Examining the Structural and Chemical Flexibility of the Active Site Base, Lys-258, of *Escherichia coli* Aspartate Aminotransferase by Replacement with Unnatural Amino Acids†

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ABSTRACT: The active site base, Lys-258, of *Escherichia coli* aspartate aminotransferase (AATase) was mutated to a unique cysteine for chemical modification in a cysteine-free AATase [Quint; Gloss *et al.* (1992) *Biochemistry 31*, 32−39]. Two homolysine analogues (γ-thiahomolysine and γ-dithiohomolysine) and the carboxylate series [glutamate, (carboxymethyl)cysteine, and (carboxyethyl)cysteine] were prepared. The k_{cat} and k_{cat}/K_M values for amino acids exhibited by the 258-homo-Lys AATases are *ca.* 1000-fold less than those of Quint. However, the k_{cat}/K_M values for keto acids are only reduced by 2.5−10-fold. The 258-side-chain carboxylates bind amino acids 1−4 orders of magnitude more tightly than Quint, but have first-order catalytic rate constants 5−7 orders of magnitude less than Quint. The 258-homo-Lys AATases stabilize the E·PLP *vs* the E·PMP species by ~3 kcal/mol relative to Quint; the 258-side-chain carboxylate variants stabilize E·PMP *vs* E·PLP by 5.8 kcal/mol relative to WT. The C_α-^{2}H kinetic isotope effects for the reactions of 258-homo-Lys and 258-side-chain carboxylate AATases demonstrate that transaldimination is equally or more rate-determining than C_α proton abstraction for both sets of enzymes. The values of k_{cat}/K_M calculated for the 258-side-chain carboxylate variants from the Brønsted plot of Toney and Kirsch [(1989) *Science 243*, 1485−1488] are in close agreement with those observed. This "tethered" Brønsted analysis shows that the earlier reported poor reactivity of carboxylates in chemical rescue is due to electrostatic exclusion from the active site.

A structural hallmark of pyridoxal phosphate (PLP)[1] dependent enzymes is the presence of a lysine residue, which forms an aldimine with the cofactor. The role of this lysine (Lys-258) has been well-studied by site-directed mutagenesis in *Escherichia coli* AATase (K258A, K258M: Toney & Kirsch, 1989, 1992, 1993; K258R: Toney & Kirsch, 1991a; K258C: Planas & Kirsch, 1991; K258H: Ziak *et al.*, 1993; Malashkevich *et al.*, 1995). This transaminase catalyzes the transfer of the α-amino groups of L-Asp and L-Glu to the α-keto acids, OAA and α-KG, as shown in eq 1:

$$\text{Enz−PLP} + \text{L-aspartate} \rightleftharpoons \text{Enz−PMP} + \text{oxalacetate} \quad (1)$$

$$\text{Enz−PMP} + \text{α-ketoglutarate} \rightleftharpoons \text{Enz−PLP} + \text{L-glutamate}$$

Lys-258 has multiple roles in the mechanism of AATase. The presence of the internal aldimine enhances the rate of formation of the external aldimine with amino acid substrate, because transaldimination proceeds more rapidly than Schiff base formation from an amine and aldehyde (Cordes & Jencks, 1962; Toney & Kirsch, 1993). Most importantly, the ϵ-amino group is the general base that catalyzes the 1,3-prototropic shift between the external aldimine and the ketimine (Scheme 1). Lys-258 may also play a role in ketimine hydrolysis to release the keto acid product and yield the PMP enzyme species.

Catalytic activity can be restored to the inactive mutant, K258A, by the addition of exogenous amines (Toney & Kirsch, 1989, 1992). Acetate and formate can also restore activity to this mutant. This implies that a 258 residue with a carboxylate side chain would have significantly faster rates of turnover than K258A.

Unnatural amino acids have been incorporated into many proteins by chemical modification of cysteine, *e.g.*, γ-thialysine and γ-dithiohomolysine in Rubisco (Smith & Hartman, 1988). γ-Thia-Lys was also introduced at position 258 by the combined use of site-directed mutagenesis (to generate K258C) and modification with bromoethylamine (Planas & Kirsch, 1990, 1991). A cysteineless AATase, Quint, was engineered (Gloss *et al.*, 1992), and Cys-258 was reintroduced as a unique cysteine for chemical modification with

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[1] Abbreviations: AATase, aspartate aminotransferase; WT, wild type *E. coli* AATase; Quint, *E. coli* AATase without cysteine, containing the mutations C82A, C191A, C192A, C270A, and C401A; $K258C_Q$, Quint *E. coli* AATase which contains a unique cysteine at position 258 in place of the WT Lys residue; nomenclature for the chemically modified enzymes, $K258C_Q$-X, is given in Scheme 2; $K258E_Q$, site-directed mutant of Lys-258 to Glu, in the Quint (cysteine-less) background; α-KG, α-ketoglutarate; AMPSO, 3-[1,1-dimethyl-(2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid; α-Me-Asp, α-methyl-DL-aspartate; L-CS, L-cysteine sulfinate; Br-PA, bromopropylamine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); HGDH, α-hydroxyglutarate dehydrogenase from *Peptostreptococcus asaccharolyticus*; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; KIE, primary C_α proton kinetic isotope effect; LDH, lactate dehydrogenase; MDH, *E. coli* malate dehydrogenase; OAA, oxalacetate; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate.

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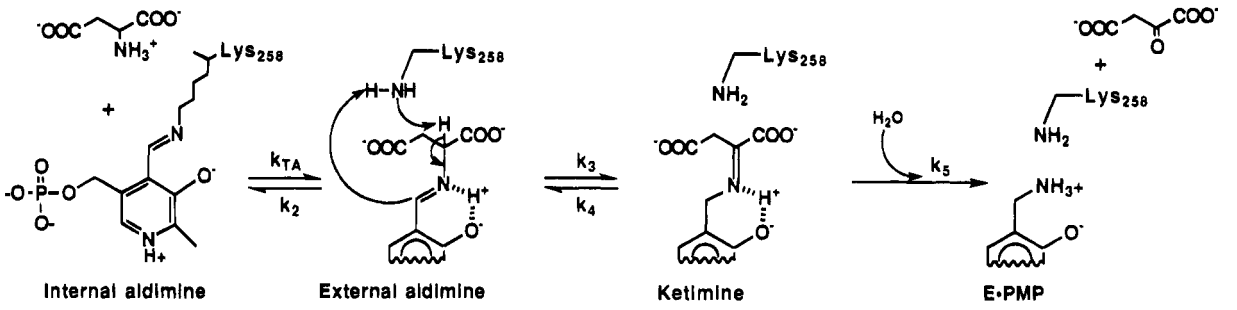
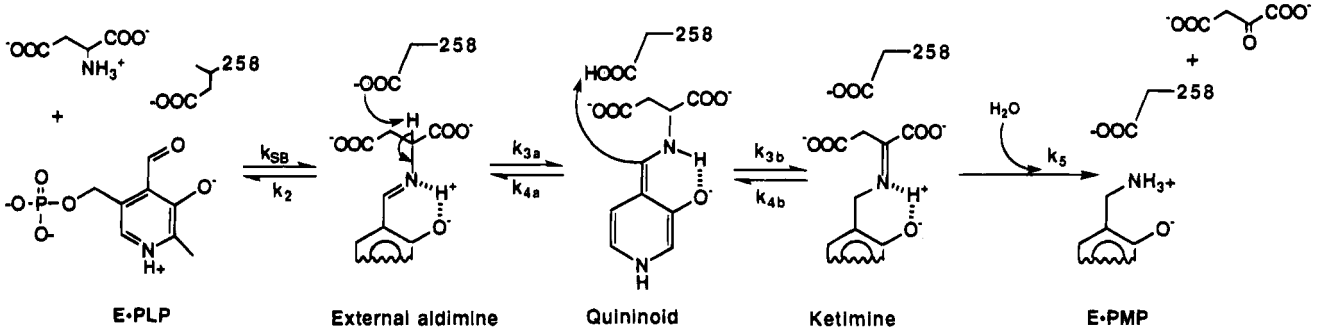
Scheme 1: Partial Mechanism of the Reaction of L-Asp with (A) Aspartate Aminotransferases with Lysine or a Lysine Analogue at Position 258 or (B) Aspartate Aminotransferases with a Side-Chain Carboxylate at Position 258[a]

1A. Reaction of enzymes with lysine or lysine analogues at position 258.



