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Aspartate β-Decarboxylase from *Alcaligenes faecalis*: Carbon-13 Kinetic Isotope Effect and Deuterium Exchange Experiments†

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ABSTRACT: We have measured the ^{13}C kinetic isotope effect at pH 4.0, 5.0, 6.0, and 6.5 and in D_2O at pD 5.0 and the rate of D–H exchange of the α and β protons of aspartic acid in D_2O at pD 5.0 for the reaction catalyzed by the enzyme aspartate β-decarboxylase from *Alcaligenes faecalis*. The ^{13}C kinetic isotope effect, with a value of 1.0099 ± 0.0002 at pH 5.0, is less than the intrinsic isotope effect for the decarboxylation step, indicating that the decarboxylation step is not entirely rate limiting. We have been able to estimate probable values of the relative free energies of the transition states of the enzymatic reaction up to and including the decarboxylation step from the ^{13}C kinetic isotope effect and the rate of D–H exchange of α-H. The pH dependence of the kinetic isotope effect reflects the pK_a of the pyridine nitrogen of the coenzyme pyridoxal 5′-phosphate but not that of the imine nitrogen. A mechanism is proposed for the exchange of aspartate β-H that is consistent with the stereochemistry suggested earlier.

Aspartate β-decarboxylase catalyzes the decarboxylation of L-aspartic acid to alanine, as in eq 1. Both a keto acid,

$$^{-}OOC\text{–}CH_2CH(NH_3^+)\text{–}COO^- + H^+ \rightarrow CO_2 + CH_3CH(NH_3^+)\text{–}COO^- \quad (1)$$

in this work α-ketoglutaric acid, and the coenzyme pyridoxal 5′-phosphate must be added to the enzyme before the addition of substrate to prevent abortive transamination (Tate & Meister, 1969). The accepted mechanism for the reaction (up to the decarboxylation step), based principally on the work of Meister and his collaborators, is shown in Scheme I (Tate & Meister, 1969; Chang et al., 1982). This enzyme is different from other pyridxal phosphate dependent decarboxylases whose kinetic isotope effects have been studied (O'Leary et al., 1970, 1981; O'Leary & Piazza, 1981) in that there is a hydrogen-transfer step prior to the decarboxylation step.

One goal of the enzymologist is to deduce the complete free energy profile for the steps of an enzyme mechanism (Knowles & Albery, 1977; Albery & Knowles, 1976a; Ray, 1983). Such a determination would permit identification of the rate-limiting, or "most sensitive", step in the sequence (Ray, 1983) and estimation of the degree to which the enzyme has evolved to an optimum catalytic efficiency (Albery & Knowles, 1976b). Isotope effects are one particularly useful way to get this information (Knowles & Albery, 1977). Particularly when multiple isotope effects are available for a reaction, it can be possible to obtain a relatively complete free energy profile. We have been able to determine patterns for the relative free energy of each of the transition states for the decarboxylation of aspartic acid up to the irreversible loss of CO_2 through a combination of measurements of the rate of D–H exchange of the α-H of aspartic acid and measurements of the ^{13}C kinetic isotope effect on V/K.

Materials and Methods

Enzymes. L-Aspartic acid β-decarboxylase (specific activity 70 IU/mg) was prepared from *Alcaligenes faecalis*, strain N (AT CC 25094), by Aghdas Laghai (Chang et al., 1981) by the method of Tate et al. (1970). Glutamic-oxaloacetic transaminase and malic dehydrogenase were obtained from Sigma Chemical Co.

Chemicals. All the L-aspartic acid used was from a single bottle (to avoid variations in isotope composition) obtained from Aldrich Chemical Co. Pyridoxal 5′-phosphate, α-ketoglutaric acid, bovine serum albumin, reduced nicotinamide adenine dinucleotide, and hydroxypropylcellulose were obtained from Sigma Chemical Co. The D_2O used was 99.8 atom % D. Other chemicals were of reagent grade.

Enzyme Assay. Aspartate β-decarboxylase activity was determined respirometrically at 37.0 °C (Umbreit et al., 1972) with a Gilson constant-pressure respirometer. The method of Novogrodsky & Meister (1964) was used, with 2.5 mL of the following solution in the flask: 0.10 M acetate buffer, pH 5.0, 2 mM α-ketoglutaric acid, 0.86 mM pyridoxal 5′-phosphate,[1] and enzyme and with 0.5 mL of 4×10^{-2} M aspartic acid, titrated to pH 5.0, in the side arm. Because acetate ion was found to be an inhibitor of the enzyme (Novogrodsky & Meister, 1964; R. M. Rosenberg and M. H. O'Leary, un-

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[1] The α-ketoglutaric acid and pyridoxal 5′-phosphate were added to prevent the abortive transamination and loss of coenzyme as pyridoxamine 5′-phosphate (Tate & Meister, 1969).

