Exchange Properties of the Activator CO_2 of Spinach Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase

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ABSTRACT

The exchange properties of the activator CO_2 of spinach ribulose-1,5-bisphosphate carboxylase/oxygenase were characterized both *in vitro* with the purified enzyme, and *in situ* within isolated chloroplasts. Carboxyarabinitol-1,5-bisphosphate, a proposed reaction intermediate analog for the carboxylase activity of the enzyme, was used to trap the activator CO_2 on the enzyme both *in vitro* and *in situ*. Modulation of ribulose-1,5-bisphosphate carboxylase/oxygenase activity in intact chloroplasts during a light/dark cycle was associated with a similar modulation in carboxyarabinitol-1,5-bisphosphate-trapped CO_2. The exchange kinetics of the activator CO_2 were monitored by activation of the enzyme to steady state in the presence of $^{12}CO_2$, followed by addition of $^{14}CO_2$ and determination of the amount of labeled CO_2 trapped on the enzyme by carboxyarabinitol-1,5-bisphosphate. Rate constants (K_{obs}) for exchange with both the purified enzyme (0.45 min^{-1}) and in illuminated chloroplasts (0.18 min^{-1}) were comparable to the observed rate constants for enzyme activation under the two conditions. A similar exchange of the activator CO_2 was not observed in chloroplasts in the dark. Kinetic analysis of the exchange properties of the purified enzyme were consistent with an equilibrium between active and inactive forms of the enzyme during steady state activation.

The activity of ribulose-1,5-bisphosphate carboxylase/oxygenase, which carries out the primary carbon fixation step during photosynthesis, has been shown to be light stimulated both *in vivo* (13) and *in situ* within isolated intact chloroplasts (1). This regulatory property of the enzyme is of some significance as the activity of RuBisCO[2] may represent a rate-limiting step in photosynthesis (14).

It has been established that *in vitro* an inactive form of purified RuBisCO can react with CO_2 and Mg^{2+} to form a catalytically active ternary complex, enzyme-CO_2-Mg^{2+} (8). Kinetic data indicate that the activation process involves the ordered binding of first CO_2 to an activator site on the enzyme and then Mg^{2+} (8). The binding of the activator CO_2 has been shown to result in carbamate formation on lysyl residue 201 of spinach RuBisCO (7). Lorimer (6) and Miziorko (12) have demonstrated that the activator CO_2 moiety is distinct from the catalytically active species in the carboxylase activity of the enzyme. The relationship between the activation of purified RuBisCO by CO_2 and

Mg^{2+} as observed *in vitro* to the modulation of activation *in vivo* remains unclear (13).

Carboxyarabinitol-1,5-bisphosphate a proposed reaction intermediate analog for the carboxylase activity of RuBisCO (15) has been shown to interact with the isolated enzyme to form an extremely stable quarternary complex of enzyme-CO_2-Mg^{2+}-CABP (12). CABP effectively traps the activator CO_2 on the purified enzyme and has been used to demonstrate that the net binding of CO_2 to the activator site on purified RuBisCO accurately reflects the activation state of the isolated enzyme at equilibrium (2). The stability of the activator CO_2, once bound to RuBisCO, has not been directly investigated.

We have used CABP trapping of the activator CO_2 to demonstrate that the light/dark modulation of RuBisCO activity *in situ* within isolated intact chloroplasts is associated with changes in activator CO_2 binding. In addition, CABP trapping was used to directly determine the exchange properties of the activator CO_2 both *in vitro* and *in situ* in intact chloroplasts. The data presented here demonstrate that the activation of RuBisCO is a dynamic rather than static process.

MATERIALS AND METHODS

Materials. RuBisCO was prepared from spinach leaves as described by McCurry *et al.* (10) and stored as a 50% saturated $(NH_4)_2SO_4$ precipitate at 4°C. Protein concentration was determined spectrophotometrically (19). Intact chloroplasts were prepared from spinach grown hydroponically as described by Heldt and Sauer (4). CABP was a kind gift from Jun-ichi Takanashi and W. L. Ogren. RuBP was purchased from Sigma.[3] $NaH^{14}CO_3$ was purchased from New England Nuclear. All other reagents were of the highest purity commercially available.

