Lysine 258 in Aspartate Aminotransferase: Enforcer of the Circe Effect for Amino Acid Substrates and General-Base Catalyst for the 1,3-Prototropic Shift†

Michael D. Toney and Jack F. Kirsch*

Department of Molecular and Cell Biology, Division of Biochemistry and Molecular Biology, Barker Hall, University of California, Berkeley, California 94720

Received August 27, 1992; Revised Manuscript Received November 18, 1992

ABSTRACT: The replacement of Lys258 by alanine (K258A) in aspartate aminotransferase reduces the rate constant for the central, 1,3-prototropic shift by 10^6–10^8-fold, confirming the role of Lys258 as the general-base catalyst for this step. The rate constant for the 1,3-prototropic shift interconverting K258A aldimine and ketimine intermediates is pH-independent, like that of the wild-type enzyme (WT-AATase). K258A binds amino acid substrates in external aldimine intermediates 10^5-fold more *tightly* than does WT-AATase. The excess amino acid binding energy observed in the mutant is sacrificed by the WT-AATase in order to increase the value of k_{cat}. The net result is that the k_{cat}/K_M values for amino acid substrates are reduced only 3–100-fold by the mutation. This provides a clear example of the Circe effect propounded by Jencks [Jencks, W. P. (1975) *Adv. Enzymol. Rel. Areas Mol. Biol. 43*, 219]. Part of the increase in k_{cat} due to the inclusion of Lys258 is accomplished by a 10^4–10^5-fold acceleration of external aldimine formation and hydrolysis. This step is partially rate-determining for K258A, but not for WT-AATase. A significant consequence of the utilization of amino acid binding energy for catalysis is the raising of the dissociation constants for these substrates to levels near the physiological concentrations of amino acids. The major product of the reaction of K258A with oxalacetate is pyruvate due to decarboxylation of the β-imine formed in the ketimine intermediate.

Aspartate aminotransferase is a PLP[1]-dependent, dimeric enzyme that reversibly interconverts the dicarboxylic substrates aspartate and α-ketoglutarate with glutamate and oxalacetate.

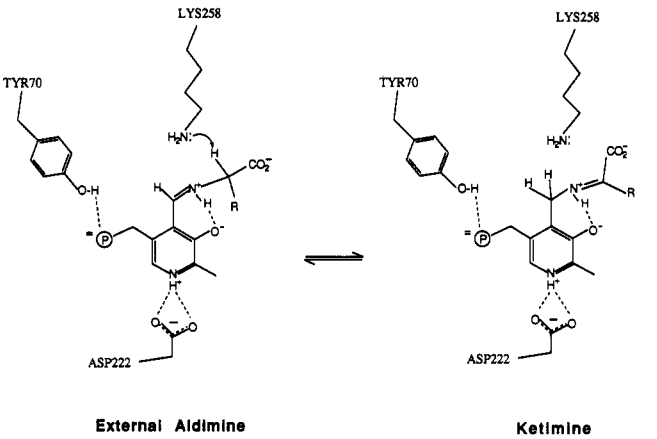
$$\text{E-PLP} + \text{aspartate} \rightleftharpoons \text{E-PMP} + \text{oxalacetate}$$

$$\text{E-PMP} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{E-PLP} + \text{glutamate}$$

The mechanism of this enzyme is relatively well understood due to the large number of classical solution [for a review, see Christen and Metzler (1985)] and recent X-ray crystallographic studies on the eukaryotic (Jansonius & Vincent, 1987; Arnone et al., 1985) and *Escherichia coli* isozymes (Smith et al., 1989; Jaeger et al., 1989; Kamitori et al., 1988). The roles of specific active site residues located by the crystallographic studies are now being tested through site-directed mutagenesis (Toney & Kirsch, 1989, 1991, 1992; Yano et al., 1992; Inoue et al., 1989, 1991; Goldberg et al., 1991; Planas & Kirsch, 1991; Hayashi et al., 1990; Cronin & Kirsch, 1988).

Transamination requires a 1,3-prototropic shift to interconvert aldimine and ketimine intermediates (Scheme I; also see Scheme II for nomenclature). This step is subject to general-base catalysis in nonenzymatic reactions (Auld & Bruice, 1967). The active site structure of AATase restricts the candidacy for general-base catalysis of the enzymatic 1,3-prototropic shift to two residues: Tyr70 and Lys258 (Kirsch et al., 1984; Jansonius & Vincent, 1987). It was previously demonstrated that the Y70F AATase mutant exhibits a k_{cat} value which is only 12-fold less than that of WT-AATase (Toney & Kirsch, 1987, 1991a). These results are interpreted as indicating that Tyr70 is not an essential component of the catalytic apparatus. It was shown in complementary studies that substitution of alanine for Lys258 results in an inactive

Scheme I: Central Intermediates in Transamination[a]



[a] The two central intermediates in transamination are the external aldimine and the ketimine. Their interconversion involves a 1,3-azaallylic rearrangement which is general-base-catalyzed in nonenzymatic reactions (Auld & Bruice, 1967), as well as in AATase reactions by Lys258.

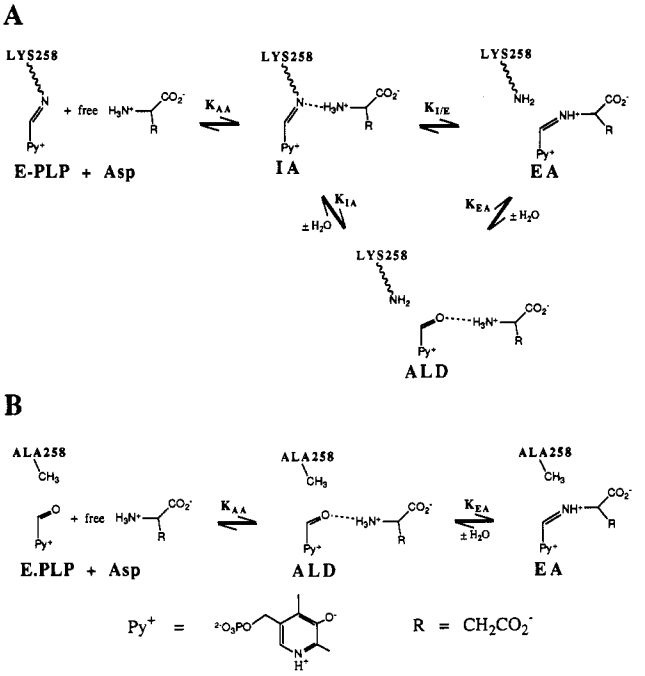
† This work was supported by NIH Grant GM35393. M.D.T. was supported in part by NIH Training Grant GM07232.

* Author to whom correspondence should be addressed.

[1] Abbreviations: AATase, aspartate aminotransferase (EC 2.6.1.1); PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; E–PLP and E–PMP, PLP and PMP forms of AATase, respectively; WT-AATase, the wild-type form of AATase; K258A, K258M, and K258C, AATase in which Lys258 has been changed to alanine, methionine, or cysteine by site-directed mutagenesis; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; MES, 4-morpholinoethanesulfonic acid; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]-1-propanesulfonic acid.

0006-2960/93/0432-1471$04.00/0

Scheme II: Equilibria Associated with Formation of the External Aldimine Intermediate[a]



[a] The reactions of (A) WT-AATase include the internal aldimine species, which is inaccessible with (B) K258A. Definitions: E–PLP + Asp, WT-AATase reactants in which PLP is covalently bound; E·PLP + Asp, K258A reactants in which PLP is noncovalently bound; IA, internal aldimine; ALD, aldehyde; EA, external aldimine. The equilibrium constants are as follow: K_{AA} = ([enzyme]$_{free}$[substrate]$_{free}$)/[IA]; $K_{I/E}$ = [IA]/[EA]; K_{IA} = [IA]/[ALD]; K_{EA} = [ALD]/[EA].

enzyme (Malcolm & Kirsch, 1985; Kirsch et al., 1987). Crystallographic studies confirm that the inactivity is not due to a major structural reorganization (Smith et al., 1989). Partial catalytic activity can be restored to K258A by simple primary amines, confirming Lys258 as the general-base catalyst (Toney & Kirsch, 1989). This report presents a comprehensive dissection of the factors responsible for the large decreases in enzymatic activity due to the K258A mutation.

