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IDENTIFICATION OF CATALYTICALLY ESSENTIAL PORTIONS OF RIBULOSEBISPHOSPHATE CARBOXYLASE AND RESULTANT MECHANISTIC IMPLICATIONS

A Dissertation

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Doctor of Philosophy

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ABSTRACT

Until recently the catalytic potential of ribulosebisphosphate carboxylase was not fully appreciated, as the enzyme's allosteric activation by CO_2/Mg^{2+} and its oxygenase activity were unknown. When fully activated by CO_2 and Mg^{2+} the enzyme plays a pivotal role in photosynthesis being responsible for CO_2 fixation and photorespiration. Despite the biological importance of ribulosebisphosphate carboxylase, relatively little is known about the makeup of its active site.

Affinity labeling is a promising approach to the characterization of ligand binding sites. In Part I several reagents, cis- and trans-2,3-epoxybutane-1,4-diol 1,4-bisphosphate, N-bromoacetyldiethanolamine bisphosphate, N-bromoacetylserine phosphate, N-bromoacetylethanolamine phosphate, phosphoglycolic acid azide, and glycidol phosphate, are examined as potential affinity labels for ribulosebisphosphate carboxylase. Of these reagents one, N-bromoacetylethanolamine phosphate, has proven to be a useful affinity labeling reagent for the enzyme. Inactivation of the spinach carboxylase by the reagent is pseudo-first-order and exhibits rate saturation, indicating reversible binding prior to chemical modification. The substrate D-ribulose 1,5-bisphosphate prevents inactivation by the reagent, suggesting that the substrate and reagent share a common binding site. Mg^{2+}, a catalytically essential cofactor, stimulates the maximal rate of inactivation approximately sixfold. Based on amino acid analyses of [^{14}C]N-bromoacetylethanolamine phosphate modified enzyme, Mg^{2+} also alters the specificity of the

reagent from cysteinyl to lysyl residues. Further analyses of the modified enzyme at the level of tryptic peptides reveal that in the absence of Mg^{2+} inactivation correlates with the modification of two different cysteinyl residues (which are protected against modification by substrate); in the presence of Mg^{2+} inactivation is a consequence of the rather selective modification of a single lysyl residue (protection of this residue is afforded by a competitive inhibitor, butane-1,4-diol 1,4-bisphosphate, or by the transition state analog, 2-carboxyribitol 1,5-bisphosphate). Isolation and sequencing of the tryptic peptides which contain the modified residues reveal that one of the two cysteinyl residues is only three residues distant in sequence from the lysyl residue. This lysyl residue is identical to one of two modified by the previously described affinity label 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate.

In Part II culture conditions are described for Rhodospirillum rubrum, a purple nonsulfur photosynthetic bacterium, which result in a large fraction of the soluble protein (> 40%) being comprised of ribulosebisphosphate carboxylase. When cells are cultured by this method sufficient quantities of the R. rubrum enzyme are obtained for affinity labeling and other studies requiring relatively large quantities of enzyme. Comparative studies between structurally and evolutionarily diverse enzymes can be helpful in determining catalytically essential features. Comparison of the spinach and R. rubrum ribulosebisphosphate carboxylases reveals substantial differences in their kinetic constants, affinities for various competitive inhibitors, and molecular architecture.

The spinach and R. rubrum enzymes thus provide an especially stringent test case for the determination of whether residues identified as essential in affinity labeling studies represent a species-invariant feature.

The enzyme from R. rubrum is inactivated by the two reagents which are affinity labels for the spinach enzyme, 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate and N-bromoacetylethanolamine phosphate, with protection afforded against inactivation by the substrate ribulosebisphosphate. As is the case with the spinach enzyme, 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate and N-bromoacetylethanolamine phosphate appear to inactivate the R. rubrum enzyme in the absence of Mg^{2+} primarily as a consequence of lysyl and cysteinyl modification, respectively. In contrast to the spinach enzyme, however, Mg^{2+} does not induce an alteration in the specificity of N-bromoacetylethanolamine phosphate, presumably reflecting structural differences in the active site region of the two enzymes.

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LIST OF SYMBOLS

ATP = Adenosine 5′-triphosphate

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

BrAcNHEtOP = N-Bromoacetylethanolamine phosphate

Br-butanone-P_2 = 3-Bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate

Butanediol-P_2 = Butane-1,4-diol 1,4-bisphosphate

Cm = Carboxymethyl

CKR-P_2 = 2-Carboxy-3-ketoribitol 1,5-bisphosphate

CR-P_2 = 2-Carboxyribitol 1,5-bisphosphate

δ = Position of the signal in an NMR spectra (in parts per million)

dd = A doublet of doublet signals (four peaks) in an NMR spectra

DEAE = Diethylaminoethyl

DTNB = 5,5′-Dithiobis(2-nitrobenzoic acid)

EDTA = Ethylenediaminetetraacetate

Epoxybutane-P_2 = cis- or trans-2,3-Epoxybutane-1,4-diol 1,4-bisphosphate

gem = Geminal

J = The coupling constant between two signals in an NMR spectra

m = A multiplet signal in an NMR spectra

mp = Melting point

NADH = β-Nicotinamide adenine dinucleotide, reduced form

NMR = Nuclear magnetic resonance

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

R_f = The distance traveled by a compound divided by the distance traveled by the solvent front on a paper or thin-layer chromatogram

SDS = Sodium dodecyl sulfate

t = A triplet signal in an NMR spectra

TNB = 5-Thio-2-nitrobenzoic acid

Tris = Tris(hydroxymethyl)aminomethane

vic = Vicinal

PART I. AFFINITY LABELING OF SPINACH RIBULOSEBISPHOSPHATE CARBOXYLASE

CHAPTER I

INTRODUCTION

Over thirty years ago Wildman and Bonner (1947) observed that a large fraction [up to 50% (Kawashima and Wildman, 1970)] of the soluble protein in plant leaves is composed of a single protein which they termed fraction 1 protein. Subsequent research concerning photosynthetic assimilation of CO_2 (for a review see Calvin, 1962) demonstrated that a single enzyme, Rbl-P_2 carboxylase (EC 4.1.1.39), is responsible for CO_2 fixation (Jacoby et al., 1956; Weissback et al., 1956). This enzyme is identical to fraction 1 protein (Kawashima and Wildman, 1970).

Because of its essentiality to the photosynthetic assimilation of CO_2, Rbl-P_2 carboxylase has been the subject of innumerable physiological, genetic, and biochemical studies in the intervening years (for a review see Jensen and Bahr, 1977). A recent surge of interest in the mechanism and regulation of its catalytic activity ensued with the realization that the carboxylase has an associated oxygenase activity which accounts for photorespiration (the Warburg effect) (Bowes et al., 1971; Tolbert, 1973). Thus, a single enzyme may determine the ratio of photosynthesis and photorespiration. Since photorespiration reduces net photosynthetic efficiency, an understanding of the structural basis for these two activities (and the possibility of altering their relative rates by external or genetic means) acquires agronomic significance (Chollet and Ogren, 1975; Zelitch, 1975).

Given the biological importance of Rbl-P_2 carboxylase and the ready availability of the enzyme, surprisingly little information about the active site of the enzyme is available. Previous studies with the affinity label Br-butanone-P_2 (Hartman et al., 1973b; Norton et al., 1975; Schloss and Hartman, 1977a) and with pyridoxal phosphate (Paech et al., 1977; Whitman and Tabita, 1976, 1978a,b) have implicated at least one lysyl residue at the active site, but other information relating to the identification of residues important in catalysis is scanty. In a search for reagents that can be used to map further the active site of the carboxylase, and thus aid in the elucidation of the reaction mechanism, I have found that BrAcNHEtOP satisfies the major criteria (Shaw, 1970a,b; Baker, 1967; Singer, 1967) of an affinity label for the enzyme from spinach.

CHAPTER II

MATERIALS AND METHODS

Materials

Spinach (Spinacia oleracea, Early Hybrid No. 7, W. Atlee Burpee Co.) was grown in a growth chamber on a 12-h light-dark cycle at 20°. Rbl-P_2 carboxylase/oxygenase was purified from freshly harvested spinach leaves by slight modification (Norton et al., 1975) of the method of Wishnick and Lane (1971). The enzyme has a molecular weight of 560,000 and contains eight protomeric units, each composed of a large (56,000 daltons) and a small (14,000 daltons) subunit (Wishnick and Lane, 1971; Rutner, 1970; Sugiyama and Akazawa, 1970).

BrAcNHEtOP, [^{14}C]BrAcNHEtOP, N-hydroxysuccinimide bromoacetate, phosphoglycolic acid azide, glycidol phosphate, butanediol-P_2, and CR-P_2 were synthesized by published procedures (Hartman et al., 1973a; Hartman et al., 1978; Rose and O'Connell, 1969; Hartman and Barker, 1965; Rabin et al., 1958). Trypsin that had been treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone was acquired from Worthington Biochemical Corporation. Carboxypeptidases A and B (that had been treated with diisopropyl-fluorophosphate), O-phospho-DL-serine, Bicine, Rbl-P_2, ATP, NADH, glutathione, glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate phosphokinase, and glycerophosphate dehydrogenase-triose phosphate isomerase were purchased from Sigma Chemical Company. Chemicals used with the amino acid analyzer and sequencer were obtained from Pierce Chemical Company. cis-2-Butenediol, m-chloroperbenzoic acid,

diethanolamine, and DTNB were obtained from Aldrich Chemical Company. 2-Butyne-1,4-diol, diphenyl chlorophosphate, and bromoacetyl bromide were purchased from Eastman Kodak. $LiAlH_4$ and platinum dioxide were products of Alfa Inorganics, Ventron and Matthey Bishop, respectively.

General Methods

Elemental analyses were performed by Galbraith Laboratories, Incorporated, Knoxville, Tennessee.

Melting points were taken with a Fisher-Jones apparatus and are uncorrected.

NMR spectra were obtained with a 100 MHz Varian XL-100 NMR spectrometer. Spectra were recorded from samples dissolved in $CDCl_3$ or D_2O and tetramethylsilane or sodium 2,2-dimethyl-2-silapentane-5-sulfonate used as an internal reference, respectively.

Thin-layer chromatography was conducted on plastic sheets coated with silica gel or cellulose containing a fluorescent indicator (MN-Polygram Sil N-HR and Cel 300 sold by Brinkman Instruments, Incorporated). The solvents used were diethyl ether (silica gel) and ethanol (cellulose). Descending paper chromatography was conducted using Whatman No. 1 paper. The solvent used was n-butyl alcohol/glacial acetic acid/water (7:2:5 v/v/v). Ion-exchange paper chromatography was conducted by use of Whatman DE 81 paper with 0.4 M ammonium bicarbonate as solvent. Phosphate esters were detected by spraying chromatograms with the ammonium molybdate reagent of Hanes and Isherwood (1949) followed by exposure to ultraviolet light. Compounds containing vicinal hydroxyl groups were visualized with periodate/benzidine (Cifonelli and

Smith, 1954). The periodate spray contained 0.05% (w/v) sodium metaperiodate (Hartman, 1975). Epoxides were detected by spraying chromatograms with saturated aqueous LiCl/1% phenolpthalein in ethanol (1:1 v/v) followed by heating chromatograms in an oven at 110°C for 5 min. Epoxides appeared as red spots on a white background and faded upon further heating. α-Halo acids and epoxides were detected with TNB (Schloss and Hartman, 1977c). Primary and secondary amines were visualized with ninhydrin/collidine (Canfield and Anfinsen, 1963). Iodine vapor was used to detect alkenes, alkynes, and epoxides.

Inorganic and total phosphate were assayed by the method of Ames and Dubin (1960).

Peracid was determined by its reaction with sodium iodide and titration of the iodine formed with sodium thiosulfate (Braun, 1932).

Synthesis of cis and trans-Epoxybutane-P_2

To a 500 mL, 3-necked round bottomed flask equipped with stirrer, condenser, and dropping funnel were added 18 g (0.474 mol) of $LiAlH_4$ and 125 mL of tetrahydrofuran. The mixture was brought to boiling, and 10.2 g (0.118 mol) of 2-butyne-1,4-diol in 50 mL of tetrahydrofuran was added dropwise during 45 min. After refluxing for 1 h, the mixture was cooled to room temperature; 100 mL of 0.1 N HCl was then carefully added during 1 h and the mixture was refluxed for an additional 15-min period. Anhydrous sodium sulfate (100 g) was added as a drying agent; it was removed by filtration through celite and washed thoroughly with two 200-mL portions of dioxane. The combined filtrate and washings were treated with a second 100-g addition of Na_2SO_4. After removal of

Na_2SO_4, rotary evaporation gave 9.05 g (87%) of a viscous syrup. Thin-layer chromatography (silica gel with diethyl ether as solvent) revealed virtually complete conversion of 2-butyne-1,4-diol (R_f 0.45) to trans-2-butene-1,4-diol (R_f 0.20), which was well resolved from the commercially available cis isomer (R_f 0.27).

To 3.62 g (0.0262 mol) of m-chloroperbenzoic acid in 50 mL of diethyl ether was added 2.11 g (0.0239 mol) of trans-2-butene-1,4-diol. After incubation of the reaction mixture on ice in the dark for 2 h, 67% of the peracid remained; incubation was continued for an additional 18 h at which time 17% remained. Crystals of trans-2,3-epoxybutane-1,4-diol, which had formed in the reaction mixture, were collected by filtration and washed with three 40-mL portions of cold diethyl ether to give 1.42 g (57%) of trans-2,3-epoxybutane-1,4-diol: mp 70-72°C [lit. (Eremenko and Korolev, 1968) 73.5-74.5°C]; ^1H NMR (D_2O) δ 3.22 (m, 2H, oxirane), 3.54 (dd, J_{gem} = 13 Hz, J_{vic} = 5.5 Hz, 2H), 3.90 (dd, J_{vic} = 2.5 Hz, 2H). Anal. Calcd for $C_4H_8O_3$ (104.11): C, 46.15; H, 7.75. Found: C, 46.23; H, 7.81. Thin-layer chromatography (silica gel with diethyl ether as solvent) revealed a single component (R_f 0.15).

To 20 g (0.116 mol) of m-chloroperbenzoic acid in 150 mL of diethyl ether was added 9.5 mL (0.115 mol) of cis-2-butene-1,4-diol. The reaction mixture was kept on ice in the dark; within 1 h a copious precipitate had developed, and only 16% of the peracid remained. After an additional 2 h, 11% of the peracid remained; the crystals of cis-2,3-epoxybutane-1,4-diol were collected by filtration and washed with cold diethyl ether to give 7.19 g (60%) of cis-2,3-epoxybutane-1,4-diol: mp

50-52°C; ^{1}H NMR (D_2O) δ 3.32 (m, 2H, oxirane), 3.60 (dd, J_{gem} = 12 Hz, J_{vic} = 7 Hz, 2H), 3.88 (dd, J_{vic} = 3.5 Hz, 2H). Anal. Calcd for $C_4H_8O_3$ (104.11): C, 46.15; H, 7.75. Found: C, 46.24; H, 7.84. Thin-layer chromatography (silica gel with diethyl ether as solvent) revealed a single component (R_f 0.19).

To 1 g (9.6 mmol) of epoxide (cis- or trans-2,3-epoxybutane-1,4-diol) in 5 mL pyridine was added 10 mL of $CHCl_3$. The mixture was cooled in an ice bath, and 6.1 mL (28.8 mmol) of diphenyl chlorophosphate was introduced. Phosphorylation was allowed to proceed at 4°C for 17 h. Ice chips (2 g) were then added to the mixture followed 3 h later by 100 mL of $CHCl_3$. The $CHCl_3$ solution was washed successively with four 100-mL portions of 1 N H_2SO_4 and saturated aqueous $NaHCO_3$. After drying (Na_2SO_4), the $CHCl_3$ solution was concentrated to dryness to give either 5.32 g (98%) of the trans isomer or 4.84 g (89%) of the cis isomer. The trans isomer crystallized spontaneously on standing, and was recrystallized twice from ethanol (70% recovery): mp 78-81°C; ^{1}H NMR ($CDCl_3$) δ 3.20 (t, 2H, oxirane), 4.20 (dd, J_{gem} = 8 Hz, J_{vic} = 3 Hz, 2H), 4.38 (dd, J_{vic} = 4 Hz, 2H), 7.28 (m, 20H, phenyl). Anal. Calcd for $C_{28}H_{26}O_9P_2$ (568.46): C, 59.16; H, 4.61; P, 10.90. Found: C, 59.26; H, 4.67; P, 10.91. Both cis (R_f 0.58) and trans (R_f 0.47) isomers were homogeneous by thin-layer chromatography (silica gel with diethyl ether as solvent). The cis isomer had the following ^{1}H NMR ($CDCl_3$) spectra: δ 3.29 (t, 2H, oxirane), 4.31 (m, 4H), 7.21 (m, 20H, phenyl).

To 2 g (3.52 mmol) of cis- or trans-2,3-epoxybutane-1,4-diol 1,4-bis(diphenyl)phosphate in 50 mL of anhydrous ethanol was added 2 g

of activated charcoal (Darco, Atlas Powder Company). Following filtration through celite, the solution was subjected to Pt-catalyzed (100 mg of PtO_2) hydrogenation which was carried out for 1.5 h at 52 psi in a Parr apparatus. After filtration through celite, the solutions were chilled on ice and neutralized with 1.9 N LiOH in methanol. The precipitates were collected by centrifugation and washed twice with 200-mL portions of ethanol. After drying for 48 h in a vacuum desiccator, 0.84 g (78%) or 0.79 g (73%) of the trans or cis isomers were recovered, respectively. Paper chromatography revealed that both compounds were homogeneous (R_f 0.14) except for a trace of inorganic phosphate (R_f 0.31), which was removed by ion-exchange chromatography. After dissolving 0.5 g of cis- or trans-epoxybutane-P_2 in 50 mL of ice-cold water, the sample was applied to a refrigerated (4°C) column (2.5 × 59 cm) of Dowex 1-X2 (Cl^-) equilibrated with glass-distilled water. The column was eluted with a 2-L linear gradient of 0 - 1 M LiCl; column elution and collection of samples were carried out at 4°C. Inorganic phosphate eluted at 0.35 M LiCl while cis- or trans-epoxybutane-P_2 eluted at 0.44 M LiCl. Pooled fractions containing cis- or trans-epoxybutane-P_2 were concentrated at 27°C to 20 mL, and the phosphate ester was then precipitated by the addition of ethanol (200 mL). The precipitates were collected by centrifugation and washed twice with 200 mL of ethanol. After drying in vacuo, 0.39 g (78%) of both isomers was obtained. The compounds were pure as judged by paper chromatography (R_f 0.14) or by ion-exchange paper chromatography (R_f 0.53). Analysis of the trans isomer gave the following results:

^{1}H NMR (D_2O) δ 3.38 (m, 2H, oxirane), 3.72 (dd, J_{gem} = 12 Hz, J_{vic} = 6 Hz, 2H), 4.12 (dd, J_{vic} = 5.5 Hz, 2H). Anal. Calcd for $C_4H_6O_9P_2Li_4 \cdot 2H_2O$ (323.83): C, 14.83; H, 3.11; P, 19.13; Li, 8.57. Found: C, 15.04; H, 3.20; P, 18.98; Li, 8.44. Analysis of the cis isomer gave the following results: ^{1}H NMR (D_2O) δ 3.47 (m, 2H, oxirane), 3.74 (dd, J_{gem} = 12 Hz, J_{vic} = 7 Hz, 2H), 4.10 (dd, J_{vic} = 6 Hz, 2H). Anal. Calcd for $C_4H_6O_9P_2Li_4 \cdot 3H_2O$ (341.85): C, 14.05, H, 3.54; P, 18.12; Li, 8.12. Found: C, 14.19; H, 3.42; P, 18.32; Li, 7.98. The elemental analyses revealed the presence of < 2% of 1-g atom of chlorine in cis- or trans-epoxybutane-P_2, thereby demonstrating that insignificant opening of the epoxide ring by chloride ions had occurred. Treatment of cis- or trans-epoxybutane-P_2 (0.05 M) with alkaline phosphatase (0.83 mg/mL, 1.5 h) followed by thin-layer chromatography (cellulose with ethanol as solvent) revealed the presence of the nonphosphorylated epoxides (R_f 0.92) and none of their hydrolysis products (threitol and erythritol). Chromatographic markers for threitol and erythritol (R_f 0.81) were obtained by base (1 N KOH, 1 h, 100°C) or acid (1 N $HClO_4$, 1 h, 100°C) hydrolysis of the nonphosphorylated epoxides. These results further verify the presence of the oxirane in cis- and trans-epoxybutane-P_2 and suggest that little hydrolysis of the epoxide occurred during their preparation.

When dissolved in D_2O, cis- and trans-epoxybutane-P_2 have half lives of approximately 8.0 and 8.5 h at 24°C, respectively, as determined by the loss of the oxirane portion of their NMR spectra. After incubation of the compounds for 18 h at 24°C, only 21% of the cis

oxirane and 23 % of the trans oxirane remained (Figure 1, Appendix B); their respective NMR spectra substantially collapsed to multiplets centered at δ 3.86 and 3.91 (presumably threitol and erythritol bisphosphates). In contrast to the lack of stability of epoxybutane-P_2, the nonphosphorylated epoxides (cis or trans) were remarkably stable in solution. After thirteen days at room temperature, no changes could be detected in the NMR spectra of cis- or trans-2,3-epoxybutane-1,4-diol dissolved in D_2O.

The reactivity of epoxybutane-P_2 and the nonphosphorylated epoxides was determined by their reaction with the sulfhydryl group of glutathione. Phosphorylated or nonphosphorylated epoxides (0.1 mmol) were dissolved in 0.95 mL of 0.5 M Bicine/1 mM EDTA (pH 8.0); to the solution was added 0.05 mL of 0.1 M glutathione. Decrease in sulfhydryl concentration in reaction mixtures and controls (lacking epoxides) was monitored colorimetrically with DTNB (Ellman, 1959). Periodically, 0.05-mL aliquots of mixtures were added to cuvets containing 2.45 mL of 0.2 M potassium phosphate/0.4 mM DTNB (pH 8.0), and the subsequent increases in A at 412 nm were measured. By this criterion, second-order rate constants for the reaction of the epoxides with the free sulfhydryl group of glutathione were obtained. Cis- and trans-epoxybutane-P_2 gave values of 2.4×10^{-4} and 2.2×10^{-4} $M^{-1}s^{-1}$, respectively, compared with 7.2×10^{-5} $M^{-1}s^{-1}$ for the nonphosphorylated epoxides.

Reaction of the epoxides with glutathione was further characterized by inspection of products on the amino acid analyzer. Mixtures comprised of 0.4 M epoxide, 0.4 M glutathione, 1.6 M $KHCO_3$, and 1 mM EDTA (pH 8)

were incubated for 20 h; samples were diluted with 100 volumes of 0.066 M sodium citrate (pH 2.2), and 0.1-mL aliquots of the dilutions were chromatographed on the long column of a Beckman 120C amino acid analyzer. Prior to dilution of reaction mixtures of epoxybutane-P_2 with sodium citrate buffer, portions were digested with alkaline phosphatase (0.4 mg/mL) for 1.5 h; these digests were also inspected on the amino acid analyzer. Control samples contained a mixture of glutathione and oxidized glutathione which eluted from the amino acid analyzer at 39 and 46 min, respectively. Reaction mixtures of nonphosphorylated epoxides were devoid of glutathione and contained oxidized glutathione and a new ninhydrin-positive product which eluted from the analyzer at 23 min. Based on the ninhydrin color constant for oxidized glutathione, the latter product (presumably an S-alkylated glutathione) was formed in 72% yield. Reaction mixtures of cis- or trans-epoxybutane-P_2 also lacked glutathione and contained oxidized glutathione and an alkylation product (50% yield with the cis epoxide and 34% yield with the trans epoxide), which eluted at 14 min. The elevation of oxidized glutathione in reaction mixtures of epoxybutane-P_2 in comparison to controls or reaction mixtures of 2,3-epoxybutane-1,4-diol suggests that the phosphorylated epoxides increase the rate of oxidation of glutathione. Treatment of epoxybutane-P_2 reaction mixtures with alkaline phosphatase resulted in the disappearance of the compound that eluted from the analyzer at 14 min and the appearance of a corresponding amount of a new component at 23 min. The latter cochromatographed with the reaction product of glutathione and 2,3-epoxybutane-1,4-diol, thereby confirming

that epoxybutane-P_2 and the corresponding nonphosphorylated epoxides react analogously with glutathione.

Synthesis of N-Bromoacetyldiethanolamine Bisphosphate

A mixture of 3 mL (0.031 mol) of diethanolamine, 12 g (0.12 mol) of orthophosphoric acid, and 10 g (0.070 mol) of phosphorous pentoxide was heated at 70°C for 16 h in a desiccator. The resulting syrup was then dissolved in 500 mL of water and the solution placed in a boiling water bath for 3 h. After cooling, the solution was neutralized with a saturated solution of LiOH, and the precipitated lithium phosphate was removed by centrifugation. The supernatant was concentrated to dryness by rotary evaporation. A small volume of water (30 mL) was added to the residue and the insoluble lithium phosphate was removed by centrifugation. The supernatant was again concentrated to dryness by rotary evaporation, then washed five times with methanol and once with diethyl ether. After drying overnight in a vacuum desiccator, 7.48 g (85%) of crude diethanolamine bisphosphate was obtained. Paper chromatography revealed that diethanolamine bisphosphate (R_f 0.17) was contaminated with small amounts of inorganic phosphate (R_f 0.31) and two unidentified ninhydrin-positive components (R_f 0.11 and 0.53) one of which (R_f 0.11) was a phosphate ester. Diethanolamine bisphosphate was freed of contaminants by ion-exchange chromatography. A small portion of diethanolamine bisphosphate (0.55 g) was applied to a column (2.5 × 27 cm) of Dowex 1-X2 and eluted with a 2-L linear gradient of 0.05 to 0.5 M LiCl which contained 1 mM HCl (prior to being applied to the column, the diethanolamine bisphosphate solution was adjusted to pH 3 with AG 50WX-4

resin). Inorganic phosphate eluted at approximately 0.16 M LiCl well resolved from diethanolamine bisphosphate which eluted at 0.26 M LiCl. Fractions containing diethanolamine bisphosphate were neutralized, concentrated by rotary evaporation, and collected by centrifugation after precipitation with ethanol. After drying overnight in a desiccator in vacuo, 0.14 g (25%) of chromatographically pure diethanolamine bisphosphate was obtained. Anal. Calcd for $C_4H_{10}O_8P_2NLi_3 \cdot 2H_2O$ (318.92): C, 15.06; H, 4.42: P, 19.42; N, 4.39. Found: C, 15.20; H, 4.75; P, 19.55; N, 4.09.

