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Deuterium Isotope Effects in the Carboxylase Reaction of Ribulose-1,5-bisphosphate Carboxylase/Oxygenase†

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ABSTRACT: The deuterium isotope effects on the carboxylase reactions of spinach and *Rhodospirillum rubrum* ribulosebisphosphate carboxylase/oxygenase by [3-^{2}H]ribulose 1,5-bisphosphate have been examined. With the spinach enzyme, the isotope effects observed at high pH on V_{max} and V_{max}/K_m do not vary with CO_2 from moderate concentrations (approximately equal to K_m) to rather high levels (up to 100 times K_m). These results are interpreted in favor of a Theorell–Chance type of kinetic mechanism in which CO_2 adds to the ene–diol of ribulose bisphosphate in a bimolecular fashion after abstraction of the C-3 proton of the sugar bisphosphate. In contrast to the lack of an effect by CO_2, the isotope effect on V_{max} is pH-dependent. The V_{max} isotope effect varies from 2 ($[^1H]V/[^2H]V$) at high pH to about 9 at low pH. The pH dependence of V and V/K for the spinach enzyme is defined by two ionizable groups over the pH range of 6–9. One group, which must be protonated for activity, exhibits a pK of 8.3 ± 0.2 in the V_{max} profile and 7.5 ± 0.4 in the V/K profile. The other group, which must be present as the free base for activity, exhibits a pK of 7.1 ± 0.1 in the V and 7.5 ± 0.4 in the V/K profile. Change in the isotope effect on V correlates with protonation of the latter enzymic group. Inhibition of the spinach enzyme by xylulose 1,5-bisphosphate, a substrate analogue, is also pH-dependent and appears to depend on the correct protonation state of the same or similar enzymic groups as those seen in the V and V/K profiles. Lack of perturbation of either pK by propylene glycol suggests both are cationic acids (lysyl or histidyl residues). In contrast to the spinach enzyme, the isotope effects on both V and V/K are pH-dependent with the corresponding enzyme from *Rhodospirillum rubrum*. The deuterium isotope effect on V varies from 1.5 at high pH to about 5 at low pH, while that on V/K varies from 1 at high pH to about 7 at low pH. Similar pK values were observed for the essential enzymic base with the *R. rubrum* enzyme in both the V and V/K profiles to those observed with the spinach enzyme. These data suggest that the enzymic group with a pK of about 7.5 in both spinach and *R. rubrum* enzymes is the essential enzymic base that abstracts the C-3 proton of ribulose bisphosphate in the first step of the reaction.

Ribulosebisphosphate carboxylase/oxygenase (EC 4.1.1.39) catalyzes the conversion of D-ribulose 1,5-bisphosphate and CO_2 to two molecules of D-3-phosphoglycerate. This reaction is the ultimate means of CO_2 fixation in most photosynthetic organisms [for review, see Akazawa et al. (1978) and Miziorko & Lorimer (1983)]. Molecular oxygen is a competing substrate (vs. CO_2) for this enzyme. Inhibition of the carboxylase reaction by O_2 accounts for the inhibition of photosynthesis by O_2, a physiological response known as the Warburg effect (Bahr & Jensen, 1974; Andrews et al., 1975; Laing et al., 1975; Chollet & Ogren, 1975; Chollet, 1977). As the oxygenase reaction is an energy wasteful process, its elimination would have potential agronomic significance. Plants grown under low oxygen tensions or elevated CO_2 concentrations, to minimize the Warburg effect, have a substantially enhanced growth rate and yield (Hardy et al., 1978). These observations have led to various speculations as to the feasibility of eliminating the oxygenase reaction by either chemical or genetic means (Andrews & Lorimer, 1978; Lorimer & Andrews,

† A preliminary report of these results has been published (Schloss, 1983).

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1973). While the possible success of genetically engineering an oxygenase-less carboxylase remains dubious, a rational approach to this problem [such as site specific mutagenesis; see Gutteridge et al. (1984)] appears not to be currently feasible primarily due to the lack of detailed information about those enzymic residues that are likely to participate in either reaction.

Various chemical modification studies have implicated lysyl (Hartman et al., 1973, 1978a,b; Norton et al., 1975; Schloss & Hartman, 1977a,b; Paech et al., 1977; Schloss et al., 1978a; Whitman & Tabita, 1976, 1978a,b; Stringer & Hartman, 1978; Paech & Tolbert, 1978; Chollet & Anderson, 1978; Robison et al., 1980; Herndon et al., 1982; Cook et al., 1984), histidyl (Saluja & McFadden, 1980, 1982; Bhagwat & Ramakrishna, 1981; Bhagwat & McFadden, 1983; Herndon & Hartman, 1984; Paech, 1984, 1985), tyrosyl (Robison & Tabita, 1979; Bhagwat, 1982), cysteinyl (Rabin & Trown, 1964a,b; Sugiyama et al., 1967, 1968; Argyroudi-Akoyunoglou & Akoyunoglou, 1968; Takabe & Akazawa, 1975; Schloss & Hartman, 1977b; Schloss et al., 1978a), methionyl (Fraij & Hartman, 1982, 1983; Christeller & Hartman, 1982), arginyl (Lawlis & McFadden, 1978; Schloss et al., 1978b; Chollet, 1978, 1981), and aspartyl or glutamyl (Valle & Vallejos, 1984) residues as being within the domain of the active site. However, the functional role of these residues, if any, remains uncertain. The only enzymic residue for which function has been assigned with certainty is the lysyl residue whose ϵ-amino group is modified to a carbamate during the obligate allosteric activation of both carboxylase and oxygenase activities by CO_2 (Lorimer & Miziorko, 1980; Lorimer, 1981; Donnelly et al., 1983).

X-ray crystallographic studies have been initiated with the carboxylase isolated from several sources (Baker et al., 1975, 1977a,b; Johal et al., 1980; Andersson et al., 1983; Meisenberger et al., 1984; Andersson & Branden, 1984; Schneider et al., 1984; Branden & Lorimer, 1984; Jansen et al., 1984). While a detailed crystal structure of the carboxylase from any source will be of great value in defining those portions of the enzyme that encompass the active site, proximity alone is not sufficient to define the functional role for a particular residue.

The pH dependence of an enzymic reaction, especially when combined with the pH dependence of kinetic isotope effects on the reaction, can be quite useful in assessing the number and type of enzymic residues that participate in the reaction (Cook & Cleland, 1981b,c). Although these data do not assign function to a particular enzymic residue, they can define the types and properties of residues that serve in substrate binding and as acid–base catalysts. Kinetic studies can complement the data obtained by crystallographic and chemical modification techniques. Together these studies can provide a coherent correlation of structure and function.

We have examined the effect of pH on the kinetic parameters (V and V/K) of the carboxylase reaction, the enzyme's affinity for the substrate analogue xylulose 1,5-bisphosphate, and the size of the kinetic isotope effects with [3-^{2}H]ribulose 1,5-bisphosphate for both spinach and *Rhodospirillum rubrum* enzymes. These data, together with several kinetic anomalies that arise in assaying the enzyme at extremes of pH, are reported here.

Materials and Methods

Materials. D-Ribulose 1,5-bisphosphate (RuBP)[1] was synthesized from ribose 5-phosphate by the method of Horecker et al. (1958); [3-^{1}H]RuBP and [3-^{2}H]RuBP were synthesized from xylulose 5-phosphate and ATP by use of xylulose-5-phosphate 3-epimerase and phosphoribulokinase in H_2O and ^2H_2O, respectively, following a similar protocol. Xylulose 5-phosphate and the epimerase were incubated for sufficient time to allow full exchange of the C-3 proton with solvent prior to the addition of kinase. By the criterion of ^{1}H NMR, negligible ^{1}H was present at C-3 in [3-^{2}H]RuBP preparations. [3-^{3}H]RuBP was synthesized by reduction of L-glyceraldehyde with NaB^3H_4 (New England Nuclear) followed by coupled enzymic synthesis using glycerokinase, α-glycerophosphate dehydrogenase, aldolase, triosephosphate isomerase, D-fructose-1,6-diphosphatase, phosphoglucose isomerase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphoribulokinase, ATP, NAD, and NADP. [^{14}C]RuBP was prepared from D-[^{14}C(U)]glucose (New England Nuclear) enzymically by using hexokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphoribulokinase, NADP, and ATP.

The various samples of labeled RuBP were purified by anion-exchange chromatography on AG 1-X8 (Bio-Rad). In a typical preparation, 0.74 mmol of [3-^{2}H]RuBP (free acid) was applied to a 58 × 2.4 cm column of AG 1-X8 (chloride form). The column had been prepared by prior elution with 1 N HCl followed by equilibration with water. After application of RuBP, the column was eluted isocratically with 0.1 N HCl, and 700-drop fractions (27 mL) were collected. Fractions 45–65, which contained the majority of eluted RuBP, were pooled (567 mL), and the pH of the solution was adjusted on ice to 6.5 with 62 mL of 1 N LiOH. Precipitation of the RuBP was obtained by the addition of 10 mL of 10% barium acetate and 1 L of ethanol. The resultant precipitate was collected by centrifugation and washed several times with small portions of ethanol (30 mL). After the precipitate was dried in vacuo over phosphorus pentoxide and Drierite, 0.35 g (0.6 mmol, 81% yield) of the barium salt of [3-^{2}H]RuBP was recovered.

