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Isolation of a Stable Enzyme·$^{14}CO_2$·Mg^{2+}· Carboxyarabinitol Bisphosphate Complex with Ribulosebisphosphate Carboxylase from *Chromatium vinosum*†

HUGH M. BROWN‡ AND RAYMOND CHOLLET*

Department of Agricultural Biochemistry, University of Nebraska, Lincoln, Nebraska 68583-0718

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Higher plant-type ribulosebisphosphate carboxylase from *Chromatium vinosum* formed a stable, nonexchangeable complex with activator $^{14}CO_2$ in the presence of Mg^{2+} and 2-carboxyarabinitol bisphosphate, an analog of the proposed transition-state intermediate. The response of the procaryotic enzyme to this analog was indistinguishable from that of the higher-plant carboxylase, which should permit comparative analysis of the activator site amino acid sequence in the two proteins.

2-Carboxyarabinitol 1,5-bisphosphate (CABP) is a potent irreversible inhibitor of ribulosebisphosphate (RuBP) carboxylase (EC 4.1.1.39; RuBPCase), which binds tightly to the catalytic site by virtue of its structural resemblance to 2-carboxy-3-ketoarabinitol 1,5-bisphosphate, the proposed six-carbon transition-state intermediate of the carboxylase reaction (3, 11). It was recently demonstrated that the exposure of $^{14}CO_2$·Mg^{2+}-activated spinach leaf RuBPCase to CABP yields an enzyme·$^{14}CO_2$·Mg^{2+}·CABP complex which contains 1 mol each of $^{14}CO_2$, Mg^{2+}, and CABP per mol of 69-kilodalton enzyme protomer after gel filtration (9, 10). Since CABP is thought to occupy simultaneously the RuBP- and substrate CO_2-binding sites, these results indicated that CABP binding greatly retards the exchange of activator CO_2 and Mg^{2+} with free ligands at a site distinct from the catalytic site (9, 10). Tight linkage between a CABP-occupied catalytic site and the stability of the activator site carbamate·Mg^{2+} complex on the catalytic subunit (7) may have interesting implications concerning the mechanism of CO_2·Mg^{2+} activation of RuBPCase. Furthermore, the extreme stabilization of activator $^{14}CO_2$ by bound CABP has allowed this activator carbamate to be trapped with diazomethane, leading to the recent amino acid sequence analysis of the activator site peptide containing this trapped $^{14}CO_2$ (6).

Preliminary experiments with highly purified RuBPCase from the nonsulfur purple photosynthetic bacterium *Rhodospirillum rubrum* have indicated that bound CABP does not stabilize activator CO_2 as effectively as with the spinach leaf enzyme; substoichiometric molar ratios of CABP-stabilized $^{14}CO_2$ per 55-kilodalton enzyme protomer were obtained after gel filtration (G. H. Lorimer, personal communication). RuBPCase from *R. rubrum* has a relative molecular weight (M_r) of approximately 114,000 and is a dimer of catalytic subunits, whereas higher-plant RuBPCase has an M_r of about 550,000 and is a hexadecamer composed of eight 55-kilodalton catalytic subunits and eight 14-kilodalton noncatalytic subunits (3). Less stringent stabilization of activator CO_2 by CABP in *R. rubrum* RuBPCase may result from the unique quaternary structure of this enzyme or may reflect a difference in the way the catalytic and activator sites are coupled. For instance, if other procaryotic RuBP carboxylases which possess higher plant-type quaternary structures also exhibit weak CABP stabilization of activator CO_2, one might infer that the extremely tight coupling displayed by eucaryotic RuBPCase represents an evolutionary refinement in enzyme structure and function.

In the following report we demonstrate that CABP fully stabilizes activator site $^{14}CO_2$ in higher plant-type RuBPCase isolated from the purple sulfur photosynthetic bacterium *Chromatium vinosum*. The response of this microbial enzyme to CABP is indistinguishable from that of spinach leaf RuBPCase. These results indicate that weak stabilization, as exhibited by the *R. rubrum* enzyme, is not a general feature of procaryotic RuBP carboxylases.

