Substitution of His-181 by alanine in yeast phosphoglycerate mutase leads to cofactor-induced dissociation of the tetrameric structure

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The structure and stability of a mutated yeast phosphoglycerate mutase in which His-181 has been replaced by alanine have been studied. The secondary, tertiary and quaternary structures of the mutant enzyme in the absence of ligands are essentially identical to those of the wild-type enzyme as revealed by c.d., fluorescence and cross-linking studies. The mutant enzyme is slightly less stable than the wild-type enzyme towards denaturation by guanidium chloride (GdnHCl). On addition of cofactor 2,3-bisphosphoglycerate, the wild-type enzyme shows increased stability towards GdnHCl. However, addition of cofactor causes dramatic changes in the structure of the mutant enzyme, leading to dissociation of the tetrameric form to dimeric and monomeric species.

INTRODUCTION

The enzyme phosphoglycerate mutase (EC 5.4.2.1) catalyses the interconversion of 2- and 3-phosphoglycerate in the glycolytic/gluconeogenic pathways. This enzyme has been very well characterized, particularly the enzyme from *Saccharomyces cerevisiae* (yeast) whose amino acid sequence and crystal structure have been determined (Watson, 1982; White and Fothergill-Gilmore, 1988). The active site of the yeast enzyme was located using crystals soaked in the substrate 3-phosphoglycerate (Winn et al., 1977). It is formed entirely by the residues of one subunit and is distant from either of the two subunit interfaces (Figure 1). There is no evidence for cooperativity for phosphoglycerate mutase. The crystal structure displays the striking feature of two histidine residues (His-8 and His-181) in close proximity at the active site. It has not so far proved possible to obtain crystals of the catalytically competent phosphorylated form of the enzyme, and therefore direct evidence for detailed structural changes during catalysis is not yet available. A catalytic mechanism has been postulated based on the currently available structural and kinetic information (reviewed by Fothergill-Gilmore and Watson, 1989). Briefly, a round of catalysis is initiated when a monophosphoglycerate substrate molecule binds at the active site of the phosphorylated enzyme. The phospho group on His-8 is then transferred to the substrate to form 2,3-bisphosphoglycerate (2,3-BPGA). It is proposed that 2,3-BPGA then changes its orientation in the active site before transferring its other phospho group to His-8, regenerating the active phosphorylated form of the enzyme, and the product is released to allow another round of catalysis. The enzyme possesses a flexible C-terminal 'tail' which is essential for its activity.

In a previous paper (White and Fothergill-Gilmore, 1992) we have reported the purification and kinetic analysis of a mutant form of phosphoglycerate mutase in which His-181 has been substituted by an alanine residue. This mutation results in a 10^4-fold drop in k_{cat}, compared with the wild-type enzyme. However, this activity is still dependent on the presence of the cofactor 2,3-BPGA and shows two-substrate Ping Pong kinetics, thus indicating that as in the wild-type enzyme His-8 undergoes phosphorylation and dephosphorylation during the catalytic cycle. It is likely that His-181 plays an important role as a proton donor/acceptor in the catalytic cycle.

In this paper we report the results of a number of studies aimed at characterizing the structure and stability of the mutant enzyme, using spectroscopic and cross-linking techniques. Our results show that addition of 2,3-BPGA to the wild-type enzyme leads to a significant degree of protection against unfolding by guanidinium chloride (GdnHCl). In contrast, the cofactor causes structural changes in the mutant enzyme, including dissociation of the tetramer into dimers and monomers. It is thus clear that perturbation of the active site (i.e. substitution of His-181 by Ala and phosphorylation of His-8) leads to widespread, long-range changes in intra- and inter-subunit contacts.

EXPERIMENTAL

The wild-type and H181A mutant phosphoglycerate mutases were prepared as described previously (White and Fothergill-Gilmore, 1992). GdnHCl (Ultrapure grade) was purchased from Gibco-BRL, Paisley, U.K. The concentrations of solutions were checked by refractive index measurements (Nozaki, 1972). The bisphosphonomethyl analogue of 2,3-BPGA, [DL-4-phosphono-2-(phosphonomethyl)butanoic acid], prepared as described previously (McAleese et al., 1987), was a gift from Dr. G. M. Blackburn, University of Sheffield, Sheffield, U.K.

