Decreasing the Basicity of the Active Site Base, Lys-258, of *Escherichia coli* Aspartate Aminotransferase by Replacement with γ-Thialysine†

Lisa M. Gloss‡ and Jack F. Kirsch*

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

Received August 23, 1994; Revised Manuscript Received December 2, 1994®

ABSTRACT: Alkylation of the K258C mutant of the wild-type aspartate aminotransferase (AATase) with bromoethylamine to give γ-thialysine 258 was complicated by partial reaction with the five native cysteines [Planas, A., & Kirsch, J. F. (1991) *Biochemistry 30*, 8268−8276]. This problem is now overcome by carrying out the alkylation with K258C$_Q$, in which Cys-258 is a unique cysteine residue in Quint, an engineered AATase in which the five cysteines have been converted to alanine [Gloss, L. M., *et al.* (1992) *Biochemistry 31*, 32−39]. The kinetics and spectral properties of the resulting enzyme, K258C$_Q$-EA, have been examined and compared to those of WT and Quint. The replacement of Lys-258 by γ-thia-Lys results in an acidic shift of 1.3 pH units in the pK_a of the internal aldimine. The C_α hydrogen kinetic isotope effects for Quint are 2.1 and 1.5 on $^D(k_{cat}/K_M^{Asp})$ and $^Dk_{cat}$, respectively. Replacement of Lys-258 by the weaker base, γ-thia-Lys, increases these values to 3.3 and 2.6, respectively The changes of K258C$_Q$-EA in ligand affinities and the keto acid half-reaction are minor; however, the k_{cat}/K_M values for amino acids are decreased by an order of magnitude. The K_D values for PMP of K258C$_Q$-EA and Quint are equal to each other (0.2 nM) and are 7-fold lower than that of WT. These combined effects are illustrated in the free energy diagrams of the reaction with L-Asp with K258C$_Q$-EA, relative to WT (and Quint). The E·PLP and E·PMP complexes of Quint are 0.9 and 1.1 kcal/mol, respectively, more stable than those of WT. The E·PLP form of K258C$_Q$-EA is 1.4 kcal/mol more stable than that of Quint, while the corresponding E·PMP complexes are equally stable.

While site-directed mutagenesis is a powerful tool for evaluating the contribution of individual residues to a catalytic mechanism, the number of possible replacements is limited to the 20 naturally occurring amino acids. There is, for example, no alternative primary amine with which to replace the side chain of lysine. Unnatural amino acids can be incorporated into proteins through the combined use of site-directed mutagenesis and chemical modification, taking advantage of the unique chemistry of the cysteine residues. Several investigators have modified cysteine residues with bromoethylamine (Br-EA)[1] or cysteamine to produce the lysine analogues to study catalytic mechanisms (Smith & Hartman, 1988; Planas & Kirsch, 1990, 1991). Recently, the reversible modification of cysteine by cysteamine was used to study the thermodynamics of protein folding (Lu *et al.*, 1992). Examples of other chemical elaborations of introduced cysteines have been reported by Lukac and Collier (1988), Sutton *et al.* (1993), Wynn and Richards (1993), and Dhalla *et al.* (1994).

While diverse and powerful, a possible limitation of this method is that multiple modifications may occur if the protein contains more than one cysteine. Planas and Kirsch (1990) devised a method of sequential protection and modification that allows selective derivatization of an introduced active site thiol in a protein with multiple cysteine residues, using *Escherichia coli* aspartate aminotransferase (AATase). An alternative route to selective elaboration of an introduced cysteine is through replacement of the natural cysteines with other amino acids by site-directed mutagenesis, prior to the introduction of the target cysteine.

The present study is an application of this approach to *E. coli* AATase. The cysteine-less mutant, Quint, is described in Gloss *et al.* (1992). The K258C mutation of Planas and Kirsch (1991) has been introduced into this Quint background. This cysteine is now a unique target for thiol-modifying reagents, allowing the preparation of milligram quantities of a homogeneously modified enzyme for physical and mechanistic characterization.

† This work was supported by NIH Grant GM35393 and by a gift from Warner-Lambert, Corp. L.M.G. was supported in part by a National Science Foundation predoctoral fellowship and a University of California at Berkeley Fellowship for Graduate Studies.

* Corresponding author.

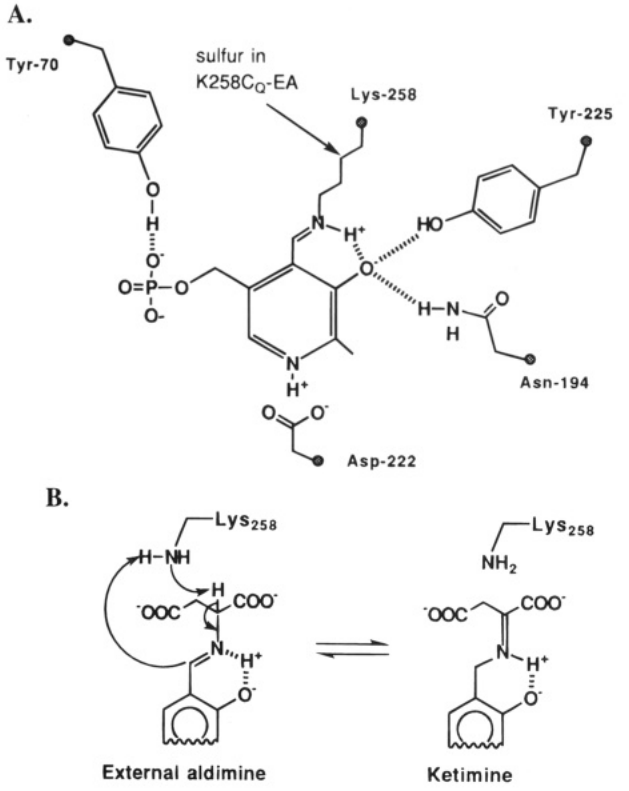
‡ Present address: Department of Chemistry, The Pennsylvania State University, State College, PA.

® Abstract published in *Advance ACS Abstracts,* March 1, 1995.

[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, containing a unique cysteine at the 258 position in place of the WT Lys residue; Br-EA, 2-bromoethylamine; K258C$_Q$-EA, K258C$_Q$ enzyme modified with Br-EA to generate γ-thia-Lys at position 258; α-KG, α-ketoglutarate; AMPSO, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid; α-Me-Asp, α-methyl-D,L-aspartate; L-CS, L-cysteinesulfinate; DTNB, 5,5-dithiobis-(2-nitrobenzoic acid); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HGDH, α-hydroxyglutarate dehydrogenase from *Peptostreptococcus asaccharolyticus*; KIE, primary C_α kinetic isotope effect; LDH, lactate dehydrogenase; MDH, *E. coli* malate dehydrogenase; MES, 2-morpholinoethanesulfonic acid; MHP buffer, a tricomponent buffer with constant ionic strength, $\mu = 0.2$, containing 25 mM MES, 25 mM HEPES, 50 mM 4-hydroxy-*N*-methylpiperidine, and 150 mM KCl; OAA, oxalacetate; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; E, enzyme.

0006-2960/95/0434-3990$09.00/0

Scheme 1: (A) Schematic View of Selected Active Site Residues of Aspartate Aminotransferase (The Targeted Lys to γ-Thia-Lys Substitution Is Indicated) and (B) Minimal Mechanism for the 1,3-Prototropic Shift between the External Aldimine and the Ketimine Intermediates



Aspartate aminotransferase is a PLP-dependent enzyme that catalyzes the transfer of the α-amino groups of L-Asp, L-CS, and L-Glu to the α-keto acids OAA and α-KG, as shown in eq 1. The enzyme from mammalian sources has

$$\text{E•PLP} + \text{amino acid 1} \rightleftharpoons \text{E•PMP} + \text{keto acid 1} \quad (1)$$

$$\text{E•PMP} + \text{keto acid 2} \rightleftharpoons \text{E•PLP} + \text{amino acid 2}$$

been well-characterized, mechanistically and structurally [Julin & Kirsch, 1989; McPhalen *et al.* (1992) and references therein]. Extensive studies have been conducted on site-directed mutants of the *E. coli* enzyme [e.g., Onuffer & Kirsch, 1994; Yano *et al.* (1992) and references therein].

