**Active Site Phosphohistidine Peptides from Red Cell Bisphosphoglycerate Synthase and Yeast Phosphoglycerate Mutase**

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Bisphosphoglycerate synthase (glycerate-1,3-P2 → glycerate-2,3-P2) and phosphoglycerate mutase (glycerate-3-P → glycerate-2-P) are both phosphorylated by substrates at a histidine residue forming covalent intermediates which have been shown to function in the phosphoryl transfer reactions catalyzed by these enzymes (Rose, Z. B., and Dube, S. (1976) J. Biol. Chem. 251, 4817–4822). We have phosphorylated bisphosphoglycerate synthase from horse red blood cells with [U-32P]glycerate-2,3-P2, digested with trypsin, and purified the phosphopeptide. The amino acid sequence of the phosphohistidine peptide has been determined to be: His-Gly-Gln-Gly-Ala-Trp-Asn-Lys. In like manner, a phosphohistidyl peptide has now been purified from yeast phosphoglycerate mutase, for which the amino acid sequence is known (Wian, S. L., Watson, H. C., Fothergill, L. A., and Harkins, R. N. (1977) Biochem. Soc. Trans. 5, 657–659). The amino acid composition of the phosphopeptide indicates that histidine-8 was phosphorylated. The sequence of this peptide is closely homologous with the active site peptide from bisphosphoglycerate synthase. In yeast phosphoglycerate mutase, the denatured phosphoenzyme hydrolyzes with a single rate constant of $2.02 \times 10^{-4}$ s$^{-1}$ at pH 3, 45°C. The relevance of these observations to the enzymatic mechanism is discussed.

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**Experimental Procedures**

**Materials**

[U-32P]Glycerate-2,3-P2 was isolated from red blood cells that had been incubated with 32P (15). Radioactivity was determined with a high liquid scintillation spectrometer in mixtures of H2O:ethanol:LSC complete scintillation fluid (Yorktown Research) in the proportions 1:1:9. Amino acid analyses were performed on a Beckman model 890B (updated) sequenator equipped with an undercut cup for the sequence analysis. High pressure liquid chromatography was performed on a Waters Associates chromatographic system consisting of two model 600A pumps, a model UK6 solvent programmer.

**Methods**

The extent of incorporation of 32P into the enzymes was determined by extraction of the phosphoenzyme into phenol and counting the washed phenol phase (6, 16). Acid lability of the phosphoryl bond was demonstrated by exposure of the enzyme to low pH prior to the phenol treatment (6). After proteolytic digestion of the enzyme, the nature of the phosphoryl bonds was analyzed using the procedure of Berenblum and Chain (17) in which inorganic phosphate is extracted by isobutyl alcohol as the acid molybdate complex. The phosphohistidyl bond of the peptide is sufficiently stable under the conditions of extraction to resist hydrolysis if the extraction is done rapidly (18). Acid-labile phosphate was determined in a similar sample for which the extraction was delayed 10 min to allow complete hydrolysis. Ammonia was determined with Nessler's reagent (19).

**Preparation of Phosphohistidine-containing Peptide from Red Cell Bisphosphoglycerate Synthase**

Red cell bisphosphoglycerate synthase was prepared from horse red blood cells (20). The specific activity for bisphosphoglycerate phosphatase activity was 9 to 10 units/mg. A 1% solution of enzyme containing sodium dodecyl sulfate (21) the enzyme, with $M_r = 28,000$, comprised about 95% of the protein.

