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Phosphoglycerate Mutase from Wheat Germ: Studies with Isotopically Labeled 3-Phospho-D-glycerates Showing That the Catalyzed Reaction Is Intramolecular†

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Appendix: Phosphoglycerate Mutase from Wheat Germ: Isolation, Crystallization, and Properties†

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ABSTRACT: The isomerization of 3-phospho-D-glycerate and 2-phospho-D-glycerate catalyzed by the cofactor-independent phosphoglycerate mutase from wheat germ (the isolation and crystallization of which is described in the Appendix) has been shown to be intramolecular by two methods. Mass-spectrometric analysis of the products from the isomerization of a mixture of 3-phospho-D-[2-^{2}H]glycerate and 3-[^{18}O]phospho-D-glycerate shows that there is no exchange of labeled phosphoryl group between carbon skeletons in the mutase-catalyzed reaction. Analysis of the products from the isomerization of a mixture of 3-phospho-D-[2-^{2}H]glycerate and 3-[^{32}P]phospho-D-glycerate by a method involving the kinetic discrimination between 2-^{2}H and 2-^{1}H species using the enolase isotope effect similarly shows that the wheat germ phosphoglycerate mutase mediates an intramolecular transfer of the phosphoryl group.

The phosphoglycerate mutase from animal sources and from yeast requires 2,3-bisphospho-D-glycerate as a cofactor, and it is probable that this cofactor maintains the enzyme in a phosphorylated form and allows the *inter*molecular shuttling of phosphoryl groups between the two substrates 3- and 2-phospho-D-glycerate and the bisphospho compound (Ray and Peck, 1972). In contrast, the enzyme from wheat germ and other plant sources shows no cofactor dependence (Grisolia and Joyce, 1959), and it is of some importance to determine whether the isomerization mediated by phosphoglycerate mutase from wheat germ is an intermolecular or an intramolecular process.

In this paper, we report the isolation and crystallization of the wheat germ enzyme, and the results of experiments in which the enzyme is allowed to isomerize a mixture of two substrate species, one of which is isotopically labeled on the carbon skeleton. The products are then analyzed to see if there has been any exchange of labeled phosphoryl group between the two kinds of carbon skeleton.

Materials and Methods

Phosphoglycerate mutase was prepared from wheat germ as described in the Appendix. Before use the enzyme was treated with 0.1 mM *p*-chloromercuribenzoate to remove any traces of enolase activity. Yeast enolase and all other enzymes were from Sigma. [^{32}P]Orthophosphate and sodium [^{14}C]bicarbonate were obtained from Amersham-Searle. Normalized $H_2{}^{18}O$ (95% enriched) was from Monsanto, and $^{2}H_2O$ (99.8%) was from Bio-Rad. Other reagents were of the highest grade available commercially. Buffers were made up using distilled–deionized–distilled water. ADP was purified by chromatography on DEAE-cellulose[1] in NH_4HCO_3 buffer, pH 8.0, to remove contaminating ATP and AMP, and stored as a frozen solution.

Instruments. All spectrophotometric measurements were made on a Pye-Unicam SP 1800 spectrophotometer equipped

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

with a thermostatted cell block. Mass spectra were taken on an A.E.I. MS9 mass spectrometer. Scintillation counting was done on a Beckman LS 233 scintillation counter using a scintillation cocktail containing toluene (1400 mL), ethanol (640 mL), naphthalene (120 g), and 2,5-diphenyloxazole (6 g).

Assays. Phosphoglycerate mutase was assayed as described in the Appendix. Phospho-D-glycerate was assayed enzymatically, either by conversion to glyceraldehyde 3-phosphate by the method of Czok and Eckert (1963) or by conversion to lactate by the method of Britton and Clarke (1972). ATP and ADP were assayed by the methods of Adam (1963).

Isotopically Labeled Substrates

3-Phospho-D-[2-^2H]glycerate. This was prepared from dihydroxyacetone phosphate by a method similar to that used previously (Leadlay et al., 1976). To a mixture of Na_2HAsO_4 (30 μmol) and EDTA (30 μmol) in 0.5 M Tris-HCl buffer in ^2H_2O (20 mL), "pH" (meter reading) 8.1, was added dihydroxyacetone phosphate (70 μmol) and NAD^+ (72 μmol), followed by a mixture of triosephosphate isomerase (50 μL of a solution of 10 mg/mL) and glyceraldehyde-3-phosphate dehydrogenase (5 mg) in ^2H_2O (1 mL). The reaction was allowed to proceed for 5 h at room temperature, after which time it was essentially complete. The mixture was then freeze-dried, and the 3-phospho-D-[2-^{2}H]glycerate was isolated by chromatography on a column (70 mL) of DEAE-cellulose (DE-52) equilibrated with 50 mM NH_4HCO_3, using a linear concentration gradient of NH_4HCO_3 (175 + 175 mL; 50–250 mM). The fractions containing phosphoglycerate were contaminated with NADH, and the pool was treated with sodium pyruvate (6 mg) and lactate dehydrogenase (10 μL of a solution of 10 mg/mL). Rechromatography of the mixture yielded 3-phospho-D-[2-^{2}H]glycerate. Mass-spectrometric analysis of this material showed it to be >92% deuterated (see later).

[^{18}O]Phospho-D-glycerate. A mixture of 2- and 3-phospho-D-glycerate, labeled with ^{18}O in the phosphoryl group, was prepared by ring opening 2,3-cyclic phospho-D-glycerate with $^{18}OH^-$. 2,3-Cyclic phospho-D-glycerate was prepared by the method of Pizer and Ballou (1959) and freeze-dried as its lithium salt. This material (90 mg), which contained less than 0.1% of the phosphate mono esters by enzymatic assay, was dissolved in $H_2{}^{18}O$ (95% enriched, 200 μL) and solid NaOH (40 mg) was added. The solution was incubated for 6 h at 37 °C. After neutralization and dilution with water, the material was purified by chromatography on a column (200 mL) of DEAE-cellulose equilibrated with 5 mM ammonium bicarbonate. Elution with a linear concentration gradient (5–300 mM; 800 + 800 mL) gave a mixture of 3-phospho-D-glycerate (48%) and 2-phospho-D-glycerate (52%) in 80% yield. The mass spectrum showed the extent of ^{18}O enrichment to be 81.6% (Table I), which is close to that expected on the basis of the $H_2{}^{18}O$ used and the known dilution with ^{16}O.

