Directed evolution relieves product inhibition and confers in vivo function to a rationally designed tyrosine aminotransferase

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Abstract

The *Escherichia coli* aspartate (AATase) and tyrosine (TATase) aminotransferases share 43% sequence identity and 72% similarity, but AATase has only 0.08% and 0.01% of the TATase activities (k_{cat}/K_m) for tyrosine and phenylalanine, respectively. Approximately 5% of TATase activity was introduced into the AATase framework earlier both by rational design (six mutations, termed HEX) and by directed evolution (9–17 mutations). The enzymes realized from the latter procedure complement tyrosine auxotrophy in TATase deficient *E. coli*. HEX complements even more poorly than does wild-type AATase, even though the (k_{cat}/K_m) value for tyrosine exhibited by HEX is similar to those of the enzymes found from directed evolution. HEX, however, is characterized by very low values of K_m and K_D for dicarboxylic ligands, and by a particularly slow release for oxaloacetate, the product of the reaction with aspartate and a TCA cycle intermediate. These observations suggest that HEX exists largely as an enzyme–product complex in vivo. HEX was therefore subjected to a single round of directed evolution with selection for complementation of tyrosine auxotrophy. A variant with a single amino acid substitution, A293D, exhibited substantially improved TATase function in vivo. The A293D mutation alleviates the tight binding to dicarboxylic ligands as K_m s for aspartate and α -ketoglutarate are >20-fold higher in the HEX + A293D construct compared to HEX. This mutation also increased $k_{\text{cat}}/K_m^{\text{Tyr}}$ threefold. A second mutation, I73V, elicited smaller but similar effects. Both residues are in close proximity to Arg292 and the mutations may function to modulate the arginine switch mechanism responsible for dual substrate recognition in TATases and HEX.

Keywords: aminotransferase; protein engineering; directed evolution; pyridoxal phosphate; substrate specificity

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Abbreviations: AATase, aspartate aminotransferase (EC 2.6.1.1); TATase, tyrosine aminotransferase (EC 2.6.1.5); HEX, a mutant of AATase with the substitutions V39L/K41Y/T47I/N69L/T109S/N297S; 8–2, a directly evolved mutant of AATase with the substitutions A13T/A26V/N69S/G72D/S139G/T167A/R282C/A293V/N297S/N339S/A381V/N396D/A398V; HO-HxodH, 2-hydroxyisocaproate (2-hydroxy-4-methylpentanoate) dehydrogenase (EC 1.1.1.-); MDH, malate dehydrogenase (EC 1.1.1.37); PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine 5'-phosphate; α KG, α -ketoglutarate; HPP, hydroxyphenylpyruvate; PP, phenylpyruvate, OAA, oxaloacetate.

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The last few years have seen several successful examples of introduced changes in enzyme specificity realized from targeted mutagenesis and from directed evolution; see Chen (2001) and Bornscheuer and Pohl (2001) for reviews. Rational redesign utilizes structural and sequence alignment information to suggest potentially beneficial replacements, which are then evaluated through site-directed mutagenesis and subsequent characterization of the expressed protein. Directed evolution entails multiple iterations of random mutagenesis and screening. This approach requires neither structural information nor an understanding of the underlying structure function relationships, but depends on a reliable method to identify rapidly variants that exhibit im-

Scheme 1. Transamination reactions catalyzed by PLP-dependent AATase and TATase. All reactions are reversible. AATase catalyzes only the reaction on the *left* while TATase catalyzes all of the reactions shown in the scheme.

provements in a targeted property. The two methods are not mutually exclusive, and there are reports in which changes in protein stability and in enzyme substrate specificity were achieved through a combination of targeted and random mutagenesis (Chen et al. 1997; Cherry et al. 1999).

How would an enzyme engineered by rational redesign of a given starting protein compare with variants derived from parallel directed evolution experiments from the same starting protein and selected for the same novel function? *Escherichia coli* aspartate aminotransferase (AATase), which has near absolute specificity for Asp, Glu, and their corresponding α -keto acids, has been converted to an enzyme with tyrosine aminotransferase (TATase) activity (Scheme 1), first by rational redesign (Onuffer and Kirsch 1995), and later by directed evolution (Rothman and Kirsch 2003). Rationally engineered AATase mutants were evaluated with an in vitro assay for TATase activity, while an in vivo selection for TATase function was employed in the directed evolution experiment. Both approaches yielded variants that exhibit much greater activities with phenylalanine and tyrosine, yet retain activity towards aspartate.

Six positions in wild-type *E. coli* AATase were changed to *E. coli* TATase residues in the rational redesign. The resulting HEX mutant displayed a 310-fold increase in $k_{\text{cat}}/K_m^{\text{Phe}}$ and only a threefold decrease in $k_{\text{cat}}/K_m^{\text{Asp}}$. However, $k_{\text{cat}}^{\text{Asp}}$ and K_m^{Asp} are, respectively, 5% and 12% of the wild-type AATase values, and are also much lower than the wild-type TATase figures for its reaction with aspartate. (Onuffer and Kirsch 1995; Shaffer et al. 2002). Directed evolution, employing DNA shuffling and in vivo selection, generated AATase variants that exhibit 100–270-fold increases in $k_{\text{cat}}/K_m^{\text{Phe}}$ (Rothman and Kirsch 2003). These evolved TATases contain 9–17 amino acid replacements, but include only two of the HEX substitutions: T109S and N297S. Thus, the rational redesign as judged by the in vitro $k_{\text{cat}}/K_m^{\text{Phe}}$ value exhibited by HEX was slightly more successful than that realized from directed evolution, although there are fewer mutations in HEX. The AATase variants obtained from directed evolution complement Tyr and Phe auxotrophies in TATase deficient *E. coli* strains, but it was not known whether HEX would function similarly in vivo. The following results show that HEX only poorly complements aromatic amino acid auxotrophy. However, when HEX was subjected to the pressures of directed evolution, it

acquired a single mutation that allows it to confer viability to the deficient strains. The kinetics were analyzed in detail.

