microsomal membrane enzyme [i.e., HMG CoA reductase (Goldfarb, 1978)].

Whether estradiol and/or Triton acts on 7α-hydroxylase via changes in microsomal membrane lipid structure in a manner analogous to that found for their effects on Na^+,K^+-ATPase (Davis et al., 1976b) remains to be proven. However, our results showing a direct effect of these agents on microsomal 7α-hydroxylase provide valuable information toward being able to control bile acid synthesis and cholesterol homeostasis.

Registry No. Estradiol, 57-63-6; 7α-hydroxylase, 9037-53-0; Triton, 25301-02-4; cholesterol, 57-88-5.

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Kinetic Mechanism of Ribulosebisphosphate Carboxylase: Evidence for an Ordered, Sequential Reaction

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ABSTRACT: NMR measurements and isotope-trapping experiments indicate that activated ribulosebisphosphate carboxylase binds bicarbonate anion but *not* carbon dioxide in the absence of ribulose bisphosphate. In the presence of suboptimal amounts of carbon dioxide, the enzyme also catalyzes the exchange of H-3 of RuBP with solvent faster than it catalyzes product formation. At saturating concentrations of carbon dioxide, the rate of exchange is dramatically reduced. These results indicate that the carboxylation of ribulose bisphosphate proceeds via the ordered addition and enolization of ribulose bisphosphate followed by reaction with the gaseous substrate.

Ribulosebisphosphate carboxylase/oxygenase (RuBP carboxylase)[1] catalyzes two reactions, the oxygenation and carboxylation of RuBP, which are the initial steps in the competing metabolic pathways of photorespiration and photosynthesis in higher plants. A variety of evidence suggests that the relative flux of carbon through these pathways may well determine the rate of plant growth in a wide variety of agronomically useful plant species. Accordingly, the manipulation of the kinetic properties of RuBP carboxylase is often viewed as a potentially attractive means for increasing the relative rate of photosynthesis relative to that of photorespiration.

Jordan and Ogren have shown that the specificity of RuBP carboxylase for CO_2 and O_2 is species-specific (Jordan &

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[1] Abbreviations: RuBP, ribulose 1,5-bisphosphate; XuBP, xylulose 1,5-bisphosphate; CABP, 2-*C*-(phosphohydroxymethyl)-D-ribonic acid 5-phosphate (carboxyarabinitol bisphosphate); PGA, glyceric acid 3-phosphate; Tris, tris(hydroxymethyl)aminomethane; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

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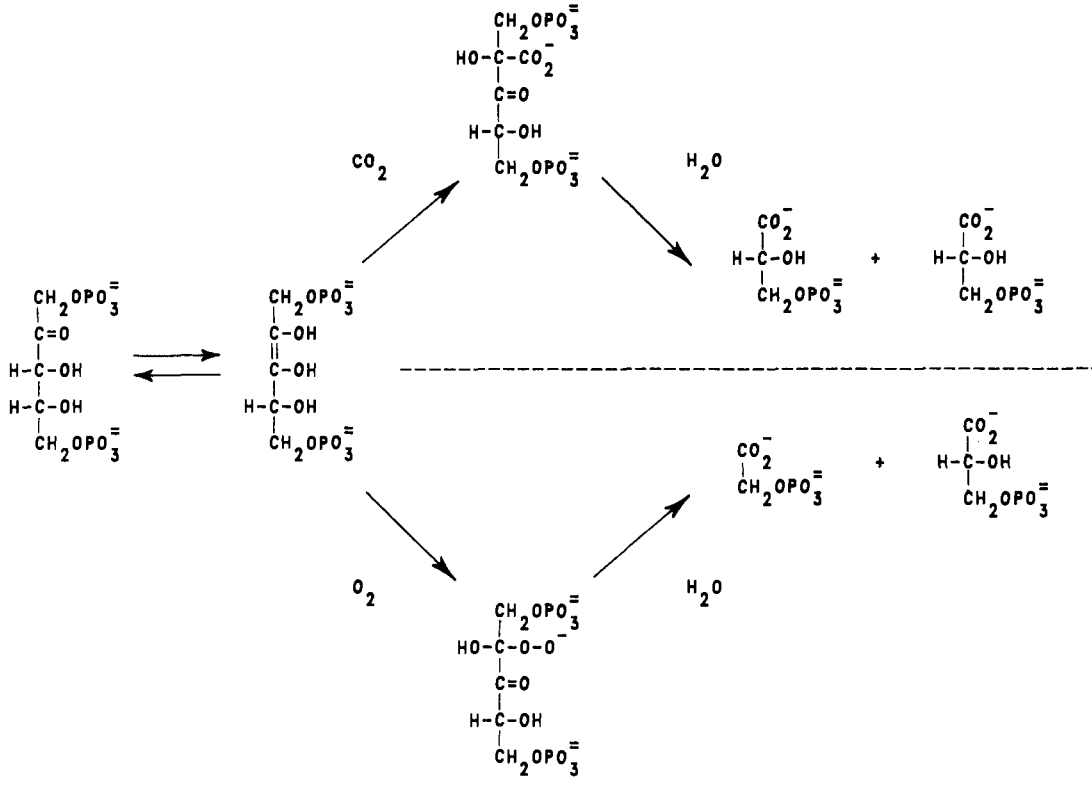
Scheme I



Ogren, 1981, 1983). That is, the ratio of carboxylation to oxygenation at identical CO_2 and O_2 concentrations varies from ~9 for photosynthetic bacterial RuBP carboxylases to ~80 for enzymes from higher plant species. It appears as if the most sensitive parameter associated with changes in the enzyme's specificity is $K_m(CO_2)$. However, it is not clear whether the values for $K_m(CO_2)$ are determined by any specific CO_2 binding step or whether the Michaelis constant for CO_2 is wholly determined by kinetic constraints. The same considerations apply to the apparent affinity of the enzyme for O_2.

We may briefly summarize our understanding of the processes involved in the oxygenation and carboxylation of RuBP. The enzyme is rendered catalytically active upon reaction with CO_2 to form a carbamate residue (Lorimer & Miziorko, 1980), which is in close proximity to the catalytically essential, divalent metal atom (Pierce & Reddy, 1985). The active, enzyme–carbamate–metal complex catalyzes the carboxylation reaction via the intermediacy of the *enol* form of RuBP (Saver & Knowles, 1982; Jaworowski et al., 1984) and a carboxylated 6-carbon β-keto acid intermediate (Schloss & Lorimer, 1982) deriving from RuBP and an additional CO_2 molecule (Scheme I). This intermediate is stabilized via interaction with the metal ion (Miziorko & Sealy, 1984) and is subsequently converted to two molecules of glycerate 3-phosphate (PGA), by the stereospecific hydrolysis of the C-2–C-3 bond of the 6-carbon intermediate (Pierce et al., 1980). A similar, though much more speculative mechanism has been proposed for the oxygenase reaction (Lorimer et al., 1973) wherein the formation and subsequent hydrolysis of a 5-carbon hydroperoxide intermediate (Scheme I) results in the formation of PGA and 2-phosphoglycolate.

