INACTIVATION OF RIBULOSEBISPHOSPHATE CARBOXYLASE/OXYGENASE FROM SPINACH WITH THE AFFINITY LABEL N-BROMOACETYLETHANOLAMINE PHOSPHATE

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Summary: N-Bromoacetylethanolamine phosphate irreversibly inactivates both activities of spinach ribulosebisphosphate carboxylase/oxygenase. Loss of enzymic activity follows pseudo first-order kinetics, shows rate saturation, and can be prevented by saturating levels of ribulosebisphosphate. The catalytically essential cofactor Mg^{2+} stimulates the rate of inactivation but decreases the enzyme's affinity for reagent. Mg^{2+} appears to change the specificity of the reagent from cysteinyl to lysyl residues. Inactivation correlates with modification of 1.6 residues (predominantly cysteinyl) per protomer in the absence of Mg^{2+}, and 1.1 residues (predominantly lysyl) in the presence of Mg^{2+}.

Rbl-P_2‡ carboxylase/oxygenase (EC 4.1.1.39) is a key enzyme in plant metabolism, being essential both to the photosynthetic fixation of CO_2 (1) and to photorespiration (2,3). Although the reaction pathway is reasonably well understood (4), the enzyme's active site has not been characterized thoroughly. Previous studies with the affinity label Br-butanone-P_2 (5–7) and with pyridoxal phosphate (8,9) 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/oxygenase, and thus aid in the elucidation of the reaction mechanism, we have found that BrAcNHEtOP satisfies the major criteria (10–13) of an affinity label for the enzyme from spinach.

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‡The abbreviations used are: Rbl-P_2, D-ribulose 1,5-bisphosphate; BrAcNHEtOP, N-bromoacetylethanolamine phosphate; butanediol-P_2, butane-1,4-diol 1,4-bisphosphate; Br-butanone-P_2, 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate.

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TABLE I

Incorporation

Inactivation conditions	Protector	Initial enzymic activity remaining (%)	Incorporation mol reagent/ mol enzyme	Difference between unprotected and protected	Extrapolated to 100% inactivation	Mol reagent/ mol protomer (70,000 daltons)
No Mg^{2+}	None	7.8	19.4	12.2	13.2	1.65
	1 mM Rbl-P_2	103	7.2			
Plus Mg^{2+}	None	45	6.87	4.78	8.69	1.09
	20 mM butanediol-P_2	94	2.09			

No Mg^{2+}: Mg^{2+}-free 66 mM $NaHCO_3$/50 mM bicine (pH 8.0); 5 mM reagent, 26-hr reaction time.

Plus Mg^{2+}: 5 mM $MgCl_2$/ 66 mM $NaHCO_3$/50 mM bicine (pH 8.0); 1 mM reagent, 16-hr reaction time.

Materials and Methods

Rbl-P_2 carboxylase was purified from freshly harvested spinach leaves by the slight modification (6) of the method of Wishnick and Lane (14). The enzyme (MW 560,000) is composed of eight 70,000-dalton protomers, each consisting of one small (14,000-dalton) and one large (56,000-dalton) subunit (4). Concentrations of carboxylase were determined from A_{280} nm, assuming an $E^{1\%}_{1cm}$ of 16.4 (14). The spectrophotometric assay of Racker (15) was used to determine carboxylase activity. Assay conditions were the same as those described earlier (6). Oxygenase activity was measured at 30°C with an oxygen electrode by the method of Lorimer et al. (16). 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.

All biological materials used in the carboxylase and oxygenase assays were products of Sigma Chemical Co. BrAcNHEtOP, [^{14}C] BrAcNHEtOP, and butanediol-P_2 were synthesized by published procedures (17, 18). In the case of [^{14}C] BrAcNHEtOP, [^{14}C] bromoacetic acid (1.23 mCi/mmol) was purchased from New England Nuclear.

Enzyme (10 mg/ml) was incubated with BrAcNHEtOP at 30°C under the conditions specified in the table and figure legends. Where necessary, buffers were freed of Mg^{2+} by passage through Chelex-100 resin (BioRad). Incorporation of ^{14}C reagent was determined by measuring radioactivity and protein concentrations after exhaustive dialysis against 0.05 M potassium phosphate (pH 7.6)/10 mM 2-mercaptoethanol. Radioactivity was measured with the Triton X-100/toluene (1:2 v/v) cocktail of Patterson and Greene (19) in a Packard 3003 liquid scintillation spectrometer. The cocktail contained 5.5 g/liter of Permablend I (Packard) as scintillant.

Complete acid hydrolysis was achieved in an evacuated (<50 μm Hg) sealed tube with 6 N HCl/0.1 M 2-mercaptoethanol for 21 hr at 110°C. Amino acid analyses were carried out according to Spackman et al. (20) with a Beckman 120C amino acid analyzer.