3-[^{32}P]Phospho-D-glycerate. γ-[^{32}P]ATP (10 μmol) was prepared from $^{32}P_i$ (1 mCi) and unlabeled ATP by the method of Glynn and Chappell (1964). The labeled ATP was isolated by chromatography on DEAE-cellulose in NH_4HCO_3, pH 8.0, using a linear gradient (50–250 mM) of NH_4HCO_3. After freeze-drying the fractions containing ATP, this material was used to phosphorylate dihydroxyacetone by incubation with glycerol kinase by a modification of the method of Hajra (1968). The resulting dihydroxyacetone [^{32}P]phosphate was purified chromatographically on DEAE-cellulose in triethanolamine hydrochloride buffer, pH 7.0, using a linear concentration gradient (5–300 mM) of the same buffer. The fractions containing dihydroxyacetone [^{32}P]phosphate were freeze-concentrated and immediately converted to 3-[^{32}P]phospho-D-glycerate using triosephosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase as described above in the preparation of 3-phospho-D-[2-^{2}H]glycerate, except that ^1H_2O was used. After purification by ion-exchange chromatography, 3-[^{32}P]phospho-D-glycerate (3.3 μmol) of specific radioactivity 9.84×10^7 cpm/μmol was obtained.

3-Phospho-D-[1-^{14}C]glycerate. ^{14}C-Labeled 3-phospho-D-glycerate was prepared from ribulose 1,5-bisphosphate and sodium [^{14}C]bicarbonate by a method based on that described by Sjödin and Vestermark (1973) using ribulose-1,5-bisphosphate carboxylase. The reaction mixture contained EDTA (0.5 mM), $MgCl_2$ (25 mM), glutathione (12 mM), ribulose 1,5-bisphosphate (5 mg, 12.6 μmole), and ribulose-1,5-bisphosphate carboxylase (34 mg, 1 unit) in 1.5 mL of 80 mM triethanolamine hydrochloride buffer, pH 7.95, to which 0.5 mL of sodium [^{14}C]bicarbonate (16.6 μmol, 0.98 mCi) was added. The solution was incubated for 2 h at 30 °C, after which the reaction was stopped by the addition of 1 N HCl. The labeled 3-phospho-D-[^{14}C]glycerate (21.8 μmol) was isolated by chromatography on Dowex-1 (chloride form), and had a specific radioactivity of 4.3×10^7 cpm/μmol.

Mass-Spectrometric Analysis of Phosphoglycerate. The sample of phosphoglycerate was converted to the free acid by treatment with Dowex-50 (H^+ form), and after removal of the resin by filtration, the material was freeze-dried. The resulting solid was dissolved in dry MeOH, and treated with an excess of an ethereal solution of diazomethane. After 30 min, the solvents were removed under reduced pressure, to yield a mixture of trimethyl and tetramethyl phospho-D-glycerates. The mass spectrum (see Fisher et al., 1976, for details) shows a peak at $M^+ - 59$ for each species, corresponding to loss of the carbomethoxy group from the molecular ion. The $M^+ - 59$ peak preserves both ^2H at C-2 and ^{18}O in the phosphoryl group, and this peak for the trimethyl phospho-D-glycerate, at *m/e* 169, was used for the analysis of the isotopic composition of phosphoglycerate in experiments involving ^2H- and ^{18}O-labeled material. Since 2-phosphoglycerate and 3-phosphoglycerate give ions of the same atomic composition at $M^+ - 59$ (M^+ minus $COOCH_3$), the equilibrium mixture of isomers obtained after incubation with phosphoglycerate mutase was analyzed directly.

Analysis of Phosphoglycerate Exploiting the Kinetic Isotope Effect of Enolase. Mixtures of phospho-D-[2-^{2}H]glycerate and [^{32}P]phospho-D-glycerate were partially converted to pyruvate and ATP by the action of enolase and pyruvate kinase–ADP. To a mixture of 3-phospho-D-[2-^{2}H]glycerate and 3-[^{32}P]phospho-D-glycerate (ca. 5 μmol, in 0.61 mL of water) was added 5.0 mL of 50 mM triethanolamine hydrochloride buffer, pH 7.12, containing $MgCl_2$ (1 mM), KCl (50 mM), ADP (5.25 μmol in 150 μL of water), wheat-germ phosphoglycerate mutase (12.8 units in 100 μL of triethanolamine hydrochloride buffer), and pyruvate kinase (10 μL of a suspension containing 10 mg/mL). This mixture was incubated for 20 min at 30 °C to ensure the complete equilibration of 3- and 2-phospho-D-glycerate by the mutase, and enolase (20 μL of a solution of 0.1 mg/mL) was then added. The reaction was stopped after the appropriate time (depending upon the extent of reaction desired) by the addition of EtOH (10 mL). After removal of the ethanol under reduced pressure, the mixture was subjected to chromatography on a column (18 mL) of DEAE-cellulose in 50 mM NH_4HCO_3, pH 8.0, using a linear concentration gradient (45 + 45 mL; 50–300 mM) of NH_4HCO_3. The elution profile for a typical separation is shown in Figure 1. Fractions containing phosphoglycerate,

ADP, and ATP were separately pooled and freeze-dried. Each pool was then assayed and counted.

Determination of the Kinetic Isotope Effect of Enolase. The kinetic isotope effect of the enolase reaction was determined under the conditions used for the partial conversion experiments. To a solution of 3-phospho-D-glycerate or 3-phospho-D-[2-^{2}H]glycerate in 50 mM triethanolamine hydrochloride buffer, pH 7.12 (2.5 mL), containing $MgCl_2$ (1 mM) and KCl (50 mM), was added ADP (50 μL of a solution of 24 mg/mL), NADH (50 μL of a solution of 7 mg/mL), pyruvate kinase (2 μL of a suspension containing 10 mg/mL), lactate dehydrogenase (2 μL of a suspension containing 10 mg/mL), and phosphoglycerate mutase (50 μL containing 6.4 units). The mixture was thermostated at 30 °C, and the reaction was initiated by the addition of enolase (10 μL of a solution of 0.1 mg/mL). The initial rate of the reaction was determined from the decrease in absorbance due to NADH at 340 nm.

