Evidence for the Existence of Discrete Activator and Substrate Sites for CO₂ on Ribulose-1,5-bisphosphate Carboxylase*  

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When incubated with CO₂ and Mg²⁺, ribulose-1,5-bisphosphate carboxylase forms a ternary complex of enzyme·CO₂·Mg. This complex was prepared using high specific activity [¹⁴C]CO₂ and injected into a solution containing a large (50- to 112-fold) molar excess of [¹³C]O₂ and sufficient ribulose 1,5-bisphosphate to permit the catalytic site to turn over several times. The enzyme was then rapidly separated from the other components by gel filtration and its radiospecific activity was determined to be 30 to 60 times that of the medium.

If the CO₂ activator and the CO₂ substrate sites were one and the same, then, following turnover, the enzyme should have been in isotopic equilibrium with the medium. The finding that this was not the case, by a factor of about 40, indicates that the CO₂ activator site is physically distinct from the CO₂ substrate site.

The activity of ribulose-bisphosphate carboxylase (EC 4.1.1.39) has long been known to be stimulated by preincubation with CO₂ and Mg²⁺ (3). Kinetic studies indicate that the activation of the enzyme is associated with the ordered formation of a ternary enzyme·CO₂·Mg complex (1, 2, 4, 5). Enzyme reacts first with CO₂ in a rate-determining step, the resultant enzyme·CO₂ complex being stabilized by the subsequent rapid addition of Mg²⁺. An enzyme·[¹⁴C]O₂·Mg complex could be isolated with about 33% yield by gel filtration chromatography provided that the column was equilibrated with Mg²⁺ (1). This result suggested that the dissociation of the enzyme·[¹⁴C]O₂·Mg complex was a slow process. However, attempts to demonstrate such a complex using isotope trapping techniques (6, 7) were essentially negative. These observations lead us to suggest that the activator CO₂ molecule might well be distinct from the substrate CO₂ molecule (for a discussion of this, see Ref. 8). Miziorko (9) has recently provided the first physical (as opposed to purely kinetic) evidence for the existence of discrete sites for the activator CO₂ and the substrate CO₂ molecules. Carboxypentitol bisphosphate (CPBP), a potent competitive (with respect to ribulose 1,5-bisphosphate) inhibitor of both the carboxylase and oxygenase reactions, was shown to stabilize an enzyme·[¹⁴C]O₂·Mg complex to such an extent that the [¹⁴C]O₂ could no longer exchange with an excess of added [¹³C]O₂. Assuming that the carboxyl group of CPBP occupies the substrate CO₂ binding site, it follows that the [¹⁴C]O₂ of the enzyme·[¹⁴C]O₂·Mg·CPBP complex must occupy another site.

This report describes evidence of a quite different nature which further strengthens the proposition that the sites for the activator CO₂ and substrate CO₂ are separate entities.

**EXPERIMENTAL PROCEDURES**  

**Materials**  

RuBP carboxylase from spinach leaves was purified, activated, and assayed as previously described (1, 2). Protein was determined at 280 nm as reported elsewhere (10). The specific activity of the enzyme used in the experiments reported here was 1.3 to 1.4 units mg⁻¹ at 25°C. RuBP was purchased from Sigma Chemical Co., Bio-Gel P-4 (100 to 200 mesh) from Bio-Rad Labs., and NaH¹⁴CO₃ from Amer sham/Searle. Other reagents were of the highest purity commercially available. Microliter additions were made with precision microliter syringes from Scientific Glass Engineering, Houston, Tex.

**Methods**  

**Standardization of NaH¹⁴CO₃**  

To 300 μl of 0.194 M NaHCO₃ was added 50 μl of carrier-free NaH¹⁴CO₃ containing 0.25 μCi of CO₂ and 4.2 μmol of bicarbonate. Several 1- and 2-μl aliquots were diluted into 100 μl of 10% (v/v) ethanolamine in ethanol, scintillation mixture was added and the entire mixture was counted using the cross-channels ratio method to determine counting efficiency.

