To the Graduate Council:

I am submitting herewith a dissertation written by Cinda S. Herndon entitled "Ribulosebisphosphate Carboxylase/Oxygenase: Synthesis of Potential Affinity Labels and Characterization of Active-Site Regions." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

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RIBULOSEBISPHOSPHATE CARBOXYLASE/OXYGENASE: SYNTHESIS OF POTENTIAL AFFINITY LABELS AND CHARACTERIZATION OF ACTIVE-SITE REGIONS

A Dissertation

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ABSTRACT

Ribulosebisphosphate carboxylase/oxygenase catalyzes both the carboxylation and oxidative cleavage of ribulose 1,5-bisphosphate, opposing reactions in photosynthetic carbon assimilation and photorespiration, respectively. One approach to understanding this bifunctionality entails identification of active-site regions of the enzyme by affinity labeling. Comparison of active-site residues in enzymes from evolutionarily distant sources should reveal conserved molecular features which are likely to function in catalysis.

Irregularities in a published report from another laboratory prompted the reexamination of the binding site for pyridoxal 5'-phosphate in the carboxylase from Rhodospirillum rubrum, described in Part I. A single pyridoxal 5'phosphate-labeled peptide was obtained. The reactive lysyl residue lies in a seven-residue sequence common to all known carboxylases.

Part II describes the synthesis and characterization of four potential affinity labels for the carboxylase. Reductive amination of ribulose 1,5-bisphosphate with bifunctional amines by cyanoborohydride and bromoacetylation of the isolated amino bisphosphates gave 2-(4-bromoacetamido)anilino-2-deoxypentitol 1,5-bisphosphate (reagents A and B, epimeric at C2), 2-[2-(bromoacetamido)ethyl]amino-2-deoxypentitol 1,5-bisphophate (reagent C), 2-[4-(bromoacetamido)butyl]amino-2-deoxypentitol 1,5-bisphosphate (reagent D), and 2-{4-[4-(bromoacetamido)phenylmethyl]-anilino}-2-deoxypentitol 1,5-bisphosphate (reagent E).

The interactions of the epimeric reagents A and B with the carboxylase were consistent with the documented effect of configuration at C2 on

binding to the enzyme. Of the two, only reagent B irreversibly inactivated the R. rubrum enzyme in a time-dependent manner. Isolation of reagent B-modified tryptic peptides revealed two approximately equal sites of labeling, His^{44} and Cys^{58}, both of which were subject to protection by 2-carboxyribitol 1,5-bisphosphate. Although these residues are not conserved in the six known carboxylase sequences, they lie in a region of homology not previously known to be at or near the active site of the enzyme.

Reagents C and D also fulfilled criteria of affinity labeling; the former alkylated Met^{335} while the latter alkylated His^{44}, Cys^{58}, and Met^{335}. The methionyl residue in the R. rubrum enzyme, previously modified by two affinity labels, is replaced by leucine in the other carboxylases. Reagent E, although a competitive inhibitor, exhibited no time-dependent effect on carboxylase activity. The implications of these findings to the active-site geometry of the carboxylase are discussed.

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ABBREVIATIONS

Amino Acids = A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

ATP = Adenosine 5'-triphosphate

Bicine = N, N-Bis(2-hydroxyethyl)glycine

Carboxyarabinitol-P_2 = 2-C-Carboxy-D-arabinitol 1,5-bisphosphate

Carboxyribitol-P_2 = 2-C-Carboxy-D-ribitol 1,5-bisphosphate

Cm-Cys = S-Carboxymethylcysteine

Cm-Hcy = S-Carboxymethylhomocysteine

τ-Cm-His = τ-Carboxymethylhistidine

DEAE = Diethylaminoethyl

EDTA = Ethylenediaminetetraacetic acid

Hepes = N-(2-Hydroxyethyl)piperazine-N'-2-ethanesulfonic acid

NADH = β-Nicotinamide adenine dinucleotide, reduced form

PLP = Pyridoxal 5'-phosphate

PLP-Lys = N^6-(Phosphopyridoxal)lysine

QAE = Diethyl-(2-hydroxypropyl)-aminoethyl

Quadrol = N,N,N',N'-Tetrakis(2-hydroxypropyl)ethylenediamine

Reagent A, Reagent B = Ribo and arabino epimers of 2-(4-bromoacetamido) anilino-2-deoxypentitol 1,5-bisphosphate

Reagent C = 2-[2-(Bromoacetamido)ethyl]amino-2-deoxypentitol 1,5-bisphosphate

Reagent D = 2-[4-(Bromoacetamido)butyl]amino-2-deoxypentitol 1,5-bisphosphate

Reagent E = 2-{4-[4-(Bromoacetamido)phenylmethyl]anilino}-2-deoxypentitol 1,5-bisphosphate

R_f = The distance migrated by a compound divided by the distance traveled by the solvent front.

Ribulose-P_2 = D-Ribulose 1,5-bisphosphate

SP = Sulfopropyl

TPCK = Tosylphenylalanyl chloromethyl ketone

Tris = Tris(hydroxymethyl)aminomethane

INTRODUCTION

Ribulose-P_2 carboxylase/oxygenase (E.C. 4.1.1.39) catalyzes the competing reactions of ribulose-P_2 with CO_2 and O_2, the initial steps of photosynthetic carbon reduction and photorespiration, which yield two molecules of D-3-phosphoglycerate (1) and one molecule each of D-3-phosphoglycerate and phosphoglycolate, respectively (2). Photorespiration appears to be an energetically wasteful process, so that alteration of the carboxylase:oxygenase ratio has potentially beneficial effects on photosynthetic efficiency and crop productivity. With the goal of chemically manipulating this bifunctionality, numerous investigations are being directed toward the characterization of the enzyme's structural and functional properties.

Spinach carboxylase is typical of higher plant carboxylases in that it is a hexadecamer of eight large (53,000 dalton) and eight small (14,000 dalton) subunits (3-5). The large subunits are encoded by the chloroplast DNA while the small subunits are encoded by the nuclear genome (6,7). The primary sequences of carboxylases from eukaryotes and blue-green bacteria are highly conserved, showing about 85% overall homology (4,8-12). However, the carboxylase from the purple nonsulfur photosynthetic bacterium *Rhodospirillum rubrum* is markedly different in both primary and quaternary structure (13-15); the enzyme is a dimer of large (53,000 dalton) subunits, which show only about 28% homology with the spinach enzyme based on partial sequence data (16). Despite the differences in primary structure, the large subunits are functionally similar in that they contain the sites of catalysis (14,17) and CO_2/Mg^{++}-dependent activation (18-20).

The evolutionary distance between the carboxylases from R. rubrum and spinach make comparative sequence studies especially useful in identifying molecular features which have been conserved and are therefore likely to be catalytically functional. These comparisons assume that in the evolution from an ancestral carboxylase gene, the integrity of sequences performing an essential function has been conserved and that the substitution of a new sequence for one already functioning is rare (21).

As a means of identifying and comparing residues at the active site of the carboxylase, affinity labeling has been applied to the enzymes from R. rubrum and spinach. At the onset of this study, little sequence information for the enzyme was available. Recently, sequencing of cloned large subunit DNA has provided carboxylase sequences from spinach (4), tobacco (8), maize (9), Chlamydomonas (10), Anabaena (11), and Synechococcus (12). Traditional protein sequencing techniques have allowed the elucidation of the majority of the R. rubrum sequence (16). The finding of homology in itself is not a sufficient basis for assignment at the active site. However, implication of a residue's presence at the active site by affinity labeling coupled with a finding of strong interspecies sequence homology greatly strengthens the probability of a role in catalysis.

PART I

CHARACTERIZATION OF THE BINDING SITE FOR PYRIDOXAL 5'-PHOSPHATE

CHAPTER I

INTRODUCTION

Affinity labeling studies have proven informative with spinach carboxylase/oxygenase, with three different affinity labels implicating the same lysyl residue (Lys^{175})[1] as being at the active site. Of the three reagents, 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate (22), N-(bromoacetyl)ethanolamine phosphate (23), and PLP (24,25), only PLP-modified enzyme has proven amenable to characterization at the level of primary structure with the R. rubrum enzyme (26-28). The reported R. rubrum active-site peptide unexpectedly exhibited little homology with the corresponding peptide from spinach. Since both carboxylases evolved from a common ancestral gene (21), the reported lack of homology cast doubt on the assignment of the reactive lysyl residue at the active site.

Concurrent total sequence determination of the R. rubrum enzyme revealed a sequence similar to the reported active-site peptide obtained after PLP modification (28), except that the position corresponding to the reactive lysyl residue contained glycine. Since the apparent lack of homology of this peptide with the eukaryotic sequence strongly influences conclusions concerning the carboxylase active site, a reexamination of the PLP-binding site in the R. rubrum enzyme was undertaken. A unique phosphopyridoxal peptide was isolated from a carboxymethylated tryptic digest of R. rubrum carboxylase/oxygenase which had been inactivated with

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[1]Residues will be numbered according to spinach sequence (3) unless indicated otherwise.

PLP and reduced with sodium borohydride (29). In contrast to the earlier report, the peptide's sequence surrounding the PLP-reactive lysyl residue is highly homologous with the spinach enzyme, increasing the probability of a functional role for Lys^{175} in catalysis.

CHAPTER II

EXPERIMENTAL PROCEDURES

Materials

Bicine, Hepes, ATP, NADH, glutathione, glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate phosphokinase, glycerophosphate dehydrogenase/triosephosphate isomerase, and PLP were products of Sigma Chemical Company. TPCK-treated trypsin was purchased from Millipore, and NaB^3H_4 (333 mCi/mmol) was from New England Nuclear. Iodoacetic acid, obtained from Aldrich, was recrystallized before use. Pyridine was distilled over ninhydrin.

General Methods

Ribulose-P_2 was prepared enzymatically from D-ribose 5-phosphate according to published procedures (30). Ribulose-P_2 carboxylase/oxygenase from R. rubrum was purified to homogeneity as described previously; this enzyme is a dimer of 53,000-dalton subunits (14-16). Protein concentration was determined by using an $E_{280\ nm}^{1\%,1cm}$ of 12.0 (31). Enzymatic activity was assayed by the spectrophotometric method of Racker (32) as described earlier (15).

Modification with PLP for Determination of Stoichiometry and for Analytical Gels

Solutions (400 µl) of enzyme at 5 mg/ml (89 µM subunit) in 50 mM Hepes/1 mM EDTA/20 mM sodium bicarbonate/10 mM magnesium acetate (pH 7.8) were incubated with 0-100 µM PLP at 25°C for 60 minutes. The concentrations of PLP in stock solutions were determined from their absorbancies

in 0.1 M NaOH at 388 nm using an extinction coefficient of 6600 M^{-1} cm^{-1} (33). After the addition of 50 µl of octyl alcohol to prevent excessive foaming, the enzyme solutions were reduced for five minutes on ice with 5 mM $NaBH_4$. The reduced samples were assayed for carboxylase activity and were then dialyzed extensively against 0.01 M ammonium bicarbonate (pH 8.1). Aliquots of the dialyzed solutions were then withdrawn for determination of enzymatic activity and protein concentration, and the remaining samples were lyophilized prior to polyacrylamide gel electrophoresis. Due to the sensitivity of PLP to light, all procedures were carried out in the dark.

Modification with PLP for Peptide Isolation

Enzyme (50 mg per reaction) was incubated as described above at 0.5 mg/ml (8.9 µM subunit), 1.5 mg/ml (26.7 µM subunit), and 5.0 mg/ml (89 µM subunit) with PLP at a molar ratio of one, two, or four with respect to subunit. The reactions were terminated at 50-80% losses of activities. After the addition of 50 µl of octyl alcohol, the enzyme solutions were reduced for five minutes on ice with 1 mM NaB^3H_4 which had been freshly prepared by diluting labeled material about 50 to 1 with unlabeled $NaBH_4$. The modified enzyme was assayed and then extensively dialyzed in the dark at room temperature against 50 mM potassium phosphate/1 mM EDTA/1 mM 2-mercaptoethanol (pH 7.5), followed by dialysis against 0.01 M ammonium bicarbonate. Aliquots were removed for determination of protein concentration, enzyme activity, and tritium incorporation. Tritium was quantitated by using a Packard 3255 liquid scintillation spectrometer; samples were diluted with 0.5 ml of H_2O and counted in 10 ml of ACS scintillant (Amersham). Corrections were made

for tritium incorporation into the native enzyme that had not been treated with PLP.

Disc Gel Electrophoresis

The lyophilized samples were redissolved in 0.1 M glycine/0.013 M Tris (pH 8.6) containing 5% (v/v) 2-mercaptoethanol, 8 M urea, and 50% (v/v) glycerol. Discontinuous gels (stacking gel = 2.5% acrylamide, separating gel = 6% acrylamide) were run at pH 8.9 according to the manufacturer's instructions (Canalco) with modifications described by Gabriel (34), except that 8 M urea was also included in the stacking and separating gels. The gels were run for 2.5 h, stained with amido Schwarz, and destained electrophoretically.

Carboxymethylation and Trypsin Digestion

Carboxymethylation was performed essentially as described by Schloss et al. (23). Following extensive dialysis against 0.01 M ammonium bicarbonate, the PLP-modified protein was lyophilized to dryness. The residue was redissolved in 0.01 M sodium bicarbonate at a concentration of about 25 mg/ml. The solution was brought to 0.01 M in 2-mercaptoethanol, and an equal volume of 8 M guanidinium hydrochloride/0.1 M sodium phosphate/ 3 mM EDTA (pH 8.0) was added. Fifteen minutes later sodium iodoacetate, from a 1 M stock in 2 M sodium bicarbonate, was added to a final concentration of 0.03 M. After 20 minutes at room temperature in the dark, the reaction was quenched with 0.1 M 2-mercaptoethanol and dialyzed extensively against 0.01 M ammonium bicarbonate.

The carboxymethylated enzyme was digested with 1% (w/w) trypsin for 12 h at 40°C, at which time an additional 1% trypsin was added. Twelve hours later, the samples were frozen and stored at -20°C until needed.

Peptide Mapping

Peptide mapping was performed by the method of Katz et al. (35) with the following modifications. A 1-µl sample of 1% phenol red was added to the sample as a marker. Electrophoresis was carried out in an acetic acid/pyridine/water solvent (1:10:100 v/v) at pH 6.5 for 1 h at 1200 v. After the map was dried, it was subjected to descending chromatography by using the upper phase obtained from partitioning 1-butanol/acetic acid/water (4:1:5 v/v). Peptides were visualized by dipping the map in ninhydrin/collidine (36), followed by heating it at 80°C.

High Performance Liquid Chromatography

Purities of the isolated peptides were assessed by reversed phase high performance liquid chromatography (Laboratory Data Control) on a LiChrosorb RP8 (5 µM) column (Merck) using a 0.1% trifluoroacetic acid/acetonitrile solvent system.

Amino Acid Analyses

Peptides were hydrolyzed at 110°C in 6 N HCl/0.01 M 2-mercaptoethanol in sealed evacuated (<50 µM Hg) tubes for 21 h and dried on a Speed Vac Concentrator (Savant Instruments, Inc.). Amino acid compositions were determined with a Beckman 121 M amino acid analyzer using Beckman's "three-hour-single column system." Threonine and serine were increased by 5% and 10%, respectively, to correct for destruction during hydrolysis. N^6-(Phosphopyridoxal)lysine eluted between ammonia and arginine, and was quantitated using the relative color value of Forrey et al. (37).

Sequence Analyses

The purified peptide (75 nmol) was subjected to automated Edman degradation with a Beckman 890C, the vacuum system of which was modified according to Bhown et al. (38). 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 (39). The peptide was sequenced twice, once in a Quadrol buffer system and once in a dimethylallylamine buffer system with Beckman's peptide program 102974. The sequence program used with the Quadrol buffer was that of Bhown et al. (38), 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 peptide program 030176. 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 (40). Threonine and arginine appear as α-aminobutyric acid and ornithine, respectively, in base hydrolysates. Aspartic and glutamic acids were distinguished from the corresponding amides by high performance liquid chromatography of the phenylthiohydantoins.

CHAPTER III

RESULTS AND DISCUSSION

Titration of the carboxylase with PLP shows that the inactivation is directly proportional to the amount of PLP added, and suggests that one molar equivalent per subunit is required for total inactivation (Figure 1A, Appendix B). Although earlier studies also showed that both subunits were reactive toward PLP, they indicated half-of-sites reactivity, i.e., modification of one subunit per dimer sufficing to inactivate the enzyme completely (28). For clarification of this disparity, samples at intermediate levels of inactivation were subjected to polyacrylamide gel electrophoresis under denaturing conditions with the expectation that the more negatively charged PLP-derivatized subunits would be resolved from unmodified subunits. As seen in Figure 1B, Appendix B, the relative amount of unmodified subunit (the slower moving component) agrees with the percentage of initial activity remaining, and thus half-of-sites reactivity does not apply. The seemingly low incorporation values in prior publications (27,28) are explained in part by the use of 9.74 for the $E^{1\%,1cm}_{280\ nm}$ (14) rather than 12.0 (31). Additionally, the PLP stoichiometry based on tritium incorporation from [^{3}H]borohydride was less than that determined by spectrophotometric analyses, which complicates assessment of its validity.

The labeled peptide in tryptic digests of carboxylase after inactivation with PLP, reduction with NaB^3H_4, and carboxymethylation of sulfhydryls with iodoacetic acid, was purified by successive chromatography

on DEAE-cellulose, SP-Sephadex, and Sephadex G-25 as described in the legend to Figure 2, Appendix B. In initial experiments, the concentrations of enzyme (1.5 mg/ml, 26 μM subunit), PLP (50 μM), EDTA (1 mM), sodium bicarbonate (20 mM), magnesium acetate (10 mM), and protons (pH 7.8) were identical with those reported (28). Only one phosphopyridoxal peptide was found, but its amino acid composition differed significantly from the one published previously (Table 1, Appendix A, Sample A).

A possible explanation for the different results from the two laboratories is that there are two different lysyl residues that are accessible to PLP but the site of attack is dependent upon experimental conditions. If the two sites differed in affinity for PLP, the molar ratio of PLP to enzyme could be a crucial variable. Alternatively, if the "dimeric" carboxylase existed as an equilibrium mixture of monomers and dimers [for which, however, there is no evidence over the concentration range from 0.25 to 5 mg/ml (14)] which interacted differently with PLP, enzyme concentration could be a crucial variable. Thus, the phosphopyridoxal peptide was isolated from enzyme derivatized at several concentrations with different levels of PLP.

The purification of labeled peptide from a mixture of four different preparations of PLP-inactivated enzyme (protein at 0.5 and 5 mg/ml modified with both 1:1 and 4:1 molar equivalents of PLP) is shown in Figure 2, Appendix B. No evidence of a second site of modification by PLP was found, given reactant concentrations as variable parameters. The overall recovery from 20 mg (357 nmol of subunit) of mixed tryptic digests was 51% based on radioactivity and 63% based on nanomoles of peptide actually obtained.

Identical chromatographic procedures were used to purify the phosphopyridoxal peptide from several individual preparations of modified carboxylase. With every preparation examined, the sole labeled peptide emerged at the same position from each of the three columns employed, and the purified peptides' amino acid compositions were indistinguishable (Table 1, Appendix A). Based on the absence of several amino acids and the approximate whole-number molar ratios of those present, the peptides appeared essentially homogeneous. This conclusion was verified by peptide mapping (Figure 3, Appendix B), high performance liquid chromatography (data not shown), and by sequence analyses (Table 2, Appendix A). We attribute the low values for isoleucine to the stability of Ile-Ile peptide bonds. That the peptide visualized on the map with ninhydrin was indeed the phosphopyridoxal peptide was confirmed by cutting the spot out and counting it; the origin was devoid of radioactivity.

The total incorporation of PLP, extrapolated to 100% inactivation, into the enzyme calculated from the specific activities of the purified peptides ranged from 1.1 to 1.5 mol of PLP per mol of subunit. The higher levels of incorporation were observed when the enzyme at 5 mg/ml was treated with a four-fold molar excess of PLP. Since even in this instance only one labeled peptide in significant quantities was observed in tryptic digests, the excess incorporation must represent the reaction of multiple lysyl residues in very low yields. The greater than stoichiometric incorporation of PLP explains the lower yield of peptide based on radioactivity as compared to actual nanomoles (see above).

