Table IV: The Partition[a] E-FDP → FDP + E; G3P + DHAP + E

chase (s)	pellet (%)	^{3}HOH (%)	E-FDP → FDP / G3P
none	50.5	2.5	
1	0	14	83/11.5[b]
5	0	13	84/10.5[b]

[a] Each incubation contained in 30 μL E (53 μM), [5-^{3}H]FDP (3.3 μM, 5000 cpm), and DHAP (200 μM). After 3 s, either TCA was added or 200 μL of the chase solution [FDP (1 μmol) + triosephosphate isomerase (150 units)] followed after 1 or 5 s by TCA. ^{3}HOH was determined after microdistillation as in Table III. [b] These values represent the partition of all the bound FDP (corrected for 97% purity and presence of 2.5% as the ternary complex).

the problem of which of these steps is limiting would be reached if the partition of the E–eneamine–G3P, I_4, could be determined. However, its low concentration at equilibrium seems to rule out this approach to the problem.

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Active Site Histidine in Spinach Ribulosebisphosphate Carboxylase/Oxygenase Modified by Diethyl Pyrocarbonate†

Yasuo Igarashi, Bruce A. McFadden,* and Ted El-Gul

Biochemistry/Biophysics Program, Washington State University, Pullman, Washington 99164-4660

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ABSTRACT: [^{3}H]Diethyl pyrocarbonate was synthesized [Melchior, W. B., & Fahrney, D. (1970) *Biochemistry 9*, 251–258] from [^{3}H]ethanol prepared by the reduction of acetaldehyde by NaB^3H_4. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) from spinach was inactivated with this reagent at pH 7.0 the presence of 20 mM Mg^{2+}, and tryptic peptides that contained modified histidine residues were isolated by reverse-phase high-performance liquid chromatography. Labeling of the enzyme was conducted in the presence and absence of the competitive inhibitor sedoheptulose 1,7-bisphosphate. The amount of one peptide that was heavily labeled in the absence of this compound was reduced 10-fold in its presence. The labeled residue was histidine-298. This result, in combination with our earlier experiments [Saluja, A. K., & McFadden, B. A. (1982) *Biochemistry 21*, 89–95], suggests that His-298 in spinach RuBisCO is located in the active site domain and is essential to enzyme activity. This region of the primary structure is strongly conserved in seven other ribulosebisphosphate carboxylases from divergent sources.

Various chemical modifications of D-ribulose-1.5-bisphosphate carboxylase/oxygenase (RuBisCO,[1] EC 4.1.1.39) have shown that lysyl, cysteinyl, histidyl, tyrosyl, arginyl, and methionyl residues may be located in the active site domain of this enzyme [for a review, see Miziorko & Lorimer (1983)]. Among those, the locations of only lysyl (Lys-175 and Lys-334) and cysteinyl (Cys-202 and Cys-459) residues have been determined in the large subunit of spinach RuBisCO to date. The requirement of Lys-175 for activity and its placement at the active site are especially well established (Schloss et al.,

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[1] Abbreviations: DEP, diethyl pyrocarbonate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; NEM, *N*-ethylmaleimide; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, D-ribulose 1,5-bisphosphate; SBP, D-sedoheptulose 1,7-bisphosphate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

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1978; Stringer & Hartman, 1978; Spellman et al., 1979; Zurawski, 1981).

Our earlier experiments on spinach RuBisCO have shown the following: (1) The enzyme is rapidly inactivated by diethyl pyrocarbonate (DEP) under conditions in which two to three histidine residues per 66 500-dalton combination of large and small subunits are modified (Saluja & McFadden, 1980). (2) DEP-modified histidine residues are located on the large subunit, in which catalytic activity resides; RuBP and several competitive inhibitors including SBP protect the enzyme against inactivation by DEP and protect one histidine residue against modification (Saluja & McFadden, 1982). (3) RuBisCO is rapidly inactivated by photooxidation sensitized by pyridoxal phosphate, which is bound at Lys-175. Both photooxidation and ethoxyformylation of the essential histidine by DEP occur at the same histidine residue (Bhagwat & McFadden, 1983).

From the above results, we have concluded that the active site domain of spinach RuBisCO has one histidine residue that is essential for activity. In this paper, we describe the synthesis of [^{3}H]DEP, the labeling of RuBisCO in the presence of the competitive inhibitor SBP, the isolation of several tryptic peptides, one of which contains the essential histidine, and the location of this residue in the sequence of the large subunit of the spinach enzyme.

