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The Sequence of a Phosphorylated Hexadecapeptide from Rabbit Muscle Phosphoglucomutase*

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ABSTRACT: A phosphorous-containing tridecapeptide has been isolated from crystalline rabbit muscle [^{32}P]phosphoglucomutase after sequential degradation by trypsin and chymotrypsin. The sequence was established by analysis of peptide subfractions obtained after papain digestion. Amide assignments were based on peptide migrations during electrophoresis. The results of these analyses combined with data from two additional peptides establish the sequence of a phosphorus-containing hexadecapeptide to be: Thr-Ala-SerP-His-Asp-Pro-Gly-Gly-Pro-Asn-Gly-Asn-Phe-Gly-Ile-Lys.

Crystalline phosphoglucomutase (PMG)[1] (Cori *et al.*, 1938; Najjar, 1948) was shown to possess a covalently bound phosphate group that is transferable to the substrate as part of its mechanism of action (Najjar and Pullman, 1954; Sidbury and Najjar, 1957; Ray *et al.*, 1966). The phosphate group was identified as phosphoserine in acid hydrolysates of the enzyme (Anderson and Jolles, 1957). This proved to be a convenient marker for the chemical exploration of the active site, particularly that it could be labeled enzymatically by its ^{32}P-labeled substrate. It was Koshland and Erwin (1957) who made the first attempt in that direction and reported the isolation of phosphoserine peptides from acid hydrolysates of the ^{32}P-labeled enzyme. The amino acid composition of these peptides was found to mimic that of the active site of trypsin. In sharp contrast was the sequence reported by Milstein and Sanger (1961): Thr-Ala-SerP-His-Asp. It thus appeared that the enzyme might possibly have two differing active sites. This became all the more likely when phosphopeptides containing both SerP-Ala and SerP-His were isolated also from acid hydrolysates (Harshman and Najjar, 1965). In this instance, acidic and basic peptides were isolated which revealed a number of overlapping residues. This allowed the sequential arrangement of two serines, four residues apart, on the same peptide chain, either of which would be phosphorylated. By contrast, Sloan and Mercer (1964) also using acid hydrolysis, reported the occurrence of two sites based on composition analysis and concluded that these were not sequentially adjacent. Finally, extensive sequence analysis of phosphopeptides obtained by proteolytic fragmentation (Milstein and Milstein, 1968; Hooper *et al.*, 1968) failed to show other than the SerP-His site (Milstein and Sanger, 1961). We have since performed

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: PGM, phosphoglucomutase; TrF, tryptic fragments; ChF, chymotryptic fragments; CP-A, carboxypeptidase A; FDNB, fluorodinitrobenzene; LAP, leucine aminopeptidase.

enzymatic fragmentation of PGM and have also failed to identify other than the SerP-His site. It appears certain therefore, that the two sites, if any, cannot be adjacent on the same chain.

In this communication we report the amino acid sequence of rabbit muscle PGM as deduced from analysis of peptides obtained using enzymatic degradation techniques. Our data show the structure of a phosphate-containing hexadecapeptide to be: Thr-Ala-SerP-His-Asp-Pro-Gly-Gly-Pro-Asn-Gly-Asn-Phe-Gly-Ile-Lys.

Materials and Methods

Enzymes. Crystalline rabbit muscle PGM was prepared from frozen rabbit muscle (Pel-Freez, Rogers, Ark.) according to the method of Najjar (1962). Trypsin was five-times crystallized; α-chymotrypsin, purified by gel filtration; carboxypeptidase A, DFP treated; leucine aminopeptidase, DFP treated; alkaline phosphatase from *Escherichia coli;* all were obtained from Worthington Corp. (Freehold, N. J.) and used without further purification. Papain, obtained as the mercury salt in 70% ethanol, was a gift from Dr. Joe Kimmel. Pseudocollagenase was a partially purified preparation from *Clostridium welchii* and was a gift from Dr. W. M. Mitchell.

Radiolabeling and Assay. [^{32}P]PGM was prepared as described previously as were the terminally labeled [^{32}P]ATP and the glucose 6-phosphate (Harshman and Najjar, 1965). [^{32}P]-Phosphoric acid was obtained as the carrier-free, salt-free, low acid product (New England Nuclear Corp., Boston, Mass.). Radioactivity was measured using an autoscaler equipped with a gas-flow thin-window counting tube and an automatic sample changer (Nuclear-Chicago, Chicago, Ill.). Radioautography was done using Bluebrand X-ray film (Kodak Co., Rochester, N. Y.).

