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INACTIVATION OF RIBULOSEBISPHOSPHATE CARBOXYLASE/OXYGENASE FROM RHODOSPIRILLUM RUBRUM AND SPINACH WITH THE NEW AFFINITY LABEL 2-BROMO-1,5-DIHYDROXY-3-PENTANONE 1,5-BISPHOSPHATE*

Mark I. Donnelly† and Fred C. Hartman

University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences and Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

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SUMMARY

In an attempt to identify the active-site base believed to initiate catalysis by ribulosebisphosphate carboxylase, we have synthesized 2-bromo-1, 5-dihydroxy-3-pentanone 1,5-bisphosphate, a reactive analogue of a postulated intermediate of carboxylation. Although highly unstable, this compound can be shown to inactivate the carboxylases from both Rhodospirillum rubrum and spinach rapidly and irreversibly. Inactivation follows pseudo first-order kinetics, shows rate saturation and is greatly reduced by saturating amounts of the competitive inhibitor, 2-carboxyribitol 1,5-bisphosphate. The incorporation of reagent, quantified by reducing the modified carboxylases with [^{3}H]$NaBH_4$, shows that inactivation results from the modification of approximately one residue per catalytic subunit of the Rhodospirillum rubrum enzyme and less than one residue per protomeric unit of the spinach enzyme.

As the catalyst for the initial reaction in both photorespiration and the photosynthetic assimilation of CO_2, ribulose-P_2‡ carboxylase/oxygenase (EC 4.1.1.39) plays a crucial role in determining growth rates and yields of plants (reviewed in ref. 1). Consequently, intensive efforts to characterize this enzyme fully are in progress, including active-site mapping (reviewed in ref. 2). Affinity labeling studies strongly point to the presence of a particular lysyl residue at the enzyme's active-site (3-6) but its role in catalysis, if any, has not yet been defined.

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‡The abbreviations used are: ribulose-P_2, D-ribulose 1,5-bisphosphates; Br-pentanone-P_2, 2-bromo-1,5-dihydroxy-3-pentanone 1,5-bisphosphate; CR-P_2, D-2-carboxyribitol 1,5-bisphosphate; Br-butanone-P_2, 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate; 2-pentanone-P_2, 1,5-dihydroxy-2-pentanone 1,5-bisphosphate.

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According to the current model, carboxylation is initiated by abstraction of the C-3 proton of ribulose-P_2. Carboxylation of the reactive species so formed is believed to generate 2-carboxy-3-ketoarabinitol 1,5-bisphosphate (7,8). With these considerations in mind, we have prepared 2-bromo-1,5-dihydroxy-3-pentanone 1,5-bisphosphate (Br-pentanone-P_2), a reactive analogue of the putative 3-keto intermediate. In theory, binding should place the reactive carbon atom, C-2, close to the base believed to abstract the C-3 proton from ribulose-P_2. Our initial studies indicate that Br-pentanone-P_2 is a potent, active-site-directed reagent for both the R. rubrum and spinach carboxylases, enzymes from evolutionarily distant sources which differ greatly in their quaternary and primary structure (9-12).

MATERIALS AND METHODS

CR-P_2, Br-butanone-P_2 and 2-pentanone-P_2 were synthesized by published procedures (8,13,14). [^{3}H]$NaBH_4$ was procured from New England Nuclear. [^{14}C]$NaHCO_3$ and ACS scintillation cocktail were products of Amersham. QAE-Sephadex was purchased from Pharmacia, Bicine from Sigma and dimethyl 3-ketoglutarate from Aldrich.

Ribulose-P_2 carboxylases from R. rubrum and from spinach were purified as described elsewhere (3,15,16). The enzymes' concentrations were determined from A_{280} using $E^{1\%}_{1cm}$ values and protomeric molecular weights of 12.1 and 56,000, respectively, for the R. rubrum enzyme (10,12) and 16.4 and 70,000 for the spinach enzyme (9,15). All assays and manipulations of both carboxylases were performed in standard Bicine buffer (0.1 M Bicine/NaOH, pH 8.0, containing 66 mM $NaHCO_3$, 10 mM $MgCl_2$, and 0.1 mM EDTA).

Carboxylase activity was determined by measuring incorporation of [^{14}C]CO_2 into acid stable products after the method of Lorimer et al. (17) using standard Bicine buffer supplemented with 0.5 mM ribulose-P_2 and [^{14}C]$NaHCO_3$ at 0.3 μCi/μmol. Reactions were initiated by adding 1-10 μl of enzyme to 0.5 ml of assay buffer at 25° and terminated after 40 sec with 0.1 ml of 1 N HCl. Acid stable radioactivity was determined by liquid scintillation spectrometry (Packard 3255) and the data were corrected for quenching.

