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Isotope Effect Studies of the Pyruvate-Dependent Histidine Decarboxylase from *Lactobacillus* 30a†

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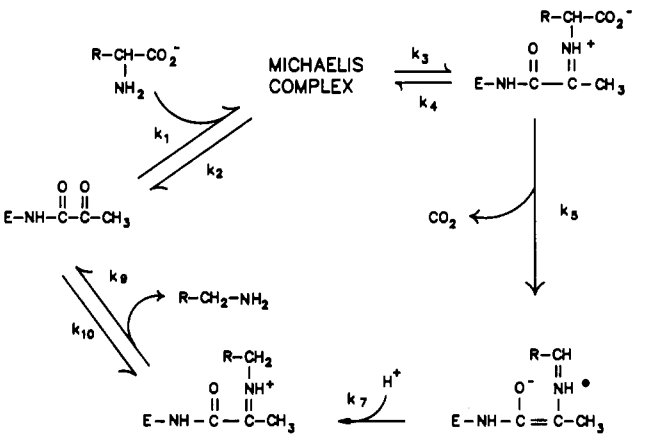
ABSTRACT: The decarboxylation of histidine by the pyruvate-dependent histidine decarboxylase of *Lactobacillus* 30a shows a carbon isotope effect $k^{12}/k^{13} = 1.0334 \pm 0.0005$ and a nitrogen isotope effect $k^{14}/k^{15} = 0.9799 \pm 0.0006$ at pH 4.8, 37 °C. The carbon isotope effect is slightly increased by deuteriation of the substrate and slightly decreased in D_2O. The observed nitrogen isotope effect indicates that the imine nitrogen in the substrate–Schiff base intermediate complex is ordinarily protonated, and the pH dependence of the carbon isotope effect indicates that both protonated and unprotonated forms of this intermediate are capable of undergoing decarboxylation. As with the pyridoxal 5′-phosphate dependent enzyme, Schiff base formation and decarboxylation are jointly rate-limiting, with the intermediate histidine–pyruvate Schiff base showing a decarboxylation/Schiff base hydrolysis ratio of 0.5–1.0 at pH 4.8. The decarboxylation transition state is more reactant-like for the pyruvate-dependent enzyme than for the pyridoxal 5'-phosphate dependent enzyme. These studies find no particular energetic or catalytic advantage to the use of pyridoxal 5′-phosphate over covalently bound pyruvate in catalysis of the decarboxylation of histidine.

In 1953, Rodwell discovered that the Gram-positive bacterium *Lactobacillus* 30a contained a large quantity of histidine decarboxylase (Rodwell, 1953). Closer examination of the properties of this enzyme by Rosenthaler et al. (1965) confirmed Rodwell's suspicion that this particular histidine decarboxylase did not require the cofactor pyridoxal 5′-phosphate (PLP)[1] for catalysis. Instead, the enzyme contains a covalently bound pyruvoyl residue as its prosthetic group (Riley & Snell, 1968). Subsequent studies have revealed the existence of a small class of enzymes, principally decarboxylases, that require covalently bound pyruvate for catalysis, rather than the more common PLP (Recsei & Snell, 1984). The mechanistic comparison of these two classes of enzymes is the subject of the present study.

The pyruvate-dependent histidine decarboxylase from *Lactobacillus* 30a contains covalently bound pyruvate, but contains no metal ion or other cofactors. The enzyme has a molecular weight of 208 000 and a hexameric subunit structure $(\alpha\beta)_6$ (Hackert et al., 1981). The hexamer contains six active sites and one pyruvoyl residue per active site. The larger α subunit contains the pyruvoyl residue, which is covalently bound to the amino-terminal phenylalanine. The smaller β subunit, which is essential for activity, does not contain pyruvate, cysteine, histidine, or phenylalanine (Huynh et al., 1984). A 3-Å X-ray crystal structure of this enzyme (Hackert et al., 1981; Parks et al., 1985) shows that lysine-155 and a glutamic acid are present in the active site, which is at the bottom of a deep cleft. Modification of lysine-155 with

Scheme I



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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; CHES, 3-(cyclohexylamino)ethanesulfonic acid.

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fluorodinitrobenzene destroys all enzymic activity (Huynh et al., 1986).

Histidine decarboxylase is active between pH 2.9 and 7.0 and has a broad optimum pH range of 4.8–6.5. The V_{max}/K_m vs pH profile shows a break at pH 4.2, below which log V/K decreases with a slope of 1.0, and a break at 6.5, above which log V/K decreases with a slope of 2.0. Above pH 7.0, sigmoidal kinetics are observed and the affinity of the enzyme for substrate decreases rapidly (Recsei et al., 1970; Chang et al., 1968; Snell & Huynh, 1986).

Recsei and Snell (1970) have proposed that the enzyme operates by the mechanism shown in Scheme I. In the first step a Schiff base is formed between the amino group of the substrate and the ketone carbonyl group of the pyruvoyl residue. The resulting Schiff base decarboxylates in the second step, apparently by using the second carbonyl group of the pyruvoyl residue as an electron sink. The enamine thus produced is reprotonated with retention of configuration at the α-carbon (Battersby et al., 1980). Hydrolysis of the Schiff base releases the product histamine and restores the enzyme to its resting state. There is strong evidence for the intermediacy of the Schiff base in this mechanism. Both N^2-(1-carboxyethyl)histidine and N^1-(1-carboxyethyl)histamine have been isolated following addition of $NaBH_4$ to a mixture of enzyme and histidine (Recsei & Snell, 1970).