The phosphopyridoxal peptide (Table 1, Appendix A) was sequenced by automated Edman degradation. Results of one run using a Quadrol buffer

system are provided in Table 2, Appendix A; a second run, in which a dimethylallylamine buffer was used, revealed the same sequence. The high degree of purity of the peptide is readily apparent at cycle 1 in which the only contaminants, glycine and leucine, are present at levels less than 5% of that of NH_2-terminal valine. Amino acids that are not listed in the table were not detected in any of the 20 cycles.

The sequence of the phosphopyridoxal peptide from R. rubrum carboxylase as deduced from the data shown in Table 2, Appendix A and as published earlier and the sequence of the corresponding peptide from spinach carboxylase are as follows (underlining denotes the labeled residue):

R. rubrum peptide (28)

Ala-Leu-Gly-Arg-Pro-Glu-Val-Asp-Lys-Gly-Thr-Leu-Val-Ile-Lys

R. rubrum peptide [present study (29)]

Val-Leu-Gly-Arg-Pro-Glu-Val-Asp-Gly-Gly-Leu-Val-Val-Gly-Thr-Ile-Ile-Lys-Pro-Lys

Spinach peptide (22,23,25)

Tyr-Gly-Arg-Pro-Leu-Leu-Gly-Cys-Thr-Ile-Lys-Pro-Lys

The differences between the R. rubrum peptides cannot be reconciled. Given the variety of conditions used for the inactivation and given the number of times the peptide isolation was repeated, confidence is felt in the correctness of the data reported herein. One notes that beyond the NH_2-terminal residue, which was reported as alanine rather than valine, the next seven amino acids are the same in both peptides. Perhaps the material sequenced previously was impure, and the last half of the sequence published represents that of an impurity remaining after

preferential washout of the phosphopyridoxal peptide from the spinning cup. In this connection, we were unable to obtain a pure peptide by the use of the two columns described by Robison et al. (28).

In comparing our sequence of the phosphopyridoxal peptide from the R. rubrum enzyme with the tryptic peptide from spinach carboxylase that contains Lys^{175} [which is reactive toward PLP, 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate, and N-(bromoacetyl)ethanolamine phosphate], a common sequence of four residues is seen encompassing the essential lysyl residue. The homology may be extended by aligning the six known carboxylase sequences (4,8-12) and the R. rubrum sequence (16), revealing seven identical residues: -Ile-Lys-Pro-Lys-Leu-Gly-Leu. The finding of this striking homology between functionally analogous, but structurally dissimilar, enzymes from such diverse species strengthens the earlier conclusions that Lys^{175} is located within the active site. Furthermore, the species invariance in primary structure adjacent to this unusually reactive lysyl residue is suggestive of a catalytic functionality.

Pierce et al. (41) have pointed out that a divalent metal ion (Mg^{++}) or a protonated lysyl side chain could function in the carbanion inversion that is necessary for development of the proper stereochemistry in the product (i.e., D- rather than L-3-phosphoglycerate) derived from carbon dioxide and C1 and C2 of ribulose-P_2. An ionized amino group could also act as a general base promoting the hydrolytic scission between C2 and C3 of ribulose-P_2. A third possible role for the lysyl ε-amino group is that of the essential base which initiates the catalytic reaction by abstraction of the C3 hydrogen atom of ribulose-P_2 as a proton. Based on the primary isotope effect observed with [3-^{3}H]ribulose-P_2, this step

is partially rate-limiting (42-44). If an amino group were to participate in this way, the very low k_{cat} of the carboxylase, 2.5 sec^{-1} for the spinach enzyme (13,45) and 8 sec^{-1} for the R. rubrum enzyme (15), might be explained. As discussed by Rose (46,47) concerning the identity of the essential base in triosephosphate isomerase, the k_{cat} for turnover cannot exceed the ionization rate of the protonated base. Irrespective of the nature of the base, this ionization rate equals the diffusion-controlled rate of protonation of the free base (10^{10} M^{-1} sec^{-1}) (48) multiplied by the ionization constant of the protonated species. Thus, an ε-amino group with a typical pK_a of 9.5, functioning as the essential base, cannot support a k_{cat} exceeding about 3 sec^{-1}. Of course, if the unusual reactivity of Lys^{175} reflects an abnormally low pK_a, the maximally allowed value for k_{cat} will be increased.

Cys^{172} in the spinach carboxylase, which is preferentially alkylated by N-(bromoacetyl)ethanolamine phosphate when the enzyme assumes its deactivated conformation (23), can now be excluded as having any essential catalytic role other than in hydrogen bonding. This conclusion follows the observation that in the R. rubrum enzyme the cysteinyl residue is replaced by threonine.

PART II

AFFINITY LABELING OF RIBULOSEBISPHOSPHATE CARBOXYLASE USING 2-(N-BROMOACETYL)AMINO SUBSTRATE ANALOGS

CHAPTER I

INTRODUCTION

Previous affinity labeling studies have established three regions of primary structure as being at or near the active site of both spinach and R. rubrum carboxylase. Lys^{175} has been labeled in the spinach enzyme by three different reagents — 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate (22), N-(bromoacetyl)ethanolamine phosphate (23), and PLP (24, 25) — and in the R. rubrum enzyme by PLP (26,27,29). An identical seven-residue sequence encompasses the reactive lysyl residue in the two species. The second region has been documented as the site of formation of a Mg^{++}-stabilized carbamate at the ε-amino group of Lys^{201}, which occurs upon activation of the enzyme (19,20). The sequence surrounding Lys^{201} exhibits considerable homology in that 10 out of 17 residues are identical in the two enzymes. The third active-site region contains Lys^{334} in spinach and Met^{335} in the R. rubrum enzyme. The former is labeled by 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate (22) and the latter by 2-[(bromoacetyl)amino]pentitol 1,5-bisphosphate (49,50) and 2-N-chloroamino-2-deoxypentitol 1,5-bisphosphate (51). Although Met^{335} is replaced by Leu in the spinach enzyme, Lys^{334} and eight other residues in its immediate vicinity are conserved.

In an attempt to identify additional active-site residues, a series of potential affinity labels for the carboxylase have been synthesized in which the keto group at C2 of ribulose-P_2 has been replaced by a reactive moiety. The merit of mapping residues in proximity to C2 of

substrate is revealed by the structure of the proposed transition state complex.

The transition state analog, carboxyarabinitol-P_2, complexed with enzyme, divalent metal ion, and activator CO_2 forms an extremely stable quaternary complex which exhibits a stoichiometry of 1:1:1:1 (52,53). The requirement for metal cation in the activation process (54) has been hypothesized at the molecular level as involving the stabilization of the carbamate (18). The single metal ion in the quaternary complex has also been placed in proximity to the carboxyl group of carboxyarabinitol-P_2. Nuclear magnetic resonance studies of spinach carboxylase complexed with Mn^{++}, estimates the distance from the substrate CO_2 to metal ion as 5.4 Å (55), near enough to visualize a role in catalysis as well as activation (41,53). A potential interaction of metal cation with both substrate and activator CO_2 is pictured in Figure 4, Appendix B. The distance between substrate and activator CO_2 molecules in such a model may be roughly estimated as being between 9 and 12 Å, depending upon the activator CO_2-metal ion distance, so that a reasonable approximation of the distance between substrate's C2 and the ε-amino group of Lys^{201} is 10 to 14 Å.

A series of ribulose-P_2 analogs bearing reactive arms at C2 would probe regions of carboxylase structure at varying distances from the ribulose-P_2 binding site. This approach would potentially reveal structural features essential for the substrate-metal-carbamate interactions as well as perhaps bridging the distance to the ε-amino group of the carbamate-forming lysyl residue. A series of such compounds have been synthesized by the reductive amination of ribulose-P_2 with various

bifunctional amines and subsequent bromoacetylation of the resultant primary amine. A summary of the structures of the reagents and the distances from C2 to the α-carbons of the N-bromoacetyl groups is shown in Figure 5, Appendix B. The interactions of these compounds with the carboxylase are described. One member of the series, 2-(4-bromoacetamido) anilino-2-deoxypentitol 1,5-bisphosphate, which satisfies the criteria of an affinity label, provides the first chemical evidence for a highly conserved region of the R. rubrum carboxylase as being at or near the active site.

CHAPTER II

EXPERIMENTAL PROCEDURES

Materials

Bicine, ATP, NADH, glutathione, glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate phosphokinase, glyceraldehyde dehydrogenase/triose phosphate isomerase, alkaline phosphatase and p-phenylenediamine were obtained from Sigma. Ethylenediamine was from Eastman. Angiotensin converting enzyme substrate (Hippuryl-L-His-L-Leu) was purchased from United States Biochemicals. TPCK-treated trypsin was from Worthington. Iodoacetic acid, 5,5'-dithiobis(2-nitrobenzoic acid), N-hydroxysuccinimide, 1,4-diaminobutane, 4,4'-methylenedianiline, and sodium cyanoborohydride were products of Aldrich; the latter was recrystallized according to Jentoff and Dearborn (56) and tritiated by exchange in tritiated water (New England Nuclear) to a specific activity of 216,000 dpm/μmol (57). [2-^{14}C]Bromoacetic acid from Amersham was used to prepare the N-hydroxysuccinimide ester as described by Hartman et al. (58). Elemental analyses were performed by Galbraith Laboratories, Incorporated, Knoxville, Tennessee. Mass spectra were recorded by the Analytical Chemistry Division of the Oak Ridge National Laboratory.

General Methods

Ribulose-P_2 was prepared enzymatically from D-ribose 5-phosphate according to published procedures (30). Carboxyribitol-P_2 was prepared and purified as described by Pierce et al. (41). R. rubrum carboxylase/oxygenase was purified to homogeneity as previously described (15); this

enzyme is a dimer of apparently identical 53,000 dalton subunits (14-16). The enzyme used in this study had a specific activity of 5.5 units/mg. Protein concentration was determined using an $E_{280\ nm}^{1\%,1cm}$ of 12.0 (31). Carboxylase activity was routinely determined by slight modification (14) of the spectrophotometric method of Racker (32); however, for the competitive inhibition studies, the $^{14}CO_2$-fixation assay was used (59). Oxygenase activity was monitored using an oxygen electrode (Hansatech) according to Lorimer et al. (59).

Polyacrylamide gel electrophoresis was carried out according to the manufacturer's instructions (Canalco) on 7.5% polyacrylamide gels in Tris-glycine at pH 8.9 and stained with amido Schwarz.

A Packard 3255 liquid scintillation spectrometer was used to quantitate radioactivity. Samples were diluted to 0.5 ml with H_2O and counted in 10 ml of ACS scintillant (Amersham). Corrections for quenching were made by the channels ratio method using a standard curve constructed from the manufacturer's quenched standards.

Descending paper chromatography was carried out on Whatman No. 1 paper with a n-butanol/acetic acid/H_2O (7:2:5, v/v/v) solvent system. Compounds were visualized with ammonium molybdate for phosphate esters (60), ninhydrin/cadmium for primary amines (61), dimethylaminobenzaldehyde for aromatic amines (62), and 5-thio-2-nitrobenzoic acid for reactive halogen (63).

Inorganic phosphate was quantitated by the method of Marsh (64) subsequent to the hydrolysis of the phosphate ester in sulfuric acid as previously described (49). The bromine contents of the reagents were quantitated by reaction with an excess of glutathione followed by

titration of the unreacted glutathione with 5,5'-dithiobis(2-nitrobenzoic acid) (65).

Synthesis of Reagents A and B

The dibarium salt of ribulose-P_2 (300 mg; 0.34 mmol based on 65% purity of ribulose-P_2) was suspended in 3.0 ml H_2O and freed of Ba^{++} ions by the addition of 2 g (2 meq) of Dowex 50 (H^+). The suspension was filtered and the resin washed successively with three, 1-ml aliquots of H_2O. To the combined filtrate and washings were added 330 mg (3.05 mmol; nine-fold molar excess) of p-phenylenediamine (free base) which had been dissolved in 6.0 ml of 80% methanol/0.6 N HCl. After adjustment of the pH to 6.9 with 5 N LiOH, the yellow solution was transferred to a round-bottom flask equipped with a magnetic stirrer and purged with N_2 for five minutes. Subsequent to the addition of 21 mg (0.34 mmol) of sodium [^{3}H]cyanoborohydride, the flask was tightly capped and the solution was stirred at room temperature for six hours. At this time, the pH was increased to 8.0 with 1 N LiOH, and the solvent was removed by rotary evaporation at 40°C. The solid residue was dissolved in H_2O, transferred to a centrifuge tube with rinsing (total volume = 4 ml), and chilled to 4°C. The phosphate esters were precipitated by the addition of four volumes of cold ethanol and were collected one hour later by centrifugation.

Following three washings of the pelleted material with 10 ml of ethanol, the residue was dissolved in H_2O (5 ml; 2.3 x 10^7 dpm; 330 µmol) and applied to a column (1 x 20 cm) of QAE-Sephadex A-25 (Cl^-) (Pharmacia) which was equilibrated with H_2O. Elution with a 400-ml linear gradient of 0-150 mM HCl resulted in the profile depicted in Figure 6A, Appendix B. Spot tests of peak fractions showed peaks A and B (11% and 10% of the

applied radioactivity, respectively) to be phosphate esters with an amino group, while peak C (70% of the applied radioactivity) was a phosphate ester lacking a primary amine.

The dibarium salts of peaks A, B, and C were collected by adding a ten-fold molar excess of $BaBr_2$ to the pooled fractions at 4°C, adjusting the pH to 8.0 with 1 N LiOH, and adding two volumes of cold ethanol. After at least one hour at 4°C, the precipitates were collected by centrifugation and washed with three successive 10-ml portions of cold ethanol.

The wet barium salts were dissolved in 0.2 ml of 1 N HCl, and the Ba^{++} was precipitated as $BaSO_4$ by the addition of a 2.2-molar excess of 1 M H_2SO_4. [Dowex 50 (H^+) could not be used as it adsorbed the desired amine.] After centrifugation, the pellet was washed twice with 0.15 ml of 0.5 N HCl and the three supernatants were then pooled. Paper chromatography revealed the major components in peaks A and B to be amino bisphosphates with identical mobilities (R_f = 0.14). A minor ninhydrin-reactive component (R_f = 0.27) was present in peak A, while peak B was slightly contaminated with inorganic phosphate (R_f = 0.44). Peak C (R_f = 0.12) contained a ninhydrin-negative bisphosphate.

The contaminants present in peaks A and B were removed by rechromatography on QAE-Sephadex (Figure 6B and C, Appendix B). The solution of peak A (30 µmol), freed of Ba^{++} as described above, was diluted with water to 5.0 ml, adjusted to pH 8.0 with 1 N LiOH, and brought to 5 mM in 2-mercaptoethanol. This solution was applied to a column (1 x 20 cm) of QAE-Sephadex A-25 (Cl^-) equilibrated with 5 mM 2-mercaptoethanol and eluted with a 400 ml-linear gradient of 0-0.5 M LiCl in 5 mM 2-mercaptoethanol. The major peak, which contained organic phosphate and a primary

amino group, was pooled, adjusted to pH 8 with 1 N LiOH, and lyophilized. The residue was suspended in 20 ml of ethanol and centrifuged. The alcohol-insoluble pellet was washed three times with 10 ml of ethanol and then dissolved in 0.2 ml H_2O and filtered. The Li^+ salt was reprecipitated by the addition of four volumes of cold ethanol, washed three times with 10 ml of ethanol, and finally dried by lyophilization; 7 mg (16 μmol, 5%) of the lithium salt were obtained. Peak B (30 μmol) was purified in an identical manner to give 12 mg (28 μmol, 8.3%). The compounds appeared homogeneous by paper chromatography, each containing an amino bisphosphate (R_f = 0.14; specific activity = 54,000 dpm/μmol) without the prior contaminants. Quantitative phosphate assays verified the presence of 2 mol organic phosphate per mol of peak A or B.

$C_{11}H_{16}Li_4N_2O_{10}P_2 \cdot 2H_2O$

C:H:N	Calculated:	4.72:0.72:1.00
Peak A	Found:	4.90:0.73:1.00
Peak B	Found:	5.07:0.72:1.00

Peaks A and B were analyzed by mass spectrometry after digestion of the phosphate groups by alkaline phosphatase and removal of P_i by chromatography on DEAE-cellulose. Mass spectra (70eV) m/e(relative intensity): peak A 242(M^+,12), 224(10), 211(5), 193(12), 151(35), 108(100); peak B 242(M^+,12), 224(20), 211(8), 193(32), 151(25), 108(100).

The amino bisphosphates were bromoacetylated to give reagents A and B, respectively, with N-hydroxysuccinimide bromoacetate as previously described (58). A solution of the phosphate ester in hydrochloric acid was freed of Ba^{++} as described above and adjusted to pH 6.5 with 5 N LiOH

after the addition of a two-fold mole excess of triethylamine. Tetrahydrofuran (1 ml) and a five-fold mole excess of N-hydroxysuccinimide bromoacetate were added, and the solution stirred at room temperature for one hour. After cooling to 4°C, the phosphate esters were precipitated by the addition of a five-fold mole excess of $BaBr_2$ dissolved in methanol, followed by 5 ml of cold ethanol. After at least 30 minutes at 4°C, the products were collected by centrifugation and washed successively with three 10-ml portions of cold ethanol.

Paper chromatography of both reagents A and B revealed the disappearance of the ninhydrin-positive amino groups with the concomitant appearance of a single phosphate-positive, halogen-positive component (R_f = 0.33). The homogeneity of the product was also demonstrated by quantitation of the ratio of total phosphate concentration to reactive halogen concentration, which was always between 1.9 and 2.1. [^{14}C]Reagent B for peptide characterization was prepared using the N-hydroxysuccinimide ester synthesized from [2-^{14}C]bromoacetic acid (specific activity of product = 1.2×10^6 dpm/μmol).

The epimeric relationship between these two compounds is supported by several lines of evidence. The compounds are formed in equal amounts (based on radioactivity in peaks A and B, Figure 6, Appendix B), the sum of which is comparable to the yield of amino bisphosphate in four other reductive aminations of ribulose-P_2 (49, C. S. Herndon and F. C. Hartman, unpublished observations). Both compounds contain a primary amine, two phosphate groups, and one tritium incorporated from [^{3}H]$NaBH_3CN$. Based on paper chromatography, the isolated amino bisphosphates migrate with the same R_f both before and after acylation of the amino function.

Elemental analyses of the amino bisphosphates are in reasonable agreement with the predicted C:H:N ratio, and the mass spectra of peaks A and B are virtually identical. This confirms the stereochemical relationship between peaks A and B since epimeric compounds give the same fragmentation patterns, only differing in the relative abundance of the fragments. Subsequent to bromoacetylation, quantitative phosphate and halogen assays establish their ratio as 2:1 in each case. In studies with model peptides, both reagents A and B show the same second order rate constant in their reactions with cysteine, and little reactivity with histidine (see Chapter IV). The adducts formed with glutathione appear identical in that they coelute slightly ahead of methionine from the amino acid analyzer upon removal of the phosphate groups with alkaline phosphatase. The differences in K_i values as competitive inhibitors of the carboxylase (see Chapter III) are consistent with discrimination in the binding of C2 epimers, as seen with carboxyribitol-P_2 and carboxyarabinitol-P_2 (41) and with 2-carboxyglucitol 1,6-bisphosphate and 2-carboxymannitol 1,6-bisphosphate (66). Although one might speculate that reagent B contains the bromoacetyl moiety and the hydroxyl groups in a cis configuration, assignment of stereochemistry at C2 awaits further chemical characterization.

Synthesis of Reagents C, D, and E

Reagents C, D, and E were synthesized similarly to reagents A and B from primary amines obtained by reductive amination of ribulose-P_2 with ethylenediamine, 1,4-diaminobutane, and 4,4'-methylenedianiline, respectively. The same protocol was used in all three syntheses, with minor differences as indicated below.

The dibarium salt of ribulose-P_2 (300 mg; 0.34 mmol, based on 65% purity of ribulose-P_2) was suspended in 3.0 ml H_2O and freed of Ba^{++} ions by the addition of 2 g (2 meq) of Dowex 50(H^+). The suspension was filtered and the resin washed successively with three, 1-ml aliquots of H_2O. To the combined filtrate and washings was added a ten-fold mole excess of amine from either an aqueous stock solution adjusted to pH 6.9 (reagents C and D) or a methanolic solution containing 1 N HCl (reagent E). After adjustment of the pH to 6.9 with 5 N LiOH, a mole equivalent of sodium [^{3}H]cyanoborohydride was added, and the reaction stirred overnight (reagents C and D) or two hours (reagent E). After adjustment of the pH to 8.0 with LiOH, the reaction was taken to dryness under a stream of nitrogen at 40°C. The residue was washed three times with 10-ml aliquots of ethanol and redissolved in a minimum volume of H_2O. The phosphate esters were reprecipitated by the addition of four volumes of ethanol and collected one hour later by centrifugation. The pellet was redissolved in 5 ml H_2O for anion-exchange chromatography.

