A Model for the Kinetics of Activation and Catalysis of Ribulose 1,5-Bisphosphate Carboxylase

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Further evidence for time-dependent interconversions between active and inactive states of ribulose 1,5-bisphosphate carboxylase is presented. It was found that ribulose bisphosphate oxygenase and ribulose bisphosphate carboxylase could be totally inactivated by excluding CO_2 and Mg^{2+} during dialysis of the enzyme at 4°C . When initially inactive enzyme was assayed, the rate of reaction continually increased with time, and the rate was inversely related to the ribulose bisphosphate concentration. The initial rate of fully activated enzyme showed normal Michaelis–Menten kinetics with respect to ribulose bisphosphate ($K_m = 10\mu\text{M}$). Activation was shown to depend on both CO_2 and Mg^{2+} concentrations, with equilibrium constants for activation of about $100\mu\text{M}$ and 1mM respectively. In contrast with activation, catalysis appeared to be independent of Mg^{2+} concentration, but dependent on CO_2 concentration, with a $K_m(\text{CO}_2)$ of about $10\mu\text{M}$. By studying activation and de-activation of ribulose bisphosphate carboxylase as a function of CO_2 and Mg^{2+} concentrations, the values of the kinetic constants for these actions have been determined. We propose a model for activation and catalysis of ribulose bisphosphate carboxylase:

where E represents free inactive enzyme; complex in parentheses, activated enzyme; R, ribulose bisphosphate; M, Mg^{2+} ; C, CO_2 ; P, the product. We propose that ribulose bisphosphate can bind to both the active and inactive forms of the enzyme, and slow inter-conversion between the two states occurs.

Ribulose bisphosphate carboxylase (EC 4.1.1.39) catalyses the carboxylation (Weissbach *et al.*, 1956) and oxygenation (Bowes *et al.*, 1971) of ribulose bisphosphate, resulting in both the fixation of CO_2 in the photosynthetic carbon-reduction cycle and the production of phosphoglycolate in the photorespiratory pathway (Tolbert, 1971).

When first purified, ribulose bisphosphate carboxylase was found to have a $K_m(\text{CO}_2)$ severalfold higher than the value for photosynthesis *in vivo* (Weissbach *et al.*, 1956). The $K_m(\text{CO}_2)$ *in vivo* for whole leaves (Goldsworthy, 1968) or isolated chloroplasts (Jensen & Bassham, 1966) was found to be about $10\mu\text{M}$, whereas the purified enzyme had a value in excess of $400\mu\text{M}$ (Weissbach *et al.*, 1956; Paulsen & Lane, 1966). Diverse reasons for this anomaly have been suggested (Walker, 1973).

Since then, Buchannan & Schurmann (1973) and Chu & Bassham (1975) have showed that $K_m(\text{CO}_2)$ can be decreased by the presence of sugar phosphates, and Bassham *et al.* (1968) and Sugiyama *et al.* (1968) have found that high Mg^{2+} concentrations also decreased the $K_m(\text{CO}_2)$. The finding that the $K_m(\text{CO}_2)$ was low immediately after the lysis of spinach

chloroplasts (Bahr & Jensen, 1974) suggested that the properties of the enzyme could change after isolation. However, Laing *et al.* (1975), in short-term kinetic studies of purified ribulose bisphosphate carboxylase, showed the enzyme was activated by either HCO_3^- or CO_2 and the activated form had a $K_m(\text{CO}_2)$ close to the value *in vivo*. Comparisons of the effect of CO_2 on the carboxylase and oxygenase reactions led these workers to conclude that the activation involves an increase in V_{max} , rather than a change in K_m .

Lorimer *et al.* (1976) have published a detailed account of the activation processes for the isolated enzyme. They proposed that CO_2 combined with the enzyme in a slow step, and then Mg^{2+} rapidly converted the enzyme– CO_2 complex into the active form of the enzyme, which reacts with ribulose bisphosphate and CO_2 to form phosphoglycerate. In addition, it was shown that CO_2 , not HCO_3^- , was the activating species.

In the present paper we report experiments that suggest that ribulose bisphosphate binds to both activated and an inactivated enzyme, decreasing the rate of change from one form into another. We also show that Mg^{2+} appears to be required for the forma-

tion of activated enzyme, but not for the catalysis of the carboxylase or oxygenase reactions. On the basis of these findings and those of Lorimer *et al.* (1976), we have proposed a model for the kinetics of activation of, and catalysis by, the enzyme.

