Modification of Active Site Histidine in Ribulosebisphosphate Carboxylase/Oxygenase†

Ashok K. Saluja and Bruce A. McFadden*

ABSTRACT: Both carboxylase and oxygenase activities of ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase from a bacterial source, *Rhodospirillum rubrum*, and a plant source, spinach, were rapidly and completely inactivated by low concentrations of diethyl pyrocarbonate (DEP) at pH 7.0 and 30 °C. Inactivation kinetics were pseudo first order for up to 80% inactivation. The reaction order with respect to inactivation by DEP was approximately 1 for both enzymes, thereby indicating the modification of a single residue per subunit. One substrate, ribulose bisphosphate, the product 3-phosphoglycerate, and two competitive inhibitors, sedoheptulose 1,7-bisphosphate and 2-carboxyhexitol 1,6-bisphosphate, protected against inactivation, thereby indicating that DEP modifies the active site. DEP-modified enzyme showed an increase in the absorption at 240 nm, which was reversed upon treatment with hydroxylamine. The activity lost by DEP modification could be partially recovered after treatment with 0.5 M hydroxylamine at 25 °C for 2.5 h. The differential absorption at 240 nm suggests that DEP modified 4.5 and 2.2 histidine residues per large subunit of the enzymes from *R. rubrum* and spinach, respectively. After dissociation with sodium dodecyl sulfate of the spinach enzyme inactivated with [^{3}H]DEP, polyacrylamide gel electrophoresis established the modification of large subunits only. Up to 65% loss of activity in the spinach enzyme was directly proportional to the number of histidine residues modified, and the extrapolation to 100% inactivation implied involvement of 1 histidine residue in activity per subunit. The differential absorption at 240 nm in the presence and absence of protectants coupled with differential labeling with [^{3}H]DEP also indicated the modification of 1 essential histidine residue per subunit in RuBP carboxylase/oxygenase from spinach and at least 1 per subunit for enzyme from *R. rubrum*.

D-Ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] is a key catalyst of the reductive pentose phosphate pathway, the Calvin cycle. This protein, located in the stroma of chloroplasts, catalyzes the reaction of RuBP with CO_2 to give two molecules of 3-phosphoglycerate or with O_2 to give one 3-phosphoglycerate and one phosphoglycolate, which are the primary steps of photosynthesis and photorespiration, respectively.

However, in spite of its biological importance, the fact that it is probably the most abundant [up to 60% of the total soluble protein in green leaves; see Ellis (1979)] protein in Nature, and its easy availability, little is known about the structure of its active site. Although sulfhydryl reagents such as *p*-mercuribenzoate and iodoacetate have been shown to inactivate the enzyme and substrate protection is also observed (Nishimura et al., 1973; Sugiyama & Akazawa, 1967), most criteria for active site modification have not been met. Recently, by the use of affinity labels 3-bromo-1,4-dihydroxybutanone bisphosphate and *N*-(bromoacetyl)ethanolamine phosphate, Hartman et al. (1978) have shown the modification of 2 lysine residues per subunit of RuBP carboxylase, which are essential for its activity. However, these reagents also modify sulfhydryl groups of the enzyme. Whitman & Tabita (1978) have confirmed the involvement of a lysyl residue at the active site by modifying the enzyme with pyridoxal 5′-phosphate. The presence of arginine at the active site of one plant and two bacterial enzymes has been suggested by Lawlis & McFadden (1978) and Purohit et al. (1979), but the stoichiometric data, obtained by borate-dependent inactivation by 2,3-butanedione, indicated the modification of 22 of 36 arginyl residues in a pseudomonad enzyme of which 3-phosphoglycerate protected 8 per repeating unit. Schloss et al. (1978) have also shown the modification of 3–5 arginine residues per subunit by using phenylglyoxal with the *R. rubrum* enzyme. The inactivation of the same RuBP carboxylase by tetranitromethane, which was correlated with the modification of tyrosine, has been described (Robinson & Tabita, 1979), but criteria to establish the involvement of tyrosine at the active site have yet to be met.

Diethyl pyrocarbonate (DEP) in aqueous solutions at neutral or slightly acidic pH values has been shown to modify histidine residues in proteins with considerable specificity (Melchior & Fahrney, 1970). For example, the specific modification of 1 essential histidine residue at the active site of succinyl-CoA synthetase (Collier & Nishimura, 1979), tryptophanyl-tRNA synthetase (Favorova et al., 1978), L-α-hydroxy acid oxidase (Meyer & Cromartie, 1980), apamin (Vincent et al., 1975), and lactate dehydrogenase (Holbrook & Ingram, 1973) has been achieved with DEP. The use of this reagent has been reviewed recently (Miles, 1977). Quite recently we have shown the inactivation of RuBP carboxylase (Saluja & McFadden, 1980) from spinach by this reagent.

In the present studies we have further investigated and characterized the modification of histidine in RuBP carboxylase/oxygenase by DEP. Enzymes from two markedly different phylogenetic sources, one of the photosynthetic nonsulfur purple bacteria, *Rhodospirillum rubrum*, and a plant, spinach, were used in these experiments. The enzyme from spinach has a molecular weight of 560 000 consisting of eight large (L, 56 000 daltons) and eight small (S, 14 000 daltons) subunits whereas that from the *R. rubrum* enzyme is an L_2 enzyme lacking small subunits and is the smallest RuBP carboxylase (Tabita & McFadden, 1974; McFadden, 1980). Because of the major differences in quaternary structure, the carboxylase/oxygenases from these two organisms provide an especially suitable system to probe for common structural features. In this context, comparisons of the

† From the Institute of Biological Chemistry and the Biochemistry/Biophysics Program, Washington State University, Pullman, Washington 99164. *Received January 2, 1981; revised manuscript received July 22, 1981.* This research was supported in part by grants from the Herman Frasch Foundation and the National Institutes of Health (GM-19,972).