C.d. spectra were recorded at 20 °C on a JASCO J-600 spectropolarimeter. Molar ellipticity values were calculated using a value of 112 for the mean residue weight (White and Fothergill-Gilmore, 1988). Analysis of the secondary structure content was undertaken by using the CONTIN procedure (Provencher and Glockner, 1981).

Fluorescence spectra were recorded at 20 °C on a Perkin–Elmer LS50 spectrofluorimeter. The spectra obtained were corrected for Raman scattering by the solvent.

The unfolding of phosphoglycerate mutases was carried out by incubating the enzyme with GdnHCl for 15 min at 20 °C before

Abbreviations used: GdnHCl, guanidinium chloride; 2,3-BPGA, 2,3-bisphosphoglycerate.
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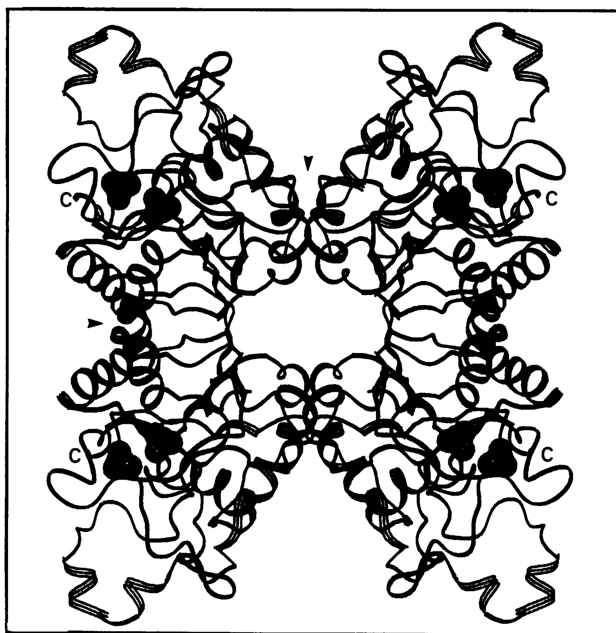

Figure 1 The yeast phosphoglycerate mutase tetramer

Two sulphate ions are indicated at each active site. It is assumed that these correspond to the binding sites of the two phospho groups of the ligand 2,3-BPGA (Fothergill-Gilmore and Watson, 1989). The two different subunit interfaces are labelled with arrows and the C-terminus of each subunit is indicated. This figure was produced using the program FRODO.

analysis (Hermann et al., 1983). Under these conditions incubation for a further 30 min gave no significant changes in the readings obtained.

The quaternary structure of phosphoglycerate mutases, both in the absence and presence of GdnHCl, was monitored using the glutaraldehyde cross-linking method (Hermann et al., 1981, 1983) modified to include trichloroacetic acid/deoxycholate precipitation (Jaenicke and Rudolph, 1986). Using this method, GdnHCl could be present up to a concentration of about 1.6 M in the cross-linking reaction.

A sample (10–50 μg) of the protein to be cross-linked was prepared in a 1 ml solution containing 50 mM sodium phosphate buffer, pH 7.0. Enzyme substrates, inhibitors and/or GdnHCl were included as required and the mixture was incubated at room temperature for 15 min. Glutaraldehyde (40 μl) [25% (w/v), Sigma grade 1] was added and samples were thoroughly mixed and incubated at room temperature for 2 min. An aliquot (50 μl) of 2 M $NaBH_4$ was then added and samples were mixed and incubated at room temperature for a further 20 min. Aqueous sodium deoxycholate [3 μl of 10% (w/v)] and 45 μl of 78% (w/v) trichloroacetic acid were then added, the samples were stored on ice for 5 min then centrifuged in a microcentrifuge for 15 min at high speed. The supernatant was discarded and the pellet was dispersed in 600 μl of ice-cold acetone, with a centrifugation step in a microcentrifuge for 10 min at high speed to re-precipitate the protein. The acetone-wash step and centrifugation were repeated a second time. Finally, the pellet was drained and re-suspended in 10–50 μl of electrophoresis loading buffer [0.1 M Tris/HCl, pH 8.0/1% SDS/50 mM dithiothreitol/10% (v/v) glycerol/0.005% Bromophenol Blue]. Samples were subjected to SDS/PAGE and the gels were scanned using a Joyce Loebel Chromoscan 3 densitometer to calculate the percentage of enzyme present in tetrameric, dimeric and monomeric forms.