The above physical depiction of reaction intermediates appears rather disjointed when viewed in kinetic terms. Although there is general agreement that the steady-state reaction mechanism is sequential, a number of studies have fallen short of establishing whether substrate addition is ordered or random. Steady-state kinetic analyses (Badger & Collatz, 1978) and product inhibition studies (Laing & Christeller, 1980) tend to favor a random mechanism. Contrarily, an ordered mechanism is most consistent with the finding that the ratio of a given enzyme's specifity for its substrates (the ratio of V_{max}/K_m values for the carboxylase and oxygenase reactions, i.e., $[V_c/K_c]/[V_o/K_o]$) is constant over a wide range of CO_2, O_2, H^+, and RuBP concentrations (Jordan & Ogren, 1984). The true reaction progression is of particular interest for studies aimed at understanding how the relative rates of carboxylation and oxygenation vary from species to species, and critical questions remain unanswered. Is gaseous substrate binding random or ordered with respect to RuBP binding? If Michaelis complexes are formed with the gaseous substrates, are the relative binding affinities of CO_2 and O_2 altered in enzymes from different species?

The availability of RuBP carboxylase enzymes with differing kinetic properties provides an opportunity for comparative studies aimed at answering these questions. In this study, we have analyzed the gaseous substrate affinities for the spinach enzyme [$K_m(CO_2)$ = 14 μM] and a modified enzyme from *Rhodospirillum rubrum* [$K_m(CO_2)$ = 89 μM] and the effects of the gaseous substrates on a partial enolization reaction catalyzed by these enzymes. Evidence for a sequential and ordered mechanism for substrate binding to RuBP carboxylase is presented, and implications of this mechanism are discussed.

Materials and Methods

Compounds and Enzymes. Published procedures were used in preparing RuBP (Weissbach et al., 1956), XuBP (McCurry & Tolbert, 1977), and CABP (Pierce et al., 1980). Sodium [^{13}C]bicarbonate (90 atom % ^{13}C) was from B.O.C. Limited, U.K., and sodium [^{14}C]bicarbonate was from New England Nuclear. RuBP carboxylase from spinach was prepared as previously described (Ryan & Tolbert, 1975). RuBP carboxylase from *Rhodospirillum rubrum* was obtained from a recombinant *Escherichia coli* strain (Somerville & Somerville,

1984), grown in large-scale culture (Pierce & Gutteridge, 1985), and purified according to Pierce and Reddy (1985). [The enzyme obtained from this preparation differs from the naturally isolated *R. rubrum* enzyme by containing an extra 25 amino acid polypeptide (Met-Thr-Met-Ile-Thr-Asn-Ser-Pro-Asp-Arg-Trp-Gly-Tyr-Ser-Ala-Pro-His-Arg-Thr-Ser-Arg-Glu-Ser-Pro-Pro) at its amino terminus. This extra polypeptide results from an in-frame fusion of a portion of the 5′ noncoding region of the *R. rubrum* carboxylase gene with the gene for β-galactosidase, the resulting fusion protein being under control of the *lac* promoter from *E. coli*. Thus, the *E. coli* derived protein is a dimer of 53 000-dalton subunits. However, its specific activity and catalytic constants are essentially indistinguishable from authentic *R. rubrum* enzyme [cf. Gutteridge et al. (1984b) and Jordan & Ogren (1981)]. Henceforth, we will refer to this protein simply as *R. rubrum* carboxylase.] Purified preparations of RuBP carboxylase were stored in 0.1 M K^+-Bicine buffer (pH 8.0) containing 1 mM dithiothreitol and 20% (v/v) glycerol as frozen peas at −80 °C.

^{13}C NMR Measurements of CO_2 and HCO_3^- Binding. Either spinach or *R. rubrum* RuBP carboxylase was exhaustively dialyzed against 0.2 M Tris-HCl buffer (pH 7.5, previously freed of metal ions by passage over Chelex resin). Solutions containing $NaH^{13}CO_3$ (90 atom % ^{13}C) and $MnCl_2$ were then added to give desired concentrations. NMR measurements (90 MHz) were performed at 25 °C in 20-mm tubes fitted with a capillary insert (containing ^2H_2O for field-frequency locking) so that there was no gaseous space over the solution. Longitudinal relaxation rates ($1/T_1$) and CO_2–HCO_3^- exchange rates were determined by magnetization-transfer NMR spectroscopy with either selective inversion or selective presaturation protocols (Campbell et al., 1978) as well as by conventional, nonselective inversion-recovery techniques. (The apparent relaxation rate of HCO_3^- is hardly affected by CO_2–HCO_3^- exchange primarily because of the preponderance of HCO_3^- over CO_2 in the solutions studied. That is, nonselective inversion-recovery experiments and selective inversion-recovery experiments gave identical recovery gradients for the HCO_3^- resonance. Conversely, the *apparent* relaxation rate of CO_2 contains contributions from both the intrinsic relaxation rate and the rate of CO_2–HCO_3^- exchange. Therefore, selective inversion and selective presaturation protocols were required to determine the intrinsic relaxation rate of CO_2.)

Isotope Trapping of the Substrate Carbon Dioxide. The general protocol described by Rose (1980) was followed. RuBP carboxylase (from *R. rubrum* and spinach) was dialyzed and concentrated with a ProDiMem M_r 15 000 cut-off membrane (Pierce Chemical Co., Rockford, IL 61105) against 0.10 M Tris-HCl–1 mM dithiothreitol, at a known pH between 8.10 and 8.20 at 23 °C. Mixing of the reagents was performed with a System 1000 rapid-mixing device from Update Instruments, Inc. (Madison, WI). Details of the reaction solutions, reaction times, and composition of the quenching solutions are given in the legend to Table II. After being quenched with HCl, samples were neutralized by the addition of about 160 mg of solid $NaHCO_3$ (plus 1 drop of octanol to control foaming). Duplicate aliquots were added to 5% formic acid and taken to dryness under vacuum. Acid-stable radioactivity was determined by liquid scintillation counting. The protein precipitate in the remainder was removed by filtration through Whatman No. 1 paper, and the PGA content of the filtrate was determined as described below. To determine the quantity of ^{14}C in solutions of $NaHCO_3$, an aliquot was added to 0.5 mL of 10% (v/v) triethanolamine in methanol followed by 5 mL of ScintiVerse (Fisher Scientific). The samples were then counted in the usual manner. This measurement permits the calculation of the radiospecific activity of the CO_2 before and after mixing.

Determination of RuBP and PGA. The basic assay mixture (total final volume 1.0 mL) contained 50 μmol of Bicine–KOH, pH 8.0, 5.0 μmol of ATP, 0.2 μmol of NADH, 10 μmol of $MgCl_2$, 50 μmol of $NaHCO_3$, 5.9 μmol of phosphocreatine, 7 units of creatine kinase (1 unit = 1 $\mu mol \cdot min^{-1}$), 31 units of PGA kinase, 13 units of phosphoglyceraldehyde dehydrogenase, 5 units of triosephosphate isomerase, and 11 units of glycerophosphate dehydrogenase. In the case of PGA, the reaction was initiated by the addition of 10–50-μL aliquots of PGA. In the case of RuBP, the assay contained additionally 0.5–1.0 unit of spinach RuBP carboxylase and was initiated by the addition of RuBP. Under these conditions with the spinach carboxylase (but not with the *R. rubrum* enzyme), the amount of oxygenation can be ignored. All assays were performed in triplicate.

