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Sequence of a Segment of Muscle Glycogen Phosphorylase Containing the Pyridoxal 5′-Phosphate Binding Site*

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ABSTRACT: This article describes the amino acid sequence of the pyridoxal 5′-phosphate binding site of rabbit muscle glycogen phosphorylase (EC 2.4.1.1). Limited chymotryptic and tryptic hydrolysis of $NaBH_4$-reduced phosphorylase yielded three phosphopyridoxyl peptides (33, 29, and 26 residues long), each terminating with the same N^{ϵ}-(P-pyridoxyl)Lys-Phe sequence at their carboxyl end. Peptic hydrolysis of these gave rise to a number of smaller fragments whose sequence was determined by a combination of Dansyl-Edman degradation, carboxypeptidases A and B, and leucine aminopeptidase digestions. Partial acid hydrolysis of the parent peptides provided the necessary overlaps from which the peptic fragments could be ordered. Cyanogen bromide degradation of $NaBH_4$-reduced phosphorylase gave rise primarily to a phosphopyridoxyl tripeptide, indicating that the sequence N^{ϵ}-(P-pyridoxyl)Lys-Phe occurs between two methionyl residues. Fortuitously, however, the methionyl peptide-bond distal to the P-pyridoxyllysyl residue was not quantitatively cleaved and an undecapeptide containing a single amino terminus and two homoseryl residues was also isolated and partially characterized. The following sequence for the pyridoxal 5′-phosphate binding site of phosphorylase was established

1 5 10 15
Arg-Val-Ser-Leu-Ala-Glx-Lys-Val-Ile-Pro-Ala-Ala-Asp-Leu-Ser-
20 25
Glx-Glx-Ile-Ser-Thr-Ala-Gly-Thr-Gln-Ala-
(5′-P-Pxy)
30 | 35
Ser-Gly-Thr-Gly-Asp-Met-Lys-Phe-Met-Gly-
42
Arg-Thr-Leu-(Glx,Asx,Thr)-Met . . .

Chymotryptic digestion of $NaBH_4$-reduced rat muscle phosphorylase also produced a 29-residue peptide identical in composition with that obtained from the rabbit, further stressing the structural analogies between the two enzymes.

Since the finding over 10 years ago (Fischer *et al.*, 1958a) that sodium borohydride reduction of muscle phosphorylase (EC 2.4.1.1) would covalently fix pyridoxal-P to the protein with little loss of enzymatic activity, several attempts were made to isolate and characterize a sizeable portion of the cofactor binding site. This task proved more difficult than originally anticipated. Exhaustive chymotryptic digestion of the reduced enzyme yielded only a substituted dipeptide identified as N^{ϵ}-(P-pyridoxyl)lysylphenylalanine (Fischer *et al.*, 1958a; Nolan *et al.*, 1964); no other phosphopyridoxyl peptide was detected, strongly supporting the assumption that $NaBH_4$ reduction had specifically fixed the cofactor at a unique site. Disappointingly, as discussed in this article, cyanogen bromide cleavage of the reduced enzyme led only to the isolation of a tripeptide, indicating that the dipeptide mentioned above was "sandwiched" between two methionyl residues.

For several reasons, it appeared essential to pursue this problem and determine the structure of a larger fragment of the cofactor binding site. First, all phosphorylases so far investigated possess stoichiometric amounts of pyridoxal phosphate, strongly suggesting that this compound is directly involved in catalysis and, therefore, that its binding site may be part of the active site of the enzyme. Second, since the exact mode of binding of P-Pyr[1] to the native molecule has not been established, characterization of the phosphopyridoxyl peptide might shed some light on this problem and explain some of the unusual spectral properties of the enzyme. Third, all glycogen phosphorylases consist of a basic subunit of very similar size (mol wt *ca.* 100,000; Seery *et al.*, 1967, 1970) suggesting that they might all have originated from the same ancestral gene. Information regarding the comparative amino acid sequence of the pyridoxal phosphate site could be of considerable in-

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[1] Abbreviations used are: Hse, homoserine; Asx, aspartic acid or asparagine; Glx, glutamic acid or glutamine; DNS or dansyl, dimethylaminonaphthalene-5′-sulfonyl; Pxy, pyridoxyl; P-Pxy, pyridoxyl 5′-phosphate.

terest from an evolutionary, as well as a mechanistic, point of view. Finally, amino acid sequences of the cofactor binding site of several types of classical pyridoxal phosphate containing enzymes involved in amino acid metabolism (*e.g.*, transaminases, decarboxylases, α- and β-eliminases, etc.) have recently been elucidated. Comparison of these sites might reveal certain structural similarities that would furnish additional information regarding the function of pyridoxal 5'-phosphate in phosphorylase.

The present manuscript describes the isolation of several large peptides obtained after limited tryptic and chymotryptic digestion of $NaBH_4$ reduced rabbit muscle phosphorylase. From these, a 38-amino acid long sequence of the pyridoxal phosphate binding site of the enzyme was elucidated. Cyanogen bromide cleavage of the protein provided further information regarding the structure of four additional residues. This is the seventh contribution of a series on the role of pyridoxal phosphate in phosphorylase; for previous publication, see Shaltiel *et al.* (1969).

Materials and Methods

Crystalline rabbit muscle phosphoyrlase *b* was prepared by the method of Fischer *et al.* (1958b) and converted into phosphorylase *a* using purified phosphorylase kinase (Krebs *et al.*, 1964). Reagents were as follows: $NaBH_4$ (Metal Hydrides, Inc.); 1-(tosylamido-2-phenyl)ethyl chloromethyl ketone treated trypsin (Gallard-Schlesinger Chemical Corp.); carboxypeptidases A and B, leucine aminopeptidase (diisopropyl phosphofluoridate treated), pepsin, soybean trypsin inhibitor, and *Escherichia coli* alkaline phosphatase (Worthington); thermolysin (Daiwa Kasei K. K., Osaka, Japan), precoated silica gel thin-layer plates F_{254} (Brinkmann Instruments, Inc.); Sephadex G-50 (Pharmacia Fine Chemicals, Inc.); and Whatman No. 3MM chromatography paper (Reeve-Angel). Dowex resins (Bio-Rad) were further treated as described by Schroeder (1967). Pyridine, *N*-ethylmorpholine, α-picoline, and phenyl isothiocyanate were redistilled. Cyanogen bromide (Eastman) was stored under vacuum at $-20°$ and used without further purification. All other chemicals were reagent grade.