The amine intermediate of reagent E was purified on QAE-Sephadex exactly as described for reagents A and B. The only difference in the elution profiles was the lack of resolution of the epimeric amino bisphosphates, which elute at about 30 mM HCl. In the case of reagents C and D, an AG1-X8 (acetate form) column (1 x 22 cm) equilibrated with H_2O was used and eluted with a 400-ml linear gradient of 0-0.15 N acetic acid. The amino bisphosphates elute at about 40 mM acetic acid. Again, no resolution of the epimeric products was seen. In all cases, the eluted amino bisphosphates were collected as the dibarium salts by adding excess $BaBr_2$, adjusting the pH to 8.0 with LiOH, and adding four volumes

of cold ethanol. Yields were 24%, 22%, and 25% for the amine intermediates of reagents C, D, and E, respectively.

The isolated amino bisphosphates were examined for purity by paper chromatography and subsequently bromoacetylated as described for reagents A and B. Reagents C, D, and E appeared homogeneous by paper chromatography, which showed in each case, a single phosphate ester containing reactive halogen which was unreactive with ninhydrin (R_f = 0.25, 0.38, and 0.62, respectively).

Reaction of Reagents A and B with Model Compounds

The second order rate constants for the reaction of reagents A and B with glutathione were determined by the incubation of 1 mM glutathione and 1 mM reagent A or B in 0.1 M Bicine (pH 8.0) at 25°C and periodically quantitating unreacted glutathione concentration with 5,5'-dithiobis(2-nitrobenzoic acid). Each glutathione adduct was subsequently treated with 1% (w/w) alkaline phosphatase for 2 h at 25°C and subjected to amino acid analysis with and without acid hydrolysis. To monitor the alkylation of histidine, 4 mM reagent A or B was incubated with 0.2 mM hippuryl-histidyl-leucine in 0.1 M Bicine (pH 8.0) at 25°C, and the concentration of histidine was then quantitated after acid hydrolysis by amino acid analysis.

Treatment of Carboxylase with Reagents A and B

For monitoring time-dependent inactivations, the enzyme was incubated at 25°C with the reagent as described in the legends to Figures 7 and 8, Appendix B, in Chelex-treated 50 mM Bicine/0.1 mM EDTA (pH 8.0) either with or without the addition of 66 mM $NaHCO_3$/10 mM $MgCl_2$. When CO_2 and Mg^{++} were included, the enzyme was preincubated to ensure full activation

prior to reagent addition. Periodically, aliquots were withdrawn and assayed for carboxylase or oxygenase activity.

For the determination of the stoichiometry of modification and for the preparation of modified enzyme to be characterized chemically, [^{14}C]-labeled reagent B was used. Two portions of activated enzyme were incubated at 5 mg/ml (94.3 μM subunit) in 50 mM Bicine/66 mM $NaHCO_3$/10 mM $MgCl_2$/0.1 mM EDTA (pH 8.0) with 250 μM reagent in the presence (reaction volume = 1 ml) and absence (reaction volume = 36 ml) of 250 μM carboxyribitol-P_2. Periodically, 10-μl aliquots were diluted into buffer containing 0.1 M 2-mercaptoethanol for enzymic assays, while 20-μl aliquots were spotted on filter paper discs (67) for determination of covalent incorporation by trichloroacetic acid precipitation. Only protein-bound reagent remains associated with the filter paper disc. The discs were placed in ice-cold 10% trichloroacetic acid immediately after sample application, and subsequently washed with two 500-ml portions of cold 5% trichloroacetic acid, two 500-ml portions of cold ethanol/diethyl ether (1:1 v/v) and two 500-ml portions of diethyl ether. After drying at room temperature, the discs were counted in 10 ml of scintillation fluid. The degree of quenching by the filter was assessed by counting an aliquot of the [^{14}C]reagent B solution which had been spotted on a filter disc and air-dried. At the desired levels of inactivation, samples (0.5 mg) from the unprotected reaction were also quenched with 0.1 M 2-mercaptoethanol for later polyacrylamide gel electrophoresis and then dialyzed against 0.01 M $NaHCO_3$. After dialysis, aliquots of the samples were withdrawn for the determination of protein concentration incorporation, and enzymic activity. In all cases, incorporations determined from the filter paper method were in agreement with those obtained after dialysis.

When the unprotected sample had lost 93% of its initial activity, it was quenched with 0.1 M 2-mercaptoethanol and then dialyzed extensively against 0.01 M NH_4HCO_3. Following the withdrawal of aliquots to quantitate protein concentration, incorporation, and enzymic activity, the protein was lyophilized and carboxymethylated with iodoacetate as described previously. Subsequent to dialysis against 0.01 M NH_4HCO_3, the carboxymethylated enzyme was digested with 1% (w/w) trypsin for 4 h at 40°C, at which time an additional 1% trypsin was added. Four hours later, the solution was divided into 60-mg aliquots and frozen at -20°C until needed for peptide purification.

Treatment of Carboxylase with Reagents C, D, and E

For monitoring time-dependent effects of reagents C, D, and E on carboxylase activity, the enzyme was incubated at 25°C with the reagent in Chelex-treated 50 mM Bicine/0.1 mM EDTA (pH 8.0) either with or without the addition of 66 mM $NaHCO_3$/10 mM $MgCl_2$. When CO_2 and Mg^{++} were included, the enzyme was preincubated to ensure full activation prior to reagent addition. Ten-µl aliquots were withdrawn and assayed for carboxylase activity.

For further characterization of reagent C-inactivated carboxylase, a 40-mg (754 nmol) sample of carboxylase (5 mg/ml, 94.3 µM subunit) was treated with 750 µM [^{14}C]reagent C in 50 mM Bicine/66 mM $NaHCO_3$/10 mM $MgCl_2$/0.1 mM EDTA (pH 8.0). A protected sample (5 mg) was treated identically except for the inclusion of 250 µM carboxyribitol-P_2. Periodically, 10-µl aliquots were diluted into buffer containing 0.1 M 2-mercaptoethanol for enzymic assays, while 20-µl aliquots were spotted on filter

paper discs for determination of covalent incorporation, as previously described.

When the unprotected sample had lost 75% of its activity, it was quenched with 75 mM 2-mercaptoethanol and dialyzed extensively against 0.01 M NH_4HCO_3. Aliquots were removed for the determination of protein concentration, incorporation, and enzymic activity, and the remainder of the protein solution was lyophilized. The residue was then carboxymethylated as described previously, dialyzed against 0.01 M NH_4HCO_3, and digested with 1% (w/w) trypsin at 37°C for 7 h. Seventeen hours after a second addition of 1% trypsin, the solution was divided into 10-mg samples and frozen.

Characterization of reagent D-modified carboxylase involved the treatment of a 15-mg sample of enzyme (5 mg/ml, 94.3 μM subunit) with 2 mM [^{14}C]reagent D at 25°C in 50 mM Bicine/66 mM $NaHCO_3$/10 mM $MgCl_2$/0.1 mM EDTA (pH 8.0) until the sample had lost 75% of its initial activity. The reaction was quenched with 0.1 M 2-mercaptoethanol, desalted on a Sephadex G-25 (fine) column (2.5 x 15 cm) in the same Bicine buffer, and aliquots removed for the determination of protein concentration, incorporation, and enzymic activity. The protein was then lyophilized, carboxymethylated, and digested with trypsin as previously described. A 1-mg aliquot of the tryptic digest was hydrolyzed for amino acid analysis in order to identify the types of amino acids which had been alkylated.

Amino Acid Analysis

Peptides were hydrolyzed at 110°C in 6 N HCl/0.01 M 2-mercaptoethanol in sealed evacuated (<50 μm Hg) tubes for 21 h and dried on a Speed Vac Concentrator (Savant Instruments, Incorporated). Amino acid compositions

were determined with a Beckman 121 M amino acid analyzer using Beckman's "three-hour-single column system." Threonine and serine were increased by 5% and 10%, respectively, to correct for destruction during hydrolysis.

Sequence Analysis

The purified peptides (50 nmol each; 6.0×10^4 dpm) were subjected to automated Edman degradation in 0.1 M Quadrol buffer with a Beckman 890C, the vacuum system of which was modified acording to Bhown et al. (38). 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 (39). The sequence program was that of Bhown et al. (38) 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 peptide program 030176. 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 (40). Threonine and arginine appear as α-aminobutyric acid and ornithine, respectively, in base hydrolysates. Aspartic and glutamic acids were distinguished from the corresponding amides by high performance liquid chromatography of the phenylthiohydantoins.

CHAPTER III

RESULTS

Reagents A and B

Kinetics of inactivation of carboxylase. The effects of the epimeric reagents A and B on R. rubrum carboxylase activity are shown in Figure 7, Appendix B. In the absence of CO_2 and Mg^{++}, neither compound inactivates the enzyme to a significant extent. However, the fully activated enzyme, although still refractory to reagent A, shows a time-dependent inactivation by reagent B which is subject to protection by the competitive inhibitor carboxyribitol-P_2 (Figure 8A, Appendix B). The inactivation is pseudo-first order, essentially complete (<2% activity remaining) upon prolonged incubation with the reagent, and is not reversed by dialysis. Oxygenase activity is lost in parallel with the carboxylase activity (data not shown). Both reagents A and B are linear competitive inhibitors of the carboxylase with K_i values of 705 and 104 μM, respectively (data not shown). The enzyme from spinach is not affected by incubation with either reagent A or B in the presence or absence of CO_2 and Mg^{++}.

The demonstration of rate-saturation, a consequence of the reversible formation of an enzyme-reagent complex prior to the covalent reaction, is an important criterion for affinity labeling. A plot of the half-time of inactivation versus the reciprocal of the reagent concentration, according to the equation on page 35, should show a positive intercept on the Y-axis, indicating a minimal half-time (T_{min}) at infinite reagent concentration.

$$t_{1/2} = (K_{inact}/T_{min})(1/[reagent]) + 1/T_{min}$$

The parameter K_{inact}, analogous to a K_m for substrate, represents the reagent concentration giving the half-maximal rate of inactivation (68, 69). Such a plot for the inactivation of the carboxylase by reagent B is shown in Figure 8B, Appendix B. The T_{min} is 7.3 minutes with a K_{inact} of 125 μM. The similarity in the K_{inact} and K_i obtained from competitive inhibition studies is consistent with the visualization of the same binary complex, and supports the earlier supposition that the difference in K_i and K_{inact} seen with 2-[(bromoacetyl)amino]pentitol 1,5-bisphosphate (85 μM versus 380 μM) arises from the use of an epimeric mixture (49).

Stoichiometry of inactivation of carboxylase. The large scale inactivation for subsequent characterization at the sequence level was monitored by the time course of activity loss as well as covalent incorporation of reagent. Figure 9, Appendix B, shows the incorporation to be directly proportional to activity remaining. Extrapolation to total inactivation correlates with an incorporation of 1.1 mol reagent/mol subunit. The inclusion of 250 μM carboxyribitol-P_2 greatly retards the inactivation ($t_{1/2}$ > 100 minutes versus 25 minutes) with a concomitant reduction in incorporation. At the time of quenching (70 minutes, 93% activity lost), the incorporation into the unprotected enzyme was 1.0 mol reagent per mol subunit.

The extent of covalent modification at varying levels of inactivation could be directly assessed by polyacrylamide gel electrophoresis of the carboxylase under nondenaturing conditions. Reaction with the reagent, due to its negative charge, sufficiently increases the rate of migration

of the enzyme toward the anode to distinguish unmodified dimers from those containing one mole (partially inactive) or two moles of reagent (fully inactivated). The distributions of these species at various stages of inactivation of the carboxylase by reagent B (Figure 10, Appendix B) are consistent with those predicted for a dimer containing two catalytic sites. For example, at 50% activity remaining, the expected ratio of native:partially inactive:fully inactive is 1:2:1. The specificity indicated by covalent incorporation is also apparent from the absence of additional bands during the later stages of the inactivation process.

Characterization of inactivated carboxylase. In order to identify the types of amino acids alkylated, a 600-μg aliquot of the [^{14}C]-labeled trypsin-digested enzyme was subjected to acid hydrolysis and amino acid analysis (Figure 11, Appendix B). Upon acid hydrolysis, residues modified by N-bromoacetyl compounds elute as carboxymethyl amino acids, the elution positions of which are well-documented (70,71). One minute fractions of the effluent from the ninhydrin reaction chamber of the analyzer were collected directly into scintillation vials and counted. The radioactivity (82% total recovery) eluted in two peaks corresponding to carboxymethylcysteine (at fraction 37; 35% of applied radioactivity) and τ-carboxymethylhistidine (at fraction 90; 36% of applied radioactivity).

Chromatography of the carboxymethylated tryptic digest of [^{14}C]-reagent B-inactivated enzyme on DEAE-cellulose (Figure 12A, Appendix B) revealed two major sites of modification, peaks I and II (accounting for 35% and 25% of the recovered radioactivity, respectively). These elute later than most of the peptides as a consequence of the negatively-charged phosphate groups. Further purification of the modified peptides

from peaks I and II was anticipated by treatment with alkaline phosphatase and rechromatography, which should selectively alter the elution positions of only the desired peptides (71). Accordingly, peaks I and II were reapplied to DEAE-cellulose after treatment with alkaline phosphatase (Figures 12B and 12C, Appendix B) and were subsequently judged pure by amino acid analysis, high performance liquid chromatography, and sequence analysis. The overall yields for peptides I and II were 17% (195 nmol) and 10% (117 nmol), respectively.

The amino acid compositions of the peptides are shown in Table 3, Appendix A. Except for the presence of τ-carboxymethylhistidine in peptide II, their compositions are identical. Both peptides contain carboxymethylcysteine, since the protein was treated with iodoacetate prior to protease digestion. However, the carboxymethylcysteine in hydrolysates of peptide I arises from [^{14}C]reagent; this was established by the corresponding elution of radioactivity from the amino acid analyzer.

The amino acid compositions agree with that of the cysteine-containing peptide T6 previously described (31); to confirm the identity, each peptide was subjected to automated Edman degradation (Table 4, Appendix A) which established the sequences as follows:

Peptide I: Ala-Gly-Tyr-Gly-Tyr-Val-Ala-Thr-Ala-Ala-His-Phe-Ala-Ala-Glu-Ser-Ser-Thr-Gly-Thr-Asn-Val-Glu-Val-Cys-Thr-Thr-Asp-Asp-Phe-Thr-Arg

Peptide II: Ala-Gly-Tyr-Gly-Tyr-Val-Ala-Thr-Ala-Ala-His-Phe-Ala-Ala-Glu-Ser-Ser-Thr-Gly-Thr-Asn-Val-Glu-Val-Cys-Thr-Thr-Asp-Asp-Phe-Thr-Arg

The peptides, positions 34 to 65 in the R. rubrum enzyme (16), [corresponding to positions 47 to 80 in spinach carboxylase (4)], contain

the modified histidine and cysteine at positions 44 and 58, respectively. The purity of the peptides was apparent from the absence of significant amounts of contaminating amino acids at cycle one. Neither the cysteinyl derivative in peptide I nor the histidyl derivative in peptide II was extracted from the reaction cup with butyl chloride. In both cases, the cycles corresponding to the modified residues were characterized by the absence of any other extracted amino acid. Moreover, the assignment of the modified residues was confirmed by the appearance of the unmodified amino acid in the sequence of the complementary peptide.

Reagent C

Kinetics of inactivation of carboxylase. The effect of reagent C on R. rubrum carboxylase activity is shown in Figure 13A, Appendix B. In the presence of CO_2 and Mg^{++}, the enzyme is inactivated in a pseudo-first order manner by reagent C; the inclusion of the competitive inhibitor, carboxyribitol-P_2, greatly retards the rate of inactivation. Neither the deactivated form of the R. rubrum enzyme nor the spinach enzyme (data not shown) is affected by reagent C. Upon prolonged incubation with the reagent, the enzyme loses essentially all of its activity.

Rate-saturation is demonstrated in the inactivation of the R. rubrum carboxylase by reagent C, as shown in Figure 13B, Appendix B. The kinetic constants derived from the plot of half-time of inactivation versus reciprocal reagent C concentration are T_{min} = 18.7 min and K_{inact} = 1.2 mM. From competitive inhibition studies, the K_i of the amine intermediate of reagent C is 165 μM. The large difference in K_i and K_{inact} values could be attributed in part to the difference in structure

between the amine and the bromoacetylated reagent, and in part to the use of an epimeric mixture to determine K_{inact}.

Characterization of inactivated carboxylase. The covalent incorporation of reagent C into the carboxylase as a function of activity remaining is shown in Figure 14, Appendix B. Total inactivation correlates with an incorporation of 1.0 mol reagent/mol subunit. In the presence of carboxyribitol-P_2, the incorporation is greatly reduced to values consistent with the observed slight inactivation.

A 500-μg sample of the [^{14}C]-labeled, carboxymethylated tryptic digest was hydrolyzed and subjected to amino acid analysis in order to identify the nature of the modified amino acids. The effluent from the analyzer, bypassing reaction with ninhydrin, was directly collected into scintillation vials and counted. Figure 15, Appendix B, shows the relative elution positions of the radioactivity. A major peak, eluting slightly ahead of glycine at fraction 67, and several small early-eluting peaks are observed, which correspond to degradation products of an alkylated methionyl residue. The methionine sulfonium salt upon acid hydrolysis breaks down to give a mixture of products including S-carboxymethylhomocysteine (major product), homoserine, glycollic acid, thioglycollic acid, and chloroacetic acid (70,71). The small peak which elutes at fraction 89 corresponds to the presence of a small amount of τ-carboxymethylhistidine.

Fractionation of the carboxymethylated tryptic digest of the [^{14}C]reagent C-inactivated enzyme on DEAE-cellulose (Figure 16, Appendix B) indicated a major peak comprising 50% of the applied radioactivity. The labeled peptide contained in this peak was purified by

successive chromatography on SP-Sephadex and Sephadex G-25. The overall yield (33 nmol, 5.6 x 10^4 dpm) was 20% by radioactivity and 18% by actual nanomoles of peptide obtained.

The amino acid composition of the reagent C-modified peptide is shown in Table 5, Appendix A. From the lack of a number of amino acids along with the whole-number ratios of the amino acids present, the peptide appears pure. The methionine sulfonium salt degradation products observed in acid hydrolysates of the entire tryptic digest also were present in acid hydrolysates of the peptide.

The amino acid composition of the peptide agrees exactly with the labeled peptide obtained after affinity labeling of the R. rubrum carboxylase by 2-[(bromoacetyl)amino]pentitol 1,5-bisphosphate (50), which also contained a modified methionyl residue (49). The sequence of the 2-[(bromoacetyl)amino]pentitol 1,5-bisphosphate-labeled peptide, and by inference that of the reagent C-modified peptide is shown below.

Leu-Gln-Gly-Ala-Ser-Gly-Ile-His-Thr-Gly-Thr-Met-Gly-Phe-Gly-Lys-
Met-Glu-Gly-Glu-Ser-Ser-Asp-Arg

The peptide contains two methionyl residues; the position of the label (underlined residue) was verified chemically in the 2-[(bromoacetyl) amino]pentitol 1,5-bisphosphate-modified peptide (50), accounting for the resistance of the internal Lys-Met peptide bond to cleavage by trypsin. The similar resistance of the reagent C-modified peptide to tryptic cleavage at this position strongly suggests that the same methionyl residue is alkylated. The peptide corresponds to positions 320 to 342 in the spinach enzyme (4) with the reactive methionyl residue at position 335.

Reagent D

Kinetics of inactivation of carboxylase. In the presence of CO_2 and Mg^{++}, reagent D inactivates the carboxylase from R. rubrum in a time-dependent, pseudo-first order manner (Figure 17A, Appendix B). The spinach enzyme is not affected by the reagent (data not shown). Affinity labeling criteria which are fulfilled by the interaction of reagent D with the carboxylase include protection by the competitive inhibitor carboxyribitol-P_2, essentially complete inactivation, and rate-saturation (Figure 17B, Appendix B) characterized by a T_{min} of 21 minutes and K_{inact} of 590 μM. The amine intermediate in the synthesis of reagent D is a competitive inhibitor of the carboxylase with a K_i of 150 μM. As seen with reagent C, a large discrepancy exists between K_{inact} and K_i values which can be attributed to structural differences along with complications arising from the use of an epimeric mixture.

