Significant Improvement to the Catalytic Properties of Aspartate Aminotransferase: Role of Hydrophobic and Charged Residues in the Substrate Binding Pocket†

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ABSTRACT: The substrate specificity of tyrosine aminotransferase (eTAT) from *Escherichia coli* has been tested by transferring the critically different residues Leu39, Glu141, and Arg293 into equivalent positions of aspartate aminotransferase (eAAT). These residues are not directly involved in the catalytic process. The single mutant eAAT V39L possesses greater values of k_{cat}/K_M not only for tyrosine but also for aspartate and glutamate. In contrast, the double mutant eAAT P141E,A293R and also the triple mutant eAAT V39L,P141E,A293R exhibit smaller changes of k_{cat}/K_M. The converse mutants of tyrosine aminotransferase, in which critical residues of eAAT (Val39) and of mitochondrial AAT (Ala39, Val37) were transferred into equivalent positions of eTAT, exhibited generally decreased values of k_{cat}/K_M for both dicarboxylic and aromatic substrates. On the basis of the known structures of eAAT and eAAT V39L as well as of a refined model of eTAT, these results indicate that the different substrate specificities of eAAT and eTAT are due to multiple side chain differences and minor rearrangements of the backbone. The generally improved catalytic efficiency of the mutant eAAT V39L appears to be due to an indirect effect, namely, the facilitated closure of the active site upon substrate binding.

Aminotransferases catalyze the reversible transfer of amino groups between different amino acids by the following two-step mechanism, using pyridoxal 5′-phosphate (PLP)[1] as coenzyme (Christen & Metzler, 1985):

$$\alpha\text{-amino acid}_1 + \text{E·PLP} \rightleftarrows \alpha\text{-keto acid}_1 + \text{E·PMP}$$

$$\alpha\text{-keto acid}_2 + \text{E·PMP} \rightleftarrows \alpha\text{-amino acid}_2 + \text{E·PLP}$$

E is the active site of the aminotransferase, and PMP is the transiently transaminated form of PLP.

Aspartate aminotransferase (AAT), which catalyzes almost exclusively the transamination between Asp and Glu, is currently the most intensely investigated member of the family of aminotransferases. The recent X-ray crystallographic studies on the eukaryotic isoenzymes cAAT and mAAT (Ford et al., 1980; Kirsch et al., 1984; Arnone et al., 1985; McPhalen et al., 1992) and the prokaryotic eAAT (Kamitori et al., 1990; Jäger et al., 1989; Smith et al., 1989; Jäger, 1991) have established to high resolution a common overall structure of these AATs and their active sites. Site-directed mutagenesis and appropriate physicochemical characterization of both wild-type and mutant enzymes are currently being used to assign specific catalytic roles to conserved amino acid side chains in the active site [for recent advances, see, e.g., Yano et al. (1992) and Toney and Kirsch (1993) and references cited therein]. Together with X-ray crystallographic studies on the mutant enzymes and their complexes with various analogues of catalytic intermediates, these data establish the catalytic mechanism of AAT in uncommon detail (Kirsch et al., 1984; Jansonius & Vincent, 1987).

The specificity of AAT for substrates carrying two carboxylate groups (Asp, α-KG, OAA, Glu) is neatly accounted for by the hydrogen bonds and salt bridges formed between R386 and the proximal carboxylate, as well as between R292* and the distal carboxylate of these substrates. Asterisks denote residues in the shared active site that are contributed by the second subunit.

Tyrosine aminotransferase from *Escherichia coli* (eTAT; Fotheringham et al., 1986) is an interesting member of an evolutionarily related subfamily of aminotransferases (Mehta et al., 1989), because it not only catalyzes the same reactions as AAT but also catalyzes the transamination between Asp (or Glu) and aromatic monocarboxylic substrates such as Tyr and *p*-hydroxyphenylpyruvate (HOPP, Figure 1). Using k_{cat}/K_M values as a quantitative measure of substrate specificity (Fersht, 1985), Table 1 documents a 10^5-fold increase of

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[1] Abbreviations: AAT, aspartate aminotransferase (EC 2.6.1.1); cAAT, cytosolic AAT; mAAT, mitochondrial AAT; eAAT, AAT from *Escherichia coli*; eAAT-ER, double mutant of eAAT with Pro141 replaced by Glu and Ala293 replaced by Arg; eAAT-L, mutant of eAAT with Val39 replaced by Leu; eAAT-LER, triple mutant of eAAT with replacements of both eAAT-ER and eAAT-L; eTAT, tyrosine aminotransferase from *E. coli*; eTAT-V, mutant of eTAT with Leu39 replaced by Val; eTAT-VA, mutant of eTAT with Ile37 replaced by Val and Leu39 replaced by Ala; mAAT, mitochondrial aspartate aminotransferase from chicken; HOPP, *p*-hydroxyphenylpyruvate; P-domain, pyridoxal 5′-phosphate binding domain of AAT, residues 47–329; α-KG, α-ketoglutarate; OAA, oxaloacetate; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate.

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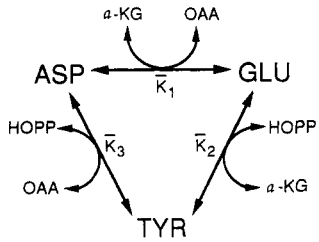

FIGURE 1: Transamination reactions catalyzed by aspartate and tyrosine aminotransferase from *E. coli*. eAAT is specific for Asp/Glu, whereas eTAT catalyzes Asp/Glu as efficiently as eTAT, but both Glu/Tyr and Asp/Tyr even more efficiently (cf. Table I).

specificity of eTAT over that of mAAT for HOPP (Mavrides & Christen, 1978; Köhler, 1990). In contrast, the specificities of mAAT, eAAT, and eTAT for Asp are almost identical. This phenomenon leads to the question of how both dicarboxylic and aromatic monocarboxylic substrates can bind productively to the active site of eTAT.