Enzyme Preparation. Isolated RuBisCO was pelleted and resuspended in dialysis buffer (25 mM Bicine, 1 mM EDTA, 10 mM 2-mercaptoethanol [pH 7.8]) (11). The enzyme was then dialyzed against 1000-fold excess of buffer for 16 h, 4°C. The dialyzed enzyme was then made up to 20 mM with respect to both $MgCl_2$ and $NaHCO_3$, and 5 mM with respect to DTT, and heated for 25 min at 50°C (9) in a sealed vial (final maximal activity with saturating substrates was approximately 2.0 μmol mg^{-1} min^{-1}). The heat activated enzyme was then deactivated as described by Lorimer *et al.* (9) and stored in CO_2-free buffer at room temperature.

Determination of Activation and the Exchange of Activator CO_2 in Isolated RuBisCO. The kinetics of activation and exchange were monitored in an activation mixture containing 50

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[2] Abbreviations: RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; CABP, carboxyarabinitol-1,5-bisphosphate; RuBP, ribulose-1,5-bisphosphate; PGA, 3-phosphoglycerate.

mM Tris buffer (CO_2-free), 1.5 mM $NaHCO_3$, 20 mM $MgCl_2$ (pH 8.2) 20°C. Assays for activation of carboxylation activity were initiated by addition of deactivated enzyme (0.1 mg ml^{-1} final concentration) to the activation mixture. Samples were removed from the activation mixture as indicated in Figure 1 and added to RuBP carboxylase assays (100 mM Tris, 5 mM $NaH^{14}CO_3$ [1 μCi μmol^{-1}], 5 mM $MgCl_2$, 0.6 mM DTT, 0.4 mM RuBP [pH 8.2], 25°C). The reaction was terminated after 20 s by addition of HCl. Acid-stable radioactivity was determined by liquid scintillation counting.

For CABP trapping experiments with purified RuBisCO the same activation mixture was used (final protein concentration in activation mix approximately 0.5 mg ml^{-1}), but was made approximately 10 μCi μmol^{-1} $NaH^{14}CO_3$ either prior to addition of enzyme (activation experiments) or 5 min after addition of enzyme (exchange experiments). Aliquots were removed from the activation mixture as indicated in Figure 1 and added to a CABP-quenching solution (300 μM CABP, 10 mM Tricine, final concentrations in quench pH 8.2). Quenched samples were stored at room temperature for 1 h (12) and frozen in liquid N_2 and stored overnight at −80°C. The samples were then thawed and acid-labile counts associated with the high mol wt fraction were determined by gel filtration through Sephadex G-50 (12). Acid and base-stable counts were determined by addition of HCl or NaOH prior to liquid scintillation counting. CABP-trapped CO_2 was calculated from the NaOH-stable, HCl-labile, CABP-dependent counts associated with the high mol wt fraction. The data presented in Figure 1 were corrected for a background level of CABP-trapped CO_2 which was found to be independent of the activation state of the enzyme. Subsequent experiments have indicated that this background can be greatly reduced by the inclusion of high bicarbonate in the gel filtration column (VJ Streusand and AR Portis, unpublished data).

Determination of RuBisCO Activation and Exchange of Activator CO_2 in Intact Chloroplasts. For determination of RuBP carboxylase activity, chloroplasts (200 μg) Chl were added to 1.5 ml of an incubation mixture containing 0.333 M sorbitol, 50 mM Hepes, 0.1 mM EDTA, 3000 units catalase, 1.5 mM $NaHCO_3$, 5 mM Pi, or 2 mM PGA as indicated (pH 7.7), 20°C. The same incubation mixture was used in the CABP trapping experiments with the exception of the addition of $NaH^{14}CO_3$ (approximately 10 μCi μmol^{-1}) either prior to chloroplast addition (activation), or as indicated in the exchange assays in Figure 3. The reaction mixtures were illuminated by a 500 W slide projector (PAR 900 μE m^{-2} s^{-1}). For determination of RuBP carboxylase activity, samples of the incubation mixture were removed as indicated in the Figures, and added to the RuBP carboxylase assay mixture described above, containing in addition 0.05% (w/v) Triton X-100 to ensure complete lysis of the chloroplasts. Acid-stable radioactivity was determined as before. For determination of CABP-trappable CO_2, samples of the incubation mixture were removed at the indicated times and quenched by addition to a quench containing 100 μM CABP, 0.05% Triton X-100, 10 mM Tris·HCl (final concentrations), pH 8. After incubation at room temperature for 1 h, samples were centrifuged to remove debris, and the supernatants were frozen and stored as described above. The acid and base stable counts associated with the high mol wt fraction were determined as described above, except that the volume of the samples was reduced by lyophilization prior to gel permeation chromatography.

