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Differential Effects of Metal Ions on *Rhodospirillum rubrum* Ribulosebisphosphate Carboxylase/Oxygenase and Stoichiometric Incorporation of HCO_3^- into a Cobalt(III)–Enzyme Complex†

Peter D. Robison,‡ Melinda N. Martin, and F. Robert Tabita*

ABSTRACT: Mg^{2+} or Mn^{2+} ions supported both the carboxylase and oxygenase activities of the *Rhodospirillum rubrum* ribulosebisphosphate carboxylase/oxygenase. For the carboxylase reaction, Mn^{2+} supported 25% of the maximum activity obtained with Mg^{2+}; oxygenase activity, however, was twice as great with Mn^{2+} as compared to that with Mg^{2+}. A further differential effect was obtained with Co^{2+}. Co^{2+} did not support carboxylase activity and, in fact, was a strong inhibitor of Mg^{2+}-dependent carboxylase activity, with a K_i of 10 μM. Co^{2+} did, however, support oxygenase activity, eliciting about 40% of the Mg^{2+}-dependent oxygenase activity. No other divalent cations supported either activity. With high concentrations of Mg^{2+} or Mn^{2+}, maximum carboxylase activity was seen after a 5-min activation period; activity decreased to about half of maximum after 30-min activation. A similar time dependence of activation was observed with Mn^{2+}-dependent oxygenase activity but was not seen for Mg^{2+}- or Co^{2+}-dependent activity. Both carboxylase and oxygenase activities were inactivated by the oxidation of Co^{2+} to Co(III) with the resultant formation of a stable Co(III)–enzyme complex. In the presence of HCO_3^- (CO_2), Co(III) modification was stoichiometric, with two cobalt atoms bound per enzyme dimer. Carbon dioxide was also incorporated into this Co(III)–enzyme complex, but only one molecule per enzyme dimer was bound, indicative of half-the-sites activity. These results thus indicate that there are substantial differences in the metal ion sites of the carboxylase and oxygenase activities of *R. rubrum* ribulosebisphosphate carboxylase/oxygenase.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) catalyzes either the carboxylation or oxygenolysis of ribulose 1,5-bisphosphate [for a review, see Jensen & Bahr (1977)]. The carboxylase activity is the primary catalyst of photosynthetic carbon reduction while the oxygenase activity catalyzes the first step in photorespiratory glycolate production (Tolbert, 1973). Thus, the enzyme catalyzes the first reaction of two competing pathways and is intimately involved in crop productivity. Preincubation with HCO_3^- (CO_2) and Mg^{2+} is required to activate both activities (Andrews et al., 1975; Lorimer et al., 1976; Laing & Christeller, 1976). While the enzyme from higher plants contains eight large (catalytic) and eight small subunits (of unknown function) and has a molecular weight of 560000 (Paulsen & Lane, 1966), a simpler enzyme, consisting of a dimer of large subunits (M_r 114000), is isolated from the photosynthetic bacterium *Rhodospirillum rubrum* (Tabita & McFadden, 1974a,b). Activation of the enzyme by HCO_3^- and Mg^{2+} is also observed with the *R. rubrum* enzyme (Tabita & McFadden, 1974a; Christeller & Laing, 1978; Whitman & Tabita, 1978a,b).

The role of the metal ion in catalysis is not clear. Some evidence has been presented (Lorimer et al., 1976) that indicates the CO_2 activation and substrate sites may be different. Carboxylase activity of the spinach enzyme can be obtained by using Mg^{2+}, Mn^{2+}, Co^{2+}, or Ni^{2+} (Weissbach et al., 1956). In the presence of HCO_3^-, a single binding site per large subunit was found for Mn^{2+} (Miziorko & Mildvan, 1974). Nuclear magnetic resonance measurements of water proton and substrate ^{13}C relaxation rates were consistent with an active quaternary enzyme–Mn^{2+}–RuBP–HCO_3^- complex with RuBP[1] in the inner sphere and HCO_3^- in the second sphere of enzyme-bound Mn^{2+} (Miziorko & Mildvan, 1974). Recently, the ratio of Mn^{2+} to Mg^{2+} supported activity of the spinach enzyme has been found to be much higher for the oxygenase than the carboxylase (Wildner & Henkel, 1978).

Based on various studies with effector metabolites, it has been assumed that the same active site is responsible for both activities (Ryan & Tolbert, 1975; Chollet & Anderson, 1976). However, it was recently found that hydroxylamine inhibits only the oxygenase activity of the spinach enzyme (Bhagwat et al., 1978). Obviously, it will be extremely important to

† From the Department of Microbiology, University of Texas at Austin, Austin, Texas 78712. *Received April 13, 1979; revised manuscript received June 8, 1979.* This work was supported by National Institutes of Health Grant GM-24497 and Robert A. Welch Foundation Grant F-691.

