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Aspartate Aminotransferase Catalyzed Oxygen Exchange with Solvent from Oxygen-18-Enriched α-Ketoglutarate: Evidence for Slow Exchange of Enzyme-Bound Water†

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ABSTRACT: Partitioning of the ketimine (or ketimine + quinonoid) intermediate(s) in the mitochondrial aspartate aminotransferase reactions was investigated by following the rates of loss of ^{18}O from carbonyl-^{18}O-enriched α-ketoglutarate together with the rate of L-glutamate formation. The ratio of these rate constants was found to equal 1 at 10 °C, implying that the above intermediate(s) face(s) equal barriers with respect to the forward and reverse reactions. This partition ratio of 1 together with that measured from the α-amino acid side of the reaction [Julin, D. A., Wiesinger, H., Toney, M. D., & Kirsch, J. F. (1989) *Biochemistry* (preceding paper in this issue)] suggests that the rate constant for exchange of α-ketoglutarate-derived $H_2{}^{18}O$ from the ketimine (or ketimine + quinonoid) form(s) of the enzyme with solvent is comparable with that for k_{cat}.

The relative energy barriers facing the first intermediate formed upon labilization of the C_α proton from the α-amino acid in the aspartate aminotransferase (AATase)[1] reaction were measured by monitoring the relative rates of C_α-deuterium exchange with solvent and of α-keto acid formation (Julin et al., 1989). This paper describes a probe of the barriers for the reverse AATase reaction (αKG plus the pyridoxamine 5′-phosphate form of the enzyme) by comparing the rate of exchange of oxygen-18 from carbonyl-^{18}O-enriched

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[1] Abbreviations: TAPS, 3-[[tris(hydroxymethyl)methyl]amino]-propanesulfonic acid; αKG, α-ketoglutarate; 2,4-DNPH, 2,4-dinitrophenylhydrazine; MDH, malic dehydrogenase; mAATase, mitochondrial aspartate aminotransferase.

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αKG with solvent to that of formation of L-glutamate. The rationale for such an experiment is that if only a single intermediate, or a family of rapidly equilibrating intermediates, is formed on a given reaction pathway, then this intermediate should partition independently of the side of the reaction from which it has been formed. The observation of unequal partition ratios requires that more than one kinetically significant intermediate exists along the reaction pathway and/or that the rate constant for exchange of the enzyme-bound isotope with solvent is not fast relative to the values of k_{cat}.

Materials and Methods

Materials. Catalase was purchased from Boehringer-Mannheim. MDH from pig heart cytoplasm, specific activity 440 units/mg, and the disodium salt of NADH were obtained from Sigma. The diazomethane precursor diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) was obtained from Aldrich. Water, 90% enriched with ^{18}O, was obtained from Bio-Rad. [U-^{14}C]-L-Glutamic acid (230 mCi/mmol) was obtained from California Bionuclear Corp. Pig heart mitochondrial mAATase was a gift from J. Foote of this department. Diazomethane was prepared by using a modification of the method of Schlenke and Gellerman (1960) in which ethanol is substituted for carbitol.

Determination of Kinetic Parameters. All spectrophotometric measurements of reaction kinetics were carried out on a Cary 118C spectrophotometer fitted with thermostatically jacketted cell holders connected to a circulating constant temperature bath. Dry nitrogen was blown across the faces of the cuvettes to avoid condensation at the lower temperatures. Glass-distilled water was used in all experiments.

The specific activity of the mAATase was determined to be approximately 230 units/mg of protein (Julin & Kirsch, 1989). The reaction was assayed in the direction of L-glutamate and oxaloacetate production by coupling to the MDH reaction (Velick & Vavra, 1962) and monitoring the decrease in the absorbance of NADH at 340 or 389.7 nm ($\epsilon_{389.7}$ = 454 M^{-1} cm^{-1}). The values of K_m and V_{max} at 10 °C were determined in reaction mixtures containing 25 mM TAPS buffer, 0.2 mM NADH, and 2.86 units/mL MDH (186 nM). The concentrations of the substrates, L-aspartate and αKG, were varied from 0.5 to 5.0 × K_m with the pH adjusted to 8.3 with NaOH. The mAATase concentration was 27.5 nM, and the assays were carried out at 340 nm.