$NaBH_4$-reduced phosphorylase was prepared according to the procedure of Strausbauch *et al.* (1967). In the handling of phosphopyridoxyl peptides, light was excluded to minimize photodecomposition of the chromophore.

Enzymatic digestions of $NaBH_4$-reduced phosphorylase were followed with a Radiometer TTTla-SBR 2U pH-Stat. Chymotryptic digestions, at protease: phosphorylase (w/w) ratios of 1:76 to 1:360, were carried out at pH 8.0 for up to 20 hr; possible tryptic activity was inhibited by addition of soybean trypsin inhibitor (1:30, w/w). Tryptic digestions were carried out at a 1:25 w/w ratio at pH 9.5 for 30 hr. At the end of each digestion, the pH was lowered to 6.0 with acetic acid and the suspension was centrifuged to remove insoluble material; the clear supernatant was concentrated by rotary evaporation or lyophilized. Peptide CB 5 was digested with thermolysin (1:20 w/w thermolysin: peptide) at pH 7.5 for 6 hr at 30°. The products of the hydrolysis were separated by high-voltage paper electrophoresis at pH 3.6. Cyanogen bromide cleavage of phosphorylase (10 mg/ml) was carried out in a sealed vessel in 70% formic acid with a 50-fold molar excess of CNBr over methionine (Steers *et al.*, 1965; Gross, 1967). After 20 hr at room temperature, the reaction was terminated by a 10-fold dilution with water followed by lyophilization.

Semimicro columns (0.6 × 60 cm) of ion-exchange resins (Dowex 1-X2 or Dowex 50-X2) were used in peptide purifications employing the volatile buffers described by Schroeder (1967).

Fractions obtained from column chromatographies were analyzed with ninhydrin after alkaline hydrolysis (Hirs, 1967); phosphopyridoxyl-containing fractions were detected by diluting aliquots with 0.1 M phosphate buffer (pH 6.0) and measuring the characteristic fluorescence emission at 395 nm following excitation at 330 nm. The emission could be distinguished from (and corrected for) the fluorescence due to tryptophanyl residues which have a characteristic excitation maximum at 280 nm and an emission maximum at 357 nm.

High-voltage paper electrophoresis (Katz *et al.*, 1959) was carried out in the pH 2.1, 3.6, and 6.5 buffers of Ryle *et al.* (1955); paper chromatography was performed in the 1-butanol–acetic acid–H_2O (4:1:5, v/v) system of Richmond and Hartley (1959) and in 1-butanol–pyridine–acetic acid–H_2O (30:20:6:24, v/v) of Waley and Watson (1953). Peptide mapping was performed as described by Sevilla and Fischer (1969).

Amino-terminal groups were determined by the dansyl-Cl method of Gray (1967). DNS-amino acids were separated by thin-layer chromatography on silica gel plates in chloroform–methanol–acetic acid (90:10:1, v/v) or 1-propanol–ammonia (80:20, v/v) (G. H. Dixon, private communication) and detected by their fluorescence. Water-soluble dansylamino acid derivatives were separated by high-voltage paper electrophoresis at pH 3.6. The amino acid sequence of the peptides was determined by the dansyl-Edman procedure (Edman, 1956; Gray, 1967).[2]

Routine amino acid analyses were carried out in a Spinco Model 120C amino acid analyzer. Analysis of N^{ϵ}-(Pxy)-lysine was also performed on this instrument according to Forrey *et al.* (1971) and Strausbauch and Fischer (1970). CNBr-peptide hydrolysates were treated with 0.05 N NaOH for 15 min at 30° immediately prior to the amino acid analysis to quantitatively convert homoserine lactone into homoserine (Tang and Hartley, 1967). The color value of homoserine was taken as the average of the threonine and serine values.

Peptic cleavages were carried out in 0.1 N acetic or formic acid at 27° for 16 hr using 2.0 mg of enzyme/μmole of peptide according to Sanger and Thompson (1953) and partial acid hydrolysis according to Schroeder *et al.* (1963). Leucine aminopeptidase was activated as described by Light (1967). Digestions by carboxypeptidase A (0.1 mg of enzyme/μmole of peptide) and leucine aminopeptidase (0.25 mg of enzyme/μmole of peptide) were carried out at 27° in 0.1 M Tris buffer (pH 8.0) for 4 and 9 hr, respectively. In both instances, the reaction mixtures were rapidly evaporated and the residue was taken up in pH 2.2, 0.2 M citrate buffer and applied to the amino acid analyzer.

Results

Isolation of the CNBr Peptides from the Pyridoxal-P Binding Site of Phosphorylase. CNBr cleavage of phosphorylase *b*, before and after sodium borohydride reduction, was carried out as indicated under the Methods. Initial attempts to fractionate the mixture by gel filtration chromatography were unsuccessful due to aggregation of the components. The problem was circumvented by separating the phosphopyridoxyl peptide from the bulk of the material by precipitation at pH 4.0. To this effect, glacial acetic acid was first added to the lyophilized

[2] The expression dansyl-Edman is used to designate the Edman degradation of the peptide followed by treatment with DNS-Cl (Hartley, 1970).

TABLE I: Composition of Peptides.