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[1] Abbreviations used: RuBP, D-ribulose 1,5-bisphosphate; Mops, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid; BME, 2-mercaptoethanol.

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inhibit, chemically modify, or otherwise selectively alter either of the two activities of this bifunctional enzyme. In this report, we show that there are distinct differences in the divalent metal dependent carboxylase and oxygenase activities of the *R. rubrum* enzyme. Moreover, there are significant differences between the complex higher plant enzyme and the dimeric enzyme from *R. rubrum*. Use was made of an exchange-inert cobalt–enzyme complex formed by oxidizing Co^{2+} to Co(III) (Kang et al., 1972) to investigate the stoichiometry of incorporation of CO_2 and Co^{2+} into the complex.

Experimental Procedures

Materials. RuBP was prepared enzymatically from ribose 5-phosphate (Horecker et al., 1958), as described earlier (Whitman & Tabita, 1976). [^{14}C]$NaHCO_3$ (20 mCi/mmol) was purchased from Amersham. H_2O_2 was obtained from Fisher; solutions were made up fresh immediately before each experiment. All other materials were of reagent-grade quality. All metals were the chloride salts.

RuBP carboxylase/oxygenase from *R. rubrum* was purified as described by Tabita & McFadden (1974a) except for minor modifications (Robison & Tabita, 1979). Sodium dodecyl sulfate gel electrophoresis (Laemmli, 1970) showed the enzyme to be homogeneous. Protein concentrations of the enzyme were calculated from the extinction coefficient at 280 nm of 0.974 L g^{-1} cm^{-1} (Tabita & McFadden, 1974b). Co(III) modification did not change this extinction coefficient. Enzyme, substrates, and buffers were treated with Chelex 100 resin (Bio-Rad Laboratories) before use to remove contaminating metal ions.

Ribulosebisphosphate Carboxylase Assay. Carboxylase activity was measured by acid-stable [^{14}C]$NaHCO_3$ incorporation (Whitman & Tabita, 1976). The assay mixture (0.25 mL) contained 40 mM K–Mops (pH 7.8), metal ion, 20 mM $NaHCO_3$, and 0.8 mM RuBP. The enzyme was activated for 5 min, unless stated otherwise, by incubation with 20 mM $NaHCO_3$ and the desired metal ion. The assay was initiated with RuBP (25 μL) and terminated after 5 min by the addition of propionic acid. Specific activity refers to micromoles of $^{14}CO_2$ fixed per minute per milligram of enzyme.

Ribulosebisphosphate Oxygenase Assay. The consumption of oxygen was measured polarographically by using a Clark-type oxygen electrode (YSI, Model 5331). The polarizing voltage across the electrode was 0.8 V, and the signal was passed through a zero suppression unit to a recorder operated at 1 mV. The sensitivity of the system allowed detection of as little as 0.5 nmol of O_2 consumed per min. The standard assay (1.7 mL) contained 1.6 mL of 50 mM K–Mops (pH 7.8), 25 μL of 8 mM RuBP, 50 μL of the desired metal ion, and 25 μL of activated enzyme (30–50 μg). All assay components except enzyme were added to the electrode chamber and allowed to equilibrate for 5 min at 30 °C prior to initiation of the reaction with activated enzyme. The enzyme was activated in the presence of 40 mM $NaHCO_3$ and the desired metal ion for 5 min unless otherwise stated. The enzyme was activated and then diluted into the assay mix because the HCO_3^- concentration which provided maximum activation was inhibitory to the oxygenase activity when present in the assay. Specific activity refers to micromoles of O_2 consumed per minute per milligram of enzyme.

Co(III) Modification. Incubations were performed at room temperature in 31–38 mM Mops (pH 7.8) in a total volume of 60–80 μL. The modifications were stopped by gel filtration on a Sephadex G-25 column in a Pasteur pipet, which also removed excess cobalt and H_2O_2. The samples were analyzed within 2–3 h since Co(III) is not exchange inert in an absolute sense (Taube, 1952). All carboxylase assays of Co(III)-modified enzyme refer to enzyme activated for 5 min with 10 mM Mg^{2+}. Oxygenase activities represent a 5-min activation using 2 mM Mn^{2+} for increased sensitivity. In the modifications with HCO_3^-, enzyme was incubated with Co^{2+} and HCO_3^- for 5 min before the addition of H_2O_2. [^{14}C]$NaHCO_3$ in the Co(III)–enzyme complex was measured by using the same liquid scintillation system employed with the carboxylase assay (Whitman & Tabita, 1976).

Atomic Absorption Spectroscopy. The concentration of cobalt in Co(III)–enzyme complexes was determined by measuring the absorption at 241 nm, using a Perkin-Elmer Model 306 spectrophotometer equipped with a HGA-70 heated graphite atomizer.

