DIFFERENTIAL REACTIVATION OF RIBULOSE 1,5-BISPHOSPHATE OXYGENASE WITH LOW CARBOXYLASE ACTIVITY BY Mn^{2+}

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1. Introduction

Ribulose 1,5-bisphosphate carboxylase-oxygenase (EC 4.1.1.39) catalyses either the carboxylation or the oxidative splitting of ribulose 1,5-bisphosphate (RuBP) [1]. Both enzyme activities are stimulated by incubation with Mg^{2+} and bicarbonate, both of which are necessary in converting the enzyme molecules to an active enzyme–CO_2–Mg^{2+} complex [2–5]. Kinetic analysis [6] suggested that the activating sites of the enzyme molecules are not identical with the substrate binding sites for CO_2.

The role of divalent cations is not confined to the formation of this ternary complex, since divalent cations are also bound [7] to the second substrate RuBP at the active site of the enzyme. Mn^{2+} can substitute for Mg^{2+} in the formation of a quaternary complex, enzyme–RuBP–Me^{2+}–CO_2, with RuBP in the inner sphere and CO_2 in the outer sphere of enzyme bound Mn^{2+}.

The studies concerning the replacement of Mg^{2+} by Mn^{2+} and its influence on the carboxylase activity showed diverging results, in some experiments the presence of Mn^{2+} could not activate the enzyme [8–10], whereas other data supported a stimulatory role for this ion [11].

The aim of this paper is to describe the influence of Mn^{2+} on the RuBP oxygenase and to re-evaluate the discrepancies on the carboxylase function. The reconstitution experiments revealed that Mn^{2+} could replace Mg^{2+} ions thereby fully restoring the oxygenase activity; however, the carboxylase activity was only slightly enhanced.

2. Materials and methods

2.1. *Enzyme purification*

RuBP carboxylase-oxygenase was isolated from spinach leaves. The purification process including ammonium sulfate precipitation (30–55% saturation), Sephadex G-200 gel filtration and ultracentrifugation on a sucrose step gradient were detailed in [12]. The enzyme fraction with the highest specific activity (1.2 U/mg protein) showed a single band in 5% acrylamide gels after disc electrophoresis [13].

2.2. *RuBP carboxylase assay*

The activity was estimated by [^{14}C]bicarbonate incorporation into 3-phosphoglycerate. The assay mixture contained in 0.2 ml: 50 mM Tris–sulfate, pH 8.0; 1 mM RuBP; 25 mM $NaH^{14}CO_3$ (1 Ci/mol); enzyme equivalent to 30 μg protein and $MgCl_2$ and $MnCl_2$ as indicated in the figure legends. The assay conditions and procedure were described in [12].

2.3. *RuBP oxygenase assay*

The enzyme was analysed polarographically with a Gilson oxygraph. The assay mixture contained in 1.5 ml: 50 mM Tris–sulfate, pH 8.0; 0.65 mM RuBP; 0.25 mM oxygen and 0.4 mg enzyme protein, $MgCl_2$ and $MnCl_2$, as indicated in the figure legends. The reaction was started by the addition of the enzyme.

3. Results

3.1. *Reactivation of RuBP oxygenase*

The enzyme was isolated in the activated state, i.e.,

in presence of Mg^{2+} and bicarbonate during the purification process. Enzyme samples were dialysed against 50 mM Tris–sulfate buffer, pH 8.0, containing 1 mM dithiothreitol and 2 mM EDTA, and finally against the same buffer without EDTA, to remove the Mg^{2+} ions. Aliquots of the enzyme samples were preincubated with different concentrations of $MnCl_2$ (0.1–4 mM) and 10 mM $NaHCO_3$ for 15 min at 25°C. After the preincubation period, aliquots were analysed for RuBP oxygenase activity. The assay mixtures contained the same $MnCl_2$ concentration as used in the preceding incubation. The initial reaction rates were measured and the results are presented in fig.1. RuBP oxygenase was fully activated at 1 mM $MnCl_2$. The activation followed Michaelis-Menten kinetics with a

