Rivier, J., Spiess, J., & Vale, W. (1983) *Proc. Natl. Acad. Sci. U.S.A.* (in press).
Spiess, J., Rivier, J., Rodkey, J. A., Bennett, C. D., & Vale, W. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2974–2978.
Spiess, J., Rivier, J., Rivier, C., & Vale, W. (1981a) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6517–6521.
Spiess, J., Villarreal, J., & Vale, W. (1981b) *Biochemistry 20*, 1982–1988.
Spiess, J., Rivier, J., Rivier, C., & Vale, W. (1982a) in *Methods in Protein Sequence Analysis* (Elzinga, M., Ed.) pp 131–138, Humana Press, Clifton, NJ.
Spiess, J., Rivier, J., Thorner, M., & Vale, W. (1982b) *Biochemistry 21*, 6037–6040.
Sutton, R. E., Koob, G. F., LeMoal, M., Rivier, J., & Vale, W. (1982) *Nature (London) 297*, 331–333.
Tarr, G. E., Beecher, J. F., Bell, M., & McKean, D. J. (1978) *Anal. Biochem. 84*, 622–627.
Vale, W., Spiess, J., Rivier, C., & Rivier, J. (1981) *Science (Washington, D.C.) 213*, 1394–1397.
Vale, W., Rivier, C., Spiess, J., & Rivier, J. (1983) in *Brain Peptides* (Krieger, D., Brownstein, M., & Martin, J., Eds.) Wiley, NY (in press).
Wittmann-Liebold, B. (1980) in *Polypeptide Hormones* (Beers, R. F., Jr., & Bassett, E. G., Eds.) pp 87–120, Raven Press, NY.

Characterization of the Activator Site of *Rhodospirillum rubrum* Ribulosebisphosphate Carboxylase/Oxygenase†

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ABSTRACT: Carbon dioxide/magnesium ion activated ribulosebisphosphate carboxylase/oxygenase from *Rhodospirillum rubrum* was incubated with the transition-state analogue 2-carboxyarabinitol bisphosphate to form the quaternary complex of enzyme·$^{14}CO_2$·Mg^{2+}·carboxyarabinitol bisphosphate, which was then isolated by gel filtration. Despite the relative instability of this complex compared to the analogous one prepared with spinach carboxylase, the $^{14}CO_2$, presumably bound to a lysyl residue as a carbamate, was trapped in high yield by methylation with diazomethane. Following treatment with base to hydrolyze methyl esters of glutamyl and aspartyl residues formed during the trapping procedure, the ^{14}C-labeled protein was digested with chymotrypsin. Two radioactive peptides were purified from the digest by preparative chromatographic procedures. Amino acid analyses, automated Edman degradations, and carboxy-terminal analyses with carboxypeptidases A and Y revealed their sequences to be identical: Leu-Gly-Gly-Asp-Phe-Ile-Lys-Asn-Asp-Glu-Pro-Gln-Gly-Asn-Gln-Pro-Phe. Residue 7, the site of bound $^{14}CO_2$, was identified at the level of its phenylthiohydantoin derivative as N^{ϵ}-(methoxycarbonyl)lysine. Although the observed sequence is similar to that described for the activator-site region of the spinach carboxylase [Lorimer, G. H. (1981) *Biochemistry 20*, 1236–1240], the isoleucyl and asparaginyl residues immediately adjacent to the carbamate-forming lysine of the *R. rubrum* carboxylase are substituted by threonine and aspartic acid, respectively.

Activation of D-ribulose 1,5-bisphosphate (ribulose-P_2)[1] carboxylase/oxygenase involves the sequential binding of CO_2 and divalent metal ion (Lorimer et al., 1976; Laing & Christeller, 1976). For the enzyme from spinach, it has been demonstrated rigorously that activating CO_2 is distinct from CO_2 utilized as substrate (Miziorko, 1979; Lorimer, 1979) and that activation occurs via the formation of a carbamate with Lys-201 of the large subunit (Lorimer & Miziorko, 1980; Lorimer, 1981; Zurawski et al., 1981). This identification entailed isolation of the quaternary complex enzyme·activator CO_2·Mg^{2+}·carboxyarabinitol-P_2. The transition-state analogue carboxyarabinitol-P_2 (Pierce et al., 1980) greatly enhanced the stability of the carbamate and permitted its permanent trapping by esterification with diazomethane; the esterified carbamate survived the conditions used for protein fragmentation and subsequent peptide fractionation.

Universality of reversible carbamylation as a regulatory mode for ribulose-P_2 carboxylases is implied by studies with the enzyme from *Rhodospirillum rubrum*. In contrast to most species of ribulose-P_2 carboxylase which consist of eight large catalytic subunits (M_r = 53 000) and eight small subunits (M_r = 14 000) of ill-defined function, the *R. rubrum* enzyme is a dimer of catalytic subunits (Tabita & McFadden, 1974). Despite this striking difference in quaternary structure, the enzyme exhibits CO_2/Mg^{2+}-dependent activation (Christeller & Laing, 1978; Whitman et al., 1979) which appears to involve the formation of a carbamate between a lysyl ϵ-amino group

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[1] Abbreviations: ribulose-P_2, D-ribulose 1,5-bisphosphate; carboxyarabinitol-P_2, D-2-carboxyarabinitol 1,5-bisphosphate; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; Quadrol, *N,N,N′,N′*-tetrakis(2-hydroxypropyl)ethylenediamine; TPCK, tosylphenylalanyl chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Gdn·HCl, guanidine hydrochloride.