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structure–function relationship for these two distinctly different enzymes may provide useful insights into the evolutionarily conserved features of RuBP carboxylase/oxygenase (McFadden, 1973).

Experimental Procedures

Materials. 3-Phospho-D-glyceric acid (3-PGA), tetrasodium salts of RuBP and sedoheptulose 1,7-bisphosphate (SBP), and DEP were purchased from Sigma Chemical Co. Carboxyhexitol 1,6-bisphosphate (CHBP) was prepared by the method of Gordon et al. (1980). $NaH^{14}CO_3$ was obtained from ICN. [^{3}H]DEP, which had been synthesized by the method of Melchior & Fahrney (1970), was kindly supplied by Professor J. S. Nishimura of the University of Texas. Other chemicals used during this investigation were of reagent quality.

Ribulosebisphosphate carboxylase used in this investigation was obtained from two different sources, *Rhodospirillum rubrum* and spinach (*Spinacia oleracea*). To procure the enzyme from *R. rubrum*, we grew the organism in the light on butyrate and purified the enzyme as described by Schloss et al. (1979), except that DEAE[1]-Sephacel (Pharmacia) was used instead of DEAE-cellulose for column chromatography. The former has the advantage of higher flow rates compared to the latter. In the first DEAE-Sephacel column, eluted with a 0–0.3 M linear gradient of NaCl dissolved in 50 mM Tris-HCl, 50 mM $NaHCO_3$, 20 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 1 mM EDTA (pH 8.0 at 25 °C), the enzyme was centered at 0.2 M NaCl, whereas in the second DEAE-Sephacel column, which was eluted with a 0.05–0.15 M linear gradient of potassium phosphate buffer (containing 10 mM 2-mercaptoethanol and 0.1 mM EDTA, pH 7.6), the enzyme was centered at 0.14 M phosphate. The enzyme was concentrated by precipitation with $(NH_4)_2SO_4$. To obtain the enzyme from spinach, we obtained the commercial preparation from Sigma Chemical Co. and further purified it by sedimenting 4 mg/mL of the crude enzyme into a 0.2–0.8 M linear sucrose gradient prepared in TEMB buffer containing 50 mM Tris, 1 mM EDTA, 20 mM $MgCl_2$, and 50 mM $NaHCO_3$ (pH 8.0 at 25 °C). Specific activities of the enzymes, which were homogeneous by the criterion of polyacrylamide gel electrophoresis, were 4.0 and 1.5 μmol of CO_2 fixed 1 min^{-1} (mg of protein)$^{-1}$ for the *R. rubrum* and the spinach enzyme, respectively. The protein concentration of the purified enzyme was determined either by the method of Lowry et al. (1951) or from the absorbance at 280 nm by assuming an $E^{1\%}_{1cm}$ of 9.74 for the *R. rubrum* enzyme and 16.4 for the spinach enzyme (Tabita & McFadden, 1974; Schloss et al., 1979).

The *R. rubrum* enzyme was stored at 2 °C in TEM buffer (50 mM Tris, 1 mM EDTA, and 20 mM $MgCl_2$, pH 7.0 at 25 °C). The spinach enzyme when stored at 2 or −20 °C in TEMB buffer containing 0.5 M sucrose lost little activity in 6 weeks.

RuBP Carboxylase Assay. Unless otherwise indicated for the assay of RuBP carboxylase, the reaction mixture (at pH 8.0, 25 °C) contained in 150 μL the following: 2.4 mol of $MgCl_2$, 0.4 μmol of EDTA, 0.1 μmol of dithiothreitol, 6 μmol of $NaH^{14}CO_3$ (sp act. 0.2 μCi/μmol), 5 μmol of Tris-HCl, 0.25 μmol of RuBP, 2.0 μmol of histidine, and 10–40 μg of the enzyme. The reaction mixture (except RuBP, which was contained in 50 μL) was preincubated for at least 10 min at 30 °C before initiating the reaction at 30 °C with RuBP. In the case of protection experiments, the reaction was initiated by a mixture of RuBP and $NaH^{14}CO_3$ after activation by 18 mM unlabeled bicarbonate in the presence of the other components specified. The reaction was terminated with 60% cold trichloroacetic acid 2 min after the addition of RuBP. An aliquot was transferred to a scintillation vial, and excess $^{14}CO_2$ was liberated at room temperature overnight or at 70 °C for 2 h. The sample was dissolved in 0.5 mL of water, 5 mL of Ready-Solv MP (Beckman) scintillation fluid added, and the radioactivity counted in a Beckman LS-9000 liquid scintillation counter. The activity was measure as RuBP-dependent incorporation of $^{14}CO_2$ into acid-stable product.