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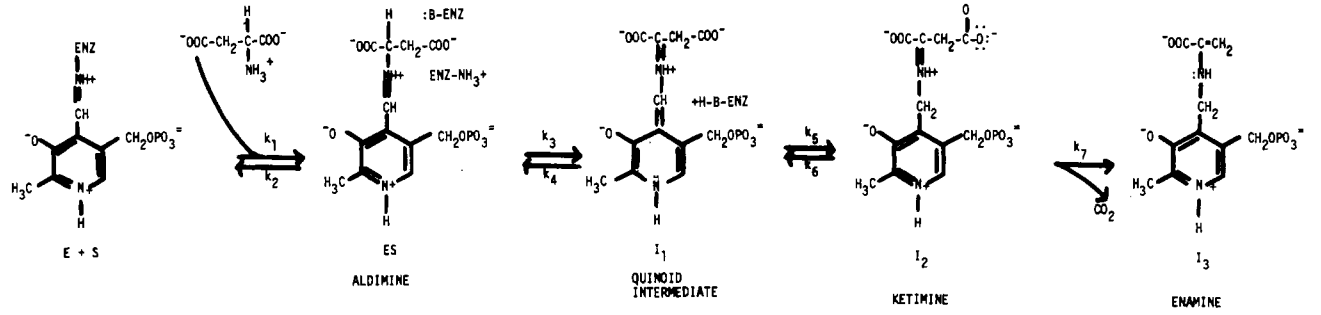
Scheme I



published results), experiments at different values of pH were carried out at constant concentration of acetate ion. Either bovine serum albumin (1 mg/mL) or hydroxypropylcellulose (0.3 mg/mL) was used to stabilize dilute solutions of enzyme (Shibatani et al., 1974).

^{13}C Kinetic Isotope Effect. ^{13}C isotope effects on the rate of decarboxylation of aspartic acid were measured by the competitive method as described by O'Leary (1980), except that the enzyme was made CO_2 free by placing it in the buffer solution in a dialysis tube while the buffer was being sparged with CO_2-free N_2. The buffers used were 0.54 M acetate at pH 4.0, 0.10 M acetate at pH 5.0, 0.082 M acetate at pH 6.0, and 0.080 M acetate at pH 6.5. In each case, the buffer was 2 mM in α-ketoglutaric acid and 0.86 mM in pyridoxal 5′-phosphate. The 40 mM aspartic acid solution was adjusted to the pH of the buffer with 1 M NaOH before sparging, and the reaction was started by forcing the aspartic acid solution into the reaction vessel under N_2 pressure after the dialysis tubing had been punctured and the enzyme mixed with the buffer. The ratio of the m/e 44 and m/e 45 peaks for the isolated CO_2 was measured relative to a tank standard CO_2 on a Nuclide Associates RMS 6-60 isotope ratio mass spectrometer (O'Leary & Marlier, 1979). Correction to the 45/44 ratio for the presence of $^{12}C^{17}O^{16}O$ was made as described by O'Leary (1980). The kinetic isotope effect was calculated from the equation (Tong & Yankwich, 1957)

$$^{12}k/^{13}k = \frac{\ln (1 - f)}{\ln [1 - f(R_f/R_{100})]}$$

where R represents the isotope ratio $^{13}C/^{12}C$ of the product CO_2 either when 100% of the substrate had been converted or when a fraction f of the substrate had been converted. Isotope effect measurements in D_2O are perturbed by the presence of excess ^{17}O in D_2O, so the CO_2 samples from reactions in D_2O were exchanged with H_2O for 40 h at 25 °C before carbon isotope ratios were measured. pD of buffers in D_2O were adjusted with 1 M NaOD before dilution to volume. A value of 0.4 was added to pH meter readings in D_2O after calibration with buffers in H_2O (Glasoe & Long, 1960). The concentration of aspartic acid remaining after completion of the "100%" reaction was monitored by a coupled enzyme method that was designed as an assay for glutamic-oxaloacetic transaminase (Sizer & Jenkins, 1962). We placed the following in a 3-mL spectrophotometer cell: 2.6 mL of 0.2 M phosphate buffer, pH 7.5, 0.1 mL of 0.2 M α-ketoglutaric acid solution in 0.2 M phosphate buffer, pH 7.5, 0.02 mL of malic dehydrogenase (11 units) dissolved in 0.2 M phosphate, pH 7.5, and 0.2 mL of either standard aspartic acid in 0.2 M phosphate, pH 7.5, or 0.2 mL of acidified reaction mixture titrated to pH 7.5 with 1 M NaOH. After incubation at 25 °C for 15 min, 0.05 mL of glutamic-oxaloacetic transaminase (14 units) in 0.2 M phosphate, pH 7.5, was added to start the reaction. The absorbance decrease at 340 nm was measured. For the 100% reaction, no change was found relative to the controls, and known aspartic acid was added to the assay mixtures to ensure that absence of aspartic acid was the only cause for lack of change.

