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THE EFFECTS OF ANIONS, SUBSTRATES, METAL IONS AND SULFHYDRYL REAGENTS ON THE PROTEOLYTIC SUSCEPTIBILITY OF YEAST PHOSPHOGLYCERATE KINASE

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With the aim of confirming our previous spectrophotometric binding studies ((1978) Eur. J. Biochem. 85, 345–350 and (1980) Eur. J. Biochem. 104, 249–254) and of ascertaining the full physiological significance of ion binding, we investigated the effects of ions and thiol reagents on the proteolysis of yeast phosphoglycerate kinase (ATP : 3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3). The single non-essential thiol of the enzyme was modified with 5,5′-dithiobis(2-nitrobenzoic acid) or 2-chloromercuri-4-nitrophenol. Both modifications greatly increased the susceptibility of the kinase to inactivation by trypsin or yeast proteinase A, when compared with that of the native kinase. Electrophoresis in sodium dodecyl sulfate (SDS) revealed that limited proteolysis had occurred. The time courses for the proteolysis and loss of catalytic activity were followed and the active and inactive fragments identified. The molecular masses of the major proteolytic fragments differed with the two endopeptidases. Substrate and non-substrate anions in a concentration-dependent fashion, protected the native and mercurial-labelled kinase from inactivation by trypsin or yeast proteinase A. However, Zn^{2+}, in a concentration-dependent fashion, increased the susceptibility of the native kinase to inactivation by each endopeptidase. The time courses for the inactivation and for the proteolysis allowed the active and inactive fragments to be identified. Zn^{2+} decreased the rate of inactivation of the mercurial-labelled kinase by proteinase A. The effects of these ions were detected at concentrations compatible with occupancy of an anion binding site and a low affinity Zn^{2+} binding site, both of which have been indicated from our previous binding studies.

Introduction

Thiol-directed reagents do not alter substrate K_m values of yeast phosphoglycerate kinase (ATP: 3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) [1,2]. We have previously described the use of Nbs_2 and 2-chloromercuri-4-nitrophenol reacted with the single thiol of this enzyme to monitor spectrophotometrically the binding of substrate and non-substrate anions [3]. This study suggested the presence of a nonspecific anion binding site that was not at the active site and complemented several other studies that involved physical and kinetic experiments supporting anion binding sites [4–10]. Whereas Scopes believes that anions bind only at the active site [5], Khamis and Larsson-Raźnikiewicz [8], and ourselves [3] have concluded that there is another site as well. In any case, anions are both inhibitors and activators of the enzyme.

We have also used Nbs_2 and the mercurial to monitor metal ion binding to yeast phosphoglycerate kinase [11]. These data suggested two binding sites for Zn^{2+} with considerably differing affinities.

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Abbreviations: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); Tes, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid.

Physical and enzyme kinetics studies had earlier shown binding of several different metal ions to the enzyme [10,12,13].

The lack of specificity for anion binding suggested to us that the ions may have a more important physiological role than an alteration of enzyme activity. We therefore investigated the effects of anions and metal cations on the proteolytic susceptibility of yeast phosphoglycerate kinase. Anions had little effect on the native kinase but protected the thiol-modified kinase from proteolysis. This system also allowed us to confirm certain of our spectrophotometric binding studies. Zn^{2+} had little effect on the modified kinase but greatly enhanced the rate of inactivation of the native enzyme. The molecular masses of the polypeptides generated during these incubations with either trypsin or yeast proteinase A were determined and equated with the loss of enzyme activity.

Materials and Methods

Materials

Yeast phosphoglycerate kinase, yeast glyceraldehyde-3-phosphate dehydrogenase (EC 1.1.1.8), bovine pancreatic trypsin (EC 3.4.21.4), yeast proteinase A (EC 3.4.23.6), triethanolamine · HCl, Tes, Nbs_2, the monosodium salt of D(-)3-phosphoglycerate (free of D-glycerate-2,3-bisphosphate), β-NADH, the bistris salt of D-glycerate-2,3-bisphosphate, Coomassie blue R-250, and the sodium salts of citrate, ATP, ADP and succinate were from Sigma Chemical Co. (St. Louis, MO). Sodium hydroxide, hydrochloric acid, $ZnCl_2$, $MgCl_2$, NaCl, ammonium persulfate, glycerol, 2-propanol, acetic acid, ethanol and trichloroacetic acid were products of Fisher Scientific (Fairlawn, NJ). Bromphenol blue was obtained from BDH Chemicals (Toronto, Canada). Carrier ampholytes (pH 7–9) were purchased from LKB-Produkter A.B. (Broma, Sweden) and 2-mercaptoethanol was a product of Eastman Kodak (Rochester, NY). Acrylamide, bisacrylamide, sodium dodecyl sulfate, *N,N,N′,N′*-tetramethylethylenediamine and the subunit molecular mass markers were obtained from Bio-Rad Laboratories (Richmond, CA). The 2-chloromercuri-4-nitrophenol was the generous gift of Dr. D.R. Trentham (University of Pennsylvania, U.S.A.).

