Primary Structure of Pyridoxal Phosphate Binding Site in the Mitochondrial and Extramitochondrial Aspartate Aminotransferases from Pig Heart Muscle. Chymotryptic Peptides*

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ABSTRACT: For a comparison of the primary structures of the coenzyme binding sites in aspartate aminotransferases from the mitochondrial and extramitochondrial fractions of pig heart muscle, phosphopyridoxyl peptides were isolated from the chymotryptic digests of these two enzymes after borohydride reduction followed by carboxymethylation. The peptides containing the reduced coenzyme were then identified by their fluorescence characteristics. A single phosphopyridoxyl peptide was obtained from the extramitochondrial enzyme and its amino acid sequence was determined as Ser-(ε-phosphopyridoxyl)-Lys-Asn-Phe. Several pyridoxyl peptides were obtained from the mitochondrial enzyme. Some of them presumably resulted from side splits of a typical peptide, or from the oxidation of methionine to its sulfoxide during isolation procedures. The sequence, Ala-(ε-phosphopyridoxyl)-Lys-Asn-Met, was assigned to one of these typical peptides.

Aspartate aminotransferase (EC 2.6.1.1) present in the extramitochondrial fraction and that present in mitochondria of mammalian tissues represent two distinct entities as evidenced by a number of investigations of their comparative kinetic properties (Boyd, 1961; Katsunuma *et al.*, 1962; Morino and Wada, 1963; Morino *et al.*, 1963; Wada and Morino, 1964; Nisselbaum and Bodansky, 1964, 1966) and protein structures (Morino *et al.*, 1964a,b; Martinez-Carrion *et al.*, 1967; Martinez-Carrion and Tiemeier, 1967; Wada *et al.*, 1968; Kagamiyama *et al.*, 1968). The reaction catalyzed by these two isoenzymes is, however, identical. Therefore, it is logical to assume that the structure of the two active sites should show some essential common characteristics. A comparative study of the primary structure around the bound coenzyme of each isoenzyme would contribute to the mapping of the active site and, hence, to the understanding of the mechanism of enzymic pyridoxal catalysis on a molecular level.

Hughes *et al.* (1962) and Polyanovskii and Keil (1963) have investigated the amino acid sequence of the pyridoxyl peptide isolated from chymotryptic digests of reduced pig heart aspartate aminotransferase. Several criteria suggest that this enzyme is of extramitochondrial origin (Wada and Morino, 1964). Only incomplete determinations of the amino acid sequence were reported by the former group and the results of the latter workers show some ambiguity.

The present communication describes a comparative study on the amino acid sequences of active-site tetrapeptides isolated from the chymotryptic digests of the extramitochondrial and mitochondrial aspartate aminotransferases of pig heart.

Experimental Procedures

Materials. m- and s-GOT[1,2] were prepared by the procedures developed recently in our laboratory which will be published elsewhere. Iodoacetic acid was twice recrystallized from hot chloroform. Leucine aminopeptidase (C_1 = 80) was prepared by the method described by Hill *et al.* (1958). α-Chymotrypsin and carboxypeptidase A (diisopropylfluorophosphate treated) were obtained from Worthington. Phenyl isothiocyanate (Tokyo Chemical Industry Co., Ltd.) was redistilled before use.

ε-Pyridoxyllysine was kindly donated by Dr. Y. Izumi in the Institute for Protein Research, Osaka. ε-Phosphopyridoxyllysine was synthesized by phosphorylation of ε-pyridoxyllysine (50 mg) according to the procedure described for pyridoxamine by Peterson and Sober (1954). The resulting reaction mixture (1 ml) was applied to an Amberlite CG 50 (H^+ form) column (1.2 × 100 cm) which was washed thoroughly with distilled water. After washing the column with 200 ml of distilled water, ε-phosphopyridoxyllysine was eluted by 0.1 N acetic acid. Other chemicals were from commercial sources and were used without further purification.

Methods. m- or s-GOT in 0.5 M Tris-HCl buffer (pH 8.0) was reduced by the addition of 10 mg of sodium borohydride/100 mg of the enzyme. Upon reduction, the aldimine linkage formed between 4-aldehyde group of pyridoxal phosphate and ε-amino group of a lysine residue in the enzyme is converted into a bond resistant to acid hydrolysis (Fischer *et al.*, 1958).