D–H Exchange. Proton NMR spectra of aspartate β-decarboxylase reaction mixtures were obtained on an IBM WP200SY 200-MHz Fourier transform nuclear magnetic resonance (FTNMR)[2] spectrometer with a deuterium lock. All chemical shifts were measured relative to that for the acetate methyl group of the buffer. Areas were measured relative to HDO. The buffer for NMR experiments was 0.1 M CH_3COOH, 2.4 mM α-ketoglutaric acid, and 0.76 mM pyridoxal 5′-phosphate, titrated to pD 5.0 in D_2O, and it contained 0.3 mg/mL hydroxypropylcellulose. The enzyme used was dialyzed against this buffer before use, both to obtain exchange of D for H in the enzyme solution and to prevent inactivation of the enzyme by abortive transamination (Tate & Meister, 1969). Control spectra were obtained with 6 mM aspartic acid and 6 mM alanine in the buffer. Each spectrum was the result of 64 acquisitions of data, each of which accumulated 5 s of free induction decay. For each spectrum, integrals were obtained for the HDO peak, for the α-H and β-CH_2 of aspartic acid, and, when sufficient alanine had been produced, for the alanine β-CH_3.

Results

Carboxyl carbon isotope effects for the decarboxylation of aspartate have been measured by comparison of the isotopic composition of CO_2 isolated after about 10% of reaction with the isotopic compositions of CO_2 isolated after 100% reaction. Multiple measurements at pH 5.0 (Table I) show the same high reproducibility of the isotope ratio for 100% reaction and for the isotope effect as has been obtained in earlier studies in this laboratory (O'Leary et al., 1981). Measurements of the isotope effect at other pH values (Table I) also show a high degree of reproducibility and give the same isotope ratio for the 100% reaction sample.

H/D Exchange Experiment. The proton NMR spectrum of aspartic acid shows the ABX pattern expected for the pair of enantiotopic β protons adjacent to a single α proton, as shown in Figure 1 (Becker, 1980). The chemical shifts were measured relative to the methyl group of the acetate buffer. Figure 2 shows the NMR spectrum of alanine in the absence of enzyme. The spectrum of aspartate was stable in the absence of enzyme, but when aspartate β-decarboxylase was added, the α-H and β-H peaks decreased in area and a peak for product alanine methyl appeared and grew in area. The peak for α-H retained its characteristic spin–spin splitting, whereas the peak for β-H lost its characteristic pattern; the

[2] Abbreviations: NMR, nuclear magnetic resonance; FT, Fourier transform.

Table I: ^{13}C Kinetic Isotope Effects[f]

pH	R_{100} (cor)	R_{10} (cor)		$^{12}k/^{13}k$
5.0 (D_2O)	0.010166	0.010104[c]		1.0065
5.0 (D_2O)	0.010153	0.010104[c]		1.0051
			mean:	1.0058 ± 0.0007[e]
6.5 (H_2O)	0.010156[a]	0.010093		1.0066
6.5 (H_2O)	0.010156[a]	0.010082		1.0077
			mean:	1.0072 ± 0.0005[e]
6.0 (H_2O)	0.010153[d]	0.010079		1.0077
6.0 (H_2O)	0.010153[d]	0.010076		1.0081
6.0 (H_2O)	0.010155[b]	0.010076		1.0083
6.0 (H_2O)	0.010155[b]	0.010085		1.0073
			mean:	1.0079 ± 0.0002[e]
4.0 (H_2O)	0.010153[d]	0.010027		1.0133
4.0 (H_2O)	0.010153[d]	0.010042		1.0117
4.0 (H_2O)	0.010146	0.010020		1.0133
4.0 (H_2O)	0.010146	0.010035		1.0117
			mean:	1.0125 ± 0.0005[e]
5.0 (H_2O)	0.010153	0.010057		1.0101
5.0 (H_2O)	0.010145	0.010057		1.0092
5.0 (H_2O)	0.010157	0.010062		1.0100
5.0 (H_2O)	0.010152	0.010059		1.0098
5.0 (H_2O)	0.010151	0.010060		1.0095
5.0 (H_2O)	0.010153	0.010053		1.0105
			mean:	1.0099 ± 0.0002[e]

[a] Same sample. [b] Same sample. [c] Same sample. [d] Average of other values for R_{100} (cor). [e] Standard deviation of mean at 95% confidence level. [f] $R = (R/R_{tank})(0.010869)$, $R(cor) = R - 0.0007$, and $^{12}k/^{13}k = \ln(0.90)/\ln[1 - (0.10)(R_{10}(cor)/R_{100}(cor))]$.

