Do metal ions promote the re-activation of the 2,3-bisphosphoglycerate-independent phosphoglycerate mutases?

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It has been reported [Smith, McWilliams & Hass (1986) *Biochem. Biophys. Res. Commun.* **136**, 336–340] that addition of certain metal ions, notably Co^{2+} and Mn^{2+} , promoted the refolding of denatured phosphoglycerate mutase from wheat germ. We have re-investigated these experiments and have shown that, when precautions are taken to avoid artefacts in the assay system, the metal ions do not promote any re-activation of the denatured wheat-germ or *Aspergillus nidulans* enzymes. An alternative explanation is offered for the observations of Smith *et al.* (1986).

INTRODUCTION

Phosphoglycerate mutase catalyses the interconversion of 2- and 3-phosphoglycerate. The enzymes from mammalian sources, baker's yeast, *Escherichia coli*, insects and crustaceans are dependent on the cofactor 2,3-bisphosphoglycerate for activity, whereas the enzymes from plant sources (e.g. wheat germ), filamentous fungi (e.g. *Aspergillus nidulans*), *Bacillus* sp., coelenterates and arachnids are active in the absence of this cofactor (Grisolia & Joyce, 1959; Ray & Peck, 1972; Carreras *et al.*, 1982; Price *et al.*, 1983). In contrast with the cofactor-dependent enzymes, for which much structural and mechanistic knowledge is available (see, e.g., Winn *et al.*, 1981), very little is known about the cofactor-independent enzymes. Using isotopically labelled chiral substrate, Blättler & Knowles (1980) showed that the reaction catalysed by the wheat-germ enzyme proceeds with retention of configuration at phosphorus and suggested that a very short-lived phosphoenzyme intermediate might be involved.

Various lines of evidence have indicated that metal ions may play an important structural and/or functional rôle in the 2,3-bisphosphoglycerate-independent enzymes. The enzymes from two species of *Bacillus* are dependent on Mn^{2+} for activity (Singh & Setlow, 1979; Watabe & Freese, 1979). Smith & Hass (1985) showed that the enzyme from wheat germ lost activity on incubation with various chelating agents such as EDTA and HQSA. We have also shown that some other 2,3-bisphosphoglycerate-independent enzymes are inactivated by these chelating agents (Johnson *et al.*, 1987). Although these results suggest that a metal ion may be important for activity, it should be noted that several polyanions, including EDTA, have been reported to act as competitive inhibitors of 2,3-bisphosphoglycerate-dependent enzymes (Ray & Peck, 1972; Rose, 1980).

Smith *et al.* (1986) have reported that, after denaturation of the wheat-germ phosphoglycerate mutase by GdnHCl, activity could be only regained on dilution of the denaturing agent if metal ions, notably Co^{2+} and Mn^{2+} were added. Two aspects of the results reported by Smith *et al.* (1986) seem puzzling.

(i) The rate of re-activation on addition of the metal ions was very rapid. It has been shown that the rate of re-activation of many large polypeptide chains (> 300 amino acids) is slow and often inefficient, owing to improper pairing of domains (Jaenicke, 1984; Teschner *et al.*, 1987). The M_r of the monomeric wheat-germ enzyme is approx. 60 000 (Leadlay *et al.*, 1977), corresponding to approx. 540 amino acids.

(ii) The experiments of Smith *et al.* (1986) were performed at alkaline pH (8.7), where many transition-metal ions are known to give rise to insoluble hydroxyl complexes (see, e.g., Kragten, 1978).

As part of our investigations of the structure and function of 2,3-bisphosphoglycerate-independent phosphoglycerate mutases, we have re-examined the experiments of Smith *et al.* (1986). The present work has involved the development of an assay system which can be reliably used in the presence of metal ions. Our results show that metal ions such as Mn^{2+} and Co^{2+} do not promote the refolding of the enzymes after denaturation. This conclusion is reached for the enzymes from wheat germ and from *A. nidulans*; the purification of the latter enzyme is also described in this paper.

