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FORMATION OF AN ACTIVE PHOSPHOENZYME BY DIPHOSPHOGLYCERATE-DEPENDENT PHOSPHOGLYCEROMUTASES FROM MUSCLE, KIDNEY AND YEAST

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SUMMARY

1. The reactions of D-2,3-diphosphoglycerate with phosphoglyceromutases (2,3-diphospho-D-glycerate:2-phospho-D-glycerate phosphotransferase, EC 2.7.5.3) from rabbit muscle, yeast and pig kidney have been examined by observing spectrophotometrically the formation of monophosphoglycerate in the presence of excess enolase (EC 4.2.1.11), pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27). Monophosphoglycerate was produced in an initial burst and thereafter in a steady rate until hydrolysis of 2,3-diphosphoglycerate was complete. The initial burst was interpreted as the formation of a phosphoenzyme (2 moles phosphate per mole enzyme in rabbit muscle and pig kidney; >3 moles phosphate per mole yeast enzyme) and successive additions of 2,3-diphosphoglycerate indicated that the phosphoenzymes were unstable with half-lives of the order of 5, 8 and 1–2 min for rabbit muscle, pig kidney and yeast, respectively. The 2,3-diphosphoglycerate phosphatase (EC 3.1.3.13) activity could be attributed to the instability of the phosphoenzymes. The K_m values for 2,3-diphosphoglycerate were low ($\leqslant 1$ μM).

2. The reactions of 2,3-diphosphoglycerate with the enzymes from rabbit muscle and pig kidney were also examined spectrophotometrically in the presence of rate-limiting quantities of enolase. The initial velocities indicated that little, if any, monophosphoglycerate associated with the phosphoenzymes to form enzyme–2,3-diphosphoglycerate complexes at monophosphoglycerate concentrations of about 11 μM. With rapid dialysis the dephosphoenzymes were shown not to bind monophosphoglycerate under these conditions.

3. The rabbit muscle, pig kidney and yeast enzymes are shown to obey Michaelis–Menten kinetics down to 3-phosphoglycerate concentrations of the order of 1 μM. In view of the evidence that little, if any, of the enzyme–2,3-diphosphoglycerate species is present in solution under these conditions, and since there is no activation in the

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enolase coupled mutase assay as D-2-phosphoglycerate accumulates, it is concluded that the phosphoenzymes must themselves be catalytically active. The possibility that enzyme–2,3-diphosphoglycerate complexes if they exist, may also be catalytically active is not excluded.

4. After incubation with ^{14}C- and ^{32}P-labelled D-2,3-diphosphoglycerate and gel filtration the enzymes were obtained labelled with ^{32}P but not with ^{14}C. 2 moles phosphate per mole enzyme were bound by rabbit and pig kidney. The ^{32}P bound to the enzymes exchanged with the substrate and was liberated as P_i with $HClO_4$. After denaturation with sodium dodecyl sulphate, the phosphoproteins from yeast and pig kidney were stable to alkali but hydrolysed by acid suggesting the involvement of phosphohistidine.

5. The evidence presented for the formation of active phosphoenzymes supports the hypothesis that D-2,3-diphosphoglycerate-dependent phosphoglyceromutases are closely related to D-2,3-diphosphoglycerate diphosphatases.

INTRODUCTION

The phosphoglyceromutases (2,3-diphospho-D-glycerate:2-phospho-D-glycerate phosphotransferase, EC 2.7.5.3) from rabbit muscle, pig kidney and yeast require D-2,3-diphosphoglycerate for activity. Recent isotopic studies strongly indicate that all of these enzymes possess a pathway involving phosphoenzymes as intermediates (ping–pong mechanism) (rabbit muscle[1,25], kidney (H. G. Britton, J. Carreras and S. Grisola, unpublished results) and yeast[2]). On the basis of conventional initial velocity measurements an alternative sequential mechanism has been proposed for the yeast enzyme involving an intermolecular transfer of phosphate from 2,3-diphosphoglycerate[3], although as pointed out with phosphoglucomutases (EC 2.7.5.1)[4], such data may also be consistent with the ping–pong mechanism.

Kinetic evidence for the mechanism however does not necessarily indicate the nature of the active form of the enzyme. With the sequential mechanism, phosphate is transferred by the enzyme from 2,3-diphosphoglycerate to D-monophosphoglycerate, so that the molecule of 2,3-diphosphoglycerate is converted into a molecule of product and the original molecule of D-monophosphoglycerate becomes a molecule of 2,3-diphosphoglycerate. Radioactive exchange data[28,29] indicate that the molecule of 2,3-diphosphoglycerate must usually remain trapped within the enzyme and that it is this complex that reacts with the substrate. The active form of the enzyme is therefore an enzyme–2,3-diphosphoglycerate complex (Eqn 1). With the phosphoenzyme (ping–pong) mechanism, the enzyme possesses a phosphate group which it donates to the substrate. Transiently a molecule of 2,3-diphosphoglycerate is probably formed; and then one of the phosphates of the 2,3-diphosphoglycerate (the phosphate in the original substrate molecule) is returned to the enzyme to reform the phosphoenzyme and to yield a molecule of product. The phosphoenzyme may be a true phosphoenzyme in that the phosphate may be covalently attached to the enzyme protein. In this case the phosphoenzyme will be generated from 2,3-diphosphoglycerate as shown in Eqn 2. However, there is the alternative possibility that the enzyme may bind a molecule of 2,3-diphosphoglycerate, and one of the phosphates of the cofactor may be rendered labile, so that it may function as the phosphate of a phosphoenzyme. In this case the

active form of the enzyme will be an enzyme–2,3-diphosphoglycerate complex (Eqn 1). There is also the further possibility that this complex may partly break down into a phosphoenzyme (which may or may not be catalytically active) and monophosphoglycerate. This sequence of reactions is summarised by Eqn 3. The phosphoglyceromutases may be related to 2,3-diphosphoglycerate diphosphatases (EC 3.1.3.13), (Britton and Clarke[25]). Modification of a diphosphatase could produce a mutase with a phosphoenzyme mechanism in which the active form of the enzyme is either a true phosphoenzyme or an enzyme–2,3-diphosphoglycerate complex.

