Reengineering the Catalytic Lysine of Aspartate Aminotransferase by Chemical Elaboration of a Genetically Introduced Cysteine†

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ABSTRACT: The active-site essential catalytic residue of aspartate aminotransferase, Lys 258, has been converted to Cys (K258C) by site-directed mutagenesis. This mutant retains less than 10^{-6} of the wild-type activity with L-aspartate. The deleted general base was functionally replaced by selective (with respect to the other five cysteines in wild type) aminoethylation of the introduced Cys 258 with (2-bromoethyl)amine following reversible protection of the nontarget sulfhydryl groups at different stages of unfolding. The chemically elaborated mutant (K258C-EA) is 10^5 times more reactive than is K258C and has a k_{cat} value of ~7% of that of wild type (WT). K_m and K_I values are similar to those for WT. The acidic pK_a controlling V/K_{Asp} is shifted from 7.3 (WT) to 6.0 (mutant). V/K values for amino acids are ~3% of those found for WT, whereas they are ~20% for keto acids. The value of ^DV increases from 1.6 for WT to 3.4 for the mutant, indicating that C_α proton abstraction constitutes a more significant kinetic barrier for the latter enzyme. A smaller, but still significant, increase in $^D(V/K_{Asp})$ from 1.9 in WT to 3.0 in the mutant shows that the forward and reverse commitment factors are inverted by the mutation. The acidic limb of the V/K_{Asp} versus pH profile, is lowered by 1.3 pH units, probably reflecting the similar difference in the basicity of the ϵ-NH_2 group in γ-thialysine versus that in lysine. The decreased basicity of this group in K258C-EA is also considered to be principally responsible for the lower values of V_{max} and the larger kinetic isotope effects characterizing K258C-EA when compared to wild-type enzyme.

Aspartate aminotransferase (AATase,[1] EC 2.6.1.1) catalyzes the transfer of the α-amino group from L-Asp and L-Glu to α-KG and OAA, respectively. The kinetics obey a ping-pong bi-bi mechanism where the enzyme shuttles between the PLP and the PMP forms. The central step in enzymatic transamination is the reversible tautomerization of the aldimine to the ketimine (Scheme I), where the transfer of a proton between C_α of the amino acid and $C_{4'}$ of the cofactor is at least partly rate-determining for the overall process (Julin & Kirsch, 1989). X-ray crystallographic analysis (Kirsch et al., 1984) and site-directed mutagenesis studies (Toney & Kirsch, 1987, 1989) have shown that Lys 258 is the essential general base catalyzing the 1,3-prototropic shift [see Arnone et al. (1985), Gehring (1985), and Jansonius and Vincent (1987) for recent reviews].

Cloning and expression of AATase from *Escherichia coli* (Malcolm & Kirsch, 1985) have allowed the application of site-directed mutagenesis to test the well-documented features of the reaction mechanism (Velick & Vavra, 1962; Braunstein, 1973; Kiick & Cook, 1983; Julin et al., 1989; McLeish et al., 1989; Julin & Kirsch, 1989), and the individual roles of the active-site residues involved in catalysis, cofactor binding, and substrate specificity (Malcolm & Kirsch, 1985; Toney & Kirsch, 1987, 1989; Cronin & Kirsch, 1988; Hayashi et al., 1989; Inoue et al., 1989).

Deletion of the amino group of Lys 258 by substitution to Ala (K258A mutant; Malcolm & Kirsch, 1985) resulted in an enzyme that is essentially incapable of catalyzing transamination. The current limitation of site-directed mutagenesis technology to replacements by the other 19 amino acids found in proteins restricts more detailed investigations of the role of Lys 258, since no other α-amino acids bearing a primary amine side chain are available. This restriction has recently been partially overcome by combining genetic engineering with chemical modification by specifically alkylating introduced cysteine residues. Lysine analogues have been realized by reaction with BEA and cystamine (Smith & Hartman, 1988; Smith et al., 1988; Pease et al., 1987), and carboxylate groups have been generated by carboxymethylation of an engineered thiol (Lukac & Collier, 1988).

E. coli AATase has five cysteines per subunit of the α_2 dimer in positions 82, 191, 192, 270, and 401 (Kondo et al., 1987), none of which are in or near the active site. In this study, Lys 258 is mutated to Cys in order to introduce an unnatural lysine analogue, *S*-(2-aminoethyl)cysteine, by selective aminoethylation of the engineered sulfhydryl group. The properties of the inactive K258C mutant and of the chemically elaborated enzyme are reported.

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[1] Abbreviations: AATase, aspartate aminotransferase; WT, wild-type *E. coli* AATase; K258C, *E. coli* AATase in which Lys 258 has been changed to Cys; K258C-EA, aminoethylated K258C at Cys 258; K258C-CM, carboxymethylated K258C at Cys 258; MDH, porcine heart cytoplasmic malate dehydrogenase; PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine 5'-phosphate; CS, L-cysteine sulfinate; α-KG, α-ketoglutarate; OAA, oxalacetate; α-MeAsp, α-methyl-DL-aspartate; BEA, (2-bromoethyl)amine; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; TPCK, tosylphenylalanine chloromethyl ketone; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid); Tris, tris(hydroxymethyl)aminomethane; TAPS, 3-[[tris(hydroxymethyl)methyl]propanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; CAPS, 3-(*N*-cyclohexylamino)-1-propanesulfonic acid; C_α-KIE, C_α hydrogen primary kinetic isotopic effect; ^DV, C_α-KIE on k_{cat}; $^D(V/K)$, C_α-KIE on k_{cat}/K_m.

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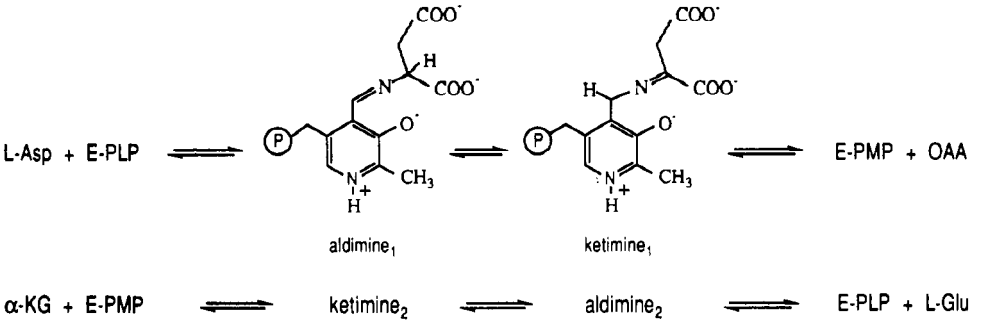
Scheme I