EXPERIMENTAL

The following companies supplied the reagents listed: BDH Chemicals, GdnHCl (AristaR grade), ZnCl_2 , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (all AnalaR grade), $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (General Purpose Reagent); Fisons Scientific Equipment, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (both Analytical Reagent grade); Sigma Chemical Co., Reactive Blue 2–Sepharose CL-6B, Dalton mark VII-L M_r marker set, phenylmethanesulphonyl fluoride, HQSA; Boehringer, 3-phosphoglycerate (Grade I), 2-phosphoglycerate, 2,3-bisphosphoglycerate, ADP, NADH, enolase, phosphoglycerate mutase, pyruvate kinase/lactate dehydrogenase mixture (all from rabbit muscle). Except where indicated, solutions of metal salts were made up in double-distilled water.

Protein concentrations were determined by the method of Sedmak & Grossberg (1977), with bovine serum albumin as a standard.

* Abbreviations used: HQSA, 8-hydroxyquinoline-5-sulphonic acid; GdnHCl, guanidinium chloride.

Assays of 2,3-bisphosphoglycerate-independent phosphoglycerate mutase activity were initially performed under the conditions described by Smith & Hass (1985), i.e. with 20 mM-3-phosphoglycerate, 8.3 mM-MgSO₄ and 40 µg of enolase in 0.1 M-Tris/HCl buffer, pH 8.7 (total volume 1.0 ml). Some later experiments were performed in the assay system described by Grisolia (1962), i.e. with 10 mM-3-phosphoglycerate, 3 mM-MgSO₄ and 10 µg of enolase in 0.03 M-Tris/HCl buffer, pH 7.0 (total volume 1.0 ml). A unit of enzyme activity causes an increase in A_{240} of 0.1/min under the stated conditions; according to McAleese *et al.* (1985), this corresponds to the conversion of 0.084 µmol of 3-phosphoglycerate/min. Phosphoglycerate mutase from rabbit muscle was assayed as described by Grisolia (1962).

Phosphoglycerate mutase from *A. nidulans*

A. nidulans (strain BWB 272) was grown in liquid culture as described previously (Price *et al.*, 1983). All subsequent steps were performed at 4 °C. Mycelia (75 g wet wt. from 4 litres of culture) were harvested by filtration, ground with an equal weight of acid-washed sand and extracted into 2 vol. of 50 mM-sodium phosphate buffer, pH 7.5, containing 1 mM-phenylmethanesulphonyl fluoride. The slurry was centrifuged (15000 g for 20 min) to give the initial extract. In the next step, the material precipitated between 67% and 95% saturation of (NH₄)₂SO₄ was dissolved in 10 ml of 50 mM-sodium phosphate buffer, pH 7.5, and dialysed overnight against 2 litres of 10 mM-Tris/HCl buffer, pH 8.0. The dialysis residue was applied to a column of QAE (quaternary aminoethyl)-cellulose (3.8 cm² × 30 cm) previously equilibrated against the Tris buffer. After washing the column with 100 ml of 10 mM-Tris/HCl buffer, pH 8.0, containing 75 mM-NaCl, enzyme activity was eluted by a gradient formed from 100 ml of 10 mM-Tris/HCl buffer, pH 8.0, containing 0.1 M-NaCl and 100 ml of 10 mM-Tris/HCl buffer, pH 8.0, containing 0.3 M-NaCl. The fractions containing the peak of enzyme activity were pooled and freeze-dried, and the residue was dissolved in 0.2 vol. of water. After dialysis against 2 litres of 10 mM-Tris/HCl buffer, pH 8.0, the residue was applied to a column of Reactive Blue 2-Sepharose CL-6B (4.9 cm² × 5 cm) equilibrated against this buffer. Enzyme activity was eluted with 10 mM-Tris/HCl buffer, pH 8.0. Peak fractions were pooled and applied to a column of DEAE-cellulose (1.8 cm² × 15 cm). After washing with 100 ml of 10 mM-Tris/HCl buffer, pH 8.0, enzyme activity was eluted by a gradient formed from 75 ml of the Tris buffer containing 25 mM-NaCl and 75 ml of the Tris buffer containing 150 mM-NaCl.

