The Phosphorylation of Diphosphoglycerate Mutase*

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SUMMARY

Diphosphoglycerate mutase, purified to apparent homogeneity from human erythrocytes, was found to have a molecular weight of 60,000 (gel filtration) and subunit weight of 32,000 (electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate). One \(^{32}P\)phosphoryl group is covalently bound per subunit upon incubation of enzyme with either the \(^{33}P\)-labeled substrate, 1,3-diphosphoglycerate, or the product, 2,3-diphosphoglycerate. The phosphate group is transferred to either 2- or 3-phosphoglycerate or to water if glycolate-2-P is added to phosphorylated enzyme. The phosphoryl group was stable at alkaline pH but was liberated from the denatured phosphoprotein in the acid range at rates consistent with a phosphoramidate linkage to histidine. Since a similar phosphorylation reaction had been shown previously with monophosphoglycerate mutase, it was necessary to achieve complete separation in the preparation of the enzyme.

Diphosphoglycerate mutase catalyzes the intermolecular transfer of the acyl phosphate of 1,3-DPG\(^{1}\) to an acceptor molecule of monophosphoglycerate with the formation of 2,3-DPG. Either 2-PGA or 3-PGA can act as the phosphoryl acceptor (Equation 1) with higher rates observed in the presence of 2-PGA (1). Kinetic studies failed to show the participation of a phosphoryl enzyme intermediate (1).

\[
1,3\text{-DPG} + 3\text{-PGA (or 2-PGA)} \rightarrow 2,3\text{-DPG} + 3\text{-PGA} \quad (1)
\]

Intersecting lines were obtained in double reciprocal plots of either substrate as a function of the second, constant substrate. In addition, attempts to show the equilibration shown in Equation 2 were unsuccessful.

\[
1,3\text{-DPG} + 3\text{-[\text{U}^{14}C]PGA} = 1,3\text{-[\text{U}^{14}C]DPG} + 3\text{-PGA} \quad (2)
\]

Although these results indicate that a ternary complex of enzyme, phosphoryl donor, and phosphoryl acceptor is an intermediate, they do not rule out the subsequent phosphorylation of the enzyme as a necessary step.

In the present study the possible participation of phosphorylated enzyme has been reinvestigated by the method of direct isolation used previously with the monophosphoglycerate mutases of muscle (2) and yeast (3). The cofactor, 2,3-DPG, phosphorylates these enzymes forming an acid-labile linkage identified as 3-phosphohistidine (2, 3). It has also been shown that 1,3-DPG, the substrate of diphosphoglycerate mutase, can phosphorylate monophosphoglycerate mutase (2, 4). Therefore, to investigate the possible phosphorylation of diphosphoglycerate mutase, it was necessary to obtain a preparation free of monophosphoglycerate mutase. The best preparation obtained previously contained monophosphoglycerate mutase activity (1). In the present study an apparently homogeneous preparation of diphosphoglycerate mutase has been obtained, its phosphorylation shown, and the role and nature of the phosphoryl group examined. The monophosphoglycerate mutase activity remaining in the diphosphoglycerate mutase appears to be an intrinsic property of the enzyme.

EXPERIMENTAL PROCEDURE

Materials—\(\text{H}^{+}\)-Drosylapatite was from Bio-Rad. Ampholytes were obtained from LKB. Sephadex and blue dextran were from Pharmacia. Carbowax (flaked polyethylene glycol, 20,000) was from Union Carbide. \[^{32}P\]Phosphoric acid, carrier-free, was purchased from New England Nuclear. 3-PGA (barium salt), containing 1% 2,3-DPG, was from Schwarz. 2-PGA (sodium salt) and 2,3-DPG (cyclhexylamine salt) were from Boehringer. Glycolate-2-P (phosphoglycolate) was from General Biochemicals.

