$$[S_{tot}] = [S°_{tot}] - k^{p}_{cat}[E_T]t \quad (16)$$

For the minimal mechanism (eq 8) $k^{p}_{cat} = k_2$ and under these conditions eq 14 will become

$$-\frac{dX^*}{dt} = \frac{k^{e}_{cat}[E_T]X^*}{[S°_{tot}] - k^{p}_{cat}[E_T]t} + k'X^* \quad (17)$$

Rearrangement and integration from $t = 0$ to $t = t$ gives

$$\ln\left(\frac{X^*_0}{X^*}\right) = \frac{k^{e}_{cat}}{k^{p}_{cat}}\left[\ln\left(\frac{[S_{tot}]}{[S°_{tot}] - k^{p}_{cat}[E_T]t}\right)\right] + k't \quad (18)$$

Further rearrangement leads to that form of eq 3 given under Materials and Methods, a form that can be fit by standard nonlinear regression, adjusting X^*_0 and k^{e}_{cat}.

Registry No. mAATase, 9000-97-9; αKG, 114019-72-6; ^{18}O, 14797-71-8.

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Kinetic Isotope Effect Studies on Aspartate Aminotransferase: Evidence for a Concerted 1,3 Prototropic Shift Mechanism for the Cytoplasmic Isozyme and L-Aspartate and Dichotomy in Mechanism†

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ABSTRACT: The C_α primary hydrogen kinetic isotope effects (C_α-KIEs) for the reaction of the cytoplasmic isozyme of aspartate aminotransferase (cAATase) with [α-^{2}H]-L-aspartate are small and only slightly affected by deuterium oxide solvent ($^{D}V = 1.43 \pm 0.03$ and $^{D}V/K_{Asp} = 1.36 \pm 0.04$ in H_2O; $^{D}V = 1.44 \pm 0.01$ and $^{D}V/K_{Asp} = 1.61 \pm 0.06$ in D_2O). The D_2O solvent KIEs (SKIEs) are somewhat larger and are essentially independent of deuterium at C_α ($^{D_2O}V = 2.21 \pm 0.07$ and $^{D_2O}V/K_{Asp} = 1.70 \pm 0.03$ with [α-^{1}H]-L-aspartate; $^{D_2O}V = 2.34 \pm 0.12$ and $^{D_2O}V/K_{Asp} = 1.82 \pm 0.06$ with [α-^{2}H]-L-aspartate). The C_α-KIEs on V and on V/K_{Asp} are independent of pH from pH 5.0 to pH 10.0. These results support a rate-determining concerted 1,3 prototropic shift mechanism by the multiple KIE criteria [Hermes, J. D., Roeske, C. A., O'Leary, M. H., & Cleland, W. W. (1982) *Biochemistry 21*, 5106]. The large C_α-KIEs for the reaction of mitochondrial AATase (mAATase) with L-glutamate ($^{D}V = 1.88 \pm 0.13$ and $^{D}V/K_{Glu} = 3.80 \pm 0.43$ in H_2O; $^{D}V = 1.57 \pm 0.05$ and $^{D}V/K_{Glu} = 4.21 \pm 0.19$ in D_2O) coupled with the relatively small SKIEs ($^{D_2O}V = 1.58 \pm 0.04$ and $^{D_2O}V/K_{Glu} = 1.25 \pm 0.05$ with [α-^{1}H]-L-glutamate; $^{D_2O}V = 1.46 \pm 0.06$ and $^{D_2O}V/K_{Glu} = 1.16 \pm 0.05$ with [α-^{2}H]-L-glutamate) are most consistent with a two-step mechanism for the 1,3 prototropic shift for this isozyme–substrate pair. Primary C_α-hydrogen and SKIEs on the mAATase plus L-aspartate, cAATase plus L-glutamate, and cAATase plus L-alanine reactions are consistent with either a one- or two-step mechanism. Solvent isotope effects on the competitive inhibition constants for maleate and α-methyl-D,L-aspartate are $^{D_2O}K_i = 1.35 \pm 0.06$ and 1.11 ± 0.03, respectively. The KIEs together with the previous results [Julin, D. A., Wiesinger, H., Toney, M. D., & Kirsch, J. F. (1989) *Biochemistry* (first of three papers in this issue); McLeish, M. J., Julin, D. A., & Kirsch, J. F. (1989) *Biochemistry* (second of three papers in this issue)] provide the basis for the partial construction of free energy profiles for these reactions.

The key step in the transamination reactions catalyzed by the pyridoxal phosphate (PLP)[1] dependent enzyme AATase is the 1,3 prototropic shift interconverting the external aldimine and ketimine forms of the cofactor substrate complex (Julin et al., 1989). This process is generally depicted as occurring in two steps with proton abstraction from the C_α position of the amino acid followed by protonation at $C_{4'}$ of the cofactor, with a quinonoid intermediate (Kirsch et al., 1984; Julin et al., 1989; Jansonius & Vincent, 1987). The latter form has

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[1] Abbreviations: cAATase and mAATase, the cytosolic and mitochondrial isozymes of aspartate aminotransferase; KIE, kinetic isotope effect; SKIE, D_2O solvent KIE; ^{D}parameter or ^{D_2O}parameter, the primary deuterium KIE or SKIE, respectively, on the kinetic parameter (Northrop, 1977); MDH, malic dehydrogenase; LDH, lactic dehydrogenase; OAA, oxaloacetate; αKG, α-ketoglutarate; PMP, pyridoxamine 5′-phosphate; PLP, pyridoxal 5′-phosphate; α-MeAsp, α-methyl-D,L-aspartate; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid.

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an absorbance maximum at ca. 490–500 nm in equilibrium mixtures of cAATase with L-aspartate (Jenkins & Taylor, 1965; Michuda & Martinez-Carrion, 1969) and L-glutamate (Jenkins & D'Ari, 1966) and is more intense when *erythro*-β-hydroxyaspartate is the substrate (Jenkins, 1961).

Although the quinonoid has been established as a kinetically competent intermediate with the substrate *erythro*-β-hydroxyaspartate (Jenkins & Harruff, 1979), there is little evidence that it is an obligatory intermediate along the primary pathway for product formation with the natural substrates L-aspartate and L-glutamate. An alternative mechanism is a concerted one involving simultaneous partial breakage of the C_α-H bond and partial formation of the $C_{4'}$-H bond in a single transition state with N^ϵ of Lys-258 serving as both acid and base. Thus the quinonoid, if formed, would be off the primary reaction pathway. These possibilities can, in principle, be distinguished by the double kinetic isotope effect criteria introduced by Hermes et al. (1982) and Belasco et al. (1983) in which the mutual dependence or independence of the two KIEs is used to differentiate the alternatives. KIEs are expected on both proton transfers in the 1,3 prototropic shift. Deuterium at the C_α position of amino acid substrates leads to a KIE on C_α-hydrogen abstraction while $C_{4'}$ protonation should be susceptible to a D_2O SKIE since protons on the ϵ-amino group of Lys-258, the presumptive proton-transfer agent (Kirsch et al., 1984), are solvent exchangeable. Measurement of the effects of C_α-deuterium and D_2O solvent on the reaction kinetics thus can potentially elucidate the nature of the 1,3 prototropic shift mechanism in AATase. The present paper contains the results of C_α primary and solvent KIE measurements for the reactions of both AATase isozymes with L-aspartate and L-glutamate substrates, the pH dependence of the C_α-KIE for cAATase and L-aspartate, and KIEs for cAATase and the reaction with L-alanine. The multiple KIE experiments provide evidence for a concerted mechanism in the cAATase plus L-aspartate case and for a stepwise one when L-glutamate is the substrate for mAATase. The KIEs taken together with results reported in the two accompanying papers (Julin et al., 1989; McLeish et al., 1989) provide the basis for partial construction of free energy profiles for these reactions.