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and CO_2 (O'Leary et al., 1979). However, since partial sequence information about the *R. rubrum* enzyme (Hartman et al., 1982) shows it to be only 28% homologous with the catalytic subunits of the maize and spinach enzymes whose complete sequences have been established (McIntosh et al., 1980; Zurawski et al., 1981), it remains unclear whether the lysyl residue corresponding to position 201 of the spinach carboxylase forms the carbamate involved in activation. One cysteine-containing tryptic peptide (designated T4C) from *R. rubrum* ribulose-P_2 carboxylase appears to correspond to residues 184–201 of the spinach enzyme, ending at the lysine expected to be involved in activation, but the homology preceding this residue is not extraordinarily high, and the peptide's assignment to this location must be considered tentative (Hartman et al., 1982). Furthermore, homology does not prove functional equivalence. Therefore, we have now prepared the esterified carbamate, labeled with $^{14}CO_2$, of *R. rubrum* ribulose-P_2 carboxylase and have isolated and sequenced a chymotryptic peptide that encompasses the derivatized lysyl residue. The peptide, which overlaps tryptic peptide T4C, is homologous with the activator-site peptide previously isolated from the spinach carboxylase. We also show in this report that the enzyme·activator $CO_2 \cdot Mg^{2+}$·carboxyarabinitol-P_2 complex in the case of *R. rubrum* carboxylase is far less stable than the analogous complex of the spinach enzyme.

Experimental Procedures

Materials

Bicine, ATP, NADH, glutathione, glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate phosphokinase, glycerophosphate dehydrogenase/triosephosphate isomerase, phosphoriboisomerase, phosphoribulokinase, carboxypeptidase A, carboxypeptidase Y, and ribose 5-phosphate were products of Sigma Chemical Co. TPCK-treated trypsin was purchased from Worthington and chymotrypsin from Calbiochem. Methylurea was a product of Eastman Organic Chemicals. N^{α}-(*tert*-Butoxycarbonyl)-L-lysine was purchased from Vega Biochemicals. $NaH^{14}CO_3$ (53 mCi/mmol) was from Amersham and $Na^{14}CN$ (3.1 mCi/mmol) from New England Nuclear. Pepstatin was a product of United States Biochemical.

Methods

Ribulose-P_2 was prepared enzymatically from D-ribose 5-phosphate according to published procedures (Horecker et al., 1958). Ribulose-P_2 carboxylase/oxygenase from *R. rubrum* was purified to homogeneity as described previously (Schloss et al., 1982); this enzyme is a dimer (Tabita & McFadden, 1974) of subunits estimated to be 53 000 daltons on the basis of similarities to spinach carboxylase. Protein concentration was determined by using an $E_{280nm}^{1\%,1cm}$ of 12.0 (Stringer et al., 1981). Enzymatic activity was assayed by the spectrophotometric method of Racker (1963) as described earlier (Norton et al., 1975) or by the fixation of $^{14}CO_2$ (Lorimer et al., 1977). Carboxyarabinitol-P_2 was synthesized and purified as described by Pierce et al. (1980). N^{ϵ}-(Methoxycarbonyl)-L-lysine and its phenylthiohydantoin derivative were prepared following published procedures (Lorimer & Miziorko, 1980; Edman, 1950). *N*-Methyl-*N*-nitrosourea[2] was synthesized by the method of Arndt (1943). All experiments were performed at room temperature (22 °C) unless specified otherwise.

Isolation and Analysis of the Quaternary Complex. The quaternary complex was formed by adding a 1.8-fold excess of carboxyarabinitol-P_2 (relative to active sites) to activated *R. rubrum* carboxylase in 50 mM Bicine/66 mM $NaHCO_3$/10 mM $MgCl_2$/1 mM 2-mercaptoethanol (pH 8.0). Greater than 99% of the enzymatic activity was lost within 10 min. The complex was isolated by gel filtration through Sephadex G-50 equilibrated with the buffer described above modified to contain 10 instead of 66 mM $NaHCO_3$. To determine stoichiometries of binding, we used $NaH^{14}CO_3$ or [^{14}C]carboxyarabinitol-P_2 to form the quaternary complex. Kinetic parameters involved in the formation and dissociation of the complex were determined as described by Pierce et al. (1980).

Trapping of Activator $^{14}CO_2$ by Methylation. To a 13-mL solution at pH 8.0 of ribulose-P_2 carboxylase (130 mg, 2.5 μmol of subunit) in 50 mM Tris-HCl/10 mM $MgCl_2$/0.1 mM EDTA/10 mM $NaH^{14}CO_3$ (sp act. 4900 cpm/nmol) was added 1.3 mg (3.4 μmol) of carboxyarabinitol-P_2. A 1.5-mL solution of the enzyme, under precisely the same conditions but with unlabeled $NaHCO_3$, received 0.18 mg of carboxyarabinitol-P_2. Loss of carboxylase activity was greater than 99% within 5 min of addition of carboxyarabinitol-P_2. The two samples were dialyzed separately against 50 mM Tris-HCl/10 mM $MgCl_2$/0.1 mM EDTA/10 mM $NaHCO_3$ (pH 8.0) for 16 h during which time the dialysis buffer was changed twice. The labeled sample (13 mL) had a specific activity of 5900 cpm/nmol, indicative of the binding of 1.2 mol of $^{14}CO_2$ per mol of catalytic subunit. To the small unlabeled sample (1.5 mL) was added 1 μL of 35 mM $NaH^{14}CO_3$ (sp act. 53 μCi/μmol) so as to give about the same number of cpm per milliliter as found in the labeled protein. This sample which received exogenous, labeled bicarbonate was designed to ascertain the degree of nonspecific labeling that could ensue if $^{14}CO_2$ were to dissociate from the quaternary complex during the methylation process and become refixed randomly. Esterification with diazomethane was begun within 30 min of termination of dialysis.

Diazomethane[2] was prepared by the addition of 8.4 g of *N*-methyl-*N*-nitrosourea in 500-mg portions during a 30-min period to 45 mL of agitated, ice-cold diethyl ether over 15 mL of 40% (w/v) aqueous KOH in a 125-mL Erlenmeyer flask. As determined by titration with 0.2 M benzoic acid in 2-methoxyethanol, the diazomethane concentration was 0.65 M. The ethereal layer of diazomethane (36 mL) was decanted onto KOH pellets for drying and was then mixed with 24 mL of ethyl alcohol. The 13- and 1.5-mL solutions of the quaternary complex of ribulose-P_2 carboxylase at 0 °C were added dropwise with stirring during a 60-s period to 39- and 4.5-mL portions, respectively, of the diazomethane in ether/alcohol. Immediately thereafter, a few drops of a saturated aqueous solution of glycine were added to the methylation reaction mixtures in order to consume the excess diazomethane, as indicated by the disappearance of the yellow color.