Except where otherwise noted, all reagents and enzymes used in the synthesis of various labeled RuBP preparations were obtained from Sigma Chemical Co. 4-Carboxy-D-arabinitol 1,5-bisphosphate and spinach and *Rhodospirillum rubrum* ribulosebisphosphate carboxylases were prepared by published procedures (Schloss & Lorimer, 1982; Lorimer et al., 1976; Schloss et al., 1982). Mes, Heppso, Ampso, Mopso, Hepes, Bistris, Taps, Ches, bicine, tricine, Mops, Bes, Dipso,

[1] Abbreviations: RuBP, D-ribulose 1,5-bisphosphate; XuBP, D-xylulose 1,5-bisphosphate; RuBisCO, ribulosebisphosphate carboxylase/oxygenase; D_V, a deuterium isotope effect on V_{max} ($[^1H]V/[^2H]V$); $D_{(V/K)}$ and $T_{(V/K)}$, a deuterium isotope effect or tritium isotope effect, respectively, on V_{max}/K_m for RuBP [($[^1H]V/K$)/($[^2H]V/K$) or ($[^1H]V/K$)/($[^3H]V/K$)]; $13_{(V/K)}$, a ^{13}C isotope effect on V/K for CO_2 [($[^{12}C]V/K$)/($[^{13}C]V/K$)]; 13_V, a ^{13}C isotope effect on V ($[^{12}C]V/[^{13}C]V$). The following abbreviations used for buffers are the same as used by the vendor (Research Organics Inc.) from which they were purchased: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Heppso, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-hydroxypropanesulfinic acid; Ampso, 2-acrylamido-2-methylpropanesulfonic acid; Mopso, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; Taps, 3-[[tris(hydroxymethyl)methyl]amino]-propanesulfonic acid; Ches, 2-(cyclohexylamino)ethanesulfonic acid; bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Mops, 3-(*N*-morpholino)propanesulfonic acid; Bes, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; Dipso, 3-[bis(hydroxyethyl)amino]-2-hydroxypropanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Popso, piperazine-*N*,*N*′-bis(2-hydroxypropanesulfonic acid); Tes, 2-[[tris(hydroxymethyl)methyl]-amino]ethanesulfonic acid.

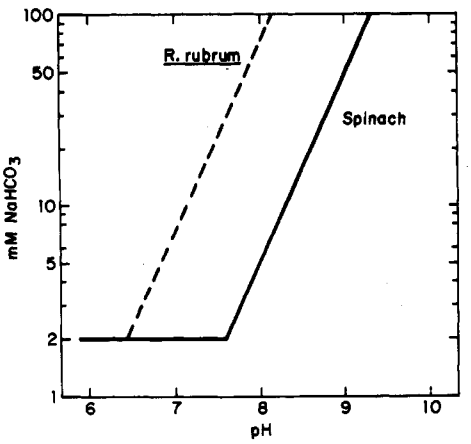


FIGURE 1: Concentration of $NaHCO_3$ ($NaHCO_3 + Na_2CO_3 + CO_2$) used in assays of spinach (—) and *R. rubrum* (- - -) carboxylases at various pH values.

Pipes, Popso, and Tes were obtained from Research Organics Inc.

Assays. The spectrophotometric assay of Racker (1965), as described previously (Schloss et al., 1982), was used for the routine assay of RuBisCO from spinach or *R. rubrum*. Each routine assay contained 50 mM bicine–KOH, pH 8, 50 mM $NaHCO_3$, 0.5 mM RuBP, 10 mM $MgCl_2$, 5 mM ATP, 0.2 mM NADH, 81 units of phosphoglycerate kinase, 17 units of glyceraldehyde-3-phosphate dehydrogenase, 31 units of triosephosphate isomerase, and 3 units of glycerolphosphate dehydrogenase. All kinetic data were collected at 25 °C. In determining the effect of pH on the kinetic parameters of either enzyme, various buffers (most buffers were obtained from Research Organics Inc.; for a listing see Materials; other buffers used but not listed include acetate and pyridine) were substituted for bicine. All buffers were prepared as 1 M stock solutions at their p*K*s, either as sodium or chloride salts (1 M buffer and 0.5 N NaOH or 0.5 N HCl, for monoprotic buffers). These were diluted to 0.1 M final concentration in the assays, and for intermediate pH values, between that of stock buffer solutions, two buffers were mixed. The combined concentration of buffers in the mixture was maintained at 0.1 M. To minimize the effect of variations in ionic strength, 0.1 M KCl was included in all assay mixtures. Where possible, zwitterionic buffers were employed, so that constant ionic strength was maintained. No specific buffer effects were encountered, as deduced by correspondence between data sets obtained at the same (or very close) pH with different buffers. Except where CO_2 was varied, sufficient $NaHCO_3$ was added to saturate the reaction with respect to this substrate. At each pH, the optimal quantity of $NaHCO_3$ was determined and a saturating, noninhibitory concentration used. Although not examined in detail, the K_m for CO_2 did not appear to be pH dependent. The concentration of $NaHCO_3$ employed in assays for spinach and *R. rubrum* carboxylases at different pH values is shown in Figure 1. At higher pH, the concentrations of $NaHCO_3$ employed give constant CO_2 concentrations of 0.11 mM for the spinach carboxylase and 1.6 mM for the *R. rubrum* enzyme. Assays were initiated by use of an adder mixer containing preactivated carboxylase (0.1 M bicine, pH 8, 10 mM $MgCl_2$, and 50 mM $NaHCO_3$) and coupling enzymes. Especially at low pH, where deactivation of the carboxylase is rapid (half-time for spinach carboxylase at pH 6 was about 1 min), it is important to obtain good estimates of initial reaction rates.

When radiometric assays were employed, enzymic reaction mixtures were quenched at 15 s with 0.1 volume of 50% trifluoroacetic acid and dried under a stream of N_2 at 100 °C. After a second addition of acid and redrying, the residue was dissolved in 1 mL of water and 10 mL of Scinti Verse I (Fisher Chemical Co.) scintillation cocktail.

Data Reduction. The MLAB data modeling program (Knott, 1979), available from the National Institutes of Health, was used to fit the equations to data:

$$v = VA/(K + A) \quad (1)$$

$$v = VA/[K(1 + I/K_I) + A] \quad (2)$$

$$v = VA/[(K + A)(1 + A/K_I)] \quad (3)$$

$$v = VA/[K(1 + F_iE_{(V/K)}) + A(1 + F_iE_V)] \quad (4)$$

$$y = C/(1 + H/K_1 + K_2/H) \quad (5)$$

$$y = [Y_L + Y_H(K/H)]/(1 + K/H) \quad (6)$$

$$y = C/(1 + H^m/K_1^m + K_2^n/H^n) \quad (7)$$

In eq 1–4, v is velocity, V is maximal velocity, A is substrate concentration, K is the concentration of substrate which gives half-maximal velocity, or the Michaelis constant, K_I is an inhibition constant for an inhibitor or in eq 3 for substrate, I is the concentration of inhibitor, F_i is the fraction of isotopic substitution, and E_V and $E_{(V/K)}$ are the isotope effects minus one on V and V/K, respectively. In eq 5–7, y is a pH-dependent variable [V, V/K, E_V, or $E_{(V/K)}$], C is the pH-independent value of y, K is an ionization constant, K_1 is the more acidic ionization constant (essential base) and K_2 the more basic ionization constant (essential acid), Y_L and Y_H are the low and high pH values for y, and m and n are the number of ionizing groups, where more than one group ionizes at the same pH.

RESULTS

pH Dependence of Spinach Carboxylase. When the fixed-time assay was used to determine the pH dependence of V and V/K for the spinach carboxylase, the data shown in Figure 2 were obtained. A single essential basic and acidic residue adequately defines the data for both V and V/K. Apparent pK values of 7.1 ± 0.1 and 8.3 ± 0.2 were obtained for V, and V/K was best fit with two equivalent pK values of 7.5 ± 0.4.

When the spectrophotometric assay was used to determine the activity of spinach RuBP carboxylase, the results obtained in Figure 3 were obtained. V appears to be dependent on two essential basic groups, each with an apparent pK of 6.85 ± 0.03, and two essential acidic groups, each with an apparent pK of 8.58 ± 0.04, when fit to eq 7 (Materials and Methods). V/K is equally complex and appears to be dependent on three essential basic groups, each with an apparent pK of 6.76 ± 0.02, and three essential acidic groups, each with an apparent pK of 8.37 ± 0.03. The ratio of the fitted parameters for [^{1}H]RuBP and [^{2}H]RuBP were used to generate the dependence of D_V and $D_{(V/K)}$ on pH (Figure 3). While D_V varies from 2 at high pH to 9.5 at low pH, $D_{(V/K)}$ appears pH-independent and has a value of 2.5. However, on comparing the results obtained with the spectrophotometric assay with those obtained by the $^{14}CO_2$-fixation assay (above), the absolute rates obtained are increasingly disparate at the pH extremes.

