^{2}H, ^{13}C, and ^{15}N Kinetic Isotope Effects on the Reaction of the Ammonia-Rescued K258A Mutant of Aspartate Aminotransferase†

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ABSTRACT: Deuterium isotope effects at C2 of aspartate and heavy atom isotope effects at C2, C3, and the amino group of aspartate were determined for the reaction of the lysine-258 to alanine mutant of *Escherichia coli* rescued with exogenous ammonia. We were able to calculate an ^{15}N intrinsic isotope effect of 1.034. The intrinsic ^{13}C isotope effect at C3 is 1.0060, and the ^{13}C isotope effect at C2 is 1.0016. These isotope effects reveal that collapse of the carbinolamine (or *gem*-diamine) to give the final product is the rate-determining step in this system. Furthermore, these results indicate that lysine-258 is critical to the catalysis of the final breakdown to give product, and in fact this step is more strongly affected by mutation of lysine-258 than the deprotonation of the external aldimine.

Aspartate aminotransferase (AATase)[1] catalyzes the pyridoxal phosphate (PLP)-dependent transamination of aspartate with 2-ketoglutarate to give glutamate and oxaloacetate. Numerous kinetic and model experiments, and especially the determination of the crystal structure, have led to a detailed chemical understanding of the roles of the enzymic side chains in the active site (*1*−*3*). The role of one critical side chain, Lys-258, has been further studied by site-directed mutagenesis in a number of experiments (*4*−*8*).

Lys-258 has been identified as the side chain that catalyzes the deprotonation of C2 of aspartate, which leads to transamination. The importance of this step has been demonstrated by mutation of Lys-258, which results in an enzyme that cannot catalyze this step (*9*). However, mutants lacking Lys-258 can be rescued by the inclusion of amines or ammonia in the reaction (*4*). The rescued enzyme is then able to carry out the critical deprotonation step, showing that it is the chemical reactivity of the Lys-258 side chain that is important to the mechanism and explaining its role. However, the role of Lys-258 in other steps of the mechanism is not as clear. In this work, we report the results of kinetic isotope effect experiments with the ammonia-rescued K258A mutant of AATase.

Kinetic isotope effects are an excellent tool to use to understand the effect of mutation of Lys-258 on the chemistry of AATase since they give information about any step of the mechanism that is or becomes rate-determining. On the basis of previous determinations of deuterium isotope effects with the ammonia-rescued K258A AATase, we had hoped to study the transition state of the deprotonation of C2 of aspartate, as the nature of this transition state has been the subject of much research (*10*, *11*). Multiple heavy atom isotope effects on porcine heart wild-type AATase at pH 7.5 revealed that $C_{\alpha}-H$ cleavage, ketimine hydrolysis, and oxaloacetate dissociation all contribute to the rate limitation, and for the *Escherichia coli* Y225F mutant, ketimine hydrolysis is the major rate-limiting step (*11*). However, our isotope effect data showed that mutation of Lys-258 is even more detrimental to catalysis of a step following deprotonation of C2 and reveals the importance of Lys-258 to that later step.

MATERIALS AND METHODS

Materials. Aspartic and 2-ketoglutaric acids were from Aldrich. Oxidized glutathione was from Acros. Glucose 6-phosphate, NADP, NADH, *n*-octane, malic enzyme, aspartate aminotransferase, glutathione reductase, glucose-6-phosphate dehydrogenase, pyridoxine, and ninhydrin spray reagent were from Sigma. Sodium hypobromite was prepared by the 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. Malate dehydrogenase from *E. coli* was purified according to the previously published scheme (*12*). The purification of K258A aspartate aminotransferase was performed as previously described (*13*). K258A AATase, as isolated, contained approximately equal amounts of PLP and PMP bound at the active site. Reconstitution of the apoenzyme with PLP was performed as described elsewhere (*8*).

2-d-Aspartic Acid. Aspartic acid (800 mg) and dibasic potassium phosphate (180 mg) were dissovled in 30 mL of D_2O. The solution was titrated to pD 7.9 with KOD. Oxaloacetic acid (5 mg) and aspartate aminotransferase (50 units) were added. The reaction mixture was sealed and stirred for 48 h. 2-*d*-Aspartate was synthesized by the sterospecific incorporation of deuterium from the exchangeable hydrogens of Lys-258 into the substrate during enzymic

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[1] Abbreviations: AATase, aspartate aminotransferase; K258A, *Escherichia coli* aspartate aminotransferase with lysine 258 replaced by alanine; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; OAA, oxaloacetate.

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turnover. The reaction was monitored by the disappearance of the 2-proton resonance at 3.9 ppm by ^{1}H NMR. The enzyme was removed by Amicon filtration (10000 MW cutoff). 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 2-*d*-aspartate eluted as soon as the resin had been converted to the ammonium ion form. Fractions containing 2-*d*-aspartate were repeatedly evaporated to dryness, taken up in a small portion of water, and then lyophilized.

Characterization of AATase under Reaction Conditions. Each assay contained sodium pyrophosphate (2.2 mg) and 35 μM AATase. The ammonia concentration was varied from 10 mM to 2 M. The solution was titrated to pH 9.0, and the UV−vis spectrum was obtained from 200 to 800 nm.

