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Interaction of Sugar Phosphates with the Catalytic Site of Ribulose-1,5-bisphosphate Carboxylase†

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ABSTRACT: The activated and catalytically competent form of ribulose-1,5-bisphosphate carboxylase is a ternary complex of enzyme·activator CO_2·Mg. The effectors NADPH and 6-phosphogluconate promoted activation by formation of a rapid equilibrium quaternary complex of enzyme·activator CO_2·Mg·effector; i.e., the effectors did not activate the enzyme per se but promoted the basic activation process by stabilizing the activated enzyme·activator CO_2·Mg complex. Kinetic and gel filtration studies showed that the effectors stabilized the binding of the activator CO_2 and Mg^{2+} (or Mn^{2+}), thereby decreasing the rate of deactivation. Binding studies indicated the presence of one 6-phosphogluconate binding site per protomer. The binding of 6-phosphogluconate and NADPH to the enzyme·activator CO_2·Mg complex was (a) completely prevented when the catalytic site for ribulose bisphosphate was occupied by the transition-state analogue, 2-carboxyarabinitol 1,5-bisphosphate, and (b) competitively diminished in the presence of 3-phosphoglycerate, the product of the carboxylation reaction. NADPH, 6-phosphogluconate, and 3-phosphoglycerate acted as linear competitive inhibitors of carboxylation with respect to ribulose bisphosphate. These results demonstrate that the effectors elicit their response through interaction at the catalytic site for ribulose bisphosphate and that their effect is secondary to the basic CO_2-Mg^{2+}-dependent activation reaction. An enzyme molecule cannot be simultaneously catalytically competent (capable of binding and carboxylating ribulose bisphosphate) and activated by an effector, since the latter involves occupancy of the ribulose bisphosphate binding site.

Previous studies (Lorimer et al., 1976, 1977; Miziorko & Mildvan, 1974) established that the activation of ribulose 1,5-bisphosphate (RuBP)[1] carboxylase involves the ordered addition of CO_2 and Mg^{2+}, with the addition of CO_2 being the rate-determining step (eq 1). Kinetic turnover (Lorimer,

$$\underset{\text{inactive}}{E\text{–}NH_2} + {}^{A}CO_2 \underset{\pm H^+}{\overset{\text{slow}}{\rightleftharpoons}} E\text{–}NH\text{–}{}^{A}COO^- + Mg^{2+} \overset{\text{fast}}{\rightleftharpoons} \underset{\text{active}}{E\text{–}NH\text{–}{}^{A}COO^{-}\cdots Mg^{2+}} \quad (1)$$

1979) and CO_2 binding (Miziorko, 1979) experiments demonstrate that the activator carbon dioxide ($^{A}CO_2$)[2] is distinct from the substrate carbon dioxide ($^{S}CO_2$) which becomes fixed during carboxylation. $^{A}CO_2$ is bound to the enzyme in the form of a carbamate on the ϵ-amino group of a lysine residue located within the large (catalytic) subunit (Lorimer et al., 1976; O'Leary et al., 1979; Lorimer & Miziorko, 1980).

A number of investigators reported the stimulatory effects of a variety of sugar phosphates upon the activation process (Buchanan & Schürmann, 1973; Chu & Bassham, 1975; Ryan & Tolbert, 1975; Chollet & Anderson, 1976; Lendzian, 1978; Vater & Salnikow, 1979; Whitman et al., 1979). Two compounds in particular, 6-phosphogluconate and NADPH, stand out as being especially effective, and physiological significance has been attributed to the effects they elicit (Buchanan & Schürmann, 1973; Chu & Bassham, 1975). Various models have been advanced to explain these effects (Chu & Bassham, 1975; Vater & Salnikow, 1979). A common feature is the involvement of allosterism, the binding of the effector at one or more sites distinct from the catalytic site. In this report, we present kinetic and physical evidence that the effects of 6-phosphogluconate and NADPH are exerted through interaction at the catalytic site to which RuBP binds. A model which accounts for many of the rather diverse observations concerning the effects of various sugar phosphates on the activation of RuBP carboxylase is presented.

Experimental Procedures

Materials

RuBP carboxylase from spinach leaves was purified, activated, and assayed as previously described (Lorimer et al., 1976, 1977). Care was taken to ensure that contaminating enzymes such as phosphatases or NADPH oxidase were absent from the preparations of carboxylase used in this study. Enzyme protomer was determined at 280 nm by using an extinction coefficient of 1.15×10^5 M^{-1} cm^{-1} (Paulsen & Lane, 1966). Biochemicals were obtained from either Sigma

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[1] Abbreviations used: RuBP, ribulose 1,5-bisphosphate; CABP, 2-carboxyarabinitol 1,5-bisphosphate; Bicine, *N,N*-bis(2-hydroxyethyl)-glycine.

[2] The superscripts $^{A}CO_2$ and $^{S}CO_2$ are used to distinguish between the molecule of CO_2 involved in activation and that used as substrate, respectively.

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Chemical Corp. or Boehringer-Mannheim Co. Bio-Gel P-4 and ion-exchange resins were purchased from Bio-Rad Labs. Radiochemicals were supplied by either Amersham-Searle Co. or New England Nuclear Corp. Prochem US Services Inc. (Summit, NJ) was the source of the K[^{13}C]N. Other chemicals were of reagent grade or of the highest purity commercially available.

Methods

Synthesis of 2-Carboxyarabinitol 1,5-Bisphosphate. Both [2-^{13}C]- and [2-^{14}C]CABP were synthesized and purified by the method of Pierce et al. (1980). ^{13}C NMR indicated the CABP to be at least 95% pure.