Experimental

Enzyme isolation

Ribulose biphosphate carboxylase was isolated as described by Christeller *et al.* (1976). The purified enzyme was stored at 4°C in a 55% satd. (NH₄)₂SO₄ solution and prepared for use by centrifugation (10 min, 20000g) and by dissolving the pellet in 10 mM-Tris/HCl/0.1 mM-EDTA/5 mM-cysteine, pH 7.7. Before assay, the enzyme was de-activated by dialysis at 4°C for 3–4 h in the same buffer continuously bubbled with N₂. The dialysed enzyme showed essentially no initial activity, but was activated at a rate dependent on the Mg²⁺ and CO₂ concentrations. If the enzyme was to be assayed in an activated state, it was first dialysed as described above and then activated by pretreatment at 20°C with CO₂ and Mg²⁺ as described in the Results section. Fully active enzyme had a specific activity of 0.5–1 μmol of CO₂ fixed/min per mg of protein at 30°C and saturating concentration of substrates. Phosphoglycollate phosphatase and glycollate oxidase, used in the ribulose biphosphate oxygenase assay, were isolated by the procedures described by Laing (1974).

Enzyme assay

Ribulose biphosphate was prepared as described by Horrecker *et al.* (1958), but further purified by Dowex chromatography (Dowex 1 X8; 200–400 mesh; Cl[−] form) by using a 0–0.2 M-HCl gradient to remove the P_i and traces of ribose 5-phosphate and phosphoglycollate otherwise found in the preparation. NaH¹⁴CO₃ was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Ribulose biphosphate carboxylase was assayed at 20°C in reaction mixtures containing 50 mM-Tris/HCl, pH 8.0, and variable quantities of Mg²⁺, CO₂ and ribulose biphosphate were added in the order described for each experiment.

For assays sampled at 1 min intervals or longer, 100 μl portions were taken by micro-syringe. For short-time assays (less than 40 s total duration), the reaction mixture and enzyme were simultaneously injected into a 5 ml ratchet-controlled syringe and 0.5 ml portions were then dispensed at 3 s intervals. Samples in both cases were injected into 0.5 ml of methanolic 1 M-HCl in scintillation vials, the mixture was dried, the residue dissolved in 0.3 ml of water and radioactivity (c.p.m.) determined by using a scintillation fluid modified from that of Anderson & McClure (1973), in that *p*-terphenyl replaced 2,5-diphenyloxazole and Triton X-100 replaced Triton

X-114. Radioactivity as c.p.m. was converted into d.p.m. by using the channels-ratio technique. Ribulose biphosphate oxygenase and carboxylase were assayed simultaneously as described by Laing (1974).

Dissolved CO₂ present from equilibrium with the air was determined by using the Bunsen coefficient given by Hodgeman *et al.* (1958). The equilibrium between CO₂ and HCO₃[−] was calculated by using the constant tabulated by Laing (1974) ($K = 4.13 \times 10^{-7}$ M at 20°C). Assays to determine the constants for the activation process were performed as described by Lorimer *et al.* (1976), except that 10 μl of the enzyme, pretreated as described in the Tables and Figures, was injected into 0.5 ml of reaction mixture containing 50 mM-Tris/HCl, pH 8, 0.5 mM-ribulose biphosphate, 2 mM-H¹⁴CO₃[−] and either 20 mM- or no MgCl₂. The reaction was terminated after 15 or 30 s.

Results

Characterization of the activation reaction

Initially, the activation reaction was conceived as a CO₂- and Mg²⁺-dependent conversion of inactive enzyme into active enzyme as described by Laing *et al.* (1975) and Lorimer *et al.* (1976). Ribulose biphosphate carboxylase was assayed at 16 CO₂ concentrations by taking samples about every 3 s for 30 s as described in the Experimental section. A parabola was fitted to the data and the rate determined at 10, 20 and 30 s from the first differential of the parabola. The rate was linearly related to CO₂ concentration up to the highest value (590 μM at pH 8.0) at the ribulose biphosphate concentration used (333 μM).