It should be noted that when higher concentrations of GdnHCl were included in the cross-linking reaction mixture the resulting gels showed broad, indistinct bands, presumably as a result of the poor recovery of protein adsorbed on to the highly insoluble guanidinium dodecyl sulphate.

RESULTS

Comparison of the wild-type and H181A mutant enzyme structures

The structures of the wild-type and H181A mutant enzymes (in the absence of GdnHCl) have been compared using far- and near-u.v. c.d., protein fluorescence and cross-linking with glutaraldehyde. No significant differences in the secondary, tertiary or quaternary structures between the wild-type and mutant enzymes could be observed in the absence of ligands. Under the standard conditions used in this study for glutaraldehyde cross-linking each of the enzymes appears to exist entirely in a tetrameric form. This is in agreement with previous studies of the quaternary structure of the wild-type enzyme from yeast (Ray and Peck, 1972; Hermann et al., 1983).

Stability of enzymes towards GdnHCl

The changes in wild-type and mutant enzymes on addition of GdnHCl have been investigated by far-u.v. c.d. and fluorescence spectroscopy and by glutaraldehyde cross-linking. The results are summarized in Figures 2(a) and 2(b) for the wild-type and mutant enzymes respectively. The ordinate shows the change in ellipticity at 225 nm and fluorescence at 330 nm expressed as a percentage of the total change observed between 0 M and 4 M GdnHCl. At the latter concentration the wild-type enzyme has been shown to be completely unfolded (Hermann et al., 1983). The percentage of the enzyme existing as a monomer is also plotted on the ordinate. There was no evidence for significant accumulation of dimers during the dissociation of the tetrameric enzymes, as shown by the cross-linking pattern of the mutant enzyme in Figure 2(c).

For both enzymes, the changes in the far-u.v. c.d. and in the cross-linking pattern as a function of GdnHCl concentration are well correlated, suggesting coordinate loss of secondary, tertiary and quaternary structures in each case. There was no evidence for significant accumulation of dimeric or folded monomeric species on GdnHCl-induced denaturation. The wild-type enzyme appears to be somewhat more stable than the mutant, with 50% of the total c.d. change occurring at 1.05 M and 0.9 M GdnHCl respectively. The changes in fluorescence on addition of GdnHCl are rather more complex and may reflect differential effects on the environments of the five tryptophan residues in each subunit, one of which (Trp-162) is in a subunit contact region. In the wild-type enzyme, the changes in F_{330} lag behind those in the far-u.v. c.d. below 1.1 M GdnHCl, but above this concentration run ahead of the changes in c.d. In the case of the mutant, a decrease in fluorescence, representing some 20% of the total change at low concentrations of GdnHCl (0.05 M), was consistently observed. If the fluorescence value measured over the range 0.05–0.5 M GdnHCl is used as a baseline then the subsequent change in fluorescence on further addition of GdnHCl matches those observed by c.d. and cross-linking very closely. While the reason for the effect of low concentrations of GdnHCl on the fluorescence of the H181A mutant is not clear, analogous effects have been observed on the flexibility and activity of other enzymes (Strambini and Gonnelli, 1986; Johnson and Price, 1987).

