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Structure of the Binding Site of Pyridoxal 5′-Phosphate to *Escherichia coli* Glutamate Decarboxylase*

Paul H. Strausbauch† and Edmond H. Fischer

ABSTRACT: Pyridoxal 5′-phosphate is bound to *Escherichia coli* glutamate decarboxy-lyase (EC 4.1.1.15) in two spectral forms with absorption maxima at 340 and 415 nm. The second species which is formed at low pH and is attributed to a Schiff base was reduced with $NaBH_4$ to produce an inactive enzyme in which pyridoxal 5′-phosphate was found to be covalently bound to an ε-amino group of a lysyl residue. This derivative was digested with trypsin and the peptide containing the 5′-phosphopyridoxyl residue was isolated by a combination of gel filtration and ion exchange chromatography. Its amino acid sequence was determined from a sequential Edman degradation coupled with end group determinations using Dansyl·Cl and from a carboxypeptidase A digestion; the following structure was obtained: Ser-Ile-Ser-Ala-Ser-Gly-His-(PylP)Lys-Phe. An improved procedure for the isolation of 5′-phosphopyridoxyl containing peptides, based on the "diagonal" procedure of Brown and Hartley (Brown, J. R., and Hartley, B. S. (1963), *Biochem. J. 89*, 59P), is described; it takes advantage of the altered elution of these peptides from Dowex after digestion with alkaline phosphatase.

The spectral properties of PLP[1] in glutamate decarboxylase were studied in detail by Shukuya and Schwert (1960b). They found that at pH values below 5, the enzyme has an absorption maximum at 415 nm which diminishes as the pH is increased, with the concomitant appearance of a new peak at 340 nm. This spectral shift was similar to those described earlier for other PLP-containing enzymes (Kent *et al.*, 1958; Jenkins and Sizer, 1959) and was attributed to the formation of a substitution aldimine from a Schiff base. Anderson and Chang (1965) were able to bind PLP covalently to an ε-amino group of a lysyl residue of glutamate decarboxylase by reducing the "415 nm" form of the enzyme with $NaBH_4$ according to the procedure of Fischer *et al.* (1958). Since, in the case of glycogen phosphorylase, it was found that $NaBH_4$ reduction caused no transfer of PLP to other sites on the protein, and no gross change in the architecture of the enzyme (Fischer *et al.*, 1958), a unique tool appeared to be available for deter-

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[1] The abbreviations used for pyridoxal 5′-phosphate and its derivatives are those tentatively accepted by the Commission on Biochemical Nomenclature, IUB, namely, PLP, pyridoxal 5′-phosphate; Pxy, pyridoxyl; 5′-*P*-Pxy, pyridoxyl 5′-phosphate. Dansyl is dimethylaminonaphthalenesulfonyl.

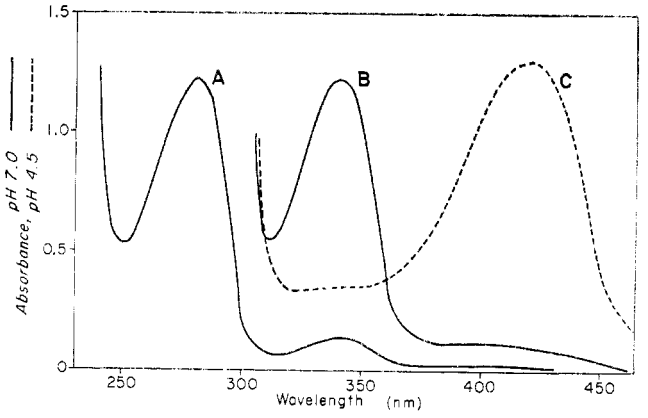


FIGURE 1: Absorption spectra of crystalline *E. coli* glutamate decarboxylase, determined at 0.72 mg/ml of enzyme (curve A) and 7.20 mg/ml of enzyme (curve B) in 0.1 M sodium phosphate buffer, pH 7.0; curve C, 6.57 mg/ml of enzyme in 0.1 M pyridine·HCl buffer (pH 4.5).

