Estimation of Free Energy Barriers in the Cytoplasmic and Mitochondrial Aspartate Aminotransferase Reactions Probed by Hydrogen-Exchange Kinetics of C_α-Labeled Amino Acids with Solvent†

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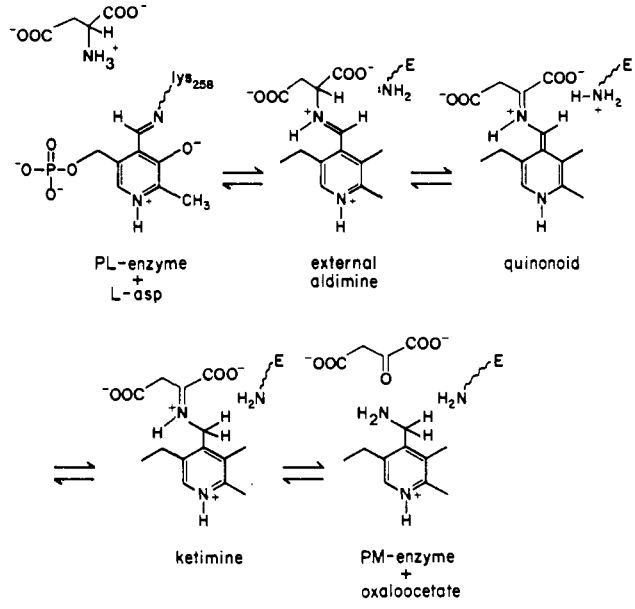
ABSTRACT: The existence of the postulated quinonoid intermediate in the cytoplasmic aspartate aminotransferase catalyzed transamination of aspartate to oxaloacetate was probed by determining the extent of transfer of tritium from the C_α position of tritiated L-aspartate to pyridoxamine 5′-phosphate in *single turnover* experiments in which washout from the back-reaction was obviated by product trapping. The maximum amount of transferred tritium observed was 0.7%, consistent either with a mechanism in which a fraction of the net transamination reaction proceeds through a quinonoid intermediate or with a mechanism in which this intermediate is formed off the main reaction pathway. It is shown that transfer of labeled hydrogen from the amino acid to cofactor cannot be used to differentiate a stepwise from a concerted transamination mechanism. The amount of tritium transferred is a function of the rate constant for torsional equilibration about the ϵ-amino group of Lys-258, the presumptive abstractor of the C_α proton; the relative rate constants for hydrogen exchange with solvent versus cofactor protonation; and the tritium isotope effect on this ratio. The free energy barriers facing the covalent intermediate between aldimine and keto acid product (i.e., ketimine and possibly quinonoid) were evaluated relatively by comparing the rates of C_α-hydrogen exchange in starting amino acid with the rates of keto acid formation. The value of θ ($=k_{exge}/k_{prod}$) was found to be 2.6 for the reaction of cytoplasmic isozyme with aspartate and ca. 0.5 for that of the mitochondrial form with glutamate.

Aspartate aminotransferase (AATase)[1] (EC 2.6.1.1) catalyzes the transamination of L-aspartate and L-glutamate to their corresponding α-keto acids OAA and αKG, respectively. This enzyme is the only PLP-dependent enzyme of amino acid metabolism to have its structure determined to atomic resolution by X-ray crystallography. The structures of the chicken heart mitochondrial (Ford et al., 1980), chicken heart cytosolic (Harutyunyan et al., 1984) and pig heart cytosolic (Arnone et al., 1984) subforms have all been described. The general features of the reaction mechanism have been elucidated from the results of inter alia steady-state (Velick & Vavra, 1962; Kiick & Cook, 1983) and rapid reaction kinetics (Fasella & Hammes, 1967; Hammes & Haslam, 1968, 1969), studies of interactions with inhibitors (Jenkins & D'Ari, 1966; Michuda & Martinez-Carrion, 1970), NMR (Mattingly et al., 1982; Scott et al., 1984; Morino, 1984), and UV–visible spectrophotometry studies (Johnson & Metzler, 1970; Bonsib et al., 1975). An earlier mechanistic model for this enzyme (Ivanov & Karpeisky, 1969) has been followed by an analysis based additionally on more recent information including the crystal structure (Kirsch et al., 1984; Arnone et al., 1985; Jansonius & Vincent, 1987).

The principal intermediates along the transamination pathway—external aldimine, quinonoid, and ketimine—are shown in Scheme I. The important 1,3 prototropic shift is believed to be catalyzed by Lys-258 as indicated (Kirsch et al., 1984). While the external aldimine and ketimine must exist as covalent entities formed from reaction of amino acid with PLP enzyme or keto acid with PMP enzyme, respectively, the kinetic competence of the quinonoid has yet to be established for the natural substrates L-aspartate and L-glutamate. The quinonoid is, however, definitely on the reaction pathway for the transamination of *erythro*-β-hydroxyaspartate (Jenkins & Harruff, 1979). The visible absorbance spectrum of PLP

Scheme I



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[1] Abbreviations: c- and mAATase, cytoplasmic and mitochondrial isozymes, respectively, of aspartate aminotransferase; MDH, malate dehydrogenase; ADH, alcohol dehydrogenase; αKG, α-ketoglutarate; KIE, kinetic isotope effect; OAA, oxaloacetate; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; PM, pyridoxamine; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid.

