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Activation and CO_2 Exchange Kinetics of Ribulose-1,5-bisphosphate Carboxylase/Oxygenase: Negative Cooperativity with Respect to Mg^{2+}

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ABSTRACT: We have reexamined the activation kinetics of spinach ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) with respect to CO_2 and Mg^{2+}. Negative cooperativity (Hill coefficient = 0.65) with respect to the Mg^{2+} dependence of rubisco activation was observed both in equilibrium studies and in kinetic determinations of the pseudo-first-order rate constants. No cooperativity effects (Hill coefficient = 1) were observed with respect to the CO_2 dependence of activation. The effects of Mg^{2+} and CO_2 on the exchange kinetics of the activator CO_2 of rubisco were also analyzed by utilization of the transition-state analogue 2-carboxyarabinitol 1,5-bisphosphate to trap the CO_2 moiety on the enzyme. Rate constants for exchange at steady-state activation determined in the presence of different Mg^{2+} and CO_2 concentrations were also consistent with negative cooperativity with respect to Mg^{2+} binding and indicate an equilibrium between active and inactive forms of the enzyme during steady-state activation. The presence of negative cooperativity in activation with respect to Mg^{2+} but not with respect to CO_2 may indicate that the observed cooperativity effects are due to allosteric interactions in activation rather than a heterogeneity of the activator sites on the purified enzyme.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) (EC 4.1.1.39) is a bifunctional enzyme that catalyzes either the carboxylation or the oxygenation of the substrate ribulose 1,5-bisphosphate (RuBP). The enzyme from higher plants is large (550 kDa) and is composed of eight large and eight small subunits (Miziorko & Lorimer, 1983). Both the carboxylation and oxygenation activities are associated with the large subunit. The function of the small subunit, while proposed to be regulatory, is unknown.

The activation state of the enzyme has also been shown to be modulated in vivo, and the extent of rubisco activation may limit overall photosynthesis (Perchorowicz et al., 1981). Kinetic evidence (Lorimer et al., 1976; Laing & Christeller, 1976) has established that an inactive form of the enzyme can be activated in vitro by the ordered addition of first CO_2 and then Mg^{2+}. The activating CO_2 has been shown to bind to an activator site on the enzyme, which results in carbamate formation on a lysyl residue (Lorimer & Miziorko, 1980). The CO_2 bound during the activation process has been shown to be distinct from that involved in catalysis (Lorimer, 1979; Miziorko, 1979).

In their pioneering studies on the kinetics of rubisco activation, Lorimer et al. (1976) obtained evidence that the binding of CO_2 to the enzyme is the rate-limiting step in the activation process, followed by a rapid equilibration of the enzyme–CO_2 complex with Mg^{2+} to form the catalytically competent enzyme–CO_2–Mg^{2+} complex. The following model was proposed to account for the data obtained:

$$E_i + CO_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{–}CO_2 + Mg^{2+} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_a \qquad (1)$$

where E_i represents an inactive form of the enzyme and E_a represents the catalytically competent ternary complex (enzyme–CO_2–Mg^{2+}). Laing and Christeller (1976) derived values for the individual rate constants of the soybean enzyme by combining the pseudo-first-order rate constants for activation and equilibrium measurements at various CO_2 and Mg^{2+} concentrations with measurements of the decay rate of the activated enzyme in the absence of Mg^{2+}. Mg^{2+}-dependent variation in the derived rate constants was reported. This variation was attributed to possible changes in rubisco acti-

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vation state during the course of the assays, even through the presence of RuBP in the assay was shown to substantially reduce the rates of interconversion between the different forms of the enzyme.

The active form of rubisco interacts with 2-carboxyarabinitol 1,5-bisphosphate (CABP), a proposed transition-state analogue for the carboxylase activity of the enzyme (Pierce et al., 1980), resulting in the formation of an extremely stable quaternary complex of enzyme–CO_2–Mg^{2+}–CABP (Miziorko, 1979). CABP has been used to demonstrate a correlation between activation and CO_2 binding (McCurry et al., 1981).

Recently, we showed that CABP can be utilized to follow the exchange kinetics of the activator CO_2 (Belknap & Portis, 1986). The observed kinetics were consistent with the model for activation proposed by Lorimer et al. (1976). However, in further studies presented here, in which CO_2 and Mg^{2+} were varied, the exchange data were not adequately accounted for by this model. We reinvestigated the activation kinetics of the purified spinach enzyme and present data that indicate that a distinct negative cooperativity exists in the Mg^{2+} dependence of activation. Our exchange data were also adequately described by this model if negative cooperativity with respect to Mg^{2+} binding was introduced.

