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The Reactions between Active and Inactive Forms of Wheat Ribulosebisphosphate Carboxylase and Effectors

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The processes of activation and deactivation of ribulose-1,5-bisphosphate carboxylase purified from wheat have been investigated. Two forms of the enzyme are indistinguishable in terms of ribulose-1,5-bisphosphate carboxylation and oxidation but exhibit different rates of activation. One form is slowly activated in saturating CO_2 and Mg^{2+} at moderate temperatures ($t_{0.5} \approx 120$ min at 25 °C), the other form rapidly activated ($t_{0.5} \approx 8$ s).

In the presence of the effectors 6-phosphogluconate or NADPH, significantly lower concentrations of the activating co-factors can achieve full activation of both enzyme species. However, with another effector, fructose 1,6-bisphosphate, for the slowly activating species the mode of action is the same as with 6-phosphogluconate or NADPH, whereas the activation of the rapidly activating species is significantly inhibited. The substrate, ribulose 1,5-bisphosphate, also inhibits this rapid activation process.

A mechanism is proposed for the reactions involving activation that accounts for the differential rates of activation and the response to effectors.

Ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) catalyses two competing reactions, the carboxylation of ribulose-P_2 to two molecules of 3-phosphoglycerate and, in the presence of molecular oxygen, the oxidation of ribulose-P_2 to 2-phosphoglycolate and 3-phosphoglycerate. The two catalytic reactions are considered to involve the same site on the enzyme, because they exhibit a similar response to activating co-factors, and certain effectors and inhibitors. The enzyme requires activation with CO_2 and Mg^{2+} ion both for full carboxylase and oxygenase activities.

Activation of the enzyme is an ordered and reversible process involving, firstly, the binding of a CO_2 molecule to an ε-amino group of a lysine residue on the enzyme and, then, addition of the Mg^{2+} to the enzyme-CO_2 complex [1]. The activating CO_2 molecule is thus quite distinct from the substrate CO_2 consumed during the carboxylase reaction [2]. Details of the involvement of the activating CO_2 and Mg^{2+} ion in the catalytic reactions are lacking. However, most recent proposals suggest that the co-factors bind within the active-site region of the enzyme [3].

The activation of the carboxylase from a number of sources has been studied [4–6] particularly the spinach enzyme with, most recently, details of the effects of sugar phosphates on the activation process [7,8]. In this report we give details of the activation of the carboxylase purified from wheat leaves. Previous work [9] has shown that two forms of the wheat enzyme can exist that exhibit different activation kinetics. One is a slowly activating species requiring prolonged incubation in CO_2 and Mg^{2+}, the other is a rapidly activating form similar to the carboxylases from other sources. In the present work, we have investigated both forms of the wheat enzyme and in particular the effects of sugar phosphates and inhibitors on the reactions of the enzymes and activating co-factors.

Abbreviations. Ribulose-P_2, ribulose 1,5-bisphosphate; fructose-P_2, fructose 1,6-bisphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

Enzyme. Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39).

MATERIALS AND METHODS

Materials

Ribulose-P_2 (≈ 98% pure), 6-phosphogluconate and other sugar phosphates were obtained as the sodium salts from Sigma Ltd (Poole, UK), as were the various nicotinamide derivatives and zwitterionic buffers. All other materials were the best grade commercially available.

Extraction and Purification of the Carboxylase

Step 1. Seedlings of *Triticum aestivum* (var. Maris Dove) were grown for three weeks in a glasshouse, with supplementary lighting, when required, to give a 16-h day. The third and fourth leaves to emerge were removed and finely chopped into a 20 mM Tris/Cl buffer (pH 8.0) containing 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 1.0 mM EDTA, 1 mM dithiothreitol and 0.002% (w/v) chlorhexidine diacetate (Hibitane from ICI Ltd, Macclesfield, Cheshire) with 1% (w/v) insoluble polyvinylpolypyrrolidone (Sigma Ltd, UK) in a ratio of 1 ml buffer: 7 g tissue fresh weight. The leaves were homogenized for four 15-s periods (with 15-s intervals) at top speed with a Waring blender (MSE Automix). The homogenate was filtered through two layers of muslin and the filtrate containing the carboxylase made 35% saturated with solid $(NH_4)_2SO_4$ and centrifuged at $20000 \times g_{av}$ for 15 min (MSE HS21).