Finally, the peak fractions from the DEAE-cellulose column were pooled, freeze-dried and redissolved in 0.2 vol. of water. The redissolved material was dialysed against 2 litres of 50 mM-sodium phosphate buffer, pH 7.5, and applied to a column of Sephacryl S-300 (4.9 cm² × 38 cm). The peak fractions were dialysed individually against several changes of 10 mM-Tris/HCl buffer, pH 8.0, and then freeze-dried. The residues were dissolved in 0.1 vol. of water and analysed by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970). Those showing > 80% homogeneity were pooled and stored at -20 °C.

Phosphoglycerate mutase from wheat germ

Phosphoglycerate mutase was purified from wheat germ by the methods of Leadlay *et al.* (1977) and of Smith & Hass (1985). In both cases the final specific activity, assayed at pH 8.7 (Smith & Hass, 1985), was approx. 3000 units/mg. Although this value is comparable with the previous results (Leadlay *et al.*, 1977; Smith & Hass, 1985), the preparations showed various minor bands of lower M_r on SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) in addition to the major band at an M_r of 60000 ± 3000. The presence of these minor bands has been referred to previously (Smith & Hass, 1985; McAleese *et al.*, 1985).

Phosphoglycerate mutase from *Neurospora crassa*

Phosphoglycerate mutase, purified from *N. crassa*, was kindly given by Dr. S. McAleese, Department of Biochemistry, University of Aberdeen, Aberdeen, U.K. The specific activity of the purified material was 3400 units/mg when assayed under the conditions described below. The results obtained with the metal-chelating agents (see Fig. 2) were essentially identical for the purified enzyme and for an (NH₄)₂SO₄-fractionated crude extract of specific activity 480 units/mg. From the position of elution of the latter material from a Sephacryl S-300 column (Johnson *et al.*, 1987), the M_r of the phosphoglycerate mutase was estimated to be 50000 ± 5000.

Phosphoglycerate mutase from *N. crassa* was assayed in 0.5 M-Tris/HCl, pH 8.0, containing 10 mM-3-phosphoglycerate, 5 mM-MgSO₄ and 40 µg of enolase in a volume of 1.0 ml.

RESULTS

Purification of phosphoglycerate mutase from *A. nidulans*

The progress of the purification of phosphoglycerate mutase from *A. nidulans* is shown in Table 1. The final yield of enzyme was approx. 0.7 mg from 75 g wet wt. of mycelia, corresponding to 23% of the activity in the initial extract, and a purification factor of 120-fold. On SDS/polyacrylamide-gel electrophoresis the material was > 85% homogeneous as judged by Coomassie Blue staining; the subunit M_r was estimated to be 60000 ± 3000 (Fig. 1). In some preparations the band occurred as a doublet (M_r values 60000 ± 3000 and 56000 ± 3000), which might be the result of proteolysis by traces of endogenous proteinases. However, there was no difference in the specific activity of the material, suggesting that any amino acids removed by proteolysis are not essential for activity. Despite numerous attempts to improve the isolation procedure, we were unable to obtain material of greater specific activity.

Chromatography on Reactive Blue-Sepharose was included in the purification scheme in order to remove some contaminating proteins (probably kinases and dehydrogenases) which interfered with the subsequent ion-exchange step. In accordance with previous observations (Price & Stevens, 1983), it was found that phosphoglycerate mutase from *A. nidulans* was not retained by the Reactive Blue-Sepharose. In the final (gel-filtration) step of the purification, it was found necessary to use 50 mM-sodium phosphate buffer, pH 7.5, rather than 0.1 M-Tris/HCl buffer, pH 8.0, as the

Table 1. Purification of phosphoglycerate mutase from *A. nidulans*

The data refer to mycelia from 4 litres of liquid culture (75 g wet wt. of mycelia). Enzyme assays were performed at pH 8.7, under the assay conditions described by Smith & Hass (1985). Further details are given in the Experimental section.

