The Amino Acid Sequence at the Catalytic Site of Phosphoglucomutase from Rabbit Muscle*

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ABSTRACT: The sequence of the amino acids at the active site of phosphoglucomutase from rabbit muscle has been constructed from analysis of ^{32}P-labeled phosphopeptides. These were derived from partial acid hydrolysis of labeled enzyme. The catalytic site contains two functional serines on one peptide chain separated by four amino acid residues.

Each of the serine residues has been isolated as the phosphate ester. Since the enzyme contains only one phosphate group per mole, it must then exist in two forms depending on which of the two serine residues is phosphorylated.

The two forms are presumed to be in dynamic equilibrium. The proposed structure is as follows: Glu-(Asp, Leu)-Gly-Val-Thr-Ala-*Ser*-His-Asp-Gly-Glu-*Ser*-Ala-Gly-Leu-Asp-Leu.

Phosphoglucomutase (PGM),[1] which catalyzes the reversible conversion of glucose 1-phosphate (G-1-P) to glucose 6-phosphate (G-6-P) (Cori *et al.*, 1938), is particularly suited for various active site studies. It can be obtained easily in pure crystalline form (Najjar, 1948), the mechanism of its action has been elucidated (Najjar and Pullman, 1954), and the existence of a single phosphate group per mole of enzyme that is reversibly transferrable to the substrate has been established (Sidbury and Najjar, 1957). It is therefore possible to label the enzyme through its natural catalytic function utilizing either of the two ^{32}P-labeled glucose phosphates without altering the enzymatic activity. A number of laboratories have exploited these properties. It was reported (Anderson and Jolles, 1957) that the phosphate group was bound by ester linkage to the amino acid serine. This finding was followed by the isolation of a number of peptides (Koshland and Erwin, 1957), all of which contained serine phosphate. Using a novel and elegant technique based on electrophoretic and chromatographic mobilities (Milstein and Sanger, 1961), the amino acid sequence Thr-Ala-SerP-His-Asp was proposed. This sequence contrasted with that reported from this laboratory (Harshman and Najjar, 1962) in which Asp-Gly-Glu-SerP-Ala-Gly was obtained. It is the purpose of this paper to present an extension of the sequence which also resolves this apparent discrepancy. Preliminary reports of these findings have already appeared (Harshman *et al.*, 1964; Bocchin *et al.*, 1964).

Materials and Methods

Crystalline PGM was prepared either from freshly excised or frozen rabbit muscle (Pel-Freez Corp., Ark.) by a slightly modified method (Najjar, 1962).

PGM-^{32}P was prepared by mixing G-6-^{32}P with a tenfold molar excess of PGM dissolved in 1×10^{-3} M $MgCl_2$ and 0.04 M imidazole pH 7.5 buffer. After 10 min of equilibration at 30° the mixture was cooled in an ice bath and the PGM-^{32}P was precipitated by adding three volumes of a solution of saturated ammonium sulfate. The precipitate was collected by centrifugation and then dialyzed overnight against water at 0°.

Hydrolytic fragmentation of PGM-^{32}P was achieved by refluxing in 2 N HCl at 100° for 120 min. Under these conditions 85% of the bound ^{32}P and only about 50% of the total protein were solubilized. Following hydrolysis, the reaction mixture was chilled on ice and the insoluble residue was removed by centrifugation. The supernatant fluid, containing the phosphopeptides, was dried by flash evaporation and the excess HCl was removed by two successive additions of 10 ml each of water, followed by redrying.

Prompted by the recent warning (Ikawa and Snell, 1961) that the two amino acids, glutamic acid and serine, are prone to form *O*-glutamylserine esters during prolonged contact in dilute acids, we made a direct evaluation of the hydrolytic and drying procedures used in these studies. Thus, when a mixture containing 1.0 μmole of glutamic acid and 1.0 μmole of serine was submitted to the standard procedure of hydrolysis in 2 N HCl followed by flash evaporation, no glutamylserine was formed. The possibility of inversion in the amino acid sequence of certain peptides during hydrolysis in 2 N HCl at 100° (Sanger and Thompson,

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[1] Abbreviations used: PGM, phosphoglucomutase; G-1-P, glucose 1-phosphate; G-6-P, glucose 6-phosphate; FDNB, fluorodinitrobenzene; TPD, triosephosphate dehydrogenase; PGK, phosphoglycerate kinase.

1952; Schaffer *et al.*, 1955) was also ruled out. No evidence for diketopiperazine formation or inversion of any of the amino acid sequence was detected during any of the various procedures used. Consistent with this observation were the findings following hydrolysis in 12 N HCl at room temperature for 72 hr, a procedure in which inversion of amino acid sequence is minimized (Sanger, 1952). This type of hydrolysis yielded peptides similar in column fractionation and electrophoretic mobilities as well as in amino acid composition to those obtained by our standard method.

The amino acid composition of each peptide was determined after 18 hr of hydrolysis at 100° in 6 N HCl by a two-dimensional paper-separation technique. The separation of amino acids was achieved by high-voltage electrophoresis at pH 2.0, 0.75 M formic acid (Gross, 1959), in the first direction followed by descending chromatography in 80% pyridine in the second direction. When proline was to be separated from glutamic acid, or valine from leucine and isoleucine, 1-butanol–acetic acid–water (4:1:5) was used in the second dimension. Control amino acids were added to the paper for each run. The controls added during electrophoresis were removed by cutting a strip from the chromatogram, and fresh controls were applied before the second dimension was run. This procedure permitted the identification of each amino acid by comparing its mobility in each dimension with the controls. The amino acids, which were visualized by lightly spraying with ninhydrin, were submitted to quantitative analysis (Yemn and Cocking, 1955) with some modification as follows. An area of paper to be analyzed was cut out, minced, placed in a test tube, and wetted with 0.1 ml of a solution of methanol containing 0.3 M boric acid and 0.4 M KOH (Connell *et al.*, 1955). The papers, so treated, were dried *in vacuo* after which 0.7 ml of the ninhydrin reagent was added. The latter was composed of 3.3×10^{-3} M ninhydrin in 0.3 M acetate buffer, pH 4.7, containing 10% Methyl Cellosolve (ethylene glycol monomethyl ether). This was followed by 1.0 ml of 2×10^{-4} M KCN in 10% Methyl Cellosolve. The mixture was capped and heated at 100° for 15 min, cooled to 20°, and read at 570 mμ. Proline was read at 440 mμ. The statistical analysis of seventeen replicate determinations gave standard deviation values for the amino acids, Glu, Asp, Thr, Ala, Ser, Gly, His, Val, Lys, and Arg, that ranged from 5 to 13% of the mean values. A comparison was made between the values obtained by the two-dimensional paper method with those obtained by the modification (Piez and Morris, 1960) of the column method (Spackman *et al.*, 1958) developed by Technicon Corp. It was found that the two procedures gave equivalent quantitative values. All values for the amino acids listed in the text are uncorrected including those of serine and threonine.