Step 2. The precipitate was discarded and the carboxylase precipitated from the supernatant with 55% saturated $(NH_4)_2SO_4$ and collected as a pellet after a further centrifugation at $20000 \times g_{av}$ for 15 min. The pellet was dissolved in a 20 mM

Tris/Cl (pH 8.0) resuspension buffer containing 1 mM dithiothreitol, 1 mM EDTA, 1 mM $MgCl_2$ and 0.002% Hibitane and insoluble material removed by centrifugation at $77000 \times g_{av}$ for 30 min.

Step 3. 4-ml portions of the supernatant were layered onto linear sucrose gradients (8–25% w/v in 32 ml of the resuspension buffer) and centrifuged at $215000 \times g_{av}$ (Beckman L2-65B) for 2.5 h using a 60 Ti rotor.

Step 4. The fractions from the eight gradients containing the carboxylase were combined and loaded onto a DEAE-Sephacel column (25 × 360 mm) equilibrated with resuspension buffer. The protein was eluted from the column with a linear NaCl gradient (0–0.5 M). Those protein fractions exhibiting ribulose-P_2 carboxylase activity were combined and desalted on a column of Sephadex G-25 (fine, 50 × 310 mm) equilibrated with a 5 mM Hepes buffer pH 8.0. The protein eluting at the void volume of the column was collected, shell-frozen and freeze-dried. The enzyme as a freeze-dried powder was stored over dessicant at 5 °C and in this form remained stable for several months. From 100 g of leaf material, this procedure consistently produced 200 mg of purified protein exhibiting a carboxylase activity of 1.0–1.2 μmol CO_2 fixed min^{-1} (mg protein)$^{-1}$ and oxygenase activities in aerated solutions of 0.17–0.22 μmol O_2 min^{-1} mg^{-1}, at pH 8.2 and 25 °C.

A number of preparations were subjected to gel electrophoresis on polyacrylamide gradient gels (4–20%) using Tris/glycine electrode buffers (50 mM Tris, pH 8.3) and a 0.375 M Tris/Cl gel buffer. After staining the gel with Coomassie blue and destaining [14] the enzyme was judged to be at least 96% pure and the molecular weight of 560000 consistent with that of carboxylases from higher plants [15]. Electrophoresis of the purified enzyme as denatured samples on 16% polyacrylamide gel containing 0.1% sodium dodecyl sulphate [16] indicated only two protein bands corresponding to the large (M_r 56000) and small (M_r 14500) subunits of the enzyme.

Activation and Assay of the Carboxylase

For routine measurements of the catalytic activities of the purified enzyme from freeze-dried powders, powder was dissolved directly into a 0.1 M Hepes activation buffer pH 8.2 containing 10 mM $NaHCO_3$ and 20 mM $MgCl_2$, without any detectable loss of activity or protein (see [9]). Different temperatures were employed for activating the enzyme (see Results) but at 40 °C maximum carboxylase and oxygenase activities were achieved within 40 min (160 min at 30 °C). The enzyme could then be stored at 25 °C without a significant decrease in activity for at least 4 h.

CO_2 and O_2 are inhibitors of the oxygenase and carboxylase activities of the enzyme, respectively; therefore CO_2-free or O_2-free solutions were used where appropriate. For both assays a stoppered oxygen electrode vessel (Hansatech Ltd, Kings Lynn, UK) was used, which for carboxylase measurements allowed contaminating oxygen to be maintained at a minimum and constant amount (≈ 0.02 mM) before each assay; also there was no large gas space above the solution. Thus the need for equilibration of the CO_2 in the assay solution with any gas space [10] before initiating the reaction was avoided.

Generally, and unless otherwise stated, the carboxylase activity was measured in a 0.1 M O_2-free Bicine buffer, pH 8.2, containing 10 mM $NaH^{14}CO_3$ (1 Ci/mol), 20 mM $MgCl_2$ and 0.6 mM ribulose-P_2 in a 1.0-ml volume at 25 °C. To ensure adequate conversion of HCO_3^- to CO_2 during the assays, carbonic anhydrase (10 μg/ml final concentration) was included [10]. The reaction was stopped after 60 s by addition of 0.1 ml 2 M formic acid and half the contents (0.55 ml) removed for drying in an oven at 60 °C. The carboxylase activity was calculated from the amount of $^{14}CO_2$ fixed into acid-stable products as previously described [7, 11].