Synthesis of 6-Phospho[^{14}C]gluconate. This was synthesized from [^{14}C]glucose by using hexokinase and glucose-6-phosphate dehydrogenase, with a phosphocreatine–creatine kinase ATP regenerating system. The progress of the reaction was followed at 340 nm. The change in absorbance at 340 nm was within 4% of that calculated on the basis of the quantity of glucose used, indicating the quantitative conversion to 6-phosphogluconate. The product was purified as its γ-lactone by using the same ion-exchange chromatography system as was used for the purification of CATP (Pierce et al., 1980). More than 90% of the ^{14}C applied to the column emerged as a single peak. This was pooled, adjusted to neutrality, concentrated under vacuum, and precipitated with $Ba(Ac)_2$ in 50% (v/v) ethanol. The barium salt was converted to the free acid form by treatment with Dowex 50, H^+ form, and adjusted to pH 8.0 with NH_4OH. This solution was held at room temperature for 24 h before use to ensure the complete hydrolysis of the γ-lactone.

The ^{14}C content of the 6-phosphogluconate was checked by scintillation counting and the quantity determined from the change in absorbance at 340 nm in the presence of $NADP^+$ and 6-phosphogluconate dehydrogenase. The ^{14}C specific activity of the 6-phosphogluconate determined in this manner was within 5% of the starting [^{14}C]glucose. These tests confirm the chemical and radiochemical purity of the 6-phospho[^{14}C]gluconate.

Activation Kinetics. These were performed essentially as described before (Lorimer et al., 1976, 1977).

Quantitative Binding Studies. When quantitative information was required, the gel filtration technique (Hummel & Dreyer, 1962; Ackers, 1973) was used. Aliquots (500 μL) of enzyme were equilibrated before being applied to a column (1.5 × ~15 cm) of Bio-Gel P-4 (200–400 mesh) equilibrated with the appropriate solution. The flow rate was about 30 mL h^{-1}. Fractions (1 mL) were collected. The peak of ligand enrichment and the trough of ligand depletion were within 5% of being equal. The peak and the trough were always clearly separated by a plateau region, confirming that the system had reached equilibrium. An example of the exact procedure followed is given in the legend to Figure 6A.

Qualitative Binding Studies. When it was desired merely to demonstrate that the effectors stabilize the binding of either [^{14}C]O_2 or ^{54}Mn to the enzyme (Table II), the following protocol was adopted. The enzyme was first incubated with either [^{14}C]O_2 and Mg^{2+} or CO_2 and $^{54}Mn^{2+}$ with or without the effector ligand of choice. After equilibration, the mixture was applied to a 1.5 × 15 cm column of Bio-Gel P-4 (200–400 mesh) equilibrated at 4 °C with 50 mM CO_2-free Bicine–NaOH, pH 8.2, with or without the appropriate effector. Elution of the enzyme was performed at a flow rate of about 30 mL h^{-1}.

Measurement of Radioactivity. ^{14}C radioactivity was determined by liquid scintillation counting by using the cross-channel ratio method to determine efficiency. ^{54}Mn radioactivity was determined with a γ solid scintillation counter. Counting efficiency was about 6%.

Other experimental details are included in the legends to the tables and figures.

Table I: Activation of RuBP Carboxylase: Effects of NADPH and 6-Phosphogluconate and Their Dependence upon CO_2 and Mg^{2+}

preincubation treatment (mM)[a]				
HCO_3^-	Mg^{2+}	6-PGlu	NADPH	% activation
10	20	0	0	100[b]
10	20	0.5	0	101.3
10	20	0	0.5	102.7
1	2	0	0	35.1
1	2	0.5	0	95.9
1	2	0	0.5	93.2
0	0	0	0	3.5
0	0	0.5	0	6.3
0	0	0	0.5	5.6
0	2	0	0	10.5
0	2	0.5	0	53.5
0	2	0	0.5	54.6
1	0	0	0	7.8
1	0	0.5	0	10.2
1	0	0	0.5	13.6

[a] Enzyme (192.5 μg or 2.75 nmol of protomer) was incubated for 15 min at 25 °C in 50 mM Bicine–NaOH, pH 8.2, containing the components listed above. A 5-μL aliquot of enzyme (19.25 μg) was added to 500 μL of reaction mixture to initiate carboxylation. Assay duration, 1 min at 25 °C. The effectors were therefore diluted 100-fold to a concentration of 5 μM. The use of 550 μM RuBP (25 times the K_m for RuBP) in the reaction mixture served to reduce any inhibitory effects of the effectors to negligible proportions. The reaction mixture contained 50 mM Bicine–NaOH, pH 8.2, 20 mM $MgCl_2$, 1.21 mM NaH[^{14}C]O_3, 550 μM RuBP, and ambient (250 μM) O_2. [b] 100% corresponds to 0.74 unit/mg of protein.

Results

Influence of NADPH and 6-Phosphogluconate on the Activation Reaction: Equilibrium Effects. In order to study the influence of the effectors on the activation reaction, two conditions must be satisfied. The assay of catalytic activity must be sufficiently short that the enzyme does not undergo a significant change in its activation state during the assay. The procedures used here have been previously shown to satisfy this requirement (Lorimer et al., 1976). Secondly, the effectors are linearly competitive inhibitors of catalysis with respect to RuBP (Figure 7). Therefore, the activation experiments had to be performed in a manner which enabled us to distinguish between the effects on activation and those on catalysis. By dilution of the effector at least 30-fold on introduction of it into the catalytic reaction mixture, and by use of saturating (0.3–0.5 mM) levels of RuBP (K_m = 20 μM) during catalysis, the inhibitory effects were minimized to a point where they could be ignored. This strategy presupposes that the equilibrium between the enzyme and the effector is rapidly established. The linearity of the time course of catalysis (data not shown) and the nature of the inhibition elicited by these effectors, rapid equilibrium competitive (Figure 7), suggest that this assumption is valid.