RuBP Oxygenase Assay. The oxygenase activity was monitored at 30 °C by measuring RuBP-dependent oxygen consumption with an oxygen electrode (Hansatech Ltd.) with a continuous recording of oxygen uptake from a solution that had been equilibrated with air. The reaction mixture (in a 0.75-mL chamber) consisted of buffer containing 72 mM Tris-HCl, pH 8.0 (25 °C), 13.3 mM $MgCl_2$, 1.3 mM EDTA, 2 mM $NaHCO_3$, and 5 mM histidine, the enzyme, and 0.94 mM RuBP unless otherwise specified. The reaction was initiated with enzyme (150 μL) that had been preincubated at 30 °C for 20 min in TEMB buffer containing 10 mM $NaHCO_3$ and 25 mM histidine, and the initial linear time course of oxygen consumption was used to calculate the reaction velocity.

Inactivation with Diethyl Pyrocarbonate. For a typical inactivation experiment the enzyme was concentrated (1–2 mg/mL) by dialysis in vacuo against TEM buffer (pH 7.0 at 25 °C). The enzyme was incubated with the indicated concentration of DEP dissolved in ethanol at 30 °C. The ethanol concentration did not exceed 3% by volume and was found to have no effect on the activity and stability of the enzyme during the incubation time. After an appropriate time, an aliquot of the incubation mixture was added to an equal volume of a quenching buffer [TEM buffer containing 50 mM hisitidine, 20 mM $NaHCO_3$, and 2 mM dithiothreitol (pH 8.0, 25 °C)], and the remaining activity was assayed for RuBP carboxylase and/or oxygenase as described above. In the experiments with [^{3}H]DEP, the excess reagent was removed by extensive dialysis against TEM buffer. The small amounts (200 μL) were easily dialyzed by using Spectra/Por 2 semimicrodialysis tubing. For determination of the concentration of DEP, 5 μL of the [^{3}H]DEP reagent used was added to 995 μL of TEM containing 50 mM histidine, and the absorbance at 240 nm was measured. A molar extinction coefficient of 3200 M^{-1} cm^{-1} was used (Miles, 1977).

In the protection experiments, the enzyme was preincubated with the substrate, product, or the competitive inhibitor at the concentrations indicated in the table or figure legends for at least 5 min prior to the addition of DEP.

Reactivation with Hydroxylamine. Recovery of the enzyme activity, after inactivation with DEP, was tested by adding NH_2OH (pH 7.0) at a final concentration of 0.5 M and incubating at 25 °C for the indicated time or at 2 °C for 24–48 h. Controls were compared in which no NH_2OH had been added. Before the assay for reactivated enzyme, NH_2OH was removed either by passage through a Sephadex G-25 column (PD-10 column of Pharmacia) or by extensive dialysis against TEM buffer.

Spectroscopic Studies. The differential spectra of carbethoxylated vs. untreated enzyme was obtained on the Cary Model 14 spectrophotometer. Diethyl pyrocarbonate did not contribute to the absorption. The kinetics of modification of histidine residues were followed at 30 °C by differential absorption at 240 nm. The number of modified histidine residues

[1] Abbreviations: DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; NaDodSO$_4$, sodium dodecyl sulfate.

was determined by using 3200 M^{-1} cm^{-1} as the molar absorption coefficient for carbethoxyhistidine residues.

Electrophoresis. To determine the site of DEP modification of RuBP carboxylase/oxygenase, we treated 0.26 mg of the spinach enzyme with [^{3}H]DEP, quenched the reaction, and removed excess [^{3}H]carbethoxyhistidine by dialysis against TEM buffer with at least a 1000-fold volume excess present during each of two changes of buffer. The modified enzyme was dissociated by incubation with 2.5% $NaDodSO_4$ and 4 mM 2-mercaptoethanol at 25 °C for 2 h. The dissociated enzyme (17 or 25 μg) was loaded in replicate on each of several lanes of a 0.4% $NaDodSO_4$–12.5% polyacrylamide slab gel. The sample was allowed to pass through the spacer gel at a current of 12 mA, and the electrophoresis was then continued for 7 h at 25 mA. After electrophoresis, the 90 mm long slab gel was cut vertically into two parts, and one part was fixed in 50% trichloroacetic acid and stained with Coomassie brilliant blue R by the procedure of Laemmli (1970). After the excess of dye was destained, this portion of the slab gel was scanned by using Beckman scanning and computing densitometers (Models R-112 and R-115). The other part of the gel was further cut vertically into lanes. Each of these lanes was then cut horizontally into small slices of 3-mm length and was processed for radioactive counting by the method of Goodman & Matzura (1971).

Results

Inactivation of Active and Inactive Enzymes. RuBP carboxylases from spinach and *R. rubrum* were rapidly inactivated by 0.6 and 1.7 mM DEP in Tris–EDTA–Mg^{2+} (TEM, pH 7.0) and Tris–EDTA–Mg^{2+}–HCO_3^- (TEMB, pH 8.0) buffers, respectively. Thus, both the inactive state and the fully active state, favored by the inclusion of 50 mM HCO_3^- and by pH 8.0, were vulnerable to modification. The modifications of both states were closely similar, as indicated by the stoichiometry in the presence and absence of the competitive inhibitor sedoheptulose 1,7-bisphosphate (see Stoichiometry of DEP Inactivation).

DEP is much less stable at a pH of 8.2 than at 7.5 (Miles, 1977) although activation of RuBP carboxylase is strongly favored as the pH is raised in this interval and higher (Lorimer et al., 1976). To minimize the decomposition of DEP and turnover of the substrate RuBP in protection studies, we obtained the following results, except as noted, with enzyme in TEM buffer at pH 7.0.