Proteins purchased were: monophosphoglycerate mutase (rabbit muscle) and cytochrome \(c\) from Boehringer, ovalbumin (lyophilized) from Sigma, alcohol dehydrogenase (yeast) from Worthington. \[^{14}C\]Ketodeoxygluconate-6-P aldolase was the gift of Dr. H. P. Meloche. Bovine serum albumin was obtained from Pentex.

Methods—1,3-[\text{U}^{32}P]DPG (1) and 2,3-[\text{U}^{32}P]DPG (5) were prepared as reported previously. Protein was determined by the procedure of Warburg and Christian (6). 2,3-DPG was determined with glycolate-2-P to stimulate the diphosphoglycerate phoshatase activity of monophosphoglycerate mutase; the resulting phosphoglycerate was determined enzymatically (7). Phosphorylated protein was separated from small molecules by extraction with phenol (8) as follows: to each 0.2 ml sample was added 1.0 ml of phenol, saturated with Tris-Cl buffer, 0.05 M, pH 8, containing 1 mM sodium phosphate. This

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1 The abbreviations used are: 1,3-DPG, 1,3-diphosphoglycerate; 2,3-DPG, 2,3-diphosphoglycerate; 2-PGA, 2-phosphoglycerate; 3-PGA, 3-phosphoglycerate.
was extracted with 2 ml of the same buffer mixture, saturated with phenol, followed by two 4-ml washes with the same buffer. When the distribution of phosphate between inorganic and organic phosphate compounds was determined, the first aqueous extracts were treated with acid molybdate and extracted with isobutyl alcohol-phenol-benzene (1:1:1) (9).

Diphosphoglycerate mutase was assayed spectrophotometrically. The observed rates have been multiplied by 3 in order to obtain a true rate of formation of products (1). Monophosphoglycerate mutase was determined according to the method of Cowgill and Pizer (10) by coupling to enolase and measuring the increase in absorbance at 240 nm due to the conversion of the 2-PGA formed to P-enolpyruvate. A change of 0.975 per min indicated the formation of 1 µmole of substrates to products per min at 25°C under the conditions of the assay.

**RESULTS**

**Enzyme Purification**—For the phosphorylation studies it was necessary to obtain enzyme free of monophosphoglycerate mutase. The purification procedure used was essentially that reported previously (1). Although there was separation of the two enzymes upon fractionation with ammonium sulfate as well as upon chromatography on hydroxypatite, the best preparation obtained at that time contained 1% monophosphoglycerate mutase activity. In the present study, in spite of efforts to improve the separations obtained at each step in the preparation, the diphosphoglycerate mutase obtained after fractionation through the hydroxylapatite step had 3% monophosphoglycerate mutase activity.

**Second Hydroxylapatite Column**—In order to determine whether the monophosphoglycerate mutase activity in the diphosphoglycerate mutase was due to trailing of the former peak (Fig. 1A), the diphosphoglycerate mutase was rechromatographed on hydroxylapatite. Enzyme with 19 units of diphosphoglycerate mutase activity was applied to a hydroxylapatite column, 1.5 ml, equilibrated with potassium phosphate, 5 mM, pH 7.2, containing 1 mM dithiothreitol. The enzyme was eluted with a 100-ml linear gradient in which potassium phosphate, 5 mM, pH 7.2, containing 0.5 mM dithiothreitol, increased from 5 to 100 mM. The flow rate of the column was 6.7 ml per hour and 2-ml fractions were collected. There was no monophosphoglycerate mutase detectable in the fractions preceding the diphosphoglycerate mutase peak, which eluted with 41 to 67 mM phosphate (Fig. 1B). As little monophosphoglycerate as 0.002 unit per ml would have been detected. The recovery of diphosphoglycerate mutase was 16 units or 84%. Monophosphoglycerate mutase activity was found in the fractions containing diphosphoglycerate mutase with the peak activity for both enzymes in the same fractions. The monophosphoglycerate mutase activity was 3% of the diphosphoglycerate mutase level, as in the starting material for this step.

