^{13}C and ^{15}N Kinetic Isotope Effects on the Reaction of Aspartate Aminotransferase and the Tyrosine-225 to Phenylalanine Mutant†

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ABSTRACT: Heavy atom isotope effects at C-2, C-3, and the amino nitrogen of aspartate were determined for the reaction of porcine heart cytosolic aspartate aminotransferase and the tyrosine-225 to phenylalanine mutant of *Escherichia coli* aspartate aminotransferase. The effects of deuteration at C-2 of aspartate and of D_2O on the observed heavy atom isotope effects were determined. The multiple isotope effects support the contribution of $C_\alpha-H$ cleavage, ketimine hydrolysis, and oxaloacetate dissociation to the rate limitation with the wild-type enzyme. The existence of a quinonoid intermediate could not be determined due to the kinetic complexity of the enzyme. For the tyrosine-225 to phenylalanine mutant, we are able to conclude that ketimine hydrolysis is the major rate-determining step.

Aspartate aminotransferase (AATase)[1] catalyzes the pyridoxal phosphate-(PLP-) dependent transamination of aspartate with α-ketoglutarate to give glutamate and oxaloacetate. AATase employs a ping-pong kinetic mechanism in which the PLP form of the enzyme reacts with the amino acid substrates to give the pyridoxamine phosphate (PMP) form of the enzyme and a keto acid. The amino group is then transferred from the PMP cofactor to a different keto acid, resulting in transamination (*1*−*3*).

AATase is one of the best studied of all enzymes. Numerous spectrophotometric, kinetic, structural, and model experiments have been done with substrates and substrate analogues (*4*, *5*). These experiments, especially determinations of the crystal structure, have led to a detailed chemical mechanism including the roles of most of the enzymic side chains in the active site (*6*). The role of most of these side chains has been further probed by site-directed mutagenesis, especially Lys-258 (*7*−*11*) and Tyr-225 (*12*−*16*).

The chemical mechanism of AATase is generally considered to employ a quinonoid intermediate in the proton shift from C_α to C-4′. However, it is possible that this proton shift is concerted and that quinonoid formation is not necessary. Multiple deuterium isotope effects have been used to address this question. These experiments suggested a dichotomy in the mechanism, where the reaction of cAATase with aspartate and of mAATase with glutamate involved a concerted proton shift, while in other cases the mechanism involved a quinonoid intermediate or was indeterminate (*17*). In this work, we have determined heavy atom isotope effects at C-2, C-3, and the amino nitrogen of aspartate to determine the viability of the quinonoid intermediate. In addition, these heavy atom isotope effects were determined with the Y225F mutant to study the effect of this mutation on the chemical mechanism of AATase.

EXPERIMENTAL PROCEDURES

Materials. Aspartic acid, α-ketoglutaric acid, and D_2O were from Aldrich. Oxidized glutathione was from Acros. Glucose-6-phosphate, NADP, NADH, *n*-octane, malic enzyme, aspartate aminotransferase, malate dehydrogenase, glutathione reductase, glucose-6-phosphate dehydrogenase, and ninhydrin spray reagent were from Sigma. Sodium hypobromite was prepared by slow addition of bromine to cold 17 N NaOH. This solution was kept at 4 °C for 5 days and the precipitate was removed by filtration. The overexpression and purification of Y225F was performed as previously described (*18*). The wild-type transaminase was the porcine heart cytosolic enzyme, while the mutant was from *Escherichia coli*. The active-site side chains involved in the chemical mechanism are identical in the porcine heart cytosolic and *E. coli* wild-type enzymes (*19*).

