REACTION OF RIBULOSEBISPHOSPHATE CARBOXYLASE FROM RHODOSPIRILLUM RUBRUM WITH THE POTENTIAL AFFINITY LABEL 3-BROMO-1,4-DIHYDROXY-2-BUTANONE 1,4-BISPHOSPHATE

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SUMMARY

Under mild conditions, 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate rapidly and irreversibly inactivates ribulosebisphosphate carboxylase from Rhodospirillum rubrum. The substrate ribulosebisphosphate protects the enzyme against inactivation. Incorporation of reagent has been quantitated by reduction of the modified carboxylase with [^{3}H]NaBH$_4$. Based on the difference in the levels of incorporation found in the inactivated enzyme as compared with the protected enzyme, loss of enzymic activity results from the modification of about 0.4 residue per catalytic subunit. Analyses of hydrolysates demonstrate that both cysteinyl and lysyl derivatives are present in the inactivated carboxylase; the protected sample contains about the same amount of modified cysteine but little of the modified lysine. Thus, inactivation appears to correlate with derivatization of lysyl residues.

Br-butanone-P$_2$‡ is an affinity label for Rbl-P$_2$ carboxylase (EC 4.1.1.39) from spinach (1,2). Inactivation results from the modification of two different lysyl residues that appear to be located within the binding domain for Rbl-P$_2$. Although the previous studies provided evidence for the presence of lysyl residues at or near the active site, they neither verified nor excluded the possibility that these residues play a role in catalysis. Since interpretation of chemical modification data in terms of catalytic functionality is fraught with uncertainties (3), we wished to

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‡Abbreviations used: Br-butanone-P$_2$, 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate; Rbl-P$_2$, D-ribulose 1,5-bisphosphate; KES, 50 mM potassium phosphate/10 mM 2-mercaptoethanol/1 mM EDTA (pH 7.3).

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ascertain if the presence of essential lysyl residues is a property common to Rbl-P_2 carboxylases from diverse species. Therefore, we have investigated the reaction of Br-butanone-P_2 with the carboxylase from R. rubrum (a purple non-sulfur bacterium), an organism evolutionarily distant from spinach and a member of the most primitive Family (Athiorhodaceae) of organisms that contain Rbl-P_2 carboxylase (4-6). If active-site lysyl residues have been conserved during evolution, the likelihood is greater that such residues are important in catalysis.

Aside from evolutionary arguments, comparisons of carboxylases from spinach and R. rubrum, because of gross differences in structure and enzymatic properties of the two enzymes, provide an especially stringent test case for the determination of whether essential lysyl residues represent a species-invariant feature. Some of the more striking differences are the following: (a) Spinach and R. rubrum carboxylases are immunologically distinct (6). (b) Spinach carboxylase has a molecular weight of 560,000 with two types of subunits (56,000 and 14,000 daltons) and eight protomeric units (7), whereas the carboxylase from R. rubrum is a simple dimer of identical subunits of 56,000 daltons (6). (c) The absolute requirement for divalent metal ions is satisfied only with Mg^{2+} in the case of the R. rubrum enzyme (5), but Co^{2+}, Ni^{2+}, and Mn^{2+} can substitute for Mg^{2+} in the spinach enzyme (7). (d) Spinach carboxylase is strongly inhibited by 6-phosphogluconate while the enzyme from R. rubrum is not (6,8). The observed inhibition of the spinach enzyme is competitive with Rbl-P_2, and therefore the lack of inhibition of the R. rubrum enzyme may reflect differences in active-site topology (6,8). (e) The amino acid compositions of the two enzymes differ significantly (6).

MATERIALS AND METHODS

Br-butanone-P_2 was synthesized as previously described (9). All biologicals used in carboxylase assays were purchased from Sigma Chemical Co. [^{3}H]$NaBH_4$ was purchased from Amersham/Searle Corp; hydroxyapatite was a product of Clarkson Chemical Co.; and DEAE-cellulose (DE-52) and DEAE-Sephadex (A-50) were obtained from Whatman and Pharmacia, respectively.

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. (10). The nitrogen source was 0.1% $(NH_4)_2SO_4$ instead of L-glutamate. A heterotrophically grown culture (1.5 liters) was used as inoculum for a 40-liter autotrophic culture, that consisted of the Ormerod medium without malate and was sparged with 7% CO_2 in H_2 at 30 liters/hr. A New Brunswick MicroFerm Laboratory Fermentor was used to maintain the temperature of the culture between 31 and 33°C and to stir the culture continuously at 60 rpm. Two sides of the 30 X 30 X 60 cm^3 glass carboy were lined with twelve 60-watt Lumiline lamps, and a 200-watt medium flood lamp was used to illuminate a third side. Cultures were usually harvested 2 weeks after inoculation.