The enzymes are not extracted as phosphoenzymes and they do not contain 2,3-diphosphoglycerate[5–7]. When the rabbit muscle enzyme was incubated with [2,3-$^{32}P_2$]-diphosphoglycerate and separated by filtration through a Dowex column, approximately two atoms of ^{32}P were bound per mole enzyme[8]. The label exchanged with the substrates and was liberated as P_i with $HClO_4$. Labelled enzyme was again obtained when the enzyme was separated by gel filtration[9]. However, in this case although the label was released as P_i with both $HClO_4$ and KOH, chromatography yielded approximately equal quantities of P_i and monophosphoglycerate and it was suggested that the labelled material was an enzyme–2,3-diphosphoglycerate complex rather than a phosphoenzyme. An electrophoretic study on the products of reaction of the enzyme with [2,3-$^{32}P_2$]diphosphoglycerate also suggested that an enzyme–2,3-diphosphoglycerate complex is formed[27]. More recently Rose[10] has confirmed with gel filtration and with phenol extraction that the rabbit muscle enzyme can be obtained with two molecules of bound phosphate; in this study the complex was not chromatographed but it was assumed to be a phosphoenzyme rather than an enzyme–2,3-diphosphoglycerate complex because of the release of P_i with $HClO_4$. With sodium dodecyl sulphate it dissociated into two equal fragments each with a mole of phosphate bound to histidine. Rose[11] has very recently reported that the yeast enzyme incubated with [2,3-$^{32}P_2$]-diphosphoglycerate can also be isolated containing exchangeable phosphate which appears to be bound to histidine after sodium dodecyl sulphate treatment. In both studies, however, Rose concludes that her findings do not necessarily establish that a phosphoenzyme is the kinetic intermediate in the mutase reactions. No studies appear to have been reported on the kidney enzyme.

In view of the uncertain nature of the product formed in the reactions between 2,3-diphosphoglycerate and muscle enzymes and the relative lack of evidence with the yeast and kidney enzymes, additional studies have been carried out.

METHODS AND MATERIALS

Yeast and pig kidney phosphoglyceromutases were prepared by published methods[7,12]. Rabbit muscle phosphoglyceromutase was obtained from Boehringer Mannheim. 2,3-Diphospho-[^{14}C]glycerate was obtained from Calbiochem. By paper chromatography (2-methoxyethanol–ethylmethyl ketone–3 M NH_4OH (7:2:3, by vol.)[13] and radioactive scanning it was approximately 80% pure. The major impurity appeared to be monophosphoglycerate with a trace of glycerate. When incubated with an excess of monophosphoglycerate in the presence of yeast phosphoglyceromutase followed by conversion of monophosphoglycerate to lactate[14] at least 95% of the radioactivity was found in the lactate. Other reagents were obtained and [2,3-$^{32}P_2$]diphosphoglycerate was prepared as described[14]. The [2,3-$^{32}P_2$]diphospho-

glycerate was chromatographed twice with isopropylether–88% formic acid[15]. Mutase activity was assayed with enolase[16]. ^{14}C and ^{32}P were assayed by liquid scintillation[14]. $^{32}P_i$ was determined by adding 0.1-ml samples to 0.85 ml of 0.6 M $HClO_4$. 0.1 M sodium phosphate (5 μl) and (0.4 ml) phosphate precipitating mixture (0.2 M triethylamine hydrochloride–0.08 M ammonium molybdate–H_2O, 1:2:5, v/v/v[17]) were then added and after standing overnight the precipitate was washed, dissolved and counted[14]. Exchangeable phosphate was determined by adding 5 μl of 100 mM 3-phosphoglycerate to 0.2 ml of the phosphoenzyme eluted from the column, warming the mixture briefly to room temperature, adding 0.85 ml of 0.6 M $HClO_4$ and precipitating P_i. [3-^{32}P]monophosphoglycerate in the supernatant was detected by chromatography[9]. After treatment with sodium dodecyl sulphate the stability of the phosphoprotein was determined by phenol extraction[10] with half quantities of reagents. For rabbit muscle phosphoglyceromutase protein concentrations were determined from the absorbance at 280 nm assuming 1.48 cm^2/mg protein[18]. For the other enzymes biuret methods[19,20] were used. Dialysis studies on the dephosphoenzymes were carried out in a cell (model 295) obtained from Bolab Inc., 395 Main St., Reading, Mass. 01867 U.S.A. The crystalline enzymes in $(NH_4)_2SO_4$ were centrifuged before use.