Oxygen-Exchange Reactions. The preparation of [*carbonyl*-^{18}O]αKG was carried out by the method of Viswanathan et al. (1982). The extent of enrichment was measured by rapidly dissolving a 1-mg sample of the enriched acid in 0.5 mL of 30% H_2O_2 in 25 mM, pH 7.6 imidazole buffer. The resulting succinic acid was esterified with diazomethane and analyzed for ^{18}O content by mass spectrometry.

The ^{18}O content was quantitated by measurement of the relative ion intensities of the peaks at *m/e* 115 and 117 in the mass spectrum of dimethyl succinate. The peak at *m/e* 115 represents the $^{+}COCH_2CH_2CO_2CH_3$ fragment from the dimethyl ester (Viswanathan et al., 1982). This fragment has a 75% probability of retaining the ^{18}O from the parent compound, neglecting an isotope effect on fragmentation. Quantitative analysis showed that 67.3% of the carbonyl groups of αKG contained ^{18}O and that enrichment in the carboxyl oxygens was insignificant.

The exchange reactions were carried out in the following manner: A 20-mL solution containing 25 mM TAPS buffer, 2.5 mM NADH, 10 mM L-aspartate, and 5–25 units/mL MDH was temperature equilibrated at 10 °C. Sufficient solid αKG, enriched with ^{18}O in the carbonyl group, was added with rapid stirring to produce a 2.5 mM solution and the pH adjusted to 8.3 with NaOH. mAATase (0–100 μL, containing 0–45 units) was then added, with stirring. At appropriate time intervals 1.0-mL aliquots of the reaction mixture were withdrawn and quenched into 0.5 mL of a mixture containing hydrogen peroxide (38%, w/w) in 25 mM, pH 7.6 imidazole buffer. The samples were mixed thoroughly with a vortex stirrer and cooled in an ice bath. The subsequent lyophilization, esterification, and analysis were carried out as described by Viswanathan et al. (1982), the only modification being that the samples were taken to pH 3.7 ± 0.2 prior to lyophilization. Mass spectrometric measurements were carried out with a Du Pont Model 21-491 mass spectrometer coupled to a Varian Series 1400 gas chromatography. A Supelco SP-2340 column (6 ft × $^1/_8$ in.) was employed for separation. The column was operated isothermally at 175 °C with helium as the carrier gas. Mass spectrometric data were acquired at an ionizing voltage of 70 eV and the relative ion intensities measured with a Columbia Scientific Industries Model 260/720 data system and digital printer. An automatic, repetitive scan attachment enabled the peak intensities to be measured at 1–2-s intervals.

Radiolabeling Experiments. Ten solutions, each 20 mL in volume and containing 2.5 mM αKG, 10 mM L-aspartate, 2.5 mM NADH, and 9.15 units/mL MDH in 25 mM, pH 8.3 TAPS buffer, were incubated with [U-^{14}C]-L-glutamic acid (ca. 200 000 cpm) at 10 °C. After thermal equilibrium had been attained, two of these solutions were quenched with 20 mL of a solution containing 0.3% 2,4-DNPH in 4 M HCl. The reaction mixtures were allowed to stand for 75 min at 5 °C after which time the 2,4-DNPH derivative of αKG had precipitated (Dancis et al., 1963). This was collected by vacuum filtration, washed with cold 2 M HCl (2 × 15 mL), and air-dried. Samples of the filtrate containing the [^{14}C]-L-glutamic acid (50–500 μL) were added to 9.5 mL of Liquiscint (National Diagnostics), the volume was adjusted to 10 mL with 2 M HCl, and the samples were counted. The solid 2,4-DNPH derivative was taken up in sufficient ethyl acetate to produce a solution of approximately 3 mg/mL. Aliquots of this solution (50–500 μL) were added to 4.5 mL of Liquiscint, and the samples were counted after the volume was adjusted to 5.0 mL with ethyl acetate. mAATase was added, in turn, to each of the remaining eight solutions and the reaction allowed to proceed for time periods ranging from 30 to 360 s (2.5–30% reaction). The reactions were quenched with the acidic 2,4-DNPH solution, and the 2,4-DNPH derivative of αKG was processed as described above. No enrichment in radioactivity over that obtained in the minus enzyme control was observed in the isolated 2,4-dinitrophenylhydrazone of αKG when [^{14}C]-L-glutamate was included in the incubation mixture. This result obtains at least to 30% reaction proving that the MDH trapping of oxaloacetate is quantitative; therefore, no significant amount of the enzyme-catalyzed carbonyl-^{18}O depletion in αKG is due to the de novo formation of αKG from the back-reaction of L-glutamate with oxaloacetate. Control experiments showed that no precipitation occurred if αKG was omitted from the reaction mixture. The derivative showed only a single spot on thin-layer chromatography (EtOH/silica), and a sample recrystallized from aqueous ethanol had mp 211–212 °C (lit. 213 °C) (*Dictionary of Organic Compounds*, 1982).