Materials and Methods

Materials. c- and mAATase, MDH, NADH, [α-^{2}H]-L-aspartic and -glutamic acids, and deuterium oxide were prepared or obtained as described in Julin et al. (1989). Mass spectrometric analysis of the diethyl ester of [α-^{2}H]-L-glutamate indicated the presence of 10% undeuterated L-glutamic acid diethyl ester. Some experiments were performed with cAATase purchased from Calbiochem-Behring. The KIEs determined with this enzyme were in all cases identical within the experimental uncertainty with those obtained with cAATase purified as described in Julin et al. (1989). α-MeAsp, LDH from pig heart, type XVIII, and pig heart alanine aminotransferase (glutamate pyruvate transaminase) were obtained from Sigma.

Preparation of D,L-*Aspartic Acid and* [α-^{2}*H*]-D,L-*Aspartic Acid.* Solid OAA (0.5 g, 3.8 mmol) was added in several portions to 12 mL of 10 M ammonia adjusted to pH 11.5 with 50% NaOH, and the mixture was stirred for 1 h. Sodium borohydride or sodium borodeuteride (Sigma, 98 atom % deuterated) was added in two 0.5-g portions (total of 26 mmol) over a 15-min period and the mixture stirred an additional hour. Excess ammonia was removed under vacuum and the remaining borohydride destroyed by slow addition of concentrated HCl. After evolution of hydrogen gas ended, the pH was lowered to 1.6 with HCl and the D,L-aspartic acid purified by chromatography on Dowex 50 (H^+ form) and crystallized from ethanol/water as described by Dougherty et al. (1972). The purified material (0.35 g, 70% yield) had an NMR spectrum and elemental analysis consistent with those expected for aspartic acid. No residual protium at C_α was evident in the NMR spectrum of the [α-^{2}H]-D,L-aspartate.

Preparation of [α-^{2}*H*]-L-*Alanine.* L-Alanine (2 g, 22 mmol), potassium dihydrogen phosphate (0.27 g, 1.98 mmol), and 0.31 mL of 5 M KOH were dissolved in 20 mL of water, and the pH was adjusted to 7.9 with 5 M KOH. The solution was dried at reduced pressure and the solid residue dissolved and dried twice from 20 mL of D_2O (95 atom % D). The residue was finally dissolved in 20 mL of D_2O (99 atom % D), sodium pyruvate (3.3 mg, 0.03 mmol) added, and the exchange reaction initiated by adding alanine aminotransferase (6.6 units, 0.29 mg in 0.1 mL). The mixture was kept at room temperature and three more 0.10-mL aliquots of enzyme were added over a 5-day period. The L-alanine was isolated by ion-exchange chromatography on Dowex 50 (H^+ form). The product (1.29 g, 58% yield) had an NMR spectrum and elemental analysis consistent with those of partially deuterated L-alanine. The extent of deuteration was 82% at the C_α position and 84% at the C_β (2.5 deuteriums/methyl group) as determined by NMR. A second preparation was 75% deuterated at C_α. The substantial exchange of the β-hydrogens of L-alanine catalyzed by alanine aminotransferase has been observed previously (Babu & Johnston, 1976; Walter et al., 1975; Cooper, 1976).

One batch of [α-^{2}H]-L-alanine prepared by this procedure contained a substance that strongly inhibited cAATase in its reactions with both L-alanine and L-aspartate. L-Alanine itself at concentrations up to 1 M did not inhibit the reaction with L-aspartate. This inhibitor, apparently a metal ion, was removed by recrystallization from ethanol/water in the presence of 5 mM dithiothreitol.

Enzyme Concentrations. The active-site concentration of the cAATase stock solution (ammonium sulfate suspension) was determined by measuring the absorbance at 362 nm of an aliquot that had been dialyzed against 0.05 M Tris-HCl, pH 8.5 [$\epsilon_{362} = 8200\ M^{-1}\ cm^{-1}$ (Martinez-Carrion et al., 1965)]. The dialyzed solution was assayed as in Julin et al. (1989). Specific activities of 14.6 and 13.7 units/nmol of active sites were found for two different enzyme preparations. The mitochondrial enzyme concentration was estimated from its activity in the standard assay, and specific activities of 350 (cAATase) and 240 units/mg (mAATase) were found under similar assay conditions (Sonderegger et al., 1977). A specific activity of 10 units/nmol was calculated for the mitochondrial enzyme by using subunit molecular weights of 47 000 and 44 000 for the cytoplasmic and mitochondrial isozymes (Barra et al., 1980), respectively.

Kinetics. (A) Reactions with L-*Aspartate and* α*KG.* Kinetic parameters were determined by initial rate measurements at 340 nm in a coupled assay with MDH (2 units/mL) and 0.14 mM NADH. Reaction mixtures (2-mL total volume) were equilibrated to 25 °C before AATase (2–10 pmol) was added. The pH of the reaction mixture was measured after the run.

(B) Deuterium Oxide SKIEs on the Reaction of AATase with L-*Aspartate and* α*KG.* The pH dependence of the kinetic parameters for both isozymes was determined before SKIEs were measured. Runs were done at constant αKG and varying L-aspartate concentrations over the range pH 7.1–9.5, at intervals of 0.4 pH unit. The results with cAATase (not shown) are in general agreement with those reported by Kiick and Cook (1983), except that the pK_a of 9.3 which they report in

the V/K_{Asp} profile was not detected, probably because insufficient data were collected in the high-pH region in the present study. The kinetic parameters in D_2O were measured at three pD values, to verify that the maximum is not shifted significantly in D_2O. Most subsequent SKIE experiments with both isozymes were done at pH 8.3, in the pH-independent region of the V/K profile, and at pD 8.7 (pD = meter reading + 0.4).

Initial rates were measured as described above in reaction mixtures of 1-mL total volume at 25 °C. Buffer salts were sometimes lyophilized at least once from D_2O (90 atom % D) to remove exchangeable hydrogens. This was not done for all experiments, since the low concentrations of buffer (25 mM) and other reagents introduced only a small amount of ^1H (at most 4–5 atom %, mostly due to the use of enzyme solutions in H_2O) into the solution. No correction was made for this residual protium. The buffers were HEPES or TAPS, pH 8.3 (pD 8.7), and potassium phosphate, pH 7.5 (pD 7.9). Solutions of L-aspartate, αKG, and MDH/NADH were prepared in each solvent, while only one AATase solution, in either H_2O or D_2O, was used for all runs in both solvents. Identical results, to within experimental uncertainty, were obtained with cAATase diluted into H_2O as compared with that in D_2O.

Solvent isotope effects on the competitive inhibition constants of maleate and α-MeAsp were measured in 0.025 M TAPS, pH 8.3 (pD 8.7). L-Aspartate was the varied substrate while the αKG concentration was held constant at inhibitor concentrations of 0, 1, or 4 × K_i.