RESULTS

The phosphoglyceromutases may react with 2,3-diphosphoglycerate to give an enzyme–2,3-diphosphoglycerate complex or this complex may break down into a phosphoenzyme and monophosphoglycerate. The different possibilities may be represented by the following equations*:

$$E + \text{2,3-diphosphoglycerate} \rightleftharpoons E\text{–2,3-diphosphoglycerate} \quad (1)$$

$$E + \text{2,3-diphosphoglycerate} \rightleftharpoons E\text{–2,3-diphosphoglycerate} \rightarrow \text{phosphoenzyme} + \text{monophosphoglycerate} \quad (2)$$

$$E + \text{2,3-diphosphoglycerate} \rightleftharpoons E\text{–2,3-diphosphoglycerate} \rightleftharpoons \text{phosphoenzyme} + \text{monophosphoglycerate} \quad (3)$$

The reversible reaction of monophosphoglycerate with phosphoenzyme envisaged in Eqn 3 involves binding of the monophosphoglycerate at a site different to the substrate binding site in the mutase reaction (see Introduction). If the *E*–2,3-diphosphoglycerate complex is catalytically active, the enzyme substrate complex will have the composition *E*–2,3-diphosphoglycerate, monophosphoglycerate.

If reactions should occur according to Eqns 2 or 3 monophosphoglycerate will be formed. Experiments were therefore carried out in the presence of enolase (EC 4.2.1.11), pyruvate kinase (EC 2.7.1.40), lactic dehydrogenase (EC 1.1.1.27), ADP and

* In these equations the reaction of the phosphoenzyme or the enzyme–2,3-diphosphoglycerate complex, with monophosphoglycerate as a substrate in the mutase reaction is not considered. This is justified by the fact that the monophosphoglycerate concentration in the experiments to be described was about 11 μM whereas the K_m for 3-phosphoglycerate is of the order of 200 μM (ref. 2) and the K_m for 2-phosphoglycerate is about 40 μM (H. G. Britton and J. B. Clarke, unpublished results and H. G. Britton, J. Carreras and S. Grisolia[2] and unpublished results). The equilibrium constant [3-phosphoglycerate]/[2-phosphoglycerate] is 11.3 (H. G. Britton and J. B. Clarke, unpublished results).

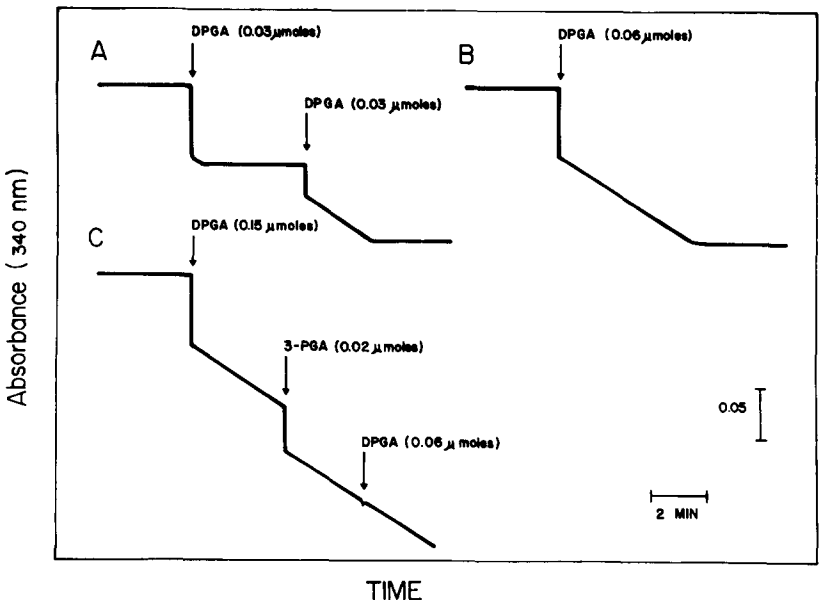

Fig. 1. Changes in absorbance (1 cm light path) at 340 nm on addition of 2,3-diphosphoglycerate (DPGA) and 3-phosphoglycerate (3-PGA) to substrate quantities of rabbit muscle mutase. The reaction mixture contained in a total volume of 2.65 ml at 25 °C: mutase, enolase, and pyruvate kinase, 0.38 mg/ml each; lactate dehydrogenase, 0.19 mg/ml; NADH, 0.13 mM; ADP, 0.19 mM; KCl, 6 mM; $MgCl_2$, 6.4 mM; ethanolamine–Cl^- (pH 7.6), 40 mM. 2,3-diphosphoglycerate (DPGA) and 3-phosphoglycerate were added as indicated (vol. approx. 5–10 μl). A, B and C are consecutive observations at 40-min intervals. Approximately 20 s elapsed between each addition and the first record of absorbance.

NADH, to convert monophosphoglycerate to lactate. In an experiment with rabbit muscle phosphoglyceromutase (Fig. 1), the addition of 0.03 μmole 2,3-diphosphoglycerate to substrate quantities of the enzyme caused an immediate drop in absorbance (A) of 0.065 at 340 nm followed by a short approximately linear decline to a constant $A_{340\text{ nm}}$. The total change in $A_{340\text{ nm}}$ corresponded closely with complete hydrolysis of the 2,3-diphosphoglycerate to monophosphoglycerate. A second addition of 2,3-diphosphoglycerate (0.03 μmole) approximately 3 min later gave a smaller initial drop and a correspondingly longer linear decline to yield the same total change in $A_{340\text{ nm}}$. 40 min later a third addition of 2,3-diphosphoglycerate (0.06 μmole) gave the same initial drop (0.065 $A_{340\text{ nm}}$) as with the first addition; and a linear decline with the same slope as with previous additions until hydrolysis of the 2,3-diphosphoglycerate was complete. A fourth addition of 2,3-diphosphoglycerate (0.15 μmole) after a further 40 min again gave the same initial drop as with the first addition and a linear decline of the same slope. The addition of 2,3-diphosphoglycerate (0.06 μmole) during the linear decline caused no further drop in $A_{340\text{ nm}}$ whereas the addition of 3-phosphoglycerate (0.02 μmole) caused an immediate stoichiometric fall in $A_{340\text{ nm}}$.

