(1974) *Nature (London) 252,* 326.
Sundaralingam, M. (1975) in *Structure and Conformation of Nucleic Acids and Protein-Nucleic Acid Interactions* (Sundaralingam, M., & Rao, S. T., Eds.) p 487, University Park Press, Baltimore, Md.
Topal, M. D., & Warshaw, M. M. (1976) *Biopolymers 15,* 1755.
Warshaw, M. M., & Cantor, C. R. (1970) *Biopolymers 9,* 1079.
Weinstein, I. B., Jeffrey, A. M., Jennette, K. W., Blobstein, S. H., Harvey, R. G., Harris, C., Autrup, H., Kasai, H., & Nakanishi, K., (1976) *Science 193,* 592.
Yagi, H., Thakker, D. R., Hernandez, D., Koreeda, M., & Jerina, D. M. (1977) *J. Am. Chem. Soc. 99,* 1604.
Yang, S. K., Gelboin, H. V., Trump, B. F., Autrup, H., & Harris, C. C. (1977) *Cancer Res. 37,* 1210.

Modification of *Rhodospirillum rubrum* Ribulose Bisphosphate Carboxylase with Pyridoxal Phosphate. 1. Identification of a Lysyl Residue at the Active Site†

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ABSTRACT: Ribulose 1,5-bisphosphate carboxylase isolated from *Rhodospirillum rubrum* was strongly inhibited by low concentrations of pyridoxal 5′-phosphate. Activity was protected by the substrate ribulose bisphosphate and to a lesser extent by other phosphorylated compounds. Pyridoxal phosphate inhibition was enhanced in the presence of magnesium and bicarbonate, but not in the presence of either compound alone. Concomitant with inhibition of enzyme activity, pyridoxal phosphate forms a Schiff base with the enzyme which is reversible upon dialysis and reducible with sodium borohydride. Subsequent to reduction of the Schiff base with tritiated sodium borohydride, tritiated N^6-pyridoxyllysine could be identified in the acid hydrolysate of the enzyme. Only small amounts of this compound were present when the reduction was performed in the presence of carboxyribitol bisphosphate, an analogue of the intermediate formed during the carboxylation reaction. Therefore, it is concluded that pyridoxal phosphate modifies a lysyl residue close to or at the active site of ribulose bisphosphate carboxylase.

Ribulose bisphosphate carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] is the primary carbon dioxide fixing enzyme in most photosynthetic and chemosynthetic organisms (McFadden & Tabita, 1974). Considering the importance of this enzyme in autotrophic metabolism, little is known about the structure of the active site. In part, this is due to the lack of reagents specific for the active site of this enzyme. While sulfhydryl directed reagents have long been known to inhibit enzymatic activity (Rabin & Trown, 1964), they also modify nonessential sulfhydryl groups and cause the dissociation of the quaternary structure (Sugiyama et al., 1967, 1968; Nishimura et al., 1973). More recently, the affinity label, 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate, has been used to modify essential lysyl residues at the active site of the spinach and *Rhodospirillum rubrum* ribulose bisphosphate carboxylase (Norton et al., 1975; Schloss & Hartman, 1977). However, this reagent also modifies sulfhydryl groups and spontaneously decomposes under the conditions used for modification (Hartman et al., 1973). Therefore, it seemed worthwhile to investigate other reagents known to react with amino acids in proteins for their potential to specifically modify the active site of ribulose bisphosphate carboxylase.

Pyridoxal 5′-phosphate has been shown to selectively modify primary amino groups in or near phosphate binding sites of many proteins (Columbo & Marcus, 1974). Low concentrations of pyridoxal phosphate are inhibitory for the ribulose bisphosphate carboxylase from *Rhodospirillum rubrum, Chlamydomonas reinhardtii, Alcaligenes eutrophus* (*Hydrogenomonas eutropha*), and spinach leaf (Whitman & Tabita, 1976). The enzyme from *R. rubrum* is protected from pyridoxal phosphate inhibition by the substrate, ribulose bisphosphate (Whitman & Tabita, 1976). Pyridoxal phosphate is a competitive inhibitor with respect to carbon dioxide and noncompetitive with respect to ribulose bisphosphate for the spinach enzyme (Paech et al., 1977). The spinach enzyme also binds 2 mol of pyridoxal phosphate/mol of catalytic subunit, presumably 1 mol at the catalytic site and 1 mol at a regulatory site. Pyridoxal phosphate was also reported to inhibit the oxygenase activity of the spinach enzyme as well as the carboxylase activity (Paech et al., 1977).