(C) Reactions with L-Glutamate and OAA. Conditions for the reverse reaction were identical with those described above except that the reactions were followed by the decrease in absorbance at 257 nm (ϵ = 1153 M^{-1} cm^{-1}) due to the enol tautomer of OAA (Kokesh, 1976). A lag observed in the time course of the reaction (Henson & Cleland, 1964) was eliminated by including 54 mM *N*-ethylmorpholine.[2] The decarboxylation of OAA, indicated by a slow, enzyme-independent, absorbance decrease, is not accelerated significantly by *N*-ethylmorpholine nor are the kinetics of the reaction of cAATase with L-aspartate and αKG affected. Isotope effect experiments were done at saturating (0.5 or 1.0 mM) OAA and varying L-glutamate concentrations in 1.0-cm cuvettes thermostated at 25 °C.

(D) Reactions of cAATase with L-Alanine and αKG. The reaction of cAATase with L-alanine was followed at 340 nm in a coupled assay with LDH and NADH in 25 mM HEPES (pH 7.5) or TAPS (pH 8.3) buffer, 1.0 mM αKG, 0.14–0.70 M L-alanine, 1.0 unit of LDH, and 0.16 mM NADH, at 25 °C in 1-mL total volume. The reaction rate was shown in a control experiment to be independent of the amount of LDH and NADH present and linearly dependent on the amount of cAATase. The reaction was initiated by adding 2–10 units (0.13–0.72 nmol) of cAATase and followed for 30–40 min.

A very slow change in the absorbance at 340 nm was observed in mixtures of L-alanine, αKG, LDH, and NADH in the absence of cAATase. This blank rate may be due to the slight activity of LDH with αKG as the substrate (Meister, 1950) or possibly to contaminating enzyme activities in the commercial LDH. The blank slope, between 10% and 25% of the cAATase-catalyzed slope at the lowest L-alanine concentrations used, was essentially independent of the L-alanine concentration and was subtracted from the cAATase-dependent slope.

[2] Tertiary amines catalyze the tautomerization of OAA (Bruice & Bruice, 1978), but it is not clear that slow enolization accounts for the lag.

(E) KIE Experiments with L-Alanine. The primary α-deuterium KIE experiments were performed as described above except that 5 mM EDTA was included in the reaction mixtures to guard against the effects of any inhibitor that might have remained in the [α-^2H]-L-alanine (see above).

The values of V/K_{Ala} are virtually identical at pH 8.0 (V/K_{Ala} = 0.0314 ± 0.0001 M^{-1} s^{-1}), 8.3 (0.0354 ± 0.0008 M^{-1} s^{-1}), and 8.6 (0.0333 ± 0.0023 M^{-1} s^{-1}), in 0.025 M TAPS containing 5 mM EDTA, 1 mM αKG, and 0.2–0.8 M L-alanine (data not shown). Subsequent SKIE experiments were done at pH 8.3 (pD 8.7) in 0.025 M TAPS buffer. Stock solutions of buffer, substrates, and LDH/NADH were prepared in H_2O and in D_2O (99 atom % D). Exchangeable protons in the L-alanine and TAPS buffer were removed by successive cycles of dissolution in D_2O followed by removal of the solvent under reduced pressure.

Calculations. Rate data were fit to the equation

$$\frac{v_i}{[E_T]} = \frac{V}{1 + K_A/[A] + K_O/[O]} \quad (1)$$

where v_i is the initial rate, V is $V_{max}/[E_T]$ ($=k_{cat}$), K_A and K_O are the K_m's for amino acid and keto acid, respectively, and [A], [O], and [E_T] are the concentrations of amino acid, keto acid, and enzyme. Plots of v_i vs [Ala] are linear up to [Ala] = 0.8 M, indicating that the K_m for L-alanine (K_{Ala}) is very large for cAATase (Azaryan et al., 1978; Saier & Jenkins, 1967). The rate of the reaction of AATase with L-alanine is thus

$$v_i/[E_T] = V[Ala]/K_{Ala} \quad (2)$$

Competitive inhibition of AATase is described by the equation (Segel, 1975)

$$\frac{v_i}{[E_T]} = \frac{V}{1 + K_A(1 + [I]/K_i)/[A] + K_o(1 + [I]/K_i')/[O]} \quad (3)$$

in which K_i and K_i' are the dissociation constants for the inhibitor bound to the PLP and PMP forms of the enzyme, respectively, [I] is the inhibitor concentration, and the other terms are defined in eq 1. It was assumed in calculating K_i for α-MeAsp that the D isomer does not bind to the enzyme (Fasella et al., 1966). The bell-shaped V/K_{Asp} vs [H^+] curve was fit to

$$V/K_{obs} = (V/K)_{lim}/(1 + [H^+]/K_1 + K_2/[H^+]) \quad (4)$$

where K_1 and K_2 are the acid dissociation constants at low and high pH, respectively, and $(V/K)_{lim}$ is the pH-independent, limiting value of V/K.

KIEs on reactions with [α-^2H]-L-glutamate and [α-^2H]-L-alanine were corrected for incomplete deuteration by the equation (Dahlquist et al., 1969)

$$KIE_{cor} = (KIE_{obs} - 1 + f)/f \quad (5)$$

where KIE_{obs} is the observed isotope effect, f is the extent of deuteration, and KIE_{cor} is the corrected isotope effect. Isotope effects measured with L- or D,L-aspartate were not corrected for the 2–3% residual protium detected by mass spectrometry (Julin et al., 1989).

Error Analysis. Standard errors supplied by the curve-fitting programs are reported in cases where a parameter was determined only once. Weighted-average values (Clifford, 1973) are listed for parameters determined several times. Other errors were calculated by the usual error propagation methods (Rosenberg & Kirsch, 1981) with the exception of

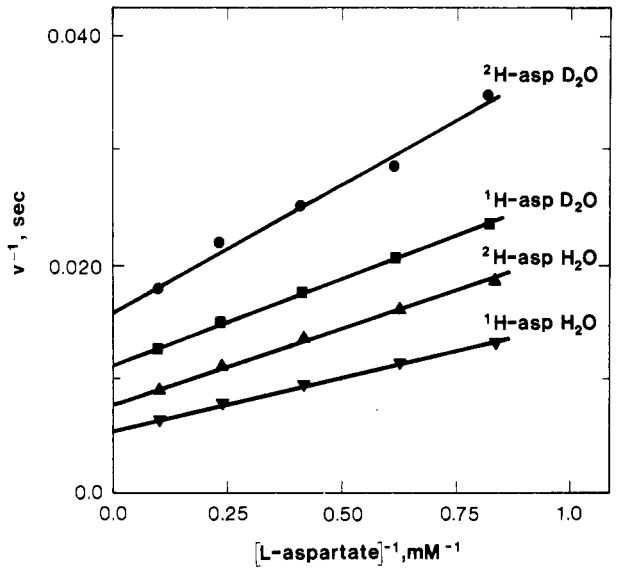


FIGURE 1: Plot of v_i^{-1} vs [L-aspartate]$^{-1}$ for the reaction catalyzed by cytoplasmic aspartate aminotransferase with [α-^{1}H]-L-aspartate in H_2O (▼), [α-^{2}H]-L-aspartate in H_2O (▲), [α-^{1}H]-L-aspartate in D_2O (■), and [α-^{2}H]-L-aspartate in D_2O (●). Each point represents the average of two determinations. The lines are the computer-calculated best fits of the data to eq 1 (see text). The reaction conditions were 0.025 M TAPS, pH 8.3 (pD 8.7), 1.2–10.0 mM L-aspartate, 1.0 mM α-ketoglutarate, 0.15 mM NADH, 2.1 units/mL MDH, and 2.6 nM (H_2O) or 2.6–5.2 nM (D_2O) cAATase, at 25 °C.

those on V/K. Since V and K are highly correlated (correlation coefficients generally were about 0.8–0.9), the error in V/K is calculated from the equation (Clifford, 1973)

$$\frac{\sigma(V/K)}{V/K} = \sqrt{\frac{\sigma^2(V)}{V^2} + \frac{\sigma^2(K)}{K^2} + \frac{2[\mathrm{cov}(V,K)]}{VK}} \quad (6)$$

where $\sigma(i)$ is the standard error in parameter i and cov(V,K) is the covariance of V and K.

