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Effects of Manganese Ions and Magnesium Ions on the Activity of Soya-Bean Ribulose Bisphosphate Carboxylase/Oxygenase

By John T. CHRISTELLER and William A. LAING
Plant Physiology Division, D.S.I.R., Palmerston North, New Zealand

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The Michaelis constants of soya-bean ribulose bisphosphate carboxylase for CO_2 in the carboxylation reaction and for O_2 in the oxygenation reaction depend on the nature of the bivalent cation present. In the presence of Mg^{2+} the K_m for bicarbonate is 2.48mM, and the K_m for O_2 is 37% (gas-phase concentration). With Mn^{2+} the values decrease to 0.85mM and 1.7% respectively. For the carboxylation reaction $V_{max.}$ was 1.7μmol/min per mg of protein with Mg^{2+} but only 0.29μmol/min per mg of protein with Mn^{2+}. For the oxygenation reaction, $V_{max.}$ values were 0.61 and 0.29μmol/min per mg of protein respectively with Mg^{2+} and Mn^{2+}.

Ribulose bisphosphate carboxylase (EC 4.1.1.39) catalyses the reaction of D-ribulose 1,5-bisphosphate with CO_2 to yield two molecules of 3-D-phosphoglycerate per molecule of ketose. In the presence of O_2, however, the same enzyme catalyses a competing reaction (Bowes *et al.*, 1971) in which the products of cleavage of D-ribulose 1,5-bisphosphate are 2-phosphoglycollate and 3-D-phosphoglycerate. Wildner & Henkel (1978) reported differential responses of these two reactions to Mg^{2+} and Mn^{2+}. Under their conditions carboxylase activity with Mn^{2+} was 10-fold inhibited relative to that with Mg^{2+} and oxygenase activity was 1.5-fold stimulated. In the present paper we explain these results in terms of the Michaelis constants by using a simultaneous assay of both activities.

Materials and Methods

Enzyme purification

Our usual isolation procedure for ribulose bisphosphate carboxylase (EC 4.1.1.39) was followed [Laing & Christeller (1976); modified by G. H. Lorimer (personal communication)]. Soya-bean (*Glycine max* L. cultivar Amsoy) leaves were ground in 25mM-Hepes/10mM-$MgCl_2$/10mM-$NaHCO_3$, pH7.2, with 5% polyvinylpolypyrrolidone at 2°C, and the homogenate was filtered and centrifuged at 10000*g* for 20min. The supernatant was immediately made 37% saturated with respect to $(NH_4)_2SO_4$ by adding the correct volume of saturated $(NH_4)_2SO_4$ solution, immediately centrifuged, and the supernatant made 45% saturated with respect to $(NH_4)_2SO_4$ as above and re-centrifuged. The pellet was dissolved in 10mM-sodium phosphate buffer, pH7.6, and the purification was continued as previously described (Laing & Christeller, 1976).

Phosphoglycollate phosphatase (EC 3.1.3.18) (Christeller & Tolbert, 1978) was prepared from tobacco (*Nicotiana tabacum* L. var. *xanthium*) leaves. Leaves were ground in 10mM-Hepes, pH7.2, with 10% polyvinylpolypyrrolidone at 2°C, and the homogenate was filtered and centrifuged as above. Acetone at −20°C was added to the supernatant up to a concentration of 20% (v/v) acetone and the mixture was centrifuged. The supernatant was adjusted to 40% (v/v) acetone, and after centrifugation the pellet was dissolved in 25mM-Tris/HCl buffer, pH8.0, applied to a DEAE-cellulose column equilibrated in the same buffer, and eluted with a 0–0.3M-KCl gradient. The active fractions were concentrated by ultrafiltration and stored at 2°C in 50mM-Hepes/50mM-sodium citrate/25mM-$MgCl_2$, pH7.0.

Glycollate oxidase (EC 1.1.3.1) was prepared as for ribulose bisphosphate carboxylase with the following modifications. The pellet was obtained from the 25–37%-saturation-$(NH_4)_2SO_4$ fraction, the lower limit being determined as that required to remove the chlorophyll complexes from the suspension. $(NH_4)_2SO_4$ was added as solid. The glycollate oxidase was eluted from the DEAE-cellulose column in the void volume.