Jencks (1975) has emphasized that the intrinsic affinity of an enzyme for its substrate can be orders of magnitude greater than that realized in the measured value of the dissociation constant and that this excess binding energy can be utilized to increase k_{cat} values. This means of catalysis is called the Circe effect. The present studies comparing K258A and WT-AATase provide a particularly clear experimental verification of this hypothesis by demonstrating that the binding energy available from amino acid–PLP imine formation in WT-AATase (external aldimine intermediate) is sacrificed to lower the activation barrier of the external aldimine formation step in the reaction sequence.

EXPERIMENTAL SECTION

Materials. The construction of K258A has been reported (Malcolm & Kirsch, 1985). K258C (Planas & Kirsch, 1990) and K258M were prepared similarly except that the *aspC* gene was in pUC119 (J. J. Onuffer and J. F. Kirsch, unpublished results). The bacteria were grown and the protein was purified as described by Cronin and Kirsch (1988). Typically, 12 g of cell paste yielded ~300 mg of pure protein (purity >95% as judged from SDS–PAGE). L-Cysteine sulfinic acid, *o*-phthaldialdehyde, and Brij-35 were purchased from Aldrich Chemical Co. Other chemicals and lactate dehydrogenase were from Sigma Chemical Co. Conventional kinetic data and absorbance spectra were measured with a Kontron Uvikon 860 spectrophotometer interfaced to an IBM-compatible personal computer, which facilitated kinetic data reduction by the nonlinear regression program ENZFITTER (R. J. Leatherbarrow, Biosoft Publishing Co.). Rapid kinetics were followed with a Union-Giken RA-401 stopped-flow spectrophotometer interfaced to an OLIS data acquisition–reduction system (On-Line Instrument Systems). Circulating water baths maintained the reaction vessels of both instruments at 25 °C. Amino acid analysis was performed by HPLC with a Knaur pump–mixing system and an Axxiom workstation.

Kinetic Assays of WT-AATase. The transamination of cysteine sulfinate and α-ketoglutarate catalyzed by WT-AATase was monitored by coupling the production of pyruvate (the product of rapid desulfination of β-sulfinylpyruvate, the transamination product of cysteine sulfinate) to lactate dehydrogenase and NADH and following the decrease in absorbance at 340 nm. The alanine/α-ketoglutarate reaction was similarly assayed. The glutamate/oxalacetate reaction was monitored by the decrease in A_{260}, resulting from the disappearance of oxalacetate (Velick & Vavra, 1962; Julin & Kirsch, 1989).

Preparation of K258A–PLP. As isolated, K258A contains approximately equal amounts of PLP and PMP bound at the active site. The PMP form of K258A is readily obtained by reaction with cysteine sulfinate. The PLP-free aldehyde form is more difficult to realize since reaction with either oxalacetate or α-ketoglutarate leads to a very stable external aldimine (see below). Resolution of the PMP form and reconstitution of apoenzyme with PLP was therefore necessary. Removal of PMP from WT-AATase is readily accomplished by precipitation with ammonium sulfate at pH 4.9 (Wada & Snell, 1962; Toney & Kirsch, 1991a), but this procedure only partially removes PMP from K258A [Kochhar, et al. (1987); M. D. Toney and J. F. Kirsch, unpublished results]. Complete resolution requires more severe conditions. Typically, cold, saturated ammonium sulfate was added to K258A–PMP (~25 mg/mL in ~10 mM potassium phosphate buffer, pH 7.2) to 70% saturation. The apparent pH was lowered to 3.0 with 1 M hydrochloric acid. The precipitate was left at 4 °C for 20 h, harvested by centrifugation, resuspended in unbuffered 70% ammonium sulfate, and reharvested. The precipitate was dispersed in a solution of 70% saturated ammonium sulfate, 100 mM HEPES–KOH (pH 7.5); the pH was adjusted to 7.0 by addition of 1 M KOH. (It is necessary to keep the protein in ammonium sulfate while at low pH to prevent denaturation.) The precipitate was harvested again and resuspended in 100 mM HEPES–KOH (pH 7.5). PLP was directly added to give a 10-fold excess over apoenzyme monomer concentration, and the association of apoenzyme with PLP was allowed to proceed for 30 min at room temperature. This solution was dialyzed against 2 mM potassium phosphate (pH 7.2) at 4 °C, to remove excess PLP and ammonium sulfate. This procedure typically replaced >95% of the initially bound PMP with PLP. The protein was stored either in the dialysis buffer at 4 °C (for immediate use) or frozen in 10 mM HEPES–KOH, 5 mM dithiothreitol, and 1 mM ethylenediamine tetraacetate in dry ice–2-propanol and stored at –80 °C. This freezing procedure causes no loss in the activity of WT-AATase and no change in the kinetic behavior of K258A.

Half-Reaction Transamination Kinetics. AATase transamination half-reactions are readily monitored by the change

in the coenzyme absorbance spectrum (Cronin & Kirsch, 1988). All reactions were conducted under pseudo-first-order conditions, with substrate in at least 10-fold molar excess. K258A concentrations were ~10 μM. The absorbance data conformed well to single-exponential processes typically for ~3 half-lives in reactions with amino acid substrates and ~4 half-lives for keto acid reactions. Exponential curve fitting was performed with the program ENZFITTER. The reactions of K258A–PLP with amino acids were monitored at 330 nm (formation of K258A–PMP), and the reactions of K258A–PMP with keto acids were followed at 430 nm (formation of K258A–amino acid aldimine). The rate constants for the reactions of K258A–PLP with dicarboxylic amino acids were independent of substrate concentration under the conditions employed, and 5 mM amino acid concentrations were used to obtain values of k_{obs} (=k_{max}; see eq 1). The values of k_{obs} for the reactions of K258A–PMP with keto acids are dependent on substrate concentration under the present conditions. The k_{obs} vs keto acid concentration data were fitted to a rectangular hyperbola, eq 1, where k_{max} is the half-reaction maximal rate constant, and K_{app} is the apparent Michaelis constant.

$$k_{obs} = k_{max}[S]/(K_{app} + [S]) \quad (1)$$

The values of K_I for maleate inhibition of the K258A–PMP reactions with oxalacetate were determined by varying the oxalacetate concentration at several fixed concentrations of maleate. The values of k_{obs} were fitted to eq 2, where [I] is the inhibitor concentration and K_I is the inhibition constant.

$$k_{obs} = k_{max}[S]/(K_{app}(1 + [I]/K_I) + [S]) \quad (2)$$

The pH dependence of k_{max} for the cysteine sulfinate reaction with K258A was measured in MES, HEPES, TAPS, and CHES buffers half-neutralized with potasssium hydroxide. The ionic strength was held constant at 0.2 by the addition of potassium chloride.