The present study will further elucidate the inhibition by pyridoxyl phosphate of the *R. rubrum* ribulose bisphosphate carboxylase. Unlike other carboxylases hitherto purified, the enzyme from *R. rubrum* is a dimer of large, catalytic type subunits (Tabita & McFadden, 1974a), as compared with the spinach enzyme which is a hexadecamer of eight large and eight small subunits (Rutner, 1970). This relative structural simplicity may be used to advantage in determining the site of pyridoxal phosphate modification and the manner in which it interacts with the enzyme. In addition, comparisons of structure–function relationships between the ribulose bisphosphate carboxylase from photosynthetic microorganisms such as *R. rubrum* and the modern higher plant enzyme may provide useful insights into the evolutionarily conserved features of the carboxylase molecule.

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Experimental Procedures

Materials. Ribulose 1,5-bisphosphate was prepared as previously described (Whitman & Tabita, 1976). 2-Carboxy-D-ribitol 1,5-bisphosphate was prepared as described by Wishnick et al. (1970). Tritiated poly(N^6-phosphopyridoxyllysine) was prepared as described by Schnackerz & Noltmann (1971) for poly(N^6-pyridoxyllysine), except that pyridoxal phosphate was substituted for pyridoxal and tritiated sodium borohydride (18.6 Ci/mol) was substituted for unlabeled sodium borohydride. Tritiated N^6-pyridoxyllysine was prepared from tritiated poly(N^6-phosphopyridoxyllysine) by acid hydrolysis in 6 N HCl at 108 °C in vacuo, for 18 h. The identity of N^6-pyridoxyllysine was confirmed by paper chromatography in isopropyl alcohol, pyridine, acetic acid, and water (30:20:6:24) (Forrey et al., 1971). Tritiated sodium borohydride (186 Ci/mol) was obtained from New England Nuclear, Boston, Mass. Other biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo.

Ribulose bisphosphate carboxylase from *Rhodospirillum rubrum* was purified as described by Tabita & McFadden (1974a) except for the following modifications: DEAE[1]-cellulose chromatography was followed by chromatography on Sephadex G-200 (Pharmacia, Inc.) and in some cases DEAE-Sephadex A-25 (Pharmacia, Inc.).The resulting enzyme preparation was electrophoretically homogeneous in the triethanolamine-*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TEA-TES) system of Orr et al. (1972) and the sodium dodecyl sulfate system of Laemmli (1970). Protein concentrations of the purified enzyme were estimated from the extinction coefficient at 280 nm of 0.974 L g^{-1} cm^{-1}. The molecular weight of the dimer was assumed to be 114 000 (Tabita & McFadden, 1974b).

Assays. Homogeneous enzyme was filter sterilized and stored in 50 mM potassium phosphate buffer, pH 7.5, and 1 mM EDTA at 4 °C. Prior to an experiment, aged enzyme was activated by a 3-h incubation in 10 mM dithiothreitol and the storage buffer at 30 °C, followed by dialysis against 10 mM potassium Mops, pH 7.5, and 1 mM EDTA for 3 h. When fresh enzyme was used, the activation step with dithiothreitol was omitted. In all cases, the unmodified enzyme had a specific activity greater than 1.0 μM of carbon dioxide fixed per min per mg of protein. Except where noted, incubations of enzyme with inhibitors or other compounds were for 60 min at 30 °C in 10 mM potassium Mops, pH 7.5, and 1 mM EDTA. Incubations with pyridoxal phosphate or related compounds were always performed in the dark or subdued light. At the conclusion of the incubation, enzyme was added to the complete assay mixture containing 0.5 mM ribulose bisphosphate, 20 mM $NaH^{14}CO_3$, 20 mM magnesium acetate, 1 mM EDTA, and 0.15 M potassium-Mops, pH 7.8. After 10 min, the reaction was terminated and acid-stable radioactivity determined as described previously (Whitman & Tabita, 1976).

Incorporation of Pyridoxal Phosphate. Ribulose bisphosphate carboxylase, 1.4 mg/mL, was incubated with 80 μM pyridoxal phosphate, 6.7 mM magnesium acetate, 13 mM sodium bicarbonate, 10 mM potassium-Mops, pH 7.5, and 1 mM EDTA at 30 °C in a total volume of 0.6 mL. After 60 min, the enzyme was divided into 120-μL portions. Five microliters of octanol was added to each portion, followed promptly by 20 μL of either ice-cold water or 70 mM sodium borohydride in water and 50 μL of 10 mM potassium phosphate, pH 7.5, and 1 mM EDTA. The test tubes containing the enzyme solutions were incubated for 40 min in an ice-water bath prior to exhaustive dialysis against 10 mM potassium phosphate, pH 7.5, and 1 mM EDTA at room temperature. After dialysis, the samples were diluted to 0.25 mL with buffer and the optical density at 280 nm was determined with a Beckman 25 dual beam spectrophotometer and matched, raised-bottom quartz microcuvettes (Precision Instruments, Hicksville, N.Y.). The protein concentration was determined from the extinction coefficient at 280 nm of the unmodified enzyme. The contribution of the phosphopyridoxylamino acid to the absorption at 280 nm was neglected because it was shown to be insignificant with the extent of incorporation achieved.

