Noncoded Amino Acid Replacement Probes of the Aspartate Aminotransferase Mechanism†

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ABSTRACT: The primary role of Tyr225 in the aspartate aminotransferase mechanism is to provide a hydrogen bond to stabilize the 3′O$^-$ functionality of bound pyridoxal phosphate. The strength of this hydrogen bond is perturbed by replacement of Tyr225 with 3-fluoro-L-tyrosine (FlTyr) by *in vitro* transcription/translation. This mutant enzyme exhibits k_{cat}/K_M^{Asp} values that are near to those of wild type enzyme; however, the k_{cat}/K_M^{Asp} *vs* pH profile is much sharper with similar pK_as of ∼7.5 for both the ascending and descending limbs. The pK_as are assigned to the endocyclic proton of the internal aldimine and to the bridging hydrogen bond, respectively. The pK_as in the k_{cat} *vs* pH profile of 7.2 and 8.7 are assigned to the ϵ-NH_3^+ of lysine 258 and to the endocyclic protons of the ketimine complex, respectively. Arginine 292 forms a salt bridge with the β-COOH of the substrate, aspartate. An improvement on the earlier attempt to invert the substrate charge specificity via R292D mutation-induced arginine transaminase activity [Cronin, C. N., & Kirsch, J. F. (1988) *Biochemistry 27*, 4572−4579] is described. Here Arg292 is replaced with homoglutamate (R292hoGlu). This construct exhibits 6.8×10^4-fold greater activity for the cationic substrate D,L-[C_α-^{3}H]-α-amino-β-guanidinopropionic acid (D,L-[C_α-^{3}H]AGPA) than does wild type enzyme. The gain in selectivity for this substrate is at least 4500-fold greater than that achieved in the 1988 experiment, *i.e.*, $\{(k_{cat}/K_M)^{R292hoGlu}/(k_{cat}/K_M)^{WT}$ (D,L-[C_α-^{3}H]AGPA)$\} \geq 4500 \times \{(k_{cat}/K_M)^{R292D}/(k_{cat}/K_M)^{WT}$ (L-arginine)$\}$. The value of $(k_{cat}/K_M)^{R292D}$ is 0.43 M^{-1} s^{-1} with L-Arg while $(k_{cat}/K_M)^{R292hoGlu}$ is 29 M^{-1} s^{-1} with D,L-[C_α-^{3}H]AGPA (it is assumed that the D-enantiomer is unreactive). The latter value is the lower limit because of the uncertain value of ^{3}H kinetic isotope effect.

Aspartate aminotransferase (AATase,[1] EC 2.6.1.1) catalyzes the interconversion of the dicarboxylic amino and keto acids [eq 1; see Goldberg and Kirsch (1996), Kirsch *et al.* (1984), and Jansonius and Vincent (1987)].

L-amino acid **1** + AATase-PLP ⇌ α-ketoacid **1** + AATase-PMP

α-ketoacid **2** + AATase-PMP ⇌ L-amino acid **2** + AATase-PLP (1)

The proton bridging 3′O$^-$ of the cofactor and Tyr225 is essential to the maintenance of the catalytically active conformation of AATase [Chart 1, form (2)]. Removal of the hydrogen bond via the Y225F mutation results in a 450-fold decrease in k_{cat} and a 30−50-fold decrease in K_M (Goldberg *et al.*, 1991; Goldberg & Kirsch, 1996; Inoue *et al.*, 1991). No natural amino acid substitution exists that would maintain this hydrogen bond, although the Y225R mutant provides electrostatic stabilization for the negatively charged 3′O$^-$ (Inoue *et al.*, 1991; Graber *et al.*, 1995). The distance between the 3′O$^-$ and the guanidino nitrogen is 3.67 Å (NH_2) or 3.16 Å (NH) in the Y225R mutant. In the present study 3-fluoro-L-tyrosine (FlTyr) is substituted for Tyr225, decreasing the pK_a of the phenolic OH by *ca.* 1.0 unit. This substitution should result in a stronger hydrogen bond in the bridging position because the pK_as of the cofactor and the substituted tyrosine are more nearly matched. The kinetic consequence is a predicted lowering of the pK_a governing the rising limb of the k_{cat}/K_M *vs* pH profile (equilibria 1 ⇌ 2 *vs* 5 ⇌ 6, Chart 1).

The specificity for the anionic amino acid substrate is dictated by a salt bridging interaction between the guanidino

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[1] Abbreviations: AATase, aspartate aminotransferase; WT, the wild type AATase from *Escherichia coli*; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; Y225F, mutant AATase with Tyr225 replaced by phenylalanine; FlTyr, 3-fluoro-L-tyrosine; Y225FlTyr, mutant AATase with Tyr225 replaced by FlTyr; hoGlu, L-homoglutamate; R292D and R292hoGlu, mutant AATases in which arginine 292 has been replaced by aspartic acid or hoGlu, respectively; TATase, tyrosine aminotransferase; L-AGPA, L-α-amino-β-guanidinopropionic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; α-KG, α-ketoglutarate; MDH, *E. coli.* malate dehydrogenase; OAA, oxaloacetate; DEAE-Sepharose, diethylaminoethyl Sepharose; Tris, tris-[hydroxymethyl]aminomethane; TAPS, 3-[[tris(hydroxymethyl)methyl]-amino]propanesulfonic acid; TCA, trichloroacetic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid.