Experimental Procedures

Materials. Sodium salts of SBP, MOPS, RuBP, and TPCK-treated trypsin (type XIV) were purchased from Sigma Chemical Co. Acetonitrile was of HPLC grade from J. T. Baker Chemical Co. $NaH^{14}CO_3$ was obtained from ICN. ^{3}H-Labeled DEP (2.73 Ci/mol) was synthesized as described in the next section. All other reagents were the highest grade available. PD-10 filtration columns (5 cm long) were purchased from Pharmacia Fine Chemicals, Inc.

Ribulosebisphosphate carboxylase/oxygenase (RuBisCO) was purified from 125 g of fresh spinach (Berhow et al., 1982), and 200 mg of the enzyme was stored in 5–10-mL batches (2–3 mg of RuBisCO/mL) at −120 °C. Before each modification, solid $(NH_4)_2SO_4$ was added to one batch to 70% saturation at 2 °C, and the precipitate was collected by centrifugation in the cold and dissolved at 2 °C in 1 mL of 50 mM MOPS, pH 7.0, containing 1 mM EDTA and 20 mM $MgCl_2$ (MEM buffer). The solution was then passed through a PD-10 column at 4 °C that had been equilibrated with MEM buffer. Fractions that contained RuBisCO were collected and used for further experiments after adjustment to the desired concentration by MEM buffer. The protein concentration was determined by using the relationship that an absorbance of 1 (in 1.0 cm path cell) at 280 nm corresponds to a RuBisCO concentration of 0.61 mg/mL (Wishnick and Lane, 1971).

Synthesis of [^{3}H]Ethanol. A mixture of 2.5 mL of anhydrous triglyme (freshly distilled) and 76 mg of sodium borotritide (2 mmol, 100 Ci/mol; from Research Products International Corp., Mt. Prospect, IL) was gently stirred in a 5-mL round-bottom flask attached to a Vigreux column. After this was stirred at 2 °C for 30 min during which most of the NaB^3H_4 dissolved, a solution of 0.44 g (10 mmol) of acetaldehyde in 1.5 mL of triglyme was injected through a septum over a 5-min period during which the mixture was stirred and cooled in ice. After replacement of the ice bath with a water bath at 20 °C, the mixture was incubated for 2 h, cooled again in ice, and stirred as 0.2 mL of water was carefully added dropwise. After this was heated gently to 60 °C for 25 min and cooled to room temperature, 0.5 mL of ethanol was added, and 1 mL of ethanol was removed by distillation at 78–79 °C.

Synthesis of [^{3}H]DEP. Sodium metal pieces (506 mg, 22 mmol) were stirred in 15 mL of anhydrous ether (from a freshly opened can) in a three-necked round-bottom flask equipped with a dropping funnel and condenser. After this was flushed with N_2 for 20 min and then with a stream of CO_2 that had been passed through H_2SO_4, 1 mL of [^{3}H]ethanol prepared as described was added dropwise over a 5-min period while stirring the mixture in an ice bath. After the ice bath was removed, the mixture was incubated at 22 °C for 6 h. The flow of CO_2 was then stopped and most of the ether removed by rotary evaporation. Then, 3.1 mL of ethyl chloroformate (33 mmol) from a freshly opened bottle was added and the mixture stirred gently at 60 °C for 24 h. After cooling to 22 °C, the mixture was centrifuged and the supernatant transferred to a 5-mL round-bottom flask attached to a Vigreux column. Excess ether was removed in vacuo at room temperature, and the product was distilled at low pressure (0.29 mmHg) and 40 °C. The distillate (1.54 g) contained [^{3}H]-DEP, which was sealed in ampules in 50-μL portions and stored at −120 °C and further characterized as described.

RuBP Carboxylase Assay. Carboxylase activity was assayed according to the general method of McFadden et al. (1975) by measuring the RuBP-dependent incorporation of $^{14}CO_2$ into acid-stable product, after quencing with glacial acetic acid.

Radioactivity. Aliquots (50–200 μL) of samples were added to 5 mL of aqueous counting scintillant (Amersham Corp.). Radioactivity was measured with a Beckman LS9000 liquid scintillation counter.

