Role of Arginine-292 in the Substrate Specificity of Aspartate Aminotransferase As Examined by Site-Directed Mutagenesis†

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ABSTRACT: X-ray crystallographic data have implicated Arg-292 as the residue responsible for the preferred side-chain substrate specificity of aspartate aminotransferase. It forms a salt bridge with the β or γ carboxylate group of the substrate [Kirsch, J. F., Eichele, G., Ford, G. C., Vincent, M. G., Jansonius, J. N., Gehring, H., & Christen, P. (1984) *J. Mol. Biol. 174*, 497-525]. In order to test this proposal and, in addition, to attempt to reverse the substrate charge specificity of this enzyme, Arg-292 has been converted to Asp-292 by site-directed mutagenesis. The activity (k_{cat}/K_M) of the mutant enzyme, R292D, toward the natural anionic substrates L-aspartate, L-glutamate, and α-ketoglutarate is depressed by over 5 orders of magnitude, whereas the activity toward the keto acid pyruvate and a number of aromatic and other neutral amino acids is reduced by only 2-9-fold. These results confirm the proposal that Arg-292 is critical for the rapid turnover of substrates bearing anionic side chains and show further that, apart from the desired alteration, no major perturbations of the remainder of the molecule have been made. The activity of R292D toward the cationic amino acids L-arginine, L-lysine, and L-ornithine is increased by 9-16-fold over that of wild type and the ratio $(k_{cat}/K_M)_{cationic}/(k_{cat}/K_M)_{anionic}$ is in the range 2-40-fold for R292D, whereas this ratio has a range of $[(0.3-6) \times 10^{-6}]$-fold for wild type. Thus, the mutation has produced an inversion of the substrate charge specificity. Possible explanations for the less-than-expected reactivity of R292D with arginine, based in part on the preliminary X-ray crystallographic data of D. Ringe and D. Smith, are discussed.

Aspartate aminotransferase (AATase;[1] EC 2.6.1.1) catalyzes the reversible transamination of α-amino and α-keto acids. The enzyme operates solely via binary enzyme–substrate complexes and utilizes bound pyridoxal 5′-phosphate (PLP) as an amino group carrier [see Braunstein (1973)].

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

Although AATase is active toward aromatic amino/keto acids, the preferred substrates for reaction are the dicarboxylic amino acids L-aspartate and L-glutamate and their respective keto acids, oxaloacetate and α-ketoglutarate.

The amino acid sequences of both the mitochondrial and cytoplasmic isozymes from a number of eukaryotes have been determined [e.g., Doonan et al. (1975), Barra et al. (1980), and Graf-Hausner et al. (1983)] as well as that of the *Escherichia coli* enzyme (Kondo et al., 1987). A comparison of the primary sequences indicates that all active-site residues present in the eukaryotic enzymes, identified by X-ray crystallography (Ford et al., 1980; Arnone et al., 1984; Harutunyan et al., 1984; Jansonius et al., 1985; Jansonius & Vincent, 1987), are also present in the prokaryotic enzyme. Preliminary crystallographic data have been obtained with the *E. coli* AATase (Smith et al., 1986), and a recently obtained 3.0-Å map shows the same general folding pattern as obtains for the eukaryotic isozymes (D. L. Smith and D. Ringe, personal communication). The recent cloning of the *E. coli* AATase gene in this (Malcolm & Kirsch, 1985) and other laboratories (Kuramitsu et al., 1985; Fotheringham et al., 1986) as well as that coding for the mouse isozymes (Obaru et al., 1986) provides the opportunity of using the technique of site-directed mutagenesis to examine specifically the roles of those active-site residues that have been suggested, on the basis of crystallographic, kinetic, and other data, to be involved in the catalytic mechanism of the enzyme [see Kirsch et al. (1984)]. The production of the first of these mutant AATases in which the active-site Lys-258 has been replaced with alanine has been reported (Malcolm & Kirsch, 1985).

