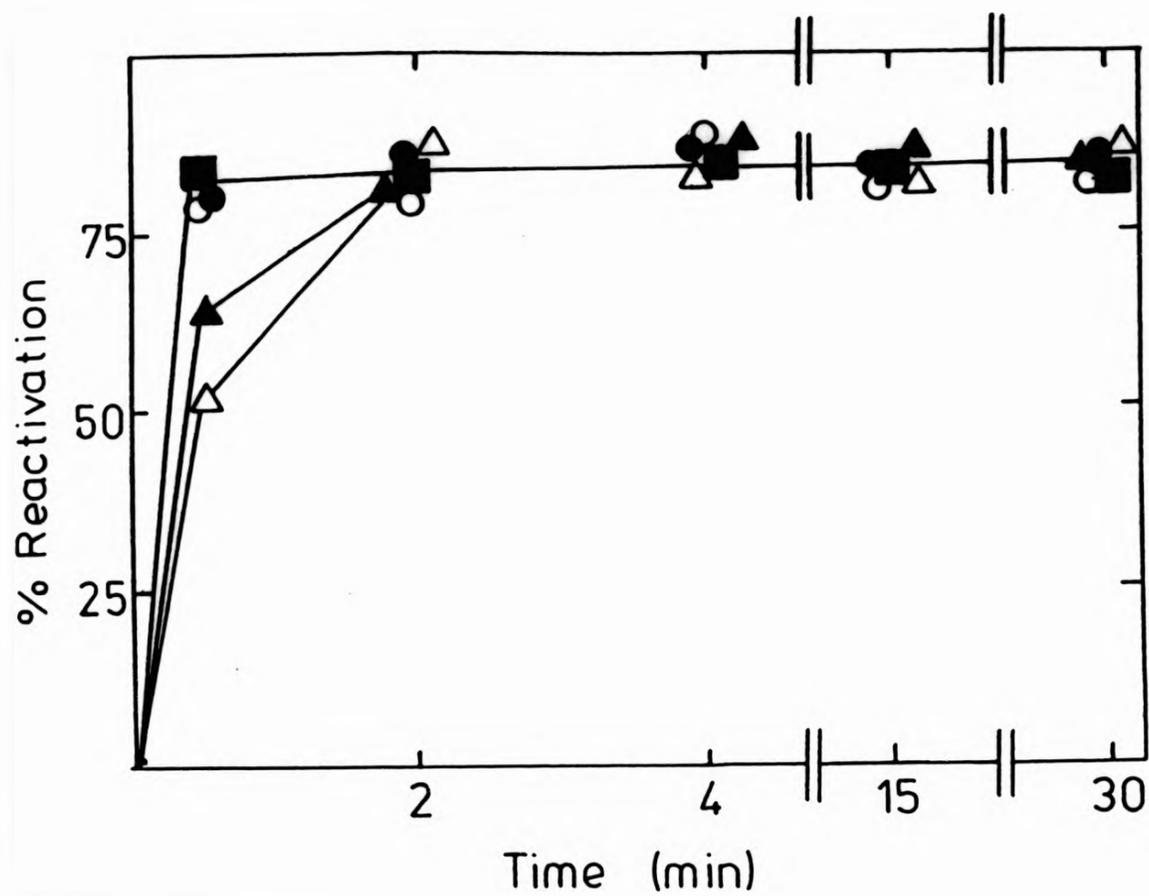
Figure 42.   Reactivation of S pombe phosphoglycerate mutase at
             12 µg ml^{-1} assessed following a one minute proteinase
             pulse of 20 µg ml^{-1}.

S. pombe apoenzyme renaturing in 50 mM sodium phosphate buffer,
pH 7.5, at 20°C was diluted to a standard concentration of
10 µg ml^{-1} at the indicated times and incubated for 1 min at 20°C
prior to assay in the presence of the following proteinases at
20 µg ml^{-1};   ●   , trypsin;   ▲   , chymotrypsin;
     ○   , thermolysin and   △   ▲   , proteinase K.   ■   ,
activity measured following dilution and incubation in the
absence of proteinase.

control system in which samples of renaturating enzyme were incubated in buffer for 1 min. Chymotrypsin and proteinase K produced a small reduction in activity regained at the earliest time points (0.5 min) of renaturation. These findings clearly indicate that the reactivation of the *S. pombe* apoenzyme is accompanied by the rapid formation of a tertiary structure which resembles the native enzyme in terms of its resistance to proteolytic inactivation. Only at very early time points during this process is there any evidence of the presence of a species with marginally differing proteinase susceptibility. This form presumably has a more open or unfolded structure than the native enzyme thereby exposing normally buried sites to proteolytic attack.

The structural changes occurring during reactivation of the *S. pombe* apoenzyme might be more readily examined at lower temperatures of renaturation. Under these conditions the rapid events occurring within the first minutes would be slowed down and would therefore be more accessible to study using the proteinase pulse technique. It might also be possible to use alternative techniques, such as intramolecular glutaraldehyde cross-linking, to freeze species during the renaturation of the *S. pombe* enzyme. The susceptibility of the activity and structure of these species to proteolysis could then be examined in more detail.

Careful comparison of the reactivation of the *S. pombe* apoenzyme shown in Fig. 41 with the control (buffer incubated) reactivation in Fig. 42 reveals that a higher level of reactivation is obtained after 0.5 min

of renaturation in the latter case. This difference results from the technique of incubating the enzyme in buffer for 1 min as a control to the proteinase "pulses" used in the other treatments shown in Fig. 42. Clearly this pulse allows an additional 1 min of renaturation. In comparison during the reactivation experiments described in section 5.3.5.1. samples were assayed directly from the renaturation mixture. After 4 min of renaturation the data in both Figs. 41 and 42 and show equivalent reactivation levels of 85%.

5.3.6. Amino acid analysis of *S.pombe* phosphoglycerate mutase.

The tryptophan content of the *S.pombe* enzyme was determined by the spectro photometric method of Edelhoch (1967) as described in section 5.2.6.   A value of 5.1 moles of tryptophan per mole of enzyme was obtained. The cysteine content of the enzyme was determined by titration with Ellmans reagent as described in section 5.2.6. Under native conditions there was one titratable cysteine residue per mole of enzyme and this value was not increased under denaturing conditions in the presence of SDS. Price *et al*. (1985b) have reported that the *S.pombe* enzyme is not inactivated by the cysteine specific modifying reagent potassium tetrathionate. It thus appears that the cysteine residue titrated in the native enzyme is not essential for activity. The remainder of the amino acid composition of the *S.pombe* enzyme was determined by Prof.J.Fothergill at the University of Aberdeen.

The complete amino acid composition of the *S.pombe* enzyme is given in Table II A of Appendix II. This data is based on an assumed Mr of 23,000 and a mean residue weight of 112. The methods of Cornish-Bowden (1983) for comparison of protein relatedness from amino acid composition are of little use where the proteins differ considerably in size (N_A > N_B ± 18, section 4.2.4.4.). It was therefore not appropriate to compare the *S.pombe* enzyme data with the bakers yeast or rabbit muscle amino acid compositions. However, it was considered possible that the interesting differences in quaternary structure between the three enzymes (*S.pombe*; monomeric, bakers yeast; tetrameric and rabbit muscle dimeric) might originate from their relative "hydrophilic" and "hydrophobic" amino acid compositions. It has been suggested that a high hydrophobic amino acid component may favour oligomer formation (Fisher, 1964). In situations where all hydrophobic residues cannot be buried within the interior of a protein, the formation of an oligomeric structure provides additional shielding of hydrophobic sites in the inter subunit contact regions. A number of indices of amino acid hydrophobicity have been reported (Rose, 1978, Zimmerman *et al.*, 1968, von Heijne and Blomberg, 1979) however the hydropathy scale of Kyte and Doolittle (1982) is reported to overcome some problems associated with these earlier models. The hydropathy of each amino acid is calculated with reference to its water-vapour transfer free energy and its documented distribution within a range of proteins. The hydropathy values for each amino acid are given in Table II A of Appendix II with the more negative values implying more hydrophilic polar residues. By summing the hydropathy

144

values of all the amino acid within a protein and dividing by the number of residues in the sequence a GRAVY score may be obtained (Kyte and Doolittle, 1982). Application of this technique to the amino acid compositions of the bakers yeast, rabbit muscle and *S.pombe* enzymes gives values of -0.262, -0.259 and -0.371. Kyte and Doolittle have examined 84 soluble proteins in their studies and calculated GRAVY values of between approximately 0 and -0.6. Membrane embedded proteins examined had values between 0.5 and 1.5. The less negative GRAVY scores in the bakers yeast and rabbit muscle enzymes are therefore indicative of a slightly higher hydrophobic component relative to the *S.pombe* enzyme. It is thus tempting to speculate that this difference might be related in part to the observed differences in quaternary structure between the three enzymes.

5.3.7. Amino acid sequence of *S.pombe* phosphoglycerate mutase.

A proportion of the amino acid sequence of the *S.pombe* enzyme was determined by Prof.J.Fothergill at the University of Aberdeen. The sequence of the clostripain and chymotrypsin peptides obtained following proteolysis of the *S.pombe* enzyme are given in Table III A of Appendix II. These peptide sequences were compared with the published sequence of bakers yeast phosphoglycerate mutase (Fothergill and Harkins, 1982) and arranged to give optimal alignment of residues. The complete sequence of the bakers yeast enzyme with the aligned peptides is given in Fig. 43. Areas of homology between the two enzymes are indicated by "blocking" and correspond to 50 residues of

```
                    10                    20                        30
1.  H2N-P-K-L-V-L-V-R-H-G-Q-S-E-W-N-E-K-N-L-F-T-G-W-V-D-V-K-L-S-A-K-G-
2.                    H-G-E-S-E-W-N- -K-E-L-A-T-G-V-P-I-V-Y-Y-L-Q-K-
                          (1)                    (2)

        40                    50                60
1.  Q-Q-E-A-A-R-A-G-E-L-L-K-E-K-G-V-N-V-L-V-D-Y-T-S-K-L-S-R-A-I-Q-T-
2.


            70                    80                    90
1.  A-N-I-A-L-E-K-A-D-R-L-W-I-P-V-N-R-S-W-R-L-N-E-A-H-Y-G-D-L-Q-G-L-
2.                                            Y-H-G-D-L-Q-G-L-
                                                              (4)

            100                   110                   120
1.  D-K-A-Q-T-L-K-K-F-G-E-E-K-F-N-T-Y-R-R-S-F-D-V-P-P-P-P-I-D-A-S-S- . .
2.  N-K-D-D-A-R-K-K-W-G-A-E-Q-V-Q-I-W-R-S-Y-D-I-A-P-P-N-G-E-S-L-K-
                      (5) 140                          (7)

        130                   140                   150
1.  P-F-S-Q-K-G-D-E-R-Y-K-Y-V-D-P-N-V-L-P-E-T-E-S-L-A-L-V-I-D-R-L-L-
2.  D-T-A - - - - -E-R-


        160                   170                   180                   190
1.  P-Y-W-Q-D-V-I-A-K-L-V-G-K-T-S-M-I-A-A-H-G-N-S-L-R-G-L-V-K-H-L-E-
2.          S-T-I-V-A-A-I-L-K-G-V-K-V-L-I-A-A-H-G-N-S-L-R-A-L-I-P-D-L-E-
                              (8)        210                   220

                    200
1.  G-I-S-D-A-D-I-A-K-L-N-I-P-P-G-T-I-L-V-F-E-L-D-E-N-L-K-P-S-K-P-S-
2.  G-L-T-G-D-Q-I-V-K-
        (9)     230                   240
1.  Y-Y-L-D-P-E-A-A-G-A-A-V-A-N-Q-K-K-G-COOH
2.
```

Figure 43 . Complete amino acid sequence of bakers yeast phosphoglycerate mutase showing the S.pombe peptide sequences arranged for optimal alignment of residues .

1. Bakers yeast phosphoglycerate mutase (Fothergill and Harkins , 1982)
2. S.pombe peptide sequences (Appendix II)

Peptide labelling is as detailed in Appendix II with - indicating a proposed deletion in the S.pombe sequence .

the 105 sequenced in total. Unfortunately there are no overlaps between the peptides from clostripain and chymotrypsin and the N- and C-terminal regions remain undefined. The sequencing of the *S. pombe* enzyme is being pursued by the University of Aberdeen group and therefore the complete sequence of the enzyme should soon be available.

The homology between the bakers yeast enzyme and the partial *S. pombe* sequence is approximately 48%. This high degree of homology implies a close phylogenetic relationship between the enzymes from the two yeasts. Of particular interest is the considerable homology around histidine residues 8 and 179 of the bakers yeast enzyme sequence since these areas are known to form the active site of this enzyme (Winn *et al.*, 1981). Histidine 8 is thought to mediate the phosphoryl transfer between the 3- and 2-carbon positions of the substrate (section 1.2.1.). It thus seems possible that the active site and mechanism of action in *S. pombe* phosphoglycerate mutase may be similar to that of the bakers yeast enzyme. Further sequence information and mechanistic studies would hopefully verify this tentative conclusion.

In the bakers yeast enzyme two types of intersubunit contact are present (Campbell *et al.*, 1974). Contact I, which is thought to be present in both dimeric and tetrameric phosphoglycerate mutases, is comprised of the residues from loop 3 (133-140) and helix 5 (153-165) of the bakers yeast sequence. Contact II, which is only thought to exist in the tetrameric enzyme is comprised of the residues from helix

2 and adjoining chain sequences (54-76). These regions are as yet largely unmapped in the *S.pombe* enzyme but would be expected to show considerably less homology than other areas in view of the enzymes monomeric configuration. Furthermore, since the *S.pombe* enzyme has an Mr approximately 4000 to 5000 lower than the bakers yeast subunit then its polypeptide chain must be some 35-45 residues shorter. Assuming an evolutionary relationship between bakers yeast and *S.pombe* then deletion of 35-45 amino acids from the former enzymes subunit contact regions would both lower the protein Mr and also produce a its monomeric *S.pombe* "type" configuration. Once again the confirmation of this proposal will depend on further sequence information.

Clearly the *S.pombe* and bakers yeast phosphoglycerate mutases provide a suitable vehicle to study the process of oligomer formation. Since deletion of residues in the subunit contact regions may have led to the formation of the *S.pombe* monomer then it could be possible to introduce suitable deletions or mutations into the bakers yeast sequence (defined by the changes observed in the *S.pombe* enzyme) which might be expected to produce active bakers yeast enzyme in structural conformations of dimers and monomers.

CHAPTER SIX : METAL ION

REQUIREMENT AND REFOLDING

OF COFACTOR INDEPENDENT

PHOSPHOGLYCERATE MUTASES

6.1. INTRODUCTION.

The cofactor independent and cofactor dependent phosphoglycerate
mutases do not appear to be closely related structurally or
mechanistically. This fact may have some evolutionary significance in
relation to the phylogenetic distribution of these two groups (section
1.1.1.). Cofactor independent phosphoglycerate mutases catalyse the
intramolecular transfer of the phosphoryl group between 3- and 2-
phosphoglycerates. A phosphoenzyme intermediate has never been
isolated and therefore the nature of the amino acid residue mediating
this transfer is unknown. In contrast, the cofactor dependent enzymes
catalyse an intermolecular phosphoryl transfer mediated by a histidine
residue (section 1.1.2.).

Cofactor independent phosphoglycerate mutases seem to have a common
structural pattern characterised by a monomeric configuration, Mr of
approximately 60,000, inability to bind Cibacron Blue F3GA and
resistance to vanadate inhibition (section 1.3.4.). In contrast the
cofactor dependent enzymes have a range of structural configurations
(monomer, dimer, tetramer), a basic subunit Mr of between 20,000 and
30,000, bind Cibacron Blue F3GA and are inhibited by vanadate (section
1.2.5.). The pH optima of the two enzyme groups also differ, being pH
9 for the cofactor independent and pH 7 for the cofactor dependent
enzymes (Grisolia and Carreras, 1975). Finally there is some evidence
that the cofactor independent phosphoglycerate mutases have a metal

ion requirement for activity and possibly for structural regain following denaturation in GdnHCl (sections 1.3.1. and 1.3.2.).

On the basis of these mechanistic and structural differences it was considered of interest to further examine the pattern of chelator sensitivity (and thus metal ion requirement) and native Mr of a range of cofactor independent phosphoglycerate mutases. These studies were performed on partially purified extracts from various plant and fungal sources. Refolding of purified phosphoglycerate mutase from wheat germ and *Aspergillus nidulans*, and the effect of metal ions on these reactions, were also examined. Purification of the wheat germ enzyme was performed using two previously published procedures (Leadley *et al.*, 1977, Smith and Hass, 1985) while that for *A nidulans* was designed and developed. It was also hoped to define the nature of the metal ion requirement of these two enzymes by inhibition with chelator followed by the addition of various metal ions in excess. A similar approach had proved successful in implicating a Mn^{2+} ion requirement for the activity of the phosphoglycerate mutases from *Bacillus subtilis* and *Bacillus megaterium* (Watabe and Freese, 1979, Singh and Setlow, 1979).

6.2. METHODS.

6.2.1. Preparation of partially purified phosphoglycerate mutases.

Crude extracts from various sources were fractionated using $(NH_4)_2SO_4$ to give partially purified enzyme preparations for chelator sensitivity and Mr determinations. Standard $(NH_4)_2SO_4$ fractionation involved the addition of solid $(NH_4)_2SO_4$ to the required saturation, stirring for 30 min at 4°C followed by centrifugation at 25,000 g for 20 min to recover precipitated protein. Unless otherwise stated standard 50 mM sodium phosphate buffer, pH 7.5, was used throughout. In addition, all buffers used for the isolation and storage of enzyme preparations contained 1 mM phenylmethylsulphonyl fluoride and 1 mM benzamidine (both freshly prepared and added). The source of the bacterial and fungal strains was as indicated in section 2.1.1.

6.2.1.1. Preparation of 2,3-bisphosphoglycerate dependent phosphoglycerate mutases.

Cofactor dependent activity was isolated from the following sources;

Klebsiella aerogenes;   *K. aerogenes* was grown for 24 h at 37°C in nutrient broth. Cells were obtained by centrifugation of the culture at 23,000 g for 30 min. The cell pellet was ground with an equal weight of alumina in a pre-cooled mortar at 4°C. Two volumes of buffer were subsequently added and the crude extract recovered by

150

centrifugation at 23,000 g for 30 min. The crude extract was fractionated by $(NH_4)_2SO_4$ and phosphoglycerate mutase activity was precipitated between 60 and 80% saturation.

Escherichia coli; *E.coli* was grown for 24 h at 37°C in tryptic soy broth. Cells were obtained and processed as for *K.aerogenes* with phosphoglycerate mutase activity again precipitating between 60 and 80% saturation.

Candida utilis; *C.utilis* was grown in the liquid culture medium used for *S.pombe* (section 5.2.1.) for 60 h at 25°C. Cells were harvested by centrifugation at 22,000 g for 20 min and ground with an equal weight of acid washed sand in a pre-cooled mortar at 4°C. Two volumes of buffer were subsequently added and the extract recovered by centrifugation at 22,000 g for 30 min. The crude extract was fractionated by $(NH_4)_2SO_4$ and phosphoglycerate mutase activity was precipitated between 70 and 90% saturation.

6.2.1.2. Preparation of 2,3-bisphosphoglycerate independent phosphoglycerate mutases.

Cofactor independent activity was isolated from the following sources;

Potato; Potato tuber was homogenized in a one third volume of buffer. The homogenate was filtered through a muslin cloth and centrifuged at 13,000 g for 20 min. The resultant supernatant was fractionated by

$(NH_4)_2SO_4$ and phosphoglycerate mutase activity was precipitated between 60 and 80% saturation.

Mung bean seedlings; Mung beans were germinated in the dark for 7 days. The etiolated shoots were harvested and homogenized with an equal volume of buffer. The homogenate was centrifuged at 13,000 g for 20 min and the resultant supernatant fractionated by $(NH_4)_2SO_4$. Phosphoglycerate mutase activity was precipitated between 60 and 80% saturation.

Wheat germ; Flaked wheat germ was stirred for 60 min at 4°C in two volumes of 10 mM triethanolamine-HCl buffer, pH 8.0. Crude extract was recovered by centrifugation at 20,000 g for 30 min and fractionated by $(NH_4)_2SO_4$. Phosphoglycerate mutase activity was precipitated between 50 and 65% saturation.

Aspergillus nidulans; *A.nidulans* was grown in a liquid culture medium and the mycelium harvested by filtration as described in section 6.2.4. The mycelium was ground for 5 min with an equal weight of acid washed sand in a pre-cooled mortar at 4°C. An equal volume of buffer was added and the extract recovered by centrifugation at 20,000 g for 30 min. The crude extract was fractionated by $(NH_4)_2SO_4$ and phosphoglycerate mutase activity was precipitated between 60 and 95% saturation.

__Nasturtium seedlings__;   A   crude   extract   of   nasturtium   seedlings
containing   phosphoglycerate   mutase   activity   was   supplied   by
Dr.J.S.G.Reid, University of Stirling, Scotland.

Neurospora crassa;   Purified phosphoglycerate mutase and a $(NH_4)_2SO_4$
fractionated   crude   extract   from   _N.crassa_   were   the   kind   gift   of
Dr.S.M.McAleese,   University   of   Aberdeen,   Scotland.   Phosphoglycerate
mutase from _N.crassa_ was assayed at 30°C in 0.5 M Tris-HCl buffer, pH
8.0, containing 10 mM 3-phosphoglycerate, 5 mM $MgSO_4$ and 40 μg (1.6
units) of enolase in a volume of 1 ml. A unit of enzyme activity was
defined as producing an increase in A_{240} of 0.1 min^{-1}. The specific
activities   of   the   purified   and $(NH_4)_2SO_4$ fractionated   enzyme
preparations were 3400 units mg^{-1} and 480 units mg^{-1} respectively.

6.2.2. Chelator sensitivity of cofactor dependent and independent
phosphoglycerate mutases.

The   sensitivity   of   phosphoglycerate   mutases   to   the   chelators
ethylenediaminetetraacetate   (EDTA)   and   8-hydroxyquinoline   sulphonate
(HQSA)   were   examined   in   100   mM   Tris-HCl   buffer,   pH   8.7,   at   20°C
essentially as described by Smith and Hass (1985). Enzyme preparations
were dialysed into this buffer and diluted to give between 25 and 50
activity units ml^{-1}. After equilibration at 20°C EDTA and HQSA were
added to a final concentration of 1 mM and phosphoglycerate mutase
activity   monitored   over   180   min   incubation.   Enzyme   activity   was
determined   using   the   standard   pH   7.0   enolase   coupled   assay

(section 2.2.3.1.) or for *N.crassa* using the assay described in section 6.2.1.2.. Control measurements indicated that the maximal carry over concentration of EDTA and HQSA (0.02 mM) into either assay had no effect on the determination of phosphoglycerate mutase activity. Percentage activity in the presence of chelators was calculated relative to a control sample incubated in the absence of chelators and under identical conditions. Where this control activity was unstable (section 6.3.1.) the percentage activity was calculated relative to the initial control activity.

In addition to the partially purified phosphoglycerate mutase preparations described in sections 6.2.1.1. and 6.2.1.2., the purified enzymes from bakers yeast, rabbit muscle and *S.pombe* were included in the chelator sensitivity studies.