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enzyme incubated with L-aspartate does show a minor absorption at ca. 495 nm, consistent with a quinonoid structure (Jenkins & Taylor, 1965), but this observation does not establish whether the quinonoid is on the reaction pathway. Similarly, the temperature-jump reaction data, which monitored a change in absorbance at 490 nm, were not analyzed in terms of a model with the quinonoid off the reaction pathway (Fasella & Hammes, 1967).

The present series of papers reports the results of experiments designed to investigate the kinetic competence of the quinonoid, to determine the quantitative barrier heights facing each intermediate, and to analyze some of the transition-state structures. The first paper is concerned with the extent of tritium transfer from [C_α-^{3}H]-L-aspartate to cofactor in a single turnover experiment and with the partitioning between forward and reverse reactions of intermediates formed after C_α-H abstraction, thus defining the relative barrier heights facing these intermediates.

The second paper (McLeish et al., 1989) evaluates the partition ratios for the ketimine formed from αKG by simultaneously monitoring the rates of L-glutamate formation and ^{18}O depletion in carbonyl-^{18}O-enriched αKG. The third paper (Julin & Kirsch, 1989) is concerned with the magnitudes of C_α and solvent hydrogen kinetic isotope effects and addresses the question of whether the 1,3 prototropic shift is a concerted or stepwise process with the corollary of the existence of the quinonoid as an intermediate along the reaction pathway.

Materials and Methods

Materials

MDH from pig heart cytoplasm, ADH from yeast, and the disodium salt of NADH were purchased from Sigma. Porcine heart cAATase used in the ^{3}H transfer reactions was purchased from Calbiochem. Calf intestinal alkaline phosphatase (molecular biology grade, 19 units/μL) was from Boehringer-Mannheim. The mitochondrial isozyme of pig heart AATase was a gift of J. Foote of this department. Deuterium oxide (99.8 atom % D) was from Bio-Rad and was glass-distilled before use. [2,3-^{3}H]-L-Aspartic acid (25 Ci/mmol) and NaB^3H_4 (1.4 Ci/mmol) were obtained from ICN. To determine the specific activity at C_α of [2,3-^{3}H]-L-aspartic acid, a ^{3}H washout experiment was performed according to the procedure of Gehring (1984), which gave a value of 50% of the ^{3}H at this position.

Deuterated L-aspartic and L-glutamic acids were prepared essentially as described by Dougherty et al. (1972), except that the corresponding keto acid (OAA for aspartate and αKG for glutamate) was added at a concentration of 1–2 mM. The product was purified by ion-exchange chromatography on Dowex 50 (H^+ form) and recrystallized from ethanol/water. Mass spectrometric analysis of the diethyl esters indicated ca. 2% residual C_α-hydrogen in aspartate and 12% deuteration at the C_β position, while the glutamic acid contained 9% residual C_α-hydrogen and 5–6% deuteration at C_β and/or C_γ (see below). A second preparation of deuterated glutamate, which was incubated with the enzyme for 2 days, contained about 1.5% undeuterated and 22% dideuterated glutamate.

Methods

All spectrophotometric measurements were made on a Cary 118C spectrophotometer with a thermostated cell holder connected to a circulating constant temperature bath (Lauda K-2/R). Mass spectra were obtained on a Du Pont Model 21-491 mass spectrometer equipped with a Columbia Scientific Model 260/722 digitizing data system and printer (Rosenberg & Kirsch, 1979).

AATase Assay. AATase activity was measured in a standard assay mixture containing 100 mM L-aspartate, 6 mM αKG, 0.15 mM NADH, and 2 units of MDH in 2.0 mL of 0.05 M potassium phosphate buffer, pH 7.5, at 25 °C. Reactions were initiated by adding 0.01–0.05 mL of enzyme and were followed at 340 nm.

cAATase Purification. The α-subform of cAATase was purified from pig heart muscle by using minor modifications of the procedures described by Jenkins et al. (1959), Martinez-Carrion et al. (1965), and Martinez-Carrion et al. (1967) (see paragraph at end of paper regarding supplementary material). The product had a specific activity of 160–185 units/mg. Electrophoresis on SDS–polyacrylamide gels showed one major band of MW 45 000–50 000, with several contaminating minor bands.

Tritium Transfer from [C_α-^{3}H]-L-Aspartate to Enzyme. Reactions were initiated by the addition of approximately 1 nmol of [2,3-^{3}H]-L-aspartic acid (3.25×10^7 cpm at C_α). After 100 s at room temperature, 70 μL of 25% perchloric acid was added to quench the reactions. They were then mixed vigorously for 30 s and allowed to stand at room temperature for 5 min. The suspensions were next brought to pH 8 with 2 N KOH, followed by 5-min incubation on ice and centrifugation to pellet the precipitated protein and potassium perchlorate. The supernatants were removed and assayed for PMP as described below.