The presence of cooperativity effects with respect to the Mg^{2+} in both the activation and exchange kinetics with purified rubisco could be interpreted as indicating binding site heterogeneity in the purified enzyme (Dalquist, 1978). However, one would expect such heterogeneity to be reflected in the binding of both substrates of the activation process. The observed Hill coefficient of 1 for the CO_2 dependence of activation and exchange may indicate that the negative cooperativity observed for Mg^{2+} is due to allosteric interactions between activator sites on the enzyme.

Experimental Procedures

Materials

Rubisco was prepared from spinach leaves as described by McCurry et al. (1982) and stored as a 50% saturated $(NH_4)_2SO_4$ precipitate at 4 °C. Protein concentration was determined spectrophotometrically (Wishnick & Lane, 1971). CABP was a kind gift of Jun-ichi Takanashi and W. L. Ogren. RuBP was purchased from Sigma Chemical Co.[1] All other reagents were of the highest purity commercially available.

Methods

Enzyme Preparation. Isolated rubisco was pelleted and resuspended in dialysis buffer [25 mM *N*,*N*-bis(2-hydroxyethyl)glycine (Bicine), 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM 2-mercaptoethanol, pH 7.8] (McCurry et al., 1982). The enzyme was then dialyzed against a 1000-fold excess of buffer for 16 h, 4 °C. The dialyzed enzyme was then made 20 mM with respect to $MgCl_2$ and $NaHCO_3$ and 5 mM with respect to dithiothreitol (DTT), followed by heating for 25 min at 50 °C (Lorimer et al., 1977) in a sealed vial. The heat treatment resulted in an approximate 2-fold increase in carboxylase activity (final maximal activity approximately 2.0 μmol min^{-1} mg^{-1}). The heat-activated enzyme was then deactivated by gel filtration through a 10-mL Sephadex G-50 column equilibrated with a CO_2-free 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.2 (Lorimer et al., 1977). The deactivated enzyme was placed in CO_2-free buffer at room temperature prior to use.

Enzyme Activation Assays. Activation kinetics were determined at 20 °C in an activation mixture consisting of CO_2-free Tris buffer supplemented with $NaHCO_3$ and $MgCl_2$ as indicated in the figure legends. Assays were initiated by addition of deactivated enzyme (0.1 mg ml^{-1} final concentration) to the activation mixture. Activation assays were run in sealed vials with minimal air space above the solution to avoid contamination with atmospheric CO_2. Samples were removed from the activation mixture as indicated in the figures and added to RuBP carboxylase assays (100 mM Tris, 5 mM $MgCl_2$, 0.6 mM DTT, 0.4 mM RuBP, 20 mM $NaH^{14}CO_3$ (1 μCi μmol^{-1}, pH 8.2, 25 °C). The reaction was terminated after 20 s by addition of HCl. Acid-stable radioactivity was determined by liquid scintillation counting. Activities were corrected for activity detected by preincubation with $NaHCO_3$ or $MgCl_2$ (<0.1 μmol min^{-1} mg^{-1}) in the $MgCl_2$ or $NaHCO_3$ experiments, respectively.

CABP Trapping Experiments. These experiments were carried out with the same activation mixture described above, supplemented with $NaH^{14}CO_3$ (10 μCi μmol^{-1}) either prior to addition of enzyme (activation experiments) or 5 min after addition of enzyme (exchange experiments). The rubisco concentration used in these experiments was approximately 0.3 mg mL^{-1} (final concentration in activation mixture). Aliquots were removed from the activation mixture as indicated in the figures and added to a CABP quenching solution (300 μM CABP and 10 mM *N*-[tris(hydroxymethyl)-methyl]glycine (Tricine), final concentrations in quench, pH 8.2). Quenched samples were kept at room temperature for 1 h (Miziorko, 1979) and then frozen in liquid N_2 and stored at −80 °C. The samples were thawed, and blue dextran (2 mg mL^{-1}) was added in order to visualize the high molecular weight fraction during gel filtration. The acid-labile counts associated with the high molecular weight fraction were determined by gel filtration through Sephadex G-50. Control assays for determination of the time course for activation of RuBP carboxylase activity under the conditions shown in the figures were also run (data not shown). The data presented in the figures were corrected for a background level of CO_2 that was found to be independent of the activation state of the enzyme. Subsequent experiments have indicated that this background can be greatly reduced by the inclusion of high bicarbonate in the gel filtration column (V. J. Streusand and A. R. Portis, Jr., unpublished results).