Calculation of the Variation in Specific Radioactivity of Remaining Substrate and of Product after Partial Conversion. Consider the two competing pathways, where S and S′ refer to protonated and deuterated substrate, respectively, and the primed rate constants relate to the reaction of the deuterated species:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} \text{products}$$

$$E + S' \underset{k_{-1'}}{\overset{k_{1'}}{\rightleftharpoons}} ES' \xrightarrow{k_{2'}} \text{products}$$

We have, assuming that the Michaelis equation is obeyed:

$$v = [ES]k_2 = \frac{k_2[E]}{\left[1 + \frac{K_m}{[S]}\left(1 + \frac{[S']}{K_{m'}}\right)\right]}$$

and

$$v' = [ES']k_{2'} = \frac{k_{2'}[E]}{\left[1 + \frac{K_{m'}}{[S']}\left(1 + \frac{[S]}{K_m}\right)\right]}$$

So:

$$\frac{v}{v'} = \frac{dx/dt}{dx'/dt} = \frac{dx}{dx'} = \frac{k_2}{k_{2'}} \frac{\left[1 + \frac{K_{m'}}{[S']}\left(1 + \frac{[S]}{K_m}\right)\right]}{\left[1 + \frac{K_m}{[S]}\left(1 + \frac{[S']}{K_{m'}}\right)\right]}$$

where x and x' are the amounts of protonated and deuterated product formed at time t. Since $k_2/k_{2'} = I$ (the observed kinetic isotope effect), and $K_{m'} = K_m$ (see Figure 2), we have

$$\frac{dx}{dx'} = I\frac{[S]}{[S']}$$

Now let $[S] = a - x$ (where a is the initial concentration of S) and $[S'] = a' - x'$ (where a' is the initial concentration of S′) and integrate, to give:

$$I \ln \frac{a' - x'}{a'} = \ln \frac{a - x}{a}$$

If p is the fractional extent of reaction, and is $(x + x')/(a + a')$, we find:

$$I \ln \left[1 - p\left(1 + \frac{a}{a'}\right) + \frac{x}{x'}\right] - \ln\left(1 - \frac{x}{a}\right) = 0$$

from which, by an iterative procedure, we obtain values of specific radioactivities of remaining starting material and of product at different values of p.

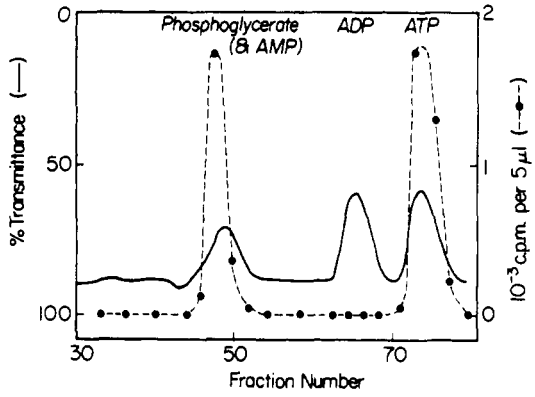


FIGURE 1: Elution profile for the separation of reaction components in the partial conversion of phospho-D-glycerate to pyruvate + ATP. For conditions, see the text.

Results and Discussion

Incubation of 3-[^{18}O]Phospho-D-glycerate and 3-Phospho-D-[2-^2H]glycerate with Phosphoglycerate Mutase. When a mixture of 2- and 3-[^{18}O]phospho-D-glycerate and 3-phospho-D-[2-^{2}H]glycerate is incubated with phosphoglycerate mutase, if the phosphoryl transfer is intramolecular the heavy isotope labels remain separate and the isotopic distribution in the phosphoglycerate mixture remains the same as it was before incubation. If, however, the phosphoryl transfer is intermolecular (for a number of intermolecular possibilities, see Britton et al. (1971)), some molecules will be produced that contain both ^{18}O and ^{2}H labels and the proportion of molecules containing both ^{18}O and ^{1}H will decrease. This furnishes a method for determining the molecularity of the phosphoryl group transfer. Typical results from such experiments are set out in Table I. It is clear that incubation of a mixture of the singly labeled species with phosphoglycerate mutase causes no significant change in the distribution of ions at *m/e* 169–172. There has therefore been no scrambling of phosphoryl groups between carbon skeletons, and *the phosphoglycerate mutase catalyzed reaction must be intramolecular.* As a control, incubation of the same mixture with the cofactor-dependent mutase from rabbit muscle, which is known to catalyze an *inter*-molecular phosphoryl transfer, results in the complete scrambling of ^{18}O and ^{2}H labels (see Table I). The starting ratio of 3- and 2-phosphoglycerate was not the equilibrium ratio (this is because varying amounts of 3-phospho-D-[2-^{2}H]glycerate were added to the 60:40 mixture of 2-[^{18}O]phospho-D-glycerate and 3-[^{18}O]phospho-D-glycerate: see Methods section), and the mixture was assayed for 2- and 3-phosphoglycerate after incubation with mutase to confirm that the mutase had effected the turnover of the substrates. The results of this experiment would be invalidated, of course, if phosphoglycerate mutase catalyzes the exchange of ^{18}O in the phosphoryl group with ^{16}O of the solvent water. That this does not occur has been checked both by allowing the mutase reaction to occur in $H_2{}^{18}O$ and searching for ^{18}O in the substrates, and by incubating ^{18}O-labeled substrate with mutase in $H_2{}^{16}O$ (Breathnach and Knowles, 1977).

Incubation of 3-[^{32}P]Phospho-D-glycerate and 3-Phospho-D-[2-^2H]glycerate with Phosphoglycerate Mutase. The nature of the phosphoryl transfer catalyzed by phosphoglycerate mutase may also be investigated by determining whether any of the radiolabeled phosphoryl group in 3-[^{32}P]phospho-

TABLE I: Mass Spectra of Methylated Labeled Phospho-D-glycerates, before and after Incubation with the Phosphoglycerate Mutases from Wheat Germ and from Rabbit Muscle.[a]

m/e	Phospho-D-[2-^{2}H]-glycerate[b]	[^{18}O]Phospho-D-glycerate[b]	Unlabeled phospho-D-glycerate	Mixture of [2-^{2}H]- and [^{18}O]substrates	Mixture after incubation with wheat germ mutase[c]	Mixture after incubation with rabbit muscle mutase[c,d]	Expd for *intra*molecular transfer[e]	Expd for *inter*molecular transfer[f]
169	0.08	0.22	1.00	0.27	0.33	0.75	0.27	0.75
170	1.00	0.04	0.05	1.00	1.00	1.00	1.00	1.00
171	0.08	1.00	0.02	0.73	0.70	0.55	0.73	0.56
172	0.00	0.06	0.00	0.06	0.05	0.45	0.06	0.42