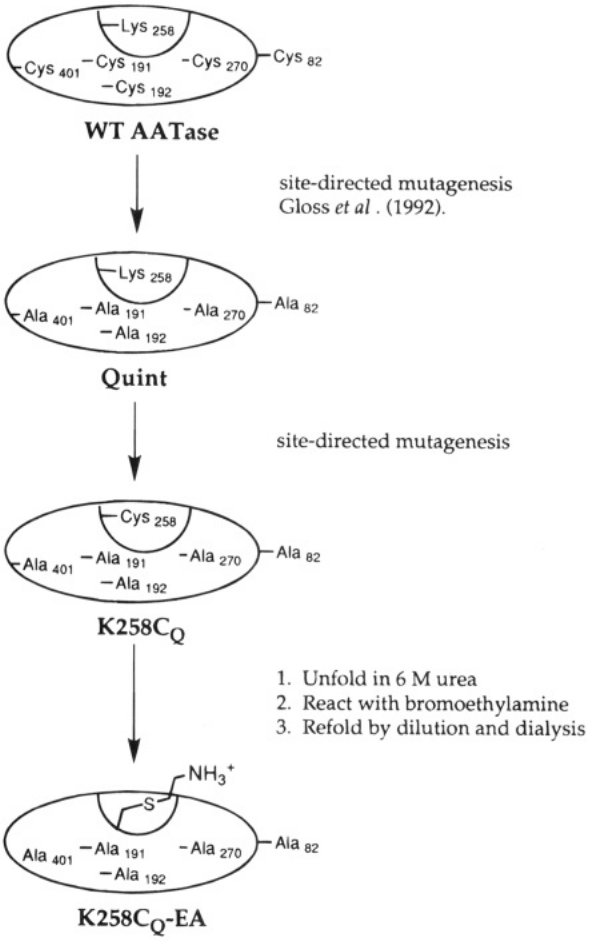
The position targeted in this study is Lys-258. The ϵ-amino group of this residue forms the aldimine linkage to the PLP cofactor (Scheme 1A) and subsequently acts as the base in the transamination reaction (Scheme 1B). The mutant enzyme K258C$_Q$ was reacted with Br-EA, replacing the WT lysine with γ-thialysine, as outlined in Scheme 2. γ-Thia-Lys is a nearly isosteric substitution for Lys, whose ϵ-amino group has a pK_a value 1.1 pH units less than that of Lys (Hermann & Lemke, 1968).

MATERIALS AND METHODS

Materials

[C_α-^{2}H]-L-Asp was a gift from Dr. D. A. Julin. MDH was purified from an AATase-deficient *E. coli* strain (Onuffer & Kirsch, 1994). HGDH was purified from *Peptostreptococcus asaccharolyticus* by the procedure of Yagi *et al.* (1979). LDH was from Boehringer Mannheim. 4-Hydroxy-

Scheme 2: Overview of the Site-Directed Mutagenesis and Chemical Modification Steps for the Generation of the K258C$_Q$-EA Aspartate Aminotransferase



N-methylpiperidine was purchased from Lancaster and vacuum-distilled before use as a buffer. All other chemicals and reagents were of the highest purity available from Sigma or Aldrich.

Methods

Site-directed mutagenesis was carried out as described previously (Gloss *et al.*, 1992; Onuffer & Kirsch, 1994). Purification and quantification of WT and mutant AATases were performed according to Gloss *et al.* (1992).

Modification of K258C$_Q$ with Br-EA. A 10 mg/mL (0.23 μM monomer) solution of K258C$_Q$ was unfolded in 6 M urea, 200 mM AMPSO (pH 9.0), and 15 mM EDTA, and Br-EA was added to 37.5 mM. The reaction proceeded for 3 h at room temperature, in the dark, under N_2. The extent of modification was assessed by reaction with DTNB (Planas & Kirsch, 1990). The K258C$_Q$-EA enzyme was diluted to 0.5 mg/mL in a solution of 6 M urea, 100 mM HEPES (pH 7.5), 15 mM EDTA, 200 mM KCl, and 20 μM PLP at 4 °C. Refolding was accomplished at 4°C by dialyzing this urea solution against 100 mM HEPES (pH 7.5), 100 mM KCl, 5 mM EDTA, and 20 μM PLP, with or without an intermediate dialysis step against 3 M urea, HEPES, KCl, EDTA, and PLP. The refolded protein was concentrated with an Amicon flow cell to ~1 mg/mL, and the protein was precipitated by the addition of ammonium sulfate to 90%. The pellet was resuspended with and dialyzed against 20 mM potassium phosphate (pH 7.5), 5 mM EDTA, and 20 μM PLP. The

final enzyme solution was filtered and stored in the dark at 4 °C.

Spectrophotometric Titrations. The titrations of the internal aldimine formed between Lys-258 (or γ-thia-Lys-258) and the PLP cofactor were performed by the method of Goldberg *et al.* (1991). The data were fitted to eq 2 (for increasing ϵ_{430} with decreasing pH) and eq 3 (for decreasing ϵ_{356} with decreasing pH):

$$\epsilon = \frac{\epsilon_1 - \epsilon_2}{1 + 10^{(pH - pK_a)}} + \epsilon_2 \quad (2)$$

$$\epsilon = \frac{\epsilon_1 - \epsilon_2}{1 + 10^{(pK_a - pH)}} + \epsilon_2 \quad (3)$$

where ϵ_1 and ϵ_2 are the upper and lower limits, respectively, of the extinction coefficients for that wavelength.

Steady-State Kinetics. Steady-state kinetics was monitored by the decrease in NADH absorbance at 340 nm at 25 °C with a Kontron Uvikon 860 spectrophotometer. Steady-state kinetics at pH 7.5 (Tables 1 and 3) was performed in 200 mM HEPES, 100 mM KCl ($\mu = 0.2$), 150 μM NADH, and 5−10 units/mL of the appropriate coupling enzyme (MDH for the L-Asp/α-KG substrate pair; LDH for the L-CS/α-KG pair; HGDH for the L-Glu/OAA pair). For steady-state kinetics in which the pH of the experiment was varied (Table 2 and Figure 3), the buffer was a tricomponent system designed to give a pH-independent, constant ionic strength (Ellis & Morrison, 1982). This MHP buffer contained 25 mM MES, 25 mM HEPES, 50 mM 4-hydroxy-*N*-methylpiperidine, and 150 mM KCl ($\mu = 0.2$), with 150 μM NADH and 5−10 units/mL of the coupling enzyme. The MHP buffer is discussed in more detail in Gloss and Kirsch (1995).

Matrices of data were fitted by nonlinear regression with the NLIN program of the SAS statistical package (SAS Institute, Cary, NC). The errors on the k_{cat}/K_M parameters were determined by the method of Julin and Kirsch (1989). Data sets of only x and y variables were fitted by nonlinear regression with the program, *Enzfitter* (Biosoft Publishing Co.).

To determine the kinetic parameters of the L-Asp/α-KG and L-CS/α-KG substrate pairs, matrices of initial rate data at five or six concentrations of each substrate were collected. The data were fitted to the equation describing the ping-pong bi-bi mechanism (Velick & Vavra, 1962):

$$\frac{v}{E_t} = \frac{k_{cat}}{1 + K_M^{AA}/[AA] + K_M^{\alpha\text{-KG}}/[\alpha\text{-KG}]} \quad (4)$$

where AA is the amino acid. For the L-Glu/OAA substrate pair, data were collected at a saturating concentration of OAA ((15−30) K_M) and 8−10 concentrations of L-Glu. The data were fitted to eq 5, which is the reduced form of eq 4, where [OAA] > K_M^{OAA}:

$$\frac{v}{E_t} = \frac{k_{cat}[AA]}{K_M^{AA} + [AA]} \quad (5)$$

pH Profile Kinetics. The data for the k_{cat}/K_M^{Asp} profile were collected as described elsewhere (Gloss & Kirsch, 1995) and fitted to a bell curve model:

$$k_{cat}/K_M^{Asp} = \frac{(k_{cat}/K_M^{Asp})_{lim}}{1 + 10^{(pK_1 - pH)} + 10^{(pH - pK_2)}} \quad (6)$$

Competitive Inhibition. In these studies, subsaturating substrate concentrations were used to avoid substrate inhibition. For indicated data sets, fixed K_M values from L-Asp/α-KG matrices in MHP buffer at the appropriate pH (K_M values given in Table 2) were used in fitting to eqs 7 and 8. The steady-state kinetic data used to determine the competitive inhibition constants for α-Me-Asp were collected as matrices of five concentrations each of L-Asp and α-Me-Asp at a fixed concentration of α-KG ((3−5)$K_M^{\alpha\text{-KG}}$). The data were fitted to

$$\frac{v}{E_t} = \frac{k_{cat}}{1 + (K_M^{Asp}/[Asp])(1 + [I]/K_i) + K_M^{\alpha\text{-KG}}/[\alpha\text{-KG}]} \quad (7)$$

where [I] is the concentration of inhibitor, α-Me-Asp, and K_i is its dissociation constant. $K_M^{\alpha\text{-KG}}$ (Table 2) was used as a constant in the fitting.