Kinetics of Inactivation by Diethyl Pyrocarbonate. RuBP carboxylases from spinach and *R. rubrum* were rapidly inactivated when incubated with low concentrations of DEP at pH 7.0 and 30 °C. The inactivation rates for the *R. rubrum* enzyme at varying concentrations of DEP obeyed pseudo-first-order kinetics until the loss of activity exceeded 80%. Similar inactivation was observed with the spinach enzyme (Saluja & McFadden, 1980). Complete inactivation was obtained upon longer incubations at 1 mM DEP. For example, 5- and 10-min incubations resulted in complete loss of activity of the bacterial and plant enzyme, respectively. A second-order rate constant (k) and the reaction order with respect to DEP for inactivation were determined from a plot of log k' vs. log (DEP) as described by Levy et al. (1963):

$$\log k' = \log k + n \log (\mathrm{DEP})$$

where k' is the apparent first-order rate constant at a particular DEP concentration. From the intercept of such a plot, shown in Figure 1, values of 39 and 14 M^{-1} s^{-1} were obtained for second-order rate constants for the *R. rubrum* and spinach enzymes, respectively. Slopes of the lines in this plot yielded

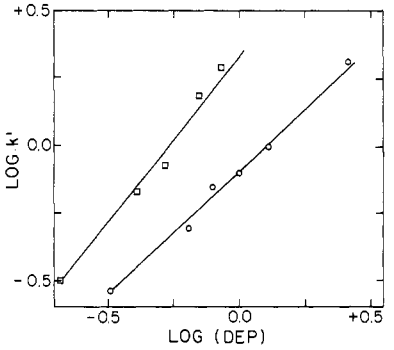

FIGURE 1: Kinetic order of the reaction between RuBP carboxylase and DEP with respect to DEP. Units of time and concentration were minutes and millimolar, respectively. The data for the *R. rubrum* (□) enzyme were obtained as follows: The enzyme in TEM buffer, pH 7.0 (25 °C), was incubated with DEP. After incubation at 30 °C, 20-μL aliquots were removed and treated with an equal volume of 50 mM histidine contained in TEMB buffer, pH 8.0 (25 °C). The RuBP carboxylase activity was measured as described under Experimental Procedures. The data for the spinach (○) enzyme are from Saluja & McFadden (1980). The slope of the plots gives n equal to 1.23 ± 0.21 and 0.93 ± 0.17 for the bacterial and plant enzymes, respectively.

reaction orders of 1.23 ± 0.21 and 0.93 ± 0.17 for the bacterial and plant enzymes, respectively.

Although the inactivation was somewhat faster at pH 6.2 and 6.6, a pH of 7 was chosen for detailed study because the solubility of the spinach enzyme was appreciably greater at this pH. There was also substantial inactivation at pH 8.1, but higher concentrations of DEP were required to achieve comparable inactivation to that observed at lower pH values. The nature of the buffer (Tris or phosphate) did not (data not shown) make any significant difference in the inactivation nor did the omission of $MgCl_2$. Under the conditions of inactivation used in the present investigation (30 °C in 50 mM Tris, 20 mM $MgCl_2$, and 1 mM EDTA, pH 7.0), DEP was found to undergo a spontaneous hydrolysis, which followed first-order kinetics with a half-life of 10 min, which appears to be slightly longer than the reported value (Berger, 1975) of 9 min for DEP hydrolysis in 27 mM sodium phosphate, pH 7.0, at 25 °C.

The oxygenase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase from *R. rubrum* and spinach was also inactivated by DEP (Table I).

Protection of RuBP Carboxylase against Inactivation. To characterize the modification of RuBP carboxylase by DEP, we preincubated the *R. rubrum* enzyme at pH 7.0 with the substrate RuBP, the product 3-phosphoglyceric acid, or the competitive inhibitors SBP (Saluja & McFadden, 1978) and carboxyhexitol 1,6-bisphosphate (Gordon et al., 1980) for 5 min at 30 °C prior to the addition of DEP (Figure 2). All of these compounds showed a marked protection against DEP inactivation at all the time intervals tested. The protection by RuBP was observed under conditions (no added bicarbonate) in which substrate turnover should have been minimal.

Protection with 3-phosphoglyceric acid was much less than with RuBP, which may reflect the much weaker binding of 3-phosphoglycerate than of RuBP. However, with all the protectants tested, some inactivation by DEP occurred, which is in accord with other active site modification studies reported in literature [see, for example, Miles (1977)]. The spinach RuBP carboxylase was also similarly protected by these ligands against inactivation by DEP (data not shown). There was less protection at pH 6.4 by RuBP but similar protection at pH 7.6 and 8.1 (data not shown). A control experiment in which

Table I: Recovery of RuBP Carboxylase/Oxygenase Activity after NH_2OH Treatment[a]

concn of DEP (mM)	time of incubation (min)	activity (%) remaining before and after treatment with hydroxylamine[b] RuBP carboxylase before	RuBP carboxylase after	RuBP oxygenase before	RuBP oxygenase after
spinach					
0	5	(100)	(100)	(100)	(100)
0.2	2	82	107	83	109
0.4	1.5	68	100	68	89
0.5	2	27	41	40	63
R. rubrum					
0	5	100	100	100	100
0.2	2	51	83	42	92
0.2	5	34	79	25	84
0.4	2	18	51	19	60
0.4	5	6	33	0	40

[a] The DEP-modified (and ethanol-treated control) enzyme was treated with 25 mM histidine before incubation in the presence of 0.5 M NH_2OH in TEM, pH 7.0, buffer for 2.5 h at 25 °C. After the incubation period, the enzyme was dialyzed twice against at least a 1000-fold excess of TEMB buffer (pH 8.0) and assayed for both activities. [b] The RuBP carboxylase assays were done under conditions very similar to those of RuBP oxygenase assays (as described under Experimental Procedures) except that the concentration of bicarbonate during assay was more (22 mM; due to added $NaH^{14}CO_3$) than in the oxygenase assays.