The binding of dicarboxylic amino/keto acids to AATase is believed to involve the electrostatic neutralization of the side-chain and α-carbon carboxylate groups of each substrate through their interaction with two arginine residues, Arg-292[2] and Arg-386, respectively, that are located at the entrance to active site (Ford et al., 1980; Arnone et al., 1984; Harutunyan et al., 1984; Jansonius et al., 1985). In order to define the role of Arg-292 in the substrate specificity of AATase and to attempt to invert the substrate charge specificity of the enzyme, Arg-292 has been replaced with an aspartate residue by site-directed mutagenesis. A stereo model of L-aspartate bound to the active site of wild-type AATase, based on the coordinates supplied by J. N. Jansonius of the maleate complex with the mitochondrial enzyme, is shown in Figure 1A. Figure 1B shows the modeled structure of the mutated enzyme with Asp-292 replacing Arg-292 and with L-arginine bound in the Michaelis complex. The kinetic properties of this mutant

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[1] Abbreviations: AATase, aspartate aminotransferase (EC 2.6.1.1); R292D AATase, aspartate aminotransferase with arginine-292 replaced with aspartate; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; TRIZMA, tris(hydroxymethyl)aminomethane; MDH, malate dehydrogenase (EC 1.1.1.37); IPTG, isopropyl β-D-thiogalactopyranoside; SDS, sodium dodecyl sulfate.

[2] The residue number is that of the pig cytoplasmic AATase sequence, in keeping with previously accepted convention (Ford et al., 1980).

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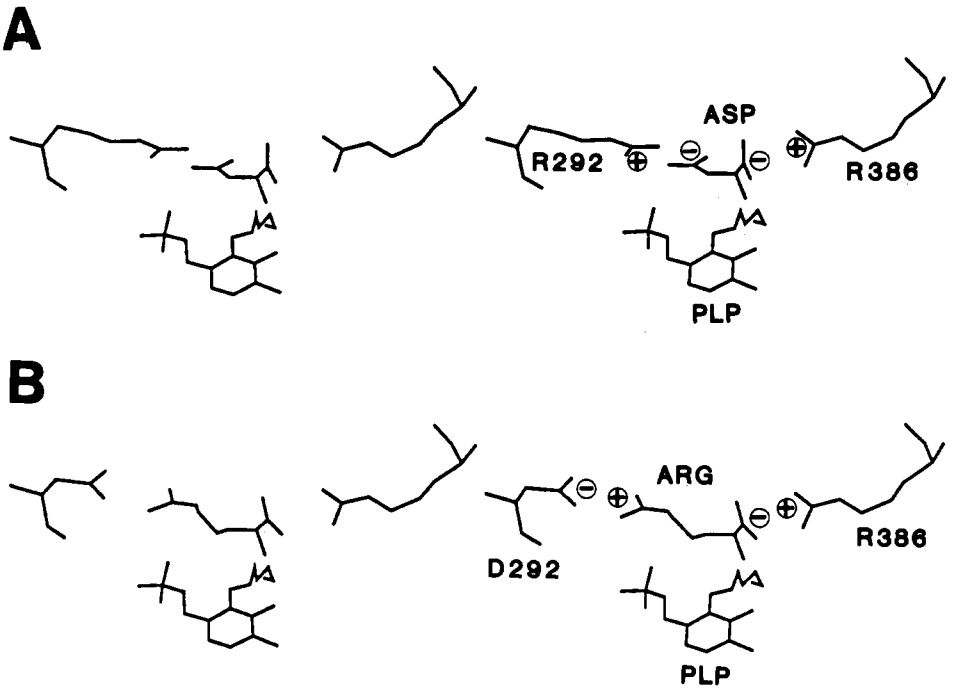

FIGURE 1: (A) Stereoview of the active-site structure of chicken heart mitochondrial AATase with bound L-aspartate and (B) a computer graphics model of the hypothetical active-site structure of *E. coli* R292D AATase with L-arginine bound. The structure of the chicken enzyme, determined at 2.8-Å resolution (J. N. Jansonius, personal communication), is shown with aspartate superimposed on the location found for the competitive inhibitor maleate.

AATase are reported. Part of this work has been described previously (Cronin et al., 1987).