Amino Acid Analysis. Amino acid analyses were done following a modification of the method of Piez and Morris (1960). The Technicon analytical system used in these analyses was equipped with both B and C-2 resins as well as photomultiplier equipment (Technicon Corp., Ardsley, N. Y.).

Performic Acid Oxidation. Performic acid oxidation was done essentially as described by Hirs (1967) except that the lyophilized protein was dissolved directly in the performic acid mixture and the reaction was run in an ice bath at 0° (Dr. I. Harris, personal communication).

End-Group Analysis. The Edman degradation procedure was a modification of the Dopheide method (Dopheide *et al.*, 1967) recommended by Dr. S. Moore (personal communication). The modification essentially involves a reduction in reagent volumes. Approximately 0.05 μmole of peptide, dissolved in 0.2 ml of 60% pyridine, is mixed with 0.005 ml of phenyl isothiocyanate (Pierce Chemicals, Rockford, Ill.) and incubated under N_2 at 37° for 2 hr and lyophilized; 100 μl of trifluoroacetic acid is added and incubation at 23° is continued for 1 hr under N_2. After lyophilization, 0.4 ml of 0.4 M acetic acid is added and the N-terminal amino acid phenylthiohydantoin is extracted with two volumes of *n*-butyl acetate saturated with H_2O. The FDNB reaction was done as described previously (Harshman and Najjar, 1965) except that the water phase, after extraction of the DNP derivative, was submitted to amino acid analysis.

Enzymatic Methods. Carboxypeptidase A digestion was run in 0.025 M Tris buffer (pH 7.7), containing 0.5 M NaCl 5×10^{-4} M $MgCl_2$, and 5% by weight LiCl; 20 μg of enzyme was added to 0.024 μmole of peptide in a total volume of 0.25 ml. Alkaline phosphatase hydrolysis was run in 0.05 M Tris buffer at pH 8.8; 11 μg of enzyme was added to 0.1 μmole of peptide in a total volume of 0.5 ml. Leucine aminopeptidase digestion was carried out in 0.1 M Tris buffer (pH 8.8) containing 1×10^{-3} M $MgCl_2$; 25 μg of enzyme was added to 0.05 μmole of peptide in a volume of 0.25 ml. Papain digestion was done after preactivation of the enzyme. In a typical experiment 0.1 ml of the enzyme suspension (12.4 mg/ml as the mercury salt in 70% ethanol) was centrifuged and the pellet was dissolved in 0.6 ml of water containing 1×10^{-3} M EDTA, 5×10^{-3} M 2-mercaptoethanol, and 3×10^{-5} M 2,3-dimercaptopropanol. After 2 min of activation, 0.3 ml of enzyme (0.62 mg) was added to 1 μmole of peptide which had been dissolved in 1.0 ml of water containing 2×10^{-3} M 2-mercaptoethanol and 4×10^{-4} M EDTA. To this mixture 1.0 ml of 2.0 M NaCl was added and the pH was adjusted to 6.4 with 0.1 M NaOH. The reaction proceeded for 16 hr at 23°.

Results

Preparation of the ^{32}P-Labeled Tryptic Fragments from [^{32}P]-PGM. In a typical preparation, 1.2 g of rabbit muscle PGM (first crystals) (Najjar, 1962) was equilibrated with 0.25 μmole of G-6-^{32}P and the [^{32}P]PGM product was collected by centrifugation after precipitation with $(NH_4)_2SO_4$. This procedure has been found to be useful in removing nonspecifically bound G-1,6-^{32}P and is more rapid than dialysis. Following dialysis to remove salts and lyophilization, the dried [^{32}P]PGM was subjected to performic oxidation (Hirs, 1967) and again lyophilized. The [^{32}P]PGM was suspended next in 20 ml of H_2O and 8.7 mg of trypsin, dissolved in 3.0 ml of 1.0 M Tris (pH 8.86), was added and the mixture was stirred gently at 23°. After 3 hr, an addition of 11 mg of trypsin and 0.1 ml of toluene was made, the flask was sealed with Parafilm, and the reaction was allowed to proceed overnight. By ^{32}P analysis, 79.3% of the radioactivity was converted into a soluble form. The supernatant was made acidic (25% by volume) by the addition of cold glacial acetic acid and chromatographed on Sephadex G-50 course (3.75 × 46 cm). The "trypsin" precipitate was resuspended in 15 ml of H_2O to which 1.0 ml of 1.0 M Tris (pH 8.8) was added followed by 10 mg of trypsin. The reaction proceeded at 23° for an additional 24 hr after which the soluble material was collected and chromatographed on Sephadex as above. The ^{32}P fractions from both Sephadex columns, containing 94% of the initial radioactivity, were combined, lyophilized, redissolved in 30 ml of 0.05 M ammonium acetate, adjusted to pH 8.5 with 1.0 M Tris buffer, and again digested with 10 mg of trypsin for 6 hr at 23°. The products were rechromatographed on Sephadex G-50 fine in 0.05 M ammonium acetate buffer. The radioactive fraction was combined, lyophilized, and designated "trypsin fragments" (TrF).

