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3-Bromo-2-Butanone 1,4-Bisphosphate as an Affinity Label for Ribulosebisphosphate Carboxylase

(active site/reactive analog)

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ABSTRACT **3-Bromo-2-butanone 1,4-bisphosphate has been synthesized in an attempt to find a reactive analog of ribulose 1,5-bisphosphate for labeling the active site of ribulosebisphosphate carboxylase (EC 4.1.1.39). The reagent irreversibly inactivates the carboxylase from spinach, and several observations suggest that the inactivation results from modification of an active-site residue: (1) Ribulose 1,5-bisphosphate protects against inactivation. (2) The extent of reagent incorporation shows that modification of one residue per catalytic site can account for the inactivation. (3) Comparisons of autoradiograms of peptide maps prepared from carboxylase treated with the ^{32}P-labeled reagent in the absence and presence of substrate indicate that inactivation results from a fairly selective modification. (4) Although the reagent's greatest inherent reactivity is toward sulfhydryl groups, inactivation of the enzyme is due to alteration of an amino-acid residue other than cysteine.**

The initial step in the photosynthetic carbon cycle (1) is the carboxylation of ribulose 1,5-bisphosphate (ribulose 1,5-P_2) to form 3-phosphoglycerate, a reaction catalyzed by ribulosebisphosphate carboxylase (EC 4.1.1.39) (2–4). A thorough characterization of the carboxylase is of particular interest in view of recent evidence that suggests that the enzyme is also responsible for photorespiration in plants; i.e., it possesses oxygenase activity and catalyzes the oxygenation of ribulose 1,5-P_2 to form phosphoglycolate and 3-phosphoglycerate (5, 6). The proposed mechanism (see ref. 7 for a detailed description) for the carboxylation may also be applicable to the oxygenation (6). The initial step presumably involves isomerization of ribulose 1,5-P_2 to the corresponding C2-C3 enediol (8, 9), thereby developing a nucleophilic center at C2 to which either CO_2 or O_2 could add. The products are then formed by hydrolytic cleavage between C2-C3. Strong support for the existence of a six-carbon intermediate in the carboxylation reaction is that 2-carboxy-D-ribitol 1,5-P_2 (a stable analog of the proposed intermediate) is a potent inhibitor of the carboxylase (10). Most recently the proposed intermediate, 2-carboxy-3-ketoribitol 1,5-P_2, has been chemically synthesized and shown to undergo spontaneous hydrolytic cleavage to yield 3-phosphoglycerate (11).

Despite the partial elucidation of the mechanism of action of ribulosebisphosphate carboxylase, the functional groups of the enzyme that are essential to the catalytic process have not been identified. This probably reflects the difficulty of using general protein reagents to characterize the active site of such a complex enzyme. The enzyme from spinach has a molecular weight of 560,000 (12) and contains two kinds of subunits (13). Since a high degree of specificity for active-site residues can be obtained with affinity-labeling reagents, we have attempted to design such a reagent for ribulosebisphosphate carboxylase. In this report, we describe the reaction of a newly designed alkylating agent, 3-bromo-2-butanone 1,4-bisphosphate, with the carboxylase from spinach.

Abbreviations: Bicine, *N,N*-bis(2-hydroxyethyl)glycine; ribulose 1,5-P_2, ribulose 1,5-bisphosphate.

MATERIALS AND METHODS

Ribulosebisphosphate carboxylase was isolated from spinach by the method of Wishnick and Lane (14), except that the final step (chromatography on hydroxylapatite) was omitted. The preparations were at least 90% pure as judged by disc-gel electrophoresis. Sodium [^{14}C]bicarbonate, diphenyl [^{32}P]-phosphorylchloridate, and sodium [^{3}H]borohydride were obtained from Amersham/Searle Corp. Bicine [*N,N*-bis(2-hydroxyethyl)glycine] and biological materials used in assays for carboxylase activity were products of Sigma Chemical Co. Trypsin treated with L-(tosylamido-2-phenyl)ethyl chloromethyl ketone was purchased from Worthington Biochemical Corp.

Carboxylase activity was determined either by the spectrophotometric method of Racker (15) or by the [^{14}C]bicarbonate method described by Wishnick and Lane (14). By the latter procedure, the freshly prepared carboxylase used in this study had a specific activity of 1.3 units/mg, in reasonable agreement with values of 1.5 units/mg reported previously (14). Protein sulfhydryl groups were quantitated with Ellman's reagent (16). Peptide mapping and autoradiography were carried out as described (17). Radioactivity was assayed with a Packard 3003 liquid scintillation spectrometer. Protein hydrolysates, prepared by hydrolysis under reduced pressure for 21 hr with 6 N HCl containing 0.1 M 2-mercaptoethanol, were chromatographed on a Beckman 120C amino-acid analyzer (18).