Thus, both the PLP-dependent enzyme and the pyruvate-dependent enzyme operate by way of a Schiff base intermediate and use the prosthetic group as an electron sink in the decarboxylation step. The Schiff base formation steps are significantly different in the two cases: the pyruvate-dependent enzyme forms the Schiff base directly from a carbonyl group, whereas the PLP enzyme forms the Schiff base from a previous Schiff base. The aromatic ring of the cofactor serves as an electron sink for the PLP enzyme, whereas the amide carbonyl group serves this function for the pyruvoyl enzyme. Despite these differences, the two enzymes have essentially identical turnover numbers (Tanase et al., 1985).

The two enzymes differ in their sensitivity to inhibitors. α-(Fluoromethyl)histidine and α-methylhistidine, which are good substrates and potent inactivators of the PLP enzyme, are not even competitive inhibitors of the pyruvoyl enzyme (Recsei & Snell, 1984; Kollonitsch, 1982). Potential substrates with substituents at the α-position appear to be unable to bind to the pyruvoyl enzyme. α-Methylhistidine inactivates PLP-dependent histidine decarboxylase through the occasional occurrence of a decarboxylation-dependent transamination, behavior that has been observed for other PLP-dependent enzymes (O'Leary & Herreid, 1978). Pyruvate-dependent *S*-adenosylmethionine decarboxylase has also been found to be inactivated by a similar transamination reaction with substrate, but a similar inactivation was not observed for pyruvoyl histidine decarboxylase (Tanase et al., 1985).

In this paper we report results of carbon, nitrogen, and hydrogen isotope effect studies of the histidine decarboxylase from *Lactobacillus* 30a. Comparison of these results with results for the corresponding PLP-dependent enzyme (Abell & O'Leary, 1988b) provides interesting insight into the comparative efficiencies of the two cofactors.

Materials and Methods

Most materials and procedures used in these experiments are described in the previous paper (Abell & O'Leary, 1988b).

Histidine decarboxylase from *Lactobacillus* 30a was initially provided by Prof. E. E. Snell's laboratory at the University of Texas and later was isolated from acetone powder of this same organism (Sigma Chemical Co.) by using the literature procedure (Chang et al., 1968).

The enzyme was assayed manometrically by using an all-glass Gilson differential respirometer at 37 °C using the reaction buffer of choice. Activity measurements above pH 7.0 were made by the acid-quench method described previously (Abell & O'Leary, 1988b).

Carbon Isotope Effects. Isotope effects were measured by comparing the ^{13}C contents of CO_2 isolated after ca. 10% reaction and 100% reaction (O'Leary, 1980; Abell & O'Leary, 1988b). Isotope ratio measurements were made either on a Nuclide Associates RMS 6-60 isotope ratio mass spectrometer or else on a Finnigan Delta-E isotope ratio mass spectrometer. For earlier experiments using the Nuclide instrument, isotope effects were calculated from each pair of results from partial and complete reactions from the same stock solution. For later results using the Finnigan instrument, $\delta^{13}C$ values for 100% reaction samples were quite constant from week to week and isotope effects were calculated from an individual partial reaction and the average value for all 100% reactions measured.

Reaction solutions contained 10 mM histidine in a mixed buffer containing 0.1 M MES, 0.1 M HEPES, and 0.1 M acetate at the desired pH or, in some cases, 10 mM histidine in 0.2 M ammonium acetate buffer, pH 4.8. All buffers contained 0.03 mg/mL (hydroxypropyl)cellulose. Partial reactions were quenched with 1.0 mL of concentrated H_2SO_4. Complete conversion reactions were carried out overnight with excess enzyme and were quenched with 1 mL of 1 M H_2SO_4. All complete conversion reactions were carried out at the optimum pH of 4.8 and were assayed for remaining histidine with histidase. No residual histidine was ever detected.

Carbon isotope effect measurements in D_2O were carried out with enzyme that had been extensively dialyzed in D_2O as described below. Reaction solutions contained 50 mM histidine and 0.2 M ammonium acetate buffer, pD 4.8, containing 0.03 mg/mL (hydroxypropyl)cellulose. pD values were determined by the method of Glasoe and Long (1960), where pD = pH meter reading + 0.40. CO_2 from these reactions was isotopically normalized by exchanging with water overnight before isotope ratio analysis.

Carbon isotope effect experiments with L-[α-^2H]histidine were carried out in 0.2 M ammonium acetate buffer, pH 4.8, containing 5 mM substrate and 0.03 mg/mL (hydroxypropyl)cellulose. The substrate used in this experiment was dissolved in water and lyophilized to dryness prior to use in order to remove any residual ethanol that might interfere with the isotope ratio analysis.