Step	Enzyme activity (units)	Protein (mg)	Specific activity (units/mg)	Purification factor	Yield (%)
Initial extract	16 500	379	43.5	1.0	100
67–95 %-satn.-(NH ₄) ₂ SO ₄ precipitate	10 080	50.1	201	4.6	61
QAE-cellulose chromatography	7540	6.2	1218	28.0	46
Reactive Blue–Sephacryl chromatography	5400	2.6	2050	47.1	33
DEAE-cellulose chromatography	4440	1.19	3723	85.6	27
Sephacryl S-300 gel filtration	3750	0.71	5250	120.7	23

Fig. 1. SDS/polyacrylamide-gel electrophoresis of phosphoglycerate mutase from *A. nidulans*

Enzyme (3 μ g of protein) was loaded on a 12 %-acrylamide gel. After staining with Coomassie Blue and destaining, the gel was scanned at 600 nm. The horizontal M_r calibration scale was determined with the Dalton mark VII-L M_r marker set.

eluting buffer, since enzyme activity was irreversibly lost in the latter buffer. However, for subsequent concentration of the enzyme (by freeze-drying) and storage of the enzyme, Tris buffer was found to minimize inactivation (< 5 % activity lost on storage at -20°C for 4 weeks).

Characterization of phosphoglycerate mutase from *A. nidulans*

The M_r of native phosphoglycerate mutase as judged by gel filtration on Sephacryl S-300 was $60\,000 \pm 5\,000$. Taken in conjunction with the value of the subunit M_r given above, it is clear that the enzyme is monomeric.

The enzyme activity is sensitive to the metal-chelating agents EDTA and HQSA. Fig. 2 shows the loss in activity of the enzymes from *A. nidulans* (and from wheat germ and *N. crassa*) on incubation with 1 mM chelating agents. Since the loss in activity in each case is time-

Fig. 2. Inactivation of phosphoglycerate mutases by the metal-chelating agents EDTA and HQSA

The enzymes were incubated at 20°C in 0.1 M-Tris/HCl buffer, pH 8.7, in the presence of 1 mM chelating agents. Activities are expressed relative to control samples incubated in the absence of chelating agents: ●, ■, ▲ refer to the enzymes from wheat germ, *N. crassa* and *A. nidulans* incubated at concentrations of 50, 5 and 10 $\mu\text{g/ml}$ respectively in the presence of EDTA; ○, □, △ refer to the same enzymes incubated in the presence of HQSA.

dependent, it is unlikely that the inactivation is due to competition between binding of the chelators and substrate at the active site of the enzymes. The different rates of inactivation of the three enzymes suggest that any important metal ions have different degrees of accessibility to the reagents. The results of the M_r determinations and experiments with chelating agents

confirm the pattern previously noted for some other 2,3-bisphosphoglycerate-independent phosphoglycerate mutases (Johnson *et al.*, 1987).

Stability of metal ions under assay conditions

It is well recognized (see, e.g., Kragten, 1978) that a number of transition-metal ions are unstable in aqueous solutions at alkaline pH. Experiments were therefore undertaken to investigate the stability of these metals in the buffer used for assay by Smith *et al.* (1986), namely 0.1 M-Tris/HCl, pH 8.7, and in the assay mixture, which contains additionally 20 mM-3-phosphoglycerate, 8.3 mM-MgSO₄ and 40 µg of enolase (total volume 1.0 ml).