Amino Acid	C1	C2	C2 (Rat)	T1	T1P1	C2P1	C1P2[b]	C2P3	C2P4
Lysine	0.84 (1)	0.85 (1)	0.87 (1)			1.01 (1)			
Histidine									
Arginine	0.61 (1)								
(P-Pxy)-lysine	0.68 (1)	0.70 (1)	0.70 (1)	0.62 (1)					0.89 (1)
Aspartic acid	2.10 (2)	2.13 (2)	2.15 (2)	2.14 (2)	1.05 (1)	1.01 (1)			1.00 (1)
Threonine	2.89 (3)	3.04 (3)	2.85 (3)	3.04 (3)				1.62 (2)	0.81 (1)
Serine	3.76 (4)	2.89 (3)	3.00 (3)	2.73 (3)			0.93 (1)	0.79 (1)	0.77 (1)
Homoserine									
Glutamic acid	4.04 (4)	4.28 (4)	4.15 (4)	3.36 (3)		1.13 (1)	2.04 (2)	1.04 (1)	0.23 (0)
Proline	1.00 (1)	1.00 (1)	1.39 (1)	1.00 (1)	1.04 (1)	1.03 (1)			
Glycine	2.96 (3)	3.05 (3)	3.00 (3)	3.27 (3)				1.02 (1)	1.80 (2)
Alanine	4.97 (5)	5.22 (5)	5.23 (5)	4.45 (4)	2.21 (2)	3.00 (3)		1.00 (1)	1.25 (1)
Valine	1.42 (2)	0.59 (1)	0.61 (1)	0.59[a] (1)	0.52[a] (1)	0.81 (1)			
Methionine	0.94 (1)	0.97 (1)	1.00 (1)	0.79 (1)					0.66 (1)
Isoleucine	1.61 (2)	1.66 (2)	1.77 (2)	1.56[a] (2)	0.60[a] (1)	0.83 (1)		0.89 (1)	0.21 (0)
Leucine	2.04 (2)	1.20 (1)	1.31 (1)	1.29 (1)		0.35 (0)	0.25 (1)		
Tyrosine									
Phenylalanine	0.77 (1)	0.96 (2)	0.92 (1)	0.99 (1)					0.89 (1)
Total residues	33	29	29	26	6	9	4	7	9

[a] The valyl–isoleucyl bond in this peptide was incompletely hydrolyzed in 24 hr. [b] The low recovery of the amino-terminal

CNBr-peptide mixture (0.8 ml/100 mg of peptide) to produce a uniform slurry. A clear solution was obtained upon addition of water to a final acetic acid concentration of 30% and the pH of this solution was adjusted to 4.0 with 1 M ammonia. The resulting suspension was stirred for 10 min, then centrifuged. The supernatant was collected; the pellet was resuspended in water and recentrifuged and the combined supernatant solutions were lyophilized.

This procedure yielded 60–70% of the fluorescent material present in the original digest, free of the large peptides probably responsible for the aggregation that occurred during chromatography.

Sephadex and Ion-Exchange Chromatography of the CNBr Peptides. As shown in Figure 1A, one major fluorescent fraction was obtained from G-50 Sephadex chromatography of the sodium borohydride reduced enzyme. The minor fluorescent peak may have been derived from an incomplete CNBr cleavage of the protein as will be discussed later. The pattern for the unmodified (nonreduced) enzyme (Figure 1B) was similar to that of the reduced enzyme except that, of course, no fluorescent fraction was present. The major fluorescent fraction and its nonsubstituted counterpart obtained from the nonreduced enzyme were separately lyophilized and applied to identical Dowex 50 columns. The elution patterns shown in Figure 2 are, once more, qualitatively similar except that one of the peptides (CB 3) originating from the unmodified enzyme was absent from the elution diagram of the reduced protein. This immediately suggested that peptide CB 3 contained the unsubstituted lysyl residue involved in the binding of pyridoxal phosphate. Peptide CB 3 was pure as judged by peptide mapping and amino acid analysis; the latter procedure showed that it contained only three amino acids: namely, lysine, phenylalanine, and homoserine in stoichiometric amounts (see Table I).

The sequence Lys-Phe-Hse for this tripeptide was confirmed by the finding that lysine occupied the amino-terminal position; it is consistent with the sequence (P-Pxy)Lys-Phe previously determined for the pyridoxal phosphate binding site of phosphorylase. The fluorescent fraction CB 3′, obtained from the reduced enzyme, was not further purified since its amino acid composition (Table I) indicated that it was identical with peptide CB 3, except that it contained the phosphopyridoxyl substituent on the lysyl residue.[3]

Since this project was part of a more general study of the primary structure of phosphorylase, all the peptides that emerged from the Dowex 50 column were isolated and their amino acid compositions determined. Fortuitously, two other peptides (CB 3-5 and CB 5) isolated from nonreduced phosphorylase proved to be related to the pyridoxal phosphate binding site of the enzyme.

Peptide CB 3-5 (11 amino acids; see Table I) contained 2 homoseryl residues but only one amino-terminal group (lysine), as if the CNBr treatment had modified one of the methionyl side chains without peptide-bond cleavage. Furthermore, carboxypeptidase A digestion released only 1 equiv of homoserine. The amino acid composition of this peptide was identical with the sum of CB 3 (3 residues) and CB 5 (8 residues), suggesting that the latter two peptides together constituted CB 3-5; CB 5 would then occupy a position immediately adjacent and distal to the tripeptide CB 3 (Lys-Phe-Met)

[3] In an alternate purification procedure, the fluorescent fraction obtained from the Sephadex G-50 column was chromatographed twice on a Dowex 1 column, before and after removal of the 5′-phosphate group by treatment with *E. coli* alkaline phosphatase according to the procedure of Strausbauch and Fischer (1970). Removal of the phosphate group altered the charge of this peptide and, hence, its elution volume, without affecting the elution pattern of contaminating peptides it emerged as a pure material. This further demonstrates the general applicability of this procedure for the purification of P-pyridoxyl peptides.