Maleate binds predominantly to the PMP−enzyme at pH values above the pK_a of the internal aldimine of AATase (Kiick & Cook, 1983). At these pH values (pH 8.2 for Quint; pH 8.2 and 7.1 for K258C$_Q$-EA), the concentration of inhibitor was varied under two substrate regimes: (1) [L-Asp] = (3−4.5)K_M^{Asp} and [α-KG] = (0.5−1)$K_M^{\alpha\text{-KG}}$; (2) [L-Asp] = (0.4−0.7)K_M^{Asp} and [α-KG] = 10$K_M^{\alpha\text{-KG}}$. The data were fitted to eq 8, where K_{iL} and K_{iM} are the inhibition constants for the PLP and PMP enzyme forms, respectively, with values fixed for the K_M^{Asp} and $K_M^{\alpha\text{-KG}}$ terms:

$$\frac{v}{E_t} = k_{cat}/[1 + (K_M^{Asp}/[Asp])(1 + [I]/K_{iL}) + (K_M^{\alpha\text{-KG}}/[\alpha\text{-KG}])(1 + [I]/K_{iM})] \quad (8)$$

Maleate binds strongly to both the PLP and PMP enzyme forms at pH values less than or equal to the pK_a of the internal aldimine. The concentration of maleate was varied at five different [L-Asp]/[α-KG] ratios: (1) both substrates at their K_M values; (2) [L-Asp] = (3−4)K_M and [α-KG] = K_M; (3) [L-Asp] = (3−4)K_M and [α-KG] = 0.5K_M; (4) [α-KG] = (2.5−5K_M and [L-Asp] = K_M; (5) [α-KG] = (2.5−5)K_M and [L-Asp] = 0.5 K_M. The data were fitted to eq 8, with fixed values for K_M^{Asp} and $K_M^{\alpha\text{-KG}}$.

[C_α-^{2}H]-L-Asp Kinetic Isotope Effects. The data were collected at near-saturating concentrations of α-KG ([α-KG] = 5K_M) and varied concentrations of [C_α-^{1}H]- and [C_α-^{2}H]-L-Asp. The concentrations of L-Asp were the same for both the ^{1}H and ^{2}H data sets and varied from 0.75 to 15 mM (exact range depending on the enzyme being assayed). The data were fitted to eq 5, and the values of $^D k_{cat}$ and $^D(k_{cat}/K_M)$ were determined.

Kinetics of PMP Dissociation and Association. The rate constants for PMP dissociation (k_{diss}) from the WT, Quint, and K258C$_Q$-EA enzymes were measured by the steady-state kinetic method of Toney and Kirsch (1987, 1991). The conditions were 200 mM HEPES (pH 7.5), 100 mM KCl, 50 mM L-Asp (100 mM for K258C$_Q$-EA), and 150 μM PLP. The [AATase monomer] was varied from 1 to 10 μM, and the rates of decrease in absorbance at 410 nm were linearly dependent upon the enzyme concentration. The rate con-

stants reported are the slope of the secondary plot of observed rate *vs* [monomer]. The potential complication of the competing reverse reaction (E·PMP + OAA → E·PLP + Asp) was addressed by measuring the rates in the presence and absence of 100 mM morpholine, which catalyzes the decarboxylation of OAA to pyruvate. The latter is a very poor substrate for AATase.

The rate constants for PMP association (k_{assoc}) with WT, Quint, and K258C$_Q$-EA were measured as described in Toney and Kirsch (1991). The quenching of protein fluorescence upon PMP association (Churchich & Farrelly, 1969) was monitored with an Applied Photophysics stopped-flow spectrophotometer (SV.17 MV). The conditions were pseudo-first-order, with the [apoenzymes] = 5 μM and the [PMP] varied from 50 to 200 μM. The observed rate constants were fitted to

$$k_{obs} = k_{assoc}[PMP] + k_{diss} \quad (9)$$

with k_{diss} fixed at the value determined independently by the steady-state method described earlier.

RESULTS

Conversion of Cys-258 to γ-Thia-Lys. The K258C$_Q$-EA enzyme was constructed as outlined in Scheme 2. The preparation and properties of Quint, the cysteine-free AATase, are described in Gloss *et al.* (1992). The replacement of Lys-258 by a unique cysteine, in the Quint background, is denoted as K258C$_Q$. K258C$_Q$, like K258C (Planas & Kirsch, 1991), has low catalytic activity. Single-turnover kinetic measurements with L-CS were performed under the conditions described in Planas and Kirsch (1991). The values of the first-order rate constant for the L-CS half-reaction, k_{max}, and the apparent dissociation constant, K_D, are 3.7×10^{-3} s^{-1} and <50 μM, respectively, for K258C$_Q$. The K258C enzyme has a k_{max} of 2.0×10^{-3} s^{-1} and a K_D < 50 μM.

The previously reported modification of K258C in the WT background required a laborious procedure of partial unfolding, protection of the five natural cysteines, complete unfolding and modification of Cys-258 by Br-EA, deprotection of the five natural cysteines, and refolding (Planas & Kirsch, 1990). The preparation was heterogeneous, with only 50% selectivity in the modification of Cys-258 over the other five cysteine residues. Active site titration showed that only 25% of the final material was significantly more active than the starting K258C enzyme (Planas & Kirsch, 1991).

The modification conditions described in the Methods subsection resulted in >99% alkylation of K258C$_Q$ as judged by thiol assays with DTNB. No loss of thiol was observed when K258C$_Q$ was incubated under similar conditions in the absence of Br-EA. The extent of modification was verified by active site titrations. Ancillary reaction of important residues (other than Cys-258) would result in an underestimate of the K258C$_Q$-EA k_{cat} value. The consistent value of k_{cat} (±0.2%) for the L-Asp/α-KG substrate pair from several preparations of K258C$_Q$-EA demonstrates that the extent and site(s) of modification are quite reproducible. The modification conditions were applied to Quint, and the resulting preparation was assayed with the L-Asp/α-KG substrate pair. No change greater than 9% was observed in the value of any kinetic parameter from those previously published (Gloss *et al.*, 1992). This result demonstrates that Cys-258 is the only catalytically important site of modification by Br-EA.

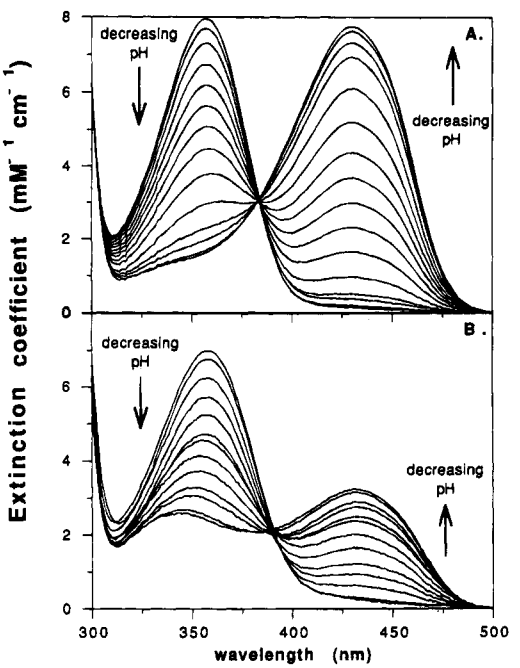


FIGURE 1: pH dependence of the UV−visible spectra of Quint and K258C$_Q$-EA aspartate aminotransferases titrated by the method of Goldberg *et al.* (1992) (μ = 0.2): (A) the Quint enzyme at pH 9.70, 8.90, 8.56, 8.21, 7.84, 7.57, 7.37, 7.19, 7.05, 6.85, 6.65, 6.34, 6.00, 5.76, 5.37, and 5.12; (B) the K258C$_Q$-EA enzyme at pH 9.80, 8.15, 6.66, 6.45, 6.16, 5.92, 5.79, 5.64, 5.47, 5.32, 5.22, 4.99, and 4.73.