When portions of solutions of the metal chlorides made up in distilled water were added to 0.1 M-Tris/HCl buffer at pH 8.7, there was immediate precipitation in the case of the Co²⁺ and Mn²⁺ salts, the A_{240} rising to > 2.0 within 5 s at a metal ion concentration of 2 mM. For Ni²⁺ the increase in A_{240} was very slow (≤ 0.0025 /min).

In the presence of the other components of the assay mixture, the rate of precipitation of the metal ions was moderated. Fig. 3 shows the rate of increase in A_{240} as a function of metal ion concentration for Co²⁺ and Mn²⁺. For Ni²⁺, Zn²⁺, Cu²⁺ and Ca²⁺ the rate of increase in A_{240} was ≤ 0.001 /min (at 2 mM concentration). By varying the composition of the solution, it was possible to conclude that 3-phosphoglycerate was responsible for moderating the rate of precipitation of the metal ions, presumably via complex-formation. It is noteworthy that, at the concentrations of Mn²⁺ and Co²⁺ (i.e. 0–3 mM) studied by Smith *et al.* (1986) in their re-activation experiments, the rate of increase in A_{240} on addition of the metal ions to the assay mixture was of the same order of magnitude as that observed in a typical enzyme assay as a result of phosphoenolpyruvate formation.

With the assay system of Grisolia (1962) at lower pH (i.e. 30 mM-Tris/HCl buffer, pH 7.0), there was no observable precipitation of any of the bivalent metal ions studied.

Fig. 3. Rate of increase in A_{240} on addition of metal ions to an assay mixture

The assay mixture of Smith & Hass (1985) consisted of 0.1 M-Tris/HCl buffer, pH 8.7, with 20 mM-3-phosphoglycerate, 8.3 mM-MgSO₄ and 40 µg of enolase/ml, at 30 °C. ▲, Addition of Co²⁺; ●, addition of Mn²⁺.

Effects of metal ions on enzyme assays

The effects of metal ions on the assays of phosphoglycerate mutase activity were initially studied under the conditions described by Smith *et al.* (1986), i.e. in 0.1 M-Tris/HCl buffer, pH 8.7. These experiments were performed by adding enzyme (from wheat germ or *A. nidulans*) to an assay mixture, observing the rate of increase in A_{240} for 1 min, then adding metal ions and observing the new rate. Fig. 4 shows the data for the wheat-germ enzyme on addition of Co²⁺ or Mn²⁺; in this Figure the new rate is expressed relative to the rate before the addition of metal ions. An essentially similar pattern was observed for the *A. nidulans* enzyme. At high concentrations of metal ions there was an increase in rate; this was due to the precipitation that occurs under these conditions (see Fig. 3). The decrease in rate at lower concentrations of metal ions implies that either phosphoglycerate mutase or enolase (or both) was inhibited. By conducting assays at pH 7.0, where no problems owing to precipitation occur, it was possible to investigate these possibilities. By using a fixed amount of phosphoglycerate mutase from *A. nidulans* (0.75 µg) and a fixed concentration of Co²⁺ (0.5 mM), it was found that the rate of increase in A_{240} rose from 0.028/min to 0.053/min as the amount of enolase was raised from 10 µg to 40 µg in the assay volume of 1.0 ml. In the absence of Co²⁺, there was no variation in the observed rate (0.09/min) as the amount of enolase was varied over this range. These findings imply that enolase is inhibited by Co²⁺. This could arise from metal ion binding to 'catalytic' or 'conformational' sites on the enzyme, as discussed by Brewer (1981). It has been reported (Wold, 1971; Brewer, 1981) that for yeast enolase Mn²⁺, Zn²⁺ and Cd²⁺ can serve as activators in place of Mg²⁺. The situation with regard to the muscle enzyme (which was used in these experiments) is not clear, though the data in Fig. 4 suggest that Mn²⁺ as well as Co²⁺ inhibits the latter enzyme.