At low pH, both assays gave equivalent rates when identical assay solutions were used for each. However, when the required components of the coupled-spectrophotometric assay were omitted from the radiometric assay (ATP, NADH, and coupling enzymes), substantially higher rates were observed. This discrepancy was due to inhibition of the spinach enzyme by ATP. ATP, although not inhibitory at pH 8 (K_i > 64 mM),

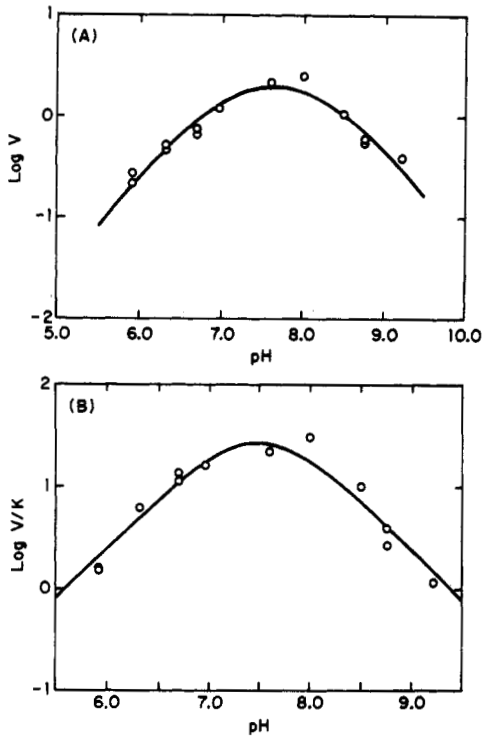


FIGURE 2: pH dependence of the spinach carboxylase reaction when the reaction is followed by the $^{14}CO_2$-fixation assay. (A) log V vs. pH. (B) log V/K vs. pH.

is a competitive inhibitor vs. RuBP at pH 6 with a K_i of 0.26 ± 0.04 mM. Increased inhibition of the enzyme by ATP at lower pH values biases the data obtained with the spectrophotometric assay, such that an overestimate of the number of essential ionizing groups is obtained. An additional complication encountered at low pH is substrate inhibition by RuBP. An apparent inhibition constant of 13 ± 4 mM was obtained for RuBP when data were fit to eq 3 (Materials and Methods). Substrate inhibition by RuBP was not observed at high pH. The concentration of RuBP required to give substrate inhibition was sufficiently high that kinetic parameters could be obtained at concentrations well below the K_i for RuBP to eliminate the need for more sophisticated data analyses.

The spectrophotometric assay exhibited a pronounced lag at more alkaline pH values (pH >8.3). Although the lag could be reduced by increasing the concentration of coupling enzymes, initial rates could not be adequately assessed at the more alkaline conditions examined. Presumably, the lack of correspondence between the two assays reflects the deterioration of the coupled assay at high pH; however, the exact problem(s) in the coupled assay has (have) not been identified.

Despite the lack of correspondence of the kinetic parameters (V and V/K) obtained by the spectrophotometric assay with those determined by the fixed-time assay, the isotope effects determined at the pH extremes by either method are in reasonable agreement (Table I). However, the useful range of the spectrophotometric assay is limited to pH 7–8.5 for the determination of other kinetic parameters (K_i, V, and V/K).

When RuBP (1 mM) was titrated at room temperature with NaOH, the last two titratable groups were unresolved and exhibited an average pK of about 6.4. When 10 mM $MgCl_2$ was included in the solution of RuBP to be titrated, in the presence or absence of 0.1 M KCl, an apparent pK of 6.0 was obtained. These data suggest that neither of the ionization constants observed in the V/K profiles is attributable to RuBP and that both of these groups are enzymic residues.

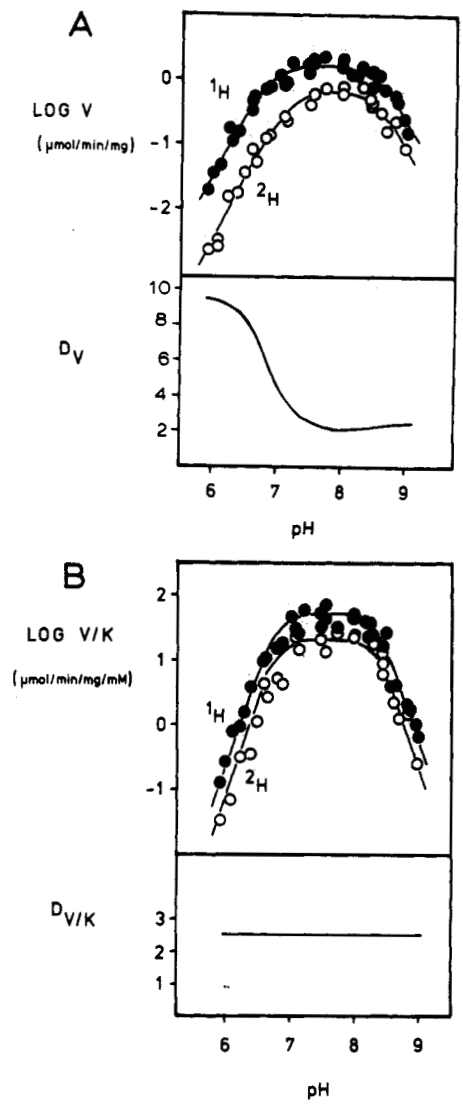


FIGURE 3: pH dependence of the carboxylase reaction of the spinach enzyme when the reaction is followed by the spectrophotometric assay. (A) The dependence of log V for [3-^{1}H]RuBP (●) and [3-^{2}H]RuBP (○). The ratio of $[^1H]V/[^2H]V$ calculated from the fitted values for these profiles is shown below. (B) The dependence of log V/K for [3-^{1}H]RuBP (●) and [3-^{2}H]RuBP (○). The ratio of $[^1H](V/K)/[^2H](V/K)$ calculated from the fitted values for these profiles is shown below.

Table I: Comparison of Isotope Effects Determined by the Continuous Spectrophotometric and Fixed-Time $^{14}CO_2$-Fixation Assays

	spectrophotometric[a]		fixed time[a]	
pH	D_V	$D_{(V/K)}$	D_V	$D_{(V/K)}$
6	10	2.4	7.7	2.6
7.5	2.8	2.3	2.8	2.2
9	2.7	2.3	1.6	2.7

[a] The average error in the fits for D_V and $D_{(V/K)}$ are 20%.

pH Dependence of Inhibition of the Spinach Enzyme by XuBP. The fixed-time assay was also used to examine the effect of pH on the inhibition of the carboxylase by the RuBP analogue XuBP. Again a single essential acidic and basic group affected the spinach carboxylase's affinity for XuBP. Apparent pK values of 7.2 ± 0.3 and 7.8 ± 0.3 were obtained from fits of the data in Figure 4. The pH-independent value of K_i for XuBP was 1.2 ± 0.6 μM. As previously reported (Ryan et al., 1975; McCurry & Tolbert, 1977), the inhibition of spinach carboxylase by XuBP was time-dependent. Only

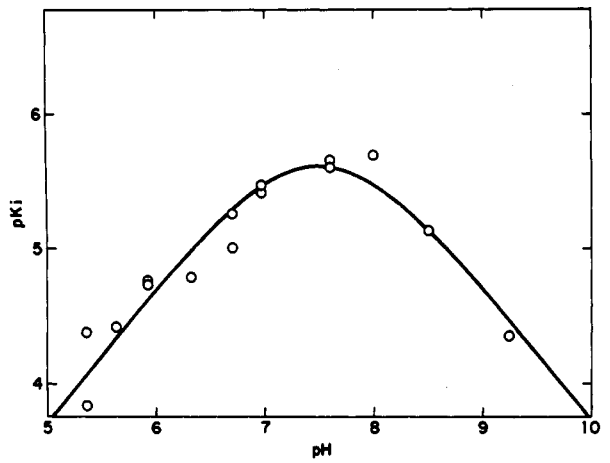


FIGURE 4: pH dependence of the affinity of the spinach carboxylase for XuBP. The negative log of the inhibition constant (pK_i) as a function of pH is illustrated.