Determination of Ligand Dissociation Constants by Direct Titration. Dissociation constants for AATase–ligand complexes can often be evaluated by monitoring spectral changes induced in the coenzyme by ligand association. The 30-nm red shift accompanying external aldimine formation was used to follow the association with amino acids. The association of K258A–PLP with maleate causes a ~7-nm blue shift in the PLP absorption maximum and a slight increase in the extinction coefficient. The association of dicarboxylate ligands with wild type–PLP induces an approximately 2 unit increase in the pK_a value of the internal aldimine (Jenkins & D'Ari, 1966) with concomitant increases in the absorbance at 430 nm under the present experimental conditions (i.e., pH 7.5). Typically, a 5–10 μM enzyme solution was titrated by directly adding small aliquots of concentrated ligand solution and the change in adsorbance measured. Absorbance values and concentration data were corrected for dilution and fitted to eq 3 by nonlinear regression to obtain values for K_D, the

$$A = A_i + \left[(A_f - A_i) \times \frac{K_D + [L] + [E] - \sqrt{(K_D + [L] + [E])^2 - 4[L][E]}}{2[E]} \right] \quad (3)$$

equilibrium dissociation constant. A is the observed absorbance; A_i and A_r are the fitted values of initial and final absorbance, respectively; [L] is the ligand concentration; and [E] is the fitted value of the enzyme concentration.

Kinetics of Aldimine Formation. The wavelength maximum in the difference spectrum of the K258A aldehyde and external aldimine (the nomenclature is defined in Scheme II), 440 nm, was used to monitor the rapid rates of aldimine formation in the stopped-flow spectrophotometer. Pseudo-first-order conditions, with 5 μM enzyme and excess amino acid, were employed. The rates of imine formation between aspartate and free PLP were monitored by the increase in absorbance at 415 nm. In the nonenzymatic case, 0.1 mM PLP and excess aspartate were reacted in 0.2 M HEPES–KOH (pH 7.5), 0.1 M potassium chloride at 25 °C.

Determination of the Transamination to β-Decarboxylation Product Ratio for the Reaction of K258A with Oxalacetate. K258A–PMP (~25 μM in 0.1 M HEPES–KOH, pH 7.5), both with and without 10 mM ammonium sulfate, was reacted with 0.5 mM oxalacetate in a 1-mL final volume. The absorbance at 430 nm was measured before oxalacetate was added in 10 μL to give the final reaction mixture. The 430-nm absorbance was again measured after 1 h of reaction at room temperature. One microliter of 0.15 M NADH was added, and the 340-nm absorbance was measured. Lactate dehydrogenase (5 units) was added, and the 340-nm absorbance was measured again after 5 min. Controls (no K258A) for both reactions (with and without ammonium sulfate) were also performed. The changes in 340-nm absorbance were corrected for the control values. Calculation of concentration changes from the absorbance changes utilized the following extinction coefficients: NADH ϵ_{340} = 6220 M^{-1} cm^{-1}; K258A-aldimine ϵ_{430} = 6800 M^{-1} cm^{-1} (Kallen et al., 1985).

Product Analysis for the Reaction of K258A with Oxalacetate. K258A–PMP was dialyzed against 2 mM potassium phosphate buffer, pH 7.2, immediately prior to the experiment. Four different conditions were tested: (1) 0.1 M HEPES–KOH (pH 7.5); (2) 0.1 M potassium phosphate (pH 7.5); (3) 0.1 M CHES–KOH (pH 9.3); (4) 0.1 M CHES–KOH, 10 mM methylamine hydrochloride (pH 9.3). In all cases 200 μM K258A was reacted with 10 mM oxalacetate in 100 μL for 1 h at room temperature. Potassium hydroxide (5 M) was added to give 0.17 M final concentration. The solutions were filtered through a Centricon 10 ultrafiltration apparatus (Amicon, Inc.) to remove the denatured protein. The filtrates were derivatized at room temperature for 1 min with an equal volume of *o*-phthaldialdehyde reagent [9 mL of H_2O, 371 mg of boric acid plus potassium hydroxide to pH 10.4, 30 μL of 30% Brij-35 detergent, 20 μL of β-mercaptoethanol, and 5 mg of *o*-phthaldialdehyde dissolved in 200 μL of ethanol (added in that order; reagent composition from the Rainin Corp.)] The derivatized filtrates were chromatographed on a Waters C18 column with a linear gradient from solvent A (90% 0.1 M sodium acetate (pH 7.2), 9.5% methanol, and 0.5% tetrahydrofuran) to 100% solvent B (methanol) over 30 min. The derivatives were quantitated by absorbance at 340 nm, using standard amino acid solutions to produce calibration curves.

RESULTS

WT-AATase Kinetic Parameters. The values of the kinetic parameters (determined at pH 7.5 and 25 °C) describing the reaction of the cysteine sulfinate/α-ketoglutarate pair are k_{cat} = 320 ± 9 s^{-1} and K^{app}_{CysSul} = 10.8 ± 0.6 mM (with α-ketoglutarate held constant at 5 mM). Those for the glutamate/oxalacetate reaction under these conditions are k_{cat} = 350 ± 7 s^{-1}, K^{app}_{Glu} = 15 ± 1 mM (at 1 mM oxalacetate) and K^{app}_{Oxal} = 70 ± 9 μM (at 75 mM glutamate). The reaction rates with alanine showed no tendency to saturate at alanine

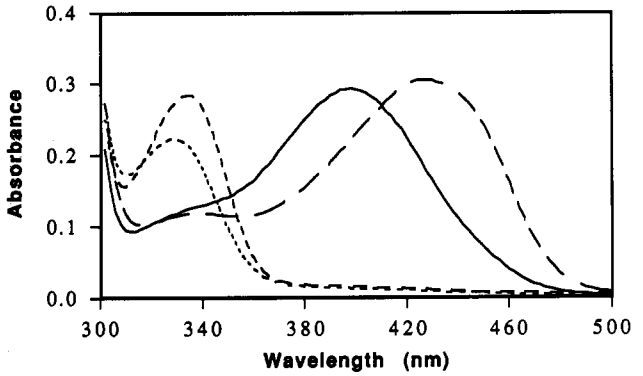


FIGURE 1: Absorption spectra of K258A derivatives. Formation of the aspartate external aldimine intermediate (5 mM aspartate; — —) from the PLP aldehyde (—) causes the absorbance maximum to shift from ~395 to 430 nm. The PMP enzyme form (– –) has λ_{max} = 335 nm. Addition of α-ketoglutarate (10 mM; - -) results in a blue shift to 324 nm and a reduced value of the extinction coefficient. Conditions: 0.2 M HEPES–KOH, 0.1 M potassium chloride (pH 7.5), 25 °C, total PLP concentration ~50 μM.

Table I: Half-Reaction Maximal Rate Constants for the Wild Type and the K258A Mutant of Aspartate Aminotransferase[a]

substrate	k_{max} (s^{-1}) wild type	K258A	wild type/K258A
Amino Acid Substrates + PLP Enzyme			
aspartate	530[b]	7×10^{-6} [d]	8×10^{7}
	(33)	(2)	(2)
glutamate	670[b]	5.8×10^{-5}	1.2×10^{7}
	(100)	(0.4)	(0.2)
		[$7.5\ (0.4) \times 10^{-5}$][d]	
cysteine sulfinate	890[c]	8.8×10^{-4}	1.0×10^{6}
	(250)	(0.1)	(0.3)
		[$9.5\ (0.1) \times 10^{-4}$][d]	
α-Keto Acid Substrates + PMP Enzyme			
oxalacetate	750[b]	9.8×10^{-4}	7.6×10^{5}
	(55)	(0.2)	(0.6)
α-ketoglutarate	500[b]	6.4×10^{-5}	7.8×10^{6}
	(47)	(0.1)	(0.7)

[a] Conditions: 25 °C, pH 7.5, 0.2 M HEPES–KOH, 0.1 M potassium chloride, except as noted. Standard errors are given in parentheses. [b] Taken from Inoue et al. (1989). Conditions: 25 °C, pH 8.0, 0.05 M HEPES–KOH, 0.1 M potassium chloride. [c] Calculated with the equation $k_{max1} = (k_{cat}k_{max2})/(k_{max2} - k_{cat})$ (Segel, 1975), where k_{max} is the half-reaction maximal rate constant, using steady-state k_{cat} = 320 ± 9 s^{-1} (cysteine sulfinate/α-ketoglutarate as substrates; see Results), and α-ketoglutarate k_{max} = 500 ± 47 s^{-1} (Inoue et al., 1989). [d] Measured in 0.1 M HEPES–KOH (pH 7.5) without added potassium chloride.

concentrations of up to 1 M, in agreement with results on cytosolic AATase (Julin & Kirsch, 1989).