The purity of the phosphoglycerate kinase was assessed by isoelectric focusing [13] and polyacrylamide gel electrophoresis in the presence of SDS (see below). The specific activity of the enzyme was 700 U/mg protein. Prior to use the kinase was extensively dialyzed against 50 mM triethanolamine, pH 7.5. Glyceraldehyde-3-phosphate dehydrogenase was dialyzed against the same buffer plus 10 mM 2-mercaptoethanol.

Lyophilized trypsin, with or without diphenyl carbamyl chloride, was prepared at a concentration of 5 mg/ml in 1 mM HCl, or 215 μM based on a molecular mass of 23281 [14]. A further dilution of this stock was made with 1 mM HCl to give a final concentration of 0.42 μM. Lyophilized proteinase A was reconstituted with water to give 5 mg protein/ml solution. A concentration of 83 μM was calculated, based on a molecular mass of 60000 [15]. All enzyme solutions were stored at 4°C.

The buffer for the proteolytic inactivation studies was 50 mM triethanolamine · HCl adjusted to pH 7.5 with NaOH.

Stock solutions of substrate or non-substrate anions were prepared in the buffer and brought to a final pH of 7.5 with sodium hydroxide. Stock solutions of metal ions were prepared in water. Stock solutions of Zn^{2+} were quantitated by atomic absorption and were kindly performed by the Department of Laboratory Medicine at the University of Alberta Hospital.

All water was deionized and double-distilled, with glass distillation as the final procedure.

Spectrophotometric procedures were carried out with a Beckman Acta CIII spectrophotometer at 30°C.

Enzyme assays

Phosphoglycerate kinase was assayed in the reverse direction as described [16]. The buffer was 50 mM triethanolamine adjusted to a final pH of 7.5 with sodium hydroxide. The reaction was monitored at 340 nm. 1 unit of activity (U) is defined as that amount of enzyme which will catalyze the phosphorylation of 1 μmol 3-phosphoglycerate/min.

The rate of hydrolysis of benzoyl arginine *p*-nitroanilide by trypsin was determined according to the method of Erlanger et al. [17] with the exceptions that the substrate was dissolved in water and the final concentration of buffer was 50 mM triethanolamine, pH 7.5.

Protein determination

Phosphoglycerate kinase protein concentration was determined using $\epsilon^{1\%}_{280nm} = 4.9$ [16] and a molecular mass of 47 000 [18]. The concentration of Nbs-modified enzyme was determined by calculating the concentration of the released 3-carboxylato-4-nitrothiophenolate anion ($\epsilon^{mM}_{412nm} = 14.1$ [19]) in the presence of 10 mM β-mercaptoethanol buffered at pH 7.5 with 50 mM triethanolamine. Excess Nbs_2 and nitrothiophenolate anion generated during the modification reaction had been removed by dialysis against 50 mM triethanolamine (pH 7.5) prior to quantitation of the Nbs-modified protein.

Labelling of phosphoglycerate kinase with mercurial

Stock solutions of 2-chloromercuri-4-nitrophenol were prepared in 0.1 M NaOH, quantitated as previously described [1], and stored at 4°C. To label the kinase with the mercurial, the enzyme at 2.0 μM was incubated with a 5–10% molar excess of mercurial for 3–5 min at 30°C in 50 mM triethanolamine, pH 7.5.

Modification of phosphoglycerate kinase with Nbs_2

A stock solution of 10.0 mM Nbs_2 was made up in 5.0 mM Tes buffer and brought to a final pH of 8.0 with 0.1 N NaOH. The reaction was carried out with 20 μM phosphoglycerate kinase and 0.4 mM Nbs_2, and the release of the nitrothiophenolate anion was monitored at 412 nm. The concentration of the Nbs-modified kinase was determined as described above.

Proteolytic inactivation of phosphoglycerate kinase

Phosphoglycerate kinase was modified witl Nbs_2 and quantitated as described above. Modification of the kinase with mercurial was according to the standard protocol (see above). Native or modified phosphoglycerate kinase (2.0 μM) in 50 mM triethanolamine, pH 7.5, was pre-incubated at 30°C for approx. 5 min. Trypsin was added to a final concentration of 0.004 μM when the kinase was modified with mercurial or Nbs_2, and to 2.0 μM when native enzyme was used. Proteinase A was added to a final concentration of 0.22 μM. All incubations were at 30°C. The final volume of the incubation mixtures was 1.0 or 2.0 ml. Aliquots were taken prior to the addition of the endopeptidase and at timed intervals thereafter, and assayed for phosphoglycerate kinase activity as described above. Percent inactivation was calculated relative to the activity determined prior to addition of the endopeptidase. When polyacrylamide gel electrophoresis in the presence of SDS (see below) was simultaneously run, a second aliquot was taken after an additional 1 min incubation and immediately prepared for electrophoresis.