Table II: ^2H Isotope Effect vs. CO_2 Concentration, Spinach Enzyme, pH 8

added $NaHCO_3$[a] (mM)	D_V[b]	$D_{(V/K)}$[b]	V[c]	K[d]
0.2	2.13	2.14	0.67	27
1.2	2.66	1.56	1.37	48
2.2	2.13	1.90	1.20	37
5.2	1.98	2.38	1.53	47
10	2.45	2.10	1.44	49
10	2.63	2.10	1.63	36
20	2.28	2.82	1.44	32
25	2.67	1.81	1.55	54
50	1.95	2.53	1.01	54
50	3.20	1.82	1.30	44
100	3.23	2.43	1.11	85

[a] The concentration of $NaHCO_3$ added to assays. Endogenous ($NaHCO_3$ + CO_2) was about 1 mM, and 0.2 mM was introduced as carryover due to preactivation of the enzyme by CO_2/Mg^{2+}. [b] The average error in the fits of data to eq 4 (Materials and Methods) was 0.2 for D_V and 0.38 for $D_{(V/K)}$. The average for all values (plus or minus one standard deviation) of D_V is 2.48 ± 0.18 and for all values of $D_{(V/K)}$ is 2.14 ± 0.12. [c] Values of V obtained for [^{1}H]RuBP are given in μmol min^{-1} mg^{-1}. The average error in the fits of data to eq 4 was 3.3% of the reported values. [d] Values for K are given in μM. The average error in the fits of data to eq 4 was 11% of the reported values.

the initial, rapidly reversible inhibition of the enzyme was examined. Given the similarity of the pK values for the inhibition profile of XuBP and those observed in the V/K profile, and the fact that only one acidic residue and one basic residue are observed, it seems likely that the identities of these groups are the same in both profiles.

Effect of CO_2 Concentration on $D_{(V/K)}$. The concentration of CO_2 had little effect on $D_{(V/K)}$ or D_V for the spinach enzyme at concentrations ranging from 1 to 100-fold that of this substrate's Michaelis constant (Table II). An average value for $D_{(V/K)}$ of 2.1 ± 0.1, and for D_V of 2.5 ± 0.2, was obtained. These experiments were conducted at pH 8.0 (Heppso buffer). The ionic strength of the assay (which is varied along with CO_2 when this substrate is supplied as $NaHCO_3$) had little effect on D_V or $D_{(V/K)}$. When the isotope effects were determined at pH 8, omitting the 0.1 M KCl normally included in all assay mixtures (0.1 M Heppso; 5 mM $NaHCO_3$), values for D_V and $D_{(V/K)}$ of 2.4 ± 0.2 and 1.6 ± 0.2, respectively, were obtained. These values are close to the average values obtained in the presence of 0.1 M KCl (Table II), with the value for $D_{(V/K)}$ somewhat smaller than at higher ionic strength.

Effect of Propylene Glycol on the Spinach pH Profiles. Determination of the ionization constants for the spinach

Table III: Apparent Ionization Constants for Spinach RuBisCO

substrate or inhibitor	V		V/K or K_i	
	pK_1	pK_2	pK_1	pK_2
RuBP	7.1 ± 0.1	8.3 ± 0.2	7.5 ± 0.4	7.5 ± 0.4
RuBP + PG[a]	6.8 ± 0.3	8.4 ± 0.3	7.3 ± 0.6	7.3 ± 0.6
XuBP			7.2 ± 0.3	7.8 ± 0.3
XuBP + PG[a]			7.3 ± 0.6	7.8 ± 0.6

[a] Values obtained in the presence of 30% propylene glycol. Only amine buffers (cationic acids) were used in these experiments.

Table IV: Tritium Isotope Effect with [3-^{3}H]RuBP and [^{14}C,3-^{1}H]RuBP

reaction time (min)	^3H (cpm)			^{14}C (cpm)		
	RuBP[a]	PGA[b]	Σ[c]	RuBP[a]	PGA[b]	Σ[c]
0	38 800	0	38 800	11800	0	11 800
0.5	35 300			8810		
2.5	26 400			3100		
300	580	9660	10 200	130	11900	12 000
300[d]	37 100	1650	38 700	9920	2300	12 200

[a] Radioactivity (^{3}H or ^{14}C) in the chromatographic position of RuBP. [b] Radioactivity (^{3}H or ^{14}C) in the chromatographic position of 3-phosphoglycerate. [c] The sum of radioactivity (^{3}H or ^{14}C) in the positions of RuBP and 3-phosphoglycerate. [d] Incubation mixture containing 4-carboxy-D-arabinitol 1,5-bisphosphate.

enzyme in the presence of 30% propylene glycol gave values that were very similar to those obtained in its absence (Table III). Although the apparent pK values in the V/K and K_i profiles are somewhat poorly defined, due to the close proximity of the ionizations of the essential acid and base, these data indicate that the groups are probably cationic acids (lysyl or histidyl residues). Consideration of the midpoints of the pH profiles, which are defined by (pK_1 + pK_2)/2, corroborates the conclusions drawn from consideration of either pK independently. V, V/K, and K_i profiles do not have higher midpoints in the presence of propylene glycol, suggesting that neither pK is perturbed to a higher value by propylene glycol. The error estimates for fits of the midpoints of these profiles are less than half the error associated with either independent pK, lending a higher degree of confidence to the interpretation.

Tritium Isotope Effect on the Spinach Carboxylase Reaction. To determine the effect of tritium substitution at C-3 of RuBP on V/K, a series of enzymic reaction mixtures were prepared with [3-^{3}H]RuBP and [^{14}C]RuBP. These reaction mixtures consisted of 0.1 M KCl, 0.1 M bicine, pH 8.3, 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 0.011 mg/mL spinach carboxylase, 0.6 μM [3-^{3}H]RuBP, and 0.1 μM [^{14}C]RuBP, in a volume of 10 mL. In one reaction mixture 0.1 mM 4-carboxy-D-arabinitol 1,5-bisphosphate was included to stop the enzymic reaction. After the enzymic reaction was allowed to proceed for the desired amount of time, 2 g of AG 50W-X8 (hydrogen form) was added to stop the reaction. The samples were then filtered, diluted to 50 mL, and applied to a 1.2 × 25 cm column of AG 1-X8 (chloride form). The column was developed with 50 mL of water, followed by 0.1 N HCl, and 1-mL fractions were collected. The results obtained are summarized in Table IV. A substantial fraction of the tritium derived from [3-^{3}H]RuBP was converted to [^{3}H]phosphoglycerate rather than water. Since previous work has demonstrated that the hydrogen abstracted from C-3 is not transferred to phosphoglycerate, but to water (Fiedler et al., 1967), the RuBP has 24.9% of the tritium label in a position other than C-3. Presumably, the commercially available L-glyceraldehyde used to prepare [3-^{3}H]RuBP was not stereochemically pure. Reduction of D-glyceraldehyde by NaB^3H_4 would lead to contamination of the [3-^{3}H]RuBP with [5-

^{3}H]RuBP (see Materials and Methods). When the incubation of ^{3}H- or ^{14}C-labeled RuBP with the enzyme was extended, virtually all of the RuBP was consumed (1.5% and 1.1% of the ^{3}H and ^{14}C remained in the position of RuBP after a 300-min incubation, respectively), and all of the ^{14}C label could be accounted for in the position of 3-phosphoglycerate. Inclusion of a potent inhibitor of the carboxylase in the incubation mixture, 4-carboxy-D-arabinitol 1,5-bisphosphate, prevented the conversion of both ^{3}H and ^{14}C, demonstrating that the reaction being followed was carboxylase-dependent. The respective first-order rate constants for loss of ^{14}C and ^{3}H from the chromatographic position of RuBP were 0.541 ± 0.021 min^{-1} and 0.152 ± 0.009 min^{-1}. These values give an apparent tritium isotope effect of 3.6. However, when the lack of specificity of ^{3}H labeling is taken into account, assuming that RuBP which contains ^{3}H at a position other than C-3 is likely to have ^{1}H at C-3, a value of 6.5 can be calculated for $T_{(V/K)}$. A previously reported value for $T_{(V/K)}$, 5.9, is between the values obtained above (Fiedler et al., 1967). In the previous report, the size of the tritium isotope effect was dependent on the extent of reaction, and extrapolation of the data of Fiedler et al. to initial reaction conditions gives a value for $T_{(V/K)}$ of 6.2. By use of the best values for $T_{(V/K)}$ (6.2–6.5) and $D_{(V/K)}$ (2.1–2.4; Tables II and I, respectively), the intrinsic deuterium isotope effect probably falls between 18 and 37, when calculated by the method of Northrop (1975). These values can be compared with the limiting values for D_V at low pH which range from 7.7 to 10 (Table I), with an extrapolated value from the pH profiles for V of 9.5 (Figure 2A). Although these values are extremely large deuterium isotope effects, they are smaller than the range of values calculated for the intrinsic deuterium isotope effect from $D_{(V/K)}$ and $T_{(V/K)}$ by the method of Northrop. Isotope effects greater than 18 exceed the generally accepted limit for deuterium effects in the absence of tunneling (Kresge, 1977). As such, it is likely that the values for D_V at low pH are equivalent to intrinsic effects and are more reliable estimates than those made from comparisons of $D_{(V/K)}$ and $T_{(V/K)}$, especially since the validity of the latter calculation requires that the reverse commitment to catalysis is very small.