RESULTS

Synthesis of 3-Bromo-2-Butanone 1,4-Bisphosphate. The title compound was synthesized as shown in Fig. 1. The diethyl ketal of bromobutanone bisphosphate (I) was isolated as a crystalline tetrakiscyclohexylammonium salt, whose elemental analysis was within experimental error of theory. To prepare the ^{32}P-labeled compound, diphenyl [^{32}P]phosphorylchloridate was used in the phosphorylation step. Incubation of the free acid of the ketal for 2 hr at 40° resulted in almost complete conversion to the corresponding ketone (II). At the end of this incubation period, paper chromatography in butanol–acetic acid–water (7:2:5) showed a trace of the ketal (R_F, 0.58) remaining and a single new phosphate ester (R_F, 0.32),

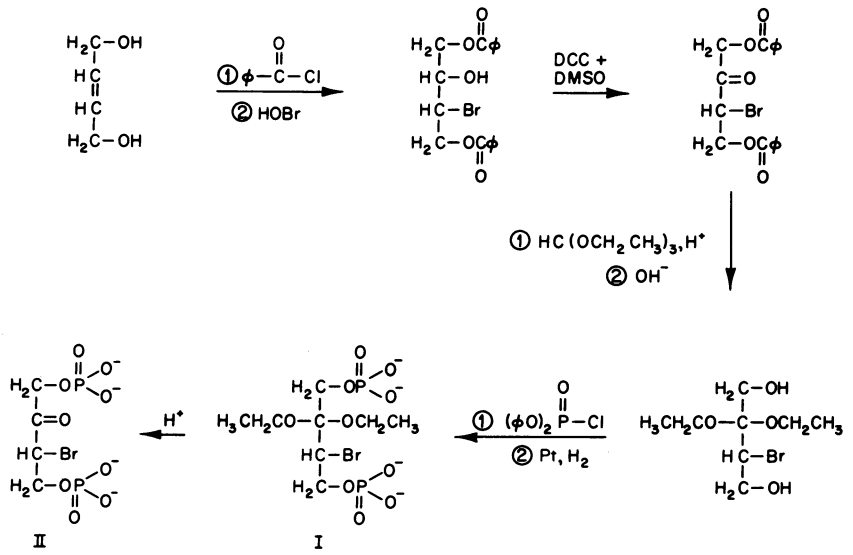

FIG. 1. Scheme for the synthesis of 3-bromo-2-butanone 1,4-bisphosphate. *DMSO*, dimethylsulfoxide; *DCC*, dicyclohexylcarbodiimide.

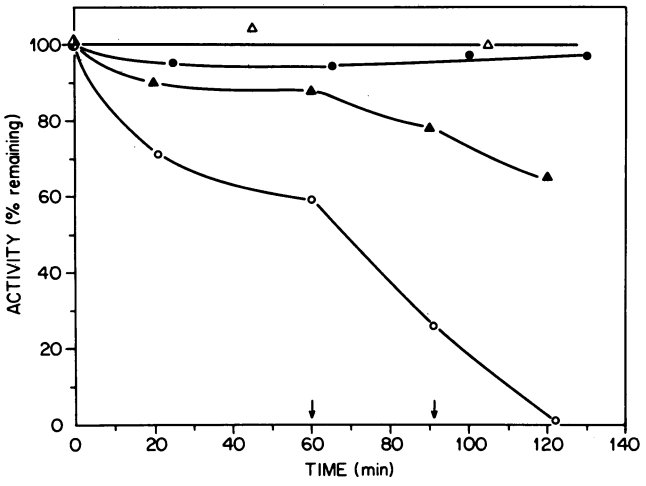

FIG. 2. Inactivation of ribulose-P_2 carboxylase by bromobutanone-P_2. Solutions of ribulose-P_2 carboxylase (5 mg/ml; 0.071 mM in protomeric units) in 0.1 M Bicine–0.06 M potassium bicarbonate–1 mM EDTA (pH 8.0) with (●) and without (○) ribulose-P_2 (0.5 mM) were incubated at room temperature with bromobutanone-P_2. In one experiment, bicarbonate was excluded from the reacture mixture (▲). Successive additions of the reagent were made at the times indicated by the *arrows;* each addition gave a final reagent concentration of 0.1 mM in the reaction mixture. Just before each addition of reagent, aliquots of the enzyme solutions were assayed for carboxylase activity. An enzyme solution under the same conditions but lacking the reagent served as control (△).

which gave a positive bromine test. Solutions of the presumed bromobutanone-P_2 contained 2 molar equivalents of total organic phosphate and 1 molar equivalent of alkaline-labile phosphate. The molar concentration of bromobutanone-P_2 was assumed to be equivalent to the concentration of alkaline-labile phosphate. No attempts were made to isolate the reagent from solution. Solutions of the free acid form of bromobutanone-P_2 could be stored in the freezer for several months without appreciable decomposition.