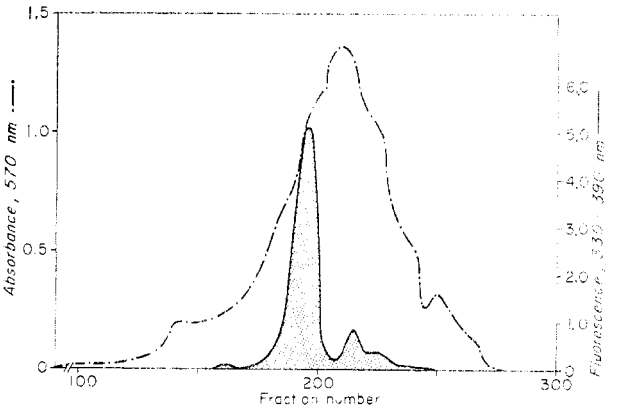


FIGURE 2: Purification of the 5′-*P*-Pxy-peptide on Sephadex G-50 in 1 N acetic acid. Fractions (2 ml) were collected and analyzed for ninhydrin-positive material and fluorescence, as indicated under Methods.

mining the structure of the PLP binding site of PLP-containing enzymes. This approach has been successful for the isolation of PLP binding peptides from glycogen phosphorylase (Fischer *et al.*, 1958) and aspartate aminotransferase (Hughes *et al.*, 1962; Polianovsky and Keil, 1963; Turano and Giartosio, 1964; Morino and Watanabe, 1969). This paper reports the isolation and structural characterization of the PLP binding site of glutamate decarboxylase. A secondary objective of this work was the development of a simplified method for the purification of 5′-*P*-Pxy-peptides since distinct difficulties have been experienced in such undertakings (Forrey, 1963). The previous manuscript (Strausbauch and Fischer, 1969) described some of the chemical and physical properties of the enzyme.

Materials and Methods

Glutamate decarboxylase was purified and crystallized three times as described by Strausbauch *et al.* (1967). The crystalline material was used exclusively for spectral studies; for the isolation of the 5′-*P*-Pxy-peptides, the fraction obtained just before crystallization was entirely adequate. Reagents were as follows: $NaBH_4$ (Metal Hydrides, Inc.); 1-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone treated trypsin (Gallard-Schlesinger Chemical Corp.); carboxypeptidase A (DFP-treated to eliminate tryptic and chymotryptic activities) and *Escherichia coli* alkaline phosphatase (Worthington); precoated silica gel thin-layer plates (F_{254}, Brinkman Instruments, Inc.); and Sephadex G-50 (Pharmacia Fine Chemicals, Inc.). Dowex resins (Bio-Rad) were further treated as described by Schroeder (1967), and pyridine, *N*-ethylmorpholine, α-picoline, and phenyl isothiocyanate were redistilled.

$NaBH_4$-reduced glutamate decarboxylase was prepared according to the general procedure of Fischer *et al.* (1958) and Strausbauch *et al.* (1967). In the handling of 5′-*P*-Pxy-peptides, light was excluded in order to minimize photodecomposition of the chromophore.

Analysis of ε-(Pxy)-lysine was carried out in a Spinco 120C amino acid analyzer according to the method of Forrey (1963) which uses a 20-cm Aminex Q-15 S column, following hydrolysis of the 5′-*P*-Pxy-peptides in twice-redistilled 5.7 N HCl for 24 hr at 108°. The column was eluted with 0.38 M sodium citrate buffer (pH 4.26) at 33° for 130 min and then with 0.35 M sodium citrate (pH 5.28) at 55°. ε-(Pxy)-lysine emerges approximately two-thirds of the way between lysine and arginine. (Pxy)-lysine was also determined fluorometrically in a Farrand spectrofluorometer: samples were diluted in 0.1–0.5 M sodium phosphate buffer (pH 6.5) and excited at 335 nm; fluorescence was measured at 390 nm. This emission could be distinguished from the fluorescence due to tryptophanyl residues which have a characteristic excitation maximum at 280 nm and an emission maximum at 360 nm. The wavelengths reported represent observed values and have not been corrected for the optical imperfections of the instrument.