Results

Michaelis–Menten Kinetic Parameters. These are k_{cat} = 237 ± 8 s^{-1}, K_{Asp} = 2.47 ± 0.10 mM, and $K_{\alpha KG}$ = 0.032 ± 0.004 mM for cAATase in the forward reaction at pH 8.3. These values are very close to those determined under similar conditions by Kiick and Cook (1983) (k_{cat} = 160 s^{-1}, K_{Asp} = 1.93 mM, and $K_{\alpha KG}$ = 0.04 mM). The figures for the reverse direction are k_{cat} = 623 ± 14 s^{-1}, K_{Glu} = 4.34 ± 0.50 mM, and K_{OAA} = 0.0138 ± 0.0015 mM. Corresponding values for mAATase in the forward direction are k_{cat} = 255 ± 6 s^{-1}, K_{Asp} = 0.523 ± 0.033 mM, and $K_{\alpha KG}$ = 0.572 ± 0.041 mM and in the reverse direction are k_{cat} = 353 ± 9 s^{-1}, K_{Glu} = 10.0 ± 0.6 mM, and K_{OAA} < 4 μM. The value of K_{OAA} is so small as to preclude an accurate determination.

Primary KIE with [α-^{2}H]-L-Aspartate and [α-^{2}H]-L-Glutamate. Sample data for the primary C_α-KIE determined with cAATase and L-aspartate are shown in Figure 1. Table I shows primary KIEs for L-aspartate measured with both isozymes. Small but significant KIEs are found for both V/K_{Asp} and V, which are largely independent of buffer type and concentration. Their magnitudes are similar to those reported previously by Banks et al. (1968) (^DV = 1.37 and $^DV/K_{Asp}$ = 1.69), at pH 7.5 and 25 °C with cAATase. The values of ^DV are about equal to those of $^DV/K_{Asp}$ throughout the pH range. The values of primary KIEs for the reactions of both isozymes with [^{2}H]-L-glutamate at pH 8.3 are collected in Table II. The values of $^DV/K_{Glu}$ are significantly greater than those of ^DV, contrasting wth the equality observed with L-aspartate. The values of ^DV are equal for both the forward and reverse directions for the individual isozymes, but this is a fortuitous agreement that is not required mathematically. The C_α-KIEs are comparable to previously reported values of $^DV/K_{Glu}$ = 2.3 and ^DV = 1.85, at pH 7.38 and 25 °C (Fang et al., 1970), and $^DV/K_{Glu}$ = 1.96 and ^DV = 1.52, at pH 7.5 and 25 °C (Banks et al., 1968), for cAATase and $^DV/K_{Glu}$ = 3.2 with mAATase from chicken heart (Gehring, 1984).

The pH independence of ^DV with cAATase and [α-^{2}H]-L-aspartate (Table I) is not surprising, given the fact that V is itself pH independent [at least as low as 5.5 (Kiick & Cook, 1983), although V may decrease at even lower pH (Velick & Vavra, 1962)]. $^DV/K_{Asp}$ also shows no substantial change with pH, although it may be slightly larger at pH 5.0 than at pH 8.3.

Solvent Isotope Effects. The solvent isotope effects on K_i for maleate and α-MeAsp are 1.35 ± 0.06 and 1.11 ± 0.03,

Table I: Primary α-^{2}H Kinetic Isotope Effects with Cytoplasmic and Mitochondrial Aspartate Aminotransferase and [α-^{2}H]-L-Aspartate at 25 °C in H_2O and D_2O

pH or pD	buffer	^DV (SE)	$^DV/K_{Asp}$ (SE)	no. of detn
	(A) Cytoplasmic AATase			
5.0[a]	acetate, fumarate	1.56 (0.08)	1.63 (0.02)	8
7.5	phosphate[b]	1.38 (0.01)	1.34 (0.07)	3
7.5	lutidine[c]	1.48 (0.01)	1.37 (0.01)	3
7.9 (D_2O)	phosphate	1.41 (0.03)	1.54 (0.06)	1
8.3[d]	TAPS	1.46 (0.02)	1.32 (0.04)	4
		1.44 (0.01)[e]	1.34 (0.03)[e]	4
8.3	HEPES	1.34 (0.04)	1.56 (0.09)	1
8.7 (D_2O)	TAPS	1.43 (0.04)	1.47 (0.10)	1
8.7 (D_2O)	HEPES	1.46 (0.02)	1.66 (0.04)	1
9.0[f]	borate	1.30 (0.08)	1.61 (0.17)	1
10.0[g]	CHES	1.45 (0.03)	1.43 (0.05)	1
	(B) Mitochondrial AATase			
8.3[h]	TAPS	1.80 (0.01)	2.13 (0.05)	3
8.7 (D_2O)		1.51 (0.03)	1.97 (0.06)	1

[a] ^DV was measured once in 25 mM potassium acetate, 50–150 mM L-aspartate, 1.3 mM αKG, and 2–4.5 nM cAATase. $^DV/K_{Asp}$ was measured in 25 or 50 mM potassium acetate or potassium fumarate, 25–150 mM L-aspartate, αKG held constant at 0.08, 0.20, or 1.3 mM, and 2–4.5 nM cAATase. [b] Conditions: 0.4, 4, and 40 mM potassium phosphate, 1.5–10 mM L-aspartate, 1 mM αKG, and 3.6 nM cAATase. Results were essentially identical at each buffer concentration and were averaged to give the results shown. [c] Conditions as in footnote *b*, except the buffer was 0.4, 4, and 40 mM 2,6-lutidine hydrochloride. No dependence of rates on buffer concentration was observed. [d] In 25 mM buffer (TAPS or HEPES), 1.5–10 mM L-aspartate, 1 mM αKG, and 1–2 nM cAATase. [e] Measured with [α-^{1}H]- and [α-^{2}H]-D,L-aspartate. [f] In 25 mM potassium borate, 2–10 mM L-aspartate, 1 mM αKG, and 1–1.6 nM cAATase. [g] Conditions: 25 mM K-CHES, 10–50 mM L-aspartate, 9 mM αKG, and 2.6–5.1 nM cAATase. [h] In 25 mM TAPS, 0.2–4 mM L-aspartate, 5, 7.6, or 10 mM αKG, and 2.2 mM mAATase.

Table II: Primary α-Deuterium Kinetic Isotope Effects in H_2O and in D_2O with Aspartate Aminotransferase Isozymes and [α-^{2}H]-L-Glutamate at 25 °C[a]

isozyme	solvent	^DV (SE)	$^DV/K_{Glu}$ (SE)	no. of detn
cytoplasmic[b]	H_2O	1.46 (0.04)	2.02 (0.09)	3
	D_2O	1.32 (0.02)	1.87 (0.05)	1
mitochondrial[c]	H_2O	1.88 (0.13)	3.80 (0.43)	3
	D_2O	1.57 (0.05)	4.21 (0.19)	1

[a] Reaction conditions were 25 mM TAPS and 54 mM *N*-ethylmorpholine, pH 8.3. Results obtained with 90% deuterated L-glutamate were corrected for incomplete deuteration by using eq 5. [b] Reactions contained 2–20 mM L-glutamate, 1 mM OAA, and 1.5 nM cAATase. [c] Reactions contained 5–50 mM L-glutamate, 0.5 or 1 mM OAA, and 3.5 nM mAATase.