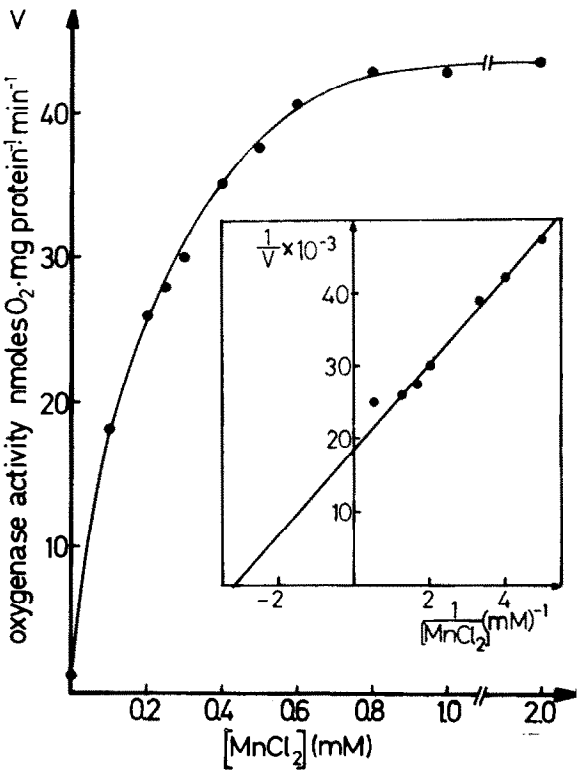

Fig.1. Reactivation of RuBP oxygenase with $MnCl_2$. The dialysed enzyme fraction was preincubated for 15 min at 25°C with 0.1–2.0 mM $MnCl_2$ and 10 mM $NaHCO_3$. Samples were taken and analysed for RuBP oxygenase activity at the appropriate $MnCl_2$ concentrations without bicarbonate. The insert shows the Lineweaver-Burk plot to determine K_a of $MnCl_2$.

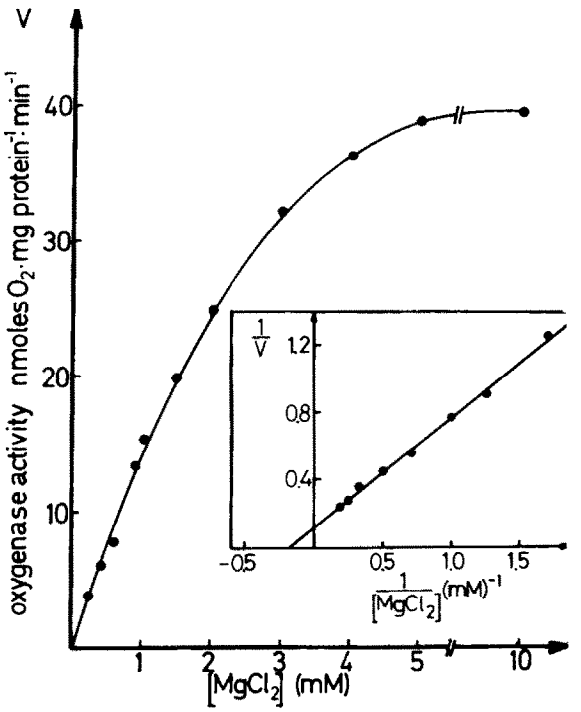

Fig.2. Reactivation of RuBP oxygenase with $MgCl_2$. The dialysed enzyme fraction was preincubated for 15 min at 25°C with 0.2–10 mM $MgCl_2$ and 10 mM $NaHCO_3$. Samples were taken and analysed for RuBP oxygenase activity at the appropriate $MgCl_2$ concentrations. The insert shows the Lineweaver-Burk plot to determine K_a of $MgCl_2$.

K_a value of 0.3 mM Mn^{2+}, the V_{max} value was equivalent to 0.055 U/mg protein (fig.1 insert).

The reactivation of RuBP oxygenase with $MgCl_2$ and bicarbonate was studied under similar conditions. The K_a value was equivalent to 6 mM Mg^{2+} and the V_{max} value was determined to be 0.083 U/mg protein (fig.2).

3.2. *Reactivation of RuBP carboxylase*

The activation of RuBP carboxylase was studied with the dialysed enzyme fraction in presence of 0.01–0.2 mM $MnCl_2$. The preincubation was carried out in the presence of 10 mM bicarbonate as described above. Figure 3 shows the dependence of the reactivation process on the $MnCl_2$ concentration. The K_a value was determined to be 0.11 mM $MnCl_2$, the V_{max} value was equivalent to 0.061 U/mg protein (fig.3 insert).