Characterization of inactivated carboxylase. The covalent incorporation of [^{14}C]reagent D into the R. rubrum carboxylase which was 75% inactivated was 0.74 mol reagent D/mol subunit, suggesting a direct correlation between the observed inactivation and incorporation of reagent D. Fractionation of the acid hydrolysate of the carboxymethylated tryptic digest (data not shown) revealed three radioactive peaks eluting in positions corresponding to S-carboxymethylcysteine (44%), S-carboxymethylhomocysteine (10%), and τ-carboxymethylhistidine (46%) (fractions 38, 67, and 90, respectively).

Reagent E

Treatment of carboxylase with reagent E. *R. rubrum* carboxylase in either the deactivated or activated conformation exhibited no time-dependent loss of activity when treated with reagent E in concentrations of up to 1 mM for a period of 24 h. The activity of the spinach enzyme was also not affected by reagent E. Inhibition studies with the amine intermediate of reagent E showed that the compound is a competitive inhibitor of both the *R. rubrum* and spinach carboxylases with K_i values of 300 μM and 780 μM, respectively.

CHAPTER IV

DISCUSSION

The selectivity of cyanoborohydride near neutral pH for imines compared to carbonyls allows for the reductive amination of aldehydes or ketones which do not readily form imines (57). The reductive amination of ribulose-P_2 with p-phenylenediamine appears similar in many respects to its reaction with NH_4^+ reported earlier (49). The combined yields of the desired epimeric amines are low (22%), and despite the more rapid reaction of ribulose-P_2 with the aromatic amine, fractionation of the reaction mixture reveals a major product which lacks an amino group. Moreover, the relative amounts of this incompletely characterized product in the five syntheses are similar and thus seem independent of the reactant amine.

Using standard bond lengths, the distances between the reagents' C2 of the ribitol chain and the α-carbon of the bromoacetyl group in the fully extended conformation may be estimated as indicated in Figure 5, Appendix B. For comparison, the corresponding distance in the related affinity label, 2-[(bromoacetyl)amino]pentitol 1,5-bisphosphate (49) is 4.3 Å. However, the dramatic increase in length and bulk at C2 of the reagents does not greatly affect the binding of the bisphosphate to the enzyme. This follows from the observation that the K_i values of the amino bisphophate intermediates of 2-[(bromoacetyl)amino]pentitol 1,5-bisphosphate and reagent D are essentially identical (150 μM). Even with the introduction of a second phenyl group in the case of reagent E, the K_i is only increased to 300 μM.

The interaction of reagent B with the R. rubrum carboxylase/oxygenase satisfies the following traditional criteria of affinity labeling: (a) essentially complete inactivation upon incubation of enzyme with reagent, (b) protection against inactivation by competitive inhibitors, (c) pseudo-first order inactivation kinetics and rate-saturation, (d) competitive inhibition by the reagent during short assay periods, and (e) correlation of total inactivation with the covalent incorporation of one mole of reagent per catalytic site.

Additional strong support, albeit indirect, for an active-site directed pathway of inactivation is provided by the following observations (a) selective alkylation by reagent B of only the catalytically competent form of the enzyme; the deactivated enzyme in the absence of CO_2/Mg^{++} is resistant; (b) differential interaction of the two epimers with the enzyme; (c) rate-enhancement of the specific alkylation of His^{44} in the enzyme as compared to alkylation of the imidazole side-chain in model compounds; (d) resistance of the enzyme to inactivation by bromoacetamide, a general alkylating agent with chemical properties similar to those of reagent B; and (e) formation of only one of the two possible isomers of alkylated histidine.

Despite their inherently equivalent alkylating potentials, reagent A does not exhibit the capacity to inactivate the carboxylase in contrast to the rapid, site-specific modification demonstrated by the corresponding C2 epimer, reagent B. One likely explanation of this striking difference is that whereas the reactive side-arm of reagent B could be penetrating an active-site fold or pocket, the corresponding side-arm of reagent A with opposite configuration of C2 could be merely exposed to solvent.

Differential interaction of the epimeric reagents with the carboxylase is also revealed at the level of competitive inhibition, with reagent B (inactivator) possessing a five-fold greater affinity for the enzyme than displayed by reagent A (not an inactivator). The effect of configuration in the binding of epimeric 2-carboxy analogs of ribulose-P_2 by the carboxylase is well documented (41,66), with tighter binding requiring the carboxyl group cis to the hydroxyl groups. The discrimination by the enzyme between reagents A and B is thus in accord with proposed active-site geometry.

Reagent B shows an enhanced reactivity with His^{44} in the enzyme relative to model peptides, again characteristic of an active-site directed reagent. The second order rate constant, k_2, of the reaction of reagents A and B with the glutathione sulfhydryl is 3.4 M^{-1} sec^{-1}. This value, measured at pH 8.0, has been corrected for actual thiolate concentration assuming a pK_a of 8.4 (73). The k_2 of the reaction of reagent B with the enzyme is 6.6 M^{-1} sec^{-1} (calculated from a $t_{1/2}$ of 17.4 minutes at 100 μM reagent under pseudo-first order conditions), thus showing only about a two-fold difference. However, attempts to measure precisely a k_2 of the reaction of reagents A and B with the imidazolium ring in hippuryl-histidyl-leucine were unsuccessful because the alkylation, if it occurs at all, is extremely slow. An 18 h incubation of the peptide (200 μM) with either reagent (4 mM) did not result in any detectable loss of histidine as determined by amino acid analysis of the acid hydrolysate. Furthermore, neither isomer of carboxymethylhistidine could be detected in the hydrolysates even when the sample size applied to the column was ten times the usual amount. Even if 5% of the reactant peptide

had been alkylated (a value considerably greater than our minimal detection level), an upper limit of 2×10^{-4} M^{-1} sec^{-1} can be placed on the k_2 of the reaction between the histidyl residue and reagent A or B, so that the corresponding reaction in the enzyme shows a minimal 33,000-fold rate enhancement. For comparison, the alkylation of His^{119} in ribonuclease A by bromoacetate is about 2,100-fold faster than that of L-histidine (74).

Also consistent with the assignment of reagent B as an affinity label is the enhanced rate of alkylation of the carboxylase by reagent B relative to the alkylation of the carboxylase by bromoacetamide. Affinity labels possess substrate-like chemical features which produce a high localized concentration of the reagent at the active site, resulting in an increased reactivity compared with general alkylating agents. Activated carboxylase treated with 0.1 M bromoacetamide under pseudo-first order conditions shows a loss of activity ($t_{1/2}$ = 80 minutes) with a k_2 of 1.4×10^{-3} M^{-1} sec^{-1} (data not shown). Thus, the reaction of the carboxylase with reagent B (k_2 = 6.6 M^{-1} sec^{-1}) is 4,600-fold greater than with bromoacetamide. This enhancement rate may be misleadingly low, because bromoacetamide does not necessarily react with either the histidyl or cysteinyl residue in question.

Fractionation of the acid hydrolysate of the [^{14}C]reagent B-alkylated carboxylase reveals roughly equal amounts of carboxymethylcysteine and τ-carboxymethylhistidine without other labeled amino acid derivatives. However, the DEAE-cellulose profile of the same modified enzyme digested with trypsin indicates less specificity than might be anticipated from the hydrolysate and the overall incorporation. Part of the heterogeneity arises due to the susceptibility of the tryptic peptide to cleavage at

the Tyr-Val bond by residual chymotryptic activity in the trypsin (31, C. S. Herndon and F. C. Hartman, unpublished observations). We have also observed slight release of P_i from the protein-bound reagent moiety that might account for some heterogeneity.

The modification of either a histidyl or cysteinyl residue per active site must preclude reaction at the other residue, since no peptide modified at both positions was detected. Whether the dimers are modified randomly or whether the alkylation in one subunit dictates the type of residue alkylated in the second subunit is not known. Our inability to electrophoretically separate the subunits containing an alkylated histidyl residue from those with an alkylated cysteinyl residue prevents unequivocal resolution of this question. However, the pseudo-first order kinetics of inactivation and the direct correlation of extent of covalent incorporation with loss of enzymic activity favor random modification of subunits. Most compatible with the existing data is the postulate that the reagent binds to the enzyme in such a manner that reaction at His^{44} and Cys^{58} occurs with approximately equal probability and that either alkylation inactivates the subunit. To invoke the alternate postulate of half-of-the-sites reactivity (75), for which compelling evidence does not exist (29), the alkylation of the second subunit rendered inactive via conformational changes induced by the initial alkylation must proceed at the same rate as the conformational changes. This unlikely event would be required by the inactivation/incorporation correlation.

The R. rubrum active-site peptide may be aligned with the corresponding sequences from spinach, tobacco, Chlamydomonas, maize, Anabaena, and Synechococcus, deduced by recombinant DNA techniques (4,8-12), as shown in Figure 18, Appendix B.

The refractivity of the spinach enzyme to reagent B is explained by the deletion of His^{44} and the substitution of Cys^{58} by Trp in the eukaryotic carboxylases.

Although the nonconservation of the reagent B-reactive residues dismisses the possibility of a catalytic functionality, the extensive homology exhibited by this region is consistent with some essential structural or functional contribution to the activity of the carboxylase. Of particular interest is the sequence Ala-Ala-Glu-Ser-Ser, which is the second-longest consecutive homology among carboxylases of known sequence, the longest being the seven residues encompassing Lys^{175} (29). Prior to this study, this region had not been implicated as being at or near the active site of the carboxylase.

A role for histidyl residues at the active sites of both the R. rubrum and spinach carboxylases has been suggested by chemical modification using diethylpyrocarbonate and rose bengal dye (76-78). Incorporation of diethylpyrocarbonate indicates that 4.5 residues/53,000 dalton protomer in the R. rubrum enzyme and 2.2 residues/67,000 dalton protomer in the spinach enzyme are modified; inclusion of 2-carboxyhexitol 1,6-bisphosphate prevents the incorporation of about one mole of reagent/protomer in each enzyme. His^{44} may be the residue in the R. rubrum enzyme which is subject to protection from diethylpyrocarbonate modification. It would then follow that the protected histidyl residue in the spinach enzyme must be located in a different region of the active site.

The interactions of the R. rubrum carboxylase with reagents C and D also fulfill several of the criteria of affinity labeling, including pseudo-first order inactivation kinetics, complete inactivation, competitive inhibition, substrate protection, and rate-saturation. In both

cases, total inactivation is accompanied by the covalent incorporation of approximately 1 mol reagent/mol subunit.

Fractionation of the acid hydrolysates of enzyme which had been inactivated with the [^{14}C]reagents, carboxymethylated, and digested with trypsin reveals differences in the types of residues alkylated by reagents C and D. In the case of reagent C, inactivation of the carboxylase is primarily associated with methionine alklyation. In contrast, reagent D alkylates histidine, cysteine, and to a small extent, methionine. Considering the structural similarities of the reagents, it is reasonable to assume that the histidyl and cysteinyl residues modified by reagent D are His^{44} and Cys^{58}, which are alkylated by reagent B. Similarly, a unique methionyl residue is probably reactive with both reagents C and D, and may be assigned as Met^{335}.

The active-site peptide isolated after affinity labeling of R. rubrum carboxylase by reagent C and 2-[(bromoacetyl)amino]pentitol 1,5-bisphosphate may be aligned with the six known carboxylase sequences (Figure 19, Appendix B). The lack of reaction of reagent C with the spinach enzyme may be explained by the replacement of Met^{335} by Leu. The conserved residue, Lys^{334}, has been labeled in the spinach enzyme by 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate (22), which further supports assignment of this region as being at the carboxylase active site. As seen with 2-[(bromoacetyl)amino]pentitol 1,5-bisphosphate, the lack of alkylation of Lys^{334} in either the R. rubrum or spinach enzyme indicates that despite the proximity in primary sequence, the proper alignment for reaction of the reagent's N-bromoacetyl moiety with the lysyl ε-amino group cannot occur.

The alkylation of Met^{335} by 2-[(bromoacetyl)amino]pentitol 1,5-bisphosphate, reagent C, and reagent D indicates that despite the varied length of the extender at C2, the reagents are able to bind to the enzyme with the alkyl group in a flexed conformation which places the electrophilic center near the attacking thioether. The existence of a similar spatial arrangement of the N-bromoacetyl group and ribitol chain in all three reagents can be demonstrated by model building studies. Additional indirect support of this argument arises from the absence of methionyl aklylation by reagent B. The phenyl ring provides a more rigid extension at C2 which lacks the rotational freedom needed to place the N-bromoacetyl group near Met^{335}. In the same context, the modification of His^{44} and Cys^{58} by both reagents B and D can be attributed to the flexibility of reagent D's extender, which is able to orient the N-bromoacetyl group similarly to reagent B. The rigidity introduced by the phenyl groups of reagent E restricts the spatial arrangements of the reactive moiety, and apparently from the lack of inactivation of the carboxylase, does not place the electrophilic center near any nucleophilic groups on the enzyme.

The inactivation of ribulose-P_2 carboxylase/oxygenase by either reagent B, C, or D requires the presence of CO_2 and Mg^{++}, conditions under which the enzyme is fully activated (79). The lack of reactivity of the reagent with the deactivated enzyme could be either a consequence of inaccessibility of the enzyme's nucleophilic side chains to the reagents' electrophilic centers or hampered binding of the reagents to the deactivated conformation. However, various phosphorylated effectors of the spinach carboxylase have affinity for both the activated and deactivated

conformations (80,81). The negative effectors ribose 5-phosphate and fructose 6-phosphate as well as ribulose-P_2 bind more tightly to the deactivated conformer. In addition, an affinity label for the spinach carboxylase, bromoacetylethanolamine phosphate, alkylates different residues in the activated and deactivated enzyme (23). In view of this, it seems more likely that the deactivated conformation lacks the appropriate juxtaposition of nucleophiles for covalent chemistry to occur.

Although several active-site nucleophilic groups in the activated conformation of the carboxylase were alkylated by the reagents, the carbamate-forming lysyl residue at position 201 remained elusive. One possibility is that the distance from the substrate binding site to activator site is merely underestimated. Another explanation of the inability of the reagents to alkylate Lys^{201} is revealed by the following description of recent studies of individual interactions in the transition state complex.

The model of metal ion interaction shown in Figure 4, Appendix B, is primarily based upon the finding of a 1:1:1:1 stoichiometry in the stable quaternary complex of enzyme-activator CO_2-metal ion-carboxyarabinitol-P_2 (52,53), coupled with the demonstrated requirement for the single metal ion in both the activation process (54,82) and catalysis (41,43,55). The role of the metal ion in the activation process has been proposed as involving the stabilization of the carbamate. Upon binding of the activator CO_2 at Lys^{201}, an additional negative charge is introduced into a region containing numerous acidic residues which could then be stabilized by the metal cation (18,19). Studies of model compounds indicate carbamate cleavage occurs via an initial N-protonation step

(83) which could be retarded by interaction with the metal cation (19). Despite the likelihood of the metal cation as being the positively charged entity responsible for carbamate stabilization, no direct evidence for this has been found. Recent electron spin resonance experiments of Miziorko (84) did not detect an inner sphere interaction of activator CO_2 with the metal cation in the R. rubrum carboxylase. Inner sphere liganding of species containing [^{17}O] to Mn^{++} will perturb the electron spin resonance signal of the quaternary complex of enzyme-activator CO_2-Mn^{++}-carboxyarabinitol-P_2. Using [^{17}O]CO_2, no perturbation of the signal was observed.

In contrast, a strong case is emerging for a direct catalytic role of the metal cation. Involvement of the metal cation in catalysis was initially indicated by the observation of varying rates of carboxylation and oxygenation in the presence of different metal ions (85-88), which has been attributed to differing efficiencies in supporting the carboxylase and oxygenase activities (53). The metal cation could function as an electron sink in several capacities, including (a) stabilization of the aci-acid form of the D-3-phosphoglycerate molecule derived from C1 and C2 of ribulose-P_2 and CO_2 (41), (b) stabilization of the enediolate formed by abstraction of the C3 proton of ribulose-P_2 by coordination to the alcoholic oxygens at C2 or C3 (55), (c) stabilization of the 2-carboxy-3-ketoarabinitol 1,5-bisphosphate transition state intermediate (41) or (d) polarization of the C-O bonds of substrate CO_2 to facilitate attack on C2 of the enediolate form of ribulose-P_2 (89).

A direct coordination of metal to substrate CO_2 has been indicated by two independent experimental approaches. First, the nuclear magnetic

resonance studies of Miziorko and Mildvan (55) demonstrated the proximity of Mn^{++} to the rapidly-exchanging CO_2 which was later identified as the substrate CO_2. Second, electron spin resonance studies of the stable quaternary complex indicated a complex inner sphere liganding of Mn^{++} in both the spinach and R. rubrum enzyme (53,84). [^{17}O]-Perturbation experiments showed inner sphere coordination of the carboxyl oxygen and the C2 alcoholic oxygen of [^{17}O]carboxyarabinitol-P_2 with Mn^{++}, and multiple H_2O liganding (84).

Finally, it appears that protein ligands are also directly coordinated to the metal cation. Oxidation of the complex formed with Co^{++}, carboxyarabinitol-P_2, CO_2 and carboxylase generated an exchange-inert cation derivative of the enzyme which was of sufficient stability to withstand preliminary chemical characterization (90). The nature and number of protein ligands have not yet been reported, but based upon the stability of the derivative, a multidentate coordination is expected. Although carboxyarabinitol-P_2 was not retained in the oxidized derivative, a bridging involving oxygen was not expected to be stable under the experimental conditions employed (84).

The seeming lack of direct coordination of metal with the carbamate along with the demonstrated intervention of protein ligands are consistent with Lys^{201} being inaccessible to the reagents described in this study. Regardless of the refinements in the transition state model, which affect only a portion of the original rationale, the affinity labeling described herein has achieved its proposed goal of identifying additional active-site regions of the carboxylase. The highly conserved sequence in the reagent B-labeled peptide suggests a functionality in the

carboxylase mechanism. Alkylation patterns observed with reagents C, D, and E place certain constraints upon the enzyme's active-site geometry. Further insights into the role and orientation of these regions of primary structure in the active site must await the generation of a three-dimensional model of the carboxylase.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Weissbach, A., Horecker, B. L., and Hurwitz, J. (1956) J. Biol. Chem. 218, 795-810.

2. Ogren, W. L., and Bowes, G. (1971) Nature (London) 230, 159-160.

3. Rutner, A. C. (1970) Biochem. Biophys. Res. Commun. 39, 923-929.

4. Zurawski, G., Perrot, B., Bottomley, W., and Whitfeld, P. R. (1981) Nucleic Acids Res. 9, 3251-3270.

5. Martin, P. G. (1979) Aust. J. Plant Physiol. 6, 401-408.

6. Chan, P. H., and Wildman, S. G. (1972) Biochim. Biophys. Acta 277, 677-680.

7. Kawashima, N. and Wildman, S. G. (1972) Biochim. Biophys. Acta 262, 42-49.

8. Shinozaki, K., and Sugiura, M. (1982) Gene 20, 92-102.

9. McIntosh, L., Poulson, C., and Bogorad, L. (1980) Nature (London) 288, 556-560.

10. Dron, M., Rahire, M., and Rochaix, J. -D. (1982) J. Mol. Biol. 162, 775-793.

11. Curtis, S. E., and Haselkorn, R. (1983) Proc. Natl. Acad. Sci. USA 80, 1385-1389.

12. Reichelt, B. Y., and Delaney, S. F. (1983) DNA 2, 121-129.

13. Siegel, M. I., Wishnick, M., and Lane, M. D. (1972) Enzymes 3rd Ed. 6, 169-192.

14. Tabita, F. R., and McFadden, B. A. (1974) J. Biol. Chem. 249, 3459-3464.

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

16. Hartman, F. C., Stringer, C. D., Omnaas, J., Donnelly, M. I., and Fraij, B. (1982) Arch. Biochem. Biophys. 219, 422-437.