Typical refolding yields were 20−30% for the preparation of K258C$_Q$-EA (Scheme 2). The yield was not affected by variations in the methods employed to remove the urea (continuous or stepwise dialysis or direct dilution), nor by the time of PLP addition to the refolding reaction (addition of 20 μM PLP as refolding was initiated or addition by dialysis at lower urea concentrations) (Gloss, 1994). Higher yields were obtained from more dilute unfolding/modification conditions (a decrease from 10 to 5 mg/mL, increased recovery to 50%). This suggested that aggregation at 6 M urea limits recovery. The apparently refolded enzyme at 4 °C was soluble; however, a precipitate formed when the solution was warmed to room temperature. The protein was precipitated by the addition of 90% ammonium sulfate at 4 °C. The aggregated material remained insoluble after dialysis and was removed by centrifugation. The final enzyme solution showed no evidence of aggregation.

Titration of the Internal Aldimine. The λ_{max} value of the internal aldimine formed between the PLP cofactor and the ε-amino group of the amino acid at position 258 is dependent on the protonation state of the aldimine (Scheme 1). The protonated forms of the wild-type enzymes (mitochondrial, cytosolic, and *E. coli*) absorb maximally at 430 nm, while the deprotonated maximum is 356−360 nm (Kallen *et al.*, 1985; Goldberg *et al.*, 1991). The spectrophotometrically determined pK_a values of Quint and K258C$_Q$-EA are 7.11 ± 0.01 and 5.83 ± 0.02, respectively. The Quint pK_a is only slightly higher than that of WT determined under identical conditions (6.95 ± 0.03, this study, data not shown; Goldberg *et al.*, 1991). An overlay of the Quint spectral data is shown in Figure 1A, and plots of $\epsilon_{356\ nm}$ and $\epsilon_{430\ nm}$ *vs* pH are shown in Figure 2. The heterogeneity of the K258C-EA (WT background) preparation precluded spectral titration (Planas & Kirsch, 1991). The pK_a of K258C$_Q$-EA

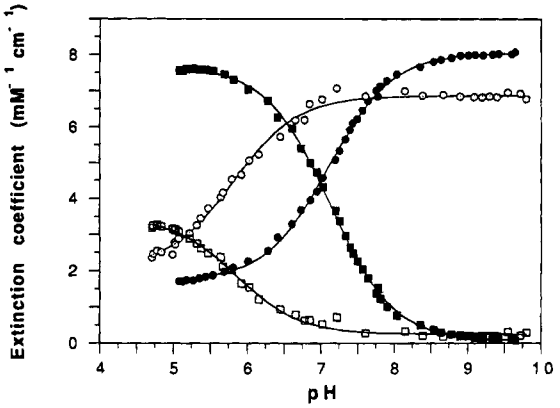


FIGURE 2: pH dependence of the extinction coefficients of quint (●, ■) and K258C$_Q$-EA (○, □) aspartate aminotransferases. Absorbance was monitored at 356 nm (●, ○) and 430 nm (■, □).

is 1.3 pH units lower than that of the parental enzyme, Quint (Figures 1B and 2).

Quint and K258C$_Q$-EA exhibit the same λ_{max} values as the *E. coli* WT enzyme. The spectral overlays of Quint and WT are largely superimposable, allowing for the small ΔpK_a of the two enzymes (data not shown for WT). However, the pH-dependent spectra of K258C$_Q$-EA are quite different from those of Quint (Figure 1). The $\Delta\epsilon$ values between the protonated and deprotonated species were calculated from the limiting values obtained from eqs 2 and 3. The exact value of $\Delta\epsilon$ can vary from titration to titration due to the inherent inaccuracy of the protein concentration changing during the course of the titration experiment. However, the ratio of $\Delta\epsilon_{430}/\Delta\epsilon_{356}$ is very reproducible, as it eliminates the protein concentration terms. The ratios of $\Delta\epsilon_{430}/\Delta\epsilon_{356}$ for Quint and WT are 1.20 and 1.15, respectively. By contrast, the ratio for K258C$_Q$-EA is 0.69. This results from a significantly decreased extinction coefficient for the protonated internal aldimine of this enzyme (Figure 1B). This effect is due to the protein environment, rather than to an inherent property of γ-thia-Lys. Spectral titration of the aldimines formed between *N*-Me-PLP and α-*N*-acetyl-Lys and α-*N*-acetyl-γ-thia-Lys yielded λ_{max} values of 402 and 350 nm for the protonated and deprotonated aldimines, respectively (Gloss, 1994). The $\Delta\epsilon_{402}/\Delta\epsilon_{350}$ ratios were 1.15 and 1.10 for the Lys and γ-thia-Lys aldimines, respectively.

Steady-State Kinetics. The steady-state kinetic parameters describing the reactions of WT, Quint, and K258C$_Q$-EA with the L-Asp/α-KG, L-CS/α-KG, and L-Glu/OAA substrate pairs are given in Table 1. The K_M values for the OAA substrate were not determined due to the technical impediment of measuring kinetics under initial rate conditions at substrate concentrations below a K_M of ~20 μM. However, [OAA] was held at 0.5 mM, which is 25-fold higher than the WT K_M value (Planas & Kirsch, 1991). Therefore, while not generated from a matrix (as were those for the L-Asp/α-KG and L-CS/α-KG pairs), the k_{cat} values listed for the L-Glu/OAA substrate pair are reasonably close approximations of the true k_{cat} values.

The most pronounced effect of replacing the active site Lys-258 with γ-thia-Lys is to decrease the values of k_{cat} and k_{cat}/K_M^{AA}. The magnitude of effects ranges from 16-fold for k_{cat}/K_M^{Glu} to 7-fold for k_{cat} for the Asp/α-KG pair. The $k_{cat}/K_M^{\alpha\text{-KG}}$ value is decreased by only 1.5-fold. The K_M values of K258C$_Q$-EA for the amino acids show small increases relative to those of Quint and WT. The K_M values for L-Asp and L-Glu, measured by steady-state kinetics, are close to the true dissociation constants for the WT enzyme [concluded from the kinetic pH profiles in Gloss and Kirsch (1995)]. K258C$_Q$-EA and WT have very similar values of $K_M^{\alpha\text{-KG}}$, while that of Quint is ~5-fold higher. This K_M value is not a dissociation constant for WT (Gloss & Kirsch, 1995). $K_M^{\alpha\text{-KG}}$ is most likely a dissociation constant for K258C$_Q$-EA (Gloss & Kirsch, 1995).

The k_{cat}/K_M^{OAA} values can be calculated from the Haldane relationship for the ping-pong mechanism (Velick & Vavra, 1962):

$$(k_{cat}/K_M^{OAA}) = \frac{K_{eq,overall}(k_{cat}/K_M^{\alpha\text{-KG}})(k_{cat}/K_M^{Asp})}{(k_{cat}/K_M^{Glu})} \quad (10)$$

The $K_{eq,overall}$ value is 6 and is independent of the enzyme (Velick & Vavra, 1962; Kuramitsu *et al.*, 1990). The calculated values of k_{cat}/K_M^{OAA} are included in Table 1. As with the $k_{cat}/K_M^{\alpha\text{-KG}}$ values, the difference between Quint and K258C$_Q$-EA is small.