Results

Equilibrium Studies. Lorimer et al., (1976) have established that preincubation of deactivated rubisco in a medium supplemented with CO_2 and Mg^{2+} results in activation of rubisco. In these and other analyses of the activation process (Laing & Christeller, 1976), no cooperativity effects were reported. The data in Figure 1 show similar experiments carried out on the enzyme preparation described here. Double-reciprocal plots of activation to equilibrium under various $NaHCO_3$ and $MgCl_2$ concentrations showed the expected kinetics with respect to $NaHCO_3$ (Figure 1A). However, distinct deviations from linearity were observed in the Mg^{2+} titration (Figure 1B). Similar deviation is apparent upon close inspection in the data presented by Lorimer et al. (1976).

The nonlinear kinetics reflect a negative cooperativity with respect to the Mg^{2+} dependence of activation, as shown more clearly by Hill plots of typical data as presented in the insert of Figure 2. As shown in Figure 2 over the range of $MgCl_2$ and $NaHCO_3$ concentrations investigated here, the Hill plots

[1] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

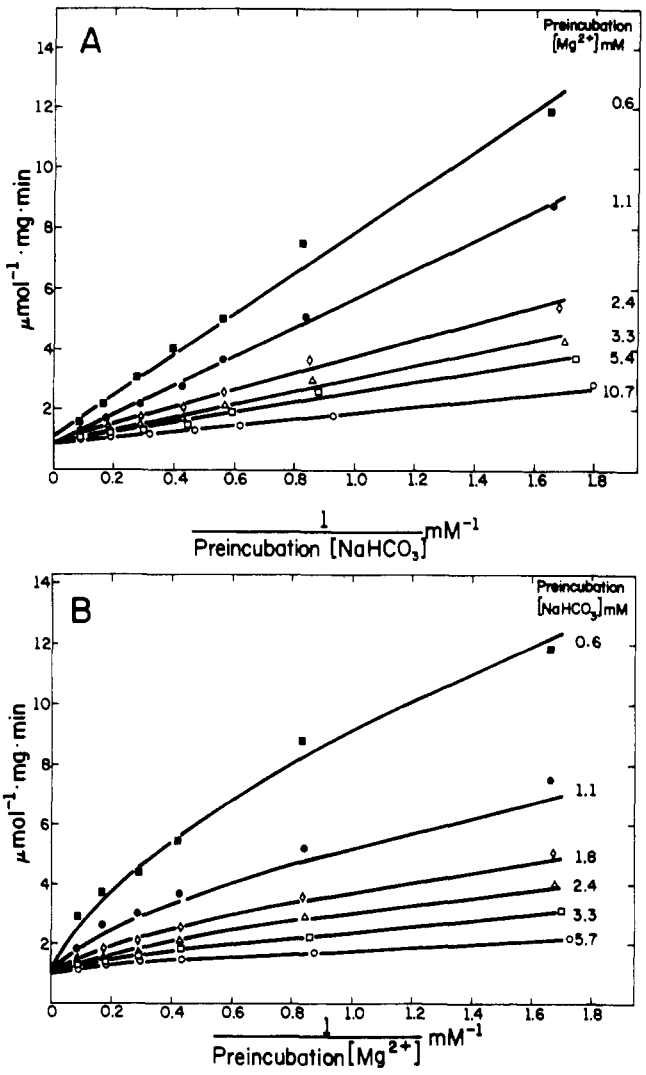

FIGURE 1: Dependence of equilibrium carboxylase activity on $NaHCO_3$ and $MgCl_2$. Double-reciprocal plots of carboxylase activity following preincubation of deactivated enzyme in the presence of different $NaHCO_3$ (A) and $MgCl_2$ (B) concentrations. Preincubation and assay conditions are described under Experimental Procedures. Slopes and intercepts in (A) determined by linear regression analysis. Lines in (B) derived from the equation described in text to correct for negative cooperatively with respect to Mg^{2+}.

with respect to $NaHCO_3$ consistently gave a Hill coefficient of 1 (correlation coefficients >0.99) while those with respect to $MgCl_2$ gave a slope of between 0.62 and 0.72 at various $NaHCO_3$ concentrations (correlation coefficients >0.99). These data indicate the presence of negative Mg^{2+} cooperativity in rubisco activation at equilibrium.