Identification of the Modified Residue. For tritium incorporation into the phosphopyridoxylated amino acid, a 12 μM concentration of enzyme was incubated with 30 μM pyridoxal phosphate in 0.25 mL of 40 mM potassium-Mops, pH 7.5, 0.8 mM EDTA, 16 mM sodium bicarbonate, and 8 mM magnesium acetate for 90 min at 30 °C. For controls, the enzyme was incubated in the absence of pyridoxal phosphate and in the presence of pyridoxal phosphate and 40 μM carboxyribitol bisphosphate. Tritiated sodium borohydride, 5 μL of a 50 mM solution in 0.01 M NaOH (18.6 Ci/mol), was added to each sample. The samples were incubated a further 30 min in an ice bath and dialyzed against deionized water until tritrium could not be detected in the dialysate. After dialysis, the samples were lyophilized, resuspended in 6 N HCl, and hydrolyzed at 108 °C, in vacuo, for 18 h in the dark. The hydrolysate was diluted with deionized water and lyophilized to dryness. This step was repeated two times and the hydrolysate was resuspended in a minimum volume of deionized water and applied onto cellulose thin-layer chromatography sheets. Chromatography was performed for 3 h with a solvent system of 1-butanol, pyridine, acetic acid, and water (30:30:6:24). After drying, the chromatographic sheet was cut into 2.5 × 0.5 cm strips. Tritium on the strips was counted in 10 mL of dioxane liquid scintillation fluid containing 5 g/L 2,5-diphenyloxazole, 100 g/L naphthalene, and 90 mL/L of 1 M sodium phosphate, pH 7.5, with a Beckman LS 100C liquid scintillation counter. Counting efficiency was determined by the external standard ratio method.

Results

The rate of inhibition of *R. rubrum* ribulose bisphosphate carboxylase activity by pyridoxal phosphate depends on the pyridoxal phosphate concentration (data not shown). Inhibition is complete after 20 min at concentrations of pyridoxal phosphate greater than 100 μM. In the presence of 3.3 μM pyridoxal phosphate, loss of activity continues for at least 60 min. Of further interest, at 1 mM pyridoxal phosphate, or 50 times the concentration of pyridoxal phosphate required for 50% inhibition, the maximal inhibition obtained is less than 100%.

The ribulose bisphosphate carboxylase from *R. rubrum*, like the enzymes from higher plants, is sensitive to the order of addition of substrates to the assay (Tabita & McFadden, 1974a). For the higher plant enzymes, this effect has been shown to be due to a required activation of the enzyme by carbon dioxide and a metal, possibly at a site other than the active site (Laing et al., 1975; Lorimer et al., 1976). To distinguish between two possible modes of pyridoxal phosphate inhibition, either on the catalytic or activation process, enzyme activity was measured in the presence and absence of pyridoxal phosphate and the substrates, ribulose bisphosphate and carbon dioxide, and the required metal activator, magnesium (Table I). When bicarbonate and magnesium were included in the preincubation mixture, inhibition was enhanced from 25%

[1] Abbreviations used: DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; Mops, 4-morpholinepropanesulfonic acid.

TABLE I: Effect of Assay Constituents on Pyridoxal Phosphate Inhibition.[a]

Assay constituents	% activity remaining[b] After 5 min	After 30 min
No additions	47	25
Ribulose bisphosphate	82	84
Ribulose bisphosphate + magnesium	81	60
Bicarbonate	67	56
Bicarbonate + magnesium	25	7.5
Magnesium	68	34

[a] Conditions: Enzyme was incubated for the indicated times in the presence and absence of 50 μM pyridoxal phosphate and the following combinations of the assay constituents: ribulose bisphosphate, 0.8 mM; bicarbonate, 20 mM; magnesium chloride, 20 mM. Assays were for 5 min. All other conditions were described in the Experimental Procedures. [b] Percent activity remaining is defined as the ratio of carboxylase activity in the presence and absence of pyridoxal phosphate in the presence of the assay constituent × 100.

TABLE II: Protection against Inhibition by Pyridoxal Phosphate.[a]

Protectant	% activity remaining[b]
None	32
Ribulose 1,5-bisphosphate	81
2-Phosphoglycolate	52
NADPH	53
Fructose 1,6-bisphosphate	49
6-Phosphogluconate	34

[a] Ribulose bisphosphate carboxylase was incubated for 60 min in the presence and absence of 50 μM pyridoxal phosphate, 1.6 mM of the protectant tested (except ribulose bisphosphate which was used at 0.8 mM), and 100 mM potassium Mops, pH 7.5, and 1 mM EDTA. Activity was measured as in the Experimental Procedures except that the protectants were included in the assay at the concentration in the incubation. [b] Percent activity is determined from the activity found in the absence of pyridoxal phosphate but in the presence of the protectant.