Experimental Procedures

Materials. L-Aspartic acid, L-glutamic acid hydrochloride, L-citrulline, L-ornithine hydrochloride, L-arginine hydrocloride, L-lysine hydrochloride, L-histidine, L-tyrosine, L-phenylalanine, L-serine, L-glutamine, L-alanine, L-valine, L-leucine, L-isoleucine, L-proline, succinic acid, L-malic acid, α-ketoglutaric acid, sodium pyruvate, tetracycline hydrochloride, ampicillin, β-mercaptoethanol, thiamin hydrochloride, nitroblue tetrazolium, phenazine methosulfate, phenylglyoxal, PLP, PMP hydrochloride, CHES, TAPS, TRIZMA, and pig heart cytoplasmic MDH were obtained from Sigma, and D-glucose was purchased from Mallinckrodt Inc. DEAE-Sephacel, Polybuffer exchanger 94, and polybuffer 74 were purchased from Pharmacia, and Ultrogel AcA 34 was from LKB. L-Cysteine sulfinic acid, 5′-sulfosalicylic acid, and 1,2-cyclohexanedione were obtained from Aldrich.

Site-Directed Mutagenesis and Cell Growth. The preparation of the R292D mutant AATase has been described (Cronin et al., 1987). The wild-type and mutant-containing clones were grown to stationary phase in LB medium,pH 7.0–7.5, supplemented with the following: 10.5 mM D-glucose, 56 μM thiamin, 0.4 mM each of L-tyrosine and L-phenylalanine, 0.8 mM each of L-aspartate, L-proline, L-valine, L-leucine, L-isoleucine, and L-arginine, 28.5 mM each of succinate and L-malate, 9.5 mM α-ketoglutarate, 25 μM tetracycline, and 140 μM ampicillin.

Enzyme Assays and Kinetics. AATase activity was measured throughout the purification procedure by coupling the reaction product oxaloacetate to the oxidation of NADH by using MDH.

Reactions were carried out at 25 °C in 50 mM TRIZMA-HCl buffer, pH 8.10, 5 mM α-ketoglutarate, 100 mM L-aspartate, 6.3 mM ammonium sulfate, 0.15 mM NADH, and 10 units/mL MDH. The decrease in the absorbance at 340 nm was recorded by using a Cary 118 spectrophotometer. Reactions were initiated with L-aspartate after the blank rate, if any, was recorded. One unit of enzyme activity is defined as that amount of enzyme required for the formation of 1 μmol of product in 1 min under the above conditions.

The substrate specificities of the purified enzymes were determined under single-turnover conditions by monitoring the spectral characteristics of the enzyme-bound cofactor. Unless otherwise indicated, these reactions were conducted at 25 °C in 100 mM TAPS–KOH buffer, pH 8.50, containing 1 mg/mL AATase (0.5 mg/mL in stopped-flow studies), substrate, and sufficient KCl to generate $\mu = 1.0$. With the exception of tyrosine, which was titrated to pH 10.0, all substrates were titrated to pH 8.50 with KOH before use. The amino acid to keto acid half-reaction was followed by monitoring the absorbance increase at 330 nm due to the formation of enzyme-bound PMP, whereas the reverse reaction, which was conducted in the presence of 10 μM additional PMP, was followed by monitoring the increase at 360 nm due to PLP formation. The PMP forms of the enzymes used were those generated by reaction of the PLP enzyme forms with the tested amino acids. The PMP enzymes were reconcentrated before use to 10 mg/mL and dialyzed against 2 L of 5 mM potassium phosphate buffer, pH 7.0, containing 100 μM PMP with three buffer changes. The majority of reactions were studied by using a Perkin-Elmer Lambda 4B spectrophotometer and were initiated by the addition of enzyme. Reaction rate constants were determined by fitting the data to a single-exponential time course by nonlinear regression. The reactions of AATase with rapidly reacting substrates were studied by using a Union-Giken RA-401 stopped-flow spectrophotometer with a 1-cm path length cuvette and a dead time of less than 2 ms. The apparatus was interfaced to an OLIS 3820 data system (On-Line Instrument Systems) for data collection, storage, and subsequent curve fitting. The enzyme and substrate under test were placed in separate reservoirs, each containing 100 mM TAPS–KOH buffer, pH 8.50, and sufficient KCl to give $\mu = 1.0$. The reaction progress curves in all cases gave excellent fits to a single-exponential time course. Values of

k_{cat}/K_M were determined by linear regression for reactions that showed a linear dependence of rate on substrate concentration, whereas such values for reactions that indicated enzyme saturation by substrate were determined by fitting the data to a rectangular hyperbola using a computer program based on the method of Wilkinson (1961), which also provided values of K_M^{app} and the maximum turnover rate. Weighting factors [1/(standard error)^2] were used in all cases.