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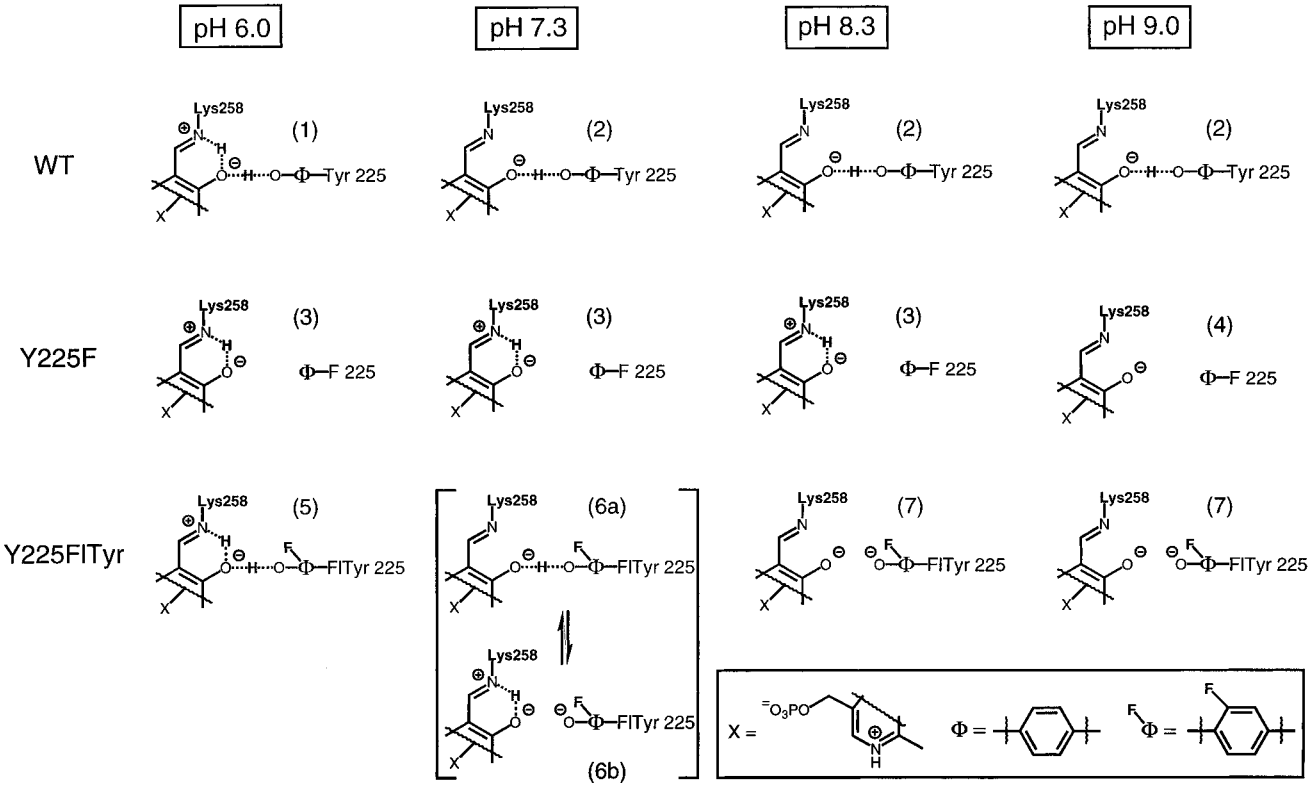
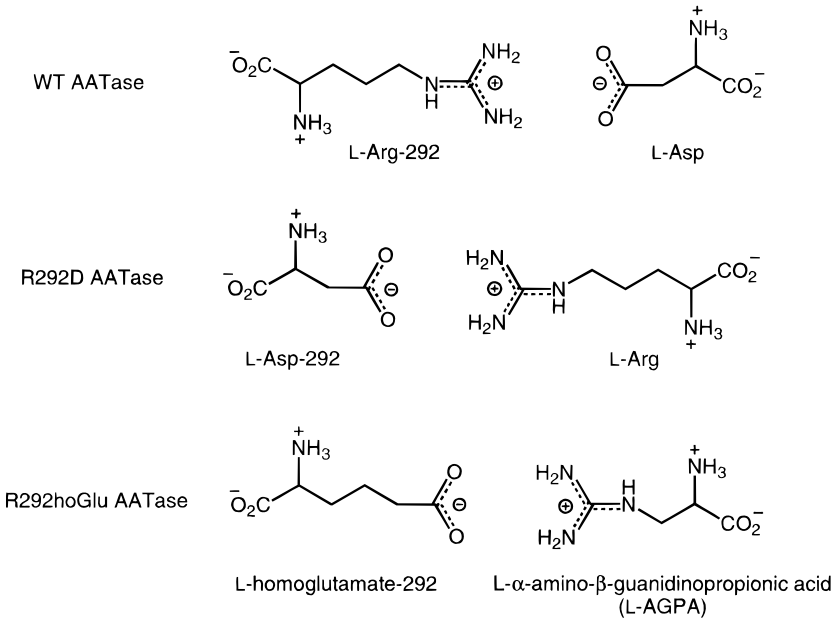
Chart 1: pH Dependence of the Protonation States at the Active Site of WT and Mutant Aspartate Aminotransferases[a]



[a] The active prototropic forms of the enzyme (2, 4, 6a) lack the proton bridging the 3′O$^-$ of the cofactor and the imino nitrogen atom. The pK_as governing these prototropic equilibria are 6.9, WT (Gloss & Kirsch, 1995); 8.6, Y225F (Goldberg *et al.*, 1991); and 7.5, Y225FlTyr (this work). Loss of the hydrogen bond binding Tyr225 or FlTyr225 with the 3′O$^-$ also leads to a catalytically incompetent form of the enzyme. The pK_as are >10 for WT (see text) and 7.5 for Y225FlTyr.

Chart 2: Diagram of the Salt Bridge between Arg292 and the Substrate L-Asp Existing in WT AATase, Together with the Hypothesized Interactions with the R292hoGlu/L-AGPA and R292D/Arg Mutant Enzyme/Alternate Substrate Pairs



moiety of Arg292 and the ω-carboxyl group of the substrate (Chart 2, Ford *et al.*, 1980). Early attempts to change the specificity of the enzyme to that of an arginine transaminase by the mutation R292D were only partly successful (Cronin & Kirsch, 1988). Hwang and Warshel (1988) have criticized protein charge reversal experiments on the basis of calculations that show that, e.g., where (− +) ion pairs are stabilized by the local dipolar protein environment, the opposite (+ −) pair is repelled from that region. They find in particular, that the local dielectric constant (ϵ) for the native (− +) pair of the WT enzyme-L-Asp complex is *ca.* 13, while the opposite R292D-L-Arg pair would exist in an environment with $\epsilon \approx 80$. To address this criticism, L-hoGlu with a negatively charged side chain is introduced at position 292 by *in vitro* transcription−translation with the objective of producing a transaminase specific for L-α-amino-β-

guanidinopropionic acid (L-AGPA), where the total number of heavy atoms separating the C_α carbons of residue 292 and the substrate is conserved, but the bridging ion pair is shifted by *ca.* 3 Å to be closer to that existing in the complex of L-aspartate with wild type enzyme (Chart 2).

EXPERIMENTAL PROCEDURES

Materials

3-Fluoro-L-tyrosine, obtained from Fluka, was N-protected as the nitroveratryloxycarbonyl derivative and converted to the corresponding cyanomethyl ester for attachment to pdCpA (Robertson *et al.*, 1991). The pdCpA dinucleotide aminoacylated with L-hoGlu was available from an earlier study (Judice *et al.*, 1993). L-AGPA, α-ketoglutarate, L-aspartic acid, pyridoxal, and PLP were purchased from Sigma. L-[U-^{14}C]aspartic acid was from Amersham. MDH, purified from an AATase-deficient *Escherichia coli* strain MG204, was available from earlier studies (Onuffer & Kirsch, 1994). All restriction enzymes were from New England Biolabs. T4 DNA ligase was from Promega. Reagents and enzymes for DNA mutagenesis and sequencing were obtained from Amersham and United States Biochemical, respectively. Pyruvate kinase and inorganic pyrophosphatase were from Sigma, RNasin was from Promega. Oligonucleotides were synthesized on an Applied Biosystems PCR mate DNA synthesizer. DEAE cellulose plates were purchased from J. T. Baker. The Centriprep apparatus was an Amicon product.