Experimental Procedures

Materials

L-Asp, α-KG,[1] L-Glu, OAA, α-MeAsp, NADH, PLP, PMP, BEA, cystamine, *S*-(2-aminoethyl)-L-cysteine, and DTNB were obtained from Sigma. Urea (crystalline, enzyme grade) and DTT were purchased from BRL, and Sephadex G25 was from Pharmacia. CS, MES, HEPES, Tris, TAPS, CHES, CAPS, and 2-mercaptoethanol were Aldrich products. Maleic acid was obtained from Matheson Coleman & Bell. [α-^{2}H]-L-Asp and [α-^{2}H]-DL-Asp were gifts from M. D. Toney and D. A. Julin and were prepared as described in Julin et al. (1989). All buffer and reagent solutions used for chemical modifications and enzyme resolutions were degassed prior to use. N^{ϵ}-Pyridoxyl-L-lysine was prepared from α-acetyl-L-lysine (from Sigma) essentially by the procedure of Dempsey and Christensen (1962) with modifications from Severin et al. (1969). Ethylenimine was obtained by reaction of BEA with concentrated KOH as described in Wystrach et al. (1955) and Reeves et al. (1951). *S*-(2-(*N*-pyridoxylamino)ethyl)-L-cysteine was synthesized as follows: α-acetyl-L-cysteine (from Sigma) was aminoethylated with ethylenimine by the procedure of Raftery and Cole (1966) to yield α-acetyl-*S*-(2-aminoethyl)-L-cysteine. It was treated, without further purification, with PLP and sodium borohydride in methanol solution as described for the preparation of other phosphopyridoxyl derivatives (Severin et al., 1969; Morino et al., 1986). Hydrolysis in 6 N HCl under the same conditions used for protein hydrolysis (see below) gave the desired product.

Methods

Spectrophotometric measurements and kinetics determinations were made on Perkin-Elmer λ 4B and Kontron Uvikon 860 instruments. Temperature was kept constant at 25 °C. Enzyme concentrations were estimated from the absorbance at 205 nm by use of A_{205} (1 mg/mL) = 31 (Scopes, 1974). The AATase concentrations are given per subunit of the dimer (MW = 43 500). AATase activity was measured by coupling the production of oxalacetate to MDH for the substrate pair L-Asp/α-KG. The assays were carried out at 25 °C in a solution of 200 mM HEPES–KOH buffer, pH 7.5, 100 mM KCl, 10 mM α-KG, 20 mM L-Asp, 150 μM NADH, and 10 units/mL MDH. Reversed-phase HPLC analyses were performed in a Knauer liquid chromatograph equipped with an ID Supercosil LC-18 column (25 cm × 4.6 mm) and a Linear Uvis 200 UV detector attached to a computerized data collection system.

Site-Directed Mutagenesis, Expression, and Protein Purification. Mutagenesis was carried out by a modification of the method described earlier (Malcolm & Kirsch, 1985; J. Onuffer, unpublished results). The WT and K258C clones were grown to stationary phase in YT medium supplemented with 200 μg/mL ampicilin, 50 μg/mL kanamycin, and 15 μg/mL tetracycline. The proteins were isolated and purified essentially by the procedure described for WT in Cronin and Kirsch (1988).

Enzyme Resolution. The apo form of K258C was prepared by two different procedures. (a) K258C(PMP) (obtained by adding 5 mM CS to the PLP holoenzyme or to the PLP–PMP mixture from the isolation procedure) was incubated in 8 M urea–100 mM Tris-HCl, pH 7.0, for 30 min at 4 °C followed by dialysis against three buffer changes of 0.5 M potassium phosphate, pH 5.5, under nitrogen. The apoprotein was concentrated to approximately 10 mg/mL and used within 24 h. (b) Solid ammonium sulfate was added to a solution of K258C(PMP) (20 mg/mL) in 20 mM phosphate buffer, pH 7.2, to 80% saturation. The suspension was adjusted to pH 3.0 with 1 N HCl at 4 °C. After incubation for 24 h at 4 °C, the precipitate was collected by centrifugation and resuspended in 20 mM sodium acetate, pH 3.0, 80% ammonium sulfate. Centrifugation and suspension were repeated three times to remove all the released cofactor. After raising the pH to 7.2 with KOH, the apoenzyme was centrifuged and redissolved in 20 mM phosphate buffer, pH 7.2. The remaining salt was removed by dialysis against the same buffer. Apo-K258C was concentrated and used within 24 h. Milder conditions are sufficient to resolve WT enzyme (Kochhar et al., 1987; Wada & Snell, 1962).

Thiol Titrations. The sulfhydryl content of the proteins was determined by the method of Ellman (1959). DTNB (500 μM) was added to the enzyme solution (approximately 10 μM subunits) in 200 mM NaTAPS (or NaHEPES) buffer at pH 8.0, either without denaturant (for determination of accessible SH groups in the native conformation) or in 8 M urea (for determination of total sulfhydryl content). The increment in absorbance at 412 nm due to the release of 5-thio-2-nitrobenzoate was measured 2–5 min after mixing. Likewise, exposure of thiol groups by urea was monitored by the extent of reaction with DTNB. Quantitation was based on a cysteine standard curve obtained under the same conditions.

Reversible Denaturation. Both K258C and WT holoenzymes were added to a solution of 8 M urea, 200 mM sodium TAPS, pH 8.0, 1 mM EDTA (sodium salt) to a final concentration of 1 mg/mL and incubated at 4 °C for 14 h. Refolding was accomplished either by 1/10 dilution in 20 mM phosphate buffer, pH 7.5, or by dialysis against the same buffer. After all the denaturant was removed, excess PLP was added, and the protein was concentrated to approximately 10 mg/mL, the refolded holoenzyme was assayed for transaminase activity. WT recovered more than 80% of its original specific activity while the refolded K258C reacted with CS with the same single-turnover rate constant as the untreated enzyme, indicating full recovery of native structure. The absorption spectra of both enzymes were identical with those of the freshly isolated proteins.

Aminoethylation of K258C by a Sequential Protection–Modification Procedure. K258C-EA was prepared by alkylation of the previously DTNB-protected K258C enzyme with BEA in the presence of urea according to the procedure recently described (Planas & Kirsch, 1990).

Selective ^{14}C-Carboxymethylation of K258C. The mutant was carboxymethylated preferentially at Cys 258 by following the same sequential protection–modification procedure described for aminoethylation (Planas & Kirsch, 1990) but substituting BEA by [1-^{14}C]iodoacetate as alkylating reagent. The DTNB-protected protein was reacted with 10 mM [1-^{14}C]iodoacetate (13 mCi/mmol) in 8 M urea at room temperature for 30 min, and the mixture was quenched with 2-mercaptoethanol. No PLP was added during the refolding step. The apo form of ^{14}C-labeled K258C-CM was concentrated to 150 μM subunits and immediately subjected to tryptic digestion (see below).

Amino Acid Analysis. Proteins were hydrolyzed at 100 °C with 6 N HCl for 24 h in evacuated sealed tubes (Allen, 1981). An aliquot of each sample was oxidized with performic acid prior to hydrolysis for determination of cysteine as cysteic acid. Hydrolysates were chromatographed in a Beckman 6300 amino acid analyzer. *S*-(2-Aminoethyl)-L-cysteine was identified by comparison with a synthetic standard.

Cyanoborohydride Reduction of PLP Enzymes. The PLP forms of WT, K258C-EA, and K258C were treated with $NaCNBH_3$ essentially according to the procedure described for WT in Kondo et al. (1987).

