Phosphoglycerate Mutase from Wheat Germ: Studies with ^{18}O-Labeled Substrate, Investigations of the Phosphatase and Phosphoryl Transfer Activities, and Evidence for a Phosphoryl-enzyme Intermediate†

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ABSTRACT: From studies using unlabeled phospho-D-glycerate in solutions enriched in $H_2{}^{18}O$, and from experiments involving [^{18}O]phospho-D-glycerate, it is shown that the intramolecular isomerization of 2- and 3-phospho-D-glycerate that is catalyzed by the phosphoglycerate mutase from wheat germ does not involve an intermediate 2,3-cyclic phosphate. It is also shown that phosphoglycerate mutase catalyzes the hydrolysis of the substrate analogues 2-phosphoglycolate, 2-phospho-D-lactate, 3-phosphohydroxypropionate, phosphoenolpyruvate, and phosphohydroxypyruvate. The substrates 3- and 2-phospho-D-glycerate are not hydrolyzed, nor are 2,3-bisphospho-D-glycerate, 2-phospho-L-lactate, 3-phospho-L-glycerate, or *sn*-glycerol 3-phosphate. Although no exchange of D-[^{14}C]glycerate into phospho-D-glycerate can be detected, the enzyme catalyzes the transfer of the phosphoryl group from "unnatural" donors such as 2-phosphoglycolate, to the "natural" acceptor, D-glycerate. It is concluded that the intramolecular phosphoryl transfer catalyzed by the wheat germ phosphoglycerate mutase follows a pathway involving a phosphoryl-enzyme intermediate.

The enzyme phosphoglycerate mutase catalyzes the isomerization of 3-phospho-D-glycerate and 2-phospho-D-glycerate. The enzyme from animal sources and from yeast requires 2,3-bisphospho-D-glycerate as a cofactor, which is likely to maintain the enzyme in a phosphorylated form and result in the shuttling of phosphoryl groups between substrates and enzyme in a way analogous to the reaction catalyzed by the more extensively studied enzyme, phosphoglucomutase (Ray and Peck, 1972). After some early confusion in the literature, the pathway of the 2,3-bisphosphoglycerate-dependent mutases has been clarified by the work of Britton on the "flux kinetics" (Britton et al., 1972a,b; Britton and Clarke, 1972) and by the unequivocal demonstrations of a chemically competent phosphoryl-enzyme for the enzymes from rabbit and chicken muscle, and from yeast, by Rose (Rose, 1970, 1971; Rose et al., 1975). The phosphoryl-enzyme may transfer the phosphoryl group either to 2-phospho-D-glycerate or to 3-phospho-D-glycerate, or more slowly to water, the last reaction resulting in the slow but specific phosphatase activity that the animal enzymes show towards 2,3-bisphospho-D-glycerate.

The enzymes from wheat germ and rice, in contrast, show no cofactor dependence (Grisolia and Joyce, 1959), and the mechanistic pathway of this potentially very simple isomerization reaction is of considerable interest. From the work of Britton et al. (1971), it appeared likely that the reaction catalyzed by the wheat germ enzyme is a true intramolecular reaction, and this has been confirmed directly by Gatehouse and Knowles (1977), who have shown that during the isomerization catalyzed by the wheat germ enzyme, the phosphoryl group is not exchanged between different isotopically labeled carbon skeletons.

The elimination of all but intramolecular processes limits the possibilities for this isomerization, and four of the more obvious options are shown in Scheme I. The first possibility (A) involves the enzyme-catalyzed formation and hydrolysis of the enzyme-bound 2,3-cyclic phosphoglycerate and provides an explicit role for water in the mutase-catalyzed reaction. In the second option (B), attack on the substrate's phosphoryl group by its neighboring hydroxyl group leads to an enzyme-bound intermediate having pentacoordinate phosphorus that must be allowed at least one pseudorotation (Westheimer, 1968) before it can collapse to give the product. The third possibility (C) is that the phosphate ester fragments to enzyme-bound glycerate and monomeric metaphosphate. There are good reasons to suppose that the monoanions of phosphate monoesters decompose via this route, and methylated monomeric metaphosphate has been observed directly (Clapp and Westheimer, 1974). Fourthly, the reaction could involve a phosphoryl-enzyme (D) from which the phosphoryl group is transferred to either the 2- or the 3-hydroxyl group of glycerate. This paper concerns attempts to distinguish amongst these four possibilities.

Materials and Methods

Alkaline phosphatase (*E. coli*), D-lactate dehydrogenase (*L. leichmanii*), phosphoglycerate kinase (yeast), D-glycerate dehydrogenase (spinach), trypsin (bovine pancreas), trypsin inhibitor (soybean), and L-lactate dehydrogenase (rabbit muscle) were obtained from Sigma. Wheat germ phosphoglycerate mutase was prepared according to the method described in the Appendix to Gatehouse and Knowles (1977). Experiments were done using enzyme from the second activity peak.

3-Phospho-L-glycerate was a gift of Dr. G. A. Orr. 2-Phosphoglycolate, D- and L-lactate, 3- and 2-phospho-D-glycerate, *sn*-glycerol 3-phosphate, D-fructose 1,6-bisphosphate, 2,3-bisphospho-D-glycerate, phosphoenolpyruvate, phosphohydroxypyruvate, and D-glucose 6-phosphate were obtained from Sigma.