The S-30 extract for *in vitro* synthesis of AATase was made from *E. coli*. MG204. The preparations of suppressor $tRNA^{Phe}_{CUA(-CA)}$, T4 RNA ligase, T4 polynucleotide kinase, and T7 RNA polymerase are described elsewhere (Ellman *et al.*, 1991; Mendel *et al.*, 1995, and references therein). The plasmid pJH for *in vitro* expression of AATase was constructed by H. H. Chung and J. Luo (unpublished results). *In vitro* expression of the *E. coli* AATase gene is under the transcriptional control of a T7 RNA polymerase promoter. The oligonucleotide (5′ GAC TTC GCT TAG CAG GGT TTT 3′) was the primer used to introduce an amber mutation in a bacteriophage vector M13mp19 containing the AspC gene (Onuffer & Kirsch, 1995). The oligonucleotide sequence (5′ AAA ACC CTG CTA AGC GAA GTC 3′) was used for amber mutation at position 225 in the vector M13mp18 containing the AspC gene. Mutagenesis was performed by the Eckstein method (Sayers *et al.*, 1988). The desired mutation(s) was confirmed by dideoxynucleotide sequencing (Sanger *et al.*, 1977). The lack of second site mutations was verified by sequencing the entire gene for the Y225 amber mutation. The AspC gene containing the amber mutations was retrieved from M13mp19 or M13mp18 replicative forms of DNA by PCR, and inserted into the expression vector prepared from pJH using *Nde*I and *Xho*I restriction sites.

D,L-[α-^{3}H]AGPA was synthesized according to Posner and Flavin (1972). The modified procedure is described briefly as follows: L-AGPA hydrochloride (0.12 mmol), pyridoxal (0.012 mmol), and $Al_2(SO_4)_3$ (0.005 mmol) were dissolved in water (H_2O, 125 μL), and the pH of the solution was adjusted to 10.3 with potassium hydroxide. Tritiated water (46 μL, 10% T_2O with 90% H_2O, v/v), generated from the reaction of platinum oxide (195 mg) with a mixture of tritium gas (T_2) in hydrogen gas (H_2) (T_2/H_2 = 10/90, v/v) at room temperature and atmospheric pressure, was added to the amino acid solution. After being stirred at room temperature for 4 h, the reaction was quenched by addition of hydrochloric acid to pH 4.5. D,L-[α-^{3}H]AGPA was purified using a Dowex 50W4-200 cation exchange column, and the product was confirmed by ^{3}H NMR. The specific activity was 28.8 Ci/mmol.

Methods

In Vitro Syntheses of Y225FlTyr and R292hoGlu AATases. Incorporation of FlTyr or hoGlu into AATase was accomplished by *in vitro* suppression of the Tyr 225→TAG or the R292→TAG amber mutation (Sayers *et al.*, 1988) using the respective chemically aminoacylated suppressor tRNA (Noren *et al.*, 1989). The details of this biosynthetic method for introducing noncoded amino acids site-specifically into proteins are described elsewhere (Ellman *et al.*, 1991; Mendel *et al.*, 1995). Suppression efficiency for the mutant proteins varied from 10% to 60%, and the protein yields ranged from 5 to 10 μg/mL of reaction. Typical volumes were 5−10 mL, yielding about 50 μg of total enzyme. AATases synthesized *in vitro* have the same chromatographic properties as the enzyme purified *in vivo* and react with anti-AATase polyclonal antibodies.

Partial Purification of Y225FlTyr AATase. The Y225FlTyr mutant was partially purified according to the literature procedure for WT AATase (Gloss *et al.*, 1992) with minor variations: 10 mM NaOAc (pH 5.2) was added to the *in vitro* protein synthesis mixture, and acetic acid was added to pH 5.0 with stirring on ice for 30 min. After removal of the precipitate by centrifugation, the supernatant was dialyzed with a buffer containing 20 mM NaOAc (pH 4.9), 0.25 mM DTT, and 20 μM PLP (buffer A), and loaded onto an equilibrated DEAE-Sepharose anion exchange column. The protein was eluted with a 2 × 200 mL linear gradient of 0−200 mM NaCl in buffer A. The elution fractions were analyzed by 15% SDS−PAGE, and fractions containing AATase were collected, concentrated using a Centriprep (Amicon), and dialyzed with a buffer containing 20 mM KH_2PO_4 (pH 7.2), 20 μM PLP, 1 mM DTT, and 0.5 mM EDTA (buffer B). This sample was loaded onto an Affi-Gel Blue 2 dye column (Sigma) equilibrated with buffer B. Y225FlTyr AATase was eluted with a linear gradient of 0−0.8 M KCl in buffer B. R292hoGlu AATase was used without purification. The enzyme was quantitated by Western blotting with anti-AATase antibodies and ^{35}S-labeled protein A.

Steady-State Kinetics for Y225FlTyr. Steady-state kinetics for the reactions with L-[U-^{14}C]Asp and α-KG were determined by a discontinuous MDH-coupled assay, in which the [U-^{14}C]OAA formed from L-[U-^{14}C]Asp was converted to radioactive malate for high-sensitivity assay. Steady-state kinetic measurements were performed as described in the legend to Figure 1. 12 μL aliquots were taken over a 3-h period and quenched with 1 μL of 100% trichloroacetic acid, and the supernatant was loaded onto a DEAE cellulose flexible plate. Malate was separated from L-Asp by eluting the DEAE plate with *n*-butanol/formic acid/H_2O (77/20/3). After autoradiography, the [U-^{14}C]malate was excised and quantitated by liquid scintillation counting. The rates of reactions were computed by linear regression of dpm *vs* reaction time.

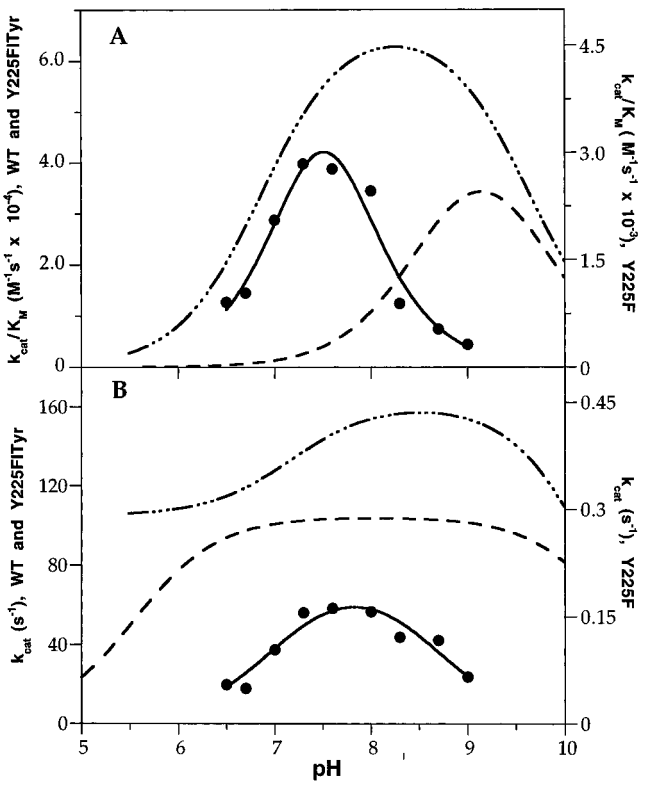


FIGURE 1: pH dependencies of k_{cat}/K_M^{Asp} (A) and k_{cat} (B) for Y225FlTyr (●), Y225F, and WT AATases. Data for the Y225FlTyr mutant were taken at constant α-KG concentrations (100 mM, ~200K_M^{WT}) under steady state conditions using a discontinuous MDH-coupled assay in 200 mM buffer at 25 °C, containing 0.2 M KCl. The buffers were Bis-Tris, pH 6.5−6.9; HEPES, pH 7.0−7.9; TAPS, pH 8.0−8.9, and CHES, pH 9.0. [L-Asp] = 0.5−50 mM containing 2.5 μCi of L-[U-^{14}C]Asp per 1 mL of reaction. [MDH] = 10 units, [NADH] = 10 mM, [PLP] = 20 μM, [AATase] = 1.0−6.0 nM, depending on the activity of the enzyme. (A) The fitted curves (— for Y225FlTyr, - - - Y225F, and — ··· — WT) were obtained by nonlinear regression on eq 2. The Y225FlTyr (—) and Y225F (- - -) k_{cat} data in B were also fitted to eq 2, while the WT curve is from eq 3. The data and curves for WT and Y225F are from Gloss and Kirsch (1995).