It appears that data of the type shown in Fig. 4 (obtained at pH 8.7) can be accounted for by a

Fig. 4. Effects of metal ions on the assay of wheat-germ phosphoglycerate mutase

The assay system used was that of Smith & Hass (1985) and the rates of increase in A_{240} are expressed relative to those observed in the absence of added metal ions. ▲, Effect of Co²⁺; ●, effect of Mn²⁺.

combination of (i) the residual enzyme-catalysed increase in A_{240} , taking into account the inhibition of enolase, and (ii) the increase in A_{240} as a result of precipitation of the metal ions. There do not appear to be any marked effects of the metal ions on the activity of phosphoglycerate mutase itself.

An assay system which can be used in the presence of metal ions

From the results mentioned above, it is clear that there are considerable problems arising from the precipitation of metal ions (especially Co^{2+} and Mn^{2+}) at pH 8.7. It was therefore decided to work at pH 7.0, where no precipitation problems arise, although the pH optima for the 2,3-bisphosphoglycerate-independent phosphoglycerate mutases are close to pH 9 (Grisolia & Carreras, 1975). (For the *A. nidulans* enzyme, the activity in the pH 8.7 assay system was approx. 3-fold higher than in the pH 7.0 system.) It was also necessary to ensure that the metal ions would not interfere with the coupling enzyme (enolase). A 'coupled quench' procedure was adopted in which a portion (0.05 ml) of the phosphoglycerate mutase-catalysed reaction mixture (enzyme added to 10 mM-3-phosphoglycerate in 0.03 M-Tris/HCl buffer, pH 7.0 at 30 °C) was added to 0.95 ml of the following mixture: 30 mM-Tris/HCl buffer, pH 7.0, 5 mM- MgSO_4 , 20 mM-KCl, 0.2 mM-ADP, 0.15 mM-NADH, pyruvate kinase (36 μg), lactate dehydrogenase (12 μg) and enolase (50 μg). The concentration of 2-phosphoglycerate formed in the original reaction mixture could then be calculated from the size of the very rapid decrease in A_{340} of the 'quench' solution (Johnson & Price, 1987). The method was calibrated by addition of portions of a solution of 2-phosphoglycerate of known concentration to the 'quench' mixture. As shown in Fig. 5(a), there was a 1:1 relationship between the 2-phosphoglycerate added and the NADH oxidized. In addition, there was no effect of Mn^{2+} , Co^{2+} or Ni^{2+} on the

'coupled quench' system at concentrations up to 0.25 mM (Fig. 5a). Taking into account the 20-fold dilution of the reaction mixture on quenching, this meant that the effects of metal ions at up to 5 mM concentration on the phosphoglycerate mutase-catalysed reaction could be studied. A previous paper (Johnson & Price, 1987) has shown that an analogous approach could be used to study the effects of GdnHCl on the phosphoglycerate mutase-catalysed reaction.

The assay system was used to determine the equilibrium concentration of 2-phosphoglycerate produced in assays performed in 30 mM-Tris/HCl buffer, pH 7.0, with the initial concentration of 3-phosphoglycerate being 10 mM. Fig. 5(b) shows that with the enzymes from *A. nidulans*, wheat germ and rabbit muscle, the equilibrium concentration of 2-phosphoglycerate is 0.80 ± 0.05 mM, giving values for the equilibrium constant and ΔG_{303}° of 0.087 ± 0.006 and 6.15 ± 0.18 kJ·mol⁻¹ respectively. The latter value is similar to the value of 5.85 kJ·mol⁻¹ reported previously at 25 °C (Bassham & Krause, 1969).

Can phosphoglycerate mutase be re-activated after denaturation?

The development of a reliable assay system for phosphoglycerate mutase permitted the study of the re-activation of the enzyme after denaturation and the effects of metal ions on this process.