Table I: Specific Activity of Carboxylase and Oxygenase with Various Divalent Metal Ions[a]

	sp act. [(μmol/min)/mg]			
	5-min activation		30-min activation	
metal ion	carboxylase	oxygenase	carboxylase	oxygenase
10 mM Mg^{2+} [b]	2.70		2.77	
10 mM Mg^{2+}	1.53	0.22	0.49	0.21
5 mM Mn^{2+}	0.38	0.43	0.26	0.19
1 mM Mn^{2+}	0.29	0.38	0.30	0.20
1 mM Co^{2+}	0	0.09	0	0.09

[a] Assays described under Experimental Procedures. [b] Assays performed in the presence of 10 mM DTT and 4 mM EDTA.

Results

Metal Ion Requirements. Both carboxylase and oxygenase activities of the *R. rubrum* enzyme utilized either Mg^{2+} or Mn^{2+} (Table I, 5-min activation time). The carboxylase activity obtained with 10 mM Mg^{2+} in Mops buffer is about 60% that found in the same buffer containing 4 mM EDTA and 10 mM DTT, conditions which yield maximum specific activity (Tabita & McFadden, 1974a). While maximum activity seen with Mn^{2+} for carboxylase activity was only about 25% of that with Mg^{2+}, the oxygenase activity with Mn^{2+} was about twice that obtained by using Mg^{2+}. A differential effect of Co^{2+} was seen on the two activities (Table I, 5-min activation time). Co^{2+} elicited no carboxylase activity while 1 mM Co^{2+} supported about 40% of the Mg^{2+} (10 mM) dependent oxygenase activity. Zn^{2+}, Ni^{2+}, and Cu^{2+} at 0.1 mM and Ca^{2+} at 5 mM supported no carboxylase activity. Zn^{2+}, Ni^{2+}, Cu^{2+}, Ca^{2+}, and Ba^{2+} at 5 mM supported no oxygenase activity.

Dependence on Activation Time. Under certain conditions, the level of either carboxylase or oxygenase activity is sensitive to the length of the activation period, the time interval during which enzyme is incubated with HCO_3^- and metal ion prior to initiation of the reaction with RuBP. Using 10 mM Mg^{2+} in the activation mixture, we saw maximum carboxylase activity after 5 min of activation; this activity decreased to about one-third to one-half of maximum activity after 30 min (Table I and Figure 1a). This time-dependent activation was not seen when carboxylase activity was determined in the presence of EDTA and DTT (Table I). A much smaller effect of activation time was seen by using 1 mM Mg^{2+} (Figure 1a). Mn^{2+}-dependent carboxylase activity also depended on the activation time at a high level of Mn^{2+} (5 mM), whereas at 1 mM Mn^{2+} no effect was seen (Table I).

Mg^{2+}- or Co^{2+}-dependent oxygenase activity was not significantly affected by the time of activation, but a large time dependence was seen for Mn^{2+}-dependent activity (Table I and Figure 1b). Similar to Mg^{2+}-dependent carboxylase activity,

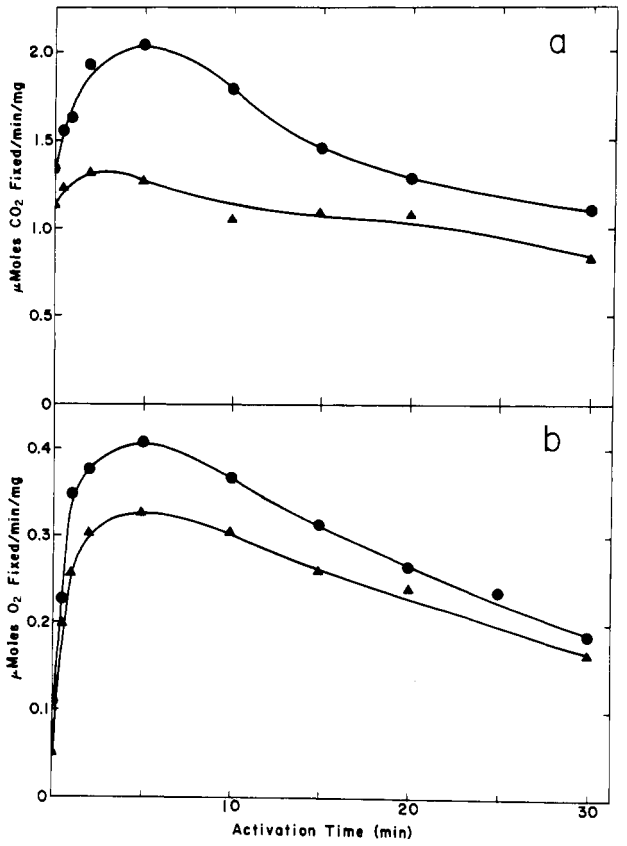

FIGURE 1: Dependence of RuBP carboxylase/oxygenase on activation time. (a) Carboxylase activity in the presence of 1 mM (▲) and 10 mM (●) Mg^{2+}. (b) Oxygenase activity in the presence of 0.5 (▲) and 5 mM (●) Mn^{2+}.