Single-Turnover Kinetics. The kinetics for the reaction of WT AATase with L-AGPA were determined under single-turnover conditions by monitoring the spectrum of the enzyme-bound cofactor. The reactions were conducted at 25 °C in 100 mM TAPS-KOH buffer, pH 8.5, I_c = 1.0 (KCl), [WT AATase] = 20 μM, and [L-AGPA] = 50−600 mM. Reactions were initiated by the addition of enzyme. The absorbance increases at 330 nm, due to the formation of the enzyme-bound PMP, or the decreases at 360 nm, due to the depletion of enzyme-bound PLP form, were monitored in a Perkin-Elmer Lambda 4B spectrophotometer. The observed reaction rates were obtained by fitting the data to single-exponential time courses by nonlinear regression.

Kinetic Activity of R292hoGlu AATase under Steady-State Conditions. AATase-mediated release of tritium from L-[2,3-^{3}H]Asp or D,L-[α-^{3}H]AGPA was measured in 100 mM HEPES buffer, pH 7.6, [PMP] = 10 μM, 25 °C. The conditions for the reaction of R292hoGlu with D,L-[C_α-^{3}H]-AGPA were [R292hoGlu] = 5.6−11.2 nM, [α-KG] = 100−300 mM, and [L-AGPA] = 50−200 mM (duplicate determinations at 50, 100, and 200 mM substrate) with 150 μCi of D,L-[α-^{3}H]AGPA/1 mL of reaction. 100 μL aliquots of the assay mixture were taken at time intervals, and the reaction was quenched with 10 μL of 100% trichloroacetic acid. After removal of the precipitate by centrifugation, the supernatant was loaded onto a small Dowex 50W4−200 cation exchange column (2 mL resin volume). The tritiated water from the reaction was eluted with water (3 mL), and the radioactivity of the eluent determined. A basal level of radioactivity, due to the washoff of the tritiated material in the absence of either the control mixture from the *in vitro* transcription−translation system lacking the AATase gene or the enzyme, was subtracted from each data point.

Data Analysis

Evaluation of the pH Dependence of the Kinetic Parameters. Data for the pH dependence of k_{cat}/K_M^{Asp} were fitted to the bell-shaped curve described by

$$Y = \frac{Y_{lim}}{1 + 10^{(pK_{a1} - pH)} + 10^{(pH - pK_{a2})}} \quad (2)$$

The Y225F and Y225FlTyr k_{cat} *vs* pH values are also described by eq 2. The k_{cat} *vs* pH profile for WT AATase was fitted to a modified bell-shaped curve described by eq 3 (Gloss & Kirsch, 1995)

$$k_{cat} = \frac{(k_2)_{lim} + (k_1)_{lim}(10^{(pK_{a1} - pH)})}{1 + 10^{(pK_{a1} - pH)} + 10^{(pH - pK_{a2})}} \quad (3)$$

which yields limiting values at low (k_1) and neutral (k_2) pH.

RESULTS

pH Profiles of k_{cat}/K_M^{Asp} for Y225FlTyr. Figure 1A shows the pH dependence of k_{cat}/K_M^{Asp} for WT, Y225F, and Y225FlTyr AATases. The data were fitted to the bell-shaped curves described by eq 2, and the calculated pK_as are collected in Table 1. The pK_{a1} value describing the pH dependence of WT has been assigned to the dissociation of the protonated internal aldimine formed between PLP and lysine 258 [Chart 1, form (1)] (Eichele *et al.*, 1978; Gloss & Kirsch, 1995). The pK_{a1} value for the reaction of Y225FlTyr with L-Asp is 7.5 ± 0.3, while the corresponding values for WT and Y225F are 6.87 and 8.59, respectively (Goldberg *et al.*, 1991; Gloss & Kirsch, 1995). The latter authors assigned the alkaline pK_{a2} = 9.6 seen in the WT and Y225F profiles to the amino acid substrate; however, the much lower figure for pK_{a2} = 7.5 ± 0.3 observed for Y225FlTyr is indicative of a second enzyme-associated prototropy unique to this mutant.[2]

pH Profiles of k_{cat} for Y225FlTyr AATase. The pH dependencies of k_{cat} for Y225FlTyr, WT, and Y225F AATases with the L-Asp/α-KG substrate pair are shown in Figure 1B. The data for Y225FlTyr and Y225F were fitted to eq 2, while the WT data were fitted to eq 3 (Table 1). An acidic pK_{a1} of 6.99 ± 0.12 was observed for Y225FlTyr,

[2] Nonlinear least-square analysis of the data shown in Figure 1A yields approximately identical pK_as of 7.5 ± 0.3 for both the ascending and descending limbs of the k_{cat}/K_M^{Asp} *versus* pH profile for the Y225FlTyr mutant. However, statistical effects normally separate the first and second pK_a values of dibasic acids by 0.6 pK_a units except where different conformations of the enzyme are separated by a significant conformational barrier (Tipton & Dixon, 1979). The values of ±0.3 in each of the standard errors are too large to permit a resolution of the two possibilities in the present case.

Table 1: pH Dependence of k_{cat} and k_{cat}/K_M^{Asp} for Y225FlTyr, Y225F, and WT Aspartate Aminotransferases[a]

	k_{cat}/K_M^{Asp}		
	pK_{a1}	pK_{a2}	$(k_{cat}/K_M^{Asp})_{Lim}$ (M^{-1} s^{-1})
Y225FlTyr[b]	7.5 ± 0.3	7.5 ± 0.3	$(1.2 \pm 0.6) \times 10^5$
Y225F[c]	8.59	9.69	3.83×10^3
WT[c]	6.87	9.64	6.78×10^4

	k_{cat}		
	pK_{a1}	pK_{a2}	k_{cat} (s^{-1})
Y225FlTyr[b]	6.99 ± 0.12	8.66 ± 0.14	76 ± 8
Y225F[c]	5.54	10.55	0.29
WT[c]	7.18	10.31	162

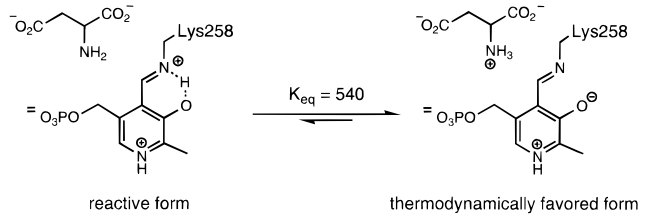
[a] The pH range covered was 5.5−9.0 for Y225FlTyr. The WT and Y225F data are from Gloss and Kirsch (1995) (see Figure 1). [b] Data for Y225FlTyr were taken under the steady state conditions described in Figure 1 legend and in the Experimental section. The pK_a values from k_{cat} *vs* pH profiles were obtained by fitting the Y225FlTyr data to eq 2. Y225F data were also fitted to eq 2, and WT data were fitted to eq 3 by Gloss and Kirsch (1995). [c] From Gloss and Kirsch, (1995).

while the values for WT and Y225F are 7.18 and 5.54, respectively (Gloss & Kirsch, 1995). The alkaline pK_{a2} of 8.66 determined for Y225FlTyr is significantly lower than the figures of 10.31 and 10.55 recorded for WT and Y225F, respectively. The low pH plateau activity seen in the WT enzyme is absent in the Tyr225 replacement constructs.

