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Reexamination of the Binding Site for Pyridoxal 5′-Phosphate in Ribulosebisphosphate Carboxylase/Oxygenase from *Rhodospirillum rubrum*†

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ABSTRACT: The high specificity of pyridoxal 5′-phosphate (PLP) for an essential lysyl residue of ribulosebisphosphate carboxylase/oxygenase was confirmed, but half-of-sites reactivity was not observed in contrast to an earlier report [Robison, P. D., Whitman, W. B., Waddill, F., Riggs, A. F., & Tabita, F. R. (1980) *Biochemistry 19*, 4848–4853]. Subsequent to reduction with [^{3}H]borohydride and tryptic digestion of the enzyme inactivated by PLP, the sole labeled peptide was purified by successive chromatography on DEAE-cellulose, SP-Sephadex, and Sephadex G-25. The peptide, recovered in good yield, appeared essentially homogeneous by amino acid analysis, peptide mapping, and sequencing. Automated Edman degradation established the peptide's sequence as Val-Leu-Gly-Arg-Pro-Glu-Val-Asp-Gly-Gly-Leu-Val-Val-Gly-Thr-Ile-Ile-(PLP)Lys-Pro-Lys instead of Ala-Leu-Gly-Arg-Pro-Glu-Val-Asp-(PLP)Lys-Gly-Thr-Leu-Val-Ile-Lys as reported by Robison et al. (1980) [Robison, P. D., Whitman, W. B., Waddill, F., Riggs, A. F., & Tabita, F. R. (1980) *Biochemistry 19*, 4848–4853]. The sequence -Ile-Lys-Pro-Lys- in the former is identical with that encompassing Lys-175 in the carboxylase/oxygenase from spinach, which reacts preferentially with PLP and two other affinity labels. This finding of homology greatly strengthens the supposition that Lys-175 in the spinach enzyme and the corresponding lysyl residue in the *Rhodospirillum rubrum* enzyme are active-site residues and furthermore increases the likelihood of their functionality in catalysis.

Three different affinity labels for D-ribulose 1,5-bisphosphate (ribulose-P_2)[1] carboxylase/oxygenase from spinach, 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate (Stringer & Hartman, 1978), *N*-(bromoacetyl)ethanolamine phosphate (Schloss et al., 1978), and PLP (Paech & Tolbert, 1978; Spellman et al., 1979), implicate the same lysyl residue as an active-site component. Based on the primary structure deduced from the sequence of the gene for the carboxylase, this lysyl residue occupies position 175 (McIntosh et al., 1980; Zurawski et al., 1981). Although catalytic functionality of the lysyl ϵ-amino group has not been proven, its unusual reactivity is consistent with an essential role (Norton et al., 1975). Comparative sequence studies are useful to discern residues involved in catalysis because of conservation of essential molecular features during evolution. Comparisons between the carboxylase from spinach and *Rhodospirillum rubrum* appear especially appropriate because these organisms are evolutionarily quite distant (McFadden & Tabita, 1974), and the two enzymes differ markedly in both primary and quaternary structure (Siegel et al., 1972; Tabita & McFadden, 1974). Total sequence homology is probably less than 20% (Akazawa et al., 1978; Stringer et al., 1981).

Of the three aforementioned affinity labels, only PLP has been thoroughly investigated in its reaction with ribulose-P_2 carboxylase from *R. rubrum* (Whitman & Tabita, 1978a,b). Since several criteria for affinity labeling were fulfilled, the reported lack of sequence homology of the phosphopyridoxal

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[1] Abbreviations: ribulose-P_2, D-ribulose 1,5-bisphosphate; PLP, pyridoxal 5′-phosphate; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Quadrol, *N*,*N*,*N*′,*N*′-tetrakis(2-hydroxypropyl)ethylenediamine; TPCK, tosylphenylalanyl chloromethyl ketone.