Identification of Pyridoxyl-Labeled Amino Acids. The cyanoborohydride-reduced enzymes were hydrolyzed with 6 N HCl for 1 h at 150 °C in evacuated sealed tubes (Allen, 1981). The hydrolysates were dissolved in 0.2 M ammonium sulfate, pH 5.2, and analyzed by reversed-phase HPLC. Samples were eluted isocratically with 0.2 M ammonium sulfate buffer, pH 5.2, at a flow rate of 1 mL/min, and the chromatogram was monitored at 320 and 254 nm. Synthetic N^{ϵ}-pyridoxyl-L-lysine and *S*-(2-(*N*-pyridoxylamino)ethyl)-L-cysteine were used as standards.

Tryptic Digestions and Peptide Mapping by HPLC. Protein solutions (2–10 mg/mL) of reduced K258C-EA(PLP), WT-(PLP), K258C(PLP), or ^{14}C-labeled K258C-CM were carboxymethylated and digested with TPCK–trypsin essentially as described in Roberts et al. (1988). The tryptic peptides were separated by reversed-phase HPLC, and the chromatograms were monitored at 320 (phosphopyridoxyl-labeled peptides) and 214 nm. The samples were eluted with a 0.33% H_3PO_4–acetonitrile gradient, with the acetonitrile concentration increasing from 0 to 12% over the first 13 min, to 28% over the next 65 min, to 42% over 12 min, and finally to 62% over the last 30 min. Radioactive peptides from the tryptic digestion of ^{14}C-labeled K258C-CM were monitored on a Flo-One-Beta radioactive flow detector (Radiomatic Inc., Tampa, FL).

Transamination Kinetics. (*a*) *Half-Reactions.* Kinetic parameters under single-turnover conditions were determined by monitoring the spectral changes of the enzyme-bound cofactor. The reactions were conducted in solutions of 100 mM HEPES buffer, pH 7.5, 50 mM KCl for K258C or 200 mM HEPES, pH 7.5, 100 mM KCl for WT and K258C-EA with L-alanine. AATase concentration was 10–15 μM subunits. The reaction progress curves gave excellent fits to the first-order rate expression. Values of k_{max} and K_m^{app} were calculated from the dependence of the pseudo-first-order rate constants (k_{obs}) on substrate concentration by nonlinear least-squares fitting to a rectangular hyperbola.

(*b*) *Steady State.* Initial rates under steady-state conditions were determined in a solution of 200 mM HEPES buffer, pH 7.5, 100 mM KCl by the MDH-coupled assay for the substrate pair L-Asp/α-KG (Cronin & Kirsch, 1988) or by the coupling of the production of pyruvate to LDH for the substrate pairs CS/α-KG and CS/OAA (Julin & Kirsch, 1989). AATase activity present in MDH preparations was reduced by prior treatment with bromopyruvate as described by Morino and Okamoto (1970). All the reactions were carried out at 25 °C, and the blank rates were recorded before the enzyme was added.

Kinetic parameters for the substrate pair L-Asp/α-KG were calculated from the initial rates determined for five concentrations of L-Asp at each of six concentrations of α-KG in the range of 0.2–10 times their K_m's by nonlinear regression fitting to eq 1 describing the ping-pong mechanism (Velick & Vavra, 1962). Kinetic parameters for the other substrate pairs were determined for the first substrate of the pair at a fixed concentration of the second ($\sim 10K_m$). Initial rates were fitted to a rectangular hyperbola by nonlinear regression given by

$$\frac{v}{[E_t]} = \frac{k_{cat}[Asp][\alpha\text{-KG}]}{K_m^{Asp}[\alpha\text{-KG}] + K_m^{\alpha\text{-KG}}[Asp] + [Asp][\alpha\text{-KG}]} \quad (1)$$

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

where [S] is the concentration of the varied substrate and K_m is its Michaelis constant.

In the pH-dependence studies of V/K for L-Asp, reactions were conducted at near-saturating α-KG concentration ($10K_m$). The 200 mM buffers were used over the following pH ranges: MES, pH 5.5–6.8; HEPES, 6.8–8.0; TAPS, 8.0–9.0; CHES, 9.0–10.0; and CAPS 10.0–10.5. No KCl was added. The bell-shaped V/K_m vs pH curve was fitted to the equation (Kiick & Cook, 1983)

$$\frac{v}{K} = \frac{(V/K)_{lim}}{1 + [H^+]/K_{a1} + K_{a2}/[H^+]} \quad (3)$$

where K_{a1} and K_{a2} are the acid dissociation constants at low and high pH, respectively, and $(V/K)_{lim}$ is the pH-independent, limiting value of V/K.

(*c*) *Kinetic Isotope Effects.* Reactions with [α-^{1}H]Asp and [α-^{2}H]Asp were carried out under steady-state conditions as described above in 100 mM KCl and 200 mM buffer at three pH values (MES, pH 6.0; HEPES, 7.5; and TAPS, 8.5). The second substrate was α-KG at a constant concentration of 10 times K_m. No difference in the measured KIEs was observed by using the L-isomers or the racemic mixtures of Asp in the runs at pH 7.5; therefore, [α-^{1}H]-L-Asp and [α-^{2}H]-DL-Asp were used at pH 6.0 and 8.5.

(*d*) *Competitive Inhibition.* Inhibition constants for maleate were determined either by varying the concentration of both substrates, L-Asp and α-KG, at various fixed concentrations of maleate or by holding α-KG constant at its K_m and varying L-Asp and maleate. The data sets obtained were fitted to the equation (Velick & Vavra, 1962)

$$\frac{v}{[E_t]} = \frac{k_{cat}}{1 + \dfrac{K_m^{Asp}(1 + [mal]/K_{I1})}{[Asp]} + \dfrac{K_m^{\alpha\text{-KG}}(1 + [mal]/K_{I2})}{[\alpha\text{-KG}]}} \quad (4)$$

where K_{I1} and K_{I2} are the inhibition constants for the PLP and the PMP enzyme forms, respectively. Data for α-MeAsp were obtained at the constant saturating α-KG concentration

Table I: Kinetic Parameters of Wild-Type and K258C Mutant Aspartate Aminotransferases

substrate[c]	K258C[a] k_{max} (10^3 s^{-1})	K258C[a] K_m^{app} (mM)	K258C[a] k_{cat}/K_m (M^{-1} s^{-1})	wild type[b] k_{cat} (s^{-1})	wild type[b] K_m (mM)	wild type[b] k_{cat}/K_m (10^{-4} M^{-1} s^{-1})	activity ratio $[(k_{max})_{K258C}]/[(k_{cat})_{wt}]$	selectivity ratio $[(k_{cat}/K_m)_{K258C}]/[(k_{cat}/K_m)_{wt}]$
L-aspartate	<0.05	$<5 \times 10^{-5}$ (K_d)[d]		142 ± 2	1.87 ± 0.07	7.6 ± 0.2	$<4 \times 10^{-7}$	
L-glutamate[e]	0.079 ± 0.001	0.012 ± 0.001	6.8 ● 0.6					
L-cysteine sulfinate	2.03 ± 0.02	<0.05	>40	363 ± 8	10.6 ± 0.6	>3.4 ± 0.1	5.6×10^{-6}	1.2×10^{-3}
α-ketoglutarate	0.048 ± 0.007	0.53 ± 0.15	$(9.2 \pm 1.5) \times 10^{-2}$	142 ± 2	0.52 ± 0.07	0.27 ± 0.03	3.4×10^{-7}	3.4×10^{-7}
oxalacetate	2.00 ± 0.03	0.51 ± 0.02	4.0 ± 0.1	196 ± 4	0.021 ± 0.002	0.093 ± 0.005	1.0×10^{-5}	4.3×10^{-7}