Absorption Spectra. Figure 1 presents absorption spectra of K258A-derived species. Addition of aspartate to K258A–aldehyde (395-nm absorption band) readily converts it to the external aldimine form (430-nm absorption band). K258A–PMP has an absorption maximum at 334 nm, similar to that observed with WT-AATase (Inoue et al., 1989). Addition of α-ketoglutarate to K258A–PMP, most probably producing a ketimine intermediate, results in a shift in the absorption maximum to 324 nm, as well as in an ~25% decrease in the extinction coefficient. Similar results were obtained with oxalacetate and K258A–PMP.

K258A Transamination Kinetics. The values of the half-reaction maximal rate constants (k_{max} in eq 1) for the reactions of K258A with amino and keto acid substrates are given in Table I. The K258A mutant exhibits a 10^6–$\sim 10^8$-fold reduction in this constant from the WT-AATase values.

The k_{cat} value for the K258A reaction with cysteine sulfinate, like that of WT-AATase with aspartate [W. L. Finlayson and J. F. Kirsch, unpublished results; Kiick and Cook (1983)], is pH-independent over the range 6.1–9.3.

Table II: Apparent Michaelis and Substrate and Inhibitor Dissociation Constants for the Wild Type and K258A Mutant of Aspartate Aminotransferase[a]

ligand	K_{app} or K_D (mM) wild type	K258A	wild type/K_{258A}
PLP Enzyme			
aspartate	4.0[b,c]	1×10^{-5} [d,e]	4×10^{5}
	(0.5)	(5)	(20)
glutamate	37[b,c]	1.9×10^{-4} [d,e]	1.9×10^{5}
	(8)	(0.5)	(0.7)
alanine	>1000	3.6[e]	>300
		(0.1)	
		[1.7 (0.2)][d,e]	
maleate	5.1	3.2[e]	1.6
	(0.2)	(0.3)	(0.2)
		[5.6 (1.6)][f]	
PMP Enzyme			
oxalacetate	0.038[b,c]	0.97[b]	0.039
	(0.008)	(0.03)	(0.008)
		[0.023 (0.002)][b,d]	
α-ketoglutarate	0.83[b,c]	0.20[b,g]	4.2
	(0.13)	(0.02)	(0.8)
maleate	5.6	0.70[h]	8.0
	(0.5)	(0.03)	(0.8)
		[0.017 (0.003)][d,h]	

[a] Conditions: 25 °C, pH 7.5, 0.2 M HEPES–KOH, 0.1 M potassium chloride, except as noted. Standard errors are given in parentheses. [b] K_{app} from single-turnover half-reactions (eq 1). [c] Taken from Inoue et al. (1989). [d] Measured in 0.1 M HEPES–KOH (pH 7.5) with no added potassium chloride. [e] Obtained by direct enzyme titration. [f] Measured as K_I for the inhibition of Schiff base formation between alanine and K258A–PLP. [g] Strong substrate inhibition was observed in the absence of potassium chloride. [h] Obtained by inhibition of single-turnover half-reactions.

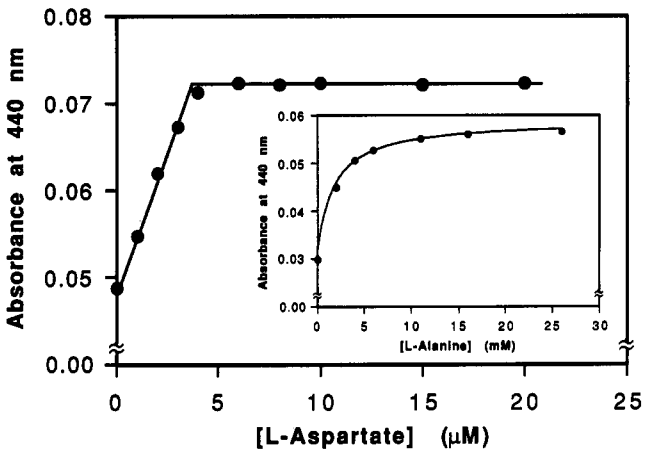


FIGURE 2: Representative K258A–amino acid association isotherms. The increase in absorbance at 440 nm due to formation of the external aldimine was monitored. The low value of K_D for the aspartate complex (<0.1 μM, Table II) results in stoichiometric association at this enzyme concentration, while the alanine complex (inset; note the difference in abcissa scales), which has K_D > 1 M for WT-AATase, exhibits K_D = 2 mM for the mutant. Amino acids were added in small aliquots directly to ~5 μM K258A in 0.1 M HEPES–KOH (pH 7.5) at 25 °C.

Ligand Affinities. Values of substrate and inhibitor dissociation constants for WT-AATase and K258A are collected in Table II. All available evidence (Kiick & Cook, 1983; Julin & Kirsch, 1989) suggests that the WT-AATase K_M values given for amino acids are true substrate dissociation constants. K258A binds both aspartate and glutamate ~10^5-fold more tightly than does WT-AATase. Figure 2 presents the results of titration experiments with K258A using aspartate

Table III: Comparison of Aspartate Aminotransferase Position 258 Mutants: Kinetic Parameters for the Reactions with Oxalacetate and Cysteine Sulfinate[a]

	oxalacetate		cysteine sulfinate
mutant	10^3k_{max} (s^{-1})	K_{app} (mM)	10^3k_{max} (s^{-1})
K258A	0.98 (0.02)	0.97 (0.03)	0.88 (0.01)
K258M	0.26 (0.01)	0.51 (0.02)	1.5[b] (0.4)
K258C	2.00[c] (0.03)	0.53[c] (0.09)	2.03[c] (0.02)

[a] Conditions: 25 °C, pH 7.5, 0.2 M HEPES–KOH, 0.1 M potassium chloride, except as noted. Standard errors are given in parentheses. [b] 0.1 M CHES–KOH, pH 9.3, 0.5 M tetramethylammonium chloride. [c] 0.1 M HEPES–KOH, pH 7.5, 0.05 M potassium chloride (Planas & Kirsch, 1991).

Table IV: Rate Constants for Schiff Base Formation between the Pyridoxal Phosphate Form of the K258A Mutant of Aspartate Aminotransferase and L-Amino Acids[a]

amino acid	k_{form} (M^{-1} s^{-1})[b]	$k_{form}^{amino\ acid}/k_{form}^{Ala}$	calc 10^5k_{diss} (s^{-1})[c]
alanine	0.31 (0.02)	1	53
glutamate	320 (10)	1030	6.1
aspartate	1850 (50)	5840	1.9

[a] Conditions: 25 °C, pH 7.5, 0.1 M HEPES–KOH, 0.2 M potassium chloride. Standard errors are given in parentheses. [b] k_{form}, the Schiff base formation rate constant, was obtained from the slope of a line fitted to k_{obs} vs amino acid concentration, where k_{obs} is the observed pseudo-first-order rate constant. [c] Calculated from $K_D = k_{diss}/k_{form}$ using the K_D values given in Table II.

and alanine as ligands. These reactions were monitored by the spectral change that accompanies external aldimine formation from the PLP aldehyde and amino acid. Aspartate is bound so tightly as to preclude an accurate determination of its dissociation constant by direct titration. The K258A–alanine complex has a much higher K_D value than does the aspartate complex, which allowed its accurate determination by this simple method. The value of the dissociation constant for the K258A–PLP complex with maleate, a noncovalently bound inhibitor, is largely unaffected by the mutation, in contrast with the amino acid results.