Data Analysis. Enzyme kinetic parameters were calculated by fitting initial velocity and substrate concentration data to the equation (Velick & Vavra, 1962)

$$\frac{v_i}{[E_T]} = \frac{V}{1 + K_{Asp}/[Asp] + K_{\alpha KG}/[\alpha KG]} \quad (1)$$

Table I: Rates of Nonenzymatic and Mitochondrial Aspartate Aminotransferase Catalyzed Carbonyl Oxygen Exchange in α-Ketoglutarate[a]

mAATase (nM)	no. of detn	$10^4 k'$ [s^{-1} (SE)]	k^e_{cat} [s^{-1} (SE)]	k^e_{cat}/k^p_{cat} (SE)
0	2	7.90 (0.36)		
0[b]	3	8.10 (0.62)		
44[b,c]	2		53.3 (2.4)	0.99 (0.05)
44[b,d]	1		47.0 (5.6)	0.94 (0.11)
88[b,d]	2		40.7 (3.5)	0.81 (0.07)

[a] Conditions: 25 mM TAPS, 2.5 mM α-ketoglutarate, pH 8.30 (±0.05), 10 °C. k' is the first-order rate constant for nonenzymatic ^{18}O exchange, and k^e_{cat} and k^p_{cat} are the value of $V_{max}/[E_T]$ for the exchange and product formation steps, respectively. [b] 10 mM L-aspartate, 2.5 mM NADH. [c] MDH, 9.15 units/mL; $k^p_{cat} = 53.52 \pm 0.49$ s^{-1}. [d] MDH, 18.3 units/mL; $k^p_{cat} = 50.25 \pm 0.36$ s^{-1}.

where $V = V_{max}/[E_T]$ ($=k^p_{cat}$), K_{Asp} and $K_{\alpha KG}$ are the K_m's for L-aspartate and αKG, respectively, and [Asp], [αKG], and $[E_T]$ are the concentrations of amino acid, keto acid, and enzyme.

The nonenzymatic exchange rates were determined by fitting the experimental data to the equation

$$(X^* - X^*_\infty)/(X^*_0 - X^*_\infty) = e^{-k't} \quad (2)$$

where k' is the first-order rate constant and X^*, X^*_0, and X^*_∞ are the fractions of ^{18}O substrate at $t = t$, 0, and ∞, respectively.

The data for the enzymatic ^{18}O exchange reactions were fit to eq 3 by nonlinear regression with k^e_{cat} and X^*_0 as adjustable parameters

$$\ln (1/X^*) = \frac{k^e_{cat}}{k^p_{cat}}\left[\ln\left(\frac{[S_{tot}]}{[S^\circ_{tot}] - k^p_{cat}[E_T]t}\right)\right] + k't - \ln X^*_0 \quad (3)$$

where k^e_{cat} and k^p_{cat} are the values of $V_{max}/[E_T]$ for the exchange and product formation steps, respectively. The derivation of this equation is given in the Appendix.

Treatment of the radiolabel exchange data required the use of a quenching curve to compensate for the color of the yellow 2,4-DNPH derivatives (Ross & Yerick, 1963). A correction factor [100/(100 − % $quenching_{obs}$)] was used in the calculation of the extent of label transfer.