17. Nishimura, M., and Akazawa, T. (1974) Biochem. Biophys. Res. Commun. 59, 584-590.

18. Lorimer, G. H., and Miziorko, H. M. (1980) Biochemistry 19, 5321-5328.

19. Lorimer, G. H. (1981) Biochemistry 20, 1236-1240.

20. Donnelly, M. I., Stringer, C. D., and Hartman, F. C. (1983) Biochemistry (in press).

21. Schwarz, R. M., and Dayhoff, M. O. (1978) Science (Washington, D.C.) 199, 395-403.

22. Stringer, C. D., and Hartman, F. C. (1978) Biochem. Biophys. Res. Commun. 80, 1043-1048.

23. Schloss, J. V., Stringer, C. D., and Hartman, F. C. (1978) Biochemistry 253, 5707-5711.

24. Paech, C., and Tolbert, N. E. (1978) J. Biol. Chem. 253, 7864-7873.

25. Spellman, M., Tolbert, N. E., and Hartman, F. C. (1979) Abstracts of Papers, 178th National Meeting of the American Chemical Society, Washington, D.C., Sept. 1979, BIOL 3.

26. Whitman, W. B., and Tabita, F. R. (1978) Biochemistry 17, 1282-1287.

27. Whitman, W. B., and Tabita, F. R. (1978) Biochemistry 17, 1288-1293.

28. Robison, P. D., Whitman, W. B., Wadill, F., Riggs, A. F., and Tabita, F. R. (1980) Biochemistry 19, 4848-4853.

29. Herndon, C. S., Norton, I. L., and Hartman, F. C. (1982) Biochemistry 21, 1380-1385.

30. Horecker, B. L., Hurwitz, J., and Weissbach, A. (1958) Biochem. Prep 6, 83-90.

31. Stringer, C. D., Norton, I. L., and Hartman, F. C. (1981) Arch. Biochem. Biophys. 208, 495-501.

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

33. Peterson, E. A., and Sober, H. A. (1954) J. Am. Chem. Soc. 76, 169-175.

34. Gabriel, O. (1971) Methods Enzmol. 22, 565-578.

35. Katz, A. M., Dreyer, W. J., and Anfinsen, C. B. (1959) J. Biol. Chem. 234, 2897-2900.

36. Canfield, R. E., and Anfinsen, C. B. (1963) in The Proteins (Neurath, R., Ed.) Vol. 1, p. 318, Academic Press, New York.

37. Forrey, A. W., Olsgaard, R. B., Nolan, C., and Fischer, E. H. (1971) Biochimie 53, 269-281.

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

39. Tarr, G. E., Beecher, J. F., Bell, M., and McKean, D. J. (1978) Anal. Biochem. 84, 622-627.

40. Smithies, O., Gibson, D., Fanning, E. M., Goodfliesh, R. M., Gilman, J. G., and Ballantyne, D. L. (1971) Biochemistry 10, 4912-4921.

41. Pierce, J., Tolbert, N. E., and Barker, R. (1980) Biochemistry 19, 934-942.

42. Fiedler, F., Mullhofer, G., Trebst, A., and Rose, I. A. (1967) Eur. J. Biochem. 1, 395-399.

43. Saver, B. G., and Knowles, J. R. (1982) Biochemistry 21, 5398-5403.

44. Sue, J. M., and Knowles, J. R. (1982) Biochemistry 21, 5404-5410.

45. Hall, N. P., McCurry, S. D., and Tolbert, N. E. (1981) Plant Physiol. 67, 1220-1223.

46. Rose, I. A. (1970) Enzymes, 3rd Ed. 2, 281-320.

47. Rose, I. A. (1975) Adv. Enzymol. Relat. Areas Mol. Biol. 43, 491-517.

48. Eigen, M. (1964) Angew. Chem., Int. Ed. Engl. 3, 1-19.

49. Fraij, B., and Hartman, F. C. (1982) J. Biol. Chem. 257, 3501-3505.

50. Fraij, B., and Hartman, F. C. (1983) Biochemistry 22, 1515-1520.

51. Christeller, J. T., and Hartman, F. C. (1982) FEBS Lett. 142, 162-166.

52. Miziorko, H. M. (1979) J. Biol. Chem. 254, 270-272.

53. Miziorko, H. M., and Sealy, R. C. (1980) Biochemistry 19, 1167-1172.

54. Lorimer, G. H., Badger, M. R., and Andrews, T. J. (1976) Biochemistry 15, 529-536.

55. Miziorko, H. M., and Mildvan, A. S. (1974) J. Biol. Chem. 249, 2743-2750.

56. Jentoft, N., and Dearborn, D. G. (1979) J. Biol. Chem. 254, 4359-4365.

57. Borch, R. F., Bernstein, M. D., and Durst, H. D. (1971) J. Am. Chem. Soc. 93, 2897-2904.

58. Hartman, F. C., Suh, B., Welch, M. H., and Barker, R. (1973) J. Biol. Chem. 248, 8233-8239.

59. Lorimer, G. H., Badger, M. R., and Andrews, T. J. (1977) Anal. Biochem. 78, 66-75.

60. Hanes, C. S., and Isherwood, F. A. (1949) Nature (London) 164, 1107-1112.

61. Dreyer, W. J., and Bynum, E. (1967) Methods Enzymol. 11, 32-39.

62. Weil-Malherbe, H. (1969) in Data for Biochemical Research (Dawson, R. M. C., Elliott, D. C., Elliott, W. H., and Jones, K. M., Eds.) p. 522, Oxford University Press, Oxford.

63. Schloss, J. V., and Hartman, F. C. (1977) Anal. Biochem. 83, 185-188.

64. Marsh, B. B. (1959) Biochim. Biophys. Acta 32, 357-361.

65. Hartman, F. C., LaMuraglia, G. M., Tomozawa, Y., and Wolfenden, R. (1975) Biochemistry 14, 5274-5279.

66. Roach, D. J. W., Gollnick, P. D., and McFadden, B. A. (1983) Arch. Biochem. Biophys. 222, 87-94.

67. Bollum, F. J. (1968) Methods Enzymol. 12B, 169-173.

68. Kitz, R., and Wilson, I. R. (1962) J. Biol. Chem. 237, 3245-3249.

69. Meloche, H. P. (1967) Biochemistry 6, 2273-2280.

70. Goren, H. J., Glick, D. M., and Barnard, E. A. (1968) Arch. Biochem. Biophys. 126, 607-623.

71. Gurd, F. R. N. (1972) Methods Enzymol. 25, 424-438.

72. Strausbauch, P. H., and Fischer, E. H. (1970) Biochemistry 9, 233-238.

73. Byers, L. D. (1977) J. Am. Chem. Soc. 99, 4146-4149.

74. Heinrikson, R. L., Stein, W. H., Crestfield, A. M., and Moore, S. (1965) J. Biol. Chem. 240, 2921-2934.

75. Levitzki, A., Stallcup, W. B., and Koshland, D. E., Jr. (1971) Biochemistry 10, 3371-3378.

76. Saluja, A. K., and McFadden, B. A. (1980) Biochem. Biophys. Res. Commun. 94, 1091-1097.

77. Saluja, A. K., and McFadden, B. A. (1982) Biochemistry 21, 89-95.

78. Bhagwat, A. S., and Ramakrishna, J. (1981) Biochim. Biophys. Acta 662, 181-189.

79. Christeller, J. T., and Laing, W. A. (1978) Biochem. J. 173, 467-473.

80. Badger, M. R., and Lorimer, G. H. (1981) Biochemistry 20, 2219-2225.

81. McCurry, S. D., Pierce, J., Tolbert, N. E., and Orme-Johnson, W. H. (1981) J. Biol. Chem. 256, 6623-6628.

82. Laing, W. A., and Christeller, J. T. (1976) Biochem. J. 159, 563-570.

83. Ewing, S. P., Lockshon, D., and Jencks, W. P. (1980) J. Am. Chem. Soc. 102, 3072-3084.

84. Miziorko, H. M., and Sealy, R. C. (1983) Fed. Proc. 42, 1924.

85. Wildner, G. F., and Henkel, J. (1978) FEBS Lett. 91, 99-103.

86. Wildner, G. F., and Henkel, J. (1979) Planta 169, 223-228.

87. Christeller, J. T., and Laing, W. A. (1979) Biochem. J. 183, 747-750.

88. Jordan, D. B., and Ogren, W. L. (1981) Pl. Physiol. 67, 237-245.

89. Lorimer, G. H. (1981) Ann. Rev. Plant Physiol. 32, 349-383.

90. Miziorko, H. M., Behnke, C. E., and Houkom, E. C. (1982) Biochemistry 21, 6669-6674.

APPENDICES

APPENDIX A

TABLES

Table 1

Amino Acid Composition of PLP-Peptide

Amino Acid	Molar Ratio						
	Robison et al. (28)	Sample A[a]	Mixture[b]	Sample B[c]	Sample C[d] 24 hr Hydrolysis	Sample C[d] 48 hr Hydrolysis	Number of Residues[e]
Asp[f]	1.00	1.00	1.00	1.00	1.00	1.00	1
Thr	0.62	1.03	0.87	0.91	0.96	0.85	1
Ser		trace	0.13	0.08	0.21	0.15	
Glu	0.91	1.15	1.13	1.11	1.15	1.09	1
Pro	0.82	1.82	1.78	1.83	1.95	1.81	2
Gly	1.72	3.81	3.56	3.73	3.85	3.80	4
Ala	1.10	0.07	0.16	0.09	0.21	0.09	
Val	1.41	3.64	3.25	3.68	3.40	3.71	4
Met				0.07			
Ile	0.76	1.85	1.35	1.39	1.51	1.56	2
Leu	1.07	1.53	1.73	1.81	1.87	1.81	2
Tyr			trace				
Phe			trace				
His		0.02	trace		trace	trace	

Table 1 (Continued)

Amino Acid	Molar Ratio: Robison et al. (28)	Sample A[a]	Mixture[b]	Sample B[c]	Sample C[d]: 24 hr Hydrolysis	Sample C[d]: 48 hr Hydrolysis	Number of Residues[e]
Lys	0.70	0.88	0.94	1.04	1.01	0.95	1
PLP-Lys[g]	0.26	0.65(1.04)	0.69(0.85)	0.70	0.79(1.05)	0.20(1.05)	1
Arg	0.61	0.86	0.88	0.92	0.84	0.90	1

[a]Purified from enzyme (1.5 mg/ml) incubated with 50 μM PLP.

[b]Purified from a mixture of 5 mg each from tryptic digests of enzyme inactivated at 0.5 mg/ml with 8.9 μM and 35.6 μM PLP and at 5 mg/ml with 89 μM PLP.

[c]Purified from enzyme (5 mg/ml) incubated with 356 μM PLP.

[d]Purified from enzyme (0.5 mg/ml) incubated with 35.6 μM PLP.

[e]Determined by sequencing.

[f]Arbitrarily set as 1.00.

[g]N^6-(phosphopyridoxal)lysine quantitated using the relative color value of Forrey et al. (37). The number in parentheses is the PLP-Lys content determined by the absorbancy at 320 nm (33).

Table 2

Sequence Analysis of PLP-Peptide[a]

Cycle	Amino Acid	Yield (nmol)	Cpm/Cycle
1	Val	58.2	280
2	Leu	39.4	150
3	Gly	38.8	130
4	Arg[b]	10.3	140
5	Pro	35.7	250
6	Glu	17.0	190
7	Val	38.5	230
8	Asp	9.0	300
9	Gly	23.0	430
10	Gly	25.0	420
11	Leu	14.8	280
12	Val	12.2	720
13	Val	20.8	600
14	Gly	10.4	610
15	Thr	—[c]	960
16	Ile	3.5	630
17	Ile	5.3	510
18	PLP-Lys	—[d]	6170
19	Pro	4.7	3910
20	Lys	2.4	2200

[a]Sample size was 75 nmol. Initial yield was 78%.

[b]Determined as ornithine in base hydrolysate.

[c]Detected as α-aminobutyric acid.

[d]Assigned by radioactivity and lack of extracted amino acid.

Table 3

Amino Acid Compositions of Reagent B-Labeled Peptides

Amino Acid	Molar Ratio		Number of Residues[a]
	Peptide I	Peptide II	
Cm-Cys	0.85	0.91	1
Asp[b]	3.00	3.00	3
Thr	5.73	6.11	6
Ser	2.24	2.04	2
Glu	2.22	2.28	2
Gly	2.93	3.06	3
Ala	5.78	5.91	6
τ-Cm-His[c]		0.83	
Val	3.08	2.97	3
Tyr	1.95	1.96	2
Phe	2.07	1.98	2
His	0.87		1
Arg	1.07	1.02	1

[a]Determined from sequencing.

[b]Arbitrarily assigned as 3.00.

[c]Calculated using color constant for glycine.

Table 4

Sequence Analyses of Reagent B-Labeled Peptides[a]

Cycle	Amino Acid	Peptide I Yield (nmol)	Peptide II Yield (nmol)
1	Ala	20.5[b]	32.3[c]
2	Gly	13.6	28.0
3	Tyr	14.2	25.0
4	Gly	11.6	24.1
5	Tyr	11.2	22.1
6	Val	15.7	28.7
7	Ala	11.8	23.1
8	Thr	—[d]	—[d]
9	Ala	11.6	20.3
10	Ala	10.8	18.9
11	His	2.5	—[e]
12	Phe	9.2	16.8
13	Ala	7.6	16.4
14	Ala	7.5	14.4
15	Glu	4.3	8.0
16	Ser	—[f]	—[f]
17	Ser	—[f]	—[f]
18	Thr	—[d]	—[d]
19	Gly	3.8	10.3
20	Thr	—[d]	—[d]
21	Asn	3.0	6.7
22	Val	4.6	11.3

Table 4 (Continued)

Cycle	Amino Acid	Peptide I Yield (nmol)	Peptide II Yield (nmol)
23	Glu	2.1	5.6
24	Val	2.8	10.5
25	Cm-Cys	—e	—f
26	Thr	—d	—d
27	Thr	—d	—d
28	Asp	1.5	3.8
29	Asp	2.0	2.9
30	Phe	2.6	5.9
31	Thr	—d	—d
32	Arg[g]	0.8	1.1

[a]The sample size was 50 nmol for each peptide.

[b]Initial yield and repetitive yield for peptide I were 41% and 92.1%, respectively.

[c]Initial yield and repetitive yield for peptide II were 64.6% and 94%, respectively.

[d]Qualitative identification based on α-aminobutyrate in the base hydrolysate.

[e]Assigned by lack of any extracted amino acid.

[f]Qualitative identification based upon degradation product in base hydrolysate coeluting with cysteic acid.

[g]Appears as ornithine in the base hydrolysate.

Table 5

Amino Acid Composition of Reagent C-Modified Peptide

Amino Acid	Molar Ratio	Number of Residues[a]
Asp	1.2	1
Thr	1.7	2
Ser	3.2	3
Hse	trace	
Glu	3.0	3
Cm-Hcy	trace	
Gly	5.7	6
Ala[b]	1.0	1
Met	1.2	2
Ile	0.7	1
Leu	0.8	1
Phe	1.3	1
His	0.7	1
Lys	0.8	1
Arg	0.9	1

[a]Determined by sequencing of 2-[(bromoacetyl)amino]pentitol 1,5-bisphosphate-modified peptide (50).

[b]Arbitrarily assigned as 1.0.

APPENDIX B

FIGURES AND FIGURE LEGENDS

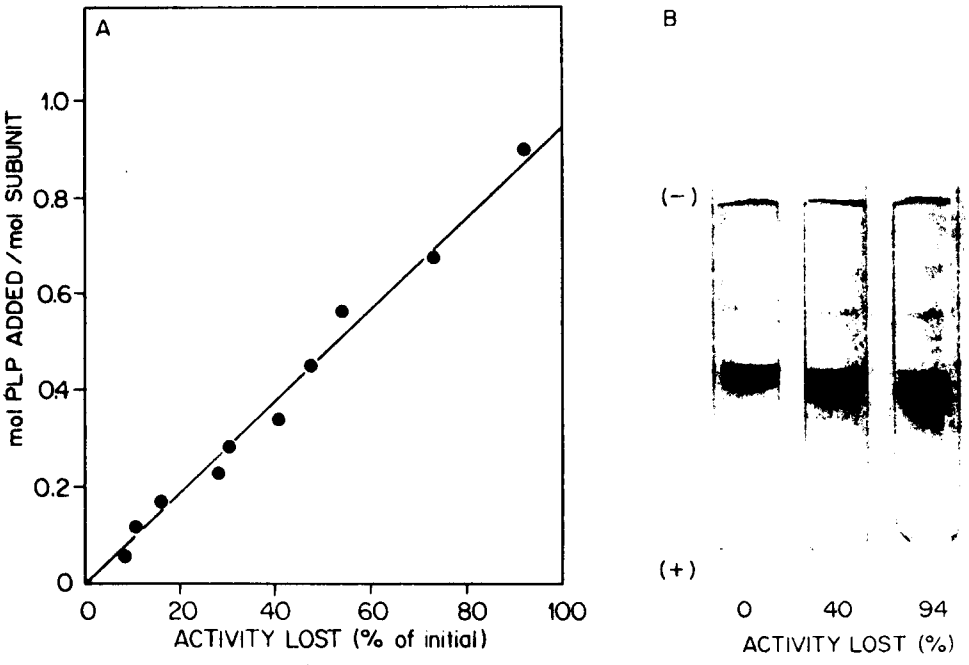


Figure 1. Stoichiometry of inactivation of ribulose-P_2 carboxylase/oxygenase with PLP. (A) Titration of the carboxylase with PLP. Conditions for modification are described in the text. (B) Polyacrylamide gel electrophoresis of PLP-modified enzyme in 8M urea at pH 8.9. Twenty micrograms of protein was applied to each gel.

Figure 2. Purification of radioactive peptide from PLP-modified ribulose-P_2 carboxylase/oxygenase. (A) Anion exchange chromatography of a tryptic digest of PLP-modified carboxylase/oxygenase. A 20-mg (5.6 ml) sample, consisting of 5 mg from four separate tryptic digests of enzyme inactivated at 0.5 mg/ml and 5.0 mg/ml, each with one and four molar equivalents of PLP to subunit, was applied to a DEAE-cellulose column (1 cm x 25 cm) in 0.01 M NH_4HCO_3/1 mM 2-mercaptoethanol (pH 8.1). A 400-ml linear gradient of 0.01-0.30 M NH_4HCO_3 in 1 mM 2-mercaptoethanol was applied at fraction 3, followed by a 0.50 M NH_4HCO_3/1 mM 2-mercaptoethanol wash at fraction 137. The flow rate was 15 ml/hr, and the fraction size was 2.5 ml. Fractions 47-52 were pooled and lyophilized. (B) Cation exchange chromatography of tritiated peak from DEAE-cellulose. The lyophilized pool was redissolved in 2.6 ml of 11 mM H_3PO_4 (adjusted to pH 3.0 with NH_4OH) and applied to a SP-Sephadex column (1 cm x 25 cm) equilibrated with the same solvent. The peptide was eluted with a 400-ml linear gradient of 0-0.3 M NaCl in 11 mM H_3PO_4 (pH 3.0). The flow rate was 16 ml/hr, and 2.7-ml fractions were collected. Fractions 92 through 100 were pooled, neutralized by addition of solid NH_4HCO_3, and lyophilized. (C) Gel filtration of tritiated peak from SP-Sephadex. The lyophilized residue was dissolved in 3.0 ml of 0.01 M NH_4HCO_3 and applied to a Sephadex G-25 column (1.7 cm x 220 cm) in 0.01 M NH_4HCO_3. The flow rate was 26 ml/hr, and the fractions were 4.3 ml. The only peak eluting before the salt region (beginning at 225 ml) was the tritiated peptide. Fractions 33-36 were pooled, lyophilized, and redissolved in a small volume of 0.01 M NH_4HCO_3.