Nitrogen Isotope Effects. Nitrogen isotope effects were measured by comparing the isotopic composition of the amino nitrogen of residual substrate after at least 50% reaction with the isotopic composition of the amino nitrogen in the initial substrate. Enzyme was prepared for these reactions by extensive dialysis against 0.2 M sodium acetate buffer with 0.03 mg/mL (hydroxypropyl)cellulose at pH 4.8. The enzyme solution was assayed for the presence of ammonia prior to use (Abell & O'Leary, 1988a). Reaction solutions contained 10 mL of 20 mM histidine in 0.2 M sodium acetate buffer with 0.03 mg/mL (hydroxypropyl)cellulose at pH 4.8. The fraction of reaction, the deamination of remaining histidine with histidase, and calculation of the nitrogen isotope effects were conducted as described in the previous paper (Abell & O'Leary, 1988b).

Solvent Isotope Effects. Decarboxylation rates were measured manometrically at 37 °C as described above. Enzyme solutions in H_2O and D_2O were prepared by dialyzing half of an enzyme stock solution in D_2O and half in H_2O

Table I: Carbon Isotope Effects on Histidine Decarboxylase from *Lactobacillus* 30a at pH 4.8, 37 °C[a]

$\delta^{13}C$ (‰)			
high conversion	low conversion	% reaction	k^{12}/k^{13}
−11.18	−41.9	6.2	1.0321
−10.92	−41.9	8.3	1.0325
−11.10	−41.9	8.1	1.0323
−12.00	−41.8	6.0	1.0312
−11.90	−44.4	7.2	1.0341
−12.80	−45.2	7.1	1.0340
−11.00	−43.5	7.2	1.0341
−9.63	−40.72	5.3	1.0333
−9.38	−41.11	6.3	1.0342
			av 1.0334 ± 0.0005

[a] Reactions in mixed buffer and value measured on Finnigan Delta E isotope ratio mass spectrometer. All other reactions carried out in 0.2 M ammonium acetate buffer at pH 4.8 and $\delta^{13}C$ values measured by using a Nuclide RMS 6-60 isotope ratio mass spectrometer.

containing 0.2 M ammonium acetate and 0.03 mg/mL (hydroxypropyl)cellulose for 3 days. The activity of each enzyme solution was measured once with substrate in H_2O and a second time with the same concentration of substrate in D_2O. No curvature in the plots of microliters of CO_2 released versus time was observed to indicate that hydrogen exchange was taking place during the course of the assay. The observed initial velocity of each enzyme solution was the same whether the enzyme was initially in H_2O or D_2O, and the rates of enzymatic reactions were proportional to enzyme concentration (Schowen & Schowen, 1982).

Kinetic constants in H_2O and D_2O were determined in side by side measurements using varying concentrations of histidine ranging from 0.12 to 3 mM in 0.2 M ammonium acetate buffer with 0.03 mg/mL (hydroxypropyl)cellulose. Concentrations of substrate solutions were confirmed by enzymatic assay. Larger reaction flasks were used for the smaller concentrations so that a linear rate could be observed for at least 5 min. The rate at each concentration was measured in triplicate. The data were plotted as microliters of CO_2 produced versus time, and rates were obtained from the slope of the linear portions of these graphs by linear regression analysis. The rates were then corrected for barometric pressure and reaction flask size by method D of Gregory and Winter (1965). Kinetic constants were obtained by use of the programs of Cleland (1967).

Secondary Deuterium Isotope Effects. Secondary deuterium isotope effects on V_{max} and V_{max}/K_m were measured manometrically by using the free base forms of L-[α-^{2}H]-histidine and L-histidine. The free base of histidine was made from histidine hydrochloride monohydrate via the literature procedure (Greenstein & Winitz, 1974) and recrystallized in the same manner as the labeled substrate. Side by side rate measurements were made using 4.7–0.53 mM L-histidine solutions or 4.8–0.50 mM L-[α-^{2}H]histidine in 0.2 M ammonium acetate buffer with 0.03 mg/mL (hydroxypropyl)cellulose at pH 4.8 and 37 °C. Exact substrate concentrations were determined enzymatically, and the rate of reaction at each concentration was determined in triplicate. The data were analyzed as described for solvent isotope effects.

Results

Carbon Isotope Effects. Carbon isotope effects on the enzymatic decarboxylation of histidine were measured by comparing the isotopic composition of product CO_2 from decarboxylation that proceeded to the extent of 10–20% with that from complete decarboxylation (O'Leary, 1980). The most extensive series of measurements was carried out at pH 4.8 and is summarized in Table I. The isotope effect, 1.0334 ± 0.0005, was independent of buffer, enzyme preparation, and mass spectrometer used. The same stock of histidine was used to prepare all reaction solutions used both in this and in the accompanying paper (Abell & O'Leary, 1988b), and the isotopic composition of the 100% reaction samples was constant throughout. Assay of 100% reaction samples with histidase confirmed that complete decarboxylation was being achieved.