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peptide from the *R. rubrum* enzyme with that from the spinach enzyme was unexpected (Robison et al., 1980). This apparent lack of homology could be explained if the reactive lysyl residue in either species of enzyme were not at the active site or if the two enzymes were not evolved from the same ancestral gene. The former possibility requires unorthodox interpretations of extensive chemical data, and the latter possibility is contrary to present theories of phylogenetic relationships among photosynthetic organisms (Fox et al., 1980; Schwartz & Dayhoff, 1978).

During current total sequence work on cyanogen bromide fragments from *R. rubrum* carboxylase, we observed a sequence similar to that reported by Robison et al. (1980) for the phosphopyridoxal peptide. However, at the position supposedly occupied by the reactive lysyl residue, we observed glycine. Since this discrepancy bears on the issue of homology between the PLP sites of ribulose-P_2 carboxylase from spinach and *R. rubrum*, which in turn is pertinent to the possible essentiality of the target lysyl residues, we have reexamined the site of modification of the *R. rubrum* enzyme by PLP. In this report, we demonstrate that the sole phosphopyridoxal peptide, obtained in good yield and at a high degree of purity from tryptic digests of *R. rubrum* carboxylase after inactivation with PLP and reduction with borohydride, is homologous with the corresponding peptide from the spinach enzyme. The peptide differs from the one previously described (Robison et al., 1980) with respect to the number of residues, sequence, and position of the PLP-reactive lysyl residue.

Experimental Procedures

Materials

Bicine, Hepes, ATP, NADH, glutathione, glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate phosphokinase, glycerophosphate dehydrogenase/triosephosphate isomerase, and pyridoxal phosphate were products of Sigma Chemical Co. TPCK-treated trypsin was purchased from Millipore, and NaB^3H_4 (333 mCi/mmol) was from New England Nuclear. Iodoacetic acid, obtained from Aldrich, was recrystallized before use. Pyridine was distilled over ninhydrin.

Methods

Ribulose-P_2 was prepared enzymatically from D-ribose 5-phosphate according to published procedures (Horecker et al., 1958). Ribulose-P_2 carboxylase/oxygenase from *R. rubrum* was purified to homogeneity as described previously (Schloss et al., 1979); this enzyme is a dimer of 56 000-dalton subunits (Tabita & McFadden, 1974). Protein concentration was determined by using an $E^{1\%,1cm}_{280\ nm}$ of 12.0 (Stringer et al., 1981). Enzymatic activity was assayed by the spectrophotometric method of Racker (1963) as described earlier (Norton et al., 1975).

Modification with PLP for Determination of Stoichiometry and for Analytical Gels. Solutions (400 μL) of enzyme at 5 mg/mL (89 μM subunit) in 50 mM Hepes/1 mM EDTA/20 mM sodium bicarbonate/10 mM magnesium acetate (pH 7.8) were incubated with 0–100 μM PLP at 25 °C for 60 min. The concentration of PLP in stock solutions was determined from their absorbancies in 0.1 M NaOH at 388 nm by assuming an extinction coefficient of 6600 M^{-1} cm^{-1} (Peterson & Sober, 1954). After the addition of 50 μL of octyl alcohol to prevent excessive foaming, the enzyme solutions were reduced for 5 min on ice with 5 mM $NaBH_4$. The reduced samples were assayed for carboxylase activity and were then dialyzed extensively against 0.01 M ammonium bicarbonate (pH 8.1). Aliquots of the dialyzed solutions were then withdrawn for determination of enzymatic activity and protein concentration, and the remaining samples were lyophilized prior to polyacrylamide gel electrophoresis. Due to the sensitivity of PLP to light, all procedures were carried out in the dark.