Results

The kinetic constants for the mAATase-catalyzed reaction of L-aspartate and αKG in 25 mM, pH 8.3 TAPS buffer at 10 °C were determined to be 51 ± 3 s^{-1}, 0.35 ± 0.05 mM, and 0.32 ± 0.05 mM for k^p_{cat}, K_{Asp}, and $K_{\alpha KG}$, respectively. The corresponding constants at 25 °C are 255 s^{-1}, 0.52 mM, and 0.57 mM (Julin & Kirsch, 1989). The results at 10 °C were obtained at a ratio of 10 units of MDH per unit of mAATase.

Table I reports the rate constants for the mAATase-catalyzed and nonenzymatic carbonyl-^{18}O exchange in αKG. Viswanathan et al. (1982) report a rate constant for nonenzymatic exchange with αKG of 0.0028 s^{-1} at 25 °C in 0.1 M imidazole buffer, pH 7.5. Results obtained in the absence of mAATase indicate that Schiff base formation between L-aspartate and αKG is negligible; i.e., the addition of L-aspartate to the reaction mixture has no effect (row 2 vs row 1) on the nonenzymatic rate of ^{18}O exchange. The addition of mAATase, on the other hand, results in a substantial increase in the rate of ^{18}O exchange. First-order plots (Figure 1) indicate that the ratio of enzymatic to nonenzymatic rates varies between 1.5 and 3 depending on the mAATase concentration used. As expected, the ratio increases at higher mAATase concentrations. The ratio of ^{18}O exchange to product formation (k^e_{cat}/k^p_{cat}) is approximately 1 (Table I).

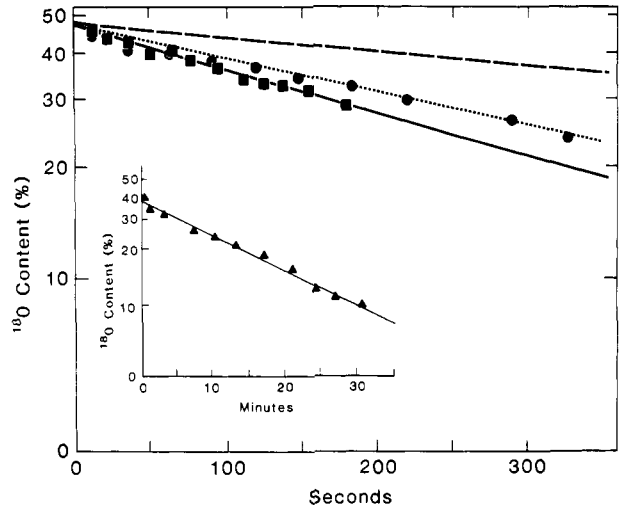
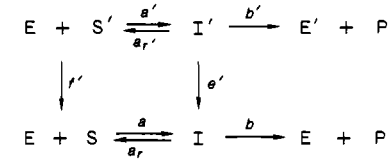


FIGURE 1: Time dependence of carbonyl-^{18}O exchange in α-ketoglutarate. Conditions as in Table I. (Insert) First-order nonenzymatic exchange. (---) Nonenzymatic-exchange rate calculated with $k' = 8.01 \times 10^{-4}$ s^{-1}; (●) mAATase, 44 nM; (■) mAATase, 88 nM. The enzymatic rate data are only approximately first order (see text).

Scheme I



Discussion

Partitioning of Intermediates Formed from the α-Keto Acid Direction. Albery and Knowles (1976) showed how information describing the partitioning of enzyme-bound intermediates, as measured by simultaneous monitoring of isotope exchange in substrate and of product formation from both directions of the reaction, can be used to define the quantitative barrier heights in the reaction coordinate profile. The specific examples explored by these workers involved hydrogen isotope exchange reactions catalyzed by triosephosphate isomerase.