The procedure for measuring oxygenase activities was to follow the initial rate of O_2 consumption with the oxygen electrode at 25 °C as the enzyme oxidized the ribulose-P_2 substrate. The Bicine buffer (0.1 M, pH 8.2) was equilibrated with CO_2-free air (BOC special gases) before use and contained 20 mM $MgCl_2$. Unless stated otherwise the reaction was started by adding the activated carboxylase to the assay solution containing 0.6 mM ribulose-P_2 in a final volume of 1.0 ml.

Where combined carboxylase and oxygenase activities were measured, the procedure for performing the oxygenase assay was used, but 2 mM $H^{14}CO_3^-$ (1 Ci/mol) was included in the aerated buffer. The reaction was stopped after 60 s as explained for the carboxylase measurements and the contents of the vessel treated likewise.

Where enzyme was used that had been activated at elevated temperatures, the sample for assay was allowed to equilibrate at 25 °C before initiating the reaction.

Activity measurements of the enzyme in crude extracts of wheat leaves used aliquots of the filtrate from step 1 of the extraction procedure after centrifugation at $100000 \times g_{av}$ for 30 min to remove insoluble debris.

Activation with $^{14}CO_2$

In experiments involving activation of the enzyme, with $^{14}CO_2$, the specific activity of the bicarbonate was 1.0 Ci/mol. The enzyme (10 mg/ml) was freed of excess and loosely bound radioactivity by addition of a large excess of non-radioactive HCO_3^- (100 mM) and then 0.5-ml aliquots rapidly gel-filtered on short columns (4.0 ml bed vol.) of Sephadex G-25 fine, using centrifugation at 2000 rev./min for 2 min [12]. The Sephadex was equilibrated with buffer containing 100 mM $NaHCO_3$ before use.

RESULTS

Solutions of the purified wheat carboxylase stored at 5 °C, or freeze-dried powders prepared from these solutions, required prolonged incubation in HCO_3^- and Mg^{2+} (10 mM and 20 mM respectively) at 25 °C and pH 8.2 to attain maximum carboxylase or oxygenase activity. Incubation of this slowly activating form of the enzyme at higher temperatures increased the rate of activation significantly, as shown in Fig. 1. At 40 °C, for example, the rate of activation is eight-times faster than at 25 °C (Fig. 1a and b respectively). We have investigated the effect of incubating the enzyme alone at elevated temperatures to determine if heating is required for the carboxylation reaction or the association with magnesium ions. Fig. 1d shows that the enzyme, when preincubated at 40 °C for 30 min, rapidly activates (within 10 s) with CO_2 and Mg^{2+} (in saturating conditions) to achieve high levels of carboxylase and oxygenase activity. However, this method produces only 70–80% of the maximum catalytic activity expected normally (cf. curve a) and further heating at 40 °C in the presence of CO_2 and Mg^{2+} is required before higher specific activities are achieved. Thus in those experiments involving the slowly activating species only, the method

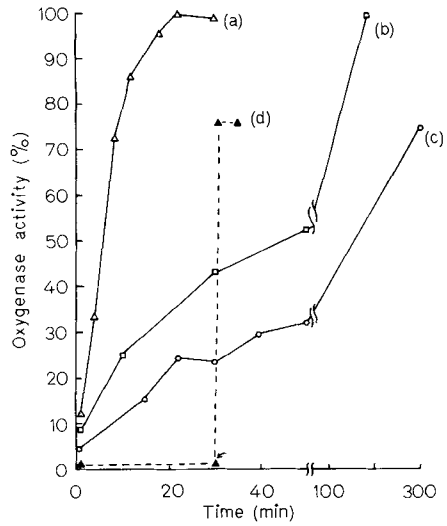


Fig. 1. *The time course of activation of the slowly activating species of the carboxylase at different temperatures.* The carboxylase (5 mg/ml) was incubated at (a) 40 °C, (b) 30 °C and (c) 25 °C in the presence of excess HCO_3^- and Mg^{2+} (10 mM and 20 mM, respectively) and the increase in oxygenase activity of the enzyme recorded at the indicated times by removing 25-µl aliquots for oxygenase assays. The effect of preincubating the enzyme at 40 °C before the addition of excess HCO_3 and Mg^{2+} (arrowed) is shown in (d). 100% corresponds to an oxygenase specific activity of 0.18 µmol O_2 min^{-1} mg^{-1}