The initial drop of $A_{340\text{ nm}}$ when 2,3-diphosphoglycerate was added (Fig. 1) is the expected result for the formation of a phosphoenzyme (Eqns 2 and 3). Since the initial drop was the same when a 40 min interval was left between additions, the phosphoenzyme must be unstable and completely hydrolysed within this time inter-

val. The smaller initial drop when the second addition of 2,3-diphosphoglycerate was added within a few minutes of the first suggests that the phosphoenzyme had only partially hydrolysed during this time and would indicate a half-life of about 5 min for the phosphoenzyme. Consistent with this interpretation of the data the addition of 2,3-diphosphoglycerate during the linear part of the hydrolysis curve (Fig. 1C) had no effect upon $A_{340\ nm}$ since the enzyme should be fully phosphorylated. If the phosphoenzyme is unstable the enzyme must exhibit 2,3-diphosphoglycerate phosphatase activity and the linear parts of the hydrolysis curves in Fig. 1 can be entirely explained with a half-life of the phosphoenzyme of about 5 min. In another experiment the phosphoglyceromutase concentration was increased to 650 μg/ml and both the initial drop (0.102 $A_{340\ nm}$) and the linear slope increased in proportion.

If a mol. wt of 60 000 is assumed for rabbit muscle phosphoglyceromutase[6,21] the initial drop in $A_{340\ nm}$ in Fig. 1 corresponds to 1.7 moles phosphate per mole of enzyme or about 17 nmoles per 1000 units. The enzyme had a specific activity of 1750 units/mg compared with a maximum of about 2100 units/mg[5] and thus was approximately 83% pure. It follows that the pure enzyme binds about 2 moles of phosphate per mole enzyme.

Similar results to those in Fig. 1 were obtained with the pig kidney and yeast enzymes. As with the rabbit muscle enzyme the initial drops in $A_{340\ nm}$ corresponded to about 17 nmoles per 1000 units, so that the turnover number per active centre appears to be similar for all three enzymes. For the kidney enzyme, if a mol. wt of 65 000 and a specific activity of 1730 units/mg for the pure enzyme are assumed[7], 2 moles phosphate per mole of enzyme were bound. With kidney enzyme the half-life of the phosphoenzyme (about 8 min) was longer than for the rabbit muscle enzyme and the phosphatase activity (as shown by the slope of the linear part of the curve) was correspondingly about 30% less. With the yeast enzyme assuming a mol. wt of 112 000 (ref. 6) and taking the measured specific activity (1250 units/mg), 2.9 moles of phosphate were bound per mole enzyme. Since the measured specific activity was taken this figure must represent a minimum value. The half-life (about 1–2 min) of the yeast enzyme was very much shorter than that of the muscle enzyme, and the phosphatase activity per unit mutase activity was about three times greater.

In Fig. 1 the linear part of the hydrolysis curves abruptly changed slope as the final density is approached. The velocity of the phosphatase reaction is therefore maintained to very low 2,3-diphosphoglycerate concentration and the K_m for 2,3-diphosphoglycerate must be very low ($\leqslant 1\ \mu$M). Similar results were obtained with the kidney and yeast enzymes although with the yeast enzyme the curvature suggested that the K_m was somewhat higher than those of the other ones.

The experiments just described provide strong evidence for the reaction of the enzyme with 2,3-diphosphoglycerate proceeding according to Eqns 2 or 3 but they do not distinguish between these possibilities since monophosphoglycerate was removed as soon as it was formed, and any equilibrium would be displaced to the right. If monophosphoglycerate is not removed and if reaction occurs according to Eqn 2, monophosphoglycerate will be formed in quantity equal to the number of moles of the enzyme that react. However, if reaction occurs according to Eqn 3 less monophosphoglycerate will be released into solution because some will be bound to the enzyme as an enzyme–2,3-diphosphoglycerate complex. Experiments similar to the one shown in Fig. 1 but in which the enolase was reduced to a rate-limiting quantity were there-