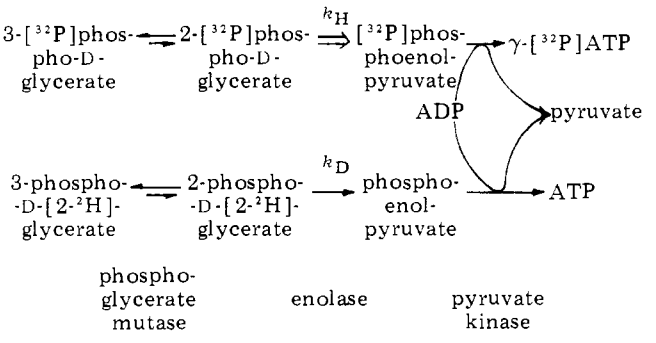
[a] Mass spectra are the means of ten determinations in each case. All observed intensities relate to the peaks for the loss of the carbomethoxy group from the trimethyl esters, and are normalized relative to the most intense peak for these ions. [b] For the preparation of these materials, see the text. [c] The mixture of labeled phospho-D-glycerates (2.7 μmol) was incubated with enzyme from wheat germ (18 units) or rabbit muscle (15 units, in the presence of 2,3-bisphosphate-D-glycerate, 0.27 μmol), in 10 mM Tris-HCl buffer, pH 7.8, at 30 °C for 8 h. [d] Corrected for unlabeled phospho-D-glycerate deriving from added cofactor. [e] That is, the original mixture. [f] Calculated from the spectra of the singly labeled species.

TABLE II: Specific Radioactivity of Remaining Phospho-D-glycerate and of Product Pyruvate after Partial Conversion of a Mixture of 3-Phospho-D-[2-^{2}H]glycerate and 3-Phospho-D-[1-^{14}C]glycerate.

	Initial			Final		
	Total μmol	10^{-6} × total cpm	10^{-6} × sp radioact (cpm/μmol)	Total μmol	10^{-6} × total cpm	10^{-6} × sp radioact (cpm/μmol)
Phosphoglycerate (substrate)	4.10	4.64	1.13	0.51	0.40	0.79
Pyruvate (product)				3.44	4.09	1.23
Phosphoglycerate plus pyruvate	4.10	4.64	1.13	3.95	4.49	1.17

D-glycerate becomes transferred to the deuterium-labeled carbon skeleton of 3-phospho-D-[2-^{2}H]glycerate during the mutase-catalyzed reaction. Following the ingenious approach that was first used by Müllhofer and Rose (1965) with ribulosebisphosphate carboxylase, the distribution of ^{32}P between ^{1}H- and ^{2}H-labeled carbon skeletons can be found using a reaction that discriminates *kinetically* between the ^{1}H and ^{2}H material. The existence of a kinetic isotope effect in the yeast enolase reaction, reported by Shen and Westhead (1973), allows this determination to be made. Consider the situation in which the mutase reaction is intramolecular, and the phosphoryl groups always remain associated with the carbon skeletons they began with. As shown in Scheme I, provided that the enolase reaction is rate limiting (which simply requires an excess of mutase and of pyruvate kinase–ADP), then, since the pyruvate kinase reaction is effectively irreversible, the kinetic isotope effect in the enolase reaction ensures that the protonated [^{32}P]phosphoglycerate will be consumed faster than the deuterated [^{31}P]phosphoglycerate. If the reaction is stopped after partial conversion of the phosphoglycerate into pyruvate, then the specific radioactivity of the remaining phosphoglycerate will be lower than its initial value. Similarly, the specific radioactivity of the ATP produced will be higher than the initial specific radioactivity of the phosphoglycerate. These are inevitable consequences of the greater flux of material through the upper pathway of Scheme I. On the other hand, if the mutase-catalyzed reaction has caused the ^{32}P label to be equally distributed between both ^{1}H- and ^{2}H-labeled carbon skeletons (which would be the result of intermolecular phosphoryl group transfer), then the specific radioactivities of remaining phosphoglycerate and of ATP will be the same as the initial specific radioactivity of the phosphoglycerate substrate.

SCHEME I: Competitive Reaction of 3-[^{32}P]Phospho-D-glycerate and 3-Phospho-D-[2-^{2}H]glycerate with Phosphoglycerate Mutase–Enolase–Pyruvate Kinase.



As a check of the feasibility of the method, a mixture of 3-phospho-D-[2-^{2}H]glycerate and 3-phospho-D-[1-^{14}C]-glycerate was incubated with mutase at pH 7.7 and then converted partially to [1-^{14}C]pyruvate and ATP under the conditions to be used in the final experiments. If the discrimination against the deuterated phosphoglycerate by enolase operates, then the specific radioactivity of the first-formed pyruvate should be higher than that of the starting mixture of 3-phosphoglycerate, and the remaining substrate should have a lower specific radioactivity than the starting mixture. That this is true is shown by the results of Table II. Further, the recovery of material and counts is satisfactory. This preliminary experiment demonstrated that the approach was indeed a viable one.

TABLE III: Specific Radioactivity of Product (ATP) and of Remaining Substrate (Phospho-D-glycerate) after the Partial Conversion of a Mixture of 3-[^{32}P]Phospho-D-glycerate and 3-Phospho-D-[2-^{2}H]glycerate to Pyruvate and ATP.[a]

Extent of reaction (%)	Material	Initial: Total substrate (μmol)	Initial: $10^{-6}\times$ total cpm[b]	Initial: $10^{-6}\times$ sp radioact (cpm/μmol)	Final: Total material (μmol)	Final: $10^{-6}\times$ total cpm[b]	Final: $10^{-6}\times$ sp radioact (cpm/μmol)	Final sp radioact/initial sp radioact
30.4	Phosphoglycerate	4.61	5.16	1.12	3.21[c]	3.00	0.935	0.83
	ATP				1.26	1.86	1.48	1.32
50.2	Phosphoglycerate	4.70	4.23	0.90	2.34[c]	1.51	0.648	0.72
	ATP				2.00	2.17	1.086	1.21
64.4	Phosphoglycerate	4.77	3.81	0.798	1.70[c]	0.628	0.370	0.46
	ATP				2.93	2.63	0.896	1.12

[a] For conditions, see the text. [b] Corrected for the decay of ^{32}P. [c] Assayed as 3- plus 2-phospho-D-glycerate.