Deuterium Isotope Effects. Deuterium isotope effects were measured by direct comparison of kinetic constants for 2-deuterated and undeuterated aspartate. The concentrations of the aspartate solutions were determined by end-point assay using an excess of 2-ketoglutaric acid and NADH with transaminase and malate dehydrogenase. Reactions were run in 50 mM sodium pyrophosphate, pH 9.0, and contained 0.1 mM NADH, saturating 2-ketoglutaric acid (0.5 mM), and 0.3 M ammonia. The aspartic acid concentration was varied from 0.1 to 100 μM. An excess of malate dehydrogenase over the transaminase was present. The initial rates were determined by observing the coupled oxidation of NADH at 340 nm.

Heavy Atom Isotope Effects. Aspartic acid (27 mg), 2-ketoglutaric acid (15 mg), glucose 6-phosphate (45 mg), NADH (5 mg), and sodium pyrophosphate (168 mg) were dissolved in 15 mL of water containing 1.5 mL of 3 M ammonia, and the solution was titrated to pH 9.0. The solution was filtered through a 0.22 μm syringe filter, and glucose-6-phosphate dehydrogenase (25 units), malate dehydrogenase (50 units), and aspartate aminotransferase (12 units) were added. The solution was allowed to stir at 4 °C 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 malic enzyme assay. Fractions containing the amino acids were located by spotting on a TLC plate followed by treatment with ninhydrin reagent. Malate eluted at the void volume by the NaCl wash, while aspartate was eluted by the citrate just prior to the glutamate.

Fractions containing either the aspartate or the glutamate were pooled, the volume was reduced by rotory evaporation, and the solution was transferred to a long-necked 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 the solutions were clear. The solution was placed on a steam still and was made basic by the introduction of an excess of 13 N NaOH. The ammonia produced was collected in 0.1 N sulfuric acid and quantified by Nessler's reagent. The ammonia was oxidized to nitrogen in vacuo by sodium hypobromite.

In addition, the ^{15}N abundance in the unreacted aspartate was determined by direct combustion. At least 30 μmol of aspartate was placed in a quartz tube (25 cm, 7 mm i.d., 9 mm o.d.) along with 2.5 g of CuO (previously baked at 850 °C for 1 h), 500 mg of copper powder (previously heated to 550 °C for 15 min under an atmosphere of hydrogen and cooled to room temperature), a 4 mm square piece of silver foil, and 500 mg of diatomaceous earth. The tube was evacuated under high vacuum and sealed. It was heated at 850 °C for 2 h, then cooled over 1 h to 550 °C, and held at that temperature for 8 h. The tube was cooled and then cracked open on a vacuum line. The nitrogen gas generated from the sodium hypobromite oxidation and the direct combustion procedures, respectively, was distilled on high vacuum lines through dry ice/2-propanol and liquid nitrogen traps and was then collected on molecular sieves chilled with liquid nitrogen. The purified nitrogen gas was analyzed by isotope ratio mass spectrometry.

Fractions from the cation-exchange column containing malate were evaporated to approximately 10 mL and were acidified with 3 drops of concentrated sulfuric acid. These solutions underwent continuous extraction with ethyl acetate for 36 h. The ethyl acetate was removed, and the residue was repeatedly taken up in 0.01 N HCl and evaporated to dryness to remove acetic acid. To the purified malate was added oxidized glutathione (92 mg), dibasic potassium phosphate (153 mg), NADP (2 mg), and 15 mL of 3 mM $MgCl_2$. Malic enzyme (1 unit) and glutathione reductase (5 units) were added, and the reaction was stirred at 4 °C for 3 h. Hydrogen peroxide (0.3 mL, 3%) was added, and the solution was stirred at 4 °C for 12 h. The solution was made acidic with 5 drops of concentrated sulfuric acid and underwent azeotropic distillation with *n*-octane (600 mL). Four drops of 5 N KOH was placed into 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 C2 of acetate or combustion to CO_2 as described elsewhere (*14*). The methane was combusted over CuO to yield CO_2. The isotopic composition of the CO_2, as well as the N_2, was determined by using a Finnigan Delta E isotope ratio mass spectrometer.

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

$$\text{isotope effect} = \frac{\log(1-f)}{\log[(1-f)(R_s/R_o)]} \qquad (1)$$

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

$$\text{isotope effect} = \frac{\log(1-f)}{\log[1-f(R_p/R_o)]} \qquad (2)$$

RESULTS

Characterization of K258A in the Presence of Ammonia. The PLP cofactor in the active site of K258A can exist as the aldehyde or as the imine in the presence of ammonia.

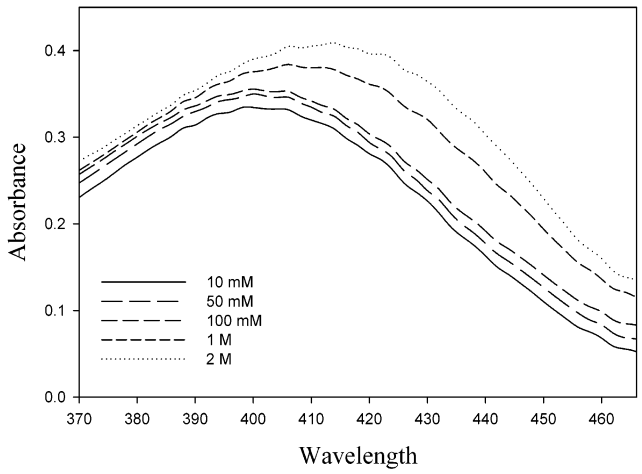


FIGURE 1: Spectra of K258A aspartate aminotransferase in the presence of 10 mM to 2 M ammonia. The enzyme concentration was 35 μM, buffered in 50 mM sodium pyrophosphate, pH 9.0. Ammonia concentrations were 0.01, 0.05, 0.1, 1, and 2 M.