Amino Acid	C2A1	T1A1	T1A2	CB 3	CB 3′	CB 5	CB 3-5	CB 3′-5	CB 5-Th-1	CB 5-Th-2
Lysine				1.01 (1)			0.82 (1)			
Histidine										
Arginine						0.81 (1)	0.75 (1)	1.00 (1)	0.70 (1)	
(P-Pxy)-lysine	NA (1)				0.73 (1)			0.58 (1)		
Aspartic acid	1.04 (1)					1.21 (1)	1.00 (1)	1.26 (1)		1.27 (1)
Threonine	2.84 (3)	0.97 (1)	1.00 (1)			1.84 (2)	1.85 (2)	2.11 (2)	1.02 (1)	1.19 (1)
Serine	2.78 (3)	1.10 (1)	0.92 (1)							
Homoserine				0.99 (1)	1.17 (1)	1.00 (1)	1.77 (2)	1.63 (2)		0.77 (1)
Glutamic acid	3.18 (3)	1.27 (1)	1.08 (1)			1.13 (1)	0.98 (1)	1.16 (1)		1.00 (1)
Proline										
Glycine	2.96 (3)	2.07 (2)	1.44 (1)			1.08 (1)	1.00 (1)	1.21 (1)	1.00 (1)	0.36 (0)
Alanine	2.20 (2)	0.70 (1)	0.84 (1)							
Valine										
Methionine	0.91 (1)									
Isoleucine	1.04 (1)									
Leucine	0.93 (1)					1.00 (1)	1.00 (1)	1.05 (1)		0.89 (1)
Tyrosine										
Phenylalanine	0.93 (1)			1.00 (1)	1.00 (1)		0.73 (1)	0.95 (1)		
Total residues	20	6	5	3	3	8	11	11	3	5

residue (leucine) results from the fact that this peptide was isolated from paper after spraying with ninhydrin.

to which pyridoxal phosphate is bound. This conclusion was confirmed by the later isolation of a fluorescent, phosphopyridoxyl peptide (CB 3′–5) containing the same 11 amino acids including 2 homoseryl residues.

Sequence of Peptide CB 5 (Residues 35–42). The amino-terminal residue of this octapeptide was found to be glycine by the dansyl chloride procedure. Thermolytic digestion (see Methods) produced two peptides, CB 5-Th-1 and CB 5-Th-2 (see Table I). Glycine was the amino-terminal residue of tripeptide (CB 5-Th-1) and one turn of the Edman degradation gave the sequence: Gly-Arg-Thr. The amino-terminal residue of the pentapeptide (CB 5-Th-2) was leucine. Repeated attempts at dansyl-Edman degradation of this peptide apparently failed in that no new DNS-amino acid could be detected. This result was verified by subtractive Edman degradation (Konigsberg, 1967); leucine was quantitatively lost after a single turn of the degradation, but no further residues could be removed by repeated cycling. Perhaps a glutaminyl residue, occurring next in the sequence, cyclized to form a pyro-

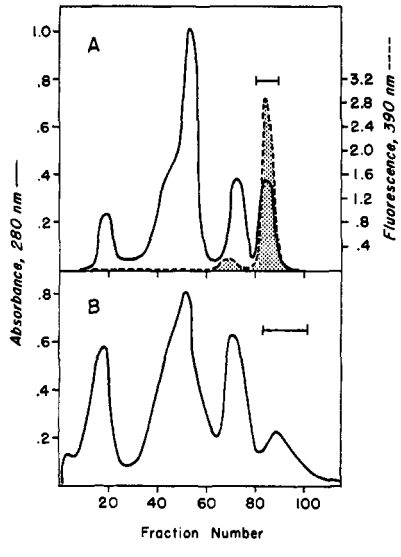


FIGURE 1: Sephadex G-50 chromatography of the pH 4 soluble CNBr peptides obtained from: (A) $NaBH_4$-reduced phosphorylase *b;* the 2.5 × 100 cm column was eluted with 1 M acetic acid at a flow rate of 30 ml/hr; aliquots of the fractions (3 ml) were analyzed for fluorescence and for absorption at 280 nm. (B) Native (nonreduced) phosphorylase *b*.

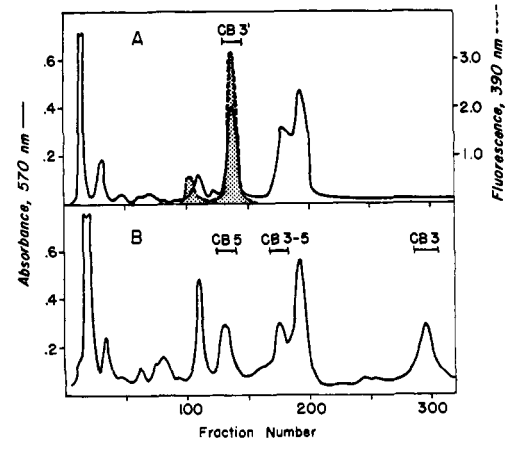


FIGURE 2: Dowex 50-X2 chromatography of the pyridoxal 5′-phosphate binding peptides after Sephadex G-50 gel filtration. In (A) the fluorescent phosphopyridoxyl fractions originated from $NaBH_4$-reduced phosphorylase *b;* in (B) the analogous (nonfluorescent) fractions were obtained from nonreduced phosphorylase *b*. Conditions are described under Methods.

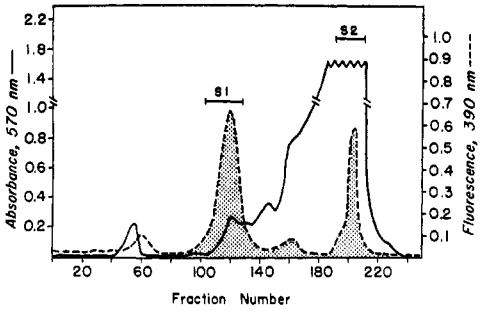


FIGURE 3: Separation on Sephadex G-50 of the 5′-P-pyridoxyl peptides from a chymotryptic digest of $NaBH_4$-reduced phosphorylase. The column (2.5 × 100 cm) was developed with 1 M acetic acid at a flow rate of 15 ml/hr; 2-ml fractions were collected and analyzed for fluorescence and ninhydrin-positive material after alkaline hydrolysis as indicated under Methods.

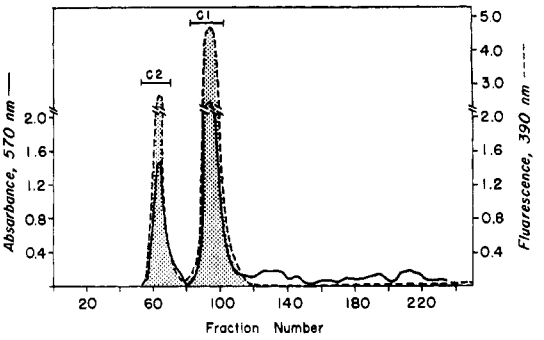


FIGURE 4: Dowex 50-X2 chromatography of Sephadex fraction S1; 1-ml fractions were collected at a flow rate of 15 ml/hr.

glutamyl peptide lacking a free amino group. A partial sequence of CB 5 is thus Gly-Arg-Thr-Leu(Glx,Thr,Asx)Hse. As expected, thermolysin readily cleaved the X-Leu–peptide bond (Ambler and Meadway, 1968; Bradshaw, 1969).