Isolation of ^{32}P-Labeled Chymotryptic Fragments (ChF) from [^{32}P]PGM. Lyophilized [^{32}P]TrF, obtained from 16 μmoles of [^{32}P]PGM, was dissolved in 9.0 ml of H_2O. The pH was adjusted to 8.6 by the addition of 1.0 ml of 1.0 M Tris and 10 mg of α-chymotrypsin was added. The mixture was incubated at 23° overnight under toluene (0.1 ml) and chromatographed on a G-25 Sephadex superfine column (5 × 70 cm) which had been equilibrated with 0.05 M ammonium acetate solution. Chymotryptic digestion of TrF resulted in the formation of a

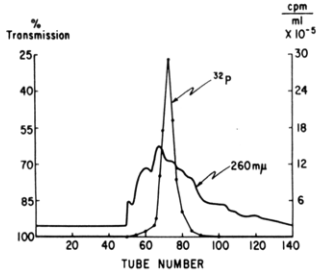

FIGURE 1: Sephadex chromatography of chymotrypsin products of TrF fraction from [^{32}P]PGM. TrF fraction from 16 μmoles of [^{32}P]-PGM was digested for 24 hr with 10.0 mg of α-chymotrypsin in 0.1 M Tris buffer (pH 8.0) and chromatographed on a G-25 superfine Sephadex K-50 column (Pharmacia, Piscataway, N. J.). The column volume was 1400 ml, the flow rate 90 ml/hr, the sample size 150 drops (10 ml), and the eluting buffer 0.05 M ammonium acetate. Per cent transmission was automatically recorded using a Uvicord (LKB, Stockholm, Sweden) and radiosiotope was measured using 50-μl samples.

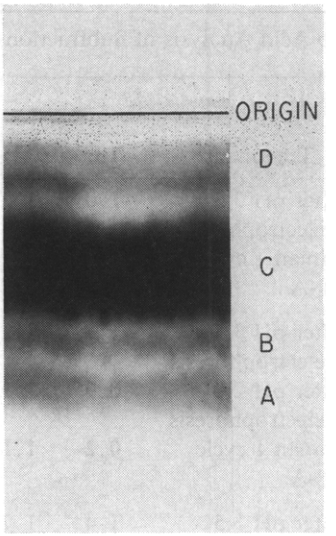

FIGURE 2: Radioautograph of ChF after chromatography on paper. The chromatogram was run with descending buffer flow using Whatman No. 3MM paper. The solvent system was butanol–acetic acid–H_2O (4:1:5, v/v) and the total running time was 48 hr at 25°.

number of nonradioactive fragments plus a single, sharp, symmetrical radioactive peak (Figure 1). The pooled radioactive peak was lyophilized and designated "chymotryptic fragments" (ChF). The recovered radioactivity of ChF represented 92% of that present in TrF which corresponds to 86.5% of the radioactivity present in the starting [^{32}P]PGM preparation.