The values of the dissociation constants for the E–PMP complexes with α-keto acids (as approximated by K_{app} values obtained from single-turnover half-reactions, eq 1) are much less perturbed by the mutation than are those of the E–PLP complexes with amino acids. As shown in Table II, both an increase and a decrease in affinity are observed. While oxalacetate is bound 14-fold more weakly to K258A–PMP, α-ketoglutarate is bound 2-fold more tightly compared to WT-AATase–PMP. At pH 9.3 in the absence of potassium chloride, the kinetic parameters for the oxalacetate reaction are $k_{cat} = (1.43 \pm 0.06) \times 10^{-3}$ s^{-1} and $K_M = 84 \pm 10$ μM (see Tables I and II for pH 7.5 values).

The value of the dissociation constant for the K258A–PMP complex with maleate was determined from competitive inhibition of the oxalacetate reaction. At pH 7.5 in the presence of potassium chloride, K258A–PMP binds maleate 8-fold more tightly than does WT-AATase. Raising the pH from 7.5 to 9.3 in the absence of potassium chloride increases the K258A K_I from 17 to 200 μM. Both phosphate and chloride ions were found to associate tightly with K258A–PMP. At pH 7.5, the values for K_I are 7.4 ± 1 and 2.4 ± 0.2 mM for phosphate and chloride ions, respectively. The effects of chloride ion on the K258A–PLP reactions are much smaller (Table II).

Comparative Kinetics of K258A, K258M, and K258C. A comparison of three position 258 mutants in their reactions with oxalacetate and cysteine sulfinate is given in Table III. All show similar decreases in activity with respect to WT-AATase, as well as similar affinity for oxalacetate.

Kinetics of Aldimine Formation. The observed pseudo–first-order rate constants for the formation of external aldimines from K258A–PLP and each of the amino acids examined, aspartate, glutamate, and alanine, were linearly dependent on amino acid concentrations up to 150, 50, and 200 mM, respectively. Table IV presents the values for the second-order rate constants describing the reactions. The rate constant for the aspartate reaction is 6- and 6000-fold larger than those for glutamate and alanine, respectively. The kinetic instability of the aldimines formed with WT-AATase prevents the direct determination of their formation rate constants by the stopped-flow method. A temperature-jump study of cytosolic AATase gave values of 10^7–10^8 M^{-1} s^{-1} for aspartate and glutamate (Fasella & Hammes, 1967). These figures are ~10^5-fold larger than the corresponding ones determined for the K258A reactions.

The rate constant for aldimine formation from aspartate and free PLP was determined to be 0.34 ± 0.06 M^{-1} s^{-1}. This value is 5400-fold less than that for K258A–PLP reacting with aspartate.

Decarboxylation of Oxalacetate Catalyzed by K258A. K258A–PMP catalyzes the decarboxylation of oxalacetate to CO_2 and pyruvate. At pH 7.5, in the absence of any added amine catalysts, the ratio of aspartate to pyruvate production is 0.47 ± 0.02. This ratio is increased to 1.5 ± 0.1 when 10 mM ammonium sulfate is included, consistent with specific catalysis of the transamination reaction by ammonia (Toney & Kirsch, 1989).

Reaction of K258A-PMP with Oxalacetate. The reaction of K258A–PMP with oxalacetate was previously reported to produce alanine as the major amino acid (Kirsch et al., 1987). This reaction was reinvestigated by HPLC analysis of the *o*-phthaldialdehyde derivatives of the amino acid products. Freshly dialyzed K258A–PMP was reacted with oxalacetate. After removal of the protein by ultrafiltration and derivatization, the only amino acid derivative detectable was that of aspartate, which was present in 74% yield. Four different buffer conditions were tested (0.1 M HEPES–KOH, pH 7.5; 0.1 M potassium phosphate, pH 7.5; 0.1 M CHES–KOH, pH 9.3; and 0.1 M CHES–KOH, 10 mM methylamine hydrochloride, pH 9.3), and all gave similar results. The earlier reported detection of alanine may perhaps be attributed to the extended time period between reaction initiation and product isolation in that work (W. L. Finlayson and J. F. Kirsch, unpublished results) and the possible presence of amines (e.g., ammonium sulfate) in solution. Amines can catalyze the interconversion of external aldimines and ketimines (Toney & Kirsch, 1989), and the latter decarboxylate oxalacetate to pyruvate (see above), providing a source for alanine.

DISCUSSION

Spectral Characteristics of K258A. The WT-AATase internal aldimine absorption spectrum exhibits characteristic maxima at 360 and 430 nm, corresponding, respectively, to the unprotonated and protonated forms with the bound proton being shared between the imino nitrogen and O3′ of the coenzyme [Kallen et al. (1985); Scheme I]. The WT-AATase

pK_a value controlling this ionization is 6.7 at I_c = 0.1. The spectrum of free PLP at neutral pH exhibits a peak at 388 nm with a shoulder at 325 nm (Peterson & Sober, 1954). The spectrum of K258A-bound PLP is pH-independent with a maximum at 395 nm (Figure 1), very similar to that of free PLP at neutral pH, confirming the presence of the free aldehyde at the active site.

The K258A–PLP complex with aspartate exhibits maximal absorbance at 430 nm (Figure 1) that is assigned to a protonated external aldimine on the basis of the similar absorption maximum of the WT-AATase protonated internal aldimine and the WT-AATase–α-methylaspartate external aldimine (Fasella et al., 1966). The K258A external aldimine converts slowly to the PMP enzyme form which has an absorption maximum at 334 nm, close to the 332-nm WT-AATase maximum but different from that of free PMP at neutral pH [325 nm; Peterson and Sober (1954)]. A decrease in the extinction coefficient and a 10-nm blue shift follow the addition of either α-ketoglutarate or oxalacetate to K258A–PMP. Given the low values of the apparent Michaelis constants (0.2 mM for α-ketoglutarate) and the high concentrations of keto acids used (10 mM α-ketoglutarate), this spectral change probably represents the formation of a ketimine intermediate, although the presence of a significantly populated Michealis complex cannot be ruled out.

Requirement for Lys258 for the 1,3-Prototropic Shift. The central and chemically most difficult step in transamination is the breaking of a carbon–hydrogen bond at either C_α of the amino acid or C4′ of the coenzyme, depending on the half-reaction in question (Julin & Kirsch, 1989). The major role of PLP is to stabilize the resulting carbanion (Braunstein & Shemyakin, 1953; Metzler et al., 1954). The 1,3-prototropic shift in PLP-catalyzed transamination in aqueous solution is accelerated by general-base catalysts (Auld & Bruce, 1967), and it is generally considered that enzyme-catalyzed transamination also depends on such catalysis (Ivanov & Karpeisky, 1969). The data in Table I show that the rate constants for the 1,3-prototropic shift catalyzed by K258A are reduced 10^6–10^8-fold for both amino and keto acid substrates. These results, taken together with those from previous crystallographic and site-directed mutagenesis studies, provide compelling evidence for the assignment of Lys258 as the sole general-base catalyst of the 1,3-prototropic shift.

The observations that K258M and K258C have kinetic parameters similar to K258A (Table III) lend weight to the proposition that the latter is not inactivated by some structural perturbation particular to the substitution of alanine. The K258M mutant effectively removes only the ϵ-amino group of Lys258, given the size and steric similarity between a methylene group and the methionine sulfur atom.