The structure of eTAT is still unknown. Because 42% of the residues of aligned sequences of mAAT and eTAT are identical (67% are similar; Mehta et al., 1989), Seville et al. (1988) built a stereochemically consistent model of eTAT based on the known structure of mAAT. Jäger et al. (1992) have recently presented a revised model of eTAT, which was based on the X-ray structures of both wild-type eAAT and a point mutant. The revised model confirms the earlier conclusion that 22 out of 28 residues in the active sites of eAAT and eTAT are identical. Because the relaxed specificity of eTAT is apparently not achieved by a major rearrangement of the main chain, the few residues in the substrate binding pocket that differ between mAAT, eAAT, and eTAT (Table I) are apparently critical to the large specificity differences of these three enzymes for HOPP.

In this work, we characterize five mutants in which several such residues of eAAT were replaced by those of eTAT, and *vice versa* (Table II). One of these mutants (eAAT-L) had generally improved specificity toward both the eAAT substrates and the eTAT substrate HOPP.

MATERIALS AND METHODS

Materials. Malate dehydrogenase and glutamate dehydrogenase were purchased from Boehringer Mannheim Corp. *p*-Hydroxyphenylpyruvate (HOPP) was purchased from Sigma and of the highest grade of purity available. α-Ketoglutarate (α-KG) and oxaloacetate (OAA) were ordered from Fluka. The purity of the α-keto acids was determined enzymatically: OAA was prepared freshly every day and the concentration determined by malate dehydrogenase, α-KG by glutamate dehydrogenase, HOPP and PP by D-2-hydroxyisocaproate dehydrogenase. The latter enzyme was a generous gift of Dr. W. Hummel, KFA Jülich, Germany.

Bacterial Strains, Plasmids, and Phages. E. coli JM101 [supE, thi, Δ(lac-proAB), [F′, traD36, proAB, lacI^q, ZΔM15] (Yanisch-Perron et al., 1985)], *E. coli* WK6 [Δ(lac-proAB), galE, strA/F′, ZΔM15, proA$^+$B$^+$], and *E. coli* WK6mutS [Δ(lac-proAB), galE, strA, mutS::Tn10/F′lacI^q, ZΔM15, proA$^+$B$^+$ (Zell & Fritz, 1987)] were used for site-directed mutagenesis. *E. coli* HW857F$^-$ [Δ(*asp*C) KanR, *tyr*B/tn 10] was used for expression of both wild-type and mutant enzymes. eAAT wt and eTAT wt were overexpressed from plasmids pIF100 and pME64, respectively (Fotheringham et al., 1986). pMa/c 5-14 was a gift from H.-J. Fritz (Stanssens et al., 1989). Plasmid pAT153 (Twigg & Sherratt, 1980) was obtained from W. Tacon. Plasmid pAT153D was derived from pAT153 by *Eco*RI digestion, filling of 5′ overhangs by polymerase, and religation to destroy the *Eco*RI site. Phage M13mp8 (Messing & Vieira, 1982) was obtained from New England Biolabs, and the helper phage M13K07 (Vieira & Messing, 1987) was purchased from Pharmacia.

Site-Directed Mutagenesis. To construct eAAT-L, the *Hin*cII–*Bgl*II fragment of pIF100, incorporating the *asp*C gene, was cloned into *Sma*I–*Bam*HI-cleaved M13mp8. Following the introduction of a unique *Acc*I site within the *asp*C gene by site-directed mutagenesis (Zoller & Smith, 1983) the *Ava*I–*Nco*I fragment of the mutated *asp*C gene was isolated and used to replace the corresponding fragment in the plasmid pIF100, yielding pIF122. The mutation eAAT-L was then introduced by cassette-mutagenesis via an *Ava*I–*Acc*I linker. The oligonucleotides used in the mutagenesis and linker replacement of the *asp*C gene are shown below. The mutagenic oligonucleotide for the creation of the *Acc*I site in *asp*C was 5′-CGTCTCATCTTTGTAGACACCAATCC-3′. The oligonucleotide linker used in the exchange of Val39 by Leu was 5′-TCGGGATTGGTCT-3′/3′-CTAACCAGAGA-5′.