Kinetic Determination of Internal Aldimine pK_a Values. The pH dependence of k_{cat}/K_M^{Asp} for WT and K258C-EA is

Table 1: Steady-State Kinetic Parameters for WT, Quint, and K258C$_Q$-EA Aspartate Aminotransferases[a]

Asp/α-KG	k_{cat} (s^{-1})	K_M (L-Asp) (mM)	K_M (α-KG) (mM)	k_{cat}/K_M(Asp) (× 10^{-4} M^{-1} s^{-1})	k_{cat}/K_M (α-KG) (× 10^{-5} M^{-1} s^{-1})
WT[b]	159 ± 2	1.75 ± 0.04	0.48 ± 0.01	9.08 ± 0.13	3.32 ± 0.05
Quint[b]	238 ± 8	2.71 ± 0.22	1.59 ± 0.14	9.04 ± 0.53	1.56 ± 0.07
K258C$_{Q\text{-EA}}$	33 ± 1	4.20 ± 0.34	0.31 ± 0.02	0.778 ± 0.074	1.05 ± 0.16
Quint/K258C$_{Q\text{-EA}}$[c]	7.2	0.64	5.1	11.6	1.5
L-CS/α-KG	**k_{cat} (s^{-1})**	**K_M (L-CS) (mM)**	**K_M (α-KG) (mM)**	**k_{cat}/K_M (L-CS) (× 10^{-4} M^{-1} s^{-1})**	**k_{cat}/K_M (α-KG) (× 10^{-5} M^{-1} s^{-1})**
Quint	404 ± 21	12.0 ± 1.2	2.58 ± 0.20	3.38 ± 0.38	1.57 ± 0.16
K258C$_{Q\text{-EA}}$	40 ± 1	11.8 ± 0.7	0.31 ± 0.02	0.34 ± 0.03	1.29 ± 0.08
Quint/K258C$_{Q\text{-EA}}$[c]	10	1.0	8.3	9.9	1.2
L-Glu/OAA	**k_{cat} (s^{-1})**	**K_M (L-Glu) (mM)**	**k_{cat}/K_M (L-Glu) (× 10^{-4} M^{-1} s^{-1})**	**k_{cat}/K_M (OAA)[d] (× 10^{-7} M^{-1} s^{-1})**	
WT	224 ± 6	19.1 ± 1.5	1.17 ± 0.06	1.55 ± 0.09	
Quint	215 ± 6	25.6 ± 1.9	0.84 ± 0.04	1.00 ± 0.09	
K258C$_{Q\text{-EA}}$	29.4 ± 0.9	57.0 ± 4.1	0.052 ± 0.002	0.9 ± 0.1	
Quint/K258C$_{Q\text{-EA}}$[c]	7.3	0.45	16.1	1.1	

[a] Conditions: 200 mM HEPES (pH 7.5) and 100 mM KCl, 25 °C. The values of k_{cat}, K_M, and k_{cat}/K_M were fitted as described in the Materials and Methods section. The L-Asp, L-CS, and L-Glu concentrations were 0.5−20 mM, 5−30 mM, and 10−150 mM, respectively. The α-KG concentrations were 0.1−6.0 mM, and [OAA] was held constant at 0.5 mM. The exact ranges depended on the enzyme being assayed. [b] From Gloss *et al.* (1992). [c] Dimensionless ratio of Quint parameter to that of K258C$_Q$-EA. [d] Calculated from eq 10.

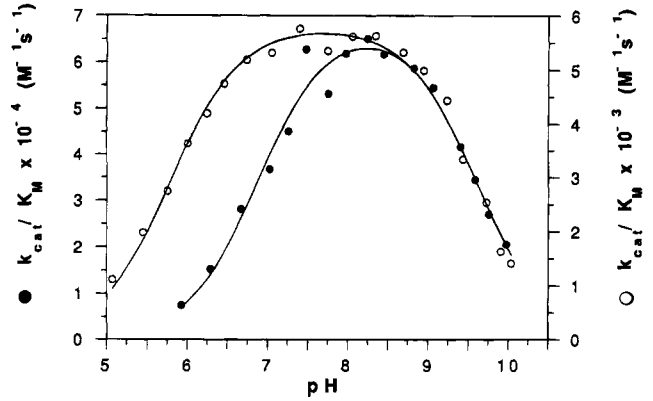


FIGURE 3: pH dependence of k_{cat}/K_M^{Asp} for WT (●) and K258C$_Q$-EA (○) aspartate aminotransferases. The fits of the data to equation 6 are shown by the solid lines. The kinetics were performed in MHP buffer (μ = 0.2).

Table 2: Competitive Inhibition Constants for the Maleate and α-Me-Asp complexes with Quint and K258C$_Q$-EA Aspartate Aminotransferases[a]

ligand	pH	Quint (mM)	K258C$_Q$-EA (mM)
		PLP−Enzyme	
K_M (L-Asp)	8.2	2.2 ± 0.1	6.1 ± 0.2
	7.1	4.4 ± 0.3	6.5 ± 0.3
	5.8	nd	10.8 ± 0.5
K_i (α-Me-Asp)	8.2	1.6 ± 0.2	2.9 ± 0.1
K_i (maleate)	8.2	16 ± 1	389 ± 98
	7.1	3.6 ± 0.1	95 ± 7
	5.8	nd	13.6 ± 0.5
	limit[b]	1.8	6.8
		PMP−Enzyme	
K_M (α-KG)	8.2	3.2 ± 0.2	0.47 ± 0.02
	7.1	2.2 ± 0.2	0.47 ± 0.02
	5.8	nd	0.40 ± 0.02
K_i (maleate)	8.2	8.6 ± 0.3	7.5 ± 0.3
	7.1	7.1 ± 0.3	7.7 ± 0.2
	5.8	nd	10.9 ± 0.4

[a] Conditions: MES−HEPES−piperdine buffer and 150 mM KCl (μ = 0.2). MDH-coupled steady-state kinetic data, collected and fitted as described in the Materials and Methods section. The α-Me-Asp and maleate data were fitted to eqs 7 and 8, respectively. [b] Calculated from eq 11, as described in the text.

shown in Figure 3. The acidic pK_a of this profile is that of the internal aldimine (Kiick & Cook, 1983). The WT and K258C$_Q$-EA values for this pK_a are 6.95 ± 0.02 and 5.83 ± 0.02, respectively. Both enzymes exhibit an alkaline pK_a of 9.6, which has been assigned to the α-amino group of the substrate, L-Asp (Gloss & Kirsch, 1995). The pH-independent limits of k_{cat}/K_M^{Asp} for WT and K258C$_Q$-EA are 68 000 ± 2 000 and 5 810 ± 80 M^{-1} s^{-1}, respectively.

Competitive Inhibition. The constants describing the complexes of Quint and K258C$_Q$-EA with α-Me-Asp and maleate are listed in Table 2. α-Me-Asp forms a dead-end complex with AATase, equivalent to the external aldimine formed with the amino acid substrates, but turnover is blocked by the methyl group replacing the C$_\alpha$ proton (Fasella *et al.*, 1966). Maleate forms noncovalent complexes with both the PLP and PMP enzyme forms.

The pH dependence of the inhibition of the PLP and PMP forms of pig heart cytosolic AATase has been described by Kiick and Cook (1983). The pH dependence of the K_i values for the α-Me-Asp·E-PLP complex is described by a bell-shaped curve with an acidic pK_a equal to that of the internal aldimine and an alkaline pK_a equal to that of the α-amino group of α-Me-Asp. Therefore, measurements at pH 8.2 provide close approximations of the minimal K_i values for this inhibitor for both Quint and K258C$_Q$-EA. The pH dependence of maleate inhibition for the PLP enzyme is described by a sigmoidal curve, with maximal inhibition below a pK_a value equal to that of the internal aldimine (Kiick & Cook, 1983; Yano *et al.*, 1993). The pH-independent, limiting K_i values for Quint and K258C$_Q$-EA are obtained from

$$K_i = (K_i)_{lim}(1 + 10^{pH-pK_a}) \quad (11)$$

and are given in Table 2. Maleate inhibition of the PMP form is pH-independent from pH 5 to ~8, but the K_i value increases above a pK_a value of 9 to 10 (Kiick & Cook, 1983; Gloss & Kirsch, 1995).

Table 3: Primary C$_\alpha$-^{2}H Kinetic Isotope Effects for the Reactions of WT, Quint, and K258C$_Q$-EA Aspartate Aminotransferases with L-Asp[a]

Asp	$^Dk_{cat}$	$^Dk_{cat}/K_M$
WT	1.47 ± 0.02	1.77 ± 0.04
Quint	1.52 ± 0.03	2.14 ± 0.09
K258C$_{Q\text{-}EA}$	2.62 ± 0.05	3.30 ± 0.10

[a] Measured by the MDH-coupled steady-state assay with [α-KG] = 5K_M, and [L-Asp] varied from 0.75 to 15 mM (exact range depending on enzyme assayed). Conditions: 200 mM HEPES (pH 7.5) and 100 mM KCl (μ = 0.2).