6.2.3. Determination of phosphoglycerate mutase relative molecular mass.

The relative molecular masses of the partially purified phosphoglycerate mutases were determined by gel filtration using a Sephacryl S-300 column (38 cm x 4.9 cm²). The operation and calibration of the column was as described in section 2.2.1.2. The elution of phosphoglycerate mutases was determined from activity measurements using the standard pH 7.0 enolase coupled assay (section 2.2.3.1.) or for the *N.crassa* enzyme using the assay described in section 6.2.1.2..

6.2.4. Isolation of phosphoglycerate mutase from *A.nidulans*.

A.nidulans was cultured in the liquid growth medium described in Appendix I at 37°C for 24 h in an orbital incubator. The mycelia were harvested from the medium by filtration, generally yielding 75 g wet weight from 4 litres of culture. All subsequent procedures were performed at 4°C unless otherwise stated.

A.nidulans mycelia were ground with an equal weight of acid washed sand in a pre-cooled mortar for 5 min. Two volumes of 50 mM sodium phosphate buffer, pH 7.5, containing 1 mM phenylmethylsulphonyl fluoride (freshly prepared and added) were mixed with the ground material and the crude extract recovered by centrifugation at 15,000 g for 20 min. The crude extract was taken to 67% $(NH_4)_2SO_4$ saturation by the addition of solid and after stirring 30 min the supernatant was recovered by centrifugation at 27,000 g for 20 min. The supernatant was taken to 95% $(NH_4)_2SO_4$ saturation and the protein precipitating in this 67-95% range was recovered by centrifugation at 27,000 g for 20 min. The precipitated protein was dissolved in 10 ml of 50 mM sodium phosphate buffer, pH 7.5, containing 1 mM phenylmethylsulphonyl fluoride and dialysed overnight against 2 litres of 10 mM Tris-HCl buffer, pH 8.0. The dialysis residue was applied to a column of QAE-cellulose (30 cm x 3.8 cm^2) equilibrated against the Tris buffer. After washing the column with 100 ml of 10 mM Tris-HCl buffer, pH 8.0, containing 75 mM NaCl, phosphoglycerate mutase activity was eluted using a gradient formed between 100 ml Tris buffer containing 100 mM

NaCl and 100 ml Tris buffer containing 300 mM NaCl. The fractions containing peak activity were pooled and freeze dried. The lyophilized material was redissolved in one fifth the volume of water and dialysed against 2 litres of 10 mM Tris-HCl buffer, pH 8.0. The dialysis residue was applied to a column of Cibacron Blue F3GA-sepharose (5 cm x 4.9 cm^2) equilibrated against the Tris buffer. Phosphoglycerate mutase activity was not retained by this affinity media and was eluted with the Tris buffer. Peak fractions were pooled and applied to a column of DEAE-cellulose (15 cm x 1.8 cm^2) and after washing with 100 ml of Tris-HCl buffer, pH 8.0, phosphoglycerate mutase activity was eluted using a gradient formed between 75 ml of Tris buffer containing 25 mM NaCl and 75 ml of Tris buffer containing 150 mM NaCl. The fractions containing peak activity were pooled, freeze dried and redissolved in one fifth the volume of water. The redissolved material was dialysed against 2 litres of 50 mM sodium phosphate buffer, pH 7.5, and applied to a Sephacryl S-300 column (38 cm x 4.9 cm^2). Phosphoglycerate mutase activity was eluted and fractions containing maximal activity were dialysed individually against several changes of 10 mM Tris-HCl buffer, pH 8.0. After dialysis the fractions were freeze dried, redissolved in one tenth the volume of water and analysed by SDS-PAGE. Those showing > 80% homogeneity after staining with Coomassie blue R250 were pooled and stored at -18°C. Prior to use the pooled enzyme preparation was dialysed against ≥ 500 volumes of appropriate buffer.

6.2.5. Isolation of phosphoglycerate mutase from wheat germ.

Phosphoglycerate mutase was isolated from flaked wheat germ by Dr.N.Price and Mrs D.Duncan using the methods of Leadley *et al.* (1977) and Smith and Hass (1985) respectively. Purified enzyme from these preparations was kindly supplied for the chelator sensitivity and reactivation studies described below.

6.2.6. Assay of phosphoglycerate mutase from *A.nidulans and wheat germ.*

The assay of 2,3-bisphosphoglycerate independent phosphoglycerate mutases has involved the use of a number of procedures. Most authors report systems based on the enolase coupled assay of Rodwell *et al.* (1957). However, Rodwell's assay was conducted at pH 7.0 and as noted by Ito and Grisolia (1959) the pH optima of the wheat germ enzyme is approximately 9. These authors therefore used a pH 8.7 assay system in monitoring their wheat germ enzyme purification scheme. In their review of cofactor independent phosphoglycerate mutases Grisolia and Carreras (1975) list a pH 8.7 assay system using 33 mM Tris-HCl buffer, 16.7 mM 3-phosphoglycerate, 3.3 mM $MgSO_4$ and 8.3 units of enolase ml^{-1}. The assay system used here for the wheat germ and *A.nidulans* enzyme is that reported by Leadley *et al.* (1977) namely 100 mM Tris-HCl buffer, pH 8.7, containing 20 mM 3-phosphoglycerate, 8.3 mM $MgSO_4$ and 40 μg (1.6 units) of enolase in a final volume of 1 ml.

The assay was performed at 30°C and this system was used at all times unless otherwise indicated.

The standard pH 7.0 enolase coupled assay (section 2.2.3.1.) was also used during studies on the cofactor independent phosphoglycerate mutases. This assay had been routinely used for the determination of the cofactor dependent phosphoglycerate mutases (chapters 3, 4 and 5). The cofactor, 2,3-bisphosphoglycerate, was omitted from the system for the assay of the cofactor independent enzymes. In addition, the amount of enolase was increased to 40 μg (1.6 units). All other conditions were as described in section 2.2.3.1. The stopped triple coupled assay system (section 2.2.3.2.) based on that of Leadley *et al.* (1977) was also used to assay the cofactor independent enzymes in the presence of metal ions. Once again the cofactor was omitted from the assay system and the concentrations of the coupling enzymes were increased to 50 μg (2.0 units) of enolase, 36 μg (15 units) of pyruvate kinase and 12 μg (14 units) of lactate dehydrogenase. All other conditions were as described in section 2.2.3.2.

6.2.7. Reactivation of *A. nidulans* and wheat germ phosphoglycerate mutase following denaturation in GdnHCl.

Reactivation of the *A. nidulans* and wheat germ enzymes following denaturation in GdnHCl was followed using the stopped triple coupled assay system. *A. nidulans* (300 μg ml⁻¹) and wheat germ (50 μg ml⁻¹) enzymes were denatured in 2 M GdnHCl for 5 min at 20°C and diluted

fifty—fold into 30 mM Tris-HCl buffer, pH 7.0 containing 10 mM 3-phosphoglycerate. After dilution (*A. nidulans* to 6 μg ml^{-1}, wheat germ to 1 μg ml^{-1}) the enzymes were incubated at 30°C and the 2-phosphoglycerate formed after 1 and 6 min was determined using the coupling assay. After 7 min incubation metal ions were added from concentrated stock solutions to give a final concentration of 3.3 mM during reactivation. The 2-phosphoglycerate formed 1 and 6 min after the addition of the metal ions was determined using the coupling assay. A control system in each case contained the enzyme at the reactivation concentration in the presence of the residual concentration of GdnHCl present in denatured samples (0.04 M).

6.2.8. Reactivation of *A. nidulans* phosphoglycerate mutase following inhibition by chelators.

Two types of experiment were used to examine the reactivation of *A. nidulans* phosphoglycerate mutase following its inhibition with chelators. The activity of the *A. nidulans* enzyme was monitored using the standard pH 7.0 enolase coupled assay in both cases.

In the first experiment the *A. nidulans* enzyme (60 μg ml^{-1}) was inhibited in 30 mM Tris-HCl buffer, pH 7.0, by EDTA and HQSA at a concentration of 0.1 mM. Following periods of incubation at 4°C the activity of the enzyme was monitored until it had declined to $\leqslant$ 5% of a control sample incubated in the absence of chelators. The inhibited enzyme was then divided into a number of aliquots and different metal

ions added to each to a final concentration of 2 mM. The activity of the control sample and inhibited enzyme in the presence and absence of the metal ions was monitored over 24 h incubation at 4°C.

In the second experiment the *A. nidulans* enzyme (270 μg ml^{-1}) was inhibited in 30 mM Tris-HCl buffer, pH 7.0, by 0.9 mM EDTA. Following periods of incubation at 20°C the activity of the enzyme was monitored until it had declined to < 5% of a control as above. The inhibited enzyme was then applied to a Sephadex G-25 column (35 cm x 2.0 cm^2) previously equilibrated against 30 mM Tris-HCl buffer, pH 7.0. The enzyme was eluted with this buffer and detected by fluorescence emission at 325 nm. Fractions exhibiting peak fluorescence were pooled and assayed for activity. The pooled enzyme was then divided into a number of aliquots and metal ions added to a concentration of 1 mM. Activity of the inhibited enzyme in the presence and absence of metal ions was monitored over 24 h incubation at 4°C.

Preliminary control measurements indicated that the Sephadex G-25 column would successfully resolve bovine serum albumin (1 mg ml^{-1}) from EDTA (2 mM). Elution of the bovine serum albumin was monitored by fluorescence emission at 325 nm and by absorbance at 280 nm and that of the EDTA was monitored by absorbance at 240 nm.

6.3. RESULTS AND DISCUSSION.

6.3.1. Chelator sensitivity of cofactor dependent and independent
   phosphoglycerate mutases.

The sensitivities of the 2,3-bisphosphoglycerate dependent
phosphoglycerate mutases to the chelators EDTA and HQSA is shown in
Fig. 44 a-f. Similarly, the sensitivities of the cofactor independent
enzymes are shown in Fig. 44 g-l. Clearly the activity of the cofactor
dependent phosphoglycerate mutases is unaffected by the presence of
chelators with $\leqslant$ 10% activity lost after 180 min incubation. In
contrast the activity of the cofactor independent enzymes is inhibited
by the chelators to varying degrees. The activity of the $A.nidulans$
enzyme is completely inhibited following 60 min incubation whereas
that of the potato enzyme is reduced by 50% and 15% following 180 min
incubation with EDTA and HQSA respectively. The general difference
between the cofactor dependent and independent enzymes in their
sensitivity to the chelators suggests that the latter group of enzymes
may have a metal ion requirement for activity. This conclusion is
supported by the report of Smith and Hass (1985) who have shown that
the wheat germ enzyme is inhibited by a range of chelators including
EDTA and HQSA. Subsequent studies by Smith et al. (1986) suggested
that the wheat germ enzyme required the presence of either Co^{2+} or
Mn^{2+} to promote reactivation following denaturation in GdnHCl. In
addition, Oh and Freese (1976) and Watabe and Freese (1979) have shown
that the cofactor independent phosphoglycerate mutase from $Bacillus$ $subtilis$ was

Figure 44.   Sensitivity of cofactor dependent and independent
             phosphoglycerate mutases to the metal chelating
             agents EDTA and HQSA.

Enzymes were incubated at 20°C in 100 mM Tris-HCl buffer, pH 8.7,
in the presence of   ◯   , 1 mM EDTA and   △   , 1 mM HQSA.
Activities are expressed relative to control samples incubated in
the absence of chelators.   The ordinate and abscissa scales
indicated in A and G apply to all figures.

Figure 44 A-F.   Cofactor dependent enzymes;   A, S. pombe;   B,
C. utilis;   C, baker's yeast;   D, rabbit muscle;   E, K. aerogenes;
F, E. coli.   The marked instability of some control samples is
indicated (--●--) and % activity was calculated relative to
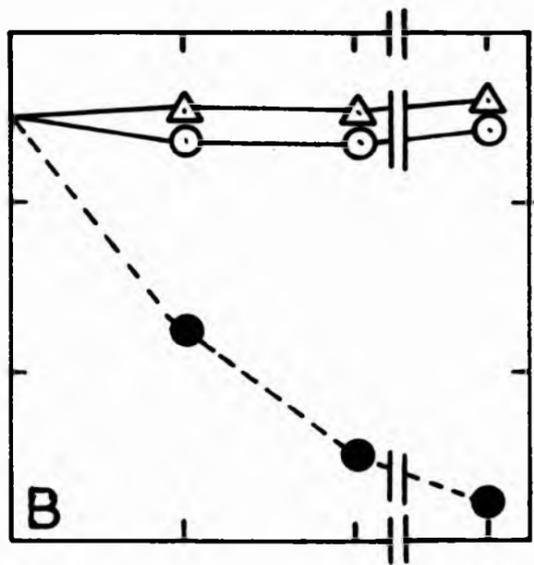the initial control activity in these cases.

Figure 44 G-L.   Cofactor independent enzymes;   G, A. nidulans;
H, mung bean;   I, potato;   J, wheat germ;   K, nasturtium;   L,
N. crassa.
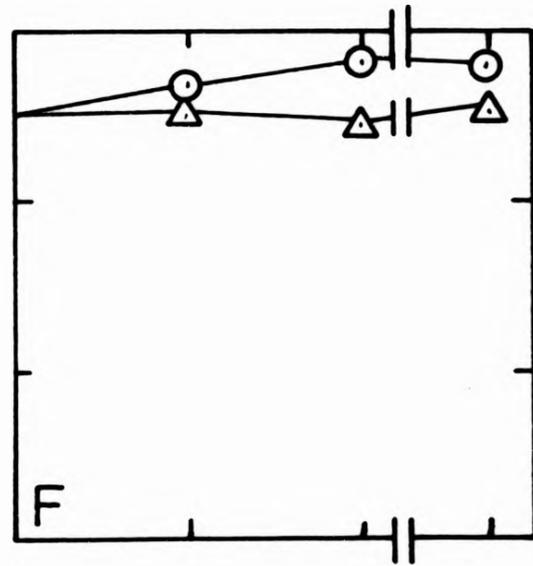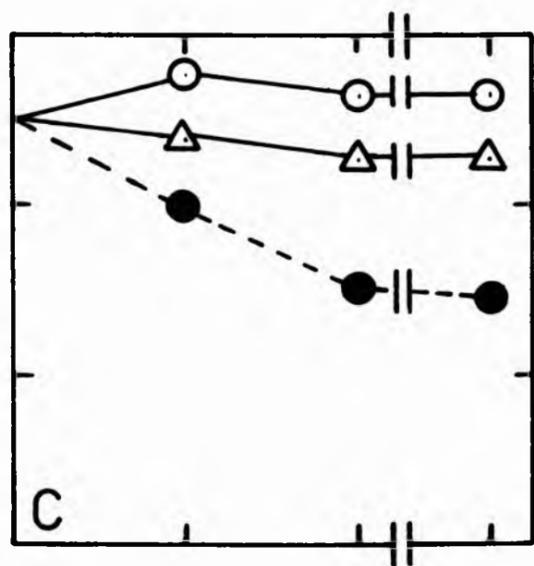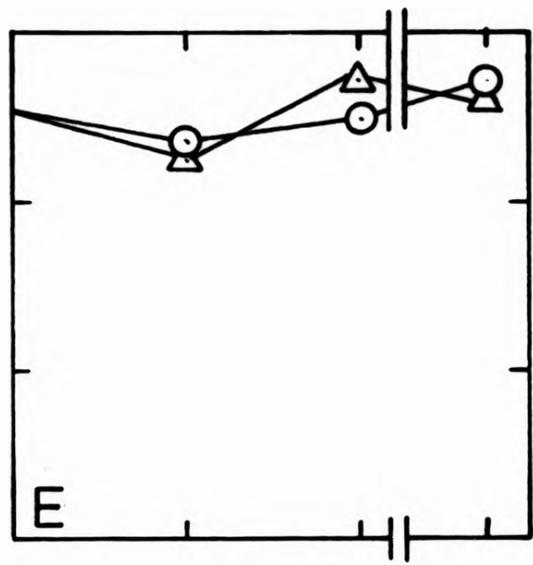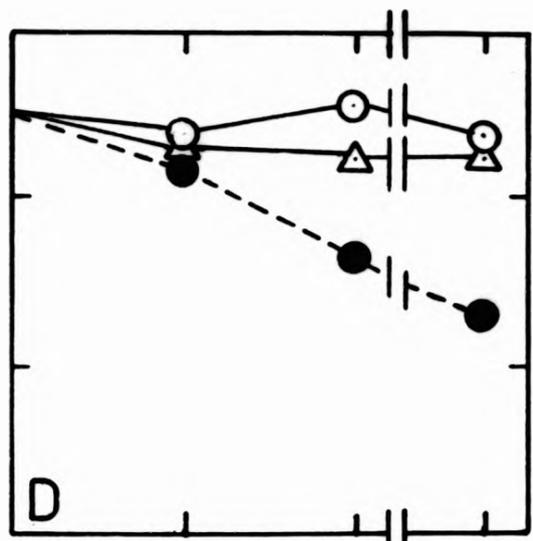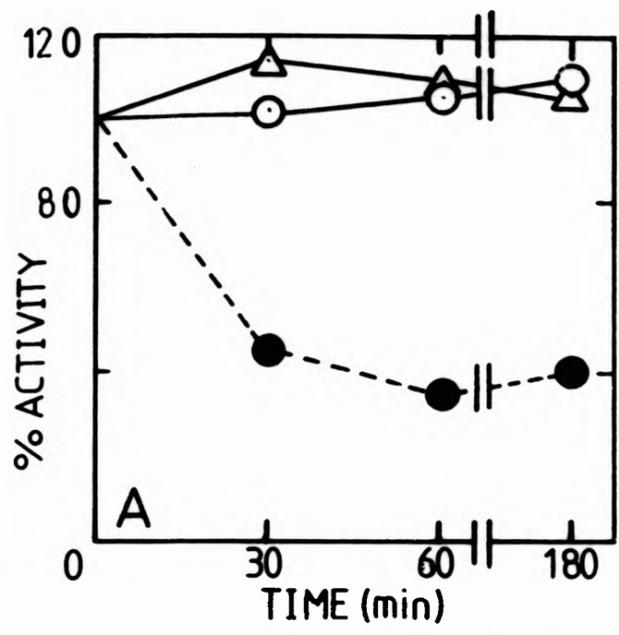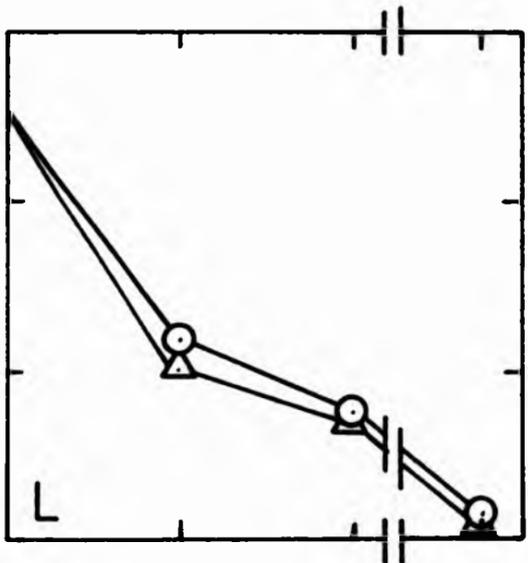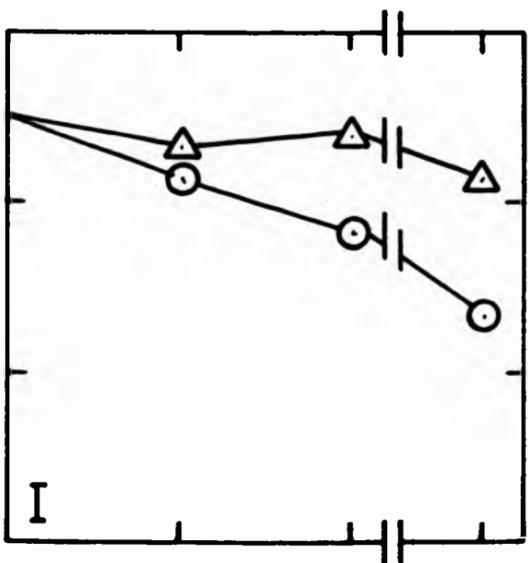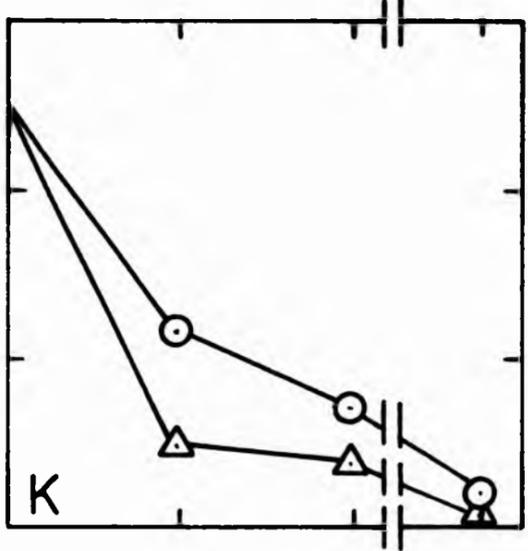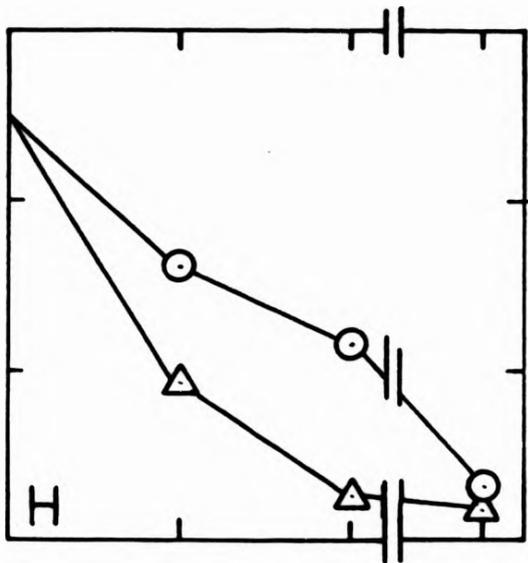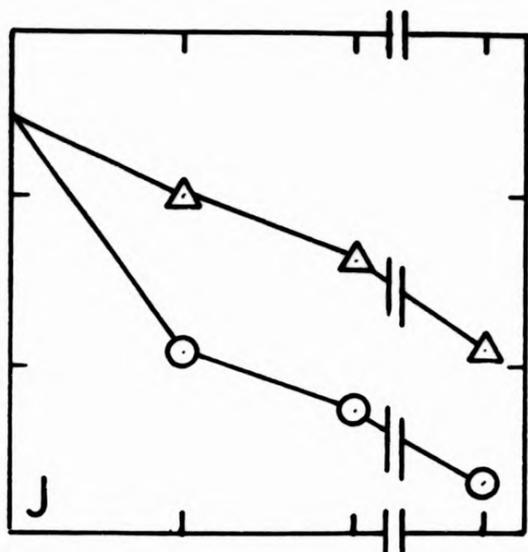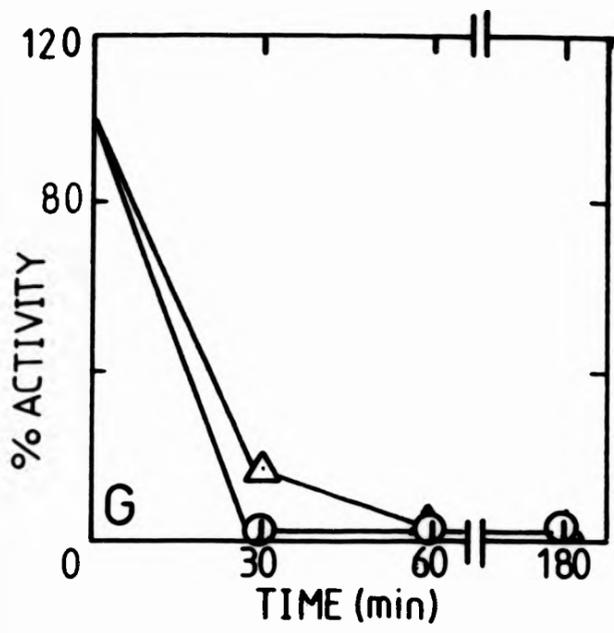
inhibited by EDTA and has an apparent Mn^{2+} requirement for activity. Singh and Setlow (1979) have reported similar observations for the phosphoglycerate mutase isolated from *Bacillus megaterium.*

The differing rates of inactivation observed for the six cofactor independent enzymes examined (Fig. 44 g-l) may be indicative of differing degrees of accessibility of, and/or strengths of association with, the enzymes and the putative metal ion(s). The material used in assessing chelator sensitivity were relatively crude extracts and the background metal ion concentration in each preparation could well have moderated the observed inhibition. However, evidence against such an effect was found on examining the chelator sensitivity of purified phosphoglycerate mutases from *A. nidulans*, *N. crassa* and wheat germ. The pattern of inhibition in these extensively purified preparations was essentially identical to that observed for the extracts fractionated by $(NH_4)_2SO_4$ shown in Fig. 44 g, j and l respectively (data not shown).