Analysis of Eluents from Column Separations. Fractions obtained from column chromatograms were analyzed with ninhydrin after alkaline hydrolysis (Hirs, 1967); 5′-*P*-Pxy-containing fractions were determined by their fluorescence, pooled appropriately, lyophilized, and stored at −20°. Isolated 5′-*P*-Pxy-peptides were analyzed both for amino acid composition and 5′*P*-Pxy content. As previously observed (Forrey, 1963), there was a 30–40% destruction of ε-(5′-*P*-Pxy)-lysine during acid hydrolysis with the formation of free lysine and other unidentified breakdown products. Quantitative determination of ε-(5′-*P*-Pxy)-lysine residues was also carried out spectrofluorometrically using the pure dipeptide, ε-(5′-*P*-Pxy)-lysylphenylalanine, isolated from reduced glycogen phosphorylase as a standard (Nolan *et al.*, 1964).

Amino-terminal groups were determined by the Dansyl-Cl method of Gray (1967). Dansyl-amino acids were separated by thin-layer chromatography on silica gel plates in chloroform–methanol–acetic acid (95:10:1) and identified by their fluorescence. In order to conserve material, carboxyl-terminal analysis was performed by carboxypeptidase A digestion and determination of the released amino acids as their Dansyl derivatives.

In some instances, it was advantageous to separate uncharged Dansyl-amino acids from the water-soluble ones, excess reagent, and side products by extraction with butyl acetate; the remaining water-soluble Dansyl-amino acids were easily separated by high-voltage electrophoresis in the pH 3.6 buffer system of Michl (1951).

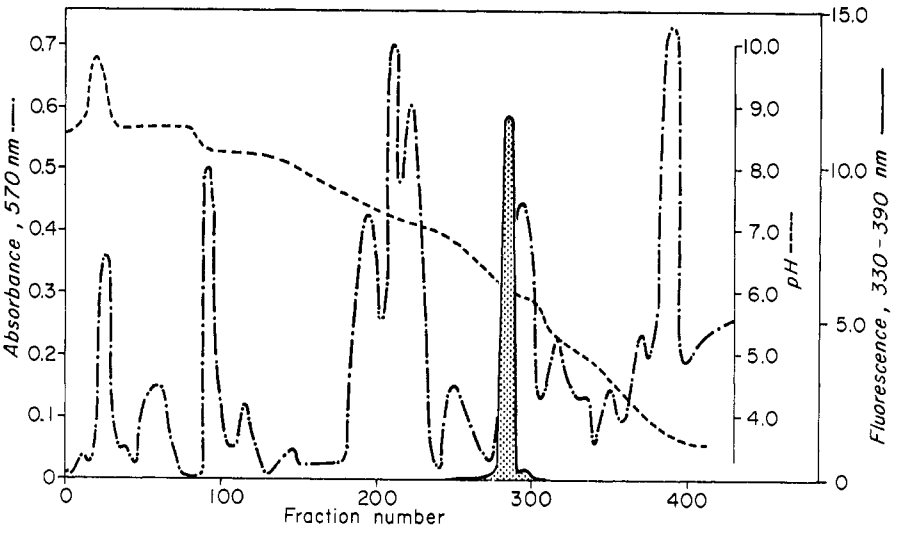


FIGURE 3: Elution pattern of a column chromatography of the 5′-*P*-Pxy-peptide on a 0.9 × 100 cm column of Dowex 1-X2. Elution was carried out with a gradient of pyridine–acetate buffer as described by Schroeder (1967). Fractions (1.5 ml) were collected and analyzed for ninhydrin-positive material and fluorescence.

The amino acid sequence of the 5′-*P*-Pxy-peptide was determined by the Dansyl-Cl–Edman procedure (Edman, 1956; Gray, 1967). At one point during the analysis, an aliquot sample was removed and analyzed for amino acid composition of the remaining peptide ("substractive" Edman procedure of Hirs *et al.*, 1960).