Effectiveness of the Coupling System. The effectiveness of the MDH/ADH trap for nascent OAA was tested by the following experiment: To 238 μL of a mixture containing OAA (final concentration = 1 μM), [^{3}H]-L-aspartate (0.8 μM), ethanol (1 mM), and ADH (11 μM) was added 12 μL of a mixture containing PMP–cAATase (2.4 μM), MDH (11.4 μM), and NADH (200 μM) in 25 mM potassium phosphate, pH 7.2. This reaction was quenched after 1 min at room temperature by adding 25 μL of 100% trichloroacetic acid. In a control experiment, PMP–cAATase was included in the first mixture and MDH and NADH were added after 1 min at room temperature, followed by trichloroacetic acid. The precipitated protein was separated by centrifugation, and the supernatant was extracted with ether and analyzed by HPLC on a Partisil SAX anion-exchange column (Whatman) using a flow rate of 0.7 mL/min with 100 mM ammonium phosphate, pH 3.4, as eluent. Radioactivity in the column effluent was measured with a Flo-one/Beta continuous flow scintillation counter (Radiomatic Instruments). No radioactivity was detected in the malate peaks (<0.5% of the ^{3}H) in the first experiment, while 23% of the ^{3}H was in the malate peak in the control experiment. The coupling system therefore is fully effective in trapping the nascent OAA formed in the cAATase reactions.

Assay for PMP. PMP concentrations in transfer reaction supernatants were determined by the restoration of transaminase activity to apo-cAATase. Typically, 5 μL of a transfer reaction supernatant containing 0–25 pmol of PMP was added to 10 μL of 10 mM Tris·HCl, pH 8.0, containing 35 pmol of apo-cAATase [prepared by the method of Arrio-Dupont and Verge (1982)]. This mixture was assayed for activity by the coupled MDH reaction after a 5-min reconstitution period at room temperature.

Conversion of PMP to PM and PM Isolation. After the concentration of PMP in the reaction supernatants had been determined (ca. 3.5 μM) and the exact volumes of the reaction supernatants measured (ca. 350 μL) with a calibrated Gilson Pipetman, 1.14 μmol of unlabeled carrier PMP followed by 20 units of alkaline phosphatase was added to each reaction

supernatant. The 5'-phosphate ester was hydrolyzed quantitatively after 30 min at 37 °C. The reaction mixtures were diluted 5-fold with water and applied to 7 × 25 mm columns of water-washed AG50W resin (Na^+ form), which were subsequently washed with 125 mL of 5 mM potassium phosphate, pH 6.9, in 2% methanol. PM was eluted with 2 mL of 30 mM ammonium carbonate, pH 8.5, in 25% methanol. The eluates were lyophilized and resuspended in 1 mL of water and then applied to 7 × 25 mm columns of DEAE-Sephadex equilibrated with water. PM was eluted with 1 mL of water and quantitated by absorbance at 325 nm. A portion of the DEAE effluent was counted by using 20 volumes of scintillant (Liquiscint, National Diagnostics). Typical recoveries of PM (based on the 1.14 μmol of carrier PMP added) were 50%. In one experiment the isolated PM was purified further by silica thin-layer chromatography with 0.2% ammonium hydroxide as eluent. The PM band was extracted into 0.1 N HCl, and the specific activity was found to be essentially identical with that determined before the thin-layer chromatography step. Control experiments in which [$C_{4'}$-^{3}H]PMP (prepared as described below) was added to apo-cAATase plus other transfer reaction components or to buffer alone showed no loss of tritium after the isolation procedure.

Synthesis of [$C_{4'}$-^3H]PMP. See supplementary material.

Enzyme-Catalyzed Amino Acid C_α-H Exchange with Solvent. Cytoplasmic AATase with [α-^2H]-L-Aspartate in H_2O. The reaction solution contained 4 mM [α-^{2}H]-L-aspartate, 2 mM αKG, 140 units of MDH, and 0.3–2.0 mM NADH in 50 mL of 25 mM TAPS buffer, pH 8.3. Twenty-five milliliters of this solution, the exchange mixture, was placed in a 25 °C thermostated bath, and 1 unit of cAATase was added to start the reaction. Product formation was followed by the change in absorbance at 340 or 375 nm. Control experiments showed that the rates were independent of the amount of NADH and MDH added (between at least 0.2 and 1.5 mM NADH and 1 and 8 units/mL MDH). When ca. 2% of the L-aspartate was consumed (calculated with molar extinction coefficients for NADH of 6220 M^{-1} cm^{-1} at 340 nm and 1890 M^{-1} cm^{-1} at 375 nm), 0.25 mL of concentrated HCl was added to terminate the reaction. The remaining 25 mL of the original solution was used as a nonenzymatic blank mixture and was similarly treated, except that cAATase was added after HCl.

L-Aspartic acid was isolated by chromatography on Dowex 50-X8 (H^+ form), 4-mL bed volume. The columns were washed with 12 mL of H_2O and the amino acid eluted with 1 M pyridine. The ninhydrin-positive fractions were dried in a vacuum desiccator over P_2O_5.