The reaction mixtures were left on ice for 30 min, at which time the flocculent white precipitates of protein were collected by centrifugation. For removal of entrapped, noncovalently bound $^{14}CO_2$, the precipitates were suspended in water (5 mL) and were again collected by centrifugation; this process was repeated once more. The two precipitates were dissolved in 4 M Gdn·HCl/0.1 M Na_2HPO_4/3 mM EDTA (pH 8.0) to give protein concentrations of about 10 mg/mL (final volumes were 12 and 1.5 mL, respectively). The smaller sample (prepared from unlabeled quaternary complex in the presence

[2] *Caution*: *N*-Methyl-*N*-nitrosourea is a potent mutagen. Also, diazomethane is toxic, volatile, and explosive. All procedures should be carried out in a well-ventilated hood using glassware free from scratches or ground-glass fittings.

of exogenous $NaH^{14}CO_3$) contained less than 1% of the added radioactivity with only 75 cpm/nmol of subunit and was not processed further; thus, the potential problem of nonselective labeling does not arise. The larger sample (prepared from labeled quaternary complex) contained 55% of the initial radioactivity with 3200 cpm/nmol or a covalent incorporation of 0.65 mol of $^{14}CO_2$/mol of subunit.

Proteolytic Digestion of Methylated Enzyme. Of the 12-mL sample of guanidine-solubilized protein, 2 mL (20 mg of protein) was dialyzed against 10 mM NH_4HCO_3 (pH 8.1). The pH of the remaining 10 mL (100 mg of protein) was adjusted to 12.0 with 10 N NaOH, and after 5 h of incubation at room temperature, this solution was also dialyzed against 10 mM NH_4HCO_3. During dialysis, both samples of protein precipitated. The 20-mg sample of protein which had not been exposed to alkali and a 10-mg portion of the protein which had been incubated at elevated pH were digested with trypsin, and the remaining 90 mg of alkali-treated protein was digested with chymotrypsin; all digestions were carried out at 40 °C. For both tryptic and chymotryptic digestions of alkali-treated protein, 1% (w/w) of the protease was added followed 12 h later by a second addition of the same amount. Complete solubilization of protein occurred within the first 2 h, and the amount of radioactivity found agreed with that observed prior to dialysis. The protein that had not been treated with alkali was digested with two additions of 3% (w/w) trypsin; solubilization of protein was only 60% completed on the basis of radioactivity. Digests were terminated by freezing after total reaction times of 24 h.

Amino Acid Analyses. Peptides were hydrolyzed at 110 °C in 6 N HCl/0.01 M 2-mercaptoethanol in sealed, evacuated tubes for 21 h. Amino acid compositions were determined with a Beckman 121 M amino acid analyzer as described earlier (Stringer et al., 1981).

Sequence Analyses. The purified peptide (19.5 nmol) was subjected to automated Edman degradation with a Beckman 890C, the vacuum system of which was modified according to Bhown et al. (1980). A liquid nitrogen cold trap was inserted between the low vacuum pump and the vacuum manifold. Polybrene (2 mg) was added to the peptide to reduce its extraction from the reaction cup (Tarr et al., 1978). The peptide was sequenced in a Quadrol buffer system by using the program of Bhown et al. (1980) with several changes. The buffer concentration was reduced from 0.5 to 0.1 M, the drying step after delivery of phenyl isothiocyanate was increased from 20 to 60 s, and the coupling steps and drying steps after delivery of benzene/ethyl acetate were from Beckman's 030176 peptide program. Of the fraction from each cycle, 10% was assayed for radioactivity. Half of the remainder was converted to the phenylthiohydantoin for identification by high-performance liquid chromatography (Laboratory Data Control) and the rest hydrolyzed in base for quantitation as free amino acids on the amino acid analyzer (Smithies et al., 1971). Threonine and arginine appear as α-aminobutyric acid and ornithine, respectively, in base hydrolysates. Aspartic and glutamic acids were distinguished from the corresponding amides and N^{ϵ}-(methoxycarbonyl)lysine was distinguished from lysine by high-performance liquid chromatography of the phenylthiohydantoins.

Digestion of Peptides with Carboxypeptidases A and Y. Carboxypeptidase A was used at room temperature and a final concentration of 100 μg/mL in 50 mM *N*-ethylmorpholine hydrochloride (pH 8.0); the peptide concentration was 18 nmol/mL. At time 0 and 24 h, 75-μL aliquots of the digestion mixture were diluted to 150 μL with 0.2 M sodium citrate (pH 2.2) and then subjected to amino acid analyses. Carboxypeptidase Y was used at 37 °C and a final concentration of 200 μg/mL in 100 mM pyridine acetate/1 mM EDTA (pH 5.5) which contained 100 μg/mL pepstatin, an endopeptidase inhibitor; the peptide concentration was 20 nmol/mL. At time 0, 2, and 24 h, 75-μL aliquots were diluted to 150 μL with 0.2 M sodium citrate (pH 2.2) and then subjected to amino acid analyses.

Results

Quaternary Complex of Enzyme·CO_2·Mg^{2+}·Carboxyarabinitol-P_2. The complex formed between *R. rubrum* carboxylase, CO_2, Mg^{2+}, and carboxyarabinitol-P_2 is stable to gel filtration. When formed with $^{14}CO_2$ or [^{14}C]carboxyarabinitol-P_2, the specific activity of the eluted complex was constant across the eluted peak (data not shown). The stoichiometries determined for CO_2 and carboxyarabinitol-P_2 upon elution were 1.2 and 1.1 nmol/nmol of protomer, respectively. Studies on the dissociation of the complex, described below, revealed that approximately 9% of the bound $^{14}CO_2$ exchanged relatively quickly with exogenous CO_2, and this portion is attributed to $^{14}CO_2$ bound nonspecifically that remains associated with the complex over the brief period (ca. 20 min) of gel filtration. Correction for this nonspecifically bound $^{14}CO_2$ gives a stoichiometry of 1.1 nmol of specifically bound CO_2 per nmol of protomer.

