FORMATION OF STOICHIOMETRIC COBALT(III)/CO_2 COMPLEXES WITH SPINACH RIBULOSE BISPHOSPHATE CARBOXYLASE/OXYGENASE

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Received 22 January 1980

1. Introduction

Ribulose 1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) catalyzes the initial carboxylation of RuBP in the Calvin reductive pentose phosphate pathway. The enzyme may also act as an internal monooxygenase and catalyze the oxygenolysis of RuBP, leading to the production of glycolic acid during photorespiration (reviewed [1]). For maximum catalytic rates, the enzyme must first be activated by preincubation with a divalent cation and CO_2 [2,3] and there is some evidence to suggest that the CO_2 activation site is distinct from the catalytic site [4,5]. With regard to the divalent cation requirement of the eucaryotic enzyme, it has been shown that Mn^{2+}, Ni^{2+} and Co^{2+} will substitute for Mg^{2+} for both carboxylase [6] and oxygenase [7] activities. However, only the oxygenase activity of the *Rhodospirillum rubrum* enzyme (a dimer of large catalytic subunits) is supported by Co^{2+}, providing the first solid evidence for a differential 'all or nothing' effect on either of the two activities of this bifunctional enzyme [8].

Because the spinach leaf enzyme (a hexadecamer of 8 large catalytic and 8 small subunits) does not show this differential activity with Co^{2+} ions, we have examined the metal and CO_2 sites of this enzyme. We have prepared exchange-inert complexes of enzyme–cobalt–CO_2, by oxidizing enzyme-bound Co^{2+} to Co(III) [9] a procedure used to examine the dimeric enzyme from *R. rubrum* [8]. These complexes are stable enough to allow quantitation of cobalt binding as well as any compound which ligates with the bound metal.

Abbreviations: RuBP, D-ribulose 1,5-bisphosphate; CRBP, 2-carboxy-D-ribitol 1,5-bisphosphate; Mops, 4-morpholinepropanesulfonic acid; SDS, sodium dodecyl sulfate

2. Materials and methods

2.1. *Materials*

Fresh spinach leaves were obtained from a local market. [^{14}C]Carbonate (20 mCi/mmol) was from Amersham. RuBP was prepared enzymatically from ribose 5-phosphate [10] as described [11]. CRBP was prepared as in [12]. H_2O_2 was from Fisher; solutions were made fresh for each experiment. All other materials were of reagent grade.

The enzyme was purified as in [12] with the exception of a BioGel A1.5M chromatography step instead of hydroxylapatite chromatrography. The enzyme was eluted from this column and stored frozen (–70°C) in 50 mM potassium phosphate (pH 7.5) containing 1 mM EDTA, 5 mM 2-mercaptoethanol and 20% glycerol. As with the enzyme from *R. rubrum* [14], the spinach enzyme is quite stable in this form and no loss in activity was seen after 6 months. The enzyme was judged homogeneous by SDS gel electrophoresis [15] and had spec. act. 2.2 μmol CO_2 fixed . min^{-1} . mg $protein^{-1}$. The protein concentration was determined using the extinction coefficient at 280 nm [13], Co(III) incorporation had no effect on this constant.

2.2. *Assay*

Acid-stable [^{14}C]O_2 incorporation was measured in 40 mM Mops (pH 7.8) as in [8] except that the assay was reduced to 1 min.

2.3. *Co(III) modification*

The incubation was performed at room temperature in 31–38 mM Mops (pH 7.8) in total vol. 60–80 μl. The reaction was stopped by gel-filtration on a Sephadex G-25 column in a Pasteur pipet. Further details are in [8].

Cobalt levels were measured by atomic absorption spectroscopy using a Perkin Elmer 306 spectrophotometer equipped with a HGA-70 heated graphite atomizer.