To prepare the phosphopeptide, bisphosphoglycerate synthase (238 nmol, 7 mg) was dissolved in 5.5 ml of cold 10 mM Tes-Na buffer (N-[Tris(hydroxymethyl)methyl-2-amino]ethanesulfonate), pH 7.8, containing 5 mM EDTA. In a 500-ml separation funnel were placed 10 ml of water, the enzyme solution, and 6 ml of [U-32P]glycerate-2,3-P2 (1.2 μmol, 7.2 × 10$^{-7}$ cpm/microgram of 32P). Previous titration had indicated that a 5-fold excess of glycerer-2,3-P2 is required for maximal...
phosphorylation. Phosphorylation occurs within the mixing time (4). Phenol (150 ml) was added to denature the enzyme and thus stabilize the phosphohistidine group. The aqueous phase, containing unreacted \[ ^{32}\text{P}\text{glycerate-2,3-P_2} \], was removed. Upon repeated extraction of the phenol phase with 0.1 M Tris(hydroxymethyl)aminomethane-Cl\text{-}N\text{H}_4 buffer, pH 8, the volume was decreased to 10 ml in the separatory funnel and subsequently to 4.6 ml in a centrifuge tube. The solution, containing 202 nmol of the phosphorylated protein, was cooled in an ice bath. To precipitate the protein, cold acetone (15 ml) was added dropwise with stirring. The mixture was kept at \(-20^\circ\text{C}\) overnight and then centrifuged at 12,000 \(\times\) \(g\) for 20 min at 0°C. The precipitate was washed twice with 1-ml portions of cold acetone and dried in a funnel.

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Purification of Phosphohistidyl Peptide

\textit{Gel Filtration on Sephadex G-25—A column (1.5 \times 140 cm) was equilibrated with 0.05 M NH}_4\text{HCO}_3. Three \(^{32}\text{P}\)-containing peaks were characterized as incompletely digested phosphorylated protein (acid-labile), phosphohistidyl peptide (acid-labile, 80% of the \(^{32}\text{P}\) applied), and peptide (Table I). The fractions containing the peptide were combined.} \textit{QAE (Quaternary Amino-Column) Sephadex A-25 Chromatography—This ion exchanger has been used previously to purify phosphohistidine-containing peptides (14, 22). A microbore column (0.3 \times 100 cm) (23) was equilibrated with 0.35 M NH\text{H}_4\text{HCO}_3 under pressure, using a peristaltic pump with a flow rate of 1.0 ml/h, which was maintained throughout the run.} The phosphohistidyl peptide from the previous step (15 ml) was applied directly to the column. Drying of the phosphorylated peptide was avoided since it resulted in some hydrolysis of the N–P bond. The column was washed thoroughly with 0.35 M NH\text{H}_4\text{HCO}_3 and a gradient of increasing NH\text{H}_4\text{HCO}_3 concentration was applied. The peptide was eluted with 0.43 M NH\text{H}_4\text{HCO}_3. The major \(^{32}\text{P}\)-peak contained the phosphohistidyl peptide (155 nmol) (Fig. 2 and Table I). This was lyophilized in preparation for analysis (the loss of the phosphoryl group at this point is not important since it hydrolyzes with subsequent procedures).

\textit{Determination of the Amino Acid Sequence}

\textit{Polybrene (24) (3 to 6 mg) was suspended in 0.2 ml of heptafluorobutyric acid and applied to the cup of a spinning cup sequenator (25). It was dried under vacuum and subjected to two complete degradation cycles with the 0.1 M Quadrol program. Lyophilized peptide (40 nmol) was dissolved in 0.15 ml of heptafluorobutyric acid, applied to the cup, and dried under vacuum. To minimize the background material, the second half-cycle was carried out prior to the fast degradative cycle. The conversion of the anilinothiazolinones to phenylthiohydantoins was effected by adding 0.2 ml of 20% aqueous trifluoroacetic acid solution and incubating the sample at 80°C for 10 min. The solution was cooled to room temperature and the phenylthiohydantoin was extracted twice with 0.5 ml of ethyl acetate.}

To identify the amino acid derivative, the ethyl acetate solution was dried under N\textsubscript{2} and the residue was redissolved in ethyl acetate containing the phenylthiohydantoin of norleucine which acted as an internal standard. An aliquot was analyzed by high pressure liquid chromatography on a \(\mu\)-Bondapak C\textsubscript{18} column (0.4 \times 30 cm).

The identity of each amino acid was verified by hydrolysis of the phenylthiohydantoin to the free amino acids. Amino acid analyses were carried out for an aliquot of each hydrolysate.