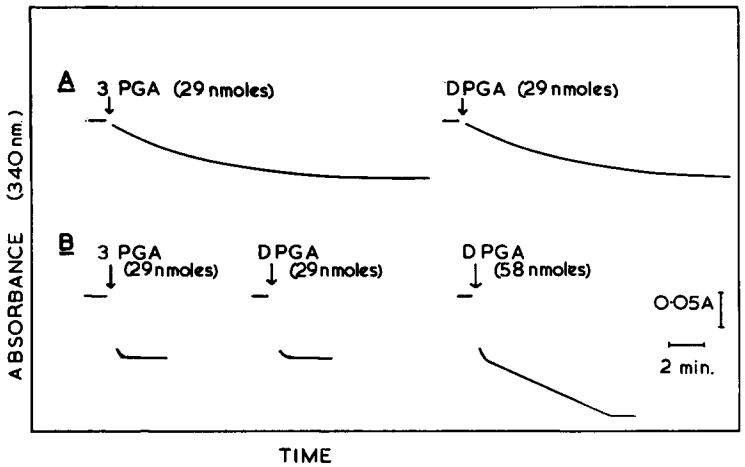

Fig. 2. Changes in absorbance (1 cm light path) at 340 nm on addition of 2,3-diphosphoglycerate (DPGA) and 3-phosphoglycerate (3-PGA) to substrate quantities of rabbit muscle phosphoglycerate mutase with rate-limiting enolase. A, the reaction mixture contained in a total volume of 2.1 ml at 22 °C: mutase, 0.715 mg/ml (a different batch of lower specific activity to that used in Fig. 1); enolase, 1.5 μg/ml; pyruvate kinase, 0.1 mg/ml; lactate dehydrogenase, 0.24 mg/ml; NADH, 0.13 mM; ADP, 0.19 mM; KCl, 6 mM; $MgCl_2$, 6.4 mM; ethanolamine–Cl^- (pH 7.6), 40 mM. B, as in A but excess enolase was added (480 μg/ml). 3-Phosphoglycerate and 2-3 diphosphoglycerate (DPGA) were added as shown in volumes of 10 μl. The 3-phosphoglycerate contained 1.4 nmoles 2,3-diphosphoglycerate.

fore carried out. In an experiment with the rabbit muscle enzyme the successive additions of 0.029 μmole 3-phosphoglycerate and 0.029 μmole 2,3-diphosphoglycerate gave curves with the same initial slopes (Fig. 2A). After excess of enolase had been added the addition of 0.029 μmole 3-phosphoglycerate led to a virtually instantaneous reaction; and the additions of 0.029 μmole and 0.058 μmole of 2,3-diphosphoglycerate showed that 0.029 μmole 2,3-diphosphoglycerate was just sufficient to phosphorylate the enzyme (Fig. 2B). Since with excess of enolase, the conversion of 3-phosphoglycerate to lactate was very rapid (the 3-phosphoglycerate contained a small quantity of 2,3-diphosphoglycerate, see legend to Fig. 2), the 3-phosphoglycerate must have been converted almost immediately into equilibrium proportions of 3- and 2-phosphoglycerate when enolase was present in rate-limiting quantity; and similarly with any monophosphoglycerate formed when 2,3-diphosphoglycerate was added. The identity of the initial slopes with 3-phosphoglycerate and 2,3-diphosphoglycerate in the first part of the experiment (Fig. 2A) therefore strongly suggests that the 2,3-diphosphoglycerate reacts with the enzyme to give a stoichiometric quantity of monophosphoglycerate. The reaction thus proceeds according to Eqn. 2 or if the reaction progresses according to Eqn 3 the equilibrium lies well to the right. This conclusion depends upon the assumption that the velocity of the enolase reaction is a function of the concentration of 2-phosphoglycerate and that the decline in velocity of the reaction (Fig. 2A) was due to the fall in 2-phosphoglycerate concentration. To exclude an alternative possibility that the fall in velocity was due to hydrolysis of the limited quantity of 2,3-diphosphoglycerate present, 2,3-diphosphoglycerate in quantity equal to that in the 3-phosphoglycerate (see legend to Fig. 2) was added when the velocity of the 3-phosphoglycerate reaction had fallen to about one half. No change in

velocity was observed. The conclusion also depends upon the assumption that the dephosphoenzyme does not bind monophosphoglycerate. 1.5 ml of a solution of the dephosphoenzyme of 380 μg/ml in ethanolamine–Cl^- buffer (40 mM, pH 7.6) was placed in the upper chamber of a dialysis apparatus[22] while the ethanolamine buffer flowed through the lower chamber at 6 ml/min. 3-ml samples were collected at 30-s intervals. 3-Phospho-[^{14}C]glycerate was added to the enzyme to give a concentration of 10 μM and at 5-min intervals 3-phosphoglycerate was added to give total concentrations of 60 and 260 μM. The radioactivity in the diffusate fell progressively over the duration of the experiment (28 min) by about 6%. Approximately half of this fall could be attributed to loss of radioactivity from the dialysis chamber. Since binding of monophosphoglycerate by the dephosphoenzyme would have led to a rise in the radioactivity in the diffusate as the monophosphoglycerate concentration was increased it is concluded that the dephosphoenzyme does not bind the monophosphoglycerate appreciably under the conditions existing in the cuvette in Fig. 2. If binding had occurred the effective monophosphoglycerate concentration when 3-phosphoglycerate was added would have been less than the assumed value.

A similar result to that shown in Fig. 2 was obtained with the pig kidney enzyme and dialysis also showed that the dephosphoenzyme did not bind monophosphoglycerate. The same experiments however were not attempted with the yeast enzyme since the short half-life of the phosphoenzyme meant that very substantial hydrolysis of the phosphoenzyme would have occurred before the initial slopes could be determined.