1B. Reaction of enzymes with sidechain carboxylates at position 258.



[a] The E•PLP form is an internal aldimine between PLP and Lys-258. PLP is bound as the aldehyde in the absence of a primary amine side chain at position 258. The E•PLP species reacts with L-Asp either by (A) transaldimination (k_{TA}) or (B) Schiff base formation (k_{SB}). This generates the external aldimine between the cofactor and the substrate. The 258-residue acts as the base catalyzing the 1,3-prototropic shift. The WT enzyme may proceed by a concerted mechanism as shown in part A (Julin & Kirsch, 1989; Goldberg, 1992). The C_α proton is abstracted (k_3 or k_{3a}), generating the quinoid as shown in part B, and reprotonation occurs at C_4' to yield the ketimine (k_3 or k_{3b}). The ketimine is hydrolyzed to release the keto acid product, OAA, and the E•PMP form (k_5).

bromoethylamine, yielding K258C_Q-EA (Gloss & Kirsch, 1995a). This report extends the latter study by additional modifications of K258C_Q to generate the following at position 258 (Scheme 2): (1) two analogues of homolysine (γ-thiahomolysine and γ-dithiohomolysine); (2) a series of carboxylates [glutamate, (carboxymethyl)cysteine, and (carboxyethyl)cysteine].

MATERIALS AND METHODS

Materials

The sources of [C_α-^{2}H]-DL-Asp, [C_α-^{2}H]-DL-CS, HGDH, LDH, and MDH are given in Gloss and Kirsch (1995a,b). Iodopropionic acid was from Kodak, and was recrystallized prior to use. All other chemicals and reagents were of the highest purity available from Sigma or Aldrich.

Methods

Site-directed mutagenesis of K258C_Q has been described elsewhere [see Gloss and Kirsch (1995a) and references cited therein]. K258E_Q was prepared by the same method. Purification and quantification of WT and mutant AATases were performed according to Gloss *et al.* (1992), with the following simplification: chromatography of the ammonium sulfate pellet over a G-100 column was replaced by dialysis against 20 mM potassium phosphate buffer, pH 7.5, 5 mM EDTA, 1 mM DTT, and 20 μM PLP. The purity of the final protein preparation was unaffected.

Modification of K258C_Q. K258C_Q (2−6 mg/mL, 45−140 μM monomer) was unfolded in 6 M urea, 200 mM AMPSO, pH 9.0, and 15 mM EDTA and reacted with 30−40 mM iodoacetate, iodopropionate, bromopropylamine, or cystamine. The reactions proceeded for 40 min (iodoreagents and cystamine) or 2 h (bromopropylamine) at room temperature, in the dark. The extent of modification was monitored by reaction with DTNB (Planas & Kirsch, 1990). The modified enzymes were refolded by the method of Gloss and Kirsch (1995a).

Steady-State Kinetics. Reaction rates were determined from the time-dependent decrease in NADH absorbance (340 nm) at 25 °C with a Kontron Uvikon 860 spectrophotometer. The assays were performed in 200 mM HEPES, 100 mM KCl (μ = 0.2), 150 μM NADH, and 5−10 units/mL of the appropriate coupling enzyme (MDH, HGDH, and LDH for the L-Asp/α-KG, L-Glu/OAA, and L-CS/α-KG substrate pairs, respectively). The kinetic parameters of the amino acid reactions were determined by holding the concentration of keto acid constant at >30 × K_M ($K_M \leq 15$ μM), and collecting data at 10−12 concentrations of amino acid. The data were fitted to eq 2:

$$v = \frac{k_{cat}[S]}{K_M + [S]} \quad (2)$$

The K_M of the keto acid is dependent upon the amino acid substrate (Segel, 1975). To verify that the keto acid reaction was fully saturated for each amino acid, the concentration of keto acid was varied from 0.2 to 1.0 mM at [amino acid] = 5 × K_M. No dependence of velocity upon [keto acid] was observed. The kinetic parameters of the α-KG reaction were determined at [L-Asp] = 5 × K_M, and the data were fitted to eq 2. The errors on the k_{cat}/K_M parameters were determined by the method of Julin and Kirsch (1989).

Single-Turnover Kinetics. K258E_Q, K258C_Q-CbMe, and K258C_Q-CbEt rate constants were determined under pseudo-first-order single-turnover conditions by monitoring the cofactor absorbance (Cronin & Kirsch, 1988; Toney & Kirsch, 1993). The assay conditions were 200 mM HEPES, pH 7.5, 100 mM KCl (μ = 0.2) with 10 μM AATase at 25 °C, with ≥8-fold excess of substrate over enzyme. Reactions with amino acids were monitored at 340 nm (PMP) and, additionally, at 430 nm (external aldimine) and 390 nm (PLP), for K258E_Q and K258C_Q-CbMe, respectively. Reactions of the PMP form of K258C_Q-CbEt with α-KG were monitored at 390 nm. The data were collected with a Kontron Uvikon 860 spectrophotometer controlled by an IBM-compatible AT computer. The exponential progress curves were fitted with the program *Enzfitter* (Biosoft Publishing Co.). The observed pseudo-first-order rate constants were dependent upon the L-CS concentrations in the range employed. The data were fitted to eq 3:

$$k_{obs} = \frac{k_{max}[S]}{K_D^{app} + [S]} \quad (3)$$

to determine the maximal rate of the half-transamination reaction (k_{max}) and the apparent Michaelis constant (K_D^{app}).

C_α-^2H Kinetic Isotope Effects. The C_α-^2H kinetic isotope effects for L-Asp and L-CS were measured by performing the reactions with [C_α-^2H]-DL-amino acid substrates in the same manner as described for the [C_α-^1H]-amino acids. D-Asp does not associate with the WT enzyme (Planas & Kirsch, 1991); it is assumed that D-Asp and D-CS do not interact with the mutants.

Spectrophotometric Determination of the Internal Aldimine pK_a. The titrations of the internal aldimine formed between the primary amine at position 258 and the PLP cofactor were performed by the method of Goldberg *et al.* (1991) at an ionic strength of 0.2. The value of ϵ_{430} as a function of pH was fitted to eq 4:

$$\epsilon = \frac{\epsilon_1 - \epsilon_2}{1 + 10^{pH - pK_a}} + \epsilon_2 \quad (4)$$

where ϵ_1 and ϵ_2 are the upper and lower limits, respectively of the extinction coefficients at 430 nm.

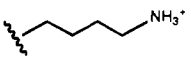
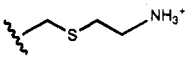
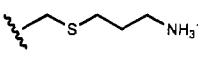
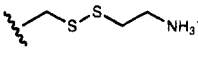
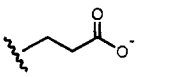
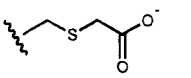
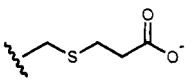
Titration of K258E_Q with L-Asp and L-Glu. The K_D values of K258E_Q for L-Asp and L-Glu are too low to be determined by single-turnover kinetics under pseudo-first-order conditions. The dissociation constants were measured by direct titration of the enzyme, monitoring the increase in absorbance at 430 nm (external aldimine); 5 and 10 μM solutions of K258E_Q·PLP were titrated with concentrated stocks of L-Asp or L-Glu. The data were fitted to eq 5 with the NLIN program of the SAS statistical package (SAS Institute, Cary, NC):

$$Abs = A_i + (A_f - A_i)\frac{K_D + [L] + [E] - \sqrt{(K_D + [L] + [E])^2 - 4[L][E]}}{2[E]} \quad (5)$$

where A_i is the initial absorbance, A_f the maximal absorbance, and K_D the dissociation constant; [L] and [E] are the concentrations of ligand and enzyme, respectively.