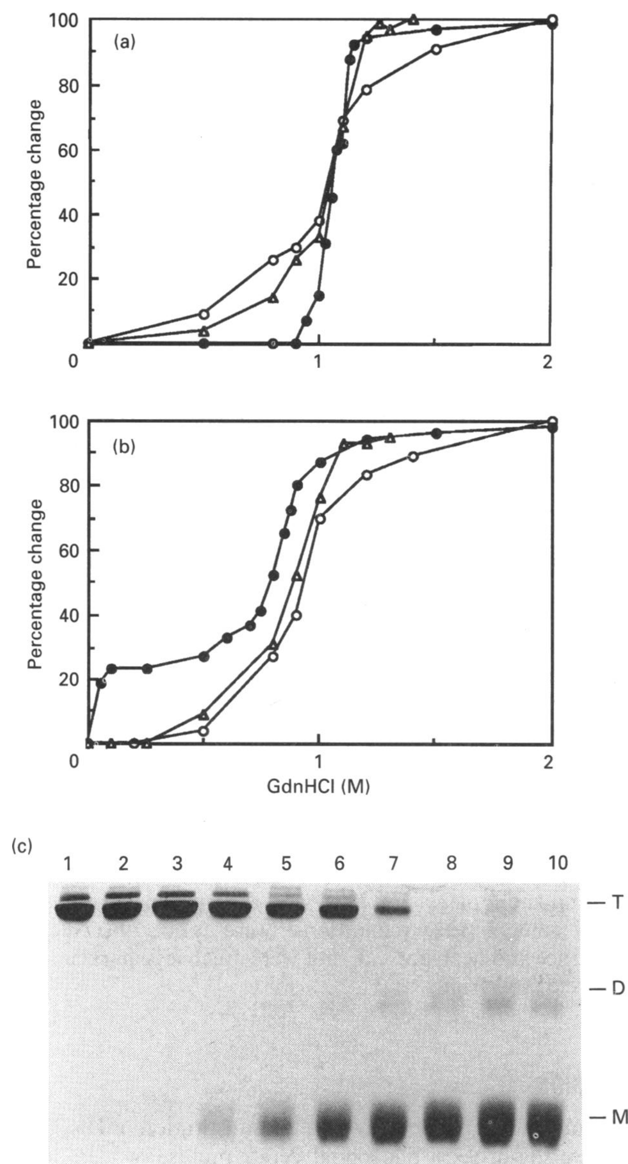

Figure 2 Denaturation of wild-type and H181A phosphoglycerate mutases

GdnHCl-induced denaturation of the wild-type (**a**) and mutant (**b**) enzymes was followed by far-u.v. c.d., fluorescence and cross-linking as described in the Experimental section. Protein concentrations were 0.1 mg/ml for the far-u.v. c.d. studies and 60 μg/ml for the fluorescence and cross-linking experiments. For cross-linking the percentage change is the percentage of the enzyme existing as monomer. In 4 M GdnHCl θ_{225} was 10% of that at 0 M GdnHCl; F_{330} was 30% of that at 0 M GdnHCl and $\lambda_{max.}$ was shifted to 355 nm. Key to symbols: ○, c.d.; ●, fluorescence; △, cross-linking. (**c**) SDS/PAGE showing the cross-linking pattern obtained after GdnHCl-induced denaturation of the H181A mutant. The pattern observed for the wild-type enzyme was qualitatively similar. The positions of tetramer (T), dimer (D) and monomer (M) are indicated. Lanes 1–10 correspond to incubation with 0, 0.1, 0.25, 0.8, 0.9, 1.0, 1.1, 1.2 and 1.3 M GdnHCl respectively.