2-D-Aspartic Acid. Aspartic acid (800 mg) and dibasic potassium phosphate (180 mg) were dissolved in 30 mL of D_2O. The solution was titrated to pD 7.9 with KOD, and oxaloacetic acid (5 mg) and aspartate aminotransferase (50 units) were added. The reaction was sealed and stirred for several days. 2-D-Aspartate was synthesized by stereospecific incorporation of deuterium from the exchangeable hydrogens of Lys-258 into the substrate during enzymatic turnover. The progress of the reaction was determined by monitoring the disappearance of the α-proton resonance at 3.9 ppm by ^1H NMR. The transaminase was removed by Amicon filtration (10 000 MW cutoff) and the solution was loaded onto a Dowex 50W-X2 cation-exchange column (2.5 × 5 cm) in the proton form. The column was washed with 250 mL of water followed by 0.4 N ammonium hydroxide. The aspartate eluted as soon as the resin had been completely converted to the ammonium ion form. Fractions containing aspartate

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[1] Abbreviations: Y225F, *Escherichia coli* aspartate aminotransferase with tyrosine-225 replaced by phenylalanine; AATase, aspartate aminotransferase; cAATase and mAATase, cytosolic and mitochondrial isoforms of aspartate aminotransferase; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; OAA, oxaloacetate.

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were repeatedly evaporated to dryness and taken up in a small portion of water, and finally lyophilized.

Deuterium Isotope Effects. Deuterium isotope effects were measured by direct comparison of kinetic constants for α-deuterated and undeuterated aspartate, or in H_2O and D_2O. Reactions were run in 3 mL quartz cuvettes with 50 mM potassium phosphate, pH 7.5 (pD 7.9), for wild-type enzyme or 50 mM sodium pyrophosphate, pH 9.5, for the Y225F mutant. Reactions contained 0.1 mM NADH and an excess of malate dehydrogenase over transaminase, allowing determination of initial rates by observing the coupled oxidation of NADH at 340 nm. With wild-type enzyme, α-ketoglutarate was held constant at 5 mM and aspartate was varied from 1 to 10 mM. With the Y225F mutant, α-ketoglutarate was held constant at 50 μM and aspartate was varied from 16.7 to 200 μM, or aspartate was held constant at 1 mM while α-ketoglutarate was varied from 5 to 50 μM. For D_2O isotope effects, solutions of phosphate buffer, aspartate, and α-ketoglutarate were prepared in D_2O and the solutions were twice evaporated to dryness and taken up in D_2O. The concentration of aspartate solutions was determined by endpoint assay with an excess of α-ketoglutarate and NADH with transaminase and malate dehydrogenase.

Heavy Atom Isotope Effects. Aspartic acid (27 mg), α-ketoglutaric acid (15 mg), glucose 6-phosphate (45 mg), NADH (5 mg), and dibasic potassium phosphate (170 mg) were dissolved in 15 mL of water and the solution was titrated to pH 7.5 or 8.0 with KOH. For reactions at pH 9.0, sodium pyrophosphate (335 mg) was used in place of potassium phosphate. For experiments in D_2O, the solution was titrated with KOD and three times evaporated to a small volume and brought back to 15 mL with D_2O. Glucose-6-phosphate dehydrogenase (25 units), malate dehydrogenase (12.5 units), and aspartate aminotransferase (3.3 units) were added and the solution was allowed to stir for 12 h. The solution was titrated to pH 2 with HCl and loaded onto a Dowex 50W-X2 cation-exchange column (3.5 × 30 cm) in the sodium form. The column was washed with 500 mL of 0.01 N HCl/0.1 N NaCl and eluted with 1 L of 0.2 N sodium citrate, pH 3.1. Fractions containing malate were located by use of malic enzyme assay, while fractions containing amino acids were located by spotting on a TLC plate followed by treatment with ninhydrin spray reagent. Malate was eluted at the void volume by the NaCl wash, while aspartate was eluted just before glutamate by the citrate.