Results

Spectral Characteristics of E. coli Glutamate Decarboxylase. Absorption spectra of the crystalline enzyme at two concentrations of protein and at two pH values are presented in Figure 1. Confirming the results of Shukuya and Schwert (1960b), the enzyme species absorbing at 340 nm is converted into a species absorbing at 420 nm by lowering the pH from 7.0 to 4.5. Absorbances were reproducible from one sample to another; calculated on the basis of PLP content, the following molar absorbance indices were obtained: at pH 7.0, ϵ(340 nm) = 9850 with a 340:420 nm ratio of 14.9; at pH 4.5, ϵ(420 nm) = 11,600 with a 340:420 nm ratio of 0.265. For comparison purposes, the molar absorbance indices of PLP are 2500 at 330 nm, pH 7.0, and 6600 at 388 nm, pH 13 (Peterson and Sober, 1954).

$NaBH_4$ Reduction of Glutamate Decarboxylase. When glutamate decarboxylase (18 mg/ml, 900 mg total) was adjusted to pH 4.5 at 0° with dilute acetic acid, the protein solution became bright yellow and exhibited the 420-nm absorption maximum characteristic of PLP–Schiff bases. Portions (5–10 ml) of a 2×10^{-2} M $NaBH_4$ solution were then added with stirring over a period of 30 min until the protein solution became colorless, indicating that reduction of the azomethine bond between PLP and the enzyme was complete (Fischer *et al.*, 1958). Analysis for free PLP derivatives (Peterson and Sober, 1954) after precipitation of an aliquot sample of the protein with 0.3 M perchloric acid showed no PLP and only a trace amount of pyridoxine 5′-phosphate, indicating that reduction was at least 95% complete. The preparation was adjusted to pH 8.0 and exhaustively dialyzed against 0.01 M ammonium bicarbonate (pH 8.7).

Trypsin Digestion of Reduced Glutamate Decarboxylase. The resulting turbid solution was diluted with 0.01 M ammonium bicarbonate (pH 8.7) to a protein concentration of 10 mg/ml, and 30 mg (1:30, w/w) of 1-(1-tosylamido-2-phenyl)-ethyl chloromethyl ketone treated trypsin was added. After digestion at 38° for 10 hr, an additional 30 mg of trypsin was added in small portions. The digest became clear after 20 hr; after 25 hr, the solution was brought to pH 6.0 with acetic acid and lyophilized. The dry product was dissolved in 40% acetic acid and centrifuged to remove a small amount of insoluble material containing negligible quantities of 5′-*P*-Pxy derivatives.

Purification of the 5′-P-Pxy-Containing Peptide. The digest was divided into three portions and each was passed through a 2.5 × 95 cm column of Sephadex G-50 equilibrated with 1 N acetic acid. The results of a typical run are shown in Figure 2. Only one major peak with fluorescence characteristic of that of 5′-*P*-Pxy-peptides was seen; the small accompanying peaks (fractions 155–168 and 207–245) had fluorescence characteristic of tryptophan-containing peptides. The fractions containing the 5′-*P*-Pxy-peptide were pooled and lyophilized; they represented only 20% of the total ninhydrin-positive material.

The combined material was dissolved in water, adjusted to pH 10.5 with NaOH, and applied to a 0.9 × 100 cm column

TABLE I: Yields in Pyridoxyl Phosphate Peptides during Purification Procedure.

Procedure	% Yield for Each Individual Step	% Overall Yield
Initial digest	100	100
Sephadex G-50 chromatography	75	75
After lyophilization	45	34
Dowex 1 chromatography	79	27
After lyophilization	90	24
Dowex 50 chromatography	93	22
After lyophilization	56	13

TABLE II: Summary of Data for the Amino Acid Sequence of the PLP Binding Site of *E. coli* Glutamate Decarboxylase.