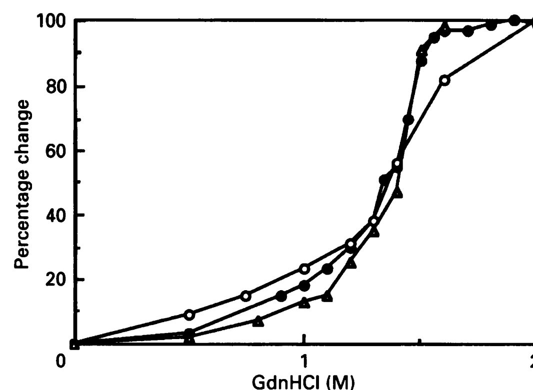

Figure 3 Denaturation of wild-type phosphoglycerate mutase in the presence of 2,3-BPGA

GdnHCl-induced denaturation of the wild-type enzyme in the presence of 2.5 mM 2,3-BPGA was followed. Details were as for Figure 2. Key to symbols: ○, c.d.; ●, fluorescence; △, cross-linking.

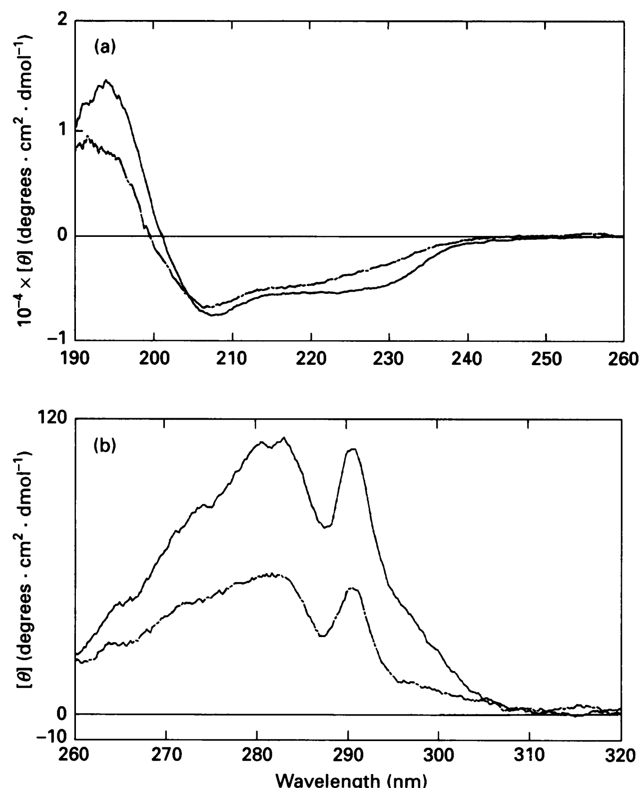

Figure 4 Effect of 2,3-BPGA on c.d. spectra of H181A mutant

The effect of 2.5 mM 2.3-BPGA on the far-u.v. (**a**) and near-u.v. (**b**) c.d. spectra of the mutant enzyme are shown. The spectra were recorded after incubation of the enzyme with cofactor for 5 min. Spectra recorded after incubation for 60 min were identical. Protein concentrations and pathlengths were: (**a**) 0.1 mg/ml, 0.1 cm and (**b**) 1 mg/ml, 0.5 cm. Solid lines show absence of 2,3-BPGA; broken lines show presence of 2,3-BPGA.

Effect of 2,3-BPGA on enzyme structure and stability

Wild-type phosphoglycerate mutase

Incubation of wild-type enzyme with 2.5 mM 2,3-BPGA [well above the K_m value of 8 μM (White and Fothergill-Gilmore, 1992)] (15 min, 20 °C), which leads to the phosphorylation of His-8 (reviewed by Fothergill-Gilmore and Watson, 1989), caused no observable change in the far- and near-u.v. c.d. and fluorescence spectra or in the cross-linking pattern (results not shown). This suggests that phosphorylation of His-8 in the wild-type enzyme does not cause gross structural changes in the enzyme. As shown in Figure 3, there is a coordinate loss of secondary, tertiary and quaternary structure as a function of GdnHCl concentration. The phosphorylated form of the wild-type enzyme is significantly more stable than the unphosphorylated form (the mid-point of c.d. change being shifted from 1.05 M to 1.35 M GdnHCl).