activity remaining with no additions to 7.5% activity remaining after 30 min (Table I). This result is inconsistent with the action of pyridoxal phosphate at a bicarbonate–magnesium activation site. A comparison of the extent of pyridoxal phosphate inhibition in the presence and absence of bicarbonate and magnesium is shown in Figure 1. In the presence of bicarbonate and magnesium, 50% inhibition is obtained at about 2.5 μM pyridoxal phosphate, while, in their absence, 50% inhibition is obtained at about 15 μM pyridoxal phosphate. This may reflect a sixfold tighter binding of pyridoxal phosphate in the presence of bicarbonate and magnesium. The maximum inhibition obtained in the presence of bicarbonate and magnesium is at about 10% activity remaining (90% inhibition) (Figure 1). Furthermore, ribulose bisphosphate protects against pyridoxal phosphate inhibition (Table I), indicating that pyridoxal phosphate binds close to or at the substrate binding site. The protection seen in the presence of bicarbonate alone may reflect protection by either the substrate, carbon dioxide, or the anion, bicarbonate, since anions have previously been shown to antagonize the inhibitory effect of pyridoxal phosphate (Whitman & Tabita, 1976). The protection evident in the presence of magnesium is due to the Cl^- present. When magnesium acetate was substituted for magnesium chloride no protection is seen.

The activity of ribulose bisphosphate carboxylase from higher plants and some bacteria has been shown to be sensitive

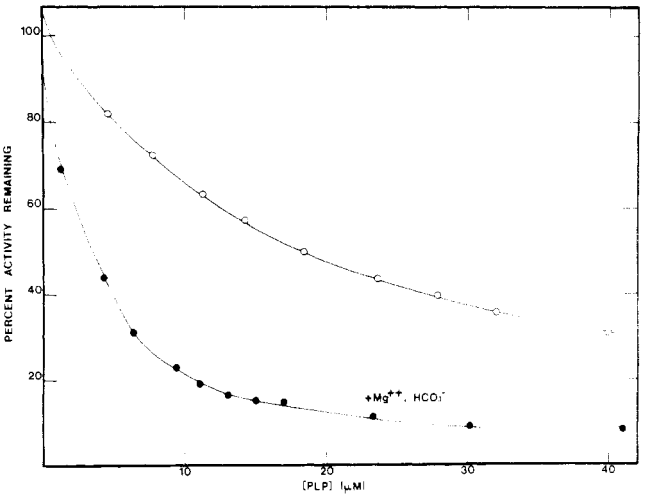

FIGURE 1: Inhibition by pyridoxal phosphate in the presence and absence of bicarbonate and magnesium. The enzyme was incubated for 3.5 h in 50 mM potassium Mops, pH 7.5, 1 mM EDTA, pyridoxal phosphate, and the presence or absence of 20 mM sodium bicarbonate and 10 mM magnesium acetate. Assays were for 5 min but otherwise as described in the Experimental Procedure.

to the concentration of a variety of metabolites including NADPH, fructose 1,6-bisphosphate, and 6-phosphogluconate (Tabita & McFadden, 1972; Bowes & Ogren, 1972; Chu & Bassham, 1972, 1974; Buchanan & Schürmann, 1973). It was therefore of interest to investigate the effect of these compounds on pyridoxal phosphate inhibition of the *R. rubrum* ribulose bisphosphate carboxylase (Table II). While NADPH and fructose bisphosphate afforded strong protection at 1.6 mM, ribulose bisphosphate gave the greatest protection of all the compounds surveyed. The protection by NADPH was specific because related compounds, NADH and $NADP^+$, gave only weak protection (39–37% activity remaining) or no significant protection (34–32% activity remaining) as seen with NAD^+. Similarly, compounds structurally related to fructose bisphosphate as fructose 6-phosphate and glucose 6-phosphate gave no significant protection. The lack of significant protection by 6-phosphogluconate is consistent with the lack of effect of this compound on *R. rubrum* ribulose bisphosphate carboxylase activity under these conditions (Tabita & McFadden, 1972). Phosphoglycolate, a product of the oxygenase reaction catalyzed by this enzyme, was also a strong protectant, while 3-phosphoglycerate, the product common to both the carboxylase and oxygenase reactions, was only a weak protectant. In general, compounds which protected against pyridoxal phosphate inhibition were themselves inhibitors of carboxylase activity in the absence of pyridoxal phosphate. Thus, under the conditions employed, phosphoglycolate inhibited enzyme activity 40%; NADPH, 30%; and fructose bisphosphate, 25%.