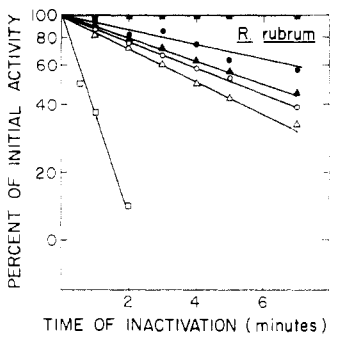

FIGURE 2: Inactivation of *R. rubrum* carboxylase in the presence of various organic phosphates. The enzyme in TEM buffer, pH 7.0 (25 °C), was incubated with the phosphates specified below 5 min prior to the addition of DEP at 30 °C. At the times indicated aliquots were removed; the reaction was quenched and assayed at described in Figure 1. In this experiment the reaction was initiated with a mixture of $NaH^{14}CO_3$ and RuBP. Enzyme + ethanol + H_2O (■); enzyme + ethanol + 0.6 mM DEP and 1.7 mM sedoheptulose 1,7-bisphosphate (●), 2.55 mM RuBP (▲), 1.5 mM carboxyhexitol 1,6-bisphosphate (○), or 15 mM 3-phosphoglyceric acid (△); enzyme + ethanol + H_2O + 0.6 mM DEP (□). The remaining activity was compared with a control lacking DEP but having the same amount of protectant and ethanol.

3 mM RuBP was incubated with 1 mM DEP under conditions similar to those for enzyme inactivation established that RuBP does not react with DEP.

Characterization of DEP-Modified Residues. Although the reaction of diethyl pyrocarbonate with proteins is relatively specific for histidine residues in the pH range of 6–7 (Miles, 1977), other residues may also be modified in neutral or weakly basic media (Melchior & Fahrney, 1970). Therefore, the modification of RuBP carboxylase resulting in inactivation was investigated by (a) following the changes in the absorption spectra in the ultraviolet region and (b) treatment of inactivated enzyme with hydroxylamine.

The reaction of diethyl pyrocarbonate with histidine residues gives an *N*-carbethoxyhistidyl moiety with an absorption maximum near 240 nm or with tyrosyl residues gives a decrease in absorption at 280 nm (Muhlrad et al., 1967). The modification of both *R. rubrum* and spinach RuBP carboxylases was accompanied by a sharp increase in the differential absorption at 240 nm. The difference spectrum of DEP-treated vs. untreated bacterial enzyme, in the ultraviolet region, showed an absorption maximum at 240 nm (Figure 3). There was no change in the ultraviolet spectrum around 280 nm. The absorption increase was essentially the same at pH 6.4 in phosphate buffer and pH 7.0 in Tris buffer. This increase in absorption at 240 nm corresponded to the modification of 4.5 histidine residues (extinction coefficient at 240 nm: 3.2×10^3 M^{-1} cm^{-1}) per 57 000-dalton subunit of the *R. rubrum* RuBP carboxylase. There was a basically similar change in the UV difference spectrum of the spinach enzyme. However, there was a marked difference in the number of histidine residues carbethoxylated between the enzyme from *R. rubrum* and spinach. In the latter case only 2.4 histidine residues per 70 000-dalton combination of large and small subunits were modified (Saluja & McFadden, 1980).

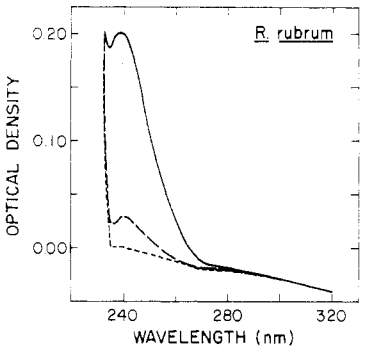

FIGURE 3: Ultraviolet difference spectra of RuBP carboxylase/oxygenase from *R. rubrum* before (---) and after (—) inactivation with DEP in both cases run against untreated enzyme. Samples consisted of 0.8 mL of the enzyme (0.95 mg/mL) in TEM buffer, pH 7.0, and contained 0.47 mM DEP. The difference spectrum was recorded after 20 min of incubation at 30 °C. -- indicates the difference spectrum after treatment of the sample (and the reference) with 0.5 M NH_2OH (contained in TEM buffer, pH 7.0).