Carboxymethylation of the reduced enzyme was performed

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[1] The abbreviations used are: m-GOT, mitochondrial aspartate aminotransferase (glutamic–oxaloacetic transaminase); s-GOT, soluble or extramitochondrial aspartate aminotransferase: CM-r-m-GOT, borohydride-reduced and then carboxymethylated m-GOT; CM-r-s-GOT, borohydride-reduced and then carboxymethylated s-GOT; PTH, phenylthiohydantoin derivatives.

[2] These preparations contained all the subforms described by Martinez-Carrion *et al.* (1967).

essentially according to the method described by Crestfield *et al.* (1963). Guanidine hydrochloride (6 M) was used as the denaturant in the present procedure. Carboxymethylated preparations were dialyzed against distilled water and the suspension was lyophilized.

In preliminary experiments, digests of CM-r-m-GOT yielded, on high-voltage electrophoresis at pH 3.7 (Katz *et al.*, 1959) on Whatman No. 3MM paper, an intense fluorescent band and several minor fluorescent bands. The appearance of the minor bands presumably resulted from incomplete or nonspecific cleavage of some peptide bonds and their intensity varied with the digestion time. The digestion time was therefore chosen to minimize the formation of these materials. Thus, the digestion of CM-r-m-GOT (600 mg) was performed by adding 6 mg of α-chymotrypsin in 60 ml of 1% ammonium bicarbonate with constant stirring at 30° in the dark. An additional 6 mg of the chymotrypsin was added after 3 hr. The digestion was continued for an additional 18 hr and terminated by bringing the solution to pH 2 with 2 N HCl, followed by lyophilization.

Contrary to the case of CM-r-m-GOT, a preliminary experiment showed that when CM-r-s-GOT was digested with chymotrypsin for 6–18 hr, the digests yielded consistently a single fluorescent spot on high-voltage electrophoresis and paper chromatography. In the large-scale experiment, 300 mg of CM-r-s-GOT suspended in 30 ml of 1% ammonium bicarbonate was digested at 30° with the addition of 3 mg of chymotrypsin, followed by the further addition of 3 mg of chymotrypsin after 3 hr. The digestion was terminated after an additional 3 hr. The digests were brought to pH 2 with HCl and lyophilized.

Column Chromatography on Dowex 1-X2. The chromatographic procedure is based on that described by Funatsu (1964). Lyophilized chymotryptic digests of CM-r-s-GOT (300 mg) were dissolved in 5.5 ml of a buffer containing 1% pyridine, 1% collidine, 0.02% acetic acid (pH 8.65), and chromatographed on a column (1 × 115 cm) of Dowex 1-X2 acetate (200–400 mesh), previously equilibrated with the same buffer at a flow rate of 12 ml/hr at 30°. Fractions of 3.3 ml were collected on a fraction collector. The buffer system employed for elution was as follows: (a) 200 ml of equilibration buffer; (b) a linear gradient between 300 ml of equilibration buffer and 300 ml of 0.1 N acetic acid; (c) 200 ml of 0.1 N acetic acid; and (d) a linear gradient between 300 ml of 0.1 N acetic acid and 300 ml of 0.5 N acetic acid.

The lyophilized chymotryptic digests of CM-r-m-GOT (600 mg) were dissolved in 16 ml of the pyridine–collidine–acetate buffer (pH 8.65) described above and applied on to a column (1.8 × 110 cm) of Dowex 1-X2 acetate (200–400 mesh) previously equilibrated with the same buffer. The chromatography was carried out at 30° at a flow rate of 50 ml/hr. Fractions of 10 ml were collected. The following buffer system was employed: (a) 320 ml of equilibration buffer; (b) a linear gradient between 1100 ml of equilibration buffer and 1100 ml of 0.1 N acetic acid; (c) a linear gradient between 1000 ml of 0.1 N acetic acid and 1000 ml of 0.5 N acetic acid; (d) 400 ml of 2 N acetic acid; and (e) 300 ml of glacial acetic acid. An aliquot (0.3 ml) of each fraction was tested by the reaction with ninhydrin (Rosen, 1957). Fractions with blue fluorescence were measured for their absorbance at 325 mμ where pyridoxyl compounds absorb.

Further Purification of Fluorescent Peptides. Fluorescent fractions under each peak were pooled and examined for homogeneity by paper electrophoresis and chromatography. High-voltage electrophoresis was performed at either pH 6.5 or 3.7 (Katz *et al.*, 1959) on Whatman No. 3MM paper at 60 V/cm for 90 min. Descending chromatography was performed on Whatman No. 3MM paper with 1-butanol–pyridine–acetic acid–water (15:10:3:12, v/v) as the solvent. When necessary, further purification of peptides was carried out either by high-voltage electrophoresis or paper chromatography or both.