The effect of substrate or nonsubstrate anions on the susceptibility of native or mercurial-labelled phosphoglycerate kinase to proteinase A or trypsin was carried out as described above. At least three concentration of anion were used so that the concentration of anion required to give 50% enzyme activity remaining after 1 h could be determined by interpolation.

The effect of Zn^{2+} on the susceptibility of native and mercurial- or Nbs-modified phosphoglycerate kinase to inactivation by trypsin or proteinase A was also examined. Experimental protocol was as described above for inactivation of the modified kinase by trypsin or proteinase A. The effect of Mg^{2+} on the inactivation of the native enzyme by trypsin was carried out as described above.

Sodium dodecyl sulfate electrophoresis

Polyacrylamide gel (10%) electrophoresis in the presence of 0.1% SDS was carried out as described [20]. Proteins were stained with Coomassie blue R-250 [21]. Molecular masses were determined from a plot of the R_F values of the marker proteins vs. the log of their subunit molecular masses. The marker proteins were: phosphorylase *b* (subunit molecular mass 94 000), bovine serum albumin (68 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (21 000) and lysozyme (14 300).

Results

Inactivation and proteolysis of phosphoglycerate kinase by trypsin

Modification of phosphoglycerate kinase with either mercurial or Nbs_2 increased its susceptibility to inactivation by trypsin when compared to that observed with the native kinase (Fig. 1). The mercurial-labelled kinase was almost 80% inactivated after 120 min incubation at a kinase-to-trypsin molar ratio of 500 to 1, but the activity of the native kinase decreased by less than 10% after incubation for the

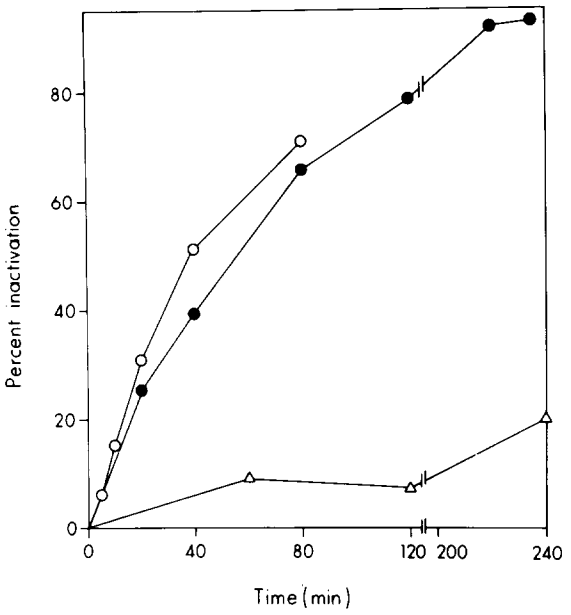

Fig. 1. Rate of inactivation by trypsin of native and mercurial- or Nbs-modified phosphoglycerate kinase. The kinase-to-trypsin molar ratio was 500 to 1 with the kinase at 0.004 μM. Activity is expressed as percent inactivation relative to the activity determined prior to the addition of trypsin. △———△, native kinase; ●———●, mercurial-labelled kinase; ○———○, Nbs-modified kinase. The buffer was 50 mM triethanolamine, pH 7.5.

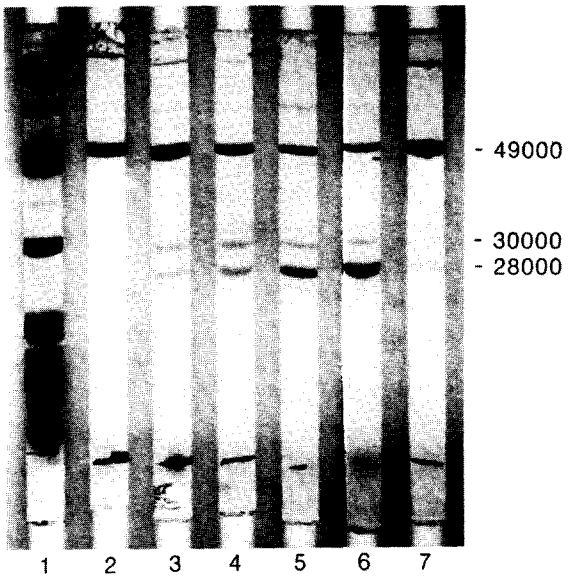

Fig. 2. Separation by polyacrylamide gel electrophoresis, in the presence of SDS, of polypeptides generated by limited tryptic digestion of native and mercurial-labelled phosphoglycerate kinase. Gel 1 is native kinase combined with the subunit molecular mass markers. The major protein bands are (top to bottom): phosphorylase *b* 94 000; bovine serum albumin, 68 000; yeast phosphoglycerate kinase, 49 000; ovalbumin, 43 000; carbonic anhydrase; 30 000; soybean trypsin inhibitor, 21 000; and lysozyme, 14 300. Gels 2–6 are aliquots of the mixture of labelled kinase and trypsin after 1, 20, 40, 80 and 120 min incubation, respectively (see Fig. 1). Gel 7 is the native kinase incubated for 120 min with trypsin.