RESULTS

Exchange Properties of the Activator CO_2 in Purified RuBisCO. The data in Figure 1 demonstrate that CABP can be effectively used to study the exchange properties of the activator CO_2 on isolated RuBisCO. Incubation of deactivated enzyme in the presence of Mg^{2+} and $NaHCO_3$ results in the activation of
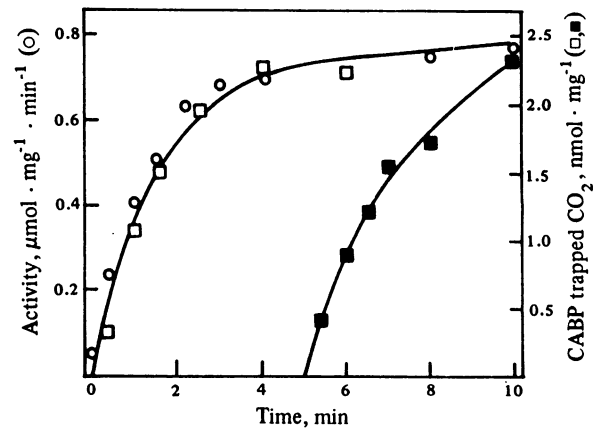

FIG. 1. Kinetics of activation, binding and exchange of CABP-trappable CO_2 with isolated RuBisCO. Reactions for determination of carboxylase activity (O) were initiated by addition of deactivated enzyme to complete reaction mixtures. Determinations of binding (□) and exchange of activator CO_2 (■) were carried out in the same activation mixtures supplemented with $NaH^{14}CO_3$ (10 μCi μmol^{-1}) either prior to enzyme addition (binding), or 5 min after enzyme addition (exchange). Assay conditions, reaction mixtures, and data analysis are described in "Materials and Methods."

the enzyme, with steady state activation being achieved within 5 min, as determined by both measurement of carboxylation activity and CABP-trappable $^{14}CO_2$ in the presence of labeled bicarbonate. Previous studies (2, 11) have demonstrated a correlation between the activation state of RuBisCO and the formation of the stable enzyme CO_2-Mg^{2+}-CABP quarternary complex under conditions of steady state activation. The data in Figure 1 show, in addition, a correlation between the kinetics of the activation process and the amount of CABP-trapped CO_2. Activation of purified RuBisCO in the presence of unlabeled bicarbonate, followed by addition of label after reaching steady state, allows observation of the exchange characteristics of the activator CO_2. Rate constants (K_{obs}) for activation and exchange of 0.60 min^{-1} and 0.45 min^{-1}, respectively, were observed under these conditions. These data clearly demonstrate the dynamic nature of the activation of the isolated enzyme.