Synthesis and analysis of Br-pentanone-P_2. Br-pentanone-P_2 was synthesized by the reactions shown in Fig. 1, details of which will be published elsewhere. The final product (IV) was purified on QAE-Sephadex A-25 with 0.1 N HCl as eluant and was stored frozen in 0.1 N HCl. Br-pentanone-P_2 thus obtained was pure as judged by the thin layer chromatography (R_f = 0.41) on cellulose plates (Polygram cel 300 UV_{254}, Brinkmann) developed at 4° with 1-butanol:acetic acid:water (7:2:5) as solvent and sprayed with reagents for phosphate esters, α-haloketones, and carbonyl groups (18-20).

The stock acidic solution of Br-pentanone-P_2 was assayed for P_i (21), organic phosphate (13), α-haloketone (22), and inorganic Br^- (by use of an Orion model 94-35 bromide selective electrode). Observed concentrations of P_i and Br^- were less than 10% of the concentration of Br-pentanone-P_2 based on organic phosphate content. The stability of Br-pentanone-P_2 with respect to release of P_i was determined in standard Bicine buffer. Periodically, 100 μl aliquots of the reaction mixtures were added to 400 μl of cold 0.1 N HCl and later assayed for P_i.

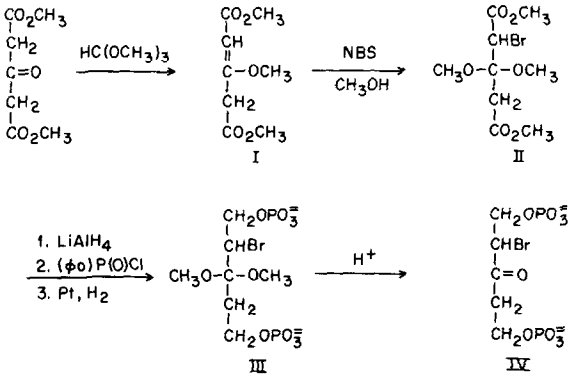

Fig. 1. Scheme for the synthesis of 2-bromo-1,5-dihydroxy-3-pentanone 1,5-bisphosphate. NBS, N-bromosuccinimide.

Modification of ribulose-P_2 carboxylases with Br-pentanone-P_2. The kinetics of inactivation were measured as described in the legend to Fig. 2. For the higher concentrations of reagent, appropriate volumes of 1 N NaOH were included in the assay media to neutralize the 0.1 N HCl in the stock reagent. For measuring the incorporation of reagent associated with inactivation, carboxylase from R. rubrum at 59 μM protomeric unit or from spinach at 33 μM was treated with Br-pentanone-P_2 in the presence or absence of CR-P_2 for 4.5 min, at which time the reagent was destroyed by adding β-mercaptoethanol to give a final concentration of 9 mM. Incorporation of reagent was quantified by reducing the modified enzymes with 0.01 M [^{3}H]$NaBH_4$ following previously described procedures, and the specific activity of the [^{3}H]$NaBH_4$ (20,700 dpm/nmol tritium) was determined by reduction and analysis of a model compound (3).

RESULTS AND DISCUSSION

Br-pentanone-P_2 (Fig. 1, IV) was synthesized from dimethyl 3-ketoglutarate (Fig. 1). Our assignment of the compound's structure is based on the method of synthesis, spectral properties of intermediates I and II (to be reported elsewhere), and the characteristics of the purified compound. The product migrated on cellulose tlc plates similarly to Br-butanone-P_2 and 2-pentanone-P_2 and gave reactions with spray reagents for phosphate esters, α-haloketones, and carbonyl groups which were consistent with the assigned structure. Quantitative analyses showed the expected 2:1 ratio for organic phosphate:haloketone.

Although stable when stored frozen in 0.1 N HCl, Br-pentanone-P_2 decomposed rapidly near neutral pH. In standard Bicine buffer, pH 8.0, phosphate was released in a bisphasic process, the first phase, accounting for one of the

compound's two phosphates, with a half-life of 4.5 min ($\underline{k}$ = 0.15 min^{-1}), the second with a half-life of 36 min ($\underline{k}$ = 0.019 min^{-1}). This second rate constant is close to that reported for phosphate loss from 3-hydroxypropionaldehyde 3-phosphate (23), a compound analogous to carbons 3-5 of Br-pentanone-P_2. Br^- is also released in a biphasic process.