2-Carboxypentitol 1,5-bisphosphate was kindly provided by Henry M. Miziorko of The Medical College of Wisconsin. The lyophilized sam-

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‡ Present address: Dupont Experimental Station, Biochemicals Department, Wilmington, DE 19898.

ple was prepared as a 1 mM solution in 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-NaOH–40 mM $MgCl_2$ (pH 8.0) and stored at −20°C. 2-Carboxypentitol 1,5-bisphosphate is actually a mixture of 2-carboxyribitol and 2-carboxyarabinitol bisphosphates, of which the latter is the potent irreversible inhibitor of RuBPCase (11). Although no attempt was made to separate the two epimers, for simplicity we will refer to the mixture as CABP (compare references 7, 9, and 10).

C. vinosum (strain D) was grown photoheterotrophically under anaerobic conditions on a succinate-NH_4Cl-Na_2S minimal salts medium (1a), and RuBPCase was purified from frozen cells by a procedure which employed selective precipitation of the enzyme by polyethylene glycol-4000, $MgCl_2$ and $(NH_4)_2SO_4$, followed by $MgCl_2$ step elution from DEAE-cellulose and linear NaCl concentration gradient elution from DEAE-Sephadex A-50 (1a). RuBPCase prepared by this protocol had a specific activity greater than 3 μmol of $H^{14}CO_3^-$ fixed per min per mg of protein at 30°C and a protein purity in excess of 97% (1a). Carboxylase activity was determined as detailed by Kung et al. (4). Protein concentration (milligrams per milliliter) was estimated by a sensitive dye-binding assay (reference 1, as modified by Bio-Rad Laboratories, January 1979) and by multiplying the absorbance of the purified protein at 280 nm (1 cm) by 0.65 (1a); the results from both methods were in excellent agreement. The molar concentrations of *C. vinosum* and spinach leaf RuBPCases were calculated by using native M_r of 525,000 (1a) and 550,000, respectively.

For the formation and gel filtration of the enzyme·$^{14}CO_2$·Mg^{2+}·CABP complex, the following procedure was adapted from that of Miziorko (9, 10). Two to three milligrams of RuBPCase was dissolved in 0.5 ml of 50 mM Tris-hydrochloride (pH 7.8) (CO_2 depleted, as in reference 12), and dialyzed for 12 h at 4°C against the same buffer with continuous N_2 bubbling to maintain CO_2-depleted conditions. The dialyzed sample was brought to 20 mM NaH-$^{14}CO_3$ (0.74 Ci/mol), 10 mM $MgCl_2$, and 50 mM Tris-hydrochloride (pH 7.8) in a final volume of 1 ml and incubated for 20 min at 30°C to fully $^{14}CO_2$·Mg^{2+}-activate the enzyme. Sufficient CABP was then added to yield a final 3:1 molar ratio of CABP to enzyme protomer, and this mixture was incubated for 1 h at 30°C. The sample was then applied to a column of Sephadex G-50 (1.5 by 25 cm) and eluted at 25°C with 50 mM Tris-hydrochloride (pH 7.8). Fractions (0.85 ml) were analyzed for protein content, RuBPCase activity, and ^{14}C radioactivity.

Highly purified RuBPCase from *C. vinosum* that had been fully $^{14}CO_2$·Mg^{2+}-activated and chromatographed on Sephadex G-50 is depicted in Fig. 1A. As expected, in the absence of CABP, activator $^{14}CO_2$ did not remain bound to the eluted protein when chromatographed through a Mg^{2+}-free gel column, and the enzyme retained full carboxylase activity when subsequently reactivated and assayed. However, pri-