The enzyme was fully activated with CO_2 and Mg^{2+} (Lorimer et al.; Table I). Addition of any of the effectors to enzyme which was already fully activated had no effect (Table I). Only when the enzyme was suboptimally activated with CO_2 and Mg^{2+} did the addition of an effector increase the activation state of the enzyme (Table I). This response was dependent upon the presence of CO_2 and Mg^{2+} in the preincubation mixture. When either CO_2 or Mg^{2+} was

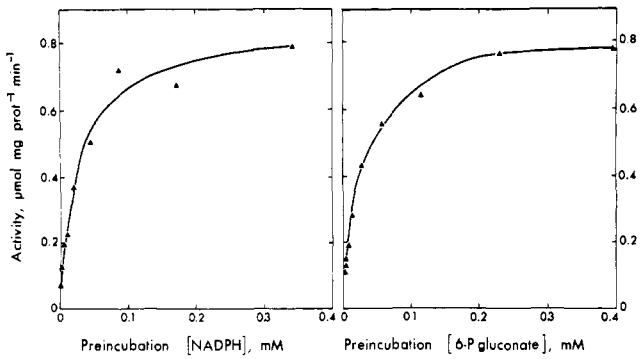

FIGURE 1: Effect of preincubation concentration of the effectors on the activation of RuBP carboxylase activity. Enzyme (0.1 mg/mL) was prepared in 100 mM Bicine, pH 7.8, 1 mM $MgCl_2$, and 1 mM $NaHCO_3$. Aliquots (10 μL) were placed in tapered 1-mL reaction vials, and 1 μL of the appropriate effector was added to bring the final concentration to that indicated in the figure. Enzyme was then incubated for 30 min at 25 °C, activity at this time was assayed by injection of 300 μL of reaction mixture [100 mM Bicine, pH 7.8, 20 mM $MgCl_2$, 1.0 mM $NaH[^{14}C]O_3$ (59 000 dpm/nmol), and 0.3 mM RuBP] equilibrated to 25 °C, and assays were then run for 20 s.

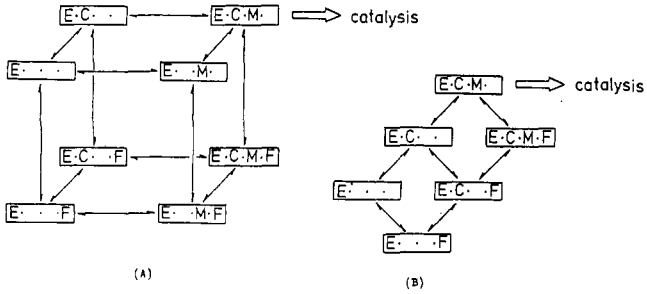

FIGURE 2: Pathways for the addition of activator CO_2 (C), Mg^{2+} (M), and effector (F) to the enzyme (E). (A) With no constraints upon the system, there is a maximum of six possible pathways from E··· to E·C·M·F. (B) with the constraint that C must add before M, the maximum number of possible pathways is reduced to three.

omitted, the enzyme did not fully respond to the effector (Table I). (The response in the absence of added CO_2 can be attributed to traces of contaminating CO_2.) The response of the activation reaction to varying concentrations of effector is shown in Figure 1. The results in Table I and Figure 1 are consistent with (but do not prove) the notion that the effectors elicit their response by displacing the equilibrium of the activation reaction to the right. That is, they do not activate the enzyme per se but achieve their effect by stabilizing the activated enzyme·activator CO_2·Mg complex.

There are six possible pathways by which CO_2, Mg, and effector can add to the enzyme to yield the quaternary complex of enzyme·$^{A}CO_2$·Mg·effector (Figure 2). However, kinetic (Lorimer et al., 1976) and spectroscopic (Miziorko & Mildvan, 1974) studies show that the metal ion reacts after the activator CO_2 has been bound. Therefore, the three pathways which involve Mg^{2+} binding before activator CO_2 can be disregarded. We are then left with the simplified scheme represented in Figure 2B.

Our results do not enable us to distinguish between the three remaining pathways. Binding studies with 6-phospho[^{14}C]-gluconate and NADPH reveal that both ligands can bind to the deactivated enzyme (data not shown). We have previously shown that the rate-determining step in activation is the addition of $^{A}CO_2$ (Lorimer et al., 1976). The presence of either 6-phosphogluconate or NADPH did not alter the initial rate of activation (Figure 3); i.e., the addition of $^{A}CO_2$ remains rate determining. Such studies do not rule out the possibility that the formation of the enzyme·effector complex precedes the

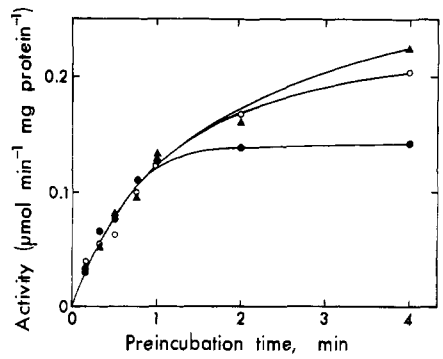

FIGURE 3: Effect of 6-phosphogluconate and NADPH on the initial rate of activation. At time zero, 2 μL of a solution containing 10 mM $NaHCO_3$, 50 mM $MgCl_2$, and, where appropriate, 5 mM NADPH or 1.25 mM 6-phosphogluconate was added to 15 μL of enzyme (8.2 mg/mL) in 100 mM Bicine–NaOH, pH 7.8. The final concentrations in the preincubation mixture were as follows: enzyme protomer, 103 μM; NADPH, 530 μM; 6-phosphogluconate, 130 μM. At the times indicated, 300 μL of reaction mixture (100 mM Bicine–NaOH, pH 7.8, 20 mM $MgCl_2$, 0.83 mM $NaH[^{14}C]O_3$, 0.4 mM RuBP) was injected, and assays were run for 30 s at 10 °C. Assays were terminated with 200 μL (10% v/v) of formic acid. Control (●), NADPH (○), 6-phosphogluconate (▲).