Roles for Lys258 in Addition to Catalysis of the 1,3-Prototropic Shift. (A) Amino Acid Dissociation Constants. The most striking characteristic of K258A, other than the reductions in the 1,3-prototropic shift rate constants, is the 10^5-fold increase in affinity for amino acid but not for keto acid substrates (Table II). Scheme II dissects the WT-AATase and K258A aldimine formation reactions. The internal aldimine (IA), the aldehyde (ALD), and the external aldimine (EA) are all accessible structures for the WT-AATase–substrate complex, but IA cannot be formed from K258A.

The observed amino acid dissociation constants (K_D's) reported in Table II are composites of the constants for bimolecular and unimolecular steps, namely, amino acid association to form the Michaelis complex and conversion of this complex to the external aldimine. The expression for K_D derived from Scheme II for K258A is given in eq 4 (constants are defined in the legend to Scheme II).

$$K_D^{K258A} = \frac{K_{AA}^{K258A}}{1 + (1/K_{EA}^{K258A})} \quad (4)$$

Solution of eq 4 for K_{EA} gives

$$K_{EA}^{K258A} = \frac{1}{(K_{AA}^{K258A}/K_D^{K258A}) - 1} \quad (5)$$

The value of K_{AA}, the Michealis complex dissociation constant, was not experimentally accessible, but is well-approximated by the values of dissociation constants for complexes of competitive inhibitors which do not form external aldimines (e.g., maleate, succinate, or malate).[2] Substitution of the value of K_I for the K258A–maleate complex (Table II) for K_{AA} allows the calculation of K_{EA} using eq 5 and the value of K_D for the K258A–aspartate complex (Table II). This procedure gives $K_{EA} = 3 \times 10^{-6}$ (=[ALD]/[EA] in the enzyme–aspartate complex).

The fact that the dissociation constants for complexes of WT-AATase with noncovalently binding inhibitors such as maleate, succinate, and malate all have values near that for aspartate [Kiick and Cook (1983), Inoue et al. (1989), and M. D. Toney and J. F. Kirsch, unpublished results] argues that formation of the covalent external aldimine bond does not greatly stabilize the WT-AATase–aspartate complex. The value of $K_{I/E}$ (=[IA]/[EA]) is thus near unity. This conclusion is supported by the following argument.

The expression for the dissociation constant of the WT-AATase–amino acid complex, derived from Scheme II, is

$$K_D^{WT} = \frac{K_{AA}^{WT}}{1 + 1/K_{IA} + 1/K_{I/E}} \quad (6)$$

which gives the following equation for $K_{I/E}$

$$K_{I/E} = \frac{1}{K_{AA}^{WT}/K_D^{WT} - 1/K_{IA} - 1} \quad (7)$$

All available spectroscopic data (Kallen et al., 1985) indicate that the value of K_{IA} is large (i.e., no free aldehyde is detectable in the WT-AATase active site). Thus, the $1/K_{IA}$ term in eq 7 is insignificant, and the value of $K_{I/E}$ calculated from K_D^{WT} and K_{AA}^{WT} (maleate K_I) is 1.7.[3]

It follows from Scheme II that $K_{IA} = K_{I/E}/K_{EA}$ (=[IA]/[ALD] in the WT-AATase–substrate complex). The calculated values of $K_{I/E}$ and K_{EA}^{K258A} give $K_{IA} = 6 \times 10^5$, in

[2] The value of K_{AA} for K258A is, in principle, obtainable from kinetic studies of external aldimine formation as K_{app} for a preassociation mechanism (eq 1), since substrate binding is expected to be rapid compared to imine formation. As discussed in the text, the free base form of the amino acid is the substrate for the aldimine formation reaction. Thus the observed linear dependence at pH 7.5 of the K258A external aldimine formation rate constant on amino acid concentration is explained by the presence of only 0.75 mM free base at the highest concentration (150 mM) of aspartate employed and the expected dissociation constant of 3.2–5.6 mM (K258A–maleate K_I, Table II).

[3] The values used here for WT-AATase–aspartate K_D and WT-AATase–maleate K_I are for the dianionic ligands binding to the protonated enzyme. They are 1.3 and 2.0 mM, respectively, obtained from pH-dependence studies (W. L. Finlayson, J. M. Goldberg, M. D. Toney, and J. F. Kirsch, unpublished results).

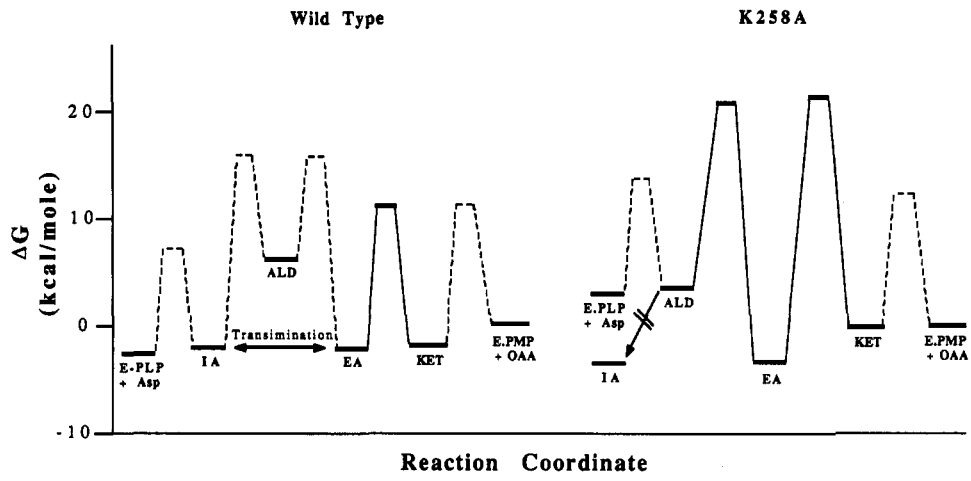


FIGURE 3: Free energy vs reaction coordinate profile for the reactions of WT-AATase and K258A with aspartate (standard state: 1 mM substrates, pH 7.5, 25 °C). Definitions: KET, ketimine intermediate; E·PMP + OAA, PMP enzyme form plus oxalacetate. The other species are defined in the legend to Scheme II. The WT-AATase mechanism as shown is hypothetical in that the ALD intermediate is not populated. Instead a more facile, direct transimination mechanism, as indicated by the double arrow and shown in Scheme II ($K_{I/E}$), is enforced. Dotted lines indicate unknown energy barriers. The positions of the PMP enzymes are arbitrarily set equal. The rate-determining step for WT-AATase is a combination of the 1,3-prototropic shift and ketimine hydrolysis steps [Julin and Kirsch (1989); J. M. Goldberg and J. F. Kirsch, unpublished results]. The K258A external aldimine is in a deep free energy well such that association or dissociation of the amino acid is partly rate-determining. This is true in spite of the fact that the free energy of activation for the aldimine to ketimine transformation is 11 kcal/mol greater than for WT-AATase. The blocked arrow for K258A indicates that the ALD intermediate cannot form an internal aldimine bond, and amino acid is dissociated directly to give E·PLP + Asp. The major effects of the mutation are to raise the kinetic barriers for both external aldimine formation and external aldimine–ketimine interconversion by factors of approximately 10^5 and 10^8, respectively, and to destabilize selectively the reactants with respect to the external aldimine intermediate.

agreement with the spectroscopic data.[4]

The greater amino acid affinity of K258A compared to WT-AATase is explained as follows. The above calculations demonstrate that the ternary complex of WT-AATase, PLP, and amino acid substrate (ALD) faces two competing reactions (Figure 3): (1) internal aldimine formation with the ε-amino group of Lys258 to give IA and (2) external aldimine formation with the amino acid to form EA. The values of the two equilibrium constants ($1/K_{IA}$ and K_{EA}) describing these reactions are approximately equal and favor the imines by 10^5-fold. The K258A mutation precludes internal aldimine formation, which stabilizes the Michaelis complex (IA) and the reactants (E–PLP + Asp) in WT-AATase, selectively destabilizing the reactants and thereby increasing the affinity for amino acids.