Inactivation and Substrate Protection. Incubation of ribulose-P_2 carboxylase with bromobutanone-P_2, under conditions of pH and ionic strength normally used to assay the enzyme, results in a rapid loss of enzymatic activity. Essentially complete inactivation is achieved by the successive addition of several aliquots of the reagent to the reaction mixture (Fig. 2). Ribulose-P_2 affords protection against inactivation, and omission of bicarbonate from the reaction solution reduces the rate of inactivation (Fig. 2).

Reagent Incorporation and Sulfhydryl Modification. Samples of carboxylase with and without ribulose-P_2 were treated with bromobutanone-P_2 and then dialyzed exhaustively to remove the unreacted reagent. After dialysis, the protein samples were assayed for radioactivity and free sulfhydryl groups (Table 1). In the case of the substrate-protected sample, the loss of sulfhydryl groups is much larger than the observed amount of reagent incorporated. Thus, it seems likely that one of the phosphate groups of the protein-bound reagent moiety is lost before the radioactivity assays. Based on the incorporation data calculated with the specific activity of the reagent (a bisphosphate) and given the possibility that these values could be as much as 100% low due to the hydrolysis of one phosphate group, the inactivation results from modification of two to four residues per mol of carboxylase.

Residues Modified. Since the ratio of reagent incorporation to sulfhydryl modification is larger in the inactivated enzyme than in the substrate-protected enzyme, we felt that inactivation might be due to alkylation of a residue(s) other than sulfhydryl. To test this possibility, we examined acid hydrolysates of modified carboxylase by chromatography on an amino-acid analyzer. To introduce an acid-stable radioactive marker we incubated the enzyme, which had been treated with unlabeled reagent, with [^{3}H]borohydride. This procedure reduces the carbonyl group of the reagent moiety to a hydroxyl group, with the concomitant incorporation of tritium. For a chromatographic marker we used the *S*-alkyl cysteine derivative prepared by treating glutathione with bromobutanone-P_2 followed by borohydride reduction and hydrolysis. Elution

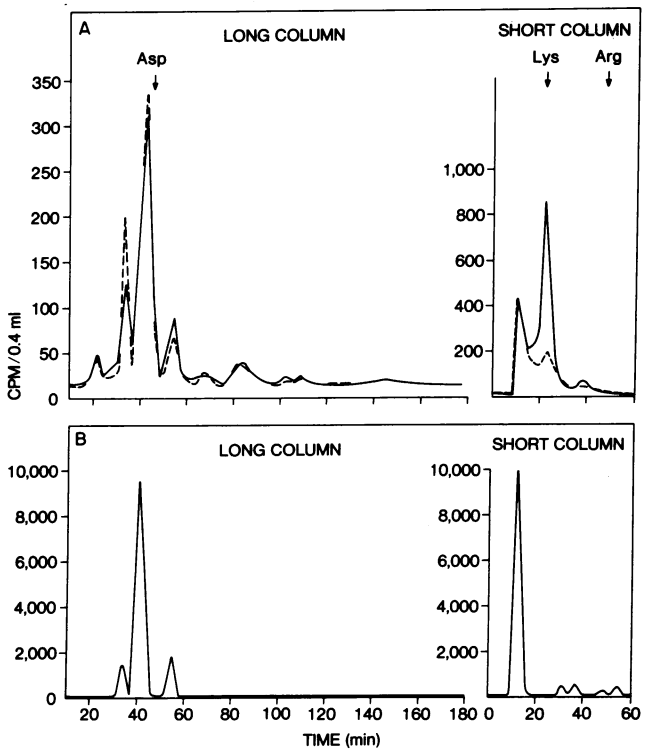

FIG. 3. Chromatographic profiles of radioactive components present in acid hydrolysates of carboxylase (*A*) and glutathione (*B*) after treatment with bromobutanone-P_2 followed by reductions with [^{3}H]$NaBH_4$. Samples of carboxylase were treated with the reagent in the absence (——) and presence (– – –) of ribulose-P_2 in a fashion identical to that described in the legend to Fig. 2. Excess reagent was removed by dialysis against 0.1 M NaCl, and then the protein solutions were made 0.1 M in $NaHCO_3$ and incubated for 30 min at 4° with 0.01 M [^{3}H]$NaBH_4$ (150 Ci/mol). After further dialysis against 0.1 M NaCl, aliquots were hydrolyzed for chromatography on the amino-acid analyzer. Fractions of the effluents, after passing through the colorimeter, were collected at 3-min intervals and assayed for radioactivity. The absorbance at 570 nm is not shown, but the elution positions of certain amino acids are indicated by *arrows*.

