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Isolation and Sequence of the Pyridoxal 5′-Phosphate Active-Site Peptide from *Rhodospirillum rubrum* Ribulose-1,5-bisphosphate Carboxylase/Oxygenase†

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ABSTRACT: Ribulose-1,5-bisphosphate carboxylase/oxygenase from *Rhodospirillum rubrum* was modified with pyridoxal 5′-phosphate and then reduced with sodium borohydride. Both carboxylase and oxygenase activities were lost when one molecule of pyridoxal 5′-phosphate was bound per enzyme dimer. Peptide maps of modified enzyme showed one N^6-(phosphopyridoxal)lysine-containing peptide. This peptide was isolated by gel filtration and cation-exchange chromatography and its sequence determined as Ala-Leu-Gly-Arg-Pro-Glu-Val-Asp-(PLP-Lys)-Gly-Thr-Leu-Val-Ile-Lys. Since activation of the enzyme with Mg^{2+}/CO_2 enhances pyridoxal 5′-phosphate modification and subsequent inactivation and the substrate ribulose bisphosphate protects against modification, the modified lysyl group is most certainly at the catalytic site and not at the activation site of the enzyme.

Ribulose-1,5-bisphosphate carboxylase/oxygenase is the primary catalyst of photosynthetic carbon fixation and also catalyzes the first step in photorespiratory glycolate production [see Jensen & Bahr (1977) for a review]. The importance of ribulose-1,5-bisphosphate carboxylase/oxygenase in carbon assimilation and crop productivity mandates a complete understanding of the enzyme's chemical structure and mode of action. While the enzyme from higher plants contains eight large (catalytic) and eight small subunits and has a molecular weight of 560 000 (Paulsen & Lane, 1966), a structurally simpler protein which is a dimer of large subunits (M_r 114 000) is easily isolated from the photosynthetic bacterium *Rhodospirillum rubrum* (Tabita & McFadden, 1974a,b).

Considering its importance, little is known about the active-site structure of this enzyme; however, recent evidence

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indicates that the Mg^{2+}/CO_2 activation site may be different from the catalytic site (Miziorko, 1979; Lorimer, 1979). The affinity label 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate (Norton et al., 1975; Schloss & Hartman, 1977a) has been used to modify essential lysyl residues at the active site of the enzymes from spinach leaf and *R. rubrum*. Another affinity label, *N*-(bromoacetyl)ethanolamine phosphate, has been used to modify a lysyl residue and two cysteinyl residues in the active-site region of the spinach enzyme (Schloss & Hartman, 1977b; Schloss et al., 1978a). Three distinct tryptic peptides containing these two affinity labels have been isolated from the spinach enzyme and sequenced (Stringer & Hartman, 1978; Schloss et al., 1978a). A lysyl group has also been modified with cyanate (Chollet & Anderson, 1978). In addition, there appears to be an essential arginine(s) (Chollet, 1978; Lawlis & McFadden, 1978; Schloss et al., 1978b) and an essential tyrosine (Robison & Tabita, 1979) at the active site of both the bacterial and plant enzyme. Each of these specific active-site residues appears to be localized on the large subunit of the enzyme.