reaction with $^{A}CO_2$. However, they do show that the addition of $^{A}CO_2$ is not influenced by the presence of the effector. Since they do not influence the forward reaction, the ability of the effectors to displace the equilibrium of the activation reaction to the right must be due to their inhibitory effect on the reverse reaction.

Influence of NADPH and 6-Phosphogluconate on the Stability of the Enzyme·$^{A}CO_2$·Mg Complex: Kinetic Studies. The key step in the deactivation process is the dissociation of $^{A}CO_2$. There is evidence (to be introduced later) which suggests that $^{A}CO_2$ is not free to dissociate from either the enzyme·$^{A}CO_2$·Mg or the enzyme·$^{A}CO_2$·Mg·effector complexes. Thus, it was to be anticipated that compounds which form addition complexes with enzyme·$^{A}CO_2$ and enzyme·$^{A}CO_2$·Mg would reduce the steady-state concentration of free E·$^{A}CO_2$, and hence slow the rate at which deactivation occurs. The rate of deactivation was determined by diluting activated enzyme into Mg^{2+}-free solution with or without an effector. At various times thereafter, Mg^{2+} and RuBP were added to initiate catalysis. As predicted, the presence of an effector decreased the rate of deactivation considerably (Figure 4).

Influence of NADPH and 6-Phosphogluconate on the Stability of the Enzyme·$^{A}CO_2$·Mn Complex: Qualitative Gel Filtration Studies. The capacity of the effectors to stabilize the binding of both $^{A}CO_2$ and Mn^{2+} (substituting for Mg^{2+}) to the enzyme was demonstrated by the experiments described in Table II. Activated enzyme, in the form of either enzyme·$^{A}[^{14}C]O_2$·Mg·effector or enzyme·$^{A}CO_2$·^{54}Mn·effector, was applied to gel filtration columns equilibrated with CO_2-free buffer containing the appropriate effector. In the control, run in the absence of effector, very little radioactivity (^{14}C or ^{54}Mn) was associated with the protein peak. However, in the presence of an effector, significant quantities of both ^{14}C and ^{54}Mn radioactivity remained associated with the protein; 0.75 mol of $^{A}[^{14}C]O_2$/mol of protomer and 0.35 mol of ^{54}Mn/mol of protomer in the case of 6-phosphogluconate, 0.13 mol of ^{A}-$[^{14}C]O_2$/mol of protomer in the case of NADPH. That less than stoichiometric quantities were recovered was anticipated since some dissociation of the radioactive ligands from the enzyme was expected under these conditions.

In a later section, it will be demonstrated that the effectors interact at a single site, the catalytic site for RuBP binding. For present purposes, however, we demonstrate how effectively

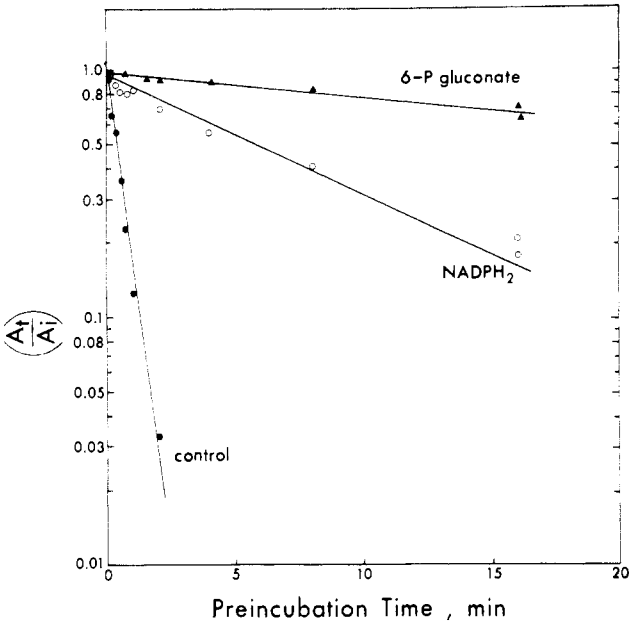

FIGURE 4: Effector-dependent inhibition of the deactivation of RuBP carboxylase. Enzyme (about 100 μM protomer) was first activated in 50 mM Bicine–NaOH, pH 8.2, 20 mM $MgCl_2$, and 5 mM $NaHCO_3$. At time zero, 5 μL (about 35 μg of enzyme) of this solution was injected into 400 μL of 0.1 M Bicine–NaOH, pH 7.8, 1 mM EDTA, and 0.6 mM $NaH[^{14}C]O_3$ at 10 °C. Where appropriate, this mixture contained 6-phosphogluconate (0.1 mM) or NADPH (0.2 mM). At the indicated times, 10 μL of 20 mM RuBP was introduced to determine the activity remaining at that time. Assays were run for 30 s before stopping with 0.2 mL of 2 N formic acid. A_t = activity at time t; A_i = initial activity.