It seems clear from the experiments just described that under the conditions existing in the cuvette with monophosphoglycerate concentrations of about 11 μM, the rabbit muscle and pig kidney enzymes are largely present as phosphoenzymes. The presence of small quantities of *E*–2,3-diphosphoglycerate complexes is however not excluded and the possibility must be considered that these complexes may be the catalytically active forms of the enzymes. The dissociation of these complexes into phosphoenzymes and monophosphoglycerate may occur with formation of 3- or 2-phosphoglycerate (Eqns 4 and 5)

$$E\text{–2,3-diphosphoglycerate} \rightleftharpoons \text{phosphoenzyme} + \text{3-phosphoglycerate} \quad (4)$$

$$E\text{–2,3-diphosphoglycerate} \rightleftharpoons \text{phosphoenzyme} + \text{2-phosphoglycerate} \quad (5)$$

If dissociation occurs according to Eqn 4, then, since the equilibrium lies well to the right, the quantity of *E*–2,3-diphosphoglycerate present, to a first approximation, will be proportional to the concentration of 3-phosphoglycerate. Further if the *E*–2,3-diphosphoglycerate complex is the active form of the enzyme, then, in the mutase reaction, the initial velocity with 3-phosphoglycerate as a substrate will be proportional to the product of the 3-phosphoglycerate concentration and the amount of *E*–2,3-diphosphoglycerate. Thus at the low concentrations of 3-phosphoglycerate existing in the cuvette the velocity of the mutase reaction should be proportional to the square of the 3-phosphoglycerate concentration. With 3-phosphoglycerate as a substrate, however, the rabbit muscle (H. G. Britton and J. B. Clarke, unpublished results) and yeast[2] and pig kidney (H. G. Britton, J. Carreras and S. Grisola, unpublished results) enzymes show Michaelis–Menten kinetics down to 3-phosphoglycerate concentrations of the order of 10–20 μM (with Michaelis constants of about 200 μM). Further in Fig. 3 an experiment with the rabbit muscle enzyme is shown which indicates that the initial

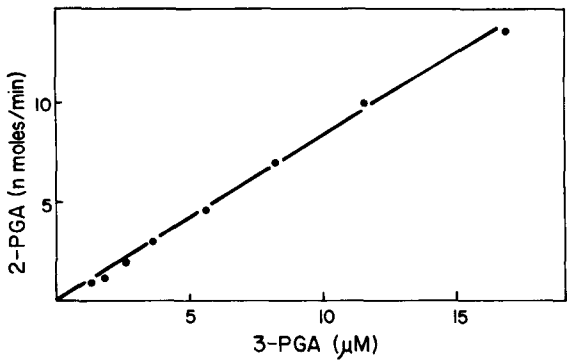

Fig. 3. Proportionality between velocity and 3-phosphoglycerate (3-PGA) concentration. The conditions were as in Fig. 1 except that 4.6 μg/ml of rabbit muscle mutase, and in different experiments, 8.4 and 32.4 μM 2,3-diphosphoglycerate, respectively, were added. The initial concentration of 3-phosphoglycerate was 24 μM and the total volume was 2.5 ml. At the indicated points the concentration of 3-phosphoglycerate was calculated from the $A_{340\ nm}$ and the velocity from slope of $A_{340\ nm}$. The velocities were independent of the concentration of 2,3-diphosphoglycerate. Similar results were obtained with the enzymes from pig kidney and yeast.

velocity remains proportional to 3-phosphoglycerate concentration down to concentrations of the order of 1 μM. Experiments with the pig kidney and yeast enzymes similar to that shown in Fig. 3 gave comparable results.

If dissociation of the *E*–2,3-diphosphoglycerate complexes occur according to Eqn 5, the amount of the *E*–2,3-diphosphoglycerate complex present under the conditions existing in the cuvette (where the equilibrium lies well to the right) will be, to a first approximation, proportional to the concentration of 2-phosphoglycerate. If the *E*–2,3-diphosphoglycerate complex is the only catalytically active form of the enzyme, the rate should therefore depend upon the product of the 3- and 2-phosphoglycerate concentrations and activation should be observed as 2-phosphoglycerate accumulates in the enolase-coupled assay[16] (at least at low concentrations of 3-phosphoglycerate). This is not found even with absorbance changes corresponding to concentrations of 2-phosphoglycerate in the range 1–3 μM*. Further, when 2-phosphoglycerate is removed with enolase, pyruvate kinase and lactate dehydrogenase, the enzyme still shows full mutase activity (rabbit muscle[25], yeast[2], and pig kidney, H. G. Britton, J. Carreras and S. Grisola, unpublished results).

If the *E*–2,3-diphosphoglycerate complexes should dissociate into 2- and 3-phosphoglycerate (Eqns 4 and 5) then both sigmoid initial velocity curves with 3-phosphoglycerate as a substrate and an increase in rate with accumulation of 2-phosphoglycerate should be observed. The absence of these findings therefore indicates that the *E*–2,3-diphosphoglycerate complexes cannot be the only active forms of the enzymes and that the phosphoenzymes must themselves be active. It may be noted that the fact that at monophosphoglycerate concentrations of the order of 11 μM (Figs 2A and 2B) the enzymes were largely present as phosphoenzymes, excludes the possibility that the *E*–2,3-diphosphoglycerate complexes had extremely high affinities for 3- and 2-phosphoglycerate, and that the sigmoid element and activation by 2-

* The K_m values for 2-phosphoglycerate for these enzymes may be calculated from the Haldane relationship to be of the order of 40 μM (H. G. Britton and J. B. Clarke, unpublished results; H. G. Britton, J. Carreras and S. Grisolia, ref. 2 and unpublished results).