Lorimer et al. (1976) derived the following equation from eq 1 to describe rubisco activation at equilibrium:

$$1/[E_a] = [k_2k_4/(k_1k_3)][E_{tot}][CO_2][Mg^{2+}] + 1/[E_{tot}] \quad (2)$$

where $[E_{tot}]$ represents total rubisco activity determined from activation at saturating CO_2 and Mg^{2+}. This equation was derived with the assumption that both the enzyme–CO_2 and the enzyme–CO_2–Mg^{2+} complexes are detected in the assay of activity but is also valid if the amount of enzyme–CO_2 complex is always much less than enzyme–CO_2–Mg^{2+}. Our data imply that the $[Mg^{2+}]$ term in eq 2 should be replaced by $[Mg^{2+}]^{0.65}$ in order to account for the observed negative cooperativity. The value of $k_2k_4/(k_1k_3)$ (=5.5 $mM^{1.65}$) can be calculated from the slopes of the double-reciprocal plots in Figure 1A or from the data in Figure 1B replotted to correct for the cooperativity effect (plot not shown). If these parameters are incorporated into eq 2, an excellent fit is obtained to data in Figure 1B as shown.

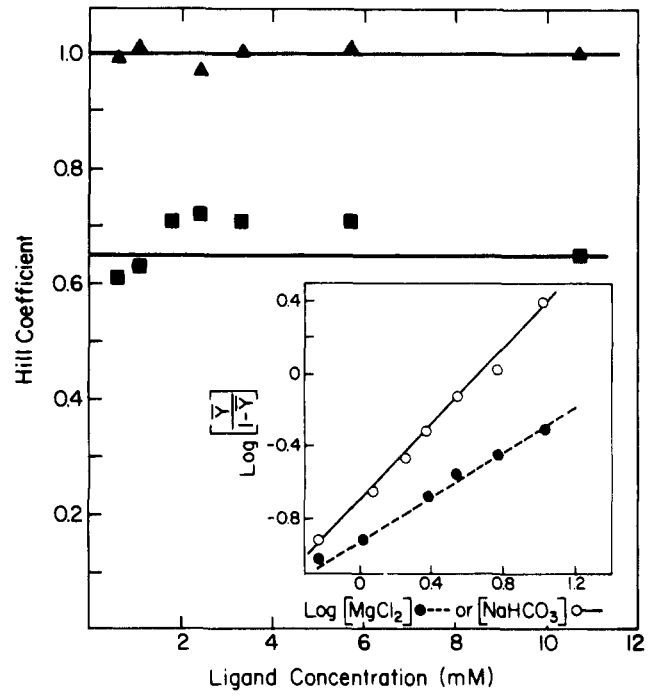

FIGURE 2: Independence of Hill coefficient for $NaHCO_3$ (▲) or $MgCl_2$ (■) in equilibrium activation from the concentration of the other ligand. Hill coefficients determined from Hill plots of equilibrium activation (log $[\bar{Y}/(1-\bar{Y})]$) vs. ligand concentration (log [S]) as shown for typical data [(●) 0.6 mM $NaHCO_3$; (○) 1.1 mM $MgCl_2$] in the insert.

Pseudo-First-Order Rate Constants. From eq 1, Lorimer et al. (1976) derived the following equation to describe the kinetics of the activation process:

$$-d[E_i]/dt = k_{act} = k_1[E_i][CO_2] - k_2k_4[E_a]/(k_3[Mg^{2+}]) \quad (3)$$

and showed that the pseudo-first-order rate constant for activation (k_{obsd}) for each CO_2 and Mg^{2+} concentration was equal to k_{act}. Equations 2 and 3 indicate that an increase in the Mg^{2+} concentration in the activation mixture should be associated with a decrease in the pseudo-first-order rate constant for activation (k_{obsd}) as well as the observed increase in equilibrium activation state. Therefore, experiments were carried out to determine the effects of Mg^{2+} and $NaHCO_3$ on the k_{act} of the enzyme preparation described here. As observed previously (Lorimer et al., 1976), the initial rate of activation was dependent upon the $NaHCO_3$ concentration, but independent of Mg^{2+}. Calculated pseudo-first-order rate constants for the kinetics of activation (k_{obsd}) were plotted vs. $NaHCO_3$ and $MgCl_2$ concentrations. As expected, the k_{obsd} varied linearly with respect to $NaHCO_3$ concentration in the activation mixture (data not shown, but slope = k_1 = 0.28 min^{-1} mM^{-1}, correlation coefficient = 0.997). However, as shown in Figure 3, a better fit was obtained with the data obtained by varying the Mg^{2+} concentration, if the k_{obsd} was plotted against $1/[Mg^{2+}]^{0.65}$ (slope = k_2k_4/k_3 = 2.7 min^{-1} $mM^{0.65}$, correlation coefficient = 0.97) rather than $1/[Mg^{2+}]$ (correlation coefficient 0.94). For a given enzyme preparation, good agreement was found between the rate constants determined from equilibrium and kinetic studies. Some variation in the calculated rate constants was observed from preparation to preparation, but in general, $k_2k_4/(k_1k_3)$ = 5.5–9 $mM^{1.65}$, k_1 = 0.2–0.3 min^{-1} mM^{-1}, and k_2k_4/k_3 = 2.5–3 min^{-1} $mM^{0.65}$.