Table II: Stabilizing Influence of Effectors on the Binding of $^{A}[^{14}C]O_2$ and $^{54}Mn^{2+}$ to the Enzyme[a]

effector (mM)	mol of ligand/mol of protomer	
	$[^{14}C]O_2$	^{54}Mn
none	<0.02	<0.05
6-phosphogluconate (0.5)	0.75	0.35
NADPH (0.5)	0.13	ND[b]
CABP[c]	1.04	1.03

[a] Enzyme (120 nmol of protomer) was first incubated at about 23 °C for 15 min in 50 mM Bicine–NaOH, pH 8.2, containing either 10 mM $NaH[^{14}C]O_3$ and 20 mM $MgCl_2$ or 10 mM $NaHCO_3$ and 20 mM $[^{54}Mn]Cl_2$ with the various effectors listed above. The solution was then chilled on ice and applied to a 1.5 × 20 cm column of Bio-Gel P-4 equilibrated at 4 °C with 50 mM Bicine–NaOH, pH 8.2, containing the effector at the concentration listed above. The protein peak was well separated from the unbound radioactive ligand. [b] Not determined. [c] In this case, the enzyme·$^{A}[^{14}C]O_2$·Mg or enzyme·$^{A}CO_2$·^{54}Mn complexes were incubated with an approximately 10-fold molar excess of CABP for 60 min, before being applied to a column equilibrated with 50 mM Bicine–NaOH, pH 8.2. The presence of CABP in the column buffer is not necessary, so tight is the binding of CABP to the enzyme.

ligands, which interact at the catalytic site, stabilize the binding of both $^{A}CO_2$ and Mn^{2+}. The transition-state analogue 2-carboxyarabinitol 1,5-bisphosphate (CABP) is ideal for this purpose since it binds most avidly ($K_d = 10^{-11}$ M) (Pierce et al., 1980). As Table II shows, occupancy of the catalytic site with CABP "locks in" stoichiometric quantities of both $^{A}CO_2$ and Mn^{2+}, so that neither was free to dissociate nor to undergo exchange with free ligand (Miziorko, 1979; Miziorko & Sealy, 1980). By analogy, we propose that the effectors act in a similar manner. However, unlike CABP, which dissociates from the enzyme very slowly if at all (Pierce et al., 1980), the effectors are in rapid equilibrium, such that upon being diluted

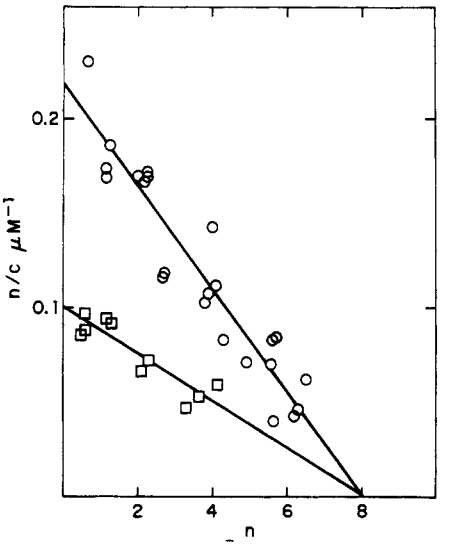

FIGURE 5: Scatchard plot demonstrating the binding of 6-phospho-$[^{14}C]$gluconate to the enzyme·$^{A}CO_2$·Mg complex. The data were obtained by running a series of experiments similar to that outlined in Figure 6A, using different concentrations of 6-phosphogluconate. The open circles indicate the data obtained in the absence of competing ligands while the open squares were obtained in the presence of 2 mM 3-phosphoglycerate. The lines were obtained from a regression analysis of the experimental data and were not forced to intersect the horizontal axis at $n = 8$. n = mol of 6-phosphogluconate bound/mol of enzyme; c = μM concentration of free 6-phosphogluconate. K_d for 6-phosphogluconate = 36.9 μM; K_d for 3-phosphoglycerate (PGA) = 1.2 mM.

into the catalytic reaction solution they readily dissociate to leave the activated complex, enzyme·$^{A}CO_2$·Mg.

Interaction of NADPH and 6-Phosphogluconate with the Catalytic Site: Competitive Binding Studies. The results of the 6-phosphogluconate binding experiments (Figure 5) indicated the presence of eight 6-phosphogluconate binding sites per enzyme or one per protomer (the 70 000-dalton species containing one large and one small subunit). With the stoichiometry of 6-phosphogluconate binding defined, competitive binding studies were performed with a view to defining the site of effector binding. CABP forms a very stable quaternary complex of enzyme·$^{A}CO_2$·Mg·CABP with a protomer-based stoichiometry of 1:1:1:1 (Miziorko, 1979; Miziorko & Sealy, 1980). It is reasonable to assume that this transition-state analogue binds at the catalytic site. Likewise, 3-phosphoglycerate, the product of carboxylation, may also be assumed to bind to the catalytic site. These two ligands, which differ in their affinity for the enzyme by 7–8 orders of magnitude, therefore represent excellent competitive ligands with which to challenge the binding of the effectors. Occupancy of the catalytic site with CABP completely abolished the binding of the effectors (Figure 6B). Although the binding of 3-phosphoglycerate is much weaker, it also displaced 6-phosphogluconate from the enzyme in a competitive manner (Figure 5). The binding of NADPH (Figure 6C) was also completely abolished when the catalytic site was occupied by CABP (Figure 6D). The results of these competitive binding experiments strongly suggest that the single site per protomer for effector binding and the catalytic site are one and the same.