Modification with PLP for Peptide Isolation. Enzyme (50 mg per reaction) was incubated as described above at 0.5 mg/mL (8.9 μM subunit), 1.5 mg/mL (26.7 μM subunit), and 5.0 mg/mL (89 μM subunit) with PLP at a molar ratio of one, two, or four with respect to the subunit. The reactions were terminated at 50–80% losses of activities. After the addition of 50 μL of octyl alcohol, the enzyme solutions were reduced for 5 min on ice with 1 mM NaB^3H_4 which had been freshly prepared by diluting labeled material about 50 to 1 with unlabeled $NaBH_4$. The modified enzyme was assayed and then extensively dialyzed in the dark at room temperature against 50 mM potassium phosphate/1 mM EDTA/1 mM 2-mercaptoethanol (pH 7.5), followed by dialysis against 0.01 M ammonium bicarbonate. Aliquots were removed for determination of protein concentration, enzyme activity, and tritium incorporation. Tritium was quantitated by using a Packard 3255 liquid scintillation spectrometer; samples were diluted with 0.5 mL of H_2O and counted in 10 mL of ACS scintillant (Amersham). Corrections were made for tritium incorporation into the native enzyme that had not been treated with PLP.

Disc Gel Electrophoresis. The lyophilized samples were redissolved in 0.1 M glycine/0.013 M Tris (pH 8.6) containing 5% (v/v) 2-mercaptoethanol, 8 M urea, and 50% (v/v) glycerol. Discontinuous gels were run according to the manufacturer's directions (Canalco) with modifications described by Gabriel (1971), except that 8 M urea was also included in the stacking and separating gels.

Carboxymethylation and Trypsin Digestion. Carboxymethylation was performed as described previously (Schloss et al., 1978). Subsequent to dialysis against 0.01 M ammonium bicarbonate, the carboxymethylated enzyme was digested with 1% (w/w) trypsin for 12 h at 40 °C, at which time an additional 1% trypsin was added. Twelve hours later, the samples were frozen and stored at −20 °C until needed.

Peptide Mapping. Peptide mapping was performed by the method of Katz et al. (1959) with modifications described earlier (Hartman & Norton, 1976). A 1-μL sample of 1% phenol red was added to the sample as a marker. Electrophoresis was carried out in an acetic acid/pyridine/water solvent (1:10:100 v/v) at pH 6.5 for 1 h at 1200 V. After the map was dried, it was subjected to descending chromatography by using the upper phase obtained from partitioning 1-butanol/acetic acid/water (4:1:5 v/v). Peptides were visualized by dipping the map in ninhydrin/collidine (Canfield & Anfinsen, 1963), followed by heating it at 80 °C.

Amino Acid Analyses. Peptides were hydrolyzed at 110 °C in 6 N HCl/0.01 M 2-mercaptoethanol in sealed, evacuated tubes for 24 h unless otherwise noted. Amino acid compositions were determined with a Beckman 121 M amino acid analyzer as described earlier (Stringer et al., 1981). N^6-(Phosphopyridoxal)lysine eluted between ammonia and arginine.

Sequence Analyses. The purified peptide (75 nmol) was subjected to automated Edman degradation with a Beckman 890C, the vacuum system of which was modified according to Bhown et al. (1980). A liquid nitrogen cold trap was inserted between the low vacuum pump and the vacuum manifold. Polybrene (2 mg) was added to the peptide to reduce its extraction from the reaction cup (Tarr et al., 1978). The peptide was sequenced twice, once in a Quadrol buffer system and once in a dimethylallylamine buffer system with Beck-