Table III: Deuterium Oxide Solvent Isotope Effects for Aspartate Aminotransferase at 25 °C

pH (pD)	buffer[a]	substrate	^{D_2O}V (SE)	$^{D_2O}V/K$ (SE)
		(A) Cytoplasmic AATase		
8.3 (8.7)	TAPS	[α-^{1}H]-L-Asp	2.19 (0.04)[b]	1.71 (0.04)[b]
	HEPES		2.23 (0.07)	1.71 (0.10)
7.5 (7.9)	phosphate		2.41 (0.13)	1.61 (0.11)
8.3 (8.7)	TAPS	[α-^{2}H]-L-Asp	2.04 (0.06)	1.68 (0.12)
	HEPES		2.42 (0.03)	1.82 (0.04)
7.5 (7.9)	phosphate		2.35 (0.05)	1.88 (0.05)
8.2 (8.8)	TAPS	[α-^{1}H]-L-Glu	2.23 (0.04)	1.31 (0.05)
		[α-^{2}H]-L-Glu	1.97 (0.04)	1.27 (0.05)
		(B) Mitochondrial AATase[c]		
8.3 (8.7)	TAPS	[α-^{1}H]-L-Asp	1.82 (0.01)	1.42 (0.12)
		[α-^{2}H]-L-Asp	1.53 (0.035)	1.39 (0.14)
8.2 (8.8)	TAPS	[α-^{1}H]-L-Glu	1.58 (0.04)	1.25 (0.05)
		[α-^{2}H]-L-Glu	1.46 (0.06)	1.16 (0.05)

[a] Buffer concentrations were 25 mM for the L-aspartate and 40 mM for the L-glutamate experiments. The latter also had 60 mM *N*-ethylmorpholine added (see Materials and Methods). [b] Weighted average of seven determinations. All other SKIEs with the cytoplasmic enzyme were measured once. [c] The SKIEs for the mitochondrial enzyme and L-aspartate are weighted averages of two measurements. The L-glutamate measurements are from single determinations.

respectively, at pH 8.3 (pD 8.7). Maleate forms a noncovalent complex with the PLP-enzyme, while α-MeAsp additionally undergoes transaldimination to form the external aldimine (Fasella et al., 1966). The solvent isotope effect on $K_i^{maleate}$ measures the effect of D_2O on the physical process of formation of the noncovalent EI complex, while the solvent isotope effect on K_i for α-MeAsp includes effects on both complex formation and transaldimination.

Sample data for the determination of SKIEs for the reactions of [α-^{1}H]-L-aspartate and [α-^{2}H]-L-aspartate are given in Figure 1, and the complete results determined with L-aspartate and L-glutamate are collected in Table III. The primary α-hydrogen KIEs measured in the two solvents are listed in Tables I and II. The value of $^{D_2O}K_{Asp} = 1.28 \pm 0.04$ (calculated from $^{D_2O}V/^{D_2O}V/K_{Asp}$) is similar to the values of ^DK_i for α-MeAsp and maleate. The primary KIEs on V for cAATase are independent of solvent (the weighted averages of the values in Table I are $^DV_{H_2O} \cong {}^DV_{D_2O} = 1.44$). $^DV/K_{Asp}$ is slightly larger in D_2O compared to H_2O [the weighted averages are $^DV/K_{Asp}(H_2O) = 1.36$ vs $^DV/K_{Asp}(D_2O) = 1.61$]. The SKIEs are also slightly greater when the substrate L-aspartate contains deuterium rather than protium (Table III) ($^{D_2O}V_{[^1H]Asp} = 2.21$ vs $^{D_2O}V_{[^2H]Asp} = 2.34$ and $^{D_2O}V/K_{[^1H]Asp} = 1.70$ vs $^{D_2O}V/K_{[^2H]Asp} = 1.82$).

Tables I and III show that the mitochondrial isozyme behaves quite differently in these experiments with L-aspartate as substrate. The C_α-KIE on V is reduced when the solvent is D_2O and the SKIE on V is reduced when the L-aspartate substrate contains deuterium rather than protium. The KIEs on V/K_{Asp} for mAATase are reduced to a much lesser extent, if at all, by changing the isotopic content of the substrate or of the solvent. The KIEs on V with either isozyme and L-glutamate are affected in a similar manner by a second isotopic substitution. ^DV with either isozyme is somewhat smaller in D_2O solvent than in H_2O (Table II), as is ^{D_2O}V with [α-^{2}H]-L-glutamate as compared to that with [α-^{1}H]-L-glutamate (Table III).

Kinetic Parameters and KIEs on the Reaction of cAATase with L-Alanine. The observed values of V/K_{Ala} are 0.04 and 0.055 M^{-1} s^{-1} at pH 8.3 and 7.5, respectively. The C_α-KIEs at the same pH values are 1.89 ± 0.10 and 1.94 ± 0.10. The SKIE with [^{1}H]-L-alanine is 1.26 ± 0.07 while that with [^{2}H]-L-alanine is 1.38 ± 0.09, at pH 8.3 (pD 8.7).

Discussion

Reaction of cAATase with L-Aspartate: Is the Quinonoid Form a Requisite Intermediate in Transamination? The measurement of multiple KIEs on an enzyme-catalyzed reaction provides a powerful means for distinguishing stepwise from concerted reaction mechanisms (Hermes et al., 1982; Belasco et al., 1983). Recent applications of the method have supported a concerted reaction mechanism for prephenate dehydrogenase (Hermes et al., 1984) while malic enzyme (Hermes et al., 1982), proline racemase (Belasco et al., 1983; Albery & Knowles, 1986), dihydroorotate oxidase (Pascal & Walsh, 1984), and 6-phosphogluconate dehydrogenase (Rendina et al., 1984) are stepwise mechanisms.

We have applied the double KIE method to the AATase problem by examining the KIE for deuterium at the C_α position of L-aspartic acid (C_α-KIE), the D_2O solvent KIE (SKIE), and the effect of each separate deuterium substitution on the KIE due to the other. Significant KIEs result from each separate substitution, and moreover, the KIEs behave independently—i.e., essentially identical C_α-KIEs on V and V/K_{Asp} are obtained for reaction in H_2O or D_2O, and reciprocally the SKIEs are independent of the isotope present at the C_α position of L-aspartate (Tables I and III).[3] The C_α-KIE probes C_α-proton abstraction from the external aldimine (Julin et al., 1989), while $C_{4'}$ protonation to produce the ketimine as well as ketimine hydrolysis and transaldimination are steps that are affected by D_2O solvent. The independence of the C_α and solvent KIEs indicates that both isotopic substitutions involve protons in a single transition state. The simplest mechanism consistent with independent KIEs is a concerted 1,3 prototropic shift with both C_α-proton abstraction and $C_{4'}$ protonation occurring in this single transition state. The 1,3 prototropic shift appears to be essentially fully rate determining, since the introduction of a second isotope does not change the value of the first KIE, allowing for experimental error (Tables I and III). This implies that the measured KIE for this concerted reaction is an intrinsic one.[4]

[3] The complexity of the AATase reaction mechanism precludes exact calculation of the degree of reduction expected in the KIEs upon a second isotopic substitution if the 1,3 prototropic shift were stepwise. However, a simplified mechanism will suffice to provide an estimate of the expected reduction, i.e.