End-group analysis on a microscale was performed using an adaptation of the fluorodinitrobenzene (FDNB) method (Sanger and Thompson, 1953). FDNB-^{14}C (specific activity 1.1–1.8 μc/μmole) was used throughout. Peptide (0.01–0.06 μmole) in 0.1 ml of water was treated with 0.01 ml containing 0.5 μmole of FDNB-^{14}C. The pH was adjusted to approximately 9.5 by adding 0.1 ml of a 0.1% v/v solution of triethanolamine and clarified by small increments of 95% ethanol. The reaction mixture was incubated overnight at room temperature. Following extraction with ether to remove any residual FDNB, the reaction mixture was dried, redissolved in 0.01 N HCl, and reextracted with ether. This last extraction was done to remove excess dinitrophenol and detect any possible contaminating free amino acids. The samples were then dried, dissolved in 6 N HCl, and hydrolyzed in two steps at 100°. After 2 hr of hydrolysis, the samples were dried, dissolved in 0.01 N HCl, and extracted with ether. Hydrolysis was then continued in 6 N HCl for 18 hr and again extracted as above. This permits the early detection of dinitrophenyl(DNP-)glycine and DNP-proline, which are labile to prolonged acid treatment. The combined ether extracts were then dried and added to a mixture of appropriate DNP amino acid controls (0.1 μmole each) and subjected to two-dimensional paper chromatography (Fraenkel-Conrat *et al.*, 1955). The ^{14}C-DNP end group was identified by radioautography.

G-6-^{32}P was prepared from terminally labeled adenosine triphosphate (ATP) ^{32}P and glucose by the reaction catalyzed by hexokinase (Colowick and Kalckar, 1941). Radioactive ATP was prepared from phosphoric acid ^{32}P ($^{32}P_i$) and adenosine diphosphate using spinach chloroplasts (Jagendorf and Avron, 1958) or by an adaptation of the enzymatic exchange of $^{32}P_i$ and adenosine triphosphate (Pfleiderer, 1961). This involved the use of a relatively large quantity of triosephosphate dehydrogenase (TPD) containing sufficient amounts of bound DPN and phosphoglycerate kinase (PGK). The latter method proved to be more convenient and is therefore given in detail. The reaction mixture was composed of 3-phosphoglycerate (3.0 μmoles), ATP (1 μmole), Mg^{2+} (5 μmoles), TPD (100 μg), PGK (20 μg), and 3–15 mcuries of neutralized carrier-free $^{32}P_i$ in a final volume of 1.0 ml of 0.10 M Tris buffer at pH 7.2. In this system no reducing agent was necessary. After 1 hr at 30°, the ATP was separated on a Dowex-1 resin column (Cohn, 1957), adjusted to pH 5.0, and stored at −50° until needed.

Results

The ^{32}P in all samples of labeled PGM used in this study was readily and completely exchangeable with either G-1-P or G-6-P. This indicates that the ^{32}P peptides obtained were derived from active ^{32}P-labeled enzyme having the substrate specificity of PGM.

Fractionation of Phosphopeptides. Phosphopeptides obtained by partial acid hydrolysis were chromatographed on Dowex-50 (H^+) resin columns. Samples corresponding to 1–2 μmoles of protein were applied to a 1.5 × 40 cm column. For larger samples (up to 10 μmoles) 4.0 × 40 cm columns were used. The peptides were then eluted first with 0.01 N HCl and then with 0.6 N HCl. The progress of the chromatography was followed by monitoring the radioactivity of the effluent

TABLE I: Isolated ^{32}P Peptides from Acid-Hydrolyzed Rabbit Muscle Phosphoglucomutase.

Peptide No.	Peptide Code	Amino Acid Composition[a]
1	III B_1	*SerP-Ala-Gly
2	II A_2	*Glu-SerP-Ala-Gly
3	III, V	Glu-SerP-Ala-Gly-Leu-Asp-Leu
4	I A_2	Glu-SerP-Ala-Gly-Leu-Asp
5	I, VII	Glu-SerP-Ala-Gly-Leu
6	I A_3	Glu-SerP-Ala-Gly
7	I B_1	*Gly-Glu-SerP
8	IV, V, VI	Gly-Glu-SerP-Ala-Gly-Leu-Asp
9	III A_2	*Gly-Glu-SerP-Ala-Gly
10	III A_3	*Gly-Glu-SerP-Ala
11	III A_1	*Asp-Gly-Glu-SerP-Ala-Gly
12	IV, VI	Asp-Gly-Glu-SerP-Ala-Gly-Leu
13	V, VI	Asp-Gly-Glu-SerP-Ala
14	IV, V, VI	Asp-Gly-Glu-SerP
15	VI, A_1	*SerP-His-Asp-Gly-Glu
16	V, B_1	*SerP-His-Asp-Gly
17	V A_2	*SerP-His-Asp
18	VI A_2	*SerP-His
19	V	Ala-SerP-His
20	V A_1	*Thr-Ala-SerP-His-Asp-Gly-Glu
21	VI	Thr-Ala-SerP-His-Asp-Gly-Glu-SerP-Ala
22	V A_3	*Thr-Ala-SerP-His
23	III B_2	*Thr-Ala-SerP
24	V B_2	*Gly-Val-Thr-Ala-SerP-His-Asp-Gly-Glu-SerP
25	I B_2	*Glu-Asp-Leu-Gly-Val-Thr-Ala-SerP
Form I		Glu-(Asp, Leu)-Gly-Val-Thr-Ala-*SerP*-His-Asp-Gly-Glu-Ser-Ala-Gly-Leu-Asp-Leu
Form II		Glu-(Asp, Leu)-Gly-Val-Thr-Ala-Ser-His-Asp-Gly-Glu-*SerP*-Ala-Gly-Leu-Asp-Leu