It should be noted that the cofactor dependent enzymes from *S. pombe*, *C. utilis*, bakers yeast and rabbit muscle exhibited a marked instability in controls, i.e; in 100 mM Tris-HCl buffer, pH 8.7, at 20°C. This effect was reversed in the presence of 1 mM EDTA or HQSA where the activity of these four enzymes was quite stable (Fig. 44 a-d). All other control samples, including the cofactor dependent enzymes from *E. coli* and *K. aerogenes*, were unaffected by the buffer system (± 5% of initial activity over 180 min incubation). The

inhibition of the cofactor dependent enzymes and its reversal by EDTA and HQSA is perhaps indicative of inhibition caused by some cationic species which can be chelated from the system. A report by Carreras *et al.* (1980) has shown that a range of 2,3-bisphosphoglycerate dependent mutases were inhibited by vanadate whereas cofactor independent enzymes were unaffected. A concentration of 10 μM vanadate was sufficient to produce approximately 70% inhibition. The effect of vanadate was more pronounced with increasing pH from 6 to 10. The pH dependence of the inhibition presumably reflects the greater formation of the pentavalent vanadium ion at alkaline pH. This species is thought to resemble the transition state of the phosphoryl group thereby inhibiting the enzyme by binding competitively to the active site (Macara, 1980). Carreras *et al.* (1980) showed that the effects of vanadate could be reversed by dilution or by the addition of EDTA or noradrenaline. The action of these chelators on vanadate seems to be directly through complexing or reduction (Cantley *et al.*, 1978). It thus seems possible that the presence of trace quantities of vanadate, or a vanadate like cationic species able to compete with the phosphoryl group, in the Tris buffer system might contribute to the inhibition of the cofactor dependent enzymes. This effect would be exacerbated at the buffer pH of 8.7 where the formation of the pentavalent vanadium ion is enhanced and the cofactor dependent enzymes are not acting at their pH optima of 7.0 (Grisolia and Carreras, 1975). The possibility also exists that inactivation of the cofactor dependent enzymes is a direct effect of the pH and that the chelators in some way stabilise the enzyme to this form of inactivation.

The activity of the cofactor dependent phosphoglycerate mutases from *E.coli* and *K.aerogenes* was unaffected in the control system. Carreras *et al.* (1980) examined a range of cofactor dependent enzymes which were all inhibited by vanadate but did not include any bacterial enzymes in these studies. Since both cofactor dependent (*E.coli*) and independent (*Bacillus sp.*) phosphoglycerate mutases have been isolated from bacteria it is possible that the cofactor dependent enzymes from *E.coli* and *K.aerogenes* differ from the mammalian and yeast phosphoglycerate mutases. Structural or mechanistic differences in the former enzymes could alter their susceptibility to the inhibition observed in the control system. It would therefore be of particular interest to examine the vanadate sensitivity of a range of bacterial phosphoglycerate mutases to confirm these tentative conclusions. However, a lack of material for those isolated and insufficient time to isolate others prevented such studies.

It has also been reported that fluoride can inhibit the activity of bakers yeast phosphoglycerate mutase (Chiba *et al.*, 1960). Curiously such inhibition can be reversed by the presence of equivalent concentrations of EDTA as if some fluoride-EDTA complex were being formed. The exact mode of inhibition is therefore unclear. However, on the basis of these observations it is possible that the instability of the cofactor dependent phosphoglycerate mutases (Fig. 44 a-d) could result from a fluoride type inhibition in the buffer system used.

6.3.2. Relative molecular mass of cofactor dependent and independent phosphoglycerate mutases.

The Mr values of the phosphoglycerate mutases examined for chelator sensitivity were determined by gel filtration on a column of Sephacryl S-300. The calibration of this column using the standards described in section 2.2.1.2. is shown in Fig. 10. The Mr values of the cofactor dependent and independent enzymes determined from Fig. 10 are given in Table 3. The published Mr values for these enzymes, where available, are included for comparison.

The results presented in Table 3, which represent a summary of the findings from the studies described in sections 6.3.2. and 6.3.1., seem to indicate a common structural pattern for the cofactor independent phosphoglycerate mutases. All the cofactor independent enzymes have a Mr of approximately 60,000 and are inhibited by the two chelators examined. The literature reports available are consistent with both the Mr values and the inhibition effect. In contrast, the cofactor dependent phosphoglycerate mutases had a range of Mr values reflecting their differing quaternary structures and their activities were unaffected by the chelators.

Phosphoglycerate mutase	Inhibition by chelators*	Mr of native enzyme[†]	Published Mr and quaternary structure	
COFACTOR DEPENDENT				
Baker's yeast	NONE	110,000	110,000 Tetramer	(1)
C. utilis	NONE	112,000	110,000	(4)
Rabbit muscle	NONE	56,000	56,000 Dimer	(1)
S. pombe	NONE	23,000	23,000 Monomer	(2)
K. aerogenes	NONE	62,000		
E. coli	NONE	57,000	56,300	(3)
COFACTOR INDEPENDENT				
Potato	YES	60,000		
A. nidulans	YES	60,000	40-60,000 Monomer	(4)(6)
Wheat germ	YES	52,000	50,000, 60,000 Monomer	(4)(5)
Nasturtium	YES	63,000		
N. crassa	YES	N.D.		
B. subtilis[‡]	YES (EDTA)		74,000 Monomer	
B. megaterium[∅]	YES (EDTA)		61,000 Monomer	

Table 3.   Chelator sensitivity and Mr of cofactor dependent and
           independent phosphoglycerate mutases.

*, sections 6.2.2. and 6.3.1.;   †, sections 6.2.3. and 6.3.2.;
N.D., not done; ‡, data from Watabe and Freese (1979); ∅, data from
Singh and Setlow (1979).

(1), Hermann et al. (1983);   (2), Price et al. (1985b);   (3), D'Alessio
and Jose (1971);   (4), Price et al. (1983);   (5), Leadley et al. (1977);
(6), section 6.3.3.

6.3.3. Isolation and characterisation of *Aspergillus nidulans* phosphoglycerate mutase.

In view of the reported metal ion requirement of the cofactor independent phosphoglycerate mutases from *Bacillus* species (Watabe and Freese, 1979, Singh and Setlow, 1979) and wheat germ (Smith and Hass, 1985, Smith *et al.*, 1986) and the chelator sensitivity observed in a range of these enzymes, the detailed purification of the *A.nidulans* enzyme was pursued with the aim of characterising in detail the relationship between metal ion and enzyme. *A.nidulans* was selected for its ease of culture and harvesting and also its pronounced sensitivity to the chelators examined (Fig. 44 g). In addition, the purification of phosphoglycerate mutase from this source had not been reported elsewhere.

Phosphoglycerate mutase was isolated from *A.nidulans* as described in section 6.2.4. Details of a typical purification are shown in Table 4. The specific activity of the purified enzyme was approximately 5000 units mg^{-1} using the pH 8.7 assay of Leadley *et al.* (1977). According to these authors one unit of activity (ΔA_{240}, 0.1 min^{-1}) under these conditions corresponds to 1.83 μmole of 3-phosphoglycerate metabolised min^{-1}. The specific activity of the enzyme preparation thus equates to 9.15 m mole of substrate metabolised min^{-1} mg $protein^{-1}$.

Analysis of the enzyme preparation by SDS-PAGE indicated > 85% homogeneity of staining with Coomassie blue R250. The Mr of the enzyme

Step	Enzyme activity (units)	Protein (mg)	Specific activity (units/mg)	Purification factor	Yield (%)
Initial extract	16500	379	43.5	1.0	100
67-95% $(NH_4)_2SO_4$ precipitate	10080	50.1	201	4.6	61
QAE cellulose chromatography	7540	6.2	1218	28.0	46
Reactive Blue-Sepharose chromatography	5400	2.6	2050	47.1	33
DEAE cellulose chromatography	4440	1.19	3723	85.6	27
Sephacryl S-300 gel filtration	3750	0.71	5250	120.7	23

Table 4. Purification of phosphoglycerate mutase from A. nidulans.

The data refer to mycelia harvested from 4 litres of liquid culture (75 g wet weight). Enzyme assays were performed using the pH 8.7 assay of Leadley et al. (1977).

$$15 \quad 30 \quad 60 \qquad Mr \, (\times 10^{-3})$$
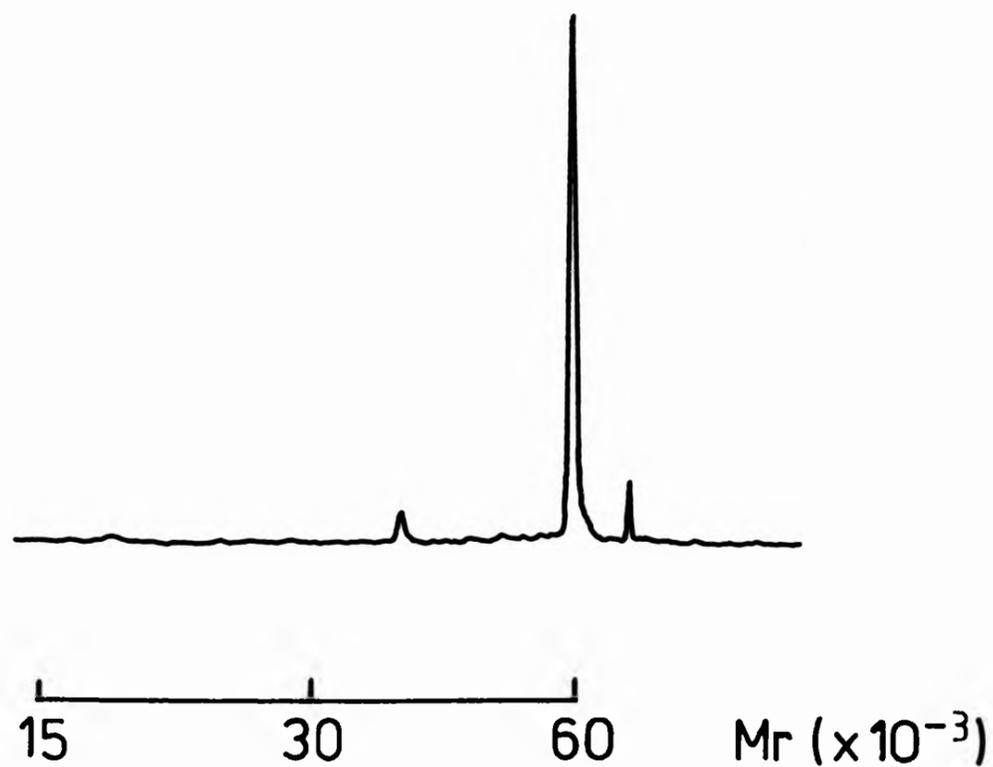
Figure 45. SDS-PAGE of phosphoglycerate mutase from A. nidulans.

Enzyme (3 µg) was analysed using a 12% acrylamide separating gel. After staining with Coomassie blue R250 and destaining, the gel was scanned at 600 nm. The horizontal Mr calibration scale was determined using the Dalton Mark VII marker set (section 2.2.1.1. and Fig. 9).

under denaturing conditions was 60,000 ± 3,000 (Fig. 45). However, in some preparations the enzyme appeared as a double band with M_r values of 60,000 ± 3,000 and 56,000 ± 3,000. In these cases the specific activity of the enzyme was not affected indicating that the probable loss of some amino acids from the polypeptide did not affect the activity of the native enzyme. The levels of conversion to the lower M_r form appeared to be related to enzyme concentration since the decrease in M_r occurred during the final gel filtration step when the total protein concentration, already lowered by the extensive purification, was further reduced during the fractionation in Sephacryl S-300. Despite several attempts using different buffer conditions for the gel filtration step the persistance of the double banding pattern was never completely eliminated. Stabilisation of the *A.nidulans* enzyme at low concentration may therefore require buffering with a protein-containing solution. A buffer containing 1% w/v ovalbumin has been used for dilution of the rabbit muscle enzyme and is reported to prevent a similar inactivation phenomenon at low enzyme concentrations (Rodwell *et al.*, 1957).

The gel filtration stage in the purification protocol was performed using 50 mM sodium phosphate buffer, pH 7.5 (section 6.2.4.). The use of 100 mM Tris-HCl buffer, pH 8.0, during this procedure resulted in irreversible inactivation of the enzyme. Leadley *et al.* (1977) have also reported that column chromatography of the wheat germ enzyme in cationic amino buffers produces inactivation. These authors therefore used sodium phosphate based buffers in such fractionations. However,

following the gel filtration step in the scheme reported here it was found that concentration of the enzyme by freeze drying in the phosphate buffer caused inactivation, possibly as a result of the high phosphate concentration generated. Thus the eluted enzyme material was dialysed into a low ionic strength Tris-HCl buffer, pH 8.0, prior to freeze drying . Following concentration by this method the enzyme preparation was stored at -18°C. The activity of the enzyme was stable over several months at this temperature.

The Mr of the native enzyme was determined using gel filtration on Sephacryl S-300 (section 6.3.2.) as 60,000 ± 6,000. In conjunction with the Mr value following SDS-PAGE it is clear that the *A.nidulans* enzyme is a monomer of Mr approximately 60,000. This value compares favourably with the report of Price *et al.* (1983) who have shown the Mr of phosphoglycerate mutases from a range of fungi (including *A.nidulans*) to be between 40,000 and 60,000.

6.3.4. Isolation and characterisation of wheat germ phosphoglycerate mutase.

Phosphoglycerate mutase was isolated from wheat germ by Dr.N.Price and Mrs D.Duncan using the methods of Leadley *et al.* (1977) and Smith and Hass (1985) respectively. Both procedures yielded material of specific activity approximately 3000 units mg^{-1} when assayed by the method of Leadley *et al.* (1977). This value equates to 5.5 m mole 3-phosphoglycerate metabolised min^{-1} mg protein^{-1}. The specific activity

was comparable with that reported by Leadley *et al.* (1977) and Smith and Hass (1985) for their preparations of the enzyme.

Analysis of both enzyme preparations on SDS-PAGE revealed a major band of Mr approximately 60,000 and a number of additional bands of lower Mr. The presence of these minor bands presumably results from some limited proteolytic or autolytic degradation during purification and their presence has been noted by other groups in their study of the wheat germ enzyme (Smith and Hass, 1985, McAleese *et al.*, 1985). In conjunction with a similar observation in the purification of the *A.nidulans* enzyme, where this effect seemed to result from excessive dilution of the enzyme, it seems that the cofactor independent enzymes may be structurally quite unstable and are able to lose a number of amino acids from their polypeptides without compromising their activity.

6.3.5. Effects of metal ions on the phosphoglycerate mutase assay system of Leadley *et al.* (1977).

It is well recognised that a number of the transition metals are unstable in aqueous solution at alkaline pH (Kragten, 1978). The basis of this instability lies in hydroxide formation in aqueous solution according to the scheme illustrated below for a divalent ion "M";

$$M^{2+} \rightleftharpoons MOH^+ \rightleftharpoons M(OH)_2 \rightleftharpoons M(OH)_3^- \rightleftharpoons M(OH)_4^{2-}$$

$$M(OH)_2 \cdot n\ H_2O \qquad SOLID$$
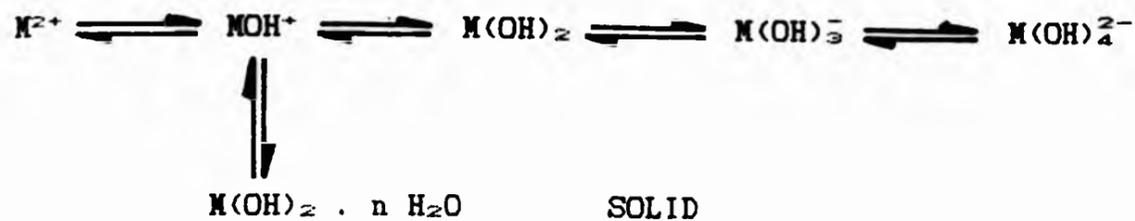
The concentration of the various species is dependent on both pH and the total concentration of "M". Clearly any reaction of the type;

$$M^i + iH_2O \rightleftharpoons M(OH)_i + iH^+$$

will be favoured by alkaline conditions. For Co^{2+} and Mn^{2+} the pH at which the $M(OH)_2$ hydroxide species predominates is 8.8 and 11.6 respectively (Kragten, 1978).

In view of the potential complications considered above it was considered necessary to examine thoroughly the effects of metal ions on the assay system of Leadley *et al.* (1977) which is performed at pH 8.7. When Co^{2+} or Mn^{2+} were added to the buffer of this assay (100 mM Tris-HCl buffer, pH 8.7) to a final concentration of 2 mM there was an almost instantaneous increase in A_{240} rising to > 2.0 within 5 s of addition. This increase was associated with visible turbidity of the buffer presumably reflecting the metal hydroxyl complex formation. In the case of Ni^{2+} added to 2 mM there was a slow increase in A_{240} of approximately 0.0025 min^{-1} whereas the other metal ions examined (Fe^{2+}, Cu^{2+}, Ca^{2+}, Zn^{2+}) produced no increase in A_{240}. Addition of Co^{2+}, Mn^{2+}, Ni^{2+} to the full assay system of Leadley *et al.* (1977) produced moderated increases in A_{240}. Fig. 46 shows the rate of
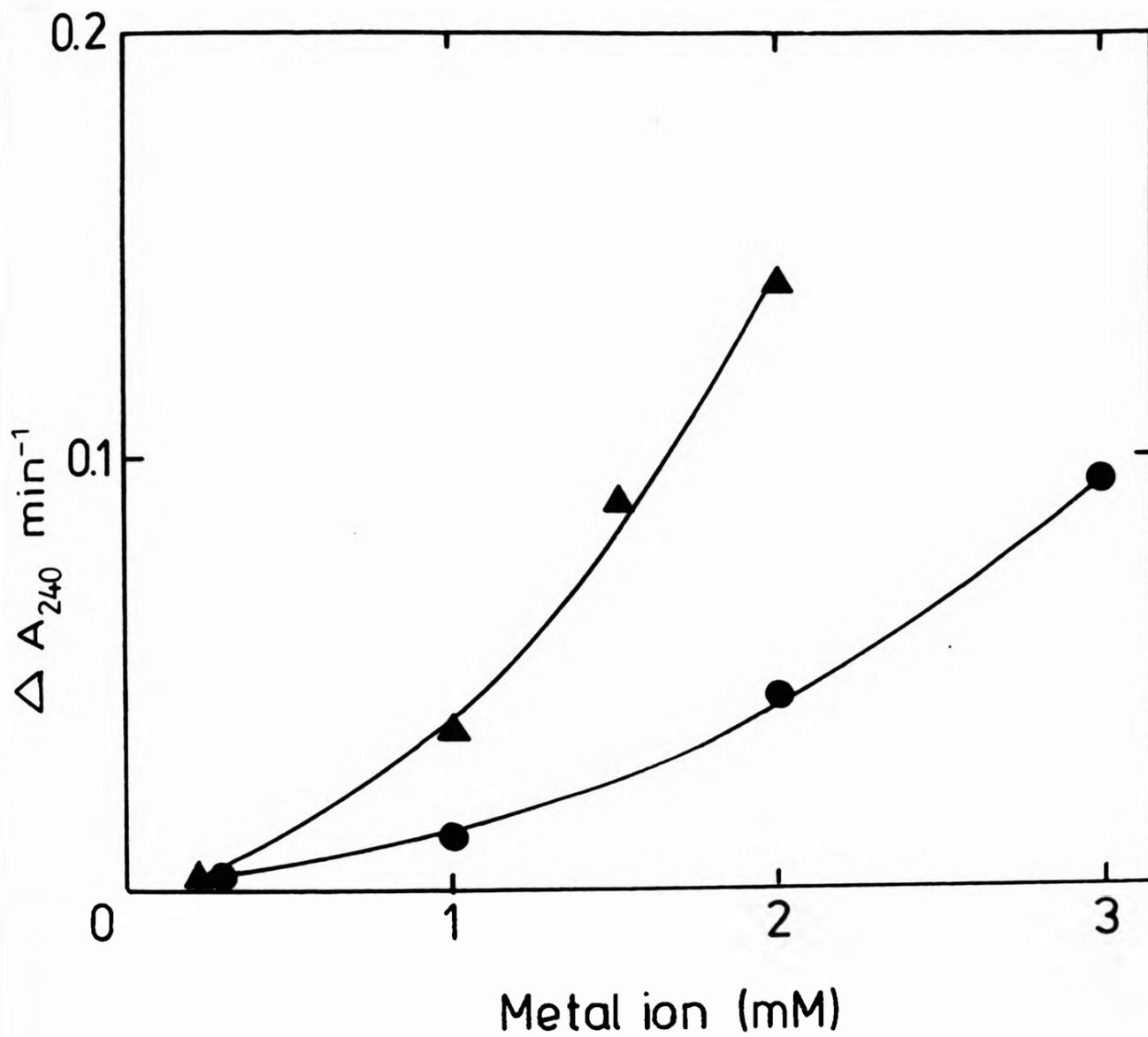
Figure 46. Rate of increase in absorbance at 240 nm of the assay
system of Leadley et al. (1977) following the addition
of Co^{2+} (▲) and Mn^{2+} (●) ions.

increase in A_{240} of the assay system as a function of added Co^{2+} and Mn^{2+} concentration. In the case of the other metal ions added to 2 mM there was no detectable increase in A_{240} of the assay mixture. Examination of each assay component in turn indicated that it was the 3-phosphoglycerate, present at a concentration of 20 mM, that was responsible for moderating the increase in A_{240}. Such an effect presumably results from complex formation between the substrate and the metal ions acting to moderate the precipitation phenomenon. Complex formation between the phosphoglycerate substrates and Mg^{2+} is well documented and in detailed kinetic and thermodynamic studies using the enolase coupled assay (which requires Mg^{2+}) allowance must be made for the effect of Mg^{2+}/phosphoglycerate complex formation on the concentration of available substrates (Ray and Peck, 1972). The dissociation constant of the Mg^{2+}/2-phosphoglycerate complex is 3.6 mM.

It is particularly noteworthy that concentrations of Co^{2+} and Mn^{2+} between 0 and 3 mM produced rates of increase in A_{240} min^{-1} of the assay system comparable to those resulting from authentic enzyme catalysed formation of phosphoenolpyruvate in standard assays (0 to 0.15 min^{-1}; Fig. 46).

