The Activation of Ribulose-1,5-bisphosphate Carboxylase by Carbon Dioxide and Magnesium Ions. Equilibria, Kinetics, a Suggested Mechanism, and Physiological Implications†

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ABSTRACT: Ribulose-1,5-bisphosphate carboxylase was activated by incubation with CO_2 and Mg^{2+}, and inactivated upon removal of CO_2 and Mg^{2+} by gel filtration. The activation process involved CO_2 rather than HCO_3^-. The activity of the enzyme was dependent upon the preincubation concentrations of CO_2 and Mg^{2+} and upon the preincubation pH, indicating that activation involved the reversible formation of an equilibrium complex of enzyme–CO_2–Mg. The initial rate of activation was linearly dependent upon the CO_2 concentration but independent of the Mg^{2+} concentration. Kinetic analyses indicated that the enzyme reacted first with CO_2 in a rate-determining and reversible step, followed by a rapid reaction with Mg^{2+} to form an active ternary complex (see eq 1 in text). The pseudo-first-order rate constant, k_{obsd}, for the activation process at constant pH was derived: $k_{obsd} = k_1[CO_2] + (k_2k_4/k_3[Mg^{2+}])$. Experimentally, k_{obsd} was shown to be linearly dependent upon the CO_2 concentration and inversely dependent upon the Mg^{2+} concentration. The activity of the enzyme after preincubation to equilibrium at constant concentrations of CO_2 and Mg^{2+} increased as the preincubation pH was raised, indicating that CO_2 reacted with an enzyme group whose pK was distinctly alkaline. It is proposed that the activation of ribulose-1,5-bisphosphate carboxylase involves the formation of a carbamate.

Ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) catalyses the carboxylation of RuBP[1] yielding two molecules of 3-phosphoglycerate. The enzyme can also act as an oxygenase, catalyzing the oxygenative cleavage of RuBP to yield one molecule each of 2-phosphoglycolate and 3-phosphoglycerate (Bowes et al., 1971; Andrews et al., 1973). One atom of molecular oxygen is incorporated into the carboxyl group of 2-phosphoglycolate during the oxygenase reaction (Lorimer et al., 1973). Oxygen inhibits the carboxylase reaction competitively with respect to CO_2 (Bowes and Ogren, 1972; Badger and Andrews, 1974) while CO_2 inhibits the oxygenase reaction competitively with respect to oxygen (Badger and Andrews, 1974).

It is known that the order of addition of the reagents to the ribulose-1,5-bisphosphate carboxylase assay affects the time-course of the subsequent fixation reaction (Pon et al., 1963). If the enzyme is first incubated with CO_2 and Mg^{2+}, and the reaction started by the addition of RuBP, the formation of 3-phosphoglycerate begins without a discernible lag. In contrast, when the enzyme is used to initiate the reaction, the fixation of CO_2 occurs only after a lag (Paulsen and Lane, 1966; Andrews and Hatch, 1971; Murai and Akazawa, 1972; Chu and Bassham, 1973). These results suggest that the enzyme is activated by CO_2 and Mg^{2+}.

Besides these order of addition effects are the unexplained kinetic responses to pH and Mg^{2+}. For example, increasing the pH reduces the observed Michaelis constants for both CO_2 (Lyttleton, 1973) and Mg^{2+} (Sugiyama et al., 1968a,b), while increasing the Mg^{2+} concentration reduces the observed $K_m[CO_2]$ (Bassham et al., 1968; Sugiyama et al., 1968a). The formation of a ternary complex of enzyme–CO_2–Mg (or Mn) may be inferred from such results. Such a complex has been physically (as opposed to kinetically) demonstrated (Akoyunoglou and Calvin, 1963a; Miziorko and Mildvan, 1974). However, the formation of such a complex has been related to the catalytic rather than the activation reaction.

Until recently the observed $K_m[CO_2]$ for the carboxylase reaction was thought to be at least an order of magnitude too high to account for the observed rates of photosynthesis in air (for a review, see Walker, 1973). Interest was therefore aroused by reports of a form of the carboxylase with a sufficiently low $K_m[CO_2]$ (Bahr and Jensen, 1974; Badger and Andrews, 1974). However, more detailed kinetic analyses revealed that this phenomenon is merely another facet of the CO_2 and Mg^{2+} induced activation (Laing et al., 1975; Andrews et al., 1975). Failure to consider this activation process has resulted in erroneously high values for the $K_m[CO_2]$ being recorded.

In addition to the effects elicited by CO_2 and Mg^{2+}, a number of sugar phosphates have been reported to modify the kinetic properties of the carboxylase (Chu and Bassham, 1972, 1973, 1974, 1975; Tabita and McFadden, 1972; Buchanan and Schürmann, 1973). But here too there seems to be an order of addition effect (Chu and Bassham, 1973, 1975).

Recognizing the double role of CO_2 and Mg^{2+}, in activating RuBP carboxylase and also as substrate and cofactor in the catalytic reaction, we felt it necessary to separate the kinetics of the activation process from those of the catalytic reaction. In this paper we report the results of experiments designed to elucidate the mechanism of activation.

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[1] Abbreviations used are: RuBP, D-ribulose 1,5-bisphosphate; EDTA, ethylenediaminetetraacetic acid; ammediol, 2-amino-2-methylpropane-1,3-diol; bicine, *N,N*-bis(2-hydroxyethyl)glycine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Table I: Requirements for the Activation of Ribulosebisphosphate Carboxylase.

Preincubation Treatment[a]	Activity[b] (nmol min^{-1})	%
Complete	5.14	100
Mg^{2+} alone, no CO_2 added[c]	0.90	18
CO_2 alone, no Mg^{2+} added	1.32	26
No Mg^{2+}, no CO_2 added[c]	0.06	1

[a] Preincubation was for 30 min at 10°. The complete preincubation mixture contained in a volume of 24 µl: 34 µg RuBP carboxylase in 0.05 *M* Tris-HCl (pH (10°) 8.2), 1 m*M* dithiothreitol, 16.7 m*M* $NaHCO_3$ (equivalent to 0.3 m*M* CO_2), and 20 m*M* $MgCl_2$. [b] Activity was determined at 10° with 1 m*M* $NaHCO_3$ (pH 8.2)–15 m*M* $MgCl_2$ as described in the Methods section. [c] We estimate the endogenous contaminating CO_2 to be about 2 µ*M*.