[a] Leu = Leu or Ileu; asterisk (*) denotes amino terminal as determined by FDNB-^{14}C.

fluid. Three fractions containing radioactive material were eluted with each solvent and recovery of total radioactivity was quantitative. The first three fractions were composed primarily of phosphorylated peptides. The last three fractions, which required 0.6 N HCl for elution, were grossly contaminated with nonphosphorylated peptide material. A typical elution pattern is shown in Figure 1. Following column chromatography, each radioactive fraction was concentrated by flash evaporation and the excess HCl was removed by repeated drying, as described above. The fractions were then submitted to descending chromatography in 2-butanol–formic acid–water (7:2:1) on Whatman 3MM paper for 18 hr at 22°. In this solvent the ^{32}P-labeled peptides were retained as a band near the origin, while significant amounts of the nonlabeled peptides moved down the paper. The ^{32}P-containing band in all instances was located by radioautography, cut out, and eluted with water. Further purification was performed by high-voltage electrophoresis at pH 3.5 in the pyridine acetate system (Ryle *et al.*, 1955). This was followed by electrophoresis at pH 2.0 in 0.75 M formic acid (Gross, 1959). The electrophoresis at each pH was repeated until discrete bands were obtained.

Two criteria for purity of peptides were used for each enzyme preparation: (a) the attainment of stable ratios among the constituent amino acid residues, and (b) the attainment of a stable ratio between ^{32}P and the serine residues. The ratio of ^{32}P to serine for a particular enzyme preparation was obtained from the isolated radioactive phosphoserine and taken arbitrarily as 1.0. Peptides containing one serine residue and those containing two attained a stable ratio of 1.0 and 0.5, respectively. This is consistent with the fact that PGM has only 1 mole of phosphorus/mole of enzyme (Sidbury and Najjar, 1957).

Considerable subfractionation of each of the column fractions occurred during purification by electrophoresis. This was particularly true in electrophoresis at pH 2.0, during which cleavage of certain peptides, in addition to subfractionation, was observed (see below).

For proper identification of the fractions reported herein, a code system of nomenclature was adopted. Column fractions are designated by Roman numerals,

subfractions achieved during electrophoresis at pH 3.5 by letters of the alphabet, and further subfractions obtained by electrophoresis at pH 2.0 by Arabic numerals. Both the letters and the numbers are assigned in an arbitrary order of increasing distance from the positive electrode. Peptides isolated at low yield or frequency are not identified beyond the column code. The bracketted number appearing to the right of a particular peptide denotes the number of enzyme preparations from which it was isolated. The total number of preparations analyzed was ten. Table I lists a series of peptides 1–25, that are arranged in such a manner that permits the consecutive ordering toward the amino terminal. It shows the manner in which the sequence of octadecapeptide can be built. The DNP peptides alone allow the construction of the sequence: Glu-(Asp,Leu)-Gly-Val-Thr-Ala-SerP-His-Asp-Gly-Glu-SerP-Ala-Gly. The remaining peptides that are listed were isolated in many enzyme preparations sufficient only for adequate determination of the amino acid composition, and consequently no FDNB reaction was attempted. These further extend the sequence toward the carboxy terminal by three additional residues: Leu-Asp-Leu. The following DNP peptides, 1, 2, 7, and 9–11, allow the unambiguous stepwise amino-terminal extension from the serine residue of peptide 1: Ser-P-Ala-Gly- to Asp-Gly-Glu-SerP-Ala-Gly. Peptides 15–18 with serine phosphate as amino terminal allow the insertion of histidine between the serine and aspartic residues. Similarly, peptides 20, 22, and 23, with threonine terminal, allow the insertion of alanine between the threonine and serine residues; peptide 24 with glycine terminal places valine between glycine and threonine. The final peptide 25 has a glutamic terminal which is separated from glycine by two unordered residues, aspartic and leucine. However, our sequence studies on peptide fragments isolated from trypsin digest followed by partial acid hydrolysis places the aspartic residue next to the amino-terminal glutamic (Harshman *et al.*, 1964).

Starting with the serine residue of peptide 1, the sequence toward the carboxy terminal is indicated by the relative length of the peptides. The three DNP-glycine peptides 7, 10, and 9, respectively, permitted the ordering of peptide 1: SerP-Ala-Gly. In like manner, peptides 5, 4, and 3, whose amino terminals are secured by the DNP peptide given, permit the extension of Leu-Asp-Leu, respectively. This is further reinforced by peptides 12 and 8.

Inasmuch as there is one phosphate and two serine residues in the sequence of the site proposed, it became necessary to postulate the existence of the active site in two forms (Form I and II). Since all of these peptides have been derived from acid hydrolysates, no distinction can be made between the dicarboxylic acids and their amides. In fact, our studies on the trypsin fragments of PGM indicate that these amino acids exist in the amide form. (Harshman and Najjar, in preparation).

The Properties of the Isolated Coded Peptides. The pertinent properties of each of the major peptides in the column fractions I–VI will now be presented in their respective order. The mole ratio is inserted following each amino acid and the amino-terminal residue for each peptide is designated by exclusion from the parenthesis. Only peptides that were subjected to the FDNB reaction are included. Those failing to react with the reagent are discussed in detail. In a few of the peptides reported, contaminating amino acids at less than integral ratios were detected. In no case did they exceed 0.2 and hence they are not reported. No yields are given for the peptide ratios since every peptide was not obtained from each preparation that was analyzed. Moreover, due to the intensive fragmentation of some of the large peptides, a final percentage yield would be misleading.

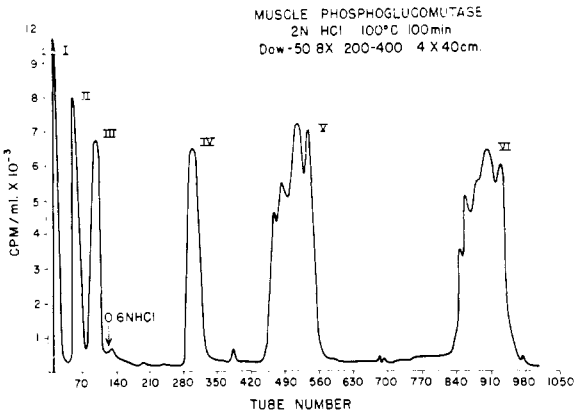


FIGURE 1: A typical elution pattern expressed as counts per minute of a partial acid hydrolysate of 10 μmoles of ^{32}P PGM. Dowex-50 in the hydrogen form was washed with water until the effluent showed a pH value about 3.5. After drying, the sample was dissolved in 5 ml of water and applied to the washed column. Aliquots (10-ml) were collected. Elution was started with 0.01 N HCl followed by 0.6 N HCl as indicated. Recovery of radioactivity approached 100%.