Treatment of RuBisCO by DEP. The enzyme (approximately 6 mg/mL of MEM buffer) was modified by DEP in the presence or absence of SBP. When SBP was used, the enzyme solution was preincubated with 2 mM SBP for 5–10 min at 30 °C before DEP modification. After 30-min incubation at 30 °C with 0.4 mM ^{3}H-labeled DEP, the solution (ca. 1.5 mL) was passed through a PD-10 column at 22 °C, which had been equilibrated with MEM buffer. In ca. 3 min, the excluded fraction which contained enzyme was obtained and used to determine protein concentration, radioactivity, and enzyme activity. The incorporation of 1.53×10^3 cpm corresponds to the modification of 1 nmol of histidine residues.

The procedure just described using the Mg^{2+} form of the enzyme was employed for all experiments unless otherwise indicated. Some CO_2 was dissolved in the buffers employed, but the concentration of large subunits of ca. 110 μM was in large molar excess (ca. 10:1) in these experiments. Under these conditions well under 10% of the RuBisCO is active (Lorimer et al., 1976). Other labeling experiments were conducted as described but in the absence of added Mg^{2+}. In addition, experiments were done as specified (in the presence of 20 mM Mg^{2+}) and in the presence of 20 mM $NaHCO_3$ at a somewhat lower enzyme concentration (4 mg/mL of MEM buffer). Under these conditions (pH 7.0), the enzyme is fully activated.

Preparation of Tryptic Digests. After PD-10 column filtration, the enzyme fraction was incubated with 5 mM DTT at 30 °C for 30 min. This solution was again rapidly passed through a preequilibrated PD-10 column at 22 °C to exlude unreacted DTT, and DTT-treated enzyme was then incubated with 0.5 mM NEM at 30 °C in MEM buffer. After 30-min incubation, the enzyme contained in ca. 1.5 mL was heated at 65 °C for 40 s and cooled to room temperature. Removal of NEM by gel filtration before heat denaturation had no effect upon the distribution of radioactivity in tryptic peptides

and was avoided to minimize dilution of labeled RuBisCO. Denatured protein was collected by centrifugation and resuspended in 750 μL of MOPS buffer (50 mM, pH 7.0). Approximately 45 μL of TPCK-treated trypsin (5% w/w) was added to the suspension and digestion carried out for 4 h at 30 °C.

HPLC of Tryptic Digests. The tryptic digest was centrifuged for 2.5 min with a Beckman microfuge B at ca. 9000 g, and 500 μL of supernatant was injected into a Pharmacia FPLC system. A RP-300 CO3-10A column (4.6 mm i.d. × 25 cm, Brownlee Laboratories Inc.) was used for peptide separation with an acetonitrile gradient to be described. Prior to a repeated chromatographic purification, the fraction of interest was lyophilized and redissolved in 750 μL of water. This solution was centrifuged by a Beckman microfuge B, and 500 μL of supernatant was loaded on the same column and purified by the same general procedure.

Amino Acid Analysis. Acid hydrolysis of peptides was achieved in evacuated sealed tubes with 6 N HCl at 110 °C for 24 h. Samples were chromatographed on a Beckman 121 MB amino acid analyzer in the "1-h single-column" system.

Results

Characterization of [^{3}H]DEP. Initially the possibility of preparing [^{3}H]DEP by Pd-catalyzed exchange of tritium gas with DEP was explored (Öberg, 1971). Unfortunately, after the exchange had been done for us commercially, almost no diethyl pyrocarbonate could be detected spectrophotometrically, indicating that this compound had decomposed during the exchange. We then considered the possibility of using an analogous exchange technique to prepare [^{3}H]ethanol as a starting material for the synthesis of [^{3}H]DEP, but the cost was too high. The possibility of using commercially available [^{14}C]ethanol was abandoned because it is very costly. The presently described synthesis was therefore developed and resulted in [^{3}H]ethanol of high specific radioactivity from which [^{3}H]DEP was synthesized. Initially, opened ampules have analyzed 60% DEP spectrophotometrically on the basis of reaction with an excess of N^{α}-acetyl-L-histidine (Miles, 1977).