Mutants eTAT-V and eTAT-VA were similarly constructed by linker replacement, following the introduction of two unique restriction sites into the *tyr*B gene. The *tyr*B gene was initially isolated on an *Eco*RI–*Bam*HI fragment and inserted into similarly cleaved M13mp8RF. The two restriction sites, *Eco*RI and *Acc*I, were then introduced by sequential site-directed mutagenesis. The entire gene was isolated on a *Hin*dIII–*Nru*I fragment and cloned into pAT153D. Replacement of the 16 bp *Eco*RI–*Acc*I fragment with synthetic DNA fragments generated the mutants eTAT-VA (I37V, L39A) and eTAT-V (L39V). The oligonucleotides used in the mutagenesis and linker replacement of the *tyr*B gene are shown below. The mutagenic oligonucleotide for the creation of the *Acc*I site in *tyr*B was 5′-CGTCTTCGTTGTAGACCAGACCGATAC-3′. The mutagenic oligonucleotide for the creation of the *Eco*RI site in *tyr*B was 5′-CCGATACTTGAATTCACTTTGTC-3′. The oligonucleotide linker used in the exchange of Leu39 by Val was 5′-AATTTAAGTATCGGTGTTTA-3′/3′-ATTCATAGCCACAAATGA-3′. The oligonucleotide linker used in the exchange of Ile37 by Val and Leu39 by Ala was 5′-AATTTAAGTGTTGGTGCTTA-3′/3′-ATTCACAACCACGAATGA-5′. For the construction of mutants eAAT-ER and eAAT-LER, the *Eco*RI–*Bgl*II fragment of plasmid pIF100 containing the *asp*C gene was subcloned into the *Eco*RI–*Bam*HI site of the polylinker of pMa 5–14 to yield pMa-AAT. The gapped duplex method described by Kramer et al. (1984) was used to generate two mutations (P141E and A293*R) in the *asp*C gene in two consecutive rounds of mutagenesis. The mutagenic oligonucleotide for the exchange of Pro141 by Glu was 5′-CTTATGGTTCTCCCAGCTTGG-3′. The mutagenic oligonucleotide for the exchange of Ala293* by Arg was 5′-GAGTAGTTACGGCGAATCGC-3′. Positive clones were then verified by sequencing. To construct the triple mutant eAAT-LER, the *Mlu*I–*Xba*I fragment of pMa-AAT was subcloned into the *Mlu*I–*Bgl*II fragment of plasmid pIF122, already carrying the V39L mutation.

Preparation of E. coli eTAT wt and Mutants. Cells were grown in 2×YT medium. The purification procedure was essentially the same as described for the P-domain (PLP binding domain, residues 47–329) of eAAT by Herold et al. (1991). Pooled fractions were stored at −70 °C after dripping into liquid nitrogen. In this form, the enzymes were stable for several months. Enzyme yields were about 0.5 mg/g of wet cells for eTAT wt, and the purity was greater than 95%

as judged by SDS–PAGE. Yields of eTAT mutant proteins were much lower, only about 7.5% of the yield of eTAT.

Purification of eAAT and Mutants. eAAT and mutants thereof were purified essentially as described by Herold and Kirschner (1990), omitting the hydroxylapatite step. Proteins were already 95% pure after the first column, as judged by SDS–PAGE. Pure proteins were stored as described for eTAT. Enzyme yields were about 12 mg of 95% pure eAAT per gram of wet cells and about 5 mg of pure protein per gram of wet cells for the mutant enzymes.

Determination of Protein Concentration. The method of Bradford (1976) was used to determine protein concentrations. Concentrations of pure eAAT and its variants were determined spectrophotometrically from the absorbance at 280 nm using an extinction coefficient of 100 000 M^{-1} cm^{-1} for the dimer and a subunit molecular weight of 43 550 (Herold et al., 1991).

Enzyme Assays. The activities of the wild-type and mutant enzymes were measured throughout the purification procedure by coupling the production of OAA from Asp and α-KG to the oxidation of NADH with malate dehydrogenase (Karmen, 1955). The standard buffer was 100 mM sodium arsenate, pH 7.6. Reactions were carried out at 37 °C in standard buffer containing 20 mM α-KG, 200 mM L-Asp, 0.2 mM NADH, and 2.5 units of malate dehydrogenase/mL.

Kinetic Analysis. For the determination of steady-state kinetic parameters, three different assays were used, depending on the substrates. (1) For Asp in conjunction with any keto acid: the malate dehydrogenase-coupled assay, as described above, was used; reactions of Asp with HOPP were recorded at 366 nm (NADH ϵ_{366nm} = 3300 M^{-1} cm^{-1}) instead of 340 nm, as HOPP absorbs strongly at 340 nm. (2) For Glu reacting with any keto acid: the glutamate dehydrogenase-linked assay was used, where α-KG is recycled to glutamate with concomitant oxidation of NADH (Akabayashi & Kato, 1989). Reaction conditions were essentially the same as in the malate dehydrogenase-coupled assay, except that NH_4Cl and ADP were added to a final concentration of 30 mM and 2 mM, respectively, and 10–100 units of glutamate dehydrogenase/mL was used. (3) For the reaction of Tyr with α-KG or OAA: The determined difference in extinction coefficient at 295 nm between Tyr and HOPP (800 M^{-1} cm^{-1}) was used to follow the conversion of aromatic amino acid to keto acid. The K_M values of Tyr are high, but its solubility is low.

Initial velocities (v_i) were determined unambiguously from the initially linear portions of the progress curves recorded with a UVIKON 800 spectrophotometer. The primary data consist of averages of initial velocities (generally triplicate runs) covering all possible combinations of five different concentrations each of the amino acid and α-keto acid substrates. Primary plots of v_i versus the varied substrate concentration ([A]) at five different concentrations of the fixed substrate ([B]) were evaluated by a program based on the direct linear plot (Cornish-Bowden & Eisenthal, 1978), yielding values of V_m^{app} and $K_M^{A,app}$ as a function of [B]. The same procedure was applied for varied [B] and fixed [A], yielding values of V_m^{app} and $K_M^{B,app}$ as a function of [A]. Subsequently, secondary plots of V_m^{app} versus the five concentrations of the fixed substrate ([B] or [A]) yielded the values of V_m, K_M^A, and K_M^B. The identity (±10%) of the two V_m values guided the decision for accepting or repeating the rate measurements. The given values of $k_{cat} = V_m/[E_0]$ represent the average. Experiments with Tyr were hampered by its low solubility (~5 mM). For $K_M^{Tyr} > 5$ mM, only the k_{cat}/K_M^{Tyr} values were obtainable from the initial slopes of plots of v_i versus [Tyr]. These were in reasonable agreement with the values of k_1k_2/k_{-1} from single-turnover progress curves of the half-reaction of EL with Tyr (data not shown; Inoue et al., 1989).