When activated enzyme was added to assay solutions containing varied concentrations of ribulose-P_2 with carboxyarabinitol-P_2 at 0, 0.5, 1.0, or 2.0 μM, activity was inhibited in a linearly competitive manner with a K_i of 0.4 μM. If enzyme was preincubated with carboxyarabinitol-P_2, activity was lost rapidly and completely. However, the first-order rate constant for the conversion of the initially formed quaternary complex EI to the inactivated complex EI* (Pierce et al., 1980) could not be determined accurately. At 0.32 μM carboxyarabinitol-P_2, the enzyme was inactivated at a rate of 0.1 s^{-1}, a rate which results in nearly 90% inactivation in 20 s. At higher concentrations, inactivation was too rapid to allow the collection of reliable data. Lower concentrations gave the appearance of second-order kinetics, as occurs when one uses concentrations of substrate below the K_m for measuring the catalytic parameters of an enzyme.

The rate of dissociation of carboxyarabinitol-P_2 and CO_2 from the inactivated quaternary complex was estimated by measuring the rate of exchange of the radiolabeled ligands in the complex with their unlabeled counterparts in solution (Pierce et al., 1980). Carboxyarabinitol-P_2 dissociated from the complex with a half-life of 2.0 days ($k_{off} = 4.0 \times 10^{-6}$ s^{-1}, Figure 1). In the absence of exogenous carboxyarabinitol-P_2, CO_2 also dissociated at this rate. In its presence, however, only 9% of the CO_2 was freely exchangeable. This fraction most likely represents CO_2 not bound at the activation site, and we therefore have corrected the estimates of CO_2 stoichiometry by the factor 0.91 (see above).

Purification of the Activator-Site Peptide. Our standard approach to peptide isolation, tryptic digestion followed by DEAE-cellulose chromatography, proved highly unsatisfactory. Profiles of eluted radioactivity so obtained were extremely complex (Figure 2A). Heterogeneity was reduced considerably by treatment of the methylated complex with alkali prior to digestion (Figure 2B), but satisfactory results were obtained only by digesting the alkali-treated complex with chymotrypsin (Figure 3). A 7.5-mL portion of the chymotryptic digest of labeled, methylated enzyme (60 mg, 1.13 μmol of subunit, 3.6×10^6 cpm) was fractionated on DEAE-cellulose at pH 8.1 (Figure 3A). Of the recovered radioactivity, 55%

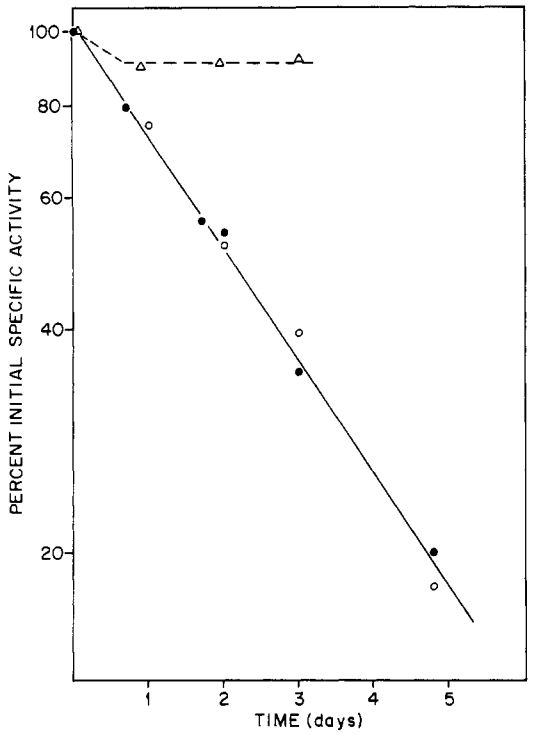

FIGURE 1: Exchange of ligands from the enzyme·CO_2·Mg^{2+}·carboxyarabinitol-P_2 complex. Complexes formed with [^{14}C]-carboxyarabinitol-P_2 (●) or $^{14}CO_2$ (△) were purified by gel filtration through Sephadex G-50 (fine, 1 × 22 cm) equilibrated with 50 mM Bicine, 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithioerythritol, pH 8.0, following which unlabeled carboxyarabinitol-P_2 was added to a final concentration of 3 mM. Initial specific radioactivities of the complexes were 290 and 9600 cpm/nmol of protomer, respectively. Periodically, portions were rechromatographed, and their specific activities were redetermined. A second complex formed with $^{14}CO_2$ [(○) initial specific activity 2100 cpm/nmol of protomer] was processed identically except unlabeled carboxyarabinitol-P_2 was not added. Exchange of $^{14}CO_2$ in the absence of exogenous unlabeled carboxyarabinitol-P_2 (○) and of [^{14}C]-carboxyarabinitol-P_2 in its presence (●) occurred with a half-time of 2.0 days ($k = 4 \times 10^{-6}$ s^{-1}).

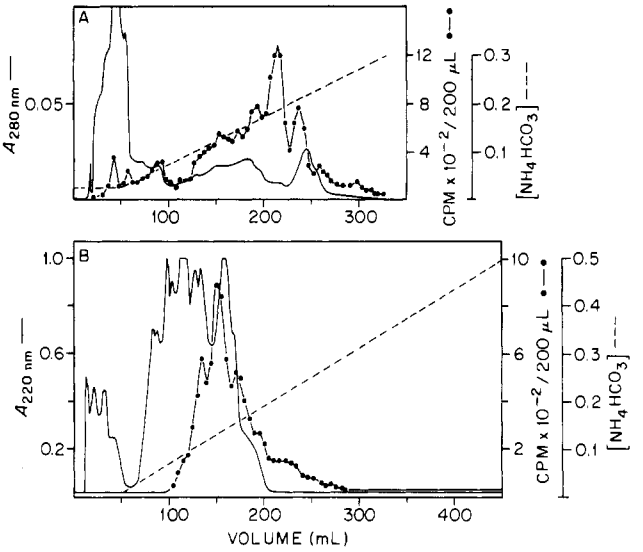

FIGURE 2: (A) Fractionation of a tryptic digest (20 mg) of methylated ribulose-P_2 carboxylase on a Whatman DE52 column (1.5 × 28 cm) equilibrated with 0.025 M NH_4HCO_3 (pH 8.1). The column was eluted with 50 mL of equilibration buffer followed by a 300-mL linear gradient of 0.025–0.3 M NH_4HCO_3 (pH 8.1). (B) Fraction of a tryptic digest (5 mg) of alkali-treated, methylated enzyme on a Whatman DE52 column (1 × 25 cm) equilibrated with 0.01 M NH_4HCO_3 (pH 8.1). The column was first eluted with 50 mL of equilibration buffer followed by a 400-mL linear gradient of 0.01–0.5 M NH_4HCO_3 (pH 8.1).