Because bromobutanone bisphosphate decomposes during modification and lacks specificity (Hartman et al., 1973), as does (bromoacetyl)ethanolamine phosphate (Schloss et al., 1978a) and cyanate (Chollet & Anderson, 1978), more detailed chemical modification and kinetic studies have been carried out with pyridoxal 5′-phosphate, (PLP),[1] a reagent known to modify selectively the ϵ-amino group of a lysyl residue in or near phosphate-binding sites of many proteins (Columbo & Marcus, 1974). Moreover, the Schiff base formed is easily reduced to a stable secondary amine with sodium borohydride (Fisher et al., 1958), a procedure which has been used to advantage in isolating and sequencing peptides containing active-site lysyl residues (Tanase et al., 1979; Parsons & Preiss, 1978). With the spinach enzyme, 2 lysines/large subunit were modified by PLP (Paech et al., 1977; Paech & Tolbert, 1978). Previous work from our laboratory has shown that PLP modified an essential lysine of the *R. rubrum* carboxylase (Whitman & Tabita, 1978a,b). Activity was protected by the substrate RuBP, but the addition of Mg^{2+} and CO_2 enhanced the rate of inactivation (Whitman & Tabita, 1976, 1978a). Such enhanced inactivation of the activated form of the enzyme was also found with the spinach enzyme (Whitman et al., 1979a), and subsequent studies have shown that the enhancement of the reaction with PLP is due to an increased affinity of the enzyme for the noncovalent intermediate formed prior to the Schiff base (Whitman et al., 1979b). Studies with the *R. rubrum* enzyme have also demonstrated that there are two PLP binding sites per enzyme dimer. However, the binding of PLP to just one of these sites is sufficient to inactivate the entire dimer (Whitman & Tabita, 1978b).

In the present study, we have isolated and determined the amino acid sequence of a 15-residue peptide containing the PLP-modified lysyl residue at the active site from tryptic digests of the *R. rubrum* enzyme. This is the first report of the sequence surrounding an active-site residue of RuBP carboxylase/oxygenase from a prokaryotic source.

Experimental Procedures

Materials. RuBP carboxylase/oxygenase from *R. rubrum* was purified as described by Tabita & McFadden (1974a) except for minor modifications (Robison & Tabita, 1979). Sodium dodecyl sulfate gel electrophoresis (Laemmli, 1970) showed the enzyme to be homogeneous. The protein concentration of enzyme preparations was calculated from the extinction coefficient at 280 nm of 0.974 L g^{-1} cm^{-1} (Tabita & McFadden, 1974b). [^{3}H]$NaBH_4$ (222 Ci/mmol) was purchased from New England Nuclear. Fluorescamine, PLP, iodoacetic acid, and trypsin (diphenylcarbamyl chloride treated) were from Sigma Chemical Co., St. Louis, MO, and carboxypeptidases A and B were from Worthington Biochemical Corp., Freehold, NJ. Materials for the Edman degradation were of sequenator grade. Pyridine was redistilled over ninhydrin prior to use. All other materials were of the highest grade commercially available.

PLP Modification. The reaction was carried out in 50 mM K–Mops (pH 7.8) containing 1 mM EDTA, in the presence of 20 mM sodium bicarbonate and 10 mM magnesium acetate to increase the rate of modification (Whitman & Tabita, 1978a). Enzyme (13 μM) was incubated in the dark with 1–50 μM PLP for 30 min at 30 °C and then reduced with 1 mM [^{3}H]$NaBH_4$ for 5 min. The modified enzyme was then extensively dialyzed against 50 mM potassium phosphate (pH 7.5) containing 1 mM EDTA and 1 mM BME. Tritium incorporation was determined by liquid scintillation counting using a dioxane cocktail containing 5 g/L 2,5-diphenyloxazole and 100 g/L naphthalene or a toluene cocktail (Whitman & Tabita, 1976). Stock [^{3}H]$NaBH_4$ solutions were prepared by diluting the radioactive material with cold $NaBH_4$ in 0.01 N NaOH. Any tritium incorporated in the absence of PLP was subtracted. Since the PLP modification is light sensitive (Ritchey et al., 1977), all procedures were performed in the dark.

Fluorography. For fluorographic analysis, the modified enzyme was dialyzed against distilled water. Tryptic digestion was carried out (Kostka & Carpenter, 1964) except that three cycles of treatment were performed instead of two cycles and the ratio of trypsin to enzyme was reduced to 1% (w/w). The tryptic peptides were resuspended in 10% pyridine–1% acetic acid (pH 6.0) and centrifuged to remove the insoluble material. The peptides were subjected to mapping (Imamura & Riggs, 1972) on 46 × 57 cm Whatman 3 MM paper. The first dimension was high-voltage electrophoresis at pH 6.0. The second dimension was descending paper chromatography with 1-butanol–acetic acid–water–pyridine (15:3:12:10) as the solvent. Fluorography was performed by dipping the chromatogram in 7% 2,5-diphenyloxazole in petroleum ether for 3 s and drying in a fume hood. The chromatogram was completely covered with four sheets of SB-54 X-ray film (Kodak) and exposed for 1 month at −70 °C.