same length of time under the same conditions. At the kinase-to-trypsin molar ratio of 500 to 1 the rate of inactivation of Nbs-modified phosphoglycerate kinase was similar to that observed with the mercurial-labelled kinase incubated under the same conditions (Fig. 1). In contrast, a kinase-to-trypsin molar ratio of 0.7 to 1 was required to obtain the same inactivation of the native kinase over a similar time span (data not shown). Inactivation curves of native or mercurial-labelled phosphoglycerate kinase determined in the presence of trypsin were similar, regardless of the presence of the chymotrypsin inhibitor diphenyl carbamyl chloride in the trypsin preparation.

During incubation of the native or mercurial-labelled kinase with trypsin (Fig. 1), samples were taken for SDS-polyacrylamide gel electrophoresis. A molecular mass of 49 000 was determined for the native or 'mercurial-labelled' kinase (Fig. 2, gels 1 and 2). Fig. 2 shows the mobilities of the polypeptides which were generated during incubation of native (gel 7) or mercurial-labelled (gels 2–6) phosphoglycerate kinase with trypsin. During the incubation time of 120 min the concentration of the kinase decreased as indicated by the gradual disappearance of the 49 000-M_r band. This disappearance correlated with the rate of inactivation (Fig. 1).

After 20 min of incubation two peptides with molecular masses of 30 000 and 28 000 formed from the mercurial-labelled kinase and catalytic activity decreased by 30% (Fig. 2, gel 3 and Fig. 1). From this it can be deduced that both species are inactive. Proteolysis of native phosphoglycerate kinase (Fig. 2, gel 7) yielded the same degradation products as those resulting from the mercurial-labelled enzyme.

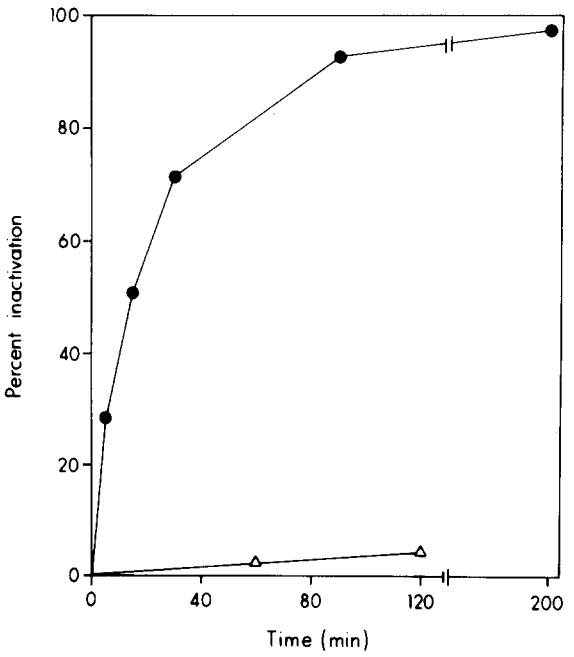

Fig. 3. Rate of inactivation by proteinase A of native and mercurial-labelled phosphoglycerate kinase. The kinase-to-proteinase A molar ratio was 9.1 to 1 and other conditions are as described in the legend to Fig. 1: △———△, native kinase; ●———●, mercurial-labelled kinase.

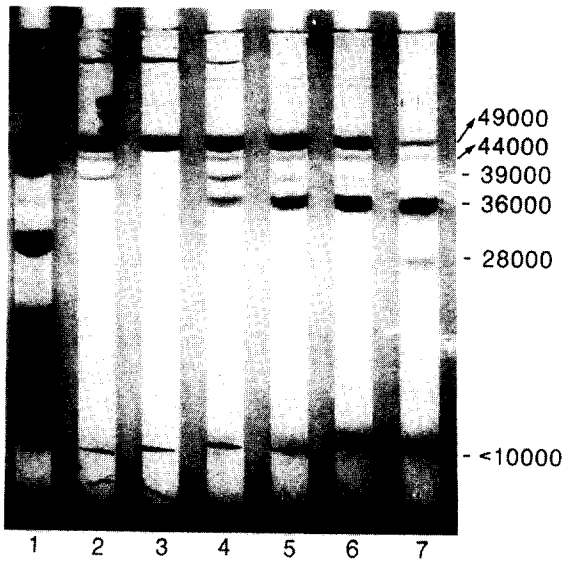

Fig. 4. Separation by polyacrylamide gel electrophoresis, in the presence of SDS, of polypeptides generated by limited proteinase A digestion of native and mercurial-labelled phosphoglycerate kinase. Gel 1 is native kinase combined with the subunit molecular mass markers (see Fig. 2). Gel 2 is the native kinase incubated for 120 min with proteinase A, Gels 3–7 are aliquots of the mixture of labelled kinase taken at 0. 5, 15, 30 and 90 min incubation, respectively (see Fig. 3).