Table 1: Correlation between Distinct Substrate Specificities of mAAT, eAAT, and eTAT and Variable Amino Acid Residues in Their Substrate Binding Pockets

	aminotransferase critical residue[a]	mAAT	eAAT	eTAT
	37	V	I	I
	39	A	V	L
	141	G	P	E
	293*	P	A	R
	297*	N	N	S
k_{cat}/K_M (mM^{-1} s^{-1})[b]	HOPP → Tyr	0.5	140	53000
	Asp → OAA	100	130	135

[a] Conventional numbering as in pig cAAT (Ovchinnikov et al., 1973). Single-letter code for amino acid residues. Asterisks denote residues in a shared active site contributed by the second subunit. [b] Involve only rate constants of the indicated half-reaction (cf. eq 9).

Determination of the Metabolic Equilibrium Constant $\bar{K}_2$. eAAT (60 nM) was added to 1-mL aliquots of 1 mM Tyr/0.1 mM α-KG in standard buffer containing variable (0.05–1 mM) concentrations of Glu. Samples were incubated for 2 h at 25 °C. The reaction was stopped by adding 40 μL of 5 N HCl and heating to 100 °C for 5 min. After neutralization with 5 N NaOH, the equilibrium concentration of α-KG was determined enzymatically with glutamate dehydrogenase. $\bar{K}_2$ was calculated from the interpolated concentration of Glu that did not affect the observed increase of [α-KG] as a function of added total [Glu].

Preparation of Apoenzymes. The wild-type and mutant enzymes were resolved to their apoforms by the method of Herold and Kirschner (1990). The quality of the apoenzyme preparation was checked spectrophotometrically for the absence of the characteristic PMP and PLP absorbance maxima at 330 and 360 nm, as well as by the standard enzyme assay, omitting PLP. The activity of the apoenzyme preparations was routinely less than 1% that of the holoenzyme.

Titration of Coenzyme with Apoenzyme. Titrations were performed in tandem cuvettes (d = 0.44 cm) with a Uvikon 800 spectrophotometer. Each cuvette contained 1 mL of 20–30 μM PLP solution (or PMP solution), in 0.05 M sodium arsenate buffer, pH 7.6, in one compartment, and 1 mL of buffer in the other compartment. Identical aliquots of a concentrated solution (~0.3 mM) of the respective apoenzyme in standard buffer were added to the appropriate compartments with an Agla Micrometer syringe. The time-independent difference absorption values between the major peak and trough in the difference spectra ($\Delta A_{355} - \Delta A_{398}$ for PLP; $\Delta A_{342} - \Delta A_{315}$ for PMP) were used for calculating the value of K_d by a nonlinear least-squares fitting procedure (Eberhard, 1990). The degree of reconstitution was checked by enzyme assay. Yields of reconstitution routinely achieved were more than 90%.

RESULTS

Design Strategy. Table 1 presents the apparently critical residues that are in analogous positions close to the active sites of both aspartate aminotransferase (mAAT and eAAT) and tyrosine aminotransferase (eTAT; Seville et al., 1988; Jäger et al., 1992). The mutants described in this work (Table 2) fall into two groups: (1) mutants of eAAT designed to relax its narrow specificity for dicarboxylic amino and keto acids by improving the transamination of aromatic monocarboxylic amino and keto acids (e.g., Tyr and HOPP); (2)

Table 2: Mutants of eAAT and eTAT

desired specificity change	mutation	designation
eAAT → eTAT	V39L	eAAT-L
	P141E,A293*R	eAAT-ER
	V39L,P141E,A293*R	eAAT-LER
eTAT → eAAT	L39V	eTAT-V
eTAT → mAAT	I37V,L39A	eTAT-VA

Table 3: Stoichiometries and Equilibrium Constants for the Binding of PLP and PMP to the Apoenzymes of eAAT and Its Mutants

	PLP		PMP	
aminotransferase	n^a	K_d (μM)	n	K_d (μM)
eAAT	0.85	0.025	0.97	0.31
eAAT-L	nd[b]	nd	1.06	0.85
eAAT-ER	0.96	0.01	1.2	0.6
eAAT-LER	1.13	0.09	1.3	3.7

[a] Number of binding sites per protomer. [b] Not determined.

mutants of eTAT designed to suppress its capacity to transaminate Tyr, while retaining efficient transamination of Asp and Glu. eAAT-L, eAAT-ER, and eAAT-LER are variants of eAAT that have one, two, and three of the critical residues of eTAT replaced by those of eTAT, whereas eTAT-V and eTAT-VA have one and two of its critical residues replaced by those of eAAT or mAAT, respectively.

Production, Purification, and Characterization of the Mutants. The plasmids used for modifying the coding sequences of both eAAT and eTAT were also suited for overexpressing the corresponding gene products constitutively. The recipient cells lacked the *asp*C gene and had a deficient *tyr*B gene. The proteins were purified to homogeneity from the cell supernatants, and their high initial concentrations allowed us to simplify the previous procedures (Powell & Morrison, 1978; Yagi et al., 1985). All of the mutant enzymes behaved similarly to the respective wild-type enzymes during purification. The visible absorption spectra of all mutant and wild-type enzymes, which depend on the protonation of the azomethine group of bound PLP (Kallen et al., 1985), varied with pH in a similar fashion (data not shown). These observations, taken together with the ability of all mutant enzymes to catalyze the transamination reaction with several substrates at reasonable rates, indicate that the mutations do no affect greatly the folding, association, and stability of the active dimer (Herold & Kirschner, 1990).