pH Dependence of the Rhodospirillum rubrum Carboxylase. Determination of deuterium isotope effects for the *R. rubrum* carboxylase at pH 8.4 with the spectrophotometric assay gave 1.5 ± 0.1 and 0.97 ± 0.07 for D_V and $D_{(V/K)}$, respectively. These values are in reasonable agreement with those previously reported for this form of the enzyme under similar conditions (Sue & Knowles, 1982a). Determination of the effects at pH 6.4, however, gave values of 3.1 ± 0.4 and 2.6 ± 0.4 for D_V and $D_{(V/K)}$, respectively. In contrast to the results obtained for the spinach carboxylase, the isotope effects for the *R. rubrum* enzyme on both V and V/K are pH-dependent. When a more detailed analysis of the pH dependence of the *R. rubrum* enzyme was carried out by using the fixed-time assay, the results shown in Figure 5 were obtained. Apparent pK values of 7.3 ± 0.1 and 7.1 ± 0.2 were obtained for an essential enzymic base in the V and V/K profiles, respectively. Values of 7.6 ± 0.2 and 7.5 ± 0.1 were obtained in the corresponding profiles for [3-^{2}H]RuBP. Although not well-defined in the accessible pH range, an essential acidic residue with a pK of 8.1 ± 0.7 and 7.8 ± 0.4 in the [3-^{1}H]-RuBP and [3-^{2}H]RuBP V/K profiles is apparent. When the deuterium isotope effects defined by these data (Figure 6) were fit to eq 6 (Materials and Methods), limiting values of 1.9 ± 0.2 and 4.7 ± 0.5 were obtained at high and low pH for D_V, and 0.8 ± 0.2 and 7 ± 1 were obtained at high and low pH for $D_{(V/K)}$. The apparent pK values for these changes in the limiting values were 6.8 ± 0.3 and 6.4 ± 0.2 for D_V and $D_{(V/K)}$, respectively. If the limiting values for D_V and $D_{(V/K)}$ at low pH approximate the intrinsic isotope effect on the bond-breaking step of proton abstraction from C-3, then they should be equal and equivalent to the intrinsic effect for the spinach enzyme. The limiting value obtained for $D_{(V/K)}$ (7) for the *R. rubrum* enzyme is in reasonable agreement with the maximal value for D_V at low pH for the spinach enzyme (about 9.5; see earlier). Although the limiting value for D_V with the *R. rubrum* enzyme is somewhat lower than these other values (5), as an extrapolated value from limited data it is probably more poorly defined than the error indicated by the fit of data to eq 6.

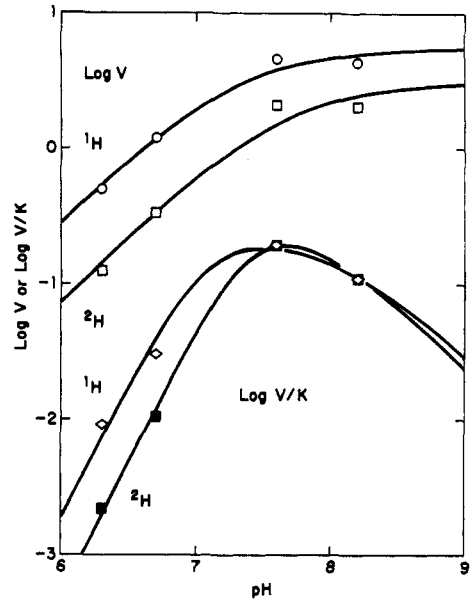


FIGURE 5: pH dependence of the carboxylase reaction of the *R. rubrum* enzyme determined by the $^{14}CO_2$-fixation assay. log V for [3-^{1}H]RuBP (○) and [3-^{2}H]RuBP (□) and log V/K for [3-^{1}H]RuBP (◇) and [3-^{2}H]RuBP (■) vs. pH are illustrated.

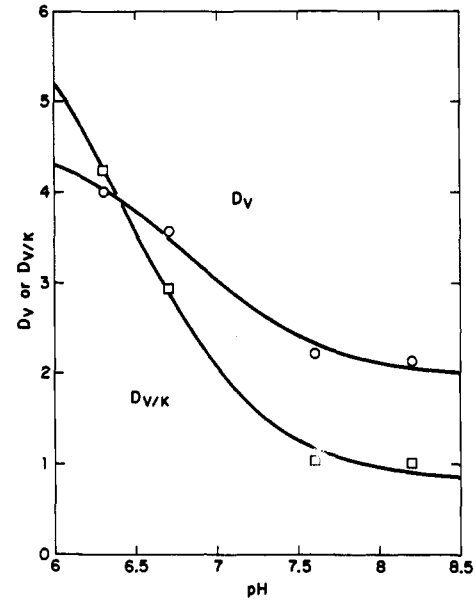


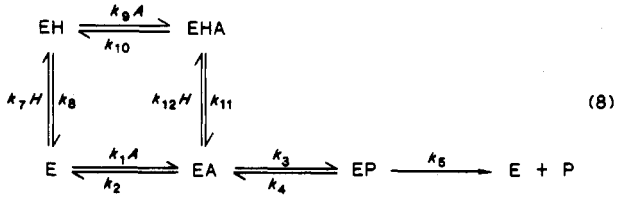
FIGURE 6: pH dependence of the deuterium isotope effects on V (○) and V/K (□) for the *R. rubrum* carboxylase.

DISCUSSION

Determination of the effect of pH on the catalytic activity

of ribulosebisphosphate carboxylase is fraught with technical difficulties. The enzyme has an absolute requirement for prior allosteric activation by CO_2 and divalent metal (Andrews et al., 1975; Lorimer et al., 1976, 1978; Badger & Lorimer, 1976; Miziorko, 1979; Lorimer, 1979), a process that is both time and pH dependent. The former attribute of activation (a relatively slow transition between active and inactive forms) allows the enzyme to be preactivated and examined briefly under nonactivating conditions. A further complication arises in that the enzyme has a competing oxygenase activity (Bowes et al., 1971; Bowes & Ogren, 1972; Andrews et al., 1973; Lorimer et al., 1973; Ryan et al., 1974; McFadden, 1974; Bahr & Jensen, 1974; Ryan & Tolbert, 1975), and unless assayed under a defined atmosphere, the reaction becomes complex at low CO_2 (inhibited by O_2 when assayed by $^{14}CO_2$ fixation or diminishes to a CO_2-independent rate when the reaction is followed by 3-phosphoglycerate production). Assay of the enzyme at saturating CO_2 (which inhibits the oxygenase activity) by the spectrophotometric method of Racker (1965) can be carried out reliably only over a rather narrow pH range. At low pH, ATP, which is a required component of this assay, inhibits the spinach enzyme. At high pH the coupled reaction exhibits a lag of increased duration and fails to accurately reflect the initial rate as determined by CO_2 fixation. Despite the lack of correspondence in absolute rate as determined by the spectrophotometric and CO_2-fixation assays, the isotope effects for [3-^{2}H]RuBP [D_V or $D_{(V/K)}$] determined by either assay are in agreement. Given the technical difficulties in determining the effect of pH on the carboxylase, divorced from the known effect of pH in altering the activation state of the enzyme, previous pH studies (Christeller, 1982; Paech, 1985) are in reasonable agreement with the current study. The somewhat lower value for the pK of the essential enzymic base in the spinach $(V/K)_{RuBP}$ profile reported previously [Paech's (1985) 6.5 vs. 7.5 presently reported] is likely to be due to bias of the data obtained at lower pH in the earlier study.

At low pH, the isotope effect on V for the spinach enzyme with [3-^{2}H]RuBP gets very large (9.5), in contrast to the effect on the V/K for RuBP, which is pH-independent. The corresponding enzyme from *Rhodospirillum rubrum* differs from the spinach enzyme in that the isotope effects on both V and V/K increase at low pH. A kinetic scheme which could account for an increase in D_V, without a corresponding increase in $D_{(V/K)}$, has been described by Cook & Cleland (1981b). The following mechanism is similar to theirs:



(8)