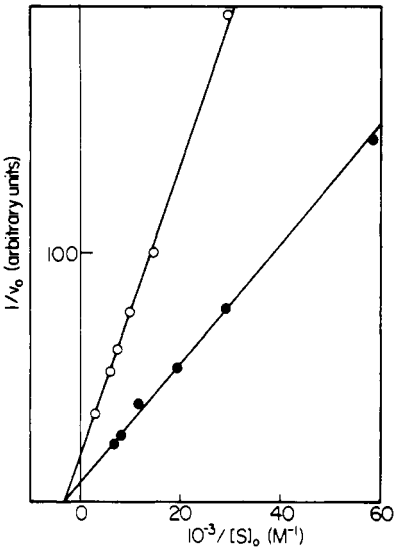


FIGURE 2: Lineweaver-Burk plot for the enolase-catalyzed reaction of 2-phospho-D-[2-^{2}H]glycerate. For conditions, see the text. 3-Phospho-D-glycerate was used as the substrate, in the presence of an excess of phosphoglycerate mutase adequate to maintain the equilibrium between 3- and 2-phospho-D-glycerate. The values of [S]$_0$ are calculated from the initial concentration of 3-phospho-D-glycerate and the equilibrium constant for the phosphoglycerate mutase reaction under these conditions (K_{eq} = [3-isomer]/[2-isomer] = 10.2).

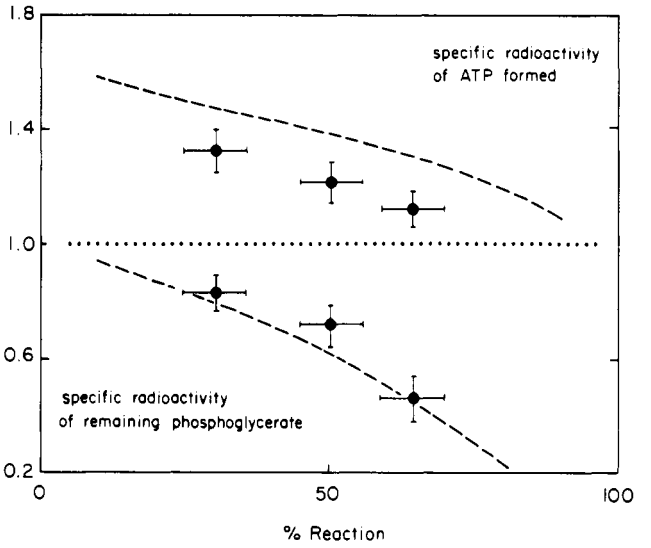


FIGURE 3: Plot of the relative specific radioactivity of ATP product (upper part) and of substrate phospho-D-glycerate remaining (lower part) against the extent of the reaction. For conditions, see the text. The dashed lines are the curves predicted for *intra*molecular transfer, on the basis of the kinetic isotope effect of the enolase reaction and the initial proportion of 3-[^{32}P]phospho-D-glycerate and 3-phospho-D-[2-^{2}H]glycerate used. If the phosphoryl group transfer had been *inter*molecular, *all* the points would have fallen on the dotted horizontal line. The ordinate represents the specific radioactivity of the product or of the remaining substrate relative to that of the substrate at the start of the reaction.

In order to define quantitatively the expected variation in the specific radioactivities of remaining phosphoglycerate and ATP, we require the kinetic isotope effect of the enolase reaction under our conditions of pH, [Mg^{2+}], temperature, etc. The Lineweaver-Burk plot for this experiment is shown in Figure 2, from which it is found (from the ratio of the vertical intercepts) that there is an isotope effect in k_{cat} of k_{cat}^{H}/k_{cat}^{D} = 2.45. Using this value, and the known initial substrate concentrations, the expected values for the specific radioactivities of remaining substrate and of product could be calculated for any percentage of substrate conversion (see Methods). This calculation takes no account of possible wash-out of isotope at C-2 of 2-phosphoglycerate during the enolase-catalyzed reaction, as has been shown to occur with muscle enolase (Dinovo and Boyer, 1971). However, Shen and Westhead (1973) have shown that for enolase from yeast there is less than 5% exchange of the C-2 hydrogen of 2-phosphoglycerate after 50% conversion at pD 7.8, even though at pD 9.2 there is about 50% exchange at 50% conversion. Their statement that there is "very slow exchange at pH 7.2 where the isotope effect is strong" allows us to neglect the possible loss of C-2 deuterium in the present experiments at pH 7.12.

The results of the experiment at three different extents of substrate conversion are shown in Table III, and the specific radioactivities are plotted in Figure 3. The dashed lines show the specific radioactivities calculated for *intra*molecular phosphoryl-group transfer, based upon the known initial rate of protonated-^{32}P and deuterated-^{31}P substrates and the observed kinetic isotope effect of the enolase reaction. For *inter*molecular phosphoryl group transfer, the specific radioactivities would remain unchanged, and all the points would fall on the dotted horizontal line. The data in Figure 3 clearly demonstrate that the phosphoryl group transfer is intramolecular. The values for the specific radioactivity of the remaining phospho-D-glycerate are very close to those calculated for intramolecular transfer. The values for the specific radioactivity of the ATP formed are all somewhat lower than predicted (though all are higher than the specific radioactivity of

the starting material and depend on the extent of reaction in the manner predicted for an intramolecular process), and this is apparently due to the disproportionation of the (nonradioactive) ADP into AMP and ATP during the course of the reaction. There is greater loss of ADP than is accounted for by the extent of the reaction (i.e., by the conversion of phosphoglycerate to pyruvate), and the known disproportionation of ADP (possibly catalyzed by an adenylate kinase contaminant) is a likely reason why the specific radioactivities of the ATP samples are all lower than the predicted values. In confirmation of this view, significant amounts of material eluting in the position expected for AMP were detected from the partial conversion incubations.

The intramolecular nature of the reaction catalyzed by the phosphoglycerate mutase both rules out a number of pathways that have been put forward by analogy with the animal and yeast mutases that require 2,3-bisphospho-D-glycerate as a cofactor, and establishes this isomerization as one of the simplest enzyme-catalyzed phosphoryl-transfer processes known. A number of possible intramolecular pathways are presented in the following paper of this issue (Breathnach and Knowles, 1977), in which further experiments are reported that allow the mechanism to be more narrowly defined.

Acknowledgments

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Appendix: Phosphoglycerate Mutase from Wheat Germ: Isolation, Crystallization, and Properties

Peter F. Leadlay, Richard Breathnach, John Gatehouse, Patricia E. Johnson, and Jeremy R. Knowles

ABSTRACT: The phosphoglycerate mutase from wheat germ has been purified to homogeneity, and crystallized. The enzyme is a monomer of molecular weight close to 60 000, and contains a single thiol group that is only accessible under denaturing conditions. With 3-phospho-D-glycerate as substrate, k_{cat} is 950 s^{-1} and K_m is 0.21 mM.