Mass Spectrometric Analysis. The deuterium content of the isolated L-aspartic acid was determined by mass spectrometric analysis of the diethyl ester. Reisolated deuterio-L-aspartate was esterified essentially as described by Biemann et al. (1961) except that 2 mL of dry ethanol and 0.4 mL of redistilled acetyl chloride were used in place of HCl gas. The ester was analyzed by injection of 0.5–1.0 μL into the liquid inlet of the mass spectrometer. Each sample was injected several times, and at least 10–20 scans of the peaks at m/e = 116–119 were taken at an ionizing potential of 70 eV and a source temperature of 200 °C. The observed mass spectrum was in agreement with that reported by Biemann et al. (1961). The esters from the exchange and blank mixtures were injected alternately.

cAATase with [α-^1H]-L-Aspartate in D_2O. Exchangeable hydrogens in TAPS were removed by dissolving 0.304 g of TAPS in 5 mL of D_2O (90 atom % D) and lyophilizing the solution. The reaction solution contained 4 mM L-aspartate, 2 mM αKG, 0.3 or 0.6 mM NADH, and 136 units of MDH in 50 mL of 25 mM TAPS buffer, pD 8.7 (pD = pH meter reading + 0.4), in D_2O (99.5 atom % D). The solution was divided into two 25-mL portions and treated as described for the previous experiment.

mAATase with [α-^2H]-L-Glutamate in H_2O. The reaction solution contained 5 mM [α-^{2}H]-L-glutamate and 2 mM OAA in 50 mL of 25 mM TAPS and 60 mM *N*-ethylmorpholine, pH 8.3. The reactions were initiated by adding 4.2 or 8.4 units of mAATase and were followed at 257 nm.

Diethyl L-glutamate was prepared as described above for diethyl L-aspartate and the deuterium content measured in the mass spectrometer by scanning the peaks at m/e = 130–132. The spectrometer had to be evacuated for 5–10 min after each injection and for 1.5–4 h before changing samples, because of memory effects for this compound.

Reaction Rates. The rate of OAA consumption in experiments with L-glutamate was calculated from the change in absorbance at 257 nm with an extinction coefficient of 1153 M^{-1} cm^{-1} for OAA, determined under the conditions of the experiment. Although only a small percentage (2–5%) of the L-glutamate and OAA was utilized, the recorder traces were nonlinear because substantial progress is made toward the final equilibrium concentrations. A slow, enzyme-independent, decrease in absorbance at 257 nm, probably due to decarboxylation of OAA to pyruvate, was subtracted.

Deuterium Content of Reisolated Amino Acid. The deuterium contents of the reisolated amino acids were determined from the relative peak intensities of the undeuterated diethyl ester spectrum (loss of $-C_\alpha O_2Et$: m/e = 116, aspartate; m/e = 130, glutamate) and those arising from deuterium substitution. The details of the method, including corrections for the natural abundance of heavy isotopes and the change in isotopic composition due to the C_α-KIE (Julin & Kirsch, 1989), are included in the supplementary material.

Results

Tritium Transfer from [^3H]-L-Aspartate to Enzyme. The results reported in Table I show that 0.72 ± 0.03% of the C_α tritons are transferred from [^{3}H]-L-aspartate to PMP in the reaction catalyzed by cAATase. The other 99.3% are presumably lost to solvent. These experiments were carried out under single turnover conditions to eliminate the potential accumulation of tritium in the active site from preceding turnovers. Such a protocol is possible in the present case because of the efficiency of the MDH/ADH trap for nascent OAA. The PMP was separated immediately after the reaction to obviate the recently demonstrated enzyme-catalyzed labilization of tritium from [$C_{4'}$-^{3}H]PMP (Tobler et al., 1986). Gehring (1984) has recently reported a range of 0.75–4.2% transfer of tritium from [C_α-^{3}H]-L-glutamate to cofactor in the mAATase-catalyzed reaction. These latter experiments were, however, conducted under conditions of multiple turnover. It is therefore possible that the original C_α-triton might remain in a slowly exchanging enzyme site and be transferred in a later turnover.

AATase-Catalyzed Exchange of C_α-Hydrogens with Solvent. The distribution of deuterium in amino acids isolated after partial reaction with AATase, calculated as described under Methods, is given in Table II. It can be seen by comparing the values of $\alpha_1\beta_0 + \alpha_0\beta_1$ vs $\alpha_0\beta_0$ present in the exchange mixture to those in the control that there is significant loss of deuterium from the deuterated L-aspartate ($\alpha_1\beta_0 + \alpha_0\beta_1 \rightarrow \alpha_0\beta_0$) in reactions done in H_2O and significant deuterium incorporation into initially unlabeled L-aspartate in the reac-

Table I: ^3H Transfer to Pyridoxamine 5′-Phosphate from [2,3-^3H]-L-Aspartate Catalyzed by Cytoplasmic Aspartate Aminotransferase[a]

expt	PMP formed by AATase (nmol)	isolated PM counted[b] (nmol)	sp act. of isolated PM (cpm/nmol)	max sp transfer (%)[c]
1	1.36	134	101	0.69
2	1.35	133	100	0.70
3	1.37	169	104	0.74
4	1.36	77	100	0.70
5	1.42	104	108	0.77
				mean 0.72 ± 0.03

[a] Reaction mixtures contained 4 μM [2,3-^3H]-L-aspartate (12.5 μCi of C_α-^3H), 10.8 μM cytoplasmic aspartate aminotransferase, 1 M ethanol, 1 mM NADH, 57 μM malate dehydrogenase, 55 μM alcohol dehydrogenase, and 10 mM Tris-HCl, pH 8.0, in a total volume of 250 μL. Reactions were quenched with perchloric acid after 100 s at room temperature. Pyridoxamine 5′-phosphate (PMP) was dephosphorylated with alkaline phosphatase and the pyridoxamine (PM) isolated by cation-exchange chromatography. [b] 1.14 μmol of unlabeled PMP was added as carrier, after the PMP in the reaction supernatant was quantified by the apoAATase assay described. [c] The maximum specific transfer was calculated from the equation % transfer = (cpm × 100)/[(μmol of PM counted/1.14 μmol of carrier PMP)(cpm of [^3H]-Asp − cpm) × 0.5 × 1000]. After the PM had been counted to determine the "cpm" variable, 25 μL of 1000-fold diluted [2,3-^3H]-L-aspartate stock (1/1000 of the amount added to each transfer reaction) was added to each of the scintillation vials which contained PM. These vials were then recounted to determine the "cpm [^3H]Asp" variable. The 25 μL of diluted [^3H]aspartate added was found to give 32 500 ± 500 cpm. This procedure corrects for internal quenching, for uncertainties in the specific activity of the aspartate, and for the C_α position containing 50% of the total ^3H.