In this mechanism A is substrate, P is product, H is hydrogen ion, E is enzyme, and k_3 and k_4 are the isotope-sensitive steps. Protonation of the EA complex results in loss of enzymic activity, as EHA is inactive. For this mechanism

$$V = k_3k_5/[k_3 + (1 + H/K_{int})(k_4 + k_5)] \quad (9)$$

$$V/K = k_3k_5[k_9H/K_f + k_1(k_{10}/k_{11} + 1)]/[[k_{10}(k_4 + k_5)H/K_{int} + (k_2k_4 + k_2k_5 + k_3k_5)(k_{10}/k_{11} + 1)][1 + H/K_f]] \quad (10)$$

$$D_V = \frac{D_k + (k_3/k_5)/(1 + H/K_{int}) + (k_4/k_5)D_{(K_{eq})}}{1 + (k_3/k_5)/(1 + H/K_{int}) + (k_4/k_5)} \quad (11)$$

$$D_{(V/K)} = \left[D_k + \frac{k_3(k_{10}/k_{11} + 1)}{k_{10}H/K_{int} + k_2(k_{10}/k_{11} + 1)} + (k_4/k_5)D_{(K_{eq})} \right] \Big/ \left[1 + \frac{k_3(k_{10}/k_{11} + 1)}{k_{10}H/K_{int} + k_2(k_{10}/k_{11} + 1)} + (k_4/k_5) \right] \quad (12)$$

where $K_{int} = k_{11}/k_{12}$, $K_f = k_8/k_7$, D_k is the intrinsic isotope effect, or the effect on the elementary step k_3, $([^1H]k_3)/([^2H]k_3)$, and $D_{(K_{eq})} = [([^1H]k_3)([^2H]k_4)]/[([^2H]k_3)([^1H]k_4)]$. The equations for D_V and $D_{(V/K)}$ conform to the general equations for isotope effects on V and V/K (Schimerlik et al., 1977; Northrop, 1977):

$$D_{(V/K)} = \frac{D_k + C_f + C_r(D_{K_{eq}})}{1 + C_f + C_r} \quad (13)$$

$$D_V = \frac{D_k + C_{Vf} + C_r(D_{K_{eq}})}{1 + C_{Vf} + C_r} \quad (14)$$

where the constants C_f and C_{Vf} are forward commitment factors and C_r is the reverse commitment factor. Mechanism 8 differs from the similar mechanism considered by Cook and Cleland (1981b) in that the isotope sensitive step (k_3) is reversible, which results in a reverse commitment factor in the equations for D_V and $D_{(V/K)}$. For this reason, the isotope effects on V or V/K do not increase to the intrinsic effect (D_k) at low pH, as in the Cook and Cleland mechanism, since C_r remains equal to k_4/k_5 rather than decreasing to zero, as do C_f and C_{Vf}, at low pH. In either mechanism, if step k_{10} becomes negligible, then $D_{(V/K)}$ is no longer pH dependent, while D_V is unaffected by the size of k_{10}. This simple scheme provides a potential explanation for the results obtained with the spinach carboxylase. If protonation of the enzyme–RuBP complex locks the substrate in the complex, preventing its release, then the isotope effect on V should remain pH dependent and increase to the intrinsic effect at low pH if the reverse commitment (k_4/k_5) is small, while the isotope effect on V/K will not vary with pH. This also provides an explanation for the difference between the spinach and *Rhodospirillum rubrum* carboxylases, since for the latter, in contrast to the spinach enzyme, both D_V and $D_{(V/K)}$ increase at low pH. With the *R. rubrum* enzyme, protonation of the enzyme–RuBP complex does not prevent release of this substrate from the complex. It is not clear why protonation should have this result with the spinach enzyme. A potential explanation may be that transfer of the C-3 proton of RuBP to the essential enzymic base normally triggers a conformational change leading to reduced solvent accessibility of the ene–diol and subsequent intermediates of the reaction. Protonation of this base by solvent would be sufficient to trigger a reduction in solvent accessibility for RuBP with the spinach enzyme but not with the *R. rubrum* enzyme.

The change in D_V with pH for the spinach carboxylase, and in both D_V and $D_{(V/K)}$ with pH for the *R. rubrum* enzyme, identifies the residue with the lower pK in the profile as the essential enzymic base that abstracts the C-3 proton from RuBP in the first step of the carboxylase reaction (Cook & Cleland, 1981b,c). This residue exhibits a pK value of 7.5 in the spinach V/K profile and, on the basis of solvent perturbation experiments, appears to be a cationic acid (Cleland, 1977), implicating either a lysyl or histidyl residue as the essential enzymic base. By diethyl pyrocarbonate modification,

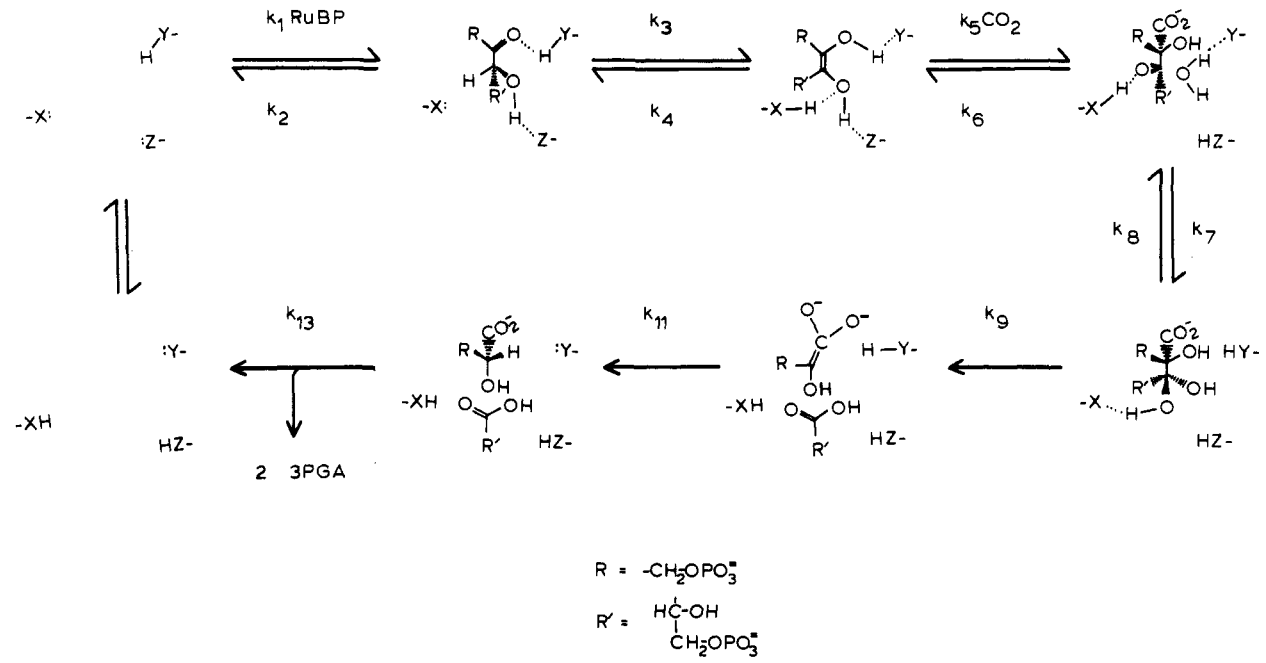


FIGURE 7: Acid–base chemistry of the carboxylase reaction. The enzymic residues that function as acids or bases are designated as X, Y, and Z in the figure.

a pK of 6.4–6.8 has been determined for an essential histidyl residue in the spinach carboxylase (Paech, 1985). Inactivation of the spinach enzyme by diethyl pyrocarbonate is due predominantly to the modification of histidine-298, which is an evolutionarily conserved residue (Igarashi et al., 1985). Both the spinach and *R. rubrum* carboxylases are inactivated by trinitrobenzenesulfonate, which is a consequence of the modification of lysine-334 in the spinach and lysine-166 in the *R. rubrum* enzyme, respectively (Hartman et al., 1985). The respective pK values for these lysyl residues based upon trinitrobenzenesulfonate modification are 9.0 and 7.9 for the activated form of the enzyme (Hartman et al., 1985). If the interconversion of EH and EHA is slow, as required to account for the observed change in D_V without a corresponding change in $D_{(V/K)}$ ($k_9 = 0$; $k_{10} = 0$), then the observed pK in the V/K profile is equal to pK_f in mechanism 8 (see eq 10). For a complete protonation scheme, the observed pK in the V/K profile will be dominated by pK_{int}. However, pK_f will be larger than pK_{int} if the substrate binds less tightly to the protonated form of the enzyme, a likely possibility since the enzyme's affinity for XuBP diminishes at lower pH. Since the pK observed in the V/K profile will be equal to or smaller than the intrinsic pK of the free enzyme (pK_f in mechanism 8, which would be the pK observed in chemical modification studies), either of the two lysyl residues, particularly lysine-166, which is highly conserved and a common site of modification by several affinity-labeling reagents, would seem to be a likely choice as the residue which abstracts the C-3 proton of RuBP. As illustrated in Figure 7, a minimum of two to three enzymic residues would be required for the expected acid–base chemistry of the carboxylase reaction.

The insensitivity of $D_{(V/K)}$ for [3-^{2}H]RuBP to CO_2 concentration provides some insight into the kinetic mechanism of the carboxylase. Several potential kinetic mechanisms have been considered: ordered binding of RuBP and CO_2 followed by proton abstraction at C-3, random addition of RuBP and CO_2 followed by proton abstraction, and proton abstraction from RuBP preceding the addition of CO_2. Ordered binding, with either RuBP or CO_2 binding first, can be eliminated as a possible mechanism by existing data. If RuBP binds first, $D_{(V/K)}$ is CO_2-dependent, and the deuterium isotope effect on $(V/K)_{RuBP}$ is abolished at high CO_2. The insensitivity of $D_{(V/K)}$ to CO_2 concentrations up to 100-fold that of this substrate's Michaelis constant rules out this kinetic mechanism. Conversely, if CO_2 binds first, $13_{(V/K)}$ is RuBP-dependent, and the ^{13}C isotope effect on $(V/K)_{CO_2}$ is abolished at high RuBP. Roeske and O'Leary (1984) have measured a large value for $13_{(V/K)}$, 1.029, at high concentrations of RuBP and found that the value obtained for $13_{(V/K)}$ was not sensitive to the concentration of RuBP used in its determination. These observations render the remaining ordered mechanism, CO_2 binding followed by RuBP addition and proton abstraction from C-3, implausible.