**Standardization of RuBP**  

A 365-d solution containing 12.5 μmol of CO₂-free Hepes/NaOH, pH 8.26, 8 μmol of MgCl₂, 4.85 μmol of NaH¹⁴CO₃ (2550 dpm/μmol), 0.1 μmol of dithiothreitol, and 373 μg (0.51 units) of RuBP carboxylase was preincubated for about 10 min at 25°C. Then, 2 μl of RuBP containing about 0.02 μmol was added and the incubation was continued at 25°C. Aliquots were withdrawn at 15, 30, and 60 min and acid-stable ¹⁴C radioactivity was determined. No significant differences in the quantity of acid-stable radioactivity at 15, 30, or 60 min were observed, from which it was concluded that the reaction had gone to completion.

Other experimental details are given in the figure and table legends.

**RESULTS**  

A solution containing 5.75 mg (82.2 nmol of protomer) or 7.3 units of RuBP carboxylase in the form of an enzyme·[¹⁴C]O₂·Mg complex, was injected into another solution at 25°C containing a 55-fold molar excess of [¹³C]O₂ and sufficient RuBP to permit an average of 10 turnovers. Thus, sufficient enzyme was present to drive the reaction to completion within about 8 s. Acid-stable ¹⁴C radioactivity, determined on aliquots withdrawn after mixing, was constant from the earliest sampling time, 20 s, confirming that the reaction proceeded rapidly to completion. The resultant mixture was rapidly transferred to a small gel filtration column equilibrated with 0.1 M Tris-HCl, 0.1 M MgCl₂, 0.01 M NaHCO₃, pH 9.02, and elution was performed with the same buffer. The result of such a procedure is shown in Fig. 1. A peak of acid-labile, alkali-stable ¹⁴C radioactivity eluted with the protein in Fractions 9 through 11 was well separated from both the acid-stable ¹⁴C (the product, 3-phosphoglycerate) and alkali stable ¹⁴C (free CO₂) in Fractions 20 to 30. The presence of Mg²⁺ during gel filtration was absolutely essential to the recovery of enzyme-bound [¹⁴C]O₂. No enzyme-bound ¹⁴C whatsoever was recovered

* The abbreviations used are: CPBP, 2-carboxypentitol 1,5-bisphosphate; RuBP, ribulose 1,5-bisphosphate; Hepes, N-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
When Mg\(^{2+}\) was omitted from the elution buffer (data not shown).

The recovery of enzyme-bound \(^{14}\)C was in the range of 20 to 75\% (Table I), depending upon the temperature at which the gel filtration step was performed. Increasing the temperature brought about greater dissociation of the enzyme-\(^{14}\)CO\(_2\)-Mg complex. The recovery of this complex was not 100\%. Some dissociation of the complex or exchange with the \(^{14}\)CO\(_2\) present in the elution buffer was to be anticipated during chromatography. Although not apparent in Fig. 1 (for reasons of scale), the acid-labile, alkali-stable \(^{14}\)C radioactivity did not quite decline to zero between the two major peaks. This indicates, as one would expect, that the enzyme-\(^{14}\)CO\(_2\)-Mg complex has undergone partial dissociation during the period of chromatography thus causing this trailing phenomenon. Nevertheless, the specific radioactivity within the four fractions encompassing the protein peak (Fractions 9 to 12) was constant at about 4700 dpm/nmol of enzyme protomer. This value is 30 times the specific radioactivity of the \(^{14}\)C in the medium after mixing. A summary of the results of six such experiments is presented in Table I.

The specific radioactivity of the product, 3-phosphoglycerate, (corrected for the formation of 2 mol of product/mol of \(^{14}\)CO\(_2\) fixed) was consistently larger than the radioactivity of the CO\(_2\) in the medium after mixing. This result implies that a small quantity of the high specific activity \(^{14}\)CO\(_2\), presumed to be present at the substrate CO\(_2\) site before mixing, was subsequently trapped with RuBP before it underwent exchange with \(^{14}\)CO\(_2\).