The reaction of an α-keto acid substrate with enzyme-bound pyridoxamine 5′-phosphate to produce the ketimine releases the carbonyl oxygen atom which can exchange with water (Scheme I). The primes indicate steps or entities with ^{18}O present as an enzyme bound species or in the keto acid. S and S′ are the carbonyl forms of the keto acid, P is the amino acid, and I and I′ represent the ketimine (or perhaps a kinetically weighted average of more than one intermediate). The net rate constants for the steps indicated are noted by a, a_r, and b. The rate constant for loss of ^{18}O from I′ to solvent is e', and the reaction with rate constant a can be neglected under initial velocity conditions. The rate constant for the nonenzymatic exchange of carbonyl ^{18}O with water is denoted by f'. Since ^{18}O kinetic isotope effects are small, we may take $b = b'$, $a_r = a_r'$, and $a = a'$.

Analysis of the enzymatic component of Scheme I by the King–Altman method gives (see Materials and Methods for definitions)

$$k^p_{cat} = b$$
$$k^e_{cat} = a_r e'/(a_r + b + e') \quad (4)$$
$$K_m = (a_r + b)/a$$

The partition ratio defining the ratio of the rate constants for loss of label to those for product formation from I is given as

$$\theta = a_r/b \tag{5}$$

The value of $\theta_{app} = (dS/dt)/(dP/dt)$ is defined by

$$\theta_{app} = \frac{a_r e'}{b(a_r + b + e')} = \frac{k^e_{cat}}{k^p_{cat}} \tag{6}$$

with

$$\theta_{app}/\theta = e'/(a_r + b + e') \tag{7}$$

The isotope-exchange experiments give $\theta = a_r/b$ (Scheme I) as measured from left to right, while $\theta = b/a_r$ for an exchange reaction measured in the reverse reaction. This relationship, $\theta_{forward} = 1/\theta_{reverse}$, is probed by C_α-hydrogen exchange from the amino acid side (Julin et al., 1989) and by C_α-oxygen exchange from the keto acid side of the reaction. The value of $\theta_{app} \cong 0.55$ was determined with mAATase and L-glutamate (Julin et al., 1989), while that determined from the αKG side of the reaction is approximately 1 (Table I), which is beyond the experimental error of the expected value of 1/0.55. The latter experiments had to be conducted at 10 °C because of the rapid nonenzymatic exchange rate at low concentrations of enzyme. In order for the temperature to account for the difference in ratios, the value of E_a for k^e_{cat} would have to equal 8 kcal/mol given that the E_a for k_{cat} is 18 kcal/mol (calculated from the values given in Table I) and $k^p_{cat}(25\ °C) = 255\ s^{-1}$ (Julin & Kirsch, 1989). Alternative possibilities accounting for the observation and $\theta_{forward} \neq 1/\theta_{reverse}$ are (a) that both the quinonoid and ketimine exist as intermediates capable of exchanging protons and/or oxygen with solvent and (b) that $\theta_{app} \neq \theta$ for one or both of the monitored exchange reactions; i.e., $a_r + b$ is comparable with or greater that e' (cf. eq 7).[2]

Although the phenomenological observation that $\theta_{forward} \neq 1/\theta_{reverse}$ might be taken to indicate the existence of two or more slowly equilibrating intermediates along a reaction pathway [cf. (a) above], this explanation cannot account for the present data. The fact that $\theta_{forward} \cong 0.55$ (Julin et al., 1989) indicates that C_α-H bond cleavage is the major committed step in the glutamate to αKG direction; i.e., *no* subsequent free energy barrier can be as high; yet the $\theta_{reverse} = 1$ measured from the αKG direction requires that there be *two* free energy barriers of equal height on the reaction pathway. This contradiction, therefore, excludes explanation a as accounting for the observation that $\theta_{forward} \neq 1/\theta_{reverse}$ in this case. We are left with the possibility that $\theta_{app} \neq \theta$ for one or both of the exchange reactions. It was shown in the previous paper (Julin et al., 1989) that θ_{app}/θ for C_α-H exchange must lie within the range 0.67–1, because the presence of lysine ϵ-amino and water protons within the active site approaches the observational equivalent of exchange with bulk solvent. No such obvious dilution effect obtains for the nascent oxygen-18 released from α-keto acid upon ketimine formation. Hydrolysis of the ketimine by enzyme-bound $H_2{}^{18}O$ occurring with a rate constant comparable to that for exchange of the isotope with bulk solvent, and/or that for the azaallylic rearrangement to aldimine, would result in an observed free energy barrier for oxygen exchange that is higher than the true one because of the "reuse" of bound $H_2{}^{18}O$. Hackney (1980) has previously considered the effect of slow exchange of $H_2{}^{18}O$ with solvent in connection with the pyrophosphatase reaction.