Table 4: Rate Constants and Equilibria for the Reaction of PMP with WT, Quint, and K258C$_Q$-EA Aspartate Aminotransferases[a]

enzyme	k_{diss} (× 10^4 s^{-1}),[b] 0 mM morpholine	k_{diss} (× 10^4 s^{-1}), 100 mM morpholine[c]	k_{assoc} (× 10^{-5} M^{-1} s^{-1})[d]	K_D (nm)[e]
WT	7.7 ± 0.3	6.8 ± 0.4	4.2 ± 0.1	1.6 ± 0.1
Quint	0.51 ± 0.02	0.56 ± 0.03	2.40 ± 0.05	0.21 ± 0.01
K258C$_{Q\text{-}EA}$	0.57 ± 0.02	0.63 ± 0.07	2.58 ± 0.07	0.23 ± 0.01

[a] Conditions: 200 mM HEPES (pH 7.5) and 100 mM KCl, 25°C. [b] The dissociation rate constants were determined by the steady-state kinetic assay of Toney and Kirsch (1987, 1991), using 150 μM PLP and 50 mM Asp for WT and Quint or 100 mM Asp for K258C$_Q$-EA. [c] Morpholine was added to catalyze the decarboxylation of the OAA product, ensuring that the observed kinetics were not perturbed by the reaction of OAA with the PMP enzyme prior to cofactor dissociation. [d] The association rate constants were determined from stopped-flow fluoresence, monitoring the quenching of protein fluorescence upon PMP binding as described in Toney and Kirsch (1991). [e] $K_D = k_{diss}/k_{assoc}$.

Kinetic Isotope Effects. The C$_\alpha$ primary hydrogen kinetic isotope effects for the reactions with L-Asp are presented in Table 3. The WT values are in good agreement with those of Planas and Kirsch (1991). The ranking of the KIE values is WT ≤ Quint ≪ K258C$_Q$-EA.

Kinetics of PMP Dissociation and Association. The rate constants describing the dissociation of PMP from WT, Quint, and K258C$_Q$-EA, in the presence and absence of morpholine, are given in Table 4. These were measured by a steady-state kinetic method in the presence of excess PLP and L-Asp, but no keto acid (Toney & Kirsch, 1987). Thus, the rate of turnover is limited by the rate of PMP dissociation after the release of OAA product. Trapping of OAA by the addition of morpholine did not significantly alter the observed rate of dissociation. The enzymes display k_{diss} values for PMP in the following relative order: WT > K258C$_Q$-EA > Quint. The rate constants for the association of PMP with

the enzymes are also given in Table 4. They only vary by a factor of 1.7 among the three enzymes. The calculated K_D values describing the enzyme•PMP complexes are included in Table 4. The relative ranking of the K_D values parallels that seen for the k_{diss} values: WT > K258C_Q-EA = Quint. The WT values in Table 4 are similar to those previously published (Toney & Kirsch, 1991).

DISCUSSION

Incorporation of Unnatural Amino Acids into Proteins. There are two techniques currently used for the introduction of unnatural amino acids into proteins: *in vitro* translation (Ellman *et al.*, 1991) and posttranslational chemical modification. The first method is potentially far more versatile than the older technique of chemical modification. In theory, almost any amino acid, even those that do not contain an α-amino group (Ellman *et al.*, 1992; Chung *et al.*, 1993), can be incorporated into the protein of interest. Chemical modification relies on the unique chemical reactivity of amino acid side chains, particularly Cys, but also Lys, His, and Tyr (Glazer, 1976). However, the use of site-directed mutagenesis to introduce a modification target residue at any site in a protein enhances the utility of this method (see introduction) over that of its initial use in the 1960s (e.g., Raftery & Cole, 1966). The two methods are compared more extensively in Gloss (1994).

Spectral Properties of K258C_Q-EA. WT, Quint, and K258C_Q-EA exhibit the same λ_{max} values for the protonated and deprotonated internal aldimines (430 and 356 nm, respectively). However, the extinction coefficient of the protonated aldimine of K258C_Q-EA is ~2.5-fold lower than those of Quint and WT. Most changes in the environment of a chromophore, such as polarity or hydrophobicity, affect λ_{max}, rather than ϵ. Electronic interactions with nearby chromophores can alter both parameters by changing the dipole strength of the electric transition dipole moment induced by the absorption of light (Urry, 1985). A classical example is the hyperchromicity associated with double-stranded DNA (Cantor & Schimmel, 1980). Hypochromic effects on the order of 10−50% are frequently observed. The other chromophore in a parallel arrangement with the PLP would have to be Trp-140. This residue forms one wall of the cofactor binding pocket (Kirsch *et al.*, 1984), and its proximity to the cofactor is responsible for quenching much of the intrinsic fluorescence of the cofactor when cofactor binds to the enzyme (Churchich & Farrelly, 1969). Electronic interactions between the two chromophores have also been shown by the phosphorescence and fluorescence studies of Cioni *et al.* (1992). A γ-thia-Lys side chain should be slightly more extended than that of Lys; the length of a thio ether bond is 1.82 Å with a C−S−C bond angle of 105°, while the values for a methylene−methylene bond are 1.54 Å and 109°, respectively (Weast, 1975). This slightly increased length may allow the protonated form of PLP in K258C_Q-EA to stack closer to Trp-140 than it does in the Quint enzyme. This is discussed more thoroughly elsewhere (Gloss, 1994).

Altering the pH Dependence and Proton Abstraction Step of AATase Activity. The spectral pK_a of the internal aldimine of K258C_Q-EA is 1.3 pH units lower than that of Quint. This difference is also reflected in the ΔpK_a of the acidic limb of the k_{cat}/K_M^{Asp} pH profiles of WT and K258C_Q-EA (Figure 3). Potentiometric titrations of the α-*N*-acetyl derivatives of Lys and γ-thia-Lys show that the pK_a of the ε-amino group of γ-thia-Lys (9.4) is 1.1 pH units lower than that of Lys (Hermann & Lemke, 1968). This difference is in good agreement with pK_a differences observed in the AATase active site. Replacement of Lys by γ-thia-Lys lowers the pH optimum of k_{cat}/K_M^{Asp} from 8.2 for WT to 7.7 for K258C_Q-EA. The limiting k_{cat}/K_M^{Asp} value of K258C_Q-EA is 12-fold less than that of WT, but their rate constants would be equal at pH 4.5 and below.

Perturbation of the pK_a of the active site base provides an important tool for dissecting the pH dependence of the kinetic parameters of an enzyme; K258C_Q-EA has been used for this purpose (Gloss & Kirsch, 1995). Decreasing the pK_a of a catalytic base results in a poorer general base catalyst, but a stronger general acid catalyst. Lys-258 abstracts the C_α proton from the external aldimine, formed between the amino acid substrate and PLP, and carries out a 1,3-prototropic shift, reprotonating at C4′ of the cofactor to yield the ketimine (Scheme 1B). γ-Thia-Lys-258, a weaker base, should be less effective in C_α proton abstraction. To the extent that this step is partially rate-determining, there will be a larger C_α-^{2}H kinetic isotope effect for K258C_Q-EA *vs* Quint.

C_α proton abstraction is partially rate-determining for WT and Quint (KIE > 1.0, Table 3), and K258C_Q-EA does exhibit higher KIE values, both on $^Dk_{cat}$ and $^D(k_{cat}/K_M)$. The values of $^Dk_{cat}$ are less than those of $^D(k_{cat}/K_M)$ for WT, Quint, and K258C_Q-EA. This is in contrast to the finding for K258C-EA (Planas & Kirsch, 1991). This difference may be due to changes between WT and Quint, in the extent to which some steps are partially rate-determining. The magnitudes of the KIEs, under identical conditions, are similar for K258C_Q-EA and K258C-EA.

Affinities for Dicarboxylate Ligands. The affinities of K258C_Q-EA for substrates and inhibitors are somewhat perturbed compared to those of Quint (Table 2). The dissociation constants for the complexes of dicarboxylates and Quint•PLP (K_M^{Asp} and K_i values for maleate and α-Me-Asp) are 1.8−3.8-fold lower than those for K258C_Q-EA. The K_i values for the E-PMP•maleate complexes are very similar to those for K258C_Q-EA and Quint. For both enzymes, the K_i for maleate is smaller for the PLP•H^+ complex than for the PMP•H^+ complex. Because of the difference in the pK_a's of the internal aldimines of K258C_Q-EA and Quint, the pH for the minimal value of K_i for maleate is lower for K258C_Q-EA•PLP than for Quint (eq 11).