**DISCUSSION**

The strategy underlying the experiments reported here depended upon bringing the CO\(_2\) at the substrate site into isotopic equilibrium with the unbound CO\(_2\) by permitting limited turnover to occur. By allowing an average of 3 to 25 turnovers/site, the possibility of "misses" occurring (a site failing to turnover) should have been minimized. If, therefore, it seems reasonable to assume that all of the substrate CO\(_2\) sites have turned over at least once. If, then, the activator CO\(_2\) and the substrate CO\(_2\) are one and the same, it follows that the radiospecific activity of the enzyme should equal that of the unbound CO\(_2\). But the observed radiospecific activity of the enzyme was some 40 times that of the unbound CO\(_2\), even after having been subject to gel filtration against a buffer containing 10 mM NaHCO\(_3\) (about 20 \(\mu\)M CO\(_2\) at pH 9.02). This result can only have occurred if the activator CO\(_2\) and substrate CO\(_2\) sites are physically distinct.

It is clear from the results reported here and elsewhere (1, 5, 9) that Mg\(^{2+}\) in some way stabilizes the activator CO\(_2\) molecule. It has been suggested that the activation of the enzyme involves the formation of a carbamate, which would provide a binding site for divalent cation and enhance the stability of the carbamate (1) (see Equation 1).

![Fig. 1. Isolation of a stable enzyme-\(^{14}\)CO\(_2\) (activator)-Mg\(^{2+}\) complex by gel filtration chromatography.](http://www.jbc.org/)

**Table I**

| Experiment | Temperature | Turnovers/site | CO\(_2\) before mixing | CO\(_2\) after mixing | Enzyme-bound CO\(_2\) after mixing and gel filtration | Fixed CO\(_2\) |
|------------|-------------|----------------|------------------------|----------------------|---------------------------------------------------|---------------|
| 1          | 28          | 4.3            | 8640                   | 99                   | 1715 (29)                                          | 502           |
| 2          | 23          | 6.6            | 8640                   | 60                   | 2681 (31)                                          | 118           |
| 3          | 23          | 3.3            | 8640                   | 60                   | 2265 (26)                                          | 129           |
| 4          | 4           | 3.8            | 8640                   | 111                  | 6354 (74)                                          | 275           |
| 5          | 4           | 25.1           | 8640                   | 74                   | 4233 (49)                                          | 159           |
| 6          | 4           | 10.0           | 8640                   | 154                  | 4679 (34)                                          | 366           |

- Each of the six experiments was performed essentially as outlined in the legend to Fig. 1. However, different quantities of enzyme or RuBP were used in each case thus giving rise to different values for the number of turnovers/ enzyme site and to the \(^{14}\)C radiospecific activity of the CO\(_2\) after mixing.
- \(^{14}\)C activity was determined from the quantity of acid-stable \(^{14}\)C radioactivity recovered after gel filtration, assuming that 8640 dpm/nmol represents 100%.

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Metal ion-enhanced stabilization of carbamates has been reported (11). A recent $^{13}$C NMR study using the enzyme from *Rhodospirillum rubrum* found evidence for the existence of such a carbamate, the detection of which was dependent upon the presence of $\text{Mg}^{2+}$ (12). The carbamate hypothesis provides a useful framework for explaining the observed stability of the enzyme-$[^{13}\text{C}]\text{O}_2$ (activator) complex in the presence of $\text{Mg}^{2+}$. Clearly, the fate of the binary enzyme-$\text{CO}_2$ complex (Species II of Equation 1) will depend upon the pH, the $\text{Mg}^{2+}$ concentration, and the relative values for $k_2$ and $k_3$.

It has been suggested that the carboxylation reaction is ordered with substrate $\text{CO}_2$ binding after RuBP (5). The ability to trap at least a small quantity of the high specific activity substrate $[^{12}\text{C}]\text{O}_2$ with RuBP (Table I) indicates that the carboxylation reaction can occur with substrate $\text{CO}_2$ binding before RuBP. This interpretation is supported by the results of $^{13}$C NMR experiments (13) which are also consistent with the idea that occupancy of the substrate $\text{CO}_2$ site may occur before RuBP binds.

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