It was observed that the enzyme could be fully activated at 20°C within a few minutes in the presence of CO₂ and Mg²⁺ without ribulose biphosphate (see also Lorimer *et al.*, 1976), but when ribulose biphosphate was present the activation process appeared to be inhibited (Fig. 1). The reaction rate of the enzyme exposed to ribulose biphosphate before activation did not approach the rate of the fully activated enzyme within a few minutes. Also, the reaction rate was inversely related to ribulose biphosphate concentration. However, the data also show the usual direct relationship between the rate of reaction and the ribulose biphosphate concentration. When the K_m for ribulose biphosphate was calculated for the initial rate of active enzyme as described by Laing *et al.* (1975), the mean K_m was 10 μM. The assay was repeated, both the oxygenase and carboxylase activities of the enzyme being measured, by initiating the reaction with either the activated or the inactivated form of the enzyme. Again, for both oxygenase and carboxylase the reaction rates were reciprocally related to ribulose biphosphate concentration for the inactive enzyme and directly

Fig. 1. Relationship between reaction rate and ribulose biphosphate concentration for both initially inactive and initially active enzyme

Enzyme was used to initiate assays in all cases. Reaction mixtures (final vol. 1.0 ml) contained 50mM-Tris/HCl, pH 8.0, 5mM-MgCl₂ and HCO₃⁻ as indicated (a-d). Ribulose biphosphate concentrations were 0.5 mM (▲), 0.1 mM (○) and 0.03 mM (△). The protein concentration was 71 µg/ml and the temperature was 20°C. In each case (a-d): (i) assays were initiated with enzyme previously inactivated by dialysis; (ii) assays were initiated with ribulose biphosphate by using inactivated enzyme that was then activated by preincubation in reaction mixture without ribulose biphosphate for 8 min.

related (Fig. 2) for the active enzyme. Note the HCO₃⁻ concentration was higher in the initially inactive case (Fig. 2a) than in the active case.

Role of Mg²⁺

Mg²⁺ appears to be involved in a second fast activation after CO₂ has converted the inactive form into an enzyme-CO₂ complex (Lorimer *et al.*, 1976).

To test whether Mg²⁺ is also necessary for catalysis, time-courses of CO₂ fixation were measured over short time-periods (samples every 3 s for 30 s) by using activated enzyme with different Mg²⁺ concentrations. The enzyme was initially inactivated by dialysis and then activated by incubation at 20°C with 1.0mM-MgCl₂ and 10mM-HCO₃⁻ for at least 10 min. Then the enzyme was assayed by rapid dilution with 60 vol. of reaction mixture containing various MgCl₂ con-

centrations and final concentrations of 10mM-HCO₃⁻ and 0.2mM-ribulose biphosphate. The range of MgCl₂ was from 16 to 500 µM. Linear-regression analysis was used to determine the reaction rate over the linear portion of the time-courses (greater than 20 s). The reaction rate when plotted against [Mg²⁺] was virtually independent of Mg²⁺ (Table 1), and a Lineweaver-Burk plot of the data gave a *K_m*(Mg²⁺) of less than 1 µM-MgCl₂.

Determination of the equilibrium and kinetic constants for HCO₃⁻ and Mg²⁺ in the activation process

Lorimer *et al.* (1976) described the activation of the enzyme as:

where C is CO₂, M is Mg²⁺ and E is enzyme.

Fig. 2. Relationship between reaction rate and ribulose biphosphate concentration for both ribulose biphosphate oxygenase and ribulose biphosphate carboxylase activities

Assays were run as described for Fig. 1. Δ , $\circ$, Oxygenase assays; $\bullet$, $\circ$, carboxylase assays. Reaction mixtures (final vol. 2.7 ml) contained 50 mM-Tris/HCl, pH 8.0, and 5 mM-MgCl₂ at 20°C. $\bullet$, $\circ$, 0.3 mM-Ribulose 1,5-bisphosphate; Δ , Δ , 0.15 mM-ribulose 1,5-bisphosphate. (a) Assays were initiated with inactive enzyme (3.05 mg/ml), which had been dialysed as described in the text. HCO₃⁻ concentration was 1.85 mM. (b) Assays were run with enzyme preincubated for 10 min in 2.36 mM-HCO₃⁻ and 5 mM-MgCl₂. Assays were initiated by adding enzyme (0.28 mg/ml) to reaction mixture. The HCO₃⁻ concentration was 0.926 mM.

They suggested that the Mg²⁺ reaction is rapid in comparison with the CO₂ reaction and can be considered to be in equilibrium. They showed that, during preincubation of the enzyme at given Mg²⁺ and CO₂

concentrations, the enzyme moves towards equilibrium between the three forms at a rate dependent on the Mg²⁺ and CO₂ concentrations. When reaction mixture containing high concentrations of ribulose biphosphate and Mg²⁺, but with a low CO₂ concentration, is added to the preincubated enzyme and the reaction stopped after 30 s, the amount of EC-plus-ECM forms of the enzyme was postulated to be proportional to the amount of ¹⁴C fixed. This was because the small amount of CO₂ in the assay was suggested to prevent activation of any more inactive enzyme (F) during assay, and the high Mg²⁺ concentration ensured rapid conversion of EC into ECM, the active form of the enzyme.

