

Inactivation of rabbit muscle phosphoglycerate mutase by limited proteolysis with thermolysin

Nicholas C. PRICE, Doris DUNCAN and John W. McALISTER

Department of Biological Science, University of Stirling, Stirling FK9 4LA, Scotland, U.K.

(Received 24 January 1985/accepted 8 March 1985)

Rabbit muscle phosphoglycerate mutase is inactivated by proteolysis with thermolysin. Inactivation is correlated with the breakage of one (or a few) bond(s) near one end of the polypeptide chain. There is no change in the overall conformation, quaternary structure or binding to Cibacron Blue on proteolysis. The possible analogy with the existence of a flexible tail in the yeast enzyme is discussed.

Phosphoglycerate mutase (EC 2.7.5.3) catalyses the interconversion of 2- and 3-phosphoglycerate. The enzyme from baker's yeast, a tetramer of subunit M_r 27000, has been well characterized in terms of amino acid sequence (Fothergill & Harkins, 1982) and three-dimensional structure (Winn *et al.*, 1981). The enzyme from rabbit muscle is a dimer of subunit M_r 28000 (Ray & Peck, 1972). Both enzymes depend on the presence of the cofactor 2,3-bisphosphoglycerate for activity. Apart from the homology of a peptide around the histidine residue that becomes phosphorylated during the reaction mechanism (Haggarty & Fothergill, 1980; Fothergill & Harkins, 1982), little comparative structural information on the two enzymes is available.

Susceptibility to proteolysis has proved to be a useful method for studying structural changes in proteins during refolding (Girg *et al.*, 1981; Price & Stevens, 1983a; Schmid & Blaschek, 1984). With its specificity for hydrophobic amino acids (Matsubara, 1970), thermolysin has proved a good choice in such work, as shown in experiments on the refolding of lactate dehydrogenase (Girg *et al.*, 1981). As part of a study of the refolding of denatured rabbit muscle phosphoglycerate mutase (Hermann *et al.*, 1983) we have explored the action of thermolysin on the native enzyme. The results indicate that there is an exposed region of the enzyme, essential for activity, near one end of the polypeptide chain. This could be analogous to the flexible tail proposed to occur at the C-terminus of the enzyme from baker's yeast (Winn *et al.*, 1981; Fothergill & Harkins, 1982).

Experimental

Rabbit muscle phosphoglycerate mutase, thermolysin (stabilized with calcium acetate and

sodium acetate), 3-phosphoglycerate (grade I), 2,3-bisphosphoglycerate and enolase were obtained from Boehringer Mannheim. Determination of the concentration of phosphoglycerate mutase and the assay of its activity were performed as described previously (Hermann *et al.*, 1983). Solutions of thermolysin were prepared freshly each day in 50mM-sodium phosphate buffer, pH 7.5. Control experiments, with the casein-digestion assay (Matsubara, 1970), showed that the solution was stable for at least 6 h at 20°C.

Reactive Blue 2-Sepharose CL-6B (containing 2 μ mol of Cibacron Blue F3G-A attached per ml of swollen gel) and the Dalton Mark VII-L Molecular Weight Marker set were obtained from Sigma Chemical Co.

The proteolysis of phosphoglycerate mutase by thermolysin was routinely studied at 20°C in 50mM-sodium phosphate buffer, pH 7.5. Samples withdrawn for assay or analysis were diluted into buffer containing 5mM-EDTA in order to inactivate the thermolysin (Girg *et al.*, 1981).

High-pressure gel-permeation chromatography was performed on a TSK G-2000 SW column (60cm \times 0.75cm diam.) as described previously (Price *et al.*, 1985). The absorbance of the column eluate was monitored at 280nm.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed on 10%-polyacrylamide slab gels with the separation system described by Laemmli (1970). Samples were withdrawn at intervals from a solution containing phosphoglycerate mutase and thermolysin; EDTA was then added to inactivate the thermolysin. A small portion of each sample was assayed for phosphoglycerate mutase activity, and the remainder was added to sample buffer and heated at 100°C for 2min before application to the gel.

C.d. spectra were recorded at 20°C in a JASCO

J-500-A spectropolarimeter equipped with a JASCO DP-500N data processor.

The binding of phosphoglycerate mutase to Cibacron Blue–Sephrose was studied by adding 0.5 ml of protein solution to 0.2 ml of immobilized dye in a conical centrifuge tube. After mixing, the contents were centrifuged (500g for 2 min) and the supernatant was analysed. With the native enzyme the supernatant was assayed for enzyme activity, and also for protein by the method of Sedmak & Grossberg (1977), with bovine serum albumin as a standard. With the enzyme treated with thermolysin only the latter method was used.