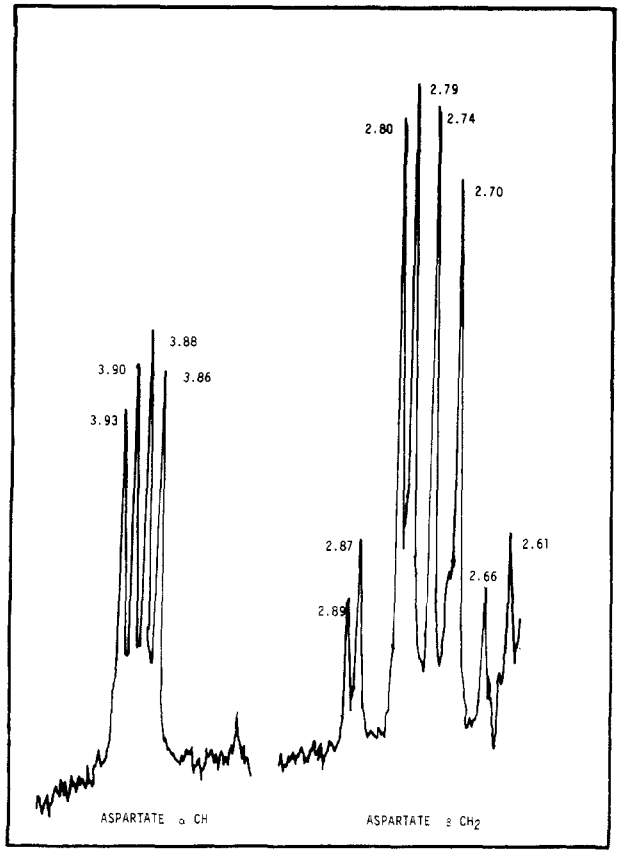


FIGURE 1: NMR spectrum of aspartic acid in pD 5.0 acetate buffer before the addition of enzyme.

alanine peak that appeared was a broad singlet instead of the doublet seen in Figure 2. A typical set of peaks, observed after 16 min of reaction, is shown in Figure 3. No alanine α-H peak could be detected. Approximately 30% of the aspartic acid was decarboxylated at this time.

The data for the decrease in area of the aspartate α-H and β-H and the increase in the area of the alanine methyl peak are shown in Figure 4. The initial slopes were obtained

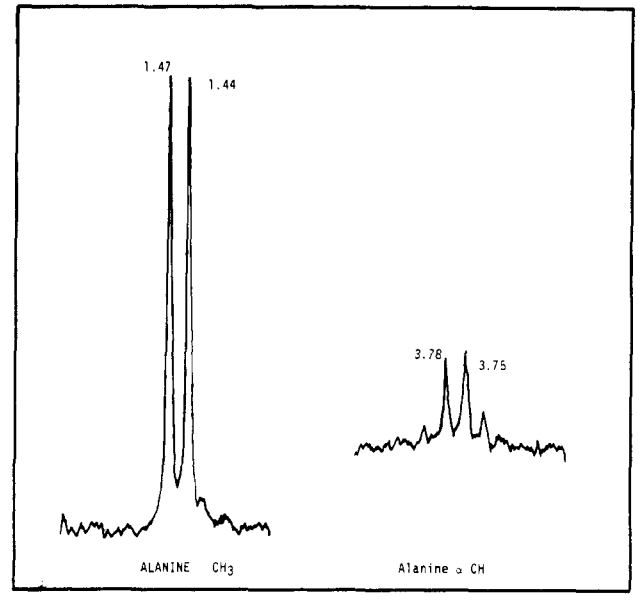


FIGURE 2: NMR spectrum of alanine in pD 5.0 acetate buffer in the absence of enzyme.