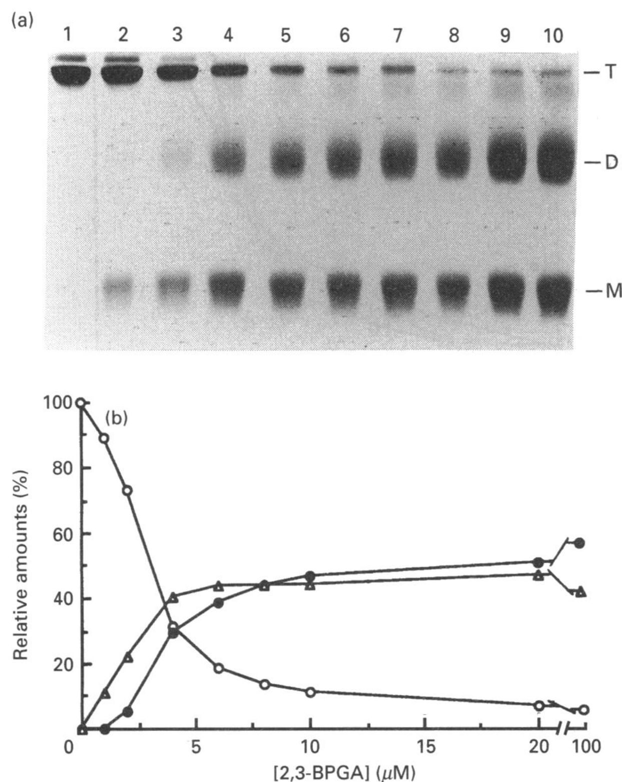

Figure 5 Effect of 2,3-3PGA on the quaternary structure of the H181A mutant

The effect of low concentrations of 2,3-BPGA on the quaternary structure of the H181A mutant was studied by glutaraldehyde cross-linking as described in the Experimental section. (**a**) The SDS/PAGE cross-linking pattern for H181A (60 μg/ml; 2.2 μM subunit concentration) after incubation with 0–100 μM 2,3-BPGA (15 min, 20 °C). The positions of tetramers, dimers and monomers are indicated. Lanes 1–10 correspond to incubation with 0, 1, 2, 4, 6, 8, 10, 20, 50 and 100 μM 2,3-BPGA respectively. (**b**) Densitometric analysis of the gel in (**a**). Key to symbols: ○, % tetramer; ●, % dimer; △, % monomer.

Similar protection was observed at a lower concentration of 2,3-BPGA (0.25 mM), which is still well above the K_m for 2,3-BPGA of 8 μM (White and Fothergill-Gilmore, 1992). No stabilizing effects were observed either by the substrate 3-phosphoglycerate or by the bisphosphonate analogue of 2,3-BPGA at concentrations of 2.5 mM. This suggests that stabilization is a specific effect of the phosphorylation of His-8.

H181A mutant

Incubation of the H181A mutant with 2.5 mM 2,3-BPGA [a concentration well above the measured K_m of 87 μM (White and Fothergill-Gilmore, 1992)] at 20 °C for 15 min leads to dramatic effects on the structure of the enzyme. The far-u.v. c.d. spectrum (Figure 4a) showed that a considerable loss of secondary structure had occurred (analysis by the CONTIN procedure showed a decrease in helical structure from 25% to 13%). A loss of tertiary structure could be inferred from the decline in the near-u.v. c.d. spectrum (Figure 4b) and from changes in the fluorescence spectrum. In the latter case, the emission maximum was shifted from 334 nm to 342 nm and the fluorescence at 330 nm declined to 38% of its initial value on addition of 2,3-BPGA. Cross-linking studies revealed that the addition of 2.5 mM, 2,3-BPGA to the mutant caused complete disappearance of the tetrameric form of the enzyme. Dimeric and monomeric species were observed to account for 57% and 43% of the total protein respectively. The formation of a monomeric species in the presence of 2,3-BPGA was also indicated in preliminary f.p.l.c. gel-filtration experiments using a Superose 13 column (results not shown).