Results

Slow product dissociation in reactions of HEX with aspartate

The kinetic parameters of the HEX mutant with substrates Phe and Asp were determined originally under single turnover conditions (Onuffer and Kirsch 1995), which monitor the first half reaction where AAT-PLP and Asp (or Phe) are converted to at least the product-ketimine complexes (Scheme 2). Both ketimine hydrolysis, the final chemical step in the first half reaction, and release of the product OAA (or PP) escape kinetic detection in such an experiment. Luong and Kirsch (1997) later devised a coupled assay to monitor TATase activity under steady-state conditions. This method, similar to the coupled assay for AATase activity, follows the overall transamination process, which includes the chemical and binding steps for both half reactions. Such steady-state kinetic analyses typically employ α KG as the substrate for the second half reaction. Certain of the steady-state parameters determined for HEX differ significantly from the single turnover values (Table 1). The predicted relationships between the steady-state rate constants k_{cat} and k_{cat}/K_m and the single turnover kinetic parameters k_f and k_f/K_D (assuming that steps prior to ketimine hydrolysis are rate determining) are shown in equations 1a and 1b.

$$k_{\text{cat}}^{\text{AA}/\alpha\text{KG}} = \frac{k_f^{\text{AA}} * k_f^{\alpha\text{KG}}}{k_f^{\text{AA}} + k_f^{\alpha\text{KG}}} \quad (1a)$$

$$k_{\text{cat}}/K_m^{\text{AA}} = k_f/K_D^{\text{AA}} \quad (1b)$$

The kinetic parameters k_f/K_D^{AA} and $k_{\text{cat}}/K_m^{\text{AA}}$ are the same as they both report exclusively on the first half reaction. However, if the rates of product dissociation or of ketimine hydrolysis were kinetically significant, the values of $k_{\text{cat}}/K_m^{\text{AA}}$ will be less than the determined k_f/K_D^{AA} (see above). The values of $k_{\text{cat}}/K_m^{\text{Asp}}$ and $k_{\text{cat}}/K_m^{\text{Phe}}$ are within threefold

Scheme 2. Mechanism for the first half reaction of the transamination of AATase, conversion of the PLP form of AATase and Asp to the PMP form and OAA. Single turnover kinetics spectrophotometrically monitor only steps through formation of the E-ketimine intermediate. Steady-state kinetics monitor the complete reaction: $\text{Asp} + \alpha\text{KG} \leftrightarrow \text{Glu} + \text{OAA}$.

Table 1. Single turnover and steady-state kinetic parameters for wild-type aminotransferases and HEX

Aminotransferase	Phe				Asp			
	Single turnover ^a		Steady state		Single turnover ^a		Steady state	
	k_f/K_D (M ⁻¹ s ⁻¹) × 10 ⁻²	k_f (s ⁻¹)	k_{cat}/K_m (M ⁻¹ s ⁻¹) × 10 ⁻²	k_{cat} (s ⁻¹)	k_f/K_D (M ⁻¹ s ⁻¹) × 10 ⁻²	k_f (s ⁻¹)	k_{cat}/K_m (M ⁻¹ s ⁻¹) × 10 ⁻²	k_{cat} (s ⁻¹)
AATase	2.5	n.s.	1.19 ^b	n.s.	2000	610	910 ^c	159 ^c
HEX	3700	37	370 ^d	28.9 ^d	3400	770	260 ^e (10)	5.1 ^e (0.1)
TATase	23,000	880	9600 ^f	250 ^f	540	240	370 ^f	140 ^f

(n.s.) No saturation.

^a From Onuffer and Kirsch (1995).^b From Luong and Kirsch (2001).^c From Gloss et al. (1992).^d From Shaffer et al. (2002).^e Conditions: 200 mM TAPS (pH = 8.0), 140 mM KCl, 0.15 mM NADH, [Asp] = 0.025–2.5 mM, 2 mM αKG, 20 μM PLP, 25°C. [MDH] ~10 units/mL. Standard errors are in parentheses.^f From Hayashi et al. (1993).

of k_f/K_D^{Asp} and k_f/K_D^{Phe} , respectively, for both wild-type AATase and wild-type TATase. In contrast, the HEX steady-state rate constants for Phe and Asp are 10-fold lower than the corresponding single turnover values. Most notable in the comparison of HEX with the wild-type enzymes is that the steady-state $k_{cat}^{\text{Asp}/\alpha\text{KG}}$ for HEX is only 5.1 sec⁻¹, whereas the corresponding wild-type AATase and TATase values are 159 sec⁻¹ and 140 sec⁻¹, respectively. The $k_{cat}^{\text{Asp}/\alpha\text{KG}}$ (defined as k_{cat} for the transamination reaction of the substrates denoted in the numerator) value for HEX is not limited by the rate for the second half reaction with αKG, because $k_{cat}^{\text{Phe}/\alpha\text{KG}}$ is nearly sixfold higher at 28.9 sec⁻¹. The particularly slow turnover for HEX with aspartate, observed under steady-state but not single turnover conditions, suggests that product dissociation or ketimine hydrolysis may be kinetically significant.

Solvent viscosity effects measure the extent to which the diffusion controlled processes of substrate association or product release determine the overall rate of an enzymatic reaction. Goldberg and Kirsch (1996) found that the k_{cat} value for wild-type AATase with Asp and αKG is dependent on the concentration of the added viscosogen, sucrose. A comparable viscosity effect was also observed for k_{cat}/K_m^{OAA} in the reverse direction. They concluded that dissociation of the product, OAA, is partially rate determining in reactions of wild-type AATase with Asp and αKG. The effects of sucrose mediated viscosity on the kinetic parameters of the HEX mutant are compared with those for wild-type AATase (Fig. 1; Table 2). The data show that the values for viscosity effects on both k_{cat} and k_{cat}/K_m for Asp are considerably greater for HEX than for wild-type AATase. These results demonstrate that the rate of release of OAA is a larger fractional component of the overall kinetic barrier for the HEX reactions, and they at least partially account for the differences in the single turnover and

steady-state parameters for the reaction with aspartate. The viscosity effects on $k_{cat}^{\text{Phe}/\alpha\text{KG}}$ and k_{cat}/K_m^{Phe} show that the release of the product, PP, is also kinetically significant in HEX, but less so than that of OAA.

Does the rationally designed HEX function in vivo?