Reaction of WT AATase with L*-α-Amino-β-guanidinopropionic Acid (*L*-AGPA).* The observed k_{cat}/K_M^{AGPA} for the reaction of WT with L-AGPA under single-turnover conditions is given in Table 2. No tendency toward saturation was observed at concentrations of up to 600 mM L-AGPA. The k_{cat}/K_M value for the reaction of WT AATase with L-AGPA is ~10^8-fold less than that observed for L-Asp, *i.e.*, <0.001 M^{-1} s^{-1} (Table 2). The rate for the reaction of WT with L-Arg was also examined under the same conditions, and the k_{cat}/K_M^{Arg} observed agrees with the literature value (Table 2; Cronin & Kirsch, 1988).

Reactions of R292hoGlu AATase with L*-Asp and* D,L*-[α-^{3}H]AGPA.* Time courses for the R292hoGlu-mediated release of tritium from L-[2,3-^{3}H]Asp (A) or from D,L-[α-^{3}H]AGPA (B) are shown in Figure 2. The total L-[AGPA] concentration (see Experimental Procedures) was varied from 50 to 200 mM, and [α-KG] was varied from 100 to 300 mM for the kinetic assay of R292hoGlu. There was no observed transamination activity in the reaction of R292hoGlu with L-Asp (Figure 2A), while the corresponding reaction with D,L-[α-^{3}H]AGPA was significantly greater than background (Figure 2B). Subtraction of the controls from the enzyme-catalyzed reaction yields the plots of Figure 2C. The calculated velocity for the reaction with L-[2,3-^{3}H]Asp is $(-8.9 \pm 1.4) \times 10^{-9}$ M s^{-1} while that for D,L-[α-^{3}H]AGPA is $(2.3 \pm 0.2) \times 10^{-8}$ M s^{-1}. These figures yield a $k_{cat}/(K_M^{Asp} + 0.2\ M)$ value of (-7.9 ± 1.2) M^{-1} s^{-1} and a k_{cat}/K_M^{AGPA} value of 29 ± 6 M^{-1} s^{-1} for the substrates L-Asp and D,L-[α-^{3}H]AGPA, respectively. The kinetic properties for the reaction of WT and R292hoGlu AATases with L-AGPA or D,L-[α-^{3}H]AGPA are summarized in Table 2. A k_{cat} of 2.5 s^{-1} and K_M^{AGPA} of 86 mM were determined for the reaction of R292hoGlu with the D,L-[C_α-^{3}H]AGPA/α-KG substrate pair on the basis of the L-enantiomer concentration. These are minimum values, as no allowance was made for tritium kinetic isotope effects (see Discussion). The parameters for the reactions of WT and the R292D mutant with other amino acids are included for comparison.

Scheme 1: Equilibrium between the Protonation States of the Internal Aldimine and the Substrate Aspartic Acid



DISCUSSION

Role of Tyr225 in Catalysis

(a) Interpretation of the k_{cat}/K_M vs pH Profile. The k_{cat}/K_M *vs* pH profile for the reaction of WT AATase with L-Asp exhibits a bell-shaped curve with acidic and basic inflection points of 6.9 and 9.6, respectively (Figure 1A). The former figure reflects the ionization of the proton shared by the 3′ hydroxyl and the Schiff's base nitrogen atom of the protonated internal aldimine [Chart 1, form (2)], while the higher value is assigned to the α-amino group of L-Asp (Gloss & Kirsch, 1995). The chemical mechanism, however, dictates that the reaction occur by condensation of the deprotonated α-NH_2 group of the substrate with the protonated Schiff's base (Scheme 1). The mechanisms are *kinetically* indistinguishable (Kirsch *et al.*, 1984) since the same number of protons is present in both configurations.

The hydrogen bond from Tyr225 that stabilizes the deprotonated internal aldimine in WT enzyme is absent in the Y225F mutant (Chart 1); therefore the pK_a is raised to 8.6 (Goldberg *et al.*, 1991; Goldberg & Kirsch, 1996; Inoue

Table 2: Amino Acid Substrate Specificities of WT and Mutant Aspartate Aminotransferases

enzyme	amino acid	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})
WT[a]	L-aspartic acid	240	6.9	3.5×10^4
WT[b]	L-α-amino-β-guanidinopropionic acid		no saturation	$(4.2 \pm 0.4) \times 10^{-4}$
WT[a]	L-arginine		2.6×10^3	0.028
R292D[a]	L-aspartic acid		no saturation	0.07
R292D[a]	L-arginine		8.0×10^2	0.43
R292hoGlu[c]	D,L-[C_α-^{3}H]AGPA	2.5 ± 0.1[d]	86 ± 15	29 ± 6[d]
R292hoGlu	L-aspartic acid	undetectable activity		

[a] Single-turnover conditions at pH 8.5, 25 °C, and I_c = 1.0. From Cronin and Kirsch (1988). [b] Single-turnover conditions at pH 8.5, 25 °C, and I_c = 1.0. Conditions: L-α-amino-β-guanidinopropionic acid (L-AGPA) concentrations were 25−600 mM, [AATase] = 20 μM, see Experimental Procedures. [c] Determined under steady state conditions at pH 7.5, 25 °C. Other conditions are given in the Figure 2 legend and in Experimental Procedures. [d] The data, based on the L-enantiomer concentration, have not been corrected for the C_α-^{3}H kinetic isotope effect. See Discussion.