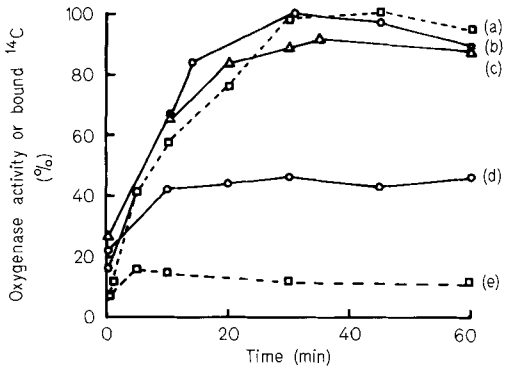


Fig. 2. *Activation of the carboxylase in low CO_2 concentrations.* The increase of the oxygenase activity (b – d) or binding of $^{14}CO_2$ (a and e) to the carboxylase incubated at 40 °C with (a) 1 mM $H^{14}CO_3^-$ plus 1 mM fructose-P_2, (b) 1 mM HCO_3^- plus 1 mM fructose-P_2, (c) 10 mM HCO_3^-, (d) 1 mM HCO_3^-, (e) 1 mM $H^{14}CO_3^-$, was recorded at different times. Curve (a) shows the concomitant increase in the amount of $^{14}CO_2$ bound to the enzyme in the presence of fructose-P_2 during this process compared to (e) the amount bound when activated in the absence of the effector. All of the activation reactions included 20 mM Mg^{2+} in the 0.1 M Hepes buffer pH 8.2. Where $^{14}CO_2$ and fructose-P_2 have been used for activation, the Sephadex G-25 column was equilibrated with 1 mM fructose-P_2 included in the buffer (see Materials and Methods). 100% corresponds to an oxygenase specific activity of 0.18 µmol O_2 min^{-1} mg^{-1}

$$E + C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EC + M \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ECM$$

Scheme 1. *The ordered and reversible activation of ribulose-P_2 carboxylase (E) by CO_2 (C) and Mg^{2+} (M)*
The ternary complex of the enzyme, ECM, is the only species that is catalytically active

adopted was to activate the enzyme with CO_2 and Mg^{2+} present throughout.

The Rate of Activation in the Presence of Effectors

The mechanism of activation of ribulose-P_2 carboxylases has been proposed [4,5] as the ordered reversible binding of CO_2 and then Mg^{2+} as shown in Scheme 1.

If activation is performed in the presence of low concentrations of certain inhibitors or 'effectors', e.g. fructose-P_2, significantly less CO_2 and Mg^{2+} are required for full activation. Fig. 2 illustrates this effect by comparing the degree of activation of the enzyme with different HCO_3^- concentrations in the presence of 0.5 mM fructose-P_2. The same concentration of 6-phosphogluconate and NADPH was found to produce the same effect. Activation in the presence of effectors did not increase the rate of activation of the enzyme above that found with saturating HCO_3^- and Mg^{2+} (i.e. 10 mM and 20 mM respectively) either at 25 °C or 40 °C. However, in sub-optimum HCO_3^- concentrations, e.g. 1 mM, the rate of activation and amount of carboxylase and oxygenase activity achieved was the same as in saturating HCO_3^-. Thus $t_{0.5}$ for activation at 25 °C or 40 °C was 120 min or 15 min respectively, when in 10 mM HCO_3^- or with sub-optimum HCO_3^- and effectors. The reason for full activation with low CO_2 and Mg^{2+} concentration is presumably due to the effectors binding to the active ternary complex (ECM) of the enzyme and hence trapping the activating CO_2 and Mg^{2+} [7,8]. To confirm this, activation was performed at 40 °C with $H^{14}CO_3^-$ in the presence of fructose-P_2 and the amount of $^{14}CO_2$ determined after non-specifically bound CO_2 had been removed by gel filtration in excess HCO_3 (see Materials and Methods). A comparison of curves (a) and (b) with (e) in Fig. 2 shows that indeed the sugar bisphosphate does trap increasing amounts of CO_2 as the enzyme progressively becomes activated. Assuming that each active protomer of the carboxylase has a molecular mass of about 70 kDa, i.e. eight functional active centres/560-kDa enzyme molecule, then the amount of radioactivity bound at maximum activation corresponds to $\approx$ 0.5 mol $^{14}CO_2$ bound/mol of protomer. This compares favourably with 0.75 mol and 0.13 mol bound when 6-phosphogluconate or NADPH (respectively) were used as effectors with the spinach carboxylase [7].