None of the metal ions studied had any detectable effect on the A_{240} of the assay buffer (30 mM Tris-HCl buffer, pH 7.0) or the assay mixture of the standard pH 7.0 enolase coupled assay.

171

At a pH of 8.0 (i.e. using 30 mM Tris-HCl buffer, pH 8.0) an
intermediate effect between that at pH 7.0 and 8.7 was observed. On
addition of 2 mM Co^{2+} to the buffer a ΔA_{240} of 0.0062 min^{-1} was
observed, compared to the instantaneous increase observed in 100 mM
Tris-HCl buffer, pH 8.7.

The effects of the metal ions on the determination of phosphoglycerate
mutase by the Leadley *et al*. (1977) assay system were also examined.
The enzymes from wheat germ and *A.nidulans* were assayed and after
observing the ΔA_{240} min^{-1}, metal ions were added to the system and the
new ΔA_{240} min^{-1} observed. The effect of Co^{2+} and Mn^{2+} addition at
concentrations up to 3 mM on the assay of the wheat germ enzyme are
shown in Fig. 47. Examining the effects of Co^{2+} it is clear that the
apparent increase in activity at concentrations above 1 mM is
explicable in terms of the precipitation induced increase in A_{240}
which occurs at these concentrations (Fig. 46) possibly in conjunction
with the enzyme catalysed ΔA_{240}. However, the fact that at lower
concentrations of Co^{2+} (<1 mM) a decrease in activity below that of
the initial assay is observed suggests that the phosphoglycerate
mutase and/or enolase is/are being inhibited. The true enzyme
catalysed ΔA_{240} at higher concentrations of Co^{2+} may therefore be
minimal. At much lower concentrations the effects of precipitation
and/or inhibition are reduced and the ΔA_{240} min^{-1} following addition
of the metal ion therefore approaches that of the initial rate. The
effects of Mn^{2+} are similar although quantitatively different (Fig.
47). However, since the precipitation of this metal ion is less marked

172
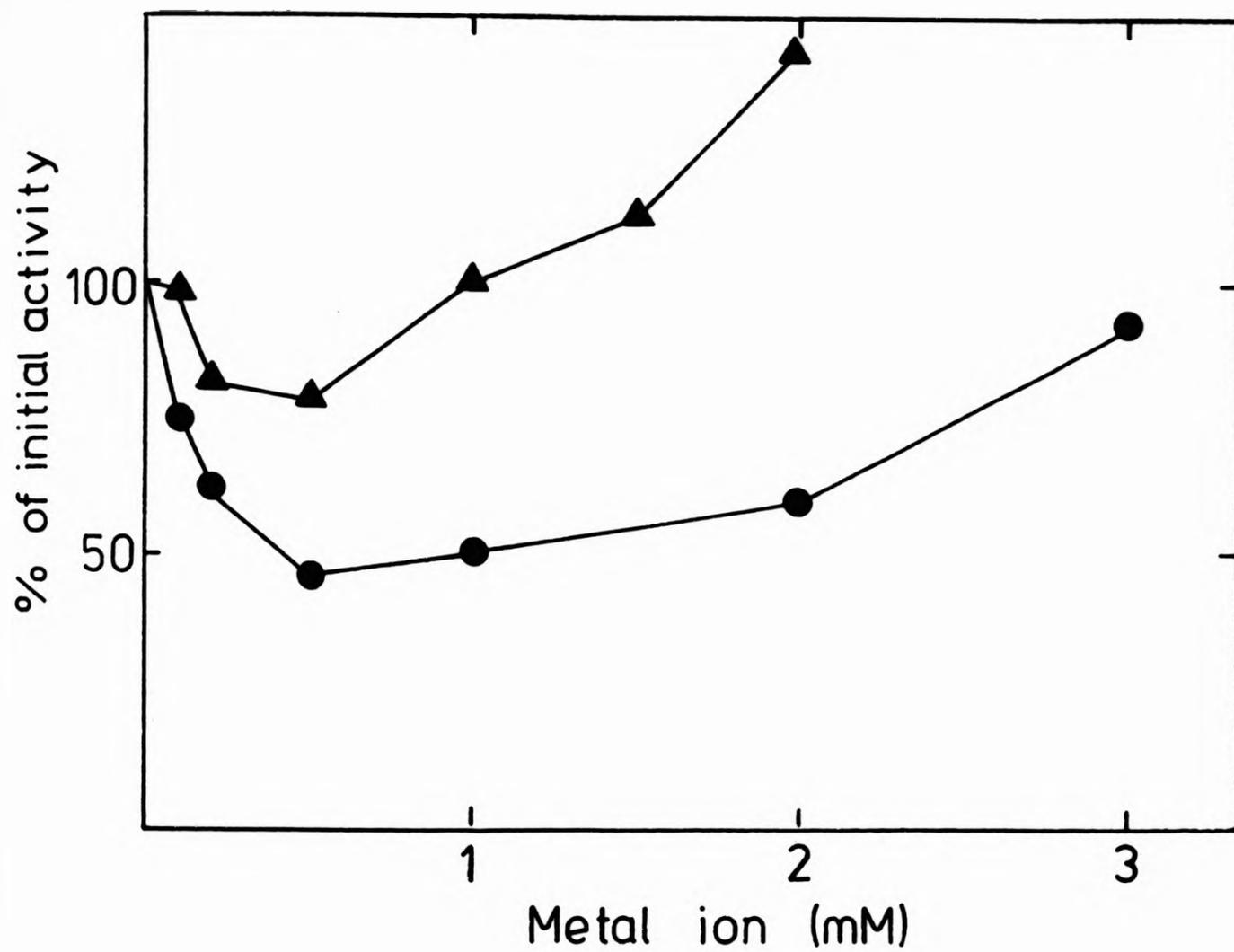
Figure 47.   Effects of metal ions on the determination of wheat germ
             phosphoglycerate mutase by the assay system of Leadley
             et al. (1977).

Activity is expressed as a percentage of the initial activity
observed prior to the addition of Co^{2+} (▲) or Mn^{2+} (●)
ions.

than that of Co^{2+} (Fig. 46) then it would be expected that a higher concentration would be required before precipitation produced apparent "activation" of the enzyme.

The effect of Co^{2+} addition on the assay of *A. nidulans* phosphoglycerate mutase at two enzyme concentrations is shown in Fig. 48. Clearly the pattern of inhibition and apparent activation is similar to that of the wheat germ enzyme. Fig. 48 also illustrates the effect of reducing the amount of enzyme assayed on the concentration dependent effects of Co^{2+}. At lower enzyme concentrations the initial ΔA_{240} min^{-1} is lower and therefore a lower precipitation induced rate is required to produce greater activation. For this reason the exact degree of inhibition and activation appears to differ between the wheat germ and *A. nidulans* enzymes (Figs. 47 and 48).

Further evidence of the inhibition of the enzyme catalysed ΔA_{240} was obtained by assaying the *A. nidulans* enzyme using the standard pH 7.0 enolase coupled assay. In this assay system there was no detectable change in A_{240} as a result of metal ion precipitation. The effect of Co^{2+} on the determination of *A. nidulans* phosphoglycerate mutase activity using this assay is shown in Fig. 49. Clearly, under these conditions, where precipitation plays no rôle, increasing Co^{2+} concentration produces increasing inhibition of the enzyme catalysed reactions.
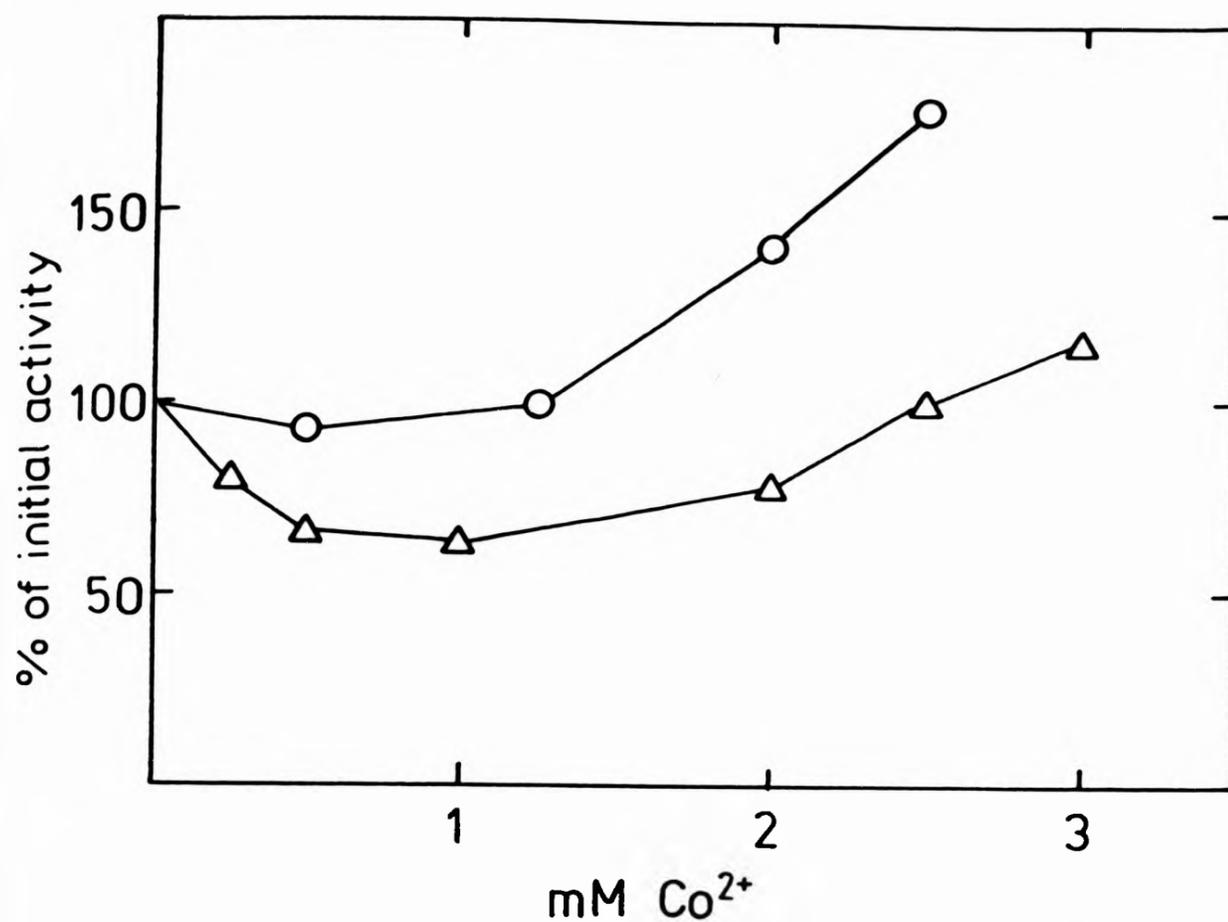
Figure 48.   Effects of Co^{2+} ions on the determination of A. nidulans
             phosphoglycerate mutase by the assay system of Leadley
             et al. (1977)

Activity is expressed as a percentage of the initial activity
observed prior to the addition of Co^{2+} ions.   O   , 0.2 µg
of enzyme assayed;   △   , 0.4 µg of enzyme assayed.
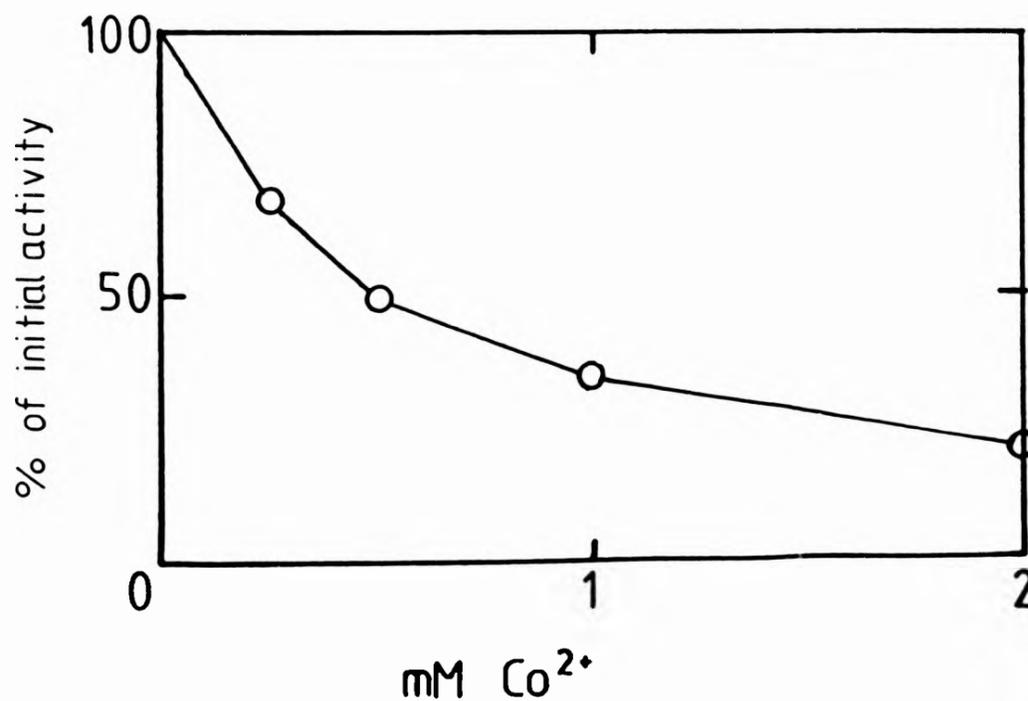
Figure 49.   Effects of Co^{2+} ions on the determination of A. nidulans
             phosphoglycerate mutase by the standard pH 7.0 assay
             system of Rodwell et al. (1957).

Activity is expressed as a percentage of the initial activity
observed prior to the addition of Co^{2+} ions.

The effect of Co^{2+} on enzyme catalysed ΔA_{240} was investigated by varying the amount of enolase present in the standard pH 7.0 assay mixture from its normal value of 40 μg down to 10 μg. In the absence of Co^{2+} this variation had no effect on the assay of a fixed amount of *A.nidulans* enzyme (0.75 μg). However, addition of Co^{2+} to a concentration of 0.5 mM reduced the ΔA_{240} min^{-1} to 60% and 31% of its initial value when between 40 and 10 μg of enolase was present respectively. This dependence on enolase concentration indicates that it is probably this enzyme which is inhibited by the Co^{2+}. Enolase has a well documented Mg^{2+} requirement for activity and in the case of the yeast enzyme this can be fulfilled by Mn^{2+}, Zn^{2+} or Cd^{2+} in the place of the Mg^{2+} (Wold, 1971). Clearly, the activity of the rabbit muscle enolase (used in the above assays) differs since the data in Figs. 47, 48 and 49 imply that this enzyme is inhibited by both Mn^{2+} and Co^{2+}.

The inhibition of the *A.nidulans* and wheat germ phosphoglycerate mutase activities cannot be totally excluded from the brief experiments involving variation of the enolase assay concentration. Indeed, bakers yeast phosphoglycerate mutase appears to be inhibited by millimolar concentrations of Zn^{2+} (Chiba and Sugimoto, 1959) and the rabbit muscle enzyme is susceptible to metal ions such as Hg^{2+}, Ag^{2+} and Cu^{2+} which can react with sulphydryl groups (Cowgill and Pizer, 1956). Therefore the effects of these metal ions and Co^{2+} and Mn^{2+} would merit further investigation as would the effects of these

metals on the enolase coupling system. Both cases could be examined by adopting a stopped coupled assay system such as described below.

6.3.6. Development of a system allowing the assay of phosphoglycerate mutases in the presence of metal ions.

In view of the complications arising from the precipitation of certain metal ions in the pH 8.7 assay of Leadley *et al.* (1977) an assay system at pH 7.0 was developed in which no such effect was observed. Unfortunately this pH is some way from the general optima (approximately 9.0) of the cofactor independent phosphoglycerate mutases (Grisolia and Carreras, 1975). The specific activity of the *A.nidulans* enzyme was 3 fold higher in the pH 8.7 assay compared with that observed in the standard pH 7.0 enolase coupled assay. In addition to this pH change a "stopped coupled quench" procedure was adopted to assay the enzyme activity. In this assay aliquots of the enzyme (maximum 50 μl) incubated with the substrate 3-phosphoglycerate were added to the coupling system which converted the 2-phosphoglycerate formed in the initial reaction mixture through to lactate with the concomitant oxidation of NADH. The rapid decline in A_{340} observed following this conversion was used to calculate the 2-phosphoglycerate formed in the initial reaction mixture. Metal ions could be included in the initial system since the carry over concentration into the coupling system (containing enolase) would be low. A similar protocol had been adopted to allow the assay of cofactor dependent phosphoglycerate mutases in the presence of GdnHCl

(section 3.3.2.1., 4.3.2.1. and 5.3.3.1.). The detailed assay protocol devised was as described in section 2.2.3.2. with the minor modifications noted in section 6.2.6.

The stopped triple coupled assay system was calibrated by addition of known concentrations of 2-phosphoglycerate to the coupling assay. Fig. 50 shows the relationship between 2-phosphoglycerate added and NADH oxidised calculated using a millimolar absorption of 6.22 for NADH at 340 nm . As could be predicted from the reaction series, the stoichiometry of the conversion was 1:1. There was no efect of Mn^{2+}, Co^{2+} or Ni^{2+} on the determination of 2-phosphoglycerate by the coupling assay at concentrations up to 0.25 mM (Fig. 50). Since the assay protocol involved a minimum 20 fold dilution from the initial assay mixture then the effects of metal ions on the phosphoglycerate mutases at concentrations up to 5 mM could be readily examined.

THe stopped triple coupled assay system was used to determine the equilibrium concentration of 2-phosphoglycerate produced in assays performed in 30 mM Tris-HCl buffer, pH 7.0, with the initial concentration of 3-phosphoglycerate being 10 mM. Two cofactor independent enzymes, namely those from *A.nidulans* and wheat germ, and the cofactor dependent enzyme from rabbit muscle enzyme were examined. The rabbit muscle enzyme has a pH optimum of 7.0 (Grisolia and Carreras, 1975) and so its inclusion would hopefully indicate any marked anomalies in the cofactor independent enzymes which were acting well below their pH optima of approximately 9.0 (Grisolia and

176
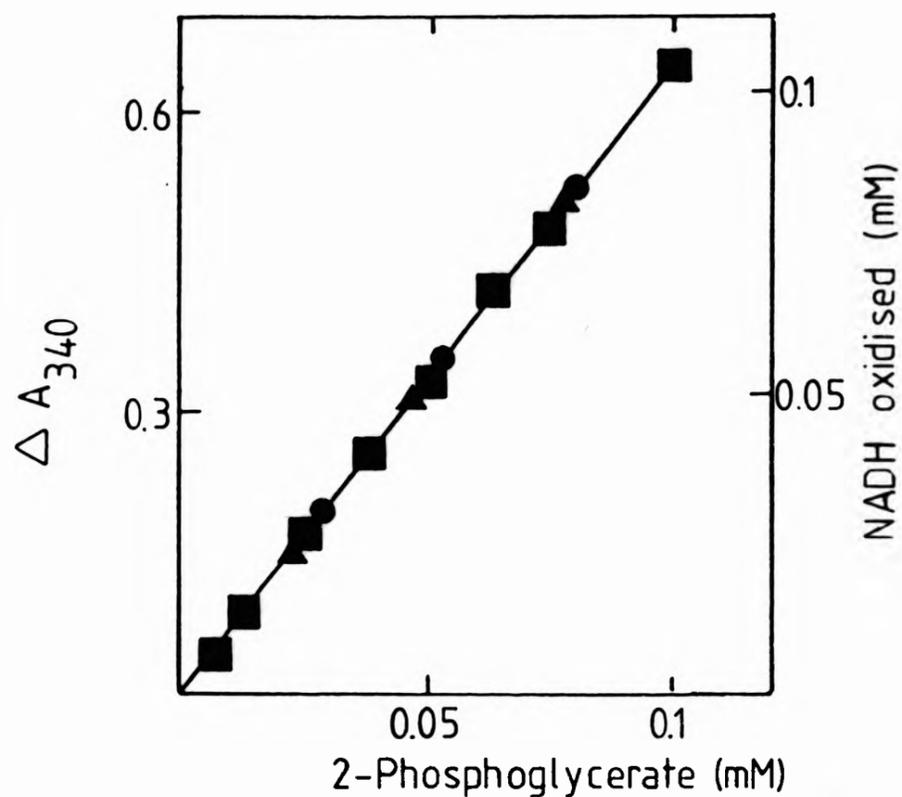
Figure 50.   Calibration of the stopped triple coupled assay system
             with additions of 2-phosphoglycerate.

■ , determinations performed in the absence of added
metal ions;   ▲   ,   ● , determinations performed in
the presence of 0.25 mM Co^{2+} or Mn^{2+} respectively.   0.25 mM Ni^{2+}
was also without effect (data not shown).
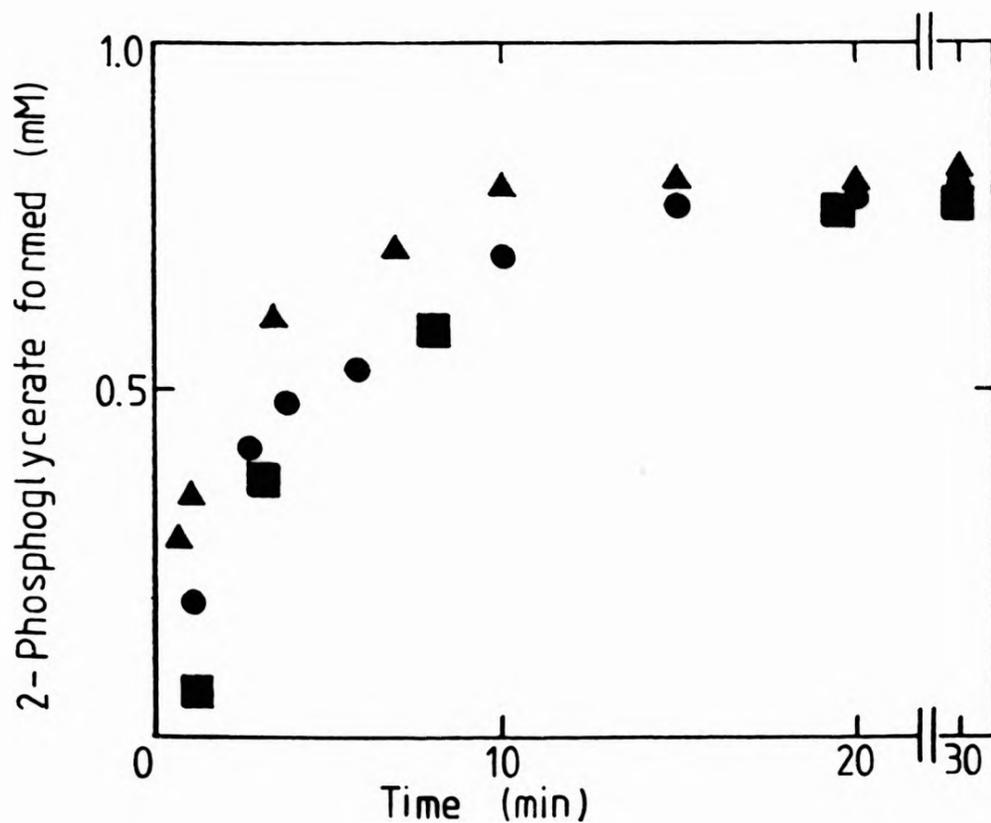
Figure 51.   Determination of the concentration of 2-phosphoglycerate
             formed from 10 mM 3-phosphoglycerate in 30 mM Tris-HCl
             buffer, pH 7.0, at 30°C using the stopped triple coupled
             assay.