Although the data presented here are consistent with the model suggested by Lorimer et al. (1976) only if a negative

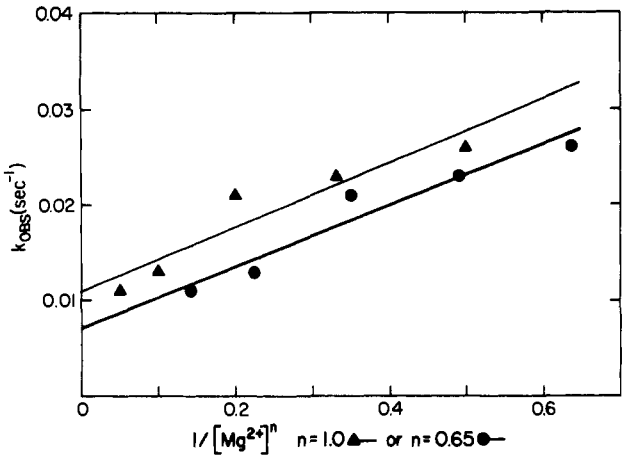

FIGURE 3: Dependence of pseudo-first-order rate constants for rubisco activation (k_{obsd}) on preincubation concentration of $MgCl_2$, assuming negative cooperativity [n = 0.65 (●)] or no cooperativity [n = 1.0 (▲)]. Slopes and intercepts were determined by linear regression analysis.

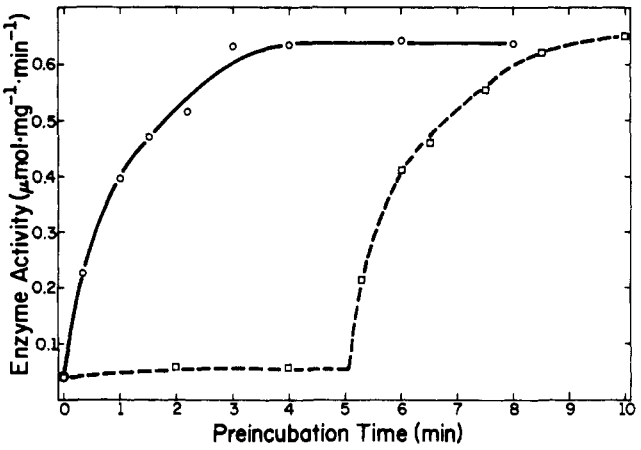

FIGURE 4: Effect of incubation of rubisco in $NaHCO_3$ on the time course of enzyme activation. Enzyme was preincubated in the presence of $NaHCO_3$ (1.5 mM) and $MgCl_2$ (20 mM) (○) or in the presence of 1.5 mM $NaHCO_3$ followed by addition of 20 mM $MgCl_2$ at 5 min (□). Activation and assay conditions are as described under Experimental Procedures.

cooperativity for the binding of Mg^{2+} is introduced, it was conceivable that the observed Mg^{2+} negative cooperativity was actually due to the measurement of both the enzyme–CO_2–Mg^{2+} and only some of the enzyme–CO_2 complexes in the assay used. We examined this possibility by comparing the activities measured when both $MgCl_2$ and $NaHCO_3$ were added together to that obtained by preincubation with 1.5 mM $NaHCO_3$ as shown in Figure 4. Only a slight increase above the background level of activity occurred during the 5-min preincubation period with $NaHCO_3$. Subsequent addition of 20 mM $MgCl_2$ resulted in activation kinetics that were almost indistinguishable from those obtained with the simultaneous addition of both ligands. Preincubation with 20 mM $NaHCO_3$ also resulted in minimal increases in activity above the initial background level, but the activation that occurred upon the addition of $MgCl_2$ was too rapid to determine if possible differences in rate existed (data not shown). The absence of a significant increase in activity during preincubation with $NaHCO_3$ indicates that very little conversion of the enzyme–CO_2 complex which should form under these conditions to the enzyme–CO_2–$MgCl_2$ complex occurs during the assay. The absence of a detectable difference in the rate of activation following Mg^{2+} addition indicates that very little enzyme–CO_2 complex is formed under these conditions. We therefore believe that both enzyme–CO_2–Mg^{2+} and probably free enzyme are much greater than enzyme–CO_2 over the range of CO_2 and $MgCl_2$ concentrations examined but, most importantly, that the enzyme–CO_2 complex is not contributing to the measured activities. The observations presented are therefore a true reflection of a negative Mg^{2+} cooperativity in activation of the enzyme.