[2] The meaning of the value of θ_{app} in terms of rate constants is changed significantly where exchange of label can occur additionally in a pathway that is off that leading to product formation. It is argued, for example in Julin et al. (1989) and Julin and Kirsch (1989), that the C_α-H exchange process in the cAATase-catalyzed reaction with L-aspartate occurs through such a mechanism. In this case θ_{app} is a measure of the ratio of the rates for product formation and of the net rate for exchange from the aldimine. The existence of an orthogonal exchange pathway for mAATase and L-glutamate would only enhance the apparent barrier height discrepancy by raising the θ_{app} compared to θ for C_α-H exchange in L-glutamate.

ACKNOWLEDGMENTS

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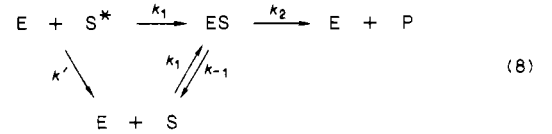
APPENDIX

Derivation of Eq 3. The minimal mechanism whereby an isotopically enriched substrate may undergo simultaneous enzymatic- and nonenzymatic-catalyzed isotope exchange with solvent, concurrent with enzyme-catalyzed product formation, is



(8)

where k' is the first-order nonenzymatic-exchange rate constant, k_2 is the rate constant for product formation, k^p_{cat}, and k_{-1} is the rate constant for the enzyme-catalyzed isotope exchange, k^e_{cat}.

Assuming no isotope effect on k^e_{cat}, which is certainly true within the present experimental precision for ^{16}O vs ^{18}O, steady-state approximations give eq 9 and 10 for the rates of loss of labeled [S*] and unlabeled substrate [S]

$$-\frac{d[S^*]}{dt} = \frac{(k_2 + k_{-1})[E_T][S^*]}{K_m + [S_{tot}]} + k'[S^*] \tag{9}$$

$$-\frac{d[S]}{dt} = \frac{k_2[E_T][S] - k_{-1}[E_T][S^*]}{K_m + [S_{tot}]} - k'[S^*] \tag{10}$$

where $[S_{tot}] = [S^*] + [S]$.

By defining X^* as the fraction of enriched material, i.e., $X^* = [S^*]/[S_{tot}]$, we may write

$$\frac{dX^*}{dt} = \frac{\partial X^*}{\partial [S^*]}\left(\frac{d[S^*]}{dt}\right) + \frac{\partial X^*}{\partial [S]}\left(\frac{d[S]}{dt}\right) \tag{11}$$

Evaluation of the partial derivatives gives

$$\partial X^*/\partial [S^*] = [S]/([S] + [S^*])^2 \tag{12}$$

$$\partial X^*/\partial [S] = -[S^*]/([S] + [S^*])^2 \tag{13}$$

Substitution of eq 9, 10, 12, and 13 into eq 11 gives

$$-\frac{dX^*}{dt} = \frac{k^e_{cat}[E_T]X^*}{K_m + [S_{tot}]} + k'X^* \tag{14}$$

The decrease in the enriched fraction will occur by a first-order process provided $K_m + [S_{tot}]$ is constant over the course of the reaction, i.e., if $K_m \gg [S_{tot}]$. The observed rate constant for the exchange is then given by

$$k_{obs} = k^e_{cat}[E_T]/K_m + k' \tag{15}$$

and the enzymatic term $k^e_{cat}[E_T]/K_m$ may be obtained by subtraction of the independently determined value of k'.

If, however, $[S_{tot}] \gg K_m$, then the substrate concentration will decrease with zero-order kinetics:

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

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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