^1H NMR Measurements of the Exchange of H-3 of RuBP with Solvent Protons. RuBP carboxylase (from *R. rubrum*) was dialyzed by continuous ultrafiltration with 0.2 M Tris-^2HCl in ^2H_2O (pH_{obsd} 7.5). At least 30 min prior to an experiment, the enzyme (1.5 $mg \cdot mL^{-1}$) was activated in a ^2H_2O solution containing 100 mM Tris-^2HCl, 25 mM $MgCl_2$, 16 mM Na^2HCO_3, and 94 mM KCl (pH_{obsd} 7.8). A ^2H_2O solution containing 110 mM Tris-^2HCl, 30 mM $MgCl_2$, and 12.2 mM RuBP (pH_{obsd} 7.8) was prepared substantially free of oxygen by bubbling with N_2 gas. All subsequent operations were performed under a stream of gaseous N_2. A 822-μL aliquot of the RuBP-containing solution was delivered into 153 μL of a ^2H_2O solution containing varying concentrations of Na^2HCO_3 and KCl such that the ionic strength was constant as the amount of Na^2HCO_3 varied from 4 to 64 μmol. The reaction was initiated by adding a 25-μL aliquot of the activated enzyme solution to 975 μL of the RuBP- and Na^2HCO_3-containing solution. The solution was dispensed into a 5-mm NMR tube, which was then capped. Spectra (360 MHz) were acquired at ambient temperature in 5-min blocks over a period of 2.5 h. The intensities of the H-3 proton of RuBP and the H-2 proton of PGA were obtained by integration. (At this temperature, the resonances of the H-1 protons of RuBP are obscured by the large residual H_2O resonance. The expected 1:1 proportionality between the loss of H-1 of RuBP and the rise of H-2 of PGA was verified by experiments at 10 °C, at which temperature the residual H_2O resonance is well separated from the resonances of H-1 of RuBP.) Similar but less extensive experiments were performed with the enzyme from spinach.

Data Analysis. Longitudinal relaxation rates were determined by fitting data from nonselective inversion-recovery experiments to

$$I(t) = (I_0 - I_f) \exp(-Bt) + I_f$$

where I_f is the intensity of the fully relaxed resonance, I_0 is the intensity of the resonance at $t = 0$ (i.e., immediately after the nonselective 180° pulse), and $B = 1/T_1$. For a perfect 180° pulse, $I_0 = -I_f$. In practice, pulse imperfections gave values for I_0 of $-0.8I_f$ to $-0.95I_f$.

The above equation was also used for determining relaxation rates and exchange rates by selective presaturation of the HCO_3^- resonance. [This assumes that the CO_2–HCO_3^- equilibrium is adequately described as a two-site exchange problem (see below).] In this instance, t is the length of time the HCO_3^- resonance was saturated prior to observing the

spectrum with a nonselective 90° pulse; I_0 is the intensity of the CO_2 resonance at $t = 0$ (i.e., without application of the saturating frequency); I_f is the intensity of the CO_2 resonance for $t = \infty$; $B = \rho + k$, where ρ is the intrinsic longitudinal relaction rate of the CO_2 resonance ($=1/T_1$) and k is the rate constant for conversion of CO_2 to HCO_3^-. Note also that for the presaturation experiment

$$I_f/I_0 = \rho/(\rho + k)$$

The interpretation of the selective inversion magnetization-transfer experiments follows from assuming that the CO_2–HCO_3^- equilibrium is adequately represented as a two-site exchange problem. Although the hydration of CO_2 involves the intermediacy of H_2CO_3, this intermediate is present at a concentration of $\sim 0.0017[CO_2]$ and is in rapid equilibrium with HCO_3^-. Under these conditions, application of the steady-state assumption, $d[H_2CO_3]/dt \ll d[CO_2]/dt + d[HCO_3^-]/dt$, reduces the three-site problem to a two-site problem, represented as

$$I \underset{k_S}{\overset{k_I}{\rightleftharpoons}} S$$

This reaction has an equilibrium constant $K = k_I/k_S = S_f/I_f$, where I_f and S_f are the unperturbed intensities of I and S. The following equations describe the two-site system (Campbell et al., 1978):

$$dI(t)/dt = -\rho_I[I(t) - I_f] - k_I I(t) + k_S S(t)$$

$$dS(t)/dt = -\rho_S[S(t) - S_f] - k_S S(t) + k_I I(t)$$

These equations may be solved for the initial conditions we used experimentally. For selective inversion of the I resonance

$$I(0) = -fI_f \qquad S(0) = S_f$$

where f allows for 180° pulse imperfections ($f = 1$ for a perfect pulse).

For selective inversion of the S resonance

$$S(0) = -gS_f \qquad I(0) = I_f$$

where g allows for imperfections in the 180° pulse.

The parameters (f, g, I_f, k_I, k_S, ρ_I, ρ_S) were estimated by simultaneously fitting the two data sets corresponding to the initial conditions above. All curve fitting was performed by varying the parameters so as to obtain the best fit between theory and observation with an unweighted, least-squares analysis.

Results

It has been shown that the catalytically essential metal ion interacts with the carboxyl group of CABP, the analogue of the carboxylated reaction intermediate (Miziorko & Sealy, 1984). In addition, for complexes of enzyme–carbamate–Mn^{2+}–CABP, the relaxation rate of the carboxyl group of CABP is so rapid that the resonance is undetectable by NMR (Pierce & Reddy, 1985). As the carboxyl group of the reaction intermediate is derived from substrate CO_2, one may be able to demonstrate binding of CO_2 by observing an enhancement of the relaxation rate of CO_2 by protein-bound Mn^{2+}. Neglecting outer sphere relaxation, the longitudinal relaxation rate of nearby nuclei in 1:1 complexes is described by [see Mildvan & Cohn (1970)]

$$1/T_{1m} = \text{const}/r^6 \qquad (1)$$

where $1/T_{1m}$ is related to observable values by

$$f(1/T_1) = 1/(T_{1m} + \tau_m) = (1/T_{1,obsd} - 1/T_1^0)[\text{nucleus}]/[Mn^{2+}] \qquad (2)$$

Table I: Paramagnetic Relaxation of HCO_3^- and CO_2 by Mn^{2+} in Complexes with RuBP Carboxylase[a]

conditions	$f(1/T_1)$ for HCO_3^- (s^{-1})[b]	$f(1/T_1)$ for CO_2 (s^{-1})[b,c]
R. rubrum enzyme	1540 (±190)	2
+XuBP[d]	<200	<3
+CABP[e]	0	0
spinach enzyme	970 (±70)	3
+XuBP[d]	<150	<2
+CABP[e]	0	0