Purification of ^{32}P-Labeled Chymotryptic Fragments. The pooled radioactivity ChF next was chromatographed in butanol–acetic acid–H_2O (4:1:5, v/v) on Whatman 3MM paper. Fresh solvent was added at 24 hr and after 48 hr the papers were dried and radioautographed. The results show that ChF, which looked homogeneous during chromatography on Sephadex G-25 superfine, could be resolved by partition chromatography into at least four radioactive components (Figure 2). These components were designated A, B, C, and D and, based on radioactivity and histidine content, contained 0.35, 0.22, 8.1, and 0.63 μmoles of peptide, respectively. Each band was eluted and further purified by electrophoresis at pH 6.5 (Gray and Hartley, 1963) at pH 3.5 (Ryle *et al.*, 1955) and in some cases at pH 2.0 (Gross, 1959). In all of these procedures extensive purification with respect to nonradiolabeled peptides was achieved as well as direct demonstrations of further heterogeneity of each of the ChF fractions. The major components of ChF fractions A, B, and D, despite this extensive purification, upon amino acid analysis were found too impure to permit unequivocal assignment of compositions and will not be discussed further. However, fraction C, the major fraction (8.1 μmoles) from 4:1:5 chromatography, could be resolved into six components by electrophoresis at pH 6.5. The major subfractions C_3, C_4, and C_5 contained 2.0, 4.9, and 0.81 μmoles of peptide, respectively (Figure 3). Each of these fractions upon electrophoresis at pH 3.5 gave a major radioactive band and a number of minor bands. The recoveries of each of the major bands at this stage were 1.4, 4.6, and 0.75 μmoles for C_3, C_4, and C_5, respectively. The amino acid composition and mole ratios of these fractions are given in Table I. It is of interest that the amino acid composition, N-terminal threonine residues, and carboxyl-terminal phenylalanine residues are identical in all three fractions. Curiously, each of the fractions initially appears to contain varying amounts of glutamic acid which is lost during purification and is therefore assumed to be a contaminant of each peptide. The basis for the observed variations in electrophoretic mobility is, at present, unexplained but may be due to variations in amide content. It is of particular interest that in all three peptides CP-A degradation stops completely after the quantitative removal of the C-terminal residue, phenylalanine. This result is true despite prolonged incubation times and additions of fresh CP-A. In view of the sequence analysis to be discussed below, this behavior with CP-A is not predicted from the known specificity of CP-A.

Enzymatic Fragmentation of ChF Subfraction C_4. Another general feature of each of the subfractions (*i.e.*, C_3, C_4, and C_5) is their remarkable resistance to proteolytic digestion by leucine aminopeptidase, carboxypeptidases A and B, subtilsin, pepsin, papain, and even pronase. For example, a major product recovered after prolonged treatment of C_4 with pronase was a peptide which differed from the parent peptide only in that the N-terminal threonine and the C-terminal phenylalanine were removed. Based on such preliminary tests we elected to submit subfraction C_4 to a vigorous attack by papain; 1.0 μmole of peptide (about 2 mg) was incubated with

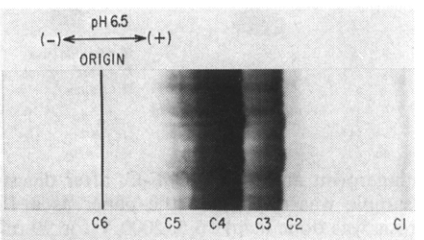

FIGURE 3: Radioautograph of ChF fraction C after electrophoresis at pH 6.5. The electrophoresis was run at 3000 V for 60 min on Whatman No. 3MM paper using refrigerated "varsol" (Humble Oil Corp., Charlotte, N. C.) as the coolant. The buffer was a pyridine–acetate–H_2O mixture at pH 6.5 (Gray and Hartley, 1963).

TABLE I: Amino Acid Analysis of Subfractions of ChF-C Peptides Derived from [^{32}P]PGM.[a]

Fraction	Treatment	Residues (mole ratios)										μmoles Analyzed
		Thr	Ala	Ser	His	Asp	Pro	Gly	Phe	PO_4	Glu	
C_3	After pH 3.5 electrophoresis	1.0	1.2	1.0	0.9	3.2	2.0	3.1	1.0	1.0	0.5	0.035
	Edman 1 cycle	**0.1**	1.0	0.8	0.8	3.2	2.1	3.0	0.9	1.0	0.2	0.031
	CP-A								1.0			0.018
C_4	After pH 3.5 electrophoresis	1.1	1.2	1.2	1.0	3.2	2.2	3.1	1.0	1.0	0.7	0.050
	After pH 2.0 electrophoresis	0.9	1.1	0.8	1.0	3.0	2.2	3.2	0.8	1.0	0.1	0.065
	Edman 1 cycle	**0.2**	1.1	0.8	1.0	3.2	2.0	3.1	1.0	1.0	0.2	0.042
	CP-A								1.0			0.046
C_5	After pH 3.5 electrophoresis	1.4	1.7	1.7	Lost	4.1	2.3	3.7	0.9	1.0	1.4	0.030
	After pH 2.0 electrophoresis	1.0	1.5	1.2	1.0	3.1	1.8	3.0	1.0	1.0	0.7	0.030
	Edman 1 cycle	**0.2**	1.1	1.2	1.0	3.0	2.0	3.1	1.0	1.0	0.3	0.040
	CP-A								1.0			0.016

[a] The ratio of amino acids per peptide is based on $^{32}PO_4$ being assigned a value of 1.0. The amino acid removed by Edman degradation is shown in boldface type.