Diethanolamine bisphosphate (3.11 g, 9.7 mmol) was dissolved in 10 mL of water, and 2.61 mL (30 mmol) of bromoacetyl bromide was added dropwise with vigorous stirring at ice temperature. The pH of the reaction mixture was maintained between 7 and 8 by the addition of 5 N LiOH. Following completion of the reaction (approximately 2 h) the solution was concentrated by rotary evaporation to less than 5 mL at 27°C and the product precipitated by the addition of 40 mL of ethanol. The precipitate was collected by centrifugation and washed three times with 40 mL of ethanol and twice with 40 mL of diethyl ether. After drying, the resultant precipitate gave 1.52 g (38%) of crude N-bromoacetyldiethanolamine bisphosphate. Inspection of the product by paper chromatography revealed substantial conversion of diethanolamine bisphosphate to a TNB-reactive phosphate ester (R_f 0.33) which was not detected by ninhydrin. Several phosphorylated products of lower mobility which were not TNB reactive were also observed [presumably polymerization products (Baker et al., 1966)]. The crude N-bromoacetyl-diethanolamine bisphosphate was estimated to be 58% pure by titration of

reactive halogen with glutathione (Hartman, 1975) and by determination of organic phosphate (Ames and Dubin, 1960). N-Bromoacetyldiethanolamine bisphosphate was purified by preparative paper chromatography (Whatman 3 MM) with n-butyl alcohol/acetic acid/water (7:2:5 v/v/v) as solvent.

Synthesis of N-Bromoacetylserine Phosphate

To 65.5 mg (0.35 mmol) of O-phospho-DL-serine in 0.85 mL of water were added 0.08 mL of triethylamine and 115 mg (0.49 mmol) of N-hydroxysuccinimide bromoacetate in 0.95 mL of tetrahydrofuran. The reaction mixture was incubated at room temperature for 15 min at which time 450 mg of $BaBr_2$ in 4 mL of methanol was added. The precipitate was collected by centrifugation and suspended in 1 mL water; 9 g of AG 50W-X4 resin (BioRad) in 5 mL of methanol were then added to the solution. The AG 50W-X4 resin was removed by filtration, and the pH of the filtrate was adjusted (on ice) to 7 with 1 N LiOH. A stream of nitrogen was then used to concentrate the sample (to less than 1 mL) followed by precipitation of the product by the addition of 7 mL of ethanol. The precipitate was washed twice with 5-mL portions of ethanol and dried in vacuo to give 24.6 mg (21%) of N-bromoacetylserine phosphate. Inspection of the product (R_f 0.45) by paper chromatography revealed the presence of a minor phosphoester component (R_f 0.40) which was not TNB reactive and did not contain a free amino group. The latter component was formed on neutralization of N-bromoacetylserine phosphate and could be avoided by the elimination of the step which converted N-bromoacetylserine phosphate to the lithium salt. O-Phosphoserine (R_f 0.30), which was a contaminant of the barium salt, could be removed

by the addition of excess AG 50W-X4 resin (hydrogen form) during conversion of N-bromoacetylserine phosphate to the free acid. When N-bromoacetylserine phosphate was tested as an affinity label, its barium salt was prepared. The barium salt of the reagent was washed twice with 4 mL of methanol, and converted to the free acid with 9 g of AG 50W-X4 resin in water.

Carboxylase and Oxygenase Assays

Carboxylase activity was determined at 25°C by the spectrophotometric method of Racker (1965) as modified by Norton et al. (1975). Oxygenase activity was measured at 30°C with an oxygen electrode by a slight modification of the method of Lorimer et al. (1975). This assay mixture consisted of 3 mL of air-saturated buffer containing 0.2 M diethanolamine (pH 9.3)/10 mM $MgCl_2$/0.1 mM EDTA/0.5 mM Rbl-P_2. Rbl-P_2 carboxylase concentrations were determined from A_{280nm}, assuming an $E^{1\%}_{1cm}$ of 16.4 (Wishnick and Lane, 1971).

Amino Acid Analyses

Total acid hydrolysis of protein or peptides was achieved in an evacuated (< 50 μm Hg) sealed tube with 6 N HCl/0.1 M 2-mercaptoethanol at 110° for 21 h. Samples were chromatographed on a Beckman 120C amino acid analyzer according to Spackman et al. (1958) or on a Beckman 121M amino acid analyzer using Beckman's "3-hour-single-column" system.

Sequence Analyses

Automated Edman degradation of peptides was carried out on a Beckman Model 890C Sequencer with Beckman peptide program #102974.

Amino acid anilinothiazolinones obtained in Edman degradation of peptides were identified indirectly as free amino acids after hydrolysis with $HCl/SnCl_2$ (Mendez and Lai, 1975) or with base (Smithies et al., 1971). Treatment of peptides with a mixture of carboxypeptidase A and B was carried out at room temperature. The digestion mixture consisted of 10 µg of carboxypeptidase A, 20 µg of carboxypeptidase B, and 50 nmol of peptide in 1.0 mL of 50 mM N-ethylmorpholine-HCl (pH 8.0). Periodically 0.1-mL aliquots of the digestion mixture were withdrawn and added to 0.1 mL of ice-cold 0.066 M sodium citrate-HCl (pH 2.2). These aliquots were then subjected to amino acid analysis.

Radioactivity

Radioactivity was measured with a Packard 3003 liquid scintillation spectrometer. Aliquots (5 to 50 µL) of samples were added to 10 mL of the Triton X-100/toluene (1:2 v/v) mixture of Patterson and Greene (1965). The mixture contained 5.5 g/liter of Permablend I (Packard) as scintillant. In those cases in which radioactivity was monitored in the effluent from the Beckman 120C amino acid analyzer, 2-min fractions (2.33 mL) were collected and mixed with 20 mL of scintillation mixture. The analyzer's ninhydrin system was not used in these experiments, since loss of ^{14}C-label from ε-[^{14}C]carboxymethyllysine results.

SDS-Gel Electrophoresis

Electrophoresis was carried out as described by Weber and Osborn (1969). Gels were frozen in powdered dry ice and sliced at 1-mm increments with a gel slicer (Mickle Laboratory Engineering Company). Solubilization of gel slices was achieved by an overnight incubation of

each slice with 0.1 mL of 30% hydrogen peroxide at 40°C. After the addition of 1 mL of water, the samples were assayed for radioactivity in 10 mL of scintillation mixture.

Treatment of Rbl-P_2 Carboxylase by BrAcNHEtOP

The enzyme (5 to 10 mg/mL) was modified at 30°C in 66 mM $NaHCO_3$/50 mM Bicine (pH 8.0) with or without $MgCl_2$ (5 mM). The buffer had been freed of trace metal contamination by passage through a column (1 × 25 cm) of Chelex 100 (BioRad). In the absence of Mg^{2+}, [^{14}C]BrAcNHEtOP (425 cpm/nmol) was used at 5 mM, and Rbl-P_2 (1 mM) served as protector. In the presence of Mg^{2+}, the reagent concentration was 1 mM, and the competitive inhibitor butanediol-P_2 (Schloss and Hartman, 1977b) at 20 mM or the transition-state analog CR-P_2 (Siegel and Lane, 1972) at 0.2 mM were used as protectors. Rbl-P_2 was not tested as a protector, since it would rapidly be converted to 3-phosphoglycerate with CO_2 and Mg^{2+} in the reaction mixture.

Periodically, aliquots were removed from the reaction mixtures and were assayed for carboxylase activity and for incorporation of radioactivity. The latter was determined by a filter-paper-disk method similar to that described by Bollum (1968). The aliquots (10 μL) were applied to 2.3-cm Whatman 3 MM filter-paper disks. The disks were immediately placed into an ice-cold solution of 10% (w/v) trichloroacetic acid, where they could remain for at least 24 h without changes in the incorporation data subsequently obtained. The disks were then washed twice with 500-mL portions of 5% trichloroacetic acid, twice with 500-mL portions of ethanol/diethyl ether (1:1 v/v), and twice with 500-mL

portions of diethyl ether. Finally, they were dried and counted in 10 mL of scintillation mixture. Only protein-bound [^{14}C]BrAcNHEtOP, because of its acid insolubility, remains associated with the paper disk.

For samples lacking Mg^{2+}, incubations were continued for 47 h at which time 4.4% of the initial enzymic activity remained in the unprotected sample; the corresponding sample containing Rbl-P_2 retained 97% of its activity. For samples containing Mg^{2+}, incubations were for 33 h, at which time 12% of the enzymic activity remained; the corresponding sample containing butanediol-P_2 retained 72% of its activity. Enzymic activity in the sample containing CR-P_2 could not be monitored due to the inactive complex formed between the transition-state analog and enzyme (Siegel and Lane, 1972; Ryan and Tolbert, 1975). At these times, reactions were terminated by the addition of 2-mercaptoethanol (10 mM) followed by the initiation of exhaustive dialysis against 50 mM potassium phosphate/10 mM 2-mercaptoethanol (pH 7.6).

Preparation of Tryptic Digests

The dialyzed samples of derivatized enzyme were made 0.12 M in sodium iodoacetate by the addition of 1 M iodoacetic acid/1.2 M $NaHCO_3$. Immediately afterwards, an equal volume of 8 M guanidine-HCl/0.1 M potassium phosphate/3 mM EDTA (pH 8.0) was added. After incubation of the samples in the dark for 20 min at 4°C, they were brought to 0.1 M 2-mercaptoethanol and dialyzed exhaustively against 50 mM ammonium bicarbonate. Following dialysis, trypsin (1% by weight of total protein) was added to the samples and digestion was carried out for 12 h at 40°C. A second addition of trypsin was then made, and digestion was allowed to proceed for 12 h more.

CHAPTER III

RESULTS

Effect of BrAcNHEtOP and Other Reagents on Rbl-P_2 Carboxylase

Of the reagents examined as potential affinity labeling reagents only BrAcNHEtOP proved to inactivate the carboxylase at a sufficiently rapid rate to warrant futher investigation. Epoxybutane-P_2 (either isomer), phosphoglycolic acid azide, and glycidol phosphate (2 - 10 mM) did not appear to inactivate the enzyme even upon extended incubation (1 - 20 h). Although N-bromoacetylserine phosphate and N-bromoacetyldiethanolamine bisphosphate inactivated the carboxylase they did so at approximately one-eighth and one-fourth of the rate obtained with BrAcNHEtOP, respectively. The synthetic routes for cis- and trans-epoxybutane-P_2, N-bromoacetylserine phosphate, and N-bromoacetyldiethanolamine bisphosphate are summarized in Figure 2, Appendix B.

Incubation of Rbl-P_2 carboxylase with BrAcNHEtOP results in a parallel loss of carboxylase and oxygenase activities in a pseudo-first-order fashion (Figure 3, Appendix B). Inactivation is irreversible, as removal of excess reagent by exhaustive dialysis does not restore enzymic activity. Rbl-P_2 and butanediol-P_2, a competitive inhibitor (Schloss and Hartman, 1977b), afford protection against inactivation; Mg^{2+}, an essential cofactor, stimulates the rate of inactivation.

Plots of the inactivation half-time (τ) against the reciprocal of reagent concentration (Figure 4, Appendix B) show rate saturation, indicative of reversible complex formation between enzyme and reagent prior to covalent modification (Meloche, 1967). The maximal rate of inactivation (1/T) has a half-time of 138 min, but this value is reduced to 24 min by the inclusion of 5 m$\underline{M}$ $MgCl_2$ in the reaction mixture. The reagent concentration that gives one-half the maximal rate of inactivation ($\underline{K}_{inact}$), i.e., the apparent dissociation constant for the reagent-enzyme complex, is 0.8 m$\underline{M}$ in the absence of Mg^{2+} and 3.0 m$\underline{M}$ in its presence. Bicarbonate (66 m$\underline{M}$) does not alter 1/T but increases $\underline{K}_{inact}$ to 2.9 m$\underline{M}$ and 11 m$\underline{M}$ without and with Mg^{2+}, respectively. The apparent effect of bicarbonate on the affinity of the enzyme for reagent was not due to CO_2 but an ionic strength effect, as an equivalent amount of NaCl had the same effect on $\underline{K}_{inact}$.

Relationship between Inactivation and Incorporation

Total incorporation found in the carboxylase samples after dialysis at the termination of incubation with [^{14}C]BrAcNHEtOP either in the presence or absence of Mg^{2+} (see Chapter II) is 1.2 and 2.7 moles of reagent per mole of protomer (70,000 g), respectively. The samples treated with reagent in the presence of butanediol-P_2, CR-P_2, or Rbl-P_2 contain 0.5, 0.3, and 0.9 moles of reagent, respectively. Thus, inactivation appears to correlate with the alkylation of 0.7 to 0.9 residue (1.2 minus 0.5 or 0.3) in the presence of Mg^{2+} and 1.8 residues (2.7 minus 0.9) in the absence of Mg^{2+}. If a correction is made for the

level of enzymic activity remaining in the unprotected sample with Mg^{2+} (12%) and for the loss of enzymic activity that occurred in the butanediol-P_2-protected sample (72% of the initial activity was retained), the apparent protection of 0.7 residue becomes 1.2.

Based on time-course data (see Chapter II), loss of enzymic activity is directly proportional to the extent of incorporation (expressed as the difference in incorporation between unprotected and protected samples) (Figure 5, Appendix B). Extrapolation of these data to complete inactivation gives values of 1.8 moles of reagent per mole of protomer in the absence of Mg^{2+} and 1.2 moles of reagent in the presence of Mg^{2+}, values that agree rather well with the end-point values obtained from the dialyzed samples (see preceding paragraph).

Identification of Modified Amino Acid Residues

The kinds of amino acid residues derivatized by [^{14}C]BrAcNHEtOP were determined by analyzing acid hydrolysates of the modified enzyme on an amino acid analyzer. Since the reagent is an N-substituted carboxamidomethyl compound, all of the derivatized residues appear in the hydrolysates as carboxymethyl amino acids, whose elution positions from the analyzer are well documented (Gurd, 1972; Goren et al., 1968). Cys(Cm) is the major derivative found in hydrolysates of enzyme after modification in the absence of Mg^{2+} (Figure 6A, Appendix B). The corresponding substrate-protected sample contains 1.0 fewer residues of Cys(Cm) per protomer. Cys(Cm) and Lys(Cm) are the major radioactive products found in hydrolysates of carboxylase after modification in the presence of Mg^{2+} (Figure 6B, Appendix B). In this case, butanediol-P_2

(which prevents inactivation) affords only slight protection of cysteinyl residues (0.2 per protomer), but essentially complete protection of lysyl residues (0.9 per protomer). Thus, without the inclusion of Mg^{2+} during the modification of enzyme, loss of enzymic activity correlates with alkylation of one SH group per protomer; whereas, with Mg^{2+}, inactivation is associated with alkylation of about one lysyl residue.

Purification of Peptides Unique to Inactivated Enzyme

Tryptic digests (see Chapter II) were brought to 0.1 M formic acid/0.25% (v/v) thiodiglycol and were then heated at 60°C for 2 h to degas. The sample (20 mg) was then subjected to ion-exchange chromatography on BioRad Aminex AG 50 W-X2 resin at 50°C. Two major radioactive peptides (designated C1 and C2, which on the basis of radioactivity represent 0.75 and 0.46 moles of peptide per mole of inactive protomer, respectively) are resolved from tryptic digests of enzyme inactivated in the absence of Mg^{2+}; these peptides are present in much smaller amounts in digests of substrate-protected enzyme (Figure 7A, Appendix B). In contrast, enzyme inactivated in the presence of Mg^{2+} yields a single major radioactive tryptic peptide (designated L1, and representing 0.73 mol of peptide per mol of inactive protomer) that is greatly reduced in samples protected by butanediol-P_2 or CR-P_2 (Figure 7B, Appendix B).

To determine the type of residue modified in the labeled peptides, an aliquot of each peak was concentrated by rotary evaporation and subjected to total acid hydrolysis. Alkylated amino acid residues were

identified as ^{14}C-labeled carboxymethyl derivatives by their elution positions from the Beckman 120C amino acid analyzer; these identifications are indicated in Figure 7, Appendix B. Both of the two major substrate-protected radioactive peptides in Figure 7A, Appendix B (from enzyme inactivated in the absence of Mg^{2+}) are derivatized at a cysteinyl residue, while the major radioactive peptide in Figure 7B, Appendix B, (from enzyme inactivated in the presence of Mg^{2+}) is derivatized at a lysyl residue.

Successive Chromatography on DEAE-Cellulose and Phosphocellulose

Fractions from the AG 50 columns that contained peptides C1 and C2 (Figure 7A, Appendix B) and L1 (Figure 7B, Appendix B) were pooled separately, lyophilized to dryness, and subjected to chromatography on DEAE-cellulose followed by phosphocellulose (Figure 8, Appendix B). The overall recoveries from these two columns were 69, 75, and 63% for peptides C1, C2, and L1, respectively.

All three peptides appear pure as judged by the integral values found for most residues in total acid hydrolysates (Table 1, Appendix A). Their purity is confirmed by the detection of a single NH_2 terminus in each during Edman degradations (see next paragraph). As expected, hydrolysates of peptides C1 and C2 contain one radioactive Cys(Cm) residue, and the hydrolysate of peptide L1 contains one radioactive Lys(ε-Cm) residue. The latter also contains one Cys(Cm) residue; this, however, is not radioactive and is therefore not reagent-derived (preceding tryptic digestion, all free SH groups in the enzyme were

carboxymethylated with iodoacetate [see Chapter II]). Peptides C2 and L1 have identical compositions, with the exception of the residue that bears the reagent moiety.

Edman Degradations

The data, presented in Table 2, Appendix A, obtained from automated Edman degradations of the three peptides establish the following sequences (the asterisk indicates the modified residues):

L1 Tyr-Gly-Arg-Pro-Leu-Leu-Gly-Cys-Thr-Ile-Lys*-Pro-Lys

C2 Tyr-Gly-Arg-Pro-Leu-Leu-Gly-Cys*-Thr-Ile-Lys-Pro-Lys

C1 Trp-Ser-Pro-Glu-Leu-Ala-Ala-Ala-Cys*-Glu-Val-Trp-Lys

Peptide L1 was carried through only seven cycles, which suffices to demonstrate that the only difference between C2 and L1 is in the residue modified. Furthermore, these two peptides are identical in sequences to a tryptic peptide isolated earlier that contains one of the two lysyl residues alkylated by the affinity label Br-butanone-P_2 (Stringer and Hartman, 1978).

Carboxypeptidase Digestion of Peptides C1, C2, and L1

As anticipated, only lysine (one residue equivalent) was liberated from L1 and C2 upon digestion with a mixture of carboxypeptidases A and B, consistent with the presence of penultimate prolyl residues as deduced from the Edman degradation studies.

Peptide C1 was extensively degraded by carboxypeptidase; digestion again stopped at a prolyl residue (in this case at position 3). The rate of release of free amino acids in consistent with the above sequence

(Figure 9, Appendix B). The alkylated cysteinyl residue eluted at the position of cysteic acid (confirmed with a standard obtained from the reaction of cysteine with BrAcNHEtOP). This position was obscured in analyses of aliquots taken at the early time-points (presumably by undigested or partially digested peptide), but the cysteinyl derivative was observed in the 24- and 48-h samples.

Subunit Location of Peptide C1

To determine whether peptide C1 is derived from the large or small subunit, enzyme which had been inactivated in the absence of Mg^{2+} by [^{14}C]BrAcNHEtOP was subjected to SDS-polyacrylamide gel electrophoresis. The distributions of radioactivity on the gels of inactivated enzyme and from a corresponding substrate-protected sample are shown in Figure 10, Appendix B. The majority of the radioactivity, and virtually all of the difference in radioactivity between inactivated and substrate-protected enzyme, appears at the position of the large subunit. This observation is consistent with the cysteinyl residues of both peptides C1 and C2 occupying positions within the large subunit. The subunit location of C2 is established independently by the previous study with Br-butanone-P_2 in which the peptide identical in sequence to that of C2 but derivatized at the internal lysyl residue was found to be a part of the large subunit (Norton et al., 1975; Stringer and Hartman, 1978). Since catalytic activity is an inherent property of the large subunit (Nishimura and Akazawa, 1973), any residue thought to be associated with the active site should be in the large subunit.

CHAPTER IV

DISCUSSION

The data obtained are consistent with the inactivation of Rbl-P_2 carboxylase by BrAcNHEtOP being an active-site-directed process. Loss of enzymic activity is pseudo-first-order, and exhibits a rate saturation with respect to concentration of reagent. The substrate Rbl-P_2 and the competitive inhibitor butanediol-P_2 protect against inactivation. With Mg^{2+}/CO_2 included in the modification reaction mixture (enzymically active conformer), inactivation of the enzyme by BrAcNHEtOP appears to be a direct consequence of a rather selective modification of one lysyl residue per protomeric unit [out of a total incorporation of 1.2 moles of reagent per mol of catalytic subunit, > 0.7 moles of a tryptic peptide containing the labeled lysyl residue was eluted from a cation exchange column (see Chapter III and Figure 7, Appendix B)]. Correlation between extent of inactivation and modification (Figure 5, Appendix B) excludes the possibility of a rapid chemical event followed by a rate-limiting conformational change with subsequent loss of enzymic activity. This correlation is important as it verifies that the rate saturation observed is due to reversible binding of the reagent prior to chemical modification. The clear demonstration of protection of this lysyl residue (at the level of tryptic profiles [Figure 7B, Appendix B]) by a competitive inhibitor and a transition-state analog is strongly suggestive of the lysyl residue occupying a position in the active-site region. The data do not

eliminate the possibility of indirect protection via a conformational change that renders the lysyl group inaccessible; however, the finding that inactivation exhibits rate saturation, thereby implicating specific binding of reagent as a prerequisite to alkylation, suggests that the observed protection reflects an affinity of substrate analogs and reagent for a common site.

Rbl-P_2 carboxylase is converted from an inactive conformer to a catalytically active conformer by CO_2 and Mg^{2+} in a time and pH dependent process (Lorimer et al., 1976; Badger and Lorimer, 1976; Laing and Christeller, 1976). That a conformational change accompanies activation by CO_2 and Mg^{2+} has been verified with fluorescent probes (Wildner, 1976; Vater et al., 1977). Consistent with the observed conformational change, specificity of BrAcNHEtOP is altered from a lysyl residue in the presence of CO_2/Mg^{2+} to two different cysteinyl residues in the absence of Mg^{2+}.

Given the likelihood that the lysyl group modified is within a binding domain for Rbl-P_2, a distinction is required between the catalytic site and the proposed allosteric site for Rbl-P_2 (Chu and Bassham, 1975). The presence of the latter site is inferred primarily from the knowledge that Rbl-P_2 binds tenaciously to the inactive conformer and inhibits activation by Mg^{2+}/CO_2 (Chu and Bassham, 1975; Laing and Christeller, 1976). These observations, however, do not rule out the existence of only a single site with nonproductive binding to the inactive conformer, as suggested by Laing and Christeller (1976) and Paech et al. (1977). In agreement with this possibility, is the

observation that in the absence of Mg^{2+} (inactive conformer) inactivation correlates with the modification of two cysteinyl residues, one of which is only three residues removed from the lysyl residue that is selectively modified in the presence of Mg^{2+} (see sequences of peptides L1 and C2 in Chapter III). Thus, it seems plausible that the conformational change induced by Mg^{2+} (Wildner, 1976; Vater et al., 1977) alters slightly the topology of a single binding site for Rbl-P_2 (or BrAcNHEtOP) so that in the reagent-enzyme complex formed from inactive conformer a sulfhydryl is accessible for alkylation, whereas in the reagent-enzyme complex formed from active conformer an ε-amino group is accessible.

Additional evidence that the selectively labeled lysyl residue is within the catalytic binding domain for Rbl-P_2 is that the same residue was implicated earlier as an active-site component with the affinity label Br-butanone-P_2 (Norton et al., 1975; Stringer and Hartman, 1978). Thus, two different reagents react preferentially with an identical ε-amino group, demonstrating that this region of the enzyme is a "hot spot" with respect to binding of phosphoesters. Since the latter reagent alkylates the lysyl residue in the absence of Mg^{2+}, the possibility is increased that the "allosteric site" for Rbl-P_2 is equivalent to, contiguous with, or overlaps the active site.