Phosphoglycerate mutases from;   ● , wheat germ (1 µg ml^{-1});
▲ , A. nidulans (6 µg ml^{-1}) and   ■ , rabbit muscle
(0.7 µg ml^{-1}).

Carreras, 1975). Fig. 51 shows that all three enzymes produced an equilibrium concentration of 0.8 ± 0.05 mM 2-phosphoglycerate within 20 min incubation at 30°C. The rate at which this equilibrium was reached differed in the three enzymes as the amounts of each in terms of enzyme units added to the initial assay mixture were not identical. The equilibrium concentration of 2-phosphoglycerate allows the calculation of the equilibrium constant and the $\Delta G^{o'}_{303}$ as 0.087 ± 0.06 and 6.15 ± 0.18 kJ mol^{-1} respectively.

The thermodynamic values for the three enzymes examined compare favourably with those reported previously for the phosphoglycerate mutase reaction. Bassham and Krause (1969) have reported a $\Delta G^{o'}_{298}$ of 5.85 kJ mol^{-1} and Hill and Attwood (1976) a $\Delta G^{o'}_{303}$ of 5.81 kJ mol^{-1}. In addition Rodwell *et al.* (1957) have reported a conversion equilibrium mixture of 8.5% 2-phosphoglycerate formed from 88.5 μmole of 3-phosphoglycerate. This obviously equates to 0.85 mM formed from 10 mM 3-phosphoglycerate. Similarly, an equilibrium constant for the 2-phosphoglycerate to 3-phosphoglycerate reaction of between 8.65 and 11.65 has been reported by Clark *et al.* (1974). It therefore appears that the stopped triple coupled assay system described here is able to successfully monitor the 3-phosphoglycerate to 2-phosphoglycerate reaction and that both cofactor dependent and independent phosphoglycerate mutases produce similar conversion equilibria at pH 7.0.

6.3.7 Reactivation of *A.nidulans.* and wheat germ phosphoglycerate mutases following denaturation in GdnHCl.

The stopped triple coupled assay system was used to monitor the reactivation of the *A.nidulans* and wheat germ enzymes after denaturation in GdnHCl. The enzymes were denatured in 2 M GdnHCl for 5 min at 20°C and diluted fifty fold into 30 mM Tris-HCl buffer, pH 7.0, containing 10 mM 3-phosphoglycerate. The rate of 2-phosphoglycerate formation following dilution was monitored using the coupling assay. After determining the activity of the enzymes, metal ions were added to a final concentration of 3.3 mM and their effects on reactivation observed. For the *A.nidulans* enzyme Fig. 52 shows the formation of 2-phosphoglycerate by denatured enzyme and also by a sample of native enzyme incubated in the residual concentration of GdnHCl (0.04 M) present during reactivation. Clearly the control example exhibits the rapid formation of the 2-phosphoglycerate equilibrium concentration of approximately 0.8 mM. The denatured enzyme shows only a minimal rate of 2-phosphoglycerate formation, either as a result of resistance to denaturation or from the rapid reactivation of a small percentage of the enzyme, and this rate is not affected following the addition of Co^{2+}, Mn^{2+}, or Ni^{2+} (indicated by the arrow in Fig. 52). The wheat germ enzyme exhibits an essentially identical pattern of activity in the control and denatured samples (Fig. 53).

Clearly these experiments give no evidence for a metal ion dependent reactivation of either the *A.nidulans* or wheat germ enzymes. This

2-Phosphoglycerate formed (mM)

1.0

0.5

0

5

10

Time (min)
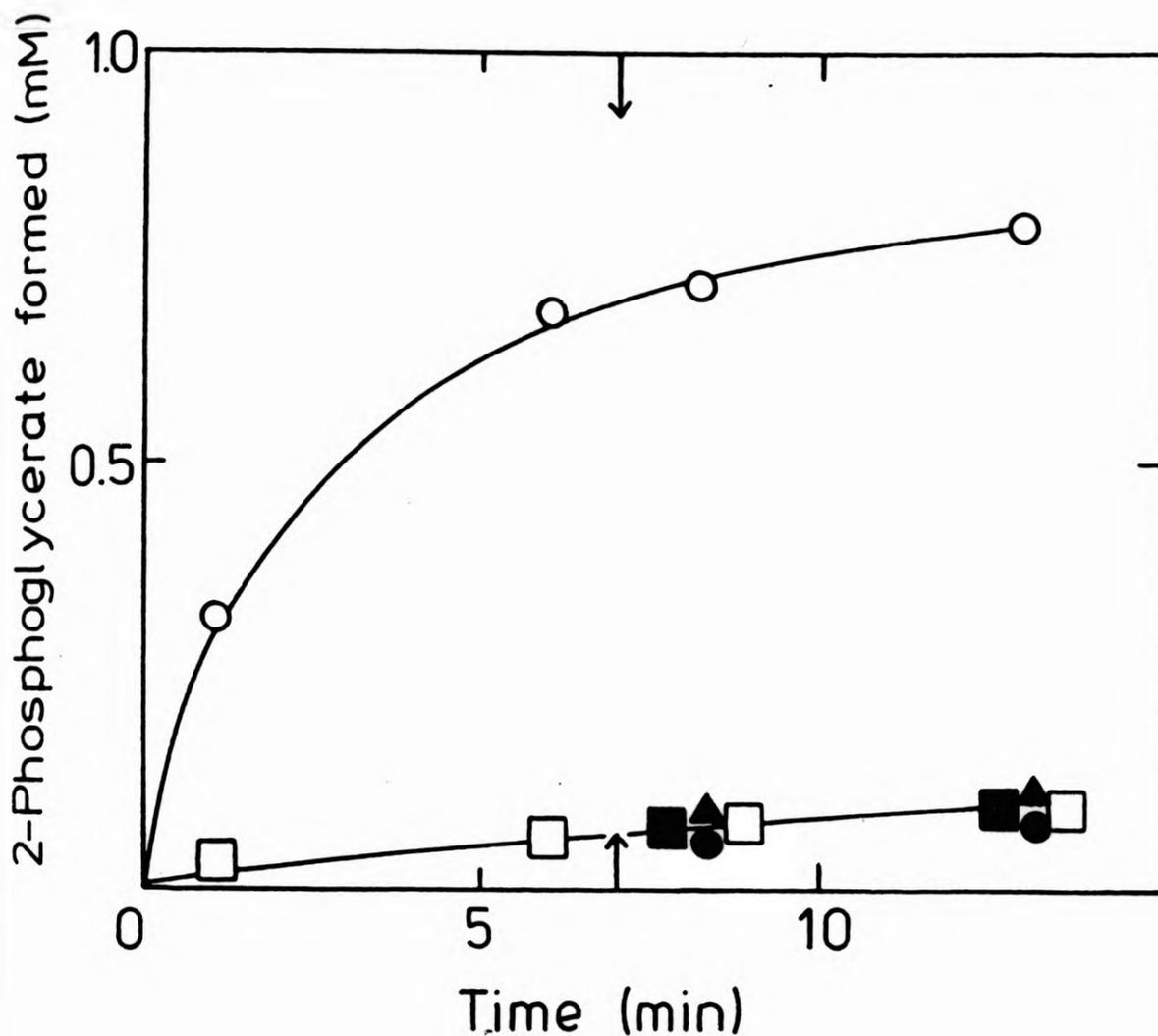
Figure 52.   Reactivation of _A. nidulans_ phosphoglycerate mutase
following denaturation in Gdn HCl.

Figures 52 and 53.   Enzymes were incubated in 30 mM Tris-HCl buffer,
pH 7.0, in the presence of 2 M Gdn HCl for 5 min at 20°C.   At zero
time, samples were diluted into 30 mM Tris-HCl buffer, pH 7.0,
containing 10 mM 3-phosphoglycerate and incubated at 30°C.   The
2-phosphoglycerate formed was determined at intervals using the
coupling assay system.   Metal ions were added to a final concen-
tration of 3.3 mM after 7 min incubation (indicated by the arrow):
□   , control;   ▲   , Co^{2+};   ●   , Mn^{2+};
■   , Ni^{2+}.   The upper curves (○   ) refer to the
native enzyme incubated in the presence of the residual concen-
tration of Gdn HCl following dilution (0.04 M).   Addition of metal
ions to these systems after 7 min incubation had no effect on the
observed 2-phosphoglycerate formation (data not shown).   Enzyme
concentrations are given in section 6.2.7.
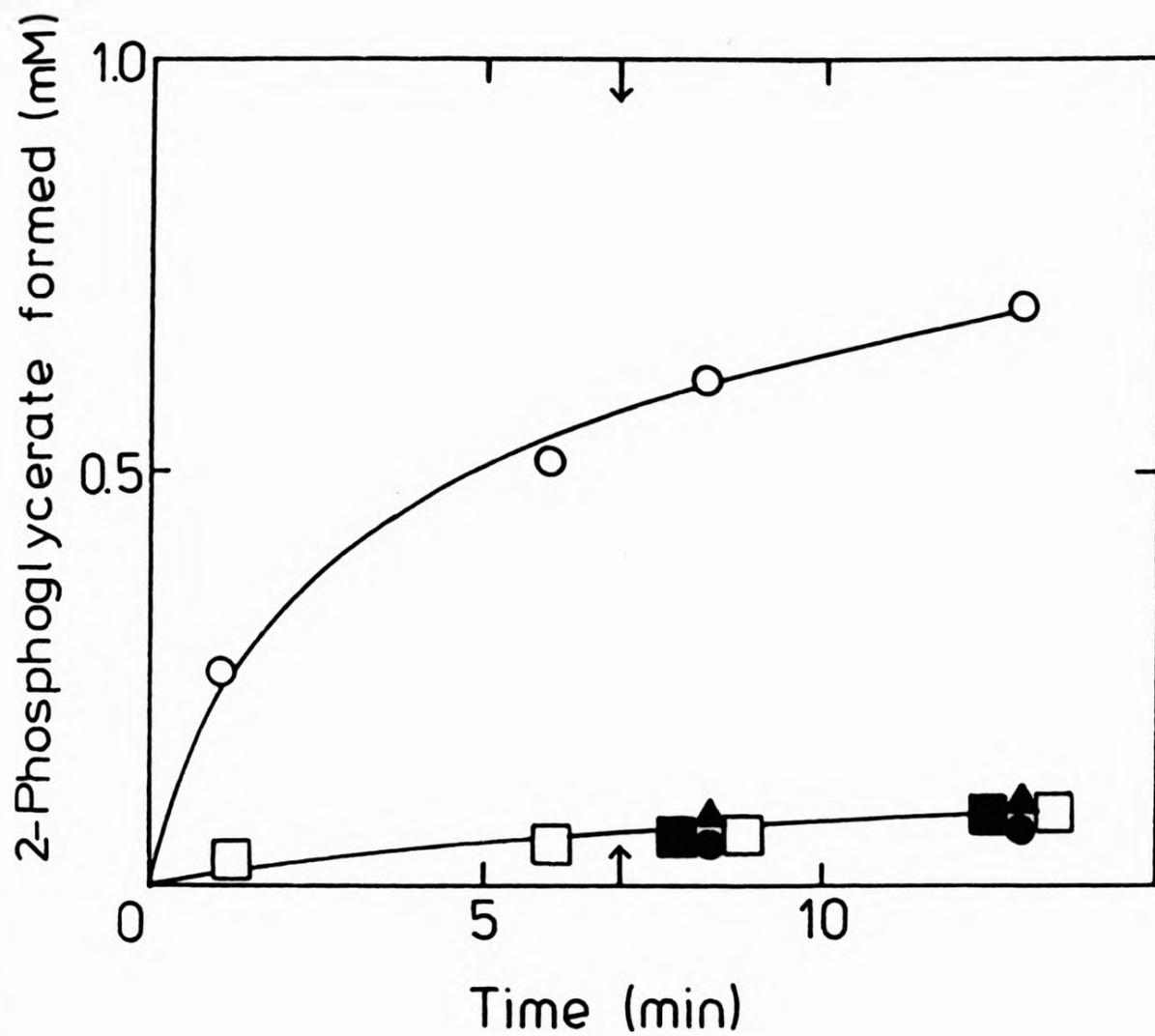
Figure 53. Reactivation of wheat germ phosphoglycerate mutase following denaturation in GdnHCl.

finding is in contrast to the report of Smith *et al.* (1986) which suggested that denaturation of the wheat germ enzyme in GdnHCl could be fully reversed following the addition of Co^{2+} or Mn^{2+} to the reactivation medium. Unfortunately the studies of Smith *et al.* (1986) were performed using the pH 8.7 assay of Leadley *et al.* (1977) at $22^{o}C$ and as shown in section 6.3.5. this system is far from reliable when used in the presence of some transition metals. The apparent reactivation of the wheat germ enzyme on addition of Co^{2+} or Mn^{2+} could thus be explicable in terms of the precipitation of these metal ions producing a ΔA_{240} min^{-1} consistent with full reactivation. The specific activity of the Smith *et al.* (1986) enzyme preparation was approximately 2,300 units mg^{-1} (Smith and Hass, 1985) and during their reactivation studies they assayed 0.84 μg of the enzyme which would therefore give an approximate ΔA_{240} min^{-1} of 0.19. Fig. 46 shows the precipitation induced ΔA_{240} min^{-1} produced by Co^{2+} when added to the pH 8.7 assay system at $30^{o}C$ (0.14 min^{-1} from 2 mM Co^{2+}). During their renaturation studies Smith *et al.* (1986) added 2.6 mM Co^{2+} to obtain complete reactivation. Clearly, at their assay temperature of $22^{o}C$ the addition of this concentration of Co^{2+} would produce a ΔA_{240} min^{-1} of approximately 0.2 in the absence of any enzyme catalysed formation of phosphoenolpyruvate. Smith *et al.* (1986) also reported that the wheat germ enzyme preferentially "bound" Co^{2+} since higher levels of Mn^{2+} were required to produce complete reactivation of the denatured enzyme. This finding is again explicable in terms of the data shown in Fig. 46 where it is evident that precipitation of Mn^{2+} in the assay system produces lower rates of ΔA_{240} min^{-1} than Co^{2+}. Therefore a

higher concentration of Mn^{2+} would be required to produce the required ΔA_{240} min^{-1} of approximately 0.2. Other metal ions examined by Smith *et al.* (1986) had no effect on the reactivation of the wheat germ enzyme. These metal ions also show no precipitation induced ΔA_{240} of the Leadley *et al.* (1977) assay system (section 6.3.5).

The presence of a reductant such as dithiothretiol during the reactivation of the *A.nidulans* or wheat germ enzymes might have produced increased levels of reactivation if either enzyme possessed a susceptible cysteine residue. However the overall failure of these enzymes to regain activity following denaturation in GdnHCl (Figs. 52 and 53) may well be a consequence of the size of their polypeptide chains. Recent reports by Jaenicke (1984) and Teschner *et al.* (1987) have indicated that polypeptides of greater than 300 amino acids in length often refold very slowly and/or inefficiently because of the failure to coordinate the processes of chain folding and domain pairing. In contrast, the polypeptides of smaller proteins or oligomeric proteins with small subunits can refold rapidly and with high efficiency such that under suitable conditions reactivation following denaturation yields almost complete regain of activity. The cofactor dependent phosphoglycerate mutases from bakers yeast (subunit 241 amino acids), rabbit muscle (subunit 250 amino acids) and *Schizosaccharomyces pombe* (205 amino acids) are examples of such proteins (sections 3.3.4., 4.3.3. and 5.3.5.). These observations raise interesting questions as to how the folding of larger proteins occurs *in vivo*. It is tempting to speculate that the folding of such

proteins may require to be regulated and coordinated as co-translational processes such that domain folding can occur correctly prior to the synthesis of remaining regions of the protein. Clearly refolding *in vitro* is without such regulation and this may therefore prevent folding as such, or allow refolding to an inactive configuration. This form of incorrect refolding has been observed for the monomeric protein octopine dehydrogenase where 30% of denatured enzyme forms an inactive species (section 1.7.2.). It is noteworthy that the Mr values for octopine dehydrogenase (45,000) and the enzymes examined here (60,000) are fairly close and therefore the refolding of these enzymes may present similar problems.

The possibility that metal ions could induce structural changes in proteins such as the *A. nidulans* and wheat germ enzymes cannot be completely excluded. A recent report by Frankel *et al.* (1987) has shown that addition of Zn^{2+} to the denatured "zinc finger domain" of transcription factor III A induces its folding. In the presence of Zn^{2+} the circular dichroism, tryptic resistance and thermostability of the denatured peptide are significantly increased implying a folded structure. However, the zinc finger is only a 30 amino acid peptide and therefore conclusions that can be drawn regarding the metal induced folding of larger proteins are necessarily limited. A more accurate description of the metal ions and their relationship to the cofactor independent phosphoglycerate mutases would be required before their effects on refolding could be more systematically investigated and defined.

181

6.3.8. Reactivation of *A.nidulans* phosphoglycerate mutase

following inhibition by chelators.

The reactivation of *A.nidulans* phosphoglycerate mutase following inhibition by the chelators EDTA and HQSA in 30 mM Tris-HCl buffer, pH 7.0 was examined. In two types of experiment metal ions were added to chelator inactivated enzyme and activity monitored using the standard pH 7.0 enolase coupled assay. Control measurements indicated that the maximum carry over concentration of metal ions (0.02 mM) had no effect on the determination of phosphoglycerate mutase by this assay system.

In the first experiment the *A.nidulans* enzyme was inhibited by a low concentration (0.01 mM) of the chelators EDTA and HQSA over a period of 16 h at 4°C. The activity of the enzyme had declined to approximately 2% of a control sample incubated in the absence of chelators. Metal ions were then added to a final concentration of 2 mM in order to saturate chelators and enzyme. No reactivation was observed following the addition of Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Ca^{2+} and Zn^{2+}.

In the second experiment the *A.nidulans* enzyme was inhibited with 0.9 mM EDTA. After 1 hour at 20°C the activity had declined to approximately 2% of a control sample incubated in the absence of EDTA. The chelator was then removed from the enzyme by desalting on Sephadex G-25 and metal ions added to a concentration of 1 mM. No reactivation was observed with Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+} or Zn^{2+} even after 24 h

incubation in the presence of these metal ions. The lack of reactivation following the desalting on G-25, either in the presence or absence of metal ions, indicated that the inactivation of the *A. nidulans* enzyme by EDTA was not reversible. In contrast, Leadley *et al.* (1977) reported that the wheat germ enzyme was reversibly inhibited by EDTA.

A similar approach to the above experiments was successfully used by Watabe and Freese (1979) in their examination of the Mn^{2+} requirement of *Bacillus subtilis* phosphoglycerate mutase. These authors have shown that EDTA at a concentration of 10 mM rapidly inhibits the enzyme. On dilution of the inhibited enzyme 100—fold (EDTA now 0.1 mM) the addition of 1 mM Mn^{2+} completely restored its catalytic activity. Singh and Setlow (1979) have used this type of technique to demonstrate the Mn^{2+} requirement of *Bacillus megaterium* phosphoglycerate mutase. In the case of the *A. nidulans* enzyme a similar type of protocol did not produce any reactivation. It is possible that the metal ion required by this enzyme was not included in these studies. However, if there were a close association between the enzyme and metal ion it could be envisaged that removal of this ion might produce irreversible structural changes in the enzyme. In such a situation the replacement of the metal ion in the medium might therefore not produce reactivation.

The studies described here and in section 6.3.7. have failed to define the putative metal ion required by the *A. nidulans* or wheat germ

enzymes. The limitations of these studies has been noted and it may be that further investigation of these enzymes using modified techniques could reveal a specific metal ion relationship. Indeed, isolation of either enzyme in sufficient quantity would allow the use of techniques such as atomic absorption spectroscopy which could readily determine their metal ion content. However, the possibility does exist that the observed inactivation of the cofactor independent enzymes (Fig. 44) is not due to chelation of a metal ion but to a slow irreversible binding of the anionic chelators to positively charged residues at the active site of these enzymes.

It is well documented that polyvalent anions can inhibit cofactor dependent phosphoglycerate mutases competitively with the substrate (Ray and Peck, 1972). Because of the high levels of substates normally present, inhibition during the assay of enzyme activity is only evident with pyrophosphate, oxalate and citrate via competition with 2,3-biphosphoglycerate. In the absence of substates, as during the chelator sensitivity studies, even low concentrations of such anions might inhibit enzyme activity. It would therefore be of particular interest to re-examine the inhibition of purified cofactor independent phosphoglycerate mutases by EDTA and HQSA in the presence of increasing concentrations of 3-phosphoglycerate. Such studies might clarify the possible rôle of non-specific inhibition in the data presented in Fig. 44. Under such a scheme of anionic inhibition the marked differences in inactivation observed between cofactor dependent and independent phosphoglycerate mutases could be explicable in terms

184

of differences in the three dimensional shape and the amino acid
residues of their active sites. Since the mechanism of phosphoryl
transfer in the two groups of enzymes appears to be quite different
(section 1.1.2.), these possibilities cannot be excluded.

CHAPTER SEVEN : GENERAL

CONCLUSIONS AND DISCUSSION

7.1. GENERAL CONCLUSIONS AND DISCUSSION.

The results presented in this thesis represent a significant contribution to the information currently available on phospho-glycerate mutases. Selected characteristics of a number of phosphoglycerate mutases have been determined and the denaturation of three 2,3-bisphosphoglycerate dependent enzymes has been extensively examined. In addition , the *in vitro* refolding characteristics of a number of cofactor dependent and independent phosphoglycerate mutases have been determined. The conclusions of this thesis will be discussed with reference to these three areas.

7.1.1. Characterisation of phosphoglycerate mutases.

The cofactor dependent and independent phosphoglycerate mutases exhibit a number of interesting differences in terms of their mechanisms of catalytic reaction (section 1.1.2.), structural configurations, ability to bind Cibacron Blue F3GA, sensitivity to vanadate inhibition, possible metal ion requirement and pH optima (sections 1.2.5. and 1.3.4.). These differences are further reflected in the observed phylogenetic distribution of the cofactor dependent and independent enzymes (section 1.1.1.) such that these two groups cannot be considered as closely related. The cofactor dependent phosphoglycerate mutases from bakers yeast, rabbit muscle and *Schizosaccharomyces pombe* and the cofactor independent enzymes from *Aspergillus nidulans*, wheat germ and other sources have been examined

186

in some detail here. The characterisation of these enzymes has contributed to a more accurate definition of the two groups.

S. pombe phosphoglycerate mutase represents the only reported monomeric cofactor dependent enzyme. The M_r of this enzyme (approximately 23,000) and its monomeric configuration have been confirmed here using a number of independent methods (sections 5.3.1. and 5.3.2.). A proportion of the sequence of the *S. pombe* enzyme has been determined (section 5.3.7.) and comparison with the published sequence for bakers yeast phosphoglycerate mutase subunit (Fothergill and Harkins, 1982) reveals a high level of homology, particularly around the two active site histidine residues (8 and 179) of the bakers yeast enzyme (Fig. 43). The areas of the sequence representing inter subunit contact regions of the bakers yeast enzyme quaternary structure have not yet been defined for the *S. pombe* enzyme. However, it might be suggested that these areas will correspond to mutations and/or deletions in the *S. pombe* sequence thereby explaining its monomeric configuration. Further sequence information for the *S. pombe* enzyme will hopefully confirm this proposal and allow future studies to be directed towards the mechanisms of oligomer formation in related enzymes. Techniques such as site-directed mutagenesis could be employed (on the basis of information gained from the *S. pombe* enzyme) to construct mutants of the bakers yeast and rabbit muscle enzymes in dimeric or monomeric quaternary configurations. Such studies would be of interest in indicating the factors controlling oligomer formation in proteins.