Experimental Procedure

Materials

Spinach (*Spinacea oleracea* L., hybrid 102) was grown as previously described (Andrews et al., 1975). Tapered 1.0-ml screw-capped Reactivials were obtained from Regis Chemical Co., Morton Grove, Ill. The vials were equipped with septa of silicone rubber (50 durometer hardness) supplied by Merco Rubber Co. Arncliffe, N.S.W. Australia. Additions to the vials were made with precision microliter syringes obtained from S.G.E. Ltd., Melbourne, Australia. Gas mixing pumps were supplied by H. Wöstoff oHG., D463 Bochum, Germany. Sephadex G-25 and Sepharose 6B were the products of Pharmacia Fine Chemicals.

Methods

Preparation of RuBP Carboxylase. RuBP carboxylase was purified from spinach leaves by methods similar to those previously described (Paulsen and Lane, 1966), except that the final hydroxylapatite column chromatography step was replaced by gel filtration on a 2.4 × 86 cm column of Sepharose 6B. The specific activity of the purified enzyme was 1.8 units mg^{-1} when fully activated and assayed at 30° with saturating substrate concentrations. The purified enzyme was homogeneous as judged by disc gel electrophoresis. Before use, the enzyme was freed of Mg^{2+} and HCO_3^- by passage through a small column of Sephadex G-25, equilibrated with 50 m*M* "CO_2 free"[2] Tris-HCl–NaOH (pH (10°) 8.2) and 1 m*M* dithiothreitol. Protein was determined as previously described (Paulsen and Lane, 1966).

Activation and Assay of RuBP Carboxylase. The activation of RuBP carboxylase and the measurement of its activity were performed at 10° in tapered 1.0-ml screw-capped Reactivials. The vials were first flushed with N_2. Then an aliquot of the inactive enzyme in 50 m*M* Tris-HCl–NaOH (pH (10°) 8.2) (typically 10 µl) was introduced. Activation of the enzyme was initiated by the addition of 2 or 4 µl of a solution containing the desired quantity of $NaHCO_3$ and $MgCl_2$. At various times thereafter, the activity of the enzyme was determined by the addition to the preincubation mixture of 300 µl of O_2-free reaction mixture. The reaction mixture contained 100 m*M* bicine–NaOH (pH (10°) 8.2), 20 m*M* $MgCl_2$, 5 m*M* dithiothreitol, 0.4 m*M* RuBP, and 1 m*M* [^{14}C]$NaHCO_3$ (1 Ci/mol). After 30 sec the reaction was terminated by the addition of about 100 µl of 2 *N* HCl. After drying, the acid-stable ^{14}C was determined by scintillation counting.

When it was necessary to maintain a constant CO_2 concentration while varying the pH, the vials were purged with the required mixture of CO_2 and N_2, obtained by mixing humidified CO_2 and N_2 with accurate gas mixing pumps. Activation was then initiated by the addition of Mg^{2+} alone.

For experiments in which the CO_2 and/or HCO_3^- preincubation concentration was varied, it was necessary to apply a small correction factor when converting observed ^{14}C dpm to nanomoles of ^{14}C fixed. This arose because different amounts of unlabeled CO_2 and HCO_3^- were carried over from the preincubation mixture to the reaction mixture. Such differences have two effects. Firstly, the carry over (CO_2 + HCO_3^-) alters the specific activity of [^{14}C]CO_2 used in the assay. Secondly, because the enzyme activity was measured at a CO_2 concentration close to the $K_m(CO_2)$, the observed activity increased as the quantity of (CO_2 + HCO_3^-) carried over increased. This effect was unrelated to the activation process and was due only to the increased substrate concentration in the assay. Therefore, we have normalized enzyme activity on the basis of Michaelis–Menten theory to a HCO_3^- concentration of 1 m*M*. The following formula, incorporating terms for both of the above effects, was used to convert uncorrected enzyme activities to normalized values:

$$\frac{K_{m(HCO_3^-)}V_f + P_a + P_b}{K_{m(HCO_3^-)}V_f + P_a}$$

where V_f = the combined volume in milliliters of the reaction and preincubation mixtures, P_a = nanomoles (CO_2 + HCO_3^-) in the reaction mixture, P_b = nanomoles (CO_2 + HCO_3^-) in the preincubation mixture, and $K_{m(HCO_3^-)}$ = the Michaelis constant expressed in terms of HCO_3^-, which was calculated, knowing the reaction pH, and assuming a K_m for CO_2 of 20 µ*M* (Badger and Andrews, 1974).

The small amount of activity observed in the absence of any preincubation was subtracted as background. Additional details are given with the data for each experiment.

Results

Requirements for the Activation of RuBP Carboxylase. To follow the conversion of inactive to active enzyme it was necessary to minimize activation during the assay. This was achieved by restricting the duration of the assay to 30 sec, by lowering the temperature to 10°, and by reducing the assay concentration of CO_2. Thus the [^{14}C]CO_2 fixed in 30 sec at 10° by the initially inactive enzyme was only 1% of that produced by the fully active enzyme (Table I).

Both CO_2 and Mg^{2+} were required for full activation (Table I). Despite stringent precautions it was difficult to completely rid buffer solutions of endogenous CO_2 (or HCO_3^-). The activation (18%) seen with Mg^{2+} alone in the absence of added CO_2 probably reflects such contamination. The activation (26%) seen with CO_2 alone, probably resulted from the formation of the enzyme–CO_2 complex which upon addition of the reaction mixture was immediately converted to the active ternary complex. The reasons for reaching this conclusion will be elaborated upon later.