[a] Single-turnover conditions at pH 7.5, 25 °C in 100 mM HEPES, 50 mM KCl. k_{max} and K_m^{app} are the single-turnover k_{cat} and apparent K_m from fitting the pseudo-first-order rate constants (k_{obs}) as a function of substrate concentration to a rectangular hyperbola. [b] Steady-state conditions at pH 7.5, 25 °C in 200 mM HEPES, 100 mM KCl. k_{cat} and K_m were determined as described in Experimental Procedures. [c] The concentration ranges of tested substrates were 0.5–20 mM for L-aspartate with both enzymes, 15–500 μM for L-glutamate with K258C, 50 μM–5 mM and 2.5–40 mM for L-cysteine sulfinate with K258C and wild type, respectively, 20 μM–5 mM and 0.1–5 mM for α-ketoglutarate with K258C and wild type, respectively, and 20 μM–5 mM and 10–190 μM for oxalacetate with K258C and wild type, respectively. Substrate pairs for steady-state measurements with wild type were L-aspartate/α-ketoglutarate, L-cysteine sulfinate/α-ketoglutarate, and oxalacetate/cysteine sulfinate. Kinetic parameters are given for the first substrate of each pair at constant saturating concentration of the second. [d] The dissociation constant [K_d (mM)] for L-aspartate with the PLP form of K258C was measured by spectrophotometric titration of the external aldimine as described in Experimental Procedures. The aspartate concentration ranged from 20 nM to 35 mM. [e] $K_d = 0.005 \pm 0.003$ mM for L-glutamate with K258C by spectrophotometric titration as described in footnote *d*. The glutamate concentration range was 1–250 μM.

($10K_m$) by varying the concentration of L-Asp and inhibitor. The inhibition constants for α-MeAsp, which binds significantly only to the PLP enzyme, were calculated by nonlinear regression to the equation (Kiick & Cook, 1983)

$$\frac{v}{[E_t]} = \frac{k_{cat}}{1 + \dfrac{K_m^{Asp}(1 + [\alpha\text{-MeAsp}]/K_i)}{[Asp]} + \dfrac{K_m^{\alpha\text{-KG}}}{[\alpha\text{-KG}]}} \quad (5)$$

It is assumed in using α-DL-MeAsp that the D isomer does not bind to the enzyme as is the case for the pig heart isozyme (Fasella et al., 1966).

Results

Physical Properties of K258C. *E. coli* AATase mutant K258C was purified according to the procedure described for WT enzyme by Cronin and Kirsch (1988). It had the same mobility as WT on SDS-polyacrylamide gels and appeared to be homogeneous. The K258C enzyme as isolated exhibits two absorption bands with maxima at 330 and 403 nm, respectively. The former was shown to be due to bound PMP, which did not exchange with the excess PLP present in the isolation procedure, by its conversion to a 430-nm band upon addition of OAA (the external aldimine of L-Asp). The same isolation procedure suffices to remove all PMP from WT enzyme. The 403-nm band represents free PLP bound at the active site, since Schiff base formation cannot occur.

Consistent with the observation of tightly bound PMP is the difficulty in resolving K258C into apoenzyme and cofactor. WT AATase can easily be resolved by dialysis of the PMP holoenzyme against 0.5 M phosphate buffer (Kochhar et al., 1987), or by ammonium sulfate precipitation in acetate buffer at pH 4.9 as reported for the cytoplasmic isoenzyme by Wada and Snell (1962). These procedures are ineffective when applied to K258C. Resolution was achieved by dialysis of the unfolded protein in 8 M urea against 0.5 M phosphate buffer at pH 5.5 or by incubation of the precipitated PMP holoenzyme at pH 3.0 for 24 h.

Reconstitution of the apoenzyme with pure PLP and PMP confirmed the original assignment of the spectral bands. K258C(PLP) shows a single band at 403 nm while K258C(PMP) absorbs maximally at 330 nm.

Catalytic Properties of K258C. The mutant retains less than 10^{-6} of the transaminase activity of WT enzyme with L-aspartate, as expected from the removal of the proposed essential lysine (Table I). The mutant enzyme, however, still undergoes some partial reactions. Changes in absorption spectra show that both amino and keto acid substrates readily form external aldimines and ketimines with the PLP and PMP forms of the enzyme, respectively (data not shown). Transamination is blocked at these intermediates except for those more reactive substrates, CS and OAA, for which that reaction does progress but with rate constants some orders of magnitude less than those exhibited by WT enzyme.

The kinetics for the reactions of the mutant were measured under single-turnover conditions following the conversion of the PLP form of the enzyme into the PMP form or vice versa upon addition of amino or keto acid substrates, respectively. The kinetic parameters for wild-type and K258C enzymes are summarized in Table I. The dependence of the single-turnover pseudo-first-order rate constant (k_{obs}) for each half-reaction as a function of substrate concentration is given by

$$k_{obs} = \frac{k_{max}[S]}{K_m^{app} + [S]} \quad (6)$$

The value of k_{max}/K_m^{app} derived from single-turnover experiments equals k_{cat}/K_m determined by steady-state methods (Cronin & Kirsch, 1988), allowing direct comparison of the specificity constants between both enzymes as shown in Table I. However, the activity ratios given as the ratio of single-turnover k_{max} for the mutant to steady-state k_{cat} for the wild type represent upper limits of relative activities. k_{cat} from steady-state considerations is a composite rate constant of the two ping-pong half-reactions (Segel, 1975) given by

$$k_{cat} = \frac{k_1/k_2}{k_1 + k_2} \quad (7)$$

where k_1 is the rate constant for the conversion of E–PLP–amino acid$_1$ complex to E–PMP and keto acid$_1$ and k_2 is the corresponding rate constant for the second half-reaction converting keto acid$_2$ to amino acid$_2$.[2] Thus, this relation establishes that k_{cat} derived from steady-state measurement is lower than or equal to the single-turnover rate constant for each half-reaction.