Carboxymethylation and Tryptic Digestion of PLP-Modified Enzyme. For sequence work, the dialyzed, modified enzyme was carboxymethylated (Schloss et al., 1978a). The enzyme was then extensively dialyzed against 50 mM NH_4HCO_3, resulting in a heavy precipitate. This was collected by centrifugation and resuspended in 20 mL of 50 mM NH_4HCO_3. Trypsin (1% w/w) was added, and the sample was incubated at 37 °C for 18 h. Another 1% aliquot of trypsin was added and digestion allowed to proceed for another 6 h. After this time, the suspension was completely clear and no remaining precipitate was seen.

Thin-Layer Chromatography. For determination of peptide purity, peptides were chromatographed upward on 20 × 20 cm cellulose thin-layer sheets (Eastman 13255) using a 50:33:1:40 1-butanol–pyridine–acetic acid–water solvent. After drying for 1 h, the chromatograms were sprayed with a 0.1%

[1] Abbreviations used: RuBP, D-ribulose 1,5-bisphosphate; PLP, pyridoxal 5′-phosphate; PTH, phenylthiohydantoin; Mops, 4-morpholinepropanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; BME, 2-mercaptoethanol.

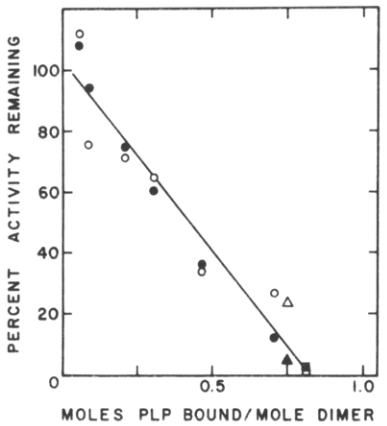

FIGURE 1: Stoichiometry of the PLP inactivation of RuBP carboxylase and oxygenase activities. Carboxylase (●, ▲, ■) and oxygenase (○, △, □) activities after modification with 1 and 50 μM PLP (●, ○), two successive modifications with 50 μM PLP (▲, △), and three successive modifications with 50 μM PLP (■, □).

fluorescamine (in acetone) solution followed by a spray of 5% pyridine in acetone and then visualized under a long-range UV lamp.

Amino Acid Analysis. The peptides were hydrolyzed at 110 °C in 6 N HCl in sealed, evacuated tubes for 24 h. Amino acid compositions of the peptides were determined with a Beckman Model 120 amino acid analyzer as described by Li & Riggs (1970). N^6-(Phosphopyridoxal)lysine eluted from the short column between lysine and histidine, and its color value was taken from Forrey et al. (1971).

Sequence Determination. Automatic Edman degradation was carried out with a Beckman protein–peptide sequencer, Model 890 updated to 890B. Beckman DMAA peptide program 111374 (revised 8/77) was used. PTH derivatives were identified with a Beckman Model G-45 gas chromatograph (Pisano & Bronzert, 1969) and by thin-layer chromatography using solvents IV and V as specified (Jeppsson & Sjoquist, 1967). PTH-arginine was identified by using a specific paper test (Yamada & Itano, 1966). The C-terminal residues were also determined by using carboxypeptidases A and B according to the method of Ambler (1967).

Other Methods. Tritium was measured in a Beckman LS-100C liquid scintillation counter. Absorption measurements were taken on a Cary 219 spectrophotometer. RuBP carboxylase and oxygenase activities were measured by established procedures (Whitman et al., 1979a,b; Robison et al., 1979).