0.64 mg of activated papain (see Methods) in a volume of 2.5 ml, and the reaction was continued overnight at 23°. After desalting on G-15 Sephadex, the peptides (the front-half of the column effluent) were lyophilized and fingerprinted (Figure 4). Repeats of the papain digestion procedure on two subsequent aliquots of C_4 showed that the fragmentation method was remarkably reproducible.

Amino Acid Sequence Analysis of Papain Fragments from ChF Subfraction C_4. The fingerprint papers were radioautographed to locate the ^{32}P-containing peptides and then sprayed lightly with ninhydrin. The fingerprint peptides, FP-2-FP-10, were eluted with water and subjected to total and sequential amino acid analysis. The results are summarized in Table II and show that the sequence of the tridecapeptide is: Thr-Ala-SerP-His-Asp-Pro-Gly-Gly-Pro-Asn-Gly-Asn-Phe. Presented below are the results of the amino acid analysis of each peptide expressed as the ratios of amino acid per peptide. The analysis after each Edman degradation step (see Table I) is listed and the amino acid removed is indicated by boldface type. In all cases the data are uncorrected for hydrolytic degradation.

Fraction FP-1 is composed of inorganic phosphate since it is both ninhydrin negative and it forms a molybdate derivative that is extractable into isobutyl alcohol–benzene. The total recovery of this fraction was 0.11 μmole.

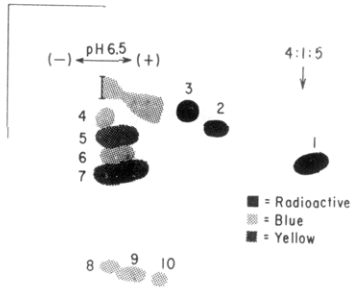

FIGURE 4: Fingerprint analysis of ChF-C_4 after digestion with papain. The sample was applied to the paper as a 1.5-cm streak. Electrophoresis was done at pH 6.5, 2000 V for 90 min; the paper was dried, turned 90°, and chromatographed in butanol–acetic acid–H_2O (4:1:5, v/v) for 20 hr at 25°. The yellow and blue refer to the colors of each peptide immediately after exposure to ninhydrin. Upon standing the yellow color changed to blue. This color change is identical with that observed with two test peptides; Gly-Pro-Ala and Gly-Pro-Gly-Gly. For details, see text.

FP-2	Thr	Ala	SerP	μmoles Analyzed
Composition	0.9	1.0	0.9	0.035
Step 1	**0**	1.1	0.9	0.037
Step 2	0	**0.6**	1.0	0.034

In the analysis of this peptide, $^{32}PO_4$ was found to be 0.035 μmole and was taken to be 1.0. The total recovery of FP-2 was 0.27 μmole.

FP-3	SerP	His	Asx	Pro	Gly	μmoles Analyzed
Composition	0.7	1.0	1.0	1.0	1.0	0.026
LAP	1.2	0.8				0.026

Analysis of this peptide showed $^{32}PO_4$ to be 0.026 μmole which was taken as 1.0. The peptide was treated with both alkaline phosphatase (10 μg) and LAP (50 μg) for 18 hr. The

TABLE II: The Mole Ratios and Amino Acid Compositions of the Fingerprint Peptides from ChF-C_4.[a]

Peptide	Thr	Ala	SerP	His	Asp	Pro	Gly	Gly	Pro	Asn	Gly	Asn	Phe
C_4 (after pH 2.0)	0.9 →→	1.1	0.9	1.1	1.1	1.1	1.0	1.0	1.1	1.1	1.0	1.1	0.9 ←
FP-2	0.9 →	1.0 →	0.9										
FP-3			0.7 →	1.0 →	1.0	1.0	1.0						
FP-4				0.7 →	0.9 →	0.9 →	1.0						
FP-6											1.1[b]	1.0	
FP-7								0.9 →	1.0 →	1.0 →	0.9	1.0	1.0 ←
FP-8	0.9 →	1.0											
FP-9												1.2 →	1.0
FP-10												1.0 →	1.0

[a] (⇢) sequence established by Edman method; (→) sequence established by the leucine aminopeptidase procedure; (←) sequence established with carboxypeptidase A. [b] Sequence established after FDNB reaction and hydrolysis in 6 N HCl, 100°, 4 hr. For further details, see text.

recoveries suggest the sequence SerP-His. The total recovery of peptide FP-3 was 0.10 μmole.