Effects of Mg^{2+} and $NaHCO_3$ on Exchange. As we have previously shown (Belknap & Portis, 1986), data obtained from the exchange kinetics of the activator CO_2 (with 1.5 mM $NaHCO_3$, 20 mM $MgCl_2$) are consistent with an equilibrium between the active and inactive forms of the enzyme during steady-state activation. However, the effects of varying Mg^{2+} and CO_2 on the exchange kinetics were not investigated. On the basis of the model for activation (Lorimer et al., 1976) as modified here

$$k_{act} = k_1[CO_2][E_i] - k_2k_4[E_a]/(k_3[Mg^{2+}]^{0.65}) \quad (4)$$

and if exchange proceeds by full reversal of rubisco activation, then exchange of labeled with unlabeled CO_2 could be described by

$$E_a\text{–}{}^{12}C \underset{k_a}{\overset{k_d}{\rightleftharpoons}} E_i + {}^{12}CO_2 \quad (5)$$

and

$$E_i + {}^{14}CO_2 \underset{k_d}{\overset{k_a}{\rightleftharpoons}} E_a\text{–}{}^{14}C \quad (6)$$

where k_a ($=k_1$) and k_d [$=k_2k_4/(k_3[Mg^{2+}]^{0.65})$] describe the association and dissociation constants for a pseudo-first-order activation process. If the extent of activation does not change following addition of the $^{14}CO_2$

$$d[E_a\text{–}{}^{14}C]/dt = -d[E_a\text{–}{}^{12}C]/dt = k_d[E_a\text{–}{}^{12}C] - k_a[E_i][{}^{12}CO_2] \quad (7)$$

However, the dissociation of the E_a–^{12}C complex (eq 5) can be viewed as an irreversible process due to the immediate mixing of the released ligand with $^{14}CO_2$ (Rose et al., 1974), and this simplifies eq 7 to

$$d[E_a\text{–}{}^{14}C]/dt = k_d[E_a\text{–}{}^{12}C] \quad (8)$$

Therefore, the pseudo-first order rate constant for exchange of ^{14}C into the activator CO_2 site can be described by

$$k_{exch} = k_d = k_2k_4/(k_3[Mg^{2+}]^{0.65}) \quad (9)$$

This implies that the rate constant for exchange should be independent of the $NaHCO_3$ concentration and vary with $1/[Mg^{2+}]^{0.65}$. The effects of Mg^{2+} and $NaHCO_3$ on exchange are shown in Figure 5. While the absolute rate of exchange was lower at the lower bicarbonate concentration, the calculated first-order rate constant (k_{exch}) was the same for both bicarbonate concentrations (Figure 5A). In contrast, the rate of exchange was faster at the lower $MgCl_2$ concentration (Figure 5B). Using eq 9 to calculate k_2k_4/k_3 from the pseudo-first-order rate constants and $MgCl_2$ concentrations in this experiment gave values of 2.5 and 2.7 min^{-1} $mM^{0.65}$ for 20 and 5 mM $MgCl_2$, respectively (Figure 5B), and 2.8 min^{-1} $mM^{0.65}$ (10 mM $MgCl_2$, 0.5 or 2 mM $NaHCO_3$) for the data in Figure 5A. Without introducing the negative Mg cooperativity, these values are 7.2 (20 mM), 6.3 (10 mM), and 4.8 (5 mM). The latter values clearly show a residual dependence on Mg. The agreement between rate constants determined from the data in Figure 5 with eq 9 and their agreement with those determined above with other methods represent further evidence for negative cooperativity with respect to Mg^{2+} binding. In addition, these data demonstrate that the exchange

