Characterization of a Phosphoenzyme Intermediate in the Reaction of Phosphoglycolate Phosphatase*

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When $^{32}$P-glycolate and phosphoglycolate phosphatase from spinach are mixed, $^{32}$P is incorporated into acid precipitated protein. Properties that relate the phosphorylation of the enzyme to the phosphatase are: the $K_{m}$ value for P-glycolate is similar for protein phosphorylation and substrate hydrolysis; the $^{32}$P in the phosphoenzyme is diluted by unlabeled P-glycolate or the specific alternative substrate, ethyl-P; the activator Cl- enhances the effectiveness of ethyl-P as a substrate and as an inhibitor of the formation of $^{32}$P-enzyme; and $^{32}$P is lost from the enzyme when $^{32}$P-glycolate is consumed. The phosphorylated protein has a molecular weight of 34,000, which is half that of the native protein and is similar in size to the labeled band that is seen on sodium dodecyl sulfate-polyacrylamide gels.

The enzyme-bound phosphoryl group appears to be an acylphosphate from its pH stability, being quite stable at pH 1, less stable at pH 5, and very unstable above pH 5. The bond is readily hydrolyzed in acid molybdate and it is sensitive to cleavage by hydroxylamine at pH 6.8.

The demonstration of enzyme phosphorylation by $^{32}$P-glycolate resolves the dilemma presented by initial rate studies in which alternative substrates appeared to have different mechanisms (Rose, Z. B., Grove, D. S., and Seal, S. N. (1986) J. Biol. Chem. 261, 10996–11002).

The fixation of carbon dioxide into organic form by ribulose bisphosphate carboxylase in the chloroplasts of leaves is always accompanied by the seemingly unproductive oxygenase reaction that forms P-glycolate. A specific phosphatase (1–5) prevents the accumulation of P-glycolate, which is a potent inhibitor of phosphofructokinase of spinach chloroplasts (6) and triose phosphate isomerase of pea chloroplasts and cytoplasm (7). Phosphoglycolate phosphatase also occurs in all mammalian tissues that have been examined (8–10). The occurrence of P-glycolate in mammalian cells was first shown by Rose and Salon (11) and has been confirmed by Spies and Vora (12). Data obtained with pyruvate kinase deficient red blood cells suggest that P-glycolate is synthesized by pyruvate kinase in vivo (13). P-glycolate inhibits rabbit muscle triose phosphate isomerase (14) and it activates the breakdown of 2,3-bisphosphoglycerate (15), which is a regulator of the oxygen affinity of hemoglobin (16).

The phosphoglycolate phosphatases from red blood cells and spinach have many properties in common. Both are highly specific enzymes, yet each hydrolyzes P-glycolate and ethyl-P with the same maximum velocity (5). Both enzymes are activated by millimolar concentrations of monovalent anions with either P-glycolate or ethyl-P as substrate (5, 17–19). With both enzymes, initial rate studies in which P-glycolate and the activating anion are varied give parallel line double-reciprocal plots (ping-pong kinetics), whereas with ethyl-P as substrate the lines intersect. The conservation of these properties in enzymes from such diverse sources suggests that the two enzymes have a common mechanism.

A mechanism that is consistent with all of the data was proposed in which phosphate transfer from P-glycolate does not require a catalyzed anion but transfer from ethyl-P does so, possibly by satisfying a function performed by the carboxyl group of P-glycolate, i.e. homosteric activation (5). The kinetic pattern of Cl- activation with P-glycolate as substrate implies an intermediate, a phosphorylated enzyme, the further hydrolysis of which requires Cl-, again to satisfy a homosteric function. In the present study it is shown that the transfer of phosphate to the enzyme and from the enzyme to water occurs during hydrolysis of P-glycolate, consistent with covalent catalysis.

EXPERIMENTAL PROCEDURES

Materials—Phosphoglycolate was from Sigma. DEAE-Sepacel and Sephacryl S-200 were from Pharmacia Biotechnology, Inc. DE52 was from Whatman. Enzyme grade ammonium sulfate was from Schwarz/Mann. Malschite green oxalate was from Harleco. Crystallize bovine albumin was from Pentex. Coomassie Blue was from Bio-Rad. The Bio-Rad protein assay reagent was used. To concentrate protein fractions, a filtration chamber with a YM-10 membrane or Centricon Microconcentrators, both from Amicon, were used. $^{32}$P, carrier-free, was obtained from Amersham Corp. $^{32}$P-Glycolate was synthesized enzymatically as reported earlier (5); 96% of the labeled product is hydrolyzed by the enzyme. $^{17}$H-Decoxyglucose was from Du Pont-New England Nuclear. An Intertechnique scintillation counter was used for Cerenkov counting when possible or with Liquifluor (Du Pont-New England Nuclear) in ethanol-toluene.