A random mechanism, random binding of CO_2 and RuBP followed by deuterium- and $^{13}CO_2$-sensitive steps, would exhibit CO_2-dependent deuterium isotope effects and RuBP-dependent ^{13}C isotope effects. The extent to which increased CO_2 decreased $D_{(V/K)}$ and increased RuBP decreased $13_{(V/K)}$ would depend on the relative rates of release of these substrates from the ternary complex, enzyme–RuBP–CO_2, in a random mechanism. A large change in the size of the observed isotope effect would be possible if the varied substrate [CO_2 for $D_{(V/K)}$ and RuBP for $13_{(V/K)}$] is released rapidly. Isotope trapping experiments have demonstrated that the rate of CO_2 release (assuming a Michaelis complex is formed with this substrate) from the carboxylase must be extremely rapid (Lorimer, 1979). To the extent that RuBP release is not equally rapid, and the reverse commitment (C_r) is not large, a substantial change in the size of $D_{(V/K)}$ would be expected for a random mechanism as CO_2 is varied. Although the observed insensitivity of $D_{V/K}$ to CO_2 concentration does not exclude a random kinetic mechanism, it reduces the likelihood of such a mechanism. Recent attempts to detect an enzyme–CO_2 complex directly by various ^{13}C NMR experiments have failed to detect such a complex (Pierce et al., 1986), further decreasing the plausibility of a random mechanism. An observation that has been reported to favor a random mechanism is the observed noncompetitive inhibition of the carboxylase by carbon oxysulfide vs. RuBP and competitive inhibition vs. CO_2 (Laing & Christeller, 1980). However, this inhibition pattern only

provides definitive evidence for a random mechanism if carbon oxysulfide is a dead-end inhibitor and *not* a substrate. Carbon oxysulfide has recently been shown to be a reasonably good substrate for the carboxylase (J. Pierce, personal communication), eliminating the only strong evidence in favor of a random kinetic mechanism.

The remaining kinetic mechanism to be considered, proton abstraction from RuBP prior to the addition of CO_2, most adequately represents the kinetic mechanism of the carboxylase. For such a mechanism, $D_{(V/K)}$ would be abolished at low CO_2 concentrations but remain constant from moderate to high concentrations of this substrate, the concentration range examined in the current study. The ^{13}C isotope effect on $(V/K)_{CO_2}$, $13_{(V/K)}$, would be insensitive to the concentration of RuBP, consistent with the observed effect (Roeske & O'Leary, 1984). For a Theorell–Chance mechanism (no Michaelis complex for CO_2), the lack of a reversible step following the addition of CO_2 would allow the level of enzyme–six-carbon reaction intermediate in steady state to be calculated from the observed D_V and the intrinsic deuterium isotope effect. The level predicted, 80% of total enzyme, is greater than 10 times the fraction obtained (6%) in intermediate trapping studies (Schloss & Lorimer, 1982). Assuming the trapping experiments accurately reflect the steady-state level of the six-carbon intermediate, a reversible step following the addition of CO_2 is required for a Theorell-Chance kinetic mechanism. A previously observed decrease in the size of the ^{13}C isotope effect with [3-^{2}H]RuBP was used to argue against substantial proton abstraction prior to the binding of CO_2 (Roeske & O'Leary, 1984; Hermes et al., 1982). For mechanisms involving proton abstraction from RuBP prior to the addition of CO_2, $13_{(V/K)}$ would be the same for [3-^{1}H]RuBP and [3-^{2}H]RuBP, if only the step involving proton abstraction from C-3 were deuterium-sensitive. This inconsistency, between kinetic mechanisms involving proton abstraction from RuBP prior to the addition of CO_2 and the observed smaller $13_{(V/K)}$ with [3-^{2}H]RuBP, can be resolved by involving the abstracted ^2H in a second step after the addition of CO_2. Although exchange can be observed between the C-3 position of RuBP and water, the abstracted proton is not readily lost to solvent (Savier & Knowles, 1982; Sue & Knowles, 1982b), especially not at the concentrations of CO_2 employed in the determination of the ^{13}C isotope effect (Pierce et al., 1986). If the proton abstracted from C-3 were retained on the enzyme and involved in a subsequent step of the mechanism, then a smaller ^{13}C isotope effect could be observed with [3-^{2}H]RuBP, even if the proton were removed prior to the addition of CO_2. The following equation represents such a mechanism:

$$E \underset{k_2}{\overset{k_1R}{\rightleftharpoons}} ER \underset{k_4}{\overset{k_3}{\rightleftharpoons}} ER' \underset{k_6}{\overset{k_5C}{\rightleftharpoons}} EK \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EK' \xrightarrow{k_9} EP'P \xrightarrow{k_{11}} EP_2 \xrightarrow{k_{13}} E + 2P \quad (15)$$

in which E is enzyme, ER is the enzyme–RuBP complex, ER′ is the enzyme complex with the ene–diol form (proton abstracted from C-3) of RuBP, EK is the complex of the keto form of the six-carbon reaction intermediate, 2-carboxy-3-keto-D-arabinitol 1,5-bisphosphate, with enzyme, EK′ is the complex of the *gem*-diol form of the six-carbon reaction intermediate with enzyme, EP′P is the complex of enzyme with 3-phosphoglycerate (P) and the carbanion of 3-phosphoglycerate (P′), and EP_2 is the complex of enzyme and two molecules of 3-phosphoglycerate. This mechanism (eq 15) corresponds to the steps illustrated in Figure 7. As illustrated in Figure 7, in this mechanism the proton abstracted from C-3 of RuBP in step k_3 is subsequently involved in the protonation of the carbonyl of the six-carbon intermediate (k_7) and then reabstracted in the next step (k_9) to effect cleavage of the *gem*-diol. Since this mechanism involves no Michaelis complex with CO_2 (Theorell–Chance), the interconversion between EK and EK′ is written as reversible so that this mechanism is consistent with the results of chemical quench experiments as described earlier. Steps k_9 and k_{11} are written as irreversible, as it is unlikely that addition of the carbanion of 3-phosphoglycerate to the carboxylate of the lower 3-phosphoglycerate would occur, and exchange of the C-2 proton of 3-phosphoglycerate (the reverse of step k_{11}) with water is not facilitated by RuBP carboxylase (Hurwitz et al., 1956; Fiedler et al., 1967). In mechanism 15 steps k_3, k_4, k_7, k_8, and k_9 would be deuterium-sensitive, while only steps k_5 and k_6 would be sensitive to $^{13}CO_2$. The equations for D_V, $D_{(V/K)}$, 13_V, and $13_{(V/K)}$ for mechanism 15 are as follows:

$$D_V = \left[D_k + \frac{k_3}{k_{13}} + \frac{k_3}{k_{11}} + \frac{k_3}{k_5C} + D_{k_9}\left(\frac{k_3}{k_9}\right) \times \left[1 + \frac{k_8}{k_7}D_{K_{eq7}}\left(1 + \frac{k_6}{k_5C}\right)\right] + D_{k_7}\left(\frac{k_3}{k_7}\right)\left(1 + \frac{k_6}{k_5C}\right) + \frac{k_4}{k_5C}\left[1 + \left(\frac{k_6}{k_7}\right)\left(D_{k_7} + \left(\frac{k_8}{k_9}\right)D_{K_{eq7}}D_{k_9}\right)\right]D_{K_{eq}}\right] \Big/ \left[1 + \frac{k_3}{k_{13}} + \frac{k_3}{k_{11}} + \frac{k_3}{k_5C} + \left(\frac{k_3}{k_9}\right)\left[1 + \frac{k_8}{k_7}\left(1 + \frac{k_6}{k_5C}\right)\right] + \left(\frac{k_3}{k_7}\right)\left(1 + \frac{k_6}{k_5C}\right) + \frac{k_4}{k_5C}\left[1 + \frac{k_6}{k_7}\left(1 + \frac{k_8}{k_9}\right)\right]\right] \quad (16)$$

$$D_{(V/K)} = [D_k + k_3/k_2 + (k_4/k_5C) \times (D_{k_7}k_6/k_7 + D_{k_9}D_{K_{eq7}}k_6k_8/k_7k_9 + 1)D_{K_{eq}}]/[1 + k_3/k_2 + (k_4/k_5C)(k_6/k_7 + k_6k_8/k_7k_9 + 1)] \quad (17)$$