COLUMN FRACTION I contained 10% of the total ^{32}P recovered. About one-half of this material I A_1 was ninhydrin negative and proved to be inorganic phosphate by mobility characteristics in chromatographic and electrophoretic systems and by extractability of the molybdate complex into isobutyl alcohol–benzene mixture (Lehninger, 1955). However, the remainder of the ^{32}P was ninhydrin positive and represented several peptides that were not extractable in this system. These were found to exhibit electrophoretic mobilities at pH 3.5 that were close to or identical with that of $^{32}P_i$. However, at pH 2.0 they fell behind and in general were readily separable. Peptide I A_2 ($Glu_{0.93}Ser\text{-}^{32}P_{1.1}Ala_{0.99}Gly_{1.2}Leu_{1.0}Asp_{1.0}$) [5] was not easily separable from inorganic phosphate. At pH 3.5, its electrophoretic mobility was identical with that of $^{32}P_i$. However, it was sufficiently retarded at pH 2.0 to allow its isolation though not completely free of $^{32}P_i$. Hence, it is the only peptide reported here in which the ^{32}P serine ratio is higher than that predicted from the analysis of the purified Ser-^{32}P. The amount of contaminating $^{32}P_i$

was determined by extraction of the phosphomolydate in isobutyl alcohol–benzene mixture. The peptide did not react with FDNB. This property along with its high acidic mobility is assumed to result from the obliteration of the terminal amino group of a pyrrolidonized glutamic acid. Peptide I A_3 ($Glu_{1.1}Ser\text{-}^{32}P_{1.0}Ala_{0.88}Gly_{0.98}$) [3] was separated from I A_2 during electrophoresis at pH 2.0. Like the previous peptide, it did not react with FDNB and exhibited strongly acidic characteristics. It was therefore presumed to have an N-terminal glutamic acid in the cyclized pyrrolidone form. Peptide I B_1 $Gly_{0.95}(Glu_{0.91}Ser\text{-}^{32}P_{1.00})$ [6] moved as a thin band immediately behind the I A fraction in electrophoresis at pH 3.5. Peptide I B_2 $Glu_{1.2}(Asp_{1.1}Leu_{1.2}Gly_{1.0}Val_{1.0}Thr_{0.76}Ala_{0.90}Ser\text{-}^{32}P_{0.39})$ [3] moved slightly behind I B_1 at pH 2.0.

COLUMN FRACTION II contained approximately 10% of the applied ^{32}P and was primarily composed of $Ser^{32}P$, II A_1. The serine phosphate was isolated from all the ten preparations analyzed and each served as the standard ^{32}P counts/ninhydrin color ratio. In contrast to the di- and monoisopropyl phosphate derivatives, very poor yields of DNP-serine phosphate were obtained. Similarly, dinitrophenylation of acidic peptides with N-terminal serine phosphate residues followed by the usual acid hydrolysis gave equally poor yields of DNP-serine. This may well result from an interaction between the α-amino group and the phosphate which may not occur with the isopropyl esters. This is supported by our finding of a pK_a of 10.5 for the α-amino group of authentic *dl*-serine phosphate as compared to a pK_a of 9.5 for the free serine. Attempts to suppress this ionic effect by carrying out the dinitrophenylation at pH 12.0 did not appreciably improve the yield of DNP-SerP. However, a significant amount of dinitroaniline was obtained. This could result only from the decomposition of a DNP-phosphoserine since DNP-serine is stable under these conditions. In contrast to all this, the FDNB reaction with the N-terminal phosphoserine adjacent to a histidyl residue yielded after acid hydrolysis expected amounts of DNP-serine. It is possible that the amino group here is more accessible to the reagent by virtue of an effect exerted by the adjacent histidyl residue. Peptide II A_2 $Glu_{1.1}(Ser\text{-}^{32}P_{1.0}Ala_{0.80}Gly_{1.1})$ [4] was separated from serine phosphate by electrophoresis at pH 2.0 where it was sufficiently retarded for proper isolation.

COLUMN FRACTION III also contained about 10% of the total radioactivity. It was resolved into two major bands during electrophoresis at pH 3.5 in which both moved toward the positive pole. Each band was further resolved at pH 2.0. Peptide III A_1 $Asp_{1.2}(Gly_{1.0}Glu_{1.0}Ser\text{-}^{32}P_{1.0}Ala_{0.89}Gly_{1.0})$ [3], like all III A peptides, had a mobility in pH 3.5 buffer that was intermediate between the serine phosphate position and the origin. However, this peptide retained a strong net negative charge at pH 2.0. Peptide III A_2 $Gly_{0.95}(Glu_{0.92}Ser\text{-}^{32}P_{1.0}Ala_{1.2}Gly_{0.95})$ [3] showed an electrophoretic mobility at pH 2.0 that placed it slightly behind peptide III A_1. Peptide III A_3 $Gly_{1.3}(Glu_{0.9}Ser\text{-}^{32}P_{1.0}Ala_{1.2})$ [2] in pH 2.0 electrophoresis fell behind both III A_1 and III A_2. Peptide III B_1 $Ser\text{-}^{32}P_{1.0}(Ala_{1.0}Gly_{0.82})$ [3] was separated from III B_2, in electrophoresis at pH 2.0. When treated with FDNB, it gave a poor yield of DNP-serine and no other DNP amino acid was detectable. However, after hydrolysis with 6 N HCl at 100° for 18 hr and retreatment with FDNB, the following DNP derivatives were readily obtained in good yield: DNP-serine, DNP-alanine, and DNP-glycine. Since all model peptides having N-terminals of serine, alanine, or glycine reacted with FDNB to give good yields of the corresponding DNP derivatives, it is concluded that the low but exclusive yield of DNP-serine in this tripeptide is indicative of an N-terminal serine phosphate (see II A_1 above). Peptide III B_2 $Thr_{1.0}(Ala_{1.0}Ser\text{-}^{32}P_{0.91})$ [4] moved slightly toward the negative pole during pH 2.0 electrophoresis.