After opening an ampule, the DEP content declined due to hydrolytic decomposition even though the opened ampule had been stored at −120 °C for 5 weeks. Nevertheless, it was possible to readily estimate the specific radioactivity of the [^{3}H]DEP (Figure 1) after reaction with N^{α}-acetyl-L-histidine from the absorbance and radioactivity in the second peak after column chromatography on a Dowex anion-exchange resin. This second peak was due to (ethoxyformyl)acetylhistidine, and from the data a specific radioactivity of 2.73 Ci/mol was computed for the [^{3}H]DEP. The first peak (Figure 1) was due largely to unreacted [^{3}H]DEP, which had been used in 20% molar excess to histidine.

DEP Modification. At the high enzyme concentrations used in the present experiments, 2.5 histidine residues per large subunit were modified by [^{3}H]DEP in the absence of SBP. In the presence of 2 mM SBP, 1.7 residues per large subunit were modified. The remaining carboxylase activity in each case was 14% and 84% of the initial activity (ca. 3.0 units/mg of protein), respectively. Thus, the modification of 0.8 histidine residue was associated with 70% inactivation. In all cases examined there was excellent concordance between histidines modified as measured either spectrophotometrically or by ^{3}H incorporation, establishing the efficacy and specificity of the synthetic [^{3}H]DEP.

HPLC of Tryptic Digests. Tryptic digests of enzyme that had been modified by DEP in the presence of 20 mM Mg^{2+} and in the presence or absence of SBP [SBP(+) or SBP(−)] were each loaded on the reverse-phase RP-300 column and eluted by changing the concentration of CH_3CN. The total radioactivity in the SBP(−) digest loaded on the column was 66 340 cpm (129 740 dpm, which corresponded to 1.15 mg of undigested enzyme) and in the SBP(+) digest was 33 900 cpm (65 900 dpm, which corresponded to 0.85 mg of undigested enzyme).

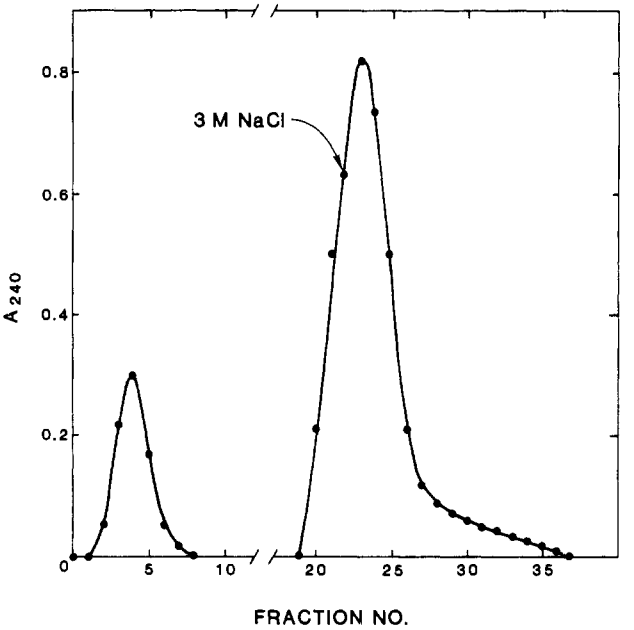

FIGURE 1: Chromatography of [^{3}H](ethoxyformyl)-N^{α}-acetyl-L-histidine on a 0.8 × 4 cm Dowex 1-X8 column (50–100 mesh). [^{3}H]DEP was adjusted to a concentration of 10 mM in ethanol, and 0.5 mL was incubated with 0.4 mL of 10 mM N^{α}-acetyl-L-histidine for 15 min at 30 °C after which the mixture was rapidly cooled to 2 °C. The mixture was then transferred to a column that had been sequentially washed with 15 column volumes of 1 N NaOH, 40 volumes of 0.1 M sodium formate, and 40 volumes of H_2O. Elution was then conducted at 2 °C at a flow rate of 0.3–0.6 mL/min; a linear 0–3 M NaCl gradient was started at fraction 16. At 0.18 M NaCl (fraction 22), elution was started with 3 M NaCl. Absorbance values are shown for each 1.0-mL fraction. For radioactivity measurements, 20 μL aliquot portions were counted and two peaks noted (fractions 2–14 and 20–39). The radioactivity per A_{240} unit (using a 1.0-cm light path) in fractions 20–26 was constant, and was used to calculate (Miles, 1977) the specific radioactivity (dpm mL mmol^{-1}) of (ethoxyformyl)acetylhistidine in each fraction from dpm/[(A_{240}/2)/3.6 × 10^3]. The A_{240} is divided by 2 because only one of the two ethoxyformyl moieties (detected by spectrophotometry) is labeled. Finally, the specific radioactivity of 2.73 mCi/mmol calculated for DEP is twice that of the ethoxyformyl moiety incorporated either into proteins or N^{α}-acetyl-L-histidine. From the radioactivity distribution in the neutral and anionic fractions, it can be shown that the radiolabeled DEP was ca. 37% pure. Presumably, the major radioactive impurity was [^{3}H]ethanol.