Results

Stoichiometry of the PLP Inactivation. The stoichiometry of PLP inactivation was determined following reduction of the Schiff base with tritiated sodium borohydride (Figure 1). This modification is stable and permits the measurement of the loss in RuBP oxygenase as well as RuBP carboxylase activity. The incorporation of about 0.8–0.9 mol of PLP per mol of enzyme dimer was sufficient to completely inactivate both enzymatic activities, substantiating previous spectral and kinetic measurements of the stoichiometry of carboxylase inactivation (Whitman & Tabita, 1978b). Successive treatments did not increase the extent of modification, as measured by tritium incorporation (Figure 1). In other experiments, levels of up to 1.2 mol of PLP per mol of enzyme dimer could be obtained by this method, whereas up to 2 mol of PLP per dimer was incorporated at high levels of PLP when the amount of PLP found was determined by the spectral method (Whitman & Tabita, 1978b).

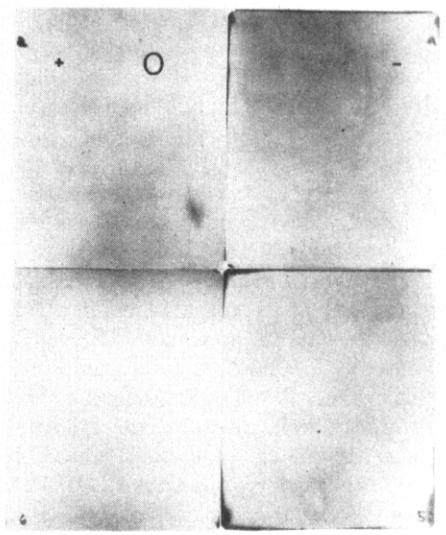

FIGURE 2: Fluorography of the peptide map of tritiated phosphopyridoxyl RuBP carboxylase/oxygenase. A composite photograph of the four sheets of SB-54 X-ray film used to cover the two-dimensional paper chromatogram is shown. The origin is labeled "O". The numbers in the corners were used to align the X-ray film with the original chromatogram. The first dimension was high-voltage electrophoresis at pH 6.0 and the cathode and the anode are labeled "+" and "–", respectively. The second dimension was descending chromatography.

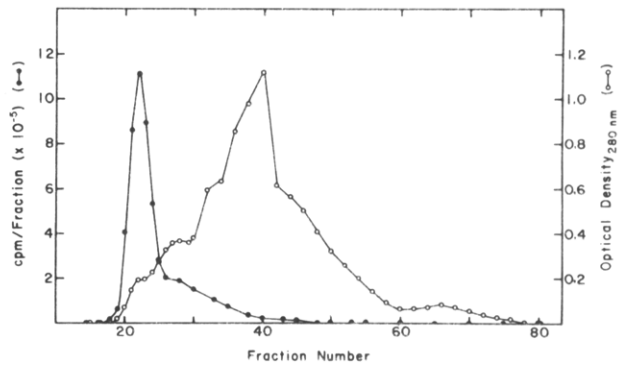

FIGURE 3: Purification of tritiated PLP peptides by Sephadex G-25 chromatography. A 10-mL tryptic digest sample was applied with a flow rate of 8 mL/h. The fraction size was 3.9 mL. Fractions 20–26 were pooled for Aminex chromatography.

Following tryptic digestion of enzyme labeled to the extent of 1 mol of tritium per dimer, 70% of the label was solubilized in 10% pyridine–1% acetic acid buffer (pH 6.0). The two-dimensional peptide map of the solubilized material showed one fluorescent peptide under a long-range UV lamp. Fluorography of the peptide map revealed one tritium-labeled peptide coincident with the fluorescent peptide (Figure 2). Therefore, at lease the majority of the phosphopyridoxal enzyme is modified at a single site.