CABP Trapping of Activator CO_2 in Intact Chloroplasts. Prior to analysis of the exchange properties of the activator CO_2 on the enzyme within intact chloroplasts, it was necessary to demonstrate that the level of CABP-trapped CO_2 reflects the activation state of RuBisCO *in situ* (18, 20). As shown in Figure 2, CABP-trapped CO_2 fluctuates with RuBisCO activation during light/dark cycles. In addition, both the light activation and light-dependent increase in CABP trapped CO_2 were markedly inhibited if the chloroplasts were incubated in the presence of PGA rather than Pi, conditions previously demonstrated to inhibit activation of RuBisCO (3). We propose that the limited increase in CABP-trapped CO_2 observed during incubation in the presence of PGA is due to exchange of the unlabeled activator CO_2 present in unilluminated chloroplasts (reflected in the RuBP carboxylase activity observed at time 0) with labeled substrate. If it is assumed that the binding of one CO_2 is associated with activation of a single RuBisCO site, then the data in this figure indicate that the turnover number of the enzyme is 2.8 s^{-1}. While this turnover number is somewhat lower than that observed for the purified enzyme in Figure 1 (5 s^{-1}), turnover numbers of approximately 3 to 5 s^{-1} have been previously reported (2, 17). The data presented here demonstrate that light modulation of RuBisCO activity in intact chloroplasts is reflected in the level of CABP-trapped CO_2.
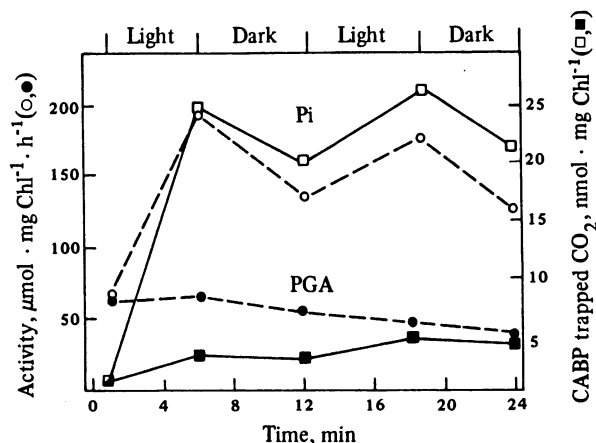
FIG. 2. Effects of light and darkness on activity of RuBisCO and CABP-trappable CO$_2$ in intact chloroplasts. For the determination of RuBP carboxylase activity (- - -/O, ●), the chloroplast incubation medium (see "Materials and Methods") contained either 5 mM Pi (O) or 2 mM PGA (●). Determinations of CABP-trapped CO$_2$ (——/□, ■) utilized the same incubation medium and Pi (□) or PGA (■) concentrations. Assay conditions, reaction mixtures, and data analysis are described in "Materials and Methods."
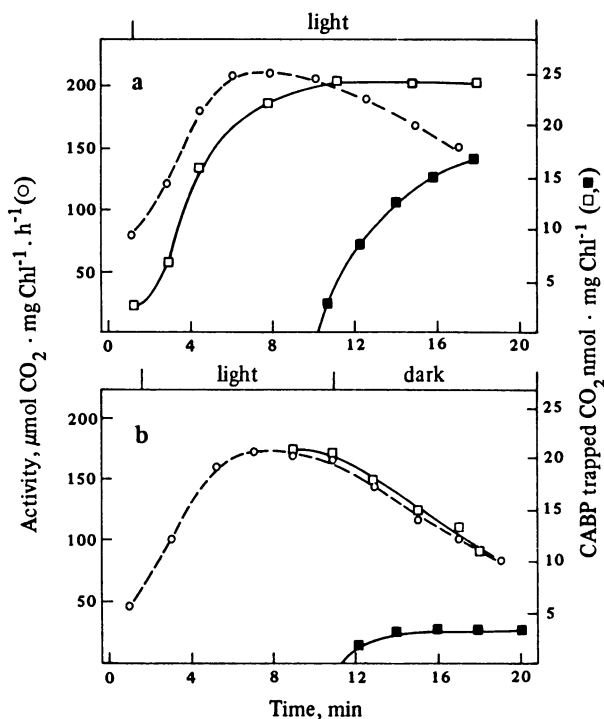

FIG. 3. Exchange of CABP-trappable CO$_2$ in intact chloroplasts in the light (a) and dark (b). For determination of RuBP carboxylase activity (- - -/O) the chloroplast incubation medium (see "Materials and Methods") contained 5 mM Pi. The same incubation medium was used in the determination of CABP-trappable CO$_2$ (——/□, ■). For CABP-trapped CO$_2$ associated with activation (□), NaH^{14}CO$_3$ was added prior to chloroplast addition. For exchange experiments (■), the same label was added after 8 min illumination (a) or 20 s into the dark period (b). Assay conditions, reaction mixtures, and data analysis are described in "Materials and Methods."

Exchange Properties of the Activating CO$_2$ in Intact Chloroplasts. The exchange properties of the activator CO$_2$ on RuBisCO in intact chloroplasts are shown in Figure 3, both in the light (Fig. 3a) and dark (Fig. 3b). The data presented in this figure clearly demonstrate that the activator CO$_2$ readily exchanges with free ligand on the enzyme *in situ* in illuminated chloroplasts. Similar rate constants were observed for activation and exchange ($K_{obs} = 0.18$ min^{-1}), but the rates were considerably lower than those observed with the isolated enzyme (Fig. 1). This may be due to the modification of the activation/exchange properties of the enzyme by metabolites or other effectors in the chloroplast which are not present in the assays employed with the isolated enzyme. Similarly, the lack of exchange of the activator CO$_2$ in darkened chloroplasts (Fig. 3b) might be accounted for by a dark-induced change in stromal conditions or metabolite levels resulting in a relative inability of the activator CO$_2$ to exchange. Further studies are clearly required to determine the biochemical basis of the lack of exchange in the dark.