Other materials were as described by Gatehouse and Knowles (1977), or were the highest grade commercially available. All buffers were made up using distilled-deion-

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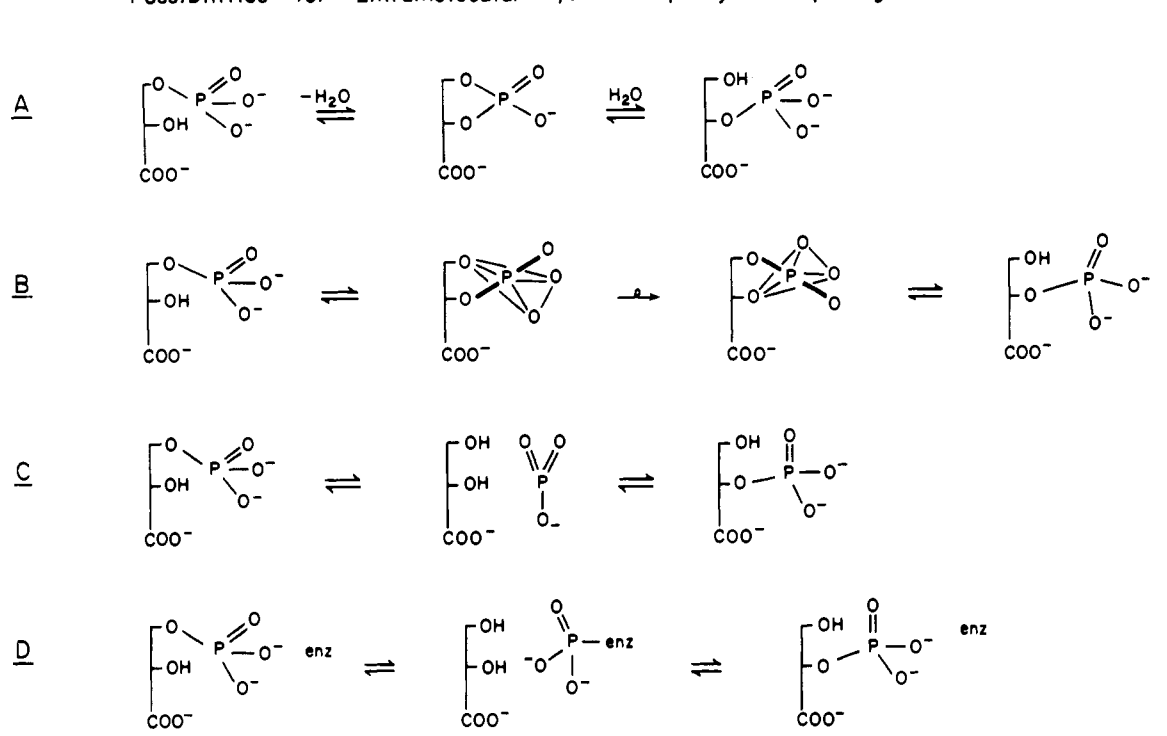
SCHEME I: Possible Pathways for Intramolecular 1,2 Phosphoryl Group Migrations.[a]



[a] All substrate species are presumed to be enzyme-bound.

ized-distilled water. ^{18}O-Enriched water (95.5 atom % ^{18}O) was from Monsanto.

[^{18}O]Phospho-D-glycerate and 3-phospho-D-[1-^{14}C] glycerate were prepared as described by Gatehouse and Knowles (1977).

D-[1-^{14}C]Glycerate was prepared by the hydrolysis of 3-phospho-D-[1-^{14}C]glycerate using alkaline phosphatase. The reaction mixture was applied to a column of DEAE[1]-cellulose equilibrated in 5 mM ammonium bicarbonate, and eluted with a linear gradient (250 + 250 mL, 5–300 mM) of ammonium bicarbonate. The D-[1-^{14}C]glycerate had a specific radioactivity of 3.1×10^7 cpm/μmol.

3-Phosphohydroxypropionate. Benzyl 3-hydroxypropionate was prepared from 3-hydroxypropionic acid by the method of Micheau and Lattes (1970) for the synthesis of benzyl glycolate. Vacuum distillation of the crude product gave a low yield of the desired benzyl ester. This material was phosphorylated with diphenyl phosphochloridate according to the procedure of Ballou and Fischer (1954). Benzyl 3-(diphenylphosphoryl)hydroxypropionate (1.5 g, 70%) was obtained: ^1H NMR (C^2HCl_3) δ 2.8 (t, 2, $CH_2COOC_7H_7$), 4.5 (sextet, 2, $CH_2CH_2OPO(OC_7H_7)_2$), 5.1 (s, 2, $COOCH_2C_6H_5$), 7.2–7.4 (m, 5, $C_6H_5CH_2$).

A solution of this compound (1.5 g) in ethanol (50 mL) was hydrogenolyzed over a mixture of 10% Pd/C and Adams' catalyst at 2 atmospheres. The catalyst was removed by centrifugation and the alcohol removed under reduced pressure. Neutralization of the resulting oil with aqueous cyclohexylamine followed by lyophilization and then recrystallization from ethanol–acetone gave the trimonocyclohexylammonium salt of 3-phosphohydroxypropionic acid (1.1 g, 60%) in 40% yield (from the benzyl ester). A solution of this salt (500 mg) in water (100 mL) was applied to a column of DEAE-cellulose (65 cm × 5.3 cm^2) and eluted with a linear gradient (800 + 800 mL, 5–300 mM) of ammonium bicarbonate. Samples (100 μL) of each fraction were treated with alkaline phosphatase and then assayed for inorganic phosphate. The major phosphate-containing peak was lyophilized repeatedly to remove ammonium bicarbonate. The ammonium salt of 3-phosphohydroxypropionic acid obtained was monodisperse on TLC (cellulose plates, eluted with 2-propanol–water–ammonia (70:25:5, v/v); R_f 0.45) and had ^1H NMR (^2H_2O) δ 2.8 (irreg t, 2, $CH_2COOC_7H_7$), 4.3 (sextet, 2, $CH_2OPO(O^2H)_2$). ^{31}P NMR (^2H_2O) gave only a triplet at −3.4 ppm from an external 85% phosphoric acid standard.