Table I: Amino Acid Composition of Activation-Site Peptide

amino acid	peptide I[a] from hydrolysate[b]	peptide I[a] from sequencer	peptide II,[a] from hydrolysate[b]
Cys[c]	0	0	0
Asp	3.5	2	3.6
Asn		2	
Thr	0.3	0	0.2
Ser	0.1	0	0.1
Glu	3.2	1	2.9
Gln		2	
Pro	1.3	2	1.6
Gly	2.7	3	2.9
Ala	0.7	0	0.3
Val	0.2	0	0
Met	0.3	0	0.4
Ile	1.0	1	1.0
Leu	1.3	1	1.1
Tyr	0.1	0	0.1
Phe	1.5	2	1.8
His	0	0	0
Lys	0.7	1[d]	0.8
Trp	0	0	0
Arg	0.2	0	0

[a] Aliquot from pooled DE52 fractions (Figure 3D). [b] Nanomoles of amino acid per nanomoles of Ile. [c] As *S*-methylcysteine. [d] As N^{ϵ}-(methoxycarbonyl)lysine.

(1.7 × 10^6 cpm) was found in the major peak with the remainder distributed between minor peaks and a slight background (Figure 3A). Minor radioactive components were not processed further. Fractions containing the major radioactive peak were pooled and lyophilized to dryness. This material was then chromatographed in succession on DEAE-cellulose at pH 4.0, Sephadex G-25, and DEAE-cellulose at pH 3.7 in the presence of 6 M urea (Figure 3). The radioactivity emerged as a single peak from the first two of these three columns and was recovered in yields of 79% and 95%, respectively. However, ion-exchange chromatography with an eluting buffer containing 6 M urea resolved two radioactive peaks (Figure 3D). Of the 9 × 10^5 cpm that was applied to this latter column, 40% (3.6 × 10^5 cpm, 85 nmol) was recovered in peak I and 47% (4.2 × 10^5 cpm, 100 nmol) was recovered in peak II. The expressed numbers of nanomoles are based on a specific activity of 4200 cpm/nmol for each peptide as was determined by amino acid analyses subsequent to the removal of urea from the samples by gel filtration on Sephadex G-25.

Amino Acid Analyses and Sequence Determination. While peptide II appeared to be very nearly pure on the basis of its amino acid composition, peptide I did not, several residues being present in amounts significantly far from integral multiples of the amount of isoleucine (Table I). In particular, alanine and leucine were high and proline and phenylalanine were low relative to peptide II. Fortunately, this material appeared to be much purer by sequencing than by amino acid analysis; each cycle of automated Edman degradation released a single amino acid with only traces of contamination (Table II). Following an initial yield of 75%, yields remained high through 15 cycles, and the sequence was unambiguously determined (Table II): Leu-Gly-Gly-Asp-Phe-Ile-Lys-Asn-Asp-Glu-Pro-Gln-Gly-Asn-Gln-Pro-Phe. The lysine residue released at cycle 7 was shown to be the site of CO_2 fixation. Of the recovered radioactivity, 90% was released at this cycle. The phenylthiohydantoin derivative of the modified lysine