Pyridoxyl-P peptide	Ser,	Ile,	Ser,	Ala,	Ser,	Gly,	His,	(5′-*P*-Pxy)-Lys,	Phe
Composition	0.86[a]	0.97	0.86[a]	1.00	0.86[a]	1.10	0.80	1.04[b]	1.00
End-group analysis	Dansyl-Ser-								-Phe
Dansyl-Edman	Ser-	Ile-	Ser-	Ala	(Ser,	Gly,	His,	(5′-*P*-Pxy)-Lys,	Phe)
Composition of remaining peptide	0	0	0	0.2	1.25	1.34	0.97	0.75[c]	1.00
Dansyl-Edman					Ser-	Gly-	His-	(5′-*P*-Pxy)-Lys-	Phe
Sequence	Ser-	Ile-	Ser-	Ala-	Ser-	Gly-	His-	(5′-*P*-Pxy)-Lys-	Phe

( ), residue determined by the Dansyl-Edman procedure.
[ ], residue determined by carboxypeptidase A attack.

[a] The value for serine (2.58) was divided by 3. [b] The value listed for (5′-*P*-Pxy)lysine was the sum (see text) of that obtained for (Pxy)lysine (0.80) plus that obtained for free lysine (0.24). [c] Sum of (Pxy)lysine (0.61) and free lysine (0.4).

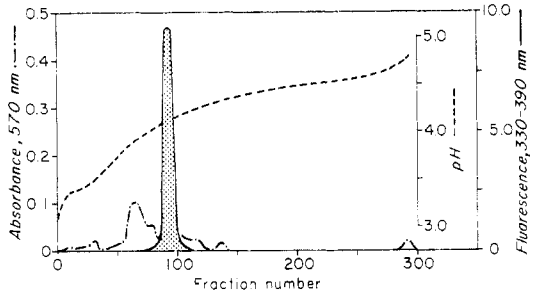


FIGURE 4: Purification of 5′-*P*-Pxy-peptide on a 0.6 × 60 cm column of Dowex 50-X2. Elution was carried out as described by Schroeder (1967). Fractions (1 ml) were collected and analyzed for ninhydrin-positive material and fluorescence.

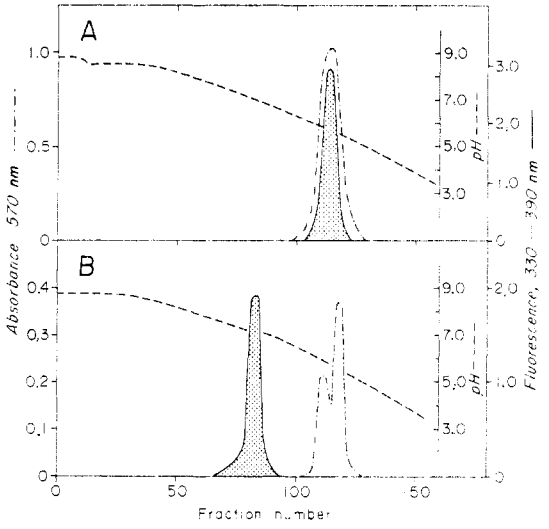


FIGURE 5: Simplified procedure for the purification of 5′-*P*-Pxy-peptides using alkaline phosphatase. The upper plot (A) shows the elution pattern of a partially purified fraction following column chromatography on a 0.9 × 60 cm column of Dowex 1-X2. Elution was carried out as described by Schroeder (1967). The fluorescent fractions were collected and subjected to alkaline phosphatase attack as described under Results. The lower plot (B) shows the elution pattern obtained following column chromatography under precisely the same conditions as in part A. Fractions (1 ml) were collected in both instances.

of Dowex 1-X2 (Figure 3). Again, there was evidence for only one 5′-*P*-Pxy-containing peptide; the fluorescent fractions were pooled and lyophilized. The residue was dissolved in water, adjusted to pH 2.0 with acetic acid, and applied to a 0.6 × 60 cm column of Dowex 50-X2 (Figure 4); once more the 5′-*P*-Pxy-containing fractions were pooled and lyophilized. Paper electrophoresis and chromatography of this material indicated that it was essentially free of contaminating peptides.