Confirming Lorimer *et al.* (1976), the activation was found to follow an exponential time-course and the pseudo-first-order rate constant k_{obs} is given by eqn. (1):

$$\ln \left(1 - \frac{n}{n_e} \right) = -k_{obs} \cdot t \quad (1)$$

where n is the number of mol fixed during the 30 s assay after preincubation for time t , and n_e is the number of mol fixed when the reactions have come to equilibrium. k_{obs} was shown by Lorimer *et al.* (1976) to be:

$$k_{obs} = k_1 \cdot [C] + \frac{k_2 k_4}{k_3} \frac{1}{[M]} \quad (2)$$

If the enzyme is allowed to come to equilibrium during preincubation, then Lorimer *et al.* (1976) also showed that in the presence of Mg²⁺ and CO₂:

$$n_e \propto [EC + ECM] = \frac{E_T \cdot [C][M]}{[C][M] + K_c K_{Mg}} \quad (3)$$

where $K_c = k_2/k_1$ and $K_{Mg} = k_4/k_3$, and $E_T = E + ECM$ (as EC is assumed to be immediately converted into ECM).

We have derived a similar equation for when Mg²⁺ is absent during preincubation:

$$n_e^* \propto [EC + ECM] = \frac{E_T \cdot [C]}{K_c + [C]} \quad (4)$$

By rearranging eqn. (2) and using the definition of K_{Mg} and K_c , it can be shown that:

$$k_1 = k_{obs} \cdot \left([C] + \frac{K_c K_{Mg}}{[M]} \right) \quad (5)$$

From measurements of the rate of activation of ribulose diphosphate carboxylase by Mg²⁺ and CO₂ as described by Lorimer *et al.* (1976), k_{obs} can be calculated (eqn. 1). Further, from measurements of the equilibrium values of enzyme preincubated in Mg²⁺ and/or CO₂, K_{Mg} and K_c can be calculated (eqns. 3 and 4). Then, from eqn. (5), k_1 can be calculated and thence k_2 .

Table 1. Independence of reaction rate on Mg^{2+} concentration

The enzyme was initially activated by incubation at 20°C for 10 min in 1 mM- $MgCl_2$ and 10 mM- HCO_3^- at pH 8.0. Then 92 μ l of activated enzyme was rapidly diluted into 6 ml of reaction mixture containing 50 mM-Tris/HCl, pH 8.0, 0.2 mM-ribulose biphosphate and 10 mM- HCO_3^- with $MgCl_2$ concentrations as specified. The final enzyme concentration was 0.021 mg/ml. Samples were taken at approx. 3 s intervals by injection into acid and treated as described in the text. Reaction rates at each $MgCl_2$ concentration were calculated by linear-regression analysis of nmol of CO_2 fixed against time over at least 20 s. The $K_m(Mg)$ was 0.91 μ M as calculated by regression of 1/rate against 1/[Mg^{2+}].

[Mg^{2+}] (μ M)	Reaction rate (μ mol/min per ml)	Correlation coefficient (<i>r</i>)
15.1	0.0137	0.999
31.5	0.0126	0.997
47.9	0.0127	0.991
64.3	0.0136	0.988
80.8	0.0132	0.996
97.2	0.0133	0.987
114	0.0136	0.987
130	0.0151	0.989
146	0.0141	0.995
163	0.0127	0.991
179	0.0142	0.996
311	0.0136	0.989
442	0.0177	0.999
508	0.0163	0.999

The kinetic constants k_3 and k_4 can be determined by another approach. The rate of decay of activated enzyme (ECM) to EC when incubated in the absence of Mg^{2+} is:

$$\frac{d[ECM]}{dt} = -k_4[ECM] \tag{6}$$

or

$$\frac{dn}{dt} = -k_4 n$$

which leads to

$$\ln \left(\frac{n}{n_0} \right) = -k_4 t$$

where n_0 is n at $t = 0$.

On incubating initially active enzyme in the virtual absence of Mg^{2+} , low CO_2 concentration and no ribulose biphosphate for a given time, the active enzyme will partly decay to the EC and E forms. By then adding high concentrations of ribulose biphosphate the quantity of ECM remaining (n) can be assayed. A plot of $\ln(n)$ against time should be linear and the slope of this line is $-k_4$ (Fig. 3).