[2] "CO_2 free" solutions were prepared by first purging the solutions with N_2 at pH 4.0–4.5, before adjusting the pH to the desired value with carbonate-free NaOH. To minimize subsequent contamination with atmospheric CO_2, all solutions were thereafter kept under N_2 in stoppered vessels. The Sephadex G-25 column was contained in a small glass-fronted box which was continuously purged with N_2.

Table II: Reversibility of the Activation of Ribulosebisphosphate Carboxylase by CO_2 and Mg^{2+}.

Treatment[a]	Activity[b] (nmol min^{-1} mg^{-1})	%
(i) I	184	100
(ii) I → GF	2	1
(iii) I → GF → I	195	106
(iv) I → GF → I → GF	3	1
(v) I → GF → I → GF → I	176	96

[a] Treatment, in the order indicated, consisted of incubation (I) with 10 mM $NaHCO_3$–20 mM $MgCl_2$ for 15 min at 10° and gel filtration (GF) to remove the $NaHCO_3$ and $MgCl_2$. Gel filtration was performed at room temperature on 7 × 200 mm columns of Sephadex G-25, equilibrated with "CO_2 free" 50 mM Tris-HCl–NaOH (pH 7.8), 2 mM EDTA, and 1 mM dithiothreitol. [b] Activity was determined at 10° with 1 mM $NaHCO_3$ (pH 8.2)–15 mM $MgCl_2$ as described in the Methods section.

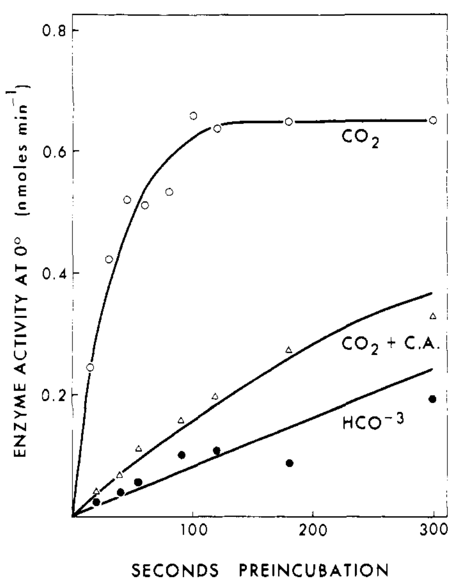

FIGURE 1: The activation of RuDP carboxylase when either CO_2 (O) or HCO_3^- (●) were added initially. The activation mixtures contained in a volume of 10 μl: 27 μg of RuBP carboxylase in 0.05 M Tris-HCl buffer, 1 mM dithiothreitol, and 2 mM EDTA (pH 8.44). At time zero 2 μl of 0.12 M $MgCl_2$ containing either 6 mM CO_2 in 0.01 M NaOAc (pH 4.1) or 6 mM $NaHCO_3$ (pH 8.5) was added. Temperature was 0–1°. At the times indicated the progress of the activation reaction was determined by the addition of 0.30 ml of ice-cold reaction mixture containing 60 μg of carbonic anhydrase. The catalytic reaction proceeded for 30 sec as described by the Methods section. The same activation reaction was performed with 5 μg of carbonic anhydrase along with CO_2 and is shown as + C.A. (Δ).

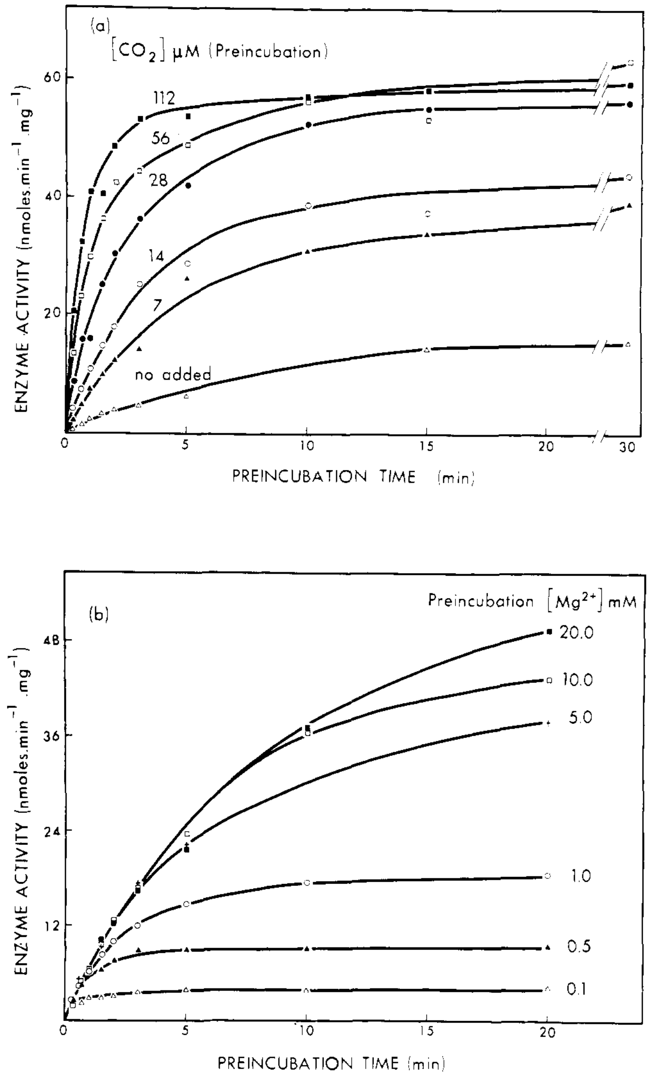

FIGURE 2: The time course for the activation of RuBP carboxylase by (a) varying concentrations of CO_2 at constant Mg^{2+} concentration, and (b) varying concentrations of Mg^{2+} at constant CO_2 concentration. (a) The activation mixture contained in a volume of 12 μl: 37 μg of RuBP carboxylase in 0.05 M Tris-HCl (pH 8.20), 1 mM dithiothreitol, 2 mM EDTA, 20 mM $MgCl_2$, and the indicated concentrations of CO_2. The activation was started with the addition of Mg^{2+} and $NaHCO_3$ and terminated after 20 sec by the addition of 0.30 ml of reaction mixture as described in the Methods section. (b) The activation mixture was the same as above except that 24 μg of RuBP carboxylase, a constant CO_2 concentration of 30 μM (1.66 mM $NaHCO_3$ pH 8.20 at 10°), and the Mg^{2+} concentrations indicated were used.