[a] Determined at pH 7.5 in 0.2 M Tris-HCl at 25 °C by nonselective inversion and saturation-transfer protocols (see Materials and Methods). The values are derived from a number of measurements at enzyme concentrations between 0.1 and 0.3 mM active sites, $[Mn^{2+}]$ from 0 to 44 μM, and total inorganic carbon ($^{13}CO_2$ + $NaH^{13}CO_3$) between 0.1 and 0.25 M. [b] Values are calculated from eq 3. In the absence of Mn^{2+}, $T_1^0(HCO_3^-) = 37$ s and $T_1^0(CO_2) = 29$ s. The rate constant for hydration of CO_2 was ~ 0.85 s^{-1}. [c] The values in this column are quite small and are essentially indistinguishable from zero. [d] A 1.5-fold molar excess of XuBP to enzyme sites was added. It was found that the XuBP slowly degraded over a period of ~6 h (reason unknown). Spectra acquired immediately after the addition of XuBP had line widths indistinguishable from those observed in the absence of Mn^{2+}. At later times, the line widths increased to the same values observed prior to XuBP addition. At this time, another aliquot of XuBP could be added, and the process was repeated. Consequently, T_1 relaxation rates were measured as quickly as possible after the addition of XuBP, thereby sacrificing signal to noise (and accuracy). As a result, these values represent upper limits for $f(1/T_1)$. The true values for quaternary complexes with XuBP may be much lower. [e] A 1.2-fold molar excess of CABP to enzymes sites was added.

In these equations, $1/T_{1m}$ is the relaxation rate of nuclei bound in a complex with the paramagnetic metal ion, $1/T_1^0$ is the relaxation rate of nuclei in the absence of the metal ion, τ_m is the half-time for exchange of the observed nuclei from the metal complex to the bulk solution, and const (under the experimental conditions used here) is 3.3×10^7 $Å^6 \cdot s^{-1}$ for the spinach enzyme (Miziorko & Mildvan, 1974) and 4.5×10^7 $Å^6 \cdot s^{-1}$ for the enzyme from *R. rubrum* (Pierce & Reddy, 1985) for the metal–nucleus distance r expressed in angstrom units. Under conditions where exchange of bound with unbound nuclei is rapid (or more precisely, when $\tau_m << T_{1m}$), then T_{1m} may be determined and a metal–nucleus distance may be estimated.

Relaxation rates of $H^{13}CO_3^-$ and $^{13}CO_2$ were determined in the presence of either spinach or *R. rubrum* carboxylase and various, substoichiometric amounts of Mn^{2+} (to ensure complete complexation of the Mn^{2+} ion). It was apparent upon inspection of the spectra that the line width of the HCO_3^- resonance was increased by the presence of the enzyme-bound Mn^{2+} atom but that of the CO_2 resonance was hardly affected, even at CO_2/Mn^{2+} ratios less than 80. These observations were correlated with a marked enhancement of the longitudinal relaxation rate of HCO_3^- and a negligible effect on the relaxation rate of CO_2 (Table I). Addition of a tight binding inhibitor, CABP, completely obliterated the relaxation enhancements, and the addition of XuBP (an RuBP substrate analogue) markedly reduced the enhancements.

The value of $f(1/T_1)$ for HCO_3^-, when corrected for the small contribution of a finite exchange rate of HCO_3^- from enzyme–carbamate–Mn^{2+}–HCO_3^- complexes [2.7×10^4 s^{-1}; see Miziorko & Mildvan (1974)], allows us to determine $1/T_{1m}$ and hence r, the metal–nucleus distance (eq 1 and 2). The metal–carbon distance for the Mn^{2+}–HCO_3^- interaction is calculated to be ~5.5 Å for the *R. rubrum* enzyme and ~5.7 Å for the enzyme from spinach. These values are in good agreement with the value of 5.4 Å reported for the spinach enzyme by Miziorko and Mildvan (1974). However, the enhancements of the relaxation rates of CO_2 are very small

Table II: Isotope Trapping of Substrate Carbon Dioxide

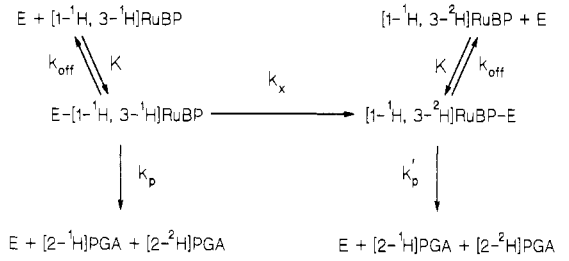
enzyme	^{14}C specific activity (dpm/nmol)[c] CO_2 before mixing	CO_2 after mixing	CO_2 fixed	no. of turnovers	fraction trapped
R. rubrum[a]	2938	144	130	2.43	−0.011
			139	5.68	−0.010
			136	6.81	−0.019
spinach[b]	3039	133	154	0.50	0.004
			124	1.69	−0.005
			122	2.31	−0.008
			118	3.53	−0.019

[a] *R. rubrum* RuBP carboxylase (0.78 μmol of protomer in 0.08 M Tris-HCl, pH 8.10, containing 18 mM $MgCl_2$, 51.4 mM $NaH^{14}CO_3$, 1 mM DTT, and 14 000 Wilbur-Anderson units of carbonic anhydrase) was mixed with 2 volumes of a freshly prepared solution containing 0.50 M $NaHCO_3$ and 31.5 mM RuBP, pH 8.13. The reaction was allowed to proceed for 0.34–1.67 s (allowing 2.4–6.8 turnovers) at which time it was quenched with 1 N HCl. [b] Spinach RuBP carboxylase (1.02 μmol of protomer in 0.08 M Tris-HCl, pH 8.10, containing 17 mM $MgCl_2$, 23.0 mM $NaH^{14}CO_3$, 1 mM DTT, and 9000 Wilbur-Anderson units of carbonic anhydrase) was mixed with 2 volumes of a freshly prepared solution containing 0.25 M $NaHCO_3$ and 47.8 mM RuBP, pH 8.06. The reaction was allowed to proceed for 0.42–3.34 s (allowing 0.5–3.5 turnovers) at which time it was quenched with 1 N HCl. [c] Acid-stable ^{14}C radioactivity and PGA were determined as described under Materials and Methods. These permit calculation of the radiospecific activity of the CO_2 fixed. The radiospecific activities of the CO_2 before and after mixing were determined as described under Materials and Methods. Values for k_{cat}, determined from the observed steady-state rates of PGA formation, were 6.25 ± 1.35 and 1.02 ± 0.12 s^{-1} for the *R. rubrum* and spinach enzymes, respectively. This method is predicated upon assumption that the chemical and isotopic equilibration of the CO_2 is rapid relative to the rate of catalysis. This was ensured by the inclusion of carbonic anhydrase in the reaction. In the absence of carbonic anhydrase, spurious trapping of $^{14}CO_2$ is observed. The very small pH differences between the pulse and the chase solutions are insufficient to account for this effect. Rather, we attribute this to the formation of nonspecific ^{14}C-labeled carbamates in the pulse solution. Upon quenching, these will be released as $^{14}CO_2$. In the absence of carbonic anhydrase, the radiospecific activity of the CO_2 in the quench will be in excess of that calculated on the basis of isotopic and chemical equilibration.