Table 2 presents calculated values of these constants, including the effect of 0.5 mM-NADPH on the measured K_s .

Fig. 3. Rate of decay of active enzyme in the absence of Mg^{2+} and ribulose biphosphate

Enzyme (0.795 mg) previously de-activated by dialysis as described in the text was activated by preincubation at 20°C at pH 8.0 in 1.0 ml of 1 mM- $MgCl_2$ /2 mM- HCO_3^- . Then 94 μ l was diluted into 6.024 ml of reaction mixture in the ratchet-controlled syringe, and 0.476 ml samples were injected every 3 s into 50 μ l of 10 mM-ribulose biphosphate. Acid was added after 15 s to stop ^{14}C incorporation. The reaction mixture contained 50 mM-Tris/HCl, pH 8.0, and 2 mM- $H^{14}CO_3^-$. The ordinate is on a logarithmic scale. The two symbols represent two experiments.

Discussion

The present paper describes the kinetics of both activation and catalysis of ribulose biphosphate carboxylase. The data can be described by a model shown in Scheme 1. The steps in the scheme with the constants k_i (for $i = 1-15$) are assumed to follow classical kinetics. The steps with constants L_i (for $i = 1-6$) are assumed to be in rapid equilibrium. We suggest that the inactive enzyme can bind with ribulose biphosphate at the same site as the active enzyme to form an enzyme-ribulose biphosphate complex that cannot be converted into the active enzyme by Mg^{2+} or CO_2 . In terms of the constants in the scheme, at least k_5 , k_9 , k_{13} and k_{14} must be essentially zero. The de-activation of the active enzyme is also slowed by ribulose biphosphate, as the binding of ribulose biphosphate to the active enzyme decreases the amount of active enzyme available to be de-activated. Consequently, ribulose biphosphate appears to stabilize the active enzyme.

The rate of reaction of enzyme initially in equilibrium with respect to CO_2 and Mg^{2+} decreases for a time after ribulose biphosphate is added to initiate the reaction (Fig. 1, and Chu & Bassham, 1975). One

Table 2. Values of kinetic and equilibrium constants calculated from the present paper and from Lorimer *et al.* (1976)

The constants were calculated as described in the text. The kinetic constant k_{obs} was determined by incubating enzyme in various concentrations of NaHCO_3 (1–8 mM) and at either 1 or 20 mM- MgCl_2 at pH 7.9 and 18°C and then assaying samples taken at regular intervals. The samples were assayed for 30 s in 50 mM-Tris/HCl, pH 8.0, 0.5 mM-ribulose biphosphate, 20 mM- MgCl_2 and 0.2 mM- $\text{H}^{14}\text{CO}_3^-$. K_c and $K_c K_m$ were determined by incubating enzyme in various MgCl_2 concentrations (0–2 mM) and NaHCO_3 concentrations (0.5–40 mM) at pH 7.9 at 18°C for 20 min and assaying in 50 mM-Tris/HCl, pH 8.0, 2 mM- $\text{H}^{14}\text{CO}_3^-$ and 0.5 mM-ribulose biphosphate for 30 s. k_4 was measured as described in the legend to Fig. 3.

Constants	Calculated from the present data	Calculated from Lorimer <i>et al.</i> (1976)
$K_c K_{Mg} (\mu\text{M}^2)^*$	1.93×10^5	1.81×10^5
$K_c K_{Mg} (\mu\text{M}^2)^\dagger$	1.03×10^5	1.46×10^5
$K_c K_{Mg} (\mu\text{M}^2)^\ddagger$	1.33×10^5	—
$K_c (\mu\text{M})$ control	91	309
+0.5 mM-NADPH	61	
$K_{Mg} (\mu\text{M})$	1130 §	529
$k_1 (\text{min}^{-1} \cdot \mu\text{M}^{-1})$ 20 mM- MgCl_2 ¶	7.11×10^{-3}	10.3×10^{-3}
1 mM- MgCl_2 ¶	1.21×10^{-3}	2.04×10^{-3}
$k_2 (\text{min}^{-1})$ 20 mM- MgCl_2 **	0.65	3.18
1 mM- MgCl_2 **	0.11	0.631
$k_3 (\text{min}^{-1} \cdot \mu\text{M}^{-1})^\dagger\dagger$	0.018	—
$k_4 (\text{min}^{-1})$	20.63	—

* Calculated from linear regression of $1/n_e$ against $1/[\text{CO}_2]$.