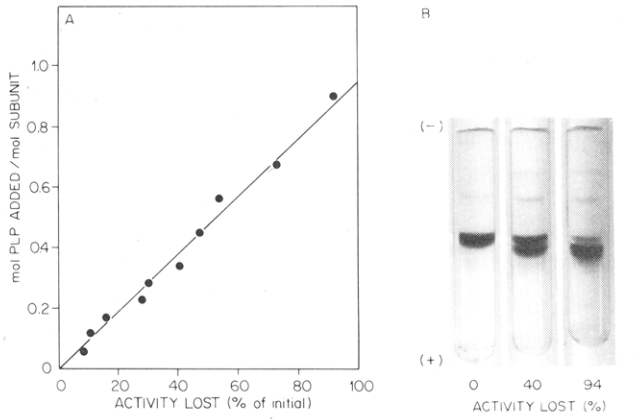

FIGURE 1: (A) Titration of ribulose-P_2 carboxylase/oxygenase with PLP. Conditions for modification are described under Experimental Procedures. (B) Polyacrylamide gel electrophoresis of PLP-modified enzyme in 8 M urea at pH 8.9. Twenty micrograms of protein was applied to each gel.

man's peptide program 102974. The sequence program used with the Quadrol buffer was that of Bhown et al. (1980) with several changes. The buffer concentration was reduced from 0.5 to 0.1 M, the drying step after delivery of phenyl isothiocyanate was increased from 20 to 60 s, and the coupling steps and drying steps after delivery of benzene/ethyl acetate were from Beckman's peptide program 030176. Of the fraction from each cycle, 10% was assayed for radioactivity. Half of the remainder was converted to the phenylthiohydantoin for identification by high-performance liquid chromatography (Laboratory Data Control) and the rest hydrolyzed in base for quantitation as free amino acids on the amino acid analyzer (Smithies et al., 1971). Threonine and arginine appear as α-aminobutyric acid and ornithine, respectively, in base hydrolysates. Aspartic and glutamic acids were distinguished from the corresponding amides by high-performance liquid chromatography of the phenylthiohydantoins.

Results and Discussion

Titration of the carboxylase with PLP shows that inactivation is directly proportional to the amount of PLP added and suggests that one molar equivalent per subunit is required for total inactivation (Figure 1A). Although earlier studies also showed that both subunits were reactive toward PLP, they indicated half-of-sites reactivity, i.e., modification of one subunit per dimer sufficing to inactivate the enzyme completely (Robison et al., 1980). For clarification of this disparity, samples at intermediate levels of inactivation, after borohydride reduction, were subjected to polyacrylamide gel electrophoresis under denaturing conditions with the expectation that the more negatively charged PLP-derivatized subunits would be resolved from unmodified subunits. As seen in Figure 1B, the relative amount of unmodified subunit (the slower moving component) agrees with the percentage of initial activity remaining, and thus half-of-sites reactivity does not apply. The seemingly low incorporation values in prior publications (Whitman & Tabita, 1978b; Robison et al., 1980) are explained in part by use of 9.74 for the $E^{1\%,1cm}_{280\ nm}$ (Tabita & McFadden, 1974) rather than 12.0 (Stringer et al., 1981). Additionally, the PLP stoichiometry based on tritium incorporation from [^{3}H]borohydride was less than that determined by spectrophotometric analyses, which complicates assessment of its validity.

Although it is not apparent with the sample loads applied to the gels shown in Figure 1, smaller samples partially resolved into two bands, which could signify nonidentity of the enzyme's