The level of sequence homology observed between the bakers yeast enzyme and partial sequence of the *S. pombe* enzyme is consistent with a moderate level of relatedness between these enzymes. Other phosphoglycerate mutases and bisphosphoglycerate mutases also show homology to the bakers yeast enzyme complete and/or active site sequences (sections 1.2.2. and 1.4., Figs. 5 and 6). Very recently the sequence of the muscle isoenzyme subunit of human phosphoglycerate mutase has been determined (Shanske *et al.*, 1987) and this shows some 50% homology to the bakers yeast phosphoglycerate mutase and human bisphosphoglycerate mutase sequences with considerable identity at the active site histidine residues identified in the bakers yeast enzyme (Winn *et al.*, 1981). Overall, it would appear that the cofactor dependent phosphoglycerate mutases and the closely related bisphosphoglycerate mutases exhibit substantial homology and this finding may allow conclusions regarding their evolutionary relationship to be drawn.

The *S. pombe* enzyme has also provided an interesting example of structural changes in a protein that can be induced by cofactor binding and phosphorylation. The presence of the cofactor produces significant increases in enzyme stability, proteinase resistance and resistance to denaturation in GdnHCl (sections 5.3.1., 5.3.3. and 5.3.4.). The sedimentation velocity of the *S. pombe* phosphoenzyme is increased implying that this form has a more compact spherical shape (section 5.3.2.). This structural difference could contribute to the observed differences between the phospho- and apo- forms. However,

further structural analysis of this system using techniques such as X-ray crystallography and high resolution NMR could define more accurately the changes occurring in the presence of the cofactor and relate these to changes in the properties of the enzyme.

Comparisons between the bakers yeast and rabbit muscle enzymes have also been reported here. These have indicated that whilst these enzymes have the same subunit Mr and show considerable homology around the histidine active site residues (sections 1.2.1. and 1.2.2.), they do differ in a number of aspects as indicated by Cleveland mapping, cysteine content, amino acid composition (section 4.3.4.), exposure of aromatic residues (section 4.3.2.2.) and helical content (section 4.3.2.3.). These findings perhaps indicate that mechanistic and active site sequence homology between enzymes is not necessarily reflected in structural and/or other measures of relatedness. As such, future comparisons between the bakers yeast and rabbit muscle enzymes should therefore be made with caution and on the basis of only moderate overall homology. It might be noted that the serine proteinases represent a group of enzymes which exhibit considerable sequence mechanistic homology round a serine, histidine, aspartate system whilst the structural and other characteristics of its members (e.g; chymotrypsin and subtilisin) are quite different (Blow, 1971, Markland and Smith, 1971).

Considerably less information is available regarding the characteristics of the cofactor independent phosphoglycerate mutases. However,

there are some reports that this group of enzymes may require a metal ion for activity (sections 1.3.1. and 1.3.2.) and structural information seems to indicate a common monomeric configuration and Mr of approximately 60,000 (section 1.3.4.). This structural and catalytic pattern has been further examined here. The sensitivity of a range of cofactor independent phosphoglycerate mutases to chelators has indicated that these enzymes may have a metal ion requirement (section 6.3.1.). The native Mr values of this range of enzymes were also found to be approximately 60,000 (section 6.3.2.). On the basis of these observations it seems that the cofactor independent phosphoglycerate mutases examined here do fit into the proposed pattern.

It has not proved possible to define the nature of this putative metal ion requirement using purified enzyme from *A. nidulans* (section 6.3.8.). Similarly it has not proved possible to demonstrate a metal ion requirement for phosphoglycerate mutase reactivation following denaturation in GdnHCl which had been reported by Smith *et al.* (1986) using the wheat germ enzyme. Studies reported here have indicated that reactivation could not be obtained either in the absence or presence of metal ions for the purified phosphoglycerate mutases from wheat germ or *A. nidulans* (section 6.3.7.). Furthermore, a number of potential complications to the procedures used by Smith *et al.* (1986) have been highlighted (section 6.3.5.) and the results presented here should allow subsequent work in this particularly interesting area to proceed without similar problems.

7.1.2. Denaturation of phosphoglycerate mutases.

The denaturation of the cofactor dependent phosphoglycerate mutases from bakers yeast, rabbit muscle and *S. pombe* have been examined in some detail here (sections 3.3.2., 4.3.2. and 5.3.3. respectively). Denaturation in GdnHCl was monitored by loss of catalytic activity and by changes in the fluorescence and circular dichroism properties of the enzymes. The results of these studies are summarised in Table 5.

Activity is generally the most sensitive indicator of an enzyme's structural integrity since inactivation generally precedes the gross conformational changes of denaturation (Tsou, 1986). The inactivation of the phosphoglycerate mutases were assessed without any period of pre-incubation of enzyme and GdnHCl. For the three enzymes examined here inactivation was complete within 0.5 min incubation in 2 M GdnHCl. At lower concentrations some residual activity was observed and the mid-points for the inactivation transitions of the rabbit muscle, bakers yeast and *S. pombe* enzymes were 0.55, 0.65 and 0.85 M GdnHCl respectively. The order of these values seems to correlate with the respective α-helical content of the three enzymes as assessed from native circular dichroism spectra (12%, 20%, 30% and 40% for the rabbit muscle, bakers yeast and *S. pombe* apo- and phosphoenzymes respectively) indicating that the presence of this structural configuration may confer increased stability and resistance to inactivation.

ENZYME	ACTIVITY	FLUORESCENCE			CIRCULAR DICHROISM	
	50% Activity loss transition GdnHCl (M)*	Native λmax (nm)	Denatured λmax (nm)	50% F325 transition GdnHCl (M)*	% helical component	50% θ225 transition GdnHCl (M)*
Baker's yeast	0.65	328	348	1.1	21 ± 4	1.2
Rabbit muscle	0.55	340	348	1.1	12 ± 1	1.1
S. pombe apoenzyme		330	350	0.8	32 ± 6	0.95
S. pombe phosphoenzyme	0.85†	330	350	0.95	43 ± 12	1.15

Table 5. Denaturation of cofactor dependent phosphoglycerate mutases.

* mid-points of the transitions between observed values in 0 and 2 M GdnHCl.

† S. pombe apoenzyme used but this became rapidly phosphorylated by the 2,3-bisphosphoglycerate present during assay (see section 5.3.3.1.).

Fluorescence measurements indicate the environment or relative exposure of a proteins fluorophores (i.e. its aromatic amino acid residues). Changes in fluorescence during denaturation are therefore evidence of structural changes such as polypeptide unfolding and/or subunit dissociation in oligomeric proteins. Fluorescence spectra of phosphoglycerate mutases were assessed following 15 min incubation of enzyme in GdnHCl. The fluorescence spectra of the three enzymes examined showed identity in the presence of 2 or 4 M GdnHCl. The denaturation of the enzymes was therefore judged to be complete following 15 min incubation at 2 M GdnHCl. The profile and λ max of these spectra were consistent with a high degree of solvent exposure of aromatic residues and the fluorescence intensity at the λ max could be roughly related to the molar tryptophan content of the proteins. At lower concentrations of GdnHCl intermediate spectra between those obtained at 0 and 2 M were observed. The mid-points of the fluorescence transitions at 325 nm for the bakers yeast, rabbit muscle, *S. pombe* apoenzyme and *S. pombe* phosphoenzyme were 1.1, 1.1, 0.8 and 0.95 M GdnHCl respectively. The correlation between α-helical content and resistance to denaturation does not seem to apply to these transitions. However, there is evidence of an increased resistance to denaturation in the *S. pombe* phosphoenzyme relative to its apo- form.

Circular dichroism between 200 and 250 nm is an indicator of the α-helical component of proteins since this structural element has a strong characteristic negative ellipticity over this range (Chen *et al.*, 1974, Chang *et al.*, 1978). Changes in circular dichroism during

denaturation can therefore be used to follow the disruption of helical structure. Circular dichroism spectra of phosphoglycerate mutases were assessed following 15 min incubation of enzyme in GdnHCl. The circular dichroism spectra of the bakers yeast and *S. pombe* enzymes in the presence of 4 M GdnHCl were similar to that of random coil structure in proteins (Chang *et al.*, 1978) indicating that helical conformation was essentially disrupted at this concentration. In 2 M GdnHCl there was some evidence for a residual helical element (approximately 10% of the native ellipticty remained in the bakers yeast enzyme following 15 min incubation at 2 M GdnHCl). The persistence of this structural element at 2 M GdnHCl may reflect its relative resistance to denaturation. The mid-points of the circular dichroism transitions at 225 nm for the bakers yeast, rabbit muscle, *S. pombe* apoenzyme and *S. pombe* phosphoenzyme were 1.2, 1.1, 0.95 and 1.15 M GdnHCl respectively. The absolute correlation between helical content and resistance to denaturation does not seem to apply to these values. However, once again there is evidence of an increased resistance to denaturation in the *S. pombe* phosphoenzyme relative to the apo- form.

It would be of interest to extend the inactivation studies reported here to include the measurement of activity loss in the three enzymes following 15 min pre-incubation in GdnHCl. The effect of substrates on the resistance to this treatment might also be revealing. Such data would allow direct comparisons between activity losses and equivalent structural changes determined by fluorescence and circular dichroism. However, since the three enzymes were all rapidly inactivated in 2 M

GdnHCl it seems likely that considerably lower concentrations of GdnHCl might produce significant activity loss over 15 min whilst producing little if any effect on the gross structural features of the enzymes. Observations of this type (i.e; inactivation preceeding structural denaturation) have been made in a number of enzymes (Tsou, 1986).

7.1.3. Refolding of phosphoglycerate mutases.

The protein refolding pathway model discussed in section 1.5. proposes that during refolding protein structure is formed via a collapse to a "molten globule state". This state resembles the conformation of the native protein but differs in that it appears to be functionally inactive and lacks certain spectral characteristics of the native configuration. It has been suggested that this state has a more "open" structure and that subsequent processes of making and breaking of non-covalent and (where appropriate) disulphide interactions lead to the formation of the native configuration.

Refolding of the phosphoglycerate mutases examined here has been followed through regain of enzyme activity. This process was probed using "pulses" of proteinases prior to assessing activity regained. These treatments had no effect on the activity of the native enzyme and could therefore reveal the presence of proteinase sensitive intermediates formed during refolding.

In the case of the tetrameric bakers yeast phosphoglycerate mutase this technique has demonstrated the presence of refolding intermediates which exhibit a partial activity sensitive to the proteinases trypsin, chymotrypsin and thermolysin (section 3.3.4.). The partial activity of these intermediates suggests considerable native-like structure whilst their sensitivity to proteolysis implies a greater exposure of sites to proteolytic attack as a result of a more open structure. These intermediates would therefore seem to represent the molten globule state of the bakers yeast phosphoglycerate mutase refolding pathway. The demonstration of partially active intermediates formed during refolding of the bakers yeast enzyme confirmed and extended similar reports by Hermann *et al*. (1983, 1985). These authors demonstrated that the monomeric and dimeric intermediates formed during refolding of the bakers yeast enzyme had 35% of native activity and that this activity was sensitive to trypsin. The studies reported here have confirmed this value and extended the observed sensitivity of these intermediates to include other proteinases. Hermann *et al*. (1983, 1985) also showed that the refolding intermediates appeared to have significant structure as assessed by circular dichroism and fluorescence. This spectral evidence is consistent with a structured native-like configuration for these molten globule state species.

Refolding studies on the dimeric rabbit muscle phosphoglycerate mutase also revealed the presence of refolding intermediates which have activity sensitive to trypsin, chymotrypsin and thermolysin (section

4.3.2.). These intermediates must have a more open structure and could therefore represent a molten globule state formed on the refolding pathway of this enzyme. The quaternary configuration of these intermediates (i.e; dimers or monomers) remains undefined as no kinetic data for the reassociation of this enzyme are currently available.

Refolding of the monomeric *S. pombe* apoenzyme has also been examined using the proteinase pulse technique. Use of the proteinases chymotrypsin and proteinase K indicated the presence of a refolding intermediate with a marginally increased proteinase sensitivity. The proteinases trypsin and thermolysin were without effect. This finding indicates the presence of a refolding intermediate with some additional sites accessible to proteolytic attack and therefore possessing a more open structure. However, these limited effects can only suggest the detection of a molten globule state intermediate and such conclusions would clearly benefit from further and more detailed examination of the refolding of this enzyme.

The refolding studies on phosphoglycerate mutases have also highlighted a number of potential complications which may be encountered during such experimental work. The bakers yeast enzyme represented perhaps the "ideal" system for refolding studies. Reactivation of this enzyme was found to be almost completely reversible at concentrations between 10 and 50 μg ml^{-1} (section 3.3.4. and Hermann *et al.*, 1983, 1985). The data presented here was found to

be a close fit to the kinetic model of Hermann *et al.* (1983) both in the presence (intermediates showing 0% activity) and absence (intermediates showing 35% activity) of a proteinase pulse prior to assay (section 3.3.4.). Reactivation of the rabbit muscle enzyme was also shown to concur with a kinetic model proposed for this enzyme by Hermann *et al.* (1983) when a proteinase pulse was included (section 4.3.3.). However, the refolding of this enzyme exhibited less than complete reversibility. The formation of intersubunit inactive aggregates represents a major competing reaction to the correct refolding pathway of the rabbit muscle enzyme. The formation of these aggregates is favoured at higher refolding concentrations and therefore the reactivation of the rabbit muscle enzyme is only completely reversible at concentrations of 5 μg ml^{-1} (Hermann *et al.*, 1983). Similar observations have been reported here with lower levels of reactivation observed at higher refolding concentrations (section 4.3.3.).

Reactivation of the monomeric *S. pombe* apoenzyme has been shown to occur very rapidly with some 85% of activity regained within 4 min of refolding (section 5.3.5.). This rapid process has parallels with the refolding of the subunits of the oligomeric bakers yeast enzyme which occurs rapidly within the first minutes of refolding (Hermann *et al.*, 1983) and emphasises that the rate limiting reactions of refolding in oligomeric systems are probably the processes of association which follow subunit refolding.

In contrast to the refolding of the *S.pombe* enzyme, reactivation of the monomeric enzymes from wheat germ and *A. nidulans* could not be demonstrated following denaturation in GdnHCl (section 6.3.7.). The inability of these enzymes to refold may result from the irreversible loss of a catalytic metal ion which may be required by these enzymes (section 7.1.1.). Alternatively, the lack of reactivation in these enzymes may indicate an inability of *in vitro* refolding conditions to mimic accurately the processes of *in vivo* folding. In the *in vivo* case processes of co-translational folding may help to co-ordinate chain folding and domain association in large proteins such as the wheat germ and *A. nidulans* enzymes so that an active conformation is correctly produced. During *in vitro* refolding these controls are absent. Similar differences between the *in vivo* and *in vitro* systems could be invoked in explaining the phenomenon of aggregate formation in the rabbit muscle enzyme and the ability of the *S.pombe* apoenzyme to regain full activity.

The use of a proteinase pulse in the refolding studies reported here has demonstrated the presence of refolding intermediates with reduced proteinase resistance and therefore a more open structure. An additional aim of this technique had been to demonstrate the presence of compact proteinase resistant fragments of structure representing domains. Such structural elements are thought to refold independently and subsequently associate to produce the native configuration (Adams *et al*., 1980). It was hoped that the use of proteinase treatments during reactivation might generate intact folding domains by cleavage

at connecting residues. These folding units could then be analysed structurally and in fragment reassociation studies such as have been applied to lactate dehydrogenase (section 1.7.3.). The search for these structural elements produced during proteinase treatment of refolding enzyme was pursued at greatest length for the bakers yeast enzyme since most structural information which could be related to the characteristics of the fragments is available for this enzyme. Evidence for low levels of fragment production was observed for this enzyme using a thermolysin treatment (section 3.3.4.3.). No other proteinase or phosphoglycerate mutase combination examined yielded further evidence of such fragments and those from the thermolysin treatment of the bakers yeast enzyme were not present at sufficient levels to allow further isolation and characterisation.

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APPENDIX I.

SDS-PAGE.

SDS-PAGE was performed essentially by the method of Laemmli (1970).
Slab gels were prepared and run using a Bethesda Research Laboratories
Inc. vertical slab gel electrophoresis apparatus. The height of the
separating gel was 10 cm and the stacking gel 4 cm. Gels were prepared
in 0.8 or 1.5 mm thicknesses. Acrylamide and bis-acrylamide were
present at a ratio of 30:0.8 respectively in both separating and
stacking gels.

Separating gels comprised 0.375 M Tris-HCl buffer, pH 8.7, 0.1% w/v
SDS and the required percentage acrylamide concentration and were
polymerised by the addition of 0.35% v/v 10% ammonium persulphate
solution and 0.075% N, N, N', N'- Tetramethylethylenediamine. Stacking
gel comprised 0.125 M Tris-HCl buffer pH 6.8, 0.1% w/v SDS and 4% w/v
acrylamide and were polymerised as for the separating gel. Samples for
SDS-PAGE were prepared by mixing with an equal volume of double
strength sample buffer comprising 0.125 M Tris-HCl buffer, pH 6.8, 3%
w/v SDS, 10% v/v glycerol and 10% v/v 2-mercaptoethanol. Samples were
boiled for 3 min and after cooling a mixture of 2-mercaptoethanol and
1% w/v bromophenol blue (50:50) was added to a final concentration of
5% v/v.

Gels were stained in a solution containing 0.1% w/v Coomassie blue R250 (CI 42660) in acetic acid: methanol: distilled water (2:2:5) for 1 h and destained in 10% acetic acid. Silver staining was performed as described below.

Silver staining.

Gels were silver stained essentially by the method of Wray *et al*. (1981) either directly following electrophoresis or after conventional Coomassie blue R250 staining. Gels were fixed in 50% v/v methanol containing 0.038% v/v formaldehyde overnight. The staining solution was prepared by mixing solution A comprising 0.8g silver nitrate in 4 ml distilled water with solution B comprising 21 ml 0.36% w/v NaOH and 1.4 ml 14.8 M NH_4OH. The staining solution was made up to 100 ml and used within 5 min. Staining was for 15 min with constant gentle agitation. After staining the gel was washed in several changes of distilled water and developed in 0.005% w/v citric acid solution containing 0.038% v/v formaldehyde until the required band intensity was achieved. Development was stopped using a 50% v/v methanol, 10% v/v acetic acid solution and the gel transfered to water for storage. Disposable gloves were used in all handling operations to avoid transfer of protein onto the gel surface.

A. *Nidulans* culture medium.

A. *Nidulans* was grown in a minimal salts growth medium containing sucrose. The culture medium contained per litre;

K_2HPO_4 . $3H_2O$, 0.74 g; KH_2PO_4, 0.93 g; Na_2SO_4, 1.6 g; $NaNO_3$, 1 g; KCl, 0.5 g; $MgSO_4$. $7H_2O$, 0.25 g; $ZnSO_4$. $7H_2O$, 10 mg; $FeSO_4$. $7H_2O$, 10 mg; $CuSO_4$. $5H_2O$, 5 mg and sucrose, 50 g and was autoclaved at 120°C, 15 p.s.i. for 15 min.

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APPENDIX II

The equation relating the slope of the ln A against r^2 plot to a proteins Mr is ;

$$\text{Slope} = \frac{0.011 \; w^2 \; Mr \; (1-\bar{v}p)}{2RT}$$

where w is the rotor speed (rev/min) , $\bar{v}$ is the partial specific volume of the protein , p is the density of the solution , R is the gas constant and T is the absolute temperature . Thus ;

$$Mr = \frac{2RT \cdot \text{slope}}{0.011 \; w^2 \; (1-\bar{v}p)}$$

| Amino acid | Bakers yeast enzyme | | Rabbit muscle enzyme |
	(1)	(2)	
ASP + ASN	28.0	29.5	20.7
THR	8.4	9.0	15.8
SER	15.1	14.7	11.5
GLU + GLN	27.5	25.9	31.6
PRO	15.9	14.2	13.1
GLY	15.6	15.8	21.5
ALA	23.0	21.5	23.3
CYS	0.0	0.0	2.1
VAL	14.8	13.0	11.9
MET	1.0	1.8	3.3
ILE	9.6	10.7	12.9
LEU	25.5	26.7	20.7
TYR	8.2	7.3	5.6
PHE	5.6	5.6	6.8
HIS	3.7	3.8	7.3
LYS	23.4	22.0	18.7
ARG	11.5	14.7	18.1
TRP	4.2	4.8	4.9
	241.0	241.0	250.0

Table I A . Amino acid composition of bakers yeast and rabbit
         muscle phosphoglycerate mutases .

Bakers yeast enzyme : (1) Published data ; Fothergill and Harkins
                          (1982) .
                     (2) Data from Prof. J. Coggins.