tions carrried out in D_2O ($\alpha_0\beta_0 \rightarrow \alpha_1\beta_0$). The results with mAATase and deuterated L-glutamate in H_2O ($\alpha_1\beta_0 \rightarrow \alpha_0\beta_0$) show that somewhat less label is lost from this substrate during the initial course of the reaction even though these reactions were carried out to approximately the same extent of product formation as were those of cAATase with L-aspartate.

The comparative rates of product formation versus α-hydrogen exchange are shown in Table III. The parameter θ_{app} is defined as the ratio of the rate of protium incorporation into substrate to the rate of keto acid formation. The corresponding parameter determined for initially unenriched L-aspartate in solvent D_2O is defined as θ'_{app}. The θ_{app} value of about 2.6 for the reaction of [α-^2H]-L-aspartate with cAATase indicates that unlabeled L-aspartate is produced more than twice as fast as are the products OAA and L-glutamate. The θ'_{app} value of ca. 4 obtained in D_2O can be compared to a range of 1.5–4.4 measured at 37.5 °C, pH 7.4, with a crude enzyme preparation (Hilton et al., 1954, 1956). The value of θ_{app} is relatively small for the reaction of mAATase with [α-^2H]-L-glutamate, indicating that the rate of hydrogen exchange is slow relative to that of product formation for this reaction.

Discussion

Transfer of Amino Acid C_α-Protons to Cofactor. The data presented in Table I show that only 0.7% of the tritons from [^3H]-L-aspartate are transferred to AATase in a single turnover experiment. These results can be evaluated within the framework of two models for the formal 1,3 prototropic shift: one, a stepwise mechanism embodying a quinonoid intermediate as a kinetically significant entity between the aldimine and ketimine, and the other, a concerted mechanism lacking this intermediate (Scheme II). A stepwise mechanism allows for the possibility of $C_\alpha \rightarrow C_{4'}$ tritium transfer while the concerted mechanism (Scheme IIB) *viewed simply* requires that tritium not be transferred to the coenzyme in a single turnover experiment, since proton abstraction from C_α and donation to $C_{4'}$ must be simultaneous. A concerted mechanism in which the aldimine and ketimine are in partial equilibrium on the enzyme (i.e., a slow step follows formation of the ketimine) and the amino group of Lys-258 rotates freely would permit retention of tritium on the amino group and subsequent transfer to $C_{4'}$; however, the results of kinetic isotope effect experiments reported in the third paper of this series (Julin & Kirsch, 1989) are most consistent with a concerted 1,3 prototropic shift with the aldimine-to-ketimine step being rate determining in the overall reaction. Thus these two inter-

Table II: Summary of Mass Spectrometric Data from Deuterium-Exchange Experiments[a]

	enzymatic exchange (%)				control (%)			
expt	$\alpha_0\beta_0$[b]	$\alpha_1\beta_0 + \alpha_0\beta_1$	$\alpha_1\beta_1 + \alpha_0\beta_2$	$\alpha_1\beta_2$	$\alpha_0\beta_0$	$\alpha_1\beta_0$	$\alpha_1\beta_1$	$\alpha_1\beta_2$
			(A) cAATase + [^2H]-L-Aspartate in H_2O[c]					
1	7.19 (0.10)	79.71 (0.14)	11.03 (0.11)	2.06 (0.04)	1.86 (0.05)	83.35 (0.17)	12.42 (0.14)	2.36 (0.04)
2	6.51 (0.09)	78.48 (0.21)	12.48 (0.15)	2.40 (0.03)	2.34 (0.20)	82.29 (0.40)	12.83 (0.19)	2.52 (0.03)
3	7.12 (0.23)	78.53 (0.34)	11.98 (0.12)	2.40 (0.02)	3.60 (0.46)	81.18 (0.40)	12.54 (0.11)	2.59 (0.04)
4	5.78 (0.13)	79.73 (0.39)	12.16 (0.25)	2.39 (0.08)	2.68 (0.10)	81.87 (0.20)	12.89 (0.07)	2.58 (0.04)
5	6.52 (0.09)	78.19 (0.17)	12.57 (0.16)	2.54 (0.07)	2.43 (0.26)	81.68 (0.20)	13.34 (0.09)	2.54 (0.03)
			(B) cAATase + [^1H]-L-Aspartate in D_2O[d]					
1	78.79 (0.20)	21.21 (0.18)			98.05 (0.26)	1.95 (0.11)		
2	94.60 (0.26)	5.40 (0.13)			99.34 (0.26)	0.66 (0.06)		
			(C) mAATase + [^2H]-L-Glutamate in H_2O[e]					
1	12.17 (0.48)	82.44 (0.52)	5.36 (0.24)		11.07 (0.42)	83.06 (0.54)	5.72 (0.18)	
2[f]	2.40 (0.28)	76.46 (0.36)	21.23 (0.14)		1.45 (0.02)	76.83 (0.08)	21.70 (0.08)	