Reversibility of the Activation of RuBP Carboxylase. The activation of RuBP carboxylase by CO_2 and Mg^{2+} was reversed upon removal of the activating reagents by gel filtration (Table II, line ii). The inactive enzyme so produced was fully reactivated by incubation with CO_2 and Mg^{2+} (Table II, line iii) and the cycle of activation–inactivation repeated (Table II, lines iv and v). These results indicate that activation is reversible.

The Activating Species, CO_2 or HCO_3^-. CO_2 rather than HCO_3^- is thought to be the species involved in the catalytic reaction (Cooper et al., 1969). However, it was not known which species is involved in the activation process or whether the HCO_3^- or CO_2 molecule involved in the activation is the same as that which is subsequently fixed during catalysis. The kinetic method of Cooper et al. (1968) allows one to distinguish between HCO_3^- and CO_2 as the activating species. The principles underlying this type of analysis have been well documented (Filmer and Cooper, 1970).

The results of such an analysis are presented in Figure 1. The initial rate of activation was much faster when CO_2 rather than HCO_3^- was used to initiate the activation reaction. Carbonic anhydrase substantially eliminated this effect. These results indicate that CO_2, and not HCO_3^-, is the species involved in the activation process.

Activation of RuBP Carboxylase—Initial Rate Studies. The initial rate of activation of RuBP carboxylase was studied as a function of the CO_2 and Mg^{2+} concentrations. The results (Figure 2) showed that the initial rate of activation was proportional to the CO_2 concentration but independent of the Mg^{2+} concentration. These results are consistent with the ordered equilibrium mechanism described by:

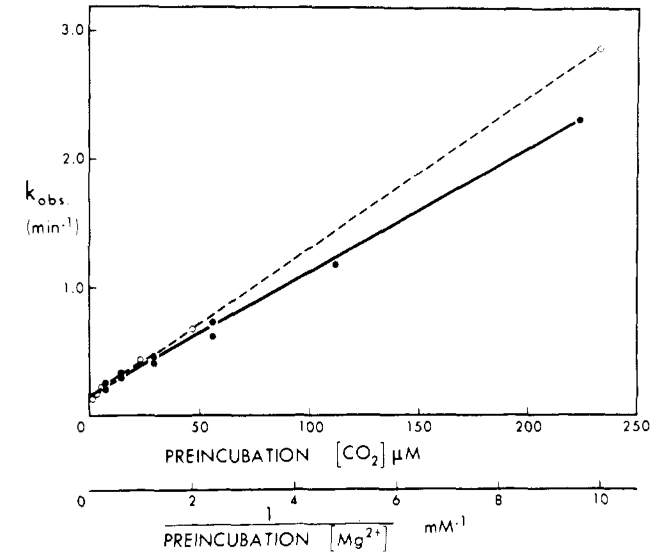

FIGURE 3: The linear dependence of the pseudo-first-order rate constant, k_{obsd}, for the activation of RuBP carboxylase upon the CO_2 concentration (●—●) and upon the inverse of the Mg^{2+} concentration (O - - - O). Values for k_{obsd} were obtained from Figure 2.

$$\underset{\text{(inactive)}}{E} + CO_2 \underset{2}{\overset{1}{\rightleftharpoons}} E\text{-}CO_2 + Mg^{2+} \underset{4}{\overset{3}{\rightleftharpoons}} \underset{\text{(active)}}{E\text{-}CO_2\text{-}Mg} \quad (1)$$

in which the reaction between the enzyme (E) and CO_2 is rate determining. In comparison, the reactions involved with the formation and dissociation of the ternary complex of enzyme-CO_2-Mg (3 and 4 of eq 1) appear to be very rapid, such that the enzyme-CO_2 complex may be assumed to be always in equilibrium with the enzyme-CO_2-Mg complex; i.e.

$$[E\text{-}CO_2] = ([E\text{-}CO_2\text{-}Mg]k_4)/(k_3[Mg^{2+}])$$

Equation 2 describes the rate of activation, assuming the ordered mechanism outlined above:

$$-d[E]/dt = k_1[E][CO_2] - (k_2k_4[E\text{-}CO_2\text{-}Mg]/k_3[Mg^{2+}]) \quad (2)$$

Since, at short times [E-CO_2-Mg] approaches zero, the right-hand side of eq 2 reduces to $k_1[E][CO_2]$, an expression in keeping with the experimental observations.

Activation of RuBP Carboxylase—Pseudo-First-Order Kinetic Studies. Since the activation process appeared to involve two sequential reversible reactions, we resorted to the use of pseudo-first-order reaction kinetics to further elucidate the mechanism of activation (Jencks, 1969).

The time course of activation at constant pH and varying CO_2 and Mg^{2+} concentrations is shown in Figure 2. From such data a series of values for the pseudo-first-order rate constant, k_{obsd}, was obtained. Replots (Figure 3) indicated that k_{obsd} was linearly dependent upon the CO_2 concentration but inversely dependent upon the Mg^{2+} concentration.