Enzyme assays

Ribulose bisphosphate carboxylase and oxygenase assays were carried out simultaneously at 30°C in 25ml Kimax flasks fitted with serum caps. Gas mixtures were prepared from combinations of O_2, air and N_2 (all humidified and CO_2-free) by using one- or two-stage gas-mixing pumps. The flasks were

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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flushed for 15min before being sealed, then 0.9ml of 50mM-Tris/HCl buffer, pH8.2, at the required O_2 saturation, containing either Mn^{2+} or Mg^{2+}, was injected through the serum cap, and the flasks were allowed to equilibrate further. Then 30s before assay ribulose bisphosphate was added, the flasks were gently agitated, and the reaction was initiated with preactivated enzyme (Laing & Christeller, 1976) containing either Mg^{2+} or Mn^{2+}, CO_2 and $^{14}CO_2$ at the assay pH. The assays were terminated after 60s with 0.1ml of 1M-HCl. Controls were treated identically other than the acid being added before the enzyme. Sampling of the gas phase above the reaction mixture showed that $^{14}CO_2$ loss from the solutions was negligible under assay conditions of high pH and short time of incubation. For assays with Mn^{2+} present, 0.1ml of 0.1M-EDTA, pH6.2, was added after 10s. The EDTA is necessary to prevent a high background colour due to the interaction of Mn^{2+} and ribulose bisphosphate. EDTA itself caused a small background colour independent of other reaction components. The O_2 concentration is expressed as that present in the gas phase under conditions where the solution is in equilibrium with the water-saturated gas phase at 30°C and normal atmospheric pressure.

Incorporation of $^{14}CO_2$ into acid-stable products was determined by removing a 50μl sample from the flask, and determining radioactivity as previously described (Laing & Christeller, 1976). Assays of the carboxylase activity alone were determined as described previously (Laing & Christeller, 1976), but the vials were capped with serum caps and flushed as described above before assay.

Production of phosphoglycollate was determined on the remaining solution after neutralization with 95μl of 1M-NaOH. The procedure described by Laing *et al.* (1974) was followed with modifications. The phosphoglycollate formed by the oxygenase reaction was determined colorimetrically after enzymic conversion of the phosphoglycollate into glycoxylate by using phosphoglycollate phosphatase, glycollate oxidase and catalase. Excess of coupling enzymes in 0.25ml of 25mM-Tris/HCl/25mM-$MgCl_2$, pH8.0, was added to samples and the mixtures were incubated at 25°C for 45min. Complete conversion was monitored by inclusion of phosphoglycollate standards run under identical conditions. Colour was developed at 25°C by addition of 0.25ml of phenylhydrazine hydrochloride (3.33mg/ml) in 0.15M-HCl, waiting 5min and then placing the samples on ice. Ice-cold concentrated HCl (1ml) was added followed by 0.25ml of $K_3Fe(CN)_6$ (16.66mg/ml), and the colour was developed at 25°C for 15min. The colour was extracted into 1.8ml of 3-methylbutan-1-ol/light petroleum (b.p. 140–160°C) (2:3, v/v) and the samples were briefly centrifuged. This modification of the method of Nirmala & Sastry (1972) left precipitated protein at the interface and the coloured layer on top of the aqueous layer, permitting easy removal of the organic layer for reading of the absorbance at 520nm.

Data analysis

All curves were analysed with computer programs that used non-linear parameter estimation (Bard, 1974) of the untransformed data. The parameter values were used to draw the lines in the Figures, which were prepared as reciprocal plots for ease of interpretation.

Results

The very different responses to O_2 concentration of the rate of ribulose bisphosphate oxygenase elicited by Mg^{2+} and Mn^{2+} are shown in Fig. 1. The presence of a low concentration of bicarbonate in the assay mixture is unavoidable since CO_2 is required to activate both the carboxylase and oxygenase activities (Laing & Christeller, 1976). The inactivation is insignificant during the assay despite the 20-fold dilution of the CO_2 concentration (Laing & Christeller, 1976). The apparent $K_{m(O_2)}$ and $V_{max.}$ in the presence of Mn^{2+} and Mg^{2+} are shown in Table 1. To calculate the true $K_{m(O_2)}$ we corrected for the small amount of CO_2 present in

