DIFFERENCES IN THE KINETIC PROPERTIES OF THE CARBOXYLASE AND OXYGENASE ACTIVITIES OF RIBULOSE BISPHOSPHATE CARBOXYLASE/OXYGENASE

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1. Introduction

D-Ribulose 1,5-bisphosphate carboxylase/oxygenase (3-phospho-D-glycerate carboxylase [dimerizing], EC 4.1.1.39) is a bifunctional enzyme which catalyzes the first step of the two competing metabolic pathways of photosynthetic carbon assimilation and photorespiratory glycolate production [1]. The carboxylase activity catalyzes the reaction of carbon dioxide with D-ribulose 1,5-bisphosphate to yield 2 molecules of 3-D-phosphoglycerate. The oxygenase activity catalyzes the reaction of O_2 with D-ribulose 1,5-bisphosphate (RuBP) to yield one molecule each of 3-phosphoglycerate and 2-phosphoglycolate [2]. Increased agricultural productivity is dependent on differential control of these 2 activities since photorespiration is a metabolically wasteful process. Thus, it is imperative that a careful examination of the properties of the carboxylase and oxygenase activities be undertaken.

In this investigation, we have found significant differences in the Michaelis constants for RuBP for the oxygenase and carboxylase activities of both spinach and *Rhodospirillum rubrum* RuBP carboxylase/oxygenase. Moreover, the concentration of fructose 1,6-bisphosphate required for maximum activation differs for the carboxylase and oxygenase activities. Since previous findings indicated that the 2 activities also differed in their affinity and requirement for divalent metal ions, our results suggest that the activation and/or catalytic sites for the 2 activities may be distinct.

Abbreviations: RuBP, ribulose 1,5-bisphosphate; FBP, fructose 1,6-bisphosphate; Mops, 4-morpholinepropanesulfonic acid; EDTA, (ethylenedinitrilo) tetraacetic acid; DTT, dithiothreitol

2. Materials and methods

2.1. *Materials*

The barium salt of RuBP was prepared as in [3] and further purified to 97% purity as estimated by enzymatic assay. The barium salt of RuBP was converted to the free acid by treatment with Dowex 50 W H^+ resin and treated with Chelex 100 (Bio-Rad Labs.) to remove contaminating metal ions. FBP was obtained from Sigma Chemical Co. (St Louis MO). Sodium [^{14}C]carbonate (20 mCi/mmol) was purchased from Amersham (Arlington Heights Il). All divalent metals were used as the chloride salt. RuBP carboxylase/oxygenase from *R. rubrum* and spinach was purified to homogeneity as in [4–6]. Protein concentrations were calculated from known extinction coefficients [5,7].

2.2. *RuBP oxygenase assay*

The consumption of oxygen was measured polarographically by using a Clark-type oxygen electrode (YSI, model 5331) as in [8]. The assay contained in 1.70 ml, 47 or 48 mM Mops (pH 7.8), 25 μl 8 mM RuBP, 50 μl desired metal ion (10 mM MgCl, 5 mM $MnCl_2$, or 1 mM $CoCl_2$), and 5–25 μl activated enzyme (25–40 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 for 5 min in the presence of 40 mM $NaHCO_3$ and the desired metal ion. Metal ion concentration was the same during the activation and assay. Small volumes of activated enzyme were added to the electrode chamber to ensure that the bicarbonate used in the activation mixture did not substantially inhibit the oxygenase activity. Rates were obtained from the first 30 s of

the reaction. Specific activity is defined as μmol O_2 fixed . min^{-1} . mg^{-1}.

2.3. *RuBP carboxylase assay*

Activity was measured as acid-stable sodium [^{14}C]-carbonate incorporation as in [9]. The assay contained in 0.25 ml, 40 mM Mops (pH 7.8), 20 mM $NaHCO_3$, 0.8 mM RuBP, 2 μCi $Na_2{}^{14}CO_3$, 3.5–4.0 μg enzyme, and divalent metal (10 mM $MgCl_2$, 5 mM $MnCl_2$, or 1 mM $CoCl_2$). Like the oxygenase assays, carboxylase assays were also initiated with enzyme that had been activated by incubation for 5 min with 40 mM HCO_3^- and the desired divalent metal ion. The divalent metal concentration was the same in the activation and assay. When assayed in the presence of Mn^{2+}, a pronounced lag was observed in the reaction. Because of this lag, all velocities were obtained from the linear region of the assay time course. Specific activity is defined as μmol CO_2 fixed . min^{-1} . mg^{-1}.