The enzyme phosphoglycerate mutase is widely distributed in nature, and activity has been detected in over 70 different plant and animal tissues (Ray and Peck, 1972). From most of these sources [EC 2.7.5.3] the mutase activity is low or nonexistent in the absence of 2,3-bisphosphoglycerate. In contrast, the mutase from certain plant sources [EC 5.4.2.1], notably wheat germ (Ito and Grisolia, 1959; Grisolia et al., 1961; Britton, et al., 1971) and rice germ (Fernandez and Grisolia, 1960), functions efficiently in the absence of 2,3-bisphosphoglycerate. Added 2,3-bisphosphoglycerate does not stimulate the reaction rate.

The phosphoglycerate mutases from mammalian muscle and from chicken muscle are dimers of subunit molecular weight 27 000 (Rose, 1970), and a symmetrical tetrameric structure has been reported for the yeast enzyme (Campbell et al., 1972) which also has a subunit molecular weight of about 27 000 (Rose, 1971). The cofactor-independent enzymes from wheat germ and rice germ have been partially purified (Ito and Grisolia, 1959; Grisolia et al., 1961; Britton et al., 1971; Fernandez and Grisolia, 1960) and Britton et al. (1971) have estimated 54 000 for the molecular weight of the wheat germ mutase. This enzyme has not been examined for possible subunit structure.

In this Appendix we report an improved purification procedure and partial characterization of the 2,3-bisphosphoglycerate-independent enzyme from wheat germ.

Experimental Procedures

Materials. The following enzymes were obtained from the Sigma Chemical Co.: lactate dehydrogenase, pyruvate kinase and enolase (as lyophilized salt-free powders for use in kinetic studies), bovine serum albumin (crystallized and lyophilized), and ovalbumin (lyophilized). The following enzymes were obtained from the Boehringer Corp.: D-glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle, cytochrome *c* from horse heart, and chymotrypsinogen A from bovine pancreas. Crystalline triosephosphate isomerase was isolated by J. Law, according to Putman et al. (1972).

3-Phospho-D-glycerate as its barium salt (from which the barium was removed by treatment with Dowex-50 [H^+ form]) and as its sodium salt (free from 2,3-bisphosphoglycerate), 2-phospho-D-glycerate as its sodium salt, 2,3-bisphosphoglycerate as its di-Tris salt, ATP, ADP, NAD^+ and NADH, and phosphoenolpyruvate were all obtained from Sigma. All other chemicals were of the highest grade available.

Diethylaminoethylcellulose in microgranular form (DE52) and unmodified cellulose were supplied by Whatman. Diethylaminoethyl-Sephadex (A50) and diethyl-(2-hydroxypropyl)aminoethyl-Sephadex (QAE-Sephadex) (A50) were obtained from Pharmacia. Dowex-1 (Cl^- form, 200–400 mesh,

TABLE IV: Purification of Phosphoglycerate Mutase from Wheat Germ.[a]

Fraction	Vol (mL)	Total protein (mg)[b]	Total act. (units)[c]	Sp act. (units/mg)	Purification	Recovery (%)
I. Extract (pH 8)	340	13 600	16 600	1.2	1	100
II. Acetone cut (43-57%)	38	822	9 970	12	10	60
III. Ammonium sulfate cut (1.7-2.4 M)	9	387	11 033	29	23	66
IV. QAE-Sephadex (pH 7.5)	230	47	8 533	180	150	52
V. Sephadex G-200	21	12	4 620	370	305	28
VI. DEAE-cellulose[d] (pH 6.6)	130	1.75	3 060	1750	1430	18

[a] From 100 g of wheat germ. A scale-up of fivefold has been found satisfactory, though in this case the yield may be lower in the early steps. [b] Assuming $E_{10\ mm}^{0.1\%}$ of 1.0 at 280 nm. [c] See the text. The simpler assay that monitors phosphoenolpyruvate formation is not very reliable in the assay of crude extracts, but suffices to identify fractions containing enzyme. [d] Data for the pool of both peaks of mutase activity (see Figure 4).

8% cross-linked) and Dowex-50 (H^+ form) were supplied by Sigma.

Prewetts "Viga-Vyte" wheat germ was purchased from Wesley Lloyd Ltd. (Oxford). General Mills wheat germ was a gift of Professor L. Bogorad.

Methods. Phosphoglycerate mutase was assayed by coupling the formation of 2-phosphoglycerate from 3-phosphoglycerate with the enolase-catalyzed reaction (Rodwell et al., 1957). The assay mixture contained 3-phospho-D-glycerate (20 mM), $MgCl_2$ (8.3 mM), and enolase (2.3 units), in 0.1 M Tris-HCl buffer, pH 8.7, and the reaction was initiated by the addition of the mutase sample (25 μL to 2.975 mL of assay mixture). The absorbance at 240 nm was monitored at 30 °C. A unit of activity is defined as the amount of enzyme required to increase the absorbance at 240 nm by 0.1/min in a cuvette of 10-mm path length. Under these conditions, 1 unit corresponds to the conversion of 5.5 μmol of 3-phosphoglycerate to 2-phosphoglycerate per min.

For the determination of kinetic parameters, the enzyme was assayed at 30 °C by further coupling with pyruvate kinase and lactate dehydrogenase. The assay mixture contained: 3-phospho-D-glycerate, sodium salt (up to 0.89 mM), ADP (0.2 mM), KCl (20 mM), $MgCl_2$ (5 mM), and NADH (0.2 mM) in 40 mM β,β-dimethylglutarate-NaOH buffer, pH 7.0. The solution also contained lactate dehydrogenase (8.3 μg/mL), pyruvate kinase (15 μg/mL), and enolase (7.5 μg/mL), and the reaction was initiated by the addition of the mutase sample (25 μL to 2.975 mL of assay mixture). The NADH absorbance at 340 nm was followed.

3-Phospho-D-glycerate was assayed as described by Maister et al. (1976).

Protein concentration was determined from the absorbance at 280 nm, assuming that the absorbance at this wavelength (in a 10-mm path-length cuvette) of a 1% (w/v) solution of protein is 10.0.

Analytical ultracentrifugation was kindly performed by Dr. P. Esnouf of the Nuffield Department of Clinical Biochemistry, Oxford.

Results

Protein Purification. All operations were done at 0-4 °C unless otherwise stated. All solutions were made up using distilled-deionized-distilled water. All buffers contained 2-mercaptoethanol (20 mM). A summary of the purification procedure is given in Table IV.