COLUMN FRACTION IV represents the first fraction eluted with 0.6 N HCl. It also contained about 10% of the ^{32}P, but was grossly contaminated with ninhydrin-positive nonradioactive peptides. This contamination was so great that purification procedures designed to isolate the radioactive peptides were, in general, unsuccessful. A major problem was that fragmentation of the ^{32}P peptides occurred during the repeated column rechromatography or exposures to the electrophoresis procedures. As a consequence of fragmentation, the ^{32}P peptides that were eventually isolated from this fraction corresponded to acidic peptides I A_2, I A_3, II A_2, III B_1, and III B_2.

COLUMN FRACTION V, in contrast to previous fractions, contained about 30% of the radioactivity and was essentially neutral in charge during initial electrophoresis at pH 3.5. Upon repeated electrophoresis, part of the band separated into a series of ^{32}P peptides, some of which moved toward the cathode and others toward the anode. The shape of the peak obtained from Dowex-50 chromatography, together with the results cited above, indicate that this fraction is heterogeneous and consists of a mixture of neutral and basic, small as well as some large, peptides. Some of these undergo fragmentation during the purification procedures. The segregation of Fraction V into two bands, A and B, as listed below was not observed with every preparation. Furthermore, a variety of acidic peptides, corresponding to those discussed above for fraction I, II, and III, were isolated during the purification procedures. These again were assumed to be fragmentation products and are not reported below. However, certain small acidic peptides, not corresponding to any found in the previous fractions, were isolated and purified. In addition, larger peptides for which end groups were not determined were also isolated. Peptide V A_1 $Thr_{1.0}(Ala_{1.2}Ser\text{-}^{32}P_{1.0}His_{1.0}Asp_{1.2}Gly_{0.92}Glu_{1.0})$ [4] proved to be essentially neutral at pH 3.5 but moved slightly to the cathode at pH 2.0. Peptide V A_2 $Ser\text{-}^{32}P_{0.80}(His_{1.0}Asp_{1.1})$ [3] showed greater mobility toward the cathode at pH 2.0 as compared to the previous peptide. Like the other histidine-containing peptides, V B_1, VI A_1, and VI A_2, it reacted readily with FDNB to yield DNP-serine as the N-terminal residue. Peptide V A_3 $Thr_{0.89}(Ala_{1.2}Ser\text{-}^{32}P_{1.0}His_{0.94})$ [3] carried a strong positive charge at pH 2.0.

TABLE II: Composition of the Uncoded ^{32}P Peptides Derived from Various Column Fractions.[a]

Column Fraction	No. of Times Isolated	Peptide No.	Mole Ratio of Amino Acids												
			Thr	Ala	SerP	His	Asp	Gly	Glu	SerP	Ala	Gly	Leu	Asp	Leu
VI	1	1	1.0	0.95	0.90	1.3	1.1	0.90	0.79	0.90	0.95				
V	1	2		1.0	0.84	1.1									
IV, V, VI	8	3					1.0	1.3	0.80	1.1					
V, VI	3	4					1.1	0.80	0.73	1.0	1.0				
III, IV, VI	4	5					0.83	1.0	1.0	0.78	1.2	1.0	0.83		
IV, V, VI	3	6						1.1	0.85	1.0	0.88	1.1	0.74	1.0	
I, III	3	7							1.0	0.92	1.1	0.92	1.0		
III, V	2	8							0.80	0.91	0.91	1.3	0.82	1.0	0.82

[a] Table II represents a list of uncoded peptides isolated either in low yields or low frequency. The value obtained for an amino acid occurring more than once in a peptide is divided by the appropriate number and recorded in each position.

Peptide V B_1 Ser-$^{32}P_{0.93}$($His_{1.0}Asp_{1.2}Gly_{0.83}$) [2] moved toward the cathode at pH 3.5 and only slightly so at pH 2.0. Peptide V B_2 $Gly_{1.0}$($Val_{1.3}Thr_{0.88}Ala_{0.94}$*$Ser_{0.9}$*-$His_{1.0}Asp_{1.0}Gly_{1.0}Glu_{1.1}$*$Ser_{0.9}$*$PO_{4\ 0.9}$) [2] was readily separable from V B_1 since it showed relatively good mobility toward the cathode at pH 2.0. It contains two serine residues per phosphate group. The ^{32}P is assumed to be in part associated with both serine residues. The mobility of this peptide at pH 2.0 reflects a strong positive charge.

COLUMN FRACTION VI also was obtained in substantial quantity and contained the final 30% of the radioactivity. It moved to the cathode during initial electrophoresis at pH 3.5, but in all other respects, concerning fragmentations and heterogeneity, it was similar to fraction V. Only two simple ^{32}P peptides, VI A_1 and VI A_2, will be discussed, as these were recovered in three separate enzyme preparations. However, a rather large group of complex ^{32}P peptides, most of which were isolated only once, were derived from this fraction. Consequently, these are listed separately. It is significant, however, that in no case did any of these exhibit amino acid compositions that were in conflict with the ordered structure derived from the analysis of the smaller ^{32}P peptides. Peptide VI A_1 Ser-$^{32}P_{1.0}$($His_{0.79}Asp_{1.1}Gly_{1.0}$-$Glu_{0.95}$) [3] exhibited only moderate mobility to the cathode during electrophoresis at pH 2.0 and was readily dinitrophenylated. Peptide VI A_2 Ser-$^{32}P_{1.0}$-($His_{1.3}$) [3] is the most basic peptide isolated after electrophoresis at pH 2.0. It too reacted readily with FDNB.

The Properties of Uncoded Peptides. In contrast to the peptides discussed above, a number of peptides shown in Table II were obtained from one or more column fractions in small yield, sufficient only for adequate determinations of amino acid composition. Consequently, no N-terminal studies were performed. These have also been listed in Table I but no properties or mole ratios have heretofore been discussed.