The column chromatography and subsequent purification of peptides were performed in the dark to prevent the possible destruction of pyridoxyl peptides.

Amino acid analysis of peptides was performed by the use of an automatic amino acid analyzer (Hitachi Perkins-Elmer KLA-3B or Yanaco LC-5S), according to the method of Spackman *et al.* (1958). Peptides were hydrolyzed in 5.7 N HCl at 105° for 36 hr.

ε-Phosphopyridoxyllysine emerged just before leucine as a peak unseparable from the latter on a long column (0.9 × 60 cm) for acidic and neutral amino acids. ε-Pyridoxyllysine appeared as a discrete, well-separated peak before the arginine peak on a short column (0.9 × 15 cm) with 0.38 M citrate buffer (pH 4.46) as the eluent at 60°.

Sequence Study. The amino acid sequence was determined by the Edman degradation method described by Konigsberg (1967) using 50% aqueous pyridine containing 2% triethylamine as the medium for coupling. The residue cleaved was identified by amino acid analysis of the residual peptide. Phenylthiohydantoin derivatives were identified by thin-layer chromatography on Eastman chromatogram sheets (type K301R) as described by Jeppsson and Sjöquist (1967). The conditions for digestion with leucine aminopeptidase and carboxypeptidase A are described in appropriate parts of Results.

Designation of Peptides. The symbols m-c- and s-c- refer to chymotryptic peptides obtained from the preparations of m-GOT and s-GOT, respectively. Arabic numerals after these letters are assigned to each peak fraction in the order of its appearance from the column. The letter following the peptide number is used when more than one peptide is obtained by paper chromatography (*cf.* Figure 2) of a single peak fraction.

Results

Isolation of Phosphopyridoxyl Peptides from the Digests of CM-r-m- and s-GOT. An elution profile of the chymotryptic digests of the CM-r-m-GOT on Dowex 1-X2 is shown in Figure 1.

Four peaks with blue fluorescence appeared on the chromatogram. All these fractions showed an absorption maximum at 325 mμ (at pH 7.0) which is characteristic of pyridoxyl compounds. Fractions under each peak were pooled, concentrated by lyophilization.

On chromatography on Whatman No. 3MM paper, each of these fractions was found to contain several peptides (Figure 2). Fractions 22 and 27 each contained a single fluorescent material, whereas fractions 23 and 29 contained two fluorescent materials.

Further purification of the fluorescent peptides in the fractions 22 and 23 was performed by paper chromatography. Those in the fractions 27 and 29 were further purified by paper chromatography and then by high-voltage electrophoresis on

TABLE I: Amino Acid Compositions of Pyridoxyl Peptides from s- and m-GOT.[a]

Amino Acid	s-c-21	m-c-22	m-c-23a	m-c-23b	m-c-27	m-c-29b
Lys	0.005	0.004	0.007			
ε-Pyridoxyl-Lys[b]	0.020 (1)	0.021 (1)	0.024 (1)	0.015 (1)	0.008 (1)	0.013 (1)
Asp	0.023 (1)	0.027 (1)	0.030 (1)	0.021 (1)	0.012 (1)	0.018 (1)
Ser	0.020 (1)				0.004	
Glu	0.005					
Gly	0.004				0.014 (1)	0.016 (1)
Ala		0.024 (1)	0.034 (1)	0.019 (1)	0.009 (1)	0.015 (1)
Met		0.023 (1)		0.020 (1)		0.013 (1)
Leu					0.013 (1)	0.019 (1)
Phe	0.021 (1)					
Recovery %	62	6	12	10	0.6	1

[a] Values for amino acids were expressed in micromoles. The assumed integral values of major components are given in parentheses. All peptides listed were negative to the Ehrlich reaction (Smith, 1953) on a paper. The values for amino acid residues present in amount less than 0.1 mole/mole of peptide were omitted. [b] The color value for this derivative was assumed to be the same as that for leucine.

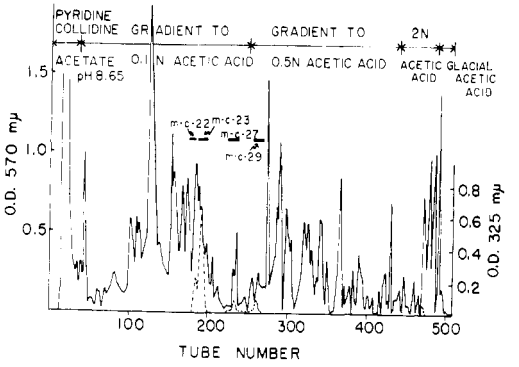

FIGURE 1: Elution of chymotryptic peptides of CM-r-m-GOT from a column (1.8 × 110 cm) of Dowex 1-X2. The dashed line indicates absorbance at 325 mμ. Bars indicate the fractions pooled. Experimental details are given in Experimental Procedure.