\textit{Preparation of the Phosphohistidine-containing Peptide from Yeast Phosphoglycerate Mutase}

Yeast phosphoglycerate mutase was prepared by autolyzing fresh yeast with toulene for 4 h in the manner of Rodwell et al. (26). Subsequent steps were according to Sasaki et al. (5). The specific activity was 750 units/mg using 1.45 as the absorbance at 280 nm of the enzyme.

## Table I

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## Table II

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\textsuperscript{a} All of the \(^{32}\text{P}\) is acid-labile.

\textsuperscript{b} Minimal value: some hydrolysis of the peptide is likely during the extraction procedure.

\textsuperscript{c} Maximal value, see footnote a.

\textsuperscript{d} Amidated.
RESULTS

Characterization of Histidyl Peptide from Bisphosphoglycerate Synthase—The two-step purification procedure allowed the isolation of phosphopeptide that subsequently met the criteria of purity. In earlier preparations, the peptide isolated at this step was acidified to hydrolyze the phosphoryl group of the histidine residue. After chromatography on QAE-Sephadex, the amino acid composition of the peptide was unchanged. The total amino acid composition was determined after hydrolysis in 6 N HCl for 24 h and quantitative analysis with an amino acid analyzer. The composition of one sample, with nanomoles in parentheses and the nearest integral number of residues in brackets, is: aspartic acid (1.39) [1], glutamic acid (1.36) [1], glycine (2.18) [2], alanine (1.23) [1], histidine (0.94) [1], lysine (1.00) [1]. The composition was unchanged after 72-h hydrolysis. After performic acid oxidation (28) there was no evidence of the presence of cysteine in the peptide. The ultraviolet spectrum of the peptide in the region 250 to 300 nm had an absorption maximum at 282 nm and a shoulder at 290 nm for which E_fnm was 270. The absorbances agree with the extinction coefficient of tryptophan reported in the literature (E_fnm, 296) (29). Therefore, the phosphoryl peptide contains 1 tryptophan molecule/mol and is an octapeptide.

The amino acid sequence of the peptide was determined with a spinning cup sequenator in the presence of Polybrene (24, 25). The phenylthiohydantoins were identified by high pressure liquid chromatography and the identification was confirmed by hydrolysis of these derivatives to the free amino acids with subsequent identification on the amino acid analyzer. One amino acid could be identified unambiguously with each degradative step. The amino acid sequence of this octapeptide is (Table III): His-Gly-Glu-Gly-Ala-Trp-Asn-Lys. In the case of the bulk preparation of peptide with high specific activity 32P described under "Methods," an additional 32P-containing degradative step. The amino acid composition of this octapeptide was observed on the QAE-Sephadex column which eluted just before the main peak and contained 12% of the radioactivity (Fig. 2, Table I). After acid hydrolysis, the amino acid composition of this peak was the same as the major peak. This less-charged peptide probably contains one more amide group than the main peak, in which case the glutamic acid residue is probably glutamine in the native enzyme. Since red cell enzymes do not turn over, it is likely that deamidations are common during the aging of the cell. Presumably this modification does not interfere with the ability of the enzyme to act catalytically since both phosphorylation and phosphoryl transfer are complete.

Phosphohistidyl Peptide from Yeast Phosphoglycerate Mutase—Yeast phosphoglycerate mutase phosphorylated with [U-32P]glycerate-2,3-P2 was digested with trypsin and the phosphohistidyl peptide was purified by the two-step procedure described for bisphosphoglycerate synthase. One active site peptide was isolated (Table II). The sequences of the histidine peptides from this enzyme are known and 2 histidine residues, 8 and 184, have been reported to be at the active site (11). After acid hydrolysis, the amino acid composition of this peptide was determined quantitatively on the amino acid analyzer to be as indicated, with nanomoles in parentheses and the nearest integral number of residues in brackets: aspartic acid (1.18) [1], serine (1.03) [1], glutamic acid (3.19) [3], glycine (1.20) [1], histidine (0.99) [1], lysine (1.00) [1]. Tryptophan was identified by its ultraviolet spectrum (20). This agrees exactly with the composition of the trypptic peptide that includes histidine-8 (Fig. 3).