3. Results

3.1. *Kinetic constants for RuBP*

Differences in the requirement and affinity for divalent metals of the *R. rubrum* and spinach oxygenase and carboxylase were reported in [8,10,11]. Kinetic studies with *R. rubrum* RuBP carboxylase/oxygenase at varying concentrations of RuBP reveal that the app. K_m for the RuBP for both activities is affected by the divalent metal present during activation and catalysis (fig.1). For both activities, The K_m for RuBP is lowest in the presence of Mn^{2+} (summarized in table 1). For the carboxylase, the difference in the K_m for RuBP of the Mg^{2+}- and Mn^{2+}-dependent activ-

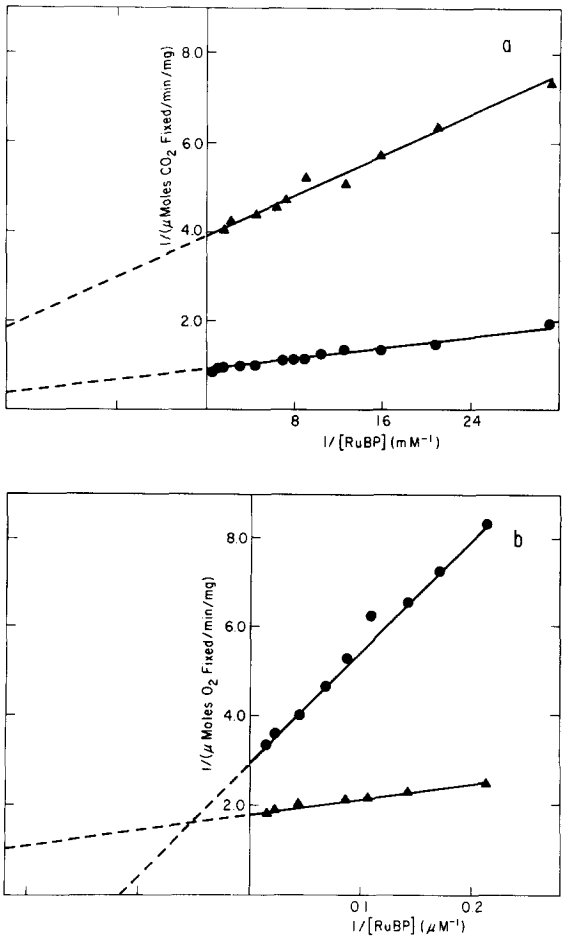

Fig.1. Double-reciprocal Lineweaver-Burk plots showing the dependence of *R. rubrum* RuBP carboxylase/oxygenase on RuBP concentrations. Assay conditions are described in the footnotes to table 1. (▲) Mn^{2+}-dependent and (●) Mg^{2+}-dependent carboxylase (a) and oxygenase (b) Mg^{2+} was 10 mM and Mn^{2+} 5 mM.

Table 1
Kinetic constants for RuBP of *R. rubrum* RuBP carboxylase and oxygenase[a]

Metal ion	Carboxylase activity		Oxygenase activity	
	V_{max}	K_m (μM)	V_{max}	K_m (μM)
10 mM Mg^{2+}	1.16	42[b]	0.34	8.6
5 mM Mn^{2+}	0.26	29[c]	0.54	1.6
1 mM Co^{2+}			0.13	1.6

[a] Assays were as in section 2: All assays were initiated with activated enzyme. Activated enzyme was added to the oxygenase assay in 5 μl to minimize the amount of HCO_3^- transferred to the assay. K_m and V_{max} values were calculated from double reciprocal Lineweaver-Burk plots. V_{max} units are defined as μmol . min^{-1} . mg^{-1}

[b] One minute assays were used; [c] Details of this assay are given in the text

ities is small. By contrast, the difference in the K_m for RuBP of the Mg^{2+} and Mn^{2+}-dependent oxygenase activity is >5-fold. Regardless of the divalent metal ion present, the K_m for RuBP of the oxygenase activity is much lower than that for the carboxylase activity (fig.1). The value for the carboxylase activity agrees well with [12]. Both the carboxylase and oxygenase assays were initiated with enzyme pre-activated by incubation for 5 min at 30°C with 40 mM HCO_3^- and a divalent metal at the concentration used in the assay (see section 2). In order to verify that the different HCO_3^- concentration in the oxygenase assay (0.1–0.4 mM) and the carboxylase assay (20 mM) was not responsible for the observed differences in the K_m for RuBP of the two activities, RuBP-initiated oxygenase assays were performed with 20 mM HCO_3^- present in the assay. While the presence of 20 mM HCO_3^- in the assay reduced the velocity, the K_m for RuBP was not affected. Thus, it is highly unlikely that differences in the K_m for RuBP of the 2 activities is an artifact which can be attributed to different bicarbonate concentrations in the 2 assays. Moreover, when simultaneous carboxylase and oxygenase assays were performed, it was obvious that the oxygenase activity had a higher affinity for RuBP.