The dissociation constants for L-Asp and L-Glu (Table I) were measured by spectrophotometric titration following the 430-nm band of the external aldimines. The values so determined are much lower than the K_m's for these amino acid substrates reacting with WT enzyme. CS, a more reactive aspartate analogue, is transaminated with a rate constant of 2×10^{-3} s^{-1}, and its K_m value is <50 μM compared to 10 mM

[2] k_1 and k_2 determined in single-turnover conditions represent upper limits for half-reaction rate constants under conditions where product dissociation is slower than transamination, i.e., $k_{max1}k_{max2}/(k_{max1} + k_{max2}) \geq k_{cat}$.

for WT. A plausible explanation for the much greater affinity of the mutant enzyme for amino acids when compared to wild type is couched in terms of competition for the aldehyde moiety of PLP by the ϵ-amino group of Lys 258 and the α-amino group of the substrate. The mutant enzyme lacks the former competitor and therefore permits tighter binding of the substrate to PLP (Kirsch et al., 1991; Toney & Kirsch, 1991). α-Keto acids, on the other hand, in accord with this hypothesis, do not bind so tightly to the PMP form of K258C and exhibit K_m values comparable to those obtained for wild type.

Aminoethylation of K258C. Attempts to convert Cys 258 to *S*-(2-aminoethyl)cysteine by direct alkylation of the native form of the enzyme with BEA according to the procedure of Raftery and Cole (1966) failed. In the presence of a denaturant, treatment with the alkylating reagent resulted in an extensive protein precipitation upon attempted refolding, although K258C and WT enzymes show reversible unfolding in urea. A good yield of the successfully aminoethylated enzyme was achieved by a stepwise protection–modification procedure at different stages of unfolding. The five nontarget cysteines of K258C were protected with DTNB or cystamine by reaction in 2 M urea followed by aminoethylation with BEA in 8 M urea. Deprotection of the initially derivatized residues was accomplished by treatment with DTT, and refolding gave a final native structure with an overall yield of 50% recovered protein. A detailed procedure has been recently reported (Planas & Kirsch, 1990).

Chemical Characterization of Aminoethylated K258C. Amino acid analysis showed 1.2 *S*-(2-aminoethyl)-L-cysteine residues/subunit and a reduction in total cysteine content from 5.6 to 4.9 residues/subunit following treatment of K258C by the stepwise BEA alkylation procedure. With the exception of a reduction in methionine from 7.9 to 7.4 residues/subunit, no other amino acids appear to be modified. The identity of the BEA-modified Cys 258 as an internal aldimine with PLP was established by reduction of the Schiff base to a hydrolytically stable secondary amine (Kallen et al., 1985). The K258C-EA–PLP complex was treated with sodium cyanoborohydride by the procedure of Kondo et al. (1987). Tryptic digestion followed by HPLC analysis (see Experimental Procedures) yielded a single peptide absorbing at 320 nm (Figure 1A). A phosphopyridoxyl-labeled peptide with approximately the same retention time was detected from WT enzyme treated similarly. The chromophoric residue was identified as *S*-(2-(*N*-pyridoxylamino)ethyl)-L-cysteine by acid hydrolysis of the reduced K258C-EA enzyme and comparison with a synthetic standard by HPLC. K258C, treated identically, yielded no 320-nm absorbing material.

The question of the distribution of the cysteine residues susceptible to alkylation following the DTNB protection step was addressed by using [1-^{14}C]iodoacetate in place of BEA in the sequential protection–modification procedure. Shown in Figure 1B are the HPLC chromatograms of a tryptic digest of the [1-^{14}C]iodoacetate-treated enzyme. Quantitation of the radioactivity incorporated in the cysteine-containing peptides revealed that 45% of the label was contained in a peptide that had the same retention time as the phosphopyridoxyl-labeled active-site peptide shown in Figure 1A, while the remaining radioactivity was found in three other peptides with a distribution of 10, 22, and 23%. Thus, out of six cysteines, Cys 258 has been selectively modified up to an extent of about 50%.

Catalytic Properties of Aminoethylated K258C. (a) Specific Activity and Titration of Active Enzyme Fraction. The specific activity measured for the natural substrate pair L-Asp/α-KG is about 5 orders of magnitude greater than the

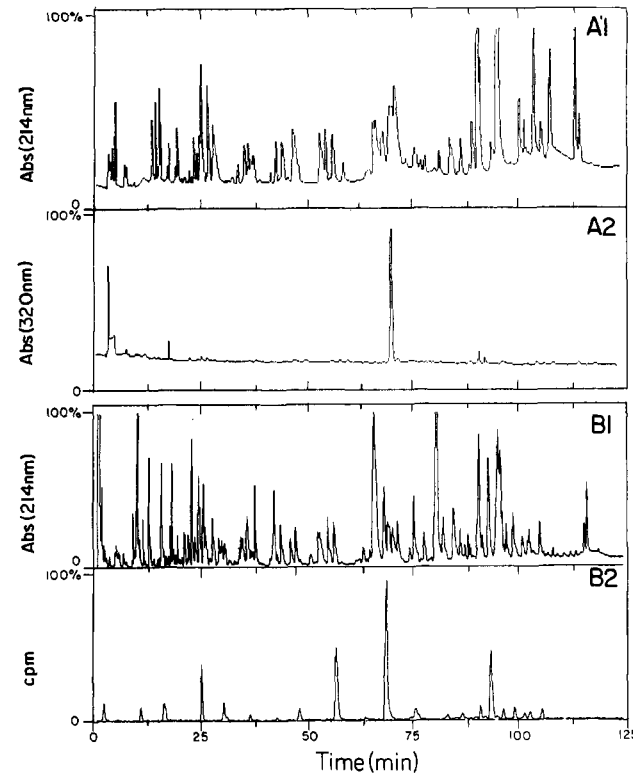


FIGURE 1: Reversed-phase HPLC of tryptic digests. Elution conditions are described in Experimental Procedures. (A) Sodium borohydride reduced aminoethylated K258C in the PLP form. Chromatogram A1: elution profile of peptides by monitoring at 214 nm. Chromatogram A2: phosphopyridoxyl-labeled peptide detected at 320 nm (active-site peptide). (B) ^{14}C-carboxymethylated K258C following the sequential protection–modification procedure (see text). Chromatogram B1: elution profile monitored at 214 nm. Chromatogram B2: radioactivity elution profile of ^{14}C-labeled peptides.

unmodified K258C mutant and is equivalent to 1.5–2% of the wild-type specific activity. On the basis of the 50% yield of BEA-modified enzyme at position 258 (see above), the lower limit of specific activity is 3–4% of WT. A more accurate estimate of functional enzyme concentration is gained by direct kinetic titration to correct for the properly aminoethylated, but otherwise damaged, protein resulting from the modification protocol. CS reacts with K258C about 5 orders of magnitude more slowly than with WT (Table I). An additional advantage is that the reaction proceeds to completion, because the primary transamination product, 3-sulfinylpyruvate, eliminates bisulfite to yield pyruvate, which is a poor substrate for the back-reaction. (Yagi et al., 1979; Griffith, 1987; Cronin & Kirsch, 1988). Upon the mixing of 25 mM CS with 90 μM K258C-EA, 25% of the total protein was converted to the PMP form within a burst period of $\leq$10 s, while the remaining 75% reacted much more slowly following an exponential time course equivalent to that exhibited by unmodified K258C. Thus, 25% of the total protein is functional.