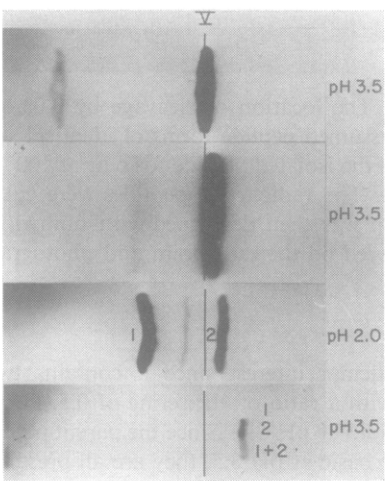


FIGURE 2: A series of autoradiograms of paper electrophoresis at the pH indicated. It shows the cleavage of a purified phospho-peptide by formic acid, 0.75 M, pH 2.0, at 20°. A highly purified peptide (see text) obtained from column fraction V is resolved at pH 3.5 into a homogeneous band. Electrophoresis at pH 2.0, 0.75 M formic acid resulted in two bands with differing charges, 1 and 2. These bands retained their respective mobilities on re-electrophoresis at pH 3.5 in pyridine acetate. Electrophoresis in all cases was performed at 40 v/cm for 70 min.

Peptides 1 and 2 were each isolated once from fraction VI and V, respectively. These moved to the cathode at both pH values, indicating the probable existence of the dicarboxylic acids in the amide form. Peptide 1

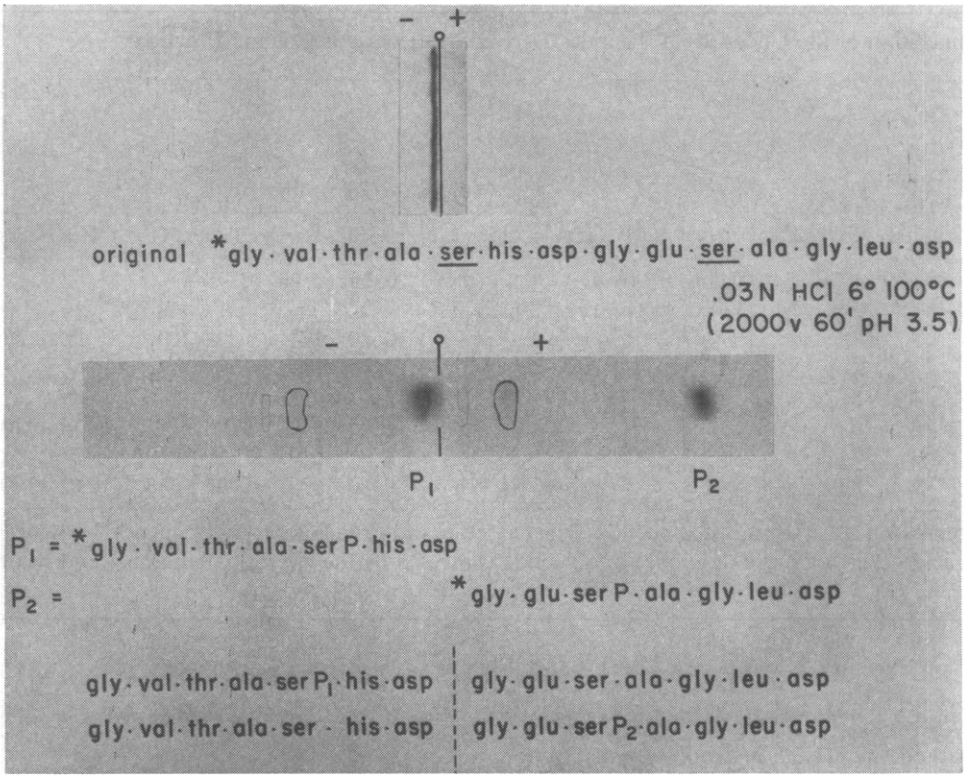


FIGURE 3: The location of cleavage by 0.03 N HCl of an isolated and ordered phospho-peptide. This figure shows the two assumed peptide forms of identical sequence except for the location of the phosphoseryl residue. After hydrolysis of the isolated peptide for 6 hr at 100° followed by drying and electrophoresis at pH 3.5, radioautograms were developed. The radioactive peptides were cut out and subjected to amino-terminal and composition analysis. The paper strip was then developed with ninhydrin and the two nonradioactive peptides were located. The strip was superimposed on the radiogram and photographed (see text for details).

is of particular interest since it contains two serine residues with a ratio of ^{32}P:serine of 0.5. Peptides 3–6 are all acidic at pH 3.5. Since the parent peptides were neutral or basic at pH 3.5, they are all presumed to be fragmentation products of larger peptides. At pH 2.0, peptide 3 is acidic, 5 is neutral, 4 and 6 are basic. Peptides 7 and 8 show a high degree of acidity at both pH values. A likely explanation is that the N-terminal glutamic residue is pyrrolidonized, thereby obliterating the positive charge on the α-amino group.

The Presence of Two Active Serine Residues at the Catalytic Site. The sequence at the active site, as derived from the peptides listed in Table I, includes two serine residues, one or the other carrying a phosphoryl group. Since the enzyme contains one transferrable phosphate per mole, it can be safely assumed that both serine phosphate residues are catalytically active. The active site therefore may exist in one of two forms: form 1, Glu-(Asp,Leu)-Gly-Val-Thr-Ala-SerP-His-Asp-Gly-Glu-Ser-Ala-Gly-Leu-Asp-Leu; form 2, Glu-(Asp,Leu)-Gly-Val-Thr-Ala-Ser-His-Asp-Gly-Glu-SerP-Ala-Gly-Leu-Asp-Leu. A number of reasons (a–d) favor this arrangement.

(a) There is a progressive overlap of the ordered amino acid residues in the various pepides 1–24 in Table I. Those peptides in which the amino-terminal residues have been determined suffice to emphasize this point.

(b) Three separate peptides have been isolated from acid hydrolysates of a number of PGM-^{32}P preparations, each possessing two serine residues to each phosphate group. These are 21 and 24 listed in the table, and a third peptide discussed below.

(c) Larger peptides from column fractions V and VI upon rechromatography on Dowex-50 broke continuously into highly acidic small peptides similar to those obtained in I, II, and III and basic peptides containing serine-^{32}P and histidine. Furthermore, upon electrophoresis in formic acid at pH 2.0, some of these peptides were also observed to break similarly into smaller peptides with higher acidic and highly basic properties. Figure 2 is illustrative of this behavior.