Inactivation and proteolysis of phosphoglycerate kinase by proteinase A

The susceptibility of mercurial-labelled phosphoglycerate kinase to inactivation by proteinase A was increased as compared with that of the native kinase (Fig. 3). Following 120 min incubation at a kinase-to-proteinase A molar ratio of 9.1 to 1, labelled kinase was 95% inactivated while the activity of the native kinase decreased by less than 5%.

During these incubations, samples were taken for SDS electrophoresis (Fig. 4). The decrease in concentration of the labelled kinase (gels 3–7) during 90 min incubation was indicated by a decrease in the amount of the 49 000-M_r band and appeared to correlate with the loss of kinase activity determined over the same time span (Fig. 3). A 44 000-M_r band was a minor component and remained at a constant concentration during incubation of the labelled kinase with proteinase A. A band corresponding to a molecular mass of 39 000 was also a minor component but its concentration decreased during the incubation. The major end product of proteolysis was a 36 000-M_r band which accumulated during the incubation. With extended incubation of the labelled kinase a band corresponding to a molecular mass of 28 000 was observed, and low molecular mass fragments (less than 10 000) were also apparent (gels 6 and 7). The 36 000-M_r species was inactive. Only very small amounts of the transient 44 000 and 39 000-M_r species were produced. Also, the closeness of their molecular size to the native enzyme and to the 36 000-M_r species did not allow their preparative purification to assess catalytic activity.

Proteolysis of the native kinase by proteinase A which caused 5% inactivation (Fig. 4, gel 2) resulted in degradation products similar to those detected during incubation of the labelled kinase with proteinase A (Fig. 4, gels 4–7).

The effect of anions on the inactivation of phosphoglycerate kinase by proteinase A

The effect of citrate on the rate of inactivation of

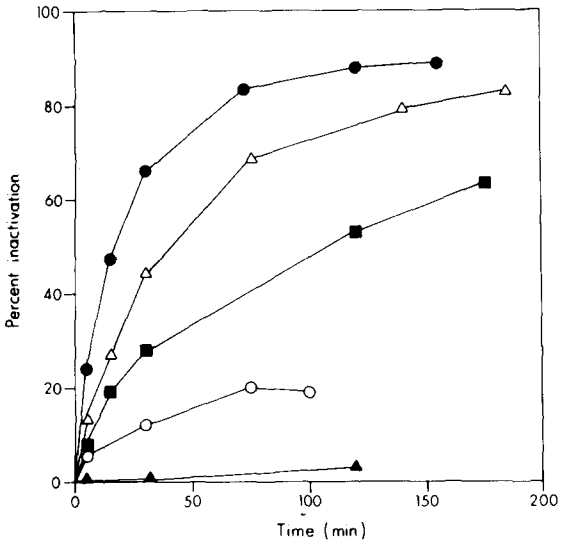

Fig. 5. Protection by citrate of the rate of inactivation by proteinase A of mercurial-labelled phosphoglycerate kinase. Conditions are as described for Fig. 1 and 3. ●——●, no citrate; △——△, 0.33 mM citrate; ■——■, 1.0 mM citrate; ○——○, 5.0 mM citrate; ▲——▲, inactivation of native kinase in the absence of citrate.

the mercurial-labelled kinase by proteinase A is shown in Fig. 5. At a kinase-to-proteinase A molar ratio of 9.1 to 1, and in the absence of citrate, 17 min were required to reach 50% inactivation. At 0.33 and 1.0 mM citrate this parameter increased to 41 and 103 min, respectively. Table I summarizes the

TABLE I

THE EFFECT OF ANIONS ON THE RATE OF INACTIVATION OF MERCURIAL-LABELLED PHOSPHOGLYCERATE KINASE BY PROTEINASE A

The kinase-to-proteinase A molar ratio was 9.1 to 1. The results are expressed as the concentration of anion required to give 50% of enzyme activity remaining after 1 h. In the absence of any added anion the enzyme was 79% inactivated in 1 h. Negative charge was determined as previously described [3].

Anion	Negative charge at pH 7.5	Concentration (mM)
ATP	3.9	0.03
3-Phosphoglycerate	3.0	0.15
Citrate	3.0	0.50
Succinate	2.0	15
Chloride	1.0	90

protective effect of various anions on the inactivation of the labelled kinase by proteinase A, and gives an approximate charge on each anion at pH 7.5. The concentrations listed in this table indicate the relative strengths of binding of the anions to the enzyme, and thus it can be seen that the greater the charge on the anion the tighter the binding. D-Glycerate 2,3-bisphosphate at 0.5 mM or ATP at 1.0 mM, concentrations that would saturate the anion binding site [3], protected the mercurial-labelled enzyme to the extent that it had the same stability to proteinase A as the native enzyme.