A typical chromatogram of the SBP(−) 4-h digest is shown in Figure 2 in terms of absorbance at 214 nm. In the separation shown in Figure 2, eight radioactive peaks, in addition to a small radioactive peak in the void volume, were observed. Amino acid analysis showed that all of these radioactive fractions contained significant amounts of histidine. Label in three of these eight radioactive peaks was markedly reduced in a chromatogram of the SBP(+) digest. Those were peaks II, III, and V (Table I). When the difference in total labeling per mg of RuBisCO with and without SBP (7700 cpm) is considered, peak III accounted for 69% of the label. Peaks absorbing at 214 nm and which corresponded to radioactive peaks II and III in the chromatogram of the SBP(−) digest were very small in the chromatogram of the SBP(+) digest

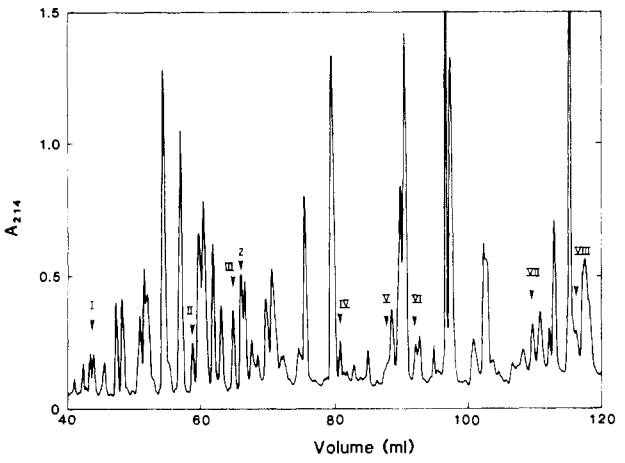

FIGURE 2: Fractionation of an SBP(−) tryptic digest. Spinach RuBisCo was modified by [^{3}H]DEP in the presence of 20 mM Mg^{2+} and in the absence of SBP. A tryptic digest of modified enzyme was chromatographed on a reverse-phase RP-300 column. Only part of the chromatogram is shown. Radioactive peaks I–VIII were collected separately to count radioactivity (Table I). Peak Z was a nonradioactive peptide whose amino acid composition was determined (Table II). Other conditions were as follows: flow rate 0.8 mL/min; eluting solvent A, 15 mM sodium phosphate buffer, pH 4.0; eluting solvent B, 60% (v/v) acetonitrile in the sodium phosphate buffer. The composition of the eluting mixture (B in A) was as follows: 0%, 0–5 mL; a linear 0–60% gradient, 5–125 mL.

Table I: Distribution of Radioactivity in a Chromatogram of Tryptic Digests[a] Derived from Enzyme Labeled in the Presence of 20 mM Mg^{2+}

radioactivity distribution	cpm incorporated/mg of RuBisCO[b]		
	SBP(−)	SBP(+)	differential labeling
peak I	4290	4250	40
peak II	2100	750	1350
peak III	5980	630	5350[c]
peak IV	2640	2150	490
peak V	1920	610	1310
peak VI	2580	2920	−340
peak VII	3370	3850	−480
peak VIII	2940	2960	−20
peaks I–VIII	25820	18120	7700

[a] Tryptic digests of RuBisCO modified by [^{3}H]DEP in the absence [SBP(−)] or presence [SBP(+)] of SBP were chromatographed under the conditions described in Figure 1, and the radioactivity of peaks I–VIII was measured. [b] Total radioactivity (cpm) loaded on the column (per mg of RuBisCO) was 57690 (−SBP) and 39880 (+SBP). [c] The percentage of differential labeling in this peak (5350/7700) × 100 was 69%.