Fractions containing aspartate or glutamate were pooled, evaporated to a small volume, and transferred to a long-neck Kjeldahl flask. To each flask was added 1.5 mL of 0.46 N HgO in 4.32 N sulfuric acid, 1.5 g of potassium sulfate, and 10 mL of concentrated sulfuric acid. The solutions were heated to reflux until all of the black color disappeared. The solution was placed on a steam still and made basic by introduction of an excess of 17 N NaOH through a septum. The ammonia produced was collected in 0.1 N sulfuric acid and quantified by Nessler's assay at 425 nm. The ammonia was oxidized to nitrogen in vacuo by sodium hypobromite. The nitrogen gas was distilled on high vacuum lines through dry ice−2-propanol and liquid nitrogen traps and collected on molecular sieves chilled with liquid nitrogen. The purified nitrogen gas was analyzed by isotope ratio mass spectrometry.

Table 1: Kinetic Parameters with Wild-Type and Y225F AATase

kinetic parameter	wild type (porcine heart cAATase)	Y225F (*E. coli*)
k_{cat} (s^{-1})	152	0.293
K_{Asp} (mM)	3.3	0.059
$K_{\alpha KG}$ (mM)	nd[a]	0.004
$^{\alpha-D}(V/K_{Asp})$	1.52	0.98
$^{D2O}(V/K_{Asp})$	1.51	nd

[a] Not determined.

Fractions from the cation-exchange column containing malate were evaporated to about 10 mL and made acidic with 3 drops of concentrated sulfuric acid. These solutions underwent continuous extraction with ethyl acetate for 36 h. The ethyl acetate was evaporated to dryness and the residue was repeatedly taken up in 0.01 N HCl and evaporated to dryness to remove acetic acid. The purified malate was taken up in a solution of oxidized glutathione (92 mg), dibasic potassium phosphate (153 mg), and NADP (2 mg) in 15 mL of 3 mM $MgCl_2$. Malic enzyme (1 unit) and glutathione reductase (5 units) were added and the reaction was stirred for 3 h. Hydrogen peroxide (0.3 mL, 3%) was added and the solution was stirred for 12 h. The solution was made acidic with 5 drops of concentrated sulfuric acid and underwent azeotropic distillation with *n*-octane (600 mL), with 4 drops of 5 N KOH in the receiver to neutralize the acetic acid. The aqueous distillate was titrated to pH 7.2 with HCl, sparged with CO_2-free nitrogen for 12 h, and evaporated to dryness. The acetate salt was divided into two portions that underwent acetate degradation to give methane from C-2 of acetate or combustion to CO_2 as described elsewhere (*20*). The methane was combusted over CuO to give CO_2.

Data Analysis. The abundance of ^{13}C or ^{15}N in unreacted aspartate (R_0), in remaining aspartate (R_s), and in the glutamate or oxaloacetate product (R_p) was determined as described, as was the fraction of reaction *f*. For comparisons between R_0 and R_s, the isotope effects were calculated from

$$\text{isotope effect} = \log (1 - f)/\log [(1 - f)(R_s/R_0)] \quad (1)$$

For comparisons between R_0 and R_p, the isotope effects were calculated from

$$\text{isotope effect} = \log (1 - f)/\log [1 - f(R_p/R_0)] \quad (2)$$

RESULTS

Characterization of Y225F Mutant. The UV−vis spectrum of Y225F showed a peak at 430 nm at pH 6.5 and at 385 nm at pH 9.5. This differed from the wild-type enzyme, which had a peak at 360 nm above pH 7. The kinetic constants for the transamination of aspartate with α-ketoglutarate were determined for the Y225F mutant by holding one substrate constant at saturating concentration while the other substrate was varied. The data were analyzed by the FORTRAN program Hypero for each substrate (Table 1) (*21*). All data show good agreement with those of Kirsch and co-workers (*12*).

Deuterium Isotope Effects. Isotope effects for deuteration of the α-carbon of aspartate were determined for wild-type enzyme and the Y225F mutant, and solvent deuterium effects were determined for the wild-type transaminase. In all cases

kinetic constants were determined by holding α-ketoglutarate constant at saturating concentrations and varying the concentration of aspartate. The data were analyzed with Hypero and are nearly identical to previously determined values (Table 1) (*17*).