2-Phospho-DL-lactate. Benzyl 2-bromopropionate (1.2 g) and tetramethylammonium dibenzylphosphate (1.8 g, obtained by neutralization of dibenzyl phosphate with tetramethylammonium hydroxide) were refluxed in glyme (70 mL, distilled from calcium hydride) for 48 h. The precipitate of tetramethylammonium bromide was removed by filtration, and the glyme was removed under reduced pressure. The residual oil was taken up in ether and washed with water, dried over $MgSO_4$, and concentrated. Benzyl 2-(dibenzylphosphoryl)hydroxypropionate (1.8 g; 80%) was obtained: NMR (C^2HCl_3) δ 1.5 (d, 3, CH_3CH), 5.1–5.5 (m, 7), 7.3–7.6 (m, 15, $CH_2C_6H_5$). This material (1.8 g) in ethanol (50 mL) was hydrogenolyzed over 10% Pd/C (100 mg) at atmospheric pressure and room temperature, overnight. The catalyst was removed by filtration and the solvent was removed under reduced pressure. The residue was neutralized with aqueous cyclohexylamine to yield the trimonocyclohexylammonium salt of 2-phospholactic acid (1.3 g, 70%). This compound was further purified by chromatography as for 3-phosphohydroxypropionate. The ammonium salt had ^1H NMR (^2H_2O) δ 1.5 (d, 3, CH_3CH); 4.6 (m, 1, CH_3CH). The ^{31}P NMR showed only a doublet at −1.1 ppm from an external phosphoric acid standard.

[1] Abbreviations used are: Tris, tris(hydroxymethyl)aminomethane; P_i, inorganic phosphate; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced form of NAD^+; DEAE, diethylaminoethyl.

Methods. Mass spectroscopic measurements were done on an A.E.I. MS9 mass spectrometer. Samples of phospho-D-glycerate were methylated with diazomethane (see Fisher et al., 1976), and the methylated derivatives were subjected to mass spectroscopic analysis with a source temperature of 40 °C.

Ultraviolet measurements were done on a Unicam SP 1800 instrument. Liquid scintillation counting was done on a Beckmann LS 233 instrument. The scintillation cocktail contained naphthalene (60 g), 2,5-diphenyloxazole (4 g), and 2,2′-*p*-phenylenebis(5-phenyloxazole) (0.2 g) in dioxane (1 L, purified by passage through alumina).

Assays. Wheat germ phosphoglycerate mutase was assayed as described by Leadlay et al. (Appendix to Gatehouse and Knowles, 1977). 2-Phospho-D-glycerate was assayed by adding a sample of this material to a mixture containing ADP (0.2 mM), NADH (0.2 mM), KCl (50 mM), $MgCl_2$ (5 mM), lactate dehydrogenase (50 μg/mL), enolase (50 μg/mL), and pyruvate kinase (50 μg/mL) in 50 mM Tris-HCl buffer, pH 7.5. The decrease in the absorption of NADH at 340 nm was followed. 3-Phospho-D-glycerate was assayed either as above (with the inclusion of wheat germ phosphoglycerate mutase in the assay mixture), or according to the method described by Maister et al. (1976). Inorganic phosphate was estimated according to Briggs (1922). D-Glycerate, D-lactate, and L-lactate were assayed by observing the production of NADH, catalyzed by the appropriate dehydrogenase in the presence of its substrate, NAD^+, and hydrazine. A solution of the compound to be assayed, NAD^+ (0.2 mM), and hydrazine (0.4 M) in 50 mM glycine–NaOH buffer, pH 8.9, was equilibrated at 30 °C and the reaction was initiated by addition of the dehydrogenase. Hydroxypyruvate was assayed by following its reduction by NADH to glycerate and NAD^+, catalyzed by glycerate dehydrogenase. Phosphohydroxypyruvate, and D- and L-phospholactate were assayed by hydrolysis of the phosphoryl ester with alkaline phosphatase, followed either by assay of the P_i released, or by assay of the carbon skeleton using the appropriate dehydrogenase, or both.

Phosphatase Activity of Wheat Germ Phosphoglycerate Mutase. The mutase-catalyzed hydrolysis of 2-phosphoglycolate, 2-phospho-DL-lactate, and 3-phosphohydroxypropionate was performed as follows. The phosphate ester (10 mM) in 50 mM Tris-HCl buffer, pH 7.6, was incubated with mutase at 30 °C. Portions were assayed for inorganic phosphate after various times. In the case of 2-phospho-DL-lactate, assays were also done for D-lactate and L-lactate. Approximately equimolar amounts of D-lactate and inorganic phosphate were liberated in the mutase-catalyzed hydrolysis. The amount of L-lactate released was less than 4% of that of the inorganic phosphate or D-lactate produced, on a molar basis. The mutase-catalyzed hydrolysis of phosphoenolpyruvate (and phosphohydroxypyruvate) was carried out by incubating the phosphate ester (10 mM), NADH (0.2 mM), L-lactate dehydrogenase (or D-glycerate dehydrogenase) (50 μg), and wheat germ phosphoglycerate mutase in 50 mM Tris-HCl buffer, pH 7.6, at 30 °C. The absorbance of NADH at 340 nm was followed. For tests of the pH dependence of the hydrolysis of phosphoenolpyruvate catalyzed by the mutase, the buffers used were 50 mM Tris-HCl (pH 6.4–8.7) and 50 mM glycine–NaOH (pH 8.9–9.6).