(or nonexistent) and are inconsistent with any CO_2 binding to the activated enzyme complex (other than in the form of the activating carbamate moiety). That is, if under the experimental conditions used (up to ~5 mM CO_2) there is one rapidly exchanging CO_2 molecule bound to the enzyme, its average distance from the metal ion must be greater than ~15 Å. (By these measurements, this calculated distance is essentially indistinguishable from an infinite distance, since the very low values observed are not reliably distinguishable from zero.) Alternatively, if it is bound at a distance of ~5.5 Å or less (as is the HCO_3^- anion), then, under the experimental conditions used, the binding site must be only fractionally occupied. The calculated occupancy (1×10^{-3}) at an assumed distance of 5.5 Å corresponds to a dissociation constant of ~5 M under the conditions used. [cf. $K_m(CO_2)$ = 14 μM for the spinach enzyme and 89 μM for the enzyme from *R. rubrum*.] Clearly, HCO_3^- binds to the activated enzyme. However, we are left with two possible conclusions regarding CO_2 binding in the absence of RuBP: either CO_2 does not bind to the activated enzyme, or if it does, then its rate of dissociation from the enzyme is too slow ($\lesssim 10^2$ s^{-1}) to allow detection of its binding by the NMR methods used here.

With the above conclusions in mind, attempts were made to trap the putative enzyme–substrate–carbon dioxide Michaelis complex by the isotope-trapping technique described by Rose (1980). The results for both *R. rubrum* and spinach enzymes (Table II) show that the specific radioactivity of the

Scheme II



CO_2 fixed was virtually identical with that of the CO_2 in the quenched solution; i.e., no substrate carbon dioxide was trapped. A similar result has already been reported for the *R. rubrum* enzyme (Jaworowski & Rose, 1984). Again, we are left with two possible conclusions; either no binary substrate CO_2 complex exists, or the dissociation of substrate CO_2 from the binary and/or ternary complexes is too fast to be trapped by this method. For an ordered mechanism with CO_2 binding first, the rate constant for dissociation of substrate CO_2 must be equal to or greater than $[RuBP]k_{cat}/K_m(RuBP)$ for a trapping efficiency of 50%. Our results allow us to estimate a lower limit for the rate constant for dissociation of substrate CO_2 from the putative binary enzyme–CO_2 complex; for the *R. rubrum* enzyme, this value is 1.2×10^4 s^{-1}, while for the spinach enzyme the value is 1.5×10^3 s^{-1}. Alternatively, a random mechanism for substrate addition allows for the release of substrate CO_2 from ternary complexes of enzyme–RuBP–CO_2. Under these circumstances, k_{cat} rather than k_{cat}/K_m modulates the trapping efficiency. We estimate that our trapping experiments are precise enough to allow us to determine bound CO_2 in ternary complexes if >5% of bound CO_2 proceeds to products. Therefore, release of substrate CO_2 from any ternary complexes occurs with a rate constant in excess of 100 s^{-1}. These values lie within the range accessible to the NMR experiments detailed above, yet no rapidly exchanging interaction between CO_2 and enzyme was observed. We conclude that neither spinach enzyme nor *R. rubrum* enzyme forms a Michaelis complex with CO_2, at least not in the absence of RuBP.

Equilibrium binding experiments were performed with the enzyme from *R. rubrum* to ascertain whether the alternate substrate O_2 can bind to the activated enzyme in the absence of RuBP. We could find no evidence for O_2 binding under conditions that would have allowed us to determine a binding constant with a magnitude of ≲10 mM [cf. $K_m(O_2)$ = 0.4 mM]. Further, no O_2 binding was observed upon addition of the RuBP analogue XuBP. Therefore, the enzyme–carbamate–Mg^{2+}–XuBP complex does not bind O_2 with a binding constant of <10 mM.

Clearly, RuBP must bind to the enzyme prior to the binding of the gaseous substrates. It has also been demonstrated that enolization of RuBP occurs during the reaction sequence (Saver & Knowles, 1982), and that enol-RuBP is present on the enzyme at low amounts during steady-state turnover (Jaworowski et al., 1984). The question arises: does the enolization of RuBP require the presence of the gaseous substrates?

Consider the reaction of RuBP with RuBP carboxylase in ^2H_2O (Scheme II). A decline in the quantity of H-1 of RuBP can occur only as a result of product formation. However, H-3 of RuBP might be consumed by two pathways: the exchange pathway characterized by k_x, the rate constant for the conversion of [3-^{1}H]RuBP to [3-^{2}H]RuBP (which is effectively irreversible in ^2H_2O solution); the catalytic pathway characterized by k_p, the rate constant for product formation. [For

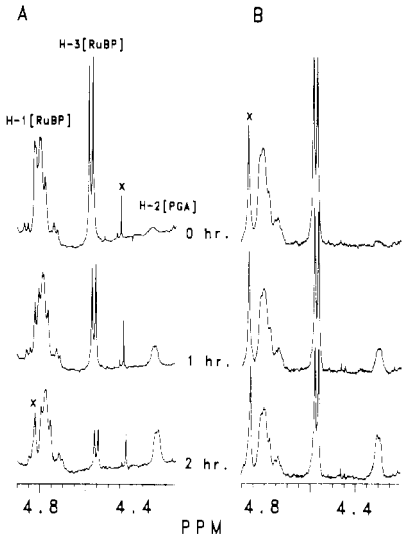


FIGURE 1: NMR determination of the exchange of H-3 of RuBP during catalysis. Proton spectra were obtained in ^2H_2O at 360 MHz and 10 °C with 10 mM RuBP and 250 $\mu g \cdot mL^{-1}$ *R. rubrum* enzyme. (See Materials and Methods for further details.) (A) Spectra were obtained in the presence of 4 mM $NaHCO_3$ at the indicated times after starting the reaction by the addition of RuBP. (B) Spectra were obtained in the presence of 32 mM $NaHCO_3$ at the indicated times after starting the reaction by the addition of RuBP. (Resonances marked with an "X" are of unknown origin.)

[3-^{2}H]RuBP, the rate constant for product formation, k_p' (Scheme II), is for our purposes essentially identical with k_p as evidenced (Sue & Knowles, 1982) by the lack of an appreciable isotope effect at H-3 of RuBP.] Under conditions where [RuBP] $\gg K$, [RuBP] $\gg$ [E_{tot}], and the rate of RuBP dissociation from the enzyme (k_{off}) $\gg k_x$ or k_p the following equations describe the time courses for appearance of [2-^{1}H]PGA (P) and disappearance of [3-^{1}H]RuBP (S):

$$P = P_0 + k_p E_{tot} t \quad (3)$$

$$S = S_0[1 - k_p E_{tot} t/S_0]^{(k_x+k_p)/k_p} \quad (4)$$

where k_p is the rate constant for product formation, k_x is the rate constant for exchange of H-3 of RuBP, and S_0 and P_0 are the concentrations of RuBP and product (PGA) at $t = 0$. At short times, the initial rates of appearance of P and loss of S reduce to

$$v_S = (dS/dt)_{t=0} = -(k_x + k_p)E_{tot}$$
$$v_P = (dP/dt)_{t=0} = k_p E_{tot} \quad (5)$$

as expected.