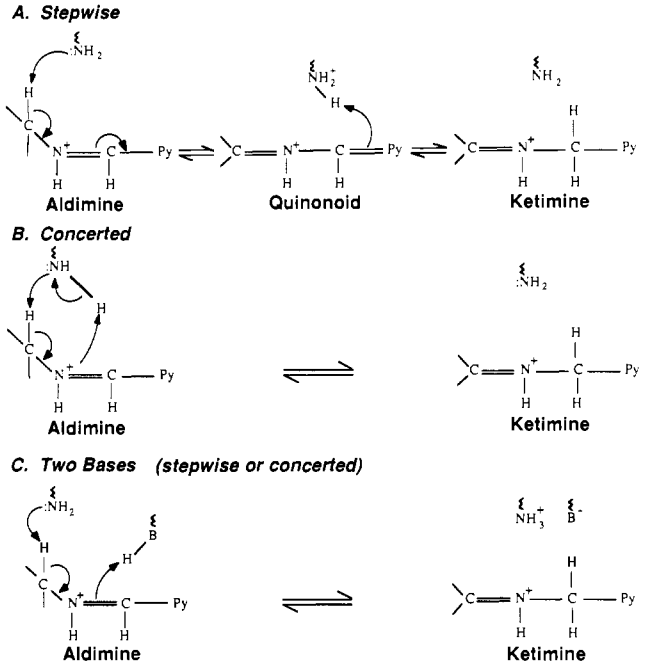
[a] The isotopic content of the diethyl esters of amino acids reisolated from the control and enzymatic-exchange mixtures was analyzed by mass spectrometry. Each sample was injected 1–6 times and scanned 10–30 times per injection. Standard errors are shown in parentheses. [b] $\alpha_i\beta_j$ represents amino acids containing i and j deuterium atoms at the α and β positions, respectively. [c] The reaction mixtures contained 4 mM [α-^2H]-L-aspartate, 2 mM α-ketoglutarate, 140 units of MDH, and 0.3–2 mM NADH in 50 mL of 25 mM TAPS buffer, pH 8.3. One unit (experiments 1–4) or 0.125 unit (experiment 5) of cAATase was added to 25 mL of this mixture (the enzymatic-exchange mixture) followed by 0.25 mL of concentrated HCl, after incubation at 25 °C for a time sufficient to allow consumption of 2% of the L-aspartate substrate. The control mixture was treated identically except that HCl was added before cAATase. The remaining L-aspartate was isolated by ion-exchange chromatography and esterified as described in the text. [d] The reaction conditions were as described in footnote *a* except that the NADH concentration was 0.3 (experiment 1) and 0.6 (experiment 2) mM and the buffer was 25 mM TAPS, pD 8.7. The mixture was divided into two 25-mL portions and treated as in footnote *a*, except that experiment 1 was allowed to proceed until 5% of the L-aspartate was consumed while experiment 2 was quenched after only 2% reaction. [e] The reaction mixture contained 5 mM [α-^2H]-L-glutamate and 2 mM oxaloacetate in 50 mL of 25 mM TAPS and 60 mM *N*-ethylmorpholine, pH 8.3. The enzymatic-exchange and control mixtures were treated as above except that 4.2 (experiment 1) or 8.4 (experiment 2) units of mAATase was added and the exchange reaction was quenched after consumption of 3.3% of the L-glutamate. [f] Experiment 2 with mAATase and glutamate was done with a much more extensively deuterated preparation than was experiment 1.

Table III: Summary of Kinetic Results of Deuterium-Exchange Experiments[a]

expt	extent of reaction[b] (%)	[NADH] (mM)	product formation rate (μM/s)	H(D) incorpn rate (μM/s)	θ_{app}[c]
		(A) cAATase + [^{2}H]-L-Aspartate in H_2O			
1	2.1	0.322	0.160	0.552	3.45 (0.21)
2	2.0	0.611	0.172	0.413	2.40 (0.30)
3	2.0	1.99	0.159	0.355	2.23 (0.31)
4	1.8	2.01	0.151	0.346	2.29 (0.34)
5[d]	1.9	2.01	0.0161	0.042	2.59 (0.23)
		(B) cAATase + [^{1}H]-L-Aspartate in D_2O			
1	5.1	0.638	0.119	0.426	3.56 (0.18)
2	1.0	0.308	0.114	0.523	4.57 (0.27)
		(C) mAATase + [^{2}H]-L-Glutamate in H_2O[e]			
1	3.3		0.280	0.182	0.65 (0.37)
2	3.3		0.413	0.189	0.45 (0.32)

[a] Reaction conditions are described in Table II. [b] Calculated as the percentage of total amino acid converted to α-keto acid. [c] θ_{app} = [H(D) incorporation rate]/(product formation rate). [d] Experiment 5 contained 8-fold less cAATase then did experiments 1–4. [e] The nonenzymatic blank rate of 0.03 μM/s, due to decarboxylation of OAA, was subtracted from the observed reaction rate to give the values shown in column 4.