Partial recovery of the RuBP carboxylase and oxygenase activities for both bacterial and plant enzymes lost at 30 °C in the presence of varying concentrations of DEP could be achieved by treatment of the modified enzyme with 0.5 M NH_2OH (in TEM buffer pH 7.0) for 2.5 h at 25 °C (Table I). Also, after 48-h incubation at 4 °C of the DEP-modified enzyme with 0.4 M NH_2OH at pH 7.0, residual spinach RuBP carboxylase activity of 55% was increased to 89%. The same incubation in the absence of NH_2OH did not restore activity. Attempts to achieve more effective reactivation were not successful. After complete prior inactivation by DEP, the carboxylase from *R. rubrum* could not be recovered upon NH_2OH treatment (data not shown). In contrast, the oxygenase activity of the enzyme from *R. rubrum* could be reactivated from 0 to 40% with NH_2OH. The differential absorption at 240 nm of DEP-treated vs. untreated enzyme almost completely disappeared during treatment with 0.5 M NH_2OH (Figure 3). Consequently, removal of ethoxyformyl groups from histidine residues was correlated with reactivation of the enzyme.

Location of DEP-Modified Histidine Residues. Since there was modification of about 2 histidine residues per combination of large and small subunits in the spinach enzyme (Saluja & McFadden, 1980), it was important to find the subunit(s) in which the reactive histidine residues are located. To locate the modified histidine residues, we modified the spinach enzyme to complete inactivation with [^{3}H]DEP, subjected it to

Table II: Stoichiometry of DEP Inactivation of RuBP Carboxylase from *R. rubrum*

additions	concn of organic phosphate (mM)	per subunit: histidine residues[b] modified	per subunit: [^{3}H]DEP incorporated	RuBP carboxylase[a] activity (%) remaining
ethanol[c]				100
DEP[d]		4.5	4.2	1
DEP + carboxyhexitol 1,6-bisphosphate	1.5	3.4	3.5	65
DEP + ribulose 1,5-bisphosphate	2.7	3.4	3.4	69

[a] The remaining activity was measured after quenching the reaction with 25 mM histidine (final concentration in TEM buffer, pH 7.0) and dialysis twice against at least a 1000-fold excess volume of TEMB buffer (pH 8.0). [b] Calculated by differential absorption at 240 nm as described in the text. [c] Ethanol equal to that furnished with DEP was added; however, there was no effect of ethanol in comparison with controls lacking ethanol. [d] DEP was dissolved in ethanol and furnished at a final concentration, during the preincubation, of 0.5 mM in all the experiments.

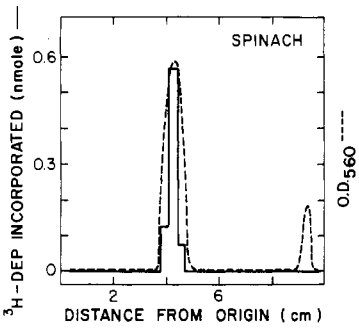

FIGURE 4: Determination of subunit location of DEP-modified histidine residues in spinach RuBP carboxylase. 25 μg of the enzyme that had been inactivated by incubation with 0.6 mM [^{3}H]DEP at 30 °C for 20 min was treated with excess histidine and dialyzed extensively to remove the excess reagent. After dissociation with 2.5% sodium dodecyl sulfate in the presence of 4 mM 2-mercaptoethanol for 2 h at 25 °C, aliquots were loaded on replicate 12.5% $NaDodSO_4$–polyacrylamide gels. After electrophoresis, the gel was analyzed for protein and [^{3}H]DEP incorporation as described under Experimental Procedures. The bars present the average amount of [^{3}H]DEP corporated (maximum: 280 dpm) per gel slice, and the dotted line indicates the relative amount of protein in each peak. The ratio of area under large and small subunit protein peaks was 3.5. A total of 2.3 [^{3}H]DEP molecules were incorporated for each molecule of large subunit.

$NaDodSO_4$–polyacrylamide gel electrophoresis, and determined the radioactivity distribution in gels. As shown in Figure 4, the radioactivity peak overlapped with the protein peak corresponding to large subunits. There was no radioactivity in the small subunit region.

Stoichiometry of DEP Inactivation of RuBP Carboxylase. Initial studies revealed the modification of two histidine residues in the spinach enzyme in the presence or absence of Mg^{2+} (TEM and TE buffers, respectively). To investigate further the number of histidine residues involved at the active site of the enzyme, we preincubated the spinach RuBP carboxylase with 1.5 mM CHBP (a known competitive inhibitor of RuBP carboxylase) and after 5 min modified the CHBP-treated enzyme with DEP. The absorption at 240 nm of the DEP-treated enzyme in the presence and absence of CHBP was measured as a function of time (Figure 5). The differential value (the dashed line) shows that the modification of 1 histidine residue was prevented by CHBP. Similar spectral differences were observed when RuBP, SBP, or PGA were used as protectants instead of CHBP. When 1.5 mM SBP was tested as a protectant against modification by 1.7 mM DEP of active enzyme (in TEMB buffer, pH 8.0), the spectrophotometrically monitored modification of histidines per large subunit was reduced from 2.6 to 1.4. In other experiments (Table II) with the *R. rubrum* enzyme in which activity loss was measured, 1.6 histidine residues per subunit were protected when a correction was made for the fact that an

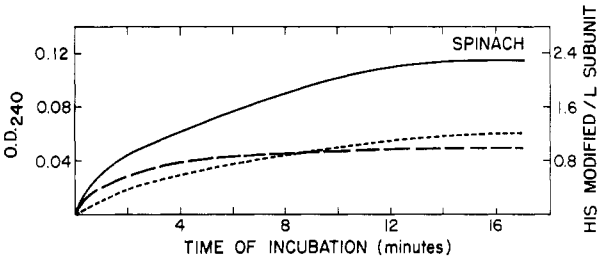

FIGURE 5: Absorption at 240 nm of DEP-inactivated enzyme in the absence (—) or presence of CHBP (---) in comparision with untreated enzyme in the presence of DEP (reference cuvette). The enzyme was preincubated at 30 °C for 5 min with 2 mM CHBP prior to treatment with 0.4 mM DEP. The number of histidine residues modified was calculated from differential absorption at 240 nm. The dashed line (--) was drawn by subtracting the absorption at 240 nm obtained in the presence of CHBP from that in the absence of CHBP.