Partial Inactivation of Wheat Germ Mutase. The enzyme (28 units/mL) was treated as follows. (a) Heat inactivation: samples of mutase were heated at 55 °C in 50 mM Tris-HCl buffer, pH 7.6, for various times until the desired extent of inactivation had been achieved. (b) Bromoacetate: samples of mutase were incubated in the above buffer in the presence of bromoacetate (10 mM) at 30 °C. (c) Trypsin: samples of mutase were incubated with trypsin (10 μg/mL) in the above buffer at 30 °C. The digestions were stopped by adding trypsin inhibitor (0.1 mg/mL).

Mutase-Catalyzed Synthesis of Phospho-D-[^{14}C]glycerate from D-[^{14}C]Glycerate. 2-Phosphoglycolate, 2-phospholactate, or 3-phosphohydroxypropionate (29 mM) and [^{14}C]-D-glycerate (0.95 mM, 3.1×10^7 cpm/μmol) were incubated in 50 mM Tris-HCl buffer, pH 7.65, with the relevant number of units of mutase in a final volume of 0.275 mL, at 30 °C for 18 h. The reaction was then stopped by dilution with water (10 mL). Unlabeled 3-phospho-D-glycerate (1 mg) was then added and the solution was applied to a column of DEAE-cellulose DE52 (15 cm × 2.5 cm^2) previously equilibrated with 5 mM ammonium bicarbonate. The column was washed with 5 mM ammonium bicarbonate (30 mL) and eluted with a linear concentration gradient of ammonium bicarbonate (150 + 150 mL, 5–300 mM). Samples (200 μL) of each fraction were subjected to scintillation counting, and assayed for phospho-D-glycerate. The amount of phosphoglycerate synthesized in the incubation was calculated from the total radioactivity eluting with the phosphoglycerate peak. For investigations of the inhibitory effect of glycerate analogues on the amount of phosphoglycerate synthesized, the relevant compound was included in the incubation mixture described above.

Results and Discussion

Is the Cyclic Phosphate a Reaction Intermediate? The intramolecular phosphoryl transfers involved in the nonenzymatic acid-catalyzed isomerizations of 2- and 3-phospho-D-glycerate (Harrison et al., 1955) and of glycerol 1- and 2-phosphate (Fordham and Wang, 1967) are believed to proceed (at least in part) via the 2,3- and 1,2-cyclic phosphate ester, respectively. It was of interest to see whether this was true of the intramolecular isomerization catalyzed by wheat germ phosphoglycerate mutase. While it is known that the 2,3-cyclic phosphate of phospho-D-glycerate is not a substrate for the wheat germ mutase (Britton et al., 1971; P. Leadlay, unpublished results), this does not rule out the possibility that the isomerization goes via such a cyclic intermediate that is tightly bound to the enzyme.

A much safer test of the possible incursion of the cyclic phosphate is to see if the enzyme catalyzes the exchange of ^{18}O between the solvent and the phosphoglycerate substrates, as depicted in Scheme I(A). Accordingly, phospho-D-glycerate was incubated with the enzyme in ^{18}O-enriched water for times sufficient to ensure complete equilibration of substrate (and of isotope, if Scheme I (A) were the reaction pathway). The mixture of 2- and 3-phospho-D-glycerates was then recovered, and methylated with diazomethane. This procedure results in a mixture of the tri- and tetramethyl derivatives of phosphoglycerate (Fisher et al., 1976). The mass spectrum of this mixture shows an intense peak at *m/e* 169, arising from the loss of the carbomethoxy group from the trimethyl (triester) species (Fisher et al., 1976). Any incorporation of ^{18}O label into the phosphoryl-group oxygens of phosphoglycerate that occurs during the incubation with enzyme in ^{18}O-enriched solvent should lead to an increase in the peak intensities at *m/e* 171, 173, and 175 (corresponding to the introduction of 1, 2, or 3 atoms of ^{18}O, respectively) relative to the ion at *m/e* 169.

In Table I, mass-spectral results are presented for samples of methylated phosphoglycerate obtained after incubation of phosphoglycerate in ^{18}O-enriched solvent, in the presence and absence of wheat germ phosphoglycerate mutase. It is evident

TABLE I: Mass-Spectral Data for Samples of Methylated Phospho-D-glycerates.

	Relative Intensity[a]			
m/e	Unlabeled substrate	Substrate after incubation without mutase[b]	Substrates after incubation with mutase[b,c]	Calcd for complete exchange of phosphoryl-group oxygens with the solvent
169	1.00	1.00	1.00	0.02
170	0.05	0.05	0.05	0.00
171	0.02	0.02	0.02	0.20
172	0.00	0.00	0.00	0.00
173	0.00	0.00	0.00	0.76
174	0.00	0.00	0.00	0.04
175	0.00	0.00	0.00	1.00

[a] Intensities relative to the most intense peak listed. Experimental results are the means of ten determinations of the mass spectrum. [b] 2-Phospho-D-glycerate (10 μmol) was incubated in the absence or presence of wheat germ phosphoglycerate mutase (10 units), in 10 mM Tris-HCl buffer, pH 8.0 (made up in $H_2{}^{18}O$, 80 atom % ^{18}O), at 30 °C for 8 h, in a total volume of 115 μL. After the incubation, the mixture was passed through a column of Dowex-50 (H^+ form) to remove enzyme and freeze-dried. The resulting solid was treated with a large molar excess of ethereal diazomethane before mass-spectral analysis. [c] The incubation was long enough to ensure *at least* 10^3 turnovers, after chemical equilibrium had been reached.