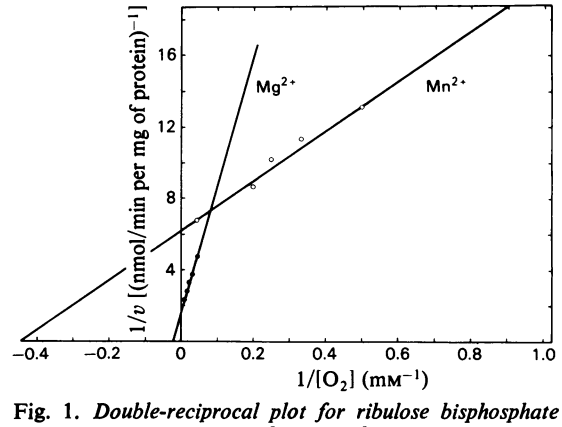

Fig. 1. *Double-reciprocal plot for ribulose bisphosphate oxygenase activity as a function of O_2 concentration*
Enzyme (120μg/assay) was preincubated in 50mM-Tris/HCl (pH8.2)/10mM-$NaHCO_3$ and either 2mM-$MnCl_2$ or 20mM-$MgCl_2$ for 10min at 30°C before assay. Metal ion concentrations were chosen at about 20-fold K_a (Wildner & Henkel, 1978). Then 50μl of enzyme was added to 0.95ml of reaction mixture containing the same concentrations of metal ion as in the preincubation mixture but with only 0.432mM-(Mn^{2+}) or 0.532mM- (Mg^{2+}) $NaHCO_3$ and 0.641mM-ribulose bisphosphate. ○, Mn^{2+} present; ●, Mg^{2+} present.

Table 1. *Kinetic constants for ribulose bisphosphate carboxylase/oxygenase*
For experimental details see the text. The results are expressed as means ± S.E.M. for three determinations.

Cation	$K_{m(CO_2)}$ (mM-HCO_3^-)	$K_{i(O_2)}$ (%) Fig. 2	$K_{i(O_2)}$ (%) Fig. 3	$V_{max.(CO_2)}$ (μmol/min per mg)	$V_{max.(O_2)}$ / $K_{m(O_2)}$ (%)	$V_{max.(O_2)}$ (μmol/min per mg)
Mn^{2+}	0.85 ± 0.07	1.00 ± 0.11	2.20 ± 0.13	0.289 ± 0.008	2.28 ± 0.28	0.289 ± 0.007
Mg^{2+}	2.48 ± 0.12	43.1 ± 3.0	60.9 ± 0.6	1.70 ± 0.035	42.2 ± 5.0	0.610 ± 0.032

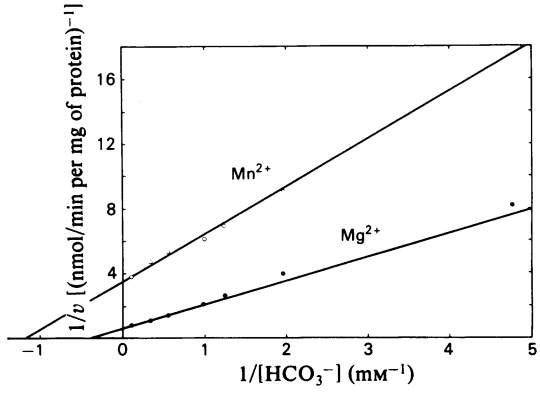

Fig. 2. *Double-reciprocal plot for ribulose bisphosphate carboxylase activity under N_2*
Enzyme (21 μg/assay) was preincubated in 50mM-Tris/HCl (pH 8.2)/10mM-HCO_3^- and either 1.86mM-$MnCl_2$ or 18.6mM-$MgCl_2$ for 10min at 25°C and then assayed at the same metal ion concentration as in the preincubation mixture, with N_2 atmosphere (shown) or 21% O_2 or 100% O_2 (not shown) and 0.42mM-ribulose bisphosphate and varied HCO_3^- concentrations.

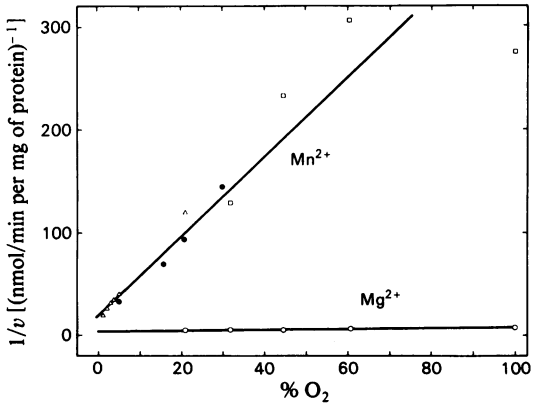

Fig. 3. *Dixon plot of O_2 inhibition of ribulose bisphosphate oxygenase activity*
Simultaneous carboxylase data are taken from Fig. 1. Three separate experiments are shown for the Mn^{2+} data at overlapping O_2 concentrations. □ and ○, 0.52mM-HCO_3^- and Mn^{2+}; △, 0.42mM-HCO_3^- and Mn^{2+}; ●, 0.52mM-HCO_3^- and Mg^{2+}. Ribulose bisphosphate concentration was 0.625mM. The curve was fitted by substituting the value of $K_{m(CO_2)}$ (corrected for temperature) into the equation for competitive inhibition.

the assay mixture by using, as an estimate of the $K_{i(CO_2)}$, the $K_{m(CO_2)}$ measured below (Laing *et al.*, 1974). After temperature correction (Badger & Collatz, 1977) the $K_{m(CO_2)}$ was inserted into the expression for the presence of a competitive inhibitor, i.e. $K_{m,app.(O_2)} = K_{m(O_2)}\{1+([CO_2]/K_{i(CO_2)})\}$. These values of $K_{m(O_2)}$ are 1.70% O_2 with Mn^{2+} and 36.9% O_2 with Mg^{2+}.