Table II: Kinetic Constants of RuBP Carboxylase/Oxygenase[a]

	carboxylase act.		oxygenase act.	
metal ion	K_m (mM)	V_{max}	K_m (mM)	V_{max}
Mg^{2+}	0.15	1.22	4.0	0.54
	0.52	1.55		
Mn^{2+}	0.05	0.22	0.22	0.61
	0.8	0.37		
Co^{2+}	0.01[b]		0.77	0.25

[a] Assays described under Experimental Procedures. For both activities the concentration of the metal ion in the assay was the same as during activation. K_m (apparent Michaelis constants) and V_{max} values were calculated from double-reciprocal Lineweaver-Burk plots. V_{max} units are as in Table I. [b] K_i value (mM; see Figure 3).

after 30 min of activation with 5 mM Mn^{2+} about 40% of the oxygenase activity was found compared to that found after 5 min of activation. Unlike the carboxylase activity, however, at low Mn^{2+} concentrations (0.5 mM), oxygenase activity also decreased 50% from 5- to 30-min activation (Figure 1b).

Kinetic Constants. Lineweaver–Burk plots of either Mg^{2+}- or Mn^{2+}-dependent carboxylase activity were biphasic. The shift in the velocity vs. metal curve occurred between 0.5 and 1 mM Mg^{2+} (Figure 2a) or between 1 and 2 mM Mn^{2+} (data not shown). Due to the biphasic kinetics, two sets of constants could be obtained for each metal ion (Table II). The lower apparent K_m for Mg^{2+} was higher than that for Mn^{2+}, whereas the reverse was true for the higher constant, reflecting the higher concentration at which the biphasic properties of the Mn^{2+}–velocity curve are obtained. Most of the contribution to the V_{max} was made by the region representing the lower K_m. Co^{2+} not only did not support any carboxylase activity but also inhibited Mg^{2+}-dependent activity. This inhibition was

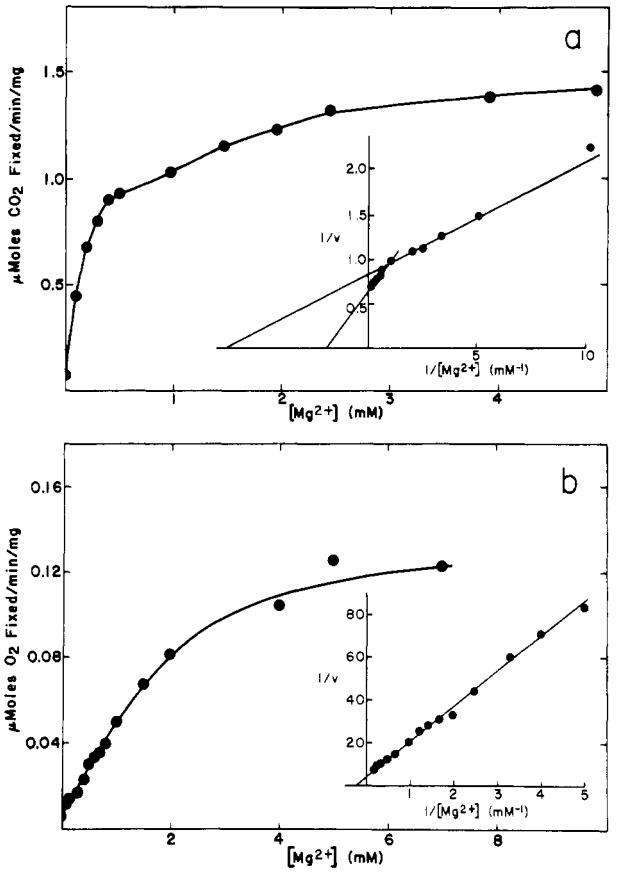

FIGURE 2: Dependence of RuBP carboxylase/oxygenase on Mg^{2+} concentration. The concentration of Mg^{2+} in the assay was the same as that during activation. (a) Carboxylase activity as a function of Mg^{2+}. The insert shows the Lineweaver–Burk plot to determine K_m and V_{max}. (b) Oxygenase activity as a function of Mg^{2+}. The insert shows the Lineweaver–Burk plot to determine K_m and V_{max}.