TABLE II: Mass-Spectral Data for Samples of Methylated [^{18}O]Phospho-D-glycerates.

	Relative Intensity[a]	
m/e	Labeled substrate after incubation without mutase[a]	Labeled substrates after incubation with mutase[a]
169	0.23	0.22
170	0.04	0.04
171	1.00	1.00
172	0.06	0.06

[a] For details, see the footnotes to Table I. A mixture of 2- and 3-[^{18}O]phospho-D-glycerates (10 μmol), enriched in ^{18}O in one of the phosphoryl group oxygens, was used.

from these data that the enzyme does not catalyze the exchange between water oxygens and the phosphoryl oxygens of the substrates. This result was confirmed by the demonstration that wheat germ mutase does not catalyze the *loss* of label from phosphoglycerate carrying ^{18}O in one of the oxygens of the phosphoryl group. This material, prepared by the hydrolysis of 2,3-cyclic phospho-D-glycerate by $^{18}OH^-$, was incubated in unlabeled solvent in the presence and absence of enzyme. Mass-spectral analysis of the methylated substrate mixture is shown in Table II. (Had loss of label occurred, the peak intensities would have been those listed for the unlabeled material in Table I.)

The results presented in Tables I and II show that no equilibration occurs between the oxygen atoms of phosphoglycerate and solvent water during the course of the intramolecular isomerization of the phosphoryl group catalyzed by phosphoglycerate mutase from wheat germ. This result complements an early study of enzymes in a rat-muscle extract, in which it was demonstrated that when the phosphoryl group of 3-[^{18}O]phospho-D-glycerate was transferred to creatine, no ^{18}O label was lost to the solvent (Harrison et al., 1955). Thus, at least under the conditions of these experiments, no oxygen-exchange processes occurred in the reactions mediated by 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase, enolase, pyruvate kinase, and creatine kinase. In contrast to the *acid*-catalyzed isomerization of 2- and 3-phosphoglycerate, therefore, the isomerization catalyzed by both kinds of phosphoglycerate mutase (i.e., those that require 2,3-bisphosphoglycerate[2] and those that do not) is unlikely to proceed via the 2,3-cyclic ester. Any pathway explicitly involving water as a cosubstrate in the enzymatic reaction is very improbable. This statement only needs qualification if the molecule of water that is expelled from either substrate as the cyclic phosphate is formed is fully retained by the enzyme without any exchange with the medium, and is incorporated back into the product after extensive motion of the enzyme relative to the substrate. This possibility seems unlikely, though since there is precedent in enzymology even for trapped protons (e.g., Rose and O'Connell, 1967), the caveat must be made.

Is a Phosphoryl-enzyme a Reaction Intermediate? Of the four possibilities for the intramolecular phosphoryl transfer reaction catalyzed by phosphoglycerate mutase set out in Scheme I, option A is very unlikely on the basis of the ^{18}O work described above. We are left with B, C, and D. One feature of C and D is that the glyceric acid is transiently dissociated from the phosphoryl group, and efforts were made to detect the exchange of the bound glyceric acid with added [^{14}C]glycerate in the medium. Although it has been reported that this exchange reaction can occur very slowly (Britton et al., 1971), in our hands the reaction was undetectable. Britton et al. (1971) used either paper chromatography or TLC to separate the large excess of glycerate from phosphoglycerate after the incubation, but in a number of systems (including all those used by these authors) the separation was incomplete when judged by strip-scanning. The fear that this exchange could be due to a phosphatase contaminant in the enzyme used by Britton et al. (1971) or due to incomplete component separation led us to repeat the exchange reaction. The mutase used contained no detectable phosphoglycerate phosphatase activity (see the Appendix to Gatehouse and Knowles, 1977), and the reaction components were separated by TLC on cellulose plates (eluted with ethanol–1 M ammonium acetate (7:3, v/v): R_f for phosphoglycerate is 0.06; R_f for glycerate is 0.52). Incubations at pH 7.5 and 8.7 in the presence of large amounts (up to 250 μg/mL) of mutase gave *no* significant exchange between [^{14}C]glycerate and phosphoglycerate in up to 6 h at 37 °C.

The absence of [^{14}C]glycerate exchange does not, of course, rule out pathways C and D, since the carbon skeleton may be very tightly bound to the phosphoryl-enzyme (in D) or to the enzyme–metaphosphate complex (in C). Indeed, if the glycerate were *not* tightly bound, the mutase would either become a wasteful phosphoglycerate phosphatase (which it certainly is not: Leadlay et al., 1977) or, since the reaction is intramolecular (Gatehouse and Knowles, 1977), fruitlessly block its active site by phosphorylation.

In an attempt to make the putative phosphoryl-enzyme (in

[2] The demonstration by Sutherland et al. (1949) of the obligatory role of 2,3-bisphospho-D-glycerate in the reaction catalyzed by the muscle enzyme and the intermolecular nature of these phosphoryl transfers effectively rules out a cyclic phosphate intermediate for the cofactor-dependent mutases, anyway.