† Calculated from linear regression of $1/n_e$ against $1/[\text{Mg}^{2+}]$.

‡ Calculated from linear regression of $1/n_e$ against $1/([\text{Mg}^{2+}] \cdot [\text{CO}_2])$.

§ Calculated from $K_c K_m / K_c$ by using method ‡ to calculate $K_c K_m$.

|| Calculated as in method § by using mean of * and ‡ to calculate $K_c K_m$.

¶ Average values of k_1 over several CO_2 concentrations.

** Calculated from $k_2 = k_1 / K_c$.

†† Calculated from $k_3 = k_4 / K_m$.

reason for this would be if the affinity of inactive enzyme for ribulose biphosphate is greater than the affinity of the active enzyme. This would push the equilibrium established by preincubation in CO_2 and Mg^{2+} towards the inactive enzyme when ribulose biphosphate is added. The data of Wishnick *et al.* (1970) indicate that the affinity of the inactive enzyme for ribulose biphosphate is greater than that of the active enzyme.

There is direct evidence for aspects of our scheme. Rabin & Trown (1964) reported a difference spectrum due to the binding of ribulose biphosphate to the enzyme. As this spectrum was measured in the absence of added Mg^{2+} and CO_2 , it suggests that ribulose biphosphate can bind to the inactive enzyme, which is in accord with the model. Further, if the analysis of Cleland (1963) and Fromm (1967) can be applied to a time-dependent activating enzyme, kinetic data of Laing (1974) show that ribulose biphosphate binds before CO_2 or O_2 . This was based on the inhibition patterns observed when the alternative substrate O_2 is present during an assay. These data also suggested that Mg^{2+} also bound before CO_2 or O_2 . However, the present activation data (Table 1), and the data of Lorimer *et al.* (1976), show that Mg^{2+} and CO_2 serve to activate the enzyme

by binding before ribulose biphosphate. This indicates that there are two binding sites for CO_2 , before and after the binding of ribulose biphosphate. There is further evidence for the existence of two binding sites for CO_2 , one for activation and one for catalysis. The activation of ribulose biphosphate carboxylase is linearly related to CO_2 concentration up to at least 600 μM - CO_2 (see the Results section), whereas the catalytic $K_m(\text{CO}_2)$ measured by using initial rates of activated enzyme is about 10 μM (Laing *et al.*, 1975). The scheme predicts both that CO_2 binds before and after ribulose biphosphate and that Mg^{2+} binds before the second or catalytic binding of CO_2 .

Role of Mg^{2+}

Mg^{2+} has always been assumed to be involved in the catalysis by ribulose biphosphate carboxylase. The data of Table 1 show that the catalysis by activated ribulose biphosphate carboxylase is almost independent of Mg^{2+} concentration, and we suggest that the small dependence observed is due to deactivation during the assay. A survey of Mg^{2+} -protein interactions yielded values of the $K_m(\text{Mg}^{2+})$ in the range of 10^{-4} – 10^{-2} M- Mg^{2+} . It therefore appears

Scheme 1. General model of activation and catalysis of ribulose biphosphate carboxylase

E represents free enzyme, ECM represents activated enzyme, and EC represents CO_2 -bound enzyme. C is CO_2 , M is Mg^{2+} , R is ribulose biphosphate, and P is phosphoglycerate. The reactions accompanied by 'L' are reactions considered to be in rapid equilibrium. In terms of the model discussed in the text, constants k_5 – k_{14} inclusive can be considered to be essentially zero.

that the value of less than 10^{-6}M in this paper is too unrealistically low to represent a $K_m(\text{Mg}^{2+})$ value. It is possible that Mg^{2+} binds in its activating site and later serves in a role in the catalysis without being displaced. The enzyme concentration in this assay was 32.3nm , so that Mg^{2+} bound to the enzyme would be negligible compared with the Mg^{2+} in the assay.