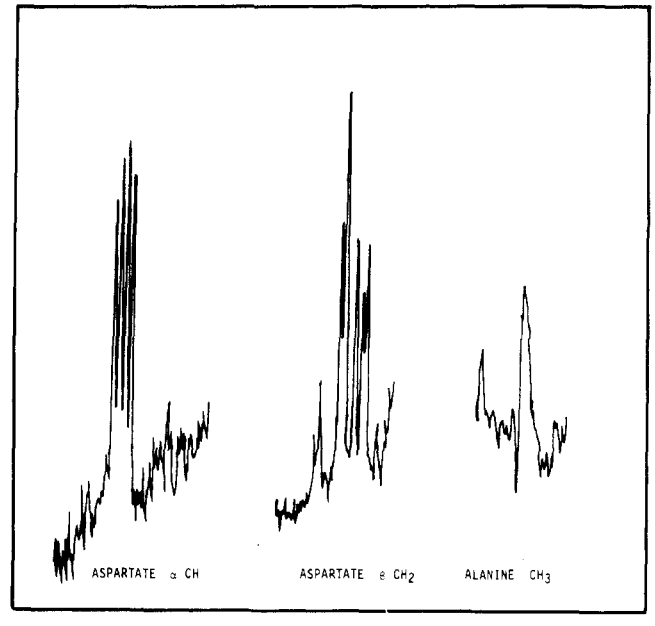


FIGURE 3: NMR peaks 16 min after the addition of enzyme for aspartate α-H, aspartate β-H, and alanine CH_3.

manually from larger scale plots and are 2.0×10^{-6} and 3.0×10^{-6} mol of H min^{-1} (enzyme unit)$^{-1}$ for α-H and β-H, respectively. The corresponding initial rate of decarboxylation in D_2O as measured by respirometry was 4.9×10^{-7} mol min^{-1} (enzyme unit)$^{-1}$. One unit of enzyme decarboxylates 1 μmol of aspartic acid min^{-1} at 37 °C (Tate et al., 1970).

The limiting concentration of alanine methyl protons observed was 3.3×10^{-3} M, compared with an initial aspartate β-proton concentration of 12.0×10^{-3} M, approximately 28% of the value expected if no β protons had been exchanged (i.e., α CH_2D group).

Discussion

Since the ^{13}C isotope effect was measured by the competitive method, it is the isotope effect on V/K that is obtained (O'Leary, 1978). The mechanism that has been established for aspartate β-decarboxylase (Novogrodsky & Meister, 1964; Chang et al., 1982) is shown in Scheme I, up to and including

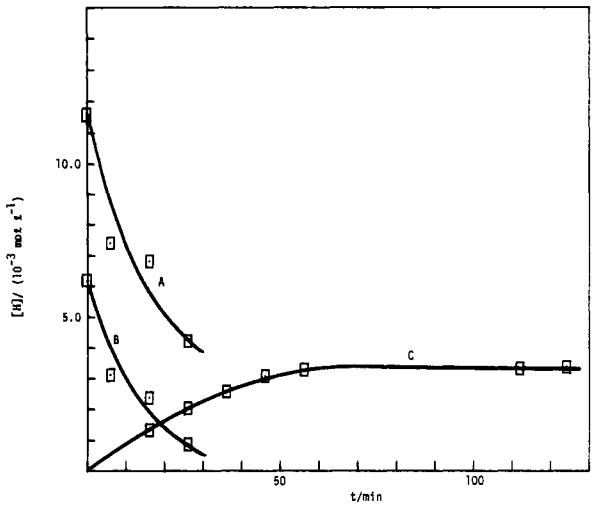


FIGURE 4: Variation with time, after addition of enzyme, of the peak areas for aspartate α-H (B), aspartate β-H (A), and alanine CH_3 (C). Proton concentration was calculated relative to the HDO peak, and the concentration of HDO was calculated from the known concentration of aspartate α-H in the absence of enzyme.

the release of CO_2. V/K for this mechanism is given by (Cleland, 1963)

$$V/K = (k_1k_3k_5k_7)/[k_2(k_4k_6 + k_4k_7 + k_5k_7) + k_3k_5k_7] \quad (2)$$

The kinetic isotope effect on V/K for this mechanism (O'Leary, 1978) is

$$\frac{^{12}k}{^{13}k} = \frac{^{12}k_7/^{13}k_7 + R}{1 + R} \quad (3)$$

and

$$R = k_7/k_6[1 + (k_5/k_4)(1 + k_3/k_2)] \quad (4)$$

Here $^{12}k_7/^{13}k_7$ is the intrinsic isotope effect on the irreversible decarboxylation step, and the other rate constants are those for the normal isotope.

Experimentally, the value of $^{12}k/^{13}k$ is found to be between 1.03 and 1.06 for nonenzymatic decarboxylations (Husang & Long, 1969; Dunn & Buccini, 1968; Seltzer et al., 1959). The value is probably relatively constant because the change in bonding at the isotopic carbon is going from the ground state to the transition state is rather independent of the nature of the rest of the substrate. Thus, if $^{12}k_7/^{13}k_7$ for the enzymatic reaction is taken as 1.03–1.06, measurement of $^{12}k/^{13}k$ permits calculation of R. If decarboxylation were entirely rate-limiting, k_7/k_6 would be effectively equal to zero, R would be equal to zero, and the observed isotope effect would be equal to the intrinsic isotope effect. Since the observed isotope effect is significantly smaller than the intrinsic isotope effect, the steps preceding decarboxylation in the mechanism are at least partly rate limiting.