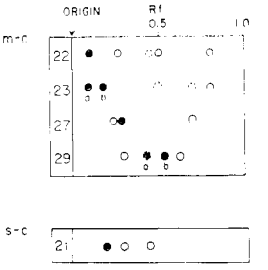

FIGURE 2: Paper chromatogram of fractions containing phosphopyridoxyl peptides. Descending chromatography on Whatman No. 3MM sheet was performed as described in the text. The hatched area indicates fluorescence and open areas indicate ninhydrin-positive spots. The sheet was dipped into 0.5% ninhydrin in acetone and the color was developed in an oven at 50° for 15 min.

Whatman No. 3MM paper at pH 6.5. Each fluorescent band was eluted with 10% acetic acid and the eluate was lyophilized. All of the peptides thus purified showed two absorption bands at 254 and 325 mμ at pH 7. The spectra are characteristic of those of pyridoxyl compounds and suggest the absence of tyrosine or tryptophan residues in these peptides.

An elution pattern of chymotryptic digests of CM-r-s-GOT was shown in Figure 3. In contrast to the case of m-GOT, single peak fractions with blue fluorescence were obtained. The pyridoxyl peptide in these fractions comprised 84% of the total amount present in the chymotryptic digests, as calculated from the absorbance at 325 mμ and pH 7.0. No further fluorescent material was found in the eluate when the column was finally washed with 200 ml of glacial acetic acid. These fluorescent fractions were concentrated by lyophilization and examined for homogeneity by paper chromatography. The chromatogram (Figure 2) showed that the fractions contained several peptide fragments, one of which was identified from its blue fluorescence as the pyridoxyl peptide. Judging from its separation from other ninhydrin-positive spots, the fluorescent band was considered to represent a fairly pure peptide. For the purification of the pyridoxyl peptide, therefore, the concentrate of the fraction 21 was applied in a band on Whatman No. 3MM paper, subjected to descending chromatography, and the fluorescent band was eluted by 10% acetic acid. The resulting pure preparation (s-c-21) showed two absorption bands at 254 and 325 mμ (pH 7.0).

Yields and Amino Acid Compositions of Pyridoxyl Peptides. Table I summarizes yields and amino acid compositions of pyridoxyl peptides purified as described above. Peptides m-c-22 and m-c-23-b showed similar amino acid compositions, whereas peptide m-c-23-a lacked methionine. Peptides m-c-27, m-c-29-a,[3] and m-c-29-b were obtained in much smaller amounts and seemed to arise from incomplete digestion by chymotrypsin.

[3] Because of its poor yield, no reliable data were obtained on the amino acid composition of this peptide.

TABLE II: Amino Acid Sequence of Peptide m-c-23b.[a]

Amino Acid	Edman Degradation (molar ratio) m-c-23b	Stage 1	Stage 2	Stage 3
ε-Pyridoxyl-Lys	0.84	0.73	0.09	0.10
Asp	1.06	1.09	1.07	0.23
Ala	1.04	0.03	0.02	0.03
Met	1.00	1.00	1.00	1.00
PTH		PTH-Ala		

	Enzymatic Degradation (molar ratio) Leucine Aminopeptidase 0.5 hr	14 hr	Carboxypeptidase A 15 min
ε-Phospho-pyridoxyl-Lys[b]	0.35	1.02	0.03
Asn	0.20	0.90	0.23
Ala	0.45	1.00	0.03
Met	0.15	0.88	0.50

[a] The procedure for subtractive Edman degradation is described in the text. The hydrolysis of the peptide (approximately 0.1 μmole) by leucine aminopeptidase (10 μg) was performed in 0.2 ml of 0.05 M Tris-HCl buffer (pH 8.5) containing 5 mM $MgCl_2$ at 37°. The hydrolysis of the peptide with carboxypeptidase A (50 μg) was carried out in 0.2 ml of 0.05 M Tris-HCl buffer (pH 7.5) at 37°. Amino acids released by the peptidase digestions were determined quantitatively on the amino acid analyzer. [b] The color value for this derivative was assumed to be the same as that for leucine.