A genetic *E. coli* selection/screen was employed in this study to compare the in vivo TATase and AATase activities

Figure 1. Effects of the added viscosogen, sucrose, on the kinetics of the reactions catalyzed by wild-type AATase and HEX. (Circles) Wild-type AATase with Asp, (squares) HEX with Phe, and (diamonds) HEX with Asp. (A) $(k_{cat}/K_m)^0 / ((k_{cat}/K_m)\eta)$ vs. relative viscosity, η/η^0 . (B) $(k_{cat})^0 / (k_{cat})$ vs. η/η^0 . The corresponding slopes for each plot are provided in Table 2. Fully diffusion-controlled reactions are represented by the dashed lines with slope = 1.

Table 2. Solvent viscosity effects on steady-state reactions catalyzed by HEX and wild-type AATase

		Solvent viscosity effects	
	Substrate	k_{cat}/K_m	k_{cat}
<i>Aminotransferase</i>			
HEX ^a	Phe	0.195 (0.008)	0.21 (0.02)
	Asp	0.64 (0.03)	0.56 (0.03)
Wild-type AATase	Asp ^a	0.00 (0.00)	0.26 (0.02)
	Asp ^b	0.10	0.26
	Ala ^b	0.00	n.s.

(n.s.) No saturation.

^a Conditions: 200 mM TAPS (pH = 8.4), 100 mM KCl, 0.15 mM NADH, 20 μ M PLP, 25°C. [Asp] = 0.1–5 mM for HEX and 1–15 mM for wild-type AATase. [Phe] = 0.25–8 mM for HEX. [α KG] = 4 mM for HEX and 7.5 mM for wild-type AATase. [MDH] ~10 units/mL for Asp coupled assays. [HO-HxoDH] ~3 μ M for Phe coupled essays. Sucrose concentrations were 0%, 12%, 24%, and 36%, providing relative viscosities in the range of 1 to 4. Viscosity effects were evaluated according to Brouwer and Kirsch (1982). Standard errors are given in parenthesis.

^b From Goldberg and Kirsch (1996).

of HEX with aminotransferases derived from the parallel direction evolution experiment. Selection for TATase activity is based on aromatic amino acid biosynthesis (Scheme 3). Engineered *E. coli* strains SR224 (RecA⁺) and SR250 (RecA⁻) are auxotrophic for Tyr and Phe because the genes encoding TATase (*tyrB*), AATase (*aspC*), and the branched chain aminotransferase (*ilvE*) are deleted (Rothman and Kirsch 2003). *TyrA* and *pheA* are also deleted in these strains. Growth in tyrosine free media is absolutely dependent on an exogenous supply of the TATase substrate, HPP, and a source of TATase activity. SR224 and SR250 are also aspartate auxotrophs, thus permitting assessment of in vivo AATase activity.

The fitness conferred by HEX, wild-type AATase, and the evolved AATase variant, 8-2 (Rothman and Kirsch 2003), were compared from relative complementation of tyrosine and aspartate auxotrophies. SR250 cells transformed with plasmid DNA encoding these enzymes were evaluated for growth rates in media lacking tyrosine, but supplemented with 0.17 mM HPP, and in media lacking aspartate. Cell density doubling times were determined in selective liquid media (Fig. 2; Table 3). The relative sizes of colonies grown from selective agar plates are also reported in Table 3. As expected, 8-2 transformants grow faster than wild-type AATase transformants in tyrosine free media. Cells harboring clone 8-2 also grow nearly as well as those containing wild-type AATase in media lacking aspartate. In contrast, HEX supports only weak growth on either tyrosine free or aspartate free media, despite the fact that it exhibits $k_{\text{cat}}/K_m^{\text{Tyr}}$, $k_{\text{cat}}^{\text{Tyr}/\alpha\text{KG}}$, and $k_{\text{cat}}/K_m^{\text{Asp}}$ values similar to those characterizing the enzyme isolated from 8-2 (Table 4).

Random mutagenesis and selection enhances HEX function in vivo

One round of mutagenesis and selection in TATase deficient strain SR224 sufficed to improve the in vivo fitness conferred by HEX. DNA shuffling was employed to generate random mutations in the HEX gene. Reassembled DNA was cloned in pBAD18 and the resultant library was transformed into SR224. Clones with beneficial mutations were identified on selective plates lacking Tyr and supplemented with 0.11–0.55 mM HPP. The selective media otherwise contained a complete set of amino acids, including aspartate and phenylalanine. Two HEX variants were picked for further analysis. Each has a single amino acid substitution, A293D or I73V. The two clones also contain one to three silent mutations in the coding sequence. The data in Figure 2 and Table 3 show that cells transformed with the HEX + A293D mutant grow substantially better than those harboring HEX. Those cells containing HEX + A293D also grow similarly to 8-2 transformants in tyrosine-free or aspartate-free media. The I73V mutation confers little advantage in tyrosine free media supplemented with 0.17 mM HPP. Additional colony size analyses with tyrosine-free agar plates supplemented with 0.42 mM HPP do indicate a modest but real growth advantage over HEX (data not shown). HEX + I73V transformants display a similar doubling time in exponential phase but a shorter lag relative to HEX in media lacking aspartate. Mutations A293D and I73V were combined by subcloning a fragment

Scheme 3. Biosynthesis of tyrosine and phenylalanine. *pheA* encodes the dual function chorismate mutase-prephenate dehydratase. *tyrA* encodes chorismate mutase-prephenate dehydrogenase. Tyrosine aminotransferase (the *tyrB* gene product) catalyzes the final step in both pathways with glutamate as the amino donor.

Figure 2. Growth curves of SR250 transformants. (Plus signs) Wild-type AATase, (solid squares) 8–2, (solid circles) HEX, (open squares) HEX + I73V, (solid diamonds) HEX + A293D, (open diamonds) HEX + I73V/A293D, (open circles) vector. (A) Selective media lacking tyrosine. (B) Selective media lacking aspartate.

of the HEX + A293D gene into the HEX + I73V clone. The resultant HEX + I73V/A293D mutant supports growth at rates that are similar to those of HEX + A293D on media lacking tyrosine, but remarkably does not support growth on aspartate-free media.