Table II: Carbon Isotope Effects on Histidine Decarboxylase from *Lactobacillus* 30a at pH 3.0, 37 °C

$\delta^{13}C$ (‰)			
high conversion	low conversion	% reaction	k^{12}/k^{13}
−9.10	−46.13	2.3	1.0391
−9.36	−45.56	2.3	1.0384
−9.31	−44.39	3.0	1.0373
−9.30	−44.95	2.1	1.0382
−9.91	−46.40	2.8	1.0383
−9.32	−43.60	3.7	1.0365
			av 1.0381 ± 0.0005

Table III: Carbon Isotope Effects on Histidine Decarboxylase from *Lactobacillus* 30a at pH 7.0, 37 °C

$\delta^{13}C$ (‰)			
high conversion	low conversion	% reaction	k^{12}/k^{13}
−9.58	−32.08	3.0	1.0290
−9.43	−35.39	9.6	1.0283
−9.31	−36.25	5.0	1.0286
−9.32	−35.87	3.0	1.0280
−9.29	−36.37	5.0	1.0290
−9.24	−36.14	5.0	1.0286
			av 1.0286 ± 0.0002

Table IV: Carbon Isotope Effects on Decarboxylation of Histidine by Histidine Decarboxylase from *Lactobacillus* 30a in D_2O at pD 4.8, 37 °C

$\delta^{13}C$ (‰)	% reaction	k^{12}/k^{13}
−8.607 ± 0.014	100	
−8.546 ± 0.026	100	
−8.144 ± 0.034	100	
−8.504 ± 0.026	100	
av −8.450 ± 0.208		
−37.028 ± 0.023	11.8	1.0316
−37.148 ± 0.018	12.7	1.0319
−33.188 ± 0.040	31.2	1.0312
−32.397 ± 0.019	28.9	1.0308
−36.896 ± 0.053	19.5	1.0330
−31.778 ± 0.018	35.2	1.0303
−35.800 ± 0.021	18.0	1.0314
−35.602 ± 0.014	18.0	1.0314
−36.272 ± 0.031[a]	13.3	1.0310
−35.676 ± 0.028[a]	13.4	1.0304
		av 1.0313 ± 0.0008

[a] Values measured with unexchanged enzyme.

The pH dependence of the carbon isotope effect was examined by measuring the carbon isotope effect at pH 3.0 and pH 7.0, where the pH profile shows V_{max}/K_m decreasing with a slope of 1 and 2, respectively. The results of isotope effect measurements at pH 3.0 are shown in Table II, and those for measurements at pH 7.0 are shown in Table III. The same mixed buffer system adjusted to the desired pH was used for all these measurements.

The results of carbon isotope effect measurements in D_2O at pD 4.8 are shown in Table IV. Carbon isotope effects

Table V: Isotope Effects on Histidine Decarboxylase from *Lactobacillus* 30a with L-[α-^{2}H]Histidine as Substrate at pH 4.8, 37 °C

δ^{13}C (‰)	% reaction	k^{12}/k^{13}
−6.802 ± 0.020	100	
−6.865 ± 0.029	100	
−6.578 ± 0.026	100	
−6.658 ± 0.025	100	
−6.448 ± 0.025	100	
av −6.670 ± 0.168		
−36.331 ± 0.010	20.9	1.0347
−35.825 ± 0.063	24.1	1.0348
−37.898 ± 0.026	10.3	1.0343
−38.342 ± 0.016	9.6	1.0347
−38.346 ± 0.021	9.1	1.0346
−38.123 ± 0.033	11.0	1.0347
−38.185 ± 0.019	9.7	1.0345
−38.256 ± 0.015	9.9	1.0346
		av 1.0346 ± 0.0002

measured with unexchanged enzyme in D_2O were not significantly different from those measured with enzyme that had been extensively exchanged with D_2O. This result indicates that exchange of the enzyme in D_2O does not fundamentally change the properties of the enzyme. The carbon isotope effect in D_2O of 1.0313 ± 0.0008 is slightly smaller than the value of 1.0334 ± 0.0005 observed in H_2O at pH 4.8.

The results of carbon isotope effect measurements with L-[α-^{2}H]histidine as substrate are shown in Table V. When the observed isotope effect of 1.0346 is corrected for the extent of deuterium incorporation in the substrate of 94%, the actual isotope effect becomes 1.0347 ± 0.0002. This value is only slightly larger than the isotope effect of 1.0334 ± 0.0005 measured with the unlabeled substrate at the same pH. The results of a *t*-test indicate that the difference in the two isotope effects, though small, is statistically significant at the 95% confidence level.

Secondary Deuterium Isotope Effects. Carefully conducted direct comparison experiments were used to measure secondary deuterium isotope effects on the catalytic parameters for pyruvoyl histidine decarboxylase. The kinetic data were fitted to eq 1 and 2. Equation 1 assumes an isotope effect on

$$v = \frac{V[A]}{K[1 + F_i E(V_{max}/K_m)] + [A]} \quad (1)$$

$$v = \frac{V[A]}{K[1 + F_i E(V_{max}/K_m)] + [A][1 + F_i E(V)]} \quad (2)$$

V_{max}/K_m only, while eq 2 assumes isotope effects on V_{max} and V_{max}/K_m. In each equation F_i is the fraction of label in the substrate A, [A] is substrate concentration, v is the observed velocity, $E(V_{max}/K_m)$ is the isotope effect on V_{max}/K_m minus 1, and $E(V)$ is the isotope effect on V_{max} minus 1. Equation 2 provided the best fit to the kinetic data, and the isotope effects shown in Table VII were calculated by using this equation.