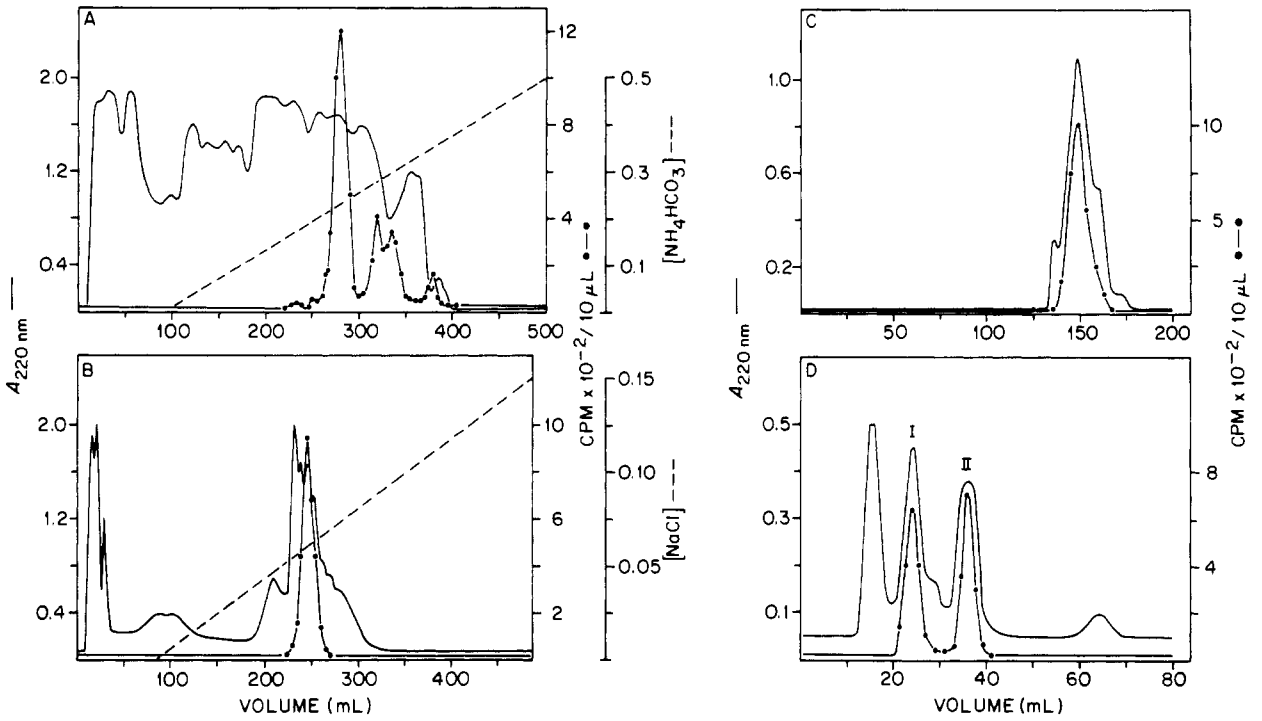

FIGURE 3: Purification of activator-site peptide from a chymotryptic digest of methylated, alkali-treated ribulose-P_2 carboxylase. (A) Chromatography of the total digest (60 mg) on a Whatman DE52 column (1 cm × 25 cm) equilibrated with 0.01 M NH_4HCO_3 (pH 8.1) and eluted with 100 mL of equilibration buffer followed by a 400-mL linear gradient of 0.01–0.5 M NH_4HCO_3 (pH 8.1). (B) Chromatography of the major radioactive peak obtained from the first column on a second column (1 cm × 25 cm) of Whatman DE52 equilibrated with 0.01 M ammonium acetate (pH 4.0) and eluted with 85 mL of equilibration buffer followed by a 400-mL linear gradient of 0–0.15 M NaCl buffered with 0.01 M ammonium acetate (pH 4.0). (C) Chromatography of the radioactive peak obtained from the second DE52 column on a column (1.7 cm × 220 cm) of Sephadex G-25 in 0.01 M NH_4HCO_3 (pH 8.1). (D) Chromatography of the radioactive peak obtained from gel filtration on a third column (1 cm × 25 cm) of Whatman DE52 equilibrated and eluted with 5 mM NaH_2PO_4/6 M urea (pH 3.7).

Table II: Sequence Analysis of Activator-Site Peptide[a]

cycle	amino acid	yield[b] (nmol)	cpm
1	Leu[c]	14.6	
2	Gly	12.7	
3	Gly	11.9	
4	Asp	6.2	
5	Phe	12.2	140
6	Ile	13.6	640
7	Lys[d]	7.3	46000
8	Asn	6.2	4400
9	Asp	4.5	400
10	Glu	5.2	90
11	Pro	7.8	
12	Gln	3.2	
13	Gly	3.8	
14	Asn	3.4	
15	Gln	2.8	
16	Pro	0.7	
17	Phe	trace	

[a] Sample size, 19.5 nmol. [b] Repetitive yield (cycle 2 → cycle 13), 90%. [c] Glu, Gly, and Ala were also present at cycle 1 but at levels less than 10% that of Leu. [d] As N^{ϵ}-(methoxycarbonyl)-lysine.

cochromatographed with the corresponding derivative of synthetic N^{ϵ}-(methoxycarbonyl)lysine on reversed-phase high-performance liquid chromatography and thin-layer chromatography on silica gel. Digestion of 2.7 nmol of peptide I with carboxypeptidase A resulted in the release of 2.5 nmol of phenylalanine and only traces of other amino acids, consistent with the assigned Pro-Phe sequence at the peptide's COOH terminus and with the presence of minor contaminating peptides inferred from the amino acid composition. Digestion with carboxypeptidase Y confirmed these assignments. After 2 h of digestion, both phenylalanine and proline had been liberated. No other amino acids were observed at levels above the trace quantities found in zero-time controls.

While the amino acid composition of peptide II is in excellent agreement with that calculated from the sequence of peptide I (Table I), we were unable to sequence peptide II completely. After eight cycles of automated Edman degradation which gave results identical with those obtained for peptide I, a precipitous drop in yield occurred. We have not determined the cause of this phenomenon (see Discussion). Carboxypeptidase A digestion of peptide II also released only phenylalanine. Thus, considering these data, the partial sequence obtained, and the amino acid composition, we conclude that peptide II possesses the same sequence as peptide I but has been modified in some unknown way by treatment with diazomethane and base that results in the abrupt fall in yield.

Discussion

The formation of a highly stable quaternary complex of spinach ribulose-P_2 carboxylase, activator CO_2, divalent metal ion, and the transition-state analogue carboxyarabinitol-P_2 permitted the specific trapping of activator CO_2 (Lorimer & Miziorko, 1980). However, some ambiguity has existed regarding the stability of the quaternary complex formed with the carboxylase from *R. rubrum* (Brown & Chollet, 1982). Stability of this complex is requisite for the specific trapping of activator CO_2. Also, since the *R. rubrum* enzyme possesses a much simpler subunit structure than most ribulose-P_2 carboxylases, the nature of the quaternary complex it forms may reflect functional differences conferred by interacting subunits.

Recently, Miziorko et al. (1982) have documented the formation of a stable quaternary complex involving stoichiometric amounts of *R. rubrum* carboxylase, CO_2, Co^{2+}, and carboxyarabinitol-P_2. The results presented here demonstrate

that magnesium can also participate in a stable complex and that the resulting complex, like that involving the hexadecameric forms of the enzyme, contains stoichiometric amounts of CO_2 and carboxyarabinitol-P_2. Analysis of the kinetics of complex formation, though incomplete, indicates that the inactivation of *R. rubrum* carboxylase by carboxyarabinitol-P_2 proceeds via a two-step mechanism as does that of the spinach enzyme (Pierce et al., 1980). Tight binding of carboxyarabinitol-P_2 is preceded by a rapid step in which carboxyarabinitol-P_2 acts as a simple competitive inhibitor with a K_i of 0.4 μM for both enzymes. In contrast, the rate constants for the second phase of inactivation are considerably higher for the *R. rubrum* carboxylase. The rate of exchange of carboxyarabinitol-P_2, 4×10^{-6} s^{-1}, is 8 times that reported for the spinach enzyme (Figure 1; Pierce et al., 1980). Also, the rate of conversion of the intitially formed complex to the tight complex is higher (see Results). Data regarding the rate of exchange of CO_2 from the quaternary complex have not been published previously. In the presence of exogenous carboxyarabinitol-P_2, exchange of CO_2 appears to be prevented (Figure 1). Apparently, activator CO_2 cannot exchange directly from the quaternary complex, as would be expected for the dissociation of a complex formed by a strictly ordered process. In the absence of exogenous inhibitor, exchange of CO_2 parallels that of carboxyarabinitol-P_2; its exchange from the ternary complex apparently is fast relative to the dissociation of carboxyarabinitol-P_2 from the quaternary complex but slower than the rebinding of carboxyarabinitol-P_2 which occurs when the latter is present in solution at a saturating concentration. This comparatively rapid exchange of CO_2 may explain the previous uncertainty regarding the stability of the *R. rubrum* quaternary complex.