Scheme II



mediates are not in equilibrium. The evidence supports a model in which the quinonoid intermediate is in equilibrium with the external aldimine but is off the main pathway for product formation. This model provides a "parking place" (i.e., the ϵ-amino group of Lys-258) for the abstracted triton. The tritium removed from C_α can be retained on Lys-258 when the quinonoid reverts to the external aldimine by transfer of a *proton* to C_α. The external aldimine then partitions between the concerted proton transfer to form the ketimine followed by hydrolysis to form PMP enzyme and OAA and transaldimination followed by release of (unlabeled) aspartate. The possibility therefore exists that the triton retained on Lys-258 could be transferred to the $C_{4'}$ position of the cofactor during the aldimine–ketimine step. The above arguments demonstrate that transfer of tritium from substrate to coenzyme can provide no evidence to differentiate a stepwise from a concerted mechanism.

There are a number of factors that can account for the relatively small amount of hydrogen transfer from C_α to $C_{4'}$. A *triton* abstracted from the C_α position of the amino acid can either be transferred to $C_{4'}$ of the cofactor, remain on the ϵ-amino group of Lys-258 while one of the two *protons* is transferred, or exchange with a solvent proton. Transfer requires rotation about the ϵ-carbon–nitrogen bond and would be discriminated against by a statistical factor of 3 (2 for a concerted mechanism) and by the kinetic isotope effect on $C_{4'}$ protonation, likely to have a magnitude within the range 4–7 (Julin & Kirsch, 1989). The potential kinetic isotope effect on C_α-hydrogen abstraction is obviated by the conditions of the experiment in which all of the L-aspartic acid is utilized. If free rotational equilibrium of the ϵ-NH_3^+ is attained and no exchange of the protein-bound triton with solvent occurs, the maximum extent of transfer in a single turnover is only about 7–12% ($=^1/_2 \times ^1/_4$ to $^1/_7$) for a concerted reaction, or 5–8% ($=^1/_3 \times ^1/_4$ to $^1/_7$) for a stepwise mechanism. The observed value of 0.7% suggests that rotational equilibrium about the ϵ-amino group is not attained and/or that significant exchange with solvent occurs. Finally, while it is considered most likely that Lys-258 acts as both the proton donor and acceptor (Kirsch et al., 1984), it is possible, in principle, that, e.g., Lys-258 could abstract the C_α proton while the $C_{4'}$ proton could be donated by Tyr 70, an amino acid residue found crystallographically to be situated within the active site (Ford et al., 1980; Arnone et al., 1984). Alternatively, a transiently bound water molecule could fulfill the latter function. This two-base mechanism, whether concerted or stepwise (Scheme IIC), would clearly also account for a submaximal level of tritium transfer, since the tritium would have to be retained on the enzyme long enough to migrate from one base to the second.

Estimation of the Exchange Rate with Solvent of Lys-258 Amino Group Protons. Proton abstraction from C_α is partly rate determining in the overall mechanism (Julin & Kirsch, 1989) and the V versus pH profile (Kiick & Cook, 1983) is flat from pH 5.5 to pH 10; therefore, the sum of the number of protons in the residues that effect the 1,3 prototropic shift must be constant over this range; i.e., the pK_a of this ensemble must be less than 5.5 or greater than 10. Because the first step involves the abstraction of either C_α-H or C_4-H, the lower pK_a model is favored (Kirsch et al., 1984; Kiick & Cook, 1983).

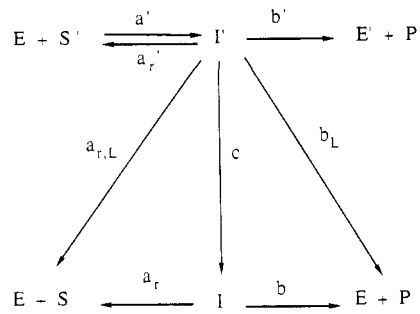
The rates of proton exchange between water and the amino group can be estimated from the work of Eigen (1964) (eq 1) with the pK_a of the ϵ-NH_3^+ group taken as 5. The value

$$RNH_3^+ + H_2O \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RNH_2 + H_3O^+ \qquad (1)$$

of k_{-1} is 4×10^{10} M^{-1} s^{-1} assuming free accessibility of water, and the rate constant k_1, determined by the pK_a value, is 4×10^5 s^{-1}. The limiting rate constant for the exchange reaction at pH 7 thus is ca. 4000 s^{-1} (i.e., 4×10^{10} M^{-1} $s^{-1} \times 10^{-7}$ M $[H^+]$), a value which is ca. 20-fold greater than that of k_{cat} for this enzyme (Julin & Kirsch, 1989). Thus this limiting model is consistent with the hypothesis that the small amount of observed tritium transfer is due to fast washout at the quinonoid stage. The exchange rate could be even greater if buffer ions have access to the active site.

