Denaturation and renaturation of the monomeric phosphoglycerate mutase from *Schizosaccharomyces pombe*

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The denaturation by guanidinium chloride of the monomeric phosphoglycerate mutase from *Schizosaccharomyces pombe* was studied. The loss in activity broadly parallels the changes in protein structure detected by fluorescence and c.d. Renaturation can be brought about by dilution of the denaturing agent. These processes were compared with those in the enzymes from baker's yeast and rabbit muscle, which are tetrameric and dimeric respectively. The effects of the cofactor 2,3-bisphosphoglycerate on the structure and stability of the *S. pombe* enzyme were also investigated.

INTRODUCTION

Phosphoglycerate mutase catalyses the interconversion of 2- and 3-phosphoglycerates. The enzyme from plants and some other sources, including filamentous fungi, shows activity in the absence of added 2,3-bisphosphoglycerate, whereas that from mammalian and baker's yeast is dependent on this cofactor (Ray & Peck, 1972; Price *et al.*, 1983). Previously (Price & Stevens, 1983) it was shown that 2,3-bisphosphoglycerate-dependent enzymes were retained by a Cibacron Blue–Sephadex matrix and could be eluted by a pulse of this cofactor, whereas 2,3-bisphosphoglycerate-independent enzymes were not bound to this matrix. Subsequently the 2,3-bisphosphoglycerate-dependent enzyme from the fission yeast *Schizosaccharomyces pombe* was purified from a crude cell extract by a single-step procedure involving Cibacron Blue–Sephadex dye–ligand chromatography (Price *et al.*, 1985a). This enzyme was shown to be monomeric, with M_r approx. 23 000; a finding of considerable interest, since all previously isolated 2,3-bisphosphoglycerate-dependent enzymes had been shown to be oligomeric (Ray & Peck, 1972). In the present paper we report studies of the denaturation of the enzyme from *S. pombe* by GdnHCl and subsequent renaturation by dilution of the denaturant. These provide complementary information to that obtained on the oligomeric enzymes from baker's yeast (tetrameric) and rabbit muscle (dimeric) (Hermann *et al.*, 1983, 1985; Johnson & Price, 1986) and thus allow comparisons to be made of a series of related enzymes of differing quaternary structures. During the course of this work, it also became clear that the presence of the cofactor 2,3-bisphosphoglycerate had a marked effect on the stability of the enzyme from *S. pombe*, and experiments have been undertaken to investigate this effect.

MATERIALS AND METHODS

The following companies supplied the reagents listed: BDH Chemicals, GdnHCl (AristaR grade), acrylamide (Electran grade); Sigma Chemical Co., Reactive Blue 2–Sephadex CL-6B, Dalton mark VII-L M_r markers set, dithiothreitol, succinimide, α -chymotrypsin [tosyl-

lysylchloromethane ('TLCK')-treated, dialysed, freeze-dried], thermolysin (stabilized with calcium acetate and sodium acetate); Boehringer Mannheim, proteinase K from *Tritirachium album* Limber, 3-phosphoglycerate (grade I), 2,3-bisphosphoglycerate, ADP, NADH, enolase, aldolase (from rabbit muscle), phosphoglycerate mutase (from rabbit muscle), pyruvate kinase/lactate dehydrogenase mixture (from rabbit muscle); Worthington Corp., trypsin [three times recrystallized, tosylphenylalanylchloromethane ('TPCK')-treated]; Bayer, aprotinin (Trasylol).

Phosphoglycerate mutase from baker's yeast was isolated as described previously (Johnson & Price, 1986). Phosphoglycerate mutase from *S. pombe* was extracted and purified as described previously (Price *et al.*, 1985a), except that a 0–4 mM gradient of 2,3-bisphosphoglycerate was used to effect elution from Cibacron Blue–Sephadex rather than a 1 mM pulse of the cofactor. The purity of the enzyme as judged by SDS/polyacrylamide-gel electrophoresis was at least 95%, and the specific activity (200 μ mol of substrate consumed/min per mg of protein) was comparable with that reported previously (Price *et al.*, 1985a). Gel-filtration (Sephadex G-150) and SDS/polyacrylamide-gel-electrophoretic studies confirmed that the enzyme was monomeric, with M_r approx. 24 000 $\pm$ 2000. The active fractions from dye–ligand chromatography were pooled and freeze-dried. After redissolution in a small volume (approx. 2 ml) of water, the preparation was gel-filtered through a column (15 cm $\times$ 0.45 cm² of Sephadex G-25 equilibrated with 5 mM-sodium phosphate buffer, pH 7.5. The enzyme-containing fractions were pooled and freeze-dried for storage. The freeze-dried preparations retained activity upon storage for at least 2 months at -18°C . For the experiments described in this paper, the freeze-dried preparation was redissolved in water (one-tenth the volume of the pooled fractions from the gel-filtration step). This procedure raised the buffer concentration to 50 mM; the pH remained at 7.5. The protein concentrations of *S. pombe* enzyme were estimated by the method of Sedmak & Grossberg (1977), with bovine serum albumin as a standard.