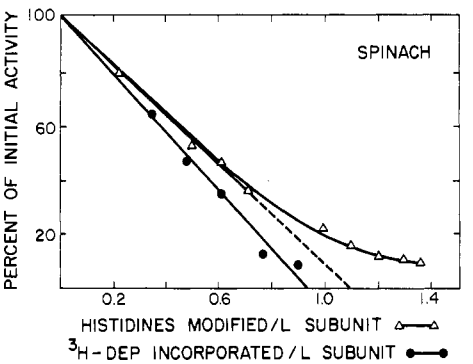

FIGURE 6: Activity as a function of stoichiometry of DEP inactivation of spinach RuBP carboxylase monitored at 240 nm (△). The enzyme was inactivated with 0.46 mM DEP. Activity as a function of stoichiometry of inactivation of spinach RuBP carboxylase by [^{3}H]DEP (●). The enzyme (1.3 mg/mL) was preincubated for 6 min (at 30 °C) with 1.7 mM sedoheptulose 1,7-bisphosphate before the addition of 0.4 mM DEP. After 4 min, the reaction was quenched with histidine and dialyzed extensively in TEM, pH 7.0, buffer, as described in the text. The dialyzed enzyme was incubated at 30 °C with 0.3 mM [^{3}H]DEP. At intervals the samples were removed, treated with histidine, and dialyzed again. The remaining activity and [^{3}H]DEP incorporation were determined for each sample.

average of 33% inactivation occurred in the presence of protective agent.

When spinach RuBP carboxylase was incubated with DEP and the percent residual activity plotted against the number of histidine residues modified (calculated by differential absorption at 240 nm), a line was obtained (Figure 6) that was linear for up to 65% inactivation but deviated from linearity as the inactivation proceeded toward complete loss of activity. When the initial portion of the curve was extrapolated to zero activity, a stoichiometry of 1.15 ± 0.17 (95% confidence level) histidines modified per subunit was obtained. The second linear portion extrapolated to a value of ca. 1.82 ± 0.19.

To test the involvement of a single histidine residue per active site of the spinach RuBP carboxylase, we first incubated the enzyme with unlabeled DEP in the presence of SBP, another competitive inhibitor of the enzyme (Saluja & McFadden, 1978), and after extensive dialysis then incubated it with [^{3}H]DEP. Aliquots were removed at different intervals to determine the residual activity and the number of [^{3}H]-ethoxyformylated groups incorporated into the enzyme. When percent residual activity was plotted against the number of [^{3}H]DEP residues incorporated per large subunit, a stoichiometry of 0.95 ± 0.10 essential histidines per active site was obtained (Figure 6).

Since about 4.5 histidines per subunit of the *R. rubrum* carboxylase were modified during inactivation (Figure 3), the stoichiometry of modification of the *R. rubrum* enzyme was further investigated. The enzyme was incubated with [^{3}H]-DEP in the presence and absence of RuBP or CHBP under conditions that would have led to complete inactivation in the absence of protectants. The amount of [^{3}H]DEP incorporated and the histidine residues modified per subunit were determined (Table II). The number of histidine residues essential for the activity of *R. rubrum* RuBP carboxylase was equal to 1.1 per subunit, correcting for 33% inactivation that occurred in the presence of protectants. Presumably this stoichiometry is more reliable than that of 1.6 obtained by spectrophotometry because unreacted and noncovalently bound [^{3}H]DEP was removed by exhaustive dialysis. In other experiments performed with 1.7 mM DEP on active RuBP carboxylase (TEMB buffer, pH 8.0), the presence of 1.5 mM SBP reduced histidine modification per subunit as measured at 240 nm from 4.6 to 3.6.

Discussion

The data presented in this paper indicate that DEP rapidly and completely inactivates both the carboxylase and oxygenase activities of ribulosebisphosphate carboxylase/oxygenase from *R. rubrum* and spinach. The protection of RuBP carboxylase activity, from both sources, by a substrate, ribulose 1,5-bisphosphate, a product, 3-phosphoglyceric acid, and the competitive inhibitors sedoheptulose 1,7-bisphosphate and 2-carboxyhexitol 1,6-bisphosphate against inactivation strongly suggests that DEP is interacting with the enzyme at or near its active site. The results also suggest that enzyme in the absence of added HCO_3^-, with or without Mg^{2+}, reacts with DEP similarly to active enzyme.