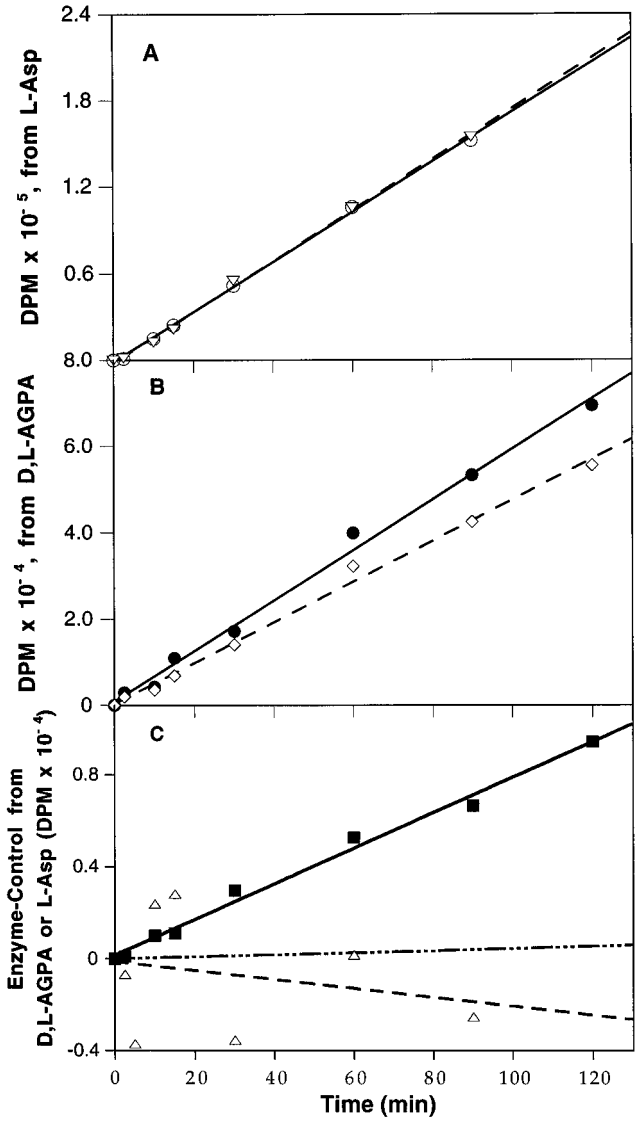


FIGURE 2: Time courses for the R292hoGlu-mediated release of tritium from L-[2,3-^{3}H]Asp (A) or D,L-[α-^{3}H]AGPA (B). General conditions: 100 mM HEPES buffer, pH 7.6, 25 °C. [R292hoGlu] = 5.6−11.2 nM, [PMP] = 10 μM, [α-KG] = 200 mM. The suppression reaction mixture containing R292hoGlu AATase was used without purification. The same mixture minus the AATase expression plasmid was used as a control for the background rate. (A) [L-Asp] = 200 mM with 150 μCi of L-[2,3-^{3}H]Asp/1 mL of reaction. (B) [L-AGPA] = 200 mM containing 150 μCi of D,L-[α-^{3}H]AGPA/1 mL of reaction. 100 μL aliquots of the assay mixture were taken at the times indicated, quenched with 10 μL of 100% trichloroacetic acid, and processed as described in Experimental Procedures. (A) R292hoGlu (○) and control (▽) reactions with L-Asp. (B) R292hoGlu (●) and control (◇) reactions with D,L-[C_α-^{3}H]AGPA. The lines represent the linear fits to the data. (C) Difference plots for enzyme catalyzed minus control reaction (solid minus dashed lines) for parts A and B above. Note expanded ordinate scale. (■ and solid line, D,L-[C_α-^{3}H]AGPA reaction; △ and dashed line, L-Asp reaction); the dash/dotted line is drawn for a $k_{cat}/(K_M + 0.2\ M) = 2\ M^{-1}\ s^{-1}$. The other lines are from linear regression fitting of the data.

et al., 1991). Curve fitting of the k_{cat}/K_M *vs* pH data for the Y225FlTyr mutant yields two pK_as with nearly the same value of 7.5 (Table 1). The pK_a of the hydroxyl moiety of FlTyr (9.0) was estimated from the pK_as of phenol (10.0, Ko *et al.*, 1964), *p*-methylphenol (10.2; Kortum *et al.*, 1961), and *o*-fluorophenol (8.8; Crimmins *et al.*, 1966) by substituent effect additivity. The hydroxyl group of FlTyr, which is a stronger acid than the corresponding entity of unsubstituted tyrosine, should therefore provide greater stabilization to the 3′O$^-$ of the deprotonated cofactor resulting in a more acidic rising limb of the k_{cat}/K_M *vs* pH profile compared to WT AATase. However, the observed value of 7.5 is 0.6 pH units *higher* than that of WT AATase. Thus the electronic effect of the fluorine substitution must be more than offset by other, presumably steric or polarity factors, that destabilize the hydrogen bond between the cofactor and FlTyr225. The distance between the introduced fluorine atom and Asn194 N_δ is 2.99 Å when modeled with the favored rotamer with FlTyr in hydrogen-bond contact with the 3′O$^-$ of the cofactor. Decreasing the fluorine to N_δ distance to form a stronger hydrogen bond would likely destabilize the interaction of the phenolic hydroxyl group with the cofactor, thus accounting for the instability of the latter interaction. However, hydrogen bonds to carbon-bound fluorine, while observed crystallographically do appear to be relatively weak (Murray-Rust *et al.*, 1983; Vermersch *et al.*, 1992; White *et al.*, 1996), so that this explanation must be considered tentative in the absence of structural confirmation. The relative populations of the accessible protonic forms of WT, Y225F and Y225FlTyr, calculated at pH 7.5, are shown in Figure 3. The catalytically active form [(2), Figure 3] is the most stable WT species, while the inactive protonated form (3) predominates for Y225F. Forms (5), (6), and (7) in Figure 3 are approximately equally populated in the Y225FlTyr mutant at this pH. Although the kinetic properties of the Y225R mutant have not been reported as a function of pH, the spectrophotometric pK_{a1} is 6.1 (Inoue *et al.*, 1991). The arginine guanidino moiety is a weaker hydrogen bond donor than is the tyrosine hydroxyl group; therefore the lower pK_{a1} compared to WT must be the result of electrostatic stabilization of the 3′O$^-$ anion in this mutant.

Dissociation of the proton from the bridging hydrogen bond [Chart 1, form (2)] has not been detected experimentally in WT or previously constructed mutant forms of the enzyme; therefore this group has a pK_a value higher than 10 as shown by the dotted arrow in Figure 3. The active form (2) predominates in the WT enzyme at pH 7.5, because the protonic dissociation yielding this species has a pK_a of 6.9. The active form is less populated at pH 7.5 in Y225F (4) because the analogous pK_a is 8.6 (Goldberg *et al.*, 1991). The pK_a controlling the (6) ⇌ (7) equilibrium for Y225FlTyr should be more than 1.0 unit lower than the corresponding value for WT as calculated from phenol substituent effects (see above). The upper shaded bar shows the calculated electronic effect of 1.4 kcal/mol of the 3-F substituent referenced to the corresponding form of the WT enzyme. The latter, which is also shown in the left part of the figure is minimally 3.4 kcal/mol less stable than the active form of the WT (2). The difference between the expected pK_a of >9 and that observed of 7.5 (−2.0 kcal/mol) reflects the noninductive further stabilization of form (7) at the expense of form (6) by the fluoro substituent. These factors are demonstrated further by consideration of the expected effect of the 3-F substituent on the (5) ⇌ (6) equilibrium. The difference in pK_a values between phenol and *o*-fluorophenol is 1.2 units ($\Delta\Delta G = 1.7$ kcal/mol). The proton in question, however, is not fully dissociated but is hydrogen bonded in form (6); therefore the rising limb of the k_{cat}/K_M^{Asp} profile should exhibit a pK_a between 5.7 and 6.9. These figures correspond to Brønsted α values of 1.0 and 0.0 for the extent