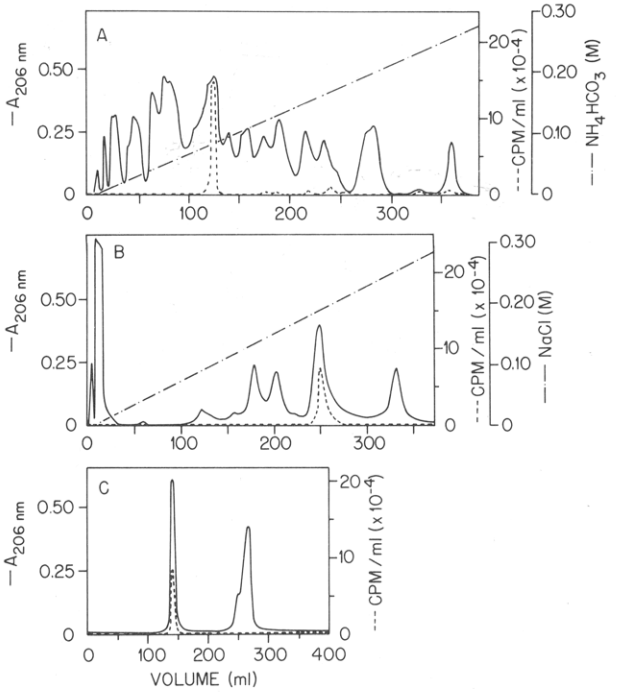

FIGURE 2: (A) Anion-exchange chromatography of a tryptic digest of PLP-modified ribulose-P_2 carboxylase/oxygenase. A 20-mg (5.6-mL) sample, consisting of 5 mg from four separate tryptic digests of enzyme inactivated at 0.5 and 5.0 mg/mL, each with one and four molar equivalents of PLP to protomer, was applied to a Whatman DE52 column (1 cm × 25 cm) in 0.01 M NH_4HCO_3/1 mM 2-mercaptoethanol (pH 8.1). A 400-mL linear gradient of 0.01–0.30 M NH_4HCO_3 in 1 mM 2-mercaptoethanol was applied at fraction 3, followed by a 0.50 M NH_4HCO_3/1 mM 2-mercaptoethanol wash at fraction 137. The flow rate was 15 mL/h, and the fraction size was 2.5 mL. Fractions 47–52 were pooled and lyophilized. (B) Cation-exchange chromatography of the tritiated peak from DE52. The lyophilized pool was redissolved in 2.6 mL of 11 mM H_3PO_4 (adjusted to pH 3.0 with concentrated NH_4OH) and applied to a SP-Sephadex column (1.0 cm × 25 cm) equilibrated with the same solvent. The peptide was eluted with a 400-mL linear gradient of 0–0.3 M NaCl in 11 mM H_3PO_4 (pH 3.0). The flow rate was 16 mL/h, and 2.7-mL fractions were collected. Fractions 92 through 100 were pooled, neutralized by addition of solid NH_4HCO_3, and lyophilized. (C) Gel filtration of the tritiated peak from SP-Sephadex. The lyophilized residue was dissolved in 3.0 mL of 0.01 M NH_4HCO_3 and applied to a Sephadex G-25 column (1.7 cm × 220 cm) in 0.01 M NH_4HCO_3. The flow rate was 26 mL/h, and the fractions were 4.3 mL. The only peak eluting before the salt region (beginning at 225 mL) was the tritiated peptide. Fractions 33–36 were pooled, lyophilized, and redissolved in a small volume of 0.01 M NH_4HCO_3.

two subunits. Heterogeneity has been suggested by isoelectric focusing (Raymond Chollet, unpublished experiments) but has not been revealed by partial sequence analyses (Stringer et al., 1981).

The labeled peptide in tryptic digests of carboxylase after inactivation with PLP, reduction with NaB^3H_4, and carboxymethylation of sulfhydryls with iodoacetic acid (see Experimental Procedures) was purified by successive chromatography on DEAE-cellulose, SP-Sephadex, and Sephadex G-25 (see legend to Figure 2 for column conditions). In our initial experiments, the concentrations of enzyme (1.5 mg/mL, 26 μM subunit), PLP (50 μM), EDTA (1 mM), sodium bicarbonate (20 mM), magnesium acetate (10 mM), and protons (pH 7.8) were identical with those reported (Robison et al., 1980). Only one phosphopyridoxal peptide was found, but its amino acid composition differed significantly from the one published previously (Table I, sample A).