Essential lysyl and cysteinyl residues have also been implicated in the Rbl-P_2 carboxylase from the evolutionarily distant organism Rhodospirillum rubrum (see Part II; Whitman and Tabita, 1978a,b). Many studies with group-specific reagents (for review see Kawashima and Wildman, 1970; Takabe and Akazawa, 1975) have also suggested that

sulfhydryls of the carboxylase are important to the maintenance of native three-dimensional structure or are associated with the catalytic site in the Rbl-P_2 carboxylases from several organisms. Two different mechanisms involving a cysteinyl residue have been proposed. Bowes and Ogren (1972) suggested that a cysteinyl residue may abstract a proton at C-3 of Rbl-P_2 in the first step of formation of the enediol intermediate [first proposed by Calvin (1954)]. Condensation of a sulfhydryl with the C-2 carbonyl of Rbl-P_2 to form a thiohemiketal followed by dehydration to an enol was proposed by Rabin and Trown (1964a,b). Recent experiments by Lorimer (1978), however, exclude the mechanism of Rabin and Trown as the oxygen at C-2 is retained. A mechanism which is consistent with the data of Lorimer and consistent with the requirement for a nucleophilic attack on CO_2 by C-2 is a concerted Michael addition of a sulfhydryl on the proposed enediol intermediate at C-3, with concomitant attack on CO_2 by the electrons localized at C-2. The resultant complex would then cleave between C-3 and C-2 to give D-3-phosphoglycerate and its thioester with the enzyme (Pathway II, Figure 11, Appendix B), or CKR-P_2 would be released as an intermediate from the enzymic thiohemiketal (Pathway I, Figure 11, Appendix B).

Sjödin and Vestermark (1973) have demonstrated the presence of a phosphate ester with the expected properties of CKR-P_2 in enzymic reaction mixtures by chromatographic methods. This observation does not necessarily exclude the thioester pathway (Pathway II, Figure 11, Appendix B) as the free keto compound may have been formed nonenzymically from the thiohemiketal during chromatography.

Siegel and Lane (1973) synthesized CKR-P_2 by oxidation of CR-P_2 over platinum on carbon. They found that the β-keto acid hydrolyzed nonenzymically to give DL-3-phosphoglycerate and suggested that the enzyme directed the stereochemistry of hydrolysis. Tight binding of the β-keto intermediate (perhaps with the participation of a thiohemiketal) by the carboxylase may be involved in the production of stereochemically pure D-3-phosphoglycerate. Based on Cram's rule, Siegel and Lane (1973) pointed out that 2-carboxy-D-ribitol 1,5-bisphosphate (rather than 2-carboxy-D-arabinitol 1,5-bisphosphate) should be the predominant epimer of CR-P_2 formed in its cyanohydrin synthesis from Rbl-P_2, and thus be the epimer which resembles the transition state. If this epimer reflects the configuration at C-2 of the transition state, then cleavage between C-2 and C-3 would have to proceed with retention rather than inversion of configuration at C-2. The enzyme's stereochemical role could then be fulfilled by preventing access of H^+ to the S-forming face at C-2, forcing the proton to approach from the direction of C-3 (as shown in Pathway II, Figure 11, Appendix B).

The transition state intermediate should be bound more tightly than substrate, and transition state theory predicts that the magnitude of this difference should reflect the relative rates of the catalyzed reaction in the presence and absence of enzyme (Wolfenden, 1976). Since carboxylation of Rbl-P_2 does not occur at measurable rates in the absence of Rbl-P_2 carboxylase, the transition state (and its analogs) should have an affinity for the carboxylase considerably greater than Rbl-P_2. The fact that CR-P_2 inactivates the carboxylase in a time

dependent manner, and binds to the enzyme so tightly (noncovalently) that it cannot be removed by gel filtration or dialysis, lends credence to the identification of CR-P_2 as a transition state analog (Siegel and Lane, 1972). CR-P_2 is not the only compound which interacts with RBl-P_2 carboxylase in such a fashion. Xylitol 1,5-bisphosphate and xylulose 1,5-bisphosphate (epimeric with Rbl-P_2 at C-3) also inactivate the carboxylase in a time-dependent manner, although it has not been previously suggested that they are transition state analogs (Ryan et al., 1975; McCurry and Tolbert, 1977). Given the possibility that these compounds are transition state analogs, then perhaps the transition state is tetrahedral at C-3 with a hydroxyl group in a threo configuration relative to those at C-2 and C-4. Such a transition state could be the hydrated ketone form of CKR-P_2 or its thiohemiketal with the enzyme which was proposed earlier (Figure 11, Appendix B).

A lysyl residue could fulfill several potential catalytic roles. An obvious possibility would be as a positively charged site for Rbl-P_2 binding. The portion of enzymic primary structure contained in peptides C2 and L1 (see sequences in Chapter III) is unusually rich in basic residues. Since C2 and L1 are tryptic peptides, in the enzyme there is a lysyl or arginyl residue adjacent to Tyr-1. These peptides also contain an arginyl and two lysyl residues at positions 3, 11, and 13, respectively. The preponderance of basic residues seen in this region of the enzyme is consistent with its serving as a binding site for the tetraanion Rbl-P_2. If this region of the enzyme does serve as a binding site for Rbl-P_2, then the enzyme should be subject to inactivation by

arginyl modification (Arg-3). Modification of the Rbl-P_2 carboxylases from barley, Pseudomonas oxalaticus, tobacco, spinach, and Rhodospirillum rubrum with the group specific arginyl reagents 2,3-butanedione or phenylglyoxal does in fact result in their inactivation (Lawlis and McFadden, 1978; Chollet, 1978; Schloss et al., 1978). Arginyl residues are generally involved in binding anionic substrates (Riordan et al., 1977).

Another possible role for a lysyl residue would be as the base which abstracts the C-3 hydrogen of Rbl-P_2 (Figure 11, Appendix B). No evidence for or against this possibility exists.

Formation of a Schiff's base between a lysyl residue and carbonyls at C-2 (in Rbl-P_2) or C-3 (in CKR-P_2) could not be detected by borohydride reduction (Wishnick and Lane, 1969). More recent experiments with ^{18}O (Lorimer, 1978) have verified that both the C-2 and C-3 oxygens are retained, excluding the possibility of Schiff's base formation.

Based on the effect of pH on activation of Rbl-P_2 carboxylase by CO_2/Mg^{2+}, Lorimer et al. (1976) concluded that a basic residue, possibly a lysyl residue, was involved in activation of the enzyme. They further proposed a carbamate as a possible product of reaction between CO_2 and the lysyl residue proposed to be involved in the activation process. Some evidence for such a carbamate exists, as a CO_2/Mg^{2+} complex with the enzyme has been isolated by chromatography on Dowex 1 (Akoyunoglou and Calvin, 1963), and the carboxylated enzyme has been stabilized by esterification with diazomethane (Akoyunoglou et al., 1967). However,

it is clear that the lysyl residue modified by BrAcNHEtOP is not involved in activation of the carboxylase by CO_2. Not only does CO_2 not protect against BrAcNHEtOP inactivation of the spinach carboxylase, but CO_2 stimulates the rate of modification of the same lysyl residue by Br-butanone-P_2 (Hartman et al., 1973b; Hartman et al., 1978).

Pyridoxal phosphate also inactivates the spinach and R. rubrum Rbl-P_2 carboxylases by modification of lysyl residues (although evidence of lysyl modification has not been provided for the spinach enzyme) (Paech et al., 1977; Whitman and Tabita, 1978a). Inactivation of the R. rubrum carboxylase by pyridoxal phosphate exhibits rate saturation and therefore appears to be a consequence of affinity labeling. Paech et al., (1977) proposed that one of the two sites modified in the spinach enzyme by pyridoxal phosphate was the catalytic binding site for CO_2; the other site was assumed to be the allosteric site for CO_2 activation. This proposal is inconsistent with the lack of protection against inactivation of the spinach enzyme afforded by saturating levels of bicarbonate.

Whether the lysyl residue(s) modified by pyridoxal phosphate is identical to those modified by BrAcNHEtOP and Br-butanone-P_2 has not yet been determined. If the same lysyl residue is selectively modified by all three chemically distinct reagents, then further evidence for its essentiality will be obtained.

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APPENDIXES

APPENDIX A

TABLES

TABLE 1

AMINO ACID COMPOSITIONS OF PEPTIDES UNIQUE TO BrAcNHEtOP-INACTIVATED Rbl-P_2 CARBOXYLASE

Amino acid	Number of residues		
	C1	C2	L1
Lys	0.9	2.0	1.0
Lys(ε-Cm)			1.0[a]
Arg		1.1	1.0
Trp[b]	2.2		
Cys(Cm)	0.9[a]	1.0[a]	0.9
Thr		0.9	1.0
Ser	0.9		
Glu	2.1		
Pro	0.9	1.9	1.9
Gly		2.0	2.2
Ala	2.9		
Val	0.9		
Ile		1.0[c]	1.0[c]
Leu	1.0[c]	2.1	2.1
Tyr		0.9	1.0

[a]Based on assays for radioactivity in the effluent from the amino acid analyzer (see Chapter III) this residue is labeled and therefore bears the reagent moiety in the intact peptide.

[b]Trp was determined from the $\underline{A}_{280.8\ nm}$ using a molar ε of 5690 (Edelhoch, 1967).

[c]This amino acid was arbitrarily assigned a value of 1.0.

TABLE 2

EDMAN DEGRADATION OF LABELED PEPTIDES[a]

Cycle	Peptide C2		Peptide L1		Peptide C1	
	Amino acid	Yield (%)	Amino acid	Yield (%)	Amino acid	Yield (%)
1	Tyr	87	Tyr	94	Trp	75
2	Gly	78	Gly	78	Ser[b]	—
3	Arg[c]	44	Arg[c]	30	Pro	35
4	Pro	43	Pro	20	Glu[d]	25
5	Leu	29	Leu	18	Leu	20
6	Leu	33	Leu	23	Ala	17
7	Gly	22	Gly	18	Ala	16
8	Cys(Cm)[e]	3			Ala	14
9	Thr[f]	6			Cys(Cm)[e]	5
10	Ile	13			Glu[d]	5
11	Lys	5			Val	7
12	Pro	4			Trp	3
13	Lys	2			Lys	2

[a]Data presented have not been corrected for losses due to incomplete extraction of anilinothiazolinones from the reaction cup or their destruction during conversion to free amino acids.

[b]Identification of serine is based on thin-layer chromatography of the corresponding phenylthiohydantoin.

[c]Arginine is identified as ornithine in base hydrolysates.

[d]Glutamic acid is distinguished from glutamine by thin-layer chromatography of the corresponding phenylthiohydantoin.

[e]Detected only in acid hydrolysates.

[f]Identified as α-aminobutyric acid in acid hydrolysates.

APPENDIX B

FIGURE LEGENDS AND FIGURES

Figure 1. ^1H NMR spectra of cis- and trans-epoxybutane-P_2 and their decomposition products.

(A) trans-Epoxybutane-P_2 in D_2O relative to DSS: δ 3.38 (m, 2H, oxirane), 3.72 (dd, J_{gem} = 12 Hz, J_{vic} = 6 Hz, 2H), 4.12 (dd, J_{vic} = 5.5 Hz, 2H). Fine splitting of the C-1 and C-4 hydrogen signals by the distal oxirane hydrogens can be observed with coupling constants of 2 and 3 Hz. (B) Same sample shown in A after 18 h at 24°C. The spectrum has collapsed into a multiplet centered at δ 3.91 and the oxirane signal at δ 3.38 is greatly reduced. (C) cis-Epoxybutane-P_2 in D_2O relative to DSS: δ 3.47 (m, 2H, oxirane), 3.74 (dd, J_{gem} = 12 Hz, J_{vic} = 7 Hz, 2H), 4.10(dd, J_{vic} = 6 Hz, 2H). Fine splitting of the C-1 and C-4 hydrogen signals by the distal oxirane hydrogens can be observed with coupling constants of 2 and 3 Hz. (D) Same sample shown in C after 18 h at 24°C. The spectrum has collapsed into a multiplet centered at δ 3.86 and the oxirane signal at δ 3.47 is greatly reduced.

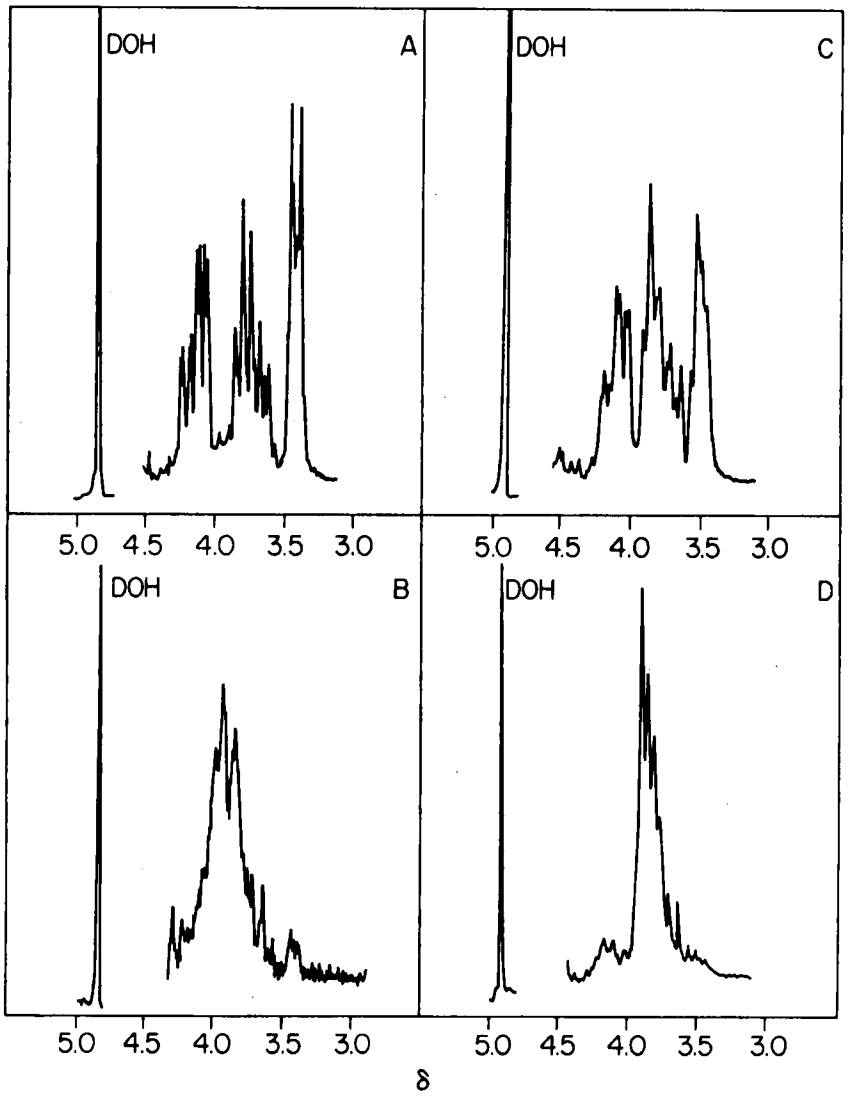
DOH
A
DOH
C
5.0 4.5 4.0 3.5 3.0
5.0 4.5 4.0 3.5 3.0
DOH
B
DOH
D
5.0 4.5 4.0 3.5 3.0
5.0 4.5 4.0 3.5 3.0
δ

Figure 1

Figure 2. The routes of synthesis of cis- and trans-2,3-epoxybutane-1,4-diol 1,4-bisphosphate, N-bromoacetyldiethanolamine bisphosphate, and N-bromoacetylserine phosphate.

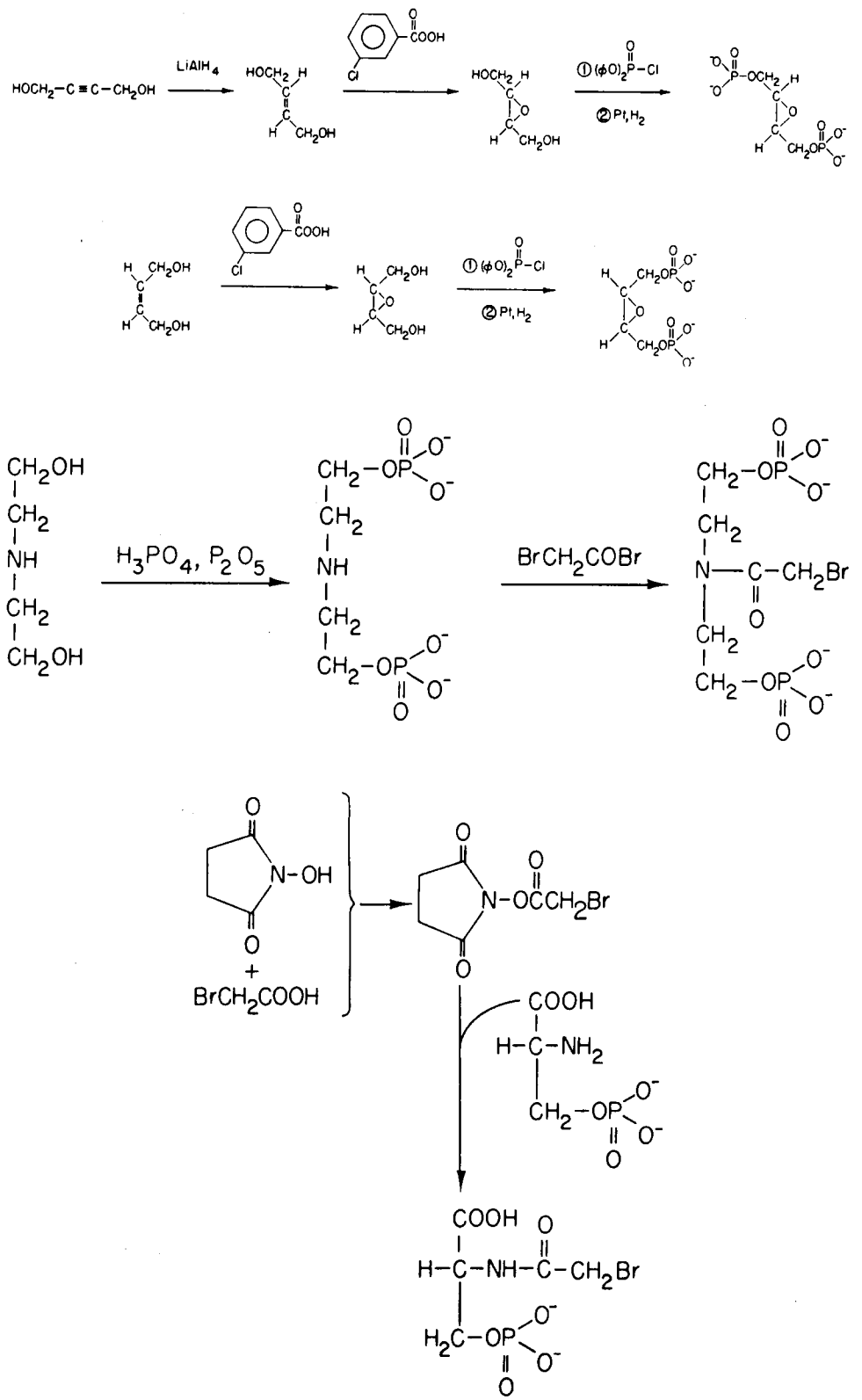

Figure 2

Figure 3. Time-course of inactivation of Rbl-P_2 carboxylase by BrAcNHEtOP.

Inactivation of carboxylase activity (30°C) by 10 mM (O), 5 mM (●), 2.5 mM (Δ), or 1 mM (▲) BrAcNHEtOP in 5 mM $MgCl_2$/50 mM Bicine (pH 8.0), and protection by 2.5 mM butanediol-P_2 in the presence of 1 mM BrAcNHEtOP (O). Inactivation of carboxylase (□) or oxygenase (■) activities (room temperature) by 5 mM BrAcNHEtOP in 50 mM Bicine (pH 8.0), and protection of carboxylase (◇) and oxygenase (◆) activities by 1 mM Rbl-P_2.

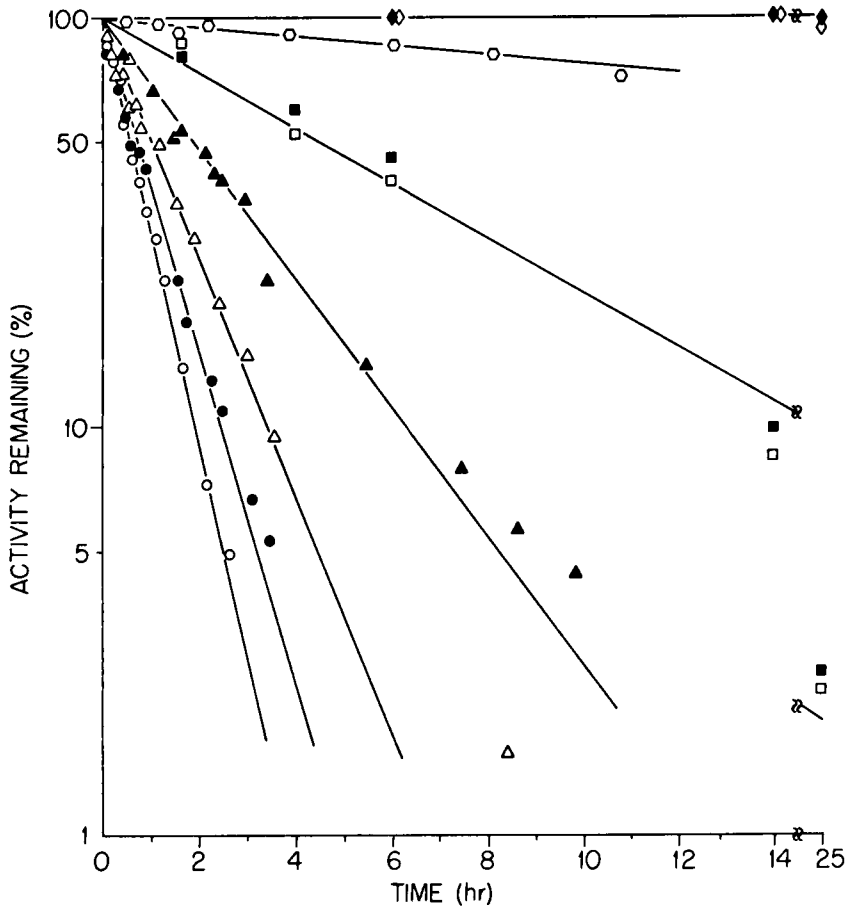

Figure 3

Figure 4. Inactivation half-time (τ) of Rbl-P_2 carboxylase as a function of the reciprocal of BrAcNHEtOP concentration.

The following conditions were used: 5 mM $MgCl_2$/50 mM Bicine (Δ); 5 mM $MgCl_2$/66 mM $NaHCO_3$/50 mM Bicine ($\blacktriangle$); Mg^{2+}-free 50 mM Bicine ($\circ$); Mg^{2+}-free 66 mM $NaHCO_3$/50 mM Bicine ($\bullet$). All buffers were pH 8.0.

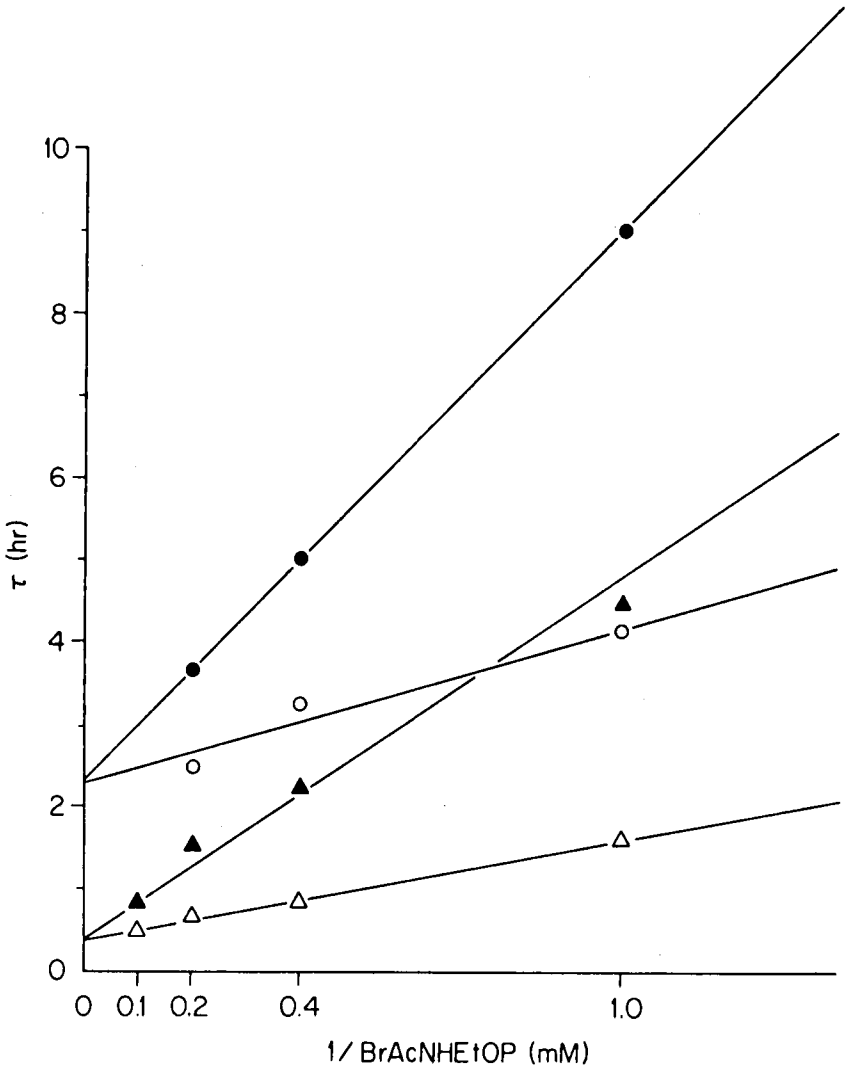

Figure 4

Figure 5. Incorporation of [^{14}C]BrAcNHEtOP as a function of enzymic activity lost.

Incorporation in the absence (□) and presence (O) of Mg^{2+}. Incorporation is expressed as the difference in incorporation between unprotected enzyme and protected enzyme (Rbl-P_2 is the protector without Mg^{2+} and butanediol-P_2 the protector with Mg^{2+}). Experimental details are given in Chapter II.