Heavy Atom Isotope Effects. All heavy atom isotope effects were determined by comparing the abundance of heavy isotope in the unreacted starting material to the abundance of heavy isotope in the product of the reaction. For nitrogen isotope effects, comparison with the remaining substrate was also possible, giving two independent determinations for each experiment. Multiple isotope effects were determined by the same method either with 2-deuterated aspartate as the substrate or with D_2O as the solvent. Reactions were run in the presence of NADH and malate dehydrogenase so that oxaloacetate produced by transamination of aspartate was irreversibly reduced to malate. No back reaction or equilibration of the products with the substrates was possible, ensuring that any measured isotope effects would be kinetic and not equilibrium ones.

Heavy atom isotope effects were measured by degrading the substrate and product molecules so as to isolate the atom of interest without fractionation and convert the samples to N_2 or CO_2 for isotopic analysis. For nitrogen effects, this simply involved Kjeldahl digestion of the amino acids and hypobromite oxidation of the resulting ammonia. The carbon effects, however, required a more involved process. Malate from the transamination reactions was enzymatically oxidized to pyruvate by malic enzyme with loss of C-4 as CO_2. This reaction was driven to completion by coupling the production of NADPH to glutathione reductase in the presence of oxidized glutathione. The initial amount of malate present was determined by end-point assay with malic enzyme and the reactions were judged to be complete when end-point assay with lactate dehydrogenase showed an equivalent amount of pyruvate. Pyruvate was oxidized by peroxide to acetate with loss of C-1 as CO_2. This reaction was judged to be complete when no pyruvate was found by lactate dehydrogenase assay. Acetate degradation resulted in the complete conversion of the methyl carbon of acetate to methane, which was combusted to CO_2 for isotopic analysis. This gave a direct determination of the abundance of ^{13}C in the C-3 carbon of the oxaloacetate product of the transamination reaction. In addition, a portion of the acetate sample was directly combusted to CO_2 for isotopic analysis, yielding an average of the abundance of ^{13}C at the C-2 and C-3 positions of the oxaloacetate product. The abundance of ^{13}C at the C-2 position of oxaloacetate could then be determined by simple subtraction. The abundance of ^{15}N in the unreacted starting material was determined by direct combustion of aspartate, while the abundance of ^{13}C at C-2 and C-3 of unreacted starting material was determined by complete transamination of aspartate with an excess of α-ketoglutarate coupled with reduction to malate. The extent of reactions was governed by the amount of α-ketoglutarate present in the reactions but was also checked by malate end-point assay and found to be as expected in all cases. Heavy atom isotope effects are shown in Table 2.

Table 2: Heavy Atom Isotope Effects with Wild-Type AATase and Y225F Mutant[a]

	aspartate, H_2O	α-D-aspartate, H_2O	aspartate, D_2O
	Wild Type (Porcine Heart cAATase), pH 7.5		
^{13}C, C-2	1.0003 (0.0003)	0.9987 (0.0015)	1.0004 (0.0006)
^{13}C, C-3	1.0022 (0.0001)	1.0018 (0.0003)	1.0024 (0.0002)
^{15}N	1.0056 (0.0001)	1.0030 (0.0003)	1.0058 (0.0004)
	Y225F (*E. coli*), pH 8.0		
^{13}C, C-2	1.0074 (0.0003)	1.0083 (0.0005)	1.0097 (0.0008)
^{13}C, C-3	1.0001 (0.0001)	1.0002 (0.0002)	1.0008 (0.0001)
^{15}N	0.9978 (0.0002)	0.9986 (0.0003)	1.0052 (0.0002)
	Y225F (*E. coli*), pH 9.0		
^{13}C, C-2	1.0105 (0.0003)	n.d.	n.d.
^{13}C, C-3	1.0010 (0.0003)	n.d.	n.d.
^{15}N	0.9998 (0.0004)	n.d.	n.d.