The activation process

From the data of Lorimer *et al.* (1976) and from our own data (Table 2) we calculate that K_c is $91\text{ }\mu\text{M}$ and K_{Mg} is $1130\text{ }\mu\text{M}$. The K_{Mg} value is similar to the values of $K_m(\text{Mg}^{2+})$ found by early investigators (see Kagawa & Wildman, 1970). However, K_c is lower than the values of the apparent $K_m(\text{CO}_2)$ initially measured for ribulose biphosphate carboxylase (200 – $400\text{ }\mu\text{M-CO}_2$; Weissbach *et al.*, 1956; Paulsen & Lane, 1966). This can be explained if the effect of ribulose biphosphate on the time-course is considered. As discussed above, ribulose biphosphate would force the equilibrium between the active and inactive forms of the enzyme towards the inactive form. This would be more significant at low rather than at high CO_2 concentrations and thus would increase the

measured $K_m(\text{CO}_2)$ value. This suggests that the $K_m(\text{CO}_2)$ would increase with increasing ribulose biphosphate concentration. This was observed by Laing (1974).

There is a discrepancy in Table 2, where the values of k_1 appear to be strongly dependent on the Mg^{2+} concentration. This is possibly due to a false assumption in the mathematics developed by Lorimer *et al.* (1976). They assumed that, when the preincubated enzyme is placed into the reaction mixture containing 20mM-Mg^{2+} , the high Mg^{2+} concentration drives the $\text{EC} \rightleftharpoons \text{ECM}$ equilibrium to the right. However, our scheme suggests that E, EC and ECM can all bind with ribulose biphosphate. If the $\text{EC} \rightleftharpoons \text{ECM}$ equilibrium is fast only with respect to the 30s of assay, but not with respect to the binding of ribulose biphosphate, then the high concentrations of ribulose biphosphate in the assay would bind much of the EC complex and consequently slow the Mg^{2+} -activation step, perhaps to a significant extent. This would result in the 30s assay not measuring the sum of EC and ECM, as postulated by Lorimer *et al.* (1976), but measuring only part of the original EC and the total ECM. Use of lower ribulose biphosphate concentrations would be unsatisfactory, as it would permit further activation of E during the 30s assay. There is strong direct evidence that ribulose biphosphate influences the EC-to-ECM step. First, Table 1 shows that in the presence of ribulose biphosphate, but low concentrations of Mg^{2+} ($16\text{ }\mu\text{M}$), the time-course of ribulose biphosphate carboxylase is linear for at least 20s, whereas in the absence of ribulose biphosphate the enzyme is essentially inactive within 12s (Fig. 3). Further, Pon *et al.* (1963) show that enzyme preincubated with CO_2 , then with ribulose biphosphate and assayed by adding Mg^{2+} , showed much less activity than when the preincubation sequence was CO_2 , Mg^{2+} and assay initiated with ribulose biphosphate. Results similar to those of Pon *et al.* (1963) were also obtained by Chu & Bassham (1973).

Effect of NADPH and other cofactors

In Table 2 we show that NADPH causes a 30% decrease in the measured K_c or dissociation constant of the reaction $\text{E} + \text{C} = \text{EC}$. Chu & Bassham (1973, 1974, 1975) have shown that various sugar phosphates and NADPH could activate the enzyme under certain circumstances and decrease the apparent $K_m(\text{CO}_2)$. They showed that preincubation of the enzyme with CO_2 , Mg^{2+} and sugar phosphate or NADPH, and assay by initiation with ribulose biphosphate, resulted in a considerable stimulation of activity compared with enzyme preincubated with CO_2 and Mg^{2+} but no cofactor. However, when the assay of the activated enzyme was initiated with ribulose biphosphate plus sugar phosphate, inhibition was

observed. NADPH did not inhibit. They showed that the activation by sugar phosphate and NADPH was a slow process, taking several minutes to reach completion, and that the affinity of the enzyme for the cofactors at the activating site was much greater than the inhibition constant observed by using activated enzyme. By using our observation that NADPH decreases the value of K_e , we can explain their data by postulating that: (1) sugar phosphate or NADPH can bind to the free inactive enzyme and increase the value of k_1 (site 1) in a slow reaction, thus decreasing the apparent dissociation constant for CO_2 ; (2) sugar phosphate additionally binds competitively with ribulose biphosphate (site 2) in a fast reaction. The affinity for sugar phosphate is greater at the first site than at the second site. Binding at the first site allows the enzyme to be activated faster and to a greater extent when the cofactor is present during the preincubation with CO_2 and Mg^{2+} . However, when inactive enzyme is assayed in the presence of cofactor without preincubation there is no stimulation of activity, as the binding of the cofactor to the enzyme at the first allosteric site is slow, and the ribulose biphosphate has already bound most of the enzyme into a form which can no longer bind the cofactor. When the cofactor is a sugar phosphate, it also binds at the ribulose biphosphate site of the active enzyme and inhibits the catalytic process in a classical competitive manner. Chu & Bassham (1974) also showed that high concentrations of sugar phosphate can inhibit the enzyme even when the enzyme is preincubated with CO_2 , Mg^{2+} and sugar phosphate. We suggest that the sugar phosphate can compete with ribulose biphosphate for the binding site of ribulose biphosphate on the inactive enzyme, and at high concentrations of sugar phosphate sufficient sugar phosphate is bound at the ribulose biphosphate site to lower the concentration of free inactive enzyme and thus decrease the rate of activation and inhibit activity. In this case the sugar phosphate is also directly competing with ribulose biphosphate for the active enzyme and inhibiting the reaction. This suggests that the inhibition of activity by high concentrations of sugar phosphate would be a time-dependent process, less inhibition occurring with longer preincubation time.