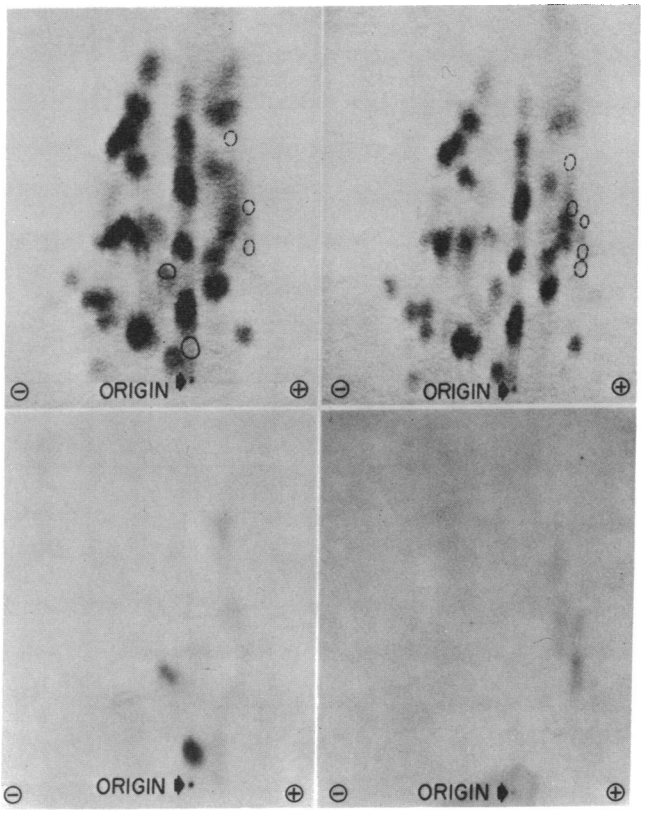

FIG. 4. Peptide maps and autoradiograms of inactivated (*left*) and substrate-protected (*right*) carboxylase. The carboxylase samples were prepared as described in the legend to Fig. 2, except that the ^{32}P-labeled reagent was used. The modified proteins were reduced with unlabeled $NaBH_4$ (see legend to Fig. 3), carboxymethylated with iodoacetate (see ref. 17), and digested with trypsin (1% by weight) in 0.1 M NH_4HCO_3 for 4 hr at 40°. The major radioactive peptides on the maps are encircled with *solid lines;* the minor radioactive peptides are encircled with *broken lines.*

profiles for radioactivity are shown in Fig. 3. Virtually all of the radioactivity in hydrolysates of the substrate-protected carboxylase elutes from the long column of the amino-acid analyzer at positions that coincide with the elution positions of components in hydrolysates of the glutathione derivative. An additional radioactive componennt, which elutes from the short column near the position of lysine and, therefore, cannot be a cysteine derivative, is found in hydrolysates of inactivated carboxylase.

Specificity of Modification. To determine the degree of specificity in the modification of ribulose-P_2 carboxylase by bromobutanone-P_2, we have used peptide mapping. The enzyme was labeled with [^{32}P]reagent, reduced with borohydride as a precautionary measure to increase the stability of the protein-reagent bond, carboxymethylated with iodoacetic acid to eliminate free sulfhydryl groups, and finally digested with trypsin. Radioactive components on the maps were detected by autoradiography (Fig. 4). The presence of about five very minor radioactive peptides in the digest of the substrate-protected sample suggests rather random modification of sulfhydryl groups. The digest of the inactivate carboxylase contains two major radioactive peptides, one present in considerably greater amount than the other. Thus, the inactivation can be accounted for by the modification of at most two amino-acid residues. A single residue may be involved in the inactivation reaction, since the proteolytic digestion can give rise to more than one peptide containing the same modified residue.