(data not shown). It is important to stress that the recovery of radioactivity in the eight chromatographic peaks was 45% in both SBP(+) and SBP(−) experiments. The remainder (39%) was recovered in the rather high and uniform background observed throughout the chromatogram but especially after 31 mL of effluent and in the earlier fractions (8–9%).

Chromatography has also been carried out after DEP modification of RuBisCO in the presence of 20 mM Mg^{2+} and in the absence of SBP followed by 24-h tryptic digestion. All eight radioactive peaks were observed (Table I), but the recovery of radioactivity was much lower (25–30%). This diminished recovery and the chromatographic recoveries described suggest that DEP-modified peptides are somewhat unstable as would be expected and decompose during the experiments reported here.

Three radioactive fractions, the major peak III and two minor peaks II and V, from the SBP(−) 4-h digest were further purified. After repeated chromatography of peak III, only the major peptide peak (peptide III-a) had radioactivity (Figure 3). By rechromatography of peak II, radioactivity was recovered in a symmetrical peak (fraction II-c), which showed absorbance at 214 nm. Upon rechromatography of peak V, a broad radioactive peak was detected, but this radioactive peak did not coincide with a 214 nm absorbing peak. Under other conditions of inactivation in the presence of 20 mM Mg^{2+} and 20 mM $NaHCO_3$ (pH 7.0) or without addition of these activators, the differential labeling patterns were closely similar to those reported in Table I.

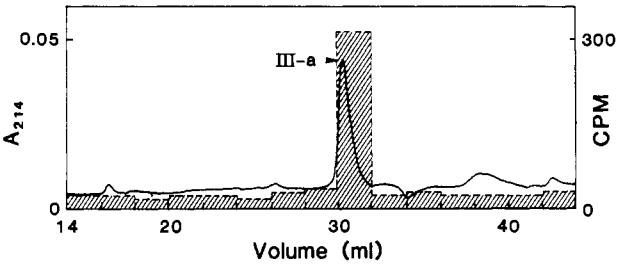

FIGURE 3: Purification of a radioactive peptide in peak III. The peak III fraction in Figure 1 was rechromatographed after lyophilization and resuspension in water. Only the part of the chromatogram containing absorbance or radioactivity peaks is shown. Radioactivity (cross-hatched areas) was determined in a 200-μL portion of each 2.0-mL fraction and was found only in the major A_{214} peak III-a (—). Other conditions were as described in Figure 1 except that the eluting mixture (B in A) was as follows: 23%, 0–35 mL; a linear 23–33% gradient, 33–55 mL.

Table II: Amino Acid Composition of Isolated Peptides

	peptide III-a	theoretical 296–303[a]	peptide Z[b]	theoretical 286–295[c]	fraction II-c[d]
Asx	1.19	1	1.74	2	1.14
Thr	0.14		0.20		0.29
Ser	trace[e]		trace		0.42
Glx	0.22		0.27		0.67
Pro	trace		trace		0.23
Gly	0.29		1.02	1	0.86
Ala	2.13	2	0.30		0.76
Cys[f]					
Val	1.12	1	0.17		1.15
Met	0.90	1	trace		0.20
Ile	0.77	1	0.85	1	0.32
Leu	0.20		2.83	3	0.25
Tyr	trace		trace		0.07
Phe	trace		0.16		0.34
Lys	trace		0.28		0.18
His	1.01	1	1.69	2	0.85
Arg	(1.0)[g]	1	(1.0)[g]	1	0.60
Trp[f]					

[a] Theoretical amino acid residues per octapeptide (the 296th–303rd positions in spinach RuBisCO). [b] A nonradioactive peptide. [c] Theoretical amino acid residues per decapeptide (the 286th–295th position in spinach RuBisCO). [d] Values are given as nanomoles of each amino acid in the sample. [e] Trace is less than 10% of the arginine value. [f] Amount not determined. [g] Assigned a value of 1.0 with all other values in the same column normalized with respect to Arg.

Amino Acid Analysis of Tryptic Peptides. The composition of the hydrolysate of peptide III-a was in good accord with that of a tryptic peptide that could be expected from the primary structure of spinach RuBisCO (Zurawski et al., 1981). This tryptic peptide coincides with the 296th–303rd positions from the N-terminus and has one histidine residue at the 298th position (Table II).