In vitro characteristics of HEX variants

The steady-state kinetic parameters for the reactions of Tyr, Asp, and α KG with HEX and the three derivative enzymes are shown in Table 4. Values for the wild-type *E. coli* AATase and TATase and for the 8-2 enzyme are included for comparison. The reactions of the HEX variants with tyrosine are characterized by 2.5- to 4.5-fold increases in $k_{\text{cat}}/K_m^{\text{Tyr}}$ over the original HEX. $k_{\text{cat}}/K_m^{\text{Tyr}}$ for HEX + I73V/A293D is 300-fold greater than that for wild-type AATase, and is within fourfold of the wild-type *E. coli* TATase value. $k_{\text{cat}}^{\text{Tyr}/\alpha\text{KG}}$ (defined as k_{cat} for the transamination reaction of the substrates denoted in the numerator) changes by less than twofold in any of the HEX variants.

Mutations A293D and I73V elicited larger effects on the kinetic parameters for dicarboxylic substrates Asp and α KG than for tyrosine. Values for $k_{\text{cat}}^{\text{Asp}/\alpha\text{KG}}$, K_m^{Asp} , and $K_m^{\alpha\text{KG}}$ are increased in each of the HEX derivatives compared to HEX, and they are closer to the corresponding values for wild-type AATase and TATase. The replacement A293D in HEX increases these three parameters over 20-fold, restoring values in HEX + A293D to within 3.5-fold of the original values in AATase. The I73V replacement elicits smaller changes of only 1.5- to 3.5-fold. $k_{\text{cat}}^{\text{Asp}/\alpha\text{KG}}$, K_m^{Asp} , and $K_m^{\alpha\text{KG}}$ values for the double mutant, HEX + I73V/A293D, are greater than those for either single mutant. The mutation A293D or I73V alters the parameter $k_{\text{cat}}/K_m^{\text{Asp}}$ by less than twofold.

The dissociation constant for maleate, an aspartate analog, was determined for the HEX complex and derivatives. The HEX complex has a very low value of K_D^{Mal} , which is 25-fold and 200-fold, respectively, less than the corresponding wild-type AATase and TATase values. The A293D mutation results in a >100-fold reduction in maleate affinity. The K_D value is close to that of wild-type TATase. The I73V mutation elicits only a small change in K_D^{Mal} , similar to the 1.5- to threefold increases observed in K_m values for dicarboxylic substrates. The K_D^{Mal} for HEX + I73V/A293D is close to that the HEX + A293D mutant.

An attempt to increase Tyr/Phe reactivity of HEX via further rational redesign was also undertaken. A HEX derivative with targeted substitutions P141E and A293R was purified and

Table 3. Growth phenotypes of wild-type and mutant aminotransferases

	-Tyr ^a		-Asp ^a	
	t_d^b (hr)	Colony size ^c	t_d^b (hr)	Colony size ^c
<i>Aminotransferase</i>				
HEX	1.54 (0.02)	–/+	4.2 (0.2)	–/–
HEX + I73V	1.42 (0.02)	+/+	4.1 (0.2)	–/–
HEX + A293D	0.98 (0.04)	++/++	1.92 (0.07)	+/+
HEX + I73V/A293D	0.96 (0.02)	++/++	11.0 (0.7)	–/–
Wild-type AATase	1.25 (0.03)	+/+	1.26 (0.04)	+++/++
8–2	1.15 (0.03)	+++/++	1.49 (0.07)	++/++
Vector	n.g.	–/–	n.g.	–/–

(n.g.) No growth observed.

^a SR250 transformants were monitored for growth rate at 37°C in defined media (described in Materials and Methods) lacking either Tyr or Asp. Tyrosine free media were supplemented with 0.17 mM HPP.

^b Cells from overnight cultures were diluted into selective liquid media. Growth was monitored over time by A_{600} (Fig. 2). Doubling times (t_d) were calculated by nonlinear regression fitting to an exponential growth function.

^c Cells from overnight cultures were streaked onto selective agar plates. Scoring is based on the relative colony sizes after 24 h growth. Scores range from – (no visible single colonies) to +++ (large colonies). Duplicate experiments—separated by a “/”—were performed with different SR250 transformants for each clone.

Table 4. Steady-state kinetic parameters and maleate complex dissociation constants for wild-type and mutant aminotransferases

	Tyr ^a			Asp ^a			αKG ^a	Maleate ^b
	k_{cat}/K_m (M ⁻¹ s ⁻¹) × 10 ⁻²	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/k_m (M ⁻¹ s ⁻¹) × 10 ⁻²	k_{cat} (s ⁻¹)	K_m (mM)	K_m (mM)	K_D (mM)
<i>Aminotransferase</i>								
Wild-type	5.50	n.s.	n.s.	910 ^c	159 ^c	1.75 ^c	0.47 ^{c,d}	19 ^g
AATase	(0.03)							
HEX	370	24	0.65	260	5.1	0.20	0.038	0.68
	(10)	(0.4)	(0.03)	(10)	(0.1)	(0.01)	(0.004)	(0.05)
HEX + I73V	880	25.6	0.29	440	16.8	0.39	0.061	1.63
	(60)	(0.6)	(0.03)	(20)	(0.3)	(0.01)	(0.003)	(0.07)
HEX + A293D	1200	39	0.33	200	105	5.3	0.8	170
	(200)	(2)	(0.07)	(20)	(4)	(0.6)	(0.1)	(30)
HEX +	1670	35.7	0.21	121	112	9	2.4	200
I73V/A293D	(70)	(0.5)	(0.01)	(9)	(4)	(1)	(0.2)	(40)
8-2 ^e	410	17.8	0.43	294	32.9	1.11	n.d.	n.d.
	(2)	(0.3)	(0.02)	(8)	(0.6)	(0.07)		
Wild-type	6500 ^f	250 ^f	0.32 ^f	370 ^f	140 ^f	3.8 ^f	1.3 ^f	140 ^g
TATase								(10)

(n.s.) No saturation.

(n.d.) Not determined.

^a Assay conditions for original kinetic data: 200 mM TAPS (pH = 8.0), 140 mM KCl, 0.15 mM NADH, 20 μM PLP, 25°C, 2–40 mM αKG for Tyr and Asp assays, 2 mM Tyr for αKG assays. [HO-HxoDH] = 0.3–3.0 μM for Tyr and αKG assays; [MDH] = 4–10 units/ml for Asp assays. Standard errors are in parentheses.