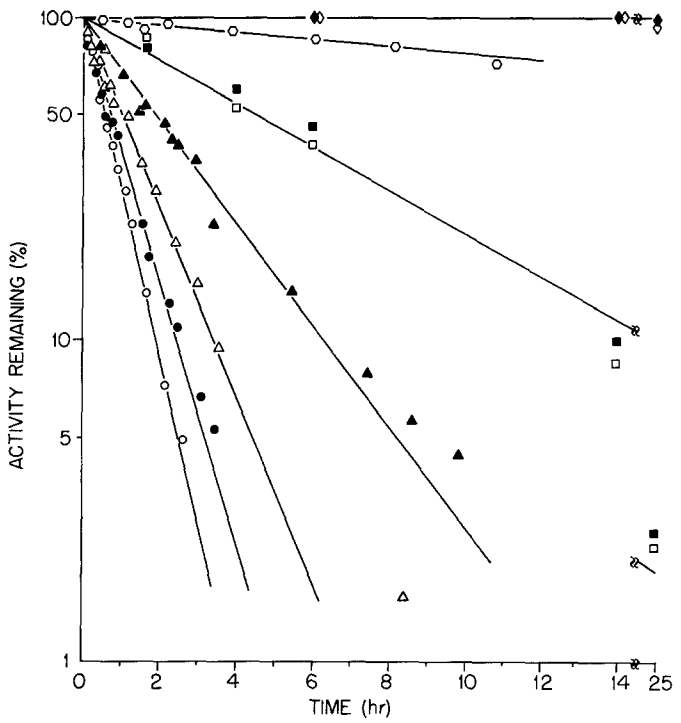

Figure 1. Time-course of inactivation of Rbl-P_2 carboxylase/oxygenase. Inactivation of carboxylase activity by 10 mM (○), 5 mM (●), 2.5 mM (△) or 1 mM (▲) BrAcNHEtOP in 5 mM $MgCl_2$/50 mM bicine (pH 8.0), and protection by 2.5 mM butanediol-P_2 in the presence of 1 mM BrAcNHEtOP (⬡). Inactivation of carboxylase (□) or oxygenase (■) activities by 5 mM BrAcNHEtOP in 50 mM bicine (pH 8.0), and protection of carboxylase (◇) and oxygenase (◆) activities by 1 mM Rbl-P_2.

Results

Incubation of Rbl-P_2 carboxylase/oxygenase with BrAcNHEtOP results in a parallel loss of both activities in a pseudo first-order fashion (Fig. 1). 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 with K_I 4.8 mM (data not shown), afford protection against inactivation; Mg^{2+}, the essential cofactor, stimulates the rate of inactivation. Butanediol-P_2 was used as the protector in all cases in which Mg^{2+} was present, because, with Mg^{2+} and even trace amounts of CO_2, Rbl-P_2 is rapidly converted enzymically to 3-phosphoglycerate.

Plots of the inactivation half-time (τ) against the reciprocal of reagent concentrations (Fig. 2) show rate saturation, indicative of reversible complex formation between enzyme

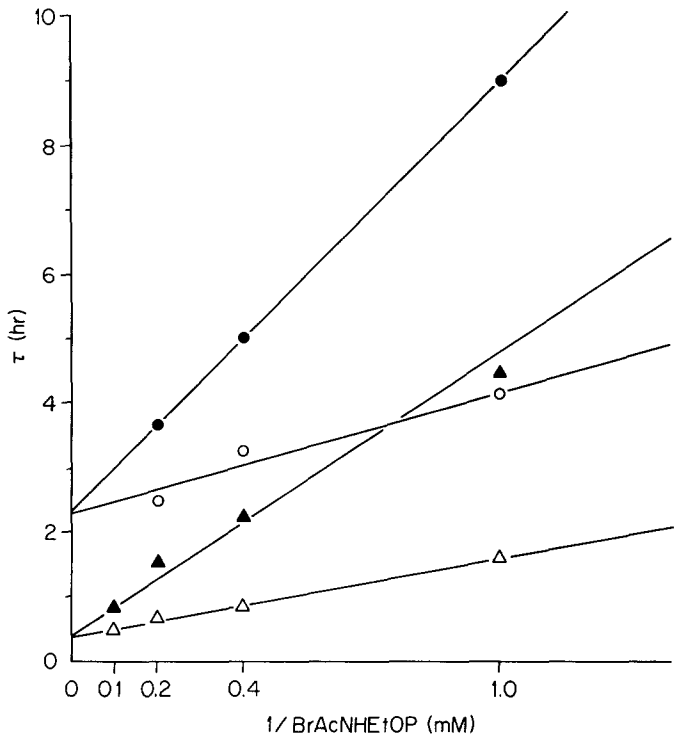

Figure 2. Inactivation half-time (τ) of Rbl-P_2 carboxylase as a function of the reciprocal of BrAcNHEtOP concentration. The following conditions were used: 5 mM $MgCl_2$/50 mM bicine (△); 5 mM $MgCl_2$/66 mM $NaHCO_3$/50 mM bicine (▲); Mg^{2+}-free 50 mM bicine (○); Mg^{2+}-free 66 mM $NaHCO_3$/50 mM bicine (●). All buffers were pH 8.0.

and reagent prior to covalent modification (21). 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 mM $MgCl_2$ in the reaction mixture. The reagent concentration that gives one-half the maximal rate of inactivation (K_{inact}), i.e. the apparent dissociation constant for the reagent-enzyme complex, is 0.8 mM in the absence of Mg^{2+} and 3.0 mM in its presence. Bicarbonate (66 mM) does not alter 1/T but increases K_{inact} to 2.9 mM and 11 mM without and with Mg^{2+}, respectively.

