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AN ELECTRON PARAMAGNETIC RESONANCE STUDY OF THE BINDING OF MANGANESE TO RIBULOSE-1,5-DIPHOSPHATE CARBOXYLASE

INHIBITION BY MAGNESIUM

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SUMMARY

Electron paramagnetic resonance (EPR) was used to measure the extent of binding of Mn^{2+} to ribulose-1,5-diphosphate carboxylase purified from spinach leaves. In the Mn^{2+} concentration range 0.60 to 0.04 mM three Mn^{2+} binding sites having $K_d = 0.06$ mM were observed. Mg^{2+} was found to competitively inhibit the binding of Mn^{2+}, and $K_d = 0.54$ mM was calculated for Mg^{2+}.

D-Ribulose-1,5-diphosphate carboxylase (3-phospho-D-glycerate carboyxlase (dimerizing), EC 4.1.1.39) catalyzes the carboxylation of D-ribulose 1,5-diphosphate to yield two moles of 3-D-phosphoglycerate in the primary carbon fixation step of photosynthesis. The reaction has an absolute requirement for a divalent metal cation [1], and it has been suggested that control of metal ion activation might play a role in the regulation of the enzyme's activity in vivo [2]. Little is known, however, about the stoichiometry of the metal ion–enzyme interaction, the nature of the groups on the protein involved in the interaction, or the mechanism of the activation. In fact, the only cases where binding of metal ions to the enzyme has been studied involved inactive ternary complexes of the enzyme with a substrate analogue or complexes with cyanide [3]. Putative evidence for the existence of an enzyme–CO_2–Mg^{2+} has also been reported [4].

We have used electron paramagnetic resonance techniques to study the binding of Mn^{2+} to the enzyme. Free Mn^{2+} in solution has an intense isotropic EPR spectrum, but the EPR of Mn^{2+} bound to small molecules and proteins is often broadened and undetectable providing a convenient means of measuring binding constants [5–7]. Electrophoretically pure ribulose-1,5-diphosphate carboxylase was

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isolated from spinach leaves as prevoiously described [8]. The specific activity was 1.2 units/mg protein, where 1 unit equals 1 μmole of carbon incorporated into acid stable compounds per min. Freshly prepared enzyme was concentrated by precipitation with $(NH_4)_2SO_4$, resuspended in 0.05 M Tris buffer (pH 7.8), and exhaustively dialyzed against this buffer. Protein concentrations were determined spectrophotometrically, taking the absorbance at 280 nm to be 1.64 $cm^{-1} \cdot mg^{-1} \cdot ml$ and assuming a molecular weight of 557 000 [9]. X-band EPR spectra were recorded on a Varian E-3 spectrometer.

When ribulose-1,5-diphosphate carboxylase was added to solutions of Mn^{2+} in buffer a quantitative reduction in the intensity of the Mn^{2+} EPR signal was observed. No changes in the EPR spectrum of the aquo complex appeared, and no evidence for any new signal was obtained. A titration of the enzyme was made assuming that the heights of the hyperfine components in the first derivative EPR spectrum are proportional to the concentration of free Mn^{2+}. The results of three experiments, using different enzyme preparations and concentrations, are presented in the form of a Hughes–Klotz plot [7, 10] in Fig. 1. In the absence of competing divalent cations the data fit a single straight line reasonably well. The x and y intercepts are equal to the reciprocals of the dissociation constant and the number of binding sites, respectively. Within the Mn^{2+} concentration range used, 0.60 to 0.04 mM, $K_d \approx 0.06$ mM and $n \approx 3$. When Mg^{2+} was added to solutions of enzyme and Mn^{2+}, an increase in the free Mn^{2+} EPR signal was observed (Fig. 1). In the presence of 0.6 and 1.2 mM Mg^{2+} the K_d for Mn^{2+} increased to 0.12 and 0.18 mM, respectively, while n remained equal to 3. Assuming competitive binding of the two cations, K_d for Mg^{2+} can be determined [11, 12]. The results of separate calculations