DISCUSSION

Previous chemical modification studies on ribulose-P_2 carboxylase have been concerned with the role of protein sulfhydryl groups. Since the first observations (2, 19) that carboxylase activity is inhibited by sulfhydryl reagents, several groups (20–22) have attempted to elucidate the function of sulfhydryls in the enzyme. Although conflicting conclusions have been reached (for a discussion, see ref. 23), one possibility is that a sulfhydryl group is close to the binding site for ribulose-P_2 and may be involved in the catalytic process (20).

In the present study on the reaction of a potential affinity-labeling reagent with ribulose-P_2 carboxylase, we also observe alkylation of sulfhydryl groups but do not believe this to be the cause of inactivation for two reasons. Firstly, although ribulose-P_2 protects against inactivation (Fig. 2), it does not prevent alkylation of sulfhydryl groups; in fact, we consistently observe a greater loss of free sulfhydryl groups in the

TABLE 1. *Extent of reagent incorporation and sulfhydryl modification during the reaction of bromobutanone-P_2 with ribulose-P_2 carboxylase*

Sample	Enzymatic activity (% remaining)	Molar equivalents of reagent per 560,000 daltons	Molar equivalents of SH per 560,000 daltons	No. of SH groups modified
Native	100	—	93	—
Substrate-protected	95	3.6	84	9
Inactivated	1	5.8	86	7

Carboxylase in the absence and presence of ribulose-P_2 was treated with bromobutanone-P_2 as described in the legend to Fig. 2, except that ^{32}P-labeled reagent (8.45×10^5 cpm/μmol) was used. Subsequent to the modification, the excess reagent was decomposed with 2-mercaptoethanol (0.01 M) and the protein samples were dialyzed exhaustively against 0.05 M NaCl–0.1 mM EDTA–0.1 mM 2-mercaptoethanol. Samples were then assayed for radioactivity and sulfhydryl content. The sulfhydryl assays were corrected for the 2-mercaptoethanol present in the dialysate.

protected samples than in the inactivated samples (Table 1). Secondly, in the substrate-protected carboxylase, modified amino-acid residues other than cysteine derivatives cannot be detected, whereas the inactivated carboxylase contains an amino-acid derivative in addition to products of cysteine alkylation (Fig. 3).

Several kinds of observations, in addition to the protection by ribulose-P_2, suggest that bromobutanone-P_2 inactivates the carboxylase by reaction with an essential residue in the vicinity of the active site. Studies on the reaction of the reagent with free amino acids (data not shown) demonstrate that the greatest reactivity by far is toward the sulfhydryl group of cysteine. The finding that modification of a functional group other than sulfhydryl is responsible for the inactivation may be taken as an indication that the reagent, as a result of its interaction with the binding site for ribulose-P_2, reacts with an essential residue with unusual properties. Consistent with this interpretation is the apparent correlation of inactivation with the modification of a small number of residues (perhaps only one per catalytic site), as indicated by the overall incorporation data (Table 1) and confirmed by peptide mapping (Fig. 4). The stimulatory effect of bicarbonate on the inactivation rate (Fig. 2) could be due to a conformational change that results in a more favorable alignment between the reagent and the residue that becomes modified. Magnesium ions, which are essential to activity (2) but not required for binding or ribulose-P_2 (24), have no effect on the rate of inactivation.

Although inactivation kinetics can be very useful for demonstrating that a reagent is active-site-directed (25), the instability of bromobutanone-P_2 (data not shown) has precluded a detailed kinetic analysis of its reaction with the carboxylase. We believe that the relatively large molar excesses of reagent needed to achieve total inactivation of the carboxylase reflect the reagent's decomposition.

Correlation of inactivation with the modification of only two to four residues per mol of an enzyme that contains eight substrate-binding sites (24) presents an enigma; however, these low levels of incorporation can be rationalized to provide additional evidence of an active-site-specific modification. Homogeneous preparations of ribulose-P_2 may not be fully active, since their turnover numbers cannot account for the rates of CO_2 fixation in intact plant cells (4). Thus, the reagent may react only with sites that are catalytically functional. Consistent with this possibility are our studies with enzyme that had lost 90% of its initial enzymatic activity during storage. In that case, inactivation was correlated with the incorporation of less than 0.5 residue per mol of enzyme. Another factor to consider is that the number of binding sites for ribulose-P_2 decreases from eight to four upon increasing the buffer concentration from 0.01 to 0.25 M (24).

The kind of amino-acid residue whose modification results in inactivation has not been identified, but the derivative's basic character (elution from the short column of the amino-acid analyzer close to lysine) suggests histidine. It is possible that the radioactive component that elutes from the short column is a degradation product and not an intact alkylated amino acid. Such a situation would nullify the hypothesis that the radioactivity represents a histidine derivative.

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