Determination of PLP vs PMP Affinities. The relative preferences for PLP and PMP of AATase mutants lacking a primary amine at position 258 were assessed by two methods: (1) reconstitution of apoenzyme with varying PLP:PMP ratios and (2) dialysis of holoenzyme against varying PLP:PMP ratios. Apoenzymes were prepared by modifications to the procedure of Toney and Kirsch (1993). K258C_Q and K258E_Q, as isolated from *E. coli*, contain 50−90% PMP. The heterogeneous mixture of enzyme·cofactor forms was precipitated by addition of a saturated ammonium sulfate solution (pH 7.0, 4 °C, 60−70% final saturation). The protein was collected by centrifugation and resuspended with saturated ammonium sulfate, pH 5.0 (in 100 mM sodium acetate buffer). The precipitate was collected, and the pellet was resuspended in saturated ammonium sulfate, pH 3.0 (in 100 mM sodium acetate buffer). This solution was incubated on ice for 10−30 min; the protein was collected and resuspended 3−5 times in saturated ammonium sulfate, pH 3.0. When the supernatant no longer contained cofactor (as determined by UV−visible spectra), the pH of the protein solution was raised by precipitations with saturated ammonium sulfate, pH 7.0. The protein was dissolved in 50 mM HEPES, pH 7.5, 5 mM EDTA, and dialyzed against this buffer.

Scheme 2: Amino Acids Incorporated at Position 258 of *E. coli* Aspartate Aminotransferase

Chemical structure	Amino acid	AATase nomenclature
	Lysine	WT or Quint
	S-(aminoethyl)-cysteine γ-thia-Lys	K258C_Q-EA
	S-(aminopropyl)-cysteine γ-thia-homo-Lys	K258C_Q-PA
	S-(aminoethylthio)-cysteine γ-dithio-homo-Lys	K258C_Q-DTA
	Glutamate	K258E_Q
	S-(carboxymethyl)-cysteine	K258C_Q-CbMe
	S-(carboxyethyl)-cysteine	K258C_Q-CbEt

The reconstitution conditions were 200 mM HEPES, pH 7.5, 100 mM KCl, and 10 μM apoenzyme. The dialysis conditions were 50 mM HEPES, pH 7.5, 100 mM KCl, and 20 μM apoenzyme. The cofactor concentrations were (a) 100 μM PLP, (b) 100 μM PMP, or (c) PLP:PMP ratios from 1:5 to 10:1, with the concentration of the underrepresented cofactor equal to 100 μM. The enzyme/cofactor solutions were incubated for 90−95 h at 4 °C. Excess (unbound) cofactor was removed by G-50 spin columns and/or Amicon Centricon ultrafiltration. The extinction coefficients of the E·PLP forms were determined from the UV−visible spectra of reconstitutions with only PLP. The ϵ_{390}^{PLP} values were used to determine the percent PLP bound in incubations with PLP and PMP. The preference for one cofactor over the other was calculated from experiments with at least two different ratios of cofactors.

RESULTS

Chemical Elaboration of Cys-258. The preparation and properties of Quint, the cysteine-free AATase, have been described previously (Gloss *et al.*, 1992). Variants constructed in the Quint background are denoted by a subscript Q, *i.e.*, K258C$_Q$ and K258E$_Q$. The preparation of K258C$_Q$-EA is described elsewhere (Gloss & Kirsch, 1995a). The modification protocols for the K258C$_Q$ variants described herein differ from that of K258C$_Q$-EA principally in the reduced time of reaction (see Methods), as the reagents used in this study are more reactive than bromoethylamine.

The modification reactions resulted in >95% alkylation of K258C$_Q$ as judged by thiol assays with DTNB (Planas & Kirsch, 1991) and active site titrations with L-CS (White & Kirsch, 1992). Quint was treated with the same reagents as was K258C$_Q$, as controls, and the resulting preparations were assayed with the L-Asp/α-KG substrate pair. Treatment of Quint with iodoacetate did not affect any subsequently determined kinetic parameter by >20%. Less than 10% effects were observed following treatment of Quint with iodopropionate or bromopropylamine.

Typical refolding yields were 50% and 15% for the preparation of K258C$_Q$-CbMe and K258C$_Q$-CbEt, respectively. K258C$_Q$-PA and K258C$_Q$-DTA refolded with 25−35% yields. Unlike the preparation of K258C$_Q$-EA (Gloss & Kirsch, 1995a), no aggregate was observed in the refolding of K258C$_Q$-CbMe. Aggregation was observed after the refolding of K258C$_Q$-CbEt, K258C$_Q$-PA, and K258C$_Q$-DTA, and the aggregates were removed by ammonium sulfate precipitation as described in Gloss and Kirsch (1995a).

Internal Aldimine pK_a Values of K258C$_Q$-DTA and K258C$_Q$-PA. The pK_a's of the internal aldimines of AATases can be determined by spectrophotometric titration (Cronin & Kirsch, 1988; Goldberg *et al.*, 1991). The protonated and deprotonated aldimines of wild-type AATases and Quint exhibit λ_{max} values of 430 and 360 nm, respectively (Kallen *et al.*, 1985; Goldberg *et al.*, 1991; Gloss & Kirsch, 1995a). The AATase variants with homolysine analogues exhibit λ_{max} values of 430 and 335 nm. The pH-dependence of the UV−visible spectra of K258C$_Q$-DTA and K258C$_Q$-PA is shown in Figure 1. The extinction coefficients at 430 nm, as a function of pH, for these enzymes are shown in Figure 2. These data fit to pK_a values of 6.21 ± 0.01 and 7.50 ± 0.01 for K258C$_Q$-PA and K258C$_Q$-DTA, respectively.

Kinetics of AATases with 258-Homo-Lys Analogues. The kinetic constants for the transamination reactions of K258C$_Q$-DTA and K258C$_Q$-PA are given in Table 1. The k_{cat}/K_M^{OAA} values could not be directly measured, because of the low K_M^{OAA} values. The rate constants were calculated from the Haldane relationship for the ping-pong mechanism (Velick & Vavra, 1962):

$$k_{cat}/K_M^{OAA} = \frac{K_{eq,overall}(k_{cat}/K_M^{\alpha\text{-KG}})(k_{cat}/K_M^{Asp})}{k_{cat}/K_M^{Glu}} \quad (6)$$

as has been done for Quint and K258C$_Q$-EA (Gloss & Kirsch,

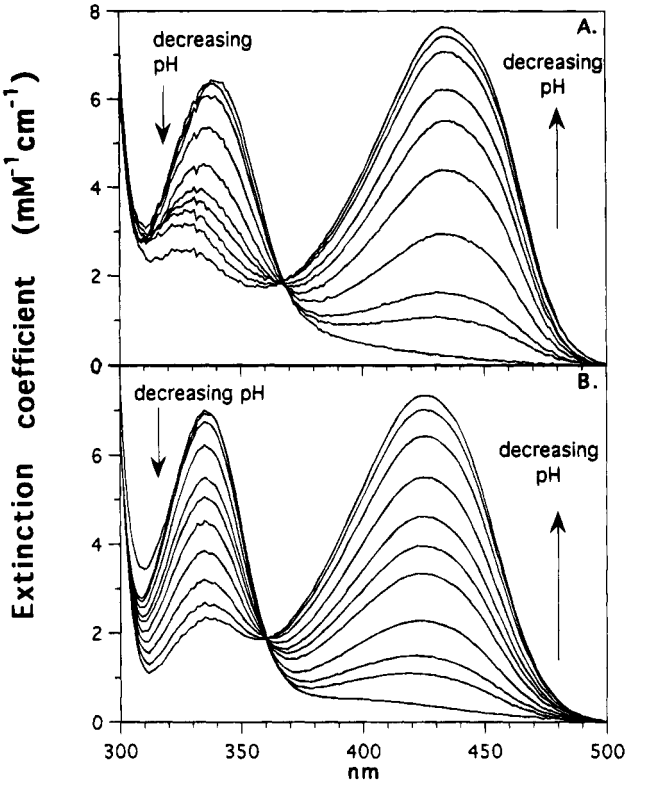


FIGURE 1: pH-dependence of the UV−visible spectra of K258C$_Q$-DTA and K258C$_Q$-PA aspartate aminotransferases titrated by the method of Goldberg *et al.* (1991). μ = 0.2. (A) K258C$_Q$-DTA at pH 9.87, 8.46, 8.19, 7.73, 7.40, 7.11, 6.88, 6.44, 5.99, and 5.04. (B) K258C$_Q$-PA at pH 9.87, 7.25, 7.03, 6.70, 6.37, 6.25, 6.03, 5.80, 5.52, 5.26, and 4.98.