DISCUSSION

We have found that the activator CO$_2$ on purified spinach RuBisCO exchanges with unbound ligand during conditions of steady state enzyme activation. In addition, CABP was shown to trap the activator CO$_2$ on RuBisCO activated *in situ* within isolated intact chloroplasts. The level of CABP-trappable CO$_2$ within the chloroplast fluctuated with the activation state of the enzyme during light/dark cycles, and the observed light-dependent increase of trapped CO$_2$ was prevented if the light activation of RuBisCO was inhibited by incubation of the chloroplasts with PGA (3). Following light activation of RuBisCO in intact chloroplasts, the activator CO$_2$ was also observed to exchange in the light, but not if illumination was discontinued. The presence of exchange may account for the disproportionately large increase in CABP-trapped CO$_2$ relative to enzyme activation during the first period of illumination (Fig. 2). The chloroplasts used in these studies had a significant level of RuBP carboxylase activity prior to illumination. Thus, the increase in CABP-trapped CO$_2$ during the initial illumination will reflect both net binding of ^{14}CO$_2$ and exchange with the previously bound unlabeled substrate.

The exchange of activator CO$_2$ could be explained by either an equilibrium between active and inactive forms of the enzyme maintained during steady state activation kinetically described in Ref. (8), or possibly a more direct exchange of the bound CO$_2$ ligand with free CO$_2$ without complete reversal of the activation process. An analysis of the data obtained with the isolated enzyme supports the first interpretation.

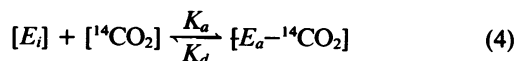The rate constants for the activation of this enzyme may be described by Lorimer *et al.* (8):

$$E + CO_2 \xrightleftharpoons[k_2]{k_1} ECO_2 + Mg^{2+} \xrightleftharpoons[k_4]{k_3} E\text{-}CO_2\text{-}Mg^{2+} \qquad (1)$$
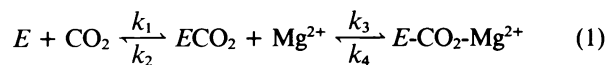
where

$$K_{obs_{Act}} = k_1 \, [CO_2] + \frac{k_2 k_4}{k_3 \, [Mg^{2+}]}. \qquad (2)$$

In this model, k_1 and k_2 represent rate constants for the forward and reverse reactions of a rate-limiting CO$_2$ binding reaction, and k_3 and k_4 rate constants for the forward and reverse reactions for the binding of Mg^{2+} to the enzyme-CO$_2$ complex. If the activation of RuBisCO is fully reversible then exchange of the label with unlabeled activator CO$_2$ may be described by:

$$[E_a\text{-}^{12}CO_2] \xrightleftharpoons[K_a]{K_{d_-}} [E_i] + [^{12}CO_2] \qquad (3)$$

and

$$[E_i] + [^{14}CO_2] \xrightarrow{\frac{K_a}{K_d}} [E_a-^{14}CO_2] \qquad (4)$$

where E_a and E_i represent active and inactive enzyme and $K_a = k_1 [CO_2]$ and $K_d = k_2 k_4/k_3 [Mg^{2+}]$. It is clear that under conditions of steady state activation

$$\frac{d[E_a-^{14}CO_2]}{dt} = \frac{-d[E_a-^{12}CO_2]}{dt} \qquad (5)$$

After addition of label, the dissociation of the $(E_a-^{12}CO_2)$ complex (Eq. 3) can be viewed as an irreversible process due to the immediate dilution of the released ligand (16), which implies that

$$\frac{-d[E_a - ^{12}CO_2]}{dt} = K_d [E_a - ^{12}CO_2] \qquad (6)$$

and therefore

$$\frac{d[E_a-^{14}CO_2]}{dt} = K_d [E_a - ^{12}CO_2] \qquad (7)$$

which implies that K_{obs} for exchange can be described by:

$$K_{obs_{exch}} = K_d. \qquad (8)$$

When an analysis of K_d was carried out on the enzyme preparation used in Figure 1, good agreement was found between K_{obs} exchange (0.45 min^{-1}) and K_d (0.41 min^{-1}) as determined from activation studies as in Refs. (5) and (8). This indicates that the exchange observed in these experiments reflects an equilibrium between active and inactive forms of RuBisCO during steady state activation.

The exchange kinetics of the enzyme *in situ* cannot be quantitatively analyzed at this time, since the effects of pH and various positive and negative effectors of activation (13) on the exchange properties of the purified enzyme have not been characterized. However, the data presented here clearly demonstrate the dynamic nature of RuBisCO activation *in situ*, as well as *in vitro*.

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