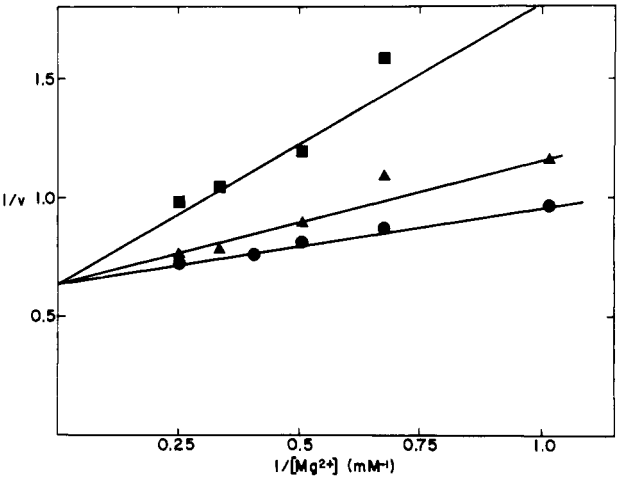

FIGURE 3: Competitive inhibition of Mg^{2+} carboxylase activity by Co^{2+}. (●) 0, (▲) 10, and (■) 20 μM Co^{2+}. The concentrations of Mg^{2+} used here are similar to the higher values in Figure 2a. Inhibition by Co^{2+} was also competitive with respect to Mg^{2+} for the lower range of Mg^{2+} in Figure 2a, and the K_i was similar to that observed here.

competitive with respect to Mg^{2+} with a K_i of 10 μM (Figure 3).

By contrast, linear kinetics were seen with Mg^{2+}-, Mn^{2+}-, or Co^{2+}-dependent oxygenase activity. Results obtained with Mg^{2+} are shown in Figure 2b. The oxygenase activity exhibits the greatest affinity for Mn^{2+}; the apparent K_m for Co^{2+} is slightly higher and the K_m for Mg^{2+} is much higher (Table II). These values for Mg^{2+}- and Mn^{2+}-dependent oxygenase activity are very similar to those reported for the more complex

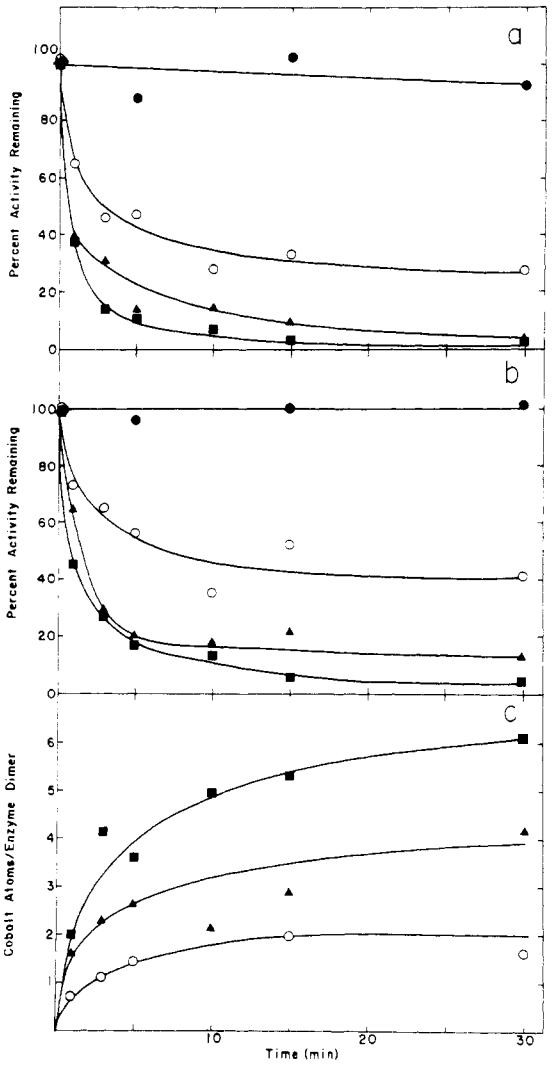

FIGURE 4: Co(III) modification of RuBP carboxylase/oxygenase. The enzyme concentration was 3.76 mg/mL and Co^{2+} was at 1 mM. (●) Control (performed in the presence of 0.5 mM H_2O_2 and 6.7 mM Mg^{2+}; one hundred percent activity remaining represents specific activities of 0.78 and 0.26 (μmol/min)/mg for carboxylase and oxygenase activities, respectively); (○) 0.1, (▲) 0.25, and (■) 0.5 mM H_2O_2. (a) Carboxylase activity. (b) Oxygenase activity. (c) Cobalt incorporation. The reaction was stopped and excess reagents and Co^{2+} were removed by gel filtration as described under Experimental Procedures.

spinach enzyme (Wildner & Henkel, 1978). The theoretical V_{max} for Mg^{2+}-dependent oxygenase activity is much higher than that obtainable in an assay (Table I) because inhibition is seen at Mg^{2+} concentrations over 10 mM, a level only about twice the K_m. The V_{max} for Mn^{2+}-dependent oxygenase activity is higher than that for Mn^{2+}-dependent carboxylase activity (Table II).