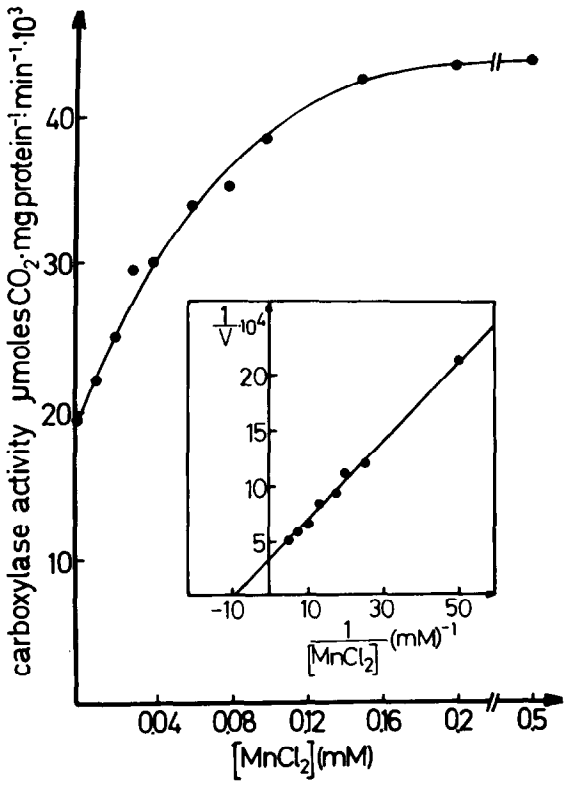

Fig.3. Reactivation of RuBP carboxylase with $MnCl_2$. The dialysed enzyme fraction was preincubated for 15 min at 25°C with 0.01–0.5 mM $MnCl_2$ and 10 mM $NaHCO_3$. Samples were taken and analyzed for RuBP carboxylase activity at the appropriate $MnCl_2$ concentrations. The insert shows the Lineweaver-Burk plot to determine K_a of $MnCl_2$.

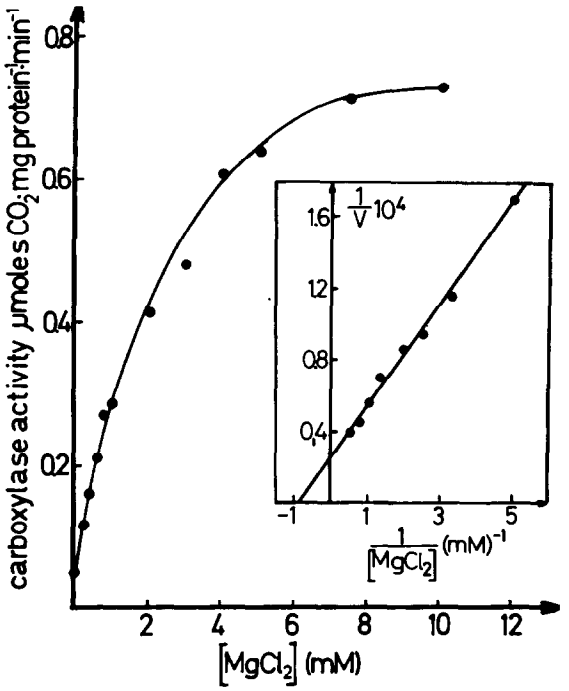

Fig.4. Reactivation of RuBP carboxylase with $MgCl_2$. The dialysed enzyme fraction was preincubated for 15 min at 25°C with 0.2–10 mM $MgCl_2$ with 10 mM $NaHCO_3$. Samples were taken and analysed for RuBP carboxylase activity at the appropriate $MgCl_2$ concentrations. The insert shows the Lineweaver-Burk plot to determine K_a of $MgCl_2$.

The dialysed enzyme fraction was also reactivated in the presence of 0.2–10 mM $MgCl_2$ and 10 mM $NaHCO_3$ for 15 min. Aliquots were removed and CO_2 fixation was estimated using assay mixtures with the same $MgCl_2$ concentration as in the preceding incubation. The results are presented in fig.4. The activation followed Michaelis-Menten kinetics with a K_a value of 1.15 mM $MgCl_2$ and a V_{max} value equivalent to 0.91 U/mg protein (fig.4 insert).