Purification of the Phosphopyridoxal Peptide. A sample containing 157 mg (1.38 μmol of dimer) of enzyme was modified with 50 μM PLP and reduced with tritiated sodium borohydride as described under Experimental Procedures. This treatment resulted in the loss of 94% of all carboxylase activity and the incorporation of 1.2 mol of PLP per dimer. After extensive dialysis, this sample was carboxymethylated and digested with trypsin, also as described under Experimental Procedures. This digest was separated into two fractions, and each was chromatographed on a 1.6 × 90 cm Sephadex G-25 column using an elution buffer of 0.2 M formic acid containing 0.02 M pyridine. The profile of one of these columns is shown in Figure 3. One radioactive peak eluting very early in the

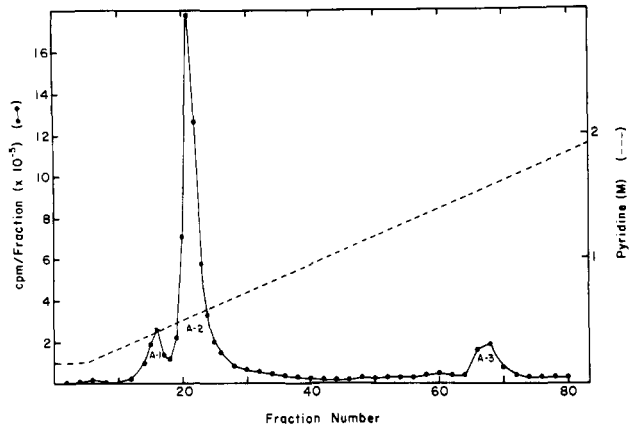

FIGURE 4: Aminex cation-exchange chromatography of the tritiated PLP peptide peak from Sephadex G-25 chromatography. The flow rate was 25 mL/h, and the fraction size was 4.75 mL. Three peaks were obtained and labeled A-1, A-2, and A-3 in order of elution in the pyridine gradient.

column was seen. When pooled, the peaks from each Sephadex G-25 column contained 80% of the radioactivity originally applied. This pooled Sephadex G-25 sample was then applied to an Aminex AG-50W-X2 cation-exchange column (1.6 × 28 cm) equilibrated with 0.2 M formic acid containing 0.02 M pyridine. The column was developed with a 400-mL 0.02–2 M pyridine gradient in 0.2 M formic acid (Figure 4). One major and two minor radioactive peaks were seen. The major peak, A-2, contained 77% of the radioactivity eluted from the column and represented a 58% yield of the amount applied to the column.

Peptides A-1, A-2, and A-3 were lyophilized and desalted on a 1.6 × 27 cm Bio-Gel P-6 column. Peptides A-2 and A-3 had an adsorption maximum at 310 nm at pH 8.0 which is characteristic of the N^6-(phosphopyridoxal)lysine residue (Forrey et al., 1971). Peptides A-2 and A-3 also had a deep blue fluorescence when spotted on paper or thin-layer cellulose sheets and viewed under a long-range UV lamp, again indicative of the (phosphopyridoxal)lysine residue. In contrast, peptide A-1 had an adsorption spectrum with a peak at 274 nm and the absence of any significant absorbance in the 310–320-nm range. Peptide A-1 had no fluorescence when spotted on thin-layer cellulose sheets. Thus, the radioactivity of peptide A-1 is not due to labeled (phosphopyridoxal)lysine. Thin-layer chromatograms of peptides A-2 and A-3 each showed a single spot, with A-3 having a slightly higher R_f than A-2. The amino acid composition of peptide A-2 is shown in Table I. Peptide A-3 had a similar composition but lacked alanine.