To see whether the mutations affect the interactions of the eAAT variants with the coenzymes, we measured the binding of both PLP and PMP by spectrophotometric titration in standard buffer. Table 3 shows that all variants of eAAT displayed high-affinity binding of the cofactors. The equilibrium dissociation constants for PLP were about 10-fold smaller than those for PMP, as observed previously for both avian and mammalian AAT's (Jenkins & Fonda, 1985). The much smaller values of K_d^{PMP} and K_d^{PLP} obtained by Toney and Kirsch (1991) for eAAT are probably due to the different buffer conditions. Phosphate ions compete with both PLP and PMP binding to cAAT ($K_d^{P_i}$ = 1.6 μM; Vergé et al., 1979). Eichele et al. (1979) have shown that arsenate binds to the subsite responsible for binding the phosphate moiety of PLP in apo-mAAT. Since arsenate and phosphate ions are isosteric, their K_i values should be similar, and 100 mM arsenate could increase the true K_d values by a factor of $\leq 10^4$ [$K_d^{PLP,app} = K_d^{PLP}(1 + [HAsO_4^{2-}]/K_d^{arsenate})$].

Specificity Changes of eAAT Mutants. The mutants designed to convert the specificity spectrum of eAAT to that

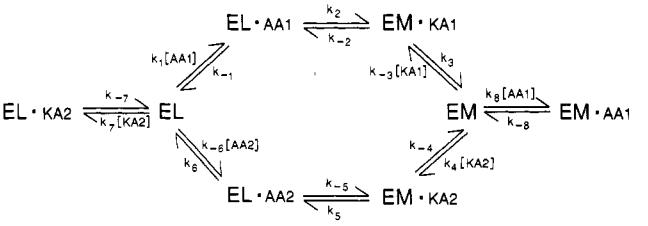

FIGURE 2: Minimal catalytic mechanism of an aminotransferase. EL, EM, AA*i*, and KA*j* represent the pyridoxal and pyridoxamine forms of the active site, and α-amino acid, and an α-keto acid pertaining to the same half-reaction. Scheme I of Inoue et al. (1989) is expanded to include the abortive complexes EL·KA2 and EM·AA1 that can lead to substrate inhibition.

of eTAT (eAAT-L, eAAT-ER, and eAAT-LER, cf. Table 2) were characterized mainly by steady-state kinetics. We chose 0.1 M sodium arsenate, pH 7.6, as the standard buffer because the most rigorous kinetic analysis of mAAT has been performed in this buffer (Velick & Vavra, 1962; Henson & Cleland, 1964).

It is well established that aminotransferases catalyze their reactions according to the ping-pong Bi-Bi mechanism depicted in Figure 2. It is expanded to show the formation of abortive complexes, for example, E·PLP·α-KG. The initial rate of the reaction (in absence of products and abortive complex formation) is given by

$$v_i = V_m\{[A][B]/(K_M^B[A] + K_M^A[B] + [A][B])\} \quad (1)$$

Upon varying [A] at different fixed [B], one obtains

$$V_m^{app} = V_m\{[B]/(K_M^B + [B])\} \quad (2)$$

$$K_M^{A,app} = K_M^A\{[B]/(K_M^B + [B])\} \quad (3)$$

The fit of the primary data to eq 2 via the direct linear plot method (Cornish-Bowden & Eisenthal, 1978) generally did not reveal systematic deviations from a hyperbola.

Figure 1 is associated with six different values of k_{cat}, and six different values of K_M (Inoue et al., 1989). A subset of these kinetic constants was determined in this work and is presented in Table 4. The specificities as expressed by k_{cat}/K_M values (Fersht, 1985) for the six substrates are presented in Figure 3 as bar diagrams. eAAT-L displays particularly interesting properties. Its k_{cat} values in four out of the six possible directions of Figure 1 are practically identical to those of eAAT, but the K_M values for the five tested substrates are smaller by factors of 2–10. As seen in Figure 3, eAAT-L is more efficient than eAAT not only for aromatic substrates, but generally so.

To check the liability of inhibition by abortive complexes, we determined the dissociation constant of α-KG to the enzyme–PLP complex (cf. Figure 2; $K_d^{\alpha\text{-KG}} = k_{-7}/k_7$) by spectrophotometric titration at 438 nm. The value of $K_d^{\alpha\text{-KG}}$ was 10.8 mM for eAAT and decreased to 2.7 mM for eAAT-L (data not shown). Similar relationships have been reported for cAAT and mAAT (Velick & Vavra, 1962).

Following the design strategy of replacing eAAT-specific in eAAT by eTAT-specific residues, we also investigated the double mutant eAAT-ER. As shown in Table 4, the values of k_{cat} were increased 2–4-fold for dicarboxylic acid substrates, but decreased by 30% for HOPP. Moreover, the K_M values were generally increased 2–7-fold. As judged by the k_{cat}/K_M values in Figure 3, the double mutant eAAT-ER is generally less efficient than eAAT. The triple mutant eAAT-LER was constructed to assess the effect of V39L in the mutant

Table 4: Kinetic Constants of Aspartate Aminotransferase and Its Variants

substrate[a]		eAAT		eAAT-L		eAAT-ER		eAAT-LER	
varied	fixed	K_M (mM)	k_{cat} (s^{-1})	K_M (mM)	k_{cat} (s^{-1})	K_M (mM)	k_{cat} (s^{-1})	K_M (mM)	k_{cat} (s^{-1})
Asp	α-KG	1.4	180	0.16	165	3.1	715	1.0	500
α-KG	Asp	0.15		0.015		1.08		0.47	
Glu	OAA	4.5	360	2.1	490	24.0	716	9.8	540
OAA	Glu	0.02		0.019		0.08		0.066	
Glu	HOPP	2.7	220	0.85	243	7.3	70	2.0	70
HOPP	Glu	1.3		0.72		4.2		3.5	
Asp	HOPP	1.0	68	0.12	85	nd[b]	nd	nd	nd
HOPP	Asp	0.78		0.19		nd		nd	

[a] Varied and fixed substrate according to eq 1–3. [b] Not determined.