^b Conditions for K_D determinations: 200 mM TAPS (pH = 8.0), 140 mM KCl, ~15–30 μM enzyme, [Maleate] = ~0.1–200 mM

^c From Gloss et al. (1992).

^d Reported K_m value determined in reactions with aspartate and αKG.

^e From Rothman and Kirsch (2003).

^f From Hayashi et al. (1993).

^g From Onuffer and Kirsch (1995).

characterized. The rationale for choosing these two substitutions is provided in the Discussion. $k_{\text{cat}}/K_m^{\text{Phe}}$ and $k_{\text{cat}}/K_m^{\text{Asp}}$ for the HEX + P141E/A293R mutant are 11,000 M⁻¹sec⁻¹ and 7000 M⁻¹sec⁻¹, respectively. Both of these values are lower than the corresponding numbers exhibited by HEX.

Discussion

Comparison of HEX with wild-type and directly evolved aminotransferases

The original rational redesign of AATase was successful in expanding its substrate specificity to include aromatic amino and α-keto acids, thus providing an archetype for understanding the basis of dual substrate recognition in TATases. The HEX mutant, incorporating only six mutations, exhibits 6% of the activity of *E. coli* TATase as measured by the steady state parameter, $k_{\text{cat}}/K_m^{\text{Tyr}}$ (Table 4). This corresponds to 60% of the free energy of activation difference between the AATase and TATase reaction. The directly evolved variant, clone 8-2, exhibits similar activity towards tyrosine. Furthermore, both HEX and 8-2 enzyme retain substantial aspartate aminotransferase activity, which is characteristic of TATase. The X-ray structure of HEX provided experimental evidence for an arginine switch

mechanism for dual recognition of dicarboxylic and aromatic substrates (Scheme 4). This model was proposed to explain how natural TATases accommodate both types of substrates (Seville et al. 1988). Arginine 292 forms a salt bridge in the active site with the side-chain carboxyl of bound aspartate analogs in both AATases and TATases. Seville et al. (1988) postulated that the arginine side chain rotates out of the active site to provide access for aromatic substrates in TATases. Crystal structures of HEX depict the Arg292 side chain adopting this alternate position when Phe and Tyr analogs are bound (Malashkevich et al. 1995). A similar arginine conformational switch has since been observed in structures of the *Paracoccus denitrificans* TATase (Okamoto et al. 1998).

AATases and the *P. denitrificans* TATase reactions proceed through an induced fit mechanism (McPhalen et al. 1992; Jager et al. 1994; Okamoto et al. 1998). Substrate association elicits an overall conformational change from an open/inactive form to a closed/active state. The enzyme likely returns to the open conformation prior to release of product (Malashkevich et al. 1993). HEX, an engineered hybrid of *E. coli* AATase and TATase, exhibits some properties that are not found in either of the wild-type enzymes. Malashkevich et al. (1995) found that the HEX mutations preferentially stabilize the closed conformation to the point

Scheme 4. The arginine switch mechanism that allows recognition of both dicarboxylic and aromatic substrates by HEX and wild-type TATase (Malashkevich et al. 1995). Wild-type *E. coli* AATase and HEX have the Arg292 side chain in the up position shown by the solid line in both the unliganded and dicarboxylate liganded forms. Arg292 adopts the down conformation (dashed side chain) when HEX is presented with aromatic α -amino or α -keto acids. The I73V and particularly the A293D mutations may stabilize the down conformation, thus lowering affinity for dicarboxylic acid ligands and improving the recognition of aromatic substrates. It is possible that the introduced Asp293 forms the indicated salt bridge with the down conformation of Arg292, while the effect of the I73V change is the result of a loss of van der Waals contacts.

that it is observed even in the absence of ligands. A thermodynamic consequence of the shift towards the closed state is manifested in lower values K_m or K_D for aromatic and dicarboxylic acid ligand complexes of HEX relative to those of AATase (Onuffer and Kirsch 1995). There is also a substantial increase in the barrier to OAA dissociation for HEX as indicated by the combination of a lower steady-state $k_{\text{cat}}^{\text{Asp}/\alpha\text{KG}}$ value (Table 1) and increased sensitivity of certain kinetic parameters to viscosity in HEX compared to wild-type AATase (Fig. 1; Table 2). In contrast to HEX, the K_D and K_m values for dicarboxylic acid complexes are larger in the *E. coli* TATase relative to *E. coli* AATase, while K_m^{Asp} for the enzyme from clone 8-2 is relatively close to the corresponding value for wild-type AATase (Table 4).

The rate of OAA dissociation is ~60% rate determining for the reactions of HEX with aspartate and α KG (Table 2), indicating that at least one chemical step is also partially rate determining. Hydrolysis of the ketimine intermediate to form the E-PMP/OAA product complex (Scheme 2) is likely responsible for this remaining kinetic barrier because this step, similar to product dissociation, eludes kinetic detection under single turnover conditions (see Results). Malashkevich et al. (1993) postulated that ketimine hydrolysis and the overall conformational change of the enzyme from closed to open state occur coordinately. In this model, the open conformation disfavors the ketimine intermediate energetically. Increasing the fraction of the closed form of HEX would stabilize the ketimine, resulting in a slower rate for its hydrolysis in addition to an increased barrier for product release.

Directed evolution of HEX improves both its *in vivo* function and kinetic properties

Initial efforts to improve the TATase characteristics of the HEX mutant entailed additional rational redesign. Seville et al. (1988) suggested that two residues in the substrate binding pocket of *E. coli* TATase might facilitate the arginine switch required for binding of aromatic substrates. They proposed that the charges provided by Glu141 and Arg293 in TATase stabilize the conformation of Arg292 where the side chain is positioned away from the active site to allow access of aromatic ligands. The amino acids at positions 141 and 293 are proline and alanine respectively in *E. coli* AATase. The construct P141E/A293R in wild-type AATase, however, did not improve recognition of aromatic substrates (Kohler et al. 1994). On the possibility that these two mutations may prove effective in the HEX context, the substrate specificity of HEX + P141E/A293R was evaluated. However, this variant exhibits even lower k_{cat}/K_m values for Phe and Asp than those found for HEX (see Results).