Interaction of NADPH and 6-Phosphogluconate with the Catalytic Site: Competitive Kinetics. The conclusion that the effectors interact at the catalytic site was reinforced by the outcome of a kinetic analysis of the influence of the effectors on catalysis. The pattern of inhibition is linearly competitive with respect to RuBP, indicating competition for a common site. The experimental results for NADPH and 3-phospho-

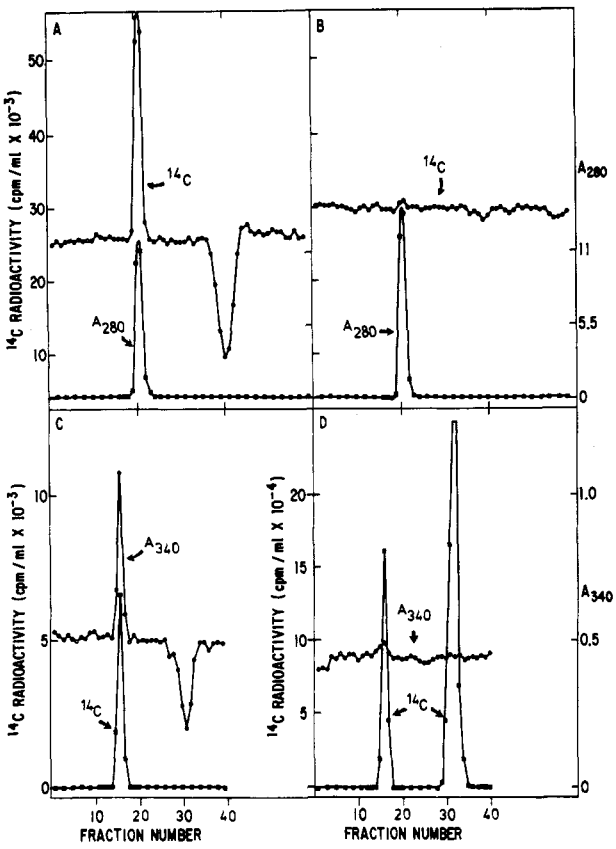

FIGURE 6: Binding of (A) 6-phosphogluconate and (C) NADPH to the enzyme·$^{A}CO_2$·Mg complex and the absence of binding of (B) 6-phosphogluconate and (D) NADPH to the enzyme·$^{A}CO_2$·Mg·CABP complex. (A) To about 200 nmol of enzyme protomer in 50 mM Bicine–NaOH, pH 8.2, 10 mM $NaHCO_3$, and 20 mM $MgCl_2$ was added sufficient 6-phospho[^{14}C]gluconate (453 dpm/nmol) to give a final concentration of 67.5 μM. The solution was incubated at 23 °C for about 15 min and then applied to a 1.5 × 23 cm column of Bio-Gel P4 (200–400 mesh) equilibrated with 50 mM Bicine–NaOH, pH 8.2, 10 mM $NaHCO_3$, 20 mM $MgCl_2$, and 67.3 μM 6-phospho[^{14}C]gluconate. Flow rate = 30 mL/h; fraction size = 1 mL. (B) To about 200 nmol of enzyme protomer in 50 mM Bicine–NaOH, pH 8.2, 10 mM $NaHCO_3$, and 20 mM $MgCl_2$ was added 2 μmol of CABP, and the mixture was incubated for 60 min at 23 °C. 6-Phospho[^{14}C]gluconate was then added to give a final concentration of 75.2 μM. After 15 min at 23 °C, the enzyme was filtered as in (A). The concentration of free 6-phospho[^{14}C]gluconate on the column was 74.7 μM. (C) A 46-nmol aliquot of the enzyme·$^{A}CO_2$·Mg·[^{14}C]CABP complex obtained from (D) was mixed with about 250 nmol of the enzyme·$^{A}CO_2$·Mg complex in 50 mM Bicine–NaOH, pH 8.2, 10 mM $NaHCO_3$, and 20 mM $MgCl_2$. The overall radiospecific activity was then determined to be 42.2 dpm of ^{14}C/nmol of enzyme protomer. (This value was used to compute the protomer content of the fractions eluting from the column.) To this enzyme solution was then added sufficient NADPH so that the A_{340} of the solution was 0.505, a value which corresponded to the A_{340} of the solution used to equilibrate the column. In addition to NADPH (A_{340} = 0.505 ≡ 81.2 μM), the column buffer contained 50 mM Bicine–NaOH, pH 8.2, 10 mM $NaHCO_3$, and 20 mM $MgCl_2$. A 1-mL sample of the enzyme solution (270 nmol of protomer) was applied to the column (1.5 × 20 cm). Flow rate = 30 mL/h; fraction size = 1 mL. (D) A 260-nmol aliquot of enzyme protomer in 50 mM Bicine–NaOH, pH 8.2, 10 mM $NaHCO_3$, and 20 mM $MgCl_2$ was incubated with 2.87 μmol of [^{14}C]CABP (3890 dpm/nmol) for 60 min at 23 °C. Then sufficient NADPH was added to give an A_{340} value of 0.45 (72.3 μM). An aliquot of this mixture containing 161 nmol of protomer was then applied to a 1.5 × 20 cm column of Bio-Gel P-4 equilibrated with 50 mM Bicine–NaOH, pH 8.2, 10 mM $NaHCO_3$, 20 mM $MgCl_2$, and 72.3 μM NADPH (A_{340} = 0.45). Flow rate = 30 mL/h; fraction size = 1 mL. The second peak of radioactivity corresponds to unbound [^{14}C]CABP. The quantity of ^{14}C radioactivity recovered in the first peak indicates the presence of the enzyme·$^{A}CO_2$·Mg·CABP complex with a 1:1 CABP/protomer stoichiometry.