Co(III) Modification. Both carboxylase and oxygenase activities were found to be inactivated upon oxidation of Co^{2+} to Co(III) by H_2O_2 with the resultant formation of a stable Co(III)–enzyme complex. No loss of activity was seen in incubations with Co^{2+} in the absence of H_2O_2, indicating that, in the time sequences examined, no air oxidation of Co^{2+} was taking place. In the presence of 1 mM Co^{2+}, both activities could be inhibited greater than 95% within 0.5 h by the addition of 0.5 mM H_2O_2 with no loss of activity in the controls (Figure 4a,b). H_2O_2 concentrations as low as 0.1 mM caused a loss of over 50% of the carboxylase and oxygenase activity (Figure 4a,b). The carboxylase activity was slightly more susceptible to Co(III) modification than oxygenase activity.

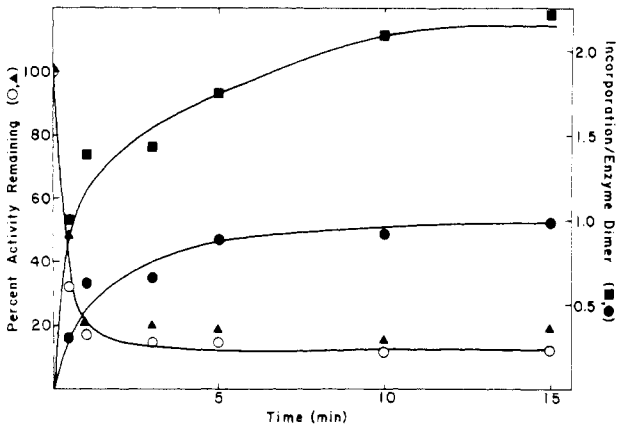

FIGURE 5: Co(III) modification in the presence of 25 mM HCO_3^-. Enzyme concentration was 3.06 mg/mL, $[Co^{2+}]$ was 0.08 mM, and $[H_2O_2]$ was 0.2 mM. (○) Carboxylase activity; (▲) oxygenase activity; (■) cobalt incorporation; (●) HCO_3^- incorporation.

Lowering the Co^{2+} concentration to 0.1 mM in an incubation with 0.25 mM H_2O_2 did not increase this reproducible differential modification, as carboxylase had 33% activity and oxygenase had 42% activity remaining after 30 min.

During the incubations with H_2O_2, up to six cobalt atoms per enzyme dimer were incorporated (Figure 4c). However, a 50% loss in carboxylase activity could be correlated with the incorporation of approximately 1.0–1.2 cobalt atoms per enzyme dimer (Figure 4c). It is thus likely that much of the additional Co^{2+} incorporation seen in Figure 4c represents nonspecific cobalt incorporation at high concentrations of H_2O_2 after long periods of incubation.

In the presence of HCO_3^- (CO_2), binding of the metal ion was greatly enhanced (Miziorko & Mildvan, 1974), and this was also reflected in Co(III) modification of *R. rubrum* RuBP carboxylase/oxygenase. Figure 5 shows the effect of 0.2 mM H_2O_2 on both enzyme activities in the presence of 0.08 mM Co^{2+} and 25 mM $[^{14}C]HCO_3^-$. Greater than 80% of the carboxylase and oxygenase activity was lost when just two cobalt atoms per enzyme dimer were incorporated. This exchange-inert complex also contained one HCO_3^- per enzyme dimer (Figure 5). Thus, a complex of enzyme–Co(III)–HCO_3^- is formed where the stoichiometry of HCO_3^- incorporation is only half the number of subunits, suggestive of half-of-the-sites reactivity.

Co(III)–protein complexes have been reported to be partially reversed by treatment with sulfhydryl reagents (Ryzewski & Takahashi, 1975; Danchin & Buc, 1973). We attempted to reverse complex formation by dialysis against BME (data not shown). Considerable removal of cobalt from the enzyme–Co(III) complex was achieved. Moreover, both HCO_3^- and cobalt were removed from the enzyme–Co(III)–HCO_3^- complex after dialysis against BME. In either case little recovery in either carboxylase or oxygenase activity was observed.

Discussion

Examination of the divalent cation dependence of both the carboxylase and the oxygenase activities of the *R. rubrum* RuBP carboxylase/oxygenase has revealed several important distinctions. The most striking result was obtained with Co^{2+}. This metal ion supported about 30% of the Mg^{2+}-catalyzed oxygenase activity while Co^{2+} did not support but rather inhibited carboxylase activity. Indeed, Co^{2+} was a powerful competetive inhibitor of Mg^{2+}-dependent carboxylase activity, with a K_i of 10 μM. It is possible that this difference is due to the coordination of the metal ions at the active site, since

it is known that Co^{2+} changes from octahedral to tetrahedral symmetry more easily than Mg^{2+} or Mn^{2+}.