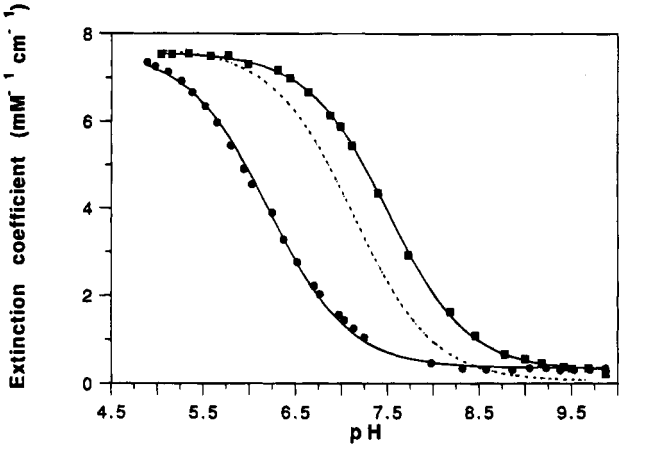


FIGURE 2: pH-dependence of the extinction coefficients of K258C$_Q$-DTA (■) and K258C$_Q$-PA (●) aspartate aminotransferases. Absorbance was monitored at 430 nm, and the solid lines represent fits of the data to eq 4. The theoretical fit of Quint (pK_a = 7.1; Gloss & Kirsch, 1995a) is shown by the dashed line.

Table 1: Kinetic Constants for the Reactions of Asparate Aminotransferases with Homolysine Analogues, K258C$_Q$-DTA and K258C$_Q$-PA[a]

substrate	parameters	K258C$_Q$-DTA	$X^{K258C_Q\text{-DTA}}/X^{Quint}$ [b]	K258C$_Q$-PA	$X^{K258C_Q\text{-PA}}/X^{Quint}$ [b]
L-Asp/α-KG[c]	k_{cat} (s^{-1})	0.191 (0.002)	8×10^{-4}	0.76 (0.05)	3×10^{-3}
	K_M(L-Asp) (mM)	1.57 (0.06)	0.58	6.0 (0.1)	2.2
	k_{cat}/K_M^{Asp} (M^{-1} s^{-1})	122 (3)	1×10^{-3}	125 (6)	1×10^{-3}
	K_m(α-KG) (mM)	0.010 (0.002)	6×10^{-3}	0.014 (0.001)	1×10^{-3}
	$k_{cat}/K_M^{\alpha\text{-KG}}$ (M^{-1} s^{-1})	16000 (4000)	0.10	45000 (7000)	0.29
L-Glu/OAA[d]	k_{cat} (s^{-1})	0.27 (0.01)	1×10^{-3}	0.308 (0.008)	1×10^{-3}
	K_M(L-Glu) (mM)	19 (2)	0.74	37 (3)	1.4
	k_{cat}/K_M^{Glu} (M^{-1} s^{-1})	13.8 (0.7)	2×10^{-3}	8.3 (0.5)	1×10^{-3}
	k_{cat}/K_M^{OAA} (M^{-1} s^{-1})	850000	0.09	4100000	0.4
L-CS[e]	k_{cat} (s^{-1})	0.114 (0.005)	3×10^{-4}	0.19 (0.01)	5×10^{-4}
	K_M (mM)	3.1 (0.3)	0.26	9.1 (0.5)	0.76
	k_{cat}/K_M (M^{-1} s^{-1})	37 (3)	1×10^{-3}	21.1 (0.1)	6×10^{-4}

[a] Conditions: 200 mM HEPES, pH 7.5, 100 mM KCl ($\mu = 0.2$), 25 °C. The parameters were determined from coupled steady-state assays as described under Methods. Standard errors are in parentheses. [b] X denotes the parameter listed in the left-hand column. The values of Quint are from Gloss and Kirsch (1995a). [c] For the L-Asp kinetic constants: [L-Asp] = 0.5−30 mM and [α-KG] = 0.5 mM. For the α-KG constants: [α-KG] = 0.025−1.0 mM; [L-Asp] = 5 × K_M. [d] [OAA] = 0.5 mM; [L-Glu] = 2.5−50 mM (K258C$_Q$-DTA) or 15−200 mM (K258C$_Q$-PA). The k_{cat}/K_M^{OAA} values were calculated as described in the text (eq 6). [e] [α-KG] = 1.0 mM; [L-CS] = 0.5−20 mM (K258C$_Q$-DTA) or 5−40 mM (K258C$_Q$-PA).

1995a). The values calculated for K258C$_Q$-DTA and K258C$_Q$-PA are given in Table 1. From these k_{cat}/K_M^{OAA} values and the measured values of k_{cat} for the L-Glu/OAA substrate pair, the estimated K_M^{OAA} values are ~300 and ~80 nM for K258C$_Q$-DTA and K258C$_Q$-PA, respectively. Similar calculations for Quint yield a K_M^{OAA} value of ~20 μM, similar to the WT value (Planas & Kirsch, 1991).

The amino acid K_M values of these enzymes are 0.26−2.2-fold of those exhibited by Quint. However, their keto acid K_M values are *ca.* 2 orders of magnitude less than those of Quint. K258C$_Q$-DTA and K258C$_Q$-PA have similar k_{cat}/K_M values for amino acids, with the relative rankings, L-Asp > L-CS > L-Glu. The k_{cat}/K_M values for amino acids are decreased, relative to Quint, by 600−1600-fold. In contrast, the k_{cat}/K_M values for the keto acid reactions of K258C$_Q$-PA and K258C$_Q$-DTA are only lower by 4- and 10-fold, respectively, from those of Quint. In general, K258C$_Q$-DTA has both lower K_M and k_{cat} values than K258C$_Q$-PA. The k_{cat} values for the two enzymes differ by 1.1−4-fold for reactions with α-KG and L-CS or L-Asp, respectively. The k_{cat} values, relative to those of Quint, are reduced by 310−3500-fold.

Kinetics of 258-Side-Chain Carboxylates. The L-amino acid K_D and K_D^{app} values of K258E$_Q$, K258C$_Q$-CbMe, and K258C$_Q$-CbEt are given in Table 2. Those determined from single-turnover reactions are likely to be true dissociation constants, as are the K_M values of Quint determined from steady-state kinetics (Gloss & Kirsch, 1995a). All three 258-side-chain carboxylate enzymes have greater affinities for the amino acid substrates than does the parent enzyme, Quint, by 1−4 orders of magnitude. As the length of the 258-carboxylate side chain is increased, the K_D^{app} value for L-CS also increases. K258C$_Q$-CbEt binds L-CS an order of magnitude less tightly than does K258E$_Q$.

The rate constants describing the reactions of 258-side-chain carboxylates with L-amino acids are presented in Table 3. The first-order rate constant, k_{max}, for the L-CS reactions increases with increasing length of the 258-side chain. The k_{max} value of K258C$_Q$-CbEt is nearly an order of magnitude higher than that of K258E$_Q$. However, this increased rate is offset by the decreased affinity for L-CS, such that the two enzymes have nearly equal k_{cat}/K_M^{CS} values. All the

Table 2: Dissociation Constants for the Complexes of L-Amino Acids and Aspartate Aminotransferases with 258-Side-Chain Carboxylates: K258E$_Q$, K258C$_Q$-CbMe, and K258CQ-CbEt[a]

enzyme	substrate	K_D (μM)	K_M^{Quint}/K_D^{mutant} [b]
K258E$_Q$	L-Asp	0.2 (0.1)[c]	15000
K258E$_Q$	L-Glu	17 (3)[c]	1500
enzyme	**substrate**	K_D^{app} (mM)	K_M^{Quint}/K_n^{mutant} [b]
K258E$_Q$	L-CS[d]	0.104 (0.005)	120
K258C$_Q$-CbMe		0.57 (0.2)	20
K258C$_Q$-CbEt		1.1 (0.1)	11

[a] Conditions: 200 mM HEPES, pH 7.5, 100 mM KCl ($\mu = 0.2$), 25 °C, and 10 μM AATase monomer. Standard errors are in parentheses. [b] The K_M^{Quint} values were determined under identical conditions by steady-state kinetics and are approximately true dissociation constants (Gloss & Kirsch, 1995a). [c] Determined by direct spectrophotometric titration as described under Materials and Methods. [d] Determined from single-turnover kinetics with L-CS. A total of 5−12 data points were collected over the range 50 μM−5 mM L-CS. The errors represent the weighted average of the kinetic parameters determined from two or more independent enzyme preparations.