Isolation of Phosphopyridoxyl Peptides from Reduced Phosphorylase Following Limited Chymotryptic Digestion. The $NaBH_4$ reduction and chymotryptic digestion of the enzyme are described under Methods. In two preparations involving 10.7 μmoles (1 g) and 30 μmoles (2.78 g) of phosphorylase (based on the molecular weight of 92,000 for the enzyme monomer; Seery *et al.*, 1970), 60 and 50%, respectively, of the expected fluorescent material was released from the enzyme in a trichloroacetic acid soluble form.

The lyophilized digest was taken up in 30% acetic acid, and centrifuged to remove insoluble material, and the clear solution was fractionated on Sephadex G-50 under the conditions described in the legend of Figure 3. Fractions corresponding to the fluorescent peak (S1), well separated from the bulk of the ninhydrin reactive material, were combined and lyophilized. A second fluorescent peak (S2) emerged from the column at the position expected for the chymotryptic phosphopyridoxyl dipeptide previously described (Fischer *et al.*, 1958a; Nolan *et al.*, 1964); it was not further investigated. For the two separate preparations, yields in fraction S1 were 34 and 24%, respectively, based on the fluorescence of the original reduced enzyme.

Dowex 50-X2 Column Chromatography of Chymotryptic Peptides. Fraction S1 was chromatographed on a column of Dowex 50-X2; the elution profile is illustrated in Figure 4. Two fluorescent peaks appeared in the effluent; fractions corresponding to each were combined and lyophilized. Fraction C2 was shown to consist of only one peptide by paper electrophoresis and chromatography, while fraction C1 was still contaminated by nonfluorescent ninhydrin-positive material. An additional chromatography on Dowex 1-X2 (not illustrated) yielded a pure peptide, C1.

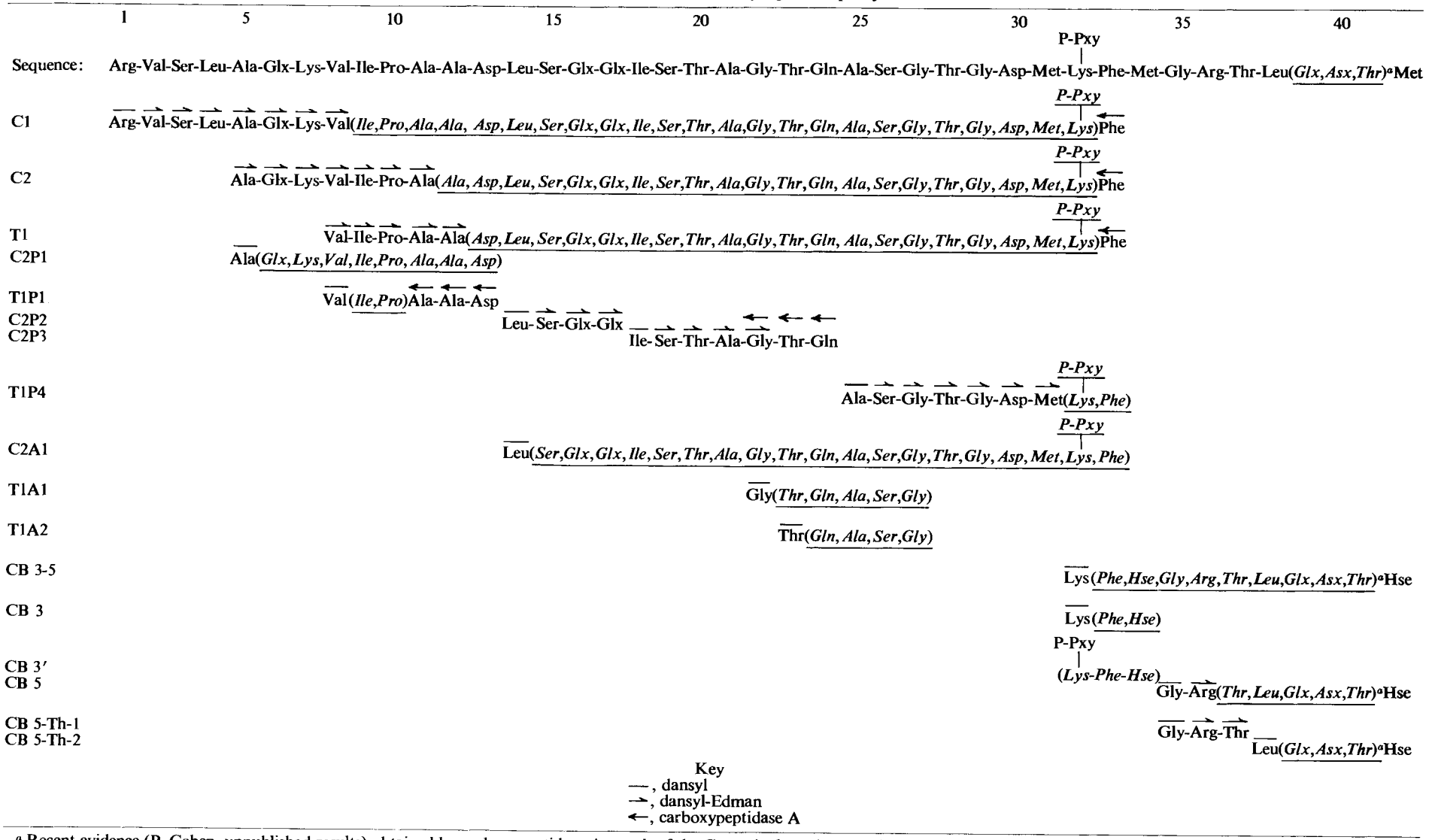
In the two preparations mentioned above, recoveries of peptides C1 and C2 were approximately 25% calculated on the basis of the total amount of phosphorylase involved, but 42 and 48%, respectively, on the basis of the amount of phosphopyridoxyl material released from the enzyme by this limited proteolysis. An additional *ca.* 25% of the fluorescent material appeared in fraction S2 of Figure 3, consisting of the dipeptide (P-Pxy)Lys-Phe. On the other hand, the relative proportion of peptides C1 and C2 varied with the conditions and time of digestion. When phosphorylase was digested for 11 hr at 27° at a 1:180 w/w ratio of chymotrypsin to phosphorylase, the ratio of peptide C1 to C2 was 3:1, while at a 1:100 w/w ratio, this proportion was reversed. As described earlier, exhaustive digestion with chymotrypsin released all the bound phosphopyridoxyl material in the form of the dipeptide (P-Pxy)Lys-Phe (Fischer *et al.*, 1958a; Nolan *et al.*, 1964).