**Isoelectric Focusing**—The column contained a 1% ampholyte solution, pH range 4 to 6, in a sucrose gradient; the anode was the lower electrode. A cooling bath at 7°C was used. The pH gradient was allowed to form overnight in order to remove any metal ions and to minimize the time the enzyme would be on the column. The concentrated sample of enzyme from the second hydroxylapatite column (1.5 ml) was adjusted to contain 10% sucrose and applied about one-fourth of the distance from the top of the column with a Pasteur pipette extended with a piece of polyethylene tubing, 1.2 mm in diameter. The current flowed again for 3½ hours by which time it had reached a constant value of 1 ma. Fractions of 2 ml were collected. The peak of diphosphoglycerate mutase activity was found in fractions of pH 6.05 to 6.40 (average value pH 6.25). Since we found in separate experiments that red cell monophosphoglycerate mutase has an isoelectric point, pl, of 6.10 to 6.40 (average value 6.25), if traces of that enzyme had been present initially, they would be removed by this procedure. The active fractions (10 ml) were combined and concentrated with Carbowax to 1 ml. In order to stabilize the activity of the still dilute protein solution, bovine serum albumin to 1 mg per ml and EDTA to 1 mM were added. The enzyme was stored in pellets in liquid nitrogen. Of 5 units of diphosphoglycerate mutase that had been applied to the column, 3 units (60%) were recovered. This material was used for the initial phosphorylation studies. Monophosphoglycerate mutase activity was still detectable at 1 to 3% of the diphosphomutase level.

**Molecular Weight of Enzyme and Subunit**—The molecular weight was determined by gel filtration on Sephadex G-150 (11, 12). A column, 2.5 × 40 cm, was equilibrated with a solution containing: potassium phosphate buffer, pH 7.2, 5 mM; glycerol, 20%, KCl, 0.1 m; and 2-mercaptoethanol, 1 mM. The flow rate was 16 ml per hour. The void volume was 72 ml as determined by the elution of blue dextran. The column was calibrated from the peaks of the elution volumes of the components of a mixture of proteins of known molecular weight: cytochrome c (12,400); ovalbumin (44,000); phosphoglycerate mutase (rabbit muscle) (57,000); alcohol dehydrogenase (yeast) (151,000). After re-equilibration of the column, a dialyzed red cell ammonium sulfate fraction containing diphosphoglycerate mutase (15 units) and monophosphoglycerate mutase (2 units), cytochrome c, and yeast alcohol dehydrogenase was applied to the column. Both the red cell monophosphoglycerate mutase and diphosphoglycerate mutase had an elution volume corresponding to a molecular weight of 60,000.

Enzyme that had been purified through the isoelectric focusing step was analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (13). Fig. 2 shows that it appears to contain a single component in addition to the bovine serum albumin which had been added to stabilize the activity. The enzyme band corresponds to a molecular weight of 32,000. It appears from this that the diphosphoglycerate mutase molecule is a dimer.

**Phosphorylation of Diphosphoglycerate Mutase**—When the substrate 1,3-[32P]DPG is incubated with diphosphoglycerate mutase, radioactivity is found associated with the enzyme in a catalytic intermediate. Phosphorylation of the enzyme also occurs when the product, 2,3-[32P]DPG reacts with the enzyme. Using enzyme purified through the isoelectric focusing step with 0.035 unit of diphosphoglycerate mutase and 1.12 × 10⁻² unit of monophosphoglycerate mutase activity, and with 2,3-[32P]DPG as the phosphorylating agent, 0.06 n mole of 32P is found associated with the enzyme. The titration with 1,3-[13C]DPG indicates maximal binding that approaches 0.03 n mole, or only half as much as observed with 2,3-DPG as phosphorylating agent.