Denaturation of enzymes in GdnHCl and subsequent renaturation were performed essentially as described

previously (Hermann *et al.*, 1983). The susceptibility 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-MgSO₄, 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 against 50 mM-sodium phosphate, pH 7.5, led to almost complete removal of 2,3-bisphosphoglycerate [$< 2\%$ activity observed in the absence of added cofactor; a value which could be accounted for by the presence of trace amounts ($< 0.005\%$) of the cofactor in Grade I 3-phosphoglycerate]. By measuring the activity after addition of known concentrations of 2,3-bisphosphoglycerate to the dialysed enzyme, the apparent K_m for the cofactor could 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-bisphosphoglycerate-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 $\geq 90\%$ 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 ($\geq 75\%$ 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. 1b). With chymotrypsin, $\leq 5\%$ 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 completely restored the resistance to proteolysis (Fig. 1b).

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

The conclusion from these experiments is that the undialysed enzyme, in which the enzyme presumably exists in its phosphorylated form, must possess a more compact, stable, structure than the dialysed (dephospho) enzyme. In previous work with the rabbit muscle enzyme (Price *et al.*, 1985b) it was also found that the phosphorylated form of the enzyme was less susceptible to proteolysis by thermolysin than the dephospho-enzyme.

Fig. 1. Effects of proteinase on *S. pombe* phosphoglycerate mutase

Phosphoglycerate mutase (75 $\mu\text{g/ml}$) was incubated with proteinase (75 $\mu\text{g/ml}$) in 50 mM-sodium phosphate buffer, pH 7.5, at 20 °C. The proteinases studied were: ●, trypsin; ▲, chymotrypsin; ○, thermolysin; △, proteinase K. (a) Undialysed phosphoglycerate mutase; (b) dialysed phosphoglycerate mutase in the absence (—) and the presence (---) of 2,3-bisphosphoglycerate (2 mM).

Fig. 2. Activity of phosphoglycerate mutase in the presence of GdnHCl

The activities of enzymes from (▲) *S. pombe*, (●) baker's yeast and (○) rabbit muscle were determined as described in the Materials and methods section. In each case the activity is expressed relative to a buffer control containing no GdnHCl.

Denaturation of *S. pombe* phosphoglycerate mutase by GdnHCl

(a) **Changes in activity.** The activity of *S. pombe* phosphoglycerate mutase in the presence of different concentrations of GdnHCl is shown in Fig. 2, where comparable data for the baker's-yeast and rabbit muscle enzymes are also shown. For the *S. pombe* enzyme there

is a small ($\leq 15\%$) but reproducible increase in activity at concentrations of GdnHCl up to 0.5 M, followed by a sharp decline in the range 0.5–1.5 M, with 50% activity lost at 0.85 M-GdnHCl. Although the reasons for the slight activation of the enzyme can only be a matter for speculation, it should be noted that work on liver alcohol dehydrogenase (Strambini & Gonnelli, 1986) has shown that low concentrations of GdnHCl can change the flexibility of the enzyme, and it is possible that such an effect could increase the activity of *S. pombe* phosphoglycerate mutase.

The changes in activity of the baker's-yeast and rabbit muscle enzymes are also shown in Fig. 2. It is clear that these oligomeric enzymes are more susceptible than is the monomeric *S. pombe* enzyme to inactivation by GdnHCl, with 50% activity lost at concentrations of 0.65 M and 0.55 M respectively.

(b) **Changes in protein fluorescence.** When excited at 290 nm, *S. pombe* phosphoglycerate mutase shows a fluorescence emission maximum at 330 nm. In the presence of 2 M-GdnHCl, the emission maximum is shifted to 345 nm (a value characteristic of tryptophan in denatured proteins) and the intensity at 325 nm is decreased by 70%. No further change in fluorescence was observed when the GdnHCl concentration was increased to 4 M. Fig. 3(a) shows a titration of the fluorescence at 325 nm as a function of GdnHCl concentration for both dialysed and undialysed enzyme. In both cases there is a sharp decline in fluorescence in the 0.5–1.5 M concentration range, with the changes being 50% complete at 0.80 M (dialysed) and 0.95 M (undialysed). These results suggest that, in the presence of 2,3-bisphosphoglycerate, the enzyme has greater conformational stability than in the absence of the cofactor. Similar experiments have also been performed for the baker's-yeast and rabbit muscle enzymes in the absence of cofactor (Johnson & Price, 1987). In both cases there are relatively small changes in fluorescence up to 0.75 M-GdnHCl, followed by a sharp decline up to 1.5 M. The mid-points of the changes are 1.1 M in both cases.