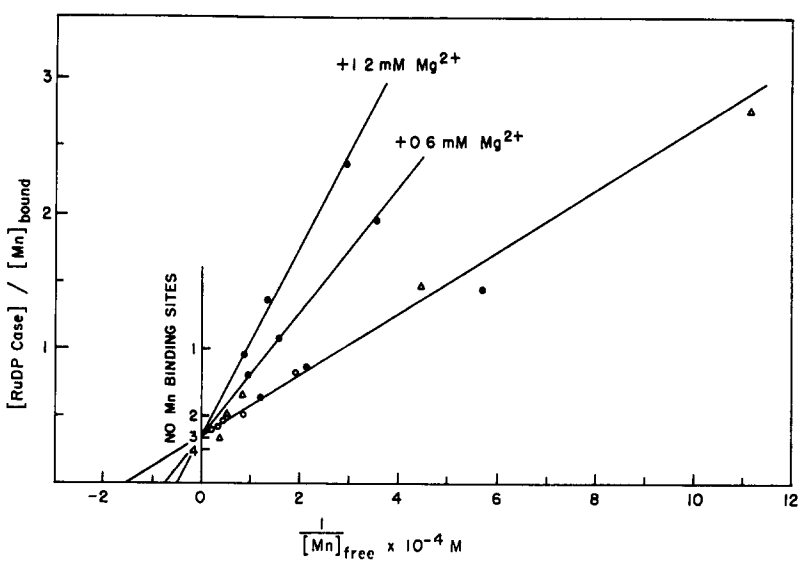

Fig. 1. Titration of D-ribulose-1,5-diphosphate carboxylase (RuDP Case) with Mn^{2+}. Hughes–Klotz plot [10] of the concentrations of free and bound Mn^{2+} determined by EPR (see text). Experimental conditions: buffer, 0.05 M Tris (pH 7.8); temperature, 22 °C; sample volume, 0.15 ml; enzyme concentrations 55 mg/ml (△), 33 mg/ml (○) and 18 mg/ml (●).

at each Mn^{2+} and Mg^{2+} concentration are shown in Table I. The average value for K_d (Mg^{2+}) = 0.54 mM.

The dissociation constant for Mn^{2+} in the absence of Mg^{2+} measured in this work is close to the Michaelis constant for Mn^{2+}, K_m = 0.04 mM, determined by others [9]. This suggests that the binding observed might yield the active enzyme-metal complex required for catalysis. The stoichiometry of the Mn^{2+}–enzyme interaction observed here, however, is unexpected. Ribulose-1,5-diphosphate carboxylase is composed of sixteen subunits of molecular weight of approx. 12 000 and 56 000 in a 1:1 molar ratio [13].

TABLE I

DISSOCIATION CONSTANTS OF Mn^{2+} AND Mg^{2+} D-RIBULOSE-1,5-DIPHOSPHATE CARBOXYLASE

Experimental conditions are given in Fig. 1 and text. K_d (Mn^{2+}) was determined from best straight line fit to data; K_d (Mg^{2+}) was calculated for each data according to ref. 11, assuming competition of Mg^{2+} with Mn^{2+}.

Mn^{2+} (mM)	Mg^{2+} (mM)	K_d (Mn^{2+}) (mM)	K_d (Mg^{2+}) (mM)
0.04–0.60	0	0.06	–
0.06	0.6	0.12	0.54
0.12	0.6	0.12	0.53
0.18	0.6	0.12	0.57
0.06	1 2	0.18	0.53
0.12	1.2	0.18	0.48
0.18	1.2	0.18	0.58
		Average	0.54

The number of binding sites for ribulose 1,5-diphosphate appears to vary from approx. 8 at low ionic strength (0.01 M Tris) to 4 ± 1 at higher ionic strength (greater than 0.1 M Tris) [3]. We have not observed any effect of ionic strength on the number of Mn^{2+} binding sites over the range of 0.01 to 0.2 M Tris although K_d may change somewhat. This effect will be investigated further.

The dissociation constant determined for Mg^{2+} competition with Mn^{2+} binding is significantly lower than the Michaelis constant for Mg^{2+}, K_m = 1.1 mM [9]. Thus, this binding may not be limiting for activation of the enzyme. The ability of Mg^{2+} to compete with Mn^{2+} in this concentration range might compensate for the lower K_m for Mn^{2+} and rule out the possibility of Mn^{2+} activation of the enzyme in vivo. Further experiments utilizing the Mn^{2+} EPR probe can be designed to determine the effects of temperature, pH, CO_2, ribulose 1,5-diphosphate and competing ions on the metal-enzyme interaction.

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