The data shown in Table I help to explain the lower apparent stoichiometry for 1,3-DPG. The experiment also illustrates that the phosphoryl group can be transferred from the enzyme in the presence of an appropriate acceptor, a necessary property for a catalytic intermediate. Phosphorylation of the enzyme is detected by the use of phenol. The addition of phenol stops the
reaction. When phenol and aqueous phases are separated, the protein remains in the phenol phase and small molecules are found in the aqueous phase. Radioactivity in the phenol phase indicates the presence of $^{32}$P covalently bound to the protein. In Table I, Experiment A, the zero time control, which lacks enzyme, illustrates the lability of the acyl phosphate of 1,3-DPG in the presence of the acid molybdate used in the procedure for the differentiation between organic and inorganic phosphates since, in this case, most of the $^{32}$P analyzes as $P_i$. As shown for the total reaction sample, when acid is added to the enzymatic system after 2 min, most of the $^{32}$P is found in acid-stable form indicating that the 1,3-DPG has been converted to the product, 2,3-DPG. A similar incubation, designated as having had no additions to indicate that no phosphoryl acceptor has been added, was stopped with phenol instead of acid, and the phenol phase extracted with aqueous buffer. The radioactivity (2054 cpm) in the phenol phase indicates the formation of 0.027 nmole of phosphoenzyme based on the specific activity of the acyl phosphate of 1,3-DPG. The radioactivity in $^{32}$P, indicates that the sample of 1,3-DPG contained 8% $P_i$. When one of the normal phosphoryl acceptors, 3-PGA or 2-PGA, is added to an incubation containing phosphorylated enzyme, the $^{32}$P is transferred
Fig. 2. Electrophoretic pattern of diphosphoglycerate mutase in polyacrylamide gel containing sodium dodecyl sulfate. Standards used for the determination of molecular weights were: ribonuclease, 13,700; phosphoglycerate mutase, 27,000; lactate dehydrogenase, 36,000; bovine serum albumin, 68,000.

from the enzyme to form 2,3-DPG as shown by the fact that no 32P is found associated with the protein in the phenol phase and no 32P is generated. When glycolate-2-P, a structural analogue of the normal acceptors, is added to phosphorylated enzyme, the 32P is also released from the enzyme, as shown by the loss of radioactivity from the phenol phase. In this case half of the radioactivity is found as P_i. Thus, glycolate-2-P induces the enzyme to behave as a phosphatase, releasing half of the phosphoryl groups of 2,3-DPG as P_i.

Observations made with 2,3-[U-32P]DPG as phosphorylating agent (Table I, Experiment B) are relevant to an understanding of the experiments with 1,3-[l-32P]DPG. After incubating 2,3-[U-32P]DPG with enzyme, 4,640 counts or 0.052 nmole of 32P were found bound to protein in the phenol phase. This radioactivity was released from the protein upon addition of 3-PGA or 2-PGA. No P_i was formed under these conditions. The addition of glycolate-2-P to an incubation containing phosphorylated enzyme resulted in the transfer of the radioactivity from the protein. Half of the 32P was found in P_i and half as P_i. 

The results are consistent with the transfer reactions shown (Equations 3 and 4) which produce singly labeled 2,3-DPG and [32P]phosphoglycerate from doubly labeled 2,3-DPG.

\[
\begin{align*}
2,3-[U-32P]DPG & \rightarrow 2,3-[2-32P]DPG + 3-[32P]PGA \\
2,3-[U-32P]DPG + 2-PGA & \rightarrow 2,3-[3-32P]DPG + 2-[32P]PGA
\end{align*}
\]

TABLE I

**Transfer of phosphoryl group from diphosphoglycerate mutase**

In incubations were at 25° in a 0.2-ml volume and contained: 0.027 nmole based on the original specific activity of the 1,3-[l-32P]DPG.

| Incubation conditions | Experiment A | Experiment B |
|-----------------------|--------------|--------------|
|                       | (1,3-[l-32P]DPG) | (2,3-[U-32P]DPG) |
| Zero time             | 10           | 25           |
|                       | 33,400       | 630,712,200  |
| Total reaction        |              |              |
|                       |              |              |
| No additions          | 2,054*       | 645,624,600  |
|                       | 2,497,314,800|              |
| Plus 3-PGA (0.2 nmole) | 18           | 540,72,600   |
|                       | 2,730,32,400 |              |
| Plus 2-PGA (0.2 nmole) | 16           | 652,71,100   |
|                       | 3,110,33,900 |              |
| Plus glycolate-2-P (0.2 nmole) | 18 | 28,800 96,200 |
|                       | 16,700,14,500|              |

* 0.027 nmole based on the original specific activity of the 1,3-[l-32P]DPG.