Results

Inactivation of phosphoglycerate mutase by treatment with thermolysin

The time course of inactivation of rabbit muscle phosphoglycerate mutase by thermolysin is shown in Fig. 1(a). At a mutase concentration of 100 µg/ml, incubation with 10 µg/ml of thermolysin/ml causes an approx. 50% loss of activity after 60 min; this loss is increased to approx. 70% and 80% by increasing the concentration of thermolysin to 50 µg/ml and 100 µg/ml respectively.

As shown in Fig. 1(b), the inclusion of cofactor 2,3-bisphosphoglycerate leads to substantial protection against inactivation. Inclusion of substrate 3-phosphoglycerate affords little if any protection and does not increase the degree of protection by 2,3-bisphosphoglycerate. Since addition of 2,3-bisphosphoglycerate leads to formation of the phospho-enzyme intermediate (Ray & Peck, 1972),

it would appear that the phospho-enzyme is less susceptible to proteolysis by thermolysin than is the native enzyme.

Conformation of phosphoglycerate mutase

The c.d. spectra of phosphoglycerate mutase over the range 260–200 nm were recorded before and after treatment with thermolysin. In order to minimize the contribution of thermolysin to the spectra, phosphoglycerate mutase (100 µg/ml) was incubated with a low concentration (10 µg/ml) of thermolysin; after 6 h, 25% activity remained. The resulting spectra (corrected for the small contribution of thermolysin) are shown in Fig. 2. In both cases there is a double-minimum pattern characteristic of α -helical structures. By using the reference values for θ_{225} and θ_{208} (Chen *et al.*, 1974; Chang *et al.*, 1978) the helical contribution to the structure of the native enzyme is calculated to be $17 \pm 2\%$, a value comparable with that determined for the baker's-yeast enzyme by this technique ($20 \pm 5\%$) (Hermann *et al.*, 1983). As shown in Fig. 2, there are only very small (<10%) changes in the values of θ on treatment with thermolysin, suggesting that proteolysis causes little change in the overall conformation of the enzyme.

Changes in M_r of phosphoglycerate mutase on proteolysis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was used to determine the pattern of fragmentation of the polypeptide chain of phosphoglycerate mutase. The changes in M_r

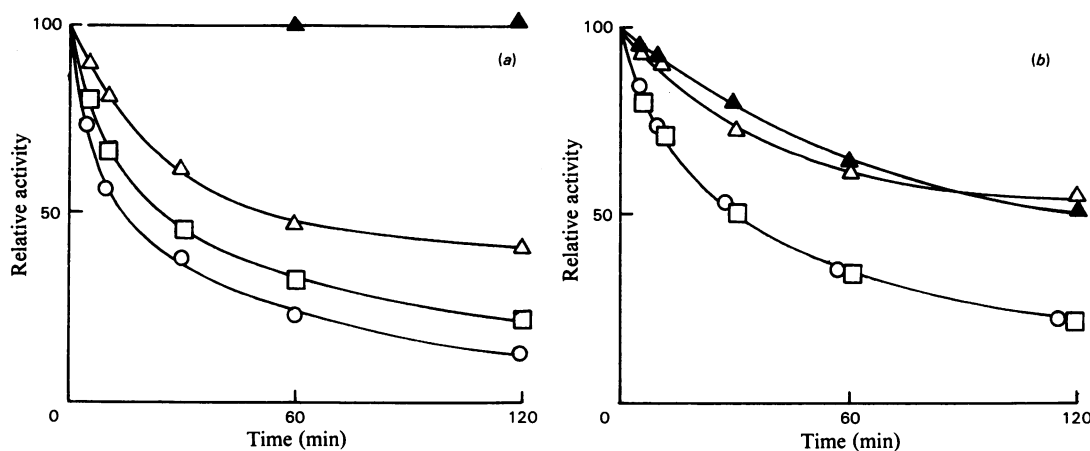


Fig. 1. Inactivation of rabbit muscle phosphoglycerate mutase by thermolysin

The reactions were performed in 50 mM-sodium phosphate buffer, pH 7.5, at 20°C. (a) Phosphoglycerate mutase concentration 100 µg/ml. Thermolysin concentrations (µg/ml): ▲, 0; △, 10; □, 50; ○, 100. (b) Phosphoglycerate mutase concentration 100 µg/ml, thermolysin concentration 50 µg/ml. Reactions were performed in the presence of: □, no added ligands; ○, 10 mM-3-phosphoglycerate; △, 0.2 mM-2,3-bisphosphoglycerate; ▲, 10 mM-3-phosphoglycerate + 0.2 mM-2,3-bisphosphoglycerate.