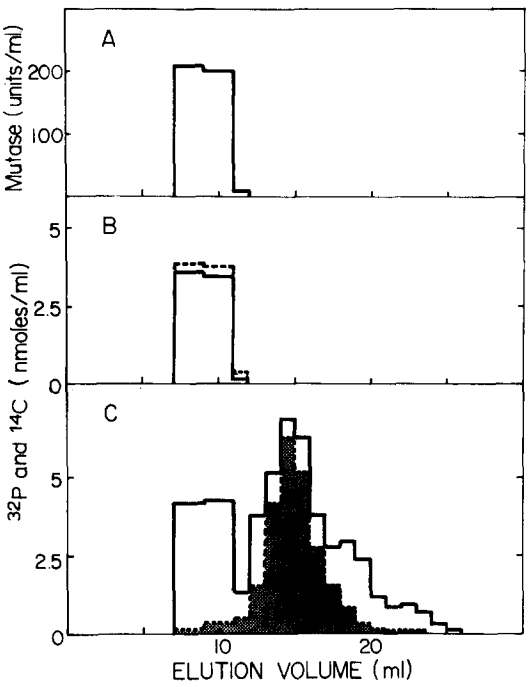

Fig. 4. Isolation of enzyme with bound radioactivity. 1400 units of pig kidney mutase were mixed with 48 μmoles of 2,3-diphospho-[^{14}C]glycerate (0.12 μCi), and [2,3-$^{32}P_2$]diphosphoglycerate (0.14 μCi) and 14 μmoles of Tris–Cl$^-$ (pH 7.4) in 0.7 ml at 25 °C. The mixture was immediately cooled to 0 °C and 0.5 ml applied to a Sephadex G-25 medium column (0.8 cm × 38 cm), and eluted at 4 °C with 20 mM Tris–Cl$^-$, pH 7.4, at 0.2 ml/min. 1-ml fractions were collected and assayed as described in the text. A, mutase activity. B, — — —, P_i released by acid; ———, fraction exchangeable with 3-phosphoglycerate. C, ———, ^{32}P; — — —, ^{14}C.

phosphoglycerate occurred at concentrations below those at which observations were made.

Gel filtration experiments

In view of the spectrophotometric evidence for phosphoenzyme formation, gel filtration experiments were carried out after incubating the enzyme with ^{32}P- and ^{14}C-labelled 2,3-diphosphoglycerate. With the pig kidney enzyme (Fig. 4) ^{32}P but essentially no ^{14}C was found in the enzyme fraction. Further, the traces of ^{14}C that were associated with the enzyme appeared to be entirely the result of overlap in the separation. Most of the ^{32}P associated with the enzyme was released as P_i with acid; the majority of this phosphate was converted to monophosphoglycerate identified by chromatography if 3-phosphoglycerate was added prior to acidification. 2.1 moles P_i and 1.9 moles exchangeable P_i were bound per mole enzyme. The other enzymes behaved similarly with no evidence of ^{14}C binding. With the rabbit muscle, the eluted enzyme solution (450 units/ml) contained 8.3 nM ^{32}P but only 0.20 nM ^{14}C (1.8 moles of P_i and 1.5 moles exchangeable P_i per mole enzyme). With yeast (eluted with 0.02 M Tris–Cl$^-$, pH 7.4, 100 mM KCl), the enzyme (210 units/ml) contained 2.02 nM ^{32}P but only 0.023 nM ^{14}C (5.1 nmoles P_i and 3.2 nmoles exchangeable P_i per 1000 units).

In a second experiment with yeast (eluted with 0.02 M Tris–Cl^-, pH 7.4) 9.7 nmoles ^{32}P and 0.9 nmole ^{14}C per 1000 units enzyme were found. The higher proportion of ^{14}C associated with the enzyme in this experiment was due to less complete separation from the phosphoglycerates. 8.16 nmoles of P_i per 1000 units enzyme were liberated with acid of which 7.4 nmoles were exchangeable. These latter values correspond to 1.14 and 1.04 nmoles of phosphate per mole enzyme, respectively, if a mol. wt of 112 000 (ref. 6) and a specific activity of 1250 units per mg are assumed. The relatively small amount of ^{32}P bound to the yeast enzyme when compared with the spectrophotometric results was presumably related to the short half-life of the phosphoenzyme.

Sodium dodecyl sulphate was added to the enzymes eluted from the column to give a concentration of 0.5% and the stability of the phosphoproteins to hydrolysis was examined. The phosphoproteins from the pig kidney and yeast enzymes were found to be completely stable at pH 11 at 47 °C for 20 min, but were partially hydrolysed (approx. 25%) at pH 2.5, and were completely hydrolysed in 0.1 M HCl under the same conditions.

DISCUSSION

Strong evidence has been obtained both by spectrophotometry and by gel filtration for the formation of phosphoenzymes by the phosphoglyceromutases of rabbit muscle, yeast and pig kidney. In retrospect it seems possible that previous chromatographic evidence for an *E*–2,3-diphosphoglycerate complex[9] may have been due to the fact that 3-phosphoglycerate was prespotted at the origin of the chromatograms. Thus when the phosphoenzyme was applied exchange may have occurred. Shorter Sephadex columns were used than in the present study and the separation of the phosphoenzyme may also have been less complete. The latter might also explain why some binding of 2,3-diphospho-[^{14}C]glycerate was found.

The spectrophotometric observations showed that the phosphoenzymes are unstable and with the rabbit muscle and pig kidney enzymes the 2,3-diphosphoglycerate phosphatase activity of the enzymes could be quantitatively attributed to their instability. With the yeast the phosphatase activity was of the appropriate order but the short half-life of the phosphoenzyme did not enable a quantitative comparison to be made. The low K_m values for 2,3-diphosphoglycerate phosphatase activity indicated by the spectrophotometric observations ($\leqslant 1\ \mu M$ for rabbit muscle and kidney enzymes) are consistent with K_m values found for activation of the mutase reaction[23–25] as is to be expected if activation involves phosphoenzyme formation. The very much higher K_m of 220 μM (at pH 5.9) reported for the 2,3-diphosphoglycerate phosphatase activity of the yeast enzyme[23] is probably related to 2,3-diphosphoglycerate binding at the substrate site rendering the phosphoenzyme less stable. In this connection unpublished experiments similar to Fig. 1 showed that phosphoglycolate (which stimulates the phosphatase activity of rabbit muscle mutase[26]) not only increases the phosphatase activity of all three enzymes but shortens the half-lives of the phosphoenzymes.