Succinate also protected the native or mercurial-labelled kinase from inactivation by trypsin (data not shown).

The effect of Zn^{2+} on the inactivation by trypsin or proteinase A of native phosphoglycerate kinase

Whereas the anions were found to protect the native or mercurial-labelled kinase against proteolytic inactivation (see above), Zn^{2+} enhanced the inactiva-

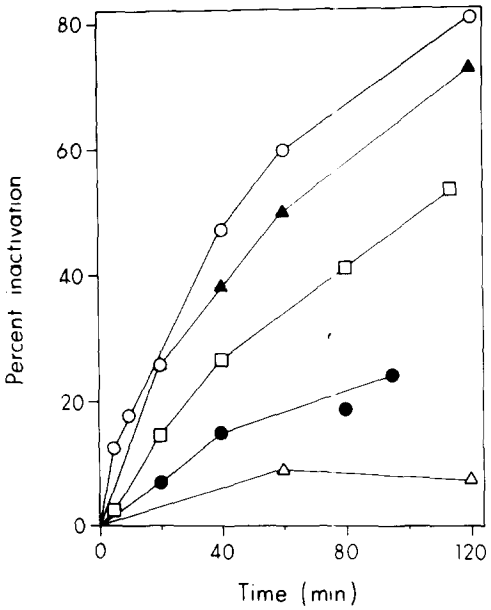

Fig. 6. The enhancement by Zn^{2+} of the rate of inactivation by trypsin of native phosphoglycerate kinase. The kinase-to-trypsin molar ratio was 500 to 1 with a kinase concentration of 2.0 μM. Other conditions are as described in the legends to Figs. 1 and 3. △——△, no Zn^{2+}; ●——●, 15.8 μM Zn^{2+}; □——□, 26.2 μM Zn^{2+}; ▲——▲, 39.4 μM Zn^{2+}; ○——○, 52.5 μM Zn^{2+}.

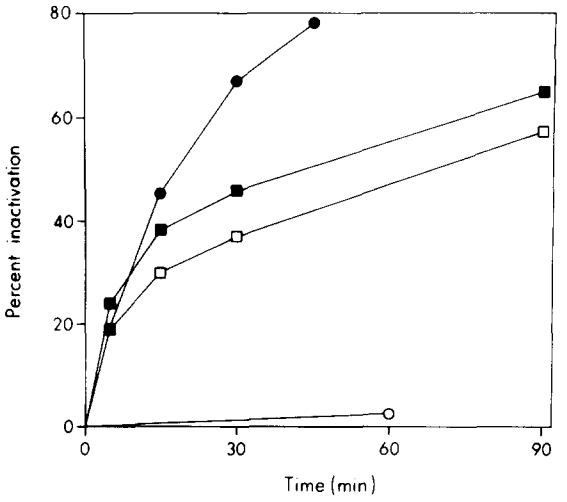

Fig. 7. The effect of Zn^{2+} on the rate of inactivation by proteinase A of native and mercurial-labelled phosphoglycerate kinase. The kinase-to-proteinase A molar ratio was 9.1 to 1 with a kinase concentration of 2.0 μM. Other conditions are as described in the legends to Figs. 1 and 3. The open symbols represent the native kinase and the closed symbols the mercurial-labelled kinase: ○ and ●, no Zn^{2+}; □ and ■, 52.5 μM Zn^{2+}.

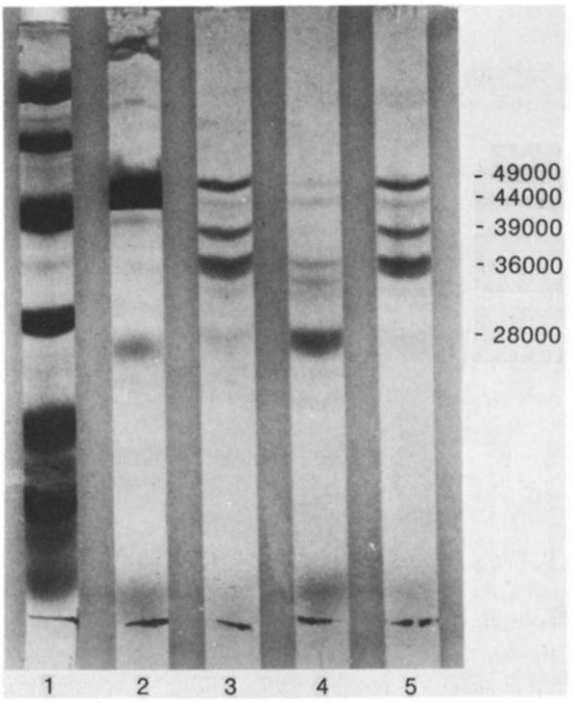

Fig. 8. Separation by polyacrylamide gel electrophoresis, in the presence of SDS, of polypeptides generated by proteinase A digestion of native and mercurial-labelled phosphoglycerate kinase in the presence and absence of Zn^{2+}. Incubations were for approx. 8 h. The samples were then frozen immediately and thawed prior to electrophoresis. Other conditions are as described for Figs. 1 and 3. Gel 1 is the subunit molecular mass markers (see Fig. 2); gel 2 is native kinase; gel 3 is native kinase plus 52.5 μM Zn^{2+}; gel 4 is mercurial-labelled kinase; gel 5 is the mercurial-labelled kinase plus 52.5 μM Zn^{2+}.