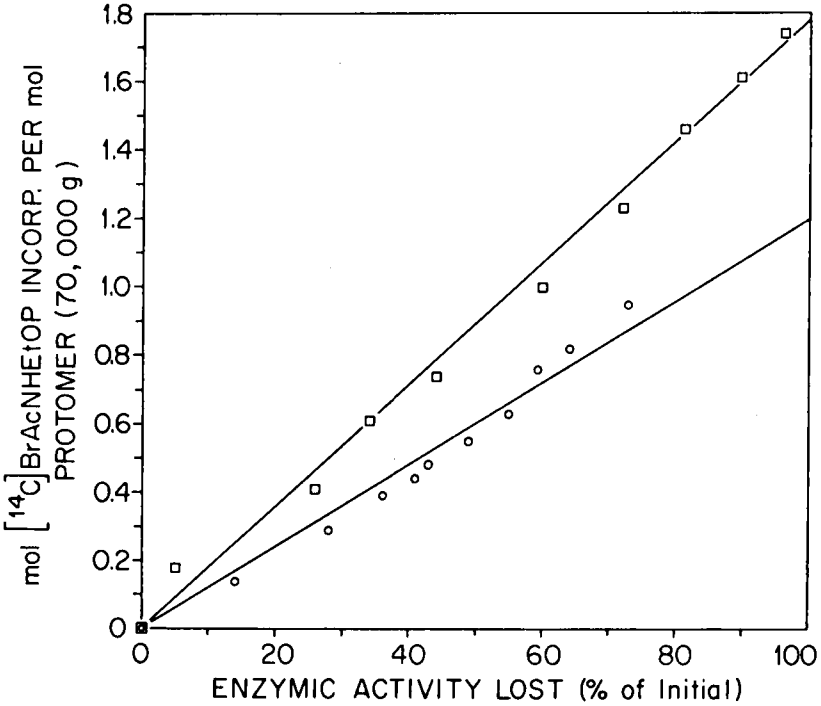
mol [14C]BrAcNHEtOP INCORP. PER mol PROTOMER (70,000 g)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
ENZYMIC ACTIVITY LOST (% of Initial)

Figure 5

Figure 6. Chromatographic profiles of hydrolysates of Rbl-P_2 carboxylase after modification by [^{14}C]BrAcNHEtOP.

The samples subjected to analysis are described in Chapter II. Chromatography was done on a Beckman 120C analyzer without use of its ninhydrin system; 2-min fractions were collected and counted. Arrows indicate the elution positions of several amino acids. (a) Modification in the absence of Mg^{2+} with (---) or without (——) protector (Rbl-P_2). (B) Modification in the presence of Mg^{2+} with (---) or without (——) protector (butanediol-P_2).

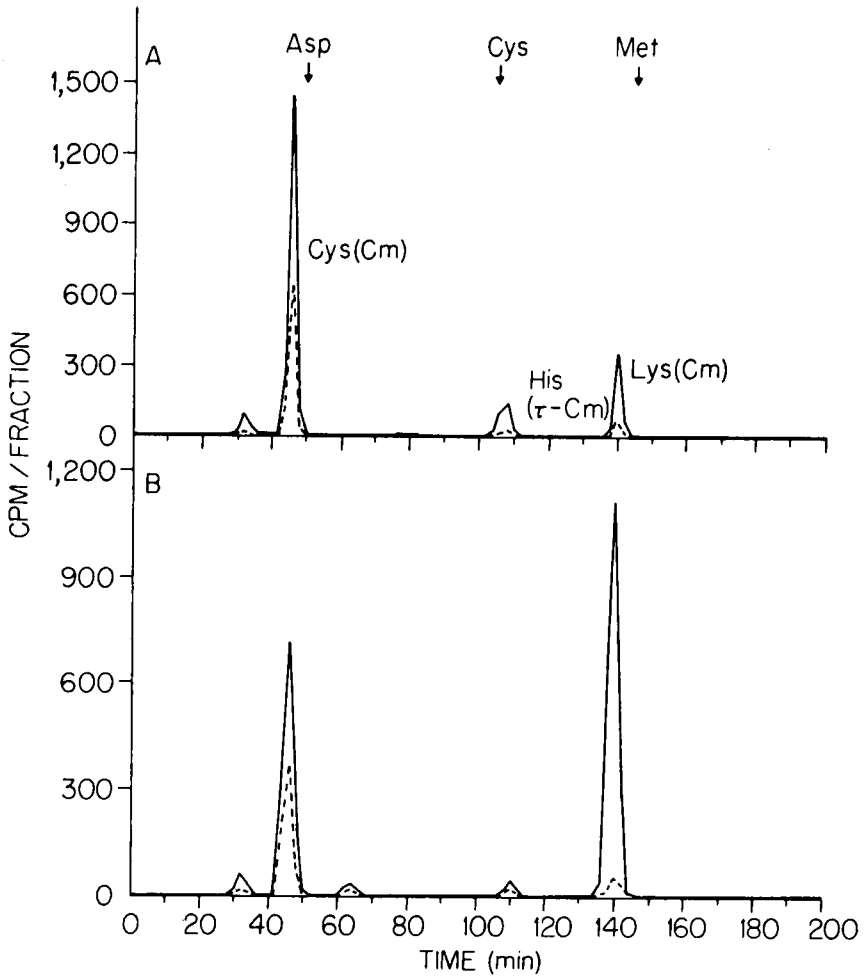
A
B
Asp
Cys
Met
Cys(Cm)
His
(τ-Cm)
Lys(Cm)
CPM / FRACTION
1,500
1,200
900
600
300
0
0
20
40
60
80
100
120
140
160
180
200
TIME (min)

Figure 6

Figure 7. Chromatography on BioRad AG 50 of tryptic digests of enzyme modified by [^{14}C]BrAcNHEtOP.

Preparation of samples is described in Chapter II. The sample (20 mg) was applied to a column (1 × 24 cm) equilibrated with 0.2 M pyridine/4.8 M acetic acid/0.25% thiodiglycol (pH 3.1). Elution was with a linear gradient composed of 200 mL of the equilibration buffer (initial) and 200 mL of 2 M pyridine/2.5 M acetic acid/0.25% thiodiglycol (pH 5.0) as the limit buffer. The kind of labeled carboxymethyl amino acid found in hydrolysates of each major radioactive peptide is indicated in the figure. (A) The enzyme was modified in the absence of Mg^{2+} with (---) or without (——) protector (1 mM Rbl-P_2). (B) The enzyme was modified in the presence of Mg^{2+} without protector (——), with 20 mM butanediol-P_2 (---), or with 0.2 mM CR-P_2 (....).

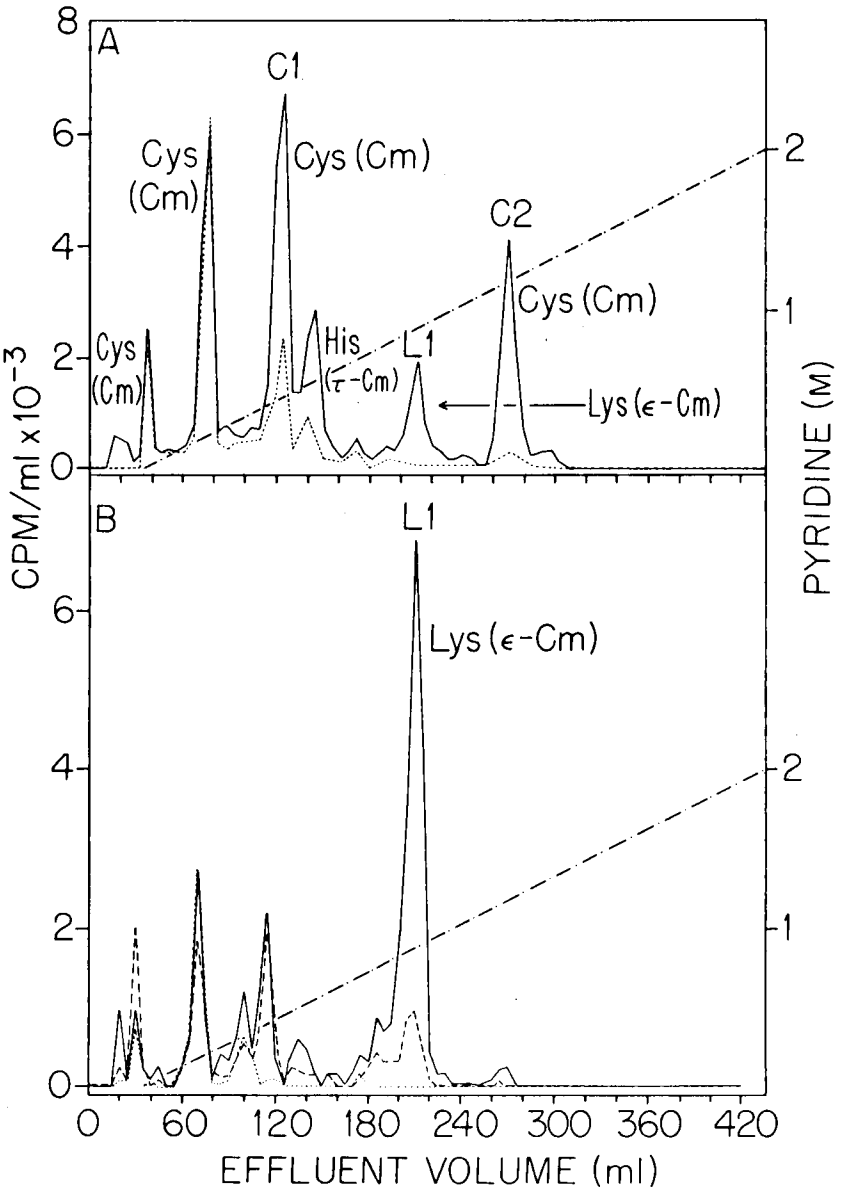
A
B
C1
C2
L1
Cys (Cm)
Cys (Cm)
Cys (Cm)
Cys (Cm)
His (τ-Cm)
Lys (ε-Cm)
Lys (ε-Cm)
CPM/ml x10⁻³
PYRIDINE (M)
EFFLUENT VOLUME (ml)
0
60
120
180
240
300
360
420
0
2
4
6
8
1
2

Figure 7

Figure 8. Purification of peptides C1, C2, and L1.

(A) Chromatography on DEAE-cellulose (Whatman DE 52). The column (1 × 25 cm) was equilibrated with 0.01 M ammonium bicarbonate (freshly prepared without adjustment of pH), and eluted with a linear gradient composed of 200 mL of the equilibration buffer (initial) and 200 mL of limit buffer. The limit buffers were 1 M ammonium bicarbonate for peptide C1, 0.07 M ammonium bicarbonate for peptide C2, and 0.3 M ammonium bicarbonate for peptide L1. All buffers contained 0.25% (v/v) thiodiglycol. (B) Chromatography on phosphocellulose (Whatman P-11). Peak fractions from the DE 52 column were pooled and lyophilized, and the residues were then dissolved in water and made 0.01 M ammonium formate (pH 4.0). The samples were chromatographed on a column (1 × 23 cm) equilibrated with 0.01 M ammonium formate (pH 4.0). In the cases of peptides C2 and L1, the column was eluted with a gradient consisting of 200 mL of equilibration buffer (initial) and 200 mL of 0.3 M ammonium formate (pH 4.0) for peptide C2, or 200 mL of 0.2 M ammonium formate (pH 4.0) for peptide L1, as the limit buffers. Peptide C1 was eluted from the column with the equilibration buffer. All buffers contained 0.25% (v/v) thiodiglycol.

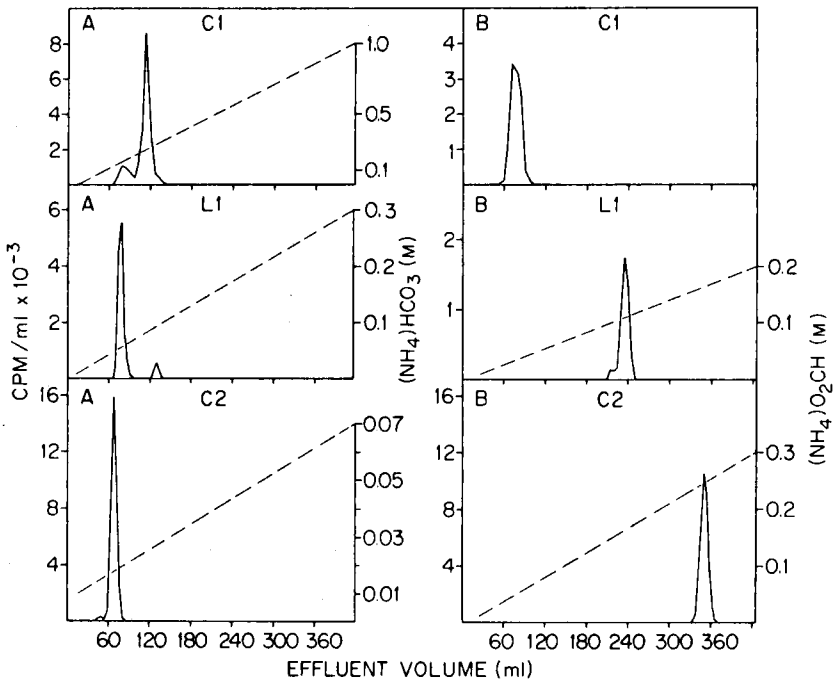
A
C1
B
C1
A
L1
B
L1
A
C2
B
C2
CPM/ml x 10⁻³
(NH4)HCO3 (M)
(NH4)O2CH (M)
60 120 180 240 300 360
EFFLUENT VOLUME (ml)

Figure 8

Figure 9. Release of amino acids from peptide C1 by digestion with a mixture of carboxypeptidases A and B.

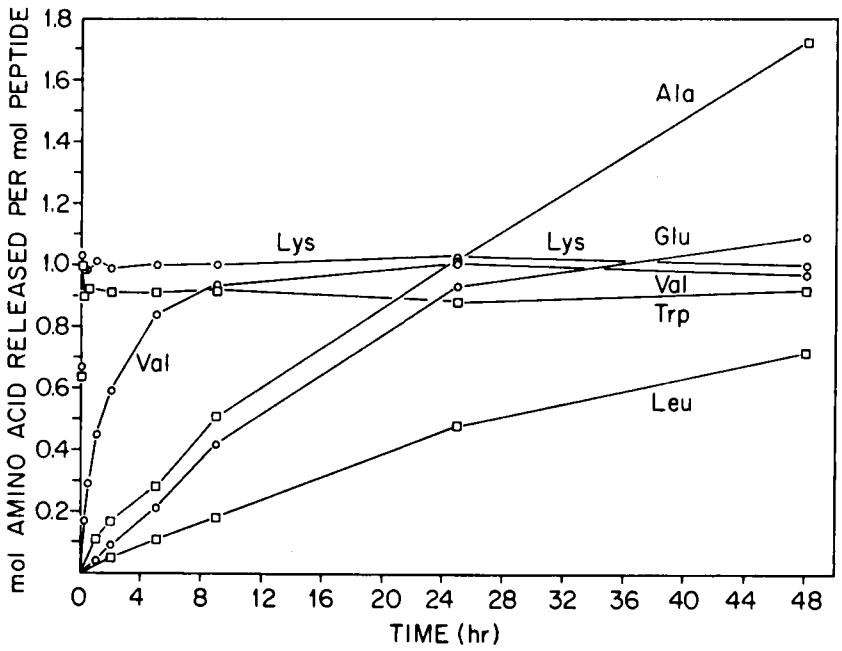
mol AMINO ACID RELEASED PER mol PEPTIDE
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
4
8
12
16
20
24
28
32
36
40
44
48
TIME (hr)
Ala
Lys
Lys
Glu
Val
Trp
Val
Leu

Figure 9

Figure 10. Distribution of radioactivity on SDS gels of enzyme after alkylation with [^{14}C]BrAcNHEtOP in the absence of Mg^{2+}.

Profiles are shown for enzyme modified (see Chapter II for conditions) in the presence (---) and absence (——) of 1 mM Rbl-P_2. Duplicate gels were stained with coomasie blue to locate the large and small subunits.

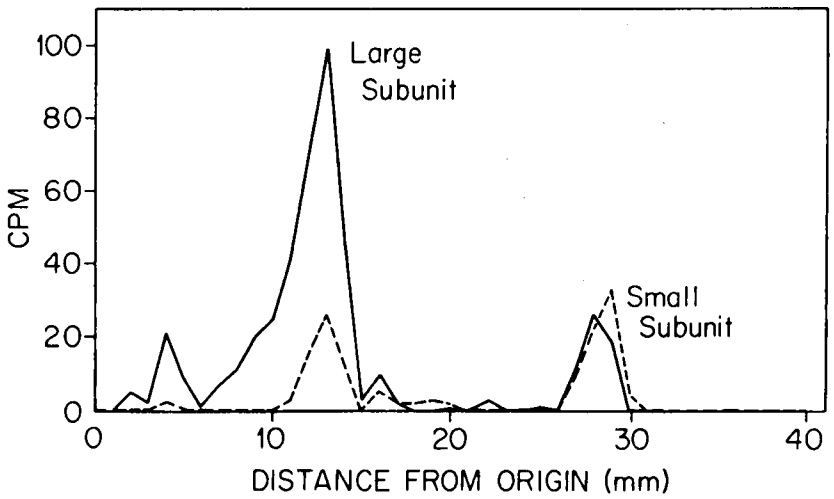
100
80
60
40
20
0
CPM
Large
Subunit
Small
Subunit
0
10
20
30
40
DISTANCE FROM ORIGIN (mm)

Figure 10

Figure 11. A proposed mechanism for Rbl-P_2 carboxylase.

In the figure Rbl-P_2 carboxylase is represented by lines (——) drawn to the right and left of chemical structures. The symbols, -B: and -SH, attached to these lines represent a basic residue and the sulfhydryl group of a cysteinyl residue, respectively. Arrows show the fate of electrons in each chemical structure during the following step. In the structure shown prior to Pathways I and II the fate of electrons in the first step of Pathway II is illustrated. Counter ions for the negatively charged Rbl-P_2 (presumably the tetraanion) are shown as two positively charged centers in the enzyme.

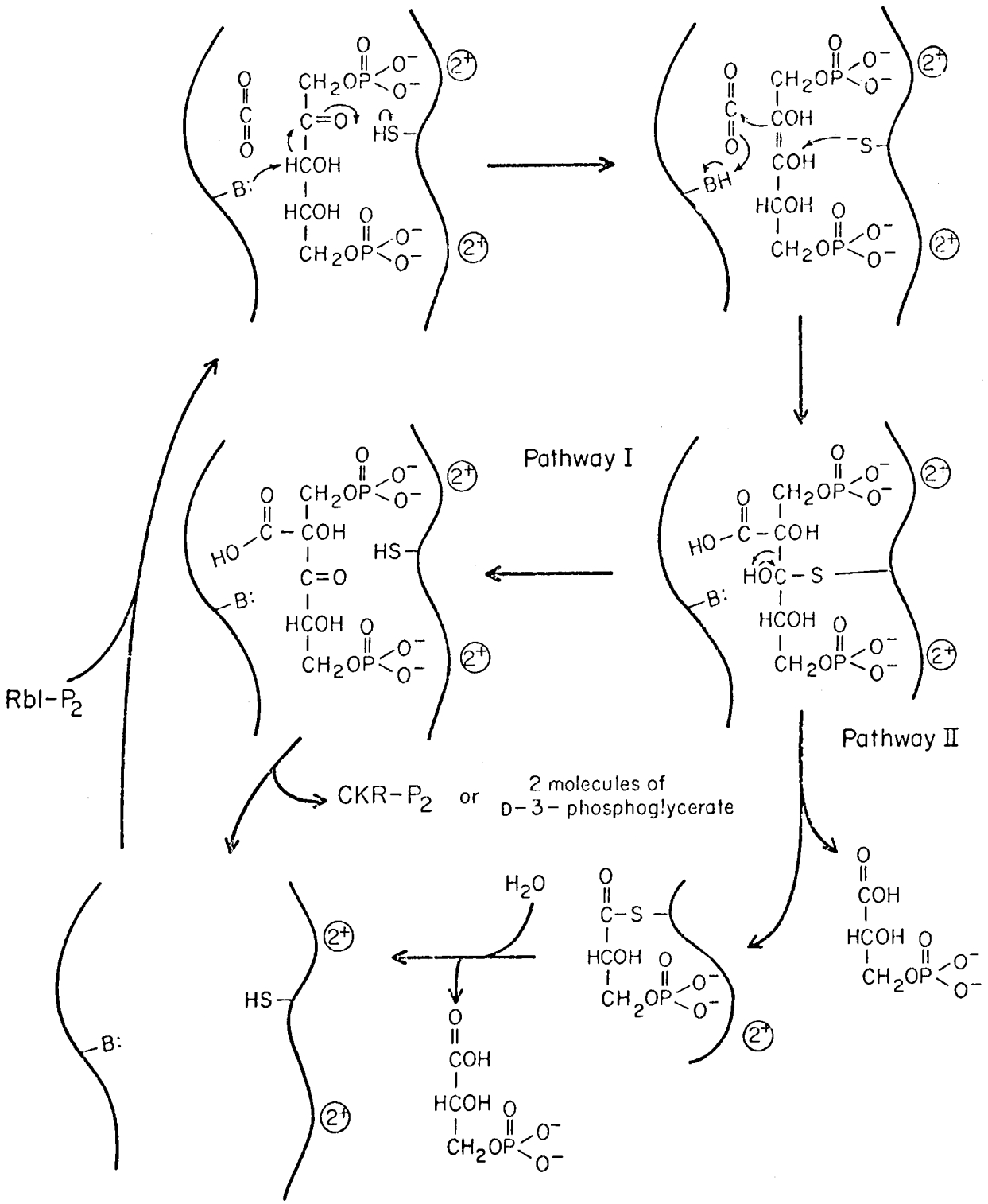
Pathway I
Pathway II
Rbl-P2
CKR-P2
or
2 molecules of
D-3-phosphoglycerate
H2O

Figure 11

PART II. RIBULOSEBISPHOSPHATE CARBOXYLASE FROM *RHODOSPIRILLUM RUBRUM*: OPTIMIZATION OF CULTURE CONDITIONS, CHARACTERIZATION, AND AFFINITY LABELING

CHAPTER I

INTRODUCTION

In determining the features of Rbl-P_2 carboxylase essential for catalysis, comparative studies between evolutionarily distant and structurally diverse Rbl-P_2 carboxylases should be helpful. The carboxylase from Rhodospirillum rubrum is especially noteworthy in this regard as it appears to have the simplest structure of any known Rbl-P_2 carboxylase (McFadden and Tabita, 1974). While the carboxylase from spinach (typical of the higher plant Rbl-P_2 carboxylases and one of the most thoroughly studied enzymes) has a molecular weight of 560,000 and is composed of eight 70,000-dalton protomers each consisting of one large (56,000 daltons) and one small (14,000 daltons) subunit (Rutner, 1970; Siegel et al., 1972), the carboxylase from R. rubrum is a simple dimer of 56,000-dalton subunits (Tabita and McFadden, 1974b). In addition to being useful in studies concerning structural-functional relationships because of its simpler structure, the R. rubrum enzyme may also aid in the determination of the role of the small subunit found in many prokaryotic and all eukaryotic Rbl-P_2 carboxylases (see Shively et al., 1978, and references cited therein). Some data suggest that the small subunit plays a regulatory role (Nishimura and Akazawa, 1974; Takabe and Akazawa, 1975).

Comparative studies between R. rubrum and spinach Rbl-P_2 carboxylases have been somewhat restricted by the limited availability of the R. rubrum enzyme. The carboxylase from higher plants comprises up to

50% of the soluble protein in green leaves (Kawashima and Wildman, 1970) and thus is probably the most abundant protein (certainly the most abundant enzyme) on earth. In R. rubrum the quantity of the carboxylase varies with the conditions of culture (Anderson and Fuller, 1967); higher levels of enzyme are obtained when cells are grown with CO_2 in H_2 (Anderson and Fuller, 1967, 1969), or more recently butyrate (Tabita and McFadden, 1974a), as the carbon source. Even under these culture conditions, only 6 to 8% of the soluble protein in R. rubrum is the carboxylase (Tabita and McFadden, 1974a).

Although the properties of the carboxylase from R. rubrum grown on butyrate have been examined in some detail (Tabita and McFadden, 1974a, b), information pertaining to the enzyme derived from autotrophically grown cells is more limited (Anderson and Fuller, 1969), primarily due to difficulties in obtaining sufficient quantities of the enzyme. While it may be assumed that the enzyme from cells grown under both sets of conditions is the same, several considerations seem to warrant a more detailed investigation: (i) The reported properties (e.g., the requirement for Mg^{2+}) of the carboxylase from autotrophically grown R. rubrum are not in complete agreement with those of the enzyme from cells grown on butyrate (Anderson and Fuller, 1969; Tabita and McFadden, 1974a). (ii) It has been reported that the closely related organisms Rhodopseudomonas sphaeroides and capsulata can produce two species of Rbl-P_2 carboxylase when grown on butyrate, one with and one without the small subunit (Gibson and Tabita, 1977a,b). (iii) The alga Chlamydomonas mundana can produce either one of two different forms of fructosebis-phosphate aldolase (Class I or II) depending upon the conditions

(autotrophic or heterotrophic) used to grow the cells (Russell and Gibbs, 1967).