Thus, the effect of substitutions P141E and A293R are likely context dependent, and several additional amino acid replacements in the surrounding regions may be required to elicit the proposed changes in specificity. The rational approach was therefore abandoned in favor of one of directed evolution, starting with HEX. The HEX mutant poorly complements tyrosine auxotrophy in aminotransferase deficient *E. coli*. A single iteration of DNA shuffling with HEX followed by selection for variants conferring faster growth was performed. The randomly generated mutations A293D, and to a lesser extent I73V, were found to improve the HEX phenotype. Interestingly, substitution A293V was observed in several clones, including 8-2, realized from the directed evolution of wild-type AATase for TATase function (Rothman and Kirsch 2003). I73T was also found in that experiment, but only in a single clone. None of these mutations represents changes at these positions to amino acids found in known TATases. Yet, I73V and A293D in particular enhance the kinetic parameters in HEX for reactions with tyrosine and with aspartate (Table 4). The affinity for dicarboxylic acid ligands is reduced by one to two orders in magnitude by the A293D mutation, easing the tight binding burden observed in HEX and thus increasing the rates for turnover. Further, every measured *in vitro* parameter for HEX + A293D and HEX + I73V/A293D is within sevenfold of the corresponding TATase value, a substantial improvement over HEX. It is improbable that purely rational redesign of HEX could have achieved this large effect with such a small number of mutations, particularly as the observed substitutions are absent in either wild-type sequence. Thus, a total of only seven amino acid replacements introduced into wild-type AATase (six rational and one from directed evolution) suffice to provide 75% ($k_{\text{cat}}/K_m^{\text{Tyr}}$) of the free energy of activation changes describing the differ-

ences between the catalytic characteristics of wild-type AATase and TATase ($\Delta\Delta G^\ddagger$ values were calculated from the data of Table 4). The wild-type enzymes are 43% identical and have 220 amino acid differences. Moreover, the HEX + A293D construct functions well in vivo.

A proposed structural role for the effects of substitutions I73V and A293D

Ile73 and Ala293 are in close proximity to Arg292 in X-ray structures of *E. coli* AATase. Amino acid replacements A293D and I73V, consequently, might alter substrate specificity via modulation of the energetics of the arginine switch, as had been originally proposed for substitutions P141E and A293R. A model for how the modifications could favor the conformation in which the Arg292 side chain is positioned away from the active site is shown in Scheme 4. Such preferential stabilization of this conformation can account for improved recognition of aromatic substrates, as less binding energy would be needed to elicit the repositioning. The change would also account for the reduced affinity for dicarboxylic ligands, which requires that the arginine side chain be positioned in the upper configuration for tight binding.

Mutations in evolved HEX variants might alleviate inhibition by dicarboxylic acids in vivo

The poor growth conferred by HEX in tyrosine free media was unexpected given the Tyr/Phe kinetic parameters for HEX compared to those for wild-type AATase. There are multiple possibilities as to why the rationally engineered enzyme confers poor fitness. Weak expression or poor stability may reduce levels of active enzyme in cells. However, yields of active enzyme with the high expression plasmids in strain MG204 following ~30 h growth at 37°C and subsequent protein purification were always similar for HEX and wild-type AATase (data not shown). Additionally, the yield from a HEX + A293D preparation based on its high expression plasmid was less than that for HEX. The HEX + I73V/A293D double mutant was generated via cloning of a fragment (encoding C-terminal protein) from HEX + A293D (strong growth) into the HEX + I73V plasmid (weak growth). This variant displays fitness on media lacking tyrosine substantially improved over HEX + I73V and similar to HEX + A293D. That fragment responsible for the enhancement contains only the A293D encoding substitution and a silent mutation at Glu234, a change from GAA to the less common codon GAG. These observations together would suggest that the HEX mutations do not impair expression or that the improved fitness in the HEX + A293D derivative is due to elevated levels of active aminotransferase. Enzymatic analyses of crude extracts were performed to directly compare active enzyme levels for the

wild-type AATase, HEX, and HEX + A293D in strain SR250 with low expression plasmids under conditions employed in growth analyses. However, expression levels were insufficient to detect any activities above background. A Western blot was also attempted to analyze expression, but the available AATase antibodies were of insufficient quality to detect low levels of expressed protein. Thus, while indirect evidence suggests that low expression of active enzyme is not likely the explanation for the particularly poor fitness exhibited by HEX relative to wild-type AATase and HEX + A293D, a definitive statement cannot be made.

We favor the interpretation that the particularly poor in vivo fitness of HEX is a consequence of intracellular metabolite concentrations that serve to inhibit HEX activity. In vivo TATase function requires efficient conversion of the intracellular pool of HPP to Tyr. This enzymatic reaction can be inhibited by competing dicarboxylic acids. α KG and OAA are both TCA cycle intermediates, and are thus maintained at relatively high concentrations. The total in vivo α KG concentration has been estimated to be 0.45 mM (Zhao and Winkler 1996). This is 10-fold higher than the $K_m^{\alpha\text{KG}}$ value of 0.038 mM found for HEX. A similar situation for OAA is expected. Thus, it is likely that the majority of HEX in vivo exists as an inactive α KG or OAA complex. Consistent with this model of in vivo inhibition are the observations that the improved HEX variants, particularly A293D, have sharply higher K_m and K_D values for dicarboxylic ligands (Table 4). $k_{\text{cat}}/K_m^{\alpha\text{KG}}$ is also reduced 10-fold in HEX + A293D relative to HEX (values not shown but are derived from $k_{\text{cat}}^{\text{Tyr}/\alpha\text{KG}}$ and $K_m^{\alpha\text{KG}}$). This correlation between dicarboxylic acid affinity and fitness would seem to provide a more compelling explanation for the in vivo results than the proposal that the A293D substitution enhances fitness through increased levels of active enzyme. The higher $k_{\text{cat}}/K_m^{\text{Tyr}}$ values in variants HEX + I73V and HEX + A293D may also be relevant to the improved phenotype. However, A293D confers a much greater growth advantage under selective conditions (Table 3) while the increases in $k_{\text{cat}}/K_m^{\text{Tyr}}$ are similar for the two mutants (Table 4). The poor fitness of the HEX + I73V/A293D double mutant in media lacking aspartate was an unexpected finding, but may result from K_m^{OAA} in the mutant possibly being larger than the intracellular OAA concentration.