FP-4	His	Asp	Pro	Gly	μmoles Analyzed
Composition	0.7	0.9	0.9	1.0	0.090
Step 1	**0.2**	0.7	0.9	1.0	0.028
Step 2	0.2	**0.6**	1.0	1.0	0.023
Step 3	0.1	0.3	**0.6**	1.0	0.059
LAP	1.0				0.011

Electrophoresis showed that the peptide was neutral at pH 6.5; hence, in view of the histidine content, the aspartic acid residue is not amidated. Treatment with leucine aminopeptidase quantitatively released histidine only. The total recovery of FP-4 was 0.23 μmole.

Despite repeated efforts to purify fraction FP-5, the amino acid composition could not be established with sufficient clarity to permit sequence work. This fraction, therefore, was not considered further.

FP-6	Gly	Asn	μmoles Analyzed
Composition	1.1	1.0	0.058
After FDNB and 4 hr of acid hydrolysis	0.8	1.0	0.058

Since electrophoresis showed that this peptide was neutral at pH 6.5, the aspartic acid residue is amidated. The total recovery of FP-6 was 0.22 μmole.

FP-7	Gly	Pro	Asn	Gly	Asn	Phe	μmoles Analyzed
Composition	0.9	1.0	1.0	0.9	1.0	1.0	0.059
Step 1	**0.1**	1.0	1.0	1.0	1.0	1.0	0.042
Step 2	0.3	**0.3**	1.0	1.0	1.0	1.0	0.030
Step 3	0.3	0.2	**0.1**	1.0	1.0	1.0	0.024
CP-A						1.0	0.033

The peptide was neutral at pH 6.5 electrophoresis, hence both aspartic acid residues are amidated. Treatment with CP-A for 50 hr quantitatively released phenylalanine only. The total recovery of FP-7 was 0.53 μmole.

FP-8	Thr	Ala	μmoles Analyzed
Composition	0.9	1.0	0.086
Step 1	**0**	1.0	0.021

The total recovery of peptide FP-8 was 0.17 μmole.

FP-9	Asn	Phe	μmoles Analyzed
Composition	1.2	1.0	0.042
Step 1	**0.2**	1.0	0.048
FP-10			
Composition	1.0	1.0	0.015
Step 1	**0.2**	1.0	0.022

Electrophoresis at pH 6.5 showed FP-9 to be neutral and FP-10 to have a net negative charge, Since, by Edman analysis, both peptides are Asx-Phe the basis for the separation of these two peptides is not certain. Aspartic acid inversion or ring formation (Naughton *et al.*, 1960) are possibilities, as is simple deamidation of the aspartic in FP-10. The total recoveries for peptides FP-9 and FP-10 were 0.19 and 0.04 μmole, respectively.

Extension of the Sequence of Peptide ChF-C_4. During the course of our studies on the structure of [^{32}P]PGM we have isolated two peptides which permit an extension of the carboxyl end of the sequence presented above. The first of these peptides were derived from a [^{32}P]PGM preparation that had been treated with excess *p*-hydroxymercuribenzoate and subsequently with trypsin. The radiolabeled tryptic peptides were fractionated on Sephadex G-25 columns and then pu-