Phosphatase Activity of Phosphoglycerate Mutase

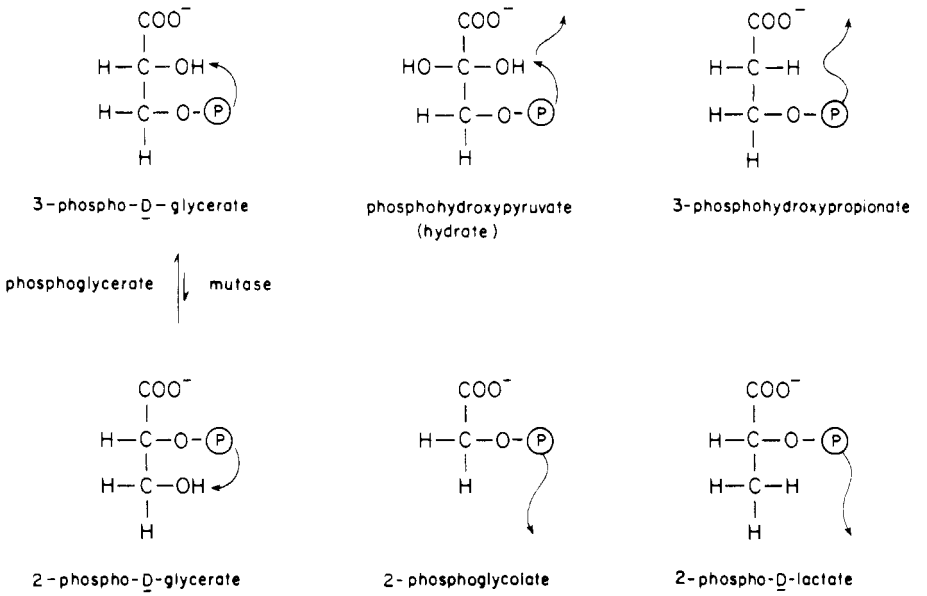


FIGURE 1: Substrates and substrate analogues for phosphoglycerate mutase. The arrows indicate (for the substrates) intramolecular phosphoryl-group transfer, and (for the substrate analogues) transfer of the phosphoryl group to water (i.e., phosphatase activity).

TABLE III: Phosphatase Activity of Wheat Germ Phosphoglycerate Mutase.

Substrate	Phosphatase act.[a]
3-Phospho-D-glycerate	<0.1
3-Phospho-L-glycerate	<0.1
Phosphohydroxypyruvate	160
Phosphoenolpyruvate	50
2-Phosphoglycolate	28
2-Phospho-D-lactate	4
2-Phospho-L-lactate	<0.1
3-Phosphohydroxypropionate	2
2,3-Bisphospho-D-glycerate	<0.1
sn-Glycerol 3-phosphate	<0.1

[a] nmol of inorganic phosphate liberated per min per 100 units of mutase. (One unit of mutase will convert 1 μmol per min of 3-phospho-D-glycerate to the 2-isomer at pH 7.6, 30 °C.) All com-ounds except phosphoenolpyruvate and phosphohydroxypyruvate were incubated at 10 mM concentration in 50 mM Tris-HCl buffer, pH 7.6, at 30 °C with mutase. The hydrolyses of phosphoenolpyruvate and phosphohydroxypyruvate were monitored by enzymatic assay of pyruvate and hydroxypyruvate, respectively.

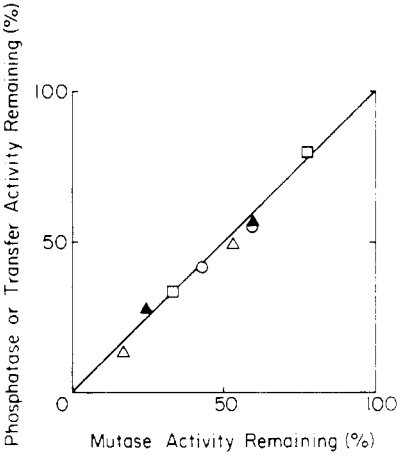


FIGURE 2: The effects of various treatments on the mutase, phosphatase, and transfer activities of phosphoglycerate mutase. (○) Bromoacetate treatment, (△) heat treatment, (□) trypsin digestion. Open symbols, mutase and phosphatase activities; filled symbols, mutase and intermolecular phosphoryl-group transfer activities. For details, see the text.

D), it was decided to provide the enzyme with phosphoryl-group donors that were not also acceptors, in the sense that they lack a free hydroxyl group. The substrate analogues chosen were 2-phosphoglycolate, phosphohydroxypyruvate, phosphoenolpyruvate, 2-phospho-D-lactate, and 3-phosphohydroxypropionate. Each of these analogues was found to be hydrolyzed slowly by the phosphoglycerate mutase (Table III and Figure 1). This phosphatase activity is specific, and is proportional to enzyme concentration. No hydrolysis of the following compounds could be detected: 3- and 2-phospho-D-glycerate, 2,3-bisphospho-D-glycerate, 3-phospho-L-glycerate, 2-phospho-L-lactate, *sn*-glycerol 3-phosphate, D-fructose 6-phosphate, and D-glucose 6-phosphate. The known existence in wheat germ of a nonspecific acid phosphatase (Joyce and Grisolia, 1960) and the possibility of a specific phosphoglycolate phosphatase (Richardson and Tolbert, 1961) must make one cautious of ascribing to mutase the hydrolysis of the five sensitive substrate analogues. However, the isolation and stability characteristics of the phosphoglycolate phosphatase are quite different from those of wheat germ phosphoglycerate mutase, and a variety of methods was used in attempts to affect the ratio of phosphatase to mutase activity. As shown in Figure 2, partial inactivation of the enzyme preparation by heat, trypsin digestion, and bromoacetate treatment all caused the hand-in-hand loss of the two activities. Further, the two peaks of mutase activity from the final DEAE-cellulose column, each of which gives rise to a single band on polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate, show identical ratios of phosphatase activity (on

TABLE IV: Rates of Phospho-D-[^{14}C]glycerate Synthesis from Various Phosphate Esters and D-[^{14}C]Glycerate.[a]