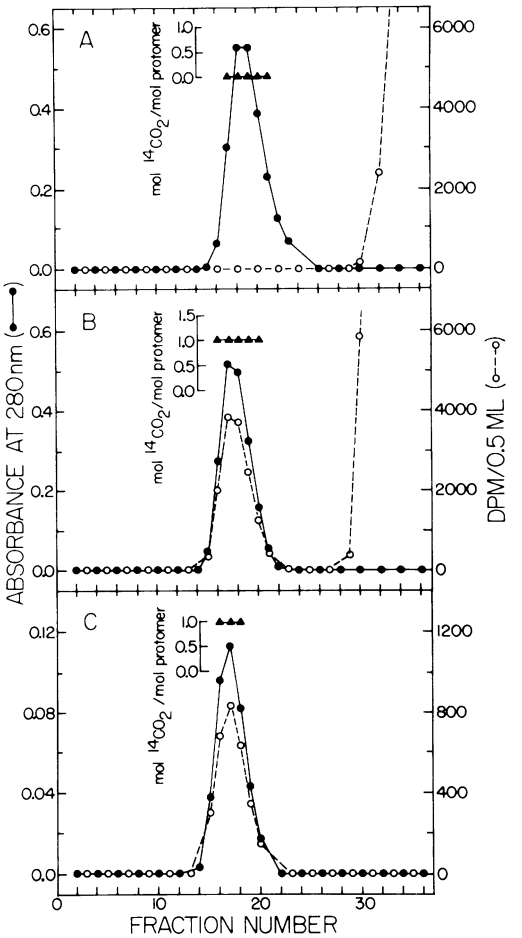

FIG. 1. Isolation of a stable, nonexchangeable enzyme·$^{14}CO_2$·Mg^{2+}·CABP complex with $^{14}CO_2$·Mg^{2+}-activated RuBP carboxylase from *C. vinosum*. (A) Gel filtration of fully $^{14}CO_2$·Mg^{2+}-activated enzyme without prior CABP treatment. (B) Gel filtration of $^{14}CO_2$·Mg^{2+}-activated enzyme after a 1-h incubation with a 3:1 molar ratio of CABP to enzyme protomer. Under identical conditions, the homogeneous spinach leaf enzyme (prepared as described in reference 2) yielded a value of 1.0 mol of $^{14}CO_2$ bound per mol of enzyme protomer through the peak protein fractions. (C) Rechromatography of pooled peak fractions from experiment B after a 1-h incubation with 50 mM unlabeled $NaHCO_3$ at pH 7.8 and 30°C. Insets show moles of $^{14}CO_2$ bound per mole of enzyme protomer (▲) through the peak protein fractions.

or addition of excess CABP (3 mol/mol of enzyme protomer) to the $^{14}CO_2 \cdot Mg^{2+}$-activated carboxylase and subsequent gel filtration yielded a protein peak to which was bound 1 mol of activator $^{14}CO_2$ per mol of protomer. This molar ratio was constant throughout the peak protein fractions (Fig. 1B). Furthermore, the enzyme was completely inhibited (>99%) by CABP, both before and after gel filtration. The tightly bound activator $^{14}CO_2$ was tested for exchangeability with excess unlabeled CO_2. Peak protein fractions were pooled and incubated with 50 mM $NaHCO_3$ for 1 h at pH 7.8 and rechromatographed. Protein-bound $^{14}CO_2$ remained at a constant ratio of 1 mol of $^{14}CO_2$ per mol of protomer through the peak fractions, and the enzyme remained completely inactive, even under optimal activation and assay conditions (Fig. 1C). These results indicate that this procaryotic, hexadecameric RuBPCase responds to CABP in a fashion identical to that of the enzyme from spinach leaves (data not shown; 8–10); bound CABP markedly stabilizes activator $^{14}CO_2$ and completely inhibits enzymatic activity. These findings have interesting implications regarding the conservation of the activator-catalytic site relationship during the evolution of autotrophy. More noteworthy, CABP stabilization of activator $^{14}CO_2$ in *C. vinosum* RuBPCase should permit analysis of the activator site amino acid sequence in comparative studies with the eucaryotic protein (6), thus providing definitive insight into the conservation of specific residues in the $CO_2 \cdot Mg^{2+}$-activator site during the evolution of this important bifunctional enzyme.

An additional advantage of the CABP stabilization of activator $^{14}CO_2$ in procaryotic RuBPCase reported here relates to the renewed interest in the subunit stoichiometry of the microbial protein which has been prompted by several reports that various bacteria have higher plant-type RuBPCases with less than eight catalytic subunits per holoenzyme (5, 13, 14). We propose that CABP stabilization of activator $^{14}CO_2$ should provide an independent, critical technique to assess the number of activator sites (and, by inference, the number of catalytic subunits) per holoenzyme. Determination of moles of $^{14}CO_2$ bound, coupled with knowledge of the protein concentration and native M_r, will yield an accurate determination of the number of catalytic subunits per holoenzyme. Care must be taken to ascertain that the bound $^{14}CO_2$ is not exchangeable with excess $^{12}CO_2$ (e.g., Fig. 1C) to ensure that bound $^{14}CO_2$ has not been lost during gel filtration.

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