In this experiment, enzyme from wheat germ or *A. nidulans* was denatured for 5 min at 20 °C in 30 mM-Tris/HCl buffer, pH 7.0, containing 2 M-GdnHCl. A 0.02 ml portion of this mixture was then diluted into 0.98 ml of assay mixture (30 mM-Tris/HCl buffer, pH 7.0, containing 10 mM-3-phosphoglycerate) at 30 °C, and the concentration of 2-phosphoglycerate formed after various times was determined by the 'coupled quench' procedure. The data are shown in Fig. 6, where the upper curves represent the formation of 2-phosphoglycerate in the

Fig. 5. 'Coupled quench' assay for phosphoglycerate mutase activity

Details of the assay procedure are described in the text. (a) Calibration of the assay using known concentrations of 2-phosphoglycerate. The concentration of NADH oxidized was calculated from the decrease in A_{340} by using a value of 6.22 for the absorption coefficient of a 1 mM solution. ■, Determinations performed in the absence of added metal ions; ▲, ●, determinations performed in the presence of 0.25 mM- Co^{2+} or - Mn^{2+} respectively. (b) Determination of the concentration of 2-phosphoglycerate formed from 10 mM-3-phosphoglycerate in 30 mM-Tris/HCl buffer, pH 7.0, at 30 °C. ●, ▲ and ■ refer to the enzymes from wheat germ (1 $\mu\text{g}/\text{ml}$), *A. nidulans* (6 $\mu\text{g}/\text{ml}$) and rabbit muscle (0.7 $\mu\text{g}/\text{ml}$) respectively. The experimental points do not lie on a single curve, because the amounts of enzyme activity added are different in the three cases; however, the final concentration of 2-phosphoglycerate formed is the same in each case.

Fig. 6. Activity of phosphoglycerate mutases after denaturation in GdnHCl and subsequent dilution

Enzymes were incubated in 30 mM-Tris/HCl buffer, pH 7.0, containing 2 M-GdnHCl for 5 min at 20 °C. At zero time, samples were diluted 50-fold into an assay mixture at pH 7.0 (see the text), and the rate of 2-phosphoglycerate formation was measured ($\square$). At 7 min after dilution (arrow), Co^{2+} ($\blacktriangle$), Mn^{2+} ($\bullet$) or Ni^{2+} ($\blacksquare$) was added to a final concentration of 3.3 mM. The upper curves ($\circ$) refer to enzymes incubated in the presence of the residual concentration of GdnHCl after dilution (i.e. 0.04 M). The concentration of 2-phosphoglycerate formed was determined by the 'coupled quench' assay as described in the text. (a) Enzyme from *A. nidulans* (6 $\mu\text{g}/\text{ml}$ after dilution); (b) enzyme from wheat germ (1 $\mu\text{g}/\text{ml}$ after dilution).

control samples incubated in the presence of the residual concentration of GdnHCl after denaturation (i.e. 0.04 M). The denatured enzymes (lower curves in Fig. 6) recovered < 5% activity on dilution. At the time indicated in Fig. 6 (i.e. 7 min after dilution), various metal ions, i.e. Mn^{2+} , Co^{2+} and Ni^{2+} , were added, to final concentrations of 3.3 mM. There was no effect of these metal ions on the activity of the diluted denatured enzymes (Fig. 6).

Are metal ions essential for the activity of phosphoglycerate mutase?