Solvent Isotope Effects. Kinetic constants in H_2O and D_2O were determined in side-by-side manometric measurements at 37 °C, pH or pD 4.8. Isotope effects were 1.43 ± 0.11 on V_{max}/K_m and 2.48 ± 0.17 on V_{max}.

Nitrogen Isotope Effects. Nitrogen isotope effects were measured by comparing the isotopic composition of the amino nitrogen of the residual substrate after 50% or more of the initial substrate was decarboxylated with the isotopic composition of the amino nitrogen in the initial substrate. The results of these experiments are shown in Table VI. The

Table VI: Nitrogen Isotope Effects on Histidine Decarboxylase from *Lactobacillus* 30a at pH 4.8, 37 °C

δ^{15}N (‰)	% reaction	k^{14}/k^{15}
10.463 ± 0.022	100	
8.444 ± 0.060	100	
10.190 ± 0.034	100	
mean 9.699 ± 1.095		
6.664 ± 0.045	59	0.9967
6.319 ± 0.063	55	0.9958
6.753 ± 0.035	56	0.9965
6.399 ± 0.036	52	0.9955
		av 0.9961 ± 0.0006
		corr[a] 0.9799 ± 0.0006

[a] Isotope effect corrected for substrate protonation.

Table VII: Summary of Isotope Effects on Histidine Decarboxylase from *Lactobacillus* 30a

k^{12}/k^{13} at pH 3.0	1.0381 ± 0.0005
k^{12}/k^{13} at pH 4.8	1.0334 ± 0.0005
k^{12}/k^{13} at pH 7.0	1.0286 ± 0.0002
k^{12}/k^{13} with [α-^{2}H]histidine, pH 4.8	1.0347 ± 0.0001
k^{12}/k^{13} in D_2O at pD 4.8	1.0313 ± 0.0008
$(V_{max}/K_m)^{H_2O}/(V_{max}/K_m)^{D_2O}$ at pH (pD) 4.8	1.43 ± 0.11
$(V_{max})^{H_2O}/(V_{max})^{D_2O}$ at pH (pD) 4.8	2.48 ± 0.17
$(V_{max}/K_m)^{H}/(V_{max}/K_m)^{D}$ with [α-^{2}H]histidine, pH 4.8	1.14 ± 0.10
$(V_{max})^{H}/(V_{max})^{D}$ with [α-^{2}H]histidine, pH 4.8	1.07 ± 0.04
k^{14}/k^{15} at pH 4.8	0.9799 ± 0.0006

observed isotope effect, 0.9961 ± 0.0006, is that for protonated histidine as the starting substrate. The observed isotope effect was divided by 1.0165, the ^{15}N equilibrium isotope effect on the protonation–deprotonation of the amino nitrogen of the substrate, to yield the corrected isotope effect of 0.9799 ± 0.001. This corrected isotope effect reflects the kinetic nitrogen isotope effect on decarboxylation of the unprotonated substrate (Hermes et al., 1985).

Discussion

Isotope effects on the pyruvoyl histidine decarboxylase are summarized in Table VII. These isotope effects are similar to those on the PLP-dependent histidine decarboxylase (Abell & O'Leary, 1988b). These isotope effects can be interpreted quantitatively by using eq 4–6 in the previous paper (Abell & O'Leary, 1988b) along with eq 3 for the secondary deu-

$$(V_{max}/K_m)^{H}/(V_{max}/K_m)^{D} = \frac{k_5^{H}/k_5^{D} + k_5/k_4}{1 + k_5/k_4} \quad (3)$$

terium isotope effect. The rate constants are defined in Scheme I. All these equations are predicated on the assumption that only k_5 has significant carbon and secondary hydrogen isotope effects, only k_3 and k_4 have significant nitrogen isotope effects, and substrate binding is at equilibrium.

Carbon Isotope Effects. The analysis of the carbon isotope effect (1.0334 at pH 4.8) is facilitated by the availability of a larger number of chemical models (Dunn, 1977) and enzymatic analogies. Values for k_5/k_4 can be calculated from the observed isotope effect by using eq 4 (previous paper) and assuming a range of possible values for the intrinsic carbon isotope effect.[2] A broader range of intrinsic isotope effects is assumed in this case than in the case of the PLP-dependent histidine decarboxylase because of the uncertainty as to

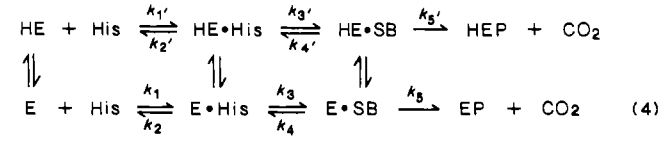
[2] An intrinsic isotope effect is an isotope effect on an individual step in a multistep reaction mechanism. The observed isotope effect is related to the intrinsic isotope effect(s) by an equation involving a collection of terms of the form k_n/k_{n-1}.