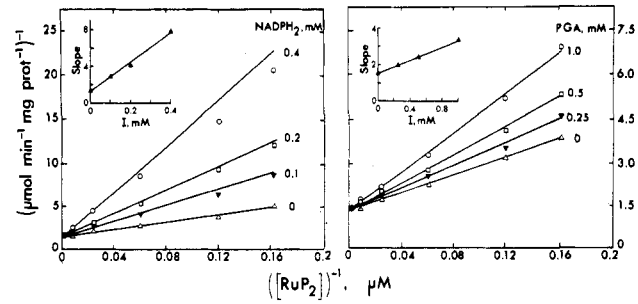

FIGURE 7: Inhibition of RuBP carboxylase activity with respect to RuBP, NADPH, and 3-phosphoglycerate. Assays were run in 1-mL stoppered reaction vials and contained a total volume of 400 μL of reaction mixture [100 mM Bicine, pH 7.8, 20 mM $MgCl_2$, and 1.50 mM $NaH[^{14}C]O_3$ (5500 dpm/nmol)] and indicated levels of RuBP and inhibitor. Reactions were initiated with 5 μL of activated enzyme, were run for 30 s at 25 °C, and were stopped with 0.2 mL of 2 N formic acid. Both reaction vials and reaction mix were flushed with N_2. Data are presented as double-reciprocal plots, with lines drawn according to the method of Wilkinson (1961). Apparent K_i values were determined from replots of slope vs. inhibitor concentration.

Table III: Competitive Inhibitors with Respect to RuBP (K_m = 20 μM)

inhibitor	K_i (μM)	ref
2-carboxyarabinitol 1,5-bisphosphate[a]	0.4	Pierce et al. (1980)
2-carboxyribitol 1,5-bisphosphate	1.5	Pierce et al. (1980)
xylulose 1,5-bisphosphate	5	McCurry & Tolbert (1977)
2-(hydroxymethyl)arabinitol 1,5-bisphosphate	5	Pierce et al. (1980)
6-phosphogluconate	8.5	this report; also Chu & Bassham (1975)
fructose 1,6-bisphosphate	40	this report; also Chu & Bassham (1975)
NADPH	70	Figure 7, this report
2-(hydroxymethyl)ribitol 1,5-bisphosphate	80	Pierce et al. (1980)
sedoheptulose 1,7-bisphosphate	75	Salujah & McFadden (1978)
3-phosphoglycerate	840	Figure 7, this report; also Paulsen & Lane (1966)
inorganic phosphate	900	this report; also Paulsen & Lane (1966)
sulfate	630	this report; also Paulsen & Lane (1966)

[a] The binding of CABP involves the reversible formation of an enzyme·CABP complex, followed by a slow, almost irreversible, conformational change to an enzyme*·CABP complex (Pierce et al., 1980): $E + I \rightleftharpoons EI \rightleftharpoons E^*{\cdot}I$ (overall $K_d \leq 10^{-11}$ M).

glycerate inhibition are shown in Figure 7 as they have not previously been reported. The remaining data are presented in Table III along with references to previous reports of the inhibitory nature of these compounds. 3-Phosphoglycerate was previously reported to be a noncompetitive inhibitor with respect to RuBP (Paulsen & Lane, 1966). However, this was before the need to fully activate the enzyme before assay was appreciated (Lorimer et al., 1976). Our observation that it is a linearly competitive inhibitor with respect to RuBP is more in line with the behavior to be expected of a reaction product which must willy-nilly occupy the same site as the substrate.

Discussion

The experimental evidence reported here permits two major conclusions concerning the role of effectors in the activation of RuBP carboxylase: (1) *The effectors elicit their response through interaction with the enzyme at the catalytic site to which RuBP binds.* (2) *The effectors do not activate the*

enzyme per se but rather influence the basic activation reaction involving CO_2 and Mg^{2+} by stabilizing the binding of these ligands to the enzyme.

On the strength of these conclusions, a model has been constructed which goes some way toward accounting for many of the rather diverse observations in the literature and which provides a framework for further discussion (Figure 2B).

The basic activation process involves the ordered addition to the enzyme of activator CO_2 with the formation of a carbamate on the ϵ-amino group of lysine residue 202 (Lorimer, 1981). This represents the rate-limiting step and is followed by the rapid addition of Mg^{2+} to form the catalytically competent ternary complex of enzyme·$^{A}CO_2$·Mg. Let us now consider the consequences of occupying the catalytic site for RuBP binding with each of the following molecules.

(a) *RuBP*: Evidence has recently been presented indicating that the binding of RuBP to the enzyme·$^{A}CO_2$·Mg complex constitutes the first step in the catalytic cycle (Badger et al., 1980). Since the enzyme cannot undergo deactivation while engaged in catalysis, one would anticipate that RuBP would stabilize the activated enzyme (i.e., slow the rate of deactivation). There is experimental evidence for this interpretation (Laing & Christeller, 1976; Badger et al., 1980). However, the influence of RuBP on the activation reaction is complicated by the fact that RuBP is also capable of binding to the deactivated enzyme (Wishnick et al., 1970). As a consequence of this formation of the enzyme·RuBP complex, the rate of activation is decreased (Laing & Christeller, 1976). The effect of RuBP upon the position of equilibrium between the activated and deactivated enzyme cannot be determined experimentally since equilibrium can never be established in the presence of any activated enzyme. However, in an imaginary experiment with constant concentrations of CO_2 and RuBP, the position of equilibrium will clearly be influenced by the relative affinities of the activated and deactivated forms of the enzyme for RuBP.

