INACTIVATION OF *RHODOSPIRILLUM RUBRUM* RIBULOSE BISPHOSPHATE CARBOXYLASE/OXYGENASE BY THE AFFINITY LABEL 2-*N*-CHLOROAMINO-2-DEOXYPENTITOL 1,5-BISPHOSPHATE

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Received 1 April 1982

1. Introduction

Ribulose-P_2 carboxylase/oxygenase (EC 7.1.1.39) catalyzes the carboxylation and oxygenation in the initial steps of photosynthetic CO_2 fixation and photorespiration, respectively (review [1]). The enzyme thus plays a pivitol role in plant growth and productivity. An increased understanding of these reaction mechanisms is being sought through identification of amino acid residues at the active site using affinity labeling techniques. Several affinity labels ([2] and citations therein) have implicated a lysyl residue at the ribulose-P_2 binding site, but the catalytic role of this residue is not known. As a probe of the region of the active site where the gaseous substrate (O_2 or CO_2) and the divalent metal ion bind, we have chosen a compound, Cl-aminopentitol-P_2, with a reactive moiety at the position corresponding to C-2 of ribulose-P_2 at which both carboxylation and oxygenation occur. The reagent is an equimolar mixture of the arabino- and ribo-epimers, as it is prepared from aminopentitol-P_2 obtained by reductive amination of D-ribulose-P_2 [3].

Halo-amines, as both oxidizing and halogenating reagents, have a wide potential for modifying amino acid residues. Under mildly acidic conditions, *N*-chlorosuccinimide oxidizes tryptophanyl, cysteinyl and methionyl residues [4], and under slightly alkaline or neutral conditions only cysteinyl and methionyl residues are attacked [5]. In [6], chlorination of an active-site tyrosine in D-amino acid oxidase by *N*-chloro-D-leucine was shown under mild conditions. Given these general chemical properties of halo-amines and the suggestion of an essential tyrosyl residue in ribulose-P_2 carboxylase based on inactivation by tetranitromethane [7], the evaluation of Cl-aminopentitol-P_2 as an affinity label seemed appropriate. Here, we show that the new reagent selectively oxidizes a methionyl residue in the active-site region of the *Rhodospirillum rubrum* carboxylase.

2. Materials and methods

Spinach and *R. rubrum* ribulose-P_2 carboxylases/oxygenases were purified as in [8,9]. Ribulose-P_2, carboxyribitol-P_2 and aminopentitol-P_2 were synthesized as in [3,10,11]. $NaH^{14}CO_3$ was obtained from Amersham and $Na^{36}Cl$ from ICN.

Carboxylase and oxygenase activities were determined by standard procedures [12]. Protein concentrations are expressed as protomer molarity; the *R. rubrum* protomer is a 56 000 M_r subunit and the spinach protomer is a 70 000 M_r combination of one large and one small subunit [8,9].

Paper chromatography was carried out at 2°C in *n*-butyl alcohol:acetic acid:water (7:2:5), and after irrigation the chromatograms were sprayed for amines with ninhydrin/cadmium [13], for phosphates with ammonium molybdate [14], or for reactive halogen with 5-thio-2-nitrobenzoic acid [15].

Cl-Aminopentitol-P_2 was generated in solution by mixing rapidly at 0°C equimolar amounts of amino-

Abbreviations: ribulose-P_2, D-ribulose 1,5-bisphosphate; Cl-aminopentitol-P_2, 2-*N*-chloroamino-2-deoxypentitol 1,5-bisphosphate; aminopentitol-P_2, 2 amino-2-deoxypentitol-P_2; carboxyribitol-P_2, 2-carboxyribitol 1,5-bisphosphate; fructose-P_2, fructose 1,6-bisphosphate

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Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

pentitol-P_2 and KOCl in 50 mM lithium borate (pH 8.0). KOCl was prepared by bubbling chlorine through 1.0 M KOH [6]. $KO^{36}Cl$ was prepared by equilibrating KOCl with a 5-fold excess of $Na^{36}Cl$ at room temperature and pH 8.0 [6]; ^{36}Cl-aminopentitol-P_2 was then synthesized as above. Solutions of both KOCl and Cl-aminopentitol-P_2 were prepared fresh immediately prior to each experiment.