These results can be explained if it is assumed that the reaction between the enzyme and CO_2 is the rate-determining step and that the reactions involving Mg^{2+} are very rapid in comparison. It follows from these assumptions that, upon addition of the reaction mixture (containing 20 m*M* Mg^{2+}), the condition, [E-CO_2-Mg] ≫ [E-CO_2], is "immediately" established. In some cases this condition may already exist at the end of the preincubation period. However, in other cases, such as the activation with CO_2 alone (Table I and Figure 4), this condition is only established after the addition of the reaction mixture. In either case, it follows that

$$[E] = [E_{total}] - [E\text{-}CO_2\text{-}Mg] \quad (3)$$

Substituting for [E-CO_2-Mg] in eq 2 and rearrangement yields

$$-d[E]/dt = [E][k_1[CO_2] + (k_2k_4/k_3[Mg^{2+}])] - (k_2k_4[E_{total}]/k_3[Mg^{2+}])$$

Integration yields

$$\ln\{[[E][k_1[CO_2] + (k_2k_4/k_3[Mg^{2+}])] - (k_2k_4[E_{total}]/k_3[Mg^{2+}])]\}/[E_{total}][CO_2]k_1 = -k_{obsd}t \quad (4)$$

where

$$k_{obsd} = k_1[CO_2] + (k_2k_4/k_3[Mg^{2+}]) \quad (5)$$

It can be shown (see Appendix) that

$$\ln[1 - ([E\text{-}CO_2\text{-}Mg]_t/[E\text{-}CO_2\text{-}Mg]_e)] = -k_{obsd}t$$

where the subscripts *t* and e refer to the enzyme activity at time *t* and equilibrium, respectively. It is evident that the experimentally observed linear dependence of k_{obsd} upon the CO_2 concentration and its inverse dependency upon the

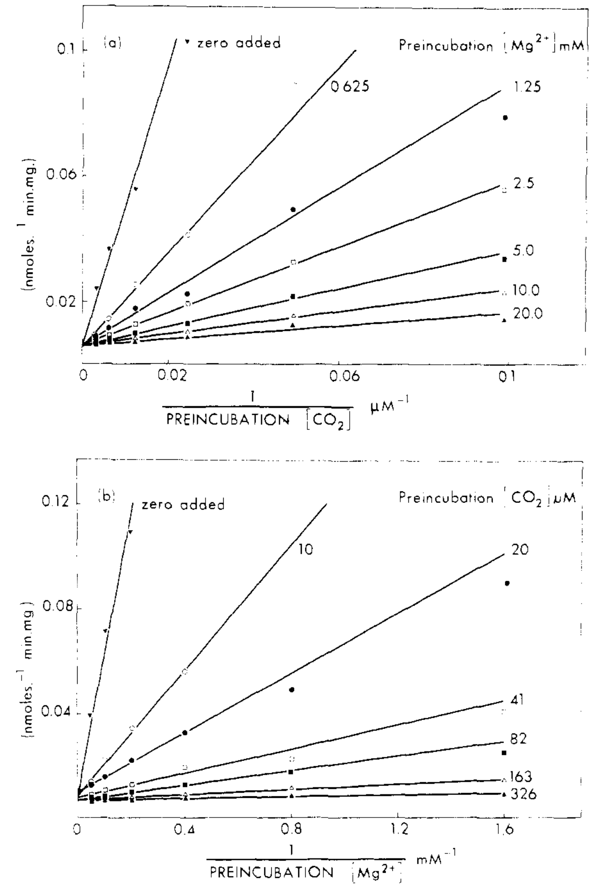

FIGURE 4: The dependency of RuBP carboxylase activity upon the preincubation concentrations of CO_2 and Mg^{2+}. Double reciprocal plots of RuBP carboxylase activity as a function of the preincubation concentrations of (a) CO_2 and (b) Mg^{2+}. Apart from the CO_2 and $MgCl_2$ concentrations, which were as indicated, the conditions for preincubation and measurement of activity were the same as those described under Table I. The slopes and intercepts of the lines were calculated according to Wilkinson (1961).

Mg^{2+} concentration can be accounted for by the expression given in eq 5.

Activation of RuBP Carboxylase—Equilibrium Studies. The preceding kinetic analyses suggested that the activation of the enzyme involved first the combination of the enzyme with CO_2 in a rate-determining, reversible step, followed by the addition of the metal ion, Mg^{2+}, to the enzyme–CO_2 complex. This being the case it was possible to develop equations to describe the equilibrium state.

Setting $K_c = k_2/k_1 = [E][CO_2]/[E\text{-}CO_2]$ and $K_{Mg} = k_4/k_3 = [E\text{-}CO_2][Mg^{2+}]/[E\text{-}CO_2\text{-}Mg]$, it follows that at equilibrium the activity of the enzyme $[E\text{-}CO_2\text{-}Mg]$ will be given by the expression, $[E][CO_2][Mg^{2+}]/K_cK_{Mg}$. Substituting for [E] from eq 3 and rearranging yields

$$1/[E\text{-}CO_2\text{-}Mg] = [K_cK_{Mg}/[Mg^{2+}][CO_2][E_{total}]] + 1/[E_{total}] \quad (6)$$

Thus, provided that the kinetic model is correct, a double reciprocal plot of the activity after preincubation to equilibrium, $[E\text{-}CO_2\text{-}Mg]$, against $[CO_2]$ should be linear with intercepts on the vertical and horizontal axes of $1/[E_{total}]$ and $-[Mg^{2+}]/K_cK_{Mg}$, respectively. A similar plot against $[Mg^{2+}]$ should likewise be linear with the intercepts $1/[E_{total}]$ and $-[CO_2]/K_cK_{Mg}$.

After preincubation to equilibrium, the activity of the enzyme was dependent upon the preincubation concentrations of CO_2 and Mg^{2+}. The resultant double reciprocal plots were of the expected form (Figure 4).

The activation observed in the absence of either added CO_2 or added Mg^{2+} represents an apparent discrepancy. The activation observed in the absence of added CO_2 is clearly trivial. Despite our precautions, it is difficult to completely eliminate CO_2 from solutions of the enzyme. The activation observed in the absence of added Mg^{2+} can be explained as before, by assuming that at high CO_2 concentrations, significant quantities of the enzyme–CO_2 complex can exist. Upon addition of the reaction mixture, the enzyme–CO_2 complex is "immediately" converted to the enzyme–CO_2–Mg complex.

Figure 5 shows the response of the activation process to pH at constant concentrations of CO_2 and Mg^{2+}. The extent of activation increased as the pH was raised. Since CO_2 rather than HCO_3^- is the activating species, such curves indicate the nature of the group on the enzyme with which CO_2 reacts. With 20 mM Mg^{2+} and 10 μM CO_2, the apparent pK of the reactive group was about 8.0. Increasing the Mg^{2+} concentration reduced the apparent pK, as would be expected given the reversible equilibrium nature of the activation process.