Deactivation of the Active Enzyme

When the fully activated carboxylase is transferred to a solution containing no Mg^{2+} ions or HCO_3, there is a rapid loss of both the carboxylase and oxygenase activities. The time course of this deactivation can be seen in Fig. 3 where activated enzyme has been transferred to an oxygen electrode vessel containing a CO_2-free buffer and then ribulose-P_2 substrate added at different times. Even in the presence of 20 mM Mg^{2+}, $t_{0.5}$ for deactivation is 15 s. The residual activity remaining at t_∞ is that supported by the HCO_3 and Mg^{2+} carryover from the activation mixture (usually 0.25 mM and 0.5 mM respectively). However, if the CO_2-free buffer in the vessel contains 6-phosphogluconate, then deactivation is significantly inhibited (Fig. 3a). If both Mg^{2+} and CO_2 are omitted from the buffer, i.e. those conditions giving most rapid loss of activity (Fig. 3f) the enzyme still remains fully activated in the presence of 6-phosphogluconate (Fig. 3b), due to the HCO_3 and Mg^{2+} transferred with the enzyme from the activation mixture. This is further evidence that effectors (fructose-P_2 and NADPH were equally as effective

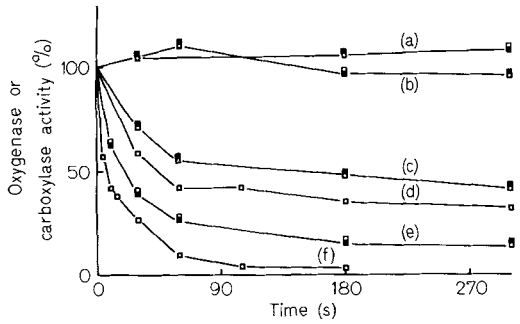


Fig. 3. *Deactivation of ribulose-*P_2 *carboxylase and oxygenase activities.* The carboxylase (5 mg/ml) was activated as explained in Materials and Methods in saturating CO_2 and Mg^{2+} concentrations and aliquots injected into the oxygen electrode vessel containing a 0.1 M Bicine buffer, pH 8.2 at 25 °C with (a) 20 mM $MgCl_2$, 0.5 mM 6-phosphogluconate, (b) 0.5 mM 6-phosphogluconate, (c, d) 20 mM $MgCl_2$ and (e, f) no Mg^{2+} or effector. Curve (d) shows the deactivation of the carboxylase in fresh leaf extracts. The loss of carboxylase and oxygenase activity of the enzyme was determined by introducing ribulose-P_2 (0.6 mM final concentration) at the indicated times. The data for (a–c, e) were determined with the combined assay method (see Materials and Methods) and (d, f) from oxygenase assays. In (e) $MgCl_2$ (20 mM) was introduced with the substrate. The 100% value was the inhibited maximum activity of the enzyme when in 0.5 mM 6-phosphogluconate or alternatively the maximum activity of the enzyme in those assays without the effector, i.e. 0.2 μmol O_2 min^{-1} mg^{-1} and 1.0 μmol CO_2 min^{-1} mg^{-1} oxygenase and carboxylase activity, respectively

as 6-phosphogluconate) maintain the enzyme in its activated state (ECM). A comparison of the inhibition of deactivation by the effectors is given in Table 1. Mg^{2+} is not very effective in maintaining the enzyme in the active form, when HCO_3^- is omitted from the buffers (Fig. 3d). Furthermore, molecules that are closely related to fructose-P_2 and NADPH, such as fructose 6-phosphate, fructose 1-phosphate, fructose, $NADP^+$, NADH or NAD^+ were unable to exhibit the same phenomena even at relatively high concentrations (2 mM). Deactivation of enzyme in freshly prepared crude extracts of wheat leaves, after activation from freeze-dried powders or from ammonium sulphate precipitates of the purified enzymes, was similar.

Reactivation of the Deactivated Enzyme

The deactivation phenomenon described above is a result of the dissociation of ECM to inactive EC and E (see Scheme 1), because addition of HCO_3 to E in the presence of Mg^{2+} or Mg^{2+} ion to E (in the presence of HCO_3) rapidly restores the oxygenase and carboxylase activities. Fig. 4d shows the rapid restoration of the carboxylase activity by addition of HCO_3^- after deactivation for 90 s in a CO_2-free buffer. The oxygenase activity (after correcting the measured rates for inhibition by 5 mM HCO_3^-) was found to respond likewise. Fig. 4c shows the reactivation of the enzyme by the addition of Mg^{2+} ion after 90 s of deactivation due to its absence. Most important, however, is that the rapid reactivation occurs at 25 °C, i.e. $t_{0.5}$ in 5 mM HCO_3^- and 20 mM Mg^{2+} is approximately 12 s.