The changes in the cross-linking patterns were studied over a range of lower 2,3-BPGA concentrations (0–100 μM). As shown in Figure 5, the dissociation of the tetramer to the dimer and monomer occurs at 2,3-BPGA concentrations of the same order of magnitude as the enzyme subunit concentration (2.2 μM). The increasing proportion of dimers (relative to monomers) over the range 0–10 μM 2,3-BPGA suggests that a monomer ⇌ dimer equilibrium is established, consistent with the kinetic analysis of the reassociation of denatured wild-type enzyme (Hermann et al., 1983). The bisphosphonate analogue [which is incapable of donating a phospho group to the enzyme (McAleese et al., 1987)] (2 mM) and substrate 3-phosphoglycerate (10 mM) were tested for their effect on the H181A mutant quaternary structure. No dissociation of the tetrameric structure was observed. Both gave protection against the dissociation of the enzyme induced by 10 μM 2,3-BPGA. These observations lend support to the hypothesis that phosphorylation of the mutant enzyme by 2,3-BPGA leads to dissociation of the tetrameric structure.

At a concentration of 2,3-BPGA equivalent to half of the subunit concentration of the H181A mutant, the dissociation of the tetramer was found to be time dependent. After 2 min no dissociation was observed. Monomer began to appear after 5 min, the proportion gradually increasing over the 4 h period of the experiment. Dimer was apparent after 15 min and thereafter increased slowly. After 240 min, the proportions of tetramer, dimer and monomer were 73, 7 and 20%, respectively. Slow changes under these conditions were also observed by fluorescence and near-u.v. c.d., but were much less marked in the far-u.v. c.d. spectrum.

DISCUSSION

The results in this paper show that the substitution of His-181 at the active site of yeast phosphoglycerate mutase by alanine has little direct effect on the structure of the enzyme as observed by c.d., fluorescence and cross-linking studies. The small decrease in the stability of the enzyme towards GdnHCl denaturation may be due to the loss of stabilizing hydrophobic contacts as a result of the mutation of this buried residue. A similar mutation in the small enzyme barnase was found to result in the loss of a stable folded structure (Sali et al., 1988).

Phosphorylation of wild-type phosphoglycerate mutase by incubation with 2,3-BPGA leads to a more stable secondary, tertiary and quaternary structure. Direct evidence for the structural details of this stabilization is not yet available from crystallographic evidence because of the lack of appropriate crystals. The presence of a phospho group at the active site may allow additional favourable electrostatic interactions to occur. Pronounced changes were observed on incubation of the mutant H181A with 2,3-BPGA. Since the reduced activity of this mutant is entirely dependent on 2,3-BPGA, and the kinetics follow the two-substrate, Ping Pong pattern (White and Fothergill-Gilmore, 1992), it is necessary to consider the consequences of these structural changes during the catalytic process. Under the conditions of the kinetic assay (i.e. low concentrations of enzyme and 50 μM–0.2 mM 2,3-BPGA) the mutant enzyme is likely to exist in a predominantly monomeric form with secondary and tertiary structures significantly different from those of the tetra-

meric form. It is thus possible that the low activity of the H181A mutant is partly due to these structural changes.

The tetrameric structure of wild-type phosphoglycerate mutase from yeast persists at very low concentrations of the enzyme (Price and Jaenicke, 1982), and is therefore likely to be the catalytically active species under normal assay conditions. Studies of the refolding of the denatured enzyme suggest that monomeric and dimeric intermediates possess partial activity, which unlike that of the tetramer is susceptible to the addition of added proteases (Hermann et al., 1985). Clearly, in the case of the wild-type enzyme the tetrameric structure imparts stability to each of the folded constituent subunits.

Our results underline the need for caution in the interpretation of structure–function studies of proteins using site-directed mutagenesis. Spectroscopic techniques such as fluorescence and c.d. may be used to examine the overall similarities in structure of wild-type and mutated enzymes; however, it is essential to perform experiments under conditions relevant to the expression of biological activity where ligand binding, conformational change and/or covalent modification must be considered.

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