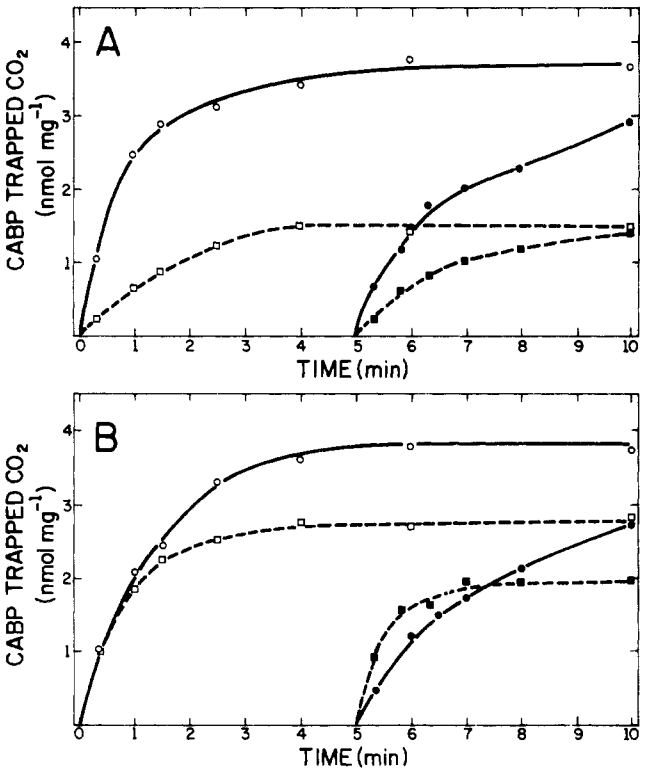

FIGURE 5: Dependence of net binding and exchange of activator CO_2 on $NaHCO_3$ (A) and $MgCl_2$ (B). Net binding of activator CO_2 (open symbols) was determined by addition of $NaH^{14}CO_3$ prior to addition of enzyme and of exchange of activator CO_2 (closed symbols) by addition of $NaH^{14}CO_3$ after 5-min preincubation in the activation mixture. Variation with $NaHCO_3$ in net binding and exchange kinetics was determined by activation of the enzyme in the presence of 10 mM $MgCl_2$ and 0.5 (□, ■) or 2 mM (○, ●) $NaHCO_3$ (A). Variation with $MgCl_2$ of net binding and exchange was determined by activation of the enzyme in the presence of 1.5 mM $NaHCO_3$ and 5 (□, ■) or 20 mM (○, ●) $MgCl_2$ (B). CABP-trapped CO_2 was determined as described under Experimental Procedures.

of the activating CO_2 proceeds via an equilibration between the E_i and E_a forms of rubisco during steady-state activation as determined by the concentrations of $MgCl_2$ and $NaHCO_3$.

Discussion

Rate Constants for Enzyme Activation. The activation kinetics of the rubisco preparation described here agree with those described previously (Lorimer et al., 1976) with the exception of the negative cooperativity observed with respect to Mg^{2+}. While independent values were not derived here for the four rate constants in eq 1, values calculated for k_1 as determined by two methods (initial rate and pseudo-first-order kinetics) and those for the combined rate constant k_2k_4/k_3 as determined by three methods (initial rate with equilibrium, pseudo-first-order, and activator CO_2 exchange kinetics) were internally consistent and did not vary with Mg. A direct comparison of the kinetic constants derived here with those calculated by Laing and Christeller (1976) is difficult due to the different methods of computation. Some comparisons, however, can be made. Values of $k_2k_4/(k_1k_3)$ (from eq 1 of approximately 5 mM^2 were obtained by these authors for the soybean and spinach enzymes, in good agreement with the values (5.5–9 $mM^{1.65}$) presented here. Also, their calculations of k_1 (approximately 0.28 min^{-1} mM^{-1} at 20 mM $MgCl_2$) are similar to ours (0.2–0.3 min^{-1} mM^{-1}). However, Laing and Christeller also observed a variation of computed values of k_1 (as well as k_2) with the concentration of Mg^{2+} in the preincubation mixture. These authors suggested that this variation was due to changes in enzyme activation during the carboxylation assay (as discussed below). However, our data indicate that the variation was due to the failure to account for the negative cooperativity described here.

Laing and Christeller (1976) calculated k_1 from the following equation:

$$k_1 = k_{obsd}/([CO_2] + k_2k_4/(k_1k_3[Mg^{2+}])) \quad (10)$$

Values of k_2 were then determined by

$$k_2 = K_c/k_1 \quad (11)$$

where K_c ($=k_2/k_1$) was independently derived. The inclusion of negative cooperativity with respect to Mg^{2+} dependence of activation (Hill coefficient = 0.65) in eq 10 results in significantly less variation in calculated values of k_1 (and therefore k_2) with Mg^{2+} concentration than indicated by these authors.

Changes in Enzyme Activation State during Assay. The problem of changes in activation state during the determination of carboxylation activity raised by Laing and Christeller (1976), and more recently encountered by Andrews and Ballment (1984) in studies of the *Synechococcus* enzyme, represents a potential source of error in determination of rubisco activation kinetics. Such changes could result in a variety of artifactual kinetic properties, including apparent cooperativity effects (Sugiyama et al., 1968). In general, one would expect a potential overestimation of activity at low concentrations of CO_2 and Mg^{2+} and the underestimation at high concentrations. We believe, however, that any changes in activity that occurred with the enzyme and under the assay conditions employed in this study were not of sufficient magnitude to account for the observed kinetics.