In an effort to obtain sufficient quantities of the R. rubrum Rbl-P_2 carboxylase for affinity labeling studies, I have found that increased levels of the enzyme result when the cells are cultured serially on CO_2 and H_2. The levels of the enzyme attained (> 40% of the soluble protein) establish this convenient culture method as the procedure of choice to obtain large quantities of the R. rubrum carboxylase. Examination of various kinetic, chemical, and physical properties suggests that the enzyme from highly derepressed autotrophically grown R. rubrum is the same enzyme as that previously obtained from cells grown on butyrate (Tabita and McFadden, 1974a,b). Direct comparison of the R. rubrum carboxylase with the corresponding enzyme from spinach confirms and expands upon previously noted differences in the structure and properties of the bacterial and spinach carboxylases (McFadden and Tabita, 1974). Br-butanone-P_2 and BrAcNHEtOP, which are affinity labels for the spinach enzyme (Norton et al., 1975; Schloss and Hartman, 1977; Schloss et al., 1978a), inactivate the Rbl-P_2 carboxylase from R. rubrum primarily as a consequence of lysyl and cysteinyl modification, respectively, thus providing additional evidence that these two types of amino acid residues play an essential role in the carboxylase reaction mechanism. Unlike the spinach enzyme, Mg^{2+} does not alter the specificity of BrAcNHEtOP from cysteinyl to lysyl residues in its reaction with the R. rubrum carboxylase, presumably due to differences in the active sites of the spinach and R. rubrum carboxylases.

CHAPTER II

MATERIALS AND METHODS

Materials

Bicine, Tris, EDTA, 3-phosphoglycerate, 6-phosphogluconate, ribulose 5-phosphate, ribose 5-phosphate, SDS, and 2-mercaptoethanol were purchased from Sigma Chemical Company. Fructose 1,6-bisphosphate was purchased from Boehringer Mannheim. DEAE-cellulose (DE-52) was a product of Whatman Incorporated. DEAE-Sephadex (A-25) and Sephadex (G-25 and G-200) were products of Pharmacia. Chelex-100 was purchased from BioRad. [^{14}C]$NaHCO_3$ was obtained from New England Nuclear. Dibarium Rbl-P_2 was purchased from Sigma Chemical Company or prepared by the method of Horecker et al. (1958) and converted to the free acid by batchwise treatment with Dowex 50W-X4 (BioRad). 2-Carboxyribitol 1,5-bisphosphate, Br-butanone-P_2, BrAcNHEtOP, and [^{14}C]BrAcNHEtOP were synthesized by published procedures (Rabin et al., 1958; Hartman, 1975; Hartman et al., 1973). Chemicals used with the amino acid analyzer and sequencer were obtained from Pierce Chemical Company. Spinach Rbl-P_2 carboxylase was purified by a slight modification (Norton et al., 1975) of the method of Wishnick and Lane (1971).

Culture of R. rubrum

An initial culture of R. rubrum (strain S-1) was obtained from Dr. Gary A. Sojka of Indiana University. Cells were maintained heterotrophically on the synthetic malate medium of Ormerod et al.

(1961) with 0.1% $(NH_4)_2SO_4$ provided as the nitrogen source. The heterotrophically grown cells were used as an inoculum for 40- to 50-liter cultures that consisted of the Ormerod medium (without malate) and the carbon source as specified. A stock potassium phosphate solution (200X concentrate) was autoclaved separately from the other salts of the Ormerod medium and added to the remaining sterile medium components after cooling to avoid precipitation of insoluble phosphates. Cultures were maintained at pH 6.5 by the addition of sterile 10 N NaOH as needed. A New Brunswick MicroFerm Laboratory Fermentor was used to maintain the temperature of cultures between 28 and 30°C and to stir them continuously at 60 rpm. Two sides of the 30 × 30 × 60 cm glass carboys were lined with twelve 60-W Lumiline lamps (General Electric Company). One 200-W medium flood (Westinghouse) and two 150-W projector spot lights (Sylvania) were used to illuminate the remaining three sides (front, back, and bottom) of each carboy.

Assay of Carboxylase

Carboxylase activity was routinely determined by the spectrophotometric assay of Racker (1965) at 25°C as described in detail previously (Norton et al., 1975). A slight modification of the $[^{14}C]CO_2$-fixation assay of Lorimer et al. (1977) was used to determine the kinetic properties of the purified carboxylase. Each assay consisted of 25 μmol (25 μCi) $[^{14}C]NaHCO_3$, 5 μmol $MgCl_2$, 50 μmol Tris-HCl (pH 8.0), and 0.25 μmol Rbl-P_2 at 25°C in a total volume of 0.49 mL and was initiated by the addition of 0.01 mL of preactivated enzyme solution. Assays were conducted for 30 to 60 s, and the reaction

rates were observed to be linear for at least 90 s. Enzyme (1 mg/mL) was preincubated in 10 mM $MgCl_2$/66 mM $NaHCO_3$/50 mM Bicine-NaOH (pH 8.0). One unit of activity in both assays represents 1 μmol Rbl-P_2 consumed per min. Protein was determined by the method of Lowry et al. (1951) or by the biuret procedure (Lyane, 1957) with crystalline bovine serum albumin (Sigma) as standard. Purified carboxylase concentrations were determined from the $A_{280\ nm}$ value by assuming an $E_1^{1\%}{}_{cm}$ of 9.74 (Tabita and McFadden, 1974a) for the R. rubrum enzyme and 16.4 (Wishnick and Lane, 1971) for the spinach enzyme. Radioactivity was measured with a Packard 3003 liquid scintillation spectrometer by use of the Triton X-100/toluene (1:2 v/v) scintillation mixture of Patterson and Greene (1965). The mixture contained 5.5 g/liter of Permablend I (Packard) as scintillant.

Cell Density and Activity Determinations

Cell density was monitored by determining the optical density of cultures at 650 nm ($OD_{650\ nm}$) with a Zeiss spectrophotometer. One $OD_{650\ nm}$ was equivalent to 1.1 g of cells per liter (wet weight), and 1 g of cells (wet weight) contained 35 mg of soluble and 160 mg of total protein. A 10- to 40-mL aliquot of the culture was centrifuged for 10 min at 10,000 × g, and the cell pellet was resuspended in 2 mL of cold (4°C) 50 mM potassium phosphate/10 mM 2-mercaptoethanol/1 mM EDTA (pH 7.6). After two sonications (Biosonik, Blackstone Ultrasonics) for 45 s each, carboxylase activity in the crude homogenate was determined by the spectrophotometric assay.

Purification of R. rubrum Carboxylase

The cell paste of R. rubrum obtained by continuous-flow centrifugation with a Sharples (T-1) centrifuge was frozen in liquid nitrogen and stored at -60°C until needed. These cells could be stored indefinitely (> 1 year) with no loss of carboxylase activity and had the same activity as unfrozen cells. The R. rubrum enzyme was purified by a modification of the method of Tabita and McFadden (1974a). Frozen cell paste (60 g) was thawed on ice after the addition of 60 mL of 20 mM Tris-H_2SO_4/50 mM $NaHCO_3$/10 mM $MgCl_2$/5 mM 2-mercaptoethanol/1 mM EDTA (pH 8.0 at 25°C). The mixture was made 1 mM in phenylmethylsulfonyl-fluoride (Sigma), and the cells were broken by five 1-min periods of sonication. This and all subsequent purification steps were carried out at 0 to 4°C. The sonicate was then centrifuged at 15,000 × g for 2 h, and the supernatant was subjected to further centrifugation at 44,000 rpm in a Beckman 60 Ti rotor for 90 min. Solid ammonium sulfate (0.39 g/mL) was added to the supernatant from high-speed centrifugation, and the resulting precipitate was collected by centrifugation. After the precipitated enzyme was dissolved in the homogenization buffer (without phenylmethylsulfonylfluoride), it was passed through a G-25 Sephadex column (2.5 × 55 cm), then absorbed on a DEAE-cellulose column (2.5 × 55 cm). The effluents of the G-25 column and all subsequent columns were monitored with a Uvicord II UV absorptiometer (LKB) at 280 nm. The DEAE-cellulose column (previously equilibrated with the homogenization buffer) was eluted with the same buffer until no more UV-absorbing material appeared in the effluent (approximately 1 liter); elution was

then carried out with buffer containing 0.1 M NaCl. Fractions containing the carboxylase (which eluted after about 600 mL) were pooled and concentrated by pressure dialysis by use of a Diaflo UM-10 (Amicon Corporation) membrane filter. The concentrated enzyme was again desalted by gel filtration and adsorbed on a DEAE-Sephadex (A-25) column (2 × 75 cm) previously equilibrated with the same buffer. After the column was washed with 1 liter of buffer, the adsorbed enzyme was eluted with 0.1 M NaCl in buffer. Fractions containing the carboxylase were collected and concentrated as before.

Electrophoresis

Polyacrylamide gel electrophoresis was carried out on standard 7% gels by use of the analytical apparatus from Canal Industrial Corporation according to the manufacturer's instructions. Gels were stained with Amido-Schwarz (Canalco) and destained electrophoretically. SDS-polyacrylamide gel electrophoresis was performed as described by Weber and Osborn (1969). Protein was visualized by staining gels with coomasie blue (RDS-L, Canalco), and the glycoprotein stain of Segrest and Jackson (1972) was used to assess the presence of carbohydrates.

Amino Acid and NH_2-Terminal Analyses

Total acid hydrolysis of purified carboxylase was achieved in an evacuated (< 50 μm Hg) sealed tube with 6 N HCl/0.1 M 2-mercaptoethanol at 110°C for 21, 48, or 96 h. Losses of amino acids due to destruction during hydrolysis were corrected by comparisons with a standard amino acid mixture (Beckman) subjected to identical hydrolytic conditions.

Cysteine-cystine was determined as cysteic acid after hydrolysis of samples with HCl/dimethylsulfoxide (Spencer and Wold, 1969). Mercaptoethanesulfonic acid (3 N) was used to hydrolyze samples for tryptophan determination (Penke et al., 1974). All hydrolysates were analyzed with a Beckman 121M amino acid analyzer by use of the manufacturer's "3-hour-single-column" system.

When amino acid hydrolysates of radioactive samples were analyzed, hydrolysates were chromatographed on a Beckman 120C amino acid analyzer according to Spackman et al. (1958) and 2-min fractions from the long column or 1-min fractions from the short column were collected and counted.

Cysteine content in protein was determined prior to acid hydrolysis by titration of free sulfhydryl groups with Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid)] (Ellman, 1959). Aliquots of enzyme (10 mg/mL) were added to cuvets containing 0.2 mM 5.5'-dithiobis(2-nitrobenzoic acid)/8 M guanidine-HCl/0.1 M potassium phosphate/3 mM EDTA (pH 8.0). The increase in $A_{412\ nm}$ was complete within 30 s, and the sulfhydryl content was calculated by using an ε of 13,600 (Ellman, 1959).

After S-carboxymethylation with iodoacetate (as described in Part I, Chapter II), the purified enzyme was subjected to Edman degradation in a Beckman 890C sequencer according to the manufacturer's instructions. Anilinothiazolinones obtained in Edman degradation were identified indirectly as free amino acids after hydrolysis with HCl/$SnCl_2$ (Mendez and Lai, 1975) or with base (Smithies et al., 1971). Asn and Gln were distinguished from Asp and Glu by thin-layer chromatography (Summers

et al., 1973). Determination of Ser was based on thin-layer chromatography and verified by the presence of Ala in acid hydrolysates (Mendez and Lai, 1975).

CHAPTER III

RESULTS

Comparison of Culture Conditions

The time courses of cell density and carboxylase activity for R. rubrum cultured on malate, CO_2 in H_2, or butyrate are shown in Figure 1, Appendix B. R. rubrum grew considerably faster on malate, the carbon source normally used, with a generation time of approximately 0.5 day. When butyrate or CO_2 was used as the carbon source, the cells had a doubling time of about 2 days. Comparisons of total enzymic activity (Figure 1B, Appendix B) or activity per cell density (Figure 1C, Appendix B) revealed that cells grown on butyrate or CO_2 produced more carboxylase than those maintained on malate, as has been seen by other investigators (Anderson and Fuller, 1967; Slater and Morris, 1973; Tabita and McFadden, 1974a). The maximum specific activity was seen for autotrophically grown cells at about 3 days, in agreement with the observations of Slater and Morris (1973); later values, although somewhat lower, remained relatively constant. The enzymic activity of cells maintained on butyrate, in contrast to that of cells grown on CO_2 in H_2 or on malate, exhibited a lag of approximately 3 days before increasing to levels comparable to those seen in autotrophically grown cells.

Serial culture of R. rubrum on CO_2 in H_2, with 10% of each culture being used as inoculum for the next culture, resulted in substantially higher levels of carboxylase activity than a single culture initiated

with cells grown on malate (Figure 2, Appendix B). Purification of the carboxylase from R. rubrum exhibiting increased levels of enzymic activity (summarized in Table 1, Appendix A) revealed that a large fraction of the soluble protein was comprised of this enzyme. In Table 2, Appendix A, the specific activity of cell extracts obtained in the current study and values reported by previous investigators are compared. Extracts (after high-speed centrifugation) from the seventh successive autotrophic culture of R. rubrum contained 42% carboxylase, based on Lowry estimations of protein (biuret determinations gave 45%), compared with 6 to 8% obtained previously (Tabita and McFadden, 1974a). A photograph of polyacrylamide gels from electrophoresis of the purified carboxylase and high-speed supernatants of crude cell extracts is shown in Figure 3, Appendix B. Inspection of these gels corroborated that the carboxylase comprised a large fraction of the protein. This fraction (42-45%) was comparable to that seen in higher plants, in which up to 50% of the soluble leaf protein can be the carboxylase (Kawashima and Wildman, 1970).

Subunit Composition

The purified R. rubrum carboxylase (10 µg) comigrated on SDS-polyacrylamide gels with the large subunit of the spinach enzyme (10 µg), placing the subunit molecular weight of the enzyme from R. rubrum at 56,000 (Rutner, 1970; Siegel et al., 1972). In contrast to the spinach enzyme, no small subunit could be detected on SDS-polyacrylamide gels of the R. rubrum enzyme (200 µg).

Analytical ultracentrifugation of the R. rubrum carboxylase (1 mg/mL) in a Beckman Model E ultracentrifuge in 50 mM potassium phosphate/10 mM 2-mercaptoethanol/1 mM EDTA (pH 7.6) or the purification buffer with or without Mg^{2+} resulted in a single homogeneous sedimentation boundary. The sedimentation constant ($S_{20,w}$) of 6.2 was in agreement with that reported for the enzyme from R. rubrum grown on butyrate by Tabita and McFadden (1974a) or from less derepressed, autotrophically grown cells by Anderson and Fuller (1969). The common sedimentation constant seen for the enzyme from R. rubrum grown autotrophically or on butyrate indicated similar native molecular weights for both enzymes [determined to be 114,000 by light scattering for the carboxylase from cells grown on butyrate (Tabita and McFadden, 1974b)].

Kinetics and Inhibitors

Kinetic parameters of the purified R. rubrum carboxylase are shown in Table 3, Appendix A. Noteworthy is the rather high K_m for CO_2 of the enzyme (1.4 mM CO_2), which was about 50- to 100-fold higher than that reported for the spinach enzyme (Jensen and Bahr, 1977). Mg^{2+} was absolutely required for the catalytic activity of the R. rubrum enzyme, as < 0.002% activity could be detected in its absence (trace Mg^{2+} contamination was removed from the assay mixture with Chelex 100 in these experiments). Mg^{2+}-dependence of the enzyme from autotrophically grown R. rubrum was not seen by Anderson and Fuller (1969), in contrast to the observations of Tabita and McFadden (1974a) and Akazawa et al.

(1970) with the enzyme from cells grown on butyrate or grown autotrophically or on malate, respectively.

A comparison of several competitive inhibitors of the spinach and R. rubrum carboxylases (Table 4, Appendix A) revealed that the R. rubrum carboxylase was in general less sensitive to anions such as phosphate and sulfate than the spinach enzyme. The R. rubrum enzyme was about twenty times less sensitive to 6-phosphogluconate than the spinach enzyme, in agreement with previous reports that the spinach, but not the R. rubrum, carboxylase was sensitive to this inhibitor (Tabita and McFadden, 1972, 1974b). In contrast to the observations of Anderson and Fuller (1969), citrate and 3-phosphoglycerate were not potent inhibitors of the R. rubrum enzyme. Ryan et al. (1974) had questioned the rather low K_I (0.3 mM) for 3-phosphoglycerate reported by Anderson and Fuller (1969) based on the lack of inhibition of a partially purified enzyme preparation by 1 mM 3-phosphoglycerate. The transition-state analog 2-carboxyribitol 1,5-bisphosphate inactivated the purified R. rubrum carboxylase in a time- and Mg^{2+}-dependent fashion, as is the case with the spinach enzyme (Siegel and Lane, 1972). Whitman and Tabita (1978a) have reported a similar inhibition of the carboxylase from R. rubrum grown on butyrate, confirming that the presence of the small subunit is not required for tight binding of the transition-state analog.

Amino Acid Composition

Comparison of the amino acid compositions of the Rbl-P_2 carboxylases from R. rubrum cells grown autotrophically or on butyrate (Table 5, Appendix A) revealed that the values for most amino acids agree within

experimental error. A few exceptions were the residues serine, tyrosine, and phenylalanine, for which the values differed substantially (25 to 50%). My values for these and the other residues (Table 5, Appendix A) were in close agreement for two different enzyme preparations isolated by the procedure detailed in Chapter II and for enzyme prepared from less derepressed R. rubrum by a different isolation procedure (Schloss and Hartman, 1977). The values reported by Tabita and McFadden (1974a) were obtained by assuming "normal" hydrolytic losses and by extrapolating serine and threonine values to zero time; in contrast, I determined losses directly under the hydrolysis conditions used by including duplicate samples of standard amino acid mixtures. However, the rather small corrections made in all cases seem insufficient to explain the differences between the two sets of data. The discrepancies seen in free sulfhydryl content between the two enzymes (Table 5, Appendix A) could be due to the use of 8 M urea in the titration of the enzyme from cells grown on butyrate (Tabita and McFadden, 1974b) instead of 8 M guanidine-HCl as in the current study, or to the omission of 2-mercaptoethanol in the later stages of enzyme purification by Tabita and McFadden (1974a). The sulfhydryl content of the enzyme seen in the present study suggests that there are no disulfides. The overall agreement between the two sets of data suggests that the enzymes from heterotrophic and autotrophic R. rubrum are identical.

Edman Degradation

Prior to Edman degradation, the spinach and R. rubrum enzymes were S-carboxymethylated (as described in Part I, Chapter II) and subjected to chromatography on a G-200 Sephadex column (2 × 82 cm) equilibrated with 1% SDS/0.25% thiodiglycol. The effluent from the column was monitored at 280 nm, and the profiles obtained for the spinach and R. rubrum enzymes are shown in Figure 4, Appendix B. As expected, the subunits of the spinach enzyme were well resolved (Rutner, 1970), but no small subunit could be seen in the R. rubrum profile; this confirms the earlier results obtained with SDS-polyacrylamide gel electrophoresis. Chromatography of the R. rubrum carboxylase could be omitted with no effect on the results of subsequent Edman degradations. The pooled fractions from the Sephadex column were lyophilized to dryness, the lyophilized powder was dissolved in a small volume of water (2.5 mL), and the protein was precipitated by the addition of ethanol (7 mL). The precipitated protein was collected by centrifugation, and the pellet was redissolved in 0.5 mL water and reprecipitated by the addition of 5 mL ethanol. After collection by centrifugation, the final pellets were dried in vacuo overnight. Based on weight, 101, 93, and 78% of the spinach small subunit, large subunit, and R. rubrum subunit were recovered, respectively. These samples were dissolved in 50% heptafluorobutyric acid and subjected to automated Edman degradation with a Beckman 890C Sequencer. The results of these analyses are shown in Table 6, Appendix A.

No amino acid anilinothiazolinone could be detected in five successive degradative cycles of the spinach large subunit. The

presence of a blocked NH_2-terminus in the spinach large subunit, which may be deduced from the results of Edman degradation, is probably a property shared with the catalytic subunit of Rbl-P_2 carboxylase from other sources. The absence of a free NH_2-terminus has been reported for the large subunit of barley (Poulsen, 1978), Chlamydomonas reinhardtii (Iwanij et al., 1974), and spinach beet (Moon and Thompson, 1971) carboxylases; surprisingly, NH_2-terminal residues were not detected for the small subunits of the latter two enzymes (Iwanij et al., 1974; Moon and Thompson, 1971) as well. A cyanogen bromide-cleavage peptide from the barley enzyme large subunit containing the blocked NH_2-terminal residue has been partially purified (Poulsen, 1978), but as yet the nature of the block has not been elucidated.

A rather striking homology was seen between the sequences of the small subunits of spinach and tobacco (Gibbons et al., 1975; Iwai et al., 1976; Strøbaek et al., 1976). Of the 12 residues determined for the small subunit of spinach, 6 were the same as residues in corresponding positions of the small subunit of tobacco (Table 6, Appendix A). The homology seen between the spinach and tobacco small subunits agreed with the estimated differences (30 to 50%) based on amino acid composition comparisons ($S\Delta Q$ = 41) made by Takabe and Akazawa (1975).

In contrast to the inability to determine an NH_2-terminal residue in the large subunit of spinach, the R. rubrum carboxylase could readily be sequenced by Edman degradation (Table 6, Appendix A). An 82% recovery (after correction for routine losses seen with a myoglobin standard) of the first residue was obtained, suggesting that little or

none of the obstruction to Edman degradation seen with the large subunit of spinach was present. The data presented are the first NH_2-terminal sequence to be determined for the catalytic subunit of any Rbl-P_2 carboxylase. No homology is seen between the small subunit sequences and that of the R. rubrum enzyme (Table 6, Appendix A).

Crystallization

Purified R. rubrum carboxylase could be crystallized by dialysis against water at 4°C (Figure 5, Appendix B). Enzyme (1 mg/mL, 2 mL) in 50 mM potassium phosphate/10 mM 2-mercaptoethanol/1 mM EDTA (pH 7.6) was dialyzed for 12 h against 1 liter of cold glass-distilled deionized water, at which time the dialysis bath was changed. Within an additional 24 h of dialysis, crystals of the R. rubrum carboxylase were obtained (Figure 5, Appendix B). Although the crystals obtained by this procedure were too small for X-ray crystallographic analysis, the relative ease with which they could be attained encourages further crystallization trials. In this regard, it should be noted that the tobacco enzyme has been crystallized by a somewhat similar dialysis of the enzyme (in high-ionic-strength buffer) against low-ionic-strength buffer (Baker et al., 1977a; Chan et al., 1972; Kawashima and Wildman, 1971), with crystal size highly dependent on pH, ionic strength, and protein concentration. Thus optimization of crystallization conditions for the R. rubrum enzyme may result in crystals of a size suitable for X-ray crystallography. Because of the less complex quaternary structure of the R. rubrum enzyme compared with that of the tobacco enzyme, the former appears to be more amenable to X-ray crystallographic studies,

which have already been undertaken with the tobacco enzyme (Baker et al., 1975, 1977a,b).

Storage

The purified R. rubrum carboxylase can be stored indefinitely (> 1 year) at -60°C in 50 mM potassium phosphate/10 mM 2-mercaptoethanol/1 mM EDTA (pH 7.6) containing 20% (v/v) glycerol with no loss of enzymic activity. Table 7, Appendix A, shows the protective effect of glycerol against denaturation of the enzyme by freezing. Denaturation of the carobxylase by freezing has been noted by previous investigators (Anderson and Fuller, 1969; Tabita and McFadden 1974a); substantial reversal by assaying the enzyme in the presence of EDTA and dithiothreitol was observed (Tabita and McFadden, 1974a). R. rubrum carboxylase stored frozen in 20% glycerol and subjected to several freeze-thaw cycles has also been inspected by polyacrylamide gel electrophoresis, and this criterion also showed that the enzyme was unaffected by storage.

At 4°C in phosphate buffer (without glycerol), the enzyme was somewhat unstable, losing 30 to 50% of its activity during 1 month. Substitution of 66 mM $NaHCO_3$/50 mM Bicine-NaOH (pH 8.0 at 25°C), which had been treated with Chelex 100 resin to remove trace metal contamination, for phosphate buffer resulted in better cold-storage properties of the enzyme, with little or no activity being lost after several months at 4°C.

Reaction of Br-butanone-P_2 with R. rubrum Carboxylase

Prior to chemical modification the purified enzyme was dialyzed exhaustively against Mg^{2+}-free (by passage through BioRad Chelex 100 Resin) 66 mM $NaHCO_3$/50 mM Bicine-NaOH/0.1 mM EDTA (pH 8.0).

Br-butanone-P_2 was added to a solution (3.3 mL) of carboxylase (0.5 mg/mL) serially, yielding an increase of 0.03 mM in reagent concentration with each addition. Sequential additions of the reagent (in stoichiometric excess) were necessary due to the instability of Br-butanone-P_2 at pH 8.0 (Hartman, 1975; Norton et al., 1975). Comparisons were made between carboxylase exposed to Br-butanone-P_2 in the presence and absence of 1 mM Rbl-P_2.

Br-butanone-P_2 inactivates Rbl-P_2 carboxylase from R. rubrum, and Rbl-P_2 protects against inactivation (Figure 6, Appendix B). Samples of the untreated, inactivated, and substrate-protected carboxylase were reduced with [^{3}H]$NaBH_4$ (Norton et al., 1975) in the presence of 4 M guanidine-HCl and thoroughly dialyzed. Based on quantitation of tritium present in the dialyzed samples, the inactivated enzyme contained 1.6 molar equivalents of reagent per mole of enzyme (114,000 g) (Tabita and McFadden, 1974b), whereas the protected enzyme contained only 0.8 molar equivalents of reagent. Since the enzyme is a dimer (Tabita and McFadden, 1974b), these data suggest that inactivation results from the modification of about 0.4 residue per subunit.