Chromatofocusing. A 13 × 0.9 cm column containing degassed Polybuffer exchanger 94 was equilibrated with a degassed solution of 25 mM piperazine hydrochloride buffer, pH 5.5, at 4 °C. Prior to the application of the protein sample, 5 mL of the developing buffer (Polybuffer 74 diluted 10-fold, adjusted to pH 3.85 with HCl, and degassed) was passed through the column. The sample (5 mL of 1 mg/mL purified R292D that had been dialyzed against 2 L of degassed equilibrating buffer) was applied and the column processed with 12 bed volumes (96 mL) of developing buffer at a flow rate of 10 mL/h. Fractions of 1 mL were collected in plastic minifuge tubes.

Gel Electrophoresis. Electrophoresis on native gels was performed by using a modification of the procedure described by Gelfand and Steinberg (1977). Slab gels were composed of 12% acrylamide, 0.32% bis(acrylamide), 0.38 M TRIZMA-HCl buffer, pH 8.30, 0.05% ammonium persulfate, and 0.0004% *N,N,N′,N′*-tetramethylethylenediamine. Gels were prerun for 1 h at 4 °C and 45 mA by using 0.38 M TRIZMA-HCl, pH 8.30, as buffer. Electrophoresis was carried out at 4 °C in 1 mM TRIZMA and 77 mM glycine, pH 8.30, and at a constant current of 45 mA for 3–4 h. Gels were stained for aminotransferase activity at 37 °C in the dark as described by Yagi et al. (1981). SDS–polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) using 12.5% slab gels. Protein staining of native and denaturing gels was carried out as follows: Gels were fixed by immersion in a solution containing 3.5% (w/v) 5′-sulfosalicylic acid, 11.5% (w/v) trichloroacetic acid for 30 min followed by protein staining in a solution containing 25% (v/v) ethanol, 8% (v/v) glacial acetic acid, and 0.12% (w/v) Coomassie Brilliant Blue R250 at 60 °C for 15 min. Gels were destained at 60 °C by using several changes of the previous solution without dye.

Enzyme Titrations. All enzyme titrations were performed at 25 °C by using a Cary 118 spectrophotometer. Purified enzymes (2.5 mg/mL) were dialyzed overnight against 2 L of 5 mM CHES–KOH buffer, pH 9.5, containing either 97 mM KCl (μ = 0.10) or 1.0 M KCl (μ = 1.0), at 4 °C. Following dialysis, the enzymes were diluted to a protein concentration of 0.40 mg/mL with the postdialysis buffer. Two milliliters of each solution was titrated by the addition, while stirring, of small aliquots (2.5–80 μL) of 6 mM sodium acetate, μ = 0.1 or 1.0 with KCl as appropriate, that had been adjusted to pH 3.75 with acetic acid. Following each addition of acid the pH was recorded and the spectrum (500–300 nm) obtained immediately. The pH electrode was not rinsed between scans, and the changes in absorbance were corrected for dilution. Data were recorded throughout the pH range 9.5–6.0.

Protein Determinations. The concentration of purified AATase was determined by using A_{205} (1 mg/mL) = 31 (Scopes, 1974).

Purification of Wild-Type and R292D AATases. All steps were performed at 4 °C. The same procedure was used to purify both the wild-type and the mutant enzymes. Where practical, solutions were surrounded by aluminum foil to protect against photodegradation of PLP.