To determine if the cofactor was in the aldehyde or imine form, the UV−vis spectrum of the enzyme was obtained at pH 9.0 in the presence of ammonia from 10 mM to 2 M (Figure 1). In the absence of ammonia, the spectrum showed a peak at 400 nm, which shifted to 414 nm with 2 M ammonia. These wavelengths are consistent with a shift from the aldehyde form of PLP to the imine form (*15*). For all kinetic experiments performed in this work, the cofactor in free enzyme was predominantly in the aldehyde form.

Deuterium Isotope Effects. The isotope effect resulting from deuteration of the 2-carbon of aspartate on V/K_{asp} was determined for the reaction of the ammonia-rescued K258A mutant by the method of direct comparison. Initial rates were determined for the reaction of either aspartate or 2-*d*-aspartate at a range of concentrations surrounding the K_m of these substrates. The data were analyzed using an Enzyme Kinetics software package, adapted from the programs of Cleland (*16*), and the isotope effect on V/K_{asp} was determined to be unity.

Heavy Atom Isotope Effects. Heavy atom isotope effects at the amino nitrogen of aspartate and at C2 and C3 of aspartate were determined by the method of internal competition. The amount of ^{13}C or ^{15}N in the products of the transaminase reaction was determined and compared to the amount of heavy atom in the unreacted starting material. For the ^{15}N isotope effects, the amount of heavy isotope in the remaining substrate after partial reaction could also be determined, allowing two independent determinations of this isotope effect. Transaminase reactions were run in the presence of NADH and malate dehydrogenase so that the oxaloacetate produced by the transaminase was removed, making the reaction irreversible. Back-reaction of product and equilibration of substrates and products were therefore impossible, ensuring that the observed isotope effects were kinetic and not equilibrium ones.

To determine heavy atom isotope effects, the substrate and product molecules were degraded such that the nuclei of interest were converted to N_2 or CO_2 without isotopic fractionation. The CO_2 and N_2 gas produced was then purified on high vacuum lines and analyzed by isotope ratio mass spectrometry.

Table 1: Isotope Effects with Ammonia-Rescued K258A AATase[a]

^{2}H, C2	0.999 ± 0.02
^{13}C, C2	0.9930 ± 0.0017
^{13}C, C3	1.0007 ± 0.0005
^{15}N (uncorrected)	1.0143 ± 0.0004
^{15}N (corrected for deprotonation of aspartate at pH 9.0)	1.0168 ± 0.0004

[a] All isotope effects are on V/K_{asp}.

For nitrogen effects, degradation of the aspartate substrate or glutamate product simply involved Kjeldahl digestion of the amino acids followed by hypobromite oxidation of the resulting ammonia. The carbon effects, however, required a more involved process. Malate from the transamination reactions was oxidatively decarboxylated by malic enzyme, resulting in the loss of C4 as CO_2. This reaction was driven to completion to avoid fractionation by coupling the production of NADPH to glutathione reductase in the presence of oxidized glutathione. The amount of malate present was determined by end-point assay with malic enzyme, and the reactions were judged to be complete when an equivalent amount of pyruvate was found with lactate dehydrogenase end-point assay. The pyruvate was then oxidized to acetate by chemical oxidation with peroxide with loss of C1 as CO_2. This reaction was judged to be complete when no pyruvate was detectable with lactate dehydrogenase. The acetate was divided into two portions. Acetate degradation of one portion resulted in complete conversion of the methyl carbon of acetate to methane, which was combusted to CO_2 to reveal the isotopic abundance of ^{13}C at C3 of the oxaloacetate product of the transamination reaction. The remaining portion of the acetate sample was directly combusted to give CO_2 from both C2 and C3 of the original oxaloacetate product. The isotopic abundance at C3, determined above, was subtracted from the combined ^{13}C abundance of C2 and C3 to yield the ^{13}C content of C2 alone. In all cases, the fraction of reaction was controlled by the amount of 2-ketoglutarate present and was also checked by malic enzyme end-point assay of the completed reaction.

The ^{15}N abundance in the unreacted aspartate was determined by direct combustion of the aspartate, while the ^{13}C abundances at C2 and C3 of unreacted aspartate were determined by driving transamination of the aspartate to completion by using an excess of 2-ketoglutarate coupled with reduction of the oxaloacetate product to malate. The isotopic abundances of the products, and of ^{15}N in aspartate after partial reaction, were compared to the isotopic abundances in the unreacted starting materials to determine the isotope effects on V/K_{asp}, which are shown in Table 1.