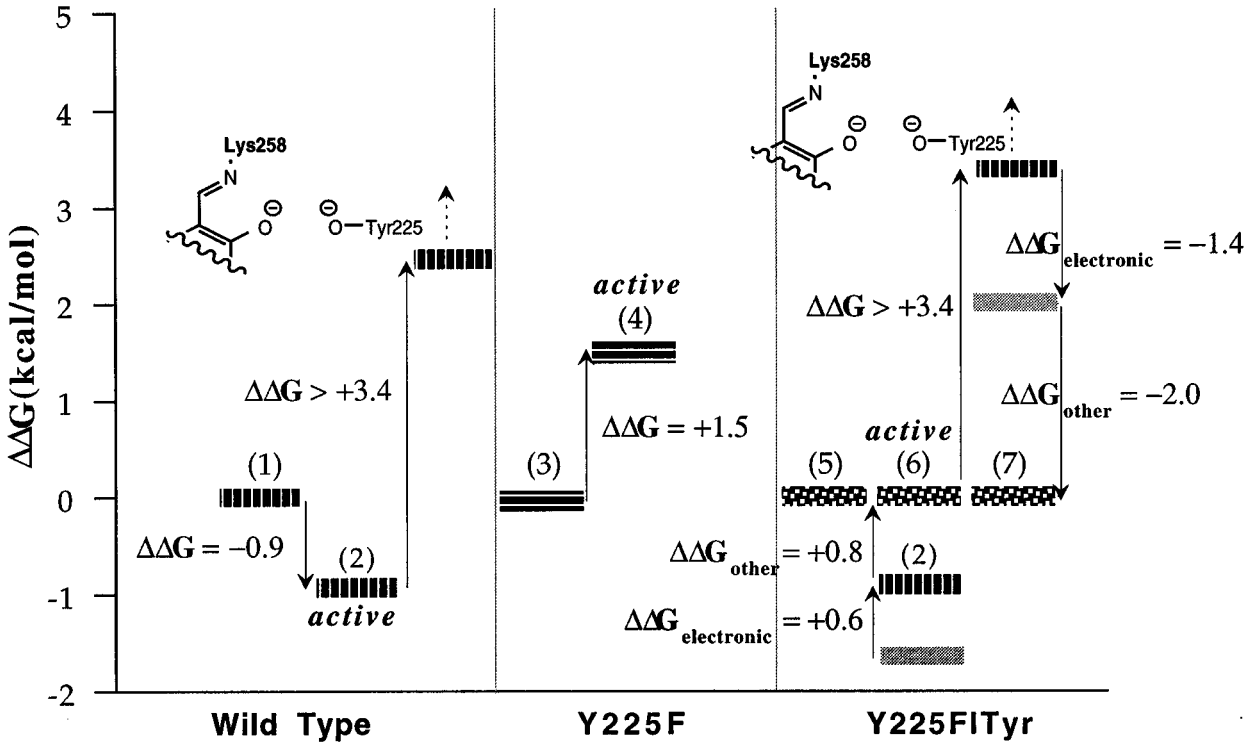
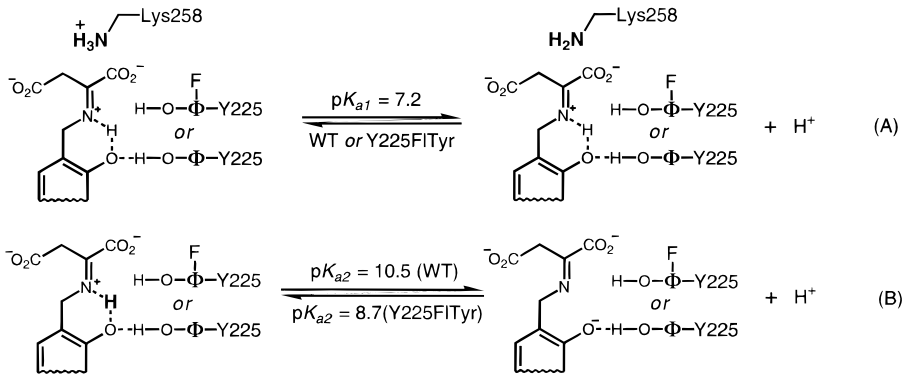


FIGURE 3: Free energy diagram representing the effects of steric and electronic contributions of the 3-F substitution in the Y225FlTyr mutant of AATase: thick vertical bars, WT; thick horizontal bars, Y225F; flecked boxes, Y225FlTyr. The catalytically active forms are (2), (4), and (6). The numbers in parenthesis are keyed to the structures shown in Chart 1. The free energy levels (kcal/mol) are calculated at pH 7.5. The hydrogen bond bridging the 3′O^- and the phenol-OH is stabilized electronically but is destabilized sterically by the 3-F substituent, gray boxes (see text).

Scheme 2: Ionization States of the Active Residues of AATase Accounting for the k_{cat} *vs* pH Profiles



of proton transfer from tyrosine or 3-fluorotyrosine to the cofactor respectively. The α value for hydrogen bond contribution of a series of fluorotyosine substituents to the stability of staphylococcal nuclease is 0.35 (Thorson *et al.*, 1995), and the free energy difference between the lower shaded bar and (2) based on an α value of 0.35 is 0.6 kcal/mol. The fact that the observed pK_a for the (5) ⇌ (6) dissociation is 0.6 pK_a units *higher* than WT (0.8 kcal/mol) illustrates further the noninductive effect of the 3-fluoro substitution on the destabilization of form (6) ($\Delta\Delta G = +1.4$ kcal/mol).

(b) Tentative pK_a Assignments in the k_{cat} vs pH Profiles. While the pH dependencies of k_{cat}/K_M profiles normally reflect only ionization of free enzyme and free substrate, those of k_{cat} are a function of all the populated enzyme−substrate or enzyme−product complexes weighted according to their relative contributions to the observed value of k_{cat}. Moreover the rate-determining step may change as a function of pH. Gloss and Kirsch (1995) showed that the value of the C_α-deuterium isotope effect on k_{cat} decreases from 1.5 at neutral pH to unity at pH extremes; therefore C_α proton abstraction is partly rate-determining only between pH 7.0 and 9.5. These workers tentatively assigned pK_{a1} to the deprotonation of the ϵ-NH_3^+ group of lysine-258 in the ketimine complex based on analysis of the reactions with alternate substrates and mutant enzyme constructs (Scheme 2). The present results are consistent with and lend further weight to the previous assignment. The ϵ-NH_3^+ functionality is reasonably isolated from the phenolic hydroxyl group in the ketimine complex. The N_ζLys258-O_ηTyr225 distance in the chicken mitochondrial L-aspartate ketimine complex of the AAT X-ray crystal structure is 3.72 Å (Malashkevich *et al.*, 1993), and therefore pK_{a1} from WT and Y225FlTyr should be similar as observed (Figure 1, Table 1).

Gloss and Kirsch (1995) assigned pK_{a2} to the dissociation of the endocyclic proton of the ketimine (Scheme 2). This pK_{a2} of 10.3 in WT is reduced to 8.7 in Y225FlTyr. This is a large change reflecting the full calculated ΔpK_a between

the phenolic ionization of L-tyrosine and that of FlTyr. The hydrogen bond formed between the 3′O^- of the cofactor and the hydroxyl group of FlTyr225 must therefore be strong, and importantly the fluorine substituent is sterically tolerated in the hydrogen-bonded ketimine complex as opposed to the internal aldimine complexes as described above.