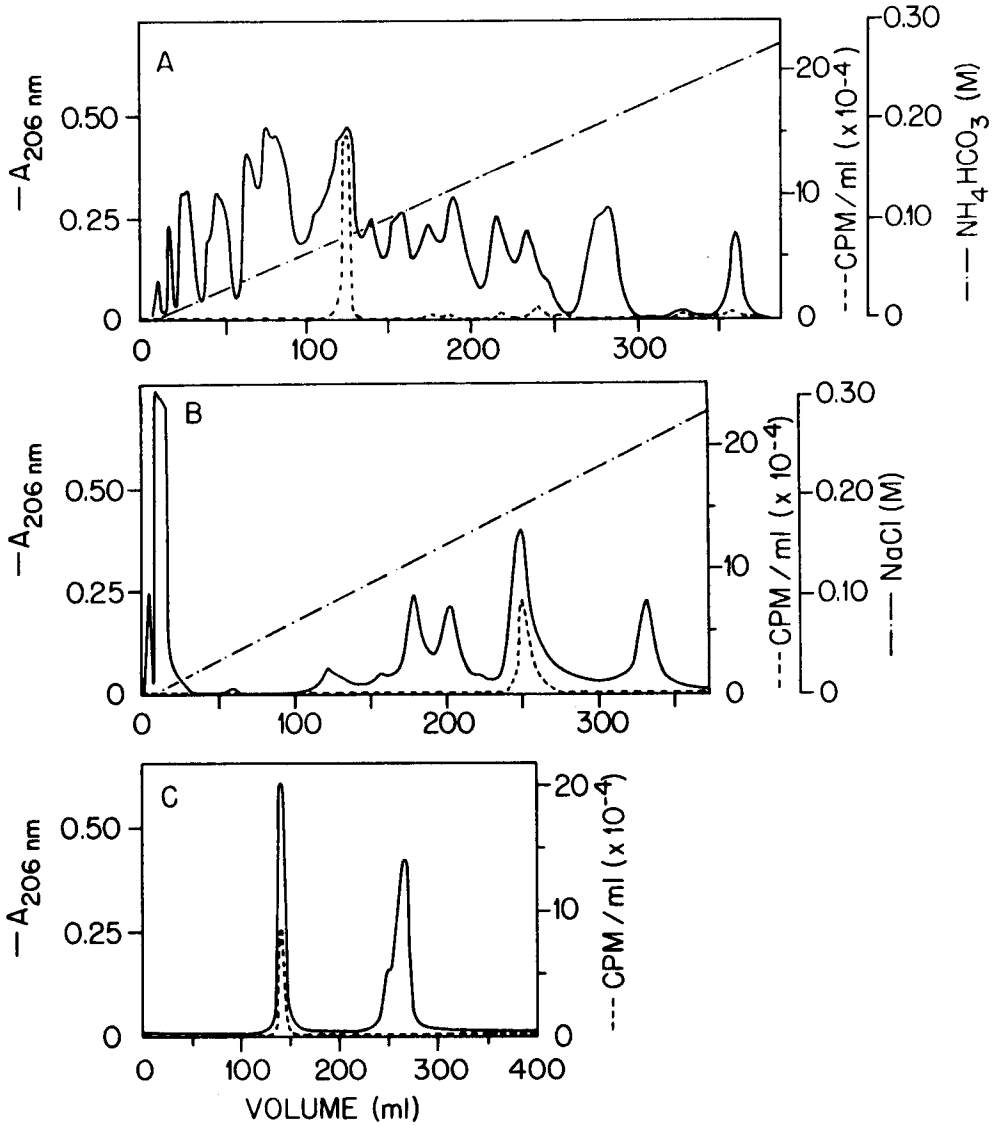

A
B
C
$-A_{206\ nm}$
0.50
0.25
0
100
200
300
400
20
10
0.30
0.20
0.10
---CPM/ml (x10-4)
— NH_4HCO_3 (M)
— NaCl (M)
VOLUME (ml)

Figure 2

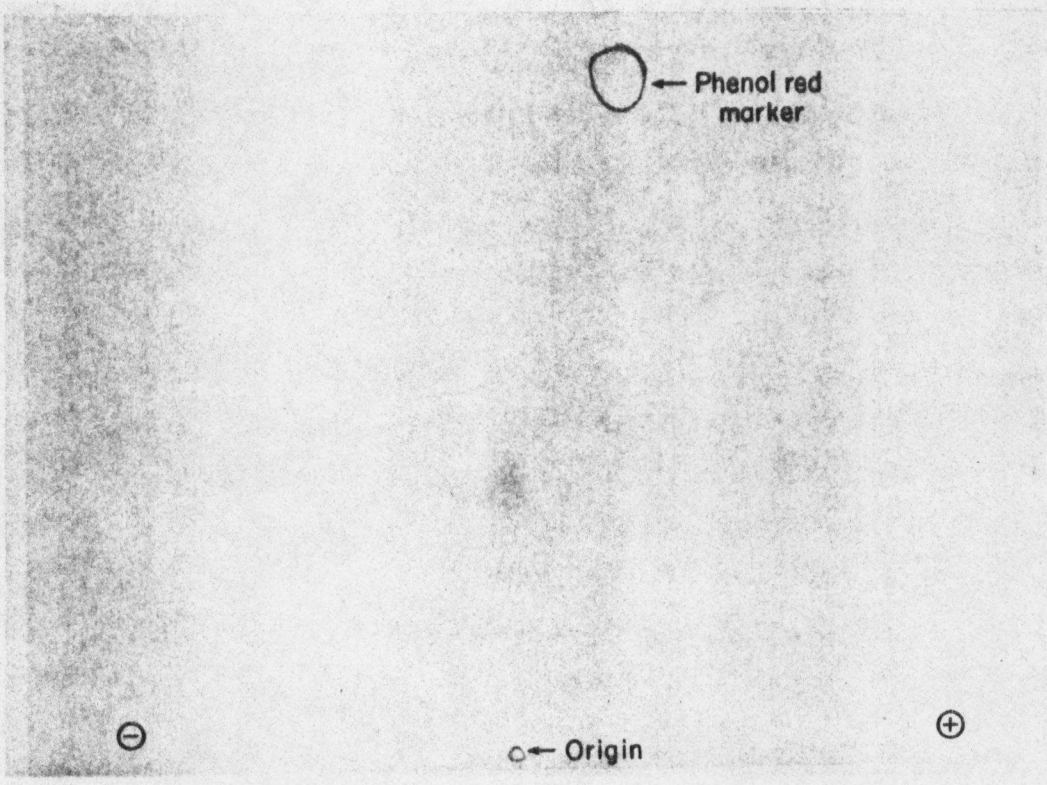


Figure 3. Peptide map of PLP-peptide. The sample size was 60 nmol. The first dimension was electrophoresis at pH 6.5; the second dimension was descending chromatography.

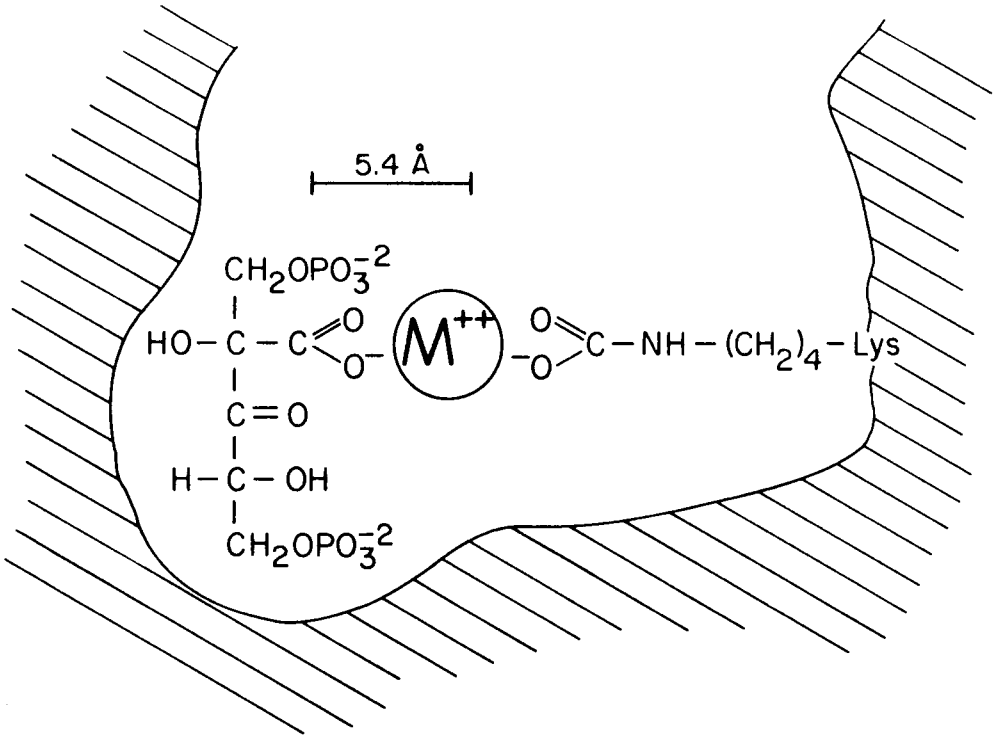
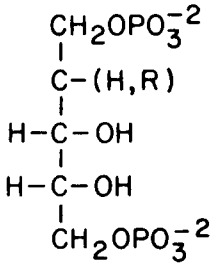


Figure 4. Proposed model of the transition state complex of ribulose-P_2 carboxylase/oxygenase. A possible interaction of the divalent metal cation with the carbamate at Lys^{201} and the 2-carboxy-3-keto-arabinitol 1,5-bisphosphate reaction intermediate is shown.



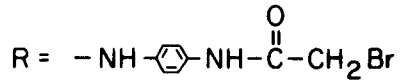
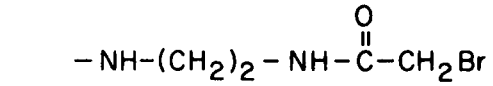
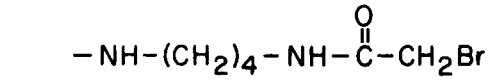
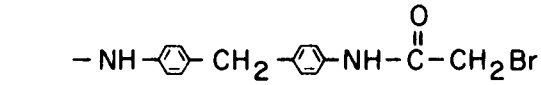
REAGENT	R =	DISTANCE (Å) C2→CH_2Br
A,B		10.0
C		8.8
D		11.9
E		15.8

Figure 5. Potential affinity labels for ribulose-P_2 carboxylase/oxygenase. The structures of the reagents synthesized by reductive amination of ribulose-P_2 and subsequent bromoacetylation of the primary amine are shown. Reagents A and B are the C2 epimers (ribo and arabino configurations) of the first structure, while reagents C, D, and E are each epimeric mixtures. The distance from C2 to the α-carbon of the bromoacetyl group indicated is calculated from standard bond lengths in the fully extended conformation.

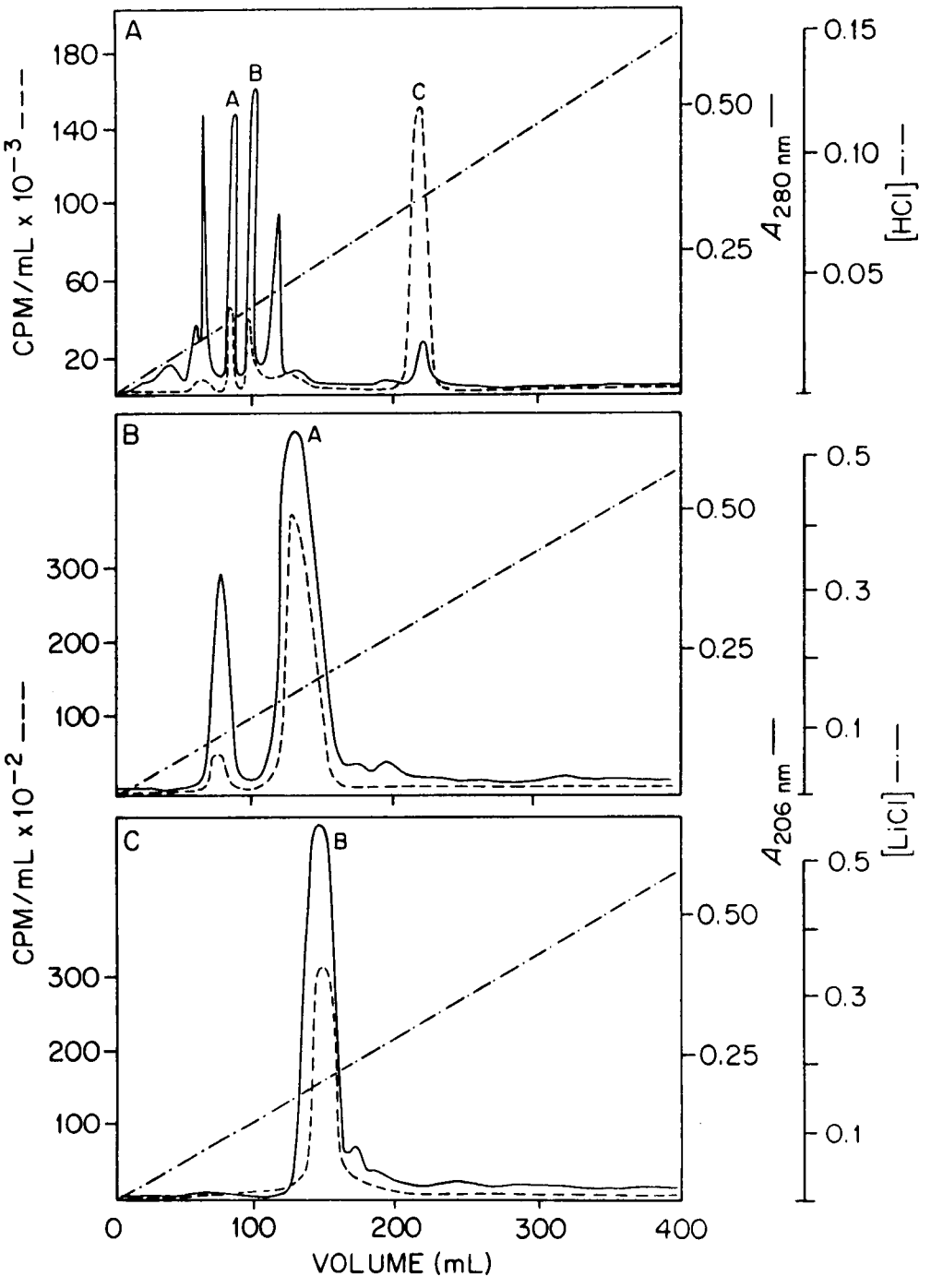


Figure 6. Purification of amino bisphosphates from reductive amination of ribulose-P_2 with p-phenylenediamine. (A) Fractionation of reaction mixture on QAE-Sephadex. (B,C) Rechromatography of peaks A and B, respectively, on QAE-Sephadex.

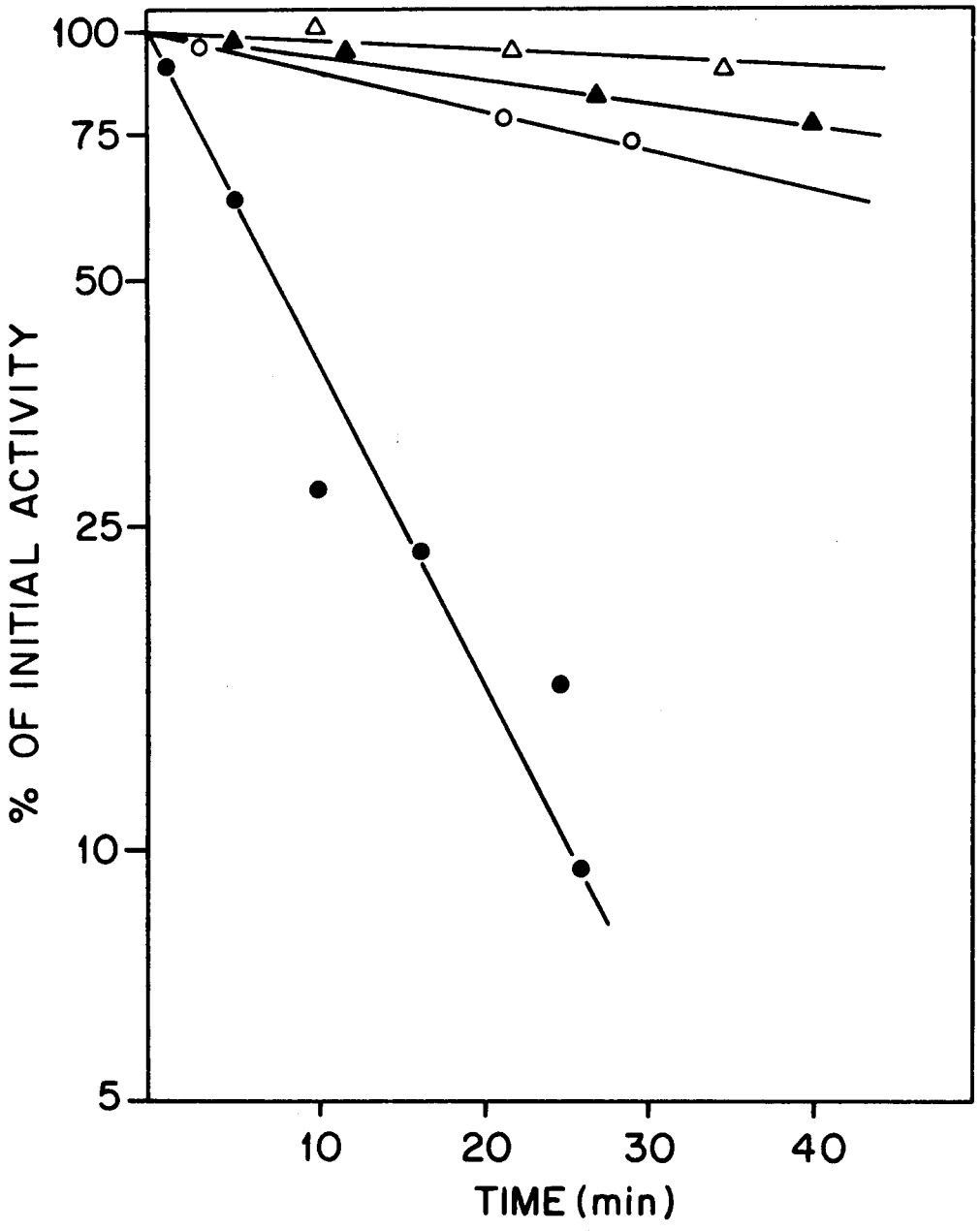


Figure 7. Incubation of ribulose-P_2 carboxylase/oxygenase with reagents A and B. Deactivated (open symbols) or activated (closed symbols) carboxylase (0.2 mg/ml, 4.7 μM subunit) was incubated with 0.5 mM reagent A (▲,△) or 0.5 mM reagent B (●,○) as described in the text.

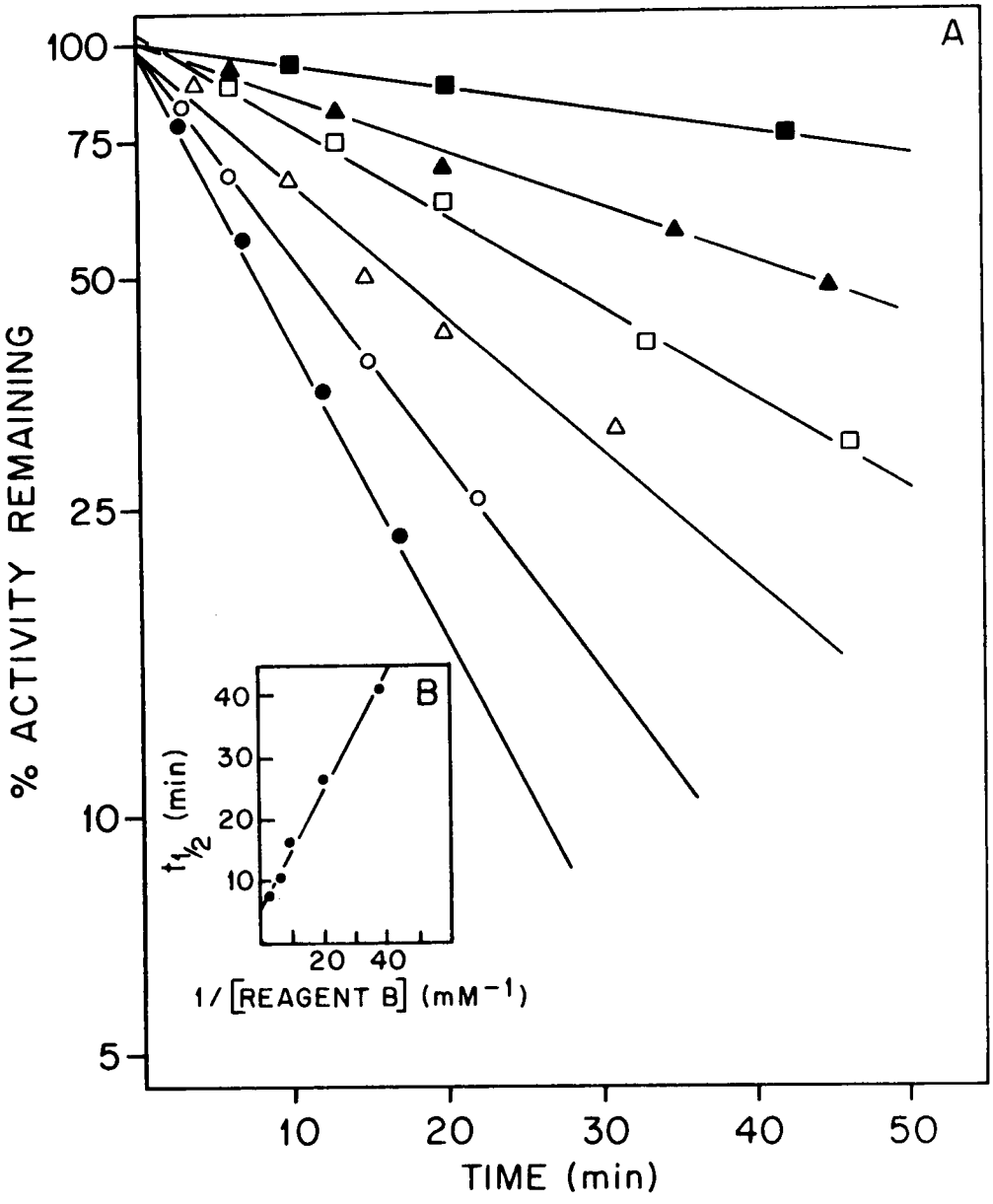


Figure 8. Rate saturation and protection by carboxyribitol-P_2. (A) Activated carboxylase (0.25 mg/ml, 4.7 μM subunit) was incubated as described in the text with either 400 μM (●), 200 μM (o), 100 μM (Δ), 50 μM (□), 25 μM (▲) reagent B or 250 μM reagent B in the presence of 250 μM carboxyribitol-P_2 (■). (B) Secondary plot of $t_{1/2}$ versus reciprocal reagent B concentration. The data in (A) are replotted according to the equation on page 35. Kinetic constants derived from this graph are given in the text.