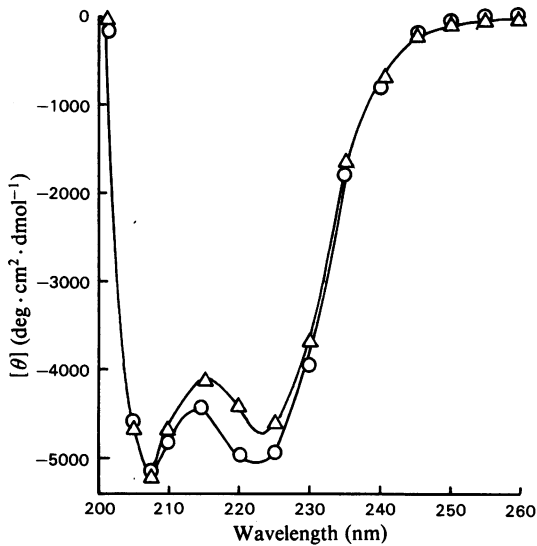


Fig. 2. *C.d. spectra of phosphoglycerate mutase*
Spectra were recorded in 50 mM-sodium phosphate buffer, pH 7.5, at 20°C, in a cell of pathlength 5 mm. ○, Native enzyme, 100 µg/ml; △, enzyme (100 µg/ml) incubated with thermolysin (10 µg/ml) for 6 h. (The spectrum has been corrected for the small contribution of thermolysin.)

under non-denaturing conditions were examined by high-pressure gel-permeation chromatography.

The results of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis are shown in Fig. 3(a). Lanes 1, 8 and 9 represent the M_r markers, phosphoglycerate mutase and thermolysin respectively. Although the sample of phosphoglycerate mutase is >95% homogeneous by this technique, there is evidence for a small amount of a species of slightly lower subunit M_r [probably reflecting the action of some endogenous proteinase(s) during isolation]. Thermolysin, in addition to the main component of M_r 35 000, contains two minor species with M_r values in the range 21 000–23 000 and a third minor species with M_r 14 000. The experiment shows quite clearly that with increasing times of proteolysis (lanes 2–7) there is a conversion of native phosphoglycerate mutase (subunit M_r 28 000) into a species of slightly lower subunit M_r (27 000) concomitant with the inactivation of the enzyme (Fig. 3b). Under these conditions there is no evidence for the formation of significant quantities of fragments of lower M_r , suggesting that proteolysis is limited to the breakage of one (or very few) bond(s) near one end of the polypeptide chain.

For the high-pressure gel-permeation experiments, phosphoglycerate mutase (500 µg/ml) was incubated with 167 µg of thermolysin/ml at 20°C in 50 mM-sodium phosphate buffer, pH 7.5, for 3 h;

19% activity remained at this point. The sample was then applied to the gel-permeation column, and the main peak was found to be eluted at a position corresponding to M_r 46 000 by reference to the elution position of the standard proteins bovine serum albumin, ovalbumin, chymotrypsinogen and ribonuclease. The second, smaller, peak (M_r 33 000) was shown in a control experiment to correspond to thermolysin. The M_r of native phosphoglycerate mutase was determined to be 48 000 by this technique. It is clear from these data that proteolysis does not cause the dissociation of the subunits of the dimeric enzyme.

Binding of phosphoglycerate mutase to Cibacron Blue–Sephadex

In a previous paper (Price & Stevens, 1983b) it was shown that 2,3-bisphosphoglycerate-dependent phosphoglycerate mutases, e.g. that from rabbit muscle, bound to Cibacron Blue–Sephadex, and that the enzyme–dye complex could be dissociated by the addition of 2,3-bisphosphoglycerate or salt. Although there has been considerable debate about the structural reasons for binding to dyes such as Cibacron Blue, it is generally regarded as characteristic of proteins with an α -helix/ β -sheet structural pattern (Beissner *et al.*, 1979).

With the method described in the Experimental section it was found that binding of native phosphoglycerate mutase (200 µg/ml) to Cibacron Blue was very much weaker in 50 mM-sodium phosphate buffer, pH 7.5 (25% of enzyme bound), than in 10 mM-Tris/HCl buffer, pH 8.0 (85% bound). Apart from general ionic-strength effects, the weaker binding in phosphate buffer could reflect competition by the buffer for those cationic sites on the enzyme that would bind to the sulphate groups on the dye (Beissner *et al.*, 1979). All subsequent experiments were performed in Tris buffer. After the addition of 2,3-bisphosphoglycerate (final concn. 1 mM) or NaCl (final concn. 1 M) to the supernatant the enzymes remaining bound to the immobilized dye decreased to 30% or 35% respectively.