A summary of the purification is given in Table I. The composition of the purified 5′-*P*-Pxy-peptide is given in Table II. The small amount of lysine observed in the analysis was assumed to be due to partial breakdown of ε-(5′-*P*-Pxy)-lysine (Forrey, 1963), and was therefore added to the value obtained for ε-(5′-*P*-Pxy)-lysine.

Simplified Procedure for the Isolation of 5′-P-Pxy-peptides. A simplified method for the purification of 5′-*P*-Pxy-containing peptides, based on the "diagonal" procedure of Brown and Hartley (1963), was carried out as follows.[2]

A crude peptide mixture obtained from a trypsin–chymotrypsin digestion of $NaBH_4$-reduced glutamate decarboxylase was first passed through a 0.9 × 60 cm column of Dowex 1-X2 (Figure 5A). The 5′-*P*-Pxy-peptide emerged when the pH of the eluting buffer was 5.9. The fluorescent fractions, containing 0.38 μmole of 5′-*P*-Pxy-peptide as well as many contaminating peptides, were pooled and lyophilized; the residue was dissolved in 2 ml of 0.05 M $NaHCO_3$ (pH 8.2) and treated with 25 μg of crystalline *E. coli* alkaline phosphates for 2.5 hr at room temperature. This treatment caused the hydrolysis of the 5′-phosphate group of the bound cofactor, thereby altering the charge of the substituted peptide. The phosphatase digest was lyophilized and the residue was dissolved in water and chromatographed on the same column of Dowex 1-X2 exactly

[2] This approach was suggested by Dr. Kenneth A. Walsh and Mr. Ralph Kenner from this department, who used it for the paper chromatographic detection of PylP-peptides.

as described above. Figure 5B shows that the elution pattern has been altered because two negatively charged groups of the Pxy-peptide have been removed; the latter now emerges at pH 7.2 while the contaminating peptides emerge, as before, at about pH 5.9.

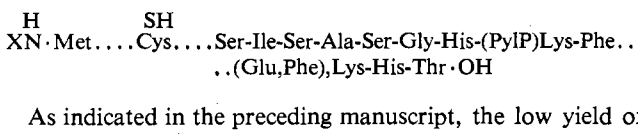
Analysis of the amino-terminal group of the peptide was carried out by the Dansyl-Cl procedure, as described under Methods, and showed only Dansyl-serine. The substituted amino acid was identified by both thin-layer chromatography and high-voltage electrophoresis. For analysis of the carboxyl-terminal group, carboxypeptidase A was added to 10 nmoles of 5′-*P*-Pxy-peptide in 0.01 M ammonium bicarbonate (pH 8.7) at a molar ratio of 1:380. After 3 hr at 37°, the reaction was terminated by the addition of acetic acid and the products were analyzed by the Dansyl-Cl procedure. Thin-layer chromatography showed two fluorescent spots corresponding to Dansyl-serine, identified above as the amino-terminal group, and Dansyl-phenylalanine, the newly released carbonyl-terminal group. No other Dansyl-amino acid was detected.

Amino Acid Sequence of the PLP Binding Site. Sequence analysis of the purified 5′-*P*-Pxy-peptide was carried out by the Dansyl-Edman procedure and is summarized in Table II. After the sequential removal of four amino acid residues from the amino terminus, an aliquot of the remaining material was analyzed for amino acid composition (Table II). This analysis confirmed the data thus far obtained by the direct Dansyl-Edman approach. After four additional cycles of the Dansyl-Edman procedure, only the carboxyl-terminal phenylalanine remained. There appears to be no ambiguity in the proposed sequence of the PLP binding site.

Discussion

It is apparent from Table I that only small losses of the 5′-*P*-Pxy-peptide occur during the various chromatographic steps, and that most of the destruction takes place during lyophilization; whether this destruction is due to the very acidic conditions prevailing during lyophilization or to the oxidation of the lyophilized peptide is not known. In spite of the poor overall yield (13%), it is apparent that PLP is bound to a unique site in *E. coli* glutamate decarboxylase. There is no indication of nonspecific binding of the cofactor to other regions of the protein which would give rise to additional substituted peptides.