rified by chromatography on Dowex 50 ion-exchange resin using 0.17 M pyridine adjusted to pH 4.6 with acetic acid as the eluent (Guidotti *et al.*, 1962). The major ^{32}P-containing tryptic peptide was next chromatographed in butanol–acetic–water (4:1:5, v/v) and subsequently digested with chymotrypsin. The products were again fractionated on Dowex 50 as described above. The main ^{32}P-containing chymotryptic fraction was purified by chromatography in 2-butanol–formic acid–water (7:2:1, v/v) and submitted to electrophoresis at pH 3.5. The recovery at this point was 0.132 μmole from the 20 μmoles of starting [^{32}P]PGM. Amino acid analysis gave the following composition: Thr (0.9), Ala (1.0), SerP (0.9), His (1.3), Asx (2.9), Pro (1.9), Gly (4.0), Phe (1.1), Ile (1.1), and Lys (1.1). Thus, it differs from ChF-C_4 in that it contains an additional residue each of Gly, Ile, and Lys. Analysis of the peptide after treatment with both carboxypeptidases A and B, 50 μg each for 1 hr at 23°, demonstrated a quantitative loss of Lys and Ile. Since the peptide was obtained after digestion with trypsin, although it was followed by chymotrypsin digestion, Lys is assumed to be the C-terminal residue, thus suggesting that the extended C-terminal sequence is···· Phe-Gly-Ile-Lys. This impression was supported by the isolation of a second peptide which permitted the unequivocal assignment of Gly as the residue on the C-terminal side of···· Phe.

The second peptide was obtained from a [^{32}P]PGM preparation that was digested with trypsin, purified by chromatography on Sephadex G-50 and then oxidized with performic acid. The ^{32}P-containing peptides were then digested with papain, 5 mg for 5 hr at 23°, and chromatographed on Sephadex G-25. The ^{32}P fraction obtained was sequentially submitted to electrophoresis at pH 3.5, chromatography in butanol–acetic acid–water (4:1:5, v/v), electrophoresis at pH 6.5, and chromatography in 2-butanol–formic acid–water (7:2:1, v/v). The recovery was 0.65 μmole from 10 μmoles of starting [^{32}P]PGM. Amino acid analysis gave the following composition: Ala (1.1), SerP (0.9), His (1.0), Asx (3.3), Pro (2.3), Gly (4.1), and Phe (1.0). This composition differs from ChF-C_4 by a single glycine residue. Leucine aminopeptidase after pretreatment with alkaline phosphatase released Ala, Ser, and His at 0.030, 0.032, and 0.022 μmole, respectively, which is consistent with the assigned N-terminal sequence of Ala-SerP-His····. Carboxypeptidase A (50 μg for 3 hr at 25°) released Gly and Phe at 0.063 and 0.068 μmole, respectively. Taken together with the sequence established for the ChF-C_4 peptide these data extend the C-terminal sequence to ····Phe-Gly. Combining the data obtained from these two peptides with the data from peptide CHF-C_4 the over-all sequence of the hexadecapeptide may now be written as: Thr-Ala-SerP-His-Asp-Pro-Gly-Gly-Pro-Asn-Gly-Asn-Phe-Gly-Ile-Lys.

Discussion

The data reported in this communication establishes the sequence of a phosphorylated hexadecapeptide from rabbit muscle PGM to be: Thr-Ala-SerP-His-Asp-Pro-Gly-Gly-Pro-Asn-Gly-Asn-Phe-Gly-Ile-Lys. This structure is in agreement with the sequence reported by Milstein and Milstein (1968): Ala-Ile-Gly-Gly-Ile-Ile-Leu-Thr-Ala-SerP-His-Asx-Pro-Gly-Gly-Pro-(Asx_2,Gly)-Phe-Gly-Ile-Lys. Additionally, our structure resolves the sequence (Asx_2,Gly) which is shown to be Asn-Gly-Asn and also establishes aspartic acid as the amino acid adjacent to the histidine residue. Although there are marked similarities, our sequence differs in detail both with the sequence of the flounder muscle PGM reported by Hashimoto *et al.* (1966): Thr-Ala-SerP-His-Asx-Pro-Gly-Gly-Pro-Asx-Asx-Gly-Phe and with the more generalized PGM sequence reported by Hooper *et al.* (1968): Ala-Ile-[Gly-Glu-Thr-Ala-SerP-His-Asp-Pro-Gly-Pro-Gly-Asp-Gly-Asp-Phe]-(Ile,Leu)-Lys.

The hexadecapeptide contains the collagen-like partial sequence ···Pro-Gly-Gly-Pro. We attempted to confirm this sequence by submitting the peptide to hydrolysis with the enzyme pseudocollagenase. No evidence of enzymatic fragmentation was obtained. The basis for the observed resistance of this peptide to collagenase attack is unknown. In this connection, however, it is of interest that no intact Pro-Gly-Gly-Pro sequences were recovered in the fingerprint analysis of ChF-C_4 after vigorous attack by papain.