Divalent metal ion-dependent differences in the K_m for RuBP of the spinach leaf carboxylase and oxygenase activities were also observed (table 2). Like the *R. rubrum* enzyme, the K_m for RuBP of the spinach oxygenase is much lower than the K_m for RuBP of the carboxylase with either Mg^{2+} or Mn^{2+}. The spinach Mn^{2+}-dependent carboxylase and oxygenase activities had K_m values for RuBP 5-fold higher than those reported for the *R. rubrum* Mn^{2+}-dependent activities. By contrast, there was a <2-fold difference in the Mg^{2+}-dependent activities of the two enzymes.

3.2. *Activation by HCO_3^-*

The divalent cation present during activation of the oxygenase was found to affect the amount of HCO_3^- required for full activation (fig.2). K_{act} values, defined as the amount of HCO_3^- required for half-maximal activation, are >18-fold larger for the Mg^{2+}-dependent oxygenase than the Mn^{2+}-dependent oxygenase (13 mM *vs* 0.7 mM). In each case, 5 min was sufficient time for maximum activation.

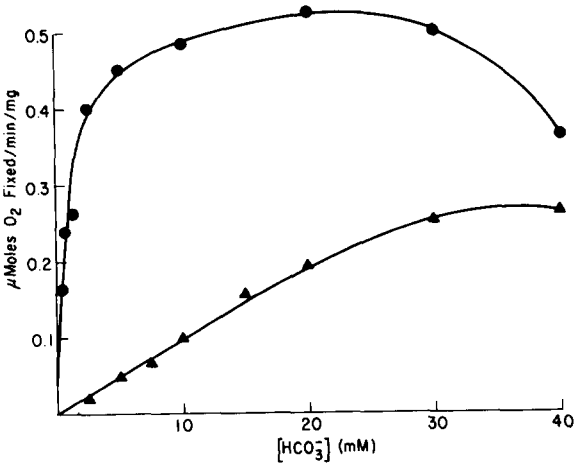

Fig.2. Effect of divalent metal ions on the activation of *R. rubrum* RuBP oxygenase by $NaHCO_3$. (•) Mn^{2+}-dependent and (▲) Mg^{2+}-dependent oxygenase as a function of HCO_3^- concentration. Assay conditions were as in section 2. Assays were initiated with enzyme which had been activated for 5 min with the divalent metal and varying concentrations of HCO_3^-. Enzyme was added to the assay in a 25 µl volume. Mg^{2+} was 10 mM and Mn^{2+} 5 mM.

3.3. *Activation by FBP*

Several phosphorylated effectors of both the *R. rubrum* and higher plant enzyme have been described which activate both the oxygenase and carboxylase activities under conditions of limiting HCO_3^- [9,13]. Activation of the enzyme is approximately to the level which is obtained by activation with high concentra-

Table 2
Kinetic constants for RuBP of spinach RuBP carboxylase and oxygenase[a]

Metal ion	Carboxylase activity		Oxygenase activity	
	V_{max}	K_m (µM)	V_{max}	K_m (µM)
10 mM Mg^{2+}	1.47	70[b]	0.16	16
5 mM Mn^{2+}	0.19	130[c]	0.27	7.3

[a] Assay conditions were as in section 2 and the footnote to table 1
[b] One minute assays were used; [c] Details of this assay are given in the text

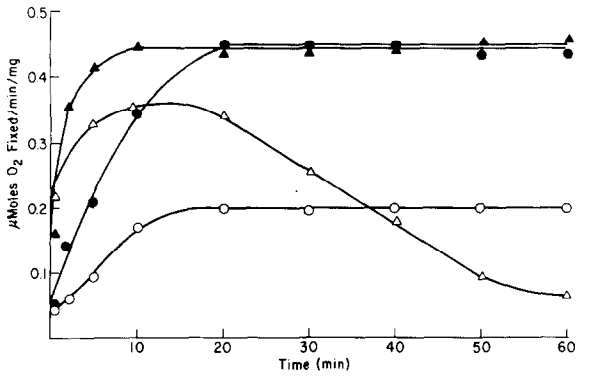

Fig.3. Dependence of *R. rubrum* RuBP oxygenase on activation time. Oxygenase was activated in the presence of 5 mM Mn^{2+} and (○) 1 mM HCO_3^- (△) 40 mM HCO_3^-, (●) 1 mM HCO_3^- plus 1.5 mM FBP, or (▲) 40 mM HCO_3^- plus 1.5 mM FBP.

tions of HCO_3^- (20–40 mM). The effect of FBP on the enzyme fully activated with HCO_3^- is not to provide further activation, but rather to maintain the enzyme in a fully activated state (fig.3). The fully activated Mg^{2+}-dependent carboxylase and Mn^{2+}-dependent oxygenase lost 50% of the activity within 30 min [8]. The time-dependent decay of the activated state does not occur with the partially activated enzyme (i.e., at 1 mM HCO_3^-). In the presence of high concentrations of HCO_3^-, full activation of the enzyme is rapid (~5 min). At low concentrations of HCO_3^- (1 mM), activation of the enzyme required 20 min. FBP does not reduce the time required for activation when HCO_3^- was limiting. Data shown in fig.3 is for the Mn^{2+}-dependent oxygenase activity. Identical results were obtained with the Mg^{2+}-dependent carboxylase activity.