Table VIII: Calculated Values of Kinetic Parameters for the Decarboxylation of Histidine Catalyzed by Pyruvoyl Histidine Decarboxylase at pH 4.8, 37 °C, for Various Assumed Values of the Intrinsic Carbon Isotope Effect

	assumed value of k_5^{12}/k_5^{13}			
derived parameters	1.040	1.050	1.060	1.070
k_5/k_4	0.198	0.497	0.796	1.096
k_3^{14}/k_3^{15}	1.045	1.006	0.996	0.992
k_4^{14}/k_4^{15}	1.081	1.040	1.030	1.026
k_5^H/k_5^D (from carbon isotope effects)	1.29	1.12	1.09	1.08
$(V_{max}/K_m)^H/(V_{max}/K_m)^D$ (from carbon isotope effects)	1.24	1.09	1.05	1.04

whether or not the model used for the PLP system (Marlier & O'Leary, 1986) is appropriate for the pyruvate system. Table VIII shows the values of k_5/k_4 derived for a range of values of the intrinsic isotope effect. As in the case of the PLP-dependent enzyme, the ratio k_5/k_4 is in the range 0.2–1.0, indicating that decarboxylation is slightly more rate-limiting than Schiff base formation.

pH Dependence of Carbon Isotope Effects. V_{max}/K_m for the decarboxylation of histidine shows a bell-shaped pH–rate profile (Recsei et al., 1970; Chang et al., 1968; Snell & Huynh, 1986). Below a pK of 4.2, the plot of log V_{max}/K_m vs pH has a slope of 1. Above a pK of 6.5, the plot has a slope of −2. Over this same range, the carbon isotope effect shows a monotonic decrease with increasing pH. Assuming that the intrinsic isotope effect is pH independent,[3] this indicates that the ratio k_5/k_4 decreases with increasing pH. This pH dependence is probably due to the existence of a parallel pathway for one or more kinetically significant steps, eq 4, in which



(4)

SB is the Schiff base and EP represents the decarboxylated product. The pH dependence of the isotope effect requires that $k_5'/k_4' < k_5/k_4$.

The pH–rate profile shows a break at pH 6.5. Such breaks are often attributed to catalytic histidine residues. However, in this case of this enzyme, there is no histidine at the active site (M. Hackert, personal communication), and an alternative explanation must be sought both for the pH dependence of the kinetics and for the pH dependence of the carbon isotope effect.

An attractive possibility is that protonation of the amino nitrogen of the enzyme–substrate Schiff base is responsible for the pH dependence of the isotope effects. Only the protonated Schiff base could hydrolyze to regenerate free histidine ($k_3 = k_4 = 0$), whereas the unprotonated Schiff base might be able to undergo decarboxylation ($k_5 \neq 0$); thus, the isotope effect would be expected to decrease with increasing pH. As we shall see below, this explanation also fits well with the nitrogen isotope effect results.

Nitrogen Isotope Effects. Intrinsic nitrogen isotope effects can be derived from eq 5 (previous paper) by using values of k_5/k_4 calculated from the carbon isotope effects. Calculation of intrinsic nitrogen isotope effects on k_5 and k_4 begins with an assumption about whether or not the Schiff base nitrogen is protonated. As in previous cases (Abell & O'Leary 1988a,b), values of k_4^{14}/k_4^{15} derived on the assumption that the Schiff base is unprotonated are consistently inverse (data not shown), and this is at variance with our expectations for this reaction. More reasonable values for k_4^{14}/k_4^{15} are obtained when the Schiff base is assumed to be protonated and K_{eq}^{14}/K_{eq}^{15} is equal to 0.9669. The results of these calculations are shown in Table VIII. From the limited model studies that are available, k_4^{14}/k_4^{15} is expected to be in the range of 1.02–1.03 (Abell & O'Leary, 1988a). For an assumed intrinsic carbon isotope effect of 1.04, k_4^{14}/k_4^{15} is 1.08. There is no precedent for such a large nitrogen isotope effect on k_4; thus, we can eliminate this set of values from further consideration. For an assumed intrinsic carbon isotope effect of 1.05, k_4^{14}/k_4^{15} is 1.04. This value is somewhat larger than is expected for such a reaction, but this possibility cannot be eliminated with confidence. Larger values of the intrinsic carbon isotope effect give reasonable values for k_4^{14}/k_4^{15}, and the best overall fit is obtained with an intrinsic carbon isotope effect of 1.05–1.06, as is true with the PLP-dependent enzyme.

These calculations reveal an important point concerning the mechanism of pyruvoyl histidine decarboxylase. The Schiff base linkage between the substrate and the prosthetic group that is formed during the course of this reaction is protonated at nitrogen. Since there is no convenient spectroscopic probe for pyruvoyl enzymes like that for PLP-dependent enzymes, the only insight into the protonation state of this intermediate is provided by these nitrogen isotope effect experiments. The conclusion that the Schiff base is protonated is consistent with the pH dependence of the carbon isotope effects.

Solvent Isotope Effects. Substitution of D_2O for H_2O has only a minor effect on kinetic parameters and isotope effects for the pyruvoyl histidine decarboxylase (Table VII). The carbon isotope effect decreases slightly, and V_{max} and V_{max}/K_m show small solvent isotope effects. The difference between the latter two solvent isotope effects is presumably due to an approximately twofold equilibrium solvent isotope effect on substrate binding, as has been observed with glutamate decarboxylase (O'Leary et al., 1981). Although solvent isotope effects are very difficult to model convincingly, these results are consistent with the interpretation of the carbon isotope effects above. As in the case of the PLP-dependent glutamate decarboxylase (O'Leary et al., 1981), the principal effect of D_2O is probably to slow down the Schiff base interchange step (k_3 and k_4), resulting in a decrease in the carbon isotope effect, as observed.