DISCUSSION

The chemical mechanism employed by the wild-type AATase is shown in Figure 2. The amino acid substrate transfers a proton to the unprotonated internal aldimine and is then able to act as a nucleophile, releasing the Lys-258 side chain in a transaldimination reaction (*17*). The subsequent role of Lys-258 has been the subject of numerous studies (*4*−*8*), and it is well established that it abstracts a proton from the 2-carbon of aspartate and reprotonates C4′. The resulting ketimine is hydrolyzed, presumably with general base assistance from Lys-258, although its role in

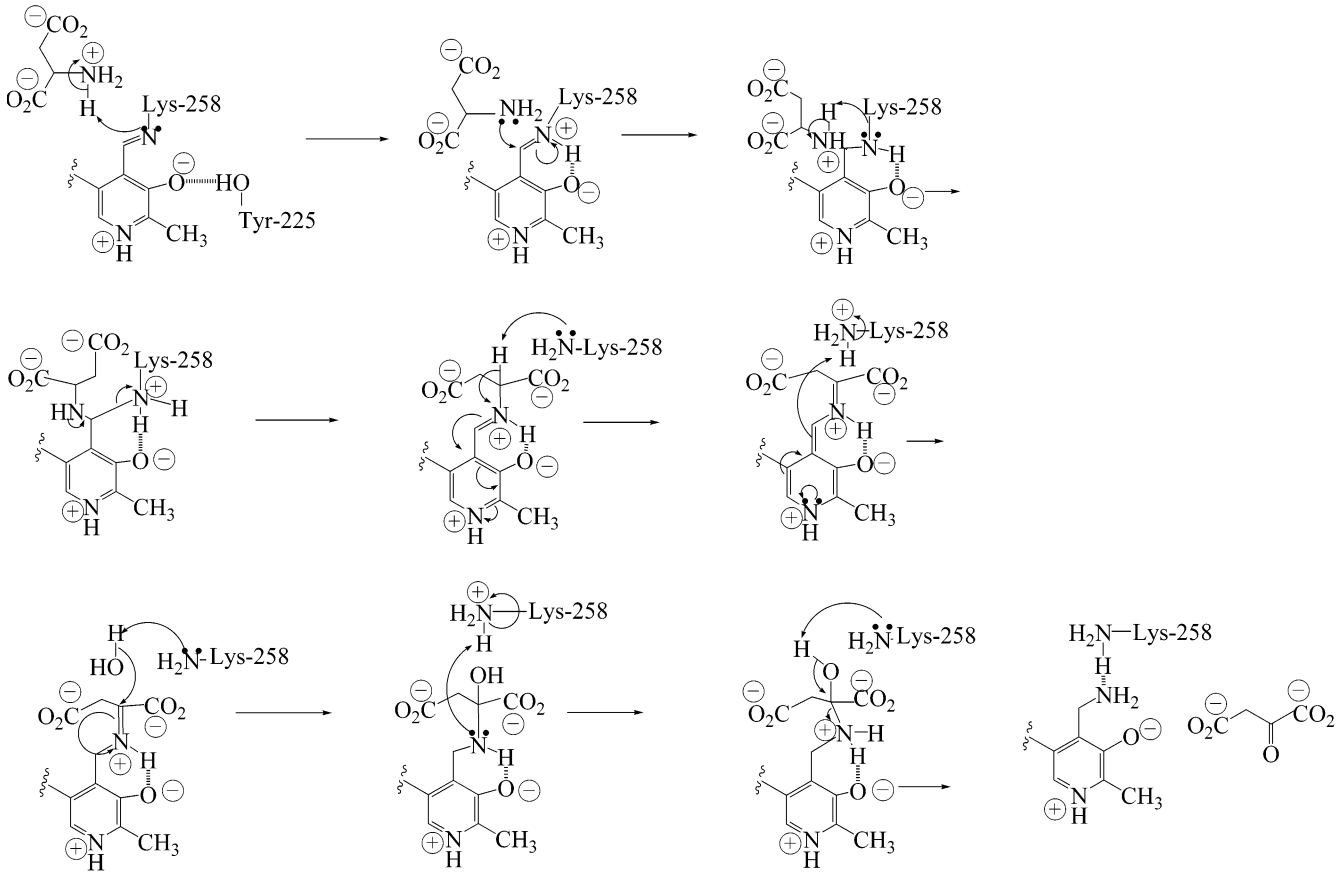


FIGURE 2: Mechanism of wild-type aspartate aminotransferase.

this step is less clear. Ketimine hydrolysis yields a product keto acid and the PMP form of the enzyme (*9*).

Mutation of Lys-258 to alanine results in a reduction of activity by a factor of 10^6-10^8 (*7*). This tremendous loss of activity shows the importance of Lys-258 to the catalytic mechanism of the transaminase. However, the mutant enzyme can be rescued if ammonia or amines are added to the reaction (*4*). The ability to rescue the K258A mutant with amines was exploited to gain further insight into the role of Lys-258 (*8*).

The chemical mechanism of ammonia-rescued K258A differs slightly from that of the wild-type in that the free enzyme cannot contain an internal aldimine with Lys-258. The cofactor must instead exist as the free aldehyde. However, in the presence of ammonia, the active form of the enzyme could instead be the imine of PLP. On the basis of the titration of the PLP enzyme with ammonia, we conclude that the cofactor was predominantly in the aldehyde form, although we cannot rule out that a small portion of enzyme in the imine form was responsible for part or all of the catalysis. The K258A mutation prevents internal aldimine formation and thus destabilizes the reactants (PLP−enzyme + aspartate), thereby increasing the affinity for the amino acid substrates by 10^5-fold. The proposed chemical mechanism up to the ketimine for ammonia-rescued K258A in the aldehyde form is shown in Figure 3, while the proposed mechanism up to the ketimine for the enzyme in the imine form is shown in Figure 4.