Phosphoryl-group donor	Rate of phospho-D-glycerate synthesis[b]
3-Phospho-D-glycerate	<0.01
Phosphoenolpyruvate	0.73
2-Phosphoglycolate	0.26
2-Phospho-D-lactate	0.45
3-Phosphohydroxypropionate	0.13
2,3-Bisphospho-D-glycerate	<0.01
sn-Glycerol 3-phosphate	<0.01

[a] The phosphoryl-group donor (5 μmol) was incubated with D-[1-^{14}C]glycerate (0.25 μmol; 3.1×10^7 cpm/μmol) and wheat germ phosphoglycerate mutase (2.8 units) in 50 mM Tris-HCl buffer (250 μL), pH 7.6, at 30 °C for 18 h. The mixture was then chromatographed on DEAE-cellulose. [b] nmol min^{-1} (100 units of mutase)$^{-1}$.

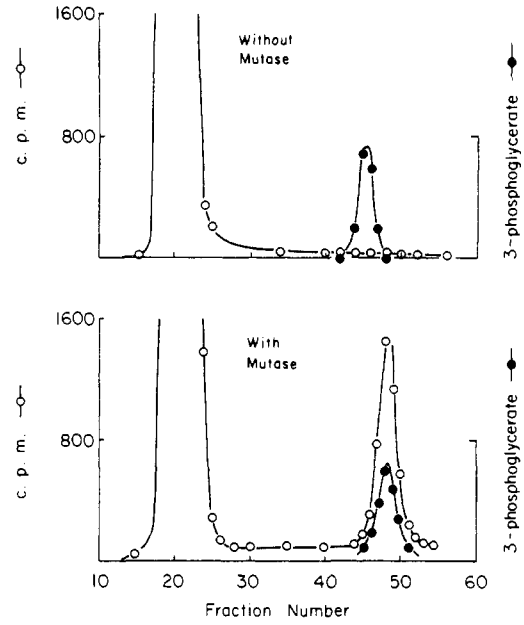


FIGURE 3: Elution profiles of incubations of 2-phosphoglycolate and D-[^{14}C]glycerate with and without phosphoglycerate mutase, from DEAE-cellulose. 2-Phosphoglycolate (5 μmol), D-glycerate (0.25 μmol; 3.1×10^7 cpm/μmol), and phosphoglycerate mutase (2.8 units, if present) were incubated in 50 mM Tris-HCl buffer (250 μL), pH 7.6, for 18 h at 30 °C. Unlabeled phospho-D-glycerate (2 μmol) was added after quenching the reaction, and the mixture was subjected to chromatography on DEAE-cellulose (50 mL), equilibrated with 5 mM ammonium bicarbonate, and eluted with a linear gradient (150 + 150 mL) of ammonium bicarbonate (5–300 mM). Fractions of approximately 4 mL were collected and assayed for phosphoglycerate (●) and radioactivity (○).

phosphoglycolate) to mutase activity. Indeed, the mutase activity and the phosphatase activity (on phosphoenolpyruvate) copurified during the isolation, the ratio of mutase to phosphatase activity remaining constant after the ammonium sulfate fractionation (see Appendix to Gatehouse and Knowles, 1977). (This phosphatase activity was not due to pyruvate kinase contamination, since it was not stimulated by Mg^{2+}, K^+, and ADP.) Finally, the ratio of mutase to phosphatase activity was closely similar for material derived from three different preparations of the wheat germ mutase, and the pH optima for the phosphatase and mutase activity are very similar. We conclude, therefore, that the phosphatase activity is an intrinsic and specific activity of phosphoglycerate mutase from wheat germ.

The phosphatase activity toward substrates lacking an acceptor hydroxyl group is, of course, consistent with any of the mechanisms B, C, and D (Scheme I). In principle, a water molecule could occupy, at least approximately, the site of the missing acceptor hydroxyl group, and the phosphoryl group could be transferred to it. However, discrimination among these three mechanisms is possible. If pathway D is in fact followed by the enzyme then, although we know that the glycerate would have to be bound tightly to the phosphoryl-enzyme, it is possible that a less specific carbon skeleton such as glycolate would be less tightly bound, and allow detection of the phosphoryl-enzyme. Accordingly, D-[^{14}C]glycerate was added to an incubation of 2-phospho-D-lactate and mutase, and the synthesis of phospho-D-[^{14}C]glycerate was observed (Figure 3). That is, the enzyme catalyzes the transfer of a phosphoryl group from 2-phospho-D-lactate to D-glycerate. The same is true for the substrate analogues 2-phosphoglycolate, 3-phosphohydroxypropionate, and phosphoenolpyruvate. (Phosphohydroxypyruvate was not tested.) This transfer activity is reduced by heat inactivation in parallel with mutase activity (Figure 2), in just the same way that the phosphatase activity is concomitantly reduced. As is evident from Figure 3, no phosphoglycerate is synthesized in the absence of the enzyme, nor can 2,3-bisphospho-D-glycerate replace any of the four phosphoryl group donors. The rates of phosphoglycerate synthesis using the four donors are listed in Table IV.

The observation of intermolecular phosphoryl-group transfer rules out pathway B (Scheme I), and leaves D or its variant C, as possible modes of the group transfer mediated by the mutase. If pathway C or D is followed, the amount of phosphoglycerate synthesized in a transfer experiment should decrease in the presence of glycerate analogues that can bind to and be phosphorylated by the phosphoryl-enzyme (D) or the enzyme with tightly bound metaphosphate (C). The steady-state rate of phosphoglycerate synthesis should then depend on the concentration of glycerate analogue, according to:

$$\text{rate of phosphoglycerate synthesis} = \frac{k_0 k_1 [\text{glycerate}]}{k_1[\text{glycerate}] + k_2[\text{glycerate analogue}]}$$

where k_0 is the steady-state constant for phosphoryl-enzyme formation, and k_1 and k_2 are the rate constants for the reaction of phosphoryl-enzyme with glycerate and glycerate analogue, respectively.