Differences between the carboxylase and oxygenase activities also were seen when Mn^{2+} ions were employed. Mn^{2+} supported twice as much oxygenase activity as Mg^{2+}, yet Mn^{2+} supported only about 40% of the Mg^{2+}-dependent carboxylase activity. A high ratio of Mn^{2+}- to Mg^{2+}-dependent oxygenase activity has also been reported for the spinach enzyme; the opposite is true for spinach carboxylase activity (Wildner & Henkel, 1978). The apparent K_m values for Mg^{2+} and Mn^{2+} for both carboxylase and oxygenase activities seen here are somewhat similar to the constants recently obtained for the spinach enzyme, with those for Mg^{2+} being much higher than those for Mn^{2+} (Wildner & Henkel, 1978). The significance of the biphasic metal ion kinetics observed here for the carboxylase activity is not yet clear. It may be related to the activation process. The K_m values for Mg^{2+} are close to the single value (0.21 mM) reported earlier for *R. rubrum* carboxylase activity (Tabita & McFadden, 1974a). This value is several-fold lower than the K_m for Mg^{2+} (1.15 mM) obtained with the spinach enzyme (Wilder & Henkel, 1978), whereas the two K_m values for Mn^{2+} reported here surround the spinach Mn^{2+} value (0.11 mM).

Ni^{2+} has been reported to support the carboxylase activity from spinach to the same extent as Mg^{2+} whereas Co^{2+} supported about 50% as much activity (Weissbach et al., 1956). Different carbon isotope fractionation values for spinach carboxylase have been obtained when either Mg^{2+}, Mn^{2+}, or Ni^{2+} was used (Estep et al., 1978). Since both Ni^{2+} and Co^{2+} were unable to support any *R. rubrum* carboxylase activity, there may be significant differences in the metal ion active sites between the multisubunit, eucaryotic enzyme and the structurally simple dimer obtained from *R. rubrum*. This interpretation is consistent with observations made with the two forms of RuBP carboxylase from *Rhodopseudomonas sphaeroides* (Gibson & Tabita, 1977). Form I enzyme has a subunit structure similar to the spinach enzyme and utilizes Mg^{2+}, Mn^{2+}, Co^{2+}, or Ni^{2+} for carboxylase activity, while form II enzyme, containing only large subunits, is active only with Mg^{2+} or Mn^{2+} (J. L. Gibson and F. R. Tabita, unpublished observations).

This study has also revealed a dependencee of both carboxylase activity and oxygenase activity on the activation time. A previous report for the *R. rubrum* enzyme indicated a time of greater than 100 min was necessary for activation (Christeller & Laing, 1978). However, these authors activated the enzyme at subsaturating (1 mM) HCO_3^- and at a significantly lower (18 °C) temperature (Christeller & Laing, 1978). In this report, by use of enzyme that is saturated for HCO_3^- in the activation and the assay (at 30 °C), Mg^{2+}- and Mn^{2+}-dependent carboxylase activities were sensitive to the time of activation. Activity was maximized after 5 min of activation and declined thereafter. Interestingly, this effect was not seen when the enzyme was assayed in the presence of EDTA and DTT, ligands known to substantially stimulate Mg^{2+}-dependent carboxylase activity (Tabita & McFadden, 1974a). Since the enzyme also shows cooperative kinetics with respect to CO_2 under these conditions (Tabita & McFadden, 1974b), these ligands must impose changes in the enzyme which may be reflective in both activation and catalysis. By contrast, only Mn^{2+}-dependent oxygenase activity is sensitive to the time of activation. No time dependence is seen with Mg^{2+} or Co^{2+}, cations which support less activity than Mn^{2+}. The reason for this effect is unclear, but the differences in the two activities are substantial.

Both activities of *R. rubrum* RuBP carboxylase/oxygenase are inactivated by oxidation of Co^{2+} to Co(III) by H_2O_2. The concentration of H_2O_2 required (<0.5 mM) is much less than that used in other similar studies [10–20 mM; Ryzewski & Takahashi (1975) and Anderson & Vallee (1977)]. The carboxylase activity was slightly, but consistently, more sensitive to Co(III) modification than oxygenase activity. Under no conditions, however, including a modification with a low Co^{2+} concentration, could this differential effect be increased.

When oxidizing Co^{2+} to Co(III) in the presence of enzyme alone, nonspecific as well as specific cobalt incorporation is seen. In the presence of 25 mM HCO_3^-, modification can be obtained by using a much lower Co^{2+} concentration, undoubtedly reflecting an increase in the specificity and affinity of Co^{2+} binding. It should be noted that the presence of HCO_3^- increases the binding of Mn^{2+} to the spinach enzyme (Miziorko & Mildvan, 1974). HCO_3^- is also incorporated into an exchange-inert Co(III)–enzyme complex which is stable to gel filtration. Interestingly, the stoichiometry of HCO_3^- incorporation is half the stoichiometry of Co^{2+} incorporation into the enzyme. The complex formed has two cobalt atoms and one HCO_3^- molecule per enzyme dimer. Thus, HCO_3^- incorporation into Co(III)–enzyme complexes shows half-of-the-sites reactivity (Levitzki & Koshland, 1976) similar to the pyridoxal phosphate modification of a specific lysyl residue of the *R. rubrum* enzyme (Whitman & Tabita, 1978a). Indeed, the modification of an active-site arginine residue in both the spinach and *R. rubrum* enzymes by phenylglyoxal shows a similar stoichiometry (Schloss et al., 1978).