A possible explanation for the different results from the two laboratories is that there are two different lysyl residues that are accessible to PLP but the site of attack is dependent upon

Table I: Amino Acid Composition of PLP-peptide

amino acid	molar ratio: Robison et al. (1980)	sample A[a]	mixture[b]	sample B[c]	sample C[d] 24-h hydrolysis	sample C[d] 48-h hydrolysis	no. of residues[e]
CM-Cys							
Asp[f]	1.00	1.00	1.00	1.00	1.00	1.00	1
Thr	0.62	1.03	0.87	0.91	0.96	0.85	1
Ser		trace	0.13	0.08	0.21	0.15	
Glu	0.91	1.15	1.13	1.11	1.15	1.09	1
Pro	0.82	1.82	1.78	1.83	1.95	1.81	2
Gly	1.72	3.81	3.56	3.73	3.85	3.80	4
Ala	1.10	0.07	0.16	0.09	0.21	0.09	
Val	1.41	3.64	3.25	3.68	3.40	3.71	4
Met				0.07			
Ile	0.76	1.85	1.35	1.39	1.51	1.56	2
Leu	1.07	1.53	1.73	1.81	1.87	1.81	2
Tyr			trace				
Phe			trace				
His		0.02	trace		trace	trace	
Lys	0.70	0.88	0.94	1.04	1.01	0.95	1
PLP-Lys[g]	0.26	0.65 (1.04)	0.69 (0.85)	0.70	0.79 (1.05)	0.20 (1.05)	1
Arg	0.61	0.86	0.88	0.92	0.84	0.90	1

[a] Purified from enzyme (1.5 mg/mL) incubated with 50 μM PLP. [b] Purified from a mixture of 5 mg each from tryptic digests of enzyme inactivated at 0.5 mg/mL with 8.9 and 35.6 μM PLP and at 5 mg/mL with 89 and 356 μM PLP. [c] Purified from enzyme (5 mg/mL) incubated with 356 μM PLP. [d] Purified from enzyme (0.5 mg/mL) incubated with 35.6 μM PLP. [e] Determined by sequencing. [f] Arbitrarily set as 1.00. [g] N^6-(Phosphopyridoxal)lysine quantitated by using the relative color value of Forrey et al. (1971). The number in parentheses is the PLP-lysine content determined by the absorbancy at 320 nm (Peterson & Sober, 1954).

experimental conditions. If the two sites differed in affinity for PLP, the molar ratio of PLP to enzyme could be a crucial variable. Alternatively, if the "dimeric" carboxylase existed as an equilibrium mixture of monomers and dimers [for which, however, there is no evidence over the concentration range from 0.25 to 5 mg/mL (Tabita & McFadden, 1974)] which interacted differently with PLP, enzyme concentration could be a crucial variable. Thus, we isolated the phosphopyridoxal peptide from enzyme derivatized at several concentrations with different levels of PLP.

The purification of labeled peptide from a mixture of four different preparations of PLP-inactivated enzyme (protein at 0.5 and 5 mg/mL modified with both 1:1 and 4:1 molar equivalents of PLP) is shown in Figure 2. We find no evidence of a second site of modification by PLP, given reactant concentrations as variable parameters. The overall recovery from 20 mg (357 nmol of subunit) of mixed tryptic digests was 51% based on radioactivity and 63% based on nanomoles of peptide actually obtained.

Identical chromatographic procedures were used to purify the phosphopyridoxal peptide from several individual preparations of modified carboxylase. With every preparation examined, the sole labeled peptide emerged at the same position from each of the three columns employed, and the purified peptides' amino acid compositions were indistinguishable (Table I). Based on the absence of several amino acids and the approximate whole-number molar ratios of those present, the peptides appeared essentially homogeneous. This conclusion was verified by peptide mapping (Figure 3) and by sequence analyses (Table II). We attribute the low values for isoleucine to the stability of Ile–Ile peptide bonds. That the peptide visualized on the map with ninhydrin was indeed the phosphopyridoxal peptide was confirmed by cutting the spot out and counting it; the origin was devoid of radioactivity.