Rabbit muscle enzyme : Data from Prof. J. Coggins.
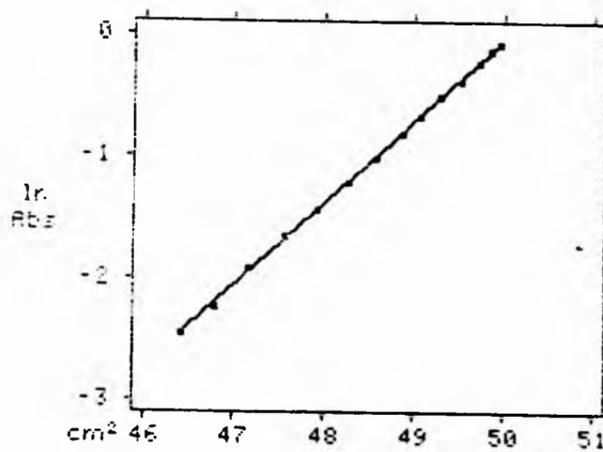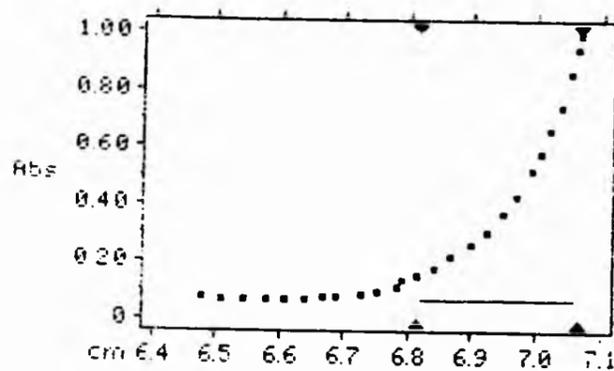
Calculated parameters:

Molecular weight : 19904	Formal correlation coeff.: .999691
Slope of graph : .66944	Baseline left margin (E): .07
Total data number: 26	Baseline right margin (E): .07
Data number used : 14	Slope of baseline (E/mm): 0
Arithmet. average: 6.9412	Number of left margin : 13
Geometric average: 6.9649	Number of right margin : 26

Fig. 1A

Calculated parameters:
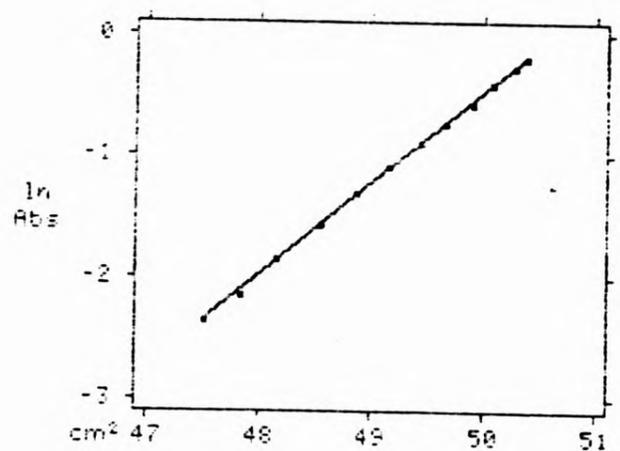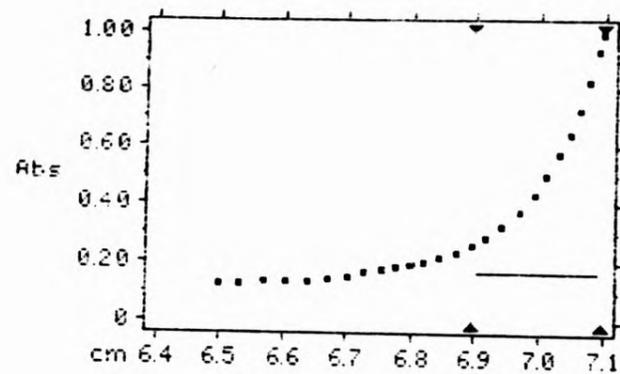
Molecular weight : 22453   Formal correlation coeff.: .999793
Slope of graph : .75795   Baseline left margin (E): .17
Total data number: 27   Baseline right margin (E): .17
Data number used : 12   Slope of baseline (E/mm): 0
Arithmet. average: 6.9961   Number of left margin : 16
Geometric average: 7.0112   Number of right margin : 27

Fig. 2A

Amino acid	Hydropathy	Number	Mole %	(Mole % X Hydropathy)
ASP + ASN	-3.5	22.1	10.8	-37.8
GLU + GLN	-3.5	25.5	12.5	-43.8
SER	-0.8	11.0	5.4	-4.3
GLY	-0.4	19.6	9.6	-3.8
HIS	-3.2	6.8	3.3	-10.6
ARG	-4.5	11.3	5.5	-24.8
THR	-0.7	9.0	4.4	-3.1
ALA	1.8	13.1	6.4	11.5
PRO	-1.6	7.1	3.5	-5.6
TYR	-1.3	6.5	3.2	-4.2
VAL	4.2	8.6	4.2	17.6
MET	1.9	1.3	0.6	1.1
ILE	4.5	11.9	5.8	26.1
LEU	3.8	21.3	10.4	39.5
PHE	2.8	3.8	1.9	5.3
LYS	-3.9	19.9	9.7	-37.8
TRP	-0.9	5.1	2.5	-2.3
CYS	2.5	0.9	0.4	1.0

205.0   (102.1-178.1) = -76.0

GRAVY SCORE = $\frac{-76.0}{205.0}$ = -0.371

Table II A . Amino acid composition and calculated hydropathy

values for S.pombe phosphoglycerate mutase .

Amino acid hydropathy values from Kyte and Doolittle (1982) .

```
Peptide (1) CL 11/1 : H-S-E-S-E-W-N - K
Peptide (2) CL 31/2 : E-L-A-T-G-V-P-I-V-Y-Y-L-Q-K
Peptide (3) CH 36/B :       A-T-G-V-P-I-V-Y
Peptide (4) CL 22/1 : Y-H-S-D-L-Q-G-L-N-K-D-D-A-R
Peptide (5) CL 19/1 : K-K-W-G-A-E-Q-V-Q-I-W-R
Peptide (6) CH 38/B :         G-A-E-Q-V-Q-I-W
Peptide (7) CL 23/2 : S-Y-D-I-A-P-P-N-G-E-S-L-K-D-T-A - - - - - E-R
Peptide (8) CL 23/1 : S-T-I-V-A-A-I-L-K-G-V-K-V-L-I-A-A-H-G-N-S-L-R
Peptide (9) CL 34/2 : A-L-I-P-D-L-E-G-L-T-G-D-Q-I-V-K
```

Table III A . Sequence of clostripain (CL) and chymotrypsin (CH) peptides
            obtained following proteolysis of <u>S.pombe</u> phosphoglycerate
            mutase .

The position of these peptides in alignement with the published sequence
of bakers yeast phosphoglycerate mutase are indicated in Fig 43 .

The two chymotrypsin peptides (3) and (6) confirm areas of the sequence
of the clostripain peptides (2) and (5) but do not indicate any areas of
ssquence overlap .

PUBLISHED WORK

The susceptibility towards proteolysis of intermediates during the renaturation of yeast phosphoglycerate mutase

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The renaturation of the tetrameric enzyme phosphoglycerate mutase from baker's yeast after denaturation in guanidinium chloride was studied. Three proteinases (trypsin, chymotrypsin and thermolysin) cause extensive loss of activity of samples taken during the early stages of refolding. As judged by SDS/polyacrylamide-gel electrophoresis, the proteinases cause substantial degradation of the polypeptide chain with no evidence for large quantities of fragments of M_r greater than 6500. These data suggest that the early intermediates in the refolding, especially the folded monomer, possess a number of sites that are susceptible to proteolysis.

INTRODUCTION

The renaturation of oligomeric enzymes after denaturation by agents such as guanidinium chloride has proved to be a useful experimental model for the folding and association of these enzymes during biosynthesis (Jaenicke, 1984). As shown by Hermann et al. (1981), the kinetic aspects of the re-association process and the catalytic properties of intermediates can be explored by comparing the rates of regain of enzyme activity and of quaternary structure, e.g. by the glutaraldehyde cross-linking technique. In most cases, the activity of intermediates is small, if not zero (Jaenicke, 1982; Jaenicke & Rudolph, 1983), although exceptions have been reported in the cases of rabbit muscle fructose bisphosphate aldolase (Chan et al., 1973; Rudolph et al., 1977) and rabbit muscle creatine kinase (Grossman et al., 1981). In earlier work (Hermann et al., 1983, 1985) we studied some aspects of the reconstitution of the tetrameric enzyme phosphoglycerate mutase (EC 2.7.5.3) from baker's yeast. Over a range of concentrations, the re-association process could be described by a model of the type:

$$4M \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} 2D \overset{k_{+2}}{\rightarrow} T \qquad (1)$$

where M, D and T represent monomer, dimer and tetramer respectively and where the values of k_{+1}, k_{-1} and k_{+2} are 6.26×10^3 $M^{-1} \cdot s^{-1}$, 6.0×10^{-3} s^{-1} and 2.75×10^4 $M^{-1} \cdot s^{-1}$ respectively in 50 mM-sodium phosphate buffer, pH 7.5, at 20 °C (Hermann et al., 1983). As judged by c.d., the enzyme regains approx. 85% of its native secondary structure within 30 s of the start of the refolding process, showing that M in scheme (1) represents a folded monomer (Hermann et al., 1983). M, D and T possess approx. 35%, 35% and 100% respectively of the activity of native enzyme; however, the activity of M and D differs from that of T in being sensitive to trypsin (Hermann et al., 1985).

Susceptibility to proteolysis has proved to be a valuable method for studying structural changes in proteins during refolding (Girg et al., 1981; Price & Stevens, 1983a; Schmid & Blaschek, 1984). In the present paper we have explored the sensitivity of the enzyme during refolding to three different proteinases, trypsin, chymotrypsin and

thermolysin. In all three cases there is a substantial loss of activity in the early stages of refolding. Analysis by SDS/polyacrylamide-gel electrophoresis shows that this loss of activity is associated with extensive digestion of the polypeptide chain. There was no evidence for the production of substantial quantities of fragments of M_r greater than 6500, suggesting that the early formed intermediates possess a number of sites that are susceptible to proteolysis.

MATERIALS AND METHODS

The following companies supplied the reagents listed: BDH Chemicals, guanidinium chloride (AristaR grade); Sigma Chemical Co., Reactive Blue 2–Sepharose CL-6B, Dalton Mark VII-L M_r-marker set, turkey egg-white proteinase-inhibitor protein (type II-T), α-chymotrypsin-[tosyl-lysylchloromethane ('TLCK')-treated, dialysed, freeze-dried], subtilisin BPN', elastase (type IV) and thermolysin (stabilized with calcium acetate and sodium acetate); Boehringer Mannheim, proteinase K from *Tritirachium album* Limber, 3-phosphoglycerate (grade I), 2,3-bisphosphoglycerate and enolase; Worthington Corp., trypsin [three times recrystallized, tosylphenyl-alanylchloromethane('TPCK')-treated];Bayer,aprotinin (Trasylol).

Phosphoglycerate mutase was initially prepared from baker's yeast by the method reported previously (Price & Jaenicke, 1982). However, some difficulties were encountered in reproducing the ion-exchange step with different batches of DEAE-cellulose. An alternative method of purification was devised, taking advantage of the finding that yeast phosphoglycerate mutase binds to Cibacron Blue–Sepharose and can be eluted by bisphosphoglycerate (Price & Stevens, 1983b). The purification procedure of de la Morena et al. (1968) was followed as far as fraction 3, which was then dialysed overnight (at 4 °C) against 50 mM-sodium phosphate buffer, pH 7.5. The protein precipitated between 58% and 70% saturation with $(NH_4)_2SO_4$ was collected and re-extracted by the procedure of de la Morena et al. (1968), and the fraction containing maximal activity was dialysed against 10 mM-Tris/HCl buffer, pH 8.0. The dialysis residue was then applied to a column (10 cm × 1.8 cm²) of Cibacron

Blue–Sepharose equilibrated against the Tris buffer. Unbound proteins were eluted by a wash with 20 ml of Tris buffer, and then some of the contaminating proteins (probably dehydrogenases and/or kinases) were eluted by a wash with 20 ml of Tris buffer containing 1 mM-AMP. Phosphoglycerate mutase was then eluted by a wash with 20 ml of Tris buffer containing 1 mM-bisphosphoglycerate. The fractions containing activity were combined, and the enzyme was precipitated by addition of solid $(NH_4)_2SO_4$ to 80% saturation. Analysis by SDS/polyacrylamide-gel electrophoresis showed that the enzyme was at least 95% homogeneous by staining with Coomassie Blue, and the specific activity (850 μmol of 3-phosphoglycerate consumed/min per mg of protein) was comparable with previously reported values. The assay of enzyme activity by an enolase-coupled assay and spectrophotometric determination of protein concentration were performed as described previously (Hermann et al., 1983).

Turkey egg-white proteinase-inhibitor protein was coupled to Sepharose 4B by using the CNBr activation procedure (March et al., 1974). The amount of inhibitor bound to the gel was determined as 2.0 mg/ml of packed gel by measuring the protein removed after reaction by a series of sequential washes at pH 9.5 and pH 4.0. Control experiments showed that the immobilized inhibitor protein inactivated at least 97% of the activity of chymotrypsin or trypsin in solution under the conditions used in the refolding experiments (see below).

Denaturation in guanidinium chloride and refolding of phosphoglycerate mutase were performed as described previously (Hermann et al., 1983). Samples withdrawn from the renaturation mixture at various times were diluted if necessary to a standard protein concentration of 10 μg/ml, and incubated for 1 min at 20 °C with proteinase (20 μg/ml or 5 μg/ml) before enzyme assay or analysis by SDS/polyacrylamide-gel electrophoresis with the buffer system of Laemmli (1970) with 15%-acrylamide slab gels of 1.5 mm thickness. The inhibition of thermolysin before SDS/polyacrylamide-gel electrophoresis was performed by addition of EDTA (final concentration 2 mM) (Girg et al., 1981). Trypsin and chymotrypsin could be effectively inhibited by addition of SDS to a final concentration of 0.1% followed by rapid transfer to a boiling-water bath and incubation for 2 min at 100 °C (Price & Stevens, 1982). Alternatively, the sample (3 ml) was mixed with 0.25 ml of immobilized turkey egg-white proteinase-inhibitor protein and centrifuged (1000 g for 1 min) before addition of SDS and boiling. The latter method had the advantage that trypsin and chymotrypsin could be effectively removed from solution, and hence bands due to these proteinases did not appear on SDS/polyacrylamide-gel electrophoresis. For the determination of M_r the Dalton Mark VII-L marker set was supplemented by addition of aprotinin (M_r 6500).

Staining and destaining of gels with Coomassie Blue was performed by the procedure of Laemmli (1970). The gels were scanned with a Gelman DCD 16 scanner. Subsequent silver staining of the gels was performed by the method of Wray et al. (1981).

RESULTS AND DISCUSSION

Regain of activity at different enzyme concentrations

As shown in Fig. 1, phosphoglycerate mutase activity was regained rapidly following the dilution of guanidinium
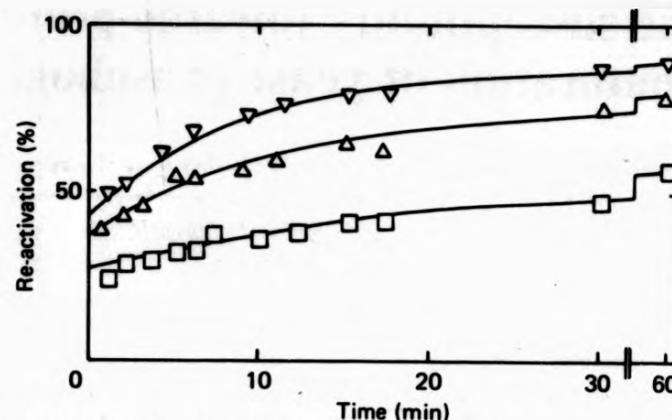

Fig. 1. Re-activation of phosphoglycerate mutase

Enzyme was denatured by incubation in 4 M-guanidinium chloride and renatured by dilution of the guanidinium chloride to 0.1 M in 50 mM-sodium phosphate buffer, pH 7.5, at 20 °C. The activity of samples taken was assayed in the absence of added proteinase. Final concentration during renaturation: ▽, 50 μg/ml; △, 30 μg/ml; □, 10 μg/ml. Continuous lines were calculated assuming the model shown in scheme (1), with the monomeric and dimeric intermediates each possessing 35% of the activity of the tetramer (Hermann et al., 1985).

chloride to 0.1 M. Data are shown for final enzyme concentrations of 10, 30 and 50 μg/ml; the continuous lines in the Figure are calculated on the basis of the model (scheme 1) in which, in the absence of proteinases, the monomeric and dimeric species possess 35% of the activity of the tetramer (Hermann et al., 1985).

Susceptibility of native enzyme to proteolysis

In order to decide which proteinases might be suitable for exploring structural changes in the enzyme during refolding, some experiments were carried out in which native enzyme (400 μg/ml) was incubated with various proteinases in 50 mM-sodium phosphate buffer, pH 7.5, at 20 °C: trypsin (40 μg/ml), chymotrypsin (40 μg/ml), thermolysin (40 μg/ml), elastase (20 μg/ml), subtilisin (10 μg/ml) and proteinase K (10 μg/ml). Under these conditions native enzyme was relatively stable towards trypsin, chymotrypsin and thermolysin ($\leq$ 10% loss of activity after 5 min, 40–65% loss after 60 min) but much less stable towards elastase, subtilisin and proteinase K (50%, 65% and 65% loss of activity respectively after 5 min). In general, these rates of loss of activity were correlated with the rates of loss of the intact subunit polypeptide chain (M_r 28000), as detected by SDS/polyacrylamide-gel electrophoresis. Since intermediates of refolding are likely to be more susceptible than native enzyme to proteolysis (Girg et al., 1981), it was concluded that, of the proteinases tested, trypsin, chymotrypsin and thermolysin afforded the best possibilities of exploring structural changes in the enzyme during refolding.

Effect of proteinases on enzyme activity during refolding

When the activities of phosphoglycerate mutase were assayed after 1 min incubation with proteinase, there was a marked decrease in activity regained, compared with control samples that had been incubated for 1 min in the absence of proteinase before assay; this decrease was especially marked at the early time points. Fig. 2 shows the results obtained at a phosphoglycerate mutase
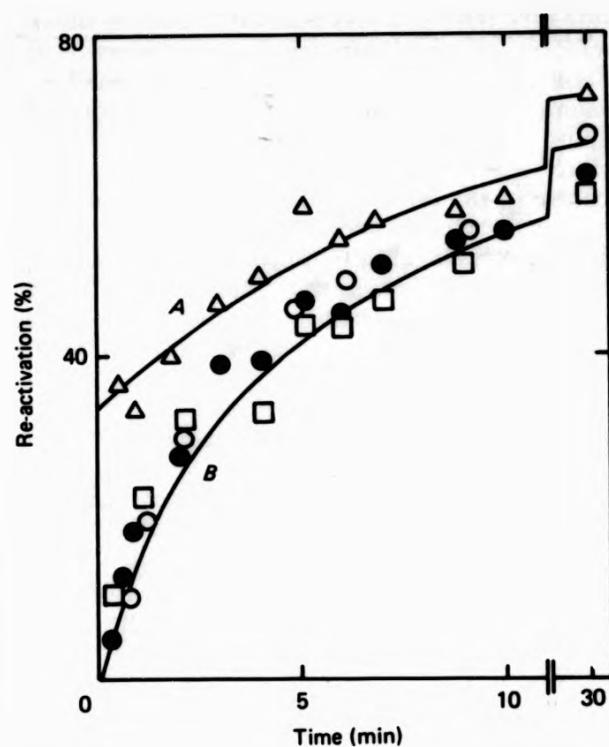
Fig. 2. Effect of proteinases on the re-activation of phosphoglycerate mutase

The final concentration of phosphoglycerate mutase during renaturation was 30 μg/ml. $\triangle$, Activity assayed in the absence of added proteinase; ●, ○ and □, activity assayed after 1 min incubation with trypsin, chymotrypsin and thermolysin respectively (each 20 μg/ml). The results obtained after incubation with proteinases at 5 μg/ml were qualitatively similar although the effects were rather less marked. Other conditions were as indicated in Fig. 1 legend. The continuous line A was calculated by using the model of scheme (1), with the monomeric and dimeric intermediates each assumed to possess 35% of the activity of the tetramer. The continuous line B was calculated by assuming these intermediates to possess zero activity.

concentration of 30 μg/ml during refolding; the results at 10 μg/ml and 50 μg/ml showed a similar effect. Under the conditions chosen, the activity of native phosphoglycerate mutase (in the absence or in the presence of 0.1 M-guanidinium chloride, the residual concentration of denaturant during refolding) was not significantly affected by incubation with the proteinases for 1 min. The continuous line A in Fig. 2 is calculated on the basis of the line in Fig. 1, i.e. that monomeric and dimeric species each possess 35% of the activity of the tetramer. The continuous line B in Fig. 2 is calculated on the basis that the monomeric and dimeric species possess zero activity. The results obtained in the presence of the proteinases show reasonable agreement with the calculated line B, thus confirming the earlier conclusion (Hermann et al., 1985) that the activities of the monomeric and dimeric species are sensitive to trypsin, and extending this conclusion to the effects of other proteinases with differing bond specificities.

Digestion of phosphoglycerate mutase by proteinases during refolding

SDS/polyacrylamide-gel electrophoresis was used to examine the digestion by proteinases of the enzyme
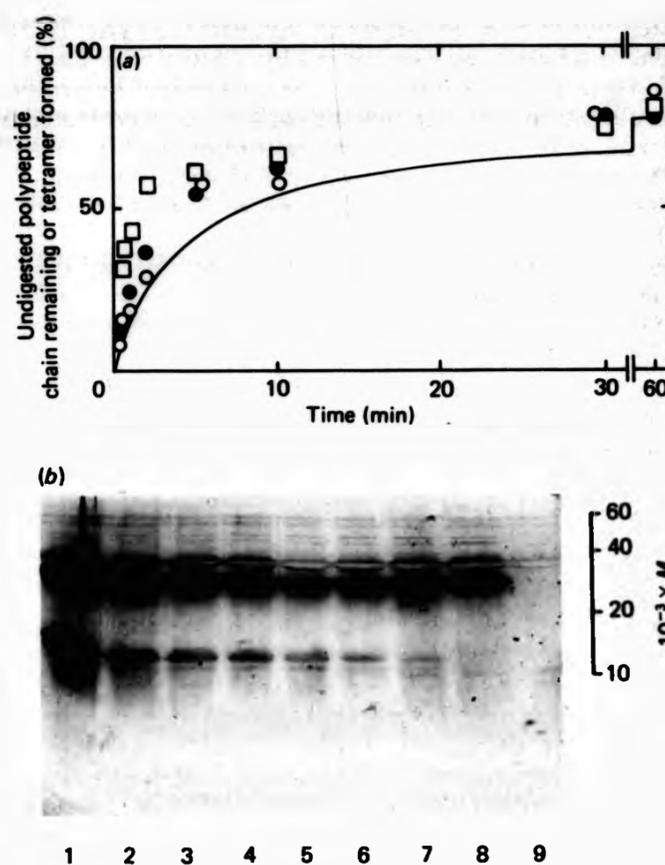

Fig. 3. Digestion of phosphoglycerate mutase by proteinases during refolding

Conditions were as indicated in Fig. 2 legend. Samples taken during renaturation were incubated for 1 min with proteinase (20 μg/ml) and then analysed by SDS/polyacrylamide-gel electrophoresis. (a) Percentage of undigested polypeptide chain (M_r 28000) remaining plotted as a function of time of renaturation. ●, ○ and □ represent digestion by trypsin, chymotrypsin and thermolysin respectively. The continuous line is the percentage of tetramer formed, calculated in accordance with scheme (1) (see line B in Fig. 2). (b) SDS/polyacrylamide gel electrophoresis of samples incubated with thermolysin. The gel was stained with silver according to the method of Wray et al. (1981). Lanes 1–7 represent samples taken after 0.25, 0.5, 1, 2, 5, 30 and 60 min renaturation respectively. Lane 8 represents native phosphoglycerate mutase incubated with thermolysin under these conditions. Lane 9 represents thermolysin. The M_r scale from standard proteins is shown on the right. Possible reasons for apparent negative silver staining of some proteins (e.g. thermolysin) have been discussed by Morrissey (1981).

during refolding. The results of these experiments are shown in Fig. 3(a) for the refolding experiments performed at a phosphoglycerate mutase concentration of 30 μg/ml and subsequent incubation with 20 μg of proteinase/ml for 1 min. The ordinate represents the percentage of intact polypeptide chain (M_r 28000) material remaining after incubation with the proteinase relative to the control sample, which represents native enzyme. (It should be noted that incubation of native enzyme with the proteinases under these conditions caused no significant digestion of the polypeptide chain.) It is clear that for all three proteinases tested (trypsin, chymotrypsin and thermolysin) there is very considerable

digestion of the polypeptide chain at the early times of refolding and relatively little digestion at the later times. In general, and especially for thermolysin, the percentage of $28000\text{-}M_r$ material remaining runs somewhat ahead of the percentage of tetramer formed (shown as the continuous line in Fig. 3a), which implies that the monomeric and dimeric species are not completely digested under these conditions. However, by increasing the concentration of proteinase, a greater degree of digestion can be obtained. Thus, by using $80 \mu g$ of thermolysin/ml, the amount of $28000\text{-}M_r$ material remaining in the sample taken at 0.25 min is reduced to 20%, compared with 32% at $20 \mu g$ of thermolysin/ml. (This higher concentration of thermolysin did not cause any digestion of native enzyme under these conditions.)