Composition of Peptides C1 and C2. The amino acid compositions of peptide C1 (33 residues) and C2 (29 residues) are presented in Table I. As can be seen, C1 has the same composition as C2 with the exception that it contains one additional residue each of arginine, serine, valine, and leucine. Both peptides contain a single phosphopyridoxyllysine residue. Amino-terminal analysis showed the presence of an arginyl residue in C1 and an alanyl residue in C2, while carboxypeptidase A released exclusively phenylalanine in stoichiometric amounts from both peptides. Since these contained only one phenylalanyl residue, this immediately suggested that the sequence (P-Pxy)Lys-Phe would represent the COOH-terminal portion of both C1 and C2.

Isolation of a Phosphopyridoxyl Peptide from Reduced Phosphorylase Following Exhaustive Tryptic Digestion. Reduced phosphorylase was also exhaustively digested with trypsin with the hope of obtaining an additional set of peptides. For the sake of simplicity, the isolation of the single tryptic peptide obtained (T1, 26 residues) will not be described here, since it could be directly generated by tryptic cleavage of the larger chymotryptic peptides C1 or C2. As can be seen from Tables I and II, it results from the deletion of 7 amino acids from the chymotryptic peptide C1 or 3 amino acids from C2. Peptide T1 generated from a tryptic attack of either the protein or the purified chymotryptic fragments had a single amino-terminal valyl residue but, unexpectedly, also a phenylalanyl residue at its carboxyl terminus. This unusual tryptic cleavage of a phosphopyridoxyllysylphenylalanyl–X bond was also observed in a study of the PLP-binding site of *E. coli* glutamic decarboxylase (Strausbauch and Fischer, 1970); other examples of chymotryptic-like activities in trypsin have been described (Cole and Kinkade, 1961; Inagami and Mitsuda, 1964).

It is of interest to note the different behavior of $NaBH_4$-reduced phosphorylase when subjected to hydrolysis by trypsin and chymotrypsin under similar sets of conditions. Proteolysis of reduced phosphorylase results in an increase (approximately 3-fold) in both absorbance at 325 nm and fluorescence at 395 nm (due to the phosphopyridoxyl moiety). With trypsin, there is a rapid increase in absorbance, fluorescence, and base uptake and yet, release of fluorescent peptide in trichloroacetic acid soluble form is slow (Figure 5A)

TABLE II: Alignment of Peptides and Sequence of Pyridoxyl 5′-Phosphate Binding Site in Rabbit Muscle Glycogen Phosphorylase.



[a] Recent evidence (P. Cohen, unpublished results) obtained by carboxypeptidase A attack of the C-terminal portion of this peptide indicates that both Asx and Glx are present in their amide forms. The sequence for the analogous peptide from dogfish phosphorylase is Met-(5′-P-Pxy)Lys-Phe-Met-Gly-Arg-Thr-Leu-Gln-Asn-Thr-Met for residues 31–42.

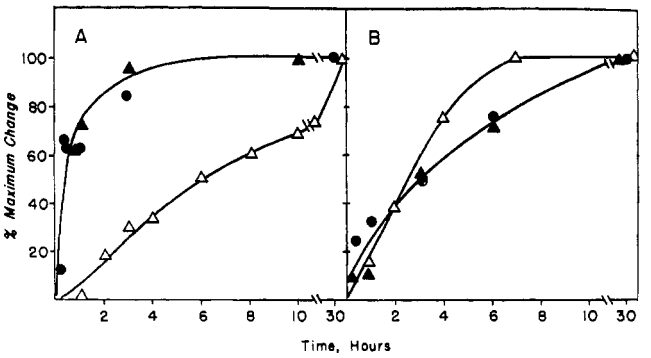


FIGURE 5: Change in absorbance and fluorescence during tryptic (A) and chymotryptic (B) attack of $NaBH_4$-reduced phosphorylase *b*. Aliquots (1 ml) of the digest were removed and diluted with 1 ml each of 2 M NaCl, 1 M sodium phosphate buffer (pH 6.1), and water. A slight turbidity was removed by centrifugation and absorbance (●) at 325 nm and fluorescence (▲) at 395 nm (λ (excitation) 325 nm) were measured. Phosphopyridoxyl-peptide release was followed by removing 2.5-ml aliquots which were precipitated with 0.25 ml of 50% trichloroacetic acid. After centrifugation, the supernatant solution was brought to pH 6.1 by addition of 2.5 ml of 1 M sodium phosphate buffer (pH 6.7) and the fluorescence (△) at 395 nm (λ (excitation) 325 nm) was measured.

By contrast with chymotrypsin (Figure 5B) phosphopyridoxyl peptide release paralleled the increase in absorbance, fluorescence, and base uptake. Peptide liberation was essentially complete in 7 hr at pH 8.0 as compared to 30 hr with trypsin at pH 9.5 even though the digestion is markedly accelerated under the latter conditions.