There is no evidence that the second ionization constant of maleate (pK_a = 6.58; Jencks & Regenstein, 1968) influences the pH dependence of maleate inhibition. The potential importance of this pK_a of maleate would be particularly relevant to the K258C_Q-EA data, as the pK_a of this enzyme's internal aldimine is lower than the second pK_a of maleate. Previous workers fit the observed K_i maleate pH dependence without considering this ionization (Kiick & Cook, 1983; Planas & Kirsch, 1991; Yano *et al.*, 1993). This implication that mono- and dianionic maleates bind with equal affinity has been discussed in detail elsewhere (Gloss, 1994). An explanation for the insensitivity of the K_i of maleate to the protonation state of maleate is that alternative isoenergetic interactions can be made with mono- and dianionic maleates. The X-ray structure of the *E. coli* PLP

maleate enzyme complex shows that there are multiple hydrogen bond donors to the carboxylates of maleate (Jäger *et al.*, 1994). These include the following: (1) the guanidino group of Arg-292, (2) the Nε1 of Trp-140, and (3) the hydroxyl of Ser-296, all within 3.6 Å of the carboxylate equivalent to the β-carboxylate of L-Asp. The α-carboxylate is within 3 Å of the guanidino group of Arg-292 and the amide nitrogen of Asn-194. The latter also donates a hydrogen bond to O3′ of the PLP cofactor (Scheme 1). At low pH, where both maleate and the internal aldimine are protonated (and thus O3′ is partially protonated), the side chain of Asn-194 could be rotated so that the carbonyl oxygen serves as a hydrogen bond acceptor for both the maleate carboxylic acid and O3′. This carbonyl oxygen is the only potential hydrogen bond acceptor within hydrogen-bonding distance of either of the maleate carboxylates.

The Half-Reaction Equilibria Are Changed by Replacement of Lys-258 with γ-Thia-Lys. The major consequence of changing the active site base, Lys-258, to γ-thia-Lys is an order of magnitude decrease in the k_{cat}/K_M values of the amino acids. The values of k_{cat} are similarly reduced, but there is only a small effect on the k_{cat}/K_M values of the keto acids. The net effect is to change the half-reaction equilibrium of K258C$_Q$-EA, relative to WT and Quint. The $K_{eq,half}$ of the half-reaction is defined by

$$K_{eq,half} = \frac{k_{cat}/K_M^{KA}}{k_{cat}/K_M^{AA}} \quad (12)$$

where KA and AA are the keto acid and amino acid, respectively. For the four-carbon substrates, L-Asp and OAA, the $K_{eq,half}$ values are 165, 110, and 1 280 for WT, Quint, and K258C$_Q$-EA, respectively. The $K_{eq,half}$ values for the five-carbon substrates, L-Glu and α-KG, are 28, 18, and 202 for WT, Quint, and K258C$_Q$-EA, respectively. The values of the L-Glu/α-KG equilibria for the WT and Quint were also determined by titration of the PMP enzymes with L-Glu, yielding values of 25 ± 4 and 16.8 ± 0.2, respectively (L. M. Gloss, *et al.*, manuscript in preparation). The equilibria of the K258C$_Q$-EA reactions lie far more toward the PLP form of the enzyme than do the reactions of the Lys-containing enzymes. This suggests that the internal aldimine formed between PLP and γ-thia-Lys-258 is 1.4 kcal/mol more stable than that between PLP and Lys-258. The formation constants of PLP Schiff bases with a variety of amines [collected in Kallen *et al.* (1985)] show that more basic amines form more stable Schiff bases. From the correlation of these data alone, the Schiff base of γ-thia-Lys and PLP should be less stable by ~1.1 kcal/mol than that formed between Lys and PLP.

An alternative interpretation of the $K_{eq,half}$ data is that the PMP form of K258C$_Q$-EA is destabilized by 1.4 kcal/mol relative to the PMP form of Quint. The K_D values for the E·PMP complexes of K258C$_Q$-EA, Quint, and WT were determined. The destabilization of the E·PMP form of K258C$_Q$-EA can be disregarded as Quint and K258C$_Q$-EA exhibit the same K_D for PMP (Table 4). The rate constants for PMP association are very similar for WT, Quint, and K258C$_Q$-EA. The changes in the K_D values for the E·PMP complexes result from much lower values of k_{diss} for the Quint and K258C$_Q$-EA complexes, relative to WT (11−15-fold). This was also observed for Y70F (Toney & Kirsch, 1991), whose increased K_D^{PMP} (24-fold), relative to WT, derived almost exclusively from an increase in k_{diss} (27-fold).

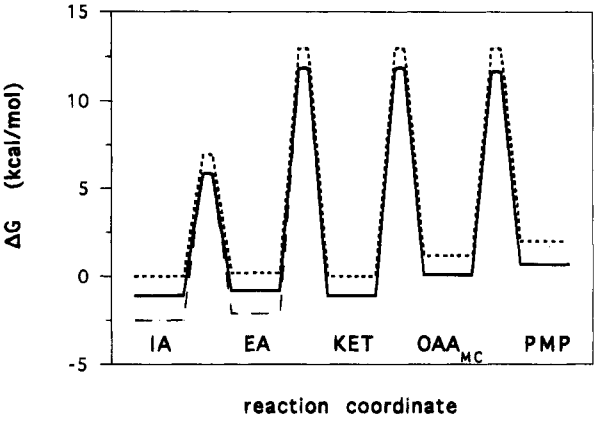


FIGURE 4: Free energy *vs* reaction coordinate diagram for the reactions of WT (dotted line), Quint (solid line), and K258C$_Q$-EA (dashed line) with L-Asp (standard state: 1 mM substrates). The position of WT·PLP + Asp (IA) was arbitrarily set to 0 kcal/mol. The relative positions of the WT barriers are from Goldberg (1992) and Toney and Kirsch (1993); the barriers for ketimine hydrolysis and external aldimine formation are estimates from computer simulations (Goldberg, 1992). The ΔΔ*G* values are described in the text. Definitions: IA, internal aldimine of the PLP enzyme; EA, external aldimine; KET, ketimine; OAA$_{MC}$, Michaelis complex with oxalacetate; PMP, PMP form of the enzyme.

Effect of the Quint Mutations on the Free Energy Profile of the L-Asp Reaction. The k_{cat} value for the L-Asp/α-KG substrate pair is greater for Quint than for WT: 238 and 159 s^{-1}, respectively (Gloss *et al.*, 1992). The C_α hydrogen kinetic isotope effects of Quint and WT are presented in Table 3. The $^Dk_{cat}$ values are the same within experimental error, while the $^D(k_{cat}/K_M)$ values are marginally higher for Quint. Therefore, the higher k_{cat} value exhibited by Quint is not due to an enhanced rate of C_α proton abstraction, but is the result of a reduced barrier for some other step. The L-Asp half-reaction is largely rate-determining for WT (Goldberg, 1992; Julin & Kirsch, 1989; Gloss & Kirsch, 1995). This is a valid assumption for Quint also, given the similar values of $^Dk_{cat}$ for the two enzymes. As shown in Figure 4, the WT L-Asp reaction is partially rate-limited by C_α proton abstraction (EA → KET), OAA dissociation (OAA$_{MC}$ → PMP), and possibly ketimine hydrolysis (KET → OAA$_{MC}$) (Goldberg, 1992). Because the Quint $^D(k_{cat}/K_M)$ value is slightly increased, proton abstraction is more fully rate-determining for Quint in the L-Asp half-reaction than for WT. Therefore, the enhanced rate component of the Quint reaction must be either ketimine hydrolysis or OAA dissociation.

The Quint mutations stabilize the E·PMP form by 1.3 kcal/mol relative to WT (calculated from the respective PMP K_D values, Table 4). However, the Quint $K_{eq,half}$ values are only 1.5-fold lower than those of WT. This indicates only a minor shift of the Quint equilibrium toward the PMP form, relative to WT. Therefore, the Quint·PLP complex must be ~1.1 kcal/mol more stable than that of WT. These cumulative effects are illustrated in the free energy profile of Figure 4.