The data shown in Fig. 2 indicate that metal ions may be essential for the activity or structural integrity of 2,3-bisphosphoglycerate-independent mutases such as those from *A. nidulans*, wheat germ or *N. crassa*. However, this tentative conclusion would be on much firmer ground if it was possible to add metal ions back to metal-depleted enzyme and observe regain of activity. Two types of experiment to examine this possibility were undertaken with the *A. nidulans* enzyme. In the first of these, enzyme (40 $\mu\text{g}/\text{ml}$) was incubated at 4 °C with 0.1 mM-EDTA or -HQSA in 30 mM-Tris/HCl buffer, pH 7.0. After 16 h, the activity had declined to 2% of that of a control sample incubated in the absence of chelating agents. Excess (2 mM) metal ions were then added to this sample and assays of enzyme activity performed at pH 7.0. No re-activation was observed with Mn^{2+} , Co^{2+} , Fe^{2+} , Cu^{2+} , Ni^{2+} , Ca^{2+} or Zn^{2+} . In the second experiment, the *A. nidulans* enzyme (190 $\mu\text{g}/\text{ml}$) was incubated at 20 °C with 0.9 mM-EDTA in 30 mM-Tris/HCl buffer, pH 7.0. After 1 h, the activity had declined to 2% of the control value. EDTA was then separated from enzyme by gel filtration on a column of Sephadex G-25. Metal ions (Mn^{2+} , Co^{2+} , Fe^{2+} , Cu^{2+} , Ni^{2+} , Zn^{2+}) were added at 1 mM, but no re-activation was observed in any case. It should be noted that control experiments had shown that addition of the metal ions had no effect on the subsequent determination of phosphoglycerate mutase activity by the enolase coupled assay at pH 7.0 (Grisolia, 1962), the maximum 'carry-over' of metal ions to the assay being 0.02 mM.

DISCUSSION

The 2,3-bisphosphoglycerate-independent phosphoglycerate mutase from *A. nidulans* is a monomeric enzyme of M_r approx. 60000 and is inhibited by metal-chelating agents such as EDTA and HQSA. In these respects it fits into the structural and functional pattern which seems to be emerging for this group of enzymes (Johnson *et al.*, 1987). The failure of experiments designed to re-activate the enzyme after treatment with the chelating agents means that it is not yet possible to state that metal ions are essential for the catalytic activity of this enzyme, and thus their possible role in the reaction mechanism remains a matter for speculation. It is possible that the failure of the re-activation experiments could be due to the occurrence of irreversible structural changes in the enzyme after removal of the metal ion(s). The data shown in Fig. 2 indicate that the inactivation caused by the chelating agents is not due to simple competition between the anionic chelators and substrate for the active sites of the enzymes, as seems to be the case for several polyanions which inhibit 2,3-bisphosphoglycerate-dependent phosphoglycerate mutases (Ray & Peck, 1972; Rose, 1980).

The data outlined in the present paper illustrate the need to exercise considerable caution in investigating the effects of metal ions on enzymes especially in alkaline conditions. We have been able to repeat the observations reported by Smith *et al.* (1986) relating to the increase in A_{240} on addition of metal ions such as Co^{2+} and Mn^{2+} to wheat-germ phosphoglycerate mutase after denaturation by GdnHCl. However, it seems clear that their results could be explained by precipitation of the metal ions under the assay conditions, rather than by enzyme-catalysed production of 2-phosphoglycerate (and hence phosphoenolpyruvate via the enolase-catalysed reaction). The results in Fig. 6 show that addition of Co^{2+} or Mn^{2+} does not, in fact, cause the enzymes to regain activity after dilution of the denaturing agent. The apparent metal-ion specificity of the re-activation reported by Smith *et al.* (1986) seems to be due to differences in the rates of precipitation of the metal ions under the alkaline conditions used; thus, for example,

Ni^{2+} shows only a very slow increase in A_{240} on addition to the buffer or assay mixture at pH 8.7, and gave no re-activation according to Smith *et al.* (1986).

The failure of the 2,3-bisphosphoglycerate-independent enzymes to regain activity after denaturation in GdnHCl (Fig. 6) may be a consequence of the size of the polypeptide chain. Other work (Jaenicke, 1984; Teschner *et al.*, 1987) has shown that polypeptide chains more than about 300 amino acids in length often refold very slowly and/or inefficiently because of the failure to co-ordinate the processes of chain folding and domain pairing.

By contrast, the smaller (M_r -23000–27000) polypeptide chains of the 2,3-bisphosphoglycerate-dependent enzymes from baker's yeast, rabbit muscle and *Schizosaccharomyces pombe* refold after denaturation with relatively high efficiency (Hermann *et al.*, 1983; Johnson & Price, 1987).

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