Pyridoxal phosphate has been shown to form a Schiff base with primary amino groups near phosphorylated substrate or effector binding sites on proteins. Consistent with this idea, pyridoxal is a poor inhibitor of the *R. rubrum* ribulose bisphosphate carboxylase. The interpretation of this result is complicated by the fact that in aqueous solution most of the pyridoxal forms an unreactive hemiacetal (Heyl et al., 1951). The concentration of the free aldehyde in the buffer used (10 mM potassium–Mops, pH 7.5, and 1 mM EDTA) was estimated from the optical density at 392 nm and the extinction coefficient (1990 M^{-1} cm^{-1}; Harris et al., 1976). By this method, the aldehyde accounted for 5–10% of the total pyridoxal. This may be compared with an estimate for the aldehyde as 2.5% of the total pyridoxal at neutral pH (Metzler & Snell,

TABLE III: Reduction of the Ribulose Bisphosphate Carboxylase–Pyridoxal Phosphate Complex with Sodium Borohydride.[a]

Treatment	% activity remaining
Prior to reduction and dialysis:	
− pyridoxal phosphate	100
+ pyridoxal phosphate	7.9
Subsequent to dialysis:[b]	
− pyridoxal phosphate, −$NaBH_4$	100
− pyridoxal phosphate, +$NaBH_4$	79
+ pyridoxal phosphate, −$NaBH_4$	117
+ pyridoxal phosphate, +$NaBH_4$	2.3

[a] The conditions were as described in the Experimental Procedures. Prior to reduction and dialysis, 100% activity corresponds to a specific activity of 2.2 μmol of CO_2 fixed per min per mg of enzyme. Subsequent to dialysis, 100% activity remaining corresponds to a specific activity of 3.5. [b] Assays were performed in the presence of 10 mM dithiothreitol.

1955). Even by the lower estimate of the concentration of aldehyde, the aldehyde form of pyridoxal is a poorer inhibitor than is pyridoxal phosphate. In the presence of 10 mM pyridoxal, or 0.5 mM of the aldehyde form (if 5% of the total pyridoxal is in the aldehyde form), the enzyme was inhibited by 41%. From Figure 1, it is evident that the concentration of pyridoxal phosphate in the absence of bicarbonate and magnesium required for 41% inhibition is about 10 μM. This is 50-fold lower than the concentration of the aldehyde form of pyridoxal necessary for the same extent of inhibition. This result reflects the importance of the phosphate moiety in the action of pyridoxal against the *R. rubrum* ribulose bisphosphate carboxylase.

Several lines of evidence indicate that a Schiff base forms during pyridoxal phosphate inactivation. Pyridoxamine, pyridoxamine phosphate, and pyridoxine, analogues of pyridoxal phosphate which lack an aldehyde, failed to inhibit even at high concentrations (10 mM). Furthermore, while full activity could be recovered upon dialysis of the pyridoxal phosphate treated enzyme, activity was irreversibly lost after treatment with sodium borohydride (Table III). Direct evidence for the formation of a Schiff base and its subsequent reduction by sodium borohydride can be obtained spectrally. When 30 μM pyridoxal phosphate is incubated with 14 μM *R. rubrum* ribulose bisphosphate carboxylase, two new absorption maxima are found (Figure 2). The major peak at 415 nm may reflect Schiff base formation with a primary amino group on the protein. The minor peak with a maximum of 334–340 nm may represent formation of an additional protein–pyridoxal phosphate complex. After reduction with sodium borohydride and dialysis, a new absorption maximum appears at 325 nm (Figure 2). These data are consistent with Schiff base formation at a primary amino group on the enzyme which is reducible by sodium borohydride.

The ribulose bisphosphate carboxylase from spinach is strongly inhibited by stoichiometric amounts of 2-carboxy-D-ribitol 1,5-bisphosphate (Siegel & Lane, 1972). This compound is believed to be an analogue of 2-carboxy-3-keto-D-ribitol 1,5-bisphosphate, the intermediate formed during the enzymatic carboxylation of ribulose bisphosphate (Calvin, 1954; Sjödin & Vestermark, 1973; Siegel & Lane, 1973). The difference spectrum of the *R. rubrum* ribulose bisphosphate carboxylase, pyridoxal phosphate, and carboxyribitol bisphosphate against the enzyme and pyridoxal phosphate alone demonstrates that carboxyribitol bisphosphate prevents pyridoxal phosphate binding (Figure 3). This is indicated by the