Inactivation of the enzyme was carried out at 0°C following activation of the enzyme at 25°C in 50 mM Bicine, 10 mM $MgCl_2$, 50 mM $NaHCO_3$ (pH 8.2). Solutions of the inhibitor (1–2 mM, 0°C) were added to the enzyme, aliquots (1–5 μl) were removed periodically with an ice-cold syringe, and assays for activity were then performed at 25 °C. The cold treatment did not affect the activity compared to controls held at 25°C throughout. Further details are given in the figure and table legends.

Methionine sulfoxide was determined directly following base hydrolysis of the protein [16] or indirectly following cyanogen bromide cleavage and total acid hydrolysis [5]. Amino acid analyses were carried out with a Beckman 121M amino acid analyzer.

3. Results and discussion

3.1. *Synthesis of Cl-aminopentitol-P_2*

Addition of aminopentitol-P_2 to KOCl abolished the absorbance peak due to KOCl ($\epsilon_{291\ nm}$ = 300) and produced two others ($\epsilon_{310\ nm}$ = 250 and $\epsilon_{250\ nm}$ = 450). These latter two absorbances decreased simultaneously when the reaction mixture was warmed to 25°C ($t_{1/2}$ = 60 min) but remained constant when the solution was maintained at 0°C. Chromatography revealed a new compound (R_F = 0.25) that was positive for phosphate and reactive halogen; the starting material was absent. Ninhydrin produced a yellow spot with a faint pink tail due presumably to breakdown of Cl-aminopentitol-P_2 during chromatography (aminopentitol-P_2 was also pink, R_F = 0.17).

3.2. *Inactivation of ribulose-P_2 carboxylase*

Rapid inactivation of *R. rubrum* enzyme was observed with low concentrations of Cl-aminopentitol-P_2 (fig.1A,B), whereas the spinach enzyme was considerably more resistant (fig.1B). KOCl and chloramine (equimolar solution of KOCl and NH_3) inactivated