When the reaction is carried out in D_2O, as in the NMR experiment, H–D exchange of the aspartate α proton occurs in the reverse reaction from the quinoid intermediate, I_1. If we assume that the reaction for which k_2 is the rate constant is irreversible for the observation of H–D exchange (since the solvent is D_2O, there is essentially no reverse exchange of H for D), the rate of H–D exchange can be calculated by Cleland's method of net rate constants (Cleland, 1975); the resulting expression is

$$\text{rate of H–D exchange} = k_4'[I_1] \quad (5)$$

Table II: Free Energy Differences for Transition States

k_5/k_4	$G_5 - G_4$ (kJ mol^{-1})	k_3/k_2	$G_3 - G_2$ (kJ mol^{-1})
$^{12}k_7/^{13}k_7 = 1.03$	$k_7/k_6 = 3.39$	$G_7 - G_6 = -3.1$ kJ mol^{-1}	
0.005	13.6	62.2	−10.6
0.20	4.1	0.58	1.4
$^{12}k_7/^{13}k_7 = 1.06$	$k_7/k_6 = 7.77$	$G_7 - G_6 = -5.3$ kJ mol^{-1}	
0.005	13.6	54.1	−10.3
0.20	4.1	0.38	2.5

in Cleland's notation, while the rate of decarboxylation, by the same method, is equal to k_7 [I_2]. Thus

$$\frac{\text{rate of D–H exchange}}{\text{rate of decarboxylation}} = \frac{k_4'[I_1]}{k_7[I_2]} = \frac{[(k_2k_4)/(k_2 + k_3)][I_1]}{k_7[I_2]} = [(k_2k_7)/(k_2 + k_3)][(k_6 + k_7)/k_5][I_2]/(k_7[I_2]) = \left[\frac{k_2k_4/(k_2 + k_3)}{k_7}\right]\frac{k_6 + k_7}{k_5} = \frac{1 + k_6/k_7}{(k_5/k_4)(1 + k_3/k_2)} \quad (6)$$

Equations 6 and 4 can be solved for the two variables k_7/k_6 and $(k_5/k_4)(1 + k_3/k_2)$. Furthermore, the maximum value of k_5/k_4 is equal to the value of the variable $(k_5/k_4)(1 + k_3/k_2)$, since the minimum value of k_3/k_2 is zero. Thus, values of k_5/k_4 over the range from zero to the maximum can be used to calculate corresponding values of k_3/k_2 consistent with the experimental kinetic isotope effect values and H–D exchange rates. One can calculate from the rate constant ratios, which are for reactions in the opposite direction from the same intermediate, the difference in Gibbs' energy between the corresponding transition states.

Since there is no evidence for the presence of α-H in the product alanine, it is reasonable to assume that D–H exchange at that position is complete before decarboxylation. Therefore, the rate of disappearance of the aspartate α-H is equal to the rate of D–H exchange at that position or 2.0×10^{-6} mol min^{-1} (enzyme unit)$^{-1}$. The ratio of the rate of D–H exchange to the rate of decarboxylation is then 4.1. Figure 5 shows the free energy profiles calculated from eq 4 and 6 for two different values of k_5/k_4 less than the maximum, with each calculation repeated for the maximum and minimum values of $^{12}k_7/^{13}k_7$ (1.03 and 1.06). The numerical values from which Figure 5 was constructed are given in Table II.

The transition state with the highest free energy is that for the conversion of the quinoid intermediate I_1, to the ketimine, I_2, in all four examples. According to the nomenclature of Ray (1983), this step is the most "sensitive" step in the sequence, a term that does not have the same implications as the "rate-determining" step. The results in all four examples are consistent with the qualitative conclusion from the kinetic isotope effect that the decarboxylation step is not solely rate limiting. All the examples are also consistent with the observation that the rate of H–D exchange, which occurs in the conversion of the quinoid intermediate to the original substrate, is more rapid than that of the decarboxylation, which must involve passage through higher energy transition states.

pH Dependence of Kinetic Isotope Effect. Three steps in Scheme I are dependent on the protonation state of a nitrogen base: the formation of the quinoid intermediate in either the forward or reverse direction (k_3 or k_6) requires a protonated