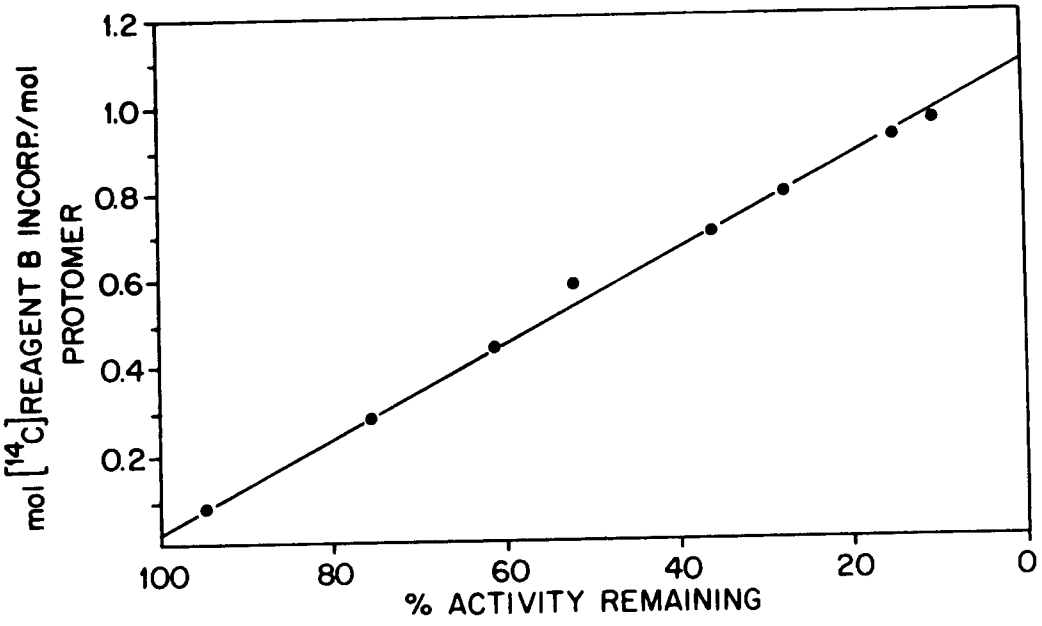


Figure 9. Covalent incorporation of reagent B as a function of ribulose-P_2 carboxylase activity remaining. Aliquots of enzyme incubated with [^{14}C]reagent B were periodically withdrawn for the determination of carboxylase activity and protein-bound radioactivity.

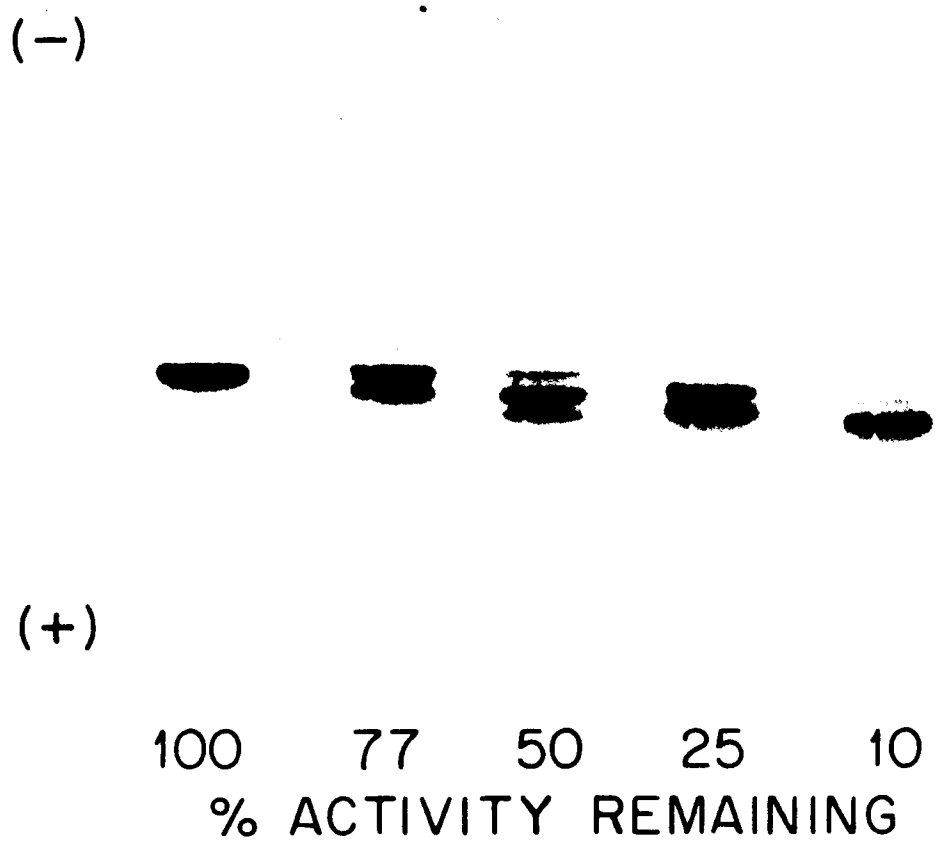


Figure 10. Polyacrylamide gel electrophoresis of reagent B-modified carboxylase/oxygenase. Enzyme (20 µg/gel), inactivated with reagent B and dialyzed against 0.01 M $NaHCO_3$, was electrophoresed on standard 7.5% polyacrylamide gels at 4°C in Tris-glycine (pH 8.9) for 1.25 h.

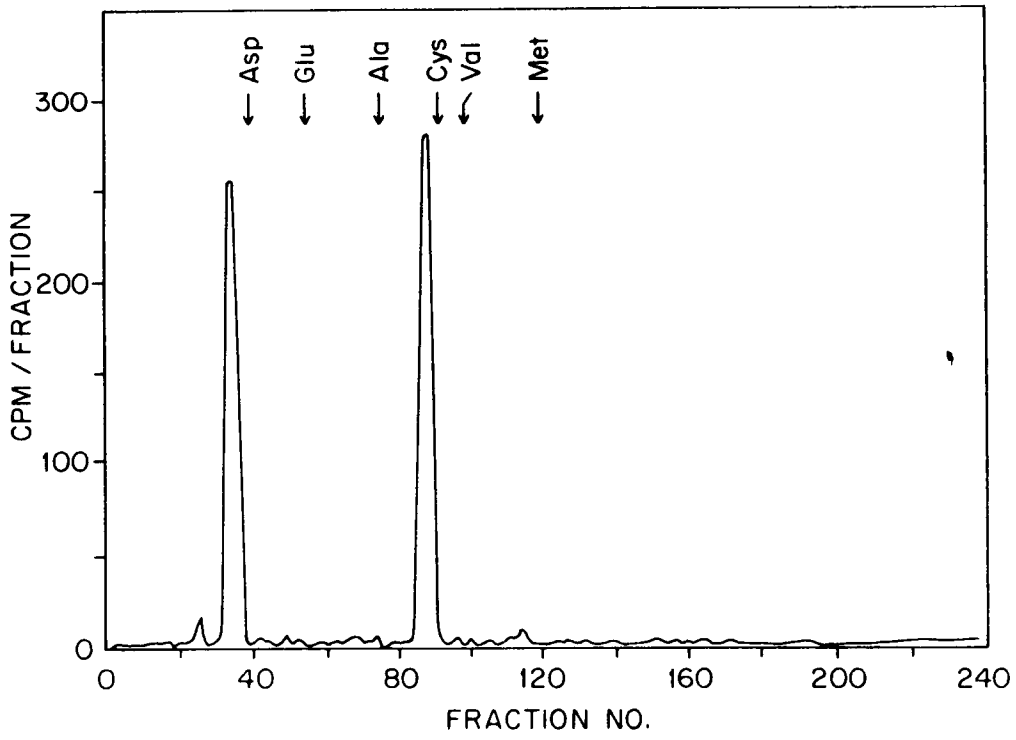


Figure 11. Amino acid analysis of [^{14}C]reagent B-modified carboxylase/oxygenase. An acid hydrolysate of enzyme inactivated with [^{14}C]reagent B, carboxymethylated, and digested with trypsin was subjected to amino acid analysis. One minute fractions of the effluent were collected in scintillation vials and counted. The elution positions of representative amino acids are indicated by arrows. S-carboxymethylcysteine and τ-carboxymethylhistidine elute at fractions 37 and 90, respectively.

Figure 12. Purification of radioactive peptides from a tryptic digest of [^{14}C]reagent B-modified carboxylase/oxygenase. (A) Anion-exchange chromatography on DEAE-cellulose. A 60-mg aliquot of the frozen, carboxymethylated tryptic digest (1.14 μmol subunit, 1.4 x 10^6 dpm) in 0.01 M NH_4HCO_3 was brought to 1 mM in 2-mercaptoethanol and applied to the column (1 x 22 cm) equilibrated with 0.01 M NH_4HCO_3/1 mM 2-mercaptoethanol (pH 8.1). Elution, at a flow rate of 13 ml/h, was effected by a 400-ml linear gradient of 0.01-0.50 M NH_4HCO_3/1 mM 2-mercaptoethanol. Total radioactivity recovered was 87%, of which 35% and 25% were contained in peaks I and II, respectively. The appropriate fractions under the peaks were pooled and lyophilized. Each peak was then redissolved in 2.5 ml of 0.01 M NH_4HCO_3/1 mM 2-mercaptoethanol and digested with 1% (w/w) alkaline phosphatase at 25°C for 3 h. (B) Rechromatography of peak I after alkaline phosphatase treatment. A portion (72 nmol, 8.5 x 10^4 dpm, 25% of total pool) of the alkaline phosphatase-treated peak I was applied to a column (1 x 22 cm) of DEAE-cellulose equlibrated with 0.01 M NH_4HCO_3/1 mM 2-mercaptoethanol. A 400-ml linear gradient of 0.01-0.30 M NH_4HCO_3/1 mM 2-mercaptoethanol was applied at a flow rate of 20 ml/h. The radioactive peak (6.0 x 10^4 dpm, 71% of applied radioactivity) was pooled and lyophilized three times. The residue was redissolved in 1.0 ml of 0.01 M NH_4HCO_3 for amino acid analysis. The remainder of the peak I pool was purified in an identical manner. (C) Rechromatography of peak II after alkaline phosphatase treatment. Subsequent to the removal of the phosphate groups, peak II (225 nmol, 2.7 x 10^5 dpm) was applied to a column (1 x 22 cm) of DEAE-cellulose equilibrated with 0.01 M NH_4HCO_3/1 mM 2-mercaptoethanol. The peptide was eluted as described for peak I at a flow rate of 16 ml/h. The radioactive fractions (1.5 x 10^5 dpm; 56% of the applied radioactivity) were pooled, lyophilized three times, and redissolved in 1.0 ml of 0.01 M NH_4HCO_3 for amino acid analysis.

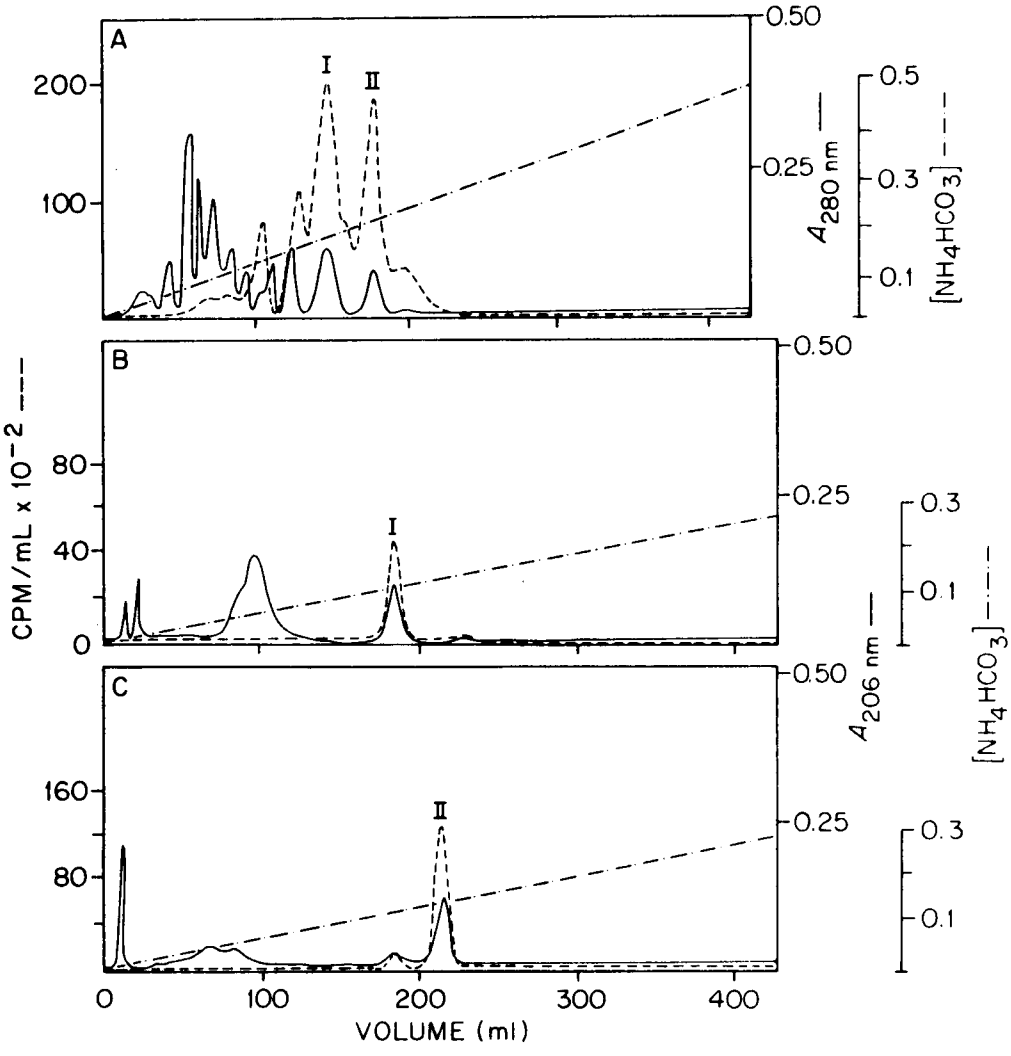

A
B
C
I
II
200
100
80
40
0
160
0.50
0.25
0.5
0.3
0.1
$A_{280\ nm}$ —
$[NH_4HCO_3]$ - - -
CPM/mL x 10^{-2} ---
$A_{206\ nm}$ —
$[NH_4HCO_3]$ -·-·-
0
100
200
300
400
VOLUME (ml)

Figure 12

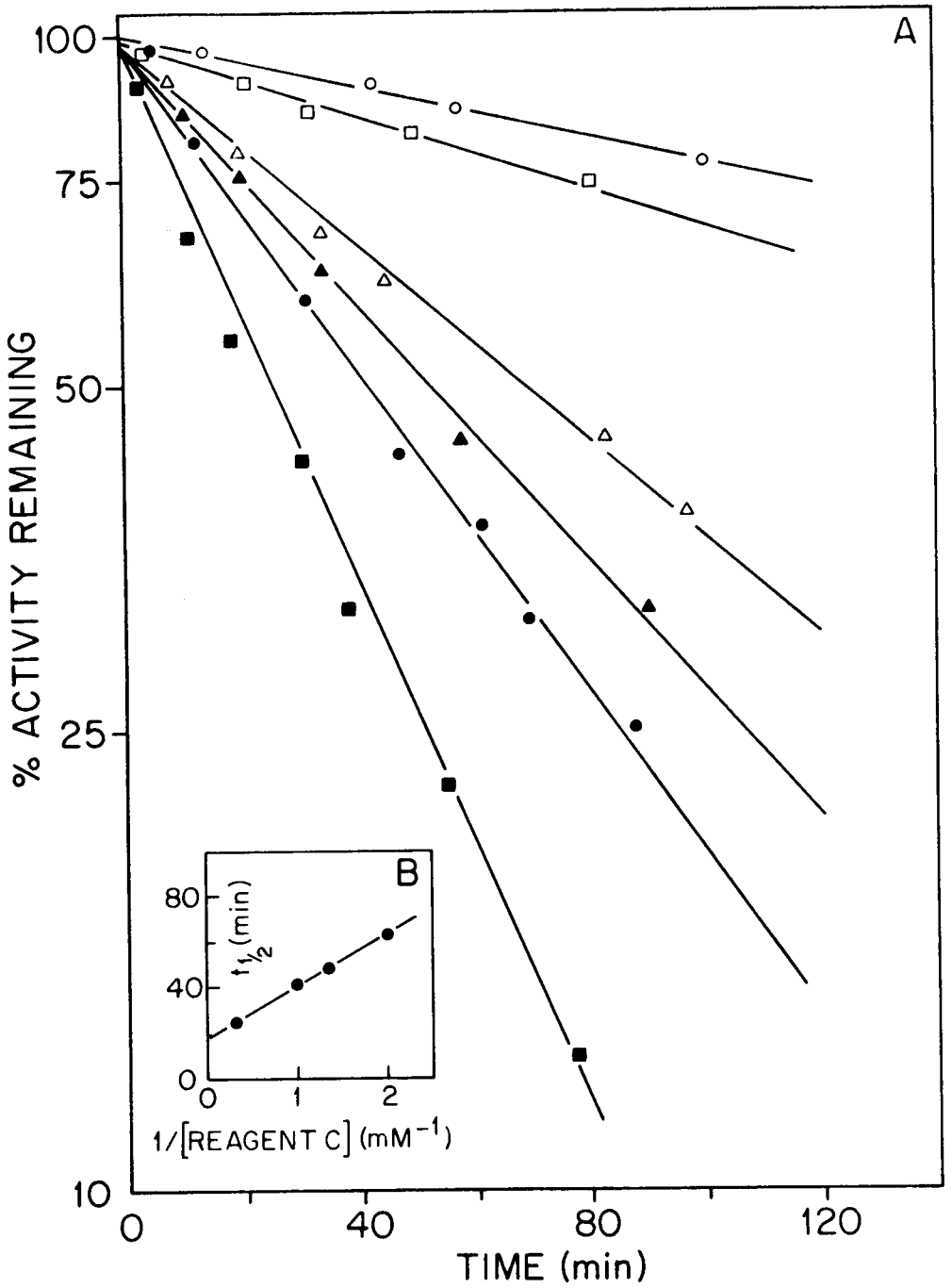


Figure 13. Inactivation of ribulose-P_2 carboxylase/oxygenase by reagent C. (A) Carboxylase (0.25 mg/ml, 4.7 μM subunit) was incubated as described in the text in the presence of CO_2 and Mg^{++} with either 3 mM (■), 1 mM (●), 0.75 mM (▲), 0.5 mM (△) reagent C or 1 mM reagent C/250 μM carboxyribitol-P_2 (□), and in the absence of CO_2 and Mg^{++} with 3 mM reagent C (○). (B) Secondary plot of $t_{1/2}$ versus reciprocal reagent C concentration of the data represented in (A). Kinetic constants derived from this graph are given in the text.