The rates of C_α proton abstraction, ketimine hydrolysis, and OAA dissociation are all kinetically significant for WT enzyme while the sole rate-determining step is ketimine hydrolysis in Y225F (Goldberg & Kirsch, 1996). The value of the limiting rate constants for both k_{cat} and k_{cat}/K_M in the Y225FlTyr construct do not differ significantly from those exhibited by WT; therefore it is likely that there are multiple rate-determining steps for the former enzyme as well. Correspondingly the pK_a assignments in the k_{cat} *vs* pH profile for Y225FlTyr remain somewhat tentative.

Role of Arg292 in Substrate Recognition

X-ray crystallographic studies showed that the guanidino group of Arg292 makes a salt bridge with the ω-carboxyl moiety of the substrates or of maleate in the closed form of the enzyme−ligand complexes (Ford *et al.*, 1980; Arnone *et al.*, 1977) (Chart 2). An early attempt to invert the substrate specificity from anionic to cationic amino acids through the mutation R292D was qualitatively successful in that L-arginine is the preferred substrate for the mutant construct. The exercise was quantitatively disappointing, however, in that the value of k_{cat}/K_M^{Arg} for R292D is only 10^{-5} of that exhibited by WT enzyme for the natural substrate, L-aspartate. The X-ray structure of R292D (Almo *et al.*, 1994) shows that the side chain of Asp292 is positioned properly to interact with the guanidino group of the substrate L-arginine, and thus provides no definitive explanation for the reduced activity. There are no significant conformational changes save for a shift of the backbone of residues 11−24, which form part of the environment of residue 292. This experimental result appears to be in disagreement with expectations from the calculations of Hwang and Warshel, who state "that the same environment which is folded to stabilize an ion pair will 'repel' an ion pair of the opposite polarity". Possible explanations in terms of stabilization of the reactive configuration have been carefully evaluated by Almo *et al.* (1994). Hayashi *et al.* (1989) converted Arg292 to valine and leucine. The resulting mutant enzymes exhibited large reductions in k_{cat}/K_M^{Asp} for the dicarboxylic substrates and 5−10-fold increases in that parameter for the transamination of aromatic amino acids.

Some of the limitations inherent in attempts to modify specificity in this enzyme significantly by single amino acid changes were addressed successfully by Onuffer and Kirsch (1995), who showed that only six amino acid changes (hexamutant) nearly sufficed to convert the kinetic parameters of AATase to those of the only 43% identical tyrosine aminotransferase (TATase). The transamination activity of the latter enzyme is also coupled to α-ketoglutarate, and TATase is nearly as effective an aspartate aminotransferase as is the eponymous enzyme. It was shown crystallographically that, while the position of Arg292 is fixed in AATase, it acts as a "gatekeeper" in the hexamutant with TATase properties (Malashkevich *et al.*, 1995). The guanidino group forms salt bridges in complexes with ligands bearing side chain carboxyl groups but moves aside to allow access of nonpolar ligands in the corresponding complexes. This movement is proscribed in WT AATase.

Table 3: Selectivity of Aspartate Aminotransferase Variants for Cationic and Anionic Amino Acid Substrates

	k_{cat}/K_M ratios		
substrate	R292D/ WT	R292hoGlu[b]/ WT	{R292hoGlu$^{(L-AGPA)}$/ R292D$^{(L-Asp)}$}
L-Asp	2×10^{-6} [a]	$<5.7 \times 10^{-5}$	
L-Arg	15[a]	ND	4500
D,L-[C_α-^{3}H]AGPA	ND	6.8×10^4	

[a] pH 8.5, 25 °C, I_c = 1.0. From Cronin and Kirsch (1988). [b] Calculated from the values in Table 2. The true values of k_{cat}/K_M D,L-[C_α-^{3}H]AGPA for R292hoGlu are lower limits because of the uncertainties in the estimation of the tritium kinetic isotope effect (see Discussion).

The original attempt to invert substrate charge specificity by charge switching between the substrate and enzyme recognition elements was criticized on the basis that the microenvironment of AATase active site that stabilizes the (+ −) ion pair disfavors the (− +) configuration (Hwang & Warshel, 1988). There are two variables in charge reversal experiments: the first is the (− +)→(+ −) mutation/substrate switch just discussed, while the second is the positioning of the resultant reversed ion pair with respect to that existing in WT enzyme (Chart 2). Replacement of Arg292 with hoGlu (R292hoGlu) moves the ion pair two carbon atoms closer to that of the WT enzyme from that existing in the R292D/Arg experiment. The complementary substrate for R292hoGlu is L-AGPA. This substitution is relatively successful compared to the R292D/Arg result. Although the k_{cat}/K_M value for the reaction of R292hoGlu with D,L-[α-^{3}H]AGPA is 1200-fold lower than that for WT with L-aspartate, it is 70-fold higher than the value for the R292D mutant with L-arginine (Cronin & Kirsch, 1988). The selectivity ratios are given in Table 3. The selectivity ratio between R292hoGlu and WT with D,L-[α-^{3}H]AGPA, $\{k_{cat}/K_M^{AGPA}$ (R292hoGlu)/k_{cat}/K_M^{AGPA} (WT)$\} = 6.8 \times 10^4$, while the ratio with L-Asp, $\{k_{cat}/K_M^{Asp}$ (R292hoGlu)/k_{cat}/K_M^{Asp} (WT)$\}$ is $<2\ M^{-1}\ s^{-1}/35\ 000\ M^{-1}\ s^{-1}\} = <5.7 \times 10^{-5}$. The latter is a conservative estimate of the upper limit since the negative value obtained for $k_{cat}/(K_M + 0.2$ M) for the reaction of L-Asp with R292hoGlu is a result of the subtraction of enzymatic and control reactions of nearly identical slope (Figure 2A). This precludes an accurate calculation of the selectivity for D,L-[α-^{3}H]AGPA over L-Asp. A lower estimate of this ratio can be calculated with the aid of the dash/dotted line in Figure 2C whose arbitrarily drawn slope of 2 $M^{-1}\ s^{-1}$ is clearly above that defined by the collected data. Furthermore, the selectivity ratio between R292hoGlu and WT toward D,L-[α-^{3}H]AGPA is 4500-fold higher than that between R292D and WT toward L-Arg, $\{k_{cat}/K_M^{Arg}$ (R292D)/k_{cat}/K_M^{Arg} (WT)$\} = 15$. The constants k_{cat}/K_M^{AGPA} and k_{cat} listed for D,L-[α-^{3}H]AGPA have not been corrected for the C_α-^{3}H kinetic isotope effect; therefore the listed figures for k_{cat}/K_M^{AGPA} and k_{cat} are minimum values. $^Dk_{cat}/K_M$ for AATase-catalyzed reactions range from a minimum of 1.4 for a good substrate with WT enzyme to a maximum of *ca.* 7 for reactions of substrates with mutant enzymes (Onuffer & Kirsch, 1994). Substitution of these figures into the Swain−Schaad equation (Swain *et al.*, 1958) yields a range of 1.6−16 for the $^Tk_{cat}/K_M$ values. It is reasonable to conclude that the observed values of for D,L-[α-^{3}H]AGPA

should be multiplied by a factor that is closer to the upper end of this range.

True isosteric positioning of the reverse ion pair with wild type enzyme would require replacement of Arg292 with 2-aminoheptanedioic acid. The substrate would be L-α-guanidinoglycine. Synthesis of the latter compound has not been reported, and it would be expected to be unstable under the assay conditions.

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