3. Results

Oxidation of Co^{2+} to Co(III) by H_2O_2 in the presence of RuBP carboxylase resulted in the formation of a stable Co(III)–enzyme complex with the concurrent loss of carboxylase activity (fig.1A). Loss of all activity could be correlated with the formation of a complex containing 1 atom cobalt/protomer. There was no loss of activity when the enzyme was incubated with the same level of H_2O_2 in the presence of Mg^{2+}, and there was no incorporation of cobalt without H_2O_2.

In the presence of CO_2, a stoichiometric Co(III)–enzyme complex could be formed using a much lower concentration of Co^{2+} (fig.1B). Again, loss of activity occurred with the incorporation of ~1 cobalt atom/protomer. CO_2 was also incorporated into this Co(III)–enzyme complex, but to a lesser degree than cobalt (fig.1B). In several experiments, CO_2 incorporation varied from 0.5–0.7 molecules/protomer, but never reached one.

While attempting to maximize CO_2 incorporation into the Co(III)–enzyme complex, it was found that the higher levels of CO_2 actually inhibited CO_2 incorporation (table 1a). In the presence of 25 mM carbon-

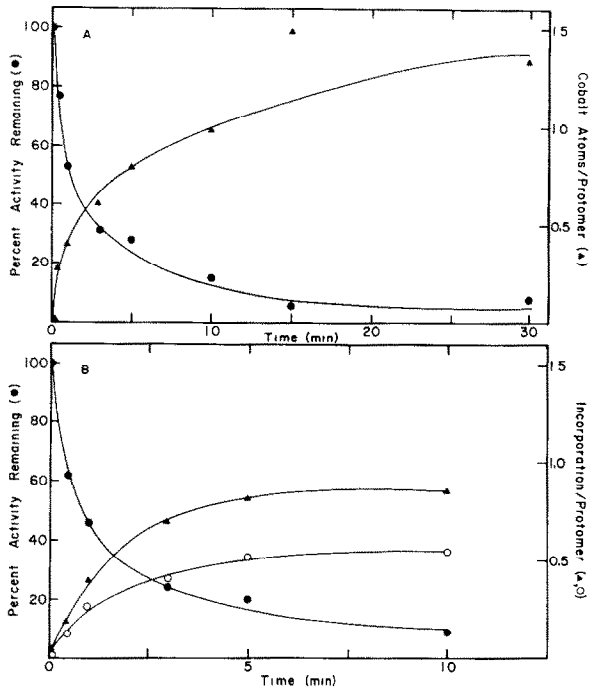

Fig.1. Incorporation of Co(III) and CO_2 into enzyme complexes. (●) Carboxylase activity; (▲) cobalt incorporation; (○) CO_2 incorporation. (A) Cobalt incorporation. The enzyme (31 μM, protomers) was incubated with 1 mM Co^{2+} and 0.1 mM H_2O_2 for various times before gel-filtration as in section 2. (B) Cobalt and CO_2 incorporation. The enzyme (35 μM in protomers) was incubated with 0.1 mM Co^{2+} and 7.6 mM [^{14}C]carbonate for 5 min, then 0.1 mM H_2O_2 was added for the indicated periods.

Table 1
Effect of CO_2 concentration (a) and CRBP (b) on cobalt/CO_2 enzyme complexes

Samples	Carbonate (mM) in incubation	Percent carboxylase activity remaining	[^{14}C]Carbonate/ protomer	Cobalt/protomer
(a) Enzyme–CO_2–Co^{2+}–H_2O_2	0.63	93	0.03	0.09
	1.25	68	0.05	0.11
	2.50	46	0.22	0.33
	6.25	9	0.67	0.88
	25.0	47	0.34	1.09
(b) Enzyme–Co^{2+}–CRBP	–	71	–	0.28
Enzyme–CO_2–Co^{2+}–CRBP	7.10	40	0.54	0.51
Enzyme–CO_2–Co^{2+}–CRBP–H_2O_2	7.10	12	0.85	1.09