258-side-chain carboxylate k_{max} values are 5−7 orders of magnitude less than those of an enzyme with Lys-258. The k_{max}/K_D^{app} values are less compromised, only 3−4 orders of magnitude lower than the k_{cat}/K_M values of Quint.

The velocity of the reaction of α-KG with K258C$_Q$-CbEt is independent of [α-KG] from 2.5 to 15 mM. The k_{max} value of K258C$_Q$-CbEt is $(2.0 \pm 0.3) \times 10^{-5}$ s^{-1}. After 24 h incubation, <2% turnover was observed for the reaction of K258E$_Q$ with α-KG. The upper limit of the k_{max} value for this mutant is therefore 2×10^{-7} s^{-1}, at least 2 orders of magnitude slower than that of K258C$_Q$-CbEt. The reaction of K258C$_Q$-CbMe with α-KG could not be studied as the PMP form of this enzyme aggregated upon concentration or incubation times greater than 12 h.

Spectral Properties of the 258-Side-Chain Carboxylate Enzymes. The spectral time courses of K258E$_Q$ and K258C$_Q$-CbEt reactions with L-CS are shown in Figure 3. The external aldimine intermediate (Scheme 1) of K258E$_Q$ could be spectrophotometrically observed with all three amino acid substrates (data not shown for L-Asp and L-Glu). The rate constants for the reaction of K258E$_Q$ with L-CS measured at 340 nm (appearance of E·PMP) agreed well with those

Table 3: Rate Constants for the Reactions of L-Amino Acids with K258E$_Q$, K258C$_Q$-CbMe, and K258C$_Q$-CbEt Aspartate Aminotransferases[a]

enzyme	substrate	$k_{max} \times 10^3$ (s^{-1})	k_{max}/K_D^{app} ($M^{-1}\ s^{-1}$)	$k_{max}^{mutant}/k_{max}^{WT}$ [b]	$(k_{max}/K_D^{app})^{mutant}/(k_{max}/K_D^{app})^{WT}$ [b]
K258E$_Q$	L-Asp[c]	0.025 (0.002)	125 (63)	1×10^{-7}	1×10^{-3}
K258E$_Q$	L-Glu[c]	0.012 (0.005)	0.7 (0.3)	5×10^{-8}	1×10^{-4}
K258E$_Q$	L-Cs[d]	0.97 (0.01)	9.5 (0.4)	3×10^{-7}	3×10^{-4}
K258C$_Q$-CbMe	L-Cs[d]	1.59 (0.02)	1.8 (0.9)	5×10^{-7}	5×10^{-5}
K258C$_Q$-CbEt	L-Cs[d]	7.8 (0.8)	6.8 (0.0)[e]	3×10^{-6}	2×10^{-4}

[a] Conditions are given in the legend of Table 2. [b] The k_{max} values (eq 3) are compared to those of WT [Onuffer & Kirsch (1995) and calculations of Gloss and Kirsch (1995b)] as k_{max} values have not been determined for Quint. The k_{cat}/K_M values of Quint are from Gloss and Kirsch (1995a). [c] The concentrations of L-Asp and L-Glu were 5 and 25 mM, respectively. The assays were performed in triplicate, and the error represents the weighted average of the observed rate constants. [d] Data were collected as described in footnote *d* of Table 2. [e] The same value was obtained in both experiments.

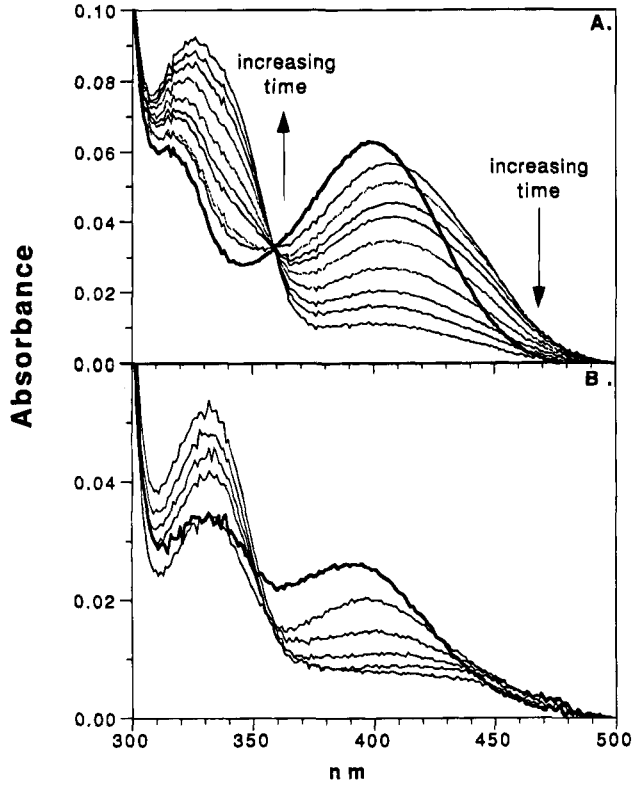


FIGURE 3: Time-dependence of the absorption spectra of K258E$_Q$ and K258C$_Q$-CbEt after the addition of L-CS. Conditions: 10−12 μM enzyme, [L-CS] = K_D (Table 2), 200 mM HEPES, pH 7.5, 100 mM KCl, μ = 0.2, 25 °C. Both enzyme preparations contain a small amount of an unreactive cofactor form with a λ_{max} of 318 nm. This may be pyridoxic acid (Sober, 1968). (A) K258E$_Q$: no L-CS (boldface); 4, 8, 15, 19, 27, 40, 55, 70, and 100 min. The external aldimine (λ_{max} = 430 nm) is formed within 2 min from enzyme-bound PLP (λ_{max} = 390 nm). The subsequent decrease at 430 nm and concomitant increase at 330 nm are due to transamination to the ketimine. (B) K258C$_Q$-CbEt: no L-CS (boldface); 1, 2, 5, 12, and 40 min. The enzyme-bound PLP (λ_{max} = 390 nm) reacts with L-CS; the external aldimine intermediate is not significantly populated as judged by the failure of the 430 nm band to appear. The decrease at 390 nm is concomitant with the increase at 330 nm.

measured at 430 nm (disappearance of the external aldimine). The reactions of K258C$_Q$-CbMe and K258C$_Q$-CbEt with L-CS did not proceed through a significantly-populated external aldimine (Figure 3B, data not shown for K258C$_Q$-CbMe). The rates measured at 340 and 390 nm (disappearance of E·PLP) agreed well for K258C$_Q$-CbMe. For K258C$_Q$-CbMe and K258C$_Q$-CbEt, the external aldimine does accumulate with the less reactive substrate, L-Asp, or the inhibitor, α-Me-Asp (Figure 4).