The requirement for rapid catalysis of the transamination of aromatic keto acids in the presence of competing dicarboxylic keto acids thus provided a simultaneous selection for tyrosine activity and a counterselection against dicarboxylic acid ligand binding. It may thus be possible to narrow enzyme specificity generally via a strategy that takes advantage of effects realized by addition of competitive inhibitors or alternate substrates to the selection culture or to in vitro screens. This would be a potentially important embellishment to the directed evolution protocol as the most

commonly observed variants exhibit broader specificity than that found in their progenitors (Matsumura and Ellington 2001).

Materials and methods

Strains and plasmids

E. coli strain SR224 (Rothman and Kirsch 2003) has the genotype $\Delta(\text{pheA-tyrA-aroF})$, $\Delta(\text{argF-lacZYA})U169$, thi1 , endA1 , hsdR17 , supE44 , hpr^+ , $\Delta\text{tyrB::spc}^r$, $\Delta\text{aspC::tet}^r$, $\Delta\text{ilvE::kan}^r$. SR250 is isogenic to SR224, except for the following modifications: $\Delta\text{ilvE::gen}^r$ and recA::kan^r . *E. coli* strain MG204 [his23 , proB , trpA-605 , lacI3 , lacZ118 , gyrA , rpsL , $\Delta\text{aspC::kan}^r$, tyrB , ilvE , recA::m10] was a gift of Ian Fotheringham, Nutrasweet Corp. Plasmid pBAD18 was obtained from American Type Culture Collections. pHEX and pJO2, containing high expression versions of HEX and wild-type AATase, respectively, in pUC119, were generated by Jim Onuffer (Onuffer and Kirsch 1994, 1995). A low expression version of wild-type AATase in pBAD18 was prepared from pJO2 by Meghan Imrie. Clone 8-2, an evolved AATase variant, was available (Rothman and Kirsch 2003).

Directed evolution

DNA shuffling was performed according to the method of Stemmer (1994), as modified by Lorimer and Pastan (1995). The HEX gene was initially amplified from pHEX with primers 5'-CAGC TGGCGAAAGGGGATGTGCTGC-3' and 5'-GCTTTACACT TTATGCTTCCGGCTCGTATGTTGTGTGG-3'. The PCR step after reassembly employed primers 5'-AAAGGTACCGGAGT GCCTCGTCATGTTTGAGAACATTACCG-3' and 5'-AAAGC ATGCTTATTATTACAGCACTGCCACAATCGC-3'. Amplified products were cloned into the KpnI and SphI sites of pBAD18 to generate a library of HEX variants. Selection in strain SR224 was performed as described by Rothman and Kirsch (2003). Selective plates lacked tyrosine and were supplemented with 0.11–0.55 mM HPP. The sequences for clones conferring improved growth were determined by automated sequencing (UC Berkeley DNA Sequencing Facility or Elim Biopharmaceuticals, Hayward, CA).

Site-directed mutagenesis and subcloning

HEX + I73V/A293D was prepared by cloning a 0.6-kb fragment containing the A293D and N297S mutations from HEX + A293D into the NcoI and HindIII sites of the HEX + I73V. High expression versions of clones coding for HEX + I73V, HEX + A293D, and HEX + I73V/A293D were generated through PCR based site-directed mutagenesis of pHEX. Mutagenic fragments were cloned into the NcoI and either EcoRI or HindIII sites of pHEX. A low expression version of HEX was prepared via PCR amplification, employing the template pHEX and primers 5'-AAAGGTACCG GAGTGCCTCGTCATGTTTGAGAACATTACCG-3' and 5'-AA AGCATGCTTATTATTACAGCACTGCCACAATCGC-3'. The amplified product was cloned into the KpnI and SphI sites of pBAD18. A version of clone 8-2 suitable for growth analyses was prepared via subcloning the evolved gene into the KpnI and HindIII sites of pBAD18.

Enzyme purification and characterization

Wild-type AATase and mutants were overexpressed in aminotransferase deficient strain MG204. Transformed cells were grown ~30 h at 37°C in 2YT containing 0.1% pyridoxine and ampicillin (100 µg/mL). Proteins were purified as previously described (Rothman and Kirsch 2003). Steady-state kinetic parameters and maleate complex dissociation constants were determined as described by Shaffer et al. (2002). Transamination reactions were monitored in spectrophotometric HO-HxoDH or MDH coupled assays respectively at 340 nm, while maleate affinities were evaluated spectrophotometrically at 430 nm. The conditions are given in Tables 1 and 4. Solvent viscosity effects were measured under steady-state conditions according to Goldberg and Kirsch (1996; see Table 2 for conditions). Controls with variable concentrations of coupling enzymes were performed at 36% sucrose to verify that reaction rates were dependent only on aminotransferase activity.

Growth analysis

Growth analyses were performed in aminotransferase deficient strain SR250. Cells were transformed with pBAD18-based low-expression plasmids containing wild-type and mutant aminotransferase genes. Expression was under the control of an inducible arabinose promoter. Transformants were grown overnight at 37°C in 5 mL LB + ampicillin (100 µg/mL) + 0.2% arabinose. Stationary phase cells (1 mL) were washed two times in 1 mL of a M9 minimal salt solution (Sigma). Cells were diluted 100- to 500-fold (dilution based on A_{600} of 5 mL cultures) into 40 mL selective liquid media (in 125-mL flasks). Washed cells were also streaked on agar (15 g/L) plates containing the same media. The growth media were derived from M9c as described by Kast et al. (1996). The media consisted of M9 minimal salts (Sigma), thiamine (10 µg/mL), 4-aminobenzoic acid (5 µg/mL), 4-hydroxybenzoic acid (5 µg/mL), 2,3-dihydroxybenzoate (1.6 µg/mL), 0.4% glycerol, 0.2% arabinose, 0.1 mM CaCl_2 , 2 mM MgSO_4 , ampicillin (100 µg/mL), Phe (40 µg/mL) and Glu (40 µg/mL). Tyr was supplemented at 40 µg/mL in media lacking aspartate, while Asp was provided at 240 µg/mL in media lacking tyrosine. Other amino acids were supplemented at 20 µg/mL.