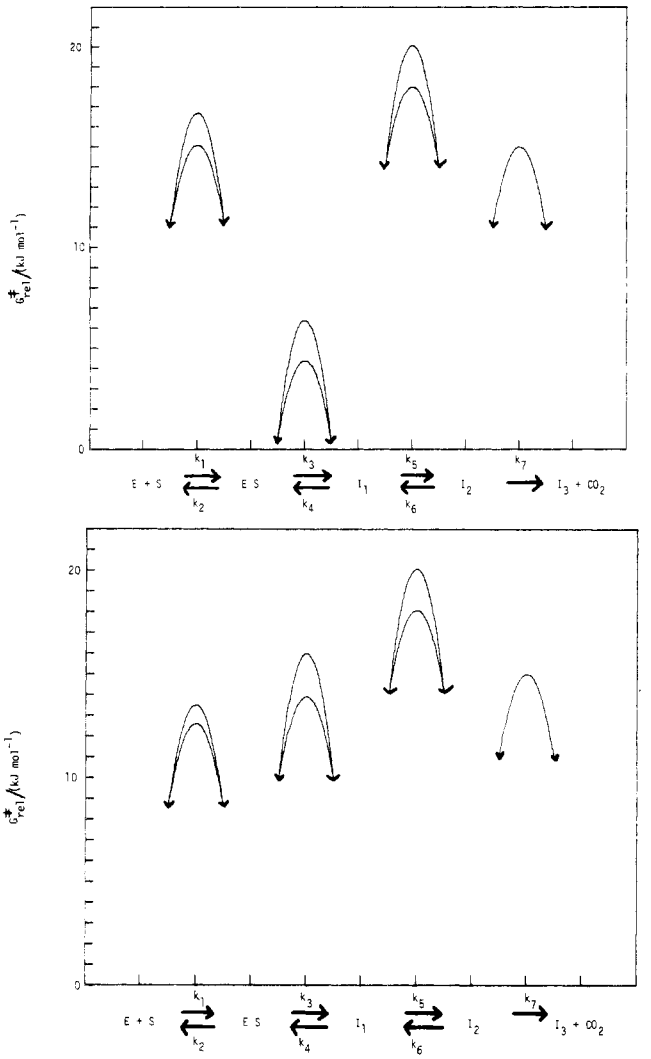


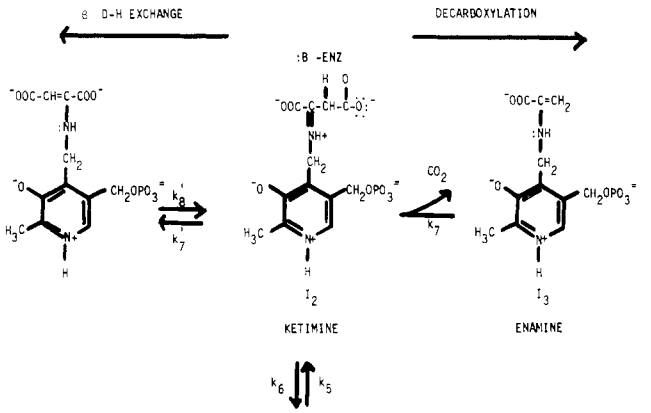
FIGURE 5: Free energy profiles for aspartate β-decarboxylase for rate of H–D exchange equal to 4.1 times the rate of decarboxylation. (Top) $k_5/k_4 = 0.005$; (bottom) $k_5/k_4 = 0.2$. The upper curve in each case is $^{12}k_7/^{13}k_7 = 1.06$, and the lower curve is for $^{12}k_7/^{13}k_7 = 1.03$.

pyridine nitrogen to act as an electron sink, and the decarboxylation step requires a protonated imine nitrogen to act as an electron sink. The pH dependence of the kinetic isotope effect enables us to decide which of these groups has a pK in the pH range used in these experiments.

If the pyridine nitrogen were changing protonation state in the pH range of these experiments, then k_3 and k_6 would decrease with increasing pH. From eq 3 and 2, we can see that a decrease of k_3 and k_6 by the same factor would lead to an increase in the value of R and a decrease in the observed isotope effect, in agreement with our results. If the imine nitrogen were changing protonation state in the pH range of these experiments, then k_7 would decrease with increasing pH, R would decrease, and the isotope effect would increase, contrary to our results. Our conclusions are consistent with other determinations of the pK of the pyridine nitrogen (pK_a = 6.5) and the pK of the imine nitrogen (pK_a = 11.9) (Weng & Leussing, 1983; Metzler et al., 1980).