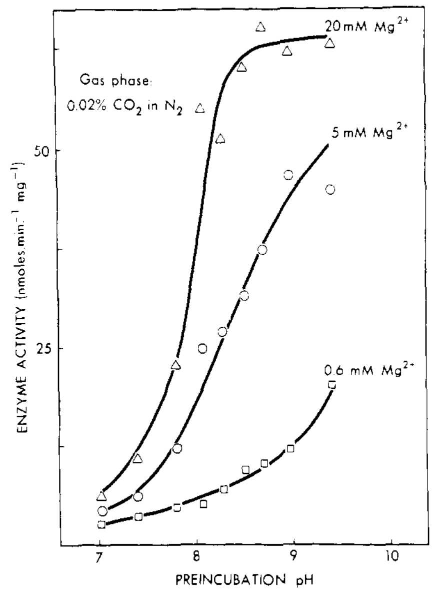

FIGURE 5: The effect of pH on the activation of RuBP carboxylase at a constant CO_2 concentration; 23 μg RuBP carboxylase (20 μl) were incubated at 10° for 30 min with 0.1 M buffer (Hepes–NaOH for pH's 7.03, 7.40, and 7.81, Tris-HCl for pH's 8.08, 8.29, 8.51, and 8.71, and ammediol-HCl for pH's 8.98 and 9.41), 0.5 mM dithiothreitol, $MgCl_2$ as indicated, 10 μM CO_2, and 3 μg of carbonic anhydrase to facilitate equilibration. The gas phase contained 200 ppm of CO_2 in N_2, giving a constant solution concentration of CO_2 of 10 μM (Umbreit et al., 1972). Activity was measured as described in the Methods section.

Discussion

Our results show that the activation of RuBP carboxylase involves the reversible formation of a ternary complex of enzyme–CO_2–Mg. The enzyme thus responds to CO_2 and Mg^{2+} in two distinct ways. It responds firstly by becoming activated by CO_2 and Mg^{2+}, and secondly it responds to CO_2 and Mg^{2+} as substrate and cofactor in the usual Michaelis–Menten manner.

Previous kinetic studies have generally been conducted by preincubating the enzyme at the pH, CO_2, and Mg^{2+} concentrations to be used in the subsequent reaction. Thus, when the reactions were initiated by the addition of RuBP, different quantities of active enzyme would have been present. Less active enzyme would have been present following preincubation at a low CO_2 concentration than at a high CO_2 concentration. Such experimental procedures have generated sigmoid reaction kinetics and erroneously high values for $K_m[CO_2]$ (Sugiyama et al., 1968b; Andrews and Hatch, 1971). When care was taken to initiate each assay with the same quantity of active enzyme, not simply the same quantity of enzyme protein, no sigmoid kinetics were observed and the $K_m[CO_2]$ was sufficiently low to account for photosynthetic CO_2 fixation rates in air; i.e., 10–20 μM CO_2 (Bahr and Jensen, 1974; Badger and Andrews, 1974). A similar explanation for the $K_m[CO_2]$ anomaly has already been proposed (Laing et al., 1975; Andrews et al., 1975). The results reported in this paper permit us to expand upon this explanation and in doing so to rationalize several diverse kinetic phenomena. In each case the enzyme was preincubated at the pH, CO_2, and Mg^{2+} concentration used in the subsequent reaction.

(a) Increasing the pH reduces the apparent $K_m[CO_2]$ (Sugiyama et al., 1968b; Lyttleton, 1973). At pH 8.4 with 20 mM Mg^{2+}, the observed $K_m[CO_2]$ was 100 μM while at pH 9.3 it was 17 μM (Lyttleton, 1973). It is apparent from the form of Figure 5 that the enzyme becomes increasingly activated as the pH is raised. Thus, in the above pH experiments different quantities of active enzyme would have been present. Less active enzyme would have been present following preincubation at low pH than at high pH. The result would then be that as the pH was raised, the observed $K_m[CO_2]$ would decrease until a limiting and true value was reached, this point being when the enzyme was fully activated. For the same reason, the reaction kinetics would become less sigmoidal as the pH was raised, as has been observed (Sugiyama et al., 1968b).

(b) Increasing the pH reduces the apparent $K_m(Mg^{2+})$ (Sugiyama et al., 1968a). It is apparent from Figure 5 that the requirement for Mg^{2+} for the activation process can be reduced by increasing the pH. For reasons similar to those outlined above, the observed $K_m(Mg^{2+})$ would be expected to decrease as the pH is raised until a limiting and true value is reached.

(c) Increasing the Mg^{2+} concentration decreases the apparent $K_m[CO_2]$ (Sugiyama et al., 1968a,b). The reaction kinetics also become less sigmoidal as the Mg^{2+} concentration is increased (Sugiyama et al., 1968b). These effects are similar to those elicited by increasing the pH, and can be explained in an analogous manner.

(d) The pH-activity profile of the enzyme was previously considered to be quite sharp, with an optimum at pH 7.8 (for a review of this and other properties of the enzyme, see Siegel et al., 1972). However such pH-activity profiles were prepared without considering that (i) the enzyme is activated by CO_2 and Mg^{2+} and (ii) CO_2 rather than HCO_3^- is the catalytically active species. Thus the sharp drop on the acid side of the pH optimum was probably due to failure to activate the enzyme while the sharp drop on the alkaline side of the pH optimum was probably due to failure to maintain a saturating concentration of CO_2 as the pH was raised. Increasing the Mg^{2+} concentration was found to shift the pH-activity profile to more acidic pH's, the effect being most marked on the acid side of the optimum (Bassham et al., 1968; Sugiyama et al., 1968a). This response is consistent with the explanation of the pH-activity profile offered above. The activity-pH profile of the fully activated enzyme is quite broad (Andrews et al., 1975) unlike that described above.