Sequence of the Phosphopyridoxal Peptide. Automated Edman degradation of peptide A-2 was carried out for all 15 steps (Table II). The PTH amino acids from steps 1–3, 5–8, and 10–15 were found in the ethyl acetate phase and identified by using gas chromatography and thin-layer chromatography. Background was noted in several steps but could be attributed to a preview of the next step (cycle 1) or trailing of the previous step (cycles 3, 4, 7, and 8). Other preparations of peptide A-2 had the same initial sequence without the leucine background seen in cycles 1 and 3. The fourth step was found in the aqueous phase and identified as arginine. A small amount of radioactivity was found in the aqueous phase of step 9 and exhibited fluorescence under a long-range UV lamp. Thus, step 9 was identified as the (phosphopyridoxal)lysine. After the sequence determination, a large amount of radioactivity remained in the cup. Apparently, the PTH-(phosphopyridoxal)lysine residue is too polar to be extracted from the cup by the 1-chlorobutane employed. C-Terminal analyses using carboxypeptidases A and B and digestion times up to 80 min confirmed that the last five residues of peptide A-2 were Thr-Leu-Val-Ile-Lys (data not shown). Thus, the sequence of the PLP active-site peptide is Ala-Leu-Gly-Arg-Pro-Glu-Val-Asp-(PLP-Lys)-Gly-Thr-Leu-Val-Ile-Lys.

Table I: Amino Acid Composition of Peptide A-2

amino acid	total (μmol)[a]	molar ratio[b]
Lys	1.18	0.9 (1)
PLP-Lys[c]	0.75	0.6 (1)
Arg	1.03	0.8 (1)
Asp	1.69	1.3 (1)
Thr	1.06	0.8 (1)
Glu	1.54	1.2 (1)
Pro	1.39	1.1 (1)
Gly	2.91	2.3 (2)
Ala	1.86	1.4 (1)
Val	2.38	1.8 (2)
Ile	1.29	1.0 (1)
Leu	1.81	1.4 (2)

[a] Since starting material was 2.75 μmol, this represents an average yield of 46% for the peptide purification. [b] Ile was arbitrarily assigned a value of 1.0. The values in parentheses are the number of residues determined by sequencing. [c] N^6-(Phosphopyridoxal)-lysine.

Table II: Sequence Analysis of Peptide A-2[a]

cycle	amino acid assigned	nmol obtained by GC	background
1	Ala	130	76 nmol of Leu
2	Leu	324	
3	Gly	157	45 nmol of Leu
4	Arg[b]		56 nmol of Gly
5	Pro	145	
6	Glu	112	
7	Val	142	61 nmol of Glu
8	Asp	46	53 nmol of Val
9	PLP-Lys[c]		
10	Gly	89	
11	Thr	20	
12	Leu	21	
13	Val	67	
14	Ile	16	
15	Lys	14	

[a] 251 nmol of peptide was applied to the sequenator. [b] Arginine was identified by a paper test. [c] Identified by the absence of any other residue plus the presence of radioactivity.

Discussion

The stoichiometry of PLP modification of *R. rubrum* RuBP carboxylase/oxygenase, measured by $[^3H]NaBH_4$ reduction, indicates that one molecule of PLP reacts per dimer of enzyme, causing the loss of all carboxylase as well as oxygenase activity. This agrees with previous stoichiometry measurements using spectral and kinetic procedures (Whitman & Tabita, 1978b). The yield of radioactivity in counts per minute was 42% for peptide A-2. This agrees very well with the yield (46%) of peptide A-2 calculated from the amino acid analysis (Table I). This indicates that both lysines per dimer were modified with PLP when the stoichiometry measured by $[^3H]NaBH_4$ was 1.2. Apparently, at high PLP concentrations, $[^3H]NaBH_4$ measurements underestimate the amount of PLP modified. The amount of PLP incorporation measured by $[^3H]NaBH_4$ incorporation at the lower levels of PLP (Figure 1, upper part of curve) agrees well with spectral and kinetic stoichiometry determinations (Whitman & Tabita, 1978b), and it can be