A highly purified phospho-peptide from column fraction V (Ser, His, Asp, Gly, Glu, Ser, Ala, Gly, Leu, Asp) that had gone through repeated electrophoresis at pH 3.5 and subsequently was subjected to electrophoresis at pH 2.0 in 0.75 N formic acid was noted to split into two major radioactive peptides, (Gly, Glu, Ser, Ala, Gly, Leu, Asp) and (Ser, His, Asp). It is clear that the cleavage had occurred after exposure to 0.75 M formic acid pH 2.0.

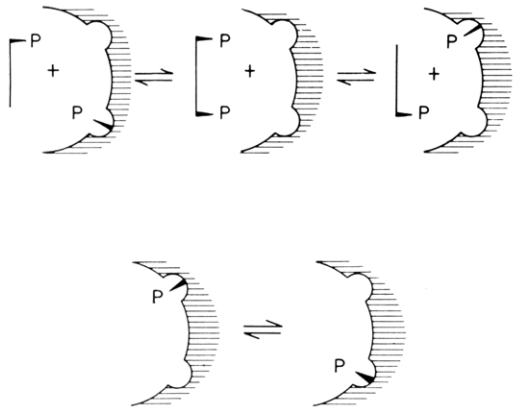


FIGURE 4: A diagrammatic sketch of the active site showing the two phosphoserine loci. It illustrates the mechanism of the transfer of phosphate between enzyme and substrate. G-1-P is shown reacting with the serine phosphate group on one locus to form G-1,6-P, followed by a transfer of the glucosyl phosphate of G-1,6-P to the serine residue at the other locus. The third step represents an equilibrium state between the phosphorylated serines.

Further, upon mixing the two separated bands and again subjecting this mixture to electrophoresis at pH 3.5, the bands, nevertheless, remained separated. Their mobility was such that one was strongly acidic and the other distinctly basic. This observation was repeatedly made on larger peptides that included the two serine residues. It was observed that treatment with 0.75 M formic acid at room temperature effected maximal splitting in a few minutes. The mechanism of this reaction is presently under study.

(d) An isolated phospho-peptide Gly-(Val, Thr, Ala, Ser, His, Asp, Gly, Glu, Ser, Ala, Gly, Leu, Asp) was purified in the usual manner including repeated electrophoresis at pH 3.5 at which pH it was essentially neutral in charge. It was then treated with 0.03 N HCl for 6 hr at 100° and again subjected to electrophoresis at pH 3.5. Figure 3 shows the resulting bands. The single band had split predictably into four ninhydrin-positive bands, two of which were radioactive, P_1 and P_2. The split occurred at the aspartyl–glycine bond as illustrated in the figure and yielded two peptides with glycine amino terminal. P_1 was basic: Gly-(Val, Thr, Ala, SerP, His, Asp) at pH 3.5; P_2 proved to be acidic: Gly-(Glu, SerP, Ala, Gly, Leu, Asp). The other two peptides, not containing phosphate, showed mobilities that differed from the phosphorylated counterparts, in a manner attributable to the difference in net charge imparted by the phosphate group. Thus one of these was more basic and moved to the cathode to a much greater extent than P_1. The other moved to the anode but to a much lesser extent than P_2.

These observations lend strong support to the proposition that the catalytic site of PGM displays two active serine residues cooperatively involved in phosphate transfer to the substrate. Implicit in this is the conclusion that the two forms of a particular phosphopeptide are indistinguishable in their chemical and physical characteristics regarding behavior in both the chromatographic and electrophoretic systems studied. This does not necessarily exclude the possibility, however unlikely, that the PGM preparation might contain two different and distinct enzymes catalyzing the same reaction but differing in the location of the phosphoserine. This is unlikely because the enzyme is homogenous in its sedimentation and electrophoretic properties (Najjar, 1962). In addition, it has no detectable amino-terminal residues (Milstein and Sanger, 1961; Harshman *et al.*, 1963; Joshi and Handler, 1964), nor any free carboxy terminal available to carboxypeptidase A or B (Harshman *et al.*, 1963). It would therefore be difficult to imagine two distinct enzymes possessing such similar characteristics.

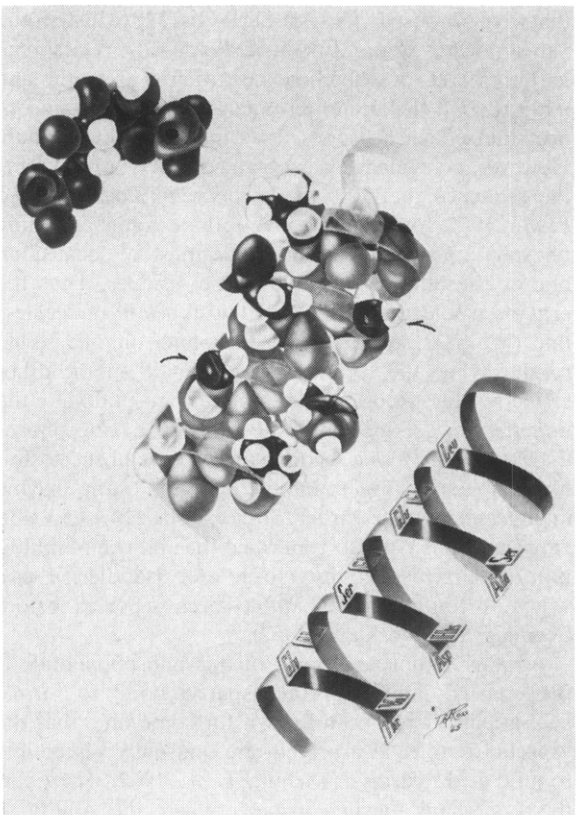


FIGURE 5: An artist's representation of the active site constructed from lapine atomic models, in the center. The helical ribbon on the lower right depicts the spatial relationships and orientations of the residues from glycine through leucine. G-1,6-P is shown on the upper left with the oxygen of the two phosphates exposed. These can readily be superimposed on the exposed side-chain carbons of the two serine residues (arrows).