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References

- Bornscheuer, U.T. and Pohl, M. 2001. Improved biocatalysts by directed evolution and rational protein design. *Curr. Opin. Chem. Biol.* **5**: 137–143.
- Brouwer, A.C. and Kirsch, J.F. 1982. Investigation of diffusion-limited rates of chymotrypsin reactions by viscosity variation. *Biochemistry* **21**: 1302–1307.
- Chen, R. 2001. Enzyme engineering: Rational redesign versus directed evolution. *Trends Biotechnol.* **19**: 13–14.
- Chen, R., Greer, A.F., and Dean, A.M. 1997. Structural constraints in protein

- engineering—The coenzyme specificity of *Escherichia coli* isocitrate dehydrogenase. *Eur. J. Biochem.* **250**: 578–582.
- Cherry, J.R., Lamsa, M.H., Schneider, P., Vind, J., Svendsen, A., Jones, A., and Pedersen, A.H. 1999. Directed evolution of a fungal peroxidase. *Nat. Biotechnol.* **17**: 379–384.
- Gloss, L.M., Planas, A., and Kirsch, J.F. 1992. Contribution to catalysis and stability of the five cysteines in *Escherichia coli* aspartate aminotransferase. Preparation and properties of a cysteine-free enzyme. *Biochemistry* **31**: 32–39.
- Goldberg, J.M. and Kirsch, J.F. 1996. The reaction catalyzed by *Escherichia coli* aspartate aminotransferase has multiple partially rate-determining steps, while that catalyzed by the Y225F mutant is dominated by ketimine hydrolysis. *Biochemistry* **35**: 5280–5291.
- Hayashi, H., Inoue, K., Nagata, T., Kuramitsu, S., and Kagamiyama, H. 1993. *Escherichia coli* aromatic amino acid aminotransferase: Characterization and comparison with aspartate aminotransferase. *Biochemistry* **32**: 12229–12239.
- Jager, J., Moser, M., Sauder, U., and Jansonius, J.N. 1994. Crystal structures of *Escherichia coli* aspartate aminotransferase in two conformations. Comparison of an unliganded open and two liganded closed forms. *J. Mol. Biol.* **239**: 285–305.
- Kast, P., Asif-Ullah, M., Jiang, N., and Hilvert, D. 1996. Exploring the active site of chorismate mutase by combinatorial mutagenesis and selection: The importance of electrostatic catalysis. *Proc. Natl. Acad. Sci.* **93**: 5043–5048.
- Kohler, E., Seville, M., Jager, J., Fotheringham, I., Hunter, M., Edwards, M., Jansonius, J.N., and Kirschner, K. 1994. Significant improvement to the catalytic properties of aspartate aminotransferase: Role of hydrophobic and charged residues in the substrate binding pocket. *Biochemistry* **33**: 90–97.
- Lorimer, I.A. and Pastan, I. 1995. Random recombination of antibody single chain Fv sequences after fragmentation with DNaseI in the presence of Mn²⁺. *Nucleic Acids. Res.* **23**: 3067–3068.
- Luong, T.N. and Kirsch, J.F. 1997. A continuous coupled spectrophotometric assay for tyrosine aminotransferase activity with aromatic and other non-polar amino acids. *Anal. Biochem.* **253**: 46–49.
- . 2001. A general method for the quantitative analysis of functional chimeras: Applications from site-directed mutagenesis and macromolecular association. *Protein Sci.* **10**: 581–591.
- Malashkevich, V.N., Toney, M.D., and Jansonius, J.N. 1993. Crystal structures of true enzymatic reaction intermediates: Aspartate and glutamate ketimines in aspartate aminotransferase. *Biochemistry* **32**: 13451–13462.
- Malashkevich, V.N., Onuffer, J.J., Kirsch, J.F., and Jansonius, J.N. 1995. Alternating arginine-modulated substrate specificity in an engineered tyrosine aminotransferase. *Nat. Struct. Biol.* **2**: 548–553.
- Matsumura, I. and Ellington, A.D. 2001. In vitro evolution of β -glucuronidase into β -galactosidase proceeds through non-specific intermediates. *J. Mol. Biol.* **305**: 331–339.
- McPhalen, C.A., Vincent, M.G., Picot, D., Jansonius, J.N., Lesk, A.M., and Chothia, C. 1992. Domain closure in mitochondrial aspartate aminotransferase. *J. Mol. Biol.* **227**: 197–213.
- Okamoto, A., Nakai, Y., Hayashi, H., Hirotsu, K., and Kagamiyama, H. 1998. Crystal structures of *Paracoccus denitrificans* aromatic amino acid aminotransferase: A substrate recognition site constructed by rearrangement of hydrogen bond network. *J. Mol. Biol.* **280**: 443–461.
- Onuffer, J.J. and Kirsch, J.F. 1994. Characterization of the apparent negative co-operativity induced in *Escherichia coli* aspartate aminotransferase by the replacement of Asp222 with alanine. Evidence for an extremely slow conformational change. *Protein Eng.* **7**: 413–424.
- . 1995. Redesign of the substrate specificity of *Escherichia coli* aspartate aminotransferase to that of *Escherichia coli* tyrosine aminotransferase by homology modeling and site-directed mutagenesis. *Protein Sci.* **4**: 1750–1757.
- Rothman, S.C. and Kirsch, J.F. 2003. How does an enzyme evolved in vitro compare to naturally occurring homologs possessing the targeted function? Tyrosine aminotransferase from aspartate aminotransferase. *J. Mol. Biol.* **327**: 593–608.
- Seville, M., Vincent, M.G., and Hahn, K. 1988. Modeling the three-dimensional structures of bacterial aminotransferases. *Biochemistry* **27**: 8344–8349.
- Shaffer, W.A., Luong, T.N., Rothman, S.C., and Kirsch, J.F. 2002. Quantitative chimeric analysis of six specificity determinants that differentiate *Escherichia coli* aspartate from tyrosine aminotransferase. *Protein Sci.* **11**: 2848–2859.
- Stemmer, W.P. 1994. DNA shuffling by random fragmentation and reassembly: In vitro recombination for molecular evolution. *Proc. Natl. Acad. Sci.* **91**: 10747–10751.
- Zhao, G. and Winkler, M.E. 1996. A novel α -ketoglutarate reductase activity of the serA-encoded 3-phosphoglycerate dehydrogenase of *Escherichia coli* K-12 and its possible implications for human 2-hydroxyglutaric aciduria. *J. Bacteriol.* **178**: 232–239.