The present paper has proposed a model of ribulose biphosphate carboxylase which explains both the activation and catalytic processes and the effect of certain cofactors. Preliminary studies using numerical techniques (Bates & Frieden, 1973) and non-linear-parameter estimation (Bard, 1974) have qualitatively supported this model.

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References

- Anderson, L. E. & McClure, W. O. (1973) *Anal. Biochem.* **51**, 173-179
- Bahr, J. T. & Jensen, R. G. (1974) *Plant Physiol.* **53**, 39-44
- Bard, Y. (1974) *Non-Linear Parameter Estimation*, Academic Press, New York
- Bassham, J. A., Sharp, P. & Morris, L. (1968) *Biochim. Biophys. Acta* **153**, 898-900
- Bates, D. J. & Frieden, C. (1973) *Comp. Biomed. Res.* **6**, 474-486
- Bowes, G., Ogren, W. L. & Hageman, R. H. (1971) *Biochem. Biophys. Res. Commun.* **45**, 716-722
- Buchanan, B. B. & Schurmann, P. (1973) *J. Biol. Chem.* **248**, 4956-4964
- Christeller, J. T., Laing, W. A. & Troughton, J. H. (1976) *Plant Physiol.* **57**, 580-582
- Chu, D. K. & Bassham, J. A. (1973) *Plant Physiol.* **52**, 373-379
- Chu, D. K. & Bassham, J. A. (1974) *Plant Physiol.* **54**, 556-559
- Chu, D. K. & Bassham, J. A. (1975) *Plant Physiol.* **55**, 720-726
- Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67**, 104-137
- Fromm, H. J. (1967) *Biochim. Biophys. Acta* **139**, 221-230
- Goldsworthy, A. (1968) *Nature (London)* **217**, 62
- Hodgeman, C. D., Weast, R. C. & Selby, S. M. (eds.) (1958) *Handbook of Chemistry and Physics*, 40th edn., Chemical Rubber Publishing Co., Cleveland, OH
- Horrecker, B. L., Hurwitz, J. & Weissbach, A. (1958) *Biochem. Prep.* **6**, 83-90
- Jensen, R. G. & Bassham, J. A. (1966) *Proc. Natl. Acad. Sci. U.S.A.* **56**, 1095-1101
- Kagawa, N. & Wildman, S. G. (1970) *Annu. Rev. Plant Physiol.* **21**, 325-358
- Laing, W. A. (1974) Ph.D. Thesis, University of Illinois, Urbana-Champaign
- Laing, W. A., Ogren, W. L. & Hageman, R. H. (1975) *Biochemistry* **14**, 2269-2275
- Lorimer, G. H., Badger, M. R. & Andrews, T. J. (1976) *Biochemistry* **15**, 529-536
- Paulsen, J. M. & Lane, M. D. (1966) *Biochemistry* **5**, 2350-2357
- Pon, N. G., Rabin, P. R. & Calvin, M. (1963) *Biochem. Z.* **338**, 7-19
- Rabin, B. R. & Trown, P. W. (1964) *Nature (London)* **202**, 1290-1293
- Sugiyama, T., Nakayama, N. & Akazawa, T. (1968) *Biochem. Biophys. Res. Commun.* **30**, 118-123
- Tolbert, N. E. (1971) *Annu. Rev. Plant Physiol.* **22**, 45-74
- Walker, D. A. (1973) *New Phytol.* **72**, 209-235
- Weissbach, A., Horecker, B. L. & Hurwitz, J. (1956) *J. Biol. Chem.* **218**, 795-810
- Wishnick, M., Lane, M. D. & Scrutton, M. C. (1970) *J. Biol. Chem.* **245**, 4939-4947