Wild-type c-AATase has been shown to catalyze the exchange of Cα hydrogens with solvent. For the wild-type enzyme-catalyzed exchange reaction in D_2O the ratio of exchange into initially unlabeled aspartate to product formation was 4.0, while the ratio of exchange into 2-*d*-aspartate in H_2O was 2.6 (*18*). For the K258A mutant rescued with ammonia, we found 8% consumption of aspartate and 36% exchange of deuterium into unlabeled aspartate from D_2O or a ratio of exchange to product formation of 4.5. Since ketimine hydrolysis seems to be slowed in D_2O, we presume that with the ammonia-rescued mutant the exchange ratio in H_2O with 2-*d*-aspartate may be ~3 rather than 4.5.

A simplified enzymatic mechanism for AATase can be written as

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EP \xrightarrow{k_5} E + P \qquad (3)$$

where E is the enzyme, A is aspartate, EA is the enzyme/external aldimine complex, EP is the enzyme/ketimine complex, P is oxaloacetate, k_1 is the rate constant for external aldimine formation, k_3 is the rate constant for the 1,3-protropic shift converting external aldimine and ketimine forms of the enzyme substrate complex, and k_5 is the rate constant for ketimine hydrolysis and product release.

The velocity for the forward reaction is given by eq 4, and the rate of the exchange reaction is defined by eq 5.

$$v_f = k_5(EP) \qquad (4)$$

$$v_{ex} = k_4\left(\frac{k_2}{k_2 + k_3}\right)(EP) \qquad (5)$$

The ratio of exchange to the forward reaction is given by the equation

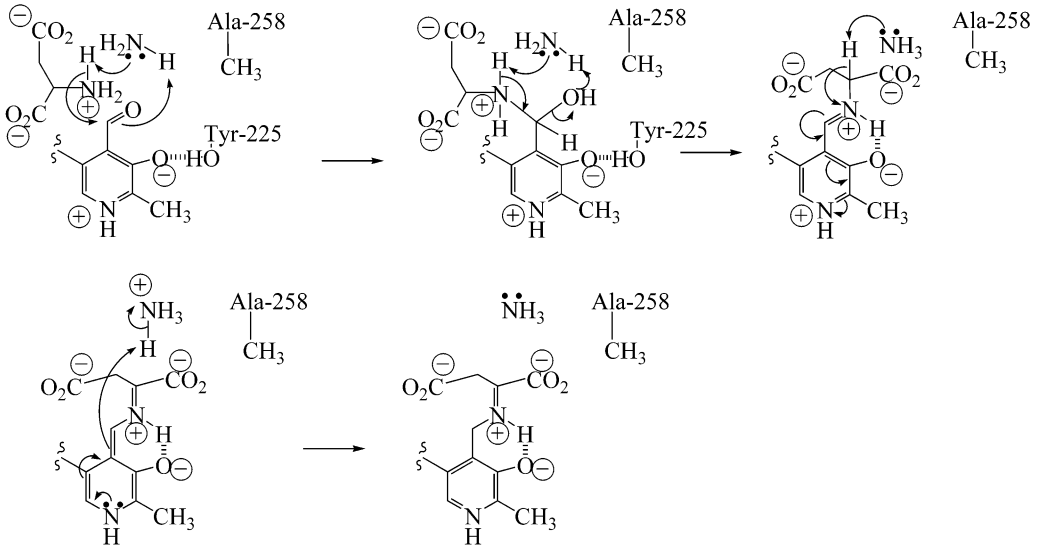


FIGURE 3: Proposed mechanism up to the ketimine for ammonia-rescued K258A in the aldehyde form.

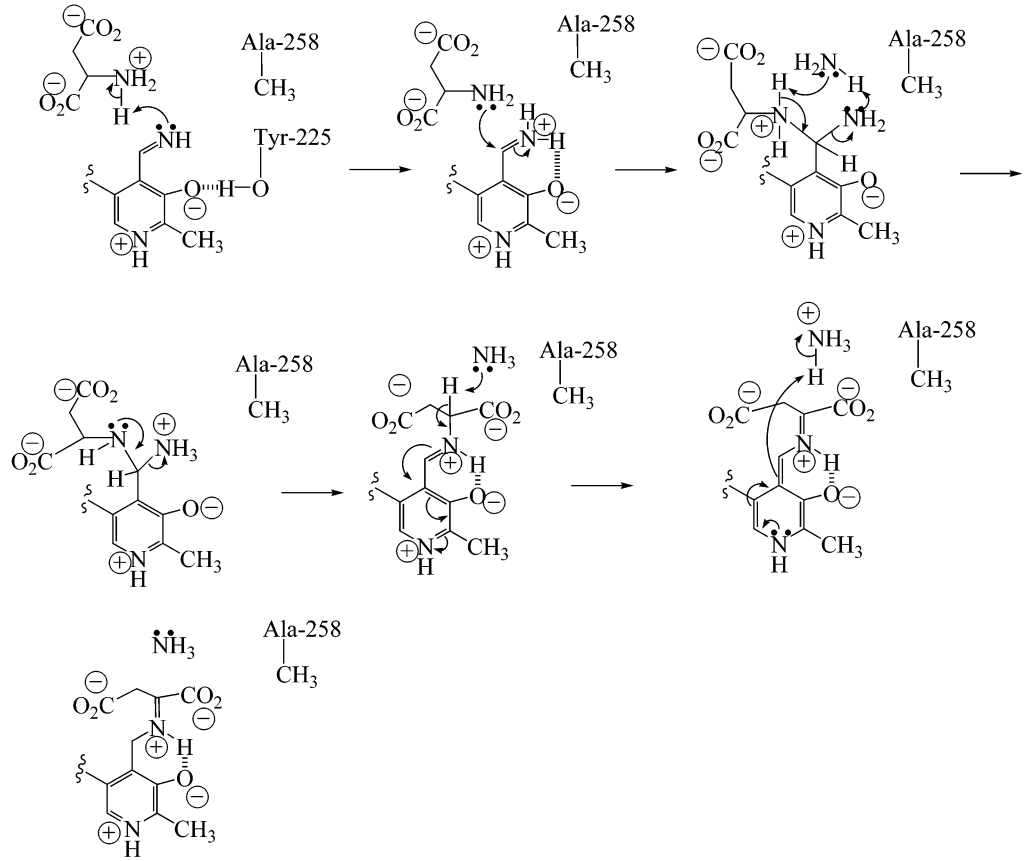


FIGURE 4: Proposed mechanism up to the ketimine for ammonia-rescued K258A in the imine form.