$$A \underset{a_r}{\overset{a}{\rightleftharpoons}} Q \xrightarrow{b} E + P$$

where A, Q, E, and P are the external aldimine, quinonoid, PMP-enzyme, and keto acid product, respectively. Step a has a C_α-KIE while step b (which includes $C_{4'}$ protonation and ketimine hydrolysis) has the solvent KIE. For the purposes of this calculation, it is assumed that the a_r step has a primary KIE but no solvent KIE (thus ignoring exchange of deuterium between Q and solvent and the polyprotonic nature of the lysine amino group). V_{max} for this mechanism is given by

$$V = ab/(a + a_r + b)$$

and the isotope effects are given by

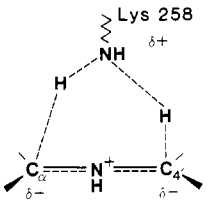
$$^DV_{H_2O} = \frac{a/a_D + [a + a_r(^DK_{eq})]/b}{1 + (a + a_r)/b}$$

and

$$^DV_{D_2O} = \frac{a/a_D + [a + a_r(^DK_{eq})]/b_{D_2O}}{1 + (a + a_r)/b_{D_2O}}$$

where $^DK_{eq}$ is the deuterium equilibrium isotope effect on the A ↔ Q step and a_D and b_{D_2O} represent rate constants for reaction with deuterated substrate or in D_2O solvent, respectively (unsubscripted rate constants are for undeuterated substrate and H_2O solvent). If $a/a_D = 4$ (see text), $b/b_{D_2O} \geq 2$ (see Table III), and $^DK_{eq} \cong 1$, the observed value of $^DV_{H_2O} = 1.4$ should decrease to $^DV_{D_2O} \leq 1.2$. The results in Table I show clearly that such a reduction is not observed.

Chart I



The double-isotope substitution method depends ultimately on the validity of the "rule of the geometric mean" (rgm) (Bigeleisen, 1955). As pointed out by Pascal and Walsh (1984), the rgm has not been found valid in all cases (Limbach et al., 1982). Deviation from the rgm in a reaction such as that studied here would be manifest as nonindependence of the C_α and solvent KIEs even for a concerted transition state. However, the present results are perfectly consistent with the rgm—i.e., the C_α and solvent KIEs are independent. Thus there is some confidence in the applicability of the rgm to this reaction, the results of Limbach et al. (1982) notwithstanding.

Transition-State Structure for the Concerted 1,3 Prototropic Shift. The transition state for the concerted 1,3 prototropic shift thus involves simultaneous C_α-proton transfer to the ϵ-NH_2 group of Lys-258 and of a second proton from the amino group to $C_{4'}$ of the cofactor. The transfers need not be synchronous (Dewar, 1984). It is likely that the abstraction of the C_α-proton has proceeded beyond the halfway point in the transition state because the small C_α-KIE is consistent with an asymmetric transition state (Westheimer, 1961) while the larger SKIE requires that the proton bridging N^ϵ and $C_{4'}$ be more nearly symmetrically placed. This cannot occur without some carbanion character being developed at $C_{4'}$ through azaallylic donation from C_α; i.e., there is some quinonoid character to the transition state, but the quinonoid itself would have a lifetime shorter than that for a molecular vibration, thus failing the criterion for the existence of an intermediate as discussed by Jencks (1980).

The geometry of the concerted transition state is such that both proton transfers cannot occur with linear arrangements of proton donor and acceptors. The mechanism therefore requires either substantial motion of the nitrogen atom or that the partial donor proton–acceptor bonds be bent significantly from linearity. Proton transfers are usually thought to proceed optimally through linear geometry, but there is evidence that reactions constrained to bent transition states can nevertheless proceed at substantial rates (Menger, 1983). Moreover, deuterium KIEs calculated for nonlinear proton transfers are smaller than for those with linear geometry (More O'Ferrall, 1970). C_α-KIEs measured for PLP-dependent enzymatic and nonenzymatic proton abstractions to produce quinonoid intermediates are typically in the range of 4–6 (Jenkins & Harruff, 1979; Ulevitch & Kallen, 1977; Banks et al., 1968; Auld & Bruice, 1967a,b; Maley & Bruice, 1968), and in one case a value of 9 has been reported (Federiuk et al., 1983). The requirement for a bent transition state may explain in part the small magnitude of the intrinsic C_α-KIEs for the cAATase–aspartate reaction. Chart I shows a transition state for the concerted 1,3 prototropic shift consistent with the above.

cAATase-Catalyzed C_α-Hydrogen Exchange from L-*Aspartate May Occur via a Quinonoid Intermediate.* One consequence of a concerted 1,3 prototropic shift mechanism is that the quinonoid form of the enzyme is not a requisite intermediate in this process. The quinonoid structure, which has been observed spectrophotometrically in equilibrium mixtures of cAATase and L-aspartate (Jenkins & Taylor, 1965) and in temperature-jump experiments (Fasella & Hammes, 1967), must therefore be off the primary reaction pathway, and little product is formed from this intermediate, but we cannot rule out the possibility that a small amount of product is formed via a stepwise mechanism. The concerted and stepwise pathways would then exist as parallel routes from the external aldimine to the ketimine, with the concerted one dominating in the cAATase–aspartate reaction. The following arguments support the contention that the primary route for C_α-hydrogen exchange (Julin et al., 1989) in the reaction of *cAATase with* L-*aspartate* is through the quinonoid formed *off* the main reaction pathway. All reaction steps between the ketimine and products must be fast if, as concluded above, the aldimine-to-ketimine conversion is fully rate determining; thus C_α-proton exchange cannot occur by incorporation of solvent-derived protons during a reverse reaction of the ketimine to aldimine. We propose instead that exchange occurs by proton abstraction to form the quinonoid, followed by incorporation of solvent hydrogens upon breakdown of the quinonoid back to the external aldimine. The partition ratio θ for cAATase-catalyzed C_α-deuterium exchange in L-aspartate (Julin et al., 1989) is then the ratio of the net rate at which the external aldimine reacts via the quinonoid to produce unlabeled L-aspartate to the net rate at which it traverses the concerted 1,3 prototropic shift leading to product formation. The value for θ of 2.6 (Julin et al., 1989) means that quinonoid formation is a facile process, at least 2.6 times faster than the 1,3 prototropic shift, but further forward progress of the quinonoid to ketimine is slow and is not a significant factor in product formation. The value of the equilibrium constant relating the external aldimine and quinonoid cannot be determined from the results of these experiments, but the spectroscopic data (Jenkins & Taylor, 1965) suggest that the equilibrium lies well to the external aldimine side. In other words, the fully formed quinonoid produced from L-aspartate is much more readily protonated on C_α than on $C_{4'}$.