$$13_V = 1 \quad (18)$$

$$13_{(V/K)} = \frac{13_k + [k_6(k_8 + k_9)/k_7k_9]13_{K_{eq}}}{1 + k_6(k_8 + k_9)/k_7k_9} \quad (19)$$

where D_{k_7} and D_{k_9} are the isotope effects on steps k_7 ($[^1H]k_7/[^2H]k_7$) and k_9 ($[^1H]k_9/[^2H]k_9$), respectively, and $D_{K_{eq7}}$ is the equilibrium isotope effect on step k_7 ($[^1H]k_7$-$[^2H]k_8/[^2H]k_7[^1H]k_8$). D_k and $D_{K_{eq}}$ retain the same definition as used previously and refer to the primary deuterium-sensitive step k_3 ($[^1H]k_3/[^2H]k_3$ and $[^1H]k_3[^2H]k_4/[^2H]k_3[^1H]k_4$, respectively). Reduction in the size of k_7 or k_9 by a deuterium isotope effect would increase the size of C_r in the equation for $13_{(V/K)}$ (eq 19). An increase in the size of C_r could decrease the size of the observed ^{13}C isotope effect obtained with [3-^{2}H]RuBP relative to the ^{13}C isotope effect obtained with [3-^{1}H]RuBP. Both D_V and $D_{(V/K)}$ are potentially dependent on the concentration of CO_2. However, if the value of k_6/k_7 is rather small, that is to say, EK is highly committed, then the dependence of the deuterium isotope effects on CO_2 would become apparent only at very low concentrations of CO_2. Direct evidence for a high degree of commitment for EK is provided by measurements of the partitioning of exogenous 2-carboxy-3-keto-D-arabinitol bisphosphate with RuBP carboxylase. When the six-carbon intermediate is prepared by large-scale chemical quench and then acted upon by fresh RuBP carboxylase, quantitative conversion of the intermediate to 3-phosphoglycerate with virtually no decarboxylation is

obtained (Lorimer et al., 1986). For 2-[^{14}C]carboxy-3-keto-D-arabinitol bisphosphate, the ratio of cleavage/decarboxylation is equal to $(k_7/k_6)[k_9/(k_7 + k_8 + k_9)]$, when decarboxylation is monitored by the release of radiolabel to CO_2. The ratio k_7/k_6 must be large (and conversely k_6/k_7 small) to account for the observed partitioning of EK in these experiments, as $k_9/(k_7 + k_8 + k_9)$ is ≤ 1. At moderate to high concentrations of CO_2, the observed deuterium isotope effect on $(V/K)_{RuBP}$ is defined by the intrinsic deuterium isotope effect, D_k, and the ratio of k_3/k_2. The value of k_3/k_2 would be approximately equal to 6 at high pH, assuming a value for the intrinsic deuterium isotope effect (D_k) of 9.5. Since the isotope effect on V can have contributions from the isotope effects on k_7 (D_{k_7}) and k_9 (D_{k_9}), as well as the effect on k_3, the actual intrinsic deuterium isotope effect on the proton abstraction step may be somewhat smaller than 9.5. Since the extrapolated value for $D_{(V/K)}$ with the *R. rubrum* carboxylase at low pH is 7, with an observed effect of >4, this somewhat smaller value may be a better estimate of the intrinsic deuterium isotope effect. The value of 9.5 is somewhat larger than expected for a deuterium isotope effect (Melander & Saunders, 1980) and may reflect contributions from the isotope effect(s) latter in the mechanism from the sequestered ^{2}H abstracted in step k_3. It should be pointed out that if the essential base that abstracts the ^{2}H is the ϵ-amino group of a lysyl residue, then only one-third of the isotopic enrichment will be maximally maintained in later steps where this deuterium may be involved. Even if a value of 7 is assumed to accurately reflect the intrinsic deuterium isotope effect, this is a rather large effect and suggests that the enzyme matches the pK values for the enzymic base and C-3 proton of RuBP during step k_3 (i.e., $\Delta G \cong 0$, for step k_3), presumably by effectively lowering the pK of RuBP (via enolate stabilization) until it is approximately equivalent to the pK of the essential enzymic base (Melander & Saunders, 1980).

In conclusion, although a random kinetic mechanism cannot be rigorously excluded on the basis of existing data, the data are consistent with a mechanism involving proton abstraction from C-3 of RuBP before addition of CO_2 in a bimolecular (Theorell–Chance) fashion. The Theorell–Chance mechanism (no Michaelis complex for CO_2) is favored due to the inability to trap radiolabeled CO_2 in product following a quench with unlabeled CO_2 (Lorimer, 1979) and the inability to detect an enzyme–CO_2 complex directly by various ^{13}C NMR experiments (Pierce et al., 1986). The extended Theorell–Chance mechanism proposed is most easily reconciled with current kinetic data for RuBP carboxylase.

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SUPPLEMENTARY MATERIAL AVAILABLE

The commitment factors of several potential kinetic mechanisms for the carboxylase reaction, including those for D_V, $D_{V/K}$, 13_V and $13_{(V/K)}$, at the limiting concentrations of the second substrate (3 pages). Ordering information is given on any current masthead page.

Registry No. RuBP, 24218-00-6; XuBP, 15565-46-5; RuBisCO, 9027-23-0; D_2, 7782-39-0; T_2, 10028-17-8.

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Rapid-Scanning Cryospectroscopy of Enzyme–Substrate–Inhibitor Complexes of Cobalt Carboxypeptidase A†

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ABSTRACT: Rapid-scanning cryospectroscopy of cobalt(II)-substituted carboxypeptidase A serves to identify and characterize ternary enzyme–substrate–inhibitor (IES) complexes formed by the interaction between the enzyme, a peptide substrate, and a noncompetitive inhibitor. A cobalt absorption spectrum distinct from any induced by peptide or inhibitor alone signals formation of the IES complex. Tight-binding noncompetitive inhibitors containing an aromatic ring, e.g., β-phenylpropionate, cause the IES complex to form much more slowly than simple binary complexes of the enzyme with either peptide or inhibitor. An inhibitor such as acetate, which binds more weakly and is less bulky, permits the IES complex to form relatively quickly. Remarkably, the cobalt spectra of the IES complexes match those previously found for the steady-state ester (depsipeptide) intermediates. Chemical quenching studies have demonstrated that in these ester intermediates the scissile bond is broken [Galdes, A., Auld, D. S., & Vallee, B. L. (1986) *Biochemistry 25*, 646–651]. This finding, in conjunction with the present studies, implies that a peptide and a noncompetitive inhibitor of its hydrolysis occupy the same binding loci as the hydrolytic products of a depsipeptide and further indicates that breakdown of an enzyme–biproduct complex is rate-determining for the turnover of depsipeptides.

Elucidation of an enzyme's mechanism requires detailed information about the transient structural changes that underlie its catalytic activity. Since these processes generally are very rapid, the response times of the methods applied to their detection must also be fast; stopped-flow and rapid-scanning spectroscopic techniques are particularly suitable (Auld, 1979). Application of such methods at subzero temperatures using an appropriate cryosolvent (Douzou, 1977) combines the advantages of shortening the analytical time frame with those of extending the duration of the process studied (Galdes et al., 1983). This approach has enabled the acquisition of absorption and EPR[1] spectra of the steady-state intermediates formed transiently during the hydrolysis of peptide and ester substrates by cobalt carboxypeptidase A, a fully active metallo derivative of this zinc protease (Geoghegan et al., 1983; Auld et al., 1984).

Kinetic studies of carboxypeptidase A inhibition have pointed to differences between the hydrolytic pathways for peptides and esters (Auld & Holmquist, 1974). Monocarboxylate anions exhibit two forms of inhibition toward carboxypeptidase substrates, one generally competitive with esters (Bunting & Myers, 1973, 1975) and one noncompetitive with peptides (Auld & Vallee, 1970; Auld & Holmquist, 1974). On the basis of the latter kinetic observation, such inhibitors are predicted to be capable of being cobound to the enzyme with a peptide substrate, forming an IES complex. The kinetics of the formation of such IES complexes and their structural basis are of interest from several viewpoints. Stopped-flow cryospectrometry has now made possible simultaneous measurements both of their rates of formation and of their absorption spectra.

Materials and Methods

Carboxypeptidase Aα (Sigma Chemical Co.) prepared by the method of Cox et al. (1964) was recrystallized and then converted to crystalline cobalt enzyme by a dialysis method (Geoghegan et al., 1983). The stopped-flow cryospectrometer has been described (Hanahan & Auld, 1980; Geoghegan et al., 1983). Scan times were 16.48 ms. Enzyme and substrate samples were prepared in 1 M NaCl for studies at 0 °C or in 4.5 M NaCl for studies at −15 to −20 °C. Precautions were taken to remove adventitious metal ions (Thiers, 1957). All solutions were buffered with 0.02–0.05 M MES. The pH values measured at +20 °C were corrected for the temperature coefficient of the buffer (Good et al., 1966).

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[1] Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; EPR, electron paramagnetic resonance.

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