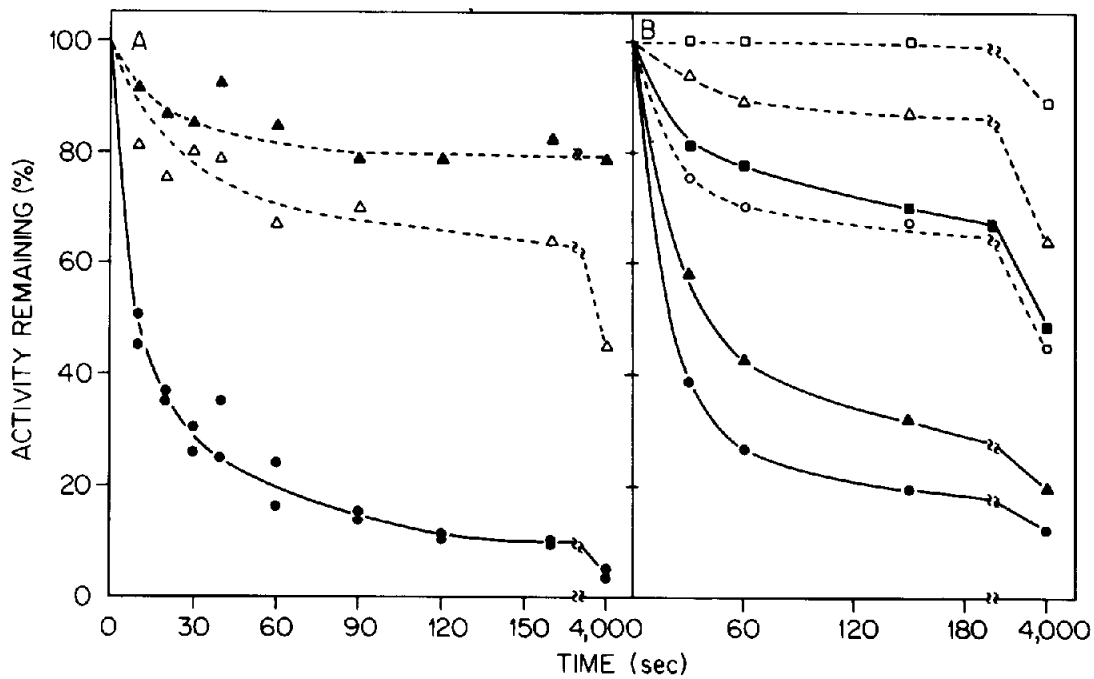

Fig.1. Inactivation of ribulose-P_2 carboxylase by Cl-aminopentitol-P_2. (A) Time course for the inactivation of the *R. rubrum* enzyme (24.8 μM) at 0°C in the presence of either the reagent (100 μM) (•), chloramine (100 μM) (△), or KOCl (100 μM) (▲). (B) Time course for the inactivation of the spinach enzyme (74 μM) in the presence of reagent (148 μM) (○), reagent (74 μM) (△), or reagent (74 μM) and carboxyribitol-P_2 (400 μM) (□); and of the *R. rubrum* ribulose-P_2 enzyme (89 μM) in the presence of reagent (178 μM) (•), reagent (89 μM) (▲), or reagent (89 μM) and fructose-P_2 (10 mM) (■).

R. rubrum carboxylase more slowly and to a more limited extent (fig.1A) than did Cl-aminopentitol-P_2, indicating that the reagent was behaving like an affinity label. A further suggestion of an affinity of Cl-aminopentitol-P_2 for the active site was that the enzyme was subject to protection from inactivation by the presence of the competitive inhibitors fructose-P_2 (K_i = 0.61 mM) [9] and carboxyribitol-P_2 (K_i = 1.5 μM) [11]. Because the inactivation rate at 25°C was much faster than the shortest possible $^{14}CO_2$ fixation assay period, it was not possible to demonstrate competition directly between Cl-aminopentitol-P_2 and ribulose-P_2 nor was it possible to obtain rates of inactivation under conditions where pseudo-first-order kinetics might have been anticipated. It is apparent from fig.2 that not only are both oxygenase and carboxylase activities diminished in parallel but that only one molecule of Cl-aminopentitol-P_2 is required per protomer for inactivation. Complex kinetics (fig.1A,B) can thus be rationalized since Cl-aminopentitol-P_2 consists of an epimeric mixture of the arabino- and ribo-forms which probably inactivate the enzyme at different rates.

When the carboxylase was modified at a 1:1 molar

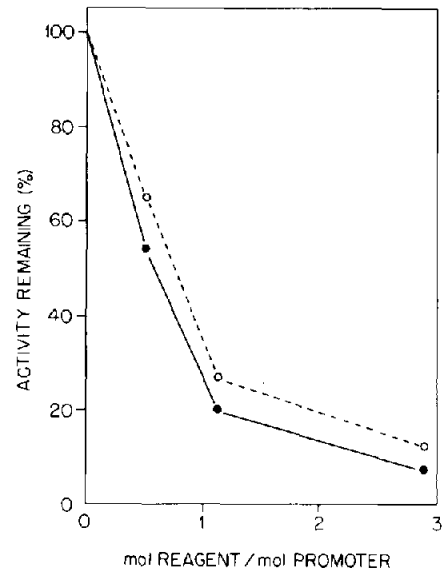

Fig.2. Titration of *R. rubrum* ribulose-P_2 carboxylase with Cl-aminopentitol-P_2. The enzyme (89.6 μM) was titrated at 25°C by successive additions of the reagent at 15 min intervals. Aliquots were removed and activities determined after each addition. The data points represent the means of triplicate carboxylase (•) and duplicate oxygenase assays (○).

Table 1
Methionine sulfoxide content of ribulose-P_2 carboxylase after inactivation with Cl-aminopentitol-P_2[a]

Sample constituents				Activity (% Initial)	No. residues/protomer		
[Enz.]	[Reagent]	[Carboxyribitol-P_2]	[Fructose-P_2]		Methionine sulfoxide (base hydrolysates)	Methionine[b] (after CNBr)	Methionine modified
88 μM	–	–	–	100[c]	ND[d]	1.5	–
88 μM	–	250 μM	–	100[c]	ND	1.7	–
88 μM	–	–	10 mM	100[c]	ND	1.6	–
88 μM	88 μM	–	–	15[c]	ND	2.8	1.2
88 μM	88 μM	250 μM	–	47[c]	ND	2.2	0.6
88 μM	88 μM	–	10 mM	62[c]	ND	1.9	0.3
88 μM	88 μM	–	–	14[e]	ND	2.9	1.3
88 μM	440 μM	–	–	1[e]	ND	5.1	3.5
20 μM	–	–	–	100[f]	ND	0.9	–
20 μM	20 μM	–	–	19[f]	ND	1.8	0.9
20 μM	–	–	–	100[f]	0.16	ND	–
20 μM	20 μM	–	–	19[f]	1.25	ND	1.1