To determine the kinds of amino acid residues that were modified, acid hydrolysates of the derivatized proteins were chromatographed on the amino acid analyzer and the effluents were assayed for radioactivity

(Figure 7, Appendix B). Hydrolysates of both the inactivated and protected samples contained about equal amounts of derivatized cysteine, represented by the breakthrough peak (16 min) from the short column and the peaks eluting at 36 and 42 min from the long column. These elution positions were established previously (Hartman, 1975; Norton et al., 1975) for the cysteine derivatives obtained from the reaction of glutathione with Br-butanone-P_2 (followed by reduction and hydrolysis). The major difference between the hydrolysates of protected and inactivated carboxylase is that the former contain less of the two derivatives that elute from the short column at 23 and 27 min, respectively. By mixing hydrolysates of the inactivated, reduced enzyme from R. rubrum with hydrolysates of the carboxylase from spinach after its inactivation by Br-butanone-P_2 and reduction with [^{3}H]$NaBH_4$, it was confirmed that the two radioactive peaks that are retarded by the short column coelute with the derivatives from the spinach enzyme that were characterized previously (Norton et al., 1975) as lysyl. Therefore, inactivation of the carboxylase from R. rubrum by Br-butanone-P_2 seems to be due primarily to modification of lysyl residues.

Reaction of BrAcNHEtOP with R. rubrum Rbl-P_2 Carboxylase

BrAcNHEtOP inactivates the R. rubrum carboxylase in a time-dependent fashion and Mg^{2+} stimulates the rate of inactivation as is the case with the spinach carboxylase (Figure 8, Appendix B). In contrast to the spinach enzyme, however, inactivation of the R. rubrum carboxylase by BrAcNHEtOP does not follow pseudo-first-order kinetics. Rbl-P_2 or

$CR\text{-}P_2$ (in the absence of Mg^{2+}) prevent inactivation of the R. rubrum carboxylase by BrAcNHEtOP (Figure 8, Appendix B).

To characterize the reaction products formed between the R. rubrum carboxylase and BrAcNHEtOP, the enzyme (100 mg, 8 mg/mL) was incubated with 1 mM [^{14}C]BrAcNHEtOP (520 cpm/nmol) in 66 mM $NaHCO_3$/50 mM Bicine/ 0.1 mM EDTA (pH 8.0) with or without 5 mM $MgCl_2$. These samples were compared to enzyme treated in an identical fashion except for the presence of 0.5 mM $CR\text{-}P_2$. Incorporation of [^{14}C]BrAcNHEtOP into the R. rubrum enzyme during the course of reactions was determined as acid-insoluble radioactivity by the paper-disc method described in Part I, Chapter II, and enzymic activity was determined concurrently at each time point. After a 48 h incubation, the sample containing Mg^{2+} (but no $CR\text{-}P_2$) retained 6.7% of its original enzymic activity; the activity in the $CR\text{-}P_2$ containing sample could not be monitored due to the inactive complex formed between the transition state analog and enzyme in the presence of Mg^{2+}. The Mg^{2+}-containing reactions were terminated at this point by the addition of 10 mM 2-mercaptoethanol, followed by exhaustive dialysis against 50 mM potassium phosphate/10 mM 2-mercaptoethanol/1 mM EDTA (pH 7.6). The samples which did not contain Mg^{2+} were incubated for 145 h; at this time the unprotected and $CR\text{-}P_2$ protected samples retained 8.8% and 114% of their initial enzymic activity respectively. Following termination of the reactions with 2-mercapto-ethanol the samples were exhaustively dialysed.

Inactivation of the R. rubrum carboxylase by BrAcNHEtOP was irreversible as dialysis did not alter the levels of enzymic activity

seen prior to removing excess reagent. Following dialysis, the unprotected enzymes contained 1.7 and 1.8 mol of [^{14}C]BrAcNHEtOP per mol of subunit while CR-P_2 containing samples contained 0.4 and 0.6 mol of reagent per mol subunit in the presence and absence of Mg^{2+}, respectively. Thus, BrAcNHEtOP inactivation of the R. rubrum carboxylase appears to be a consequence of the modification of 1.2 - 1.3 residues per protomer (1.7 minus 0.4 or 1.8 minus 0.6).

Incorporation of [^{14}C]BrAcNHEtOP as a function of enzymic activity lost is shown in Figure 9, Appendix B. Extrapolation of these data to 100% inactivation indicates complete inactivation to be a consequence of the modification of 1.3 residues per protomer in the presence or absence of Mg^{2+}, a value that agrees with the end point values obtained after dialysis.

The kinds of amino acid residues derivatized by [^{14}C]BrAcNHEtOP were determined by analyzing radioactivity present in acid hydrolysates of the modified enzyme on an amino acid analyzer. All of the derivatized residues appear in the hydrolysates as carboxymethyl amino acids (see Part I, Chapter III). Cys(Cm) is the major derivative found in hydrolysates after modification in the presence or absence of Mg^{2+} (Figure 10, Appendix B). The corresponding CR-P_2-protected samples contain 0.48 and 0.41 fewer residues of Cys(Cm) per protomer after modification in the absence and presence of Mg^{2+}, respectively. Two additional carboxymethyl amino acids, His(p-Cm) and Lys(Cm), are present in lesser amounts than Cys(Cm). The corresponding CR-P_2-protected samples contain 0.22 and 0.31 fewer residues of His(p-Cm) per protomer,

and 0.20 and 0.25 fewer residues of Lys(Cm) per protomer after modification in the absence and presence of Mg^{2+}, respectively. Thus, inactivation does not correlate with a stoichiometric incorporation of any single carboxymethyl amino acid, although the sum of all three types or protected residues is approximately one residue per protomer.

Samples of R. rubrum Rbl-P_2 carboxylase modified with [^{14}C]BrAcNHEtOP were carboxymethylated with iodoacetic acid and subject to tryptic digestion as described for the spinach enzyme (Part I, Chapter II). These tryptic digests were then further subjected to chromatography on BioRad Aminex AG 50 W-X2 resin (Part I, Chapter III). The tryptic digests of enzyme modified in the absence of Mg^{2+} (Figure 11A, Appendix B) contain one major radioactive peptide, designated as V, and numerous other labeled peptides present in lesser amounts which are protected against [^{14}C]BrAcNHEtOP modification by CR-P_2. Unlike the results obtained with [^{14}C]BrAcNHEtOP modification of the spinach carboxylase, inclusion of Mg^{2+} during modification of the R. rubrum enzyme does not enhance the reagent's specificity (Figure 11B, Appendix B). The level of only a single labeled peptide, designated as VI, appears to be altered by the inclusion of Mg^{2+} during modification. After correcting for radioactivity present in samples which contained CR-P_2, the distribution of radioactivity in the inactivated samples is as follows [the Roman numerals refer to the peak designations given in Figure 11, Appendix B, and the value following each numeral is the incorporation of reagent expressed in mol reagent per mol protomer]:

$-Mg^{2+}$ I,0.06;II,0.09,III,0.06;IV,0.06;V,0.44;
VI,0.12;VII,0.08;VIII,0.11;IX,0.06;X,0.18

$+Mg^{2+}$ I,0.02;II,0.13;III,0.06;IV,0.13;V,0.41;
VI,0.24;VII,0.08;VIII,0.10;IX,0.01;X,0.22

Summation of the differences in incorporation (with and without CR-P_2) for all peaks seen in Figures 11A and 11B, Appendix B, gives values of 1.3 and 1.4 mol reagent per mol protomer for enzyme modified in the absence and presence of Mg^{2+}, respectively. Thus, all of the incorporation of [^{14}C]BrAcNHEtOP can be accounted for in the tryptic profiles shown in Figure 11, Appendix B. As was anticipated based on the results of amino acid analysis, there are no labeled peptides which represent a stoichiometric incorporation of [^{14}C]BrAcNHEtOP per protomeric unit (56,000 daltons) of the R. rubrum carboxylase. Considering the low levels of [^{14}C]BrAcNHEtOP incorporation required for inactivation of the R. rubrum enzyme, the large number of labeled peptides is rather remarkable and an obvious disappointment. If the peak designated as V in Figures 11A and 11B, Appendix B, represents a single peptide, then it must contain a [^{14}C]BrAcNHEtOP-modified cysteinyl residue as no other modified amino acid (see preceding paragraph) is present in sufficient quantity to account for peak V. Thus, it would appear that the majority of the cysteinyl modification by [^{14}C]BrAcNHEtOP (which is subject to CR-P_2 protection) is restricted to a single, distinct residue. Peak V represents approximately one-half of one [^{14}C]BrAcNHEtOP molecule incorporated per protomer, or one reagent molecule per enzyme dimer.

CHAPTER IV

DISCUSSION

A characteristic feature of the R. rubrum carboxylase is its lack of a small subunit. Several other prokaryotes [Chlorobium thiosulfatophilum (Tabita et al., 1974a), Thiobacillus intermedius (Purohit et al., 1976), Anabaena cylindrica (Tabita et al., 1976), Agmenellum quadruplicatum (Tabita et al., 1974b), and Rhodopseudomonas sphaeroides (Gibson and Tabita, 1977a)] also have carboxylases composed solely of large subunits. However, only the carboxylase from R. rubrum is a simple dimer (Tabita and McFadden, 1974b); the other enzymes are composed of six to eight subunits (Gibson and Tabita, 1977a; Purohit et al., 1976; Tabita et al., 1974a,b, 1976). Jensen and Bahr (1977) have suggested that the absence of the small subunit may be an artifact of isolation. However, several lines of evidence argue against this possibility in the case of the R. rubrum enzyme: (i) The sedimentation constant seen for the purified enzyme (6.2 $S_{20,w}$) is the same as that seen for the enzyme in crude extracts (Anderson et al., 1968). (ii) Comparisons of polyacrylamide gels from electrophoresis of the purified enzyme and crude extracts (Figure 3, Appendix B) reveal that the mobility of the purified enzyme is not detectably different from that of the unpurified enzyme. The ability to visualize the R. rubrum carboxylase on polyacrylamide gels of crude extracts is an added benefit of its comprising such a large fraction of the total protein in the present study. If the quantity of crude protein loaded onto the gel

(as seen in Figure 3, Appendix B) is reduced to 10 to 20 μg, only a single symmetrical band with the same apparent mobility as the purified enzyme is readily detectable. (iii) Enzyme purified under a different set of conditions (Schloss and Hartman, 1977) from those described in Chapter II also lacks a small subunit. Thus, apparently either the R. rubrum carboxylase has no small subunit, or the small subunit readily dissociates from the enzyme (even under mild conditions such as those of electrophoresis and sedimentation) and probably would not be invariably associated with the enzyme in vivo.

The question remains as to whether R. rubrum possesses the genetic information for the small subunit of Rbl-P_2 carboxylase. Gibson and Tabita (1977a,b) have found that the closely related organisms Rhodopseudomonas sphaeroides and capsulata produce two different forms of Rbl-P_2 carboxylase when grown on butyrate. They have demonstrated that the enzymes from Rhodopseudomonas sphaeroides differ in that one contains small subunits while the other does not (Gibson and Tabita, 1977a). This observation raises the possibility that R. rubrum may also be able to produce a carboxylase containing small subunits under the proper set of conditions.

Based on its simple quaternary properties, McFadden and Tabita (1974) have suggested that the R. rubrum carboxylase may represent an ancestral prototype of the enzyme from which the plant-type carboxylases are derived. A similar interpretation was recognized earlier by Anderson et al. (1968) based on more limited data. However, Schwartz and Dayhoff (1978) have proposed a phylogenetic tree based primarily on

sequence data of cytochrome c, ferredoxins, and 5 S ribosomal RNA in which the Chromatiaceae and Chlorobiaceae diverge earlier than the Rhodospirillaceae. Since Chromatium vinosum (strain D), a member of the Chromatiaceae, has a plant-type carboxylase containing eight large and eight small subunits (Takabe and Akazawa, 1973a,b), this enzyme may represent the ancestral form of the carboxylase from which divergent forms of the enzyme (such as that of R. rubrum) arose. Thus, R. rubrum probably evolved from an organism possessing a carboxylase with both large and small subunits.

Although the function of the small subunit is unknown, it is not catalytically essential, as enzymically active large subunits can be isolated from enzymes that contain both large and small subunits (Nishimura and Akazawa, 1973; Takabe and Akazawa, 1973a,b). Akazawa and co-workers (Nishimura and Akazawa, 1974; Takabe and Akazawa, 1975) have suggested that the small subunit may serve a regulatory or structural function, but as yet it has only been implicated in an Mg^{2+}-dependent shift in the optimum pH of enzyme activity. The pH optimum of carboxylase activity in isolated large subunits (Nishimura and Akazawa, 1973; Takabe and Akazawa, 1973b) or enzymes not containing small subunits (Akazawa et al., 1969,1970) is independent of Mg^{2+} concentration, in contrast to small-subunit-containing enzymes. Gibson and Tabita (1977a) have also observed differences in the pH optima of the two forms of the Rhodopseudomonas sphaeroides enzyme one of which does not contain a small subunit. These observations need to be reevaluated, however, in light of the recently discovered role of Mg^{2+} (and CO_2) in a time- and pH-dependent activation of the

carboxylase (Laing and Christeller, 1976; Lorimer et al., 1976), which converts an inactive conformer of the enzyme (in the absence of Mg^{2+}/CO_2) to a catalytically active one. Thus, the different pH optima observed may reflect differing kinetics of activation between the two forms of the enzyme (with and without small subunits). In this regard it should be pointed out that the pH optimum reported in the current study for "preactivated" R. rubrum carboxylase is 1 pH unit higher than that reported by Akazawa et al. (1969).

Another feature of the R. rubrum enzyme which distinguishes it from the small-subunit-containing carboxylases is its rather high K_m for CO_2. Although V_{max} for the R. rubrum enzyme is 2.5- to 5-fold higher than plant-type carboxylases, its K_m (CO_2) is 50- to 100-fold higher (see Table 3 and Jensen and Bahr, 1977; Lorimer et al., 1977). The R. rubrum enzyme also appears to have a more selective binding specificity than the spinach carboxylase, as its affinity for various competitive inhibitors is considerably less than that of the spinach enzyme (Table 4, Appendix A). Differing affinities for some competitive inhibitors, with similar affinities for others, could reflect differences in the active-site topology between the R. rubrum and spinach enzymes, as suggested earlier by Tabita and McFadden (1974a,b).

Another property of the plant-type carboxylases not shared by the R. rubrum enzyme is reversible cold inactivation (Chollet and Anderson, 1976; Kawashima et al., 1971; Ryan and Tolbert, 1975; Wildner and Henkel, 1977). After days or months of storage at 4°C in a trace-metal-free buffer, R. rubrum enzyme loses little activity. The rate of cold

inactivation of the R. rubrum carboxylase is thus considerably slower than that of the plant-type carboxylases, or alternatively, the enzyme is activated so rapidly at assay temperatures that inactivation cannot be detected.

A common property of eukaryotic Rbl-P_2 carboxylases may be the absence of a free NH_2-terminus in their catalytic subunits (Iwanij et al., 1974; Moon and Thompson, 1971; Poulsen, 1978). The inability to detect a free NH_2-terminal residue in the large subunit of spinach carboxylase by use of the identical procedures which yielded sequence data for the small subunit of the same enzyme and for the R. rubrum enzyme suggests a blocked NH_2-terminus in the former rather than the failure of the analytical method. It will be of interest to determine the chemical nature of the NH_2-terminal derivatization and whether all chloroplastic Rbl-P_2 carboxylases carry the same blocked residue.

The reaction of Br-butanone-P_2 with Rbl-P_2 carboxylase from R. rubrum is strikingly similar to its reaction with the corresponding enzyme from spinach. Irreversible inactivation occurs, and the inactivation is subject to substrate protection. In the presence of Rbl-P_2, nonessential sulfhydryls are modified; in the absence of Rbl-P_2, not only are the sulfhydryls modified, but lysyl residues are also reactive. Thus, inactivation correlates with alteration of lysyl residues. Further, as is the case of the spinach enzyme, two chromatographically distinct lysyl derivatives are formed. Based on studies with model compounds (Hartman, 1975), one of these derivatives is thought to be the alkylation product resulting from displacement of the

bromine atom of the reagent by the ε-NH_2 group of a lysyl residue. The other derivative has not been characterized beyond the observation that upon treatment with periodate it yields free lysine. The low level of incorporation (correlation of essentially total inactivation with incorporation of 0.4 equivalent of reagent per subunit of enzyme) of reagent necessary for inactivation is not unique to the R. rubrum enzyme nor to Br-butanone-P_2. Apparent correlation of inactivation with the modification of less than one residue equivalent was also observed in the reactions of Br-butanone-P_2 with the spinach carboxylase (Norton et al., 1975), pyridoxal phosphate with the R. rubrum carboxylase (Whitman and Tabita, 1978b), and phenylglyoxal with both spinach and R. rubrum carboxylases (Schloss et al., 1978b). Possible explanations are half-of-sites reactivity (Levitzki and Koshland, 1976) or more trivially that the carboxylase preparations are only partially active. Since similar results have now been obtained with several reagents and the carboxylase from two different species, the likelihood is increased that the former explanation is correct.

In contrast to the results obtained with Br-butanone-P_2, the reaction of BrAcNHEtOP with the Rbl-P_2 carboxylase from R. rubrum is not completely analogous to its reaction with the corresponding enzyme from spinach. Although BrAcNHEtOP inactivates the R. rubrum enzyme and Rbl-P_2 or CR-P_2 protect against inactivation, inactivation of the R. rubrum carboxylase is "biphasic" in contrast to the simple pseudo-first-order inactivation seen with the corresponding enzyme from spinach. Biphasic pseudo-first-order kinetics indicates that two forms of the

enzyme are being inactivated, one (faster reaction) comprising approximately 45% of the total enzymic activity and the other (slower reaction) 55% (determined by extrapolation of the slower reaction to zero time). Since R. rubrum Rbl-P_2 carboxylase is a dimer (Tabita and McFadden, 1974b) these data could be interpreted to be the result of nonequivalent inactivation of the two subunits, each subunit contributing approximately half of the total enzymic activity. Simulated profiles for the inactivation of such a dimeric enzyme, with either an affinity label or a simple protein reagent which does not form a noncovalent complex with enzyme prior to modification, are shown in Figure 12, Appendix B (derivation of the equation which describes these profiles is given in Appendix C). These profiles are qualitatively similar to the data obtained for inactivation of the R. rubrum carboxylase (Figure 8, Appendix B), but careful comparison of the two sets of figures reveals that the initial loss of enzymic activity when the R. rubrum enzyme is inactivated by BrAcNHEtOP occurs more rapidly than is consistent with the simulated profiles. A possible explanation for this would be that initially both subunits of the enzyme are equally susceptible to inactivation by the reagent but modification (and concomitant inactivation) of one subunit induces a conformational change in the second subunit rendering it less susceptible to inactivation by the reagent. It should be pointed out that modification of more than one essential residue by the reagent will not result in biphasic kinetics, provided that modification of each residue results in complete inactivation of the enzyme (Appendix C). Another possible explanation

for nonpseudo-first-order kinetics would be partial inactivation due to modification of a critical residue, followed by a subsequent slower loss of enzymic activity remaining due to a rate limiting conformational change. This possibility is excluded, however, due to the direct proportionality seen between reagent incorporation and enzymic activity lost. Thus, the most likely explanation for the biphasic kinetics seen with inactivation of the R. rubrum enzyme by BrAcNHEtOP is nonequivalent reactivity of the two subunits (which is probably induced by modification of the first subunit). Other reagents, such as Br-butanone-P_2, pyridoxal phosphate, and phenylglyoxal, also appear to induce alterations in subunits other than those modified which, in contrast to BrAcNHEtOP, result in inactivation of subunits not chemically modified. Pyridoxal phosphate, like BrAcNHEtOP, induces allosteric changes in the R. rubrum carboxylase during inactivation which are not seen with inactivation of the spinach enzyme by this reagent. Inactivation of the spinach carboxylase (Paech et al., 1977) appears to correlate with incorporation of 1 - 2 mol of pyridoxal phosphate per mol protomer in contrast to 0.5 mol reagent per mol protomer with the corresponding enzyme from R. rubrum (Whitman and Tabita, 1978b).

Although BrAcNHEtOP inactivation of the R. rubrum enzyme does not correlate with modification of a single residue, the majority of modification prevented by the transition state analog CR-P_2 is associated with cysteinyl modification. Thus, a substantial fraction of BrAcNHEtOP inactivation of the R. rubrum enzyme is probably due to cysteinyl modification. Unlike the spinach enzyme, the R. rubrum enzyme

does not appear to be inactivated by BrAcNHEtOP in the presence of Mg^{2+} as a consequence of lysyl modification. Little change is observed between R. rubrum carboxylase modified by BrAcNHEtOP in the presence or absence of Mg^{2+} at the level of tryptic peptides. This observation is consistent with earlier conclusions suggestive of conformational differences between the active site regions of the R. rubrum and spinach carboxylases. BrAcNHEtOP appears to be a sensitive probe of conformational alterations in the spinach enzyme as inclusion of Mg^{2+} during modification of the enzyme results in a shift in reagent specificity from a cysteinyl residue (and one additional cysteinyl residue) to a lysyl residue three residues distant in sequence (see Part I, Chapter III; Schloss et al., 1978a).

Cysteinyl and lysyl residues appear to be essential for both the spinach (see Part I; Schloss et al., 1978a; Norton et al., 1975; Paech et al., 1977) and R. rubrum (see Chapter III; Whitman and Tabita, 1978a) carboxylases. Striking similarities in modification of the two enzymes by the group-specific arginyl reagent phenylglyoxal have also been noted (Schloss et al., 1978b). Both enzymes have an absolute requirement for Mg^{2+} and are inactivated in a time-dependent, Mg^{2+}-dependent fashion by the transition state analog CR-P_2. Consideration of these similarities strongly suggests that the R. rubrum and spinach carboxylases are derived from a common ancestral gene. Thus, the R. rubrum and spinach carboxylases provide a useful and valid comparison in elucidating catalytically essential features of the Rbl-P_2 carboxylase.

The R. rubrum carboxylase appears to be an attractive candidate for complete crystallographic and sequence studies. The enzyme's

crystallinity and rather simple quaternary structure are compatible with X-ray diffractometry, and the presence of a single type of subunit with a free NH_2-terminus will facilitate the elucidation of primary structure. Because of the size of the carboxylase, sequence information will be crucial in the interpretation of electron density maps (Blow and Steitz, 1970).

When the culture conditions specified in Chapter II are used, large quantities of R. rubrum carboxylase are readily obtainable. With two 50-liter fermentors, sufficient R. rubrum can be grown every 2 weeks (in serial culture) to provide approximately 1.5 g of purified carboxylase (28% recovery). Because of this availability of the enzyme comparative affinity labeling, sequence, and crystallographic studies can be undertaken.

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APPENDIXES

APPENDIX A

TABLES

TABLE 1

ENZYME PURIFICATION FROM 60 G *R. RUBRUM* (WET WEIGHT)

Step	Total Protein (mg)	Total Units	$\bar{A}_{280}/\bar{A}_{260}$	Sp act	% Recovery
Sonicate	9,840	4,800	——	0.49	100
200,000 × *g* supernatant	2,040	3,900	0.83	1.91	81
Ammonium sulfate pellet (0 to 60%)/G-25 Sephadex eluate	1,450	4,050	1.32	2.79	84
Chromatography on DEAE-cellulose	733	3,200	1.62	4.37	67
Chromatography on DEAE-Sephadex	300	1,360	1.79	4.53	28

TABLE 2

SPECIFIC ACTIVITY OF CELL EXTRACTS AFTER HIGH-SPEED CENTRIFUGATION

Carbon source	Sp act[a]	Carboxylase (% of soluble protein)
Malate	0.0035,[b] 0.008[c]	
CO_2 in H_2	0.120[b]	
Butyrate	0.128[c]	6-8[c]
CO_2 in H_2 (serially cultured, 5th transfer)	0.917	20
CO_2 in H_2 (serially cultured, 7th transfer)	1.91	42

[a]Expressed as μmol Rbl-P_2 consumed per min per mg soluble protein.

[b]Data of Anderson and Fuller (1967).

[c]Data of Tabita and McFadden (1974a).

TABLE 3

PROPERTIES OF THE PURIFIED *R. RUBRUM* Rbl-P_2 CARBOXYLASE

Property	Current study	Previously reported
Kinetic		
V_{max} (50 mM $NaHCO_3$)	4.8 units/mg	
V_{max} (0.5 mM Rbl-P_2)	9.1 units/mg	
K_m (Rbl-P_2)	0.034 mM	0.083 mM,[a] 0.053 mM[b]
K_m ($NaHCO_3$; under air)	60 mM	65 mM[a]
K_m (CO_2; under air)	1.4 mM	1.6 mM[b]
pH optimum	8.0[c]	8.1,[a] 7.0[d]
Physical		
Subunit mol wt	56,000[e]	56,000[b,e]
Carbohydrate	none[f]	
$S_{20,w}$	6.2	6.2[a,b]
$E_1^{1\%}$ cm	9.72[g]	9.74[b]

[a]Data of Anderson and Fuller (1969).

[b]Data of Tabita and McFadden (1974b).

[c]50 mM $NaHCO_3$; 0.5 mM Rbl-P_2.

[d]Data of Akazawa et al., (1969).

[e]From SDS-polyacrylamide gel electrophoresis.

[f]Carbohydrate stain for glycoproteins (Segrest and Jackson, 1972).

[g]Based on dry weight.

TABLE 4

INHIBITORS OF THE PURIFIED R. RUBRUM AND SPINACH Rbl-P_2 CARBOXYLASES

Inhibitor	K_I (mM)[a]		K_I(R. rubrum)/K_I(spinach)
	R. rubrum	Spinach	
Citrate	16	14	1
Phosphate	14	2.2	6
Sulfate	9.3	4.7	2
3-Phosphoglycerate	6.1	4.8	1
Ribulose 5-phosphate	5.9	3.4	2
Ribose 5-phosphate	13	3.9	3
6-Phosphogluconate	1.7	0.075	23
Fructose 1,6-bisphosphate	0.61	0.84	1
3-Bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate	1.2	1.0	1

[a]Rbl-P_2 was the variable substrate (0.5, 0.1, 0.05, and 0.02 mM) in 50 mM [^{14}C]$NaHCO_3$/10 mM $MgCl_2$/100 mM Tris-HCl (pH 8.0).