[a] Values in parentheses are standard errors; all isotope effects are on V/K_{Asp}.

DISCUSSION

The chemical mechanism employed by AATase is shown in Figure 1. The amino acid substrate transfers a proton from its amino group to the lysine nitrogen in the internal aldimine (*22*). The amino acid is then able to act as a nucleophile and release the lysine ϵ-amino group in a transaldimination reaction which is facilitated by use of O-3′ as a proton shuttle between the two nitrogens (*23*). The unprotonated lysine removes the α-proton and reprotonates at C-4′, either in a concerted proton shift or with formation of a quinonoid intermediate (*7*, *24*, *25*). The resulting ketimine is hydrolyzed with general base assistance from Lys-258 to yield the PMP form of the enzyme and a keto acid (*6*).

Heavy atom isotope effects were chosen as a method for determination of the nature of the proton shift from C_α to C-4′. The abstraction of the α-proton is partially rate-determining as shown by the small deuterium isotope effect with α-deuterated aspartate. Cleavage of this bond will also result in a primary ^{13}C isotope effect at C-2 of aspartate. The observed ^{13}C isotope effect at C-2 should be increased with α-deuterated aspartate as the step exhibiting the primary ^{13}C isotope effect will be slower relative to other steps. The effect on the observed ^{13}C effect with D_2O depends on the nature of the proton shift. A concerted proton shift would involve transfer of one of the exchangeable protons of lysine to C-4′ as the bond to C-2 was being broken. In D_2O, this exchangeable proton will be a deuteron, slowing the concerted proton-transfer step. Thus, in D_2O a concerted proton shift would lead to an increased observed ^{13}C effect at C-2. If a quinonoid intermediate is formed, then no exchangeable proton is involved when the bond to C-2 is broken. The observed ^{13}C effect should then be diminished as other steps sensitive to solvent deuteration are slowed. However, the ^{13}C isotope effects at C-2 with the wild-type enzyme do not conform with either of these outcomes. Instead, the ^{13}C isotope effects are at or near unity in all three experiments.

The failure of the ^{13}C effects to behave as expected is due to the kinetic complexity of AATase. The chemical mechanism in full detail has at least nine separate intermediates. The reaction of several of these could conceivably contribute to the rate limitation. Ketimine hydrolysis and breakdown of the carbinolamine will both show primary ^{13}C isotope effects at C-2, further complicating interpretation of effects at that position. To analyze the heavy atom isotope effect data, a table of fractionation factors was constructed for all intermediates in the chemical mechanism of AATase (Table 3). The equilibrium isotope effects between aspartate and oxaloacetate have been determined experimentally (*26*). The