Discussion

It is apparent that the proposed sequence of amino acids at the active site of phosphoglucomutase incorporates the two different phosphoserine peptides

that were proposed: Thr-Ala-SerP-His-Asp (Milstein and Sanger, 1961) and Gly-Glu-SerP-Ala-Gly (Harshman Najjar, 1962). In extending the structure from the and latter toward the amino terminal, we were able to confirm the sequence proposed by the Cambridge group. However, no evidence was obtained that would support the sequence that was first proposed (Koshland and Erwin, 1957) for the active site of this enzyme. The only phosphate group on the molecule must be located on one or the other of the two serine residues. Thus the enzyme would then consist of two types of molecules: one that has the functional phosphate on one serine residue while the second type carries it on the other serine residue. Repeated attempts to phosphorylate the nonesterified serine with ^{32}P diisopropylphosphorofluoridate (DFP) as a second marker were unsuccessful. No radioactivity was found incorporated in the enzyme in agreement with earlier observations (Milstein and Sanger, 1961). We also confirmed the absence of inhibition of enzyme activity (Joshi and Handler, 1964), which is contrary to what has been reported before (Kennedy and Koshland, 1957).

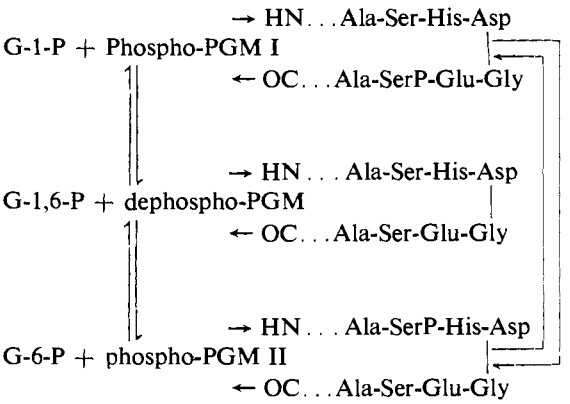
Another important aspect of this phosphopeptide is the unusual lability of the aspartyl bond to formic acid attack. It has been known for some time that the aspartic residues in a protein are unusually susceptible to mild acid hydrolysis (Schultz *et al.*, 1962). However, this is perhaps the first instance where the lability to 0.75 M formic acid at otherwise mild conditions was so evident. The mechanism of this type of cleavage is still obscure.

The possibility of two reactive loci within a single active site was first suggested at the time that the mechanism of action was formulated, and an additional reaction step was then proposed (Najjar and Pullman, 1954). The finding of two active serine residues capable of reversible transfer of the phosphate group with the substrate supports that formulation.

On the basis that one active serine transfers phosphate reversibly to the glucosyl carbon (C-1) of glucose and the other to the ester carbon (C-6), the additional step required is for the cooperative reversible transfer of phosphate between the serine residues themselves. This is obviously mandatory for the maintenance of catalysis. For clarity, one serine residue is arbitrarily assigned to C-1 and the other to C-6 of the glucose phosphate substrates as depicted in Figure 4 and detailed in Scheme I.

The formation of a transition complex between the substrate and the enzyme site can readily be constructed using Lapine atomic models (Figure 5). Under the most restrictive conditions of a helical rigid structure, it is possible, with no undue strain on the molecule, to fit the glucose diphosphate with the α-configuration of the chair form, such that the phosphates are readily apposed to the respective serine residues. No preferential fit of the phosphate on a particular serine residue was obvious. The glucosyl phosphate could be fitted on either residue and still permit good apposition of the ester phosphate on the other residue with equal ease. This does not necessarily imply lack of specificity for the phosphate toward a particular serine residue.

SCHEME I



Acknowledgment

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The Catalytic Versatility of Erythrocyte Carbonic Anhydrase. I. Kinetic Studies of the Enzyme-Catalyzed Hydration of Acetaldehyde*

Y. Pocker† and J. E. Meany‡

ABSTRACT: The present investigation establishes that the catalytic activity of erythrocyte carbonic anhydrase is not limited to the reversible hydration of CO_2 but that the enzyme very powerfully and reversibly catalyzes the hydration of acetaldehyde. pH– and pD–rate profiles were obtained at 0.0° which show points of inflection at pH 7.0 and pD 7.5 for bovine carbonic anhydrase and pH 7.4 for human carbonic anhydrase-C. K_m and V_m values are deduced in both H_2O and D_2O; in the pH range studied, K_m remains essentially constant while V_m/[bovine carbonic anhydrase] dictates the over-all change in enzymatic activity.

Evidence is presented to show that acetazolamide is a powerful noncompetitive inhibitor for the hydration of acetaldehyde; the dissociation constant, K_i, = 6.1×10^{-7} mole $l.^{-1}$ at pH 7.22. It is suggested that the active site for CO_2 and for acetaldehyde hydration is the same.

The existence of a powerful catalyst in erythrocytes, promoting the hydration of CO_2 and the dehydration of HCO_3^-, was first clearly proven by the work of Meldrum and Roughton (1933). Unlike many enzymes, carbonic anhydrase (EC 4.2.1.1) (CA[1]) appears to reversibly promote a reaction which proceeds at an appreciable rate in the absence of any catalyst and is furthermore very susceptible to general base catalysis (Booth and Roughton, 1938; Kiese and Hastings, 1940). The physiological requirement for such a powerful catalyst becomes apparent if account is taken of the short time required by the blood to pass through the living capillaries (Henriques, 1928; H. Hartridge and F. J. W. Roughton, 1925, quoted by Henderson, 1925). CA is widely distributed in the animal kingdom, but it is most readily isolated from the red blood cells where it is present in about 0.1% concentration. Recently important advances have been made in the purification and characterization of bovine carbonic anhydrase (BCA) (Lindskog and Malmstrom, 1962; Lindskog, 1963) and human carbonic anhydrase (HCA) (Nyman, 1961; Rickli and Edsall, 1962; Laurent *et al.*, 1962; Rickli *et al.*, 1964). In purified form, it is a soluble, stable, colorless protein of molecular weight of about 30,000 and contains one atom of rather firmly bound zinc per molecule of enzyme. The zinc is indispensable for enzyme activity (Keilin and Mann, 1940; Lindskog and Malmstrom, 1962). It has often been claimed (Davis, 1961; White *et al.*, 1964) that the most remarkable property of CA is its *absolute* specificity, *i.e.*, a specificity with respect to a single substrate, CO_2. In

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[1] Abbreviations used in this work: CA, carbonic anhydrase; BCA, bovine carbonic anhydrase; HCA, human carbonic anhydrase.