Kinetic Isotope Effect in D_2O. In D_2O, the hydrogen ion transferred from an enzyme base to the aldehydic carbon in the conversion of ketimine to quinoid intermediate would be deuterium, and k_6 would be smaller in D_2O than in H_2O. In addition, because α-H exchange is faster than decarboxylation, a large fraction of the hydrogen ions transferred to the enzyme base in the conversion of aldimine to quinoid intermediate

Scheme II



would be deuterium, and k_3 would be smaller in D_2O than in H_2O. Thus, the value of R would be larger in D_2O than in H_2O, and the isotope effect would be smaller in D_2O than in H_2O, in agreement with our results.

β D–H Exchange. Since the rate of disappearance of the NMR signal for β protons in mol of H min^{-1} (enzyme unit)$^{-1}$ is more than twice the rate of decarboxylation, we must assume β D–H exchange is occurring as well as decarboxylation and that the exchange is catalyzed by the enzyme. Aspartate β-decarboxylase is the only amino acid decarboxylase in which β D–H exchange has been observed (Golichowski et al., 1977). This result is not surprising, however, since this decarboxylase is unique in having an obligatory transamination step as part of the mechanism (Scheme I), and transaminases are known to catalyze β D–H exchange as well as α D–H exchange (Babu & Johnston, 1974, 1976; Golichowski et al. 1977).

The observed NMR spectra show no change in the splitting pattern of the α proton and substantial change in the splitting pattern of the β proton. Also, no α proton spectrum is observed for product alanine, and the alanine methyl peak, a broad singlet instead of a doublet, has approximately one-fourth the area it would if the methyl group were all protons. These results are consistent with the hypothesis that (1) exchange of α protons always precedes exchange of β protons and is essentially complete before decarboxylation and (2) Approximately 70% of the β protons are exchanged before decarboxylation. The mechanism for β D–H exchange shown in Scheme II is consistent with our results and the hypothesis above.

When Chang et al. (1981) studied the stereochemistry of the reaction catalyzed by aspartate β-decarboxylase, they found that 1% of the α protons from aspartate appear in product alanine. Their result is not inconsistent with our conclusion from D–H exchange observations with NMR, since we would not have been able to detect an NMR peak for the α proton of alanine for 1% of the product. They concluded from their data that β-H exchanged from product alanine on the enzyme rather than from aspartate. It is not clear that their conclusions and ours are inconsistent, since their exchange rate is strongly pH dependent, and none of their experiments were carried out at pD 5.0, the condition of our experiments. That β D–H exchange might occur from intermediate 2, the ketimine of aspartate, as an alternative to decarboxylation (Scheme II) is consistent with the geometry of the two-base mechanism proposed by Chang et al. (1981), since the enzyme base that protonates the enamine to form the ketimine of alanine could also, if not protonated, abstract the β proton of aspartate if the β-carbon of aspartate rotated by 60 deg from the position postulated for the stereospecific protonation of the enamine by Chang et al.

ADDED IN PROOF

Gehring (1984) has observed α-H and β-H exchange in mitochondrial aspartate aminotransferase.

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Registry No. H_2, 1333-74-0; ^{13}C, 14762-74-4; aspartate β-decarboxylase, 9024-57-1; pyridoxal 5′-phosphate, 54-47-7.

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Carbon Isotope Effect on Carboxylation of Ribulose Bisphosphate Catalyzed by Ribulosebisphosphate Carboxylase from *Rhodospirillum rubrum*†

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ABSTRACT: The carbon isotope effect at CO_2 has been measured in the carboxylation of ribulose 1,5-bisphosphate by the ribulosebisphosphate carboxylase from *Rhodospirillum rubrum*. The isotope effect is obtained by comparing the isotopic composition of carbon 1 of the 3-phosphoglyceric acid formed in the reaction with that of the carbon dioxide source. A correction is made for carbon 1 of 3-phosphoglyceric acid which arises from carbon 3 of the starting ribulose bisphosphate. The isotope effect is $k^{12}/k^{13} = 1.0178 \pm 0.0008$ at 25 °C, pH 7.8. This value is smaller than the corresponding value for the spinach enzyme. It appears that substrate addition with the *R. rubrum* enzyme is principally ordered, with ribulose bisphosphate binding first, whereas substrate addition is random with the spinach enzyme. The carboxylation step is partially rate limiting with both enzymes.

Ribulose bisphosphate (RuBP)[1] carboxylase, the key carbon-fixing enzyme in plants, has been isolated and characterized from a number of green plants and photosynthetic bacteria (Akazawa, 1979). The enzyme from all sources studied to date catalyzes both carboxylation and oxygenation

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[1] Abbreviations: DTT, dithiothreitol; 3-PGA, 3-phosphoglyceric acid; RuBP, ribulose bisphosphate; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

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