Enzyme-Catalyzed Isotope Exchange at C_α. The formal kinetics of isotope exchange between solvent and enzyme–substrate complexes have been evaluated by Rose (1975) and by Albery and Knowles (1976). The most general mechanism applicable to the present set of experiments is shown in Scheme III, in which the isotopic label in the substrate becomes solvent

Scheme III



exchangeable at some intermediate, I'. The primed symbols apply to rate constants (a', a'_r, b') or entities (S', I') where the original isotope, different from that contained in solvent, has not yet exchanged, i.e., [^{1}H]-L-aspartate in D_2O or [^{2}H]-L-aspartate in H_2O. The subscript L refers to rate constants for transfer of isotope from solvent through a *polyprotonic* base (e.g., the ϵ-amino group of Lys-258 in AATase) to generate S ($a_{r,L}$) or P (b_L) before the original isotope from the substrate has exchanged with solvent. The rate constant c measures the loss of label from I' to solvent. The intermediate I' represents the population-weighted sum of the enzyme-bound external aldimine, quinonoid, and/or ketimine since the C_α-hydrogen can be solvent labile at each of these intermediates. (The external aldimine with tritium label retained on Lys-258 is formed by transfer of a *proton* from Lys-258 to C_α with the original label still bound to that residue.)

Application of the King–Altman method to the mechanism shown in Scheme III gives the following expression for θ_{app} (defined under Results).

$$\theta_{app} = \frac{a_{r,L}(a_r + b) + a_r c}{(b' + b_L)(a_r + b) + bc} \quad (2)$$

The relationship of θ_{app} to θ (defined as a_r/b) depends on the values of the ratios $a_{r,L}/a_r$ and $(b' + b_L)/b$. The former would have the statistical value of 2/3 with lysine as the proton-transferring agent with no exchangeable water protons present, neglecting the secondary kinetic isotope effect, while the latter will vary from 2/3 to 1 as the primary kinetic isotope effect for this process varies from infinity to 1; therefore, the ratio

$$a_{r,L}b/[a_r(b' + b_L)] \equiv F$$

can vary only between 1 and 2/3. Substituting $\theta = a_r/b$ and $a_{r,L} = F(b' + b_L)\theta$ into eq 2 gives

$$\frac{\theta_{app}}{\theta} = \frac{F(b_L + b')(1 + \theta) + c}{(b_L + b')(1 + \theta) + c} \quad (3)$$

from which it is seen that the value of θ_{app}/θ varies from F to 1 (i.e., over a range of 2/3 to 1) depending on the relationship of c to $(b' + b_L)(1 + \theta)$. The presence of water molecules with exchangeable protons within the active site causes the observed θ value (θ_{app}) to approach even closer to the true partition ratio θ. Only one water molecule introduces two additional protons and will cause θ_{app}/θ to lie within the ratio 0.8–1 depending on the kinetic isotope effect. In the limit, the presence of many bound water molecules is exactly equivalent to fast washout.

cAATase-Catalyzed Exchange of C_α-Hydrogen Isotopes from L-Aspartate to Solvent. The data in Table III are analyzed in terms of Scheme III. Further considerations introduced above and in Julin and Kirsch (1989) will allow conclusions to be drawn regarding the identity of intermediate I' and the detailed mechanism leading to C_α-hydrogen exchange with solvent. The value of θ_{app} taken together with the limits described above for the quantity θ_{app}/θ gives $3.7 > \theta > 2.5$. The small amount of tritium transfer (0.7%) from C_α to $C_{4'}$ is the result of some combination of a kinetic isotope effect ($b' + b_L < b$) and/or washout ($c > b'$) (Scheme III).

The solvent kinetic isotope effect on the ratio $\theta'_{app}/\theta_{app}$ of 1.5 (Table III) shows that the rate constants corresponding to protonation on $C_{4'}$ by Lys-258, coupled perhaps to ketimine hydrolysis, have larger kinetic isotope effects than does protonation on C_α. These isotope effects are evaluated further in Julin and Kirsch (1989).

mAATase-Catalyzed Exchange of [C_α-^{2}H]-L-Glutamate in H_2O. The demonstration of direct ^{3}H transfer from [C_α-^{3}H]-L-glutamate to $C_{4'}$ of cofactor with mAATase by Gehring (1984) can be interpreted as above; therefore, the value of θ_{app} for this reaction may also be somewhat less than the true value of a_r/b. The limits of θ must lie between 0.45 and 0.68; i.e., the best measured value of 0.45 sets the lower limit and the upper is determined by $0.45 \times {}^3/_2 = 0.68$.

Interpretation of the θ Values. The species I (Scheme III) formed from [^{2}H]-L-aspartate in H_2O reacting with cAATase protonates C_α and releases the nascent [^{1}H]-L-aspartate approximately 2.6 times as fast as protonation on $C_{4'}$ and release of OAA occurs. The larger value of θ' recorded when the partitioning experiment is carried out in D_2O is indicative of the larger kinetic isotope effect(s) on step(s) leading to product formation versus return to substrate. The θ values determined for the reactions of [^{2}H]-L-glutamate with mAATase are substantially lower than the above, consistent with the major kinetic barrier being C_α-H abstraction for this reaction. The free energy profiles for the various half-reactions are considered further in the second (McLeish et al., 1989) and third papers (Julin & Kirsch, 1989) of this series.

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Supplementary Material Available

The procedures used in the purification of cAATase from pig heart cytoplasm, the synthesis of [$C_{4'}$-^{3}H]PMP, and the mass spectrometric determination of the deuterium content of amino acids isolated in the deuterium-exchange experiments (11 pages). Ordering information is given on any current masthead page.

Registry No. AATase, 9000-97-9; PMP, 91297-67-5; PLP, 54-47-7; [2,3-^{3}H]-L-Asp, 73024-86-9; D_2, 7782-39-0; T_2, 10028-17-8.

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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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