The effect of D_2O on the carbon isotope effect is much smaller for histidine decarboxylase than for glutamate decarboxylase. This difference arises for two reasons: First, the different values of k_5/k_4 in the two cases make the observed isotope effect less sensitive to solvent isotope effects on k_3 and k_4 in the case of histidine decarboxylase. Second, quantitative analysis of effects of D_2O on rates and isotope effects indicates that the solvent isotope effect on k_3 and k_4 is probably less than 4 for histidine decarboxylase, whereas this value is more than 6 for glutamate decarboxylase (O'Leary et al., 1981).

Secondary Hydrogen Isotope Effects. The carbon isotope effect obtained when L-[α-^{2}H]histidine is used is slightly larger than that observed with unlabeled substrate under the same conditions. This difference is smaller than that observed with the PLP-dependent histidine decarboxylase (Abell & O'Leary, 1988b). Values for k_5/k_4 that were derived from the carbon isotope effect can be used together with the carbon isotope effect with deuteriated substrate to calculate k_5^H/k_5^D by using eq 6 (previous paper). In turn, k_5^H/k_5^D can be used to calculate values for $(V_{max}/K_m)^H/(V_{max}/K_m)^D$ by using eq 3, and these calculated values (Table VIII) can be compared to the

[3] This is equivalent to assuming that the extent of bonding in the transition state is independent of external factors such as protonation of various functional groups, etc.

experimental values shown in Table VII. The comparison of experimental and calculated values is satisfactory, considering the large experimental error associated with the steady-state kinetics. Unfortunately, the experimental uncertainties in both cases prevent these results from being used to limit the range of intrinsic isotope effects as was done in the case of the nitrogen isotope effects. Instead, we have used deuterium isotope effects derived from the carbon isotope effects for a detailed analysis of the transition-state structure.

Intrinsic isotope effects for individual steps in an enzymatic reaction may sometimes be used to gain insight into details of transition-state structure. This is particularly true for secondary deuterium isotope effects, which show a smooth, monotonic increase as the transition state becomes more product-like. For the decarboxylation step in question here, a transition state that is fully product-like should show a hydrogen isotope effect near 1.29 (Melander & Saunders, 1980). The calculated hydrogen isotope effect, which is near 1.10 ± 0.04, thus indicates a relatively early transition state in which the carbon–carbon bond is roughly one-third broken. This result may be contrasted with the hydrogen isotope effect of 1.20 ± 0.05 on PLP-dependent histidine decarboxylase, which indicated that the transition state in that case is more product-like.

In spite of this difference in transition-state structures, the intrinsic carbon isotope effect for the pyruvoyl enzyme is like that for the PLP enzyme, indicating that the carbon isotope effect may not be particularly sensitive to small changes in C–C bond order in the transition state. Although there is much literature on carbon isotope effects on a variety of chemical decarboxylations (Dunn, 1977), there appear to be no systematic studies that enable us to predict whether there should be a significant change in isotope effect with transition-state structure. Available data on the subject suggest quite the opposite—carbon isotope effects in decarboxylations may be rather independent of the degree of C–C bond breaking at the transition state—and this observation is consistent with our findings.

Comparison of PLP-Dependent and Pyruvate-Dependent Histidine Decarboxylases. Now we must return to the question posed at the beginning of the previous paper: What are the fundamental differences between the two enzymes, and how do the two catalytic strategies compare? The striking result of this study is the extreme similarity of the two forms of histidine decarboxylase. Turnover numbers of the two enzymes are essentially the same (Tanase et al., 1985). Carbon and nitrogen isotope effects are nearly the same for the two enzymes. For both enzymes, the rate constant ratio k_5/k_4 for partitioning of the Schiff base intermediate is in the range 0.5–1.0.

The most noticeable difference between the two enzymes is in the intrinsic secondary deuterium isotope effects; that for the PLP enzyme is near 1.20, whereas that for the pyruvoyl enzyme is near 1.10. This indicates that the transition state for decarboxylation is more product-like for the PLP-dependent enzyme than for the pyruvoyl enzyme. However, the manner in which this difference might be of fundamental importance is not known.

Pyridoxal 5′-phosphate has a reputation among chemists as being a highly crafted, carefully optimized coenzyme. Schiff base interchange involving the ortho-hydroxyl group is commonly presented as being kinetically advantageous compared to Schiff base formation from an amine and a carbonyl group (as in the case of the pyruvoyl enzyme). The pyridine ring is considered to be an optimum electron sink for absorbing the negative charge that is generated in the decarboxylation step. Thus, we had expected to find that the pyruvoyl histidine decarboxylase was inferior to the PLP-dependent enzyme in terms of efficiency and "perfection" (Knowles & Albery, 1977). This is exactly the opposite of what we found. Both enzymes fulfill the criteria of enzymatic perfection as defined by Knowles and Albery (1977). The energy barriers for all the chemical steps are approximately the same, thus preventing any one chemical step from becoming entirely rate-limiting. By this criterion, the pyruvoyl moiety is just as effective as PLP in catalyzing the decarboxylation of histidine. Ultimately, the choice between PLP and pyruvate for catalysis of the decarboxylation of amino acids may be based on more subtle considerations. It is also possible that the significant difference is in the ability of PLP to catalyze a variety of other reactions in addition to decarboxylation—an ability to date not shared by pyruvate.