Amino Acid Sequence of Pyridoxyl Peptides. The amino acid sequence study was performed on peptides m-c-23-b and s-c-21. The stepwise Edman degradation of peptides m-c-23-b revealed that the sequence would be Ala-(ε-pyridoxyl)-Lys-Asp-Met (Table II). The amino-terminal residue of the peptide was also determined as its phenylthiohydantoin and found to be alanine. Since these results were obtained by analysis of the amino acids resulting from prolonged hydrolysis of peptides by hot acid, the possibility that the original peptide might contain asparagine and ε-phosphopyridoxyllysine instead of aspartic acid and ε-pyridoxyllysine was suspected. To test this, the digestion of the peptide by leucine aminopeptidase was performed. The analysis of the resulting digest confirmed the presence of ε-phosphopyridoxyllysine and asparagine. No trace of ε-pyridoxyllysine or aspartic acid was detected. Carboxypeptidase A digestion of the peptide confirmed methionine as its carboxyl-terminal amino acid, as expected from the specificity of chymotrypsin. Although the data are not shown, digestion of peptide m-c-22 with carboxypeptidase A produced methionine sulfoxide instead of methionine. Therefore, the difference in the mobility of peptides m-c-22 and m-c-23-b on the paper chromatogram (Figure 2) seems to result from the fact that in peptide m-c-22 methionyl residue is replaced by methionine sulfoxide.

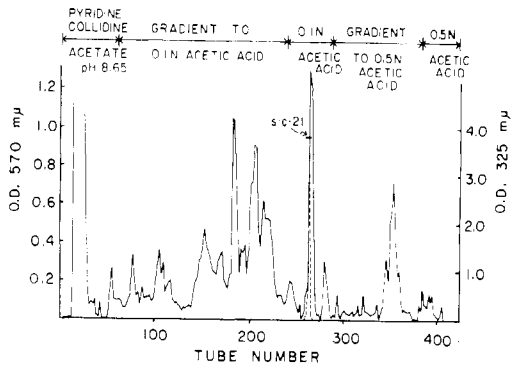

FIGURE 3: Elution of chymotryptic peptides of CM-r-s-GOT from a column (1.0 × 115 cm) of Dowex 1-X2. The dashed line indicates absorbance at 325 mμ. The bar indicates the fractions pooled. Experimental details are given in Experimental Procedures.

The exhaustive digestion of peptide m-c-23-a by carboxypeptidase yielded asparagine, alanine, and ε-phosphopyridoxyllysine in equimolar amount. Neither methionine nor its sulfoxide was found in the digests. Presumably the peptide arose from partial hydrolysis of the peptide bond between asparagine and methionine (or its sulfoxide) by chymotrypsin.

From these results, the amino acid sequence of the chymotryptic peptide (m-c-23-b) from the coenzyme binding site of m-GOT is Ala-(ε-phosphopyridoxyl)-Lys-Asn-Met.

The minor peptides, peptides m-c-27 and m-c-29-b, contained glycine and leucine in addition to the amino acids observed in peptide m-c-23-b and m-c-23-a, respectively. Therefore, it is probable that glycine and leucine are present on the amino-terminal side of the alanyl residue.

Table III shows the result of sequence analysis on peptide s-c-21. Here again, digestion with leucine aminopeptidase revealed that the peptide contained asparagine instead of aspartic acid, and phosphopyridoxyllysine instead of pyridoxyllysine. Phenylalanine was confirmed as the carboxyl-terminal amino acid from the result of carboxypeptidase A digestion. It was concluded, therefore, that the amino acid sequence of the pyridoxyl tetrapeptide from s-GOT should be Ser-(ε-phosphopyridoxyl)-Lys-Asn-Phe.

Discussion

The comparative study of the amino acid sequences of the fluorescent tetrapeptides isolated from m- and s-GOT has revealed that the residue adjacent to the lysyl residue to which pyridoxal phosphate is bound is asparagine in both enzymes. One can imagine that the presence of the neutral asparagine residue in place of a charged aspartic acid residue might contribute to an environment favorable for the binding of pyridoxal phosphate to the critical lysine residue. Alternatively, the asparagine present in these isozymes may play an unknown role in the catalytic process.

Hughes *et al.* (1962) reported the isolation and partial amino acid sequence of a pyridoxyl tetradecapeptide from chymotryptic digests of borohydride-reduced s-GOT. The discrepancy between their results and ours may result from differences in the conditions employed for digestion of the enzyme protein.