Ribulose-P_2 carboxylase is a hysteretic enzyme, undergoing a relatively slow conversion from an inactive to a fully activated form induced by CO_2 and Mg^{2+} (24,25). Therefore, our experiments with Br-pentanone-P_2 were conducted in an activating buffer at pH 8.0 despite the reagent's instability under these conditions. Data gathered only during the first 5 min of inactivation were used for kinetic analyses. Br-pentanone-P_2 inactivates ribulose-P_2 carboxylase in a pseudo first-order fashion (Fig. 2). Inactivation is irreversible, as dialysis or treatment with 50 mM β-mercapoethanol did not restore activity. Analysis of the endpoints of inactivation showed that oxygenase activity is lost in parallel. CR-P_2, a potent competitive inhibitor of both the spinach enzyme (8) and the R. rubrum enzyme ($\underline{K_i}$ = 4 μM, unpublished data), greatly reduced the rate of inactivation.

Replots of the inactivation half-times (τ) against the reciprocal of the reagent's concentration clearly show rate saturation (Fig. 2, insets), indicating that inactivation involves an initial, reversible formation of an enzyme-reagent complex. For both the R. rubrum and spinach enzymes inactivation is rapid with minimum inactivation times (T) of 9.1 and 1.25 min, respectively. The concentrations of Br-pentanone-P_2 giving half the maximum rate of inactivation ($\underline{K_{inact}}$, the apparent dissociation constant for the enzyme-reagent complex) are 70 μM and 150 μM, respectively. At higher reagent concentrations the rate of inactivation increases with time (Fig. 2A). This observation can be explained since the product(s) of degradation of Br-pentanone-P_2 inactivate the R. rubrum enzyme more rapidly than does the reagent. When reagent was preincubated in standard Bicine buffer for twenty minutes, inactivation of the