Related to the finding that K258A binds amino acids more tightly than WT-AATase is the fact that the values of k_{cat}/K_M, the second-order rate constants for the amino to keto acid half-reactions, compared to the values of k_{cat}, are only modestly decreased by the mutation. The WT-AATase–K258A k_{cat}/K_M ratios for aspartate, glutamate, and cysteine sulfinate are 97, 75, and 3.4, respectively.[5] There is little difference between WT-AATase and K258A in their rates of reaction with cysteine sulfinate under identical *second-order* conditions (e.g., 10^{-9} M). The key difference between the enzymes is that at very low amino acid concentrations the WT-AATase external aldimine is relatively unpopulated but is rapidly transformed to products, while the K258A external aldimine is significantly populated but very slowly transformed to products. This finding might have evolutionary implications if early life forms had only dilute amino acid pools available. A primitive transaminase which performed approximately as well as the present day WT-AATase could have evolved from a protein simply capable of binding an aldimine tightly in the appropriate conformation. Increasing cellular amino acid concentrations would have relaxed the requirement for tight binding, allowing the introduction of Lys258 and the progression to the mechanistic sophistication observed in present day transaminases.

The 10^5-fold higher affinity of K258A–PLP for aspartate vs alanine is a direct measure of the contribution of the β-carboxylate group toward binding. This value agrees well with the measured difference in k_{cat}/K_M for WT-AATase (Cronin & Kirsch, 1988) and coincides with the observed reduction in k_{cat}/K_M for aspartate when Arg292, the main β-carboxylate binding residue at the active site, is changed to either aspartate or leucine (Cronin & Kirsch, 1988; Hayashi et al., 1990).

(*B*) *External Aldimine Formation Rate Constants.* Cordes and Jencks (1962) demonstrated that the rate constants for reactions of imines of PLP in solution with semicarbazide are greater than those for the parent aldehyde. Their results suggest that the rate constant for formation of the external aldimine from the Lys258–PLP internal aldimine might be greater than that for enzyme-bound PLP aldehyde. Tobias and Kallen (1975) performed a study on the rate of intramolecular Schiff base interchange for the PLP–ethylenediamine imine. This transimination proceeds with a rate constant of $\sim 10^5\ s^{-1}$, which is greater than the value of k_{cat} for AATase. They postulated that the protein internal aldimine need not provide other than entropic catalysis of external aldimine formation.

Transimination in WT-AATase is facilitated by the relatively large population of the reactive Michaelis complex in

[4] The use of K_{EA}^{K258A} for K_{EA}^{WT} in calculating K_{IA} is justified since ketimine formation and inhibitor binding, which, like EA formation from ALD, do not involve Lys258, are unaffected by the mutation. Qualitative confirmation of the large magnitude of K_{IA} comes from a comparison of the PLP vs PMP equilibrium dissociation constants. The dissociation constant for PLP is 0.4 pM, while that for PMP is 1.3 nM (Toney & Kirsch, 1991b). This 3000-fold difference is similar to the value of $\sim 10^5$ calculated for K_{IA} and may be lower due to the stronger interaction between Lys258 and the PMP amino group vs the PLP aldehyde.

[5] The sources of the k_{cat}/K_M values are as follows: WT-AATase with aspartate, Toney and Kirsch (1987); WT-AATase with glutamate and cysteine sulfinate, this report (see Results); K258A with aspartate and glutamate, this report (see Tables I and II); K258A with cysteine sulfinate, this report, assuming a K_D value 0.1 μM (see Table I).

which the internal aldimine is protonated and the substrate α-amino group is in the free base form. This combination is enabled by the similarity of the pK_a values for these two bases in the complex (Kirsch et al., 1984). The pK_a value of the corresponding carbonyl oxygen protonated aldehyde of PLP is approximately −7 [based on benzaldehyde; Arnett et al. (1970)], making the analogous pathway inaccessible to K258A. K258A therefore forms external aldimines via the reactions of unprotonated substrates with PLP aldehyde. The rate constants observed at pH 7.5 (Table IV) are thus ~200-fold lower than the true constants for this mechanism due to the largely protonated state of the amino acid substrates in solution.

Fasella and Hammes (1967) estimated the external aldimine formation rate constants for the cytosolic AATase reactions with aspartate and glutamate to be 10^7–10^8 M^{-1} s^{-1} from a temperature-jump analysis. Similar values are expected for WT-AATase given the overall similarity of the isozymes in pre-steady-state kinetics (Kuramitsu et al., 1990). The rate constants for K258A external aldimine formation with the aspartate and glutamate free bases (~4×10^5 and ~6×10^4 M^{-1} s^{-1}, respectively) are significantly less than those expected for the analogous WT-AATase reactions, confirming the expectations based on model studies.

The kinetic significance of the internal aldimine bond between Lys258 and PLP at *physiological* pH is clear: it permits 10^4–10^5-fold faster (10^7–10^8 M^{-1} s^{-1} vs the values in Table IV) external aldimine formation.

Schiff base formation between *free* PLP and aspartate occurs with a rate constant of 0.34 M^{-1} s^{-1} (see Results). This value is 5400-fold less than that for the corresponding reaction of K258A–PLP with aspartate measured at the same pH. Thus, the apposition of the substrate amino and the PLP carbonyl groups at the active site, any acid–base catalysis contributed by the protein, and any protein-induced carbonyl group activation, combine to lower the free energy of activation for aldimine formation by 5.1 kcal/mol.

(C) Circe Effect. Jencks (1975) has defined the Circe effect as a mechanistic artifice whereby intrinsic enzyme–substrate binding energy is transduced into an increase in the overall rate of an enzymatic reaction. The K258A mutant of AATase provides an impressive example of the attainable magnitude of the effect. The external aldimine in K258A (Figure 3) faces two large, approximately equal kinetic barriers: (1) hydrolysis of the aldimine to E–PLP + aspartate ($\Delta G^{\ddagger}$ = 23.9 kcal/mol) and (2) conversion to the ketimine ($\Delta G^{\ddagger}$ = 24.5 kcal/mol). Therefore, both of these steps are partially rate-determining. The introduction of Lys258 (wild-type enzyme) reduces the free energy of E–PLP + aspartate relative to the E–PMP + oxalacetate reference by 7.6 kcal/mol through the formation of the internal aldimine, thereby decreasing the difference in free energy between E–PLP + aspartate and the external aldimine. The amino acid binding energy forfeited by internal aldimine formation is not wasted, but enables the lowering of the activation barrier for the E–PLP + aspartate ⇔ external aldimine transformation by 6.5 kcal/mol in the forward and by 14.1 kcal/mol in the reverse direction (Figure 3) such that this barrier is no longer rate-determining in WT-AATase. Mechanistically, the rate increase is realized via enforcement of the more facile transimination mechanism instead of Schiff base formation from the free aldehyde (see above).

The ϵ-amino group of Lys258 additionally is the catalyst that lowers the barrier for the aldimine ⇔ ketimine transformation by 10.8 kcal/mol in the forward and 8.0 kcal/mol in the reverse direction. The latter rate increase is *not* part of the Circe effect, but exemplifies the frugality of mechanism achieved by using a single residue for multiple catalytic roles.