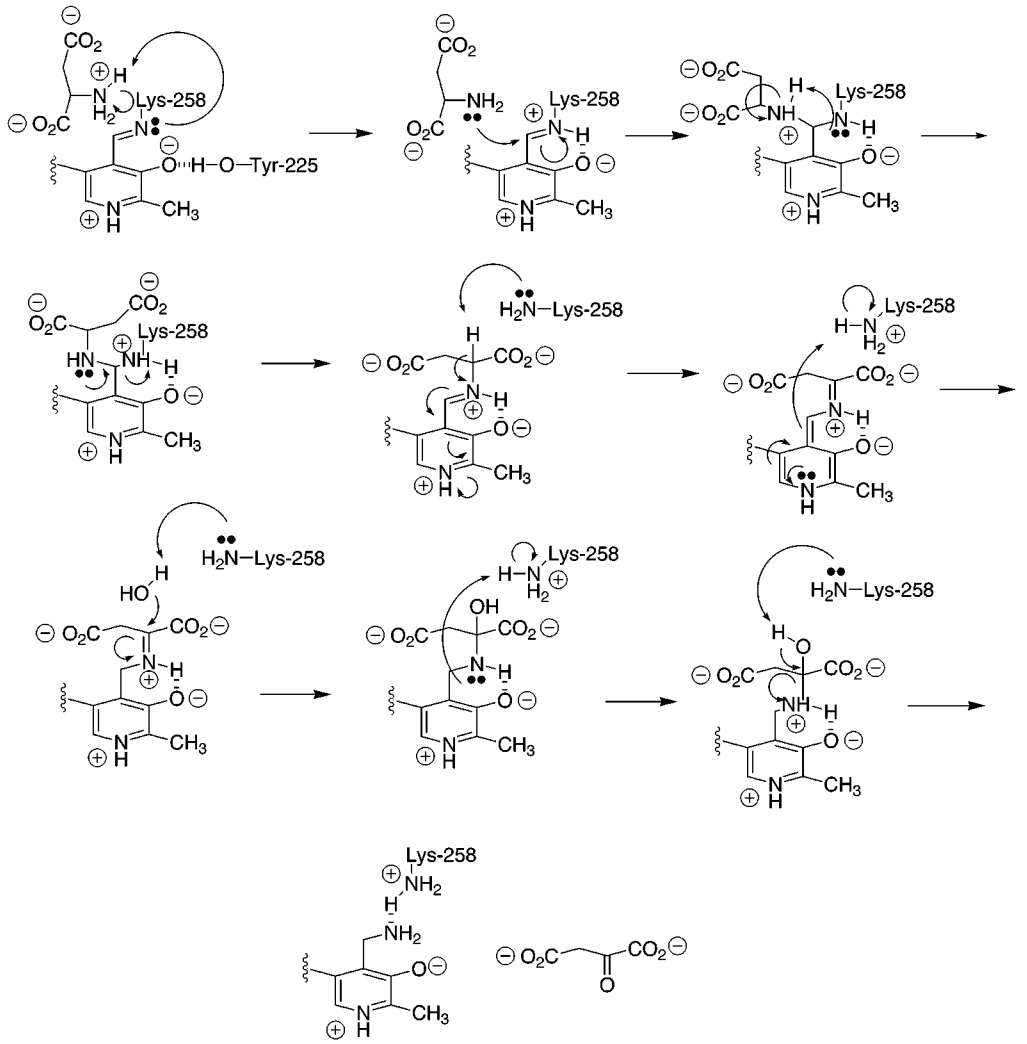


FIGURE 1: Chemical mechanism of AATase.

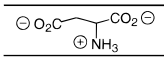
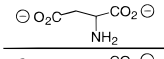
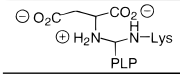
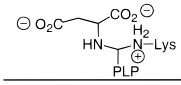
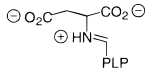
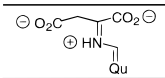
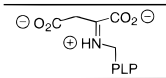
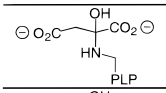
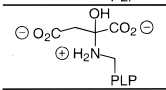
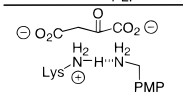
fractionation factors of the enzymic intermediates were estimated by use of the rules for the effects of substitutions on fractionation factors (*26*, *27*) or compared to similar experimentally determined equilibrium effects (*26*). The table of fractionation factors reveals the equilibrium isotope effects that will be observed for a particular intermediate if reaction of that intermediate contributes to the rate limitation.

Heavy atom isotope effects at C-3 and at the amino nitrogen of aspartate were also considered in conjunction with the table of fractionation factors to identify the rate-determining steps. The isotope effects at C-3 are the simplest to interpret as no primary effects are possible. In oxaloacetate, the fractionation factor of C-3 is reduced compared to aspartate due to hyperconjugation (*26*). Similar hyperconjugation could be present in any enzymic intermediate with a double bond at C-2. The small, normal ^{13}C effects observed at C-3 are therefore due to contributions of OAA dissociation, ketimine hydrolysis, or perhaps even reprotonation of the quinonoid intermediate to the rate limitation. The size of the observed effect is significant when compared to the size of the full equilibrium effect between aspartate and oxaloacetate. The contributions of the quinonoid intermediate, ketimine, and/or the noncovalent OAA·PMP complex to the rate must also be significant.