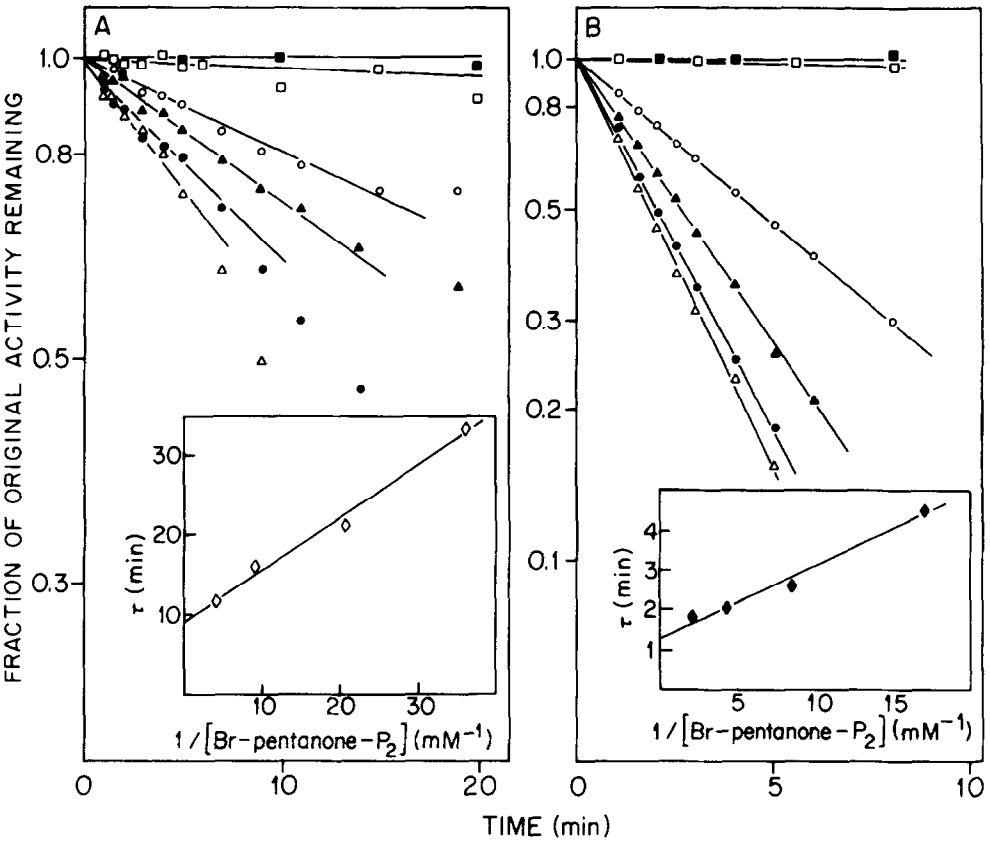

Fig. 2. Time courses for the inactivation of ribulose-P_2 carboxylases from R. rubrum (A) and spinach (B). Carboxylase from R. rubrum (A) at 17 μM protomer was treated with 220 μM (△), 110 μM (●), 55 μM (▲) and 27.5 μM (○) Br-pentanone-P_2 in standard Bicine buffer at 25°, and was protected against inactivation with 130 μM CR-P_2 in the presence of 27.5 μM reagent (□). Spinach enzyme (B) at 27 μM protomer was treated with 470 μM (△), 235 μM (●), 117 μM (▲) and 59 μM (○) Br-pentanone-P_2, and protected with 190 μM CR-P_2 in the presence of 59 μM reagent (□). Controls, either in the presence or absence of CR-P_2, were stable (■). The lines drawn are based on linear regression analyses of data gathered during the first five minutes of inactivation only, due to the demonstrated instability of the reagent (see text). In the insets, the computed half-times of inactivation (τ) are plotted as a function of the reciprocal of the Br-pentanone-P_2 concentration. Correlation coefficients determined by linear regression analyses of the replotted data were 0.995 for the R.rubrum carboxylase (◇) and 0.991 for the spinach enzyme (◆).

R. rubrum enzyme occurred by a second order process, not showing rate saturation, with a rate constant of 41 M^{-1} sec^{-1}.

To assess the extent of alkylation by Br-pentanone-P_2 required to inactivate the carboxylases, the incorporation of reagent was measured. Modified enzyme was reduced with [^{3}H]$NaBH_4$ and the specific activity of the

Table 1

Incorporation

Source of enzyme	Inactivation conditions: mol reagent/ mol protomer	protection[1]	Fraction of activity lost	Difference between unprotected and protected	Incorporation mol tritium/ mol protomer[2]	Difference between unprotected and protected	Stoichiometry of inactivation[3]
R. rubrum	2.25	-	0.164	0.107	0.143	0.097	0.91
		+	0.057		0.046		
	4.5	-	0.262	0.223	0.258	0.208	0.93
		+	0.039		0.050		
Spinach	1.8	-	0.292	0.236	0.212	0.098	0.42
		+	0.056		0.114		
	7.1	-	0.606	0.460	0.575	0.180	0.39
		+	0.146		0.395		

[1]The *R. rubrum* and spinach enzymes were protected by CR-P_2 at 490 μM and 200 μM, respectively.

[2]Values are corrected for incorporation into control samples which were 0.375 and 0.404 mol tritium/mol protomer of the *R. rubrum* and spinach carboxylases, respectively.

[3]Calculated by dividing the mol tritium incorporated/mol protomer by the fraction of activity lost after both values had been corrected for incorporation and inactivation occurring in the protected samples. This treatment of the data is based on the assumption that inactivation and incorporation not due to active-site-directed processes are the same for both the unprotected and protected samples.

resulting enzyme-reagent adduct was determined. Given the reagent's half life of 4.5 min, we limited our analyses to enzyme exposed to reagent for only 4.5 min to insure that we were evaluating inactivation by the reagent itself, not by its degradation products. However, since the minimum inactivation half-times are also within the range of minutes, we were forced to inspect enzymes only partially inactivated. Table 1 lists the extent of reagent incorporation for two reagent:protomer ratios for each carboxylase.

For both enzymes, a correlation between the fraction of activity lost and the incorporation of Br-pentanone-P_2 is apparent. For the *R. rubrum* carboxylase, the data are entirely consistent with inactivation resulting from the incorporation of a single molecule of reagent per protomer. Incorporation due to reactions occurring outside the active-site appears to be minimal.

In the case of the spinach enzyme, however, inactivation appears to result from an incorporation of reagent well below a stoichiometric amount, as was

seen previously for the incorporation of Br-butanone-P_2 (3). Although half-of-the-sites reactivity of the enzyme toward this reagent may be inferred, the high levels of incorporation which occur in protected samples precludes a definitive interpretation. Our analyses assume that reactions occurring outside the active site are identical in protected and unprotected samples, a condition shown not to be true for the reaction of Br-butanone-P_2 with this enzyme (3).

The results presented here demonstrate that Br-pentanone-P_2 acts as a high affinity, active-site-directed reagent toward the ribulose-P_2 carboxylase/oxygenases from both R. rubrum and spinach. We are currently engaged in the isolation of tryptic peptides from the modified enzymes.

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