The total incorporation of PLP, extrapolated to 100% inactivation, into the enzyme calculated from the specific activities of the purified peptides ranged from 1.1 to 1.5 mol of PLP per mol of subunit. The higher levels of incorporation were observed when the enzyme at 5 mg/mL was treated with a 4-fold molar excess of PLP. Since even in this instance only one labeled peptide in significant quantities was observed in tryptic digests, the excess incorporation must represent the reaction of multiple lysyl residues in very low yields. The greater than stoichiometric incorporation of PLP explains the lower yield of peptide based on radioactivity as compared to actual nanomoles (see above).

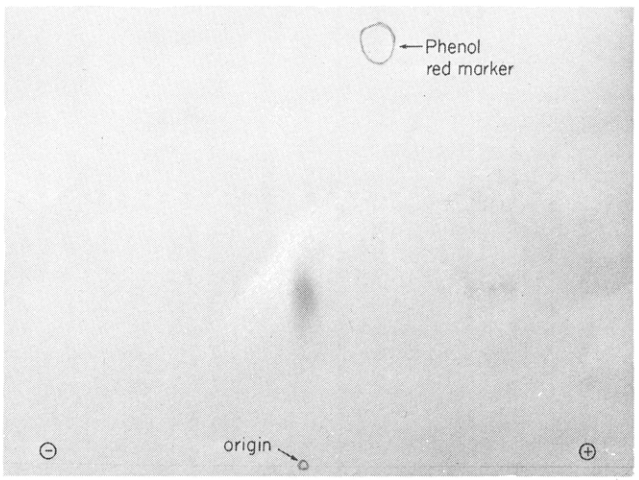

FIGURE 3: Peptide map of PLP-peptide (60 nmol). The first dimension was electrophoresis at pH 6.5; the second dimension was decending chromatography. Details are provided under Experimental Procedures.

The phosphopyridoxal peptide (Table I, sample A) was sequenced by automated Edman degradation. Results of one run using a Quadrol buffer system are provided in Table II; a second run, in which a dimethylallylamine buffer was used, revealed the same sequence. The high degree of purity of the peptide is readily apparent at cycle 1 in which the only contaminants, glycine and leucine, are present at levels less than 5% of that of NH_2-terminal valine. Amino acids that are not listed in the table were not detected in any of the 20 cycles.

The sequence of the phosphopyridoxal peptide from *R. rubrum* carboxylase as deduced from data shown in Table II and as published earlier and the sequence of the corresponding peptide from spinach carboxylase are as follows (italics denote the labeled residue):

Table II: Sequence Analysis of PLP-peptide (75 nmol)[a]

amino acid	cycle no. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Asp								*9.0*	4.2	1.2	0.6									
Thr								1.8							*ND*[c]					
Glu						*17.0*	6.6													
Pro					*35.7*														*4.7*	2.8
Gly	2.1	1.1	*38.8*	8.5	1.4	0.8	0.8	0.9	*23.0*	*25.2*		3.7	2.2	*10.4*						
Val	*58.2*						*38.5*	8.9				*12.2*	*20.8*	6.9	2.0					
Ile												0.7				*3.5*	*5.3*	3.0	1.2	
Leu	1.4	*39.4*	2.5								*14.8*	7.2	2.3	0.6	0.4		1.0	0.5	0.4	*2.4*
Lys																		0.6		
PLP-Lys																		*ND*[d]		
Arg[b]				*10.3*	2.7															
cpm/cycle	280	150	130	140	250	190	230	300	430	420	480	720	600	610	960	630	510	6170	3910	2200

[a] Given in nmol/cycle. [b] Determined as ornithine in base hydrolysate. [c] Detected as α-aminobutyric acid. [d] Assigned by radioactivity and lack of extracted amino acid.