[a] After treatment with Cl-aminopentitol-P_2, the protein was dialyzed against 10 mM ammonium bicarbonate and lyophilized prior to base hydrolysis or cyanogen bromide cleavage and acid hydrolysis

[b] The native protomer contains 15 residues of methionine [9]; the methionine present after cyanogen bromide cleavage of protein samples that had not been treated with Cl-aminopentitol-P_2 is due to incomplete reaction with cyanogen bromide

[c] Reaction terminated after 200 s by addition of mercaptoethanol to 10 mM

[d] ND, not determined

[e] Reaction terminated after 30 min by addition of mercaptoethanol to 10 mM

[f] Reaction terminated after 180 s by addition of mercaptoethanol to 10 mM

ratio with reagent, the activity plateaued at ~14% of its initial value. This treated enzyme exhibited a K_m (ribulose-P_2) of 23 ± 3 μM (as compared to 9.8 ± 0.8 μM for native enzyme) and a K_m (HCO_3^-) of 72 ± 10 mM (as compared to 12.6 ± 1.4 mM for native enzyme). With ribulose-P_2 as variable substrate, the V_{max} for modified enzyme was only 16% of that for native enzyme.

3.3. *Mode of inactivation*

Inactivation of *R. rubrum* ribulose-P_2 carboxylase/oxygenase is due to oxidation of a single methionyl residue (table 1). When samples were protected against inactivation, a corresponding decrease in the content of methionine sulfoxide was also observed. Use of a 5-fold excess of reagent led to complete loss of activity but far more extensive oxidation of methionyl residues.

The substantial inactivation that occurred at a 1:1 reagent:protomer ratio appeared to rule out oxidation of tryptophanyl and cysteinyl residues as well as dichlorination of tyrosinyl residues. Lack of modification of these residues was confirmed by several techniques. Absorption difference spectra between modified and control enzymes showed no decrease in absorption at 280 nm (tryptophan oxidation to oxyindole) [17] nor increase at 310 nm (dichlorination of tyrosine) [6]. The possibility that monochlorotyrosine was a product of modification was excluded by the failure of modified enzyme to incorporate ^{36}chlorine from ^{36}Cl-labeled reagent. Dialysis to remove non-covalently bound ^{36}Cl did not restore activity nor did incubation in 100 mM β-mercaptoethanol, conditions under which cystine should be reduced. Titration of modified and unmodified enzyme from *R. rubrum* with 5,5′-dithiobis(2-nitrobenzoate) [18] showed no differences in cysteine content.

The methionyl residue that is susceptible to preferential oxidation by Cl-aminopentitol-P_2 cannot be considered essential to catalysis since the oxidized enzyme retains residual activity. Our findings of substantially elevated K_m values for both ribulose-P_2 and CO_2 in the treated enzyme exclude the possibility of residual activity reflecting the presence of native enzyme. Modification to methionine sulfoxide presumably affects neighboring residues at the active site more intimately involved in catalysis, either through steric effects due to the introduction of the oxygen atom or electronic effects due to the accompanying dipole. 2-Bromo-acetylaminopentitol 1,5-bisphosphate, a recently designed affinity label has also provided evidence for a methionyl residue at the active site of the *R. rubrum* carboxylase [3]. If these two chemically dissimilar reactive analogues of ribulose-P_2 attack the same methionine, it almost assuredly represents an active-site component. The reported [19] sensitivity of spinach ribulose-P_2 carboxylase/oxygenase to hydrogen peroxide might reflect oxidation of a methionyl residue required for optimal catalytic activity.

Amino acid sequences of peptides surrounding lysyl residues at the catalytic and activator sites of spinach and *R. rubrum* enzymes do not contain methionine [2,20–22]. Thus, identification of the methionine sulfoxide containing peptide should locate a further portion of the primary structure in the active-site region.

Acknowledgement

Research sponsored by the Office of Health and Environmental Research, US Department of Energy, under contract W-7405-eng-26 with the Union Carbide Corp.

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