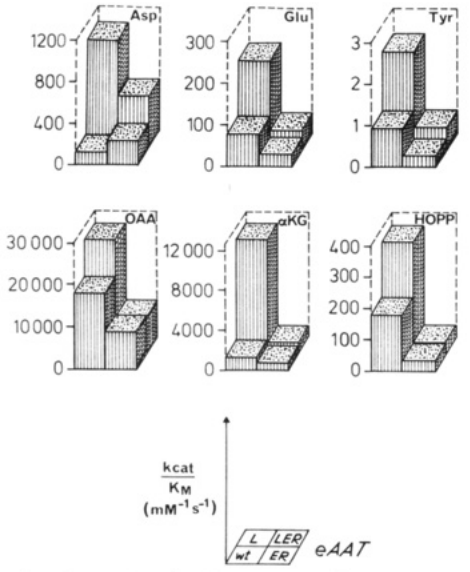

FIGURE 3: k_{cat}/K_M values for six substrates for wild-type aspartate aminotransferase are compared to those of the three mutant enzymes eAAT-L, eAAT-ER, and eAAT-LER. The assignment of the columns is indicated at the bottom.

background of eAAT-ER. In general, the kinetic constants displayed the same trends as in eAAT-L by comparison to eAAT, but the changes were smaller (Table 4 and Figure 3). These observations indicate that the L and ER mutations are not independent of each other.

We also used the Haldane relationships (Henson & Cleland, 1964) to check the self-consistency of the kinetic constants of eAAT for the three reactions of Figure 1 as follows:

$$\bar{K}_1 = \frac{[OAA][Glu]}{[Asp][\alpha\text{-KG}]} = \frac{(k_{cat}^{f})^2}{(k_{cat}^{r})^2} \frac{K_M^{OAA} K_M^{Glu}}{K_M^{Asp} K_M^{\alpha\text{-KG}}} \quad (4)$$

where k_{cat}^{f} refers to the substrate pair Asp and α-KG, and k_{cat}^{r} to OAA and Glu. The value of $\bar{K}_1 = 0.11$ for eAAT, which was calculated from the kinetic constants of Table 4, agrees well with the directly determined value [$\bar{K}_1 = 0.16$ (Velick & Vavra, 1962); $\bar{K}_1 = 0.13$ (Henson & Cleland, 1964); $\bar{K}_1 = 0.17$ (Inoue et al., 1989)].

We determined independently the value of $\bar{K}_2$ for eAAT as the catalyst as given by

$$\bar{K}_2 = \frac{[HOPP][Glu]}{[Tyr][\alpha\text{-KG}]} = 0.9 \quad (5)$$

The lower value of $\bar{K}_1$ by comparison to $\bar{K}_2$ has been attributed by Velick and Vavra (1962) to the higher energy content of OAA, which is a β-keto compound. That explanation is consistent with the calculated value of

$$\bar{K}_3 = \frac{[OAA][Tyr]}{[Asp][HOPP]} = \frac{\bar{K}_1}{\bar{K}_2} = 0.12 \quad (6)$$

which is practically identical to the value of $\bar{K}_1$.

The Haldane relationships were also used to test the self-consistency of the kinetic constants obtained for the variants of eAAT. The computed values of $\bar{K}_1$ (eq 4) obtained with eAAT-L, eAAT-ER, or eAAT-LER as catalysts were 5–10-fold larger than that of eAAT as catalyst. Since these values should be independent of the catalyst used, we conclude that some of the kinetic constants are in error. Control measurements indicate that the concentrations of NH_4Cl required as a supplement when glutamate dehydrogenase is the auxiliary enzyme can lead to increases of K_M values due to competitive inhibition by chloride, confirming earlier observations (Cronin & Kirsch, 1988; Inoue et al., 1989). We propose that the values of K_M^{OAA} and K_M^{Glu} of these mutants are overestimated approximately 2–3-fold each, and consequently the corresponding values of k_{cat}/K_M^{OAA} and k_{cat}/K_M^{Glu} in Figure 3 are underestimated approximately 2–3-fold.

Mutants of eTAT. The two mutants of eTAT designed to suppress its efficiency for aromatic monocarboxylic substrates (cf. Table 2) were investigated less extensively than the mutants of eAAT described above. As judged from the relatively low purification yields, both eTAT-V and eTAT-VA are less stable than eTAT. The steady-state kinetic analysis showed that both eTAT mutants were less active than the wild type (Table 5). It is seen that the K_M values are generally increased, more so in eTAT-VA than in eTAT-V. The corresponding k_{cat}/K_M values are presented in Figure 4 in the form of bar diagrams.

DISCUSSION

Comparison of k_{cat}/K_M Values. Figure 3 shows that, as judged from the k_{cat}/K_M values, eAAT-L is the only mutant with significantly improved specificity for HOPP and Tyr. However, this improvement is accompanied by a similar improvement of the efficiency of transamination of the other four dicarboxylic substrates. Figure 4 shows that neither of the two mutants designed to suppress the efficiency of eTAT for aromatic monocarboxylic substrates showed the desired changes, but rather a generally decreased efficiency for both classes of substrates.