R. rubrum peptide (Robison et al., 1980)

Ala-Leu-Gly-Arg-Pro-Glu-Val-Asp-*Lys*-Gly-Thr-Leu-Val-Ile-Lys

R. rubrum peptide (present study)

Val-Leu-Gly-Arg-Pro-Glu-Val-Asp-Gly-Gly-Leu-Val-Val-Gly-Thr-Ile-Ile-*Lys*-Pro-Lys

spinach peptide (Stringer & Hartman, 1978; Schloss et al., 1978; Spellman et al., 1979)

Tyr-Gly-Arg-Pro-Leu-Leu-Gly-Cys-Thr-Ile-*Lys*-Pro-Lys

We cannot reconcile the differences between our peptide compared with the one from Tabita's group. Given the variety of conditions used for the inactivation and given the number of times the peptide isolation was repeated, we feel confident of the correctness of the data reported herein. One notes that beyond the NH_2-terminal residue, which was reported as alanine rather than valine, the next seven amino acids are the same in both peptides. Perhaps the material sequenced previously was impure, and the last half of the sequence published represents that of an impurity remaining after preferential washout of the phosphopyridoxal peptide from the spinning cup. In this connection, we were unable to obtain a pure peptide by use of the two columns described by Robison et al. (1980).

In comparing our sequence of the phosphopyridoxal peptide from the *R. rubrum* enzyme with the tryptic peptide from spinach carboxylase that contains lysine-175 [which is reactive toward PLP, 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate, and *N*-(bromoacetyl)ethanolamine phosphate], we see a sequence in common, -Ile-*Lys*-Pro-Lys-, encompassing the essential lysyl residue. We believe that the finding of this striking homology between functionally analogous, but structurally dissimilar, enzymes from such diverse species strengthens our earlier conclusions that lysine-175 in the spinach enzyme, and now by inference the PLP-reactive lysine of the *R. rubrum* enzyme, is located within the active site. Furthermore, the species invariance in primary structure adjacent to this unusually reactive lysyl residue is suggestive of a catalytic functionality.

Pierce et al. (1980) have pointed out that a divalent metal ion (Mg^{2+}) or a protonated lysyl side chain could function in the carbanion inversion that is necessary for development of the proper stereochemistry in the product (i.e., D- rather than L-3-phosphoglycerate) derived from carbon dioxide and C1 and C2 of ribulose-P_2. An ionized amino group could also act as a general base promoting the hydrolytic scission between C2 and C3 of ribulose-P_2. A third possible role for the lysyl ε-amino group is that of the essential base which initiates the catalytic reaction by abstraction of the C3 hydrogen atom of ribulose-P_2 as a proton [for a discussion of the reaction pathway, see Lorimer (1981)]. Based on the primary isotope effect observed with [3-^{3}H]ribulose-P_2, this step is likely to be at least partially rate limiting (Fiedler et al., 1967). If an amino group were to participate in this way, the very low k_{cat} of the carboxylase, 2.5 s^{-1} for the spinach enzyme (Siegel et al., 1972; Hall et al., 1981) and 8 s^{-1} for the *R. rubrum* enzyme (Schloss et al., 1979), might be explained. As discussed by Rose (1970, 1975) concerning the identity of the essential base in triosephosphate isomerase, the k_{cat} for turnover cannot exceed the ionization rate of the protonated base. Irrespective of the nature of the base, this ionization rate equals the diffusion-controlled rate of protonation of the free base (10^{10} M^{-1} s^{-1}) (Eigen, 1964) multiplied by the ionization constant of the protonated species. Thus, an ε-amino group with a typical pK_a of 9.5, functioning as the essential base, cannot support a k_{cat}

exceeding $\sim 3\ s^{-1}$. Of course, if the unusual reactivity of Lys-175 reflects an abnormally low pK_a, the maximally allowed value for k_{cat} will be increased.

Cys-172 in the spinach carboxylase, which is preferentially alkylated by *N*-(bromoacetyl)ethanolamine phosphate when the enzyme assumes its deactivated conformation (Schloss et al., 1978), can now be excluded as having any essential catalytic role other than in hydrogen bonding. This conclusion follows the observation that in the *R. rubrum* enzyme the cysteinyl residue is replaced by threonine.

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