By allowing RuBP carboxylase to catalyze the carboxylation of RuBP in a ^2H_2O solution, one can use NMR to follow the change of resonance intensities associated with H-1 of RuBP, H-3 of RuBP, and H-2 of the product PGA (Gutteridge et al., 1984a). If the enzyme can catalyze the enolization of RuBP and exchange of the liberated proton with solvent protons faster than it catalyzes product formation, then H-3 will be lost at a faster rate than H-1. [H-2 of the product PGA will appear at the same rate that the H-1 proton of RuBP disappears. Although two molecules of PGA are formed per RuBP consumed, only one of them (deriving from carbons 3, 4, and 5 of RuBP) will be [2-^{1}H]PGA. The PGA molecule formed from C-1 and C-2 of RuBP and CO_2 contains a solvent-derived ^{2}H at C-2 and does not contribute to the NMR intensity at the position of H-2 of PGA.]

The data in Figure 1 are illustrative of the method applied to the *R. rubrum* enzyme. These data were acquired at 10 °C so that the residual H_2O peak would not obscure the

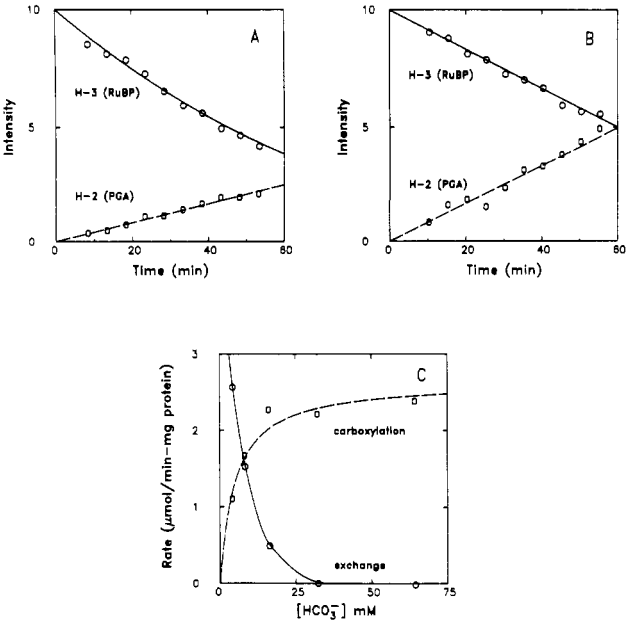


FIGURE 2: Effect of CO_2 concentration on the exchange of H-3 of RuBP with solvent during catalysis. Spectra were obtained at 25 °C. Further details are given under Materials and Methods. (A) loss of H-3 (RuBP) and gain of H-2 (PGA) signal intensities with time in the presence of 4 mM $NaHCO_3$. The open symbols are the data. The lines are drawn from eq 3 and 4 with the following parameters: S_0 = 10 mM, P_0 = 0 mM, k_p = 0.98 s^{-1}, and k_x = 2.4 s^{-1}. (B) Loss of H-3 (RuBP) and gain of H-2 (PGA) signal intensities with time in the presence of 32 mM $NaHCO_3$. The open symbols are the data. The lines are drawn from eq 3 and 4 with the following parameters: S_0 = 10 mM, P_0 = 0 mM, k_p = 2.0 s^{-1}, and k_x = 0.015 s^{-1}. (C) Variation of the rates of carboxylation and exchange of H-3 of RuBP as a function of $NaHCO_3$ (CO_2) concentration.

resonances for H-1 of RuBP. At low HCO_3^- (i.e., CO_2) concentrations (Figure 1A), it can be seen that H-3 of RuBP declines more rapidly than H-1, indicating that the exchange reaction occurs at an appreciable rate. At high CO_2 concentrations (Figure 1B), the rates are similar, indicating that the exchange rate is slow relative to the rate of conversion of RuBP to PGA.

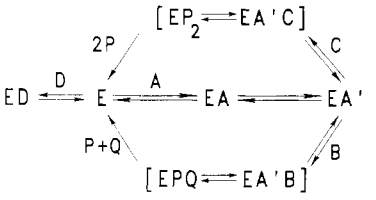
More detailed time courses were obtained at 25 °C by observing the changes in the resonance intensities for H-3 of RuBP and H-2 of PGA. Again, in the exchange experiment with the *R. rubrum* enzyme, the rate of loss of H-3 of RuBP is greater than that for formation of H-2 of PGA at low concentrations of CO_2 (Figure 2A). However, at higher CO_2 concentrations, the rates become equal, indicating that the exchange reaction does not occur (Figure 2B). When viewed over a range of CO_2 concentrations, it is apparent (Figure 2C) that ever increasing CO_2 concentrations effectively inhibit the exchange reaction. That is, k_p increases hyperbolically with [CO_2] whereas the magnitude of k_x is inversely related to [CO_2]. Extrapolation of the data to zero [CO_2] indicates that exchange and (by implication) enolization of RuBP occur in the absence of CO_2. Furthermore, the maximum rate of exchange (at low [CO_2]) is greater than the maximum rate of product formation (k_p, at high [CO_2]) (Figure 2C). Since the rate of enolization must be greater than the observed rate of exchange (see Scheme II), this result indicates that enolization can proceed, in the absence of CO_2, at rates in excess of the maximum catalytic rate for product formation. That is, CO_2 is not required for enolization at rates sufficient to account for maximum catalytic rates. Less extensive measurements with the spinach enzyme are consistent with these conclusions.[2]

Discussion

Using a variety of techniques, we have been unable to find any evidence for gaseous substrate binding to RuBP carboxylase in the absence of RuBP. Since the carboxyl group of the intermediate analogue, CABP (Sealey & Miziorko, 1984), is bound through an oxygen atom to the essential metal atom in quaternary complexes, and since the substrate CO_2 molecule is ultimately converted to the carboxyl group of the reaction intermediate, it is reasonable to assume that any CO_2 binding should likewise be nearby the metal atom. Indeed, even the phosphate atoms of CABP (Pierce & Reddy, 1985) and the HCO_3^- molecule (this report; Miziorko & Mildvan, 1974) have been found to bind within ~5–6 Å of the metal atom. If we assume the reasonable distance of 5.5 Å for CO_2 binding, our results with both the spinach and *R. rubrum* enzymes indicate that any CO_2 binding to the enzyme, in the absence of RuBP, occurs with a dissociation constant in excess of 5 M. This value is 5–6 orders of magnitude larger than the apparent K_m for CO_2 and places severe limits on the fraction of overall catalysis that could be initiated by CO_2 binding prior to RuBP. In essence, the enzyme must proceed with catalysis by first binding RuBP.[3]

In contrast, the HCO_3^- anion does bind to the enzyme in the absence of RuBP, even though HCO_3^- is not active as a substrate. As the binding distance for HCO_3^- is approximately the same as the phosphorus–metal distances determined for quaternary complexes made with CABP and since a large number of anionic species are competitive inhibitors of RuBP carboxylase with respect to RuBP (e.g., phosphate, sulfate, and glycerol phosphate, to name a few), it seems probable that HCO_3^- is no exception and that it binds to the activated enzyme species at or near the phosphate binding site. This binding by HCO_3^- is the likely cause for the apparent "CO_2" inhibition observed by many workers. In kinetic analyses, increasing CO_2 concentrations are invariably accompanied by proportional increases in the HCO_3^- concentration due to the facile hydration equilibrium for CO_2. At high concentrations, HCO_3^- binding decreases the amount of available free enzyme and may account for the experimental results.