The presence of 2 histidine residues in the active site region of yeast phosphoglycerate mutase shown by x-ray crystallography had led to the suggestion that both might participate in the phosphoryl transfer reactions (11) (see "Discussion"). Although our fractionation of the phosphorylated trypptic di-
in 2 h. At pH 3, 25°C (k = 4.27 min⁻¹), there was negligible hydrolysis of the denatured phosphorylated protein with 1.5% sodium dodecyl sulfate. At pH 7.5, 25°C, there is hydrolysis that extrapolated back to the expected initial value (error ± 1.0%). At pH 3, 45°C (k = 2.03 × 10⁴ min⁻¹), there was again no evidence for a second more reactive species. (Fig. 4). These studies would have detected no less stable species. All of the enzyme-bound ³²P is acid-labile. The experiments were done with muscle phosphoglycerate mutase with the same radioactivity of the major peak would have been detected readily. We have now shown that histidine-8 is phosphorylated by glycerate-2,3-P₂. These results gest yielded only one main peptide, we could not yet eliminate the possibility that there had been a small amount of a second, possibly more labile, peptide. To look for a second phosphorylation site directly, we phosphorylated the yeast enzyme in an ice bath. All of the protein-bound ³²P is acid-labile. The phosphoryl group was stabilized by denaturing the enzyme with 1.5% sodium dodecyl sulfate. At pH 7.5, 25°C, there is negligible hydrolysis of the denatured phosphorylated protein in 2 h. At pH 3, 25°C (k = 4.27 × 10⁻⁵ s⁻¹), a single rate of hydrolysis was observed that extrapolated back to the expected initial value (error ± 1.0%). At pH 3, 45°C (k = 2.03 × 10⁻⁴ s⁻¹), there was again no evidence for a second more reactive species (Fig. 4). These studies would have detected ≤2% of a more labile phosphoryl group. The experiments were done with muscle phosphoglycerate mutase with the same results. Therefore, all of the phosphoryl groups on the enzyme are chemically equivalent and if phosphorylation had occurred at more than one locus the ratio of the different forms should remain constant throughout the purification procedure. We detected only one ³²P-peptide. A second peptide would have been detected readily if it contained 2% as much ³²P as the isolated product.

**DISCUSSION**

The amino acid sequences of the two active site peptides analyzed in this study are remarkably similar (Fig. 3). The position of the synthase active site peptide in the whole protein is not yet established. The detection in bisphosphoglycerate synthase of a second somewhat less acidic peptide which has the same amino acid composition as the major component after acid hydrolysis implies that the 1 free acidic residue found in that peptide, glutamate, is present as the amide in the native enzyme and it is shown as such in Fig. 3. Although the deamidated form of this amino acid residue predominates in the enzyme as isolated, the enzyme did not appear markedly heterogeneous by other criteria. The enzyme was prepared from freshly drawn horse blood and it eluted from DEAE-cellulose as a well defined peak. However, it eluted from a blue dextran-Sepharose 4B column in two successive peaks, each containing similar amounts of apparently pure protein. From x-ray crystallographic studies of the nonphosphorylated form of yeast phosphoglycerate mutase, Watson and workers (11) showed that there are 2 histidine residues, 8 and 184, close enough to the glycerate-3-P binding site to participate in the phosphoryl transfer reactions catalyzed by the enzyme. It was postulated that each histidine might specifically accept and donate either the 2- or 3-phosphoryl group of the phosphoglycerates. The phosphoryl group would be transferred between the 2 histidines in the course of the reaction (Fig. 5). Britton et al. (31) showed that if isomerization of the enzyme occurs, it cannot be rate-limiting and must occur faster than 4 × 10⁸ s⁻¹. Their results were compatible with a mechanism involving a phosphoenzyme. The implications of the proposed mechanism are that there is a single binding site for all of the phosphoglycerates and that the net reaction requires phosphoryl transfer between histidine residues 8 and 184. This mechanism should give rise to two different phosphohistidine peptides from a given protein. We have looked for a biphasic rate of hydrolysis of the phosphoryl bonds in ³²P-labeled yeast and muscle phosphoglycerate mutases (Fig. 4) and can detect no less stable species. All of the enzyme-bound phosphate is acid-labile and hydrolyzes at the same rate. In our isolation of the ³²P-peptides after tryptic digestion there is no detection of an additional peptide peak, except as noted for bisphosphoglycerate synthase. An additional peptide peak at ≥2% of the radioactivity of the major peak would have been detected readily. We have now shown that histidine-8 is phosphorylated by glycerate-2,3-P₂. These results