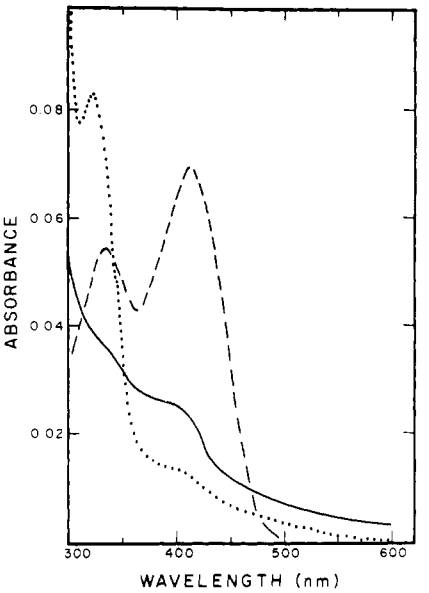

FIGURE 2: Spectra of the native and modified ribulose bisphosphate carboxylase. Spectrum of the native enzyme, 1.2 mg/mL, in 50 mM potassium phosphate, pH 7.5, and 1 mM EDTA (—). Spectrum of the enzyme, 1.2 mg/mL, modified with 60 μM pyridoxal phosphate and sodium borohydride as described in the Experimental Procedures (• • •). Difference spectrum of 1.6 mg/mL enzyme in the sample and reference cuvette and 30 μM pyridoxal phosphate in the sample cuvette (- - -). The buffer is 50 mM potassium Mops, pH 7.5, 20 mM sodium bicarbonate, 10 mM magnesium acetate, and 1 mM EDTA. Spectra were performed on a Beckman 25 recording spectrophotometer at room temperature.

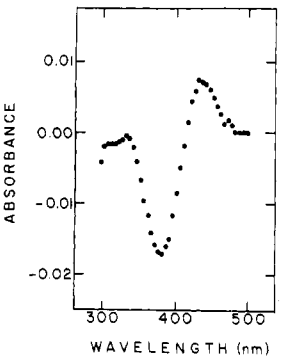

FIGURE 3: Difference spectrum of ribulose bisphosphate carboxylase and pyridoxal phosphate in the presence and absence of carboxyribitol bisphosphate. Enzyme was activated for 3 h with 10 mM dithiothreitol and dialyzed against 50 mM potassium Mops, pH 7.5, containing 1 mM EDTA, 20 mM sodium bicarbonate, and 10 mM magnesium acetate. After the addition of 30 μM pyridoxal phosphate and either 40 μM carboxyribitol bisphosphate or deionized water, the enzyme, 12 μM, was incubated for 90 min at 30 °C. The difference spectrum was recorded at room temperature on a Beckman 25 recording spectrophotometer with enzyme, pyridoxal phosphate, and carboxyribitol bisphosphate in the reference cuvette and enzyme and pyridoxal phosphate in the sample cuvette. The spectrum was corrected for fluctuations in the baseline by plotting the difference between the spectrum and a scan of the cuvettes containing water at 5-nm intervals.

large negative absorption at 380 nm of the unbound pyridoxal phosphate in the presence of carboxyribitol bisphosphate and enzyme. The positive absorption maximum at 430 and the minor maximum at 340 nm show that pyridoxal phosphate is bound under identical conditions in the absence of carboxyribitol bisphosphate. The presence of both maxima in the spectrum indicates that carboxyribitol bisphosphate replaces the pyridoxal phosphate contributing to the 340-nm absorption maximum as well as the 415-nm maximum (Figure 2). Re-

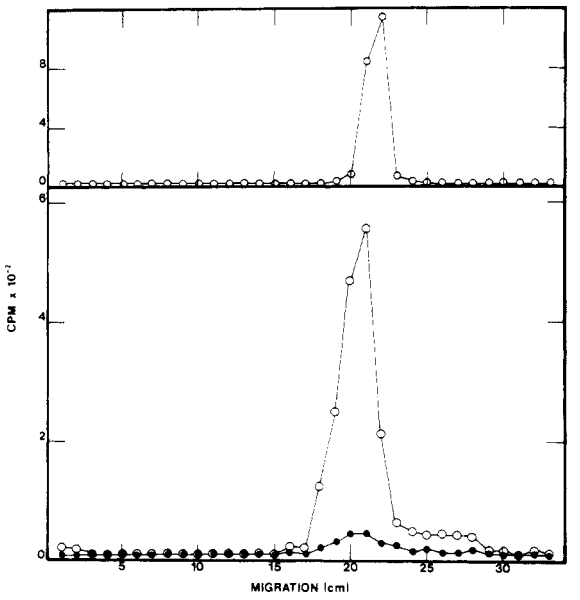

FIGURE 4: Identification of the pyridoxylated amino acid after sodium borohydride treatment of the pyridoxal phosphate–*R. rubrum* ribulose bisphosphate complex. (Lower) Profile of tritium distribution after thin-layer chromatography of the acid hydrolysate of *R. rubrum* ribulose bisphosphate carboxylase after modification with pyridoxal phosphate and tritiated sodium borohydride (○) and after modification with carboxyribitol bisphosphate, pyridoxal phosphate, and tritiated sodium borohydride (●). (Upper) Profile of tritium distribution after thin-layer chromatography of tritiated N^6-(pyridoxyl)lysine. Details are described in Experimental Procedures. The tritium counting efficiency was 0.35 as determined by the external standard ratio method.

duction of the pyridoxal phosphate–enzyme complex with tritiated sodium borohydride in the presence and absence of carboxyribitol bisphosphate demonstrates that carboxyribitol bisphosphate prevents the incorporation of tritium into the enzyme (Figure 4). Acid hydrolysis of the modified enzyme followed by cellulose thin-layer chromatography indicated only one major labeled product in the absence of carboxyribitol bisphosphate (Figure 4). This product comigrated with authentic tritiated N^6-pyridoxyllysine and was virtually absent from the chromatogram of the enzyme modified in the presence of carboxyribitol bisphosphate. Therefore, pyridoxal phosphate appears to bind a lysyl residue on the enzyme close to or at the carboxyribitol bisphosphate binding site.