Trapping of activator $^{14}CO_2$ by methylation with diazomethane using the procedures described herein was quite efficient, 65% of the specifically bound $^{14}CO_2$ being stabilized by this treatment. However, the efficacy of diazomethane as a methylating agent generated several complications in the isolation of the activation-site peptide. Diazomethane rapidly esterifies carboxyl groups, and as the isolated peptide contains three acidic residues, their partial methylation could be expected to generate heterogeneity as proposed by Lorimer (1981). The methylated protein is also quite resistant to proteolytic digestion, as was the methylated spinach carboxylase, and is not fully solubilized by prolonged digestion with high concentrations of trypsin. Saponification of the carboxyl esters eliminated this resistance to proteolysis and reduced the heterogeneity observed upon chromatography of the digest (Figure 2). Amino acid analysis of an acid hydrolysate of the methylated complex revealed extensive modification of lysyl and cysteinyl residues as well. Of the enzyme's five cysteine residues, 90% had been converted to *S*-methylcysteine, and approximately 65% of the enzyme's 20 lysine residues were modified. Recently, Poncz & Dearborn (1983) have reported that peptide bonds following N^{ϵ},N^{ϵ}-dimethyllysyl residues are highly resistant to hydrolysis by trypsin. Very likely, the heterogeneity of our tryptic digests results from variable and random methylation of lysyl residues. This problem would not have arisen with the spinach enzyme because liberation of the activation-site peptide by trypsin did not entail cleavage at lysine. The carboxyl terminus of this peptide is phenylalanine, reflective of a chymotryptic cleavage despite the use of trypsin (Lorimer, 1981). Inspection of the complete sequence of the spinach large subunit (Zurawski et al., 1981) shows that the amino terminus of the activation-site peptide was generated by tryptic cleavage of the peptide bond between Arg-194 and Gly-195. Consistent with the above rationale, chymotryptic digestion of the methylated, base-treated *R. rubrum* complex generated a single major radioactive peptide (Figure 3A). The moderate heterogeneity remaining is very likely a consequence of the use of chymotrypsin which due to its lower specificity tends to generate a more heterogeneous mixture of peptides than does trypsin.

Ion-exchange chromatography in the presence of urea resulted in separation of the radiolabeled material into two fractions. However, by amino acid analysis (acid hydrolysis), these peptides were identical. Similarly, Lorimer (1981) isolated two major radiolabeled peptides of identical amino acid composition from the spinach carboxylase. We do not know the nature of the difference between these two peptides, but the fact that they are indeed distinct was corroborated by sequencing. In the case of peptide II, a precipitous drop in yield occurred following the release of asparagine at cycle 8 whereas peptide I was sequenced in high yields through 15 cycles (Table II). Previously described examples of sudden, large drops in yield have been attributed to acid-catalyzed formation of pyroglutamate or β-aspartate from a newly formed NH_2-terminal glutamine, asparagine, or asparate residue during Edman degradation (Niall, 1973). The NH_2 terminus exposed after cycle 8 would be Asp-Glu-, so the possibility exists for intramolecular catalysis of the conversion of α- to β-aspartate, but this should have been the case for both peptides if they were identical.

The sequence determined for peptide I is highly homologous with that of the activation-site peptide of spinach characterized by Lorimer (1981). As aligned, 9 of the 17 residues match (italics denote labeled residue):

spinach peptide (Lorimer, 1981)

Gly-Gly-Leu-Asp-Phe-Thr-*Lys*-Asp-Asp-Glu-Asn-Val-Asn-Ser-Gln-Pro-Phe

R. rubrum peptide (present study)

Leu-Gly-Gly-Asp-Phe-Ile-*Lys*-Asn-Asp-Glu-Pro-Gln-Gly-Asn-Gln-Pro-Phe

Of the remaining residues, two differences can be attributed to single base pair substitutions. This degree of homology is much higher than the overall homology of 28% described by Hartman et al. (1982). It is even more striking when compared with the sequences immediately preceding the activation-site peptides. The preceding 10 residues, known from the sequence of a cysteine-containing peptide of the *R. rubrum* enzyme (Hartman et al., 1982) which overlaps the activation-site peptide and from the DNA sequence of the spinach enzyme's gene (Zurawski et al., 1981), contain only a single identity and five substitutions compatible with single base pair changes.

The finding that a high degree of precisely localized homology persists in enzymes from such evolutionarily divergent organisms, enzymes which differ dramatically in quaternary structure, argues strongly for a single, fairly rigidly constrained mechanism of activation for all species of ribulosebisphosphate carboxylase. However, this assertion must be qualified by consideration of two questions: the substitutions found immediately adjacent to the carbamate-forming lysyl residue and the role of quaternary structure in the activation process.

Both residues adjacent to lysine-201 [for the numbering of the spinach enzyme, see Zurawski et al. (1981)] are different in the two peptides. While single base pair mutations could account for these changes, the chemical nature of these residues is drastically altered; the polarities of the adjacent residues in the *R. rubrum* carboxylase are much lower.

Threonine-200 and aspartate-202 of the spinach enzyme are replaced by isoleucine and asparagine, respectively. While such changes could potentially alter the properties of the activation site significantly, the side chains of these residues might not be in close proximity to the ϵ-amino group of lysine-201. Indeed, the trans nature of the peptide bond disposes side chains of adjacent residues away from each other. Lorimer (1981) has formulated a model in which Mg^{2+} is coordinated by two carboxyl groups, one from the carbamate and another from an acidic residue of the protein. With certainty, one can say only that the carboxyl group of asparatate-202 is not an obligatory participant in such a coordination of Mg^{2+}.