Scheme III



Previous steady-state kinetic studies have suggested, contrary to our conclusions, that the enzymatic reaction proceeds via a random mechanism for substrate addition (Laing & Christeller, 1980). The conclusion of these workers rested primarily on the observation that carbon oxysulfide, COS, was a competitive inhibitor with respect to CO_2 and a noncompetitive inhibitor with respect to RuBP. Since an ordered reaction requires that a dead-end inhibitor competitive for the second substrate be uncompetitive for the first substrate, these workers concluded that the reaction involved the random addition of substrates. However, we have recently demonstrated that COS is *not* a dead-end inhibitor but rather an alternate substrate (Lorimer & Pierce, 1986) for the carboxylase enzyme. The inhibition patterns observed with COS are therefore similar to those observed with the other alternate substrate, oxygen. Consequently, the sequence of substrate binding cannot be inferred from such inhibition studies, and the apparent inhibition by COS is not discordant with an ordered reaction pathway.

Our findings concerning the partial exchange reaction of H-3 of RuBP allow us to further delineate the reaction mechanism. For the enzyme from *R. rubrum*, the exchange rate at low [CO_2] was greater than the maximum catalytic rate of the enzyme, k_{cat}. Increasing concentrations of CO_2 decreased the measured exchange rate. It appears that enolization of RuBP occurs prior to interaction of CO_2 with the enzyme–enol-RuBP complex. (One can, of course, envision more complicated, branched pathways in which the formation of the enol of RuBP in the absence of CO_2 represents a dead-end complex, incapable of proceeding to product formation. However, this would require the dubious proposition that a known intermediate, formed at rates in excess of maximum catalytic rates, is *not* on the catalytic pathway.)

The conclusion that enolization of RuBP occurs prior to gaseous substrate interaction is in accord with our results for the spinach enzyme and earlier results with the enzyme from wheat (Gutteridge et al., 1984a). However, the kinetic constants of the higher plant enzymes preclude accurate measurements over a wide range of CO_2 concentrations. So, for these enzymes, it has not been *proven* that enolization in the absence of CO_2 occurs at rates sufficient to support maximal catalytic rates. Thus, it is at least possible that the higher plant enzyme binds CO_2 prior to enolization, this mechanistic variant being favored by Roeske and O'Leary (1984) in explaining their observation that deuteration of carbon 3 of RuBP caused a slight decrease in the discrimination against $^{13}CO_2$ by the spinach enzyme. However, the corresponding experiment with the oxygenase activity indicates that there is no decrease in discrimination against $^{18}O_2$ upon deuteration of carbon 3 of RuBP (H. L. Schmidt, personal communication). We note, too, that the oxygenation of RuBP can proceed in the almost complete absence of CO_2 (some CO_2 is always present due to the requirement for CO_2 in the activation process) at rates $\sim^1/_3$ of the maximum carboxylation rate. So, CO_2 is not required for enolization to occur even at this high rate. In this

[2] It is difficult to analyze the CO_2 concentration dependence of the exchange rate with spinach enzyme due to its very low Michaelis constant for CO_2 (~10 μM) and the use of ~10 mM RuBP in the NMR experiments. (At nonsaturating concentrations of CO_2, any product formation will deplete the available supply of CO_2, and the reactions will become progressively slower due in part to lack of substrate and in part to deactivation of the enzyme via carbamate loss.) We have therefore restricted our experiments to very low (<0.2 mM) and high (20 mM) HCO_3^- concentrations. At the saturating concentration of HCO_3^-, the exchange reaction was essentially unobservable (i.e., the rate of product formation was greater than 8 times the rate of exchange). At the low HCO_3^- concentration, however, we observed the exchange reaction proceeding to the extent of 40% without any observable product formation. A similar result has been obtained with the enzyme from wheat (Gutteridge et al., 1984a).

[3] The alternative explanation for our NMR results is that CO_2 may bind to the active enzyme with a dissociation constant ≲2 mM provided that it binds at a distance in excess of ~15 Å from the metal. (Again, our results do not allow us to distinguish between this 15-Å limit and a much larger distance.) However, this possibility would require a large movement of CO_2 toward the metal ion after a subsequent binding of RuBP. Although we cannot firmly discount this possibility, it seems prudent to adopt the less complex conclusion that no binding site for CO_2 exists in the absence of RuBP. Likewise, our inability to observe any binding of O_2 to activated enzyme is certainly consistent with there being no binding site for this substrate in the absence of RuBP, although quantitatively our method allows us to state only that $K_d/K_m > 25$. This ratio yields only a rather modest constraint in determining relative fluxes through the alternate pathways in which either RuBP or O_2 binds first, so it is at least conceivable that some fraction of the oxygenase reaction may occur through the path in which O_2 binds prior to RuBP. We wish to stress, however, that there is no evidence to support the more complicated random mechanism for RuBP oxygenation.

regard, our experiments with XuBP are suggestive. The enzyme–carbamate–Mn^{2+}–XuBP complex did not enhance the relaxation rate of CO_2. Neither did XuBP promote O_2 binding to the enzyme. To the extent that XuBP mimics RuBP at the active site, one may infer that there is no binding site for CO_2 or O_2 in complexes of RuBP with activated enzyme, and indeed, this is the most straightforward explanation for our exchange experiments. Consequently, we favor the mechanism shown in Scheme III for RuBP carboxylation and oxygenation. (The symbols A, A′, B, C, D, E, P, and Q represent respectively RuBP, enol-RuBP, O_2, CO_2, HCO_3^-, activated enzyme, PGA, and phosphoglycolate.) This mechanism yields the following steady-state rate equations for initial rates of carboxylation and oxygenation (neglecting the complication due to HCO_3^- inhibition, which occurs only at high CO_2 concentrations and/or high pH) (Farquhar, 1979):

$$v_c = \frac{V_c AC}{K_c A + AC + K_c AB/K_b + K_{ac}C + K_{ab}K_c B/K_b + K_{ia}K_c} \quad (6)$$

and

$$v_b = \frac{V_b AB}{K_b A + AB + K_b AC/K_c + K_{ab}B + K_{ac}K_b C/K_c + K_{ia}K_b} \quad (7)$$

which in the absence of the alternate substrate yields

$$V_c/v_c = 1 + K_{ac}/A + K_c/C + K_{ia}K_c/(AC) \quad (8)$$

and

$$V_b/v_b = 1 + K_{ab}/A + K_b/B + K_{ia}K_b/(AB) \quad (9)$$

In these equations, K_b and K_c are the respective Michaelis constants for O_2 and CO_2, K_{ab} and K_{ac} are the respective Michaelis constants for RuBP in the oxygenase and carboxylase reactions, and K_{ia} is the kinetically determined inhibition constant for RuBP in the nomenclature of Cleland [see Segel (1975)]. V_b and V_c are the maximum velocities of the oxygenase and carboxylase reactions. [For the carboxylase reaction, the complication of a rapid equilibrium interaction of HCO_3^- modifies those terms containing A in eq 8 (i.e., $K_{ab}/A \rightarrow K_{ab}/[A(1 + D/K_d)]$; $K_{ia}K_b/(AB) \rightarrow K_{ia}K_b/[AB(1 + D/K_d)]$). This complication is absent in initial velocity studies of the oxygenase reaction in the absence of of CO_2.]