Peptic Cleavage of Peptides T1, C1, and C2. Smaller fragments of peptides C1, T1, and C2 were obtained by further digestion by pepsin; for the sake of simplicity, the isolation of peptides from T1 and C2 only will be described. Furthermore, only the major fragments that proved to be essential to the determination of a unique sequence will be discussed in detail. All other peptides that were generated were checked for purity and analyzed for amino acid composition and amino-terminal residues. Even though these data are not included in Table I, they were, in all instances, entirely consistent with the sequence presented in Table II. Pepsin was added at a 1:175 molar ratio to approximately 2.5 μmoles of peptides T1 and C2 in 5% formic acid and the digestion was allowed to proceed for 20 hr at room temperature. The digests were chromatographed on columns of Dowex 50-X2 (not illustrated); each of the peaks that emerged from the column was pooled separately, lyophilized, and further purified by paper electrophoresis and chromatography. Additional peptides were obtained after partial acid hydrolysis of C1 (Schroeder *et al.*, 1963). These peptides provided the necessary overlapping sequences required to order the peptide fragments.

Peptide Sequences. PEPTIDE C1 (RESIDUES 1–33). This 33 residue phosphopyridoxyl peptide was subjected to 7 turns of the dansyl-Edman procedure to provide the amino-terminal sequence Arg-Val-Ser-Leu-Ala-Glx-Lys-Val (see Table II). Carboxypeptidase A released only 1 equiv of phenylalanine.

PEPTIDE C2 (RESIDUES 5–33). Six successive rounds of the dansyl-Edman procedure[4] indicated an amino-terminal sequence of Ala-Glx-Lys-Val-Ile-Pro-Ala. Again carboxypeptidase A released only phenylalanine in stoichiometric amounts.

[4] DNS-proline was detected in only minute amounts due to its extreme lability during acid hydrolysis.

PEPTIDE T1 (RESIDUES 8–33). The amino-terminal sequence[4] of this 26-residue phosphopyridoxyl peptide was found to be Val-Ile-Pro-Ala-Ala. As discussed earlier, this tryptic peptide contained phenylalanine at the carboxyl end. As expected (Gray, 1967), the sequence Val-Ile was particularly resistant to acid hydrolysis (see also Table I); DNS-Val-Ile was detected in addition to DNS-valine during amino-terminal group analysis. This was confirmed by dansylation of an authentic sample of valylisoleucine (Cyclo Chemical Corp.)

Peptic Hydrolysis of Chymotryptic and Tryptic Peptides. C2 P1 (RESIDUES 5–13). Peptic digestion of C2 yielded this nonapeptide. The presence of alanine at the amino terminus and the composition of this peptide (it contained the only prolyl residue of the parent peptides) suggested that it was derived from the amino-terminal portion of peptide C2. Since the sequence was already established, it was not further investigated.

T1P1 (RESIDUES 8–13). The amino terminus of this peptide was valine, as determined by dansylation, while a time course of a carboxypeptidase A digestion provided the sequence Ala-Ala-Asp for the carboxyl end. Taken together with the sequences obtained for the amino-terminal portions of C2 and T1, these data indicate that T1P1 has the following sequence: Val-Ile-Pro-Ala-Ala-Asp.

C2P2 (RESIDUES 14–17). Three successive rounds of the dansyl-Edman procedure established the sequence: Leu-Ser-Glx-Glx.

C2P3 (RESIDUES 18–24). The sequence of this peptide was established as Ile-Ser-Thr-Ala-Gly-Thr-Gln by a combination of dansyl-Edman degradation and carboxypeptidase A digestion. The presence of a carboxyl-terminal glutaminyl residue proved to be important in establishing the order of peptides C2P2 and C2P3, as will be discussed below.

T1P4 (RESIDUES 25–33). Six turns of Edman degradation on this phosphopyridoxyl peptide yielded the sequence Ala-Ser-Gly-Thr-Gly-Asp-Met(P-Pxy)Lys-Phe. The sequence of the last two residues was already known from the determination of the carboxyl-terminal phenylalanine in the parent peptide and the previous isolation and characterization of the substituted dipeptide (P-Pxy)Lys-Phe (Fischer *et al.*, 1958a; Nolan *et al.*, 1964).

OVERLAP PEPTIDES OBTAINED BY PARTIAL ACID HYDROLYSIS, C2A1 (RESIDUES 14–33), T1A1 (RESIDUES 22–27), AND T1A2 (RESIDUES 23–27). At this point, the sequences of all fragments originating from the parent peptides had been established. The positioning of the first 13 amino acids starting from the single amino-terminal arginyl residue is unambiguous from the overlapping sequences obtained from peptides C1, C2, T1, and T1P1 (see Table II). Likewise, peptide T1P4 can be positioned unambiguously at the distal end since it contains the sole phenylalanyl residue found at the carboxyl terminus of the parent peptides C1, C2, and T1. The only ambiguity, therefore, remained in the positioning of the two inner peptides C2P2 and C2P3.

Partial acid hydrolysis of peptide C2 produced a 30-residue phosphopyridoxyl peptide (C2A1) containing the single leucyl residue of the parent peptide at its amino end. This is the first evidence that C2P2 and C2P3 must occur in this order. This conclusion was further supported by the isolation of two additional peptides (T1A1 and T1A2) also resulting from partial acid hydrolysis. T1A1 contains two of the three glycyl residues (at positions 22, 27, and 29) of the parent peptide T1; it could only arise from residues 22 to 27 or 24 to 29 which have identical composition. However, only in the former instance would glycine be found at the NH_2 terminus, as was indeed

the case. The same reasoning places peptide T1A2 at residues 23–27, again indicating that peptide C2P3 must precede the carboxyl-terminal peptide T1P4. Taken together, the above data are consistent with a single sequence as shown in Table II.

Sodium borohydride reduced rat skeletal muscle phosphorylase (Sevilla and Fischer, 1969) was also subjected to chymotrypsin attack under the same conditions as those devised for the liberation of peptide C2 from the $NaBH_4$-reduced rabbit muscle enzyme.