The data of the type shown in Fig. 3 and 4 has been used to calculate some of the rate constants of the deactivation phenomenon and these are listed in Table 2 along with similar data for the carboxylase from other sources [5, 6].

When the reactivation process was investigated in the presence of effectors, significant differences in their mode of

Table 1. *The relative stabilising influence of different effectors on the active enzyme ternary complex ECM*

The apparent K_d is that concentration of effector producing a 50% decrease in the rate of deactivation of the active enzyme. Fructose-P_2 and 6-phosphogluconate are competitive inhibitors of ribulose-P_2 binding and the K_i values are shown for comparison. n.i. = no inhibition of enzyme activity detected over the range of effector concentrations used to study the inhibition of enzyme deactivation

Effector	Apparent	
	K_d	K_i
	μM	
NADPH	40	n.i.
Fructose-P_2	30	250
6-Phosphogluconate	10	80

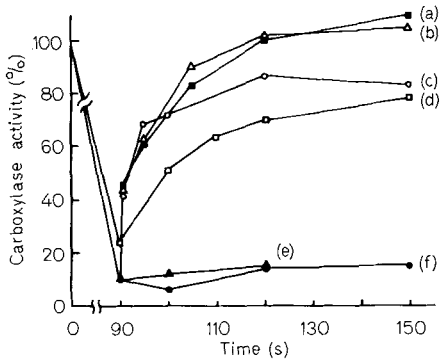


Fig. 4. *The restoration of ribulose-*P_2 *carboxylase activity by* CO_2 *and* Mg^{2+}. The carboxylase was deactivated for 90 s, as explained in the legend to Fig. 3, in a 0.1 M Bicine buffer pH 8.2 containing 5 mM $H^{14}CO_3$ without Mg^{2+}. Reactivation of the carboxylase activity of the enzyme was initiated by the addition of Mg^{2+} (as $MgCl_2$, final concentration 20 mM) in the presence of (a) 0.5 mM 6-phosphogluconate, (b) 0.5 mM NADPH, (c, d) no effector, (f) 0.5 mM fructose-P_2; the increase in carboxylase activity was determined at different times by initiating the reaction with substrate. In (e) 0.6 mM ribulose-P_2 was added about 1 s before addition of the Mg^{2+} and the amount of radioactivity incorporated into product determined by halting the reaction at the times indicated with acid (Materials and Methods). The value after 10 s is multiplied by 3 to conform to the other carboxylase measurements which were performed over 30 s. Curve (d) was obtained by deactivating the enzyme in buffer containing 20 mM Mg^{2+} but no HCO_3^- and initiating the reactivation with $H^{14}CO_3^-$ (5 mM); carboxylase activity was measured as for the other curves. Where an effector was present during activity measurements, the rates have been corrected for the inhibition it caused, e.g. 0.5 mM fructose-P_2 produces 25% inhibition

action were observed. NADPH and 6-phosphogluconate again exhibited binding to ECM by producing fully active enzyme in sub-optimum HCO_3 or Mg^{2+} concentrations (compare curves a and b with c in Fig. 4). In contrast, fructose-P_2 inhibited the rapid reactivation of deactivated enzyme by either co-factor. Furthermore, if ribulose-P_2 was introduced before either HCO_3 or Mg^{2+}, as with fructose-P_2 the process was dramatically inhibited.

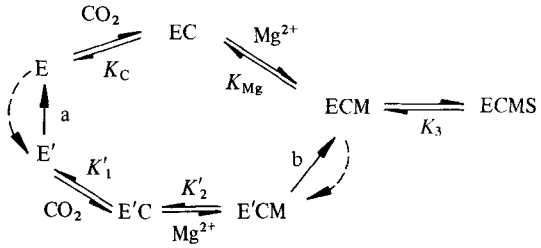
DISCUSSION

From the response of purified wheat carboxylase to effectors and activating co-factors it is clear that at least two inactive forms of the enzyme exist. The first requires prolonged incubation or elevated temperatures to attain full