Assay of Carboxylase--Rbl-P_2 carboxylase activity was determined by the spectrophotometric assay of Racker (11) under conditions described previously (2). Protein concentration was determined from the absorbance at 280 nm assuming an $E^{1\%}_{1cm}$ of 9.74 (6).

Purification of Rbl-P_2 Carboxylase--The bacterial cell paste obtained by continuous-flow centrifugation was resuspended (1:10 w/v) in cold KES buffer, then homogenized with a Gaulin press, brought to 35% saturation in ammonium sulfate, and centrifuged. The supernatant was brought to 60% saturation in ammonium sulfate, and the precipitate was collected by centrifugation. This pellet was dissolved in KES, and the solution was dialyzed exhaustively against KES. Then the sample was chromatographed on a DEAE-cellulose column (2.5 X 24 cm) with a 2-liter linear gradient of KES (0.05 – 0.15 M in potassium phosphate). Fractions containing the enzymic activity were concentrated by adding solid $(NH_4)_2SO_4$ to 90% saturation. The precipitate was collected by centrifugation and dialyzed exhaustively against KES. After dialysis the sample was applied to an A-50 DEAE-Sephadex column (2.5 X 24 cm) and eluted with a 2-liter linear gradient of 0 – 0.3 M NaCl in KES. Fractions containing carboxylase activity were pooled and concentrated by pressure dialysis using a Diaflo PM-30 (Amicon Corp.) membrane filter. During pressure dialysis the buffer was changed to 10 mM Tris-HCl/10 mM 2-mercaptoethanol/1 mM EDTA/1 mM potassium phosphate (pH 7.6). The concentrate was passed through a column of hydroxyapatite (1.2 X 9.5 cm). Rbl-P_2 carboxylase, which was not retained by the hydroxyapatite, was brought to 0.05 M in phosphate with 1 M potassium phosphate (pH 7.3) and stored at 4°C until needed. The enzyme appeared to have a minimal purity of 90% as judged by disc gel electrophoresis. The purification scheme, summarized in Table 1, is included because it provided the enzyme used in this study before the isolation procedure of Tabita and McFadden (5) appeared in the literature.

Modification of Rbl-P_2 Carboxylase with Br-butanone-P_2--Prior to chemical modification the purified enzyme was dialyzed exhaustively against Mg^{2+}-free (by passage through Bio-Rad Chelex 100 resin) 0.05 M Bicine/0.066 M $NaHCO_3$/ 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 (2,9). Comparisons were made between carboxylase exposed to Br-butanone-P_2 in the presence and absence of 1 mM Rbl-P_2.

Alkylation of the enzyme by Br-butanone-P_2 was quantitated by reducing the carbonyl of the incorporated reagent with [^{3}H]$NaBH_4$ (0.01 M), whose specific activity was determined by reduction of a model compound (the reaction product of chloroacetol phosphate and glutathione) as described previously (2). All data presented have been corrected for tritium incorporation into control (unalkylated) enzyme.

Total acid hydrolysis of native and alkylated carboxylase was achieved with 6 N HCl/0.1 M 2-mercaptoethanol in evacuated (<50 μm Hg) sealed tubes at 110°C for 21 hr. Hydrolysates were assayed on a Beckman 120C amino acid

TABLE 1

Purification Scheme for Rbl-P_2 Carboxylase from R. rubrum

Step	Total Units*	Total Protein† (mg)	Specific Activity (units/mg)	Per cent Recovery
Homogenate (from 55 g of bacteria by wet weight)	382	--	--	100
35 to 60% saturated $(NH_4)_2SO_4$ precipitate	276	7310	0.038	72
Chromatography on DEAE-cellulose	244	348	0.70	64
Chromatography on DEAE-Sephadex	93.4	52.9	1.77	24
Chromatography on hydroxyapatite	43.7	13.5	3.24	11

*One unit of activity is the carboxylation of 1 μmole of Rbl-P_2/min at 25°C; in the assay used it represents a change in $\underline{A}_{340\ nm}$ of 24.9/min.

†Protein concentration was estimated from $\underline{A}_{280\ nm}$. No corrections are made for turbidity or nucleic acids present in the crude preparations.