(e) The pH optimum of the associated RuBP oxygenase reaction was previously thought to be about pH 9.3 (Andrews et al., 1973). It is likely that this too is an artifact of the procedures adopted, and that it reflects activation of the enzyme rather than the response of the catalytic reaction to pH. CO_2 contamination of the assay buffers might also have reduced oxygenase activity at lower pH's. The activity-pH profile of the fully activated oxygenase is similar to that of the fully activated carboxylase (Andrews et al., 1975).

Chu and Bassham (1975) have proposed that the activation process involves a "sort of bootstrap operation" in which each binding of CO_2 and Mg^{2+} would increase subsequent binding of more CO_2 and Mg^{2+}. We have found no evidence to support such speculation. On the contrary, the linear response of the pseudo-first-order rate constant, k_{obsd}, to the CO_2 concentration indicates that the CO_2 binding site(s) are equivalent and independent of one another.

The proposed order of addition is consistent with the report (Miziorko and Mildvan, 1974) that Mn^{2+} (substituting for Mg^{2+}) was only tightly bound to the enzyme in the presence of HCO_3^- (or CO_2). One Mn^{2+} per 70000-dalton subunit of the enzyme was tightly bound. The authors suggested that the HCO_3^--dependent Mn^{2+} binding may be due either to tightened binding to a preexisting site or to the formation of a newly created site. We favor the latter interpretation.

The response of the activation process to pH is especially interesting (Figure 5) since it implies that CO_2 reacts with a group with a distinctly alkaline pK. While sulfhydryl groups have been implicated in the catalytic reaction (Trown and Rabin, 1964), they are known not to form CO_2 adducts (Morrow et al., 1974). Of the remaining available reactive groups, the amino group has the required alkaline pK and is known to react with CO_2 (Faurholt, 1925; Morrow et al., 1974). Since the enzyme group with which the CO_2 reacts appears to have a distinctly alkaline pK, we suggest that this group might be an uncharged amino group. If this is so, the most likely reaction between the enzyme and CO_2 is one of carbamate formation.

$$E\text{-}NH_2 + CO_2 \rightleftharpoons H^+ + ENHCOO^- \xrightarrow{CH_2N_2} ENHCOOCH_3$$

This suggestion is supported by the report (Akoyunoglou et al., 1967) that the enzyme-$^{14}CO_2$ complex was stabilized by treatment with diazomethane. The formation of the methyl ester of the carbamate would stabilize the radioactivity (Akoyunoglou and Calvin 1963b). After stabilization with diazomethane, the enzyme-$^{14}CO_2$ complex was digested with proteolytic enzymes to yield a single radioactive peptide, indicating that the CO_2 binding to the enzyme was highly specific. However, the authors argued that carbamate formation would not be sufficiently specific to account for this result. This need not be the case since the ease and specificity with which protein carbamates are formed will depend primarily upon the pK and nucleophilicity of the various amino groups. For example, in human deoxyhemoglobin, carbamate formation at pH 7.3 occurs only at the NH_2-terminal amino groups of the β subunits but not at those of the α subunits (Kilmartin et al., 1973; Arnone, 1974; Bauer et al., 1975). This, despite there being some 21 potentially reactive ϵ-lysyl amino groups.

The formation of a carbamate anion provides an explanation for the dependency upon HCO_3^- (i.e., CO_2) for tight Mn^{2+} binding (Miziorko and Mildvan, 1974). The reaction of CO_2 with the enzyme protein would effectively convert a neutral or potentially cationic amino group to an anionic group capable of interacting with Mn^{2+} (or Mg^{2+}).

Previous reports (Akoyunoglou and Calvin, 1963a,b; Akoyunoglou et al., 1967) of the activation of RuBP carboxylase by CO_2 and Mg^{2+} have assumed that the formation of the ternary complex of enzyme, CO_2, and magnesium was involved with the catalytic reaction mechanism; i.e., that the activating CO_2 molecule is the same as that which is fixed. Although this question has not been unequivocally resolved, circumstantial evidence indicates that the activating CO_2 is not that which becomes fixed. For example, the rate-pH profile for the catalytic reaction of the fully activated enzyme is quite unlike the pH profile for the activation process. Secondly, attempts to trap the complex by the isotope trapping technique of Rose et al. (1974) have been unsuccessful (G. H. Lorimer and M. R. Badger, unpublished). While this may merely indicate that the exchange of $^{12}CO_2$ with the enzyme-$^{14}CO_2$-Mg complex is faster than the catalytic reaction, it is also consistent with the idea that the activating CO_2 is not the same molecule as that which is fixed. Thirdly, if the activating CO_2 was also the reacting CO_2, i.e., occupied the active site, one would not expect to detect RuBP oxygenase activity with the activated ternary complex. Yet the oxygenase activity is also activated by preincubation with CO_2 and Mg^{2+} in a manner similar to the carboxylase activity (Badger and Lorimer, unpublished).

A number of sugar phosphates are known to modify the kinetic properties of the carboxylase (Chu and Bassham, 1972, 1973, 1974, 1975; Tabita and McFadden, 1972; Buchanan and Schürmann, 1973). However, the role of these metabolites appears to be secondary, since they are dependent upon the presence of CO_2 and Mg^{2+} for their effect (M. R. Badger and G. H. Lorimer, unpublished). We suggest that they act by modifying the basic activation reaction brought about by CO_2 and Mg^{2+}, perhaps, for example, by altering the pK of the group with which CO_2 reacts.

Finally, a number of schemes have been proposed which implicate the CO_2 and Mg^{2+} induced activation process to the light activation of photosynthetic CO_2 fixation in vivo (Walker, 1973; Chu and Bassham, 1975; Laing et al., 1975). It is known from studies with isolated chloroplasts that the stroma becomes more alkaline upon illumination (Jagendorf and Neumann, 1965; Heldt et al., 1973). It has been suggested that the light-dependent activation of the enzyme in vivo is caused by bicarbonate ions formed upon the alkalization of the chloroplast stroma (Laing et al., 1975). This is incorrect since CO_2 and not HCO_3^- is the activating species. We suggest that the in vivo activation of RuBP carboxylase is caused by the reaction of CO_2 with specific uncharged amino group(s) of the carboxylase protein formed by the alkalization of the stroma upon illumination. The reverse reactions would lead to inactivation upon darkening. The manner in which metabolites such as 6-phosphogluconate modify these reactions remains to be determined.