Table 2. *Rate constants for the reaction with activating co-factors of ribulose-P_2 carboxylase from various species*
The rate constants for the wheat enzyme have been calculated as explained by Lorimer et al. [11] and Laing and Christeller [5] and based on the mechanism illustrated in Scheme 1. Only data for the rapidly activating form of the wheat enzyme can be determined (see Discussion). The numbers given in brackets refer to the concentration of Mg^{2+} (mM) used for activation. Data for *R. rubrum* was collected at 9 °C, see [6]. Data for soya bean was collected at 18 °C [5], for spinach at 10 °C [5,11]

Rate constant	*R.rubrum*	Soya bean	Spinach	Wheat
k_1 (M^{-1} s^{-1})	4.2	20.2 (1)	34.0 (1)	140 (5)
	−15.5	−118.5 (20)	−171.7 (20)	−1000 (20)
k_{-1} (s^{-1})	–	0.002 (1)	0.011 (1)	
				0.002[b]
	–	0.011 (20)	−0.053 (20)	
k_2 (M^{-1} s^{-1})	–	300	–	–
k_{-2} (s^{-1})	–	0.34	0.03[a]	0.09
$K_c K_{Mg}$ (μM^2)	4.6×10^5	1.4×10^5	1.6×10^5	2.4×10^5

[a] Data taken from [7] presumably at 25 °C.
[b] The ratio k_{-1}/k_2 (unit M) was calculated using the values for $K_c K_{Mg}$, k_1 and k_{-2} shown in the table. k_1 and $K_c K_{Mg}$ were measured from a plot of CO_2 concentration vs k_{obs} for activation [5,11]. Only those values determined for activation in 20 mM Mg^{2+} have been used (see Discussion).



Scheme 2. *The mechanism of activation with CO_2 and Mg^{2+} of both the slowly (E') and rapidly (E) activating carboxylase and the interaction with substrate or effectors (S)*
ECM, the ternary complex of enzyme-CO_2-Mg^{2+}, is the only active form of the enzyme although EC and/or E bind substrate and fructose-P_2. The processes (a) and (b) refer to the interconversion of the two enzyme forms in the absence and presence of activating cofactors, respectively. The full arrow in (a) refers to the data at 40 °C that E' can convert to E, and the dashed arrow that E in the absence of cofactors and at 25 °C or below reverts to E' (see Discussion). It is assumed that E can also revert to E' in the presence of activators (dashed arrow in b) although data is only available for 0 °C and indicates that the rate is extremely slow [9]

carboxylase and oxygenase activities. Presumably, this is similar to the thermolabile form that has been reported for other ribulose-P_2 carboxylases, e.g. the enzyme from tobacco [13]. The second of the two enzyme forms is quite distinct exhibiting a 900-fold more rapid rate of activation at moderate (25 °C) temperatures in HCO_3 and Mg^{2+} ions. An activation scheme that incorporates both species of the wheat enzyme is shown in Scheme 2.

In this scheme, although the majority of the slowly activating species E' can be converted to E by incubation at elevated temperature, before activation with CO_2 and Mg^{2+}, the equilibria K_1' and K_2' have been retained because E' may still be able to form reversible binary and ternary complexes with CO_2 and Mg^{2+}. Furthermore, up to 30% of this form of the enzyme apparently requires the presence of CO_2 and Mg^{2+} during incubation to achieve maximum conversion to ECM.

The scheme also takes account of the reversion of E to E' previously reported for the carboxylase from wheat [9]. The rate of reversion as evidenced by the progressive loss of E, i.e. the enzyme species exhibiting rapid activation, depends on the conditions in which E is stored. For example, in solutions almost free of activating co-factors and without effectors, Mächler et al. [9] found $t_{0.5}$ at 20 °C to be about 120 min and we estimate for our particular carboxylase preparations in the absence of co-factors a $t_{0.5}$ at 25 °C of about 200 min.

The interaction of the two enzyme forms and the binary and ternary complexes with effectors indicates a possible difference between the two species. For example, the activation of E' in the presence of fructose-P_2 is not inhibited but, in fact, is enhanced both in rate and amount in sub-optimum concentrations of HCO_3^- and Mg^{2+} due to binding to ECM. Indeed the rate of effector-mediated activation including fructose-P_2 equals the rate achieved with saturating HCO_3^-. In contrast, the initial rate of activation of E by CO_2 and Mg^{2+} is not enhanced by 6-phosphogluconate or NADPH but is the same as that obtained with the HCO_3^- concentration employed. It is the final quantity of active enzyme (ECM) formed that increases. Furthermore, fructose-P_2 significantly inhibits the rapid activation of E.