The Y225F mutant of AATase results in a 430-fold decrease in k_{cat} and a 20–80-fold decrease in K_M values compared to WT-AATase (Goldberg et al., 1991). These results have also been interpreted as a manifestation of the Circe effect.

pH Dependence of the WT-AATase and K258A Reactions. The values of k_{cat} for both cytosolic and *E. coli* wild-type AATases are independent of pH between 5.5 and 10, indicating that Lys258 does not ionize in this range [Kiick and Cook (1983); W. L. Finlayson and J. F. Kirsch, unpublished results]. The value of k_{max} for the reaction of K258A with cysteine sulfinate is also pH-independent, indicating that no kinetically significant ionizations occur in the external aldimine complex and that hydroxide ion, unlike exogenous amines (Toney & Kirsch, 1989), is not an efficient catalyst of the K258A 1,3-prototropic shift.

K258A-Catalyzed Decarboxylation of Oxalacetate. Deletion of the Lys258 ϵ-amino group virtually abolishes the enzyme-catalyzed 1,3-prototropic shift, which is central to transamination, thus stabilizing ketimine intermediates formed from α-keto acids and E–PMP. The oxalacetate ketimine is potentially susceptible to decarboxylation because the imine is β to a carboxylate. An example of enzymatic catalysis of decarboxylation by β imine formation is found in acetoacetate decarboxylase (Warren et al., 1966).

The appearance of 2 mol of pyruvate per mole of aspartate yielded in the reactions of K258A–PMP with oxalacetate demonstrates that the mutant enzyme preferentially catalyzes oxalacetate decarboxylation. The addition of ~0.1 mM NH_3 (10 mM ammonium sulfate in pH 7.5 buffer) reduces this ratio to 0.7, consistent with specific catalysis of transamination by the amine (Toney & Kirsch, 1989). It is possible that WT-AATase also catalyzes the decarboxylation of oxalacetate with a rate constant similar to that of K258A, but given the large rate constant for the transamination of the WT-AATase–oxalacetate ketimine, the relative quantities of pyruvate formed would be insignificant.

REFERENCES

Arnett, E. M., Quirk, R. P., & Larsen, J. W. (1970) *J. Am. Chem. Soc. 92*, 3977.

Arnone, A., Rogers, P. H., Hyde, C., Briley, P., Metzler, C., & Metzler, M. (1985) in *Transaminases* (Christen, P., & Metzler, D., Eds.) Chapter 3, Wiley, New York.

Auld, D. S., & Bruice, T. C. (1967) *J. Am. Chem. Soc. 89*, 2098.

Braunstein, A. E., & Shemyakin, M. M. (1953) *Biochemistry (Moscow) 18*, 393.

Cordes, E. H., & Jencks, W. P. (1962) *Biochemistry 1*, 773.

Christen, P., & Metzler, D., Eds. (1985) *Transaminases*, Wiley and Sons, New York.

Cronin, C. N., & Kirsch, J. F. (1988) *Biochemistry 27*, 4572.

Fasella, P., & Hammes, G. G. (1967) *Biochemistry 6*, 1798.

Fasella, P., Giartosio, A., & Hammes, G. G. (1966) *Biochemistry 5*, 197.

Goldberg, J. M., Swanson, R. V., Goodman, H., & Kirsch, J. F. (1991) *Biochemistry 30*, 305.

Hayashi, H., Inoue, Y., Kuramitsu, S., Morino, Y., & Kagamiyama, H. (1990) *Biochem. Biophys. Res. Commun. 167*, 407.

Inoue, K., Kuramitsu, S., Okamoto, A., Hirotsu, K., Higuchi, T., & Kagamiyama, H. (1991) *Biochemistry 30*, 7796.

Inoue, Y., Kuramitsu, S., Inoue, K., Kagamiyama, H., Hiromi, K., Tanase, S., & Morino, Y. (1989) *J. Biol. Chem. 264*, 9673.

Ivanov, V. I., & Karpeisky, M. Y. (1969) *Adv. Enzymol. 32*, 21.

Jaeger, J., Kohler, E., Tucker, P., Sauder, U., Housley-Markovic, Z., Fotheringham, I., Edwards, M., Hunter, M., Kirschner, K., & Jansonius, J. (1989) *J. Mol. Biol. 209*, 499.
Jansonius, J. N., & Vincent, M. G. (1987) in *Biological Macromolecules and Assemblies* (Jurnak, F., & McPherson, A., Eds.) Vol. 3, Chapter 4, Wiley, New York.
Jencks, W. P. (1975) *Adv. Enzymol. Res. Areas Mol. Biol. 43*, 219.
Jenkins, W. T., & D'Ari, L. (1966) *J. Biol. Chem. 241*, 5667.
Julin, D. A., & Kirsch, J. F. (1989) *Biochemistry 28*, 3825.
Kallen, R. G., Korpela, T., Martell, A. E., Matsushima, Y., Metzler, C. M., Metzler, D. E., Morozov, Y. V., Ralston, I. M., Savin, F. A., Torchinsky, Y. M., & Ueno, H. (1985) in *Transaminase* (Christen, P., & Metzler, D., Eds.) Chapter 2, Wiley, New York.
Kamitori, S., Hirotsu, K., Higuchi, T., Kondo, K., Inouye, K., Kuramitsu, S., Kagamiyama, H., Higuchi, Y., Yasuoka, N., Kusunoki, M., & Matsuura, Y. (1988) *J. Biochem. 104*, 317.
Kiick, P. M., & Cook, P. F. (1983) *Biochemistry 22*, 375.
Kirsch, J. F., Eichele, G., Ford, G. C., Vincent, M. G., Jansonius, J. N., Gehring, H., & Christen, P. (1984) *J. Mol. Biol. 174*, 497.
Kirsch, J. F., Finlayson, W. L., Toney, M. D., & Cronin, C. N. (1987) in *Biochemistry of Vitamin* B_6 (Korpela, T., & Christen, P., Eds.) p 59, Birkhauser Verlag, Basel.
Kochhar, S., Finlayson, W. L., Kirsch, J. F., & Christen, P. (1987) *J. Biol. Chem. 262*, 11446.
Kuramitsu, S., Hiromi, K., Hayashi, H., Morino, Y., & Kagamiyama, H. (1990) *Biochemistry 29*, 5469.
Malcolm, B. A., & Kirsch, J. F. (1985) *Biochem. Biophys. Res. Commun. 132*, 915.
Metzler, D. E., Ikawa, M., & Snell, E. E. (1954) *J. Am. Chem. Soc. 76*, 648.
Peterson, E. A., & Sober, H. A. (1954) *J. Am. Chem. Soc. 76*, 169.
Planas, A., & Kirsch, J. F. (1991) *Biochemistry 30*, 8268.
Segel, I. H. (1975) *Enzyme Kinetics*, pp 607–608, Wiley, New York.
Smith, D., Almo, S., Toney, M., & Ringe, D. (1989) *Biochemistry 28*, 8161.
Tobias, P. S., & Kallen, R. G. (1975) *J. Am. Chem. Soc. 97*, 6530.
Toney, M. D., & Kirsch, J. F. (1987) *J. Biol. Chem. 262*, 12403.
Toney, M. D., & Kirsch, J. F. (1989) *Science 243*, 1485.
Toney, M. D., & Kirsch, J. F. (1991a) *Biochemistry 30*, 7456.
Toney, M. D., & Kirsch, J. F. (1991b) *Biochemistry 30*, 7461.
Toney, M. D., & Kirsch, J. F. (1992) *Protein Sci. 1*, 107.
Velick, S. F., & Vavra, J. (1962) *J. Biol. Chem. 237*, 2109.
Wada, H., & Snell, E. E. (1962) *J. Biol. Chem. 237*, 127.
Warren, S., Zerner, B., & Westheimer, F. H. (1966) *Biochemistry 5*, 817.
Yano, T., Kuramitsu, S., Tanase, S., Morino, Y., & Kagamiyama, H. (1992) *Biochemistry 31*, 5878.