* 0.052 nmole.
No phosphatase activity is observed under these conditions. In the presence of glycolate-2-P, phosphoryl transfer occurs and $P_1$ is released (Equation 5).

$$2,3-[\text{U}-\text{32P}]\text{DPG} \stackrel{\text{glycolate-2-P}}{\longrightarrow} 2\text{-PGA} + \text{32Pi}$$

(5)

Only half of the $\text{32P}$ in 2,3-DPG is released. Diphosphoglycerate mutase has no phosphatase activity for monophosphoglycerates, indicating that only the diphosphates can phosphorylate the enzyme.

In Experiment A of Table I it was shown that before the addition of the phosphoryl acceptors, the 1,3-DPG had been completely converted to 2,3-DPG. Therefore the results observed must be, and indeed are, the same no matter which diphosphate was used initially. The apparent lower stoichiometry observed when starting with 1.3 $[\text{U}-\text{32P}]$DPG is consistent with randomization of the radioactivity into the two phosphoryl groups of 2,3-DPG. Initially the product is 2',3'-[2-$\text{32P}$]DPG (1). Therefore randomization must occur when 2,3-DPG phosphorylates the enzyme, indicating that either phosphoryl bond of 2,3-DPG can act as the phosphoryl donor. This shows the monophosphoglycerate mutase activity of the enzyme since either 2-PGA or 3-PGA is produced. It is also in agreement with the observation that either 2-CPG or 3-CPG can act as phosphoryl acceptor in the diphosphoglycerate mutase reaction (1).

Subunit Structure of Diphosphoglycerate Mutase—After isoelectric focusing, the enzyme preparation appeared to be homogeneous on polyacrylamide gel electrophoresis in sodium dodecyl sulfate except for a band attributed to added bovine serum albumin (Fig. 2). In order to substantiate that the protein in the 32,000 molecular weight band is indeed the one that is phosphorylated by diphosphoglycerate, the migration of $\text{32P}$-enzyme, phosphorylated with 2,3-[2-$\text{32P}$]DPG, was determined under the same conditions. $[^{14}C]$Ketodeoxypyruvate aldolase, with a subunit weight of 24,000 (14), was included as an internal standard in both an unstained gel containing $[^{32P}]$diphosphoglycerate mutase and a stained gel containing diphosphoglycerate mutase and ribonuclease. The $[^{32P}]$P is lost from diphosphoglycerate mutase on destaining, making it necessary to compare two gels. It was found that relative to the $[^{14}C]$ standard, the $[^{32P}]$P ran as would a molecule of 31,200 and the stained band ran as a molecule of 31,700. From the specific activity of the $[^{32P}]$P, if one phosphoryl group combined per subunit, the gel in Fig. 2 contained 1.3 $\mu$g of a phosphorylated protein with a weight of 32,000.