Fraction II-c, which was obtained in high recovery of radioactivity (up to 70%) by rechromatography of peak II, contained almost all amino acids (Table II), including both arginine and lysine, although tryptophan and cysteine could not be measured by the method employed. The specific radioactivity (dpm/nmol of His) was 1500–2000 (theoretical

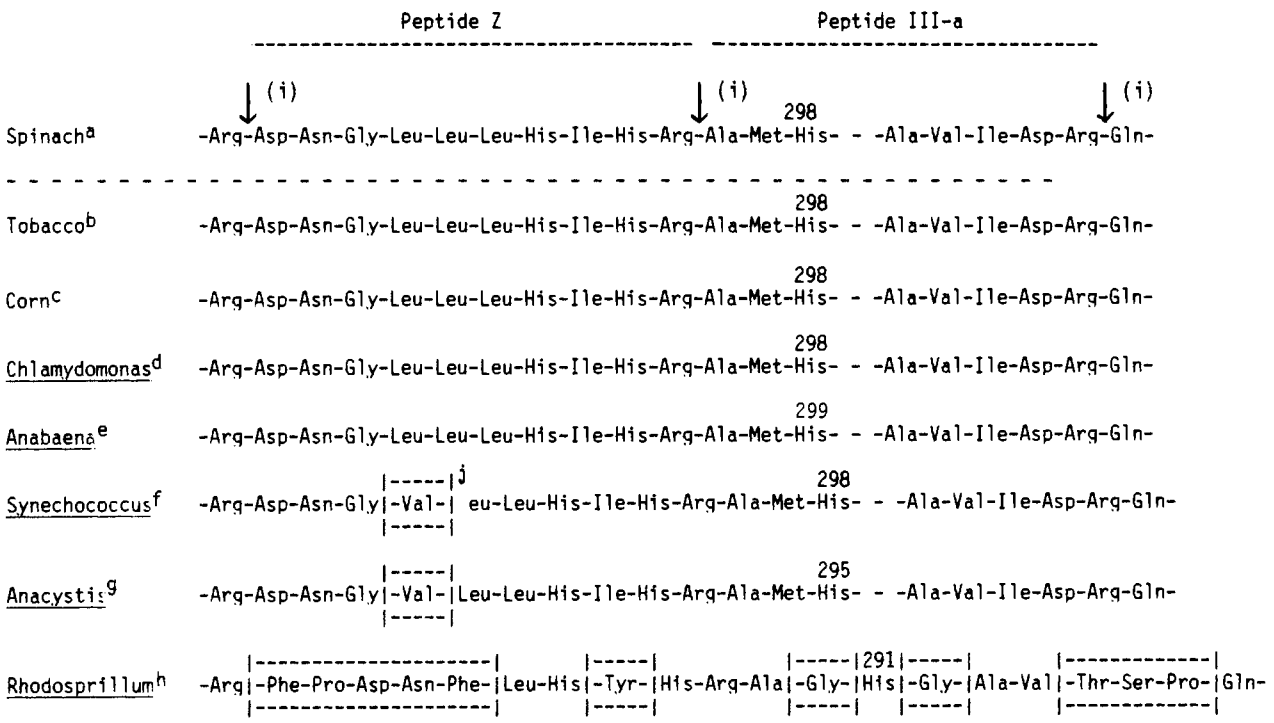

FIGURE 4: Amino acid sequence (determined or inferred) of RuBisCO from various sources in the vicinity of the essential histidine residue (in the spinach enzyme): (a) Zurawski et al., 1981; (b) Shinozaki & Sugiura, 1982; (c) McIntosh et al., 1980; (d) Dron et al., 1982; (e) Curtis & Hazelkorn, 1983; (f) Reichelt & Delaney, 1983; (g) Shinozaki et al., 1983; (h) Nargang et al., 1984; (i) arrows show cleavage sites by trypsin; (j) boxed letters show substituted amino acid residues.

3030), suggesting that some of the histidine residues had not been modified by [^{3}H]DEP. We could not assign the composition of II-c to any of the expected tryptic peptides of spinach RuBisCO. It is plausible that fraction II-c reflects incomplete tryptic digestion and/or that it contains plural peptides that cannot be easily separated.