TABLE 5

AMINO ACID COMPOSITION OF Rbl-P_2 CARBOXYLASE FROM R. RUBRUM GROWN AUTOTROPHICALLY AND ON BUTYRATE

Amino acid	Residues per 56,000 daltons	
	Present study, autotrophically grown	Previously reported, grown on butyrate[a]
Aspartic acid	50	50
Threonine	28	28
Serine	21	27
Glutamic acid	45	48
Proline	25	30
Glycine	54	55
Alanine	68	69
Half-cystine[b]	5(4.4)[c]	5(2.0)[c]
Valine	30	32
Methionine	15	13
Isoleucine	21	22
Leucine	39	39
Tyrosine	18	12
Phenylalanine	25	20
Histidine	13	11
Lysine	21	21
Tryptophan	7	9
Arginine	31	31

[a]Calculated from the data of Tabita and McFadden (1974b).

[b]Determined as cysteic acid.

[c]Value in parentheses is the cysteine content determined by titration of free sulfhydryl groups with 5,5´-dithiobis(2-nitrobenzoic acid).

TABLE 6

SEQUENCE ANALYSES OF THE SPINACH AND R. RUBRUM CARBOXYLASES

Sample	NH_2-terminal sequence
Spinach large subunit	No residues released by Edman degradation in five cycles
Spinach small subunit	Gly-Lys-Val-Trp-Pro-Thr-Glx-Asx-Met-Lys-His-Tyr-
Tobacco small subunit[a]	Met[b]—Gln-Val-Trp-Pro-Pro-(Tyr/Ile)-(Asn/Gly)-Lys-Lys-Lys-Tyr-
R. rubrum carboxylase	Met-Asp-Gln-Ser-Ser-Arg-Tyr-Val-Asn-Leu-Ala-Leu-Lys-Glu-Glu-Asp-Leu-Ile-Ala-Gly-Gly-Glx-His-Val-Leu-

[a]Data of Gibbons et al. (1975), Strøbaek et al. (1976), and Iwai et al. (1976).

[b]The NH_2-terminal Met was absent in enzyme preparations of Iwai et al. (1976).

TABLE 7

STORAGE OF PURIFIED R. RUBRUM Rbl-P_2 CARBOXYLASE

Freeze-thaw cycle	Sp act[a]	
	20% Glycerol	No glycerol
0	3.29	3.34
1	3.34	0.85
2	3.33	0.69
3	3.30	0.48
4	3.14	—
5	3.33	—

[a]Specific activity is expressed as μmol Rbl-P_2 per min per mg at 25°C, pH 8.0. The buffer in which samples were frozen was 50 mM potassium phosphate/10 mM 2-mercaptoethanol/1 mM EDTA (pH 7.6) with or without 20% glycerol. Samples were frozen in liquid nitrogen and thawed in a water bath at room temperature.

APPENDIX B

FIGURE LEGENDS AND FIGURES

Figure 1. Levels of Rbl-P_2 carboxylase and growth of R. rubrum maintained on various carbon sources.

(A) Turbidity of cultures maintained on 0.6% butyrate (■), 0.25% malate (Δ), or 2% CO_2 in H_2 (O) (12 liters of gas per h per 50 liters of culture) as carbon source. Inoculum was 3.75% of cells grown on malate. (B) Rbl-P_2 carboxylase activity levels for the same cultures shown in A. (C) Concentration of carboxylase activity divided by culture turbidity at 650 nm.

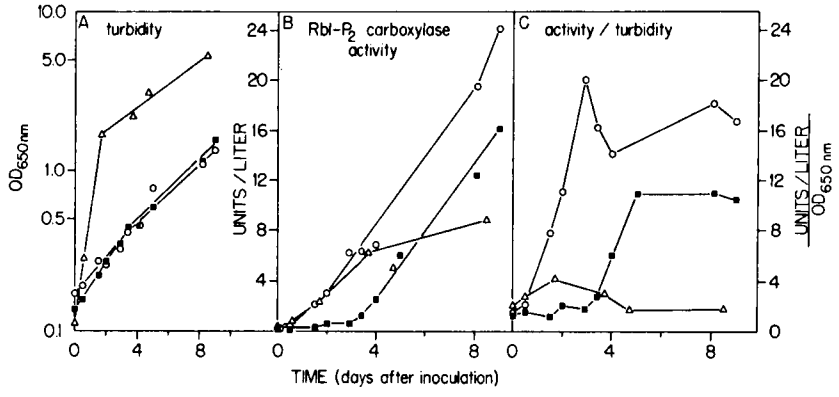
A turbidity
B RbI-P2 carboxylase activity
C activity / turbidity
OD650nm
UNITS / LITER
UNITS / LITER / OD650 nm
TIME (days after inoculation)

Figure 1

Figure 2. Specific activity of cells grown serially with 2% CO_2 in H_2.

An initial 10% inoculum of cells grown on malate was used (1st transfer) and cultures were maintained with 2% CO_2 in H_2 (12 liters of gas per h per 50 liters of culture). Each additional transfer was made by using 10% of the culture after approximately 2 weeks of growth. Cell yields averaged about 3 g per liter (wet weight).

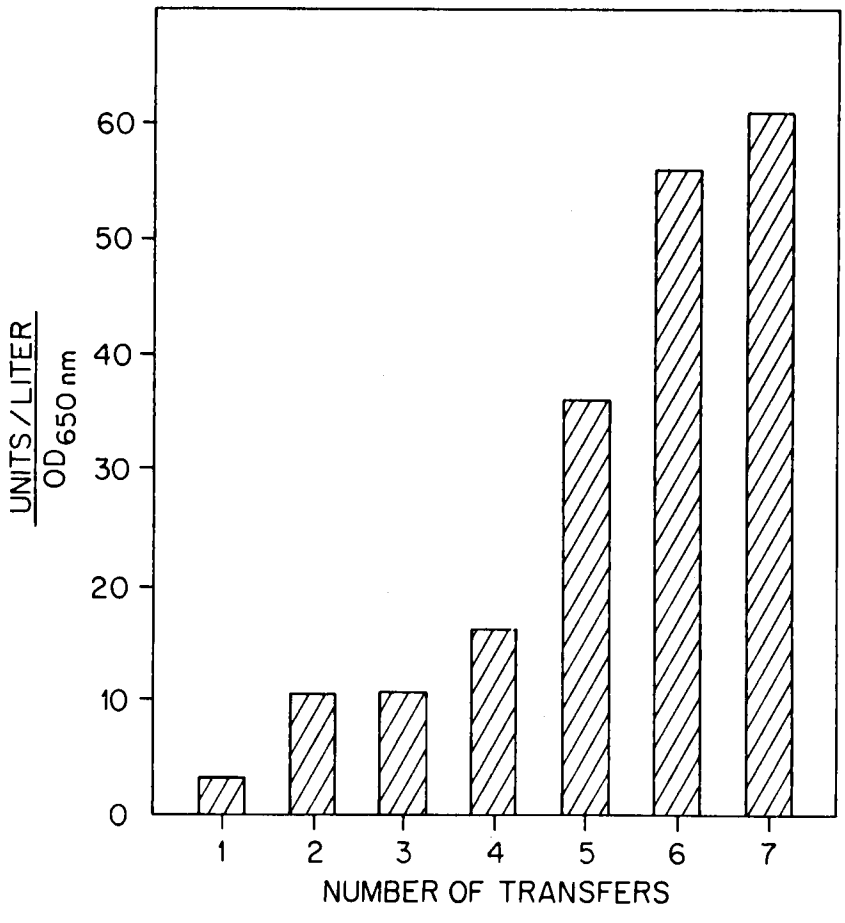
UNITS/LITER
OD650 nm
60
50
40
30
20
10
0
1
2
3
4
5
6
7
NUMBER OF TRANSFERS

Figure 2

Figure 3. Polyacrylamide gel electrophoresis (pH 9.5) of extracts from R. rubrum after high-speed centrifugation and of purified carboxylase.

(A) 20 µg pure enzyme. (B) 100 µg pure enzyme. (C) 100 µg protein, 1st transfer extract. (D) 100 µg protein, 5th transfer extract. (E) 100 µg protein, 7th transfer extract.

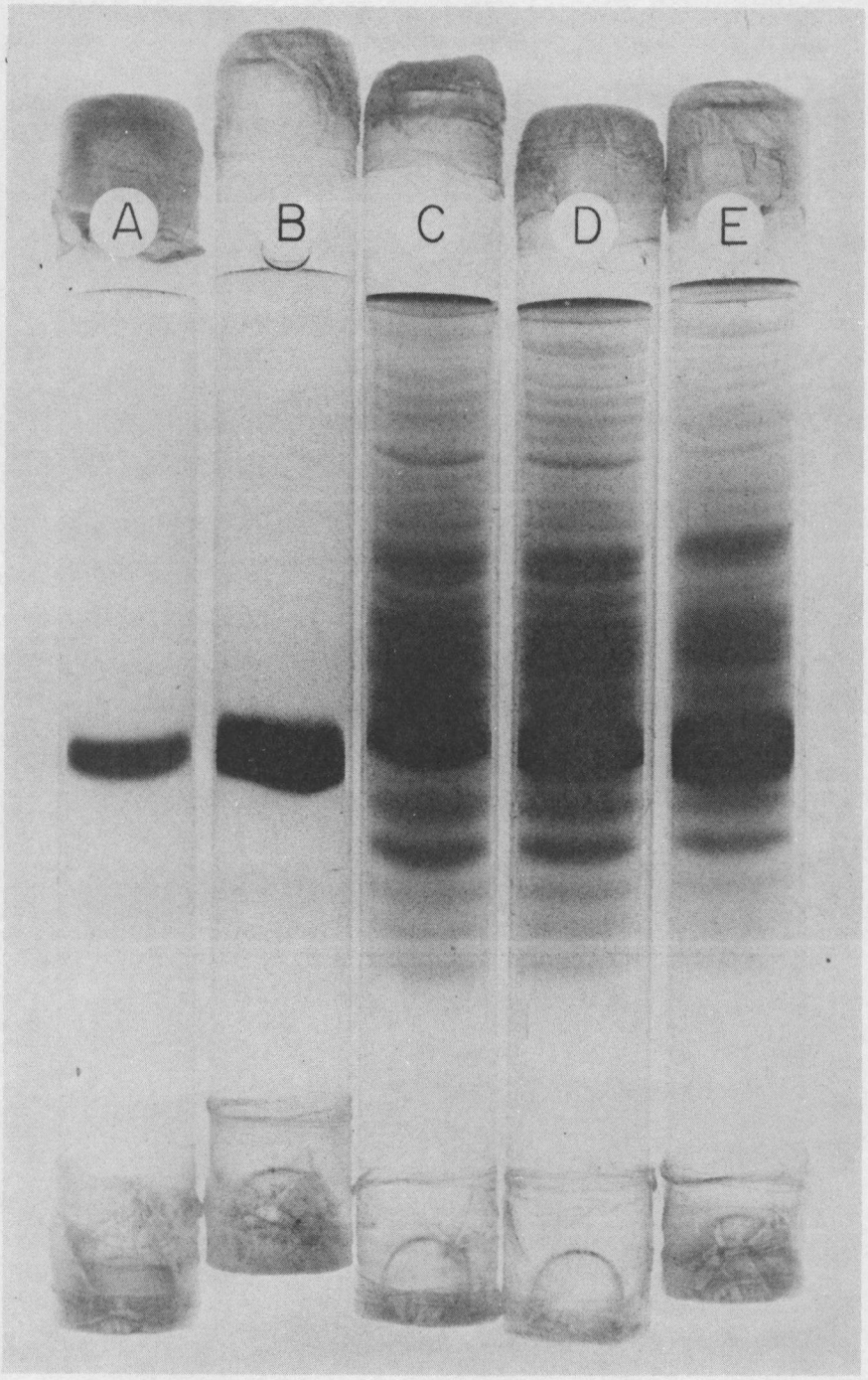

Figure 3

Figure 4. Chromatography of carboxymethylated spinach and *R. rubrum* carboxylases on a G-200 Sephadex column (2 × 82 cm) in the presence of 1% SDS/0.25% thiodiglycol.

(A) 10 mg spinach carboxylase. (B) 7 mg *R. rubrum* carboxylase. (C) Mixture of 2 mg spinach and 2 mg *R. rubrum* carboxylase.

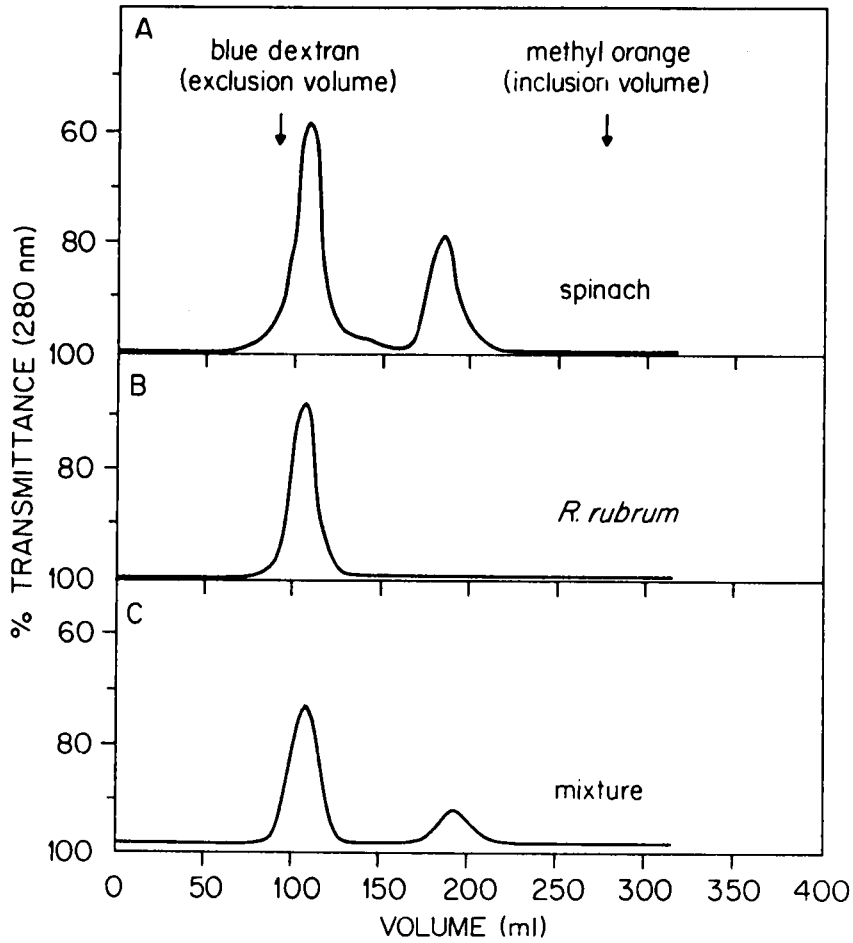
A
blue dextran
(exclusion volume)
methyl orange
(inclusion volume)
spinach
B
R. rubrum
C
mixture
% TRANSMITTANCE (280 nm)
60
80
100
0
50
100
150
200
250
300
350
400
VOLUME (ml)

Figure 4

Figure 5. Photomicrograph of crystalline R. rubrum Rb1-P_2 carboxylase with phase-contrast illumination (X200).



Figure 5

Figure 6. Inactivation of R. rubrum Rbl-P_2 carboxylase by Br-butanone-P_2.

Time-course of inactivation of Rbl-P_2 carboxylase by Br-butanone-P_2 in the absence (□) and presence (O) of 1 mM Rbl-P_2. The control (◇) lacked both reagent and substrate. Arrows indicate the times at which Br-butanone-P_2 was added. Experimental conditions are given in Chapter III.

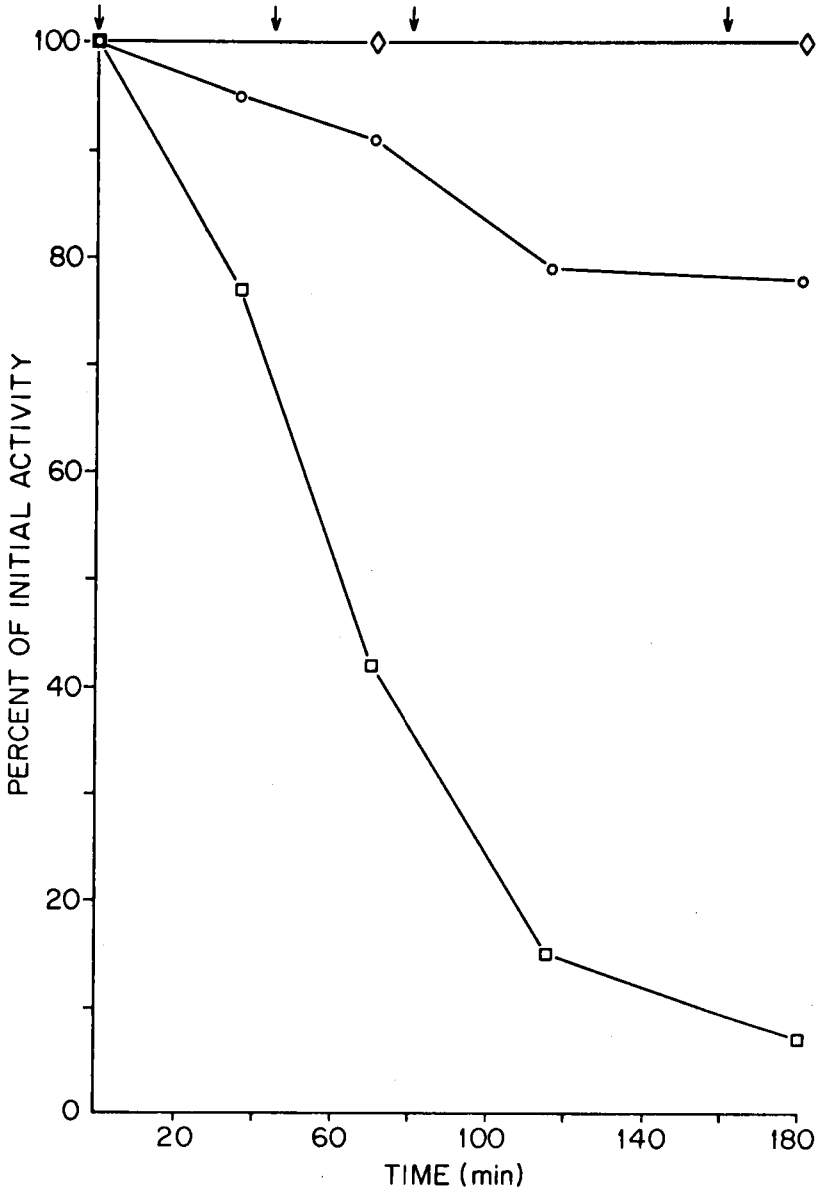
100
80
60
40
20
0
PERCENT OF INITIAL ACTIVITY
20
60
100
140
180
TIME (min)

Figure 6

Figure 7. Chromatographic analysis of acid hydrolysates of [^{3}H]$NaBH_4$ reduced R. rubrum Rbl-P_2 carboxylase modified by Br-butanone-P_2.

Radioactivity in hydrolysates of Rbl-P_2 carboxylase after treatment with Br-butanone-P_2, in the absence (——) and presence (---) of Rbl-P_2, followed by reduction with [^{3}H]$NaBH_4$. The hydrolysates were chromatographed on the Beckman 120C amino acid analyzer without use of the ninhydrin system, and fractions (1-min from the short column and 2-min from the long column) were collected and counted.

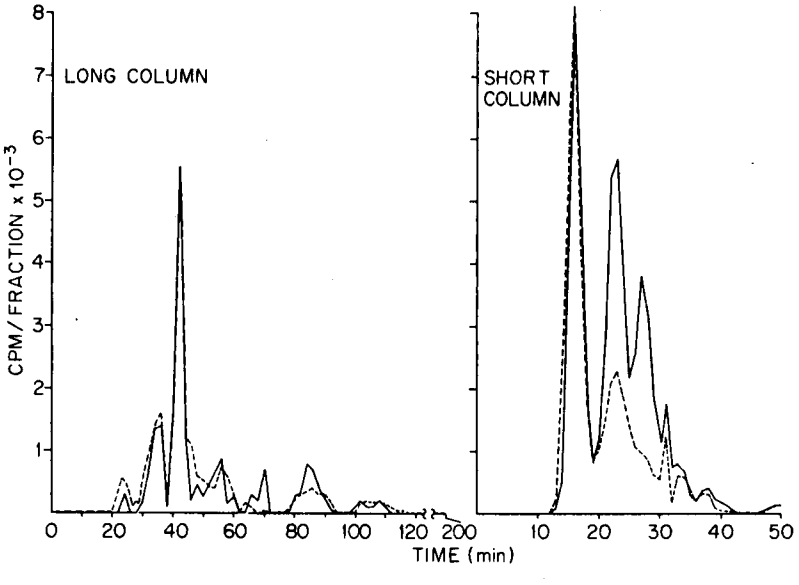
LONG COLUMN
SHORT COLUMN
CPM/FRACTION x 10⁻³
TIME (min)
0
20
40
60
80
100
120
200
10
20
30
40
50
1
2
3
4
5
6
7
8

Figure 7

Figure 8. Inactivation of R. rubrum Rbl-P_2 carboxylase by BrAcNHEtOP.

Time-course of inactivation (30°C) of carboxylase by 1 mM BrAcNHEtOP in 66 mM $NaHCO_3$/50 mM Bicine/0.1 mM EDTA (pH 8.0) with (O) or without (□) 5 mM $MgCl_2$. Protection against inactivation by 1 mM Rbl-P_2 (♦) or 0.5 mM CR-P_2 (◊) in the absence of Mg^{2+} is also shown.

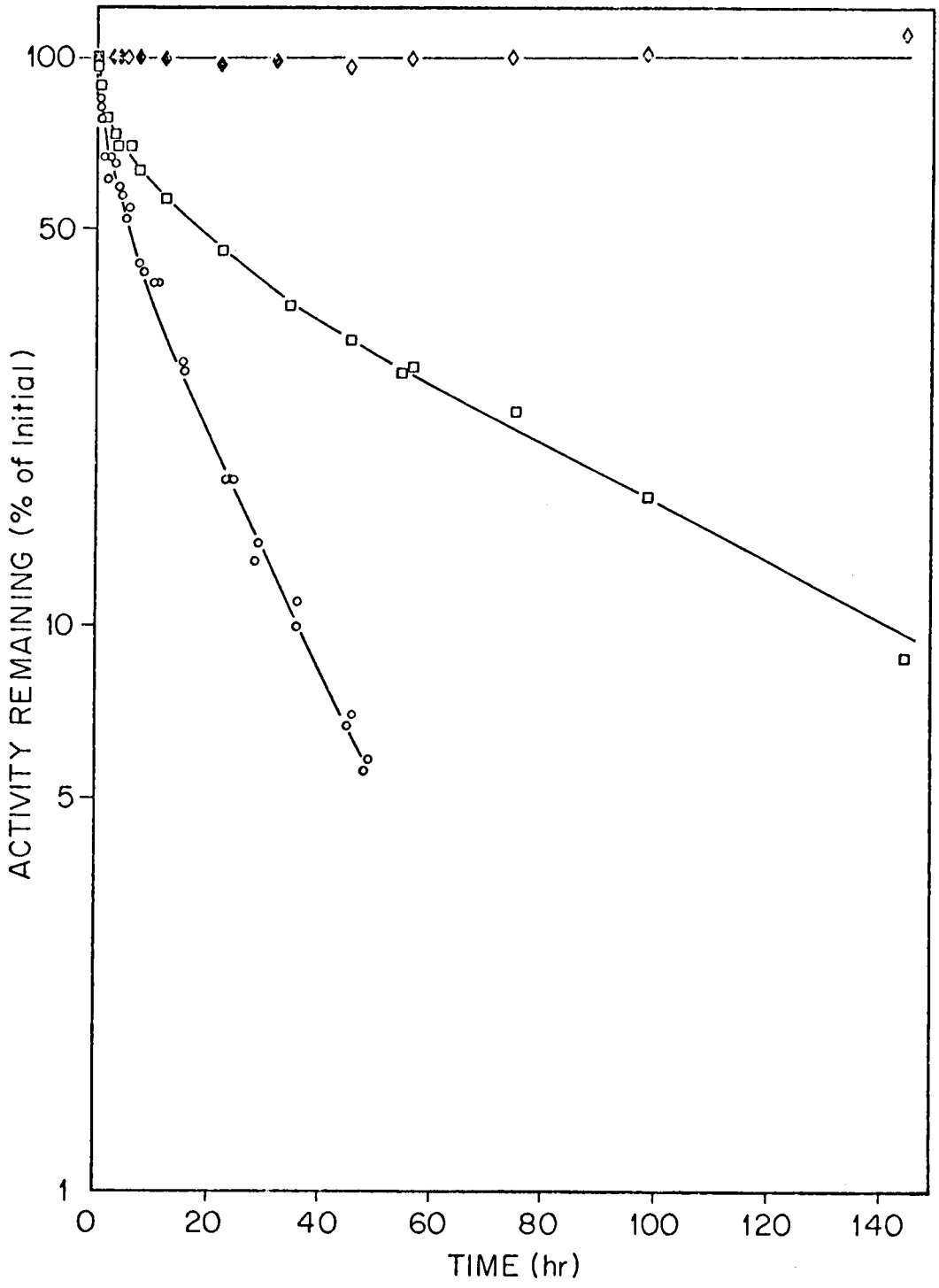
100
50
10
5
1
ACTIVITY REMAINING (% of Initial)
0
20
40
60
80
100
120
140
TIME (hr)

Figure 8

Figure 9. Incorporation of [^{14}C]BrAcNHEtOP into R. rubrum Rbl-P_2 carboxylase as a function of enzymic activity lost.