The homogeneity of this active fraction was determined by monitoring the reaction between K258C-EA and the slowly reacting substrate, L-Ala. Since the underivatized enzyme, K258C, does not react with L-Ala (data not shown), the observed single-exponential time courses over 2–3 half-lives demonstrate that at least 75–90% of the assigned activity is kinetically homogeneous. No saturation with L-Ala was observed up to 150 mM (Table II). Therefore, 25% of the total aminoethylated protein is functional and kinetically homogeneous and is taken as the active enzyme fraction (with 6–8% of wild-type activity) in the subsequent calculations of k_{cat} from maximal velocities in steady-state experiments.

Table II: Kinetic Parameters of Aminoethylated K258C and Wild-Type Aspartate Aminotransferases[a]

	k_{cat} (s^{-1})		K_m (mM)		k_{cat}/K_m (M^{-1} s^{-1})		K258C-EA/wild type		
	K258C-EA	wild type	K258C-EA	wild type	K258C-EA	wild type	k_{cat}	K_m	k_{cat}/K_m
L-aspartate	9.86 ± 0.2	142 ± 2	3.47 ± 0.14	1.87 ± 0.07	(2.84 ± 0.07) × 10^3	(7.6 ± 0.2) × 10^4	0.069	1.8	0.037
L-cysteine sulfinate	13.6 ± 0.2	363 ± 8	12.4 ± 0.5	10.8 ± 0.6	(1.10 ± 0.03) × 10^3	(3.4 ± 0.1) × 10^4	0.037	1.2	0.033
L-alanine[b]					(1.40 ± 0.20) × 10^{-2}	(6.6 ± 0.1) × 10^{-1}			0.022
α-ketoglutarate	9.86 ± 0.2	142 ± 2	0.16 ± 0.01	0.52 ± 0.07	(6.14 ± 0.16) × 10^4	(2.7 ± 0.3) × 10^5	0.069	0.3	0.22
oxalacetate	12.5 ± 0.2	196 ± 4	(7 ± 0.6) × 10^{-3}	(21 ± 2) × 10^{-3}	(1.79 ± 0.13) × 10^6	(9.3 ± 0.5) × 10^6	0.065	0.3	0.22

[a] Steady-state conditions: pH 7.5, 25 °C in 200 mM HEPES buffer, 100 mM KCl. Substrate pairs and concentration ranges for K258C-EA are the same as those for wild type and are given in the legend of Table I. k_{cat} and K_m values were determined by nonlinear least-squares fitting of the data to the ping-pong mechanism (eq 1) where both substrates were varied, or to a rectangular hyperbola (eq 2) where one of the substrates was varied at constant saturating concentration of the other. [b] Measured under single-turnover conditions. Saturation was not apparent within the substrate range used (20–150 mM with K258C-EA and 5–30 mM with wild type), and the values of k_{cat}/K_m were determined by linear least-squares regression of the data.

Table III: pH Dependence of V/K_{Asp} for Aminoethylated K258C and Wild-Type Aspartate Aminotransferases[a]

	pK_{a1}	pK_{a2}	$(V/K_{Asp})_{lim}$ (M^{-1} s^{-1})
K258C-EA	5.97 ± 0.06	8.84 ± 0.06	(5.36 ± 0.1) × 10^3
wild type	7.30 ± 0.03	9.12 ± 0.03	(1.63 ± 0.04) × 10^5
K258C-EA/WT	ΔpK = 1.33	ΔpK = 0.28	0.033
$\Delta\Delta G_{app}$[b] (kcal mol^{-1})	1.8	0.4	2.0

[a] Conditions: 200 mM buffer and no added KCl (see text). The transaminase activity was measured by the MDH-coupled assay. The pH range studied was 5.5–9.5 for K258C-EA and 5.5–10.3 for WT (see Figure 2) in the buffers listed in the Experimental Procedures section. pK_{a1}, pK_{a2}, and $(V/K_{Asp})_{lim}$ were determined by nonlinear regression fitting of the data to eq 3. [b] $\Delta\Delta G_{app} = -RT$ ln (K258C-EA/WT).

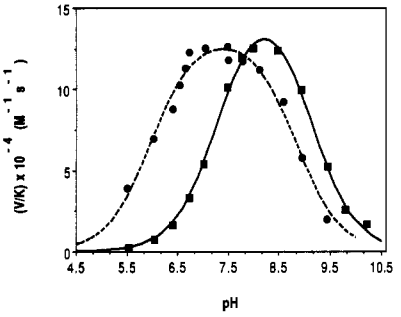


FIGURE 2: $V/K_{L\text{-aspartate}}$ vs pH profile for aminoethylated K258C (●) and wild-type (■) enzymes. Each data point was determined from the saturation curve for L-aspartate at constant α-ketoglutarate concentration (~$10K_m$) under steady-state conditions. Data for aminoethylated K258C have been multiplied by a factor of 25. Fitted curves (solid for wild type and dashed for aminoethylated K258C) were obtained as described in the legend of Table III.

(b) Steady-State Kinetics. The kinetic properties of aminoethylated K258C and wild-type enzymes are summarized in Table II. The chemically elaborated mutant has a k_{cat} (L-Asp/α-KG) equal to 7% of that of WT. Similar ratios of k_{cat} values were obtained for the other tested substrate pairs. The K_m values for amino acids are less than a factor of 2 higher for K258C-EA than for WT, while they are correspondingly 3-fold lower for keto acids. The values of k_{cat}/K_m are about 3% of the corresponding ones for WT, whereas those for keto acids are approximately 20%.

The pK_a values describing the pH dependence of V/K_m for L-Asp are given in Table III, and the experimental data are given in Figure 2. The measurements were carried out without controlling for ionic strength as opposed to the investigations of the other kinetic parameters reported in Tables I and II, where it was kept constant with added KCl. This salt was eliminated from the reaction mixtures in the pH studies in order to avoid the pH-dependent inhibition by chloride ion, which was found to give irregular results. A constant concentration of buffer was used throughout the pH range studied (see Experimental Procedures), and I_c ranged from 0.04 to 0.16. This variation had no significant effect on the steady-state kinetic parameters, as demonstrated by the varying of the buffer concentration at a fixed pH (data not shown).

Table IV: C_α Primary Hydrogen Kinetic Isotope Effect for the Reaction of L-Aspartate with Wild-Type and Aminoethylated K258C Aspartate Aminotransferases[a]

	wild type		K258C-EA	
pH	^DV	$^D(V/K)$	^DV	$^D(V/K)$
6.0	1.45 ± 0.01	1.88 ± 0.01	3.33 ± 0.05	3.15 ± 0.07
7.5	1.67 ± 0.02	1.88 ± 0.04	3.39 ± 0.05	2.90 ± 0.08
8.5	1.76 ± 0.05	2.00 ± 0.14	3.44 ± 0.07	3.13 ± 0.11

[a] Measured under steady-state conditions by the MDH-coupled assay in 200 mM buffer, 100 mM KCl at saturating α-ketoglutarate concentration (5 mM). The [α-^{1}H]- or [α-^{2}H]aspartate concentration varied from 0.5 mM to 15 mM of L-isomer. Buffers at each pH are those listed in Experimental Procedures.