The simplest explanation of these results is that E' is unable to bind fructose-P_2 and presumably must form a binding site via a conformational change of the protein structure. It is this step that limits the rate of activation of E'. In E the binding site is intact and thus the reaction with activating CO_2 is the rate-limiting reaction. Fructose-P_2 can bind to E and presumably EC, inhibiting the CO_2 or Mg^{2+} 'on' process. The substrate ribulose-P_2 clearly mimics this inhibitory effect, indicating that the region concerned is at the active site of the enzyme.

The rapidly activating species, E, corresponds closely to spinach and other carboxylases which require only a short period of exposure to CO_2 and Mg^{2+} ions to become fully activated [4,5]. The rate constants of the activation processes for the wheat enzyme are apparently different, although a direct comparison is complicated because of the different temperatures employed to study the phenomenon with other species. Furthermore, we found that applying the equations given by Lorimer et al. [4] to the activation time course data for the wheat enzyme produced values for k_1 and k_{-1} (see Scheme 1 and Table 2) that were dependent on the Mg^{2+} concentration used for the activation. This problem has been encountered with data for other ribulose-P_2 carboxylase species and has been discussed at some length (see e.g. [6]). Two factors that may contribute significantly to the Mg^{2+} dependency of the two rate constants are, firstly, that in moderate concentrations of HCO_3^-, e.g. 5 mM (59 μM CO_2 at pH 8.2), a significant amount of the enzyme may not be carboxylated. Thus on the addition of Mg^{2+}, a fraction of the enzyme has to become carboxylated before it can associate with the metal to form ECM. It is not clear whether Mg^{2+} simply favours ECM formation by trapping EC, or if there is a concomitant change in the reactivity of the lysine residue [1] toward CO_2 in the presence of Mg^{2+}. Secondly, sugar bisphosphates, such as fructose and ribulose, can effectively inhibit rapid association of the inactive enzyme with CO_2 and Mg^{2+}. Thus the oxygenase or carboxylase activities used to follow the progress of activation are not a measure of E + EC + ECM but only ECM, invalidating one of the

assumptions used previously for deriving the activation equations [4]. Unfortunately, the other states of the enzyme, i.e. E and EC, cannot be monitored directly, and therefore complete details of the equilibria amongst the various enzyme forms cannot be determined with accuracy.

The fact that sugar bisphosphates, especially the substrate ribulose-P_2, trap the activating co-factors CO_2 and Mg^{2+} tightly explains apparently contradictory observations. For example, very low magnesium concentrations can support full activity of the enzyme [5] and there is no significant loss of oxygenase activity in the absence of inhibitory CO_2, i.e. via dissociation of activating CO_2. In both cases it is clear that if all potentially functional active sites are carboxylated and bind Mg^{2+}, exposure to substrate or effectors such as 6-phosphogluconate or fructose-P_2 in conditions of low CO_2 and Mg^{2+} concentrations prevent the dissociation of the co-factors. During substrate carboxylation or oxidation, the rates of exchange of the co-factors are extremely low relative to the turnover rate, i.e. they are effectively 'irreversibly' bound.

Fructose, fructose 6-phosphate and fructose 1-phosphate are ineffective for activating the enzyme in low CO_2 concentrations or preventing the deactivation of the fully active enzymes, suggesting that the presence of two phosphate groups on the sugar is necessary for binding. Furthermore if 6-phosphogluconate and NADPH do bind to intermediate forms of the non-active enzyme, e.g. E and EC, they do so in such a way as not to interfere with activation. It is possible that both effectors require the presence of the activating Mg^{2+} ion before binding tightly.

Finally, one further significant difference between the effectors studied here is that fructose-P_2 and 6-phosphogluconate (as for other ribulose-P_2 carboxylases) are potent inhibitors of the oxygenase and carboxylase activities of the wheat enzyme, indicating their interference in catalysis. NADPH, however, did not significantly inhibit the wheat enzyme at those concentrations where it effectively prevented dissociation of activating CO_2 and Mg^{2+}. Thus, at least in the case of NADPH, its potency of inhibition is not a measure of its effectiveness in maintaining the active enzyme form. Presumably this indicates that NADPH readily dissociates in the presence of substrate to allow catalysis to proceed.

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