Differences in the quaternary structure of the two enzymes pose a more complex problem. Ribulose-P_2 carboxylase from *R. rubrum* exists as a simple dimer of catalytic subunits whereas the enzyme from higher plants consists of eight large catalytic subunits and eight small subunits. The precise role of these small subunits is poorly understood, but recent work involving separation of the subunits and reconstitution of native enzyme (Andrews & Abel, 1981; Andrews & Ballment, 1982) indicates the small subunits are absolutely required for catalytic competence of the large subunit. It is likely that in plant carboxylases the small subunits function in the activation process. Summarizing the research on the role of the small subunit, Miziorko & Lorimer (1983) conclude that the data are consistent with the hypothesis that the small subunit helps maintain the large subunit in a conformation which can be activated fully by CO_2 and Mg^{2+}. If this hypothesis is correct, activation of the *R. rubrum* carboxylase must differ in some details from that of the complex forms of the enzyme. Bowien & Gottschalk (1982) have recently characterized a large change in the sedimentation coefficient, from 17.5 to 14.3 S, which occurs upon activation of a bacterial carboxylase that has the same subunit composition as enzymes from higher plants. Such a change implies a large alteration in the molecule's shape and/or density. It is tempting to hypothesize that this change is an obligatory component of activation of carboxylases which have small subunits. The authors state that preliminary experiments with the spinach carboxylase gave results compatible with those obtained for the bacterial enzyme. However, analytical ultracentrifugation and neutron-scattering analyses performed in our laboratory failed to reveal a large conformational change associated with activation of the enzymes from either *R. rubrum* (Donnelly et al., 1983) or spinach (unpublished results).

Future studies on the properties of large subunits of the higher plant forms of ribulose-P_2 carboxylase in the absence of small subunits should help greatly to elucidate the function of the small subunit. If their essentiality and role in activation are substantiated, the ability of the *R. rubrum* carboxylase to undergo activation in the absence of small subunits poses an intriguing problem; the large subunits have diverged from a common ancestral gene (Hartman et al., 1982), yet structural changes outside the active-site regions appear to have conferred sharply different requirements for full catalytic activity of the two forms.

Registry No. Carboxyarabinitol-P_2, 27442-42-8; ribulosebisphosphate carboxylase/oxygenase, 9027-23-0; L-lysine, 56-87-1.

References

Andrews, T. J., & Abel, K. M. (1981) *J. Biol. Chem. 256*, 8445–8451.

Andrews, T. J., & Ballment, B. (1982) *J. Biol. Chem. 258*, 7514–7518.

Arndt, R. (1943) *Organic Synthesis, Collect. Vol. II*, pp 416–464, Wiley, New York.

Bhown, A. S., Cornelius, T. W., Mole, J. E., Lynn, J. D., Tidwell, W. A., & Bennett, J. C. (1980) *Anal. Biochem. 102*, 35–38.

Bowien, B., & Gottschalk, E.-M. (1982) *J. Biol. Chem. 257*, 11845–11847.

Brown, H. M., & Chollet, R. (1982) *J. Bacteriol. 149*, 1159–1161.

Christeller, J. T., & Laing, W. A. (1978) *Biochem. J. 173*, 467–473.

Donnelly, M. I., Hartman, F. C., & Ramakrishnan, V. R. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 2044.

Edman, P. (1950) *Acta Chem. Scand. 4*, 277–282.

Hartman, F. C., Stringer, C. D., Omnaas, J., Donnelly, M. I., & Fraij, B. (1982) *Arch. Biochem. Biophys. 219*, 422–437.

Horecker, B. L., Hurwitz, J., & Weissbach, A. (1958) *Biochem. Prep. 6*, 83–90.

Laing, W. A., & Christeller, J. T. (1976) *Biochem. J. 159*, 563–570.

Lorimer, G. H. (1979) *J. Biol. Chem. 254*, 5599–5601.

Lorimer, G. H. (1981) *Biochemistry 20*, 1236–1240.

Lorimer, G. H., & Miziorko, H. M. (1980) *Biochemistry 19*, 5321–5328.

Lorimer, G. H., Badger, M. R., & Andrews, T. J. (1976) *Biochemistry 15*, 529–536.

Lorimer, G. H., Badger, M. R., & Andrews, T. J. (1977) *Anal. Biochem. 78*, 66–75.

McIntosh, L., Poulsen, C., & Bogorad, L. (1980) *Nature (London) 288*, 556–560.

Miziorko, H. M. (1979) *J. Biol. Chem. 254*, 270–272.

Miziorko, H. M., & Lorimer, G. H. (1983) *Annu. Rev. Biochem. 52*, 507–535.

Miziorko, H. M., Behnke, C. E., & Houkom, E. C. (1982) *Biochemistry 21*, 6669–6674.

Niall, H. D. (1973) *Methods Enzymol. 27*, 942–1010.

Norton, I. L., Welch, M. H., & Hartman, F. C. (1975) *J. Biol. Chem. 250*, 8062–8068.

O'Leary, M. H., Jaworski, R. J., & Hartman, F. C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 673–675.

Pierce, J., Tolbert, N. E., & Barker, R. (1980) *Biochemistry 19*, 934–942.

Poncz, L., & Dearborn, D. G. (1983) *J. Biol. Chem. 258*, 1844–1850.

Racker, E. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) pp 188–190, Academic Press, New York.

Schloss, J. V., Phares, E. F., Long, M. V., Norton, I. L., Stringer, C. D., & Hartman, F. C. (1982) *Methods Enzymol. 90*, 522–528.

Smithies, O., Gibson, D., Fanning, E. M., Goodfliesh, R. M., Gilman, J. B., & Ballantyne, D. L. (1971) *Biochemistry 10*, 4912–4921.

Stringer, C. D., Norton, I. L., & Hartman, F. C. (1981) *Arch. Biochem. Biophys. 208*, 495–501.

Tabita, F. R., & McFadden, B. A. (1974) *J. Biol. Chem. 249*, 3459–3464.

Tarr, G. E., Beecher, J. F., Bell, M., & McKean, D. J. (1978) *Anal. Biochem. 84*, 622–627.

Whitman, W. B., Martin, M. N., & Tabita, F. R. (1979) *J. Biol. Chem. 254*, 10184–10189.

Zurawski, G., Perrot, B., Bottomley, W., & Whitfeld, P. R. (1981) *Nucleic Acids Res. 9*, 3251–3270.