*Intramolecular Tritium Transfer from [C_α-^3H]-*L-*Aspartate to Enzyme-Bound PMP.* The observation that a small percentage of tritium present at the C_α position of L-aspartate can be transferred during a single enzyme turnover to the cofactor is difficult to reconcile with a concerted, rate-determining, 1,3 prototropic shift mechanism (Julin et al., 1989). However, the existence of a quinonoid intermediate that forms readily but is not along the primary reaction pathway provides an avenue by which such transfer might occur. The tritium removed from C_α can be retained on Lys-258 [the "parking place" model of Julin et al. (1989)] when the quinonoid reverts to the external aldimine and thus has some probability of being transferred to $C_{4'}$ during a subsequent aldimine-to-ketimine step. The likelihood of this transfer is reduced by the statistical factor and tritium KIE (Julin et al., 1989). For a concerted mechanism, the statistical probability of placing the retained tritium at $C_{4'}$ is 1/2 and the ^3H KIE is ca. 3.8, assuming an intrinsic ^2H KIE of 2.5 (Table III) and the Swain–Schaad relationship (Swain et al., 1958). Thus, the maximum percent transfer is $100(1/2 \times 1/3.8) = 13\%$. The observed transfer

[4] It should be noted that the identity of the C_α-KIEs in H_2O and D_2O solvent might result from the fact that the SKIE could occur at more than one step of the reaction. Thus, if both $C_{4'}$ protonation (i.e., as part of the concerted 1,3 prototropic shift) and ketimine hydrolysis are each partially rate determining, it is conceivable that the *increase* in C_α KIE due to a SKIE on $C_{4'}$ protonation could be balanced by a *decrease* due to a SKIE on ketimine hydrolysis, with no overall change in the C_α-KIE. This balancing requires coincidental relative values of rate constants, which cannot be ruled out. However, the simplest and most general conclusion is that the 1,3 prototropic shift is fully rate determining.

of 0.7% is well below this value, as is expected if the aldimine-to-ketimine step is rate determining, and the external aldimine is thus most likely to break down to PLP-enzyme and unlabeled L-aspartate, with concomitant loss of tritium to the solvent. Access of water to the active site will also contribute to loss of tritium (Julin et al., 1989).

Is L-Aspartate a Sticky Substrate for cAATase? The character of the pH dependence of V/K KIEs provides a method to evaluate the extent to which the rate of an enzyme-catalyzed reaction is controlled by diffusion of the substrate to the enzyme (Cleland, 1977). The absence of any substantial increase in the KIE on V/K_{Asp} at the extreme pH values (Table I) suggests that aspartate is not a sticky substrate. This conclusion agrees with that of Kiick and Cook (1983) based on their pH-dependence studies of the cAATase reaction kinetics and with the interpretation that the observed KIEs are intrinsic. This argues that K_{Asp} is a true equilibrium constant for formation of external aldimine from free amino acid. The solvent isotope effect on K_{Asp}, 1.3 (see Results), is somewhat higher than the $^{D_2O}K_i$ measured for α-methyl-D,L-aspartate (1.1) but comparable with that recorded for maleate (1.3). The conclusion that the 1,3 prototropic shift is fully rate determining requires that $^{D_2O}V = {}^{D_2O}V/K$ when correction is made for the solvent isotope effect on binding (i.e., on K_{Asp}).

Isotope Effects for the mAATase Reaction with L-Glutamate. The much larger values of ^{D}V and $^{D}V/K$ recorded for the reaction of mAATase with [α-^{2}H]-L-glutamate (Table II) than for that of cAATase with [α-^{2}H]-L-aspartate (Table I) are indicative either of significantly different transition-state geometry or of a divergence in mechanism for the sister reactions. The magnitude of $^{D}V/K_{Glu}$ in particular is close to that expected for an intrinsic KIE on a stepwise PLP-dependent C_α-proton abstraction (see above). Since essentially the same chemistry is involved in the transamination of L-aspartate and L-glutamate (Chart I), it seems unlikely that the transition-state structure for a concerted reaction with each substrate would differ to such a degree as would be required by the KIEs. However, a small difference in the placement of the ϵ-NH_2 of Lys-258 relative to the external aldimine in the two isozyme's active sites could alter the timing of the proton transfers sufficiently to bring about a shift in mechanism, resulting in a primarily stepwise mechanism for the reaction of mAATase with L-glutamate. The large value of $^{D}V/K_{Glu}$ coupled with the small $^{D_2O}V/K_{Glu}$ argues that C_α-proton abstraction is very largely rate determining and the remainder of this half-reaction is comparatively fast. The observation that a small percentage of C_α-tritium present in L-glutamate is transferred to $C_{4'}$ of the PLP cofactor in the reaction catalyzed by the chicken heart mitochondrial enzyme, albeit under multiturnover reaction conditions (Gehring, 1984), could thus be accounted for by the stepwise mechanism in this case.

The Two Remaining Combinations—mAATase–L-Aspartate and cAATase–L-Glutamate. The KIEs for these reactions give evidence for still further kinetic variations between the isozymes in their reactions with 4-carbon vs 5-carbon substrates. ^{D}V and $^{D}V/K_{Asp}$ for mAATase are significantly larger than those found with the cytoplasmic enzyme, while the SKIEs are smaller (Tables I and III). The C_α and solvent KIEs will tend to vary inversely in reactions where they result from different chemical steps that are each partially rate determining. Thus, the C_α-KIE is large and the SKIE small when C_α-proton abstraction is the slow step (mAATase plus L-glutamate), while small C_α and large SKIEs are observed (cAATase plus L-glutamate) where the solvent-sensitive steps are the kinetically significant ones. In a concerted transition state, as proposed above for the cAATase plus L-aspartate reaction, the magnitude of the two KIEs depends on the relative extents to which the two protons are transferred in the transition state.

The C_α and solvent KIEs are not independent for the mAATase plus L-aspartate reaction, but instead a second isotopic substitution leads to a reduction in ^{D}V and ^{D_2O}V and smaller reductions in $^{D}V/K_{Asp}$ and $^{D_2O}V/K_{Asp}$ (Tables I and III). Again, multiple steps, sensitive to both C_α and solvent isotope, are partially rate determining. We are unable to draw conclusions regarding the mechanism of the 1,3 prototropic shift since the nonindependence of the C_α and solvent KIEs could result either from a stepwise 1,3 prototropic shift, by the reasoning outlined above, or from a concerted aldimine-to-ketimine step with another D_2O-sensitive step, perhaps ketimine hydrolysis, of additional kinetic significance.

The KIEs for the cAATase and L-glutamate pair are also different from those found with this substrate and the mitochondrial enzyme. Most notable is the somewhat smaller value of $^{D}V/K_{Glu}$ than is seen with mAATase. The data do not allow any conclusion as to the mechanism of the 1,3 prototropic shift.

Mitochondrial and Cytoplasmic Isozymes. Significant differences are evident in the K_m's and KIEs for the forward and reverse reactions catalyzed by the two isozymes. We have interpreted the KIEs as indicating different transamination mechanisms when L-aspartate is the substrate for cAATase than for the mAATase–L-glutamate combination. This kinetic and mechanistic variation is somewhat surprising given the chemical similarity of the reactions and the similar tertiary and active-site structures of the isozymes as discerned by X-ray crystallography (Ford et al., 1980; Arnone et al., 1984; Harutyunyan et al., 1984), despite the relatively low degree (ca. 50%) of primary sequence homology (Christen et al., 1984). Subtle structural differences must exist between the two isozymes and their interactions with 4-carbon vs 5-carbon substrates, perhaps relating to the degree of closing of the small domain upon substrate binding (Kirsch et al., 1984). A comparative summary of the properties of the two isozymes is included in Jansonius and Vincent (1987).

KIEs for the Reaction of cAATase with L-Alanine. KIEs were determined with L-alanine as the substrate with the expectation that chemical steps, rather than binding or conformation changes, would be fully rate determining with this very slowly reacting substrate and that intrinsic KIEs might therefore be observed (Northrop, 1977). $^{D}V/K_{Ala}$ is larger than $^{D}V/K_{Asp}$ while $^{D_2O}V/K_{Ala}$ is smaller than $^{D_2O}V/K_{Asp}$ (see Results). The KIEs with L-alanine as substrate are very similar to those observed for L-glutamate with this isozyme and are significantly less than expected for a fully rate-determing, stepwise, 1,3 prototropic shift. The SKIE is independent of, or slightly increased by, C_α-deuteration, raising the possibility of a concerted reaction mechanism for L-alanine as well as L-aspartate.