3.3. *Comparative studies of the reactivation process*

The carboxylase and oxygenase activity was determined for dialysed enzyme samples treated under different preincubation conditions ($MgCl_2$, $MnCl_2$, $NaHCO_3$). The results are summarized in table 1. In the presence of $MgCl_2$ and $NaHCO_3$, the carboxylase activity of the dialysed enzyme sample was almost as active as of the original undialysed fraction (92.7%), whereas the sample treated with $MnCl_2$ and $NaHCO_3$ showed only minor activity (10.5%). On the other hand the oxygenase activity was highest in the samples which were treated with $MnCl_2$ (111%) and in this case the presence of bicarbonate had no further stimulatory effect on oxygenase activity.

4. Discussion

Divalent cations participate in the carboxylation mechanism and in the activation of the enzyme molecules for both reactions (carboxylase and oxygenase) (cf. [14]). The role of Mg^{2+} in these reactions and the variation in concentration of this ion in chloroplasts

Table 1
Reactivation of RuBP carboxylase-oxygenase

Sample	carboxylase (activity in %)	oxygenase
Undialysed enzyme fraction	100[a]	100[b]
Dialysed enzyme fraction	3.4	1.7
+ 10 mM $NaHCO_3$	4.6	6.6
+ 5 mM $MnCl_2$	9.9	111
+ 10 mM $NaHCO_3$ + 5 mM $MnCl_2$	10.5	111
+ 10 mM $MgCl_2$	67.5	56.2
+ 10 mM $NaHCO_3$ + 10 mM $MgCl_2$	92.7	73

The undialysed enzyme fraction was assayed for both activities in the preactivated form in presence of 10 mM $MgCl_2$ and 10 mM $NaHCO_3$; [a] the specific activity was equivalent to 1.2 U/mg protein for the carboxylase reaction; [b] respectively, to 0.07 U/mg protein for the oxygenase reaction and the values were arbitrary set as 100%. The preincubation of the dialysed enzyme fraction with $NaHCO_3$, $MgCl_2$ and $MnCl_2$ was carried out at 25°C for 15 min. The assay mixtures were described in section 2 except that the assay reaction media contained 1 mM $MnCl_2$, 10 mM $MgCl_2$ in the appropriate assays

during light–dark transitions suggests a regulatory function in photosynthetic CO_2 fixation [14]. The necessity of Mg^{2+} for the carboxylase reaction was discovered [8] and the replacement of Mg^{2+} by other divalent cations was studied [8,9]. The results showed that Mn^{2+} had little or no effect either on the enzyme activation or the carboxylase reaction. Subsequently, however, Mn^{2+} was reported [11] to replace Mg^{2+} with a 50% loss of the enzyme activity.

The binding of Mn^{2+} to RuBP carboxylase was studied [7,15]. The enzyme, as measured by EPR showed 8 tight binding sites and a variable amount of weak binding sites for Mn^{2+}, however the influence of Mn^{2+} on enzyme activity was not measured under these experimental conditions.

It was of interest, therefore, to study the role of Mn^{2+} on the activation process for both enzyme activities (carboxylase and oxygenase).

Our data showed that Mn^{2+} can restore the oxygenase activity but failes significantly to enhance the carboxylase reaction. This observation indicated that the enzyme mechanism for the carboxylase and the oxygenase activities are different. The carboxylation of RuBP is dependent on the presence of Mg^{2+}, which participates in the binding of the positive allosteric effector CO_2, in the activation process, and on the binding of the substrate CO_2. The oxidative splitting of RuBP is catalysed in presence of Mn^{2+}, whereas in the presence of bicarbonate (CO_2) Mn^{2+} was not necessary for the activation of the enzyme.

The partial reactivation of the oxygenase activity whith $MgCl_2$ and $NaHCO_3$ could be explained by a non-reversible change of the activating sites of the enzyme molecules or by a partial loss of a cofactor, e.g., a transition metal ion, necessary for the oxygenase function. The Mn^{2+}-mediated oxygenase reaction indicated that Mn^{2+} can participate in both the activation of oxygen and the oxidative splitting of RuBP [16].

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