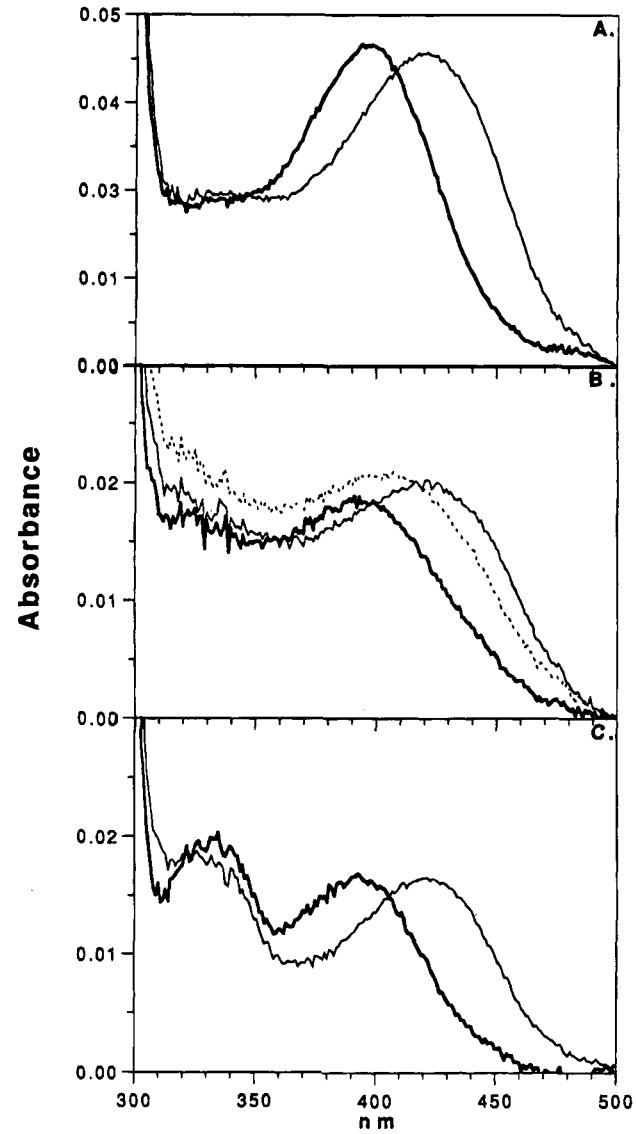


FIGURE 4: (A) K258E$_Q$·PLP with no ligand (boldface line) and 5 mM α-Me-Asp. (B) K258C$_Q$-CbMe with no ligands (boldface line), 20 mM L-Asp (solid line), and 40 mM DL-α-Me-Asp (dotted line). (C) K258C$_Q$-CbEt with no ligand (boldface line) and 40 mM α-Me-Asp. Conditions as in Figure 3.

Relative Cofactor Preference of K258E$_Q$. The time required for K258E$_Q$ and K258C$_Q$ to equilibrate with cofactors was determined by dialyzing 20 μM E·PLP and E·PMP enzymes against 100 μM solutions of the alternate cofactor. The E·PLP forms of K258E$_Q$ and K258C$_Q$ were completely converted to the E·PMP forms within 25 h at 4

Table 4: L-Asp and L-CS C_α-^2H Kinetic Isotope Effects for the Reactions of Aspartate Aminotransferases with Alternate Active Site Bases[a]

enzyme	$^Dk_{cat}$ or $^Dk_{max}$	$^D(k_{cat}/K_M)$ or $^D(k_{max}/K_D^{app})$
	L-Asp	
Quint[b]	1.52 (0.03)	2.14 (0.09)
K258C_Q-DTA[c], pH 7.5	1.91 (0.04)	1.05 (0.06)
K258C_Q-DTA,[c] pH 9.0	2.14 (0.07)	0.98 (0.08)
K258C_Q-PA[c]	4.5 (0.1)	2.2 (0.1)
K258E_Q[d]	6.7 (0.6)	ND
	L-CS	
Quint[e]	1.09 (0.06)	1.7 (0.1)
K258E_Q[d]	3.54 (0.06)	2.0 (0.1)
K258C_Q-CbMe[d]	2.2 (0.1)	1.0 (0.3)

[a] Conditions are given in the legends of Tables 1 and 2. [b] Reproduced from Gloss and Kirsch (1995a). [c] Steady-state kinetic data were collected as described in footnote *b* of Table 1. [d] Single-turnover kinetic data as described in Table 3 for the ^{1}H-L-amino acids. [e] Steady-state kinetic data at [α-KG] = 10 mM, [L-CS] = 5−30 mM.

°C. Dialysis for 85 h was sufficient to convert K258E_Q·PMP to the E·PLP form; however, after 168 h, only 30% of the K258C_Q·PMP had been converted to the E·PLP form. Therefore, the relative cofactor affinities of K258C_Q were not determined because of the long equilibration times required.

K258E_Q has a (6.7 ± 0.2)- or (6.36 ± 0.06)-fold greater affinity for PMP than for PLP as measured by reconstitution or dialysis, respectively. K258C_Q-CbEt exhibits a similar preference for PMP (6.3-fold) as determined by reconstitution. Evaluation of the relative cofactor affinities of K258C_Q-CbMe was precluded because this variant aggregates under the conditions employed in these experiments.

C_α-^2H Kinetic Isotope Effects with L-Asp and L-CS. The L-Asp C_α-^2H KIE values of K258E_Q, K258C_Q-DTA, and K258C_Q-PA (pH 7.5) are shown in Table 4. The $^Dk_{cat}$ value of WT is pH-dependent, and shows a pK_a similar to that of the internal aldimine (Gloss & Kirsch, 1995b). The KIE values of K258C_Q-DTA were also determined at pH 9.0 (Table 4), above the pK_a of its internal aldimine, demonstrating that the low KIE's observed at pH 7.5 are not due to a pH-dependence of $^Dk_{cat}$. The L-CS KIE values were determined for Quint, K258E_Q, and K258C_Q-CbMe (Table 4). The KIE values observed for the reaction of Quint with L-CS are similar to those reported for WT (Gloss & Kirsch, 1995b).

DISCUSSION

Unnatural Amino Acids in Proteins. The limitations of the genetic code have been circumvented to introduce unnatural amino acids into proteins by two methods: *in vitro* translation (Ellman *et al.*, 1991) and posttranslational chemical modification. The *in vitro* translation technique affords great versatility in the types of residues that can be introduced; in theory, almost any amino acid can be incorporated into the protein of interest. Chemical modification is limited by the chemical reactivity of the targeted amino acid side chains, usually Cys, but also Lys, His, and Tyr (Glazer, 1976), as well as by the yields of the chemical reaction.

A significant limitation to the *in vitro* translation method is that protein is usually produced in quantities sufficient only for steady-state kinetic analyses of relatively active variants. The highest published yield for this method is 40 μg/mL, using a 5 mL reaction (Ellman *et al.*, 1992; Chung *et al.*, 1993). By contrast, 10−50 mg quantities of protein can often be obtained by combined site-directed mutagenesis and chemical modification.

Table 5: Solution pK_a Values for the Side Chains of the Amino Acids Incorporated at Position 258 in Aspartate Aminotransferase[a]

amino acid	pK_a	reference
lysine	10.47	Hermann & Lemke (1968)
γ-thia-Lys	9.52	Hermann & Lemke (1968)
γ-thiahomo-Lys	10.0	calc[b]
γ-dithiohomo-Lys	9.30	Hawkins & Perrin (1962)
glutamate	4.26	Serjeant & Dempsey (1979)
S-(carboxymethyl)-Cys	3.3	calc[b]
S-(carboxyethyl)-Cys	3.9	calc[b]

[a] The conditions for the pK_a measurements were μ = 0.2, 20 °C, except for γ-dithiohomo-Lys, which was μ = 0.15. [b] From the method of Perrin *et al.* (1981) from the literature values given in the table for lysine and glutamate.

Both methods suffer from heterogeneity of the product. *In vitro* translation products can be contaminated by improper read-through of the target stop-codon, leading to incorporation from noncognate, normal tRNA's. Therefore, contaminant proteins are synthesized with a natural amino acid at the site of interest, including that of the WT protein. This results in a contaminant that is quite active, thus placing a lower limit on the activity of mutants that can be measured by steady-state kinetics.

The major contaminant of chemical modification methodology is the unmodified, usually inactive, starting material, which will not greatly affect the results, if the unmodified mutant is inactive (*e.g.*, K258C_Q; Gloss & Kirsch, 1995a). This allows study of far less active enzymes than does the *in vitro* translation methodology. Furthermore, sufficient quantities of modified protein can be obtained to permit single-turnover characterization of variants with less activity than the unmodified enzyme (*e.g.*, K258C_Q-CbMe).

Effect of Homo-Lys Analogues on the Internal Aldimine. The side-chain pK_a values of both γ-thiahomo-Lys and γ-dithiohomo-Lys (Table 5) are lower than that of lysine, the amino acid normally found at position 258 of AATase. An acidic shift of the internal aldimine pK_a from the value of Quint is only observed for K258C_Q-PA (ΔpK_a in solution −0.5, ΔpK_a of internal aldimine = −0.9). The internal aldimine pK_a of K258C_Q-DTA is 0.4 pH unit higher than that of Quint (Gloss *et al.*, 1992; Gloss & Kirsch, 1995a). The lack of correlation of the pK_a's of these internal aldimines with those of the corresponding amino acid side chains contrasts with the earlier finding that the 1.1 unit lower pK_a value of the ε-amino group of γ-thiaLys *vs* Lys is almost exactly reflected in the corresponding AATase aldimines (Planas & Kirsch, 1990; Gloss & Kirsch, 1995a).