(b) *CABP*: The binding of CABP to the activated enzyme·$^{A}CO_2$·Mg complex has recently been investigated by Pierce et al. (1980). The reversible formation of an enzyme·$^{A}CO_2$·Mg·CABP complex is evidently followed by a slower conformational change to *enzyme*·$^{A}CO_2$·Mg·CABP, where enzyme and *enzyme* represent different conformers. At least on the time scale presently under consideration, the formation of this latter complex can be regarded as irreversible. Clearly, from the data reported here and elsewhere (Miziorko, 1979; Miziorko & Sealy, 1980), the binding of CABP to enzyme·$^{A}CO_2$·Mg considerably enhances the affinity with which both the activator CO_2 and Mg^{2+} (or Mn^{2+}) are bound to the enzyme. Thus, the quaternary complex of *enzyme*·$^{A}CO_2$·Mg·CABP may be regarded as activated but catalytically incompetent.

(c) *Effectors*: Reaction of effectors (F), such as NADPH and 6-phosphogluconate, with the activated enzyme also results in the formation of an activated but catalytically incompetent quaternary complex of enzyme·$^{A}CO_2$·Mg·F. Just as the binding of CABP to the activated enzyme increases the affinity with which the activator CO_2 and Mg^{2+} are bound, so too does the binding of the effectors. The kinetic and binding studies reported here demonstrate this point. However, unlike CABP, the effectors are *reversibly* bound to the activated enzyme. Thus, when the equilibrium is perturbed, such as when the enzyme·$^{A}CO_2$·Mg·F complex is diluted into the carboxylation reaction mixture, dissociation to the activated enzyme·$^{A}CO_2$·Mg complex rapidly occurs.

The above discussion provides an explanation for both the stimulatory and the inhibitory effects elicited by compounds such as NADPH and 6-phosphogluconate without invoking multiple sites of interaction (Chu & Bassham, 1975; Ryan & Tolbert, 1975).

Stimulatory effects are almost invariably associated with the preincubation of the enzyme with the effector. Carboxylation is then initiated by the dilution of a small aliquot of enzyme plus activator into the reaction mixture. On the other hand, when the enzyme is preincubated with the effector and carboxylation is initiated by the addition of a small aliquot of RuBP, essentially no dilution of the effector occurs. Then, competitive inhibition of carboxylation ensues, depending on the concentrations of RuBP and the effector. The important point here is that the stimulatory and inhibitory effects are simply different facets of the same phenomenon—the binding of the effector to the RuBP binding site. It is also evident from the foregoing discussion that more attention must be paid to the avoidance of what are after all simple order of addition and dilution effects.

The possibility also exists that, like RuBP, the effectors might also interact with the deactivated enzyme. In this case, the final position of equilibrium will depend very much upon the relative affinities of the deactivated and activated enzyme for the effector in question. For NADPH and 6-phosphogluconate, the equilibrium is clearly displaced in favor of the activated enzyme. Therefore, we infer that the activated enzyme binds these effectors more avidly than does the deactivated enzyme.

Hatch & Jensen (1980) have recently classified the various effectors into two groups. Positive effectors, such as NADPH and 6-phosphogluconate, promote the activation reaction. Negative effectors, such as ribose 5-phosphate and fructose 6-phosphate, favor deactivation of the enzyme. Our scheme (Figure 2B) provides an explanation for these diverse responses without invoking interaction at multiple sites. It is axiomatic that compounds which promote activation must bind more tightly to the activated form of the enzyme, the enzyme·$^{A}CO_2$·Mg complex, than to the deactivated form. Conversely, those compounds which inhibit activation must bind more tightly to the deactivated form. *It is the ratio of the dissociation constants describing the binding of the effector to the activated and deactivated forms which determines whether a given effector promotes or inhibits activation, and the degree to which it does so.* Let us consider the responses to two hypothetical rapid equilibrium effectors, which differ from one another in being differentially bound to the activated and deactivated forms of the enzyme. Consider first the effector α, which binds infinitely more tightly to the activated form than to the deactivated form. It will therefore be both an efficient promoter of activation and a competitive inhibitor (vs. RuBP) of catalysis. Consider now the effector β, which binds infinitely more tightly to the deactivated form than to the activated form. It will therefore promote deactivation. But since β does not bind to the activated form, it will not behave as a competitive inhibitor of catalysis. Of course, the responses of α and β represent the extremes. A third effector, γ, might bind equally tightly to the activated and deactivated forms. In this case, γ will neither promote nor inhibit activation. But it will behave as a competitive inhibitor (vs. RuBP) of catalysis on account of its ability to bind to the activated form of the enzyme. One can readily conceive of other effectors intermediate between α and γ [positive effectors in the language of Hatch & Jensen (1980)] and between γ and β (negative effectors). Thus, a very wide variety of responses can be

elicited by using a single site of interaction and two forms of the enzyme, activated and deactivated.

The fluorescence studies (Wildner, 1976; Vater & Salnikow, 1979) as well as the kinetic study of the interaction of enzyme·$^{A}CO_2$·Mg with CABP (Pierce et al., 1980) provide evidence for conformational change(s) in the enzyme structure upon binding the various effector ligands. We do not dispute that such changes occur, and a more complete analysis would have to take them into account. The occurrence of such conformational changes does not alter the validity of the two major conclusions of the present study.

The results reported here are not without physiological significance. If one subjects the enzyme in vitro to the conditions believed to apply in vivo (about 10 μM CO_2, 5–10 mM Mg^{2+}, pH about 8.0), it remains substantially in the deactivated state. It is by no means clear how the enzyme is maintained in a substantially activated state in vivo (Lorimer et al., 1978). Clearly, effectors such as NADPH and 6-phosphogluconate stabilize the activated form of the enzyme, and it is at least conceivable that they function in vivo in this manner. However, an enzyme molecule cannot be simultaneously catalytically competent (capable of binding and carboxylating RuBP) and activatedby effector since the latter involves occupancy of the RuBP binding site. Further work aimed at resolving this in vivo problem is in progress.

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