$$\frac{\text{deuterium exchange}}{\text{forward reaction}} = \frac{\left(\frac{k_4}{k_5}\right)}{\left(1+\frac{k_3}{k_2}\right)} = \frac{c_r}{1+c_f} = x \quad (6)$$

where c_r (reverse commitment) is k_4/k_5 and c_f (forward commitment) is k_3/k_2.

When deuterium isotope effects were determined in a single turnover experiment using 2-*d*-aspartate in the reaction of K258 rescued by ammonia, a primary isotope effect of 5.2 was seen (*19*).[2] Thus in the K258A mutant rescued by ammonia the proton abstraction is rate-limiting for steps prior to ketimine formation. However, we determined a $^{D}(V/K_{asp})$ of unity for the ammonia-rescued K258A mutant in a multiple turnover study. The discrepancy between the measured isotope effects is a result of the difference in the way the deuterium isotope effects were measured. In the

[2] The deuterium isotope effect with *E. coli* wild-type enzyme was reported as 2.2 (*20*), but this was the ratio of rates of 550 s^{-1} for unlabeled and 250 s^{-1} for labeled aspartate. Since rates of this size cannot be accurately determined by stopped flow (the mixing time is several milliseconds, and the dead time of the instrument is similar), this ratio is certainly too low.

single turnover experiment, the isotope effects were determined by monitoring k_3, that is, the disappearance of the UV absorption peak corresponding to the external aldimine and the appearance of the UV absorption peak corresponding to the ketimine. In our multiple turnover experiment, we measured the rate of oxaloacetate formation. Thus, in our case the isotope effects involved all steps up to the irreversible release of oxaloacetate.

On the basis of the single turnover deuterium isotope effect of 5.2 on k_3 (*19*), we expected to see an isotope effect in the multiple turnover experiment. However, as mentioned above, we measured an isotope effect of unity. The deuterium effects from both the single turnover experiment (*19*) and our multiple turnover study can be utilized to estimate the size of the reverse commitment (c_r). To observe a deuterium isotope effect of unity in the multiple turnover experiment, c_r must be very large. Then the ketimine will return to the aldimine many times before it is hydrolyzed. Since the deuterium label is transferred to ammonia and thus presumably lost to the solvent during the first conversion of aldimine to ketimine, the interconversion of these two species prior to ketimine hydrolysis will involve rate constants k_3 and k_4 for unlabeled species and will come essentially to equilibrium.

The washout of label from deuterated aspartate results from conversion of the external aldimine to ketimine followed by complete reversal of the reaction to yield protiated aspartate. Since washout of label is faster than product formation, k_2 is greater than k_5, while the destabilization of the ground state of the enzyme discussed above decreases k_2 relative to k_3 and k_4. The aldimine and ketimine will therefore interconvert many times before forming product, and our deuterium isotope effect will equal the average effect of one conversion of a deuterated aspartate and many conversions of protiated aspartate, reducing the observed value to unity. A c_r of 200 would yield an isotope effect of 1.004, and a c_r of 400 would give 1.002. Thus c_r must be in this range for us to have seen no deuterium isotope effect. With commitments of this size, the value of x in eq 1 is simply c_r/c_f, which is 4.5 for the rescued mutant in D_2O and estimated to be ~3 in H_2O (*18*).

When the above analysis was applied to the wild-type enzyme, the exchange/product ratio in H_2O was 2.6 and the observed deuterium isotope effect on V/K_{asp} was 1.9 (*18*). Interconversion of the internal and external aldimines by transimination is fast and roughly isoenergetic, so the forward commitment for the WT enzyme is expected to be small (that is, $k_2 \gg k_3$). Setting $c_f = 0$ and equating the reverse commitment with the exchange/product ratio of 2.6, we can then write

$$1.9 = \frac{^{D}k_3 + 2.6}{1 + 2.6} \quad (7)$$

which gives $^{D}k_3 = 4.24$, a value probably not significantly different from the directly determined value of 5.2. If we assume 5.2 as the value of $^{D}k_3$ and substitute $2.6(1 + c_f)$ for c_r (from eq 6) and assume a finite value for c_f, then $c_f = 0.30$ and $c_r = 3.38$.