Enzyme Purification—This improved procedure produces nearly homogeneous enzyme (summarized in Table I). Spinach leaves (249 g) were deiced and washed with water. Subsequent steps were done at 0–4 °C. Portions of 100 g of spinach plus an equal weight of buffer 1 (10 mM triethanolamine Cl- buffer, pH 7.2, 1 mM mercaptoethanol, 0.1 mM EDTA, 0.05% Triton X-100 (included for stabilization but possibly unnecessary)) were homogenized in a Waring Blender with four 10-s bursts. The homogenate was filtered through several layers of cheesecloth and then centrifuged 10 min at 12,000 × g. To the supernatant was added solid ammonium sulfate (23 g/100 ml). After the precipitate was removed by centrifugation, the precipitate was dissolved in 20 ml of buffer 2 (buffer 1 containing 1 mM MgSO4). The enzyme was dialyzed overnight against two changes of buffer 2.

The enzyme (diluted to 20 mg/ml) was applied to a DEAE-Sepacel column (1.5 × 17 cm) equilibrated with buffer 2. The column was
washed with 120 ml of buffer 2 and eluted with a linear gradient of 200 ml each of buffer 2 alone or containing 0.3 M KCl. The active fractions were combined and concentrated by ultrafiltration. The enzyme (4.5 mg/ml) was applied to a DE52 column (1.5 x 14 cm) equilibrated with buffer 2. The column was washed successively with 40 ml of buffer 2 and 20 ml of buffer 2 containing 0.05 M KCl, and eluted with a linear gradient of 150 ml each of buffer 2 containing 0.05 M KCl or 0.25 M KCl. The active fractions were combined and concentrated as before.

The concentrated enzyme was applied to a column of Sephacryl S-200 (1.3 x 86 cm) equilibrated with buffer 2. The peak fractions were concentrated. After electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate (20), the best fraction was found by densitometry to be 94% homogeneous with the major band at M, 31,500. The molecular weight estimated on Sephacryl S-200 is 65,000-70,000, in agreement with our earlier value (5) but not that of Christeller and Tolbert (2). Therefore, the enzyme is a dimer.

Enzyme Assays—The enzyme activity was assayed by phosphate release at 30°C either colorimetrically with malachite green or by extraction of the 32P, molybdate complex into isobutyl alcohol as reported earlier (5). For the standard colorimetric assay, the incubations were at 30°C for 10 min in a 0.2-ml volume and contained 25 mM Hepes-Na+ buffer, pH 7.2, 10 mM KCl, 2 mM MgSO4, 0.1 mM mercaptoethanol, 2.5 mM P-glycolate, 5 µg of serum albumin, and enzyme. The reactions were stopped by the addition of the malachite green color reagent (0.03% malachite green in 3 N HCl plus 1.5% ammonium molybdate) and the absorbance of the molybdate complex of P, with malachite green was read at 650 nm. Under these conditions the absorbance of 1 nmol of P, was 0.250. One unit of enzyme activity releases 1 µmol of phosphate/min under these conditions, which give 0.9 of the maximum velocity with Cl- as the activating anion. Protein was determined spectrophotometrically in the crude fractions (21) and with more purified fractions by the procedure of Bradford (22) using the Bio-Rad protein assay reagent with bovine albumin as the standard. The former method gives values that are 2.3 times higher than the latter (Table I). This discrepancy accounts for the large difference between the specific activity we reported earlier (5) and that reported by Huisic and Tolbert (19).

The activity of the enzyme used to study enzyme phosphorylation was 300 units/ml of enzyme under the standard assay conditions, or a Vmax of 333 units/ml of enzyme. The specific activity was 184 units/mg, with protein determined with the Bio-Rad assay. In the absence of Cl-, the maximal rate under initial rate conditions was 25 units/ml of enzyme, not corrected for activation by the assay components, and the Kmr for P-glycolate was 4 µM.