The concentration of FBP required for full activation under conditions of limiting HCO_3^- (1 mM) is dependent on the divalent metal ion present during activation (table 3). Considerably less FBP is required for the Mn^{2+}-dependent oxygenase and carboxylase. With either divalent metal present, the concentration of FBP required to activate the oxygenase is lower than that required to activate the carboxylase (table 3). We initially suspected that the much lower concentration of FBP required to fully activate the Mn^{2+}-dependent oxygenase might be due to the fact that this activity was already half-maximally activated at 1 mM HCO_3^- (fig.2). However, the concentration of FBP required to fully activate the Mn^{2+}-dependent oxygenase activity is independent of the concentration of

Table 3
Effect of divalent metal ions on FBP concentration required for maximum activation

Metal ion	Carboxylase act. FBP (mM)	Oxygenase act. FBP (mM)
10 mM Mg^{2+}	2.0	0.85
5 mM Mg^{2+}	0.55	0.05

[a] Assay conditions were as in section 2: Assays were initiated with enzyme that had been activated by incubation for 20 min with the divalent metal, 1 mM HCO_3^-, and varying concentrations of FBP

HCO_3^- in the activation (not shown). FBP was able to fully activate the Mn^{2+}-dependent oxygenase in the absence of exogenous HCO_3^- because there was sufficient dissolved CO_2 in the reaction mixture.

4. Discussion

Because of the critical role of RuBP carboxylase/oxygenase in agricultural productivity, considerable research has been directed toward differential control of the two activities. The two activities may share the same activation and catalytic sites. For example, both oxygenase and carboxylase require prior activation with divalent metal ion and HCO_3^- [14–16] and both activities are equally stimulated or inhibited by various phosphorylated compounds [9,17–20]. Specific active site modification with 2 different probes also leads to loss of both activities [21,22], as does formation of an exchange-inert Co (III) · HCO_3^- · enzyme complex [8]. Work with enzyme from two different sources [8,10,11] has shown that there are significant differences in the requirement and affinity for divalent metals for the carboxylase and oxygenase activities. The differences are most striking with the *R. rubrum* enzyme. For the carboxylase reaction, there is a distinct preference for Mg^{2+} over Mn^{2+}; oxygenase activity was twice as great with Mn^{2+} as compared to that obtained with Mg^{2+} [8]. However, only the oxygenase activity of the *R. rubrum* enzyme is supported by Co^{2+}; Co^{2+} will not support carboxylase activity and in fact is a competitive inhibitor of Mg^{2+}-dependent carboxylase activity with a K_m of 10 μM [8]. The carboxylase and oxygenase also differed in the K_m-value for each metal. These studies provided the first real indication that differences exist and

prompted further inspection of the kinetic behavior of this enzyme.

Here, we have shown that with both the *R. rubrum* and spinach enzymes, the K_m for RuBP of both the oxygenase and carboxylase is dependent on whether Mg^{2+} or Mn^{2+} was present. The concentration of FBP required for activation also depended on whether Mg^{2+} or Mn^{2+} was present. For the oxygenase, the K_m for Mg^{2+} was found to be 18-fold greater than the K_m for Mn^{2+} [8]. Interestingly, the K_{act} for HCO_3^- and the concentration of FBP required for maximum activation of the oxygenase was also ~18-fold greater for the Mg^{2+}-dependent oxygenase activity than the Mn^{2+}-dependent oxygenase activity. The difference in the K_m for RuBP and the affinity for activators between Mg^{2+}- and Mn^{2+}-dependent carboxylase were comparatively small. However, the affinity of oxygenase for the substrate RuBP and the activator FBP appears to be significantly greater than the affinity of the carboxylase for these compounds. The effect of divalent metals on RuBP and FBP binding does not account for differences in the binding of these compounds by the oxygenase and the carboxylase. These differences might be explained by the presence of distinct oxygenase and carboxylase sites for activation and/or catalysis. Certainly, these studies invite further experimentation. During these studies, a significant difference in the K_m for RuBP for the spinach carboxylase and oxygenase activities was reported when assays were performed at sub-saturating levels of O_2 and CO_2 [23].

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