^{14}C reagent was used to determine the extent of incorporation associated with the inactivation. Based on differences in levels of radioactivity present in the inactivated samples as compared with samples protected by Rbl-P_2 or butanediol-P_2, inactivation correlates with the modification of about 1.6 residues per protomer (70,000 daltons) in the absence of Mg^{2+} or about 1.1 residues per protomer in the presence of Mg^{2+}.

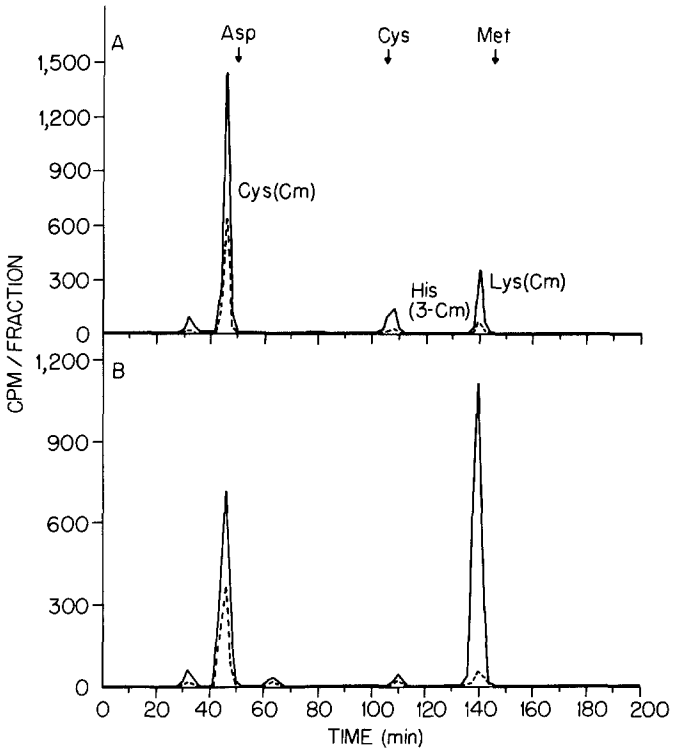

Figure 3. Chromatographic profiles of hydrolysates of Rbl-P_2 carboxylase after modification by [^{14}C] BrAcNHEtOP. The samples subjected to analysis are described in Table I. Chromatography was done on a Beckman 120C analyzer without use of its ninhydrin system; 2-min fractions were collected and counted. 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).

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 (22,23). Cys(Cm) is the major derivative found in hydrolysates of enzyme after modification in the absence of Mg^{2+} (Fig. 3). 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/oxygenase after modification in the presence of Mg^{2+} (Fig. 3). 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.

Discussion

The data obtained are consistent with the inactivation of Rbl-P_2 carboxylase/oxygenase 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. Although the reagent is not selective for a single residue, inactivation can be correlated with the alkylation of only 1–2 residues per protomer.

Interpretations other than active-site modification are possible. Chu and Bassham (24) have postulated the existence of four different types of allosteric regulatory sites for the spinach carboxylase. One of these allosteric sites is thought to be identical to the tight binding site for Rbl-P_2 observed by Wishnick et al. (25), and we cannot exclude the possibility of modification of this site with concomitant inactivation. Regulation of enzymic activity is presumably related to the activation of Rbl-P_2 carboxylase/oxygenase by Mg^{2+} and CO_2, which has recently been documented extensively (26–28). Since cysteinyl modification by reagent occurs in the absence of Mg^{2+} (where the enzyme exists in an essentially inactive conformation), loss of activity could be due to interference with the subsequent rapid activation of enzyme by the high concentrations of CO_2 and Mg^{2+} present in the assay medium. Lysyl modification, however, is seen in the presence of CO_2 and Mg^{2+}. Under these conditions inactivation of the fully activated enzyme occurs; thus prevention of the CO_2- and Mg^{2+}-dependent activation is not responsible for loss of activity. The alteration in reagent specificity (from cysteinyl to lysyl residues) brought about by Mg^{2+} is presumably a reflection of the Mg^{2+}-induced conformational change that has been detected with fluorescent probes (29, 30)

Since earlier studies with Br-butanone-P_2 (5,6) and pyridoxal phosphate (8) also implicated an essential lysyl residue in the spinach carboxylase, it will be of interest to determine whether the three reagents react with the same lysyl residue. The finding of a common site of reaction with three different types of reagents would greatly strengthen the argument that a lysyl residue is important in the catalytic process.

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