Phosphoenzyme Formation.—The enzyme and substrate were mixed in a rapid mixing apparatus from Update Instruments Co. that allows reactions to be terminated at times of 25-300 ms after mixing. The enzyme solutions contained 1 mM-deoxyglucose (2 x 102 cpm) to estimate the fraction of the enzyme recovered after mixing. Unless otherwise indicated, the reactions were quenched with cold 10% trichloroacetic acid. After centrifuging for 1 min in an Eppendorf centrifuge, the protein precipitates were washed with 1.4 ml of cold 5% trichloroacetic acid and the precipitated protein was counted in a scintillation counter by Cerenkov radiation. The supernatants were assayed for the recovery of 3H and 33P, to determine extent of 3P-glycolate hydrolysis.

The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Caes, 3-(cyclohexylamino)ethanesulfonic acid.

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2 Assayed spectrophotometrically using the equation: protein concentration (mg/ml) = 1.45 A380 - 0.74 A650 (Ref. 21).

3 Bio-Rad protein assay with bovine albumin as the standard (Ref. 22).

### TABLE I

| Activity     | Protein Specific activity |
|--------------|---------------------------|
| units mg     | units/mg                  |
| Ammonium sulfate | 1425 1520° 0.94          |
| DEAE-Sephadex | 990 54° 18                |
| DE52         | 720 15° 48                |
| Sephacryl S-200 | 128 0.124° 202          |

### RESULTS

Phosphorylation of the Enzyme—When the enzyme was mixed with 32P-glycolate and then precipitated with trichloroacetic acid, the precipitate contained much more 32P than a control with serum albumin instead of enzyme. In order to be sure that the radioactivity was associated with P-glycolate phosphatase, the denatured phosphorylated protein was applied to a molecular sieve column of Sephacryl S-200. A single sharp peak containing 87% of the phosphoenzyme applied (12,250 cpm) was found corresponding to 34 kDa (Fig. 1). This agrees well with the subunit molecular weight of 31,500 found on sodium dodecyl sulfate gels.

Effects of Substrates on Enzyme Phosphorylation—In order to isolate the phosphorylated protein, it is necessary to use short mixing times since the hydrolytic reaction occurs even in the absence of added activating anions (see Ref. 5). In these studies the enzyme and substrate were mixed in a rapid mixing device and the reactions were usually stopped in about 30 ms. We were unable to find conditions in which the enzyme was phosphorylated but did not turn over, such as were found for alkaline phosphatase (24). With P-glycolate phosphatase, the amount of phosphorylated protein isolated after a 30-ms incubation of P-glycolate with enzyme changed very little over the pH range from 5.3 to 9.1, whereas the amount of phosphate released as Pi was 3 times greater at pH 5.3 and 7.2 than at pH 9.1. Incubations of 30-100 ms duration showed no evidence for a burst or a lag in the formation of the phosphoenzyme. As the 32P-glycolate is used up, the 33P-enzyme is lost. Since the enzyme is in the steady state, the

### FIG. 1. Gel filtration of the acid-denatured [33P]phosphoenzyme. The [33P]phosphoenzyme was prepared in the usual manner and the reaction was stopped with 0.05 N HCl at 4°C. After adjusting to pH 5.0 with Mes-Na+ buffer, the mixture was applied to a column of Sephades G-25 to remove most of the small molecules. The solution that passed through the column (14,000 cpm) was applied to a Sephacryl S-200 column (1 x 46.6 cm) that had been equilibrated with 10 mM Mes-Na+, pH 5.1, containing 0.1 mM EDTA and 1 mM MgSO4. The flow rate was 7 ml/h and 0.7-ml fractions were collected. The column was calibrated with (M,) (C), bovine serum albumin (66,200); (×) ovalbumin (45,000); and (○) carbonic anhydrase (31,000).
The incubation time, as long as the substrate concentration is similar to that in the absence of C1- (Tables I and II), the observed $K_m$ value for phosphorylation is $10 \mu M$ and that for hydrolysis is $20 \mu M$, which is reasonable agreement in view of the occurrence of some experimental error in the timing of reactions in the rapid mixing device. This resulted in some variability in the calculated extent of hydrolysis, whereas there was very good reproducibility of the extent of protein phosphorylation in duplicate incubations and in successive experiments. The phosphorylation is a steady state value and is not affected by the incubation time, as long as the substrate concentration is essentially unchanged, whereas the calculation of the hydrolysis rate requires an accurate measurement of the reaction time. The observed $K_m$ value and hydrolysis rate are higher than the values obtained for the deactivated reaction under initial rate conditions and are probably due to low level activation by the assay components.