Discussion

These studies were undertaken to determine if pyridoxal phosphate was an active-site directed inhibitor of the *R. rubrum* ribulose bisphosphate carboxylase. Evidence that this is so comes largely from the identity of compounds which protect against pyridoxal phosphate inhibition of enzyme activity and protect against pyridoxal phosphate binding. The substrate, ribulose bisphosphate, protected enzymatic activity to a greater extent than did the other compounds tested. This indicates that pyridoxal phosphate may be directed at a ribulose bisphosphate binding site. The fact that a stoichiometric amount of carboxyribitol bisphosphate prevents pyridoxal phosphate binding, as measured by tritium incorporation after treatment with sodium [^{3}H]borohydride, indicates that pyridoxal phosphate acts at the catalytic ribulose bisphosphate binding site and not an allosteric ribulose bisphosphate binding site of the type suggested by Chu & Bassham (1975). Furthermore, at low concentrations, pyridoxal phosphate does not bind other sites on the enzyme in the presence of carboxyribitol bisphosphate. Therefore, under the conditions employed, pyridoxal phosphate must be specific for a particular site or sites on the enzyme.

Support for these conclusions comes from the observation that compounds which inhibit enzymatic activity generally protect against pyridoxal phosphate inhibition. Many of the phosphorylated compounds and effectors which were protectants also inhibited enzymatic activity. Carboxyribitol bisphosphate is also a potent inhibitor of *R. rubrum* ribulose bisphosphate carboxylase (Whitman & Tabita, unpublished data). Similarly, inorganic anions which inhibit activity protect from pyridoxal phosphate inhibition (Whitman & Tabita, 1976). Bicarbonate and magnesium, activators of ribulose bisphosphate carboxylase, enhance the inhibition by pyridoxal phosphate. These activators may cause a conformational change in the enzyme which increases the reactivity of the active site for pyridoxal phosphate. An alternative explanation is that pyridoxal phosphate forms a complex with magnesium at the active site in a manner analogous to ribulose bisphosphate as proposed by Miziorko & Mildvan (1974). This appears unlikely for several reasons. First, magnesium has a low reactivity in the metal catalyzed transaminations of pyridoxal and presumably is a poor chelator of the Schiff base (Longenecker & Snell, 1957). Secondly, no increase in pyridoxal phosphate inhibition is seen in the presence of magnesium without bicarbonate.

The failure of low concentrations of pyridoxal to cause significant inhibition suggests that the phosphate of pyridoxal phosphate may be important for inhibition. Inorganic phosphate is a competitive inhibitor with respect to ribulose bisphosphate for the spinach enzyme (Paulsen & Lane, 1966) and accordingly protects the *R. rubrum* enzyme from pyridoxal phosphate inhibition (Whitman & Tabita, 1976). Therefore, pyridoxal phosphate may be directed to the active site by interactions between cationic amino acids and the phosphate group. During catalysis, these basic amino acids may be necessary for ribulose bisphosphate binding. Recently, the importance of arginine residues in the binding of phosphorylated substrates in the glycolytic pathway enzymes has been demonstrated (Riordan et al., 1977). The importance of arginine in ribulose bisphosphate carboxylase has not been evaluated. However, peptides containing essential lysyl residues of the spinach enzyme obtained by Norton et al. (1975) contained arginine.

Pyridoxal phosphate forms a Schiff base with internal hydrogen bonding at a lysyl residue of the *R. rubrum* ribulose bisphosphate carboxylase. This is indicated by the absorption maximum at 415 nm of the pyridoxal phosphate–enzyme complex, the reversibility of inhibition upon dialysis, the irreversible inhibition subsequent to sodium borohydride treatment, and the identification of the labeled product after treatment with tritiated sodium borohydride. However, the minor absorption maximum at 340 nm suggests that pyridoxal phosphate may have additional interactions with the enzyme. Proposals concerning the structure of this chromophore include formation of a Schiff base in a hydrophobic environment (Shaltiel & Cortijo, 1970), a carbinolamine (Honikel & Madsen, 1972), or a *gem*-diamine or similar adduct with a second nucleophilic amino acid residue in rapid equilibrium with the Schiff base (O'Leary & Brummund, 1974). None of these possibilities could be eliminated in the present work. Because carboxyribitol bisphosphate alleviates the absorption maxima at 340 nm as well as at 415 nm, the two absorption maxima probably represent pyridoxal phosphate binding at the same or similar sites on the enzyme. Furthermore, the ability of low concentrations of carboxyribitol bisphosphate (less than 3.4 times the dimer concentration) to prevent almost

all tritium incorporation indicates that no more than two high affinity pyridoxal phosphate sites exist per subunit.