Table V: Competitive Inhibition of Wild-Type and Aminoethylated K258C Aspartate Aminotransferases

parameter	pH	KCl (mM)	K258C-EA (mM)	wild type (mM)	K258C-EA/WT
		Pyridoxal Phosphate Enzyme Forms			
K_m(L-Asp)$_{lim}$[a]		100		1.7[d]	
		0	1.8	0.9	2
K_I(maleate)[b]	7.5	100	>75	8	
	7.5	0	40	1.8	22
	6.4	0	5.7		
K_I(maleate)$_{lim}$[c]		0	1.2	0.7	1.7
K_I(α-MeAsp)[b]	7.5	100	2.3	1.3	1.8
		Pyridoxamine Phosphate Enzyme Forms			
K_I(maleate)[b]	7.5	100	5.3	6.0	0.9

[a] Limiting values determined by dividing k_{cat} by $(k_{cat}/K_m)_{lim}$. [b] Inhibition constants determined under steady-state conditions by the MDH-coupled assay as described in Experimental Procedures. Data were fitted to eq 4 for maleate and to eq 5 for α-MeAsp. [c] Limiting value estimated by eq 8. [d] Data from W. L. Finlayson and J. F. Kirsch (unpublished results).

(c) Kinetic Isotope Effects. The C_α primary hydrogen kinetic isotope effects for L-Asp are given in Table IV. The effects are essentially pH independent for both enzymes and are significantly higher for the aminoethylated mutant. Throughout the pH range investigated, $^DV > {}^D(V/K)$ for K258C-EA, whereas $^D(V/K) > {}^DV$ for WT.

(d) Competitive Inhibition. Results from competitive kinetic inhibition studies with the substrate analogues maleate and α-MeAsp are given in Table V. Maleate competes with substrates for both PLP and PMP forms of the enzyme. The K_I values correspond to the inhibition constants against the overall reaction between L-Asp and α-KG as described by eq

4. Maleate forms a noncovalent complex with the PLP enzyme binding preferentially to the protonated form of the internal aldimine (Kiick & Cook, 1983). The pH-independent $(K_{II})_{lim}$ values were calculated according to the equation

$$K_{II} = \frac{(K_{II})_{lim}}{1 + 10^{(pH-pK_{a1})}} \quad (8)$$

where pK_{a1} is taken as the pK_a of the internal aldimine as determined from the pH dependence of V/K for L-Asp (Table III). The estimates of $(K_{II})_{lim}$ from eq 8 are based on the assumption that the pK_a affecting inhibitor binding is identical with that determining k_{cat}/K_m, as is the case for the pig heart isoenzyme (Kiick & Cook, 1983). These determinations were performed by omitting potassium chloride from the assay mixtures to avoid inhibition by chloride ion. The ratio of limiting K_{II} values for aminoethylated K258C to WT is similar to the corresponding ratio of $(K_m^{L\text{-}Asp})_{lim}$ values (1.7 vs 2, respectively).

α-MeAsp forms a dead-end external aldimine complex with the PLP enzymes. The inhibition constants were calculated by fitting the data to eq 5 as described in the Experimental Procedures section. Values for K_I are less dependent on pH than are those for maleate in the range of maximal activity (Kiick & Cook, 1983). The value of $K_I(\alpha\text{-MeAsp})$ for the mutant is 1.8 times higher than that for WT at pH 7.5, a figure similar to the $K_I(\text{maleate})_{lim}$ and $K_m(\text{L-Asp})_{lim}$ ratios.

Discussion

Catalytically Deficient K258C Mutant. The spectral and kinetic properties of K258C are similar to those of the structurally related K258A mutant of AATase (Malcolm & Kirsch, 1985; Toney & Kirsch, 1989, 1991). The extreme conditions required to resolve the PMP form indicate that the cofactor is bound considerably more tightly to K258C than to WT. It is unlikely that this effect is due to a collapse of the structure around the cofactor as a result of removing the side chain of Lys 258, because the X-ray structure of K258A (Smith et al., 1989) shows no such phenomenon and the K_m values for α-keto acids are similar for K258C and WT. The lower dissociation constants for amino acids compared to those of WT may reflect the absence of competition for the cofactor carbonyl group by the ϵ-NH_2 of Lys 258 Toney & Kirsch, 1991). The 10^6-fold decrease in transamination activity sustains the postulate of the essential role of Lys 258 in catalysis (Kirsch et al., 1984).

Inaccessibility of Cys 258 to Modifying Reagents. The reactivity of the thiol groups of the eukaryotic AATase isozymes has been characterized (Gehring & Christen, 1978; Birchmeier et al., 1973; Birchmeier & Christen, 1974; Kochkina & Torchinskii, 1975). The distribution of cysteines for the bacterial enzyme is, however, different except for Cys 191, which is conserved (Kondo et al., 1987). Calculations from the X-ray structure of WT *E. coli* AATase (D. Ringe, personal communication) show that all 5 cysteines per subunit are between 11 and 24 Å away from the active site (β-sulfur to ϵ-nitrogen of Lys 258). The introduced Cys 258 in the native conformation does not react with BEA. Possible explanations include sterically restricted access into the cavity left upon removal of the lysine side chain or an unusually high pK_a for the thiol. Treatment of the enzyme with a denaturant rendered Cys 258 accessible to sulfhydryl reagents, but derivatization of some of the more easily exposed cysteines prevented refolding. Selective alkylation of the target residue was accomplished by applying a sequential protection–modification procedure (Planas & Kirsch, 1990) that takes advantage of the local protective effect exerted by the cofactor when the non-active-site cysteines are reagent exposed by a mild denaturant.

Recovery of Enzymatic Activity. Aminoethylation of Cys 258 introduces an unnatural side chain to play the role of general base in the transamination mechanism with the concomitant 10^5-fold increase in activity relative to that of K258C. Similar strategies for redesigning active-site groups have been reported. The rescue of catalytically deficient mutants of ribulosebisphosphate carboxylase/oxygenase by aminoethylation of engineered cysteines (Smith & Hartman, 1988; Smith et al., 1988), the 2500-fold activation of an inactive cysteine mutant of *Pseudomonas aeruginosa* exotoxin A after carboxymethylation (Lukac & Collier, 1988), and partial recovery of the activity of a naturally occurring cysteine-free thermostable isozyme of aspartate aminotransferase by aminoethylation of an introduced active-site cysteine (Yoshimura et al., 1990) are examples. Catalytic activity can also be partially restored by exogenous molecules as reported for the catalytically deficient AATase mutant K258A upon addition of proton-transfer catalysts in solution (Toney & Kirsch, 1989). Although the latter methodology provides a powerful tool for structure–reactivity correlation analysis by varying the catalyst, an entropic advantage is gained when the catalyst is covalently incorporated into the protein.