A reaction coordinate diagram shows the potential energy of a system along the reaction coordinate. The peaks on the reaction coordinate pathway represent transition state, while

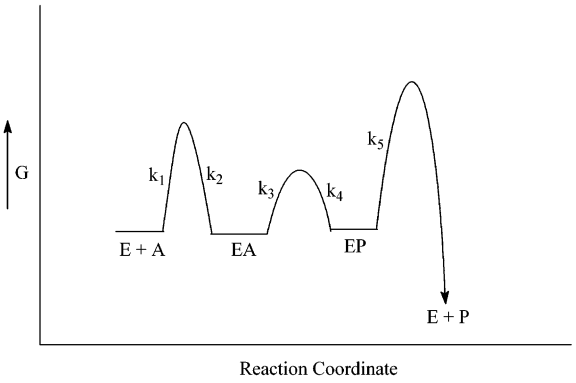


FIGURE 5: Free energy profile for the ammonia-rescued mutant. The level of substrate has been set equal to the dissociation constant for EA. The product is assumed to be absent. The deuterium isotope effect is unity, while the intrinsic ^{15}N isotope effect is 1.034 ± 0.002. The intrinsic ^{13}C isotope effects are 1.0016 ± 0.004 for C2 and 1.0060 ± 0.0007 for C3. The forward commitment (k_3/k_2) is at least 400, and the reverse commitment (k_4/k_5) is at least 90.

depressions represent intermediates. The heights of barriers are inversely proportional to the rate constants for interconversion of intermediates. From the results of the deuterium isotope effects and exchange studies, along with the calculated values for c_r and c_f, we were able to construct a free energy vs reaction coordinate profile for the rescued mutant (Figure 5). Since both c_r (k_4/k_5) and c_f (k_3/k_2) are large, we can conclude that k_3 and k_4 are large compared to k_2 and k_5. Therefore, the hydrolysis of the ketimine, represented by k_5, is the rate-determining step instead of the 1,3-prototropic shift represented by k_3.

Upon formation of the ketimine, the reaction of ammonia-rescued K258A could follow two paths to formation of oxaloacetate and PMP. In the wild-type enzyme, Lys-258 is thought to catalyze attack of water on the ketimine to give a carbinolamine. In the reaction of K258A, ammonia could replace K258 and act as a general base catalyst in the same way. The chemical mechanism from the ketimine would then be the same as that of the wild-type enzyme with ammonia in place of K258A. It is also possible that ammonia itself reacts with the ketimine to give a *gem*-diamine instead of a carbinolamine. The chemical mechanism for this pathway is shown in Figure 6. A critical difference between this pathway and that of the wild type is that no general base catalyst would be present to catalyze breakdown of the *gem*-diamine to give the imine of oxaloacetate.

For the simplified mechanism (eq 3) the ^{15}N and ^{13}C isotope effects on V/K are given by eqs 8 and 9. Note that

$$^{15}(V/K) = \frac{^{15}K_{eq1}\,^{15}K_{eq3}\,^{15}k_5 + \dfrac{^{15}K_{eq1}\,^{15}k_3}{c_r} + {}^{15}k_1\dfrac{c_f}{c_r}}{1 + \dfrac{1}{c_r}(1 + c_f)} = \frac{^{15}K_{eq1}\,^{15}K_{eq3}\,^{15}k_5 + \dfrac{^{15}k_1}{x}}{1 + \dfrac{1}{x}} \quad (8)$$

because c_r is so large, as deduced above, the second numerator and denominator terms are negligible, and thus the equations can be written with $1/x$ as the only significant

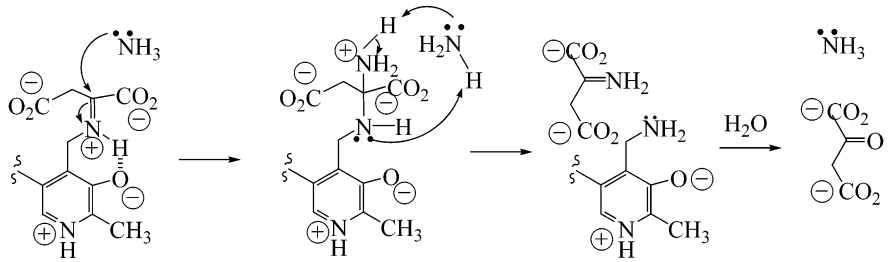


FIGURE 6: Proposed mechanism in which ammonia reacts with the ketimine to form a *gem*-diamine instead of a carbinolamine.

$$^{13}(V/K) = \frac{^{13}K_{eq1}\,^{13}K_{eq3}\,^{13}k_5 + \dfrac{^{13}K_{eq1}\,^{13}k_3}{c_r} + {}^{13}k_1\dfrac{c_f}{c_r}}{1 + \dfrac{1}{c_r}(1 + c_f)} = \frac{^{13}K_{eq1}\,^{13}K_{eq3}\,^{13}k_5 + \dfrac{^{13}k_1}{x}}{1 + \dfrac{1}{x}} \quad (9)$$

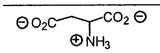
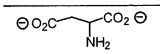
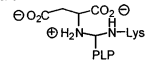
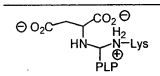
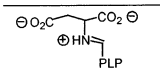
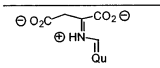
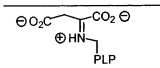
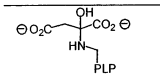
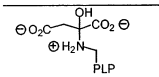
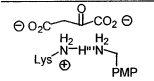
commitment. Because the ^{15}N and ^{13}C experiments were done in H_2O, we will use $x = 3$, as discussed above. The observed value of $^{15}(V/K)$ is 1.0168 (Table 1). The value of $^{15}K_{eq3}$ is 0.9931, which is the ratio of the fractionation factors of the aspartate external aldimine and the protonated carbinolamine intermediate, while $^{15}K_{eq1}$ is 0.9956, which is the ratio of the fractionation factor for an amino acid at pH 9.0 and the external aldimine (Table 2). A value of 3 was used for x, as discussed above. Equation 8 gives a value of $^{15}k_5$ of 1.034 ± 0.002. The same analysis on eq 9 for the ^{13}C isotope effects at both C2 and C3 of aspartate gives the values of $^{13}k_5$ for C2 of 1.0016 ± 0.0004 and for C3 of 1.0060 ± 0.0007. If $x = 4.5$, there is no significant difference in the$^{13}k_5$ value for C3 or the$^{15}k_5$ value. However, the $^{13}k_5$ for C2 value becomes 1.0024.