The high-pH form of E·PLP (deprotonated internal aldimine) of Quint and WT has a λ_{max} value of 360 nm (Gloss & Kirsch, 1995a). The corresponding species found in K258C_Q-DTA and K258C_Q-PA exhibits a λ_{max} value of 335 nm (Figure 1). The position of this absorbance peak is consistent with all structures that significantly reduce the extent of conjugation of the exocyclic C_4'−N double bond with the pyridine ring, such as a twisted double bond or a carbinolamine.

Effect of Lys-258 Replacements on K_M and k_{cat}/K_M Values. Replacement of Lys-258 with homo-Lys analogues results in modest changes (0.8−3.8-fold) in amino acid K_M values

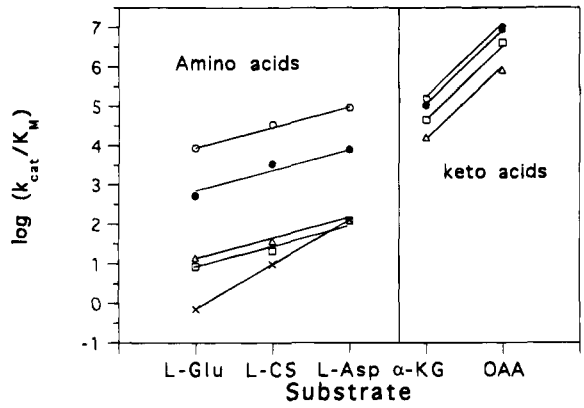


FIGURE 5: Comparison of the k_{cat}/K_M values of AATase variants for different substrates. The values for Quint (○) and K258C$_Q$-EA (●) are from Gloss and Kirsch (1995a); those of K258C$_Q$-PA (△) and K258C$_Q$-DTA (□) are from Table 1; K258E$_Q$ (×) from Table 3. Parallel lines are drawn for the four enzymes bearing side chains with a primary amino group at position 258.

(Table 1). These are close approximations to true dissociation constants (Gloss & Kirsch, 1995a,b). By contrast, the $K_M^{\alpha\text{-KG}}$ values are *decreased by more than 100-fold* for the homo-Lys analogue mutants relative to Quint. $K_M^{\alpha\text{-KG}}$ is not a true dissociation constant for WT, and it is unknown whether K_M is a dissociation constant for the mutants. However, the $K_M^{\alpha\text{-KG}}$ value of K258C$_Q$-EA, which is similar to that of WT, is a true dissociation constant (Gloss & Kirsch, 1995a,b).

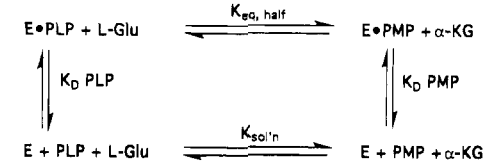
Replacing Lys-258 with a homo-Lys analogue has varying effects on the substrate K_M values; however, the relative catalytic efficiency (from the k_{cat}/K_M values) of the enzymes is similar to those observed for Quint and K258C$_Q$-EA (Gloss & Kirsch, 1995a), as shown in Figure 5. A parallel trend of k_{cat}/K_M values for both amino and keto acid substrates is observed for all four enzymes. Therefore, despite decreased catalytic rates for AATases with unnatural lysine analogues at position 258, and varied effects on K_M values, all variants similarly discriminate between substrates, *e.g.*, four *vs* five carbons or β-carboxylate *vs* β-sulfinate. The trends observed for K258E$_Q$ are similar, but the difference in the relative efficiency of transamination of the three amino acids is more marked. The reason for this enhanced discrimination is unclear.

258-Homo-Lys Analogues Alter the E·PLP:E·PMP Equilibrium. Replacement of Lys-258 with γ-thiahomo-Lys or γ-dithiohomo-Lys causes much larger decreases in the amino acid than in the keto acid k_{cat}/K_M values. The unequal effects are reflected in the $K_{eq,half}$ values (eq 7):

$$K_{eq,half} = \frac{k_{cat}/K_M^{\alpha\text{-KG}}}{k_{cat}/K_M^{Glu}} = \frac{[E\cdot PLP][Glu]}{[E\cdot PMP][\alpha\text{-KG}]} \quad (7)$$

The $K_{eq,half}$ values of K258C$_Q$-DTA and K258C$_Q$-PA are 1200 and 5400, compared to 18 for Quint (Gloss & Kirsch, 1995a). Therefore, the equilibria of the homo-Lys-containing AATases lie far more toward the E·PLP form do those of Quint. $K_{eq,half}$ values reflect the relative stability of the E·PLP and E·PMP forms of an AATase (Scheme 3). The internal aldimines formed with γ-thiahomo-Lys-258 and γ-dithiohomo-Lys-258 are stabilized by 2.5 and 3.4 kcal/mol, respectively, relative to that of Quint. An alternative interpretation of this result is that the E·PMP forms of these

Scheme 3: Relationship of the Half-Reaction Equilibrium to the Relative Stability of the E·PLP and E·PMP Species[a]



[a] $K_{eq,half}$ is the equilibrium constant as defined in eq 7. $K_{sol'n}$ is the equilibrium constant describing the transamination reaction in the absence of enzyme.

AATases are destabilized, relative to that of Quint. If the increased $K_{eq,half}$ values of the homo-Lys AATases were due, entirely or in large part, to destabilization of E·PMP, the K_D of PMP should be increased by *ca.* 2 orders of magnitude. A 10-fold increase in the K_D^{PMP} value, *e.g.*, as in Y70F (Toney & Kirsch, 1991b), is sufficient to observe cofactor dissociation during steady-state kinetics, as manifested by nonlinear kinetics at low [α-KG] in the absence of excess PMP. No such nonlinearity was observed in the steady-state kinetic assays of K258C$_Q$-DTA or K258C$_Q$-PA. The $K_{eq,half}$ value of K258C$_Q$-EA, 200, is also increased relative to that of Quint (Gloss & Kirsch, 1995a). This is due entirely to stabilization, by 1.4 kcal/mol, of the E·PLP form, with no destabilization of the PMP enzyme.

K258E$_Q$ and K258C$_Q$-CbEt Bind PMP Preferentially over PLP. The stability of the E·PLP complex of *E. coli* WT AATase is much greater than that of the E·PMP complex (K_D^{PMP}/K_M^{PLP} = 3000; Toney & Kirsch, 1991b). The opposite is found for the AATases with Glu or a Glu analogue at position 258 (PLP:PMP preference ~0.15). This translates to a stabilization of E·PMP relative to E·PLP of 5.8 kcal/mol compared to WT. The altered cofactor preference of K258E$_Q$ and K258C$_Q$-CbMe is probably the combined result of (1) a favorable electrostatic interaction between the carboxylate side chain and the amino group of PMP and (2) removal of the Schiff base linkage to PLP.

Changes in the Rate-Determining Steps for the 258-Homo-Lys Analogues. The following section summarizes the evidence from L-Asp KIE's (Table 4) showing that transaldimination (k_{TA} in Scheme 1A) is partially rate-determining for K258C$_Q$-PA and K258C$_Q$-DTA. The KIE's for Quint, WT, and K258C$_Q$-EA follow the trend $^Dk_{cat} < {}^D(k_{cat}/K_M)$, and thus $k_2 > k_5$ (Scheme 1; Gloss & Kirsch, 1995a). Therefore, C_α proton abstraction is the first partially rate-determining step in the mechanism of these enzymes, and steps after the isotope-sensitive step (k_3) are also partially rate-determining. K258C$_Q$-PA and K258C$_Q$-DTA show the opposite trend in their KIE's, *i.e.*, $^Dk_{cat} > {}^D(k_{cat}/K_M)$. Thus, steps prior to k_3 are partially rate-determining, and the steps comprising k_5 (ketimine hydrolysis, OAA dissociation, and the α-KG half-reaction) are not significantly rate-determining. For K258C$_Q$-DTA, $^D(k_{cat}/K_M)$ is essentially unity, indicating that the kinetic barrier for transaldimination is much higher than that for C_α proton abstraction. Transaldimination is less rate-determining for K258C$_Q$-PA than it is for K258C$_Q$-DTA [$^D(k_{cat}/K_M)$ = 2.2; Table 4]. The absolute values of $^Dk_{cat}$ and $^D(k_{cat}/K_M)$ differ substantially among the position 258 variants, and are, to some degree, a measure of the kinetic complexity of the reaction (*e.g.*, Northrop, 1975).