Both the spectrophotometric observations and the gel filtration experiments indicated 2 moles phosphate bound per mole by the rabbit muscle and pig kidney enzymes, confirming previous observations on the rabbit enzyme[8–10]. These enzymes

thus probably have two active centres. In the case of the yeast enzymes the present spectrophotometric observations indicated at least 2.9 moles phosphate per mole enzyme. Since this was a minimum value and since the molecular weight of the enzyme is approximately twice that of the other enzyme[6] the true value is probably 4. After denaturation with sodium dodecyl sulphate the phosphoproteins from pig kidney and yeast were stable to alkali and unstable to acid as was found for the rabbit muscle enzyme[10] and has been very recently reported for the yeast enzyme[11]. It seems likely that with these enzymes as in the rabbit[10] the phosphate is bound to histidine.

The spectrophotometric experiments with enolase reduced to rate-limiting quantity showed with the rabbit muscle and pig kidney enzymes that very little if any monophosphoglycerate associates with the phosphoenzymes to form *E*–2,3-diphosphoglycerate complexes. Furthermore, strong kinetic evidence was obtained that the phosphoenzymes are themselves catalytically active. Nevertheless since the concentration of monophosphoglycerate in these experiments was of the order of 11 μM it cannot be excluded that at higher monophosphoglycerate concentrations appreciable amounts of *E*–2,3-diphosphoglycerate may be formed and it is possible that these may also be catalytically active.

It has been suggested that the 2,3-diphosphoglycerate-dependent mutases are related to 2,3-diphosphoglycerate phosphatases which are able to hydrolyse the phosphate in either the 2 or the 3 position of 2,3-diphosphoglycerate[25]. This mechanism requires that the intermediate be a true phosphoenzyme rather than an *E*–2,3-diphosphoglycerate complex. The present experiments support this hypothesis.

REFERENCES

1 H. G. Britton and J. B. Clarke, *Biochem. J.*, 112 (1969) 10P.
2 H. G. Britton, J. Carreras and S. Grisolia, *Biochemistry*, 11 (1972) 3008
3 H. Chiba, E. Sugimoto, R. Sasaki and M. Hirose, *Agric. Biol. Chem.*, 34 (1970) 498.
4 H. G. Britton and J. B. Clarke, *Biochem. J.*, 110 (1968) 161.
5 V. W. Rodwell, J. C. Towne and S. Grisolia, *J. Biol. Chem.*, 228 (1957) 875.
6 H. Edelhoch, V. W. Rodwell and S. Grisolia, *J. Biol. Chem.*, 228 (1957) 891.
7 D. Diederich, A. Khan, I. Santos and S. Grisolia, *Biochim. Biophys. Acta*, 212 (1970) 441.
8 S. Grisolia, B. K. Joyce and M. Fernandez, *Biochim. Biophys. Acta*, 50 (1961) 81.
9 R. J. Jacobs and S. Grisolia, *J. Biol. Chem.*, 241 (1966) 5926.
10 Z. B. Rose, *Arch. Biochem. Biophys.*, 140 (1970) 508.
11 Z. B. Rose, *Arch. Biochem. Biophys.*, 146 (1971) 359.
12 E. Morena de la, I. Santos and S. Grisolia, *Biochim. Biophys. Acta*, 151 (1968) 526.
13 D. C. Mortimer, *Can. J. Chem.*, 30 (1952) 753.
14 H. G. Britton, J. Carreras and S. Grisolia, *Biochemistry*, 10 (1971) 4522.
15 L. V. Eggleston and R. Hems, *Biochem. J.*, 52 (1952) 156.
16 V. W. Rodwell, J. C. Towne and S. Grisolia, *Biochim. Biophys. Acta*, 20 (1956) 394.
17 Y. Sugino and Y. Myoshi, *J. Biol. Chem.*, 239 (1964) 1360.
18 R. Czok and Th. Bucher, *Adv. Protein Chem.*, 15 (1960) 377.
19 L. C. Mokrasch, W. D. Davidson and R. W. McGilvery, *J. Biol. Chem.*, 222 (1956) 179.
20 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
21 L. I. Pizer, *J. Biol. Chem.*, 235 (1960) 895.
22 S. P. Colowick and F. C. Womack, *J. Biol. Chem.*, 244 (1969) 774.
23 R. Sasaki, M. Hirose, E. Sugimoto and H. Chiba, *Biochim. Biophys. Acta*, 227 (1971) 595.
24 S. Grisolia and W. W. Cleland, *Biochemistry*, 7 (1968) 1115.
25 H. G. Britton and J. B. Clarke,, *Biochem. J.*, in the press.
26 Z. B. Rose and J. Liebowitz, *J. Biol. Chem.*, 245 (1970) 3232.
27 N. Zwaig and C. Milstein, *Biochem. J.*, 98 (1966) 360.
28 S. Grisolia and M. Cascales, *Biochem. Biophys. Res. Commun.*, 2 (1966) 200.
29 M. Cascales and S. Grisolia, *Biochemistry*, 5 (1966) 3116.