The size of the primary isotope effect of 1.032 indicates that a bond to the nitrogen of the amino group of aspartate is being broken in the transition state. This can be further corroborated by comparing the observed ^{15}N isotope effect (1.032) to the fractionation factors in Table 2 (*11*). Table 2 reveals that the only intermediates where the nitrogen is less stiffly bonded than in aspartate are the Michaelis complex and the PMP form of the cofactor prior to final release of the oxaloacetate product. Reaction of the Michaelis complex to give the ketimine is known not to be rate-determining from the previous deuterium isotope effect studies. Although product release appears to be slow in the wild-type enzyme (*11*, *21*, *22*), the ^{13}C isotope effects are not consistent with slow product release in the ammonia-rescued K258A mutant, as discussed below. Therefore, both the large intrinsic effect and the comparison of the observed effect to the fractionation factors suggest that a bond to the aspartate amino group nitrogen is broken during the rate-determining step of the ammonia-rescued K258A reaction. The collapse of the carbinolamine (or the *gem*-diamine in Figure 4) is the step implicated by this result as being rate-determining. Thus the EP complex in eq 3 is not the ketimine but is, in fact, the protonated carbinolamine.

The isotope effect at C2 of aspartate could include both the effects of changes in the stiffness of the bonding and bond formation or cleavage at C2. During proton abstraction from the external aldimine and reprotonation to give the ketimine, C2 is about as stiffly bonded as in aspartate (Table 2), so the only observed effects would be from bond cleavage. Proton abstraction cannot be the overall rate-determining step, since cleavage of the C2−H bond would result in a large normal ^{13}C isotope effect. The intrinsic isotope effect at C2 was found to be 1.0016. During the collapse of the carbinolamine (or *gem*-diamine), a bond is cleaved at C2, but this effect will be offset by bond formation to the oxygen atom, and thus there will essentially be no change in the bond order to the C2 of aspartate. The intrinsic effect of near unity at C2 is consistent with rate-determining collapse of the carbinolamine (or *gem*-diamine) to give oxaloacetate.

The isotope effect at the methylene (C3) carbon of aspartate is useful in describing the bonding at the adjacent C2 carbon of aspartate without the possibility of the additional factor of bond formation or cleavage, as no such

Table 2: Fractionation Factors of Heavy Atoms in Intermediates of AATase[a]

	Intermediate	C-2[b]	C-3[b]	N[c]
	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	1.012-1.0285

[a] Reference *11*. [b] Referenced to aspartate with the rules of substitution used to estimate the relative fractionation factors. [c] Fractionation factors relative to aqueous $NH_3 = 1.0000$.

changes take place at C3. In the wild-type enzyme, dissociation of oxaloacetate was found to be at least partially rate-determining, as evidenced by a small, normal observed isotope effect at C3 (*11*). This isotope effect is a result of hyperconjugation between the C3−H bonds and the carbonyl of oxaloacetate, resulting in less stiff bonding of C3 than in aspartate. No such effect is observed for the ammonia-rescued K258A mutant, showing that product release is not rate-determining. The observed $^{13}(V/K_{asp})$ at C3 is unity (Table 1), showing that the fractionation factor at C3 in the rate-determining step must be equivalent to that of aspartate. When the carbinolamine (or *gem*-diamine) collapses to give oxaloacetate, the fractionation factor at C3 drops from 1.0050 to 0.9965 (Table 2). The transition state between these two intermediates will have a fractionation factor between these two values, explaining the observed and calculated intrinsic effects of unity at C3.

In the wild-type enzyme, Lys-258 is positioned to act as a general base catalyst. If a carbinolamine is formed in the reaction of ammonia-rescued K258A, then ammonia can act in a similar role. However, if a *gem*-diamine is formed, then there will be no general base available and there will be a barrier to collapse of the diamine. Although there is no direct evidence that the ammonia-catalyzed K258A reaction proceeds through a *gem*-diamine rather than the carbinolamine, a *gem*-diamine intermediate would explain the fact that this step is even more disfavored than deprotonation of the external aldimine. In the wild-type enzyme, the amine group of Lys-258 could be held in a position so that it can catalyze carbinolamine breakdown but is unable to form a diamine. Ammonia is not so constrained, and while ammonia is able to replace the activity of Lys-258 to some extent in the deprotonation of the external aldimine, it cannot act in the same role as Lys-258 during collapse to form oxaloacetate if it has condensed with the ketimine.

The heavy atom isotope effect data lead to the identification of the breakdown of the protonated carbinolamine (or *gem*-diamine) as the rate-determining step. The proposed transition state for breakdown of the carbinolamine is shown in Figure 2 and in Figure 4 for the *gem*-diamine. These results show the importance of Lys-258, not only to the proton abstraction from the external aldimine but to the breakdown of the carbinolamine as well.

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