The C_α proton of L-CS is more acidic than that of L-Asp (Toney & Kirsch, 1992). This is reflected in the higher k_{cat}

values for the L-CS reactions *vs* those with L-Asp observed for all AATases where the residue at position 258 is no larger than γ-thia-Lys [*i.e.*, Quint, WT, K258C$_Q$-EA (Gloss & Kirsch, 1995a,b); K258A (Toney & Kirsch, 1993); K258C (Planas & Kirsch, 1991); K258H (Ziak *et al.*, 1990); K258E$_Q$ (Table 3)]. However, this order is reversed (*i.e.*, $k_{cat}^{L\text{-}Asp} > k_{cat}^{L\text{-}CS}$ for the 258-homo-Lys analogues. The most straightforward explanation invokes a sterically crowded transition state for the unnaturally extended 258-side chains with the L-CS external aldimine, which is larger than that formed with L-Asp.

Catalysis by Side-Chain Carboxylates at Position 258. The K_D values for the external aldimines formed with amino acids in K258A are 5 orders of magnitude *lower* than those of WT (Toney & Kirsch, 1993). This has been interpreted as a manifestation of the Circe effect (Jencks, 1975). Similarly, the K_D^{app} values for the AATases with carboxylates at position 258 (Scheme 2) are 2−4 orders of magnitude *lower* than that of Quint (Table 2). In accord with the interpretation of Toney and Kirsch (1993), the values of K_D^{app} increase monotonically with those of k_{max} (Tables 2 and 3), *i.e.*, the more effective the catalyst, the weaker the binding of substrate.

The WT k_{max} values for L-Asp, L-Glu, and L-CS are 214, 600, and 3000 s^{-1}, respectively (Kuramitsu *et al.*, 1990; Gloss & Kirsch, 1995b). K258A, which lacks any catalytic functionality at the 258 position, exhibits k_{max} values 6−7 orders of magnitude less than those of WT (Toney & Kirsch, 1993). The k_{max} values of K258E$_Q$ and K258C$_Q$-CbMe are not substantially different than those of K258A (Table 3). Only K258C$_Q$-CbEt, the AATase variant with the most extended carboxylate side chain, exhibits a substantial increase in the rate of L-CS transamination, 9-fold faster than K258A. This suggests that the 258-side chains of K258E$_Q$ and K258C$_Q$-CbMe are too short to abstract the C_α proton directly, but may catalyze the 1,3-prototropic shift through an intermediate water molecule, as has been demonstrated for K258H (Malashkevich *et al.*, 1995).

Transamination of the keto acid, α-KG, by a 258-side-chain carboxylate AATase was only detectable for K258C$_Q$-CbEt. In this reaction, the proton at C_4' of the cofactor is abstracted. It is possible that only the (carboxyethyl)cysteine side chain is sufficiently extended to abstract this proton. K258C$_Q$-CbEt catalyzes the transamination of α-KG ≥100-fold faster than does K258E$_Q$. This acceleration is due to a decrease in $\Delta G^{\ddagger}$, rather than a difference between the two enzymes' relative ground-state energies for E•PMP and E•PLP as the two enzymes exhibit a similar PMP:PLP preference. The $k_{max}^{\alpha\text{-}KG}$ value of K258C$_Q$-CbEt is 3-fold less than that of K258A (Toney & Kirsch, 1993) and (3×10^7)-fold slower than that of WT (Kuramitsu *et al.*, 1990).

The C_α-^{2}H kinetic isotope effect for the reaction of K258E$_Q$ with L-Asp is 6.7 (Table 4). The mutation D222A results in the same KIE value (Onuffer & Kirsch, 1994). These are the largest KIE values seen for AATase variants, and they likely reflect the intrinsic isotope effect for this reaction. Transamination of the model system, 3-hydroxypyridine 4-aldehyde and alanine, yielded a C_α-^{2}H KIE of 6.9 (Auld & Bruice, 1967).

The trend of the L-CS C_α-^{2}H kinetic isotope effects of K258E$_Q$ and K258C$_Q$-CbMe, $^D(k_{cat}) > {}^D(k_{cat}/K_M)$, is similar to that of K258C$_Q$-DTA and K258C$_Q$-PA (Table 4). Therefore, steps after C_α proton abstraction (k_{3b} and k_5 in Scheme 1B) are faster than the steps of external aldimine formation (k_{SB}) and C_α proton abstraction (k_{3a}). The $^D(k_{cat}/K_M)$ value of 1 for K258C$_Q$-CbMe demonstrates that, as for the reaction of K258C$_Q$-DTA with L-Asp, external aldimine formation is essentially irreversible. This is in accord with the observation that the external aldimine does not accumulate in the reaction of L-CS with K258C$_Q$-CbMe (Figure 3). An internal aldimine permits more efficient transamination by allowing external aldimine formation via transaldimination (k_{TA}, Scheme 1A) rather than Schiff base formation (k_{SB}, Scheme 1B), where $k_{TA} > k_{SB}$ as shown for the model reactions of PLP with semicarbazide (Cordes & Jencks, 1962). The WT value of k_{TA} is 2−3 orders of magnitude faster than is k_{SB} for K258A (Toney & Kirsch, 1993).

Two site-directed mutants, K258H and K258R, also do not populate the external aldimine species in their reactions with L-CS (Ziak *et al.*, 1990; Toney & Kirsch, 1991a). Like K258C$_Q$-CbMe and K258C$_Q$-CbEt (Figure 4), the reaction of K258H with L-Asp does accumulate an external aldimine intermediate. An external aldimine is not detected in the reactions of K258R with any L-amino acid substrate, only with the inhibitor α-Me-Asp. No KIE values are reported for K258H or K258R, precluding conclusions as to the relative extent to which Schiff base formation and C_α proton abstraction are rate-determining for these AATases.

A "Tethered" Brønsted Analysis. Construction of AATases containing active site bases with varying pK_a values (Table 5) permits application of classical Brønsted theory to the transamination reaction. The bases in this study are covalently tethered to the enzyme, unlike the Brønsted analyses of the chemical rescue of inactive enzymes with exogenous amines (*e.g.*, Toney & Kirsch, 1989, 1992; Harpel & Hartman, 1994).

Exogenous carboxylates are unexpectedly poor catalysts in the chemical rescue of K258A; acetate and formate are 100-fold less effective than predicted from the slope of the Brønsted plot constructed for the exogenous amine catalysts (Toney & Kirsch, 1992). A possible explanation is that carboxylates are selectively excluded from the active site by the electrostatic environment that includes the two substrate carboxylate groups. Covalent tethering of the catalyst circumvents this problem, to the extent that the carboxylate side chain adopts a similar position to that of Lys-258.

An L-CS k_{max}/K_D^{app} value can be calculated for the carboxylate catalysts listed in Table 5 (pK_a range 3.3−4.3) from the observed L-CS k_{cat}/K_M value of K258C$_Q$-EA and the previously reported Brønsted β value of 0.4 (Toney & Kirsch, 1989). The rate constant of K258C$_Q$-EA was used, rather than that of Quint, because the K258C$_Q$-EA $^D(k_{cat}/K_M)$ of 6.4 indicates that C_α proton abstraction is fully rate-determining for this AATase (Gloss & Kirsch, 1995b). The predicted values for the tethered carboxylates are 10−30 M^{-1} s^{-1}, compared to the observed values of 2−10 M^{-1} s^{-1} (Table 3). The latter represent lower limits for the rate constants for the 1,3-prototropic shift, given that this reaction is at most only partially rate-determining for the 258-side-chain carboxylate AATases (see above). The agreement of calculated and observed values supports the suggestion that the low reactivity of acetate and formate in the chemical rescue experiments is due to electrostatic exclusion of these ions.

The predicted L-CS k_{cat}/K_M values for K258C$_Q$-DTA and K258C$_Q$-PA are, however, 2 orders of magnitude higher than those measured. This is likely due to steric effects brought about by introduction of the larger homolysine analogue side chains.

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