In (a), the enzyme (39 μM in protomers) was incubated with 0.1 mM Co^{2+} and a variable amount of Na_2CO_3 for 5 min prior to the addition of H_2O_2 (0.1 mM). After 5 min, the sample was gel-filtered and analyzed. In (b), the enzyme (35 μM protomers) was incubated with 0.1 mM Co^{2+} plus or minus Na_2CO_3 for 5 min before the addition of CRBP (71 μM). After 5 min, the sample was gel-filtered. When H_2O_2 (0.1 mM) was added to the CRBP sample, it was incubated for another 5 min before gel-filtration

ate, the Co(III)–enzyme complex formed has only half as much CO_2 as the complex formed with 6.25 mM carbonate, as well as retaining significantly more activity (table 1a). However, the incorporation of cobalt was slightly stimulated at higher concentrations of CO_2. The time course of binding was identical for each CO_2 concentration.

The transition-state analog CRBP has been shown to form a stable complex with enzyme, Mg^{2+} or Mn^{2+}, and CO_2 [4]. Such a complex was also formed with Co^{2+} (table 1b). In this experiment, a stable complex was formed with Co^{2+} in the presence or absence of CO_2, although to a greater extent in the presence of CO_2. While the loss of activity was stoichiometric with respect to the incorporation of cobalt and CO_2, the absolute incorporation levels could not be increased over 0.6/protomer. Increasing the CRBP concentration caused a loss of most of the remaining activity without increasing the level of cobalt or CO_2 incorporated. However, if the enzyme–CO_2–Co^{2+}–CRBP complex is oxidized with H_2O_2 to form the Co(III) complex, the levels of CO_2 and cobalt incorporation are increased to ~1/protomer, with the concomitant loss of almost all the remaining activity (table 1b).

4. Discussion

RuBP carboxylase can be labeled with 1 atom Co(III)/protomer with an accompanying loss of carboxylase activity (fig.1). Thus, there appears to be 1 active metal ion site/protomer in the presence or absence of CO_2. Using the electron paramagnetic resonance signal of free Mn^{2+}, 1 tight Mn^{2+} binding site (K_d 10 μM)/protomer in the presence of CO_2 and 2–3 much weaker Mn^{2+} sites in the absence of CO_2 were found [16]. In agreement with these results, we find a lower amount of Co^{2+} is required to form a Co(III)–enzyme complex in the presence of CO_2 (fig.1B).

The findings reported here also address the question of separate CO_2 activation and catalytic sites [4,5]. The observation that higher concentrations of CO_2 prevent CO_2 labeling and loss of activity (table 1a) suggests the presence of at least a second site of interaction by CO_2, one which is not coordinated with Co^{2+}. The increased cobalt incorporation seen in the incubation at the high CO_2 concentration probably represents some non-specific labeling, since the activity is increased rather than decreased. Indeed, some non-specific Co(III) labeling is also seen in incubations without CO_2 (fig.1A). The fact that oxidation of an enzyme–CO_2–Co(III)–enzyme complex leads to further CO_2 and cobalt labeling, with increased loss of activity, suggests that the cobalt and CO_2 sites of the two types of complexes may be different.

Since Co(III)–CO_2 complexes can be formed with the *R. rubrum* enzyme [8], a protein which is a dimer of large subunits [17], it is likely that the complexes observed with the spinach enzyme are also localized on the large subunit. Interestingly, the inhibition of CO_2 incorporation into enzyme–Co(III) complexes at high CO_2 was not seen with the *R. rubrum* enzyme [8]. Thus there may be differences in the interaction of CO_2 with these two distinct structural forms of RuBP carboxylase, enzymes which are otherwise strikingly similar [18].

Acknowledgements

This research was supported by National Institutes of Health Grant GM-24497 and by Robert A. Welch Foundation Grant F-691. P.D.R. was supported by NIH postdoctoral fellowship GM-06817.

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