Polyanovskii and Keil (1963) also reported the isolation of a

TABLE III: Amino Acid Sequence of Peptide s-c-21.[c]

Amino Acid	Edman Degradation (molar ratio) s-c-21	Stage 1	Stage 2	Stage 3
ε-Pyridoxyl-Lys	0.75	0.63	0.12	0.05
Asp	0.95	1.00	0.92	0.32
Ser	0.90	0.05	0.08	0.03
Phe	1.00	1.00	1.00	1.00
PTH derivative		PTH-Ser		

Amino Acid	Enzymatic Degradation (molar ratio) Leucine Aminopeptidase 15 min	Leucine Aminopeptidase 2 hr	Carboxypeptidase A 15 min
ε-Phosphopyridoxyl-Lys	0.02	0.21	0.00
Asn + Ser	0.12[a]	0.39	0.05[b]
Phe	0.02	0.20	0.15

[a] Essentially no Asn as judged from the ratio of the color values at 570 and 440 mμ. [b] Essentially no Ser. [c] Experimental procedures are those described in Table II. The ratio of the color value at 440 mμ to that at 570 mμ was 0.6 for Asn and 0.26 for Ser.

pyridoxyl tetrapeptide from similar chymotryptic digests of s-GOT. From the amino acid compositions of five peptide fragments obtained by partial acid hydrolysis of this tetrapeptide they proposed the sequence to be (ε-pyridoxyl)-Lys-Ser-Asp-(or Asn)-Phe.

However, the quantitative results described in the present paper do not support any sequence other than Ser-(ε-phosphopyridoxyl)-Lys-Asn-Phe for the coenzyme binding site tetrapeptide from s-GOT.

Polyanovskii and Keil (1963) and, subsequently, Turano and Giartosio (1964) speculated that a lysyl residue might be present in a position adjacent to the lysyl residue to which pyridoxal phosphate is bound. In the present study, a small but significant amount of free lysine was observed in hydrolysates of several pyridoxyl peptides (Table I). One may suspect the presence of a second lysyl residue in addition to the pyridoxal phosphate combining lysyl residue. However, analytical values for free lysine in these peptides were evidently much lower than the assumed stoichiometric number of residues per molecule of peptides and are not appraised as indicating the presence of a second lysyl residue. It should rather be interpreted to mean that free lysine found in hydrolysates of these peptides arose from the partial decomposition of ε-phosphopyridoxyllysine during the purification of these peptides and/or the subsequent acid hydrolysis as suggested by Nolan *et al.* (1964). Very recently we have obtained the coenzyme binding site peptides from tryptic digests of CM-r-m- and -s-GOT's (T. Watanabe and Y. Morino, unpublished data). Both of these peptides contained 24 to 25 amino acid residues with arginine as their carboxyl-terminal amino acid and had no lysyl residue other than that to which pyridoxal phosphate is bound. A preliminary experiment indicated that the chymotryptic pyridoxyl tetrapeptide occupies the middle portion of the large tryptic peptide from each GOT.

These findings seem to rule out the possibility of the presence of a second lysine adjacent to the pyridoxal phosphate combining lysyl residue.

The s-GOT preparations employed for the present investigation showed on starch gel electrophoresis four distinct bands which result from the presence of subforms as reported by Martinez-Carrion *et al.* (1967). The fact that a single phosphopyridoxyl peptide was found in the digests indicates that these subforms are identical as far as the sequence of four amino acid residues at the coenzyme binding site is concerned. Similarly, m-GOT preparations used in the present work showed three bands separable on starch gel electrophoresis. Actually, three fractions were partially separated on preparative electrophoresis on carrier ampholine and each fraction was found to differ in its specific activity and in its absorption spectrum in the visible region (T. Watanabe and Y. Morino, unpublished data). As shown in Figure 3, we have obtained several different phosphopyridoxyl peptides on the column chromatography of chymotryptic digest of CM-r-m-GOT. Analytical comparisons indicate that the difference in their amino acid compositions very likely results from heterogeneous cleavage of a single sequence at the coenzyme binding site, and does not indicate the variation in the sequence for each m-GOT subform.

Comparisons of the amino acid sequences of the active sites in isozymes can in principal tell us which residues in the sequence are essential and which are dispensable for catalytic activity. We have presented in this paper a comparative study on primary structures of the coenzyme binding sites in two isozymes of GOT. Further comparisons of the substrate binding site and of the catalytic sites should aid in understanding the nature of the active center of these important enzymes.

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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.