**Fig. 4.** Hydrolysis of the phosphoryl bond(s) of [³²P]phosphoglycerate mutase (yeast). An incubation in 1.0-ml volume at 4°C contained: Tes-Na buffer, pH 7.5, 100 mM; phosphoglycerate mutase, 2.1 μM; [³²P]Glycerate-2,3-P₂, 4.2 μM, 2.69 × 10⁶ cpm/μg; microatom of ³²P, was added, and the mixture was incubated for 2 min. The sample was mixed with 3% sodium dodecyl sulfate (1.0 ml) and adjusted to pH 3.0 with 0.84 M glycine-HCl buffer at pH 2.2, and incubated at 45°C. At the designated time an aliquot (0.2 ml) was added to phenol and analyzed for ³²P-enzyme remaining.

**Fig. 5.** Enzyme isomerization mechanism for yeast phosphoglycerate mutase. 3-PGA, 2-PGA, and 2,3-DPG are glyceral-3-P, glycerate-2-P, and glycerate-2,3-P₂, respectively. The specificity of each histidine has been chosen arbitrarily: as shown the 2-phosphoryl enzyme occurs, it cannot be rate-limiting and must occur faster than 4 × 10⁸ s⁻¹. Their results were compatible with a mechanism involving a phosphoenzyme. The implications of the proposed mechanism are that there is a single binding site for all of the phosphoglycerates and that the net reaction requires phosphoryl transfer between histidine residues 8 and 184. This mechanism should give rise to two different phosphohistidine peptides from a given protein. We have looked for a biphasic rate of hydrolysis of the phosphoryl bonds in ³²P-labeled yeast and muscle phosphoglycerate mutases (Fig. 4) and can detect no less stable species. All of the enzyme-bound phosphate is acid-labile and hydrolyzes at the same rate. In our isolation of the ³²P-peptides after tryptic digestion there is no detection of an additional peptide peak, except as noted for bisphosphoglycerate synthase. An additional peptide peak at ≥2% of the radioactivity of the major peak would have been detected readily. We have now shown that histidine-8 is phosphorylated by glycerate-2,3-P₂. These results

**Fig. 6.** Single phosphorylation site mechanism for yeast phosphoglycerate mutase. Abbreviations are given in the legend to Fig. 5. See text for details.
strongly suggest that only histidine-8 is phosphorylated in yeast phosphoglycerate mutase and that intramolecular transfer of the phosphoryl group to another histidine residue does not occur.

As a result of inferences from kinetic data, we have made an alternative proposal in which there is phosphorylation at only one site (32). To account for the observation that KCl has opposite effects on kinetic parameters related to glycerate-2,3-P$_2$ and glycolate-2-P in the phosphatase reaction, it was proposed that the active site might have separate binding regions for glycerate-3-P and glycerate-2-P with a single overlapping phosphorylation site. Models indicate that the shapes and the charge distributions are quite different for the two monophosphoglycerates. It is suggested (see Fig. 6) that glycerate-2,3-P$_2$ can bind to either site on the nonphosphorylated enzyme and that each site is specific for the cleavage of a particular phosphoryl bond, e.g. at the site on the phosphoenzyme that binds glycerate-3-P, only the 2-phosphoryl bond is donated and re-formed. Net reaction involves re-formation of glycerate-2,3-P$_2$, which must re-orient or "creep" to the alternate binding site where the 3-phosphoryl bond is donated to the enzyme and glycerate-2-P is liberated. Phosphoenzyme is regenerated. This mechanism requires an extended binding region which would be expected to bind polyanions larger and more highly charged than any of the individual substrates. Recent studies support the hypothesis, as there is effective kinetic potencies.

lytic potencies. Better understanding of how phosphoglycerate mutase and phosphoglycerate synthase achieve their individual catalytic potencies.

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