tion of the native enzyme (Fig. 6). At a kinase-to-trypsin molar ratio of 500 to 1 in the absence of Zn^{2+}, the activity of the kinase decreased by less than 10% after 120 min incubation. Under identical conditions but in the presence of 52.5 μM Zn^{2+} the kinase was 80% inactivated after 120 min. At a concentration of 17.4 μM, Zn^{2+} did not change the rate of hydrolysis of benzoyl arginine *p*-nitroanilide (data not shown), a synthetic substrate of trypsin.

Mg^{2+} at concentrations of 50, 100 and 200 μM failed to alter the rate of inactivation of the native kinase by trypsin (data not shown).

The susceptibility of phosphoglycerate kinase to inactivation by proteinase A was also increased in the presence of Zn^{2+} (Fig. 7). At a kinase-to-proteinase A molar ratio of 9.1 to 1, the kinase activity decreased by less than 3% after 60 min incubation. Under similar conditions and in the presence of 52.5 μM Zn^{2+} degradation of the native kinase by proteinase A had taken place (Fig. 8, gel 3). The apparent molecular masses of the resulting polypeptides were similar to those detected during incubation of proteinase A with the native or mercurial-labelled kinase (Fig. 4, gels 2 and 4–7). Approx. 80% inactivation of phosphoglycerate kinase by proteinase A in the presence of Zn^{2+} had occurred at the time the sample was electrophoresed (Fig. 8, gel 3) but the relative proportions of the polypeptides were different from those detected when the mercurial-labelled kinase was 80% inactivated by proteinase A (Fig. 4, gel 6). After more than 5 h incubation with proteinase A the major end product of proteolysis of the mercurial-labelled kinase was a polypeptide with a molecular mass of 28 000 (Fig. 8, gel 4). But in the presence of Zn^{2+} and after a similar incubation time, the major product from native phosphoglycerate kinase was a polypeptide with a molecular mass of 36 000 (Fig. 8, gel 3).

In the absence of either endopeptidase, the activity of the native kinase in the presence of 52.5 μM Zn^{2+} decreased by less than 11% during a 120 min incubation (data not shown).

The effect of Zn^{2+} on the inactivation by trypsin or proteinase A of modified phosphoglycerate kinase

Zn^{2+} at a concentration of 52.5 μM Zn^{2+} did not alter the susceptibility of mercurial-labelled or Nbs-modified phosphoglycerate kinase to inactivation by trypsin (data not shown). However, Fig. 7 shows that 52.5 μM Zn^{2+} did decrease the susceptibility of the mercurial-labelled kinase to inactivation by proteinase A such that the rate of inactivation was similar to that observed with native phosphoglycerate kinase in the presence of Zn^{2+}.

The results of SDS electrophoresis suggested that in the presence of Zn^{2+}, inactivation of the mercurial-labelled kinase by proteinase A was proteolytic (Fig. 8, gel 5) and gave rise to polypeptides of mobilities similar to those which were detected during incubation of proteinase A with the native or mercurial-labelled kinase in the absence of Zn^{2+} (Fig. 4). However, the relative proportions of the polypeptides differed and this can be seen by comparing gel 6 of Fig. 4 with gel 5 of Fig. 8, both of which corresponded to 80% inactivation of the kinase. Fig. 8 also demonstrates that regardless of whether mercurial is bound to the kinase, the presence of Zn^{2+} limits proteolysis to a 36000 M_r fragment (gels 3 and 5). In the absence of Zn^{2+} and after more than 6 h incubation of labelled kinase, the enzyme was essentially inactivated and the major polypeptide had a molecular mass of 28000 (Fig. 8, gel 4).

Discussion

Although modification of the single thiol of yeast phosphoglycerate kinase with mercurial does not alter K_m values [1,2], it greatly increases the susceptibility of the kinase to inactivation by trypsin (Fig. 1). Since the proteolytic products are identical from the native or labelled enzyme it is clear that the mercurial increases the susceptibility of the same bonds that are attacked in the native enzyme (Fig. 2). The two major polypeptides appearing as a result of proteolysis, 30000 and 28000-M_r fragments, have a combined size that is larger than the native enzyme and thus the smaller of the two must be formed from the larger. The unaccounted for material (49000–30000 = 19000) must have been rapidly and extensively degraded.