The ^{15}N isotope effect is sizable and could be due to either a primary effect or an equilibrium effect with a species exhibiting a lower fractionation factor compared to the protonated amino acid. However, none of the steps that would exhibit a primary ^{15}N effect could also exhibit the observed ^{13}C effect at C-3. The ^{15}N effect is therefore probably due to partially rate-determining product release, for which there is evidence from viscosity studies with the *E. coli* AATase (*16*). In the noncovalent complex with oxaloacetate, the nitrogen is bonded to C-4′ of the pyridoxal cofactor. Depending on the location of the proton that is shared between PMP and Lys-258, the equilibrium isotope effect between protonated aspartate and PMP is between 1.0047 and 1.0210. The ketimine and external aldimine intermediates have ^{15}N fractionation factors only slightly higher than that of protonated aspartate. If reaction of the OAA·PMP complex, the ketimine, and the external aldimine all contribute to the rate limitation, then the large, normal ^{15}N isotope effect for formation of PMP will dominate the observed ^{15}N effect. The absence of a normal ^{13}C effect at C-2 from ketimine hydrolysis and $C_\alpha-H$ cleavage is due to the increased fractionation factors at C-2 in the ketimine and oxaloacetate. These contributions will cause an inverse effect, masking the primary effects.

The multiple isotope effect experiments can also be explained in terms of a model involving several partially rate-determining steps. With α-deuterated aspartate, proton abstraction will be slower and reaction of the external

Table 3: Fractionation Factors of Heavy Atoms in Intermediates of AATase

Intermediate	C-2[a]	C-3[a]	N[b]
 Aspartate	1.0000	1.0000	1.0333
 Michaelis Complex	0.9985	1.0000	1.0163
 Asp-protonated Diamine	1.0025	1.0000	1.0422
 Lys-protonated Diamine	1.0010	1.0000	1.0340
 External Aldimine	1.0000	1.0000	1.0360
 Quinonoid Intermediate	1.0060	0.9960	1.0360
 Ketimine	1.0060	0.9960	1.0360
 Neutral Carbinolamine	1.0095	1.0050	1.0350
 Protonated Carbinolamine	1.0110	1.0050	1.0432
 Bound Oxaloacetate	1.0074	0.9965	1.0123 to 1.0285

[a] Relative fractionation factors. [b] Absolute fractionation factors; aqueous $NH_3 = 1.0000$.

aldimine will make a greater contribution to the rate limitation. The ^{15}N isotope effect is diminished because the external aldimine has higher fractionation factors at this position than the ketimine or the PMP·OAA complex. The observed ^{13}C effect at C-2 should be increased due to $C_\alpha-D$ cleavage becoming more rate determining. However, ketimine hydrolysis is also subject to a primary ^{13}C effect. With α-deuterated aspartate, this step becomes less rate-determining, which will reduce the observed ^{13}C effect. These two effects appear to cancel one another exactly.