In the presence of 7.5 mM C1- and increasing concentrations of P-glycolate, the maximum extent of enzyme phosphorylation is similar to that in the absence of C1- (Tables II and III). Assuming full activity for the enzyme, the greatest extent of phosphorylation observed was 32% of the total protein as determined by absorbance at 260 nm (21) or 14% using the method of Bradford (22). The large activation shown with C1- is more than the values obtained for the nonactivated reaction under initial rate conditions and are probably due to low level activation by the assay components.

The amount of protein cannot be titrated by the extent of phosphorylation.

The amount of protein phosphorylation was studied as a function of the concentration of P-glycolate in the absence of an activating anion (Table II). There is a direct correlation between the amount of 32P-enzyme and the extent of hydrolysis. The data in Table II also show that nonlabeled P-glycolate dilutes the 32P compound. The apparent $K_m$ value for phosphorylation is $10 \mu M$ and that for hydrolysis is $20 \mu M$, which is reasonable agreement in view of the occurrence of some experimental error in the timing of reactions in the rapid mixing device. This resulted in some variability in the calculated extent of hydrolysis, whereas there was very good reproducibility of the extent of protein phosphorylation in duplicate incubations and in successive experiments. The phosphorylation is a steady state value and is not affected by the incubation time, as long as the substrate concentration is essentially unchanged, whereas the calculation of the hydrolysis rate requires an accurate measurement of the reaction time. The observed $K_m$ value and hydrolysis rate are higher than the values obtained for the deactivated reaction under initial rate conditions and are probably due to low level activation by the assay components.

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### Table II

**Phosphoenzyme formation: Effect of varying the P-glycolate concentration in the absence of C1-**

| P-glycolate | $E_{32P}$ | Hydrolysis observed | $v$ |
|-------------|-----------|---------------------|-----|
| $\mu M$ (pmol) | cpm | pmol | |
| 4 (168) | 25,811 | 7 | 46 | 18 |
| 8 (333) | 23,436 | 11 | 89 | 37 |
| 17 (667) | 26,547 | 15 | 91 | 44 |
| 33 (1,334) | 16,622 | 19 | 233 | 108 |
| 45 (1,800) | 1,502 | 18 | 242 | 112 |
| $\infty$ (from double-reciprocal plot) | 22 | | | 133 |

### Table III

**Phosphoenzyme formation: Effect of varying P-glycolate in the presence of C1-**

Experimental conditions were as in Table II except that all incubations contained 7.5 mM KCl and 1.3 units of enzyme. 32P-Glycolate (83-3962 cpm/pmol) was varied, the apparent $K_m$ value for phosphorylation is $10 \mu M$ and that for hydrolysis is $20 \mu M$, which is reasonable agreement in view of the occurrence of some experimental error in the timing of reactions in the rapid mixing device. This resulted in some variability in the calculated extent of hydrolysis, whereas there was very good reproducibility of the extent of protein phosphorylation in duplicate incubations and in successive experiments. The phosphorylation is a steady state value and is not affected by the incubation time, as long as the substrate concentration is essentially unchanged, whereas the calculation of the hydrolysis rate requires an accurate measurement of the reaction time. The observed $K_m$ value and hydrolysis rate are higher than the values obtained for the deactivated reaction under initial rate conditions and are probably due to low level activation by the assay components.

The amount of protein cannot be titrated by the extent of phosphorylation.