Correlation to Structural Differences. The structures both of eAAT (Kamitori et al., 1990; Smith et al., 1989; Jäger et al., 1993b) and of eAAT-L (Jäger et al., 1989, 1993a) are known. Thus, it is feasible to correlate the functional to the structural changes caused by the V39L mutation. For the forward direction of aspartate transamination (Asp + α-KG → OAA + Glu), the relationships between the kinetic constants and the rate constants defined in Figure 2 are given by eq 7–9

Table 5: Kinetic Constants of Tyrosine Aminotransferase and Its Variants

substrate[a]		e-TAT		eTAT-V		eTAT-VA	
varied	fixed	K_M (mM)	k_{cat} (s^{-1})	K_M (mM)	k_{cat} (s^{-1})	K_M (mM)	k_{cat} (s^{-1})
Asp	α-KG	2.1	325	5.3	250	19.1	218
α-KG	Asp	0.31		0.84		7.1	
Glu	OAA	22.4	375	41.0	308	137	53
OAA	Glu	0.036		0.072		0.075	
Glu	HOPP	28.6	800[b]	70.0	430	130	200
HOPP	Glu	0.084		0.09		0.65	
Asp	HOPP	2.4	480[b]	nd[c]	154	nd	82
HOPP	Asp	0.009		0.027		0.13	
Tyr	OAA	0.75	625[b]	1.1	219	5.5	125
OAA	Tyr	0.22		nd		nd	

[a] Varied and fixed substrate according to eq 1–3. [b] Values from Jardetzky and Seville (1988). [c] Not determined.

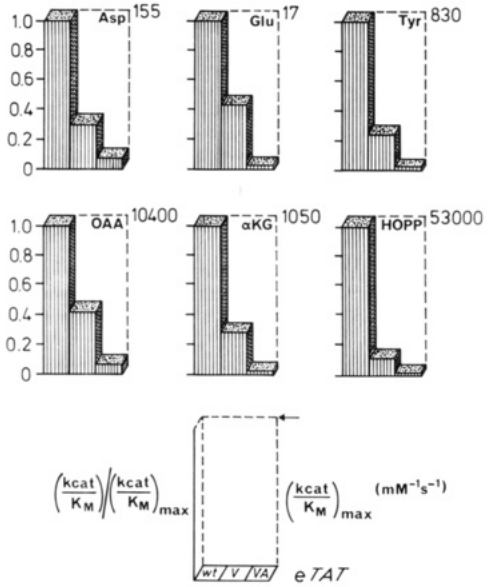

FIGURE 4: k_{cat}/K_M values for wild-type tyrosine aminotransferase and the two mutant enzymes eTAT-V and eTAT-VA. Real numbers have been normalized to a scale from 0 to 100, with the maximum value (mM^{-1} s^{-1}) indicated in the top right-hand corner for every substrate. The assignment of the columns is indicated at the bottom.

(Velick & Vavra, 1962; Inoue et al., 1989). Table 4 shows

$$k_{cat} = \frac{k_2 k_5}{k_2 + k_5} \tag{7}$$

$$K_M^{Asp} = \frac{k_{-1} k_5}{k_1 (k_2 + k_5)} \tag{8}$$

$$\frac{k_{cat}}{K_M^{Asp}} = \frac{k_1 k_2}{k_{-1}} = \frac{k_2}{K_d^{Asp}} \tag{9}$$

that, for the mutation eAAT-L, the k_{cat} values are practically unchanged. We assume that the rate constants on which k_{cat} depends (cf. eq 7) are also unchanged, and consequently the observed increases of the k_{cat}/K_M values are probably due mainly to a decrease of the corresponding K_d values (cf. eq 9).

As described in detail by Jäger (1991), and Jäger et al. (1993b), both eAAT and presumably also eAAT-L prefer a state of the active site in the absence of substrates, which is not as widely open as in the case of mAAT. The formation of the external aldimine with 2-methyl-Asp leads to closure of the active site and the exclusion of all but one water molecule. The arrangement of residues in both the PLP and substrate binding region of the closed active site is practically identical in both eAAT and eAAT-L. Importantly, closure does not lead to a direct contact between the side chain of residue 39 and maleate, the bound substrate analogue (Figure 5). In contrast, close van der Waals interactions occur between the substrate analogue and the side chains of I17, V18, and I37, whereas the side chain of L39 (in eAAT-L) interacts with the side chains of I37, T47, N69*, Y70*, and Y263. These observations support the earlier suggestion by Hayashi et al. (1991) that the V39L substitution must exert an indirect effect on the catalytic efficiency, rather than counteract a nonproductive binding mode of Tyr and HOPP as originally proposed by Seville et al. (1988). Direct evidence for preference of the closed state of the active site by eAAT-L relative to eAAT arises from the observation that the maleate complex crystallized from ammonium sulfate is in the closed state for eAAT-L but in the half-open state for eAAT (Jäger, 1991; Jäger et al., 1993b).

Why does the V39L mutation increase the catalytic efficiency for both the AAT and the TAT reactions (cf. Figure 1)? Jäger et al. (1993a,b) have shown that the relative movement of the large and small domains of the subunit of AAT, which accompanies the binding of substrate analogues, is a rather complicated structural rearrangement that can be described by a rigid-body movement only in first approximation. In contrast to successful instances of engineered specificity changes (Corbier et al., 1990; Wilks et al., 1990), a successful conversion of eAAT to eTAT apparently requires a more extensive replacement of residues as shown, for example, by Bocanegra et al. (1993) for pyruvate dehydrogenase.