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Triosephosphate Isomerase: Energetics of the Reaction Catalyzed by the Yeast Enzyme Expressed in *Escherichia coli*†

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ABSTRACT: Triosephosphate isomerase from bakers' yeast, expressed in *Escherichia coli* strain DF502(p12), has been purified to homogeneity. The kinetics of the reaction in each direction have been determined at pH 7.5 and 30 °C. Deuterium substitution at the C-2 position of substrate (*R*)-glyceraldehyde phosphate and at the 1-*pro-R* position of substrate dihydroxyacetone phosphate results in kinetic isotope effects on k_{cat} of 1.6 and 3.4, respectively. The extent of transfer of tritium from [1(*R*)-^{3}H]dihydroxyacetone phosphate to product (*R*)-glyceraldehyde phosphate during the catalyzed reaction is only 3% after 66% conversion to product, indicating that the enzymic base that mediates proton transfer is in rapid exchange with solvent protons. When the isomerase-catalyzed reaction is run in tritiated water in each direction, radioactivity is incorporated both into the remaining substrate and into the product. In the "exchange-conversion" experiment with dihydroxyacetone phosphate as substrate, the specific radioactivity of remaining dihydroxyacetone phosphate rises as a function of the extent of reaction with a slope of about 0.3, while the specific radioactivity of the product is 54% that of the solvent. In the reverse direction with (*R*)-glyceraldehyde phosphate as substrate, the specific radioactivity of the product formed is only 11% that of the solvent, while the radioactivity incorporated into the remaining substrate (*R*)-glyceraldehyde phosphate also rises as a function of the extent of reaction with a slope of 0.3. These results have been analyzed according to the protocol described earlier to yield the free energy profile of the reaction catalyzed by the yeast isomerase. The profile shows that the isomerase from yeast, like the enzyme from chicken muscle previously analyzed, is a highly efficient catalyst. The kinetic characteristics of the yeast enzyme are very close to those of the chicken isomerase and provide the necessary basis with which the behavior of mutant yeast isomerases can be compared.

To understand the relationship between structure and function in enzymology and to evaluate the role of particular amino acid side chains, it is common practice to observe the consequences of changing catalytic residues by chemical modification or by site-directed mutagenesis. To maximize our understanding, we prefer to use an enzyme system that is already well characterized in structural, mechanistic, and energetic terms. One such system is triosephosphate isomerase (TIM, EC 5.3.1.1), a glycolytic enzyme that catalyzes the interconversion of dihydroxyacetone phosphate and (*R*)-glyceraldehyde phosphate.[1] The rate of the enzymatic reaction is more than 10^9 times faster than that of the analogous reaction catalyzed by acetate ion (Hall & Knowles, 1975; Richard, 1984), and the reaction proceeds by the stereospecific abstraction of either the 1-*pro-R* proton of dihydroxyacetone phosphate or the C-2 proton of (*R*)-glyceraldehyde phosphate by an enzymic base to give a *cis*-enediol intermediate (Figure 1) (Rieder & Rose, 1959; Rose, 1962). The free energy profile of the reaction catalyzed by the isomerase from chicken muscle has been determined from a series of isotopic experiments (Albery & Knowles, 1976b) and shows that this enzyme is near to being optimal in catalytic terms (Albery & Knowles, 1976c, 1977; Knowles & Albery, 1977). Given the levels of triose phosphates in vivo and assuming that the substrates and the enzyme diffuse freely, it appears that the catalytic flux per enzyme molecule could not be increased by further modification of the protein.

The amino acid sequences of triosephosphate isomerases have been determined from a variety of organisms, both prokaryotic (Artavanis-Tsakonis & Harris, 1980; Pichersky et al., 1984) and eukaryotic (Kolb et al., 1974; Corran & Waley, 1975; Alber & Kawasaki, 1982; Lu et al., 1984; Maquat et al., 1985; Russell, 1985; Straus & Gilbert, 1985; McKnight et al., 1986; Marchionni & Gilbert, 1986; Swinkels et al., 1986). The isomerase from chicken muscle has been crystallized, and the structure has been determined to 2.5-Å resolution (Banner et al., 1975). The structure of the triosephosphate isomerase from yeast has been solved at 3-Å

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[1] Nomenclature: dihydroxyacetone phosphate, dihydroxyacetone 3-phosphate; glyceraldehyde phosphate, (*R*)-glyceraldehyde 3-phosphate (otherwise D-glyceraldehyde 3-phosphate); glycerol phosphate, (*R*)-glycerol phosphate (otherwise *sn*-glycerol phosphate); phosphoglycerate, 3-phospho-(*R*)-glycerate (otherwise 3-phospho-D-glycerate).

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