The phosphopyridoxyl peptides obtained from both animals behaved identically in all chromatographic procedures used for their isolation. Amino acid analysis of the rat peptide yielded a composition identical with that of its rabbit counterpart (see Table I). Both had alanine and phenylalanine at their amino and carboxyl ends, respectively. While scarcity of material prevented a detailed sequence analysis of the rat peptide, it is reasonable to assume that the two cofactor binding sites are homologous. Previous studies (Sevilla and Fischer, 1969) had described considerable structural similarities between the two enzymes, including the amino acid sequence of the site phosphorylated during the *b* into *a* conversion; the latter showed a single conservative substitution (an aspartyl residue in the rat replacing a glutamyl residue in the rabbit) over a sequence of 14 amino acids.

Discussion

Examination of the sequence of the pyridoxal phosphate binding site in phosphorylase (Table II) reveals several interesting structural features. First, this region of the molecule is remarkably free of basic amino acids. Whereas statistically, one would expect to find one basic amino acid every seven to eight residues in the enzyme (Sevilla and Fischer, 1969), none occurs in a sequence of *ca.* 26 amino acids except the lysyl residue substituted by the cofactor. By contrast, the seryl phosphate site which is responsible for the phosphorylase *b* into *a* conversion and covalent control of enzymatic activity displays four basic amino acids over a sequence of 14 residues; this peptide itself must belong to a highly basic region of the molecule since CNBr fragmentation of ^{32}P-labeled phosphorylase *a* led to the isolation of a large, *ca.* 88 amino acid long peptide with an isoelectric point of 10.5 (Saari, 1970). This latter fragment contains on the average one lysyl or arginyl residue for every five or six amino acids.

When the structure of the phosphopyridoxyl peptide is compared to that recently obtained from other pyridoxal-P-containing enzymes (Strausbauch and Fischer, 1970), a few common features emerge though, of course, much more information would be needed for this kind of comparison. In all instances, the cofactor is bound to the ϵ-amino group of a unique lysyl residue. In phosphorylase, as in *E. coli* glutamic acid decarboxylase (Strausbauch and Fischer, 1970), and arginine and lysine decarboxylases (B. Boeker, and D. Sabo, unpublished results from this laboratory), the portion of the peptide proximal to the phosphopyridoxyllysyl residue is predominantly hydrophilic while the distal side is hydrophobic. Actually with phosphorylase and glutamic acid decarboxylase, there is an alternating sequence of hydrophilic and hydrophobic side chains preceding the substituted lysyl residue which might suggest that this peptide segment exists in a β formation. In both structures, one finds a (P-pyridoxyl)-lysylphenylalanine sequence and the peptide bond following phenylalanine is apparently cleaved by trypsin; this peculiar behavior was discussed in more detail in Strausbauch and Fischer (1970).

In phosphorylase, the (P-pyridoxyl)lysylphenylalanine sequence is bordered by two methionyl residues. As shown in Table II, CNBr cleavage of the phosphopyridoxyl site also generates a minor (less than 10%) undecapeptide (CB 3-5) whose composition was the exact sum of that of the phosphopyridoxyl tripeptide CB 3 and octapeptide CB 5. Unfortunately, the peptide was isolated in low yield and insufficient material was available for detailed structural analysis. It is most unlikely that fraction CB 3-5 is an equimolar mixture of peptides CB 3 and CB 5, at least when first isolated. For instance, a sample of CB 3-5 migrated as a single spot on high-voltage paper electrophoresis (pH 3.6) and its mobility did not correspond to the spots obtained with peptides CB 3 or CB 5. However, upon standing for 3–4 months in 1 M formic acid, a sample of CB 3-5 clearly gave evidence for containing a mixture of its two component peptides—*i.e.*, two residues were released during the first and second turns of the Edman degradation (lysine, glycine, arginine, and phenylalanine, respectively). Prolonged storage at a low pH probably labilized the homoseryl–glycyl bond to generate a mixture of the two peptides. Nonetheless, it is quite unlikely that an equimolar mixture of peptides could have been isolated from three independent CNBr digestions of phosphorylase including one sample of $NaBH_4$-reduced enzyme. If the above assumption is correct, it then permits the placing of peptide CB 5 on the distal (COOH terminal) side of the pyridoxal phosphate tripeptide.

Several other laboratories have reported the presence of methionyl peptide bonds that appeared to be particularly resistant to CNBr cleavage (Schroeder *et al.*, 1969; Cunningham *et al.*, 1968; Narita and Titani, 1968; DeLange, 1970). In nearly all cases, the amino acid following methionine was threonine or serine. In one instance (Vanaman *et al.*, 1968) where a sequence Leu-Met-Ala occurred, methionine was modified but no homoserine formation or peptide bond cleavage was detected. In horse cytochrome *c*, incomplete cleavage of the two methionyl bonds (Met-Ile and Met-Glu) was also reported (Corradin and Harbury, 1970). CNBr cleavage of collagen (P. Bornstein and E. Click, personal communication) resulted in incomplete cleavage at several methionyl–glycyl bonds.

Finally, in phosphorylase, there is an arginyl side chain four residues away from the phosphopyridoxyl substituent that could easily interact with the 5′-phosphate group of the cofactor and, perhaps, help to maintain the latter in the proper orientation.

Data reported in Figure 5 illustrate the large increase in absorbance and fluorescence emission of the bound cofactor following enzymatic degradation of phosphorylase, suggesting that in the native enzyme, both spectra are strongly quenched. This observation supports earlier conclusions that pyridoxal phosphate is buried within the molecule, perhaps in a hydrophobic pocket (Shaltiel and Cortijo, 1970; Cortijo *et al.*, 1971). It will not react with any aldehyde trapping agent unless the protein is modified by changes in pH or distorted by salts or denaturing agents. The fact that trypsin produces a rapid increase in absorbance and fluorescence but only a slow release of the phosphopyridoxyl peptide as compared to chymotrypsin is consistent with some of the previously established structural characteristics of phosphorylase. As discussed above, the protein contains many lysyl or arginyl residues susceptible to tryptic hydrolysis but few occur in the vicinity of the pyridoxal phosphate site; furthermore, the latter might be inaccessible to trypsin, therefore preventing the early release of the substituted peptide. By contrast, two peptide

bonds adjacent to the phosphopyridoxyllysyl residue must be very susceptible to chymotryptic attack since the dipeptide (P-pyridoxyl)lysylphenylalanine seems to be liberated at the same rate as the rest of the molecule is cleaved.

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