It is not clear why the degree of inactivation caused by proteinases (Fig. 2) does not correlate exactly with the loss of the $28000\text{-}M_r$ band shown in Fig. 3(a). The discrepancy could reflect the greater degree of experimental error in the latter measurements or indicate that inactivation might be caused by the loss of only a few amino acid residues from one end of the polypeptide chain (Winn et al., 1981; Fothergill & Harkins, 1982). Some of the inactivated enzyme, which would not necessarily be resolved from native enzyme by SDS/polyacrylamide-gel electrophoresis under our conditions, could also undergo more extensive digestion.

The gels were examined carefully for evidence of distinct fragments formed by the action of proteinases on samples during refolding. Such fragments would presumably represent compact folded units, which would be resistant to digestion by proteinases. In the cases of trypsin and chymotrypsin, there was no evidence for any distinct fragments of M_r greater than 6500 in any samples during refolding (from 0.25 min to 60 min). This conclusion was reached with the use of Coomassie Blue or the more sensitive silver staining procedure. In the case of thermolysin, staining with Coomassie Blue (and more particularly with silver) revealed the presence of fragments in the $12000\text{-}15000\text{-}M_r$ range from samples taken at early time points but to a lesser extent from those taken from later time points (Fig. 3b). However, the degree of staining by Coomassie Blue of these fragments was in all cases at least an order of magnitude less than the $28000\text{-}M_r$ band of the control sample, showing that even at the early time points most of the polypeptide chain had been degraded to small fragments (M_r less than 6500).

The overall conclusion from these experiments is that, at early time points (less than 2 min) during refolding, when the folded monomeric form predominates (Hermann et al., 1983, 1985), the polypeptide chain is highly susceptible to the action of the proteinases trypsin, chymotrypsin and thermolysin. Since most of the enzyme appears to be digested to fragments of M_r less than 6500, it would appear that the folded monomeric, and probably the dimeric, species have a large number of susceptible bonds. It is noteworthy that the c.d. experiments (Hermann et al., 1983) show that the folded monomer possesses approx. 85% of the secondary structure of native enzyme. The results in the present paper imply that this folded structure is still rather 'loose', with a number of exposed sites for attack by proteinases; these sites are presumably rendered less accessible during subsequent association of the monomers (and dimers) and/or 'tightening' of the subunit structure. The small amount of fragments generated by thermolysin at short times of refolding (Fig. 3b) implies that at least one of the first bonds to be cleaved in the folded monomer is located near the centre of the polypeptide chain. Examination of the amino acid sequence of the enzyme (Fothergill & Harkins, 1982) reveals a tetraproline sequence (119–122) almost exactly in the centre of the chain of 241 amino acids. Since the initial events in the folding of polypeptide chains are thought to involve formation of secondary structures such as α-helices (Jaenicke, 1982) that cannot accommodate proline residues (Chou & Fasman, 1978), it is tempting to postulate that an early event in the refolding of the phosphoglycerate mutase subunit involves the separate folding of the two halves of the polypeptide chain, so as to leave exposed one or more bonds near the tetraproline sequence. The purification and further characterization of proteolytic fragments will be necessary to provide confirmation of this hypothesis.

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previously (Hermann *et al.*, 1983). The susceptiblity to proteolysis during refolding was measured as described by Johnson & Price (1986).

Fluorescence studies were performed at 20 °C in a Perkin–Elmer MPF 3A fluorimeter. Spectra were recorded 15 min after addition of GdnHCl; no further changes occurred after this time. The quenching of protein fluorescence by acrylamide and succinimide was performed as described by Eftink & Ghiron (1984), with the appropriate corrections described by these authors being made for dilution and 'inner filter' effects. Before use, acrylamide and succinimide were recrystallized from ethyl acetate and ethanol respectively.

C.d. spectra were recorded at 20 °C in a Jobin–Yvon Dichrographe IV, in cells of 0.1 mm path-length. The resulting spectra were analysed for secondary structure by the procedure of Siegel *et al.* (1980). Spectra were recorded 15 min after addition of GdnHCl.

The activity of phosphoglycerate mutase in GdnHCl could not be measured directly by the standard enolase-coupled-assay procedure (Grisolia, 1962), because of the pronounced effects of the denaturant at concentrations above about 0.5 M on the activity of the coupling enzyme. Accordingly, a discontinuous assay was employed, in which 0.1 ml samples of the reaction mixture (enzyme in 30 mM-Tris/HCl buffer, pH 7.0, containing 10 mM-3-phosphoglycerate and 0.3 mM-2,3-bisphosphoglycerate at 30 °C) were added to a mixture containing (final volume 1 ml) the following components: 30 mM-Tris/HCl buffer, pH 7.0, 20 mM-KCl, 5 mM-MgSO$_4$, 0.2 mM-ADP, 0.15 mM-NADH, 20 μg of enolase, 18 μg of pyruvate kinase and 6 μg of lactate dehydrogenase. The very rapid fall in A_{340} of this mixture (after correction for dilution) is a measure of the 2-phosphoglycerate formed in the sample from the phosphoglycerate mutase-catalysed reaction. By taking the difference in the fall in A_{340} between the samples withdrawn 0.5 min and 3 min after the initiation of the phosphoglycerate mutase-catalysed reaction, the rate of production of 2-phosphoglycerate could be calculated. Control experiments showed that, under the conditions employed, the rate of the mutase-catalysed reaction was constant over this period (up to 3 min reaction) and directly proportional to the amount of enzyme added. In addition, because of the greater sensitivity of the triple-coupled assay (involving oxidation of NADH) compared with the single-coupled assay (involving production of phosphoenolpyruvate), it was possible to study the mutase-catalysed reaction at GdnHCl concentrations up to 2 M, since the maximum carry-over of denaturant to the second stage was ≤ 0.2 M, which had no detectable effect on the metabolism of 2-phosphoglycerate by the coupling system.

RESULTS AND DISCUSSION

Presence of 2,3-bisphosphoglycerate in preparations of phosphoglycerate mutase from *S. pombe*

During the course of this work, it became clear that the preparations of phosphoglycerate mutase from *S. pombe* still contained a significant concentration of 2,3-bisphosphoglycerate after the Sephadex G-25 gel-filtration step. Thus the enzyme in the absence of added 2,3-bisphosphoglycerate showed approx. 50% of the activity obtained in the presence of saturating (0.3 mM)

cofactor. Extensive dialysis of the preparation agains 50 mM-sodium phosphate, pH 7.5, led to almost complet removal of 2,3-bisphosphoglycerate [< 2% activit observed in the absence of added cofactor; a value whicl could be accounted for by the presence of trace amount (< 0.005%) of the cofactor in Grade I 3-phospho glycerate]. By measuring the activity after addition o known concentrations of 2,3-bisphosphoglycerate to th dialysed enzyme, the apparent K_m for the cofactor coulc be estimated as 17 ± 1.3 μM (in the presence of 10 mM-3-phosphoglycerate). By analogous procedures, the apparent K_m values for 2,3-bisphosphoglycerate were estimated as 14 ± 1 μM and 7 ± 0.7 μM for the enzymes from baker's yeast and rabbit muscle respectively. These values lie within the range (0.1–120 μM) reported by Grisolia & Carreras (1975) for different 2,3-bisphospho-glycerate-dependent enzymes. From the observed activity of the *S. pombe* enzyme in the absence of added 2,3-bisphosphoglycerate, the concentration of cofactor in the stock solution of undialysed enzyme prepared by redissolving the freeze-dried material (0.5 mg/ml) could be calculated to be approx. 10 mM. It is reasonable to propose that the undialysed enzyme exists predominantly in the phosphorylated form, since the baker's-yeast enzyme exists in this form in the presence of 2,3-bisphosphoglycerate (Winn *et al.*, 1981).

Stability of *S. pombe* phosphoglycerate mutase

Solutions of undialysed enzyme retained ≥ 90% activity on storage for 7 days at −18 °C, even if a number of freeze–thaw cycles occurred during this period. By contrast, the dialysed enzyme was much less stable on storage. Thus four freeze–thaw cycles within a 24 h period resulted in the loss of > 90% activity. Addition of 2,3-bisphosphoglycerate (2 mM) to the dialysed enzyme restored the stability to freeze–thaw cycles.

The effects of four proteinases on the activity of *S. pombe* phosphoglycerate mutase are shown in Fig. 1(*a*). In all cases studied (chymotrypsin, trypsin, proteinase K and thermolysin) there was relatively little effect (≥ 75% activity retained) of incubation of the undialysed enzyme with proteinase [1:1 (w/w) ratio] for a 2 h period. However, the dialysed enzyme was much more susceptible to proteolysis (Fig. 1*b*). With chymotrypsin, ≤ 5% activity remained after 30 min; with trypsin, proteinase K and thermolysin, 35%, 50% and 65% activity remained respectively. Addition of 2 mM-2,3-bisphosphoglycerate to the dialysed enzyme complet ly restored the resistance to proteolysis (Fig. 1*b*).

Analysis by SDS/polyacrylamide-gel electrophor is of the mixtures in which the dialysed enzyme had b n incubated with proteinases showed that the loss of activity was associated with extensive digestion of e polypeptide chain, since there was no evidence or accumulation of fragments of M_r greater than 6500.

The conclusion from these experiments is that e undialysed enzyme, in which the enzyme presum ly exists in its phosphorylated form, must possess a n e compact, stable, structure than the dialysed (dephos]) enzyme. In previous work with the rabbit muscle enz e (Price *et al.*, 1985*b*) it was also found that e phosphorylated form of the enzyme was less suscepti e to proteolysis by thermolysin than the dephosp - enzyme.
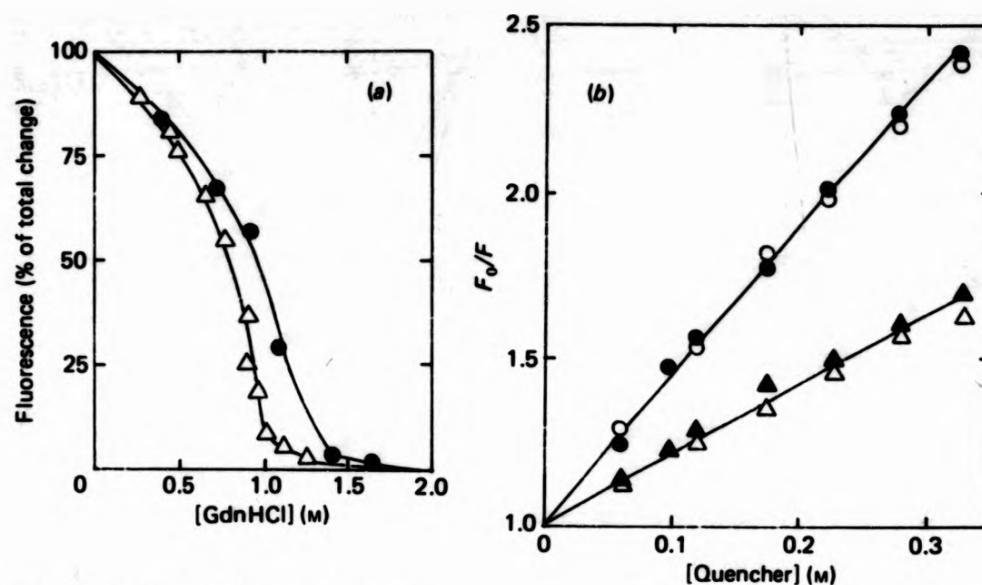
Fig. 3. Fluorescence of *S. pombe* phosphoglycerate mutase

Fluorescence was recorded in 50 mM-sodium phosphate buffer, pH 7.5, at 20 °C. The excitation and emission wavelengths were 290 and 325 nm respectively. (*a*) Changes in fluorescence in the presence of GdnHCl. The enzyme concentration was 10 μg/ml: $\triangle$, dialysed enzyme; $\bullet$, undialysed enzyme. The changes are expressed relative to the total change between 0 and 2 M-GdnHCl. (*b*) Stern–Volmer plot for quenching of fluorescence. The enzyme concentration was 17 μg/ml. On the ordinate F and F_0 refer to the fluorescence in the presence and absence of quencher respectively. $\bullet$, $\bigcirc$, Quenching by acrylamide; $\blacktriangle$, $\triangle$, quenching by succinimide; $\bullet$, $\blacktriangle$, dialysed enzyme; $\bigcirc$, $\triangle$, dialysed enzyme plus 0.3 mM-2,3-bisphosphoglycerate.

S. pombe enzyme can be estimated as 5.6; this number is similar to that found (5) in the subunits of the enzymes from baker's yeast (Fothergill & Harkins, 1982) and rabbit muscle (Johnson & Price, 1987). The average degree of exposure of the tryptophan side chains in the *S. pombe* enzyme was assessed by the relative fluorescence quenching caused by succinimide and acrylamide (Eftink & Ghiron, 1984). Fig. 3(*b*) shows the fluorescence-quenching data for the dialysed enzyme in the form of Stern–Volmer plots; from the slopes of these plots the ratio K_S/K_A (a measure of the relative effectiveness of the two quenchers and hence of the degree of exposure of the fluorophores) is estimated as 0.48. There is no significant effect on this ratio of addition of 2,3-bisphosphoglycerate, indicating that the changes in structure associated with the increased stability of the phosphoenzyme do not affect the average exposure of tryptophan side chains. The tryptophan side chains in the *S. pombe* enzyme are more exposed than those in the baker's-yeast or rabbit muscle enzymes, for which the K_S/K_A values are 0.33 and 0.43 respectively (Johnson & Price, 1987). It should also be noted that in a previous paper attention has been drawn to the changes in environments of the tryptophan side chains which may occur in association of the subunits of the baker's-yeast enzyme during renaturation (Hermann *et al.*, 1983).

(*c*) Changes in c.d. The c.d. spectra over the range 205–250 nm of *S. pombe* phosphoglycerate mutase in the absence and presence of GdnHCl (2 M) are shown in Fig. 4(*a*). There is no significant further change in the spectrum when the concentration of GdnHCl is raised to 4 M. The spectra can be compared with those previously published for the baker's yeast and rabbit muscle enzymes (Hermann *et al.*, 1983; Price *et al.*, 1985*b*). In the absence of GdnHCl the value of θ_{225}, often taken as a measure of the helical content of proteins (Chen *et al.*,

1974), for the dialysed *S. pombe* enzyme (-7800 degrees·cm^2·dmol^{-1}) is considerably greater than that for the baker's-yeast and rabbit muscle enzymes (-5100 and -4900 degrees·cm^2·dmol^{-1} respectively). For the undialysed *S. pombe* enzyme the value of θ_{225} is -9000 degrees·cm^2·dmol^{-1}.

By the analysis of Siegel *et al.* (1980), which depends on the values of θ over a range of wavelengths from 210 to 240 nm, the percentage of helical structure can be estimated as 27.9 ± 1.6, 39.8 ± 1.6 (*S. pombe* dialysed and undialysed respectively), 12.2 ± 1.9 (baker's yeast) and 9.7 ± 2 (rabbit muscle). It should be noted that the percentage helical structure for the baker's-yeast enzyme derived from c.d. is lower than that (30%) determined by X-ray crystallography (Campbell *et al.*, 1974). Possible reasons for this discrepancy have been discussed previously (Hermann *et al.*, 1983).

The loss of secondary structure of *S. pombe* phosphoglycerate mutase on addition of GdnHCl can be monitored by the changes in θ_{225} (Fig. 4*b*). The structural changes occur at somewhat higher concentrations of denaturing agent for the undialysed enzyme, again pointing to its greater conformational stability. The midpoints of the changes occur at GdnHCl concentrations of 0.95 M (dialysed) and 1.15 M (undialysed).

Analogous experiments were performed for the baker's-yeast and rabbit muscle enzymes (Fig. 4*c*). In these cases the changes in θ_{225} occur over a similar range of GdnHCl concentrations to those giving the fluorescence changes noted previously (Johnson & Price, 1987). The midpoints of the changes occur at GdnHCl concentrations of 1.20 M (baker's yeast) and 1.10 M (rabbit muscle).

(*d*) Comparison of the various measurements. When the data relating to activity (Fig. 2), fluorescence (Fig. 3*a*) and c.d. (Fig. 4*b*) of the undialysed *S. pombe* enzyme are

reproducible. The data for dialysed enzyme at a concentration of 12 μg/ml during renaturation are shown in Fig. 5(a). The effect of dithiothreitol suggested that the *S. pombe* enzyme might possess one or more thiol groups which could be damaged during the denaturation–renaturation procedure. By using the thiol-specific reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959) it was shown that the *S. pombe* enzyme possessed 0.98 mol of thiol groups/mol in the absence or presence of the denaturing agent SDS. This cysteine side chain does not appear to be essential for activity, since treatment with potassium tetrathionate causes no inactivation (Price *et al.*, 1985a), a result confirmed in the present work.

The susceptibility of the activity of the enzyme during renaturation toward proteolysis was examined by the method used in studies of the renaturation of the baker's-yeast enzyme (Johnson & Price, 1986). On incubation of samples for 1 min at a phosphoglycerate mutase: proteinase ratio of 1:2 (w/w), there was no effect of trypsin or thermolysin on the activity of the enzyme at any time point during renaturation (Fig. 5a). Chymotrypsin and proteinase K had a small effect on the activity, but only at very short times (0.5 min) of renaturation (Fig. 5a). Under the conditions employed, the proteinases had no effect on the activity of native enzyme.

The rate of regain of activity of the *S. pombe* enzyme was studied at concentrations of 1–24 μg/ml during refolding. As shown in Fig. 5(b), there is no significant effect of concentration on the rate or extent of the process.

These results can be compared with those obtained from studies of the renaturation of the tetrameric baker's-yeast enzyme (Hermann *et al.*, 1983, 1985; Johnson & Price, 1986). In this case there are profound effects of protein concentrations on both the rate and the extent of regain of activity. These effects arise respectively from second-order association steps and the limited stability of intermediates in the folding pathway (Hermann *et al.*, 1983). The folded monomeric species which predominates at early times (0.5 min) is very sensitive to proteolysis by trypsin, chymotrypsin and thermolysin (Johnson & Price, 1986), but this sensitivity is lost on subsequent association to form the native tetrameric enzyme. For the monomeric enzyme from *S. pombe*, the results of the present work show that activity is regained rapidly in a process with no detectable concentration-dependence under the conditions studied, and that this rapidly formed species resembles

the native enzyme in terms of its stability towards prc teolysis. The differences in behaviour between th baker's-yeast and *S. pombe* enzymes are presumably . consequence of their different quaternary structures since the association of subunits in the former ha been shown to make the subunits more compact an less susceptible to proteolysis (Johnson & Price, 1986).

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Co-factor-independent phosphoglycerate mutases

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Phosphoglycerate mutases catalyse the interconversion of 2- and 3-phosphoglycerates. It has been recognized for some time that the enzymes fall into two distinct categories: those which require the co-factor 2,3-bisphosphoglycerate for activity and those which are active in the absence of this co-factor. The former group includes the enzymes from mammalian sources, baker's yeast, *Escherichia coli*, insects and crustaceans, whereas the latter group includes enzymes from plant sources, filamentous fungi, coelenterates and arachnids (Grisolia & Joyce, 1959; Ray & Peck, 1972; Carreras *et al.*, 1982; Price *et al.*, 1983). The co-factor-dependent enzymes catalyse an intermolecular phosphoryl group transfer, whereas in the case of the co-factor-independent enzymes, the transfer is intramolecular (Gatehouse & Knowles, 1977).

The observations that vanadate inhibits only the co-factor-dependent enzymes (Carreras *et al.*, 1980) and that Cibacron Blue F3GA binds only to the co-factor-dependent enzymes (Price & Stevens, 1983) point to fundamental structural and mechanistic differences between the two classes of enzymes. However, in both classes the phosphoryl group transfer proceeds with retention of configuration (Blättler & Knowles, 1980), suggesting that at least some features of the reactions may be common.

At present, much more is known about the structure of the co-factor-dependent enzymes, and the amino acid sequence and X-ray structure of the tetrameric enzyme from baker's yeast have been determined, allowing a detailed mechanism of action of this enzyme to be proposed (Winn *et al.*, 1981; Fothergill & Harkins, 1982).

The enzyme from *Bacillus* sp. is co-factor-independent but appears to have an absolute requirement for Mn^{2+} for activity. The M_r of the monomeric enzyme has been determined as 61 000 (*B. megaterium*) and 74 000 (*B. subtilis*) (Singh & Setlow, 1979; Watabe & Freese, 1979). Recently, evidence has been presented that the enzyme from wheat germ may also be a metalloenzyme since (i) it is inhibited by a number of chelating agents and (ii) addition of Co^{2+} or Mn^{2+} is necessary to promote re-folding of the enzyme after denaturation by guanidine hydrochloride (Smith & Hass, 1985; Smith *et al.*, 1986). Since the wheat germ enzyme is apparently a monomer of $M_r \sim 60\,000$ (Leadlay *et al.*, 1977; Smith & Hass, 1985), it is possible that the co-factor-independent enzymes might possess a common structural pattern.

We have explored this possibility by examining the sensitivity of enzyme activity from a number of sources to the chelating agents EDTA and 8-hydroxyquinoline-5-sulphonic acid (HQSA) and by determining the M_r of the active form by gel filtration on Sephacryl S-300. Partially purified extracts obtained by ammonium sulphate fractionation followed by dialysis were studied in all cases, except for rabbit muscle, baker's yeast and *Schizosaccharomyces pombe*, which were studied as the purified enzymes (Price *et al.*, 1985). The results of these experiments are shown in Table 1. It is clear that there is a marked difference in sensitivity to the chelating agents between the co-factor-dependent and co-factor-independent enzymes. The latter group all display sensitivity to the chelating agents and have M_r values ≈ 60 000. By contrast, the co-factor-dependent enzymes are stable (< 10% activity lost in 3 h) in the presence of the chelating agents and have a range of M_r

Table 1. *Sensitivity to chelating agents and M_r values of phosphoglycerate mutases*

Stability towards the chelating agents (1 mM) was tested in 0.1 M-Tris-HCl buffer, pH 8.7 at 20°C. M_r values were determined by gel filtration on Sephacryl S-300 in 50 mM-sodium phosphate buffer, pH 7.5 at 4°C.

	Activity stable to		
Source of enzyme	EDTA	HQSA	M_r
Co-factor-dependent			
Baker's yeast	Yes	Yes	110 000
Candida utilis	Yes	Yes	112 000
Rabbit muscle	Yes	Yes	56 000
Schizosaccharomyces pombe	Yes	Yes	23 000
Klebsiella aerogenes	Yes	Yes	62 000
Escherichia coli	Yes	Yes	57 000
Co-factor-independent			
Potato	No	No	60 000
Aspergillus nidulans	No	No	60 000
Wheat germ	No	No	52 000
Mung bean	No	No	63 000
(*Bacillus*	No	N.D.	61 000, 74 000)*

*N.D. = not determined. Data taken from Singh & Setlow (1979); Watabe & Freese (1979).

values, consistent with the known differences in quaternary structure (Price *et al.*, 1985). It should be noted that the co-factor-dependent enzymes from baker's yeast, rabbit muscle, *S. pombe* and *Candida utilis* all showed a marked instability in the control solutions, in the absence of chelating agents, with ~ 50% activity lost in 1 h. There were also marked differences in the rates of loss activity of the co-factor-independent enzymes: thus, the potato enzyme lost 25% activity in the presence of HQSA in 1 h, whereas the *Aspergillus nidulans* enzyme lost 95% activity in this time.

The results obtained lends support to the proposal that there may be a common structural pattern among the co-factor-independent phosphoglycerate mutases. It remains a task for future work to study further examples of this case of enzymes; to determine the type(s) of metal ion concerned and to establish the role played by such ions in the mechanism of the enzymes.

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Abbreviation used: HQSA, 8-hydroxyquinoline-5-sulphonic acid.