The effect of various glycerate analogues (glycolate, D-lactate, and L-lactate) on the rate of phosphoglycerate synthesis from glycerate and 2-phosphoglycolate catalyzed by mutase has been investigated, and the data obtained are presented graphically in Figure 4. The slope to intercept ratio of the straight lines obtained may be used to calculate relative values of k_2/k_1 for each analogue. The values obtained are: D-glycerate, 1.00; D-lactate, 0.24; glycolate, 0.10; and L-lactate, <0.01. These numbers provide an estimate of the selectivity of the phosphoryl-enzyme, a number of k_2/k_1 of less than 1 for any analogue showing that it competes on unequal terms with glycerate for the phosphoryl-enzyme. Evidently, D-lactate competes more effectively with D-glycerate than does glycolate. L-Lactate is a very poor competitor, and it presumably binds to the phosphoryl-enzyme in an orientation where the enzyme-bound phosphoryl group cannot be transferred to it.

The phosphoryl-transfer reaction catalyzed by the enzyme must proceed by the binding of glycerate to the free phosphoryl-enzyme. If the *hydrolysis* of phosphoglycolate, phos-

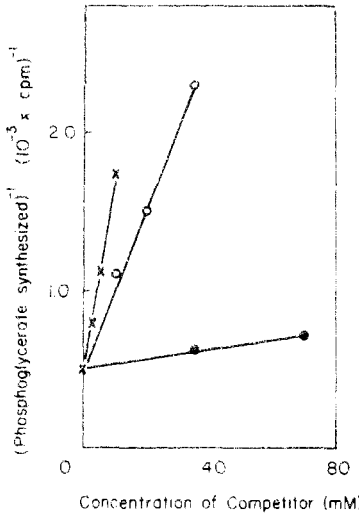


FIGURE 4: Plot of the reciprocal of the amount of 3-phospho-D-[^{14}C]glycerate synthesized from 2-phosphoglycolate and D-[^{14}C]glycerate in the presence of various acceptors. Conditions were as stated in the legend to Figure 3. (○) Added glycolate; (×), added D-lactate; (●) added L-lactate. For other details, see the text.

phoenolpyruvate, phospholactate, and phosphohydroxypropionate occurred solely by way of the free phosphoryl-enzyme, there should be a correspondence between the enzyme-catalyzed rates of hydrolysis and phosphoglycerate synthesis (in the presence of glycerate) for each compound. This is not the case (Tables III and IV), and the reason may be that, while for phosphoryl transfer the carbon skeleton of the phosphoryl donor must have left the active site (so as to allow the acceptor glycerate to bind), this may not be required for all the hydrolytic reactions. Thus, for 2-phosphoglycolate, one can envisage that a water molecule may be appropriately positioned for phosphoryl transfer (in the normal locus of the missing hydroxymethyl group) with the two-carbon glycolate skeleton still in place. In contrast, when the carbon skeleton bound to the phosphoryl-enzyme is D-lactate or 3-hydroxypropionate, water access may be so restricted that hydrolysis of the phosphoryl-enzyme can occur only in the absence of the donor's carbon skeleton. In this case, the rates of hydrolysis relative to the rate of phosphoglycerate synthesis should be similar for these two compounds, as is in fact observed.

A similar explanation can be put forward for the relatively rapid rates of hydrolysis of phosphoenolpyruvate and phosphohydroxypyruvate catalyzed by the mutase, which may proceed by transfer of the phosphoryl group from the enzyme to the relevant *gem*-diol (Pizer and Ballou, 1959; Ray and Peck, 1972), which then hydrolyzes nonenzymatically. The existence of alternative routes for hydrolysis of the compounds phosphoglycolate, phosphoenolpyruvate, and phosphohydroxypyruvate explains the lack of correspondence between the enzyme-catalyzed rates of hydrolysis and of phosphoglycerate synthesis.

The data presented suggest the intermediacy of a phosphorylated form of wheat germ phosphoglycerate mutase in some enzyme-catalyzed reactions of phosphoglycerate analogues. Compounds capable of phosphorylating the enzyme must be close structural and stereochemical analogues of the natural substrate phospho-D-glycerate. Compounds phosphorylated by the phosphoryl-enzyme must be analogues of D-glycerate. The experiments suggest that, in common with the intermolecular phosphoryl-group transfer that is catalyzed by the muscle and yeast phosphoglycerate mutases, the intramolecular shuttling of phosphoryl groups mediated by the enzyme from wheat germ also involves the formation of a phosphoryl-enzyme.

There is no way, on the basis of the available evidence, to discriminate between the phosphoryl-enzyme in D or the tightly bound metaphosphate in C (Scheme I). For the transfer reactions described above, the existence of tightly bound metaphosphate seems improbable, since it would be expected that, as soon as the carbon skeleton of the phosphoryl group donor departed from the active site, metaphosphate would react with solvent water. The fact that the transfer reaction competes reasonably with the phosphatase reaction, and is both specific and stereospecific, militates against the intermediacy of a species of such high chemical reactivity as monomeric metaphosphate. But, however unlikely this route may be for the relatively very slow reactions of substrate analogues, it cannot be ruled out for the interconversion of the natural substrates, 2- and 3-phospho-D-glycerate.

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