Catalytic Properties. Although, as discussed above, deletion of the functional side chain supports the hypothesis that Lys 258 is catalytically essential, the recovery of substantial activity (10^5-fold) by replacement with the close structural analogue, *S*-(2-aminoethyl)cysteine (γ-thialysine), provides an even stronger basis for the assignment. The subtle nature of the substitution [-S- for -CH_2-, bond length 1.54 Å (C–C) vs 1.82 Å (C–S); bond angles 109° (C–C–C) vs 105° (C–S–C) (Weast, 1975)] would indicate only a minor mispositioning of the critical ϵ-NH_2 group. The fact that the k_{cat} value of K258C-EA is only ~7% of that of WT, taken together with the observed larger values of the C_α kinetic isotope effects, is interpreted in terms of the C_α proton abstraction step being more nearly fully rate determining in K258C-EA. Although the structural characteristics differentiating the side chains of lysine from γ-thialysine are minor, there is a significant difference in the basicity of the ϵ-NH_2 groups. The pK_a of lysine is about 1 pH unit higher than that of γ-thialysine (Hermann & Lemke, 1968). The ascending limb of the V/K vs pH profile (Figure 2) reflects the pK_a of the internal aldimine (Kirsch et al., 1984). The 1.3 pH unit decrease in the pK_a of this limb most likely results from the similar lessened basicity of the ϵ-amino group of K258C-EA. A decreased basicity in the residue responsible for C_α proton abstraction also accommodates the observations described above that led to the conclusion that this step is more fully rate determining in K258C-EA.

The similarity of K_m's (within 2–3-fold) for amino and keto acid substrates (and K_I's for inhibitors) to those for WT shows that the affinity of active-site ligands has not been substantially altered by the mutation. Other mutants prepared in this laboratory and in Dr. Kagamiyama's have resulted in >50-fold changes in K_m values (Goldberg et al., 1991; Cronin & Kirsch, 1988; Inoue et al., 1989; Hayashi et al., 1989). The difference in k_{cat}/K_m values between amino and keto acids, 3% and 20% of WT, respectively, means that the equilibrium for the interconversion amino acid + E–PLP ↔ keto acid + E–PMP [$K_{eq} = (V/K_{\text{amino acid}})/(V/K_{\text{keto acid}})$] is shifted to the PLP side relative to WT.

Kinetic Isotope Effects. In addition to larger C_α-KIE values, the mutation yields an enzyme for which $^DV > {}^D(V/K)$,

whereas the reverse inequality is obtained for WT. In the minimal mechanism for the AATase reaction given by

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{-}S \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\text{-}P \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E + P$$

the only isotope-sensitive step, designated by k_3 is largely irreversible due to solvent washout of the isotope after C_α deuterium abstraction (Julin et al., 1989; Gehring, 1984). The rate constant k_5 is complex and includes ketimine hydrolysis, product release, and the second half-reaction of the ping-pong mechanism. This step is made irreversible by coupling OAA production to MDH. The kinetic isotope effect expressions are (Northrop, 1975)

$$^{D}(V/K) = \frac{(k_{3H}/k_{3D}) + (k_{3H}/k_2)}{(k_{3H}/k_2) + 1}$$

$$^{D}V = \frac{(k_{3H}/k_{3D}) + (k_{3H}/k_5)}{(k_{3H}/k_5) + 1} \quad (9)$$

By subtracting 1 from each equation, it can be shown that where $^{D}(V/K) > {}^{D}V$ as observed for WT enzyme, $k_2 > k_5$, whereas the converse, $k_5 > k_2$, must be true for K258C-EA, which has $^{D}V > {}^{D}(V/K)$. Therefore, the forward and reverse commitment factors are inverted by the mutation.

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Interaction of Lipid Vesicles with Monomolecular Layers Containing Lung Surfactant Proteins SP-B or SP-C†

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ABSTRACT: Pulmonary surfactant contains two families of hydrophobic proteins, SP-B and SP-C. Both proteins are thought to promote the formation of the phospholipid monolayer at the air–fluid interface of the lung. The Wilhelmy plate method was used to study the involvement of SP-B and SP-C in the formation of phospholipid monolayers. The proteins were either present in the phospholipid vesicles which were injected into the subphase or included in a preformed phospholipid monolayer. In agreement with earlier investigators, we found that SP-B and SP-C, present in phospholipid vesicles, were able to induce the formation of a monolayer, as became apparent by an increase in surface pressure. However, when the proteins were present in a preformed phospholipid monolayer (20 mN/m) at similar lipid to protein ratios, the rate of surface pressure increase after injection of pure phospholipid vesicles into the subphase at similar vesicle concentrations was 10 times higher. The process of phospholipid insertion from phospholipid vesicles into the protein-containing monolayers was dependent on (1) the presence of (divalent) cations, (2) the phospholipid concentration in the subphase, (3) the size of the phospholipid vesicles, (4) the protein concentration in the preformed monolayer, and (5) the initial surface pressure at which the monolayers were formed. Both in vesicles and in preformed monolayers, SP-C was less active than SP-B in promoting the formation of a phospholipid monolayer. The use of preformed monolayers containing controlled protein concentrations may allow more detailed studies on the mechanism by which the proteins enhance phospholipid monolayer formation from vesicles.

Pulmonary surfactant is a complex lipid–protein mixture that lowers surface tension at the air–water interface in the lung. Lipids comprise the majority (approximately 90%) of this surface-active material, and their composition has been studied in detail (Van Golde et al., 1988). The most abundant phospholipid components are dipalmitoylphosphatidylcholine (DPPC),[1] unsaturated phosphatidylcholine (PC) species, and phosphatidylglycerol (PG). There is general agreement that DPPC is the principal surface-active component of pulmonary surfactant. It is this compound that is responsible for decreasing the surface tension at the alveolar surfaces to low values at end expiration (Clements, 1977).

At least three families of proteins, SP-A, SP-B, and SP-C, are thought to be unique constituents of pulmonary surfactant (Possmayer, 1988). SP-A is a glycoprotein of M_r 26 000–38 000 under reducing conditions (Possmayer, 1988; Hawgood, 1989). SP-A has been shown to play a role in the formation of tubular myelin in the presence of calcium ions (Benson et al., 1984; Suzuki et al., 1989) and the hydrophobic protein SP-B (Suzuki et al., 1989). SP-A may also be important in the regulation of surfactant homeostasis (Wright et al., 1987; Rice et al., 1987; Dobbs et al., 1987; Kuroki et al., 1988) and in alveolar defense (Tenner et al., 1989; Van Iwaarden et al., 1990). SP-B and SP-C are very hydropobic proteins that copurify with the lipids during extraction of surfactant with organic solvents. SP-B has a molecular weight of 18 000 under nonreducing conditions and a molecular weight of 5000–8000 under reducing conditions (Possmayer, 1988; Hawgood, 1989). It has been shown that the presence of SP-B in phospholipid vesicles enhances the adsorption of phospholipids to an air–water interface (Hawgood et al., 1987). SP-C has a molecular weight of 5000–8000 under both reducing and nonreducing conditions (Possmayer, 1988; Hawgood, 1989) and has two palmitoyl groups covalently linked to the polypeptide chain

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[1] Abbreviations: SP-A, SP-B, and SP-C, surfactant proteins A, B, and C, respectively; PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PG, phosphatidylglycerol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid; MLV, multilamellar vesicle(s); SUV, small unilamellar vesicle(s); LUV, large unilamellar vesicle(s).

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