The amino acid composition of peptide Z, which was eluted with no radioactivity immediately after peak III in the chromatogram of the SBP(−) digest (Figure 2) in a yield of 80% of theoretical, was determined. Peptide Z corresponded to a tryptic peptide arising from the 286th–295th positions in the primary structure (Table II). Peptide Z has two histidine residues (His-292 and His-294) that are not modified by [^{3}H]DEP even in the absence of SBP, suggesting that this part of the enzyme is inaccessible to this reagent even though it is adjacent to the putative active site peptide.

Discussion

When acetaldehyde is reduced with NaB^3H_4 of high specific radioactivity, the product [^{3}H]ethanol may be used to synthesize [^{3}H]DEP. The presently described synthesis is a straightforward variation of that described by Melchior & Fahrney (1970) in which we have taken no precautions to use especially dried reagents. The product is 60% pure but must be frozen at −120 °C in aliquot portions in sealed ampules to minimize hydrolysis. The synthesis of [^{3}H]DEP as described here affords reagent of sufficient specific radioactivity to be useful in histidine modification studies.

In differentially labeling RuBisCO in the presence of 20 mM Mg^{2+} (in the presence ad absence of SBP) with [^{3}H]DEP, it is found that 0.8 histidine residue is modified per large subunit during 70% inactivation. These data are in reasonable accord with earlier experiments done at much lower enzyme concentrations (Saluja & McFadden, 1982). Although this approach may be useful in modifying an essential residue in the active site domain, rigorous proof of active site specificity must await an analysis of the three-dimensional structure of RuBisCO. In any studies of modification, it is possible that substrate (or analogue) binding renders a non active site residue inaccessible through a conformational change.

When RuBisCO is labeled in the absence of SBP, the radioactivity is distributed among eight chromatographic peaks, five of which are also almost equivalently labeled in the presence of SBP (Table I). Thus, three radioactive peaks (II, III, and V), the amounts of which are greatly reduced through protection by the competitive inhibitor SBP, are of major interest. Peak III always contains the most radioactivity and about 3 times as much label as the other two peaks (II and V), which are also reduced in a chromatogram of a SBP(+) digest. Moreover, the 10-fold decrease observed in the amount of peak III is ca. 3 times the decrease observed in peaks II and V when RuBisCO is labeled in the presence of SBP. Label in peak III also accounts for 69% of the radioactivity incorporated differentially [i.e., SBP(−) incorporation minus SBP(+) incorporation] after chromatography (Table I). The remainder is distributed almost equally between peaks II and V with a small amount (6%) found in peak IV.

The radioactive peptide from peak III can be purified with high recovery of radioactivity (approximately 80%). The resultant peptide III-a contains only one histidine residue (His-298) and no serine, cysteine, tyrosine, or lysine residues, which may also react with DEP under certain conditions (Miles, 1977). An absorbance peak at 214 nm that corresponds to peptide III-a almost disappears in a chromatogram of a SBP(+) digest. This establishes that only DEP-modified (radioactive) peptide III-a is eluted at this position. The specific radioactivity (dpm/nmol of histidine) in peptide III-a is constant (2700–3500) and is in good accord with the calculated value (3030) on the basis of the known specific radioactivity of [^{3}H]DEP. We do not know where the unmodified counterpart of peptide III-a is eluted. However, previous research has shown that the pK_a of the (ethoxyformyl)-imidazolyl moiety is more acidic by ca. 2.5 units than that of the imidazolyl group of histidine (Mühlrad et al., 1967).

Presumably, the unmodified peptide has a lower retention time under the conditions (pH 4.0) of reverse-phase HPLC used in the present work.

From these results, in combination with earlier data (Saluja & McFadden, 1980, 1982; Bhagwat & McFadden, 1983), we suggest that the essential active site histidine in spinach RuBisCO is residue 298.

Enzymes from eight different photosynthetic organisms have been directly sequenced or the sequences have been deduced from the corresponding gene structure. All of these sequences have a histidine residue at the position which corresponds to His-298 of spinach RuBisCO. Moreover, the sequence of a region containing this histidine residue is highly conservative in sources spanning from a photosynthetic bacterium to higher plants (Figure 4). This conservation plus the present data showing that His-298 is a major target of SBP-prevented DEP modification suggest that this histidine residue will prove to be essential in the function of RuBisCO.

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Registry No. RuBisCO, 9027-23-0; DEP, 1609-47-8; [^{3}H]DEP, 96617-72-0; [^{3}H]ethanol, 51628-31-0; triglyme, 112-49-2; L-histidine, 71-00-1.

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