(*1*) *Extraction.* Fresh wheat germ (100 g) was stirred with 10 mM triethanolamine hydrochloride buffer (400 mL), pH 8.0, for 1 h. After centrifugation at 16 000*g* for 30 min, the supernatant (340 mL), fraction I, was decanted.

(*2*) *Acetone Precipitation.* Acetone (250 mL of electronic grade), precooled to −20 °C, was added dropwise with continuous stirring to fraction I at −20 °C over a period of 30 min. The temperature of the mixture was maintained just above its freezing point throughout the addition. After standing for 1 h at −20 °C, the suspension was centrifuged at 4100*g* and −10 °C for 20 min, and the pellet was discarded. Acetone (160 mL), precooled to −20 °C, was added dropwise with stirring to the supernatant (490 mL) at −20 °C over a period of 30 min. The mixture was kept at −20 °C for 1 h, and the precipitate formed was then removed by centrifugation as above, and immediately suspended in 10 mM triethanolamine hydrochloride buffer (40 mL), pH 8.0, to give fraction II.

(*3*) *Ammonium Sulfate Precipitation.* To fraction II, solid ammonium sulfate was added slowly with stirring, to 1.7 M (240 g/L). After 2 h, the suspension was centrifuged at 20 000*g* for 20 min, and the pellet was discarded. To the supernatant (45 mL) a further 120 g/L of ammonium sulfate was added to bring the concentration to 2.4 M. After 2 h, the solution was centrifuged at 20 000*g* for 20 min. The resulting pellet was suspended in 40 mM sodium phosphate buffer (6 mL), pH 7.5, to give fraction III.

(*4*) *QAE-Sephadex Chromatography.* Fraction III was clarified by centrifugation at 20 000*g* for 10 min and applied to a column (5.3 $cm^2 \times 60$ cm) of Sephadex G-25 (medium) equilibrated with 40 mM sodium phosphate buffer, pH 7.5. The column was eluted with the same buffer at a flow rate of 100 mL/h. The protein-containing fractions were pooled (50 mL) and applied to a column (5.3 $cm^2 \times 40$ cm) of QAE-Sephadex (A50), equilibrated with 40 mM sodium phosphate buffer, pH 7.5. The column was then washed with the same buffer (500 mL) and the enzyme was eluted with a linear gradient (750:750 mL) of sodium phosphate buffer (40-140 mM), pH 7.5. Fractions of 15 mL were collected, at a flow rate of 30 mL/h, and those of more than twice the specific activity of fraction III were pooled, to give fraction IV.

(*5*) *Sephadex G-200 Chromatography.* The proteins in

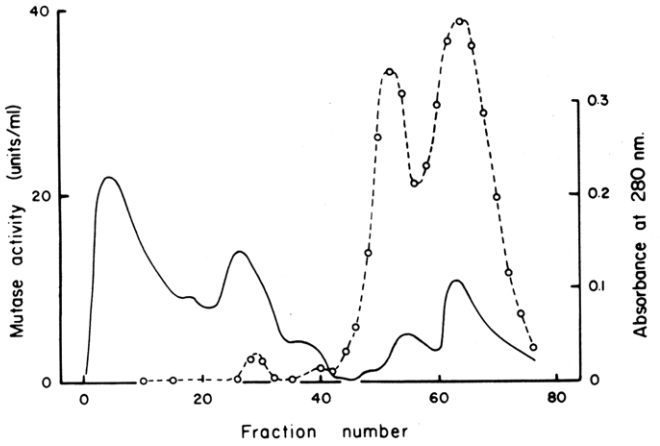

FIGURE 4: Elution profile of wheat germ phosphoglycerate mutase from DEAE-cellulose. Solid line, absorbance at 280 nm; broken line, mutase activity.

fraction IV were precipitated by the addition of solid ammonium sulfate to 3.9 M (520 g/L). The pH was maintained at 7.5 by the addition of ammonia solution (1 M). The pellet obtained after centrifugation at 13 000*g* for 30 min was suspended in 1 mL of 40 mM sodium phosphate buffer, pH 7.5. The solution was clarified by centrifugation at 20 000*g* for 10 min and applied to a column (2.0 $cm^2 \times 100$ cm) of Sephadex G-200 equilibrated with the same buffer. Fractions of 1.5 mL were collected, at a flow rate of 6 mL/h, and those of more than twice the specific activity of fraction IV were pooled, to give fraction V.

(*6*) *DEAE-Cellulose Chromatography.* The pH of fraction V was adjusted to 6.6 with HCl immediately before applying the solution to a column (0.64 $cm^2 \times 60$ cm) of DEAE-cellulose (DE-52) equilibrated with 40 mM sodium phosphate buffer, pH 6.6. The column was washed with the same buffer (100 mL) and eluted with a linear gradient (200:200 mL) of sodium phosphate buffer (40–90 mM), pH 6.6. Fractions of 4 mL were collected, at a flow rate of 45 mL/h. As can be seen from Figure 4, two peaks of mutase activity elute from the column. The specific catalytic activities of the mutase in these peaks are very similar, and each is more than 90% homogeneous on polyacrylamide gel electrophoresis. The two materials run identically on gel electrophoresis in the presence of sodium dodecyl sulfate. Unless otherwise stated, the combined pool (fractions 48–72) was used. Mutase-containing fractions were brought to pH 7.2 by addition of 1.0 M NaOH. The enzyme was precipitated by addition of ammonium sulfate to 2.4 M and stored as a suspension at 0–4 °C.

Electrophoresis. The protein from step 6 was subjected to polyacrylamide gel electrophoresis (Davis, 1964). The single protein band (from material in the second activity peak: see Figure 4) coincided with the peak of mutase activity recovered from slices of an identical but unstained gel. Recovery of activity from the gel was only 10%, but this sufficed to establish the identity of the protein. In the presence of sodium dodecyl sulfate, polyacrylamide gel electrophoresis (Shapiro et al., 1967) of the two pooled activity peaks (see Figure 4) showed a single band.