These equations provide interesting corollary relationships. The ratio of the RuBP Michaelis constants for the two reactions equals the ratio of V_{max} values for the two reactions, i.e.

$$V_c/V_b = K_{ac}/K_{ab} \quad (10)$$

Also

$$v_c/v_b = \frac{V_c/K_c}{V_b/K_b}\frac{C}{B} \quad (11)$$

The relationship in eq 10 is difficult to verify experimentally due to the very low K_m values for RuBP and the low turnover rate of the enzyme, as well as to experimental difficulties associated with the requirement for CO_2 activation in both carboxylase and oxygenase reactions, the presence of inhibitors in RuBP preparations, HCO_3^- inhibition, and a poorly understood time dependence of the reaction velocity. Equation 11, however, can be assessed by measurement of both activities simultaneously, thereby obviating the above difficulties. It has been found to hold over a wide range of CO_2, O_2, RuBP, and H^+ concentrations (Jordan & Ogren, 1984).

The ratio of V/K values in eq 11 can be taken to represent a specificity factor ratio for RuBP carboxylation and oxygenation. The elegant and informative studies of Jordan and Ogren show that this ratio varies from species to species in a manner consistent with the organism's habitat with regard to the prevailing ratio of CO_2 and O_2 concentrations available to the enzyme (Jordan & Ogren, 1981). That is, the kinetic constants of the enzyme appear to have evolved under environmental selection so as to optimize its efficiency as a carboxylase. Clearly, one way to increase this efficiency would be to preferentially lower the Michaelis constant for CO_2 via the creation of a binding site for CO_2. Our results would indicate that any such interactions must take place after the initial chemistry of enolization of RuBP. Alternatively, it may be that the apparent Michaelis constant for CO_2 is primarily determined by kinetic rather than thermodynamic parameters. This latter alternative is supported by the observation of correlated temperature dependencies (Badger & Collatz, 1978) of V_c and K_c (as well as V_b and K_b), which may arise from a common, strongly temperature-dependent rate constant in each of these terms. Under these mechanistic constraints, any further improvements in carboxylase efficiency might be expected to result from selective enhancement of the rate constants associated with carboxylation, rather than by any changes in CO_2 binding affinity, per se.

ACKNOWLEDGMENTS

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Registry No. XuBP, 15565-46-5; CABP, 27442-42-8; RuBP, 2002-28-0; RuBP carboxylase, 9027-23-0; HCO_3^-, 71-52-3; CO_2, 124-38-9; H_2, 1333-74-0.

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Isolation of Pyrophosphohistidine from Pyrophosphorylated Pyruvate,Phosphate Dikinase†

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ABSTRACT: The pyrophosphoryl form of pyruvate,phosphate dikinase was prepared by incubation with adenosine 5′-[γ-^{32}P]triphosphate and isolated by gel chromatography. Previously a phosphorylated moiety had been isolated from the enzyme and was shown to be bound through a phosphoramidate linkage to the 3′ nitrogen of a histidine residue [Spronk, A. M., Yoshida, H., & Wood, H. G. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4415]. This histidine residue has been considered to be the pyrophosphoryl and phosphoryl carrier between the three subsites of this enzyme. Previous attempts to isolate the putative [^{32}P]pyrophosphohistidine have been unsuccessful due to the lability of the [^{32}P]pyrophosphoryl-enzyme. By stabilization of the [^{32}P]pyrophosphoryl-enzyme with diazomethane, it has been possible to isolate a [^{32}P]-pyrophosphohistidine from the hydrolysates. To our knowledge this work constitutes the first direct demonstration of a pyrophosphorylated histidyl residue in an enzyme.

Pyruvate,phosphate dikinase (EC 2.7.9.1, pyruvate,orthophosphate dikinase) catalyzes the reversible formation of phosphoenolpyruvate from pyruvate as shown in eq 1.

$$\text{ATP} + \text{P}_i + \text{pyruvate} \rightleftharpoons \text{AMP} + \text{PP}_i + \text{P-enolpyruvate} \quad (1)$$

The overall reaction involves the transfer of the γ-phosphate of ATP to orthophosphate, forming pyrophosphate, and the β-phosphate of the same ATP to pyruvate to produce P-enolpyruvate. The mechanism of these transfers appears to differ depending on the source of the enzyme. Evans and Wood (1968, 1971), using the dikinase isolated from *Propionibacterium shermanii*, proposed a mechanism involving three partial reactions, each of which is catalyzed at a distinct subsite:

$$\text{enzyme} + \text{ATP} \rightleftharpoons \text{E}\sim\text{P}_\beta\text{P}_\gamma + \text{AMP} \quad (1a)$$

$$\text{E}\sim\text{P}_\beta\text{P}_\gamma + \text{P}_i \rightleftharpoons \text{E}\sim\text{P}_\beta + \text{P}_\gamma\text{P}_i \quad (1b)$$

$$\text{E}\sim\text{P}_\beta + \text{pyruvate} \rightleftharpoons \text{enzyme} + \text{P}_\beta\text{-enolpyruvate} \quad (1c)$$

Studies of the enzyme from *P. shermanii* (Evans & Wood, 1968; Milner & Wood, 1972) and from *Bacteriodes symbiosus* (Milner et al., 1978) have shown that both a phosphoryl-enzyme (E∼P)[1] and a pyrophosphoryl-enzyme intermediate (E∼PP) are formed during the reaction. Additional evidence in support of the above tri-uni-uni ping-pong mechanism has come from equilibrium exchange studies (Evans & Wood, 1968; Milner & Wood, 1972, 1976), initial velocity data (Milner & Wood, 1972, 1976), and product inhibition patterns (Milner et al., 1978). The phosphoryl moiety of the E∼P intermediate was shown to be bound to the enzyme through an acid-labile phosphoramidate linkage, and subsequently, $N^{3'}$-[^{32}P]phosphohistidine was isolated from alkaline hydro-

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[1] Abbreviations: E∼P, phosphoryl pyruvate,phosphate dikinase; E∼PP, pyrophosphoryl derivative of the enzyme; P-enolpyruvate, phosphoenolpyruvate; Diazald, *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide; TFA, trifluoroacetic acid; TEA, triethylamine; ODS, octadecylsilane; PITC, phenyl isothiocyanate; PTC-amino acid, phenylthiocarbamoyl derivative of an amino acid; HPLC, high-performance liquid chromatography; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; NADH, reduced nicotinamide adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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