### Table IV

**Effect of ethyl-P on the phosphorylation of phosphoglycolate phosphatase by 32P-glycolate**

Reactions were for 30 ms at 22 °C and contained 1.32 units of enzyme. 32P-Glycolate was 15 μM in experiment 1 and 30 μM in experiment 2.

| Ethyl-P | [Ethyl-P]/[P-glycolate] | $E_{32P}$ |
|---------|------------------------|----------|
| mM      | pmol                   |          |
| Experiment 1 (no KCl) | 0 | 18 | 44 |
| KCl     | 1.9 | 127 | 12 | 28 |
| Experiment 2 (7.5 mM KCl) | 0 | 253 | 9 | 6 |
| KCl     | 2.5 | 83 | 1.3 | 7 |
| 5.6 | 187 | 0.8 | <3 |

**FIG. 2. pH stability of 32P-phosphoenzyme.** Phosphorylated protein that had been precipitated with trichloroacetic acid was dissolved in the appropriate buffer containing 0.02 mg of serum albumin and the pH of the sample was checked. The buffers used were: 0.1 N HCl, pH 1.06; or 50 mM glycine-C1-, pH 3; Mes-Na+, pH 5.3; Hepes-Na+, pH 7.2; Ches-Na+, pH 9.1 and 10.98. Half of each sample served as a zero-time blank, whereas the remainder was incubated at 46 °C for 30 min. Trichloroacetic acid was added to 10% final concentration to precipitate the protein. 32P in the protein and the supernatant was determined. Points from three experiments are designated by different symbols. The filled circles indicate the stability at pH 5 and 10.6 after 10 min at 25 °C.
phosphocysteine (29), and phosphotyrosine (30) (reviewed in Refs. 31, 32).

The phosphonyl bond is 91% hydrolyzed in acid molybdate under the conditions used to extract inorganic phosphate (5). This lability is characteristic of N-P (33) and acyl-P bonds (34). The bond is also readily cleaved by hydroxylamine. When the phosphoenzyme was incubated for 10 min at 25 °C in 0.5 m hydroxylamine at pH 6.8, the phosphonyl bond was 95% hydrolyzed compared to a control containing 0.5 m KCl. Under these conditions the nucleophilic attack by hydroxylamine is fairly specific for carboxylic acid anhydrides (23).

**DISCUSSION**

The demonstration that 32P-enzyme can be isolated after mixing 32P-glycolate with P-glycolate phosphatase suggests that the phosphoenzyme is an intermediate in the reaction. Evidence consistent with a phosphoenzyme mechanism was reported by Christaller and Tolbert (4), who observed transphosphorylation with high concentrations of ethylene glycol, ethanol and n-propyl alcohol as well as nonphysiological levels of glycolate, glyceraldehyde, and glucose. They also observed weak inhibition by diisopropyl fluorophosphate, similar to that found with alkaline (35, 36) and acid phosphatases (37). Since alkaline phosphatases are serine phosphatases (38, 39) and many acid phosphatases are histidine phosphatases (40-42); this does not allow a conclusion as to the residue that is likely to be phosphorylated in P-glycolate phosphatase. Covariant intermediates have also been demonstrated in many other phosphatase reactions. 5'-Nucleotide phosphodiesterase (43) is phosphorylated on a serine residue. Glucose-6-phosphatase (44) is phosphorylated on a histidine residue. The Na+-K+-ATPase (25), the Ca2+-Mg2+-ATPase of sarcoplasmic reticulum (45), and two plant nucleoside phosphotransferases (46-49) have acylphosphate intermediates. Fructose bisphosphatase (50) and inorganic pyrophosphatase (51) are not known to be phosphorylated by their substrates.

The mechanism in Scheme 1 is consistent with the present observations and the different kinetic patterns observed in anion activated initial rate studies with P-glycolate and ethyl-P (5) and is similar to that proposed earlier (5). The maximum extent of phosphorylation that was observed was about 25% of the enzyme. This is consistent with a steady state rate of hydrolysis that is four times faster than the rate of formation of the phosphoenzyme.

When P-glycolate is the substrate, Cl- and other activators function only after enzyme phosphorylation has occurred. With ethyl-P as substrate, Cl- assists in achieving a favorable conformation for the phosphoryl transfer to the enzyme, possibly by binding to the carboxyl region of the active site. It is proposed that Cl- also assists the phosphoryl transfer from the enzyme to water by binding to the carboxyl site for the substrate. Since these processes occur at the active site, we designate them as "homosteric," in contrast to allosteric. Similar effects also appear to explain the activation of the 2,3-bisphosphoglycerate phosphatase activity of 2,3-bisphosphoglycerate synthase-phosphatase by specific combinations of anions or by P-glycolate (15, 52). In both of these enzymes, anions activate the reaction by substituting for portions of the substrate. Such effects may be relatively common and may be a factor in the control of reaction rates in vivo.

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