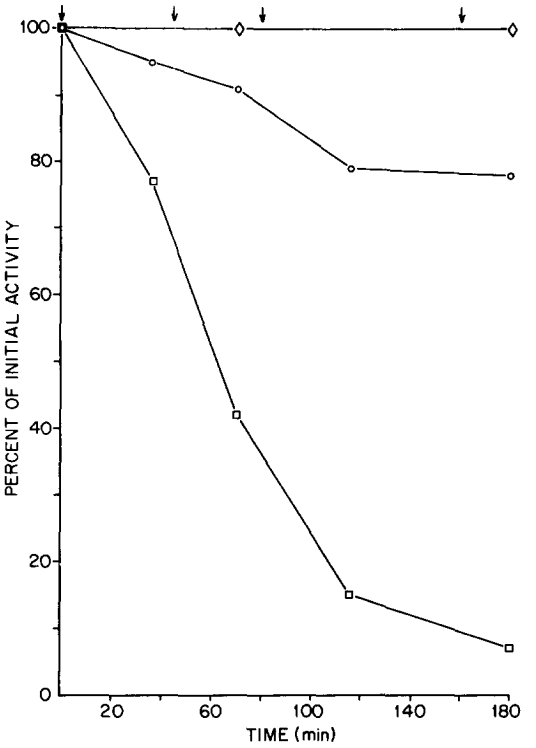

Fig. 1. Time-course of inactivation of Rbl-P_2 carboxylase by Br-butanone-P_2 in the absence (□) and presence (○) of Rbl-P_2. The control (◇) lacked both reagent and substrate. Arrows indicate the times at which Br-butanone-P_2 was added. Experimental conditions are given in Materials and Methods.

analyzer according to the procedure of Spackman et al. (12). Radioactivity was measured by use of a Triton X-100/toluene (1:2 v/v) cocktail as described by Patterson and Greene (13) with 5.5 g/liter of Permablend I (Packard).

RESULTS

Br-butanone-P_2 inactivates Rbl-P_2 carboxylase from R. rubrum, and Rbl-P_2 protects against inactivation (Fig. 1). Samples of the untreated, inactivated, and substrate-protected carboxylase were reduced with [^{3}H]$NaBH_4$ in the presence of 4 M guanidinium chloride 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) (6,14), whereas the protected enzyme contained only 0.8 molar

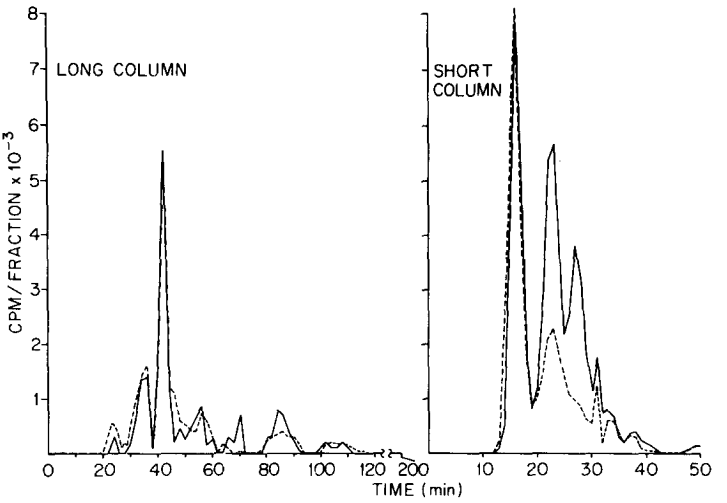

Fig. 2. Radioactivity in hydrolysates of Rbl-P_2 carboxylase after treatment with Br-butanone-P_2, in the absence (——) and presence (- - -) of Rbl-P_2, followed by reduction with [^{3}H]$NaBH_4$. The hydrolysates were chromatographed on the amino acid analyzer without use of the ninhydrin system, and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

equivalents of reagent. Since the enzyme is a dimer (6), 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 (Fig. 2). Hydrolysates of both the inactivated and protected samples contain 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 (2,9) 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]$NaBH_4$, we have 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 (2) 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.

DISCUSSION

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 in the case of the spinach enzyme, two chromatographically distinct lysyl derivatives are formed. Based on studies with model compounds (9), 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) could be due to heterogeneity in the carboxylase preparation, presence of inactive (nonfunctional) carboxylase that could be less reactive toward the reagent as is the case with the spinach enzyme (1), or half-of-sites reactivity (15).

Since the initial suggestion (2) of the presence of essential lysyl residues in spinach Rbl-P_2 carboxylase, pyridoxal 5'-phosphate has been shown

to inactivate the R. rubrum carboxylase (16), presumably by forming a Schiff base with an ε-NH_2 group of a lysyl residue in the vicinity of the active site. Lorimer et al. (17) have provided evidence that a lysyl residue is also involved in the allosteric regulation of the spinach carboxylase by CO_2, whereby activation results from CO_2 combining with an ε-NH_2 group to form a carbamate. In view of the dissimilarities of spinach and R. rubrum carboxylases enumerated in the introduction, the present finding that the R. rubrum carboxylase is subject to inactivation via lysyl modification by the same affinity label used with the spinach enzyme strongly suggests that lysyl residues are essential to catalysis. We are now attempting to isolate tryptic peptides from the R. rubrum carboxylase that contain the essential lysyl residue(s) to determine if homology exists with the peptides purified previously (2) from the spinach enzyme.

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