In a previous communication (Harshman and Najjar, 1965) we proposed a peptide sequence for the active site of rabbit muscle PGM that contained two functional phosphorylated serine residues. This sequence was inferred from an analysis of small peptides isolated from [^{32}P]PGM after partial acid hydrolysis. Although, our present data, which were obtained from enzymatically produced peptides, clearly refutes our original sequence proposal, it does not exclude the possibility of a second phosphorylated site in PGM. Since inorganic phosphate formation always accompanied proteolysis of PGM and our best recovery of ChF was only 86.5% (Figure 1) and since this fraction was clearly heterogeneous and not totally analyzable (Figures 2 and 3), it is clear that our data cannot exclude the existence of a second phosphorylated site if it forms a minor component of the total label. In this connection, it is interesting to note, that recent studies in our laboratory have shown that crystalline rabbit muscle [^{32}P]PGM can be resolved into one major and at least three minor enzymatically functional components (Harshman and Six, 1969). Multiple forms of PGM recently have been reported by others using both starch gel and ion-exchange chromatograms (Hopkinson and Harris, 1965; Dawson and Mitchell, 1969; Yankeelov *et al.*, 1964; Joshi *et al.*, 1967). These results raise the alternate possibility that the low concentrations of acidic, phosphorylated, serine-containing peptides, observed after partial acid hydrolysis of PGM, might be derived from these minor components. With this in mind, we are currently investigating the enzymatic, chemical, and physical characteristics of each of the PGM isozymes obtained by isoelectric focusing.

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Physical and Chemical Studies on Crystalline Rabbit Muscle Phosphoglucomutase. Multiple Forms*

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ABSTRACT: Crystalline preparations of rabbit muscle phosphoglucomutase have been resolved into four fractions using the technique of isoelectric focusing. The fractions contained 82, 8, 6, and 4%, respectively, of the original enzyme and each was enzymatically active and recovered in the phosphorylated form.

Similar fractionation was demonstrated using acrylamide gel electrophoresis. Physical and chemical studies on the parent crystals indicated that the phosphoglucomutase "isozymes" were not due to molecular aggregation or subunit rearrangement. The molecular weight of crystalline rabbit muscle phosphoglucomutase by sedimentation equilibrium was found to be 64,900. Sedimentation equilibrium analyses of reduced and carboxymethylated phosphoglucomutase in the presence of 5 M guanidine hydrochloride gave a molecular weight of 64,000, indicating that there are no subunit structures in rabbit muscle phosphoglucomutase. The $s_{20,w}$ value, calculated from sedimentation velocity data, was 3.82 S. Calculation of the partial specific volume from amino acid composition data gives a value of 0.737. The isoelectric pH of phosphoglucomutase, using the techniques of isoelectric focusing, was found to be 6.80.

The enzyme PGM[1] from rabbit muscle has been reported to be a single homogeneous polypeptide chain with a molecular weight of 74,000 (Taylor *et al.*, 1956) and an isoelectric point at pH 8.6 (Boser, 1955). The available physical data have been summarized (Najjar, 1962). Recently a number of laboratories have reported data that are at variance with these results. The molecular weight of PGM has been reported to be 62,000 (Filmer and Koshland, 1963; Yankeelov *et al.*, 1964; Handler *et al.*, 1965; Hashimoto and Handler, 1966). The original value of 74,000 was obtained using sedimentation velocity methods, while the lower value of 62,000 is based on data obtained by the method of Yphantis (1964). Of greater significance are the more recent reports of multiple forms of PGM. Using the technique of starch gel electrophoresis, Spencer *et al.* (1964), Hopkinson and Harris (1965), and Luan Eng (1966) have reported multiple forms of PGM from human red blood cells, all of which appear to be in the phosphorylated form (Coifman and Epstein, 1969). Similar findings have been reported by Dawson and Mitchell (1969) in a study of muscle tissues from a variety of sources. In addition, two laboratories have reported that resolution of phosphoglucomutases into two components could be achieved by ion-exchange chromatography on CM-Sephadex (Joshi *et al.*, 1967) and on CM-cellulose (Yankeelov *et al.*, 1964).

We report here that the technique of isoelectric focusing resolves crystalline preparations of PGM into four enzymatically active phosphorylated forms. We also report on studies of the parent PGM crystals by Sephadex chromatography, sedimentation velocity, sedimentation equilibrium, and amino

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[1] Abbreviation used that is not listed in *Biochemistry* 5, 1445 (1966), is: PGM, phosphoglucomutase.