Jordan and Chollet (1983) have shown that RuBP binds very tightly to the CO_2 and $MgCl_2$ free form of the spinach enzyme, which effectively prevents further addition of CO_2 and $MgCl_2$ activation if short assays are utilized. RuBP also greatly slows down the otherwise rapid deactivation of the enzyme upon dilution into media with low CO_2 and Mg concentrations (Jordan et al., 1983). Therefore, the only potential problem with an assay at different CO_2 and $MgCl_2$ concentrations than those during activation is whether RuBP also effectively inhibits the binding of Mg^{2+} to the enzyme–CO_2 complex.

Lorimer et al. (1976) obtained evidence that at least some of the enzyme–CO_2 complex was assayed under their conditions. Preincubation of the enzyme with 300 μM at 10 °C (equivalent to 17 mM $NaHCO_3$) gave 26% of maximal activity. If it is assumed that all of the complex is detected, an apparent binding constant of 850 μM CO_2 can be estimated from this data. In contrast, using preincubation at various CO_2 concentrations, Laing and Christeller (1976) calculated a binding constant of 90 μM CO_2 from their data and derived a value of 300 μM CO_2 from the data of Lorimer et al. (1976). However, they did not indicate how much activity was actually measured relative to that obtained with the fully activated enzyme. As indicated above, we attempted to determine if the formation of the complex could influence our results by examining the effects of preincubation with $NaHCO_3$ on the activity measured by our assay conditions, which differ from those of Lorimer et al. (1976), and could not detect significant differences. We would also like to emphasize that the $MgCl_2$ concentration during our assay was between the highest and lowest $MgCl_2$ concentrations used for activation. If the formation of enzyme–CO_2 was contributing significantly to our results, one would have expected nonlinear Hill plots.

Finally, it is clear that great caution must be taken in interpreting the activation kinetic data in any system in which

a substrate of the activation process (CO_2) is also a required substrate for enzyme activity. However, the most marked deviation in the kinetics observed in this study from those reported previously (Lorimer et al., 1976; Laing & Christeller, 1976) involves the cooperativity effects observed with respect to Mg^{2+}, a moiety for which activation has strict requirements but to which the actual carboxylation activity is largely insensitive (Laing & Christeller, 1976).

Exchange Kinetics. It has been previously suggested that the activating carbamate, once formed, should be stable (Lorimer & Miziorko, 1980). The exchange data presented here support this interpretation in a thermodynamic sense. The exchange kinetics of the activating CO_2 moiety are consistent with an equilibrium between the active and inactive forms of the enzyme rather than free exchange of ligand on the active form of the enzyme (Belknap & Portis, 1986). In addition, calculated values of k_2k_4/k_3 in these experiments were independent of Mg only if negative cooperativity was assumed, which provides additional evidence for negative cooperativity with respect to Mg^{2+} binding at the activator site on the enzyme.

Negative Cooperativity. Negative cooperativity with respect to substrate binding may reflect either binding site heterogeneity or true allosteric interactions between binding sites (Dalquist, 1978). The notorious instability of rubisco preparations (Hall et al., 1981) during storage may indicate a slow decay of competent activator sites, which could result in heterogeneity of Mg^{2+} sites. However, Hill coefficients of 1 with respect to the CO_2 dependence of activation are observed over a wide range of activation states (Figure 2). This implies that no heterogeneity exists with respect to the binding sites for this substrate, which has been proposed to be directly associated with the activator Mg^{2+} binding (Miziorko & Lorimer, 1981). Therefore, the observed negative cooperativity probably reflects a true allosteric interaction between the Mg^{2+} binding sites on rubisco.

The significance of negative Mg^{2+} cooperativity for the in vivo regulation of rubisco is difficult to determine at this time as various phosphorylated compounds have been shown to dramatically affect in vitro activation of the enzyme and may also be involved in vivo (Lorimer & Miziorko, 1983). Further investigation of the effects of these compounds on Mg^{2+} cooperativity in activation as well as exchange of activator CO_2 may provide useful information in this regard. However, if the light-induced changes in rubisco activation observed in vivo are only due to changes in stromal Mg^{2+} and pH (Miziorko & Lorimer, 1983), the presence of negative Mg^{2+} cooperativity implies that the activation of the enzyme is much less sensitive to such changes in Mg^{2+} than would otherwise be the case.

Registry No. CABP, 27442-42-8; CO_2, 124-38-9; Mg, 7439-95-4; HCO_3^-, 71-52-3; rubisco, 9027-23-0.

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