Crystallization. The enzyme was crystallized by the method of Jakoby (1968). Phosphoglycerate mutase (6 mg) in 20 mL of 20 mM sodium phosphate buffer, pH 7.1, containing 2-mercaptoethanol (20 mM) was precipitated by the addition of ammonium sulfate (to 3.9 M). The pellet obtained after centrifugation at 19 000*g* for 30 min was suspended in the same buffer containing ammonium sulfate (2.9 M) and allowed to equilibrate at 0–3 °C for 5 min. This suspension was centrifuged at 19 000*g* for 10 min and the supernatant was carefully removed with a Pasteur pipet. The pellet was reextracted successively with buffer containing decreasing concentrations of ammonium sulfate (2.7, 2.4, 2.2, 2.0, and 1.8 M) and the six supernatants were each left at room temperature. After several days, crystals were visible (Figure 5) in the solution containing 1.8 M ammonium sulfate. The identity of the crystals was confirmed by disc gel electrophoresis in the presence of sodium dodecyl sulfate.

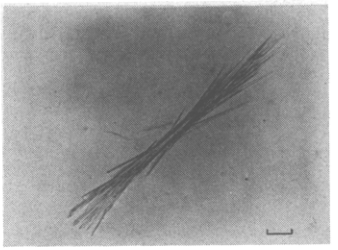

FIGURE 5: Crystalline phosphoglycerate mutase. The scale bar is 0.1 mm.

Molecular Weight. The molecular weight of the enzyme was determined by three methods. First, the electrophoretic mobility of the reduced and denatured protein in the presence of sodium dodecyl sulfate gave a value of 62 000 for phosphoglycerate mutase (average of eight determinations). Secondly, a preliminary study by equilibrium sedimentation using the method of Chervenka (1966) gave an approximate value for the native enzyme of 54 000, assuming a partial specific volume of 0.75. Thirdly, the native enzyme was subjected to gel filtration on Sephadex G-100 in 0.1 M sodium phosphate buffer, pH 6.75, containing KCl (0.5 M) and 2-mercaptoethanol (20 mM). This yielded a molecular weight of 58 000 (Andrews, 1964) (two determinations). These data indicate that the phosphoglycerate mutase from wheat germ is a monomeric protein with a molecular weight of approximately 60 000.

Thiol Content. The thiol content of the enzyme was determined by titration with 5,5′-dithiobis(2-nitrobenzoic acid) and with *p*-chloromercuribenzoate. In the first method (Ellman, 1959), phosphoglycerate mutase (0.85 mg/mL, specific activity 1100 units/mg) in 0.4 M Tris-HCl buffer, pH 8.0 (1 mL), was treated with 5,5′-dithiobis(2-nitrobenzoic acid) (0.2 mL of a 10 mM solution) and the absorbance at 412 nm was monitored. No change in absorbance was observed, and the enzyme retained all its activity. After 15 min, sodium dodecyl sulfate was added (to 0.2%, w/v), and a rapid increase in absorbance due to 5-thio-2-nitrobenzoate was observed, corresponding to 0.99 ± 0.08 thiol group per mole of mutase. In the second method (Boyer, 1954), portions (5 μL) of *p*-chloromercuribenzoate (0.5 mM) were added to a solution of phosphoglycerate mutase (0.16 mg) in 0.4 M Tris-HCl buffer, pH 8.0 (1 mL), containing sodium dodecyl sulfate (0.1 mL of a 20%, w/v, solution), and the increase in absorbance at 250 nm was measured. This method indicates the presence of 0.88 ± 0.05 thiol group per mole of enzyme.

pH Stability. Phosphoglycerate mutase (50 μg) in 0.1 M sodium phosphate buffer, pH 7.0 (0.2 mL), was added to 1 mL each of a series of buffer solutions containing 20 mM 2-mercaptoethanol (β,β-dimethylglutarate, sodium phosphate, triethanolamine hydrochloride, sodium carbonate–bicarbonate, and sodium phosphate–NaOH) of pH values between 4.5 and 11, and dialyzed against the same buffer (100 volumes)

for 10 h at 4 °C. Little or no activity loss was observed on incubation at pH values between 6.0 and 10. The sample at pH 11 retained approximately 60% of its activity, and the samples incubated at pH 5.5 and below were found to have lost all their activity.

Discussion

Enzyme Isolation. Several procedures have been published for the partial purification of phosphoglycerate mutase from wheat germ (Ito and Grisolia, 1959; Grisolia et al., 1961; Britton et al., 1971). However, even the best of these (Britton et al., 1971) could not be repeated satisfactorily, and it was necessary to devise a new method. The enzyme is readily extracted from fresh commercial wheat germ, though contrary to earlier reports the mutase is rather unstable in crude extracts. The presence of mercaptoethanol and the early acetone fractionation largely overcomes this problem. EDTA inhibits the enzyme reversibly, and was not included in the extraction buffer. An acetone cut followed by an ammonium sulfate fractionation step produces an effective purification (see Table IV). The enzyme loses activity during chromatography in cationic amine buffers, and phosphate buffers were used for the three subsequent chromatographic steps. Despite the loss of activity on the final DEAE-cellulose column at pH 6.6, it was not found possible to effect the appropriate purification at a higher pH.

The enzyme, purified 1400-fold from the crude extract, is more than 90% homogeneous on polyacrylamide gel in the absence or presence of sodium dodecyl sulfate. The purified enzyme is stable between pH 6 and 10, and its stability (when pure) is unaffected by 2-mercaptoethanol. The enzyme contains a single thiol group that is only accessible to thiol reagents under denaturing conditions. From the behavior of the purified enzyme in gel filtration, in the ultracentrifuge, and on polyacrylamide gel electrophoresis in sodium dodecyl sulfate, it is evidently a monomer of molecular weight close to 60 000.

The classical assay for phosphoglycerate mutase (Rodwell et al., 1957) involves the conversion of the product 2-phosphoglycerate to phosphoenolpyruvate by enolase. The phosphoenolpyruvate is detected by its absorbance at 240 nm. Unfortunately, the differential absorption due to phosphoenolpyruvate at 240 nm depends in a complex way on pH, Mg^{2+} concentration, and ionic strength, and this assay is not suitable for kinetic work. However, the equilibrium constants for the conversion of 2-phosphoglycerate to phosphoenolpyruvate to pyruvate to lactate allow the production of 2-phosphoglycerate to be monitored by coupling to enolase + pyruvate kinase-ADP + lactate dehydrogenase-NADH. The oxidation of NADH is then followed at 340 nm. The validity of this assay was established by demonstrating a linear dependence on mutase concentration up to 30 ng/mL, under the conditions specified under Methods. While no allowance was made for the effect of magnesium ion, k_{cat} and K_m for 3-phosphoglycerate were found to be 950 ± 80 s^{-1} and 0.21 ± 0.05 mM, respectively (pH 7.0, in 40 mM β,β-dimethylglutarate-NaOH buffer, 30 °C).

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