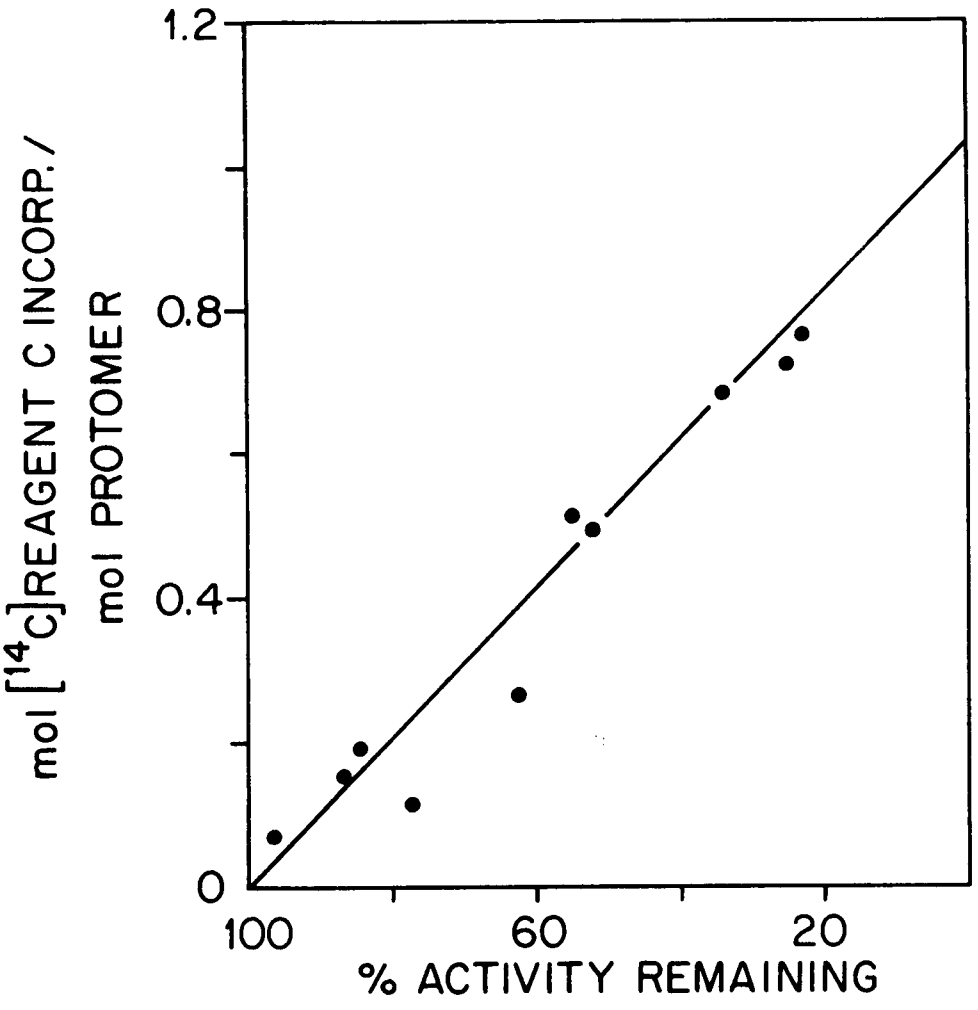


Figure 14. Covalent incorporation of reagent C as a function of ribulose-P_2 carboxylase activity remaining. Aliquots of enzyme incubated with [^{14}C]reagent C were periodically withdrawn for the determination of carboxylase activity and protein-bound radioactivity.

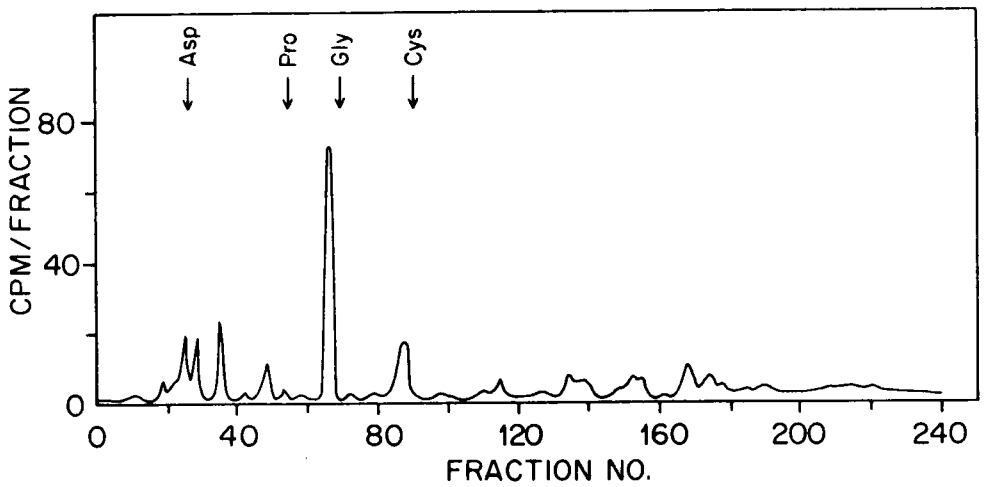


Figure 15. Amino acid analysis of [^{14}C]reagent C-modified carboxylase/oxgenase. Enzyme which had been inactivated with [^{14}C]reagent C, carboxymethylated, and digested with trypsin was hydrolyzed and analyzed for radioactive carboxymethylated amino acids. One minute fractions of the effluent, bypassing the reaction with ninhydrin, were collected. The elution positions of representative amino acids are indicated by arrows. The major peak eluting at fraction 67 is S-carboxymethylhomocysteine.

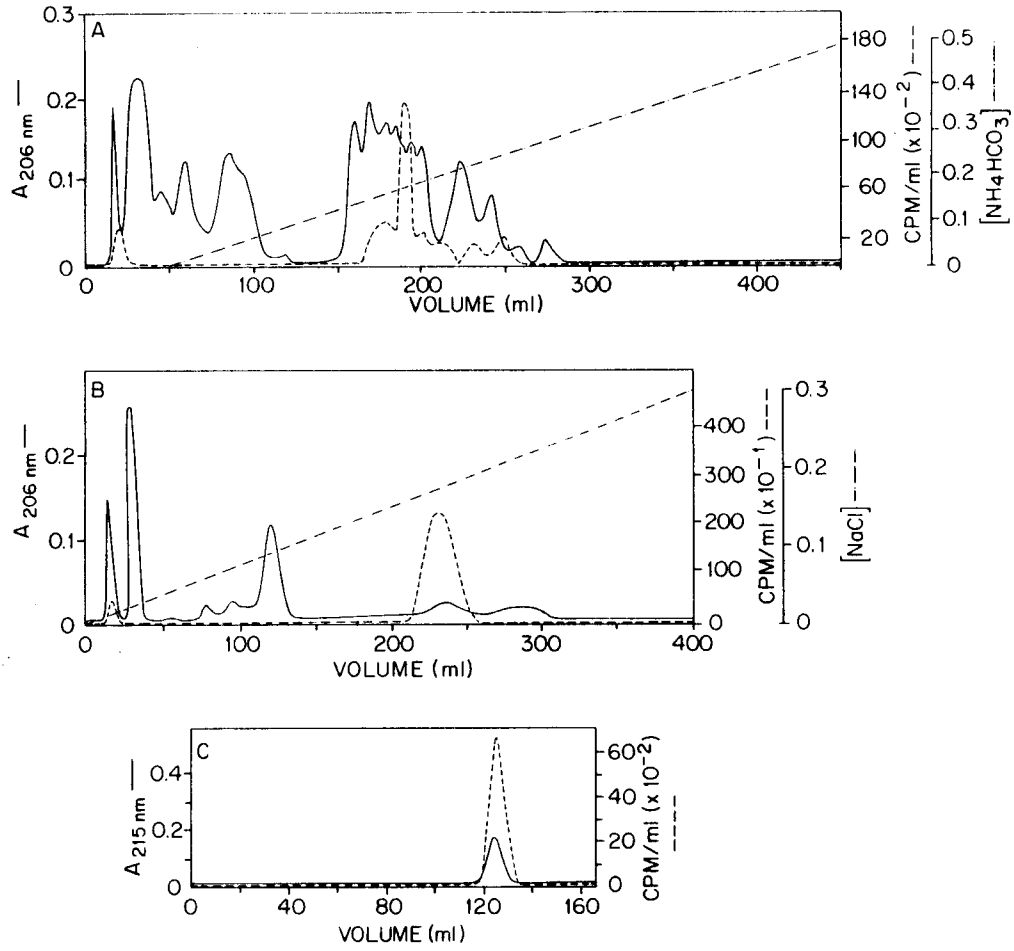


Figure 16. Purification of radioactive peptide from a tryptic digest of [^{14}C]reagent C-modified carboxylase/oxygenase. (A) Anion-exchange chromatography of a carboxymethylated tryptic digest of R. rubrum carboxylase modified with [^{14}C]reagent C. A 10-mg (188 nmol, 2.8 x 10^5 dpm) aliquot of the frozen tryptic digest was applied to a DEAE-cellulose column (1 x 22 cm) equilibrated with 0.01 M NH_4HCO_3/1 mM 2-mercaptoethanol (pH 8.1) and eluted with a 400-ml linear gradient of 0.01-0.50 M NH_4HCO_3/2-mercaptoethanol at a flow rate of 13 ml/h. The major radioactive peak (1.4 x 10^5 dpm, 50% of the applied radioactivity) was pooled and lyophilized. (B) Cation-exchange chromatography of radioactive peak from DEAE-cellulose. The lyophilized residue was redissolved in 2 ml of 11 mM H_3PO_4 (adjusted to pH 3 with NH_4OH) containing 6 M urea and applied to a column of SP-Sephadex (1 x 22 cm) equilibrated with 11 mM H_3PO_4. The column was developed at a flow rate of 15 ml/h with a 400-ml linear gradient of 0-0.3 M NaCl in 11 mM H_3PO_4. The radioactive peak (8.3 x 10^4 dpm, 60% of applied radioactivity) was pooled, neutralized by the addition of solid NH_4HCO_3, and lyophilized. A 1 M NaCl/11 mM H_3PO_4 wash of the column did not elute any additional radioactivity. (C) Gel filtration of radioactive peak from SP-Sephadex. The lyophilized residue was redissolved in 5 ml of 0.01 M NH_4HCO_3 and desalted on a column of Sephadex G-25 (fine) (2.5 x 220 cm). The radioactive peak (7.2 x 10^4 dpm, 95% of applied radioactivity) was lyophilized and redissolved in 1.5 ml of 0.01 M NH_4HCO_3 for amino acid analysis.

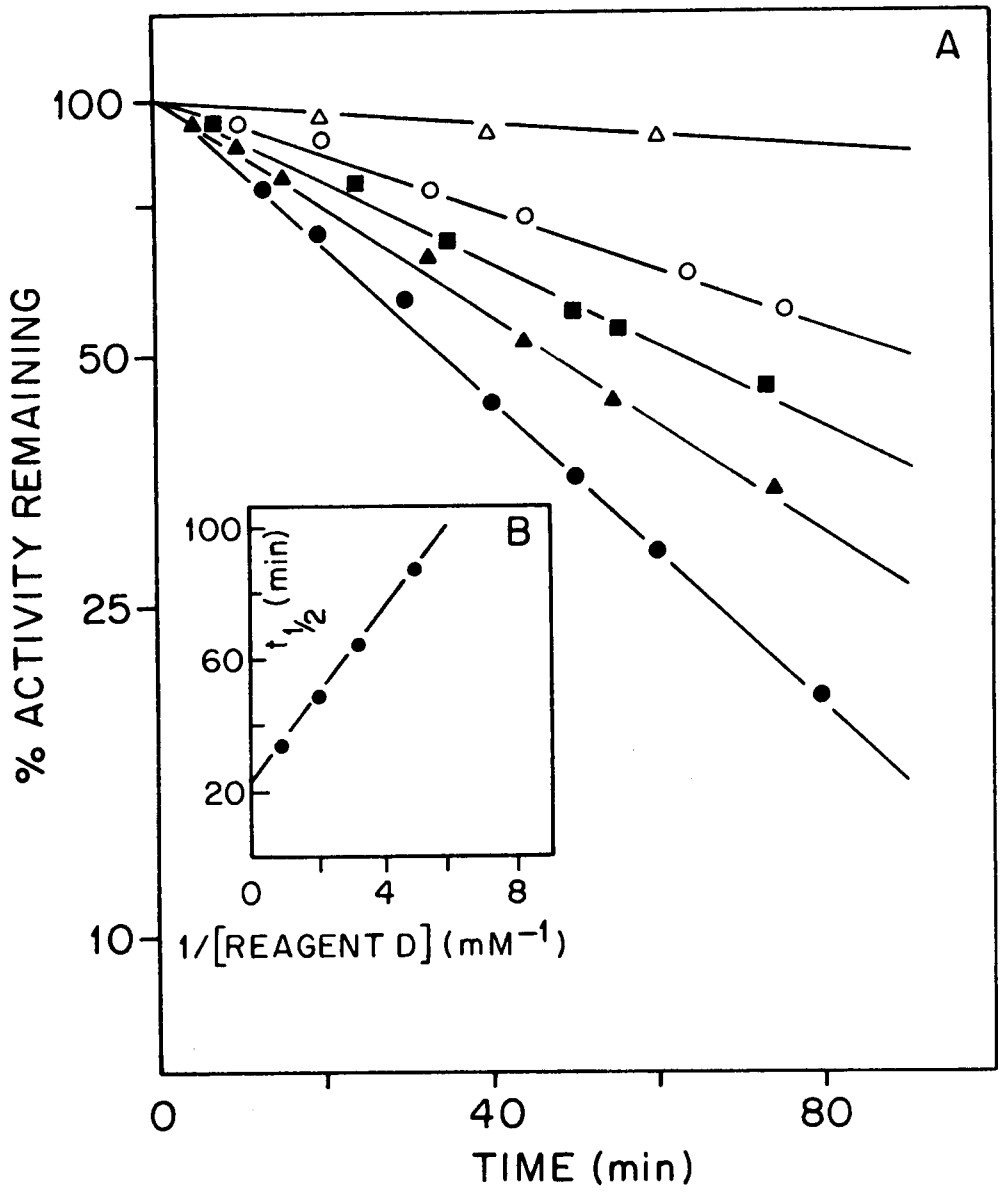


Figure 17. Inactivation of ribulose-P_2 carboxylase/oxygenase by reagent D. (A) Carboxylase (0.25 mg/ml, 4.7 μM subunit) was incubated as described in the text in the presence of CO_2 and Mg^{++} with either 1 mM (●), 0.5 mM (▲), 0.3 mM (■), 0.2 mM (o) reagent D or 1 mM reagent D/250 μM carboxyribitol-P_2 (Δ). (B) Secondary plot of $t_{1/2}$ versus reciprocal reagent D concentration of the data in (A). The kinetic constants derived from this graph are given in the text.

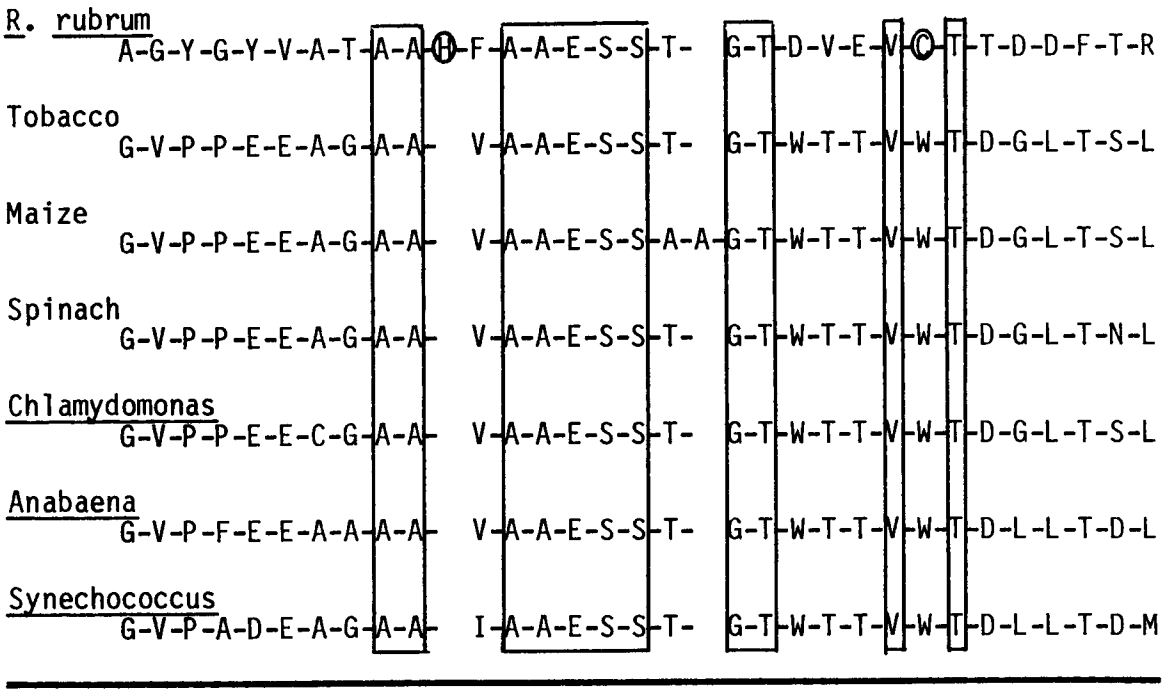


Figure 18. Sequence homology in reagent B-labeled peptides. Amino acids conserved in all seven species are enclosed by boxes while the reagent B-reactive residues, His^{44} and Cys^{58}, are circled.

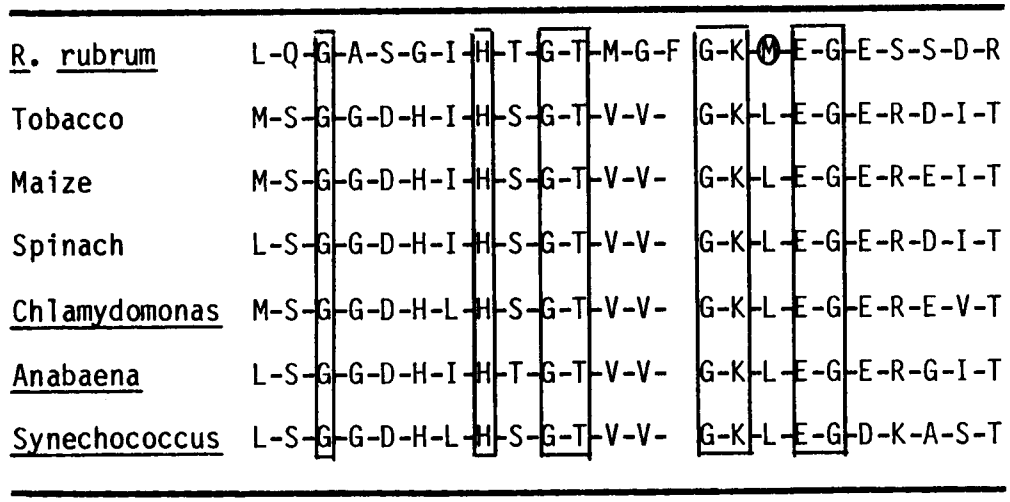


Figure 19. Sequence homology in reagent C-labeled peptide. Amino acids conserved in all seven species are enclosed by boxes while the reagent C-reactive residue, Met^{335}, is circled.

VITA

Cinda S. Herndon was born in Lancaster, Ohio, on October 20, 1956. She attended school in that city and graduated from Lancaster High School as valedictorian in June 1975. The following September, she enrolled in the Georgia Institute of Technology, transferring to Clemson University, Clemson, South Carolina, the following June. She received a Bachelor of Science degree with highest honor in Biochemistry in May 1979.

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