Chemical Nature of Phosphoryl Group—The phosphoryl linkage in $[^{32P}]$diphosphoglycerate mutase is acid-labile. Thus, when a pleural extract containing $[^{32P}]$phosphoenzyme is treated with acid molybdate and extracted in the usual way, the radioactivity analyzes as $P_1$. We had previously observed the formation of an acid-labile phosphoryl bond when rabbit muscle or yeast phosphoglycerate mutase was incubated with 2,3-[2-$\text{32P}$]DPG (2, 3). A histidyl residue was shown to have been phosphorylated. In the present study the effect of pH on the rates of hydrolysis at 46° of $[^{32P}]$monophosphoglycerate mutase (rabbit muscle) and $[^{32P}]$diphosphoglycerate mutase were compared. The observed rates of hydrolysis in 0.1 N HCl, pH 3, 4, 7.5, and 11.2, were very similar for both enzymes. Hydrolysis was rapid in 0.1 N HCl with a $t_{1/2}$of about 3 min. At pH 7.5 and 11.2 hydrolysis was not perceptible in 60 min. Hydrolysis at pH 3 was more rapid than at pH 4. This pattern of acid lability, increasing stability with increasing pH in the acid range, and stability at neutrality or in alkaline solution is consistent with the phosphorylation of diphosphoglycerate mutase having occurred on a histidyl residue.

Monophosphoglycerate Mutase and 2,3-DPG Phosphatase Activities of Diphosphoglycerate Mutase—Diphosphoglycerate mutase that had been separated completely from the main peak of monophosphoglycerate mutase contained 3% monophosphoglycerate mutase activity. This monophosphoglycerate mutase activity may be an intrinsic property of the diphosphoglycerate mutase molecule. There is evidence for kinetic differences between this activity and that shown by monophosphoglycerate mutase of red cells. As shown in Fig. 4, the red cell monophosphoglycerate mutase is not stimulated by the addition of 2,3-DPG to the standard assay system which contains 20 nmoles of 2,3-DPG. However the monophosphoglycerate mutase activity of diphosphoglycerate mutase purified through the hydroxylapatite step is enhanced by the addition of a high level of 2,3-DPG. A 4-fold increase in the 2,3-DPG from the 0.03 M of the standard assay to 0.13 M gave a 4-fold increase in the reaction rate; with 1.03 M 2,3-DPG there was an additional increase. Under these conditions the monophosphoglycerate mutase activity was about 15% of the diphosphoglycerate mutase activity.

Diphosphoglycerate mutase does not ordinarily display phosphatase activity toward 2,3-DPG. However, in the presence of glycolate-2-P, the 2-carbon analogue of the phosphoglycerates which are the normal acceptors of the phosphoryl group of the enzyme, the phosphoryl group is released from the enzyme as $P_1$ (Table I). The $K_m$ of glycolate-2-P for the release of $P_1$ (i.e. the concentration that gives half of the maximal effect) is 8 $\mu$M (Fig. 5). The maximal velocity for the activated 2,3-DPG phosphatase activity of diphosphoglycerate mutase is 20% of the diphosphoglycerate mutase activity. Glycolate-2-P shows opti...

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**Fig. 4.** Monophosphoglycerate mutase activity of diphosphoglycerate mutase. The standard assay system for monophosphoglycerate mutase included in a 1 ml volume 3.2 nmoles of 3-PGA containing 1% 2,3-DPG as an impurity (see Methods). Red cell enzymes were used: either X, a fraction high in monophosphoglycerate mutase (0.1 X 10$^{-4}$ unit containing 9 X 10$^{-4}$ unit of diphosphoglycerate mutase activity), or O, diphosphoglycerate mutase purified through the hydroxylapatite step (3.48 X 10$^{-4}$ unit containing 1.0 X 10$^{-4}$ unit of monophosphoglycerate mutase activity).
for 10 min at 25° in a 0.2 ml volume and contained: glycyglycine-K⁺ buffer, pH 7.5 (2 μmoles), 2-mercaptoethanol (1 μmole), 2,3-[U-¹⁴C]DPG (0.11 n mole, 46800 cpm per n mole of *⁴C), diphosphoglycerate mutase (7.24 × 10⁵ unit containing 1.5% monophosphoglycerate mutase activity). The reactions were stopped by the addition of H₂SO₄ and the Pi measured (see “Methods”).