K258C$_Q$-EA Stabilizes the E·PLP Complex. A partial free energy diagram for WT AATase has been previously constructed from experimental data (Goldberg, 1992; Toney & Kirsch, 1993). Solvent kinetic isotope effects and computer simulations suggest that ketimine hydrolysis may be partially rate-determining (Goldberg, 1992); therefore, the

barrier height for this step is drawn as nearly equal to those steps known to be partially rate-determining: C_α proton abstraction and OAA dissociation (Figure 4). The free energy of activation for external aldimine formation has not been measured directly, but is estimated from literature values for pcAATase (Fasella & Hammes, 1967). The free energy diagrams of Quint and K258C-EA are drawn similarly to those of WT, except where there is evidence for differences.

The effects of γ-thia-Lys, relative to Lys, are to shift the equilibrium of the enzyme further toward the PLP form and to make proton abstraction more fully rate-determining. The results reported herein indicate the following changes in the free energy diagram (Figure 4) for the reaction of L-Asp with $K258C_Q$-EA relative to those of WT and Quint: (a) the proton abstraction barrier in the forward direction is higher by 1.4 kcal/mol for $K258C_Q$-EA relative to Quint (from k_{cat}/K_M^{Asp} and $^D(k_{cat}/K_M^{Asp}$ values); (b) the reverse direction barrier from {PMP + OAA → EA} is not altered by the replacement of Lys-258 by γ-thia-Lys (from k_{cat}/K_M^{OAA} values); (c) the half-reaction equilibrium value of $K258C_Q$-EA lies 1.4 kcal/mol closer to E·PLP + amino acid relative to the corresponding WT or Quint reactions (from $K_{eq,half}$ values). The difference in the free energy of dissociation of PMP from Quint and $K258C_Q$-EA is ~0 (from the K_D^{PMP} values); the E·PMP complexes of both mutants are 1.2 kcal/mol more stable than the WT·PMP complex. Therefore, the shift in the equilibrium of $K258C_Q$-EA is due to stabilization of the E·PLP complex. (d) The K258C-EA·PLP complex is 1.4 and 2.6 kcal/mol more stable than those of Quint and WT, respectively.

The PLP conformation in the internal aldimine of wild-type structures is strained (McPhalen *et al.*, 1992; Schnackerz *et al.*, 1989). Replacing Lys with the longer side chain of γ-thia-Lys might relieve this strain. This may explain the greater stablity of the E·PLP complex formed by $K258C_Q$-EA. Such a structural rearrangement would allow the cofactor to rotate forward toward Trp-140 and enhance the interactions between Trp-140 and PLP. The decrease in the ϵ_{430} value of $K258C_Q$-EA·PLP-H^+ suggests that these interactions do occur.

ACKNOWLEDGMENT

The authors acknowledge Mr. Daniel E. Spencer for technical assistance and Dr. Jonathan M. Goldberg for critical review of the manuscript.

REFERENCES

Cantor, C. R., & Schimmel, P. R. (1980) *Biophysical Chemistry, Part II*, pp 349−404, W. H. Freeman & Co., New York.
Chung, H., Benson, D. R., & Schultz, P. G. (1993) *Science 259*, 806−809.
Churchich, J. E., & Farrelly, J. G. (1969) *J. Biol. Chem. 244*, 3685−3690.
Cioni, P., Onuffer, J. J., & Strambini, G. B. (1992) *Eur. J. Biochem. 209*, 759−764.
Dhalla, A. M., Li, B., Alibhai, M. F., Yost, K. J., Hemmingsen, J. M., Atkins, W. M., Schineller, J., & Villafranca, J. V. (1994) *Protein Sci. 3*, 476−481.
Ellis, K. J., & Morrison, J. F. (1982) *Methods Enzymol. 87*, 405−426.
Ellman, J., Mendel, D., Anthony-Cahill, S., Noren, C., & Schultz, P. G. (1991) *Methods Enzymol. 202*, 301−336.
Ellman, J., Mendel, D., & Schultz, P. G. (1992) *Science 255*, 197−200.
Fasella, P., & Hammes, G. G. (1967) *Biochemistry 6*, 1798−1804.
Fasella, P., Giartosio, A., & Hammes, G. G. (1966) *Biochemistry 5*, 197−202.
Glazer, A. N. (1976) in *Laboratory techniques in biochemistry and molecular biology* (Delange, R. J., & Sigman, D. S, Eds.) Vol. 4, Pt. I, American Elsvier Publishing Co., Amsterdam.
Gloss, L. M. (1994) Ph.D. Thesis, University of California, Berkeley CA.
Gloss, L. M., & Kirsch, J. F. (1995) *Biochemistry 34*, 3999−4007.
Gloss, L. M., Planas, A., & Kirsch, J. F. (1992) *Biochemistry 31*, 32−39.
Goldberg, J. M. (1992) Ph.D. Thesis, University of California, Berkeley, CA.
Goldberg, J. M., Swanson, R. V., Goodman, H. S., & Kirsch, J. F. (1991) *Biochemistry 30*, 305−312.
Hermann, V. P., & Lemke, K. (1968) *Hoppe-Seyler Z. Physiol. Chem. 349*, 390−394.
Jäger, J., Moser, M., Sauder, U., & Jansonius, J. N. (1994) *J. Mol. Biol. 239*, 285−305.
Julin, D. A., & Kirsch, J. F. (1989) *Biochemistry 28*, 3825−3833.
Kallen, R. G., Korpela, T., Martell, A. E., Matsushima, Y., Metzler, C. M., & Metzler, D. E. (1985) in *Transaminases* (Christen, P., & Metzler, D. E., Eds.) pp 37−108, Wiley, New York.
Kiick, D. M., & Cook, P. F. (1983) *Biochemistry 22*, 375−382.
Kirsch, J. F., Eichele, G., Ford, G. C., Vincent, M. G., Jansonius, J. N., Gehring, H., & Christen, P. (1984) *J. Mol. Biol. 174*, 497−525.
Kuramitsu, S., Hiromi, K., Hayashi, H., Morino, Y., & Kagamiyama, H. (1990) *Biochemistry 29*, 5469−5476.
Lu, J., Baase, W. A., Muchmore, D. C., & Dahlquist, F. W. (1992) *Biochemistry 31*, 7765−7772.
Lukac, M., & Collier, R. J. (1988) *J. Biol Chem. 263*, 6146−6149.
McPhalen, C. A., Vincent, M. G., & Jansonius, J. N. (1992) *J. Mol. Biol. 225*, 495−517.
Onuffer, J. J., & Kirsch, J. F. (1994) *Protein Eng. 7*, 413−424.
Planas, A., & Kirsch, J. F. (1990) *Protein Eng. 3*, 625−628.
Planas, A., & Kirsch, J. F. (1991) *Biochemistry 30*, 8268−8276.
Raftery, M. A., & Cole, R. D. (1966) *J. Biol. Chem. 241*, 3457−3461.
Schnackerz, K. D., Wahler, G., Vincent, M. G., & Jansonius, J. N. (1989) *Eur. J. Biochem. 185*, 525−531.
Smith, H. B., & Hartman, F. C. (1988) *J. Biol. Chem. 263*, 4921−4925.
Sutton, C. L., Mazumder, A., Chen, C. B., & Sigman, D. S. (1993) *Biochemistry 32*, 4225−4230.
Toney, M. D., & Kirsch, J. F. (1987) *J. Biol. Chem. 262*, 12403−12405.
Toney, M. D., & Kirsch, J. F. (1991) *Biochemistry 30*, 7461−7466.
Toney, M. D., & Kirsch, J. F. (1993) *Biochemistry 32*, 1471−1479.
Urry, D. W. (1985) in *Modern Physical Methods in Biochemistry* (Neuberger, A., & Van Deenen, L. L. M., Eds.) pp 275−346, Elsevier, Amsterdam.
Velick, S. F., & Vavra, J. (1962) *J. Biol. Chem. 237*, 2109−2122.
Weast, R. C., Ed. (1975) *Handbook of Chemistry and Physics*, 56th ed., p F214, CRC Press, Cleveland, OH.
Wynn, R., & Richards, F. M. (1993) *Protein Sci. 2*, 395−403.
Yagi, T., Kagamiyama, J., Ohtawara, S., Soda, K., & Nozaki, M. (1979) *Anal. Biochem. 100*, 20−24.
Yano, T., Kuramitsu, S., Tanase, S., Morino, Y., & Kagamiyama, H. (1992) *Biochemistry 31*, 5878−5887.
Yano, T., Minzo, T., & Kagamiyama, H. (1993) *Biochemistry 32*, 1810−1815.

BI941947E