The small D_2O effect is probably due to the effect of solvent deuteration on ketimine hydrolysis. As ketimine hydrolysis becomes more rate-determining, the observed ^{13}C effect at C-2 should increase due to the primary effect from C−O bond formation. However, $C_\alpha-H$ cleavage will become less rate-determining if no deuteron is involved in this step. This will reduce the observed ^{13}C effect, and again the two effects appear to cancel one another. The ^{15}N fractionation factor of the ketimine is slightly higher than that of protonated aspartate. However, in D_2O the relative fractionation factors of these two species will be altered. With protonated aspartate, three protons are replaced by deuterons. With the ketimine, only one proton is replaced by a deuteron. The increased mass of deuterium causes an increase of about 0.005 to the fractionation factor of nitrogen for each proton that is replaced by a deuteron (*28*). The fractionation factor of protonated aspartate will therefore increase by more than that of the ketimine in D_2O. As a result, the ^{15}N effect is actually unchanged in D_2O even though the ketimine intermediate makes a greater contribution. The heavy atom and deuterium isotope effects are best explained by the contribution of $C_\alpha-H$ cleavage, ketimine hydrolysis, and oxaloacetate dissociation to the rate limitation, which is in accord with the results of spectroscopic and kinetic studies with *E. coli* AATase[2] (*14*, *29*).

The kinetic complexity of AATase unfortunately baffles attempts to demonstrate the quinonoid intermediate. The results with α-deuterated aspartate clearly show that more than one rate-determining step exhibits a ^{13}C effect at C-2. The fact that the ^{13}C effect is unchanged in D_2O might indicate that a quinonoid intermediate must be involved. If the proton shift were concerted, then deuteration of the solvent should preferentially slow ketimine hydrolysis and the proton shift, each of which exhibits a primary ^{13}C effect at C-2. However, interpretation of multiple isotope effect experiments involving D_2O is difficult due to subtle, unexpected events that can take place (*30*). In addition, with AATase there are numerous exchangeable protons in flight throughout the mechanism. Thus, in the absence of more conclusive results, such a claim is uncertain at best.

The heavy atom isotope effects with the Y225F mutant are somewhat easier to interpret. The lack of an α-deuterium isotope effect shows that C_α proton abstraction is not rate-determining. In addition, the D_2O effect is increased to 2.3, and there is no viscosity effect on the rate (*14*, *16*). These data suggest that ketimine hydrolysis is the major rate-determining step, while product dissociation is no longer significant. This is supported by the observation of a sizable ^{13}C effect at C-2 from formation of the C−O bond. The increased ^{13}C isotope effects at both C-2 and C-3 in D_2O are due to ketimine hydrolysis becoming more rate-determining. In D_2O, the ^{15}N fractionation factor of protonated aspartate is increased relative to that of the ketimine as described above (*28*). Therefore, the observed ^{15}N effect in D_2O is considerably more normal than in H_2O. At pH 9.0, the ^{13}C effects at both C-2 and C-3 are nearly identical to those in D_2O at pD 8.4. This indicates that ketimine hydrolysis is slightly slower relative to other steps at pH 9.0 than at pH 8.0. The reason for this is unknown. The heavy atom isotope effects with α-deuterated aspartate are unchanged as expected, since proton abstraction is not rate-determining in the Y225F mutant.

The slower relative rate of ketimine hydrolysis in the Y225F mutant compared to that in the wild-type enzyme shows that Tyr-225 is important for catalysis of that step. The hydrogen bond between Tyr-225 and the 3′-O in the internal aldimine is clearly important to lowering the pK_a of the imine nitrogen, which is increased by 1.7 in the mutant (*12*, *13*). This hydrogen bond is broken in the enzymic intermediates up to the quinonoid intermediate because of tilt of the cofactor ring toward the substrate, but in the ketimine it is possible that the cofactor relaxes enough to re-form the hydrogen bond (*6*). The hydrogen bond to the ketimine could increase the electrophilicity of the ketimine or help to bind a water molecule in the correct position for hydrolysis (*15*).

[2] A reviewer has questioned whether protonation of the quinonoid intermediate by Lys-258 could be the sole rate-limiting step. This is unlikely, as the ^{15}N fractionation factor of the imino nitrogen in the quinonoid intermediate is higher than that of aspartate, and there will be no primary effect at the nitrogen during reprotonation. Rate-limiting reprotonation of the quinonoid therefore predicts an inverse ^{15}N effect, while the observed effect is normal.

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