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Appendix

$$\text{enzyme} + CO_2 \underset{k_2}{\overset{k_1}{\leftrightarrow}} \text{enzyme–}CO_2 + Mg^{2+} \underset{k_4}{\overset{k_3}{\leftrightarrow}} \text{enzyme–}CO_2\text{–Mg}$$

Let the symbols E, C, and M represent enzyme, CO_2, and Mg^{2+}, respectively. At equilibrium (e)

$$[EC_e]k_2 = [E_e][C]k_1$$

$$[EC_e][M]k_3 = [ECM_e]k_4$$

Assuming $[ECM] \gg [EC]$, therefore $[ECM] = [E_0] - [E]$, where $[E_0]$ represents the total enzyme concentration. Whence

$$[E_e] = \frac{k_2k_4[E_0]/k_3[M]}{k_1[C] + (k_2k_4/k_3[M])}$$

$$[E_0] - [E_e] = [ECM_e] = \frac{k_1[C][E_0]}{k_1[C] + (k_2k_4/k_3[M])}$$

$$[E] - [E_e] = [ECM_e] - [ECM] = ([E][k_1[C] + (k_2k_4/k_3[M])] - ([E_0]k_2k_4/k_3[M]))/(k_1[C] + (k_2k_4/k_3[M]))$$

$$([ECM_e] - [ECM])/[ECM_e] = ([E][k_1[C] + (k_2k_4/k_3[M])] - ([E_0]k_2k_4/k_3[M]))/k_1[C][E_0]$$

$$\ln [1 - ([ECM]/[ECM_e])] = \ln [([E][k_1[C] + (k_2k_4/k_3[M])] - ([E_0]k_2k_4/k_3[M]))/k_1[C][E_0]] = -k_{obsd}t$$

where $k_{obsd} = k_1[C] + (k_2k_4/k_3[M])$

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Manganese(II) and Substrate Interaction with Unadenylylated Glutamine Synthetase (*Escherichia coli* W). I. Temperature and Frequency Dependent Nuclear Magnetic Resonance Studies†

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ABSTRACT: A comprehensive study of solvent interaction with unadenylylated glutamine synthetase ($E_{1.7}$) has been conducted using the enzyme isolated from *Escherichia coli* W. The longitudinal, $(1/T_{1p})_b$, and transverse, $(1/T_{2p})_b$, proton relaxation rates were measured with various enzyme samples as a function of frequency (6-48 MHz) and temperature (1-40 °C). With Mn(II) bound at the "tight" metal ion site approximately two water molecules are rapidly exchanging with bulk solvent. This number is reduced to approximately one in the presence of glutamine. All data were successfully analyzed according to the Solomon-Bloembergen-Morgan (SBM) scheme for dipolar relaxation of water protons interacting with enzyme-bound Mn(II). The correlation time for this process varies from 1 to 3×10^{-9} for the complexes described above. Significant contributions to the correlation time arise from both $1/\tau_m$, the exchange rate for water molecules bound at the metal site, and from $1/\tau_s$, the electron spin relaxation rate for Mn(II) with the latter rate showing a frequency dependence at the magnetic field strengths used in this study. A study of Mn(II) binding to $E_{1.7}$ at 25 °C revealed two classes of metal ion sites, a "tight" set of one per subunit with $K_D = 5.0 \times 10^{-7}$ M and a "weak" set of one per subunit with $K_D = 4.5 \times 10^{-5}$ M. In the presence of glutamine the affinity of the first site for Mn(II) was unchanged but the K_D value for the weak site changed to 3×10^{-6} M. In $E_{1.7}$ samples with Mn(II) bound at both the tight and weak metal ion sites the data are interpretable with two rapidly exchanging water molecules interacting with *each* bound Mn(II) ion. With saturating amounts of glutamine or of ADP or of glutamine plus ADP plus arsenate, the proton relaxation rates progressively decreased suggesting that the substrates or inhibitors used were interacting with the bound Mn(II) ions resulting in diminished solvent accessibility to these bound ions. These results are interpretable in terms of ligand substitution into the coordination sphere of the bound Mn(II) ions. Indeed this is probably the case for Mn(II) at the weak metal ion site since Hunt et al. ((1975), *Arch. Biochem. Biophys. 166*, 102) showed that Mn(II) can bind as the Mn(II)-ADP complex to the second metal ion site. Results of proton relaxation rate data on $E_{1.7}$ with Mn(II) bound at both the tight and weak metal ion sites led to the conclusion that these metal ion sites are >6 Å apart. In comparison with proton relaxation rate data on fully adenylylated glutamine synthetase ($E_{11.8}$) as studied by Villafranca and Wedler ((1974), *Biochemistry 13*, 3286), the first "tight" metal ion site in $E_{11.8}$ has three rapidly exchanging water molecules. Mn(II) has a weaker binding constant to $E_{11.8}$ ($K_D \sim 5 \times 10^{-6}$ M) at the pH value used in both studies and a suggestion is made that an additional protein ligand is binding to Mn(II) in glutamine synthetase when the subunits are not adenylylated.

The interaction of metal ions with glutamine synthetase purified from *Escherichia coli* has been reviewed by Ginsburg (1972). Two sets of Mn(II) binding sites are observed that have important effects on the protein. Metal ion binding to 12 high affinity binding sites (one per monomer of the dodecamer) produces an ultraviolet spectral change at 290 nm in the protein (Shapiro and Ginsburg, 1968) and the release of two protons (Hunt and Ginsburg, 1972). Binding of a second set of 12 metal ions with weaker affinity than the first set releases one proton (Hunt and Ginsburg, 1972) with no accompanying ultraviolet spectral change. Full catalytic activity is seen when the second set of metal ion sites are saturated. A third much weaker set of

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