From the information presented in this and the preceding publication, the structure of a subunit of *E. coli* glutamate decarboxylase can be schematized as follows



As indicated in the preceding manuscript, the low yield of amino-terminal methionine suggests that some of these residues may be substituted. There is one reactive sulfhydryl group in the native enzyme and nine which are masked.

Three structural features of the 5′-*P*-Pxy-peptide are noteworthy. First, hydrophilic and hydrophobic side chains alternate for six residues on the amino side of the lysyl residue. This may indicate that this portion of the peptide exists in a β conformation in which hydrophilic side chains face in one direction and hydrophobic side chains in the other.

Second, the (5′-*P*-Pxy)-lysyl residue is followed by a phenylalanyl residue. The same amino acid sequence was found at the PLP binding site of rabbit muscle glycogen phosphorylase (Fischer *et al.*, 1958; Nolan *et al.*, 1964). In both instances, the peptide bond following phenylalanine is apparently cleaved by trypsin. Since both 5′-*P*-Pxy-peptides arose from an extensive tryptic digestion, this cleavage could have resulted from trace contamination by chymotrypsin even though the trypsin preparation used had been treated with 1-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone, a powerful chymotrypsin inhibitor (Schoellmann and Shaw, 1963). Alternatively, it is possible that the (5′-*P*-Pxy)-lysylphenylalanyl–X bond is susceptible to a chymotrypsin-like activity inherent in trypsin.

The probability of finding a lysylphenylalanyl sequence is 1:20 in glutamate decarboxylase and 1:22 in rabbit muscle phosphorylase, considering the frequency of phenylalanyl residues. The probability that in both these enzymes, that a particular lysyl residue is involved in the binding of the cofactor is only 1:440 assuming that PLP could be attached to any lysyl residue in both molecules. Whether or not this is a pure coincidence will have to await the sequence analysis of PLP binding sites in other decarboxylases or PLP-containing enzymes in general.

Lastly, the (5′-*P*-Pxy)-lysyl residue is preceded by a histidyl residue, itself preceded by a glycyl residue. The glycyl residue may allow relatively free rotation of the histidyl group around the peptide backbone by minimizing steric hindrances. This raises the possibility that the imidazole side chain may help in catalysis. Space-filling models of the PLP binding site show that either the histidyl or the phenylalanyl side chain, but not both, can interact with PLP. The possible involvement of an imidazole group in facilitating PLP-catalyzed reactions was discussed by Bruice and Topping (1963), although no real evidence for this has been obtained. The low optimum pH (3.8) of glutamate decarboxylase probably excludes the possibility that the imidazole ring participates in a proton transfer reaction. However, it could bind one of the two carboxyl groups of the substrate, thereby maintaining the latter in a position favorable to catalysis, or it could provide an accessory binding site for the cofactor itself. At neutral pH, the enzyme shows a small absorption maximum at 340 nm which, in the case of muscle phosphorylase, has been attributed to the formation of a substituted aldimine derivative (Kent *et al.*, 1958). If the analogous structure exists in glutamate decarboxylase as proposed by Shukuya and Schwert (1960b), the imidazole side chain could provide the second nucleophilic group required in such a structure. On the other hand, no histidyl residue was found in the vicinity of the (5′-*P*-Pxy)-lysyl residue in phosphorylase (Fischer *et al.*, 1958; Nolan *et al.*, 1964; and unpublished data from this laboratory), aspartate aminotransferase (Morino and Watanabe, 1969), and tryptophanase (personal communication from Dr. E. E. Snell) which all show the spectral characteristics generally assigned to substituted aldimine derivatives. Whether or not the histidyl or the phenylalanyl residues contribute further to the spectral characteristics of glutamate decarboxylase is not known. The study of model compounds incorporating some of these structural features should help to answer this question.

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