concluded that while two lysines per dimer may be modified with PLP, loss of activity can be correlated with the binding of just one. Care must therefore be taken when measuring the stoichiometry of PLP binding solely by [^{3}H]$NaBH_4$ reduction. This is in contrast to the spinach enzyme; using a spectral procedure, it was found that the stoichiometry of PLP modification was biphasic (Paech & Tolbert, 1978). Here, approximately 80% of both carboxylase and oxygenase activities were lost when one PLP was incorporated per large subunit, but the incorporation of a second PLP was necessary for complete inactivation (Paech & Tolbert, 1978). Thus, PLP acts as a half of the sites reagent (Levitzki & Koshland, 1976) with respect to the *R. rubrum* enzyme but perhaps not with the spinach enzyme. This indicates that there may be differences in the PLP modification of the two different structural forms of the emzyme. Interestingly, half of the site's reactivity is also seen upon phenylglyoxal modification of both the *R. rubrum* and spinach enzymes (Schloss et al., 1978b).

The peptide analysis indicates that a single lysyl residue is modified with PLP. In the final chromatographic step of the peptide purification, 80% of the radioactivity was associated with the major PLP–peptide, peptide A-2. Another 14% of the radioactivity was associated with a peptide, A-1, which was not associated with PLP modification. This was not unexpected since during the stoichiometry experiments enzyme incubated with [^{3}H]$NaBH_4$ in the absence of PLP had about 10% as much radioactivity as the samples incubated with PLP. The remaining radioactivity was associated with a peptide, A-3, which was identical in amino acid composition with that of the major peptide, A-2, with the exception of an absence of alanine, the N-terminal residue. The reason for this difference is unclear, but the peptide does appear to contain the same PLP site as the major peptide.

Tanase et al. (1979) have compared the primary structures of PLP peptides from 23 pyridoxal enzymes and found that the partial structure Ser-X-X-(PLP-Lys)- was the most commonly conserved sequence. The PLP–peptide obtained in this study does not contain this structure, but several others of those examined by Tanase et al. (1979) did not either. The active-site PLP–peptide obtained in this study may be compared with the lysine-modified peptides of the spinach enzyme obtained with the RuBP analogue affinity labels (Stringer & Hartman, 1978; Schloss et al., 1978a). One of these peptides contains a lysine modified by both affinity labels, and it has also been recently reported that this lysine of the spinach enzyme is also the lysine modified by PLP (Spellman et al., 1979). The sequence of this peptide from the spinach enzyme is Tyr-Gly-Arg-Pro-Leu-Leu-Gly-Cys-Thr-Ile-Lys*-Pro-Lys, where Lys* represents the lysine which is modified. Except for the presence of a Gly-Arg-Pro sequence early in each peptide, no obvious correlation between this and the *R. rubrum* PLP–peptide can be made.

Since Mg^{2+}/CO_2 preincubation, i.e., activation of the enzyme, enhances modification of the *R. rubrum* enzyme and the substrate RuBP protects against inactivation (Whitman & Tabita, 1976, 1978a), the lysine which is modified is probably at the catalytic site, rather than the activation site. Indeed, the protection against modification afforded by 2-carboxy-D-ribitol 1,5-bisphosphate (Whitman & Tabita, 1978a), a transition-state analogue, also indicates that PLP modifies the catalytic site. Interestingly, we have found that low concentrations of PLP activate spinach RuBP carboxylase/oxygenase in the absence of Mg^{2+}/CO_2; however, when the enzyme was activated by incubation with 10 mM $NaHCO_3$ and 10 mM Mg^{2+}, inactivation by PLP is pronounced, similar to the behavior of the *R. rubrum* enzyme (Whitman et al., 1979a). The fact that the Mg^{2+}/CO_2-activated enzyme is more susceptible to PLP modification is inconsistent with the supposed modification of a CO_2 activation site by PLP (Paech et al., 1977; Paech & Tolbert, 1978). The apparent protection of modification obtained with HCO_3^- is no doubt due to the ability of this anion (and others such as SO_4^{2-} and Cl^-) to compete nonspecifically with PLP for the cationic PLP binding site on the enzyme and is not due to protection of the CO_2 activation site by HCO_3^- (Whitman & Tabita, 1976; Whitman et al., 1979a). Thus, it is imperative that the state of activation of enzyme be defined in all modification studies as is also required for the determination of the regulatory properties of the enzyme.