Control experiments showed that the time course of inactivation of phosphoglycerate mutase by thermolysin in Tris buffer was very similar to that observed in phosphate buffer. Enzyme (730 µg/ml) was incubated with thermolysin (130 µg/ml) for 4 h in Tris buffer (20°C); at this stage 10% activity remained. After a 5-fold dilution the binding of the proteolysed enzyme was studied. About 90% of the protein bound to Cibacron Blue–Sephadex; addition of 2,3-bisphosphoglycerate or NaCl released protein to the extent that 60% or 45% of the protein remained bound respectively. A

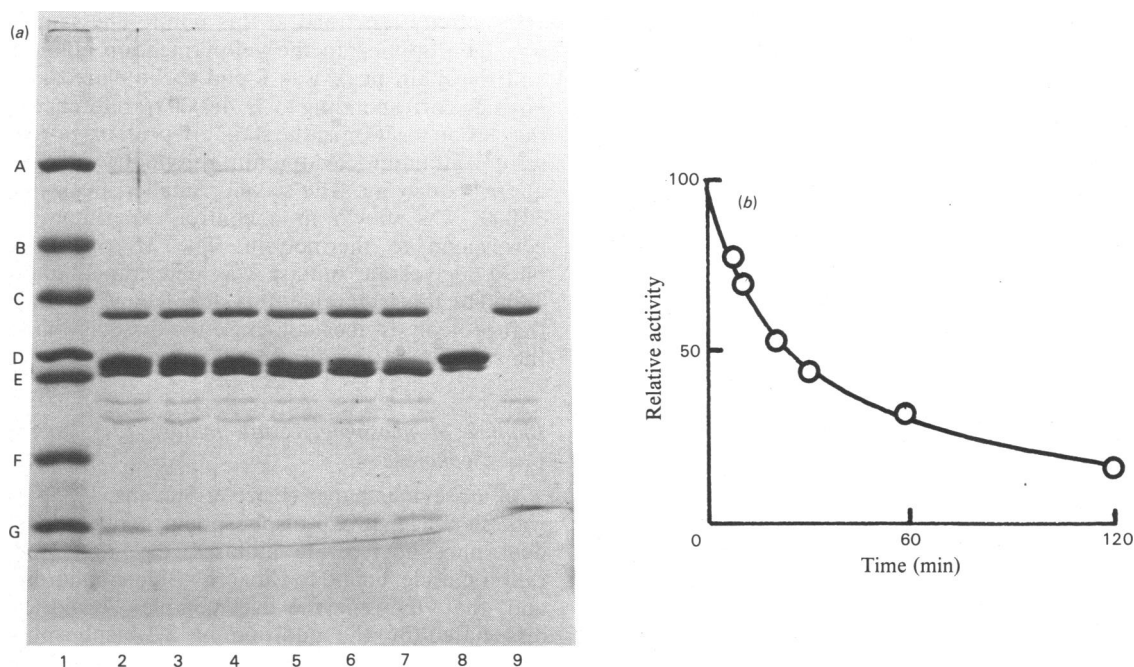


Fig. 3. Inactivation of rabbit muscle phosphoglycerate mutase by thermolysin

Phosphoglycerate mutase (500 $\mu\text{g/ml}$) was incubated with thermolysin (200 $\mu\text{g/ml}$) in 50 mM-sodium phosphate buffer, pH 7.5, at 20°C. (a) Reaction monitored by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Lane 1 contains the following marker proteins: A, bovine serum albumin; B, ovalbumin; C, glyceraldehyde-3-phosphate dehydrogenase; D, carbonic anhydrase; E, trypsinogen; F, soya-bean trypsin inhibitor; G, α -lactalbumin. Lanes 2-7 contain reaction mixtures analysed after 8, 11, 20, 30, 58 and 119 min respectively. Lanes 8 and 9 contain phosphoglycerate mutase and thermolysin respectively. (b) Reaction monitored by loss of enzyme activity.

control experiment in which thermolysin was added to the enzyme immediately before the binding determination showed that the presence of thermolysin under these conditions had no effect on the binding to or release from the immobilized dye.

Although these results should only be regarded as semiquantitative, they do clearly indicate that proteolysis of phosphoglycerate mutase by thermolysin does not affect the ability of the enzyme to bind to Cibacron Blue-Sepharose and hence does not presumably perturb the α -helix/ β -sheet structural pattern of the enzyme.