While much of the cobalt and HCO_3^- could be removed from the enzyme by dialysis against buffer containing mercaptoethanol, reducing Co(III) back to Co^{2+}, little activity is regained. Such a virtual irreversible loss of activity upon reduction of the Co(III) complex has been seen in other systems (Ryzewski & Takahashi, 1975).

A stable complex of enzyme–HCO_3^-–metal ion–carboxyribitol bisphosphate has recently been reported (Miziorko, 1979). Carboxyribitol bisphosphate (CRBP) is a transition-state analogue of RuBP carboxylase/oxygenase (Wishnick et al., 1970) and is thought to bind to both the RuBP and HCO_3^- sites simultaneously (Miziorko & Mildvan, 1974). Since this complex of spinach carboxylase/oxygenase contains one HCO_3^- and one CRBP molecule per subunit, it was concluded that there are two HCO_3^- binding sites per subunit. Whether or not the two sites proposed in this work represent distinct activation and catalytic CO_2 sites or whether these sites are the same requires much more experimentation. Since HCO_3^- and CRBP compete for a site close to enzyme-bound Mn^{2+} (Miziorko & Mildvan, 1974), it is likely that the HCO_3^- trapped in our Co(III)–enzyme complex is different from that in the complex observed by Miziorko. Alternatively, this may further reflect differences between the active sites of the enzyme isolated from spinach and *R. rubrum*. In contrast, with both the spinach and *R. rubrum* enzymes, similar stoichiometry of phenylglyoxal modifications of arginine residues was obtained (Schloss et al., 1978). Further experimentation is required to establish the degree to which the active sites of these two enzymes resemble each other.

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A Compound Representing the D-Glycerate Terminus of the Methylglucose-Containing Polysaccharide of *Mycobacterium smegmatis*†

Brian K. Hunter, Sherry L. Mowbray, and Donald J. Walton*

ABSTRACT: In order to study the structure of the methylglucose-containing polysaccharide (MGP) of *Mycobacterium smegmatis* by NMR spectroscopy, we have prepared the model compound *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid. This compound, which represents the aglycon-containing terminus of MGP, was made from leucrose [*O*-α-D-glucopyranosyl-(1→5)-D-fructopyranose] by successive treatment with sodium borohydride, lead tetraacetate, and hypobromite. The structure of *O*-α-D-glucopyranosyl-(1→2)-D-glyceric acid was confirmed by chemical and enzymic methods. ^{13}C and ^{1}H NMR spectra of this compound, together with spectra of several disaccharides, were obtained for future reference in the polysaccharide study. The nine resonances in the ^{13}C spectrum were assigned by comparison with the spectrum of methyl α-D-glucopyranoside. Analysis of the ^{1}H NMR spectrum showed that the two methylene protons on C-3 of the glycerate moiety were less equivalent in the sodium salt than in the acid. This may be attributable to hydrogen bonding between the carboxylate and the hydrogen atom of the glycerate 3-hydroxyl group.

Mycobacterium smegmatis contains two polysaccharides which stimulate fatty acid biosynthesis by interacting with acylcoenzyme A derivatives and long-chain transacylases (Yabusaki & Ballou, 1978; Wood et al., 1978). Ballou (1968) reported that one of these polysaccharides is a methylglucose-containing polysaccharide (MGP) with the structure shown in Figure 1, acylated at six to nine positions (Smith & Ballou, 1973). One of us noted that, contrary to expectations based upon this structure, exhaustive methylation, followed by acid hydrolysis, did not yield 2,4,6-tri-*O*-methylglucose.[1] This ether should have been obtained from the third glucose unit from the glyceric acid end of the chain. We therefore set out to examine the structure of MGP by a number of methods, including NMR spectroscopy. It should be possible to assign resonances of the hexose residues on the basis of comparison with spectra of suitable disaccharides. The as-

† From the Departments of Biochemistry (S.L.M. and D.J.W.) and Chemistry (B.K.H.), Queen's University, Kingston, Ontario, Canada K7L 3N6. *Received March 23, 1979.*

[1] This observation was made by D.J.W. while on a sabbatical leave in the laboratory of Professor C. E. Ballou at the University of California, Berkeley. The structural reinvestigation is proceeding in both laboratories, but the discrepancy between the structure (Figure 1) and the methylation result is still unresolved.

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