The activation by 2 PGA and glycolate-2-P of the phosphorylation of 3-PGA which was shown in the previous steady state studies (1) can be understood readily in terms of a mechanism involving a ternary complex. It had been observed that 2-PGA lowered the Kₘ of acceptor 3-PGA and increased the maximal velocity of the reaction. The activation can be visualized as resulting from the dissociation of the 3-PGA molecule from the donor site of the ternary complex to form an inactive complex. 2-PGA may add to that site to form a more reactive complex than the original one as shown.

Enzyme-P₃-3-PGA₄ + 3-PGA (or 2-PGA) → Enzyme-P₃-3-PGA₄ + 3-PGA₄ (inactive) (11)
Enzyme-P₃-3-PGA₄ + 2-PGA → Enzyme-P₃-3-PGA₄ + 2-PGA (12)

The striking parallels between the observations of the partial reactions of diphosphoglycerate mutase and monophosphoglycerate mutase suggest that the two enzymes may have similar reaction paths. Phosphorylation of muscle monophosphoglycerate mutase by 2,3-DPG or 3,4-DPG has been shown (2, 4).

The phosphorylation of the enzyme as indicated is catalytically important. Whether the transfer of the phosphoryl group to the enzyme occurs before or after the acceptor molecule of PGA is a competitive inhibitor of PGA (16, 17). It appears likely that yeast and muscle monophosphoglycerate mutases have a common mechanism which may parallel that of diphosphoglycerate mutase in involving both a ternary complex and phosphorylation of the enzyme as indicated.

Enzyme + 2,3-DPG → Enzyme-P-2,3-DPG (13)
Enzyme-P-2,3-DPG + 3-PGA (or 2-PGA) → Enzyme-P-2,3-DPG + 3-PGA (14)
Enzyme-P-PGA\textsubscript{3}·PGA\textsubscript{2} (or 2-PGA) =

\begin{equation}
\text{enzyme} \cdot 2,3\text{-DGP} \cdot \text{PGA} \textsubscript{2}
\end{equation}

(15)

\begin{equation}
\text{enzyme} \cdot \text{PGA} \textsubscript{2} + 2\text{-PGA} (or 3\text{-PGA})
\end{equation}

(16)

In view of the failure to obtain ready mixing between isotope in 2,3-DPG and PGA in the presence of muscle monophosphoglycerate mutase (IS), it appears that the phosphorylated enzyme-PG\textsubscript{h} complex generated according to Equation 16 will usually reform ternary complex instead of hydrolyzing to free enzyme and 2,3-DPG in a reversal of Reaction 13.

The conclusions reached in this study concerning the reactions of diphosphoglycerate mutase are only valid if the enzyme used was free of monophosphoglycerate mutase. If the monophosphoglycerate mutase activity present in the enzyme used to show the phosphorylation of diphosphoglycerate mutase (Fig. 3 and Table I) were due to contamination by monophosphoglycerate mutase with specific activity similar to that of the muscle enzyme, one can calculate the contribution it would make to the total observed phosphorylation, assuming one site of phosphorylation per subunit. Crystalline muscle monophosphoglycerate has a specific activity of 10\textsuperscript{10} units per mg measured under the conditions of our assay. For a subunit weight of 28,500, there are 28.8 units per nmole of subunits. In the experiments of Fig. 3 and Table I, there was 1.12 \times 10\textsuperscript{-3} unit of phosphocreatine mutase per incubation or 3.9 \times 10\textsuperscript{-3} nmole of subunits. This would make a contribution of only 0.07\% to the 0.06 nmole of phosphoprotein observed.

The specific activity of purified enzyme can be calculated from the number of units of activity of diphosphoglycerate mutase that combine with a micromole of phosphorylated enzyme, assuming one active site per subunit. With the purest enzyme obtained, the value calculated was about 20 units per mg.

These studies indicate that mono- and diphosphoglycerate mutases have striking similarities which suggest evolution from a common molecule. Future studies will consider the relationship of 2,3-DPG phosphatase to the other enzymes of 2,3-DPG metabolism.

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