Discussion

Limited proteolysis of native enzymes has been extensively used in recent years to study the domain structure of mono- and multi-functional proteins (see, e.g., Wetlaufer, 1981; Smith & Coggins, 1983; McCarthy & Hardie, 1984). Bonds particularly susceptible to proteolysis represent 'linker regions' or 'flexible loops' in the polypeptide chain.

The data in the present paper show that inactivation of rabbit muscle phosphoglycerate mutase is correlated with the cleavage of one (or a few) bond(s) near one end of the polypeptide chain. From the change in subunit M_r on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (1000 ± 400 in four experiments) it can be estimated that approximately ten amino acid residues are lost from the chain. This loss does not affect the quaternary structure (as judged by the M_r under non-denaturing conditions), the overall conformation (as judged by c.d.) or the binding site(s) for Cibacron Blue.

It is of considerable interest to note that in baker's-yeast phosphoglycerate mutase there is a flexible C-terminal tail of approximately ten amino acid residues (Fothergill & Harkins, 1982) that is susceptible to removal by endogenous proteinases (Sasaki *et al.*, 1966) and that does not appear in the electron-density map of the enzyme (Winn *et al.*, 1981). The removal of this tail leads to inactivation of the enzyme, but does not cause any significant change in the overall structure as judged by o.r.d. (Sasaki *et al.*, 1966). It has been proposed (Winn *et*

al., 1981) that a possible function of this tail is to exclude water from the active site during the mutase reaction, thus ensuring that the phospho group is transferred to the substrate rather than to a water molecule. This type of water-exclusion role has also been proposed for flexible loops in other enzymes, such as lactate dehydrogenase (Holbrook *et al.*, 1975).

Although it is tempting to conclude that the small fragment removed from rabbit muscle phosphoglycerate mutase by thermolysin plays a similar structural and functional role to the C-terminal tail in the yeast enzyme, further sequence data on the rabbit muscle enzyme are required before such a conclusion can be substantiated.

We thank Professor Rainer Jaenicke for help in determination of the c.d. spectra.

References

- Beissner, R. S., Quioco, F. A. & Rudolph, F. B. (1979) *J. Mol. Biol.* **134**, 847–850
- Chang, C. T., Wu, C.-S. C. & Yang, J. T. (1978) *Anal. Biochem.* **91**, 13–31
- Chen, Y.-H., Yang, J. T. & Chau, K. H. (1974) *Biochemistry* **13**, 3350–3359
- Fothergill, L. A. & Harkins, R. N. (1982) *Proc. R. Soc. London Ser. B* **215**, 19–44
- Girg, R., Rudolph, R. & Jaenicke, R. (1981) *Eur. J. Biochem.* **119**, 301–305
- Haggarty, N. W. & Fothergill, L. A. (1980) *FEBS Lett.* **109**, 18–20
- Hermann, R., Rudolph, R., Jaenicke, R., Price, N. C. & Scobbie, A. (1983) *J. Biol. Chem.* **258**, 11014–11019
- Holbrook, J. J., Liljas, A., Steindel, S. J. & Rossmann, M. G. (1975) *Enzymes 3rd Ed.* **11**, 191–292
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Matsubara, H. (1970) *Methods Enzymol.* **19**, 642–651
- McCarthy, A. D. & Hardie, D. G. (1984) *Trends Biochem. Sci.* **9**, 60–63
- Price, N. C. & Stevens, E. (1983a) *Biochem. J.* **209**, 763–770
- Price, N. C. & Stevens, E. (1983b) *Biosci. Rep.* **3**, 857–861
- Price, N. C., Duncan, D. & Ogg, D. J. (1985) *Int. J. Biochem.* in the press
- Ray, W. J., Jr. & Peck, E. T., Jr. (1972) *Enzymes 3rd Ed.* **6**, 407–477
- Sasaki, R., Sugimoto, E. & Chiba, H. (1966) *Arch. Biochem. Biophys.* **115**, 53–61
- Schmid, F. X. & Blaschek, H. (1984) *Biochemistry* **23**, 2128–2133
- Sedmak, J. J. & Grossberg, S. E. (1977) *Anal. Biochem.* **79**, 544–552
- Smith, D. D. S. & Coggins, J. R. (1983) *Biochem. J.* **213**, 405–415
- Wetlaufer, D. B. (1981) *Adv. Protein Chem.* **34**, 61–92
- Winn, S. I., Watson, H. C., Harkins, R. N. & Fothergill, L. A. (1981) *Philos. Trans. R. Soc. London Ser. B* **293**, 121–130