By the spectrophotometric method of Edelhoch (1967), the number of tryptophan side chains in the

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: Δ , 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. $\bullet$, $\circ$, Quenching by succinimide; $\blacktriangle$, $\triangle$, quenching by acrylamide; $\bullet$, $\blacktriangle$, dialysed enzyme; $\circ$, $\triangle$, 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.*, 1985b). 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·cm²·dmol⁻¹) is considerably greater than that for the baker's-yeast and rabbit muscle enzymes (-5100 and -4900 degrees·cm²·dmol⁻¹ respectively). For the undialysed *S. pombe* enzyme the value of θ_{225} is -9000 degrees·cm²·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. 4b). 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. 4c). 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. 3a) and c.d. (Fig. 4b) of the undialysed *S. pombe* enzyme are

Fig. 4. C.d. of phosphoglycerate mutase in the presence of GdnHCl

Spectra were recorded over the range 205–250 nm in 50 mM-sodium phosphate buffer, pH 7.5, at 20 °C, in 0.1 mm-path-length cells. (a) C.d. spectra of dialysed *S. pombe* phosphoglycerate mutase (0.31 mg/ml) (●) in the absence of GdnHCl and (○) in the presence of 2 M-GdnHCl. (b) Changes in θ_{225} of *S. pombe* phosphoglycerate mutase: △, dialysed enzyme (0.31 mg/ml); ●, undialysed enzyme (0.38 mg/ml). The changes are expressed relative to the total change between 0 and 2 M-GdnHCl. (c) Changes in θ_{225} of baker's-yeast and rabbit muscle phosphoglycerate mutase: ●, baker's-yeast enzyme (0.92 mg/ml); ○, rabbit muscle enzyme (0.56 mg/ml). The changes are expressed relative to the total change between 0 and 2 M-GdnHCl.

compared, it is clear that the changes occur broadly over the same range of denaturant concentrations, the midpoints being 0.85 M, 0.95 M and 1.15 M respectively. Since the different measurements are reflecting related but distinct aspects of the unfolding process, exact correspondence would not necessarily be expected (Tsou, 1986). For the oligomeric enzymes from baker's yeast and rabbit muscle, the changes in activity occur at substantially lower concentrations of GdnHCl than the changes in structure (the midpoints for the three different measurements are: baker's-yeast enzyme, 0.65 M, 1.10 M and 1.20 M; rabbit muscle enzyme, 0.55 M, 1.10 M and 1.10 M). These findings could imply that the subunit interactions in the oligomeric enzymes play an important role in maintaining the stability of the gross features of the tertiary structure towards denaturation.

Renaturation of *S. pombe* phosphoglycerate mutase

The regain of activity of the *S. pombe* enzyme after denaturation in 2 M-GdnHCl was studied. On dilution of

Fig. 5. Renaturation of *S. pombe* phosphoglycerate mutase

The enzyme was denatured by incubation in 50 mM-sodium phosphate buffer, pH 7.5, containing 2 M-GdnHCl for 40 min at 0 °C, and renatured by dilution of the solution with buffer to a final GdnHCl concentration of 0.2 M at 20 °C. Dithiothreitol (1 mM) was included in both denaturation and renaturation steps. Activity is expressed relative to a non-denatured control solution. (a) Effects of proteinases on the activity of samples of enzyme during renaturation: ■, activity measured in the absence of added proteinase; ●, ▲, ○ and △ represent activity measured after 1 min incubation at 20 °C with trypsin, chymotrypsin, thermolysin and proteinase K respectively. The enzyme concentration during renaturation was 12 $\mu\text{g}/\text{ml}$, and the enzyme and proteinase concentrations during proteolysis were 10 and 20 $\mu\text{g}/\text{ml}$ respectively. (b) Renaturation of enzyme at different concentrations. The concentrations of enzyme ($\mu\text{g}/\text{ml}$) during renaturation were: ■, 25; ●, 12; ○, 4; ▲, 2; △, 1.

the denaturant to a final concentration of 0.2 M, activity was regained to a rather variable extent. A rapid increase in activity (to $60 \pm 20\%$ of control) over the first 2 min after dilution was followed by a slow decline in activity over the next 1 h. In general the dialysed enzyme regained activity to a lesser extent than did the undialysed enzyme. The partial regain of activity could be correlated with a partial regain of protein fluorescence on dilution of the GdnHCl from 2 M to 0.4 M (70% and 25% for the undialysed and the dialysed enzyme respectively).

In later experiments, dithiothreitol (1 mM) was included in the denaturation and renaturation mixtures, and the extent of regain of activity was much more

reproducible. The data for dialysed enzyme at a concentration of 12 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$ 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 proteolysis. The differences in behaviour between the baker's-yeast and *S. pombe* enzymes are presumably a consequence of their different quaternary structures, since the association of subunits in the former has been shown to make the subunits more compact and less susceptible to proteolysis (Johnson & Price, 1986).

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