Phosphoglycerate kinase with bound mercurial is also much more susceptible to inactivation by proteinase A (Fig. 3). As with trypsin the products of proteolysis appear to be the same for the native and the labelled enzyme (Fig. 4). Also, the 28000-M_r product that appeared after extensive proteinase A digestion is the same size fragment that accumulated with the trypsin-degraded kinase. However, the intermediates between the 49000 and 28000-M_r species were different with the two proteolytic enzymes, and this probably reflects the different specificities of the endopeptidases. Trypsin hydrolyzes peptide bonds adjacent to arginine or lysine residues [14] while proteinase A shows properties similar to those of pepsin [15] and hydrolyzes peptide bonds adjacent to aromatic residues [14]. Based on the amino acid composition of yeast phosphoglycerate kinase (see for example Ref. 22), and the specificities of the two endopeptidases used for this study, there should be susceptible residues within the 28000-M_r fragment (Figs. 2 and 4). Since it is relatively resistant to proteolysis the fragment must have enough structure to render these potential substrate residues inaccessible.

X-ray crystallographic studies indicate that the yeast phosphoglycerate kinase molecule contains two lobes of approx. equal size [23], and it is possible that the hinge region which connects the two lobes is susceptible to proteolytic cleavage. Amino acid sequencing of the kinase isolated from horse muscle, which is very similar in primary structure to the yeast enzyme [18], contains multiple potential substrate residues in the hinge region [24] for each of the proteases used in this study.

Modification of phosphoglycerate kinase with mercurial or Nbs_2 caused a similar increase in the enzyme's susceptibility to inactivation (Figs. 1 and 3). Although the chemistries of the modification reactions differ, each introduces a substituted nitro(thio) phenolate anion adjacent to the enzyme's cysteine residue [25,26]. Thus, modification with each reagent could cause the same disturbance of local intramolecular bonding adjacent to the cysteine residue and, through conformational changes, make

critical peptide bonds available for cleavage. The use of probes structurally unrelated to the mercurial or Nbs_2 would be required to determine whether the increased susceptibility to proteolysis reported here is solely due to blocking of the enzyme's sulfhydryl group, or to the nature of the incoming reagents.

All anions examined protected the mercurial-labelled enzyme from proteinase A digestion (Fig. 5 and Table I). The relative binding affinities of the anions suggests that they accomplish this protective effect by binding to the anion binding site that we have previously identified using spectrophotometric conformational probes [3]. Zn^{2+} was the only metal ion found to alter the susceptibility of the kinase of to trypsin and the effect of this metal is shown in Fig. 6. Its effect was to increase the rate of degradation and in this respect Zn^{2+} had the same effect as modification of the enzyme thiol with mercurial or Nbs_2. Thus, it is possible the Zn^{2+} is binding to the single thiol of the kinase since reported dissociation constants for this metal ion binding to 2-mercaptoethanol and dithiothreitol are in the μM range [27]. However, our previous work has shown that Zn^{2+} very significantly enhances the rate of reaction between the thiol of yeast phosphoglycerate kinase and Nbs_2 [11]. Thus, the Zn^{2+} could be binding to the thiol but it must also be binding elsewhere. Fig. 7 shows the effects of Zn^{2+} and mercurial, added together, on the susceptibility of the kinase to inactivation by proteinase A. Although both independently increase susceptibility, Zn^{2+} behaves very much as if it were displacing the mercurial from the enzyme so that the inactivation curves for mercurial-enzyme plus Zn^{2+} and native enzyme plus Zn^{2+} are similar (Fig. 7). Also, the peptides after approx. 9 h of proteolysis are very similar in both cases (Fig. 8, gels 3 and 5). However, Zn^{2+} has no effect on the susceptibility of mercurial- or Nbs-modified kinase to trypsin and hence its effect with proteinase A is unlikely to be the result of mercurial displacement. Rather, the binding of Zn^{2+} to mercurial-enzyme has no effect on trypsin-susceptible bonds but does increase availability of proteinase A-susceptible bonds. The concentration range over which Zn^{2+} increased the susceptibility of native phosphoglycerate kinase to inactivation by trypsin is compatible with binding to a low-affinity Zn^{2+} binding site detected using spectrophotometric conformational probes [11].

In conclusion, reagents that react with the thiol group of yeast phosphoglycerate kinase, and Zn^{2+} increase the rate of proteolysis of the enzyme. In contrast, anions protect both the native and mercurial-labelled kinase from proteolysis. Whether these results are of physiological significance remains to be demonstrated. The above mentioned compounds could play a role in the in vivo turnover of this enzyme with respect to protection from, and/or enhancement of, proteolysis. It is interesting that anions at low concentrations are activators of phosphoglycerate kinase [4,6,8] and also protect the enzyme from yeast proteinase A proteolysis. Zn^{2+} on the other hand inhibits the kinase [13], and also increases its susceptibility to proteolysis.

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