References

Bowes, G., & Ogren, W. L. (1972) *J. Biol. Chem. 247,* 2171.

Buchanan, B., & Schürmann, P. (1973) *J. Biol. Chem. 248,* 4956.

Calvin, M. (1954) *Fed. Proc., Fed. Am. Soc. EXP/ Biol. 13,* 697.

Chu, D. K., & Bassham, J. A. (1972) *Plant Physiol. 50,* 224.

Chu, D. K., & Bassham, J. A. (1974) *Plant Physiol. 54,* 556.

Chu, D. K., & Bassham, J. A (1975) *Plant Physiol. 55,* 720.

Columbo, G., & Marcus, F. (1974) *Biochemistry 13,* 3085.

Forrey, A. W., Olsgaard, R. B., Nolan, C., & Fischer, E. H. (1971) *Biochimie 53,* 269.

Harris, C. M., Johnson, R. J., & Metzler, D. E. (1976) *Biochim. Biophys. Acta 421,* 181.

Hartman, F. C., Welch, M. H., & Norton, I. L. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70,* 3721.

Heyl, D., Luz, E., Harris, S. A., & Folkers, K. (1951) *J. Am. Chem. Soc. 73,* 3430.

Honikel, K. L., & Madsen, N. B. (1972) *J. Biol. Chem. 247,* 1057.

Laemmli, U. K. (1970) *Nature (London) 227,* 680.

Laing, W. A., Ogren, W. L., & Hageman, R. H. (1975) *Biochemistry 14,* 2269.

Longenecker, J. B., & Snell, E. E. (1957) *J. Am. Chem. Soc. 79,* 142.

Lorimer, G. H., Badger, M. R., & Andrews, T. J. (1976) *Biochemistry 15,* 529.

McFadden, B. A., & Tabita, F. R. (1974) *Biosystems 6,* 93–112.

Metzler, D. E., & Snell, E. E. (1955) *J. Am. Chem. Soc. 77,* 2431.

Miziorko, H. M., & Mildvan, A. S. (1974) *J. Biol. Chem. 249,* 2743.

Nishimura, M., Takabe, T., Sugiyama, T., & Akazawa, T. (1973) *J. Biochem. (Tokyo) 74,* 945.

Norton, I. L., Welch, M. H., & Hartman, F. C. (1975) *J. Biol. Chem. 250,* 8062.

O'Leary, M. H., & Brummund, W. (1974) *J. Biol. Chem. 249,* 3737.

Orr, M. D., Blakely, R. L., & Panagou, D. (1972) *Anal. Biochem. 45,* 68.

Paech, C., Ryan, F. J., & Tolbert, N. E. (1977) *Arch. Biochem. Biophys. 179,* 279.

Paulsen, J. M., & Lane, M. D. (1966) *Biochemistry 5,* 2350.

Rabin, B. R., & Trown, P. W. (1964) *Proc. Natl. Acad. Sci. U.S.A. 51,* 497.

Riordan, J. F., McElvany, K. D., & Borders, C. L., Jr. (1977) *Science 195,* 884.

Rutner, A. C. (1970) *Biochem. Biophys. Res. Commun. 39,* 923.

Schloss, J. V., & Hartman, F. C. (1977) *Biochem. Biophys. Res. Commun. 75,* 320.

Schnackerz, K.D., & Noltmann, E. A. (1971) *Biochemistry 10,* 4837.

Shaltiel, S., & Cortijo, M. (1970) *Biochem. Biophys. Res. Commun. 41,* 594.

Siegel, M. I., & Lane, M. D. (1972) *Biochem. Biophys. Res. Commun. 48,* 508.

Siegel, M. I., & Lane, M. D. (1973) *J. Biol. Chem. 248,* 5486.

Sjodin, B., & Vestermark, A. (1973) *Biochim. Biophys. Acta 297,* 165.

Sugiyama, T., Nakayama, N., Ogawa, M., & Akazawa, T. (1967) *Biochem. Biophys. Res. Commun. 28,* 431.

Sugiyama, T., Nakayama, N., Ogawa, M., & Akazawa, T. (1968) *Arch. Biochem. Biophys. 125,* 98.

Tabita, F. R., & McFadden, B. A. (1972) *Biochem. Biophys. Res. Commun. 48,* 1153.

Tabita, F. R., & McFadden, B. A. (1974a) *J. Biol. Chem. 249,* 3453.

Tabita, F. R., & McFadden, B. A. (1974b) *J. Biol. Chem. 249,* 3459.

Whitman, W., & Tabita, F. R. (1976) *Biochem. Biophys. Res. Commun. 71,* 1034.

Wishnick, M., Lane, M. D., & Scrutton, M. C. (1970) *J. Biol. Chem. 245,* 4939.