The double and triple mutants eAAT-ER and eAAT-LER had been designed to test the hypothesis of Seville et al. (1988) that E141 and R293* in eTAT set up a network of charges that facilitate the rotation of the side chain of R292* out of the active site, when aromatic monocarboxylic substrates are bound. In its normal position, R292* would interact unfavorably with the aromatic side chain of the substrates. This proposal is supported by Hayashi et al. (1991), who showed that replacement of R292* by Val improves the efficiency of the half-reaction with aromatic substrates. For the correct binding of dicarboxylic substrates, the side chain of R292* of eTAT would have to return to the active site. A decrease of k_{cat}/K_M values was observed for all substrates except Asp (cf. Figure 3). The triple mutant eAAT-LER possessed somewhat larger values of k_{cat}/K_M than eAAT-ER, but the effects of the additional V39L replacement were weaker than those observed between eAAT-L and eAAT. The effects of the simultaneous replacements of P141 by E and A293* by R both on k_{cat}/K_M (Figure 3) and on the binding of coenzymes

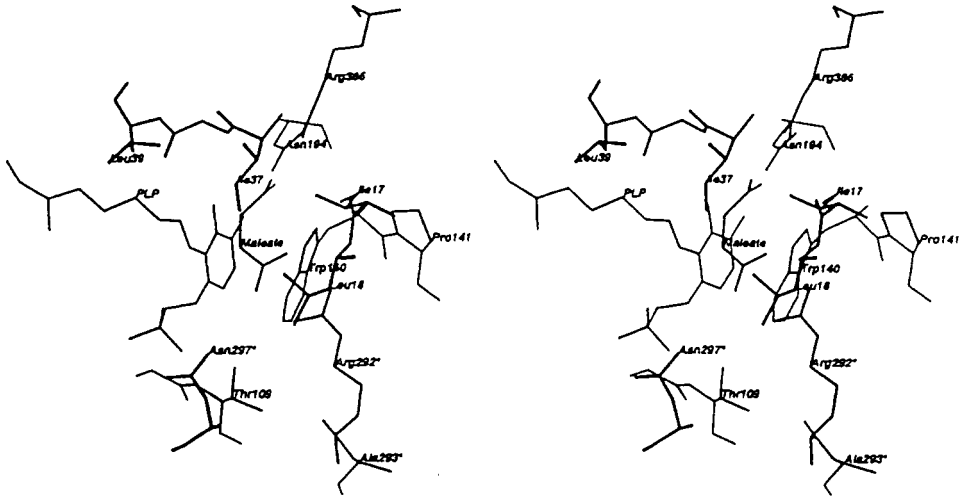

FIGURE 5: Position of L39 at the entry to the active site of the mutant eAAT-L. Stereo drawing of the maleate complex in the closed conformation, based on its known structure (Jäger, 1991).

(Table 3) are relatively small. These findings are consistent with the envisioned relatively loose packing of the active site of eAAT in the vicinity of R292*. In the refined eTAT model (Jäger et al., 1992), both E141 and N142 form hydrogen bonds with main chain atoms as well as with the side chain of R292*. Its guanidinium group is further anchored by a salt bridge to D15. These interactions direct one of the hydrogen atoms of the guanidinium group of R292* toward the phenol ring of the bound substrate. According to Levitt and Perutz (1988), this interaction is energetically favorable. In this model, the side chain of the neighboring residue R293* points away from the active site into the solvent.

The bulky $-CH_2-CH_2-CH_2-$ side chain moiety of R293* in the active site of eAAT-ER may be responsible for the suboptimal orientation of the aromatic substrates. Crystallographic studies of eAAT-ER and eAAT-LER are needed to provide a firmer basis for interpretation. In addition, S297* in eTAT is occupied by N in eAAT. According to the model of eTAT, S297* hydrogen-bonds the hydroxyl group of Tyr or HOPP. N297* in eAAT might not be able to fulfill this role. Experiments with the point mutations eAAT P141E and N297S are underway to clarify this point.

Mutants of eTAT. The above interpretation is supported by the kinetic behavior of the mutants eTAT-V and eTAT-VA. The design principle of the eTAT-V mutant is reciprocal to that of eAAT-L (cf. Table 2). These mutants have both decreased k_{cat} and increased K_M values (Table 4). It appears significant that, in terms of the efficiency parameter k_{cat}/K_M (Figure 4), the decrease observed with Tyr and HOPP is larger than that of dicarboxylic substrates. This differential effect is also observed with eAAT-VA, albeit to a lesser extent. However, among the dicarboxylic substrates, the C_5 substrates Glu and α-KG are more affected than the C_4 substrates Asp and OAA.

These behavioral changes cannot be interpreted unambiguously because little is known about the structures of these variants. However, in general the observed trend is the reverse of that observed with eAAT-L. That is, the decrease of the volume of hydrophobic side chains at the rim of the active site interferes with the energetics of closing the active site. As mentioned earlier for eAAT-L, the interactions between the mutant side chains and the bound substrates are necessarily indirect. However, unlike the case of eAAT-L, the equilibria between open and closed conformations seem to be affected differently in the eTAT-V and -VA mutants. That is, imperfect closure may affect the stability of the enzyme–substrate complex as well as the orientation and proximity of the catalytic side chains with respect to the reacting atoms.

In this picture, the hydrophobic side chains of both I37 and L39 in eTAT act as additional latches, which help to stabilize the optimally closed conformation in addition to the weak hydrogen bond between the hydroxyl groups of the aromatic substrate and that of S297* (Jäger, 1991).

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