Incorporation in the absence (□) and presence (O) of Mg^{2+}. Incorporation is expressed as the difference in incorporation between unprotected enzyme and protected enzyme (CR-P_2 is the protector with and without Mg^{2+}). Experimental details are given in Chapter III.

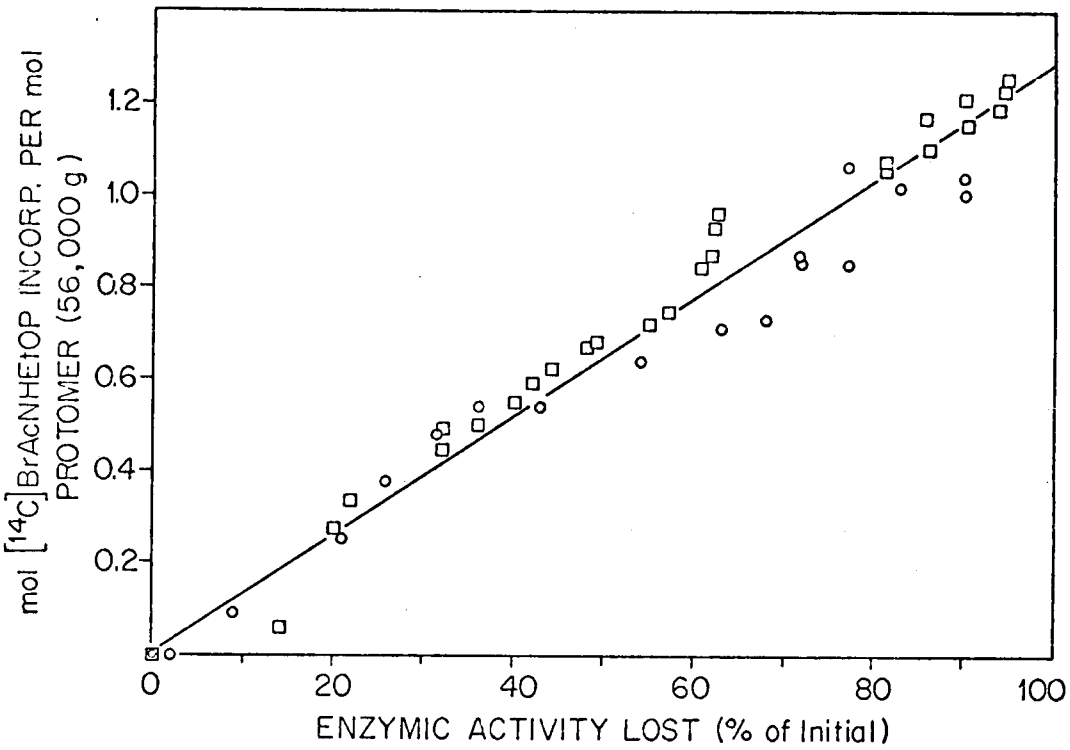
mol [14C]BrAcNHEtOP INCORP. PER mol PROTOMER (56,000 g)
1.2
1.0
0.8
0.6
0.4
0.2
0
20
40
60
80
100
ENZYMIC ACTIVITY LOST (% of Initial)

Figure 9

Figure 10. Chromatographic analysis of acid hydrolysates of [^{14}C]BrAcNHEtOP modified R. rubrum Rb1-P_2 carboxylase.

The samples subjected to analysis are described in Chapter III. Chromatography was done on a Beckman 120C analyzer without use of its ninhydrin system; 2-min fractions were collected and counted. Arrows indicate the elution positions of several amino acids. (A) Modification in the absence of Mg^{2+} with (---) or without (——) protector (0.5 mM CR-P_2). (B) Modification in the presence of Mg^{2+} with (---) or without (——) protector (0.5 mM CR-P_2).

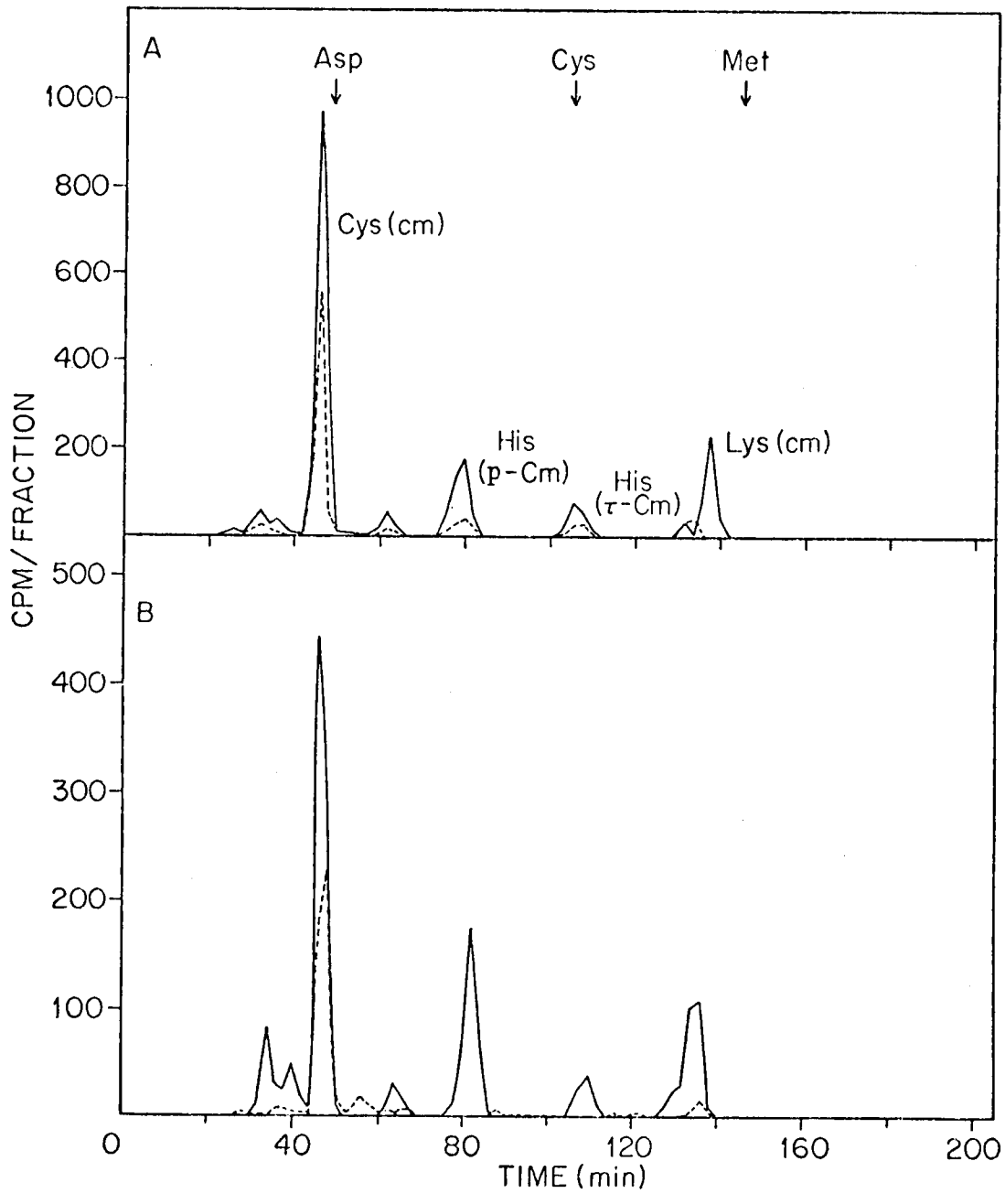
A
Asp
Cys
Met
1000
800
600
400
200
Cys (cm)
His
(p-Cm)
His
(τ-Cm)
Lys (cm)
CPM/FRACTION
500
B
400
300
200
100
0
40
80
120
160
200
TIME (min)

Figure 10

Figure 11. Chromatography on BioRad AG 50 of tryptic digests of R. rubrum Rbl-P_2 carboxylase modified by [^{14}C]BrAcNHEtOP.

Preparation of samples is described in Chapter III. The sample (20 mg) was applied to a column (1 × 24 cm) equilibrated with 0.2 M pyridine/4.8 M acetic acid/0.25% thiodiglycol (pH 3.1). Elution was with a linear gradient composed of 200 mL of the equilibration buffer (initial) and 200 mL of 2 M pyridine/2.5 M acetic acid/0.25% thiodiglycol (pH 5.0) as the limit buffer. (A) The enzyme was modified in the absence of Mg^{2+} with (---) or without (——) protector (0.5 mM CR-P_2). (B) The enzyme was modified in the presence of Mg^{2+} with (---) or without (——) protector (0.5 mM CR-P_2).

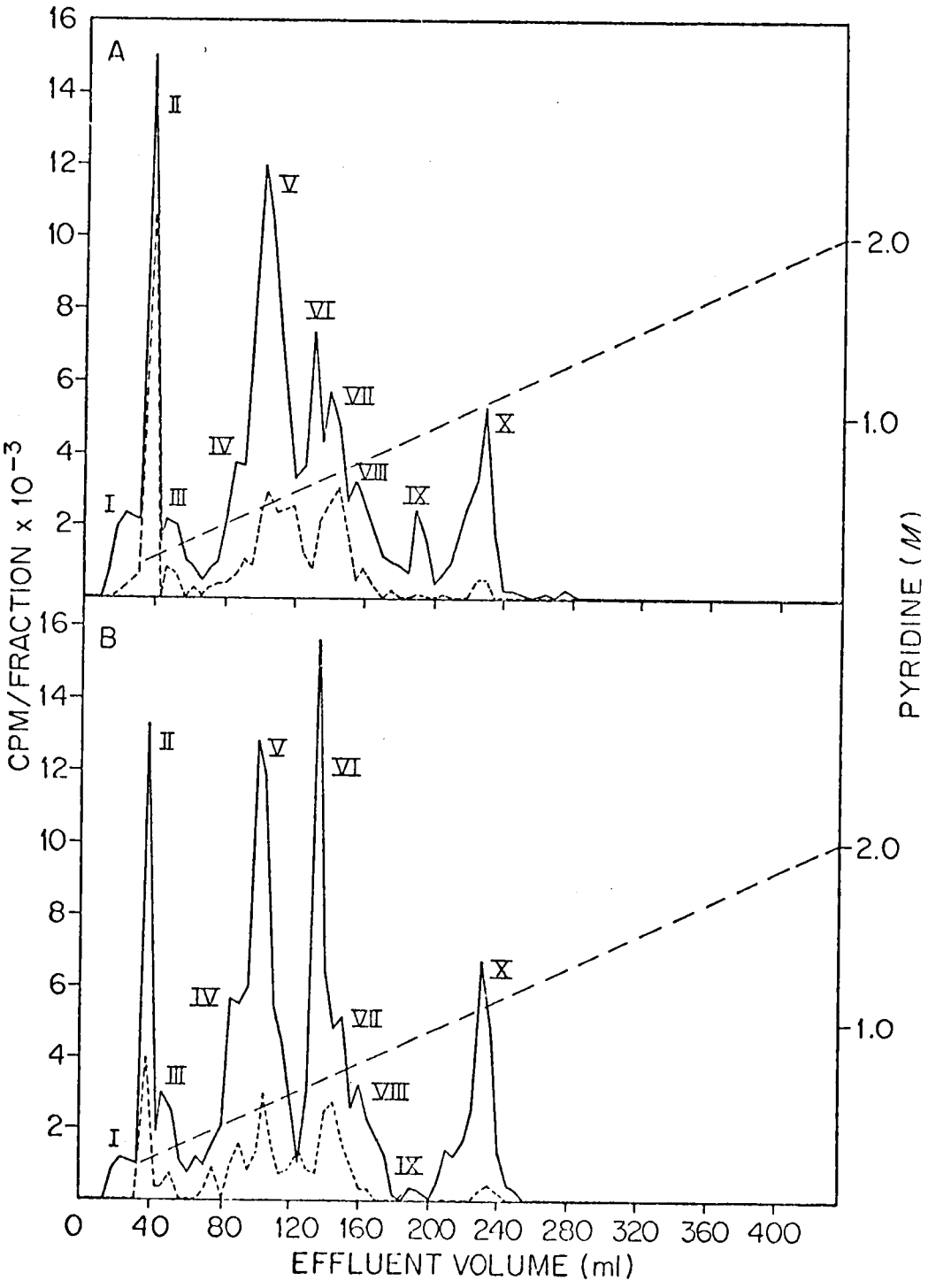
A
B
I
II
III
IV
V
VI
VII
VIII
IX
X
CPM/FRACTION x 10⁻³
PYRIDINE (M)
EFFLUENT VOLUME (ml)
0
40
80
120
160
200
240
280
320
360
400
2
4
6
8
10
12
14
16
1.0
2.0

Figure 11

Figure 12. Simulated profiles for inactivation of a dimer of dissimilar subunits by affinity labeling.

Derivation of the equation used to determine the profiles shown [Percent Activity Remaining = $(e^{-\alpha t} + e^{-\beta\alpha t})50$] is given in Appendix C.

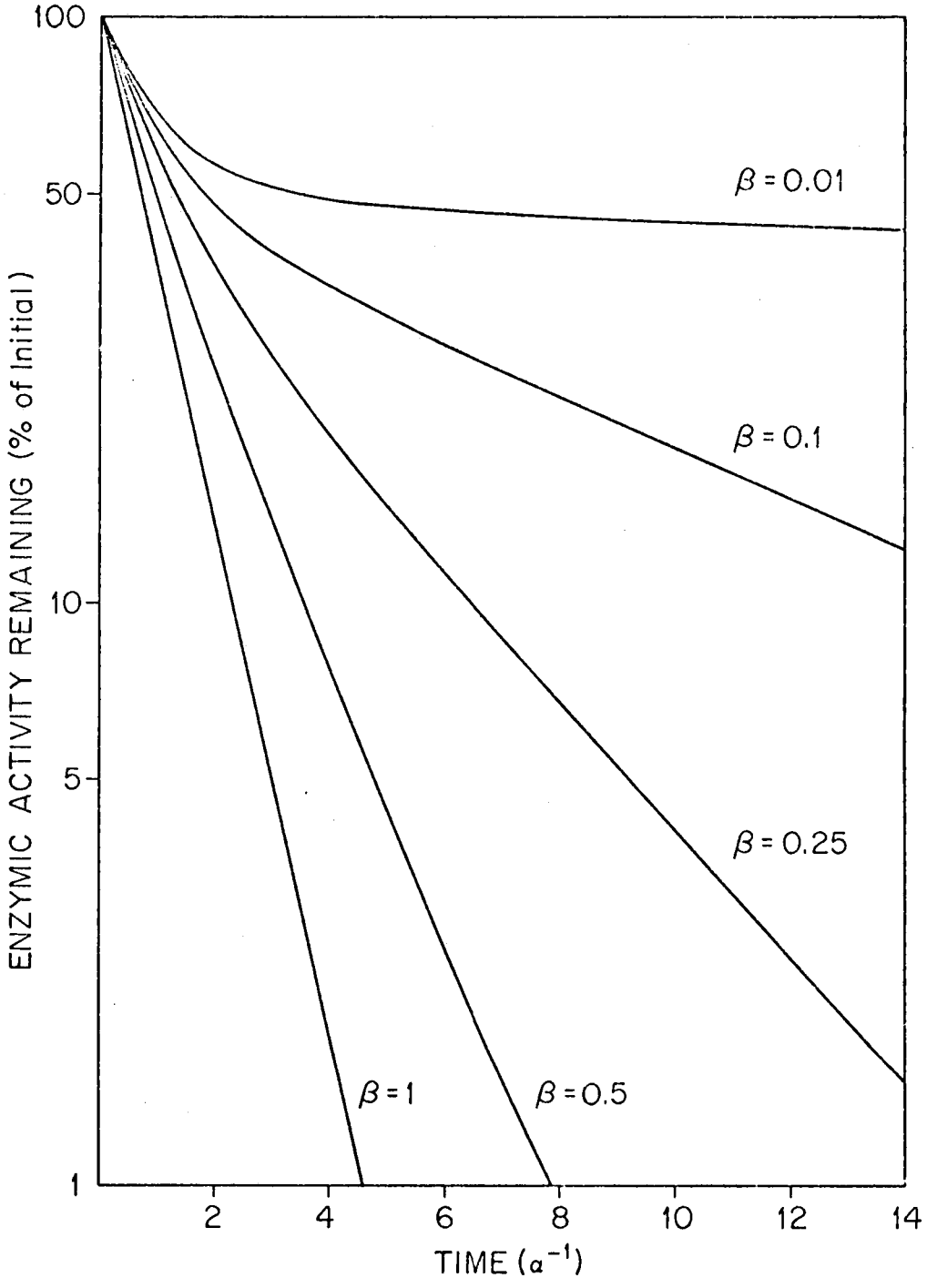
100
50
10
5
1
ENZYMIC ACTIVITY REMAINING (% of Initial)
β = 0.01
β = 0.1
β = 0.25
β = 0.5
β = 1
2
4
6
8
10
12
14
TIME (α⁻¹)

Figure 12

APPENDIX C

DERIVATION OF AN EQUATION FOR "BIPHASIC" INACTIVATION KINETICS

DERIVATION OF AN EQUATION FOR "BIPHASIC" INACTIVATION KINETICS

The kinetic equations which describe "monophasic," pseudo-first-order inactivation of an enzyme by an affinity label have been derived by making either one of two basic assumptions which have been termed "pre-equilibrium" (Roche and McFadden, 1969) and "steady-state" (Gold and Fahrney, 1964; Meloche, 1967) assumptions. These assumptions are completely analogous to those which were classically used to derive the rate equations for enzyme catalyzed reactions (Michaelis and Menten, 1913: Briggs and Haldane, 1925). The scheme for inactivation of an enzyme by an affinity label is as follows:

$$E_f + I \underset{\underline{k}_2}{\overset{\underline{k}_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{\underline{k}_3} E\text{-}I \qquad (1)$$

where E_f represents free enzyme, E·I represents a reversible complex between enzyme and affinity label (I), and E-I is covalently modified enzyme which is enzymically inactive. The total amount of active enzyme at any time, E, is equal to the sum of E_f and E·I. The initial quantity of enzyme (prior to modification by affinity labeling) will be designated by E_0. Rate constants for each step shown above have been designated $\underline{k}_1$, $\underline{k}_2$, and $\underline{k}_3$. The rate of loss of enzymic activity for such a scheme is given in equation (2).

$$\frac{dE}{dt} = -k_3 [E{\cdot}I] \qquad (2).$$

Pre-equilibrium (Baker et al., 1962; Kitz and Wilson, 1962; Schaeffer et al., 1967; Roche and McFadden, 1969) and steady-state (Gold and Fahrney, 1964; Meloche, 1967) derivations differ in the assumption which is made to determine the concentration of enzyme-reagent complex, [E•I]. Pre-equilibrium derivations assume that $\underline{k}_3 << \underline{k}_2$ or that equilibrium in binding the affinity label by the enzyme is attained much more rapidly than the rate of chemical modification. Since at equilibrium the rate of complex formation is equal to the rate of complex dissociation:

$$\underline{k}_1 [E_f][I] = \underline{k}_2 [E \cdot I] \tag{3}$$

and

$$\frac{[E_f][I]}{[E \cdot I]} = \frac{\underline{k}_2}{\underline{k}_1} \tag{4}.$$

The dissociation constant for the reagent-enzyme complex, K_I, is also defined by equation (4), and $K_I = \underline{k}_2/\underline{k}_1$. Thus, the concentration of the enzyme-reagent complex can be defined as follows:

$$K_I = \frac{[E - E \cdot I][I]}{[E \cdot I]} \tag{5}$$

$$[E \cdot I] = \left(\frac{[I]}{K_I + [I]}\right)[E] \tag{6}.$$

Steady-state derivations assume that the concentration of [E•I] is relatively constant, or

$$\frac{d[E \cdot I]}{dt} = 0 \tag{7};$$

thus the rate of formation of [E•I] must equal the rate at which it is lost:

$$\underline{k}_1[E - E\cdot I][I] = \underline{k}_2[E\cdot I]+\underline{k}_3[E\cdot I] \tag{8}$$

and

$$[E\cdot I] = \left(\frac{[I]}{K_I+[I]}\right)[E] \tag{9}$$

where $K_I = (\underline{k}_2+ \underline{k}_3)/\underline{k}_1$. Thus, the steady-state and pre-equilibrium derivations give identical equations for [E•I] which differ only in the definition of K_I. By substitution of equation (6) or (9) into equation (2) the following is obtained:

$$\frac{dE}{dt} = -\underline{k}_3\left(\frac{[I]}{K_I+[I]}\right)[E] \tag{10}$$

$$\int_{E_0}^{E} \frac{dE}{[E]} = -\underline{k}_3\left(\frac{[I]}{K_I+[I]}\right)\int_0^t dt \tag{11}$$

$$\ln {}^{E_0}/_{E} = \underline{k}_3\left(\frac{[I]}{K_I+[I]}\right)t \tag{12};$$

where $t_{1/2}$ is the time required for a 50% loss in enzymic activity:

$$t_{1/2} = \left(\frac{\ln 2}{\underline{k}_3}\right)\left(\frac{K_I}{[I]}\right) + \frac{\ln 2}{\underline{k}_3} \tag{13}.$$

Expressing equation (13) in the nomenclature of Meloche (1967), which was utilized in Part I, Chapter III, $t_{1/2}$ is expressed as τ and $(\ln 2)/\underline{k}_3$ becomes T; equation (13) takes the following form:

$$\tau = \frac{1}{[I]} (TK_I) + T \tag{14}$$

The other standard form of nomenclature is that of Kitz and Wilson (1962) in which $t_{1/2}/\ln 2$ is replaced in equation (13) by $1/\underline{k}_{app}$ (the reciprocal of the apparent pseudo-first-order rate constant):

$$\frac{1}{\underline{k}_{app}} = \left(\frac{1}{\underline{k}_3}\right)\left(\frac{K_I}{[I]}\right) + \frac{1}{\underline{k}_3} \tag{15}$$

In both derivations $E_0 << I$, as no allowance has been made for changes in [I].

To extend the above derivations to include biphasic kinetics, consider the total enzymic activity to be composed of two enzymically active populations, A and B, where

$$E = E_A + E_B \tag{16}.$$

E_A and E_B represent the amounts of enzymic activity contributed by populations A and B at time t, respectively. If A and B are both subject to independent inactivation by an affinity label, but have different affinities for reagent or maximal rates of inactivation, then by expressing both sides of equation (12) as an exponent of e:

$$\frac{E_A}{E_{AO}} = e^{-\underline{k}_A\left(\frac{[I]}{K_A+[I]}\right)t} \tag{17}$$

and

$$\frac{E_B}{E_{BO}} = e^{-\underline{k}_B\left(\frac{[I]}{K_B+[I]}\right)t} \tag{18}$$

where E_{AO} and E_{BO} are the amounts of E_A and E_B at zero time, and $\underline{k}_A$, $\underline{k}_B$, K_A, K_B are $\underline{k}_3$ and K_I for populations A and B. Then, since

$$E_O = E_{AO} + E_{BO} \tag{19}$$

and

$$E_O = f_A E_O + f_B E_O \tag{20}$$

where $f_A = E_{AO}/E_O$ and $f_B = E_{BO}/E_O$, E/E_O would equal [by dividing equation (16) by E_O]:

$$\frac{E}{E_O} = \frac{E_A}{E_O} + \frac{E_B}{E_O} \tag{21}$$

and

$$\frac{E}{E_O} = f_A\left(\frac{E_A}{E_{AO}}\right) + f_B\left(\frac{E_B}{E_{BO}}\right) \tag{22}$$

thus,

$$\frac{E}{E_O} = f_A\, e^{-\underline{k}_A\left(\frac{[I]}{K_A+[I]}\right)t} + f_B\, e^{-\underline{k}_B\left(\frac{[I]}{K_B+[I]}\right)t} \tag{23}.$$

If $\alpha = \underline{k}_A[I]/(K_A+[I])$ and $\beta = \underline{k}_B(K_A+I)/\underline{k}_A(K_B+I)$ then:

$$\frac{E}{E_O} = f_A\, e^{-\alpha t} + f_B\, e^{-\beta\alpha t} \tag{24}.$$

If [I] were not an affinity label (did not form a reversible complex prior to chemical modification), it can be shown that an equation identical to (24) can still be derived. In this case $\alpha = \underline{k}_A[I]$, where $\underline{k}_A[I]$ is the apparent first-order rate constant, and $\beta = \underline{k}_B/\underline{k}_A$, where

$\underline{k}_B/\underline{k}_A$ is the ratio of second-order rate constants for inactivation of B and A. In either case (affinity label or general protein reagent) if the two populations A and B are subunits of a dimeric enzyme, each of which contributes equal amounts of enzymic activity, then $f_A = f_B = 0.5$ and

$$\frac{E}{E_0} = (e^{-\alpha t} + e^{-\beta\alpha t})\ 0.5 \qquad (25).$$

Since the "percent enzymic activity remaining" at time t equals $(E/E_0)100$, equation (25) becomes

$$\text{percent enzymic activity remaining} = (e^{-\alpha t} + e^{-\beta\alpha t})\ 50 \qquad (26).$$

Plots of "percent enzymic activity remaining" against time according to equation (26) are shown in Figure 12, Appendix B.

If an enzyme contains two (or more) catalytically essential residues which are subject to chemical modification, such that modification of either results in complete inactivation, then pseudo-first-order inactivation would be observed. This would be true whether either or both of the residues were modified by affinity labeling or in a bimolecular (no prior complex of enzyme and reagent) fashion, since in all cases dE/dt would be directly proportional to the concentration of unmodified enzyme. Only if modification of one or both residues resulted in partial loss of enzymic activity would nonpseudo-first-order inactivation kinetics result.

VITA

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