We are currently examining tryptic digests of the enzyme from *R. rubrum* modified with tetranitromethane under conditions where one tyrosine is selectively nitrated (Robison & Tabita, 1979). Since the PLP-lysine-containing peptide does not contain tyrosine, a different active-site residue containing peptide will be obtained. Sequence information from two different active-site regions will be very important in determining the chemical structure of the site of this enzyme.

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Primary and Secondary Deuterium Isotope Effects on Equilibrium Constants for Enzyme-Catalyzed Reactions†

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ABSTRACT: Primary deuterium equilibrium isotope effects for the reaction of five secondary alcohols with nicotinamide adenine dinucleotide (DPN) to give reduced deuterionicotinamide adenine dinucleotide (DPND) (cyclohexanol-*1-d*, 1.18; 2-propanol-*2-d*, 1.175; *threo*-DL-isocitrate-*2-d*, 1.168; L-malate-*2-d*, 1.173; L-lactate-*2-d*, 1.19) are all ~1.18, while for a primary alcohol, ethanol, the value is 1.07, for an amino acid, L-glutamate-*2-d*, it is 1.14, and for a hemiacetal, glucose-*1-d*, it is 1.28. In each case deuterium becomes enriched in the alcohol, amino acid, or hemiacetal with respect to DPNH (TPNH). β-Secondary equilibrium isotope effects for reduction of ketones by DPNH (cyclohexanone-*2,2,6,6-d*$_4$, 0.81; acetone-d_6, 0.78; pyruvate-d_3, 0.83; α-ketoglutarate-*3,3-d*$_2$ reduced to glutamate, 0.898; oxaloacetate-*3,3-d*$_2$, 0.877; oxaloacetate-*3R-d*, 0.945) give an average value of 0.946/D, with deuterium becoming enriched in the alcohol or amino acid with respect to the ketone. For reduction of acetaldehyde-*1-d* by DPNH, the observed value of 0.953 includes the equilibrium effect on the hydration equilibrium in addition to that on the reduction, and the calculated values for reduction of the free aldehyde and the hydrate are 0.78 and 1.07. For reduction of benzaldehyde-*1-d*, which is not hydrated, the observed value was 0.79. The secondary equilibrium isotope effect for conversion of DPN-*4-d* to DPNH is 0.89, with deuterium becoming enriched in DPNH, and, for conversion of fumarate-*2,3-d*$_2$ to malate, the value is 0.69, with deuterium becoming enriched in L-malate. The equilibrium isotope effect for reaction of cyclohexanol-*1-d* with DPN is temperature independent over the range 15–35 °C.

Changes in the magnitude of the equilibrium constant when deuterium is substituted for hydrogen in the reactants are predicted by the calculations of Hartshorn & Shiner (1972). The magnitude of the equilibrium isotope effect reflects the different "stiffness" of the bonds in substrate and product, with deuterium becoming enriched in the stiffest bond and hydrogen in the looser one. Hartshorn & Shiner (1972) suggested that the bond stiffness depends only on the atoms bonded directly to the carbon substituted with deuterium, with more remote atoms having only small effects. There are relatively few experimental data available to test the predictions of Hartshorn & Shiner (1972), and most of these are not of sufficient precision to be useful. The aim of this report is to present simple but accurate means for obtaining deuterium isotope effects on equilibrium constants for enzyme-catalyzed reactions and to report a number of primary and secondary equilibrium isotope effects which have been determined in this laboratory.

Materials and Methods

Chemicals. DL-Isocitrate lactone, D_2O (99.8 atom %), and all enzymes were from Sigma Chemical Co. Ethanol-d_6 (99 atom %), 2-propanol-*2-d* (98 atom %), fumarate-*2,3-d*$_2$ (98 atom %), glucose-*1-d* (98 atom %), glutamate-*2,3,3,4,4-d*$_5$ (98

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