The $K_{m(CO_2)}$ was measured with Mn^{2+} and Mg^{2+} in 0%, 21% and 100% O_2. For clarity, only the data in the absence of O_2 (N_2 atmosphere) are presented (Fig. 2) to demonstrate a decrease in the $K_{m(CO_2)}$ for ribulose bisphosphate carboxylase with Mn^{2+} as compared with that with Mg^{2+}. Since the data showed, as expected, O_2 to be competitive (Bowes & Ogren, 1972), $K_{m(CO_2)}$, $K_{i(O_2)}$ and $V_{max.}$ were calculated from a simultaneous fit to the data at all three O_2 concentrations (Table 1).

The linear Dixon plots (Fig. 3) illustrate the dramatic difference in sensitivity of Mg^{2+}- and Mn^{2+}-catalysed carboxylase reactions to O_2. The former activity is only slightly affected, whereas that for the Mn^{2+}-activated enzyme is highly inhibited. The values for $K_{i(O_2)}$, calculated from the value of $K_{m(CO_2)}$ obtained above and the known CO_2 concentration in the Dixon equation, are shown in Table 1 and show good agreement with those from Fig. 2.

Discussion

The effects observed when Mn^{2+} is substituted for Mg^{2+} as a cofactor for ribulose bisphosphate carboxylase/oxygenase are due in part to the large decreases in the Michaelis constants for O_2 and CO_2. The decreases in $K_{m(O_2)}$ (22-fold) and in $K_{m(CO_2)}$ (3.0-fold) result in the Mn^{2+}-catalysed carboxylase activity being much more sensitive to O_2 concentration. The effect on $V_{max.}$ is less, the $V_{max.}$ for Mg^{2+}-

activated carboxylase being 5.9-fold higher and for Mg^{2+}-activated oxygenase being 2.1-fold higher than the corresponding Mn^{2+}-stimulated activities. From our constants we can obtain an oxygenase activity (v_o) ratio in air and 0.6mM-HCO_3^- for $v_{o(Mg^{2+})}/v_{o(Mn^{2+})}$ of 0.77 [cf. Wildner & Henkel (1978), 0.66] and a carboxylase activity (v_c) ratio in air and 25mM-HCO_3^- for $v_{c(Mg^{2+})}/v_{c(Mn^{2+})}$ of 8.95 [cf. Wildner & Henkel (1978), 8.83]. It is probable that the different affinity values obtained for a given cation saturation curve for carboxylase and oxygenase activities by Wildner & Henkel (1978) are due to the different conditions of assays, the oxygenase being assayed under low HCO_3^- concentration and requiring a higher cation concentration to maintain activity during the assay. This higher cation concentration is reflected in the higher $K_{a(cation)}$ for oxygenase than for carboxylase activities.

Our data also provide a possible explanation for the biphasic response of glycollate synthesis by isolated chloroplasts to O_2 concentration observed by Eichenbusch & Beck (1973). The low rate of synthesis at low O_2 concentrations would be supported by exogenously supplied Mn^{2+} whereas the higher rate at high O_2 concentrations would be due to the expression of activity with exogenous and endogenous Mg^{2+} (see Fig. 1).

The mechanism responsible for these differential effects is unknown. The $H^{13}CO_3^-$ n.m.r. data of Miziorko & Mildvan (1974) can be explained simply (M. H. O'Leary, personal communication) by a direct metal ion–substrate phosphate interaction. If the interaction were with the C-1 phosphate of ribulose bisphosphate, a direct effect on the gaseous substrate would be possible, the Mn^{2+} quaternary complex being more stable than the Mg^{2+} quaternary complex. Ribulose bisphosphate is acyclic and exists predominantly as a ketose in solution (Gray & Barker, 1970), whereas the enediol is the probable catalytically active form (Calvin, 1954). We would therefore expect the latter to be involved in any catalytically active complex. Alternatively, the cations could modify differentially a gas-binding site on the enzyme, since catalysis has been shown to proceed by random addition of substrates for the carboxylation reaction (Badger & Collatz, 1977; W. A. Laing & J. T. Christeller, unpublished work).

This is the first report of any variation in O_2 binding to ribulose bisphosphate carboxylase/oxygenase and the first report of induced variation in CO_2 binding that cannot be ascribed to interaction with the activation process.

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