Although DEP reacts preferentially with histidine residues, it is also known to react with a variety of amino acid side chains, including lysine, tyrosine, cysteine, and serine (Melchior & Fahrney, 1970; Miles, 1977). However, the present data suggest that the DEP inactivation of RuBP carboxylase is due to the modification of a histidine residue at the active site. This interpretation is supported by the following facts: (1) in the pH range of 6–7 in which this modification was carried out, DEP is highly specific for histidine modification; (2) the second-order rate constants of 39 and 14 M^{-1} s^{-1} for the *R. rubrum* and spinach enzyme, respectively, are in range for the reaction of imidazole with DEP in model reactions or for modification of histidine residues in other proteins (Cousineau & Meighen, 1976); (3) there is a sharp increase in the absorption at 240 nm due to DEP modification of the *R. rubrum* and spinach enzyme; and (4) the increase in OD_{240} is reversed by NH_2OH, and enzymatic activity is also restored. DEP is also known to modify amino groups, though at pH 4.0, but NH_2OH does not react with ethoxyformylated NH_2 groups. However, NH_2OH does decarbethoxylate *O*-ethoxyformyltyrosyl residues, though at a much slower rate. In the present work tyrosine residues were presumably not modified because no decrease in absorption around 280 nm was observed in the difference spectrum (Figure 3). Garrison & Himes (1975) have reported a reaction between DEP and *N*-acetylcysteine that causes an increase in the absorption at 240 nm, which is also reversed by NH_2OH. We have published data (Saluja & McFadden, 1980) that indicated that inactivation of spinach RuBP carboxylase by DEP treatment is not due to the modification of cysteine residues, which is thought to occur in carboxylate buffers. Such buffers were not employed in the present work.

The failure to realize full recovery of enzymatic activity with NH_2OH treatment and complete discharge of the ethoxyformyl moiety with the *R. rubrum* enzyme (Figure 3) may have been due in part to the reaction of 2 equiv of DEP/equiv of histidine followed by a Bamberger reaction to open the imidazole ring (Avaeva & Krasnova, 1975). Other investigators have also failed to obtain full recovery of enzymatic activity by NH_2OH after DEP inactivation (Miles, 1977). The occasional reaction of 2 DEP molecules per histidine residue under the presently employed experimental conditions may also explain the fractional (4.5) modification of histidine residues per protomer of the *R. rubrum* enzyme. Alternatively, the fractional modification may also result from uncertainties in the determination of protein or the extinction coefficient or from a progressively negative cooperativity dependent upon the order in which reactive histidines are modified. The reactivation of the *R. rubrum* enzyme appeared to be more effective than that of the spinach enzyme (cf. reactivation after 2-min treatment with 0.2 mM DEP, Table I), in spite of the fact that more histidines per subunit were modified. This may reflect the more complex quaternary structure of the higher plant enzyme.

It is worth noting that DEP does not modify all of the available histidine residues in RuBP carboxylase. Out of 12 and 18 histidine residues present in each large subunit of *R. rubrum* (Tabita & McFadden, 1974; Schloss et al., 1979) and spinach (Moon & Thompson, 1969), only 4.5 and 2.2, respectively, are modified by DEP. Our data clearly indicate that DEP exclusively modifies histidines that are located in the large subunit of spinach RuBP carboxylase (Figure 4). Although there are 4 histidine residues in each small subunit (Moon & Thompson, 1969), none of these is modified by DEP.

By using differential absorption at 240 nm in the presence and absence of various protecting agents and by differential labeling with [^{3}H]DEP, we have demonstrated that out of 4.5 and 2.2 histidine residues modified per large subunit of the *R. rubrum* and spinach enzyme only one histidine residue is essential to the catalytic activity of RuBP carboxylase/oxygenase. The kinetic observation that the reaction order with respect to DEP for inactivation is equal to 1.23 ± 0.21 and 0.93 ± 0.17 for the *R. rubrum* and spinach enzymes, respectively, when considered along with the observation that the inactivation of both the enzymes is pseudo first order, reinforces the other evidence that modification of a single histidine residue per enzyme active site is sufficient for inactivation. As indicated earlier, a total of 2 histidine residues per large subunit of the spinach enzyme are modified by DEP. These two residues appear to have different inactivation rates (Figure 6), which is consistent with the proposal that 1 histidine is especially reactive and is involved at the active site of this enzyme.

Although protection of enzymatic activity from an inactivating reagent by substrate or analogues would generally be regarded as excellent evidence in favor of modification at the

active site, it is possible that modification of histidine residues may affect the RuBP carboxylase activity in an indirect way. Diethyl pyrocarbonate may have modified histidine residues located outside the active site and thus caused a conformational change of the enzyme in such a way that it distorted the active site, which resulted in the inactivation of the enzyme. In this case protection by substrate or substrate analogus could be explained by assuming that binding caused a reduction in accessibility of the histidine residues outside the active site. This explanation is much less plausible in explaining the present data because two competitive inhibitors and a reaction product as well as the substrate, which have significantly different structures and affinities for the enzyme, all protected against inactivation by DEP.

An interesting observation is the higher number of total histidine residues modified by DEP per subunit of the bacterial enzyme in comparison with those for the plant enzyme incubated under similar conditions. DEP modifies a maximum of about 4.5 histidine residues per subunit of the bacterial enzyme as compared to 2.2 for the plant enzyme. *R. rubrum* RuBP carboxylase lacks small subunits, but its amino acid composition is similar to the large subunits of the plant enzyme (McFadden & Tabita, 1974). If these enzymes prove to have homologous regions, the interaction of small subunits with large subunits in the plant enzyme may protect histidine residues in the large subunit from modification by DEP.

In conclusion, our results suggest that the modification of one active site histidine results in a parallel loss of RuBP carboxylase and oxygenase. Whether this residue actually participates in catalysis remains to be seen, but the present work establishes that new strategies will soon be available to examine active site peptides of this important enzyme.

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