Cells were lysed by suspending 10–12 g of cell paste in 25 mL of 20 mM potassium phosphate buffer, pH 7.0, containing 20 μM PLP (buffer A) and 5 mM β-mercaptoethanol by using a Dounce homogenizer. The suspension was sonicated by using a SONIFER W185D cell disruptor at setting 6 for 10-s periods with 10-s intervals for a total time of 10 min, followed by centrifugation at 13000*g* for 10 min. The supernatant was saved and the pellet resuspended, sonicated, and centrifuged as described above. The supernatants were combined and adjusted to 30% saturation with respect to ammonium sulfate (16.4 g/100 mL) by addition of the solid over a 10-min period. The suspension was stirred for 30 min and then centrifuged at 13000*g* for 30 min and the pellet discarded. The supernatant was adjusted to 75% saturation with respect to ammonium sulfate (28.5 g/100 mL) and, after stirring for 40 min, was centrifuged as described above. The final supernatant was discarded. The pellet was resuspended in approximately 10 mL of buffer A by using a Dounce homogenizer and was applied to a 70 × 2.6 cm column of Ultrogel AcA 34 that had been equilibrated previously with buffer A and processed at a flow rate of 1 mL/min by using the same buffer. The location of eluted wild-type AATase was determined by the MDH-coupled activity assay, whereas R292D was located either by subjecting the yellow eluate fractions to SDS–polyacrylamide gel electrophoresis or by assaying these fractions for the contaminating AATase activity (see below), which elutes in a position identical with that for R292D, using the MDH-coupled assay. The peak enzyme fractions were pooled and diluted with an equal volume of 20 mM sodium acetate and 20 μM PLP adjusted to pH 4.9 with acetic acid (buffer B). The resulting suspension was titrated to pH 4.9 with acetic acid while stirring briskly. After being allowed to stand for 10 min, the solution was centrifuged at 13000*g* for 30 min to remove precipitated material.

The supernatant from the previous step was pumped through an 8 × 1.3 cm column of DEAE-Sephacel that had been equilibrated previously with buffer B, at a flow rate of 1 mL/min. The column was washed with 500 mL of buffer B prior to the elution of AATase with a linear gradient of 0–0.3M NaCl (2 × 225 mL) in buffer B. Following SDS–polyacrylamide gel electrophoresis of the yellow eluate fractions, the peak enzyme fractions were pooled, concentrated to approximately 10 mg/mL, and dialyzed overnight against 2 L of 5 mM potassium phosphate buffer, pH 7.0, containing 20 μM PLP (buffer C). The enzyme solution was dialyzed for a total of 6 h against 2 L of buffer C without PLP and with one buffer change. The purified enzymes were stored at 10 mg/mL in plastic vials at 4 °C and were surrounded by aluminum foil to protect against photodegradation of enzyme-bound PLP.

RESULTS

Purification of AATases. The specific activities of the wild-type and R292D mutant AATases, when purified by the methods outlined under Experimental Procedures, were in the ranges 200–225 and 1.6–2.3 units/mg, respectively, using the MDH-coupled activity assay. The recovery of either enzyme was 60–65% with a yield of 10–20 mg of purified protein/g of cell paste. The purified preparations migrated identically during SDS–polyacrylamide gel electrophoresis and appeared homogeneous by this criterion (data not shown).

Initial examination of the rate of transamination of L-aspartate by the R292D enzyme using the MDH-coupled assay gave an apparent k_{cat} value of 1.7 s^{-1}. This value is 60-fold greater than the value of k_{obsd} measured under single-turnover

Table I: Spectrophotometric Titration of the Internal Aldimine of Wild-Type and R292D Mutant Aspartate Aminotransferases[a]

enzyme	ionic strength	pK_a (±SE)[b]
wild type	0.1	6.74 ± 0.03
	1.0	7.16 ± 0.05
R292D	0.1	7.19 ± 0.04
	1.0	7.44 ± 0.03

[a] The PLP forms of both wild-type and R292D AATases were titrated through the pH range 9.5–6.0 as described under Experimental Procedures. [b] The increase in absorbance at 430 nm with decreasing pH, due to protonation of the internal Schiff base aldimine, was fitted by nonlinear regression to a single ionization according to $Abs = [(Abs_f - Abs_i)/(1 + 10^{pH-pK})] + Abs_i$, where Abs_i and Abs_f are the initial and final absorbance values, respectively.

conditions by direct spectrophotometric monitoring of the rate of conversion of enzyme-bound PLP to PMP (0.03 s^{-1}), at a similar concentration of L-aspartate. The excess steady-state activity was not resolvable from the R292D enzyme on nondenaturing native polyacrylamide gels but could be partially separated by chromatofocusing, as shown in Figure 2. The major protein peak absorbing at 280 nm is coincident with the 430-nm peak, indicative of the protonated internal aldimine, and contains the R292D protein. The lack of a shoulder on the 280-nm peak shown in Figure 2 indicates that the detectable MDH-coupled AATase activity constitutes only a minor fraction of the total protein. To avoid complications arising from the contaminating activity, all subsequent kinetic studies with the R292D enzyme were performed as single