Conclusions

The experiments reported here and in the two preceding papers unveil a surprisingly subtle substrate- and isozyme-dependent variation in mechanistic detail for AATase. The extreme limits are illustrated by the examples of cAATase reacting with L-aspartate versus mAATase reacting with L-glutamate. The former is a concerted 1,3 prototropic shift converting external aldimine to ketimine in a single step. This conclusion is supported by the mutual independence of the C_α-H and SKIEs. The quinonoid form of the ES complex is produced in an off-reaction pathway, leads to exchange of

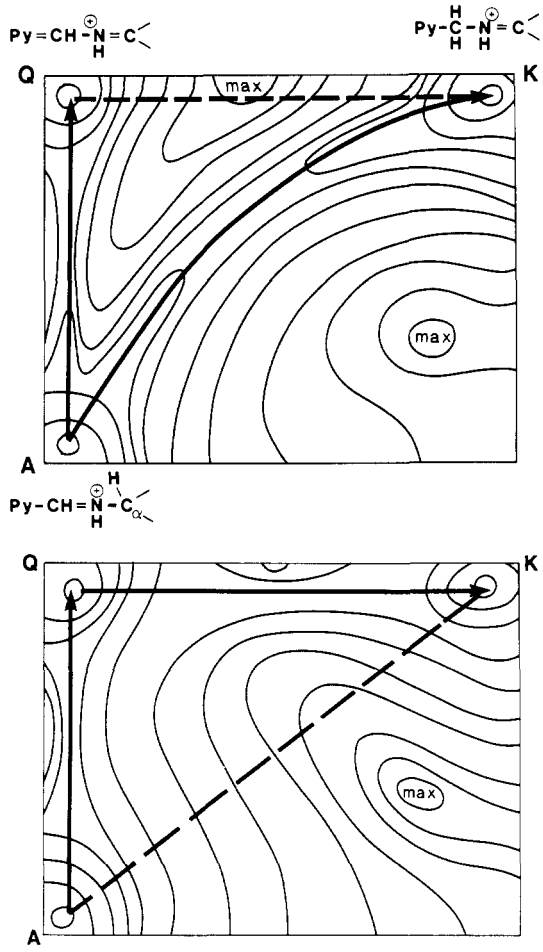


FIGURE 2: Two-dimensional More O'Farrell–Jencks projections of the free energy surfaces representing interconversion of the aldimine (A) to ketimine (K) in the aspartate aminotransferase reaction. The upper figure illustrates the concerted one-step conversion of A to K in the cAATase reaction with L-aspartate. The quinonoid (Q) is formed readily and accounts for the observed exchange of the C_α-hydrogen with solvent, but it is off the reaction pathway to K and can only return to A. The barrier separating Q and K is too great to permit this process to proceed at a kinetically significant rate. The lower figure shows Q as an important, if not obligatory, intermediate in the mAATase reaction with L-glutamate.

C_α-hydrogen with solvent, and may account for the small amount of transfer of tritium from C_α to cofactor. It is not on the main transamination pathway. The KIEs for the mAATase-catalyzed reaction with L-glutamate suggest in contrast that the 1,3 prototropic shift proceeds in a stepwise manner in this case. The large value of $^{D}V/K$ coupled with the small $^{D_2O}V/K$ supports a mechanism where C_α-H abstraction is the rate-determining step for the first irreversible step in this process. This model agrees with the conclusions reached by Gehring (1984) based on the observation that a small amount of tritium is transferred to cofactor from [C_α-^{3}H]-L-glutamate. The different pathways for these two reactions are shown diagrammatically in two-dimensional projection maps in Figure 2. Whether a given transamination reaction follows the upper or lower pathway shown in Figure 2 is a function of relatively small gradations in the free energy surface, which are in turn shaped by the fine variations in the structures of the various isozyme–substrate complexes.

ACKNOWLEDGMENTS

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Registry No. AATase, 9000-97-9; Asp, 56-84-8; Glu, 56-86-0; Ala, 56-41-7; D,L-Asp, 617-45-8; OAA, 328-42-7; α-MeAsp, 2792-66-7; D_2, 7782-39-0; [α-^{2}H]-D,L-aspartic acid, 119820-20-1; [α-^{2}H]-L-alanine, 39748-80-6; maleic acid, 110-16-7.

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Inactivation of Dopamine β-Hydroxylase by β-Ethynyltyramine: Kinetic Characterization and Covalent Modification of an Active Site Peptide

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ABSTRACT: β-Ethynyltyramine has been shown to be a potent, mechanism-based inhibitor of dopamine β-hydroxylase (DBH). This is evidenced by pseudo-first-order, time-dependent inactivation of enzyme, a dependence of inactivation on the presence of ascorbate and oxygen cosubstrates, the ability of tyramine (substrate) and 1-(3,5-difluoro-4-hydroxybenzyl)imidazole-2-thione (competitive multisubstrate inhibitor) to protect against inactivation, and a high affinity of β-ethynyltyramine for enzyme. Inactivation of DBH by β-ethynyltyramine is accompanied by stoichiometric, covalent modification of the enzyme. Analysis of the tryptic map following inactivation by [^{3}H]-β-ethynyltyramine reveals that the radiolabel is associated with a single, 25 amino acid peptide. The sequence of the modified peptide is shown to be Cys-Thr-Gln-Leu-Ala-Leu-Pro-Ala-Ser-Gly-Ile-His-Ile-Phe-Ala-Ser-Gln-Leu-His*-Thr-His-Leu-Thr-Gly-Arg, where His* corresponds to a covalently modified histidine residue. In studies using the separated enantiomers of β-ethynyltyramine, we have found the *R* enantiomer to be a reversible, competitive inhibitor versus tyramine substrate with a K_i of 7.9 ± 0.3 μM. The *S* enantiomer, while also being a competitive inhibitor (K_i = 33.9 ± 1.4 μM), is hydroxylated by DBH to give the expected β-ethynyloctopamine product and also efficiently inactivates the enzyme [k_{inact}(app) = 0.18 ± 0.02 min^{-1}; K_I(app) = 57 ± 8 μM]. The partition ratio for this process is very low and has been estimated to be about 2.5. This establishes an approximate value for k_{cat} of 0.45 min^{-1} and reveals that (*S*)-β-ethynyltyramine undergoes a slow turnover relative to that of tyramine ($k_{cat} \approx 50$ s^{-1}), despite the nearly 100-fold higher affinity of the inactivator for enzyme.

Dopamine β-hydroxylase (DBH;[1] EC 1.14.17.1) is a copper-containing mixed-function oxidase that catalyzes the hydroxylation of dopamine to norepinephrine (Scheme I; Levin et al., 1960; Skotland & Ljones, 1979; Rosenberg & Lovenberg, 1980; Villafranca, 1981; Ljones & Skotland, 1984). Despite its key physiological role in the biosynthesis of catecholamine neurotransmitters and its absolute stereochemical

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[1] Abbreviations: DBH, dopamine β-hydroxylase; Eu[(+)hfc]$_3$, tris-[3-[(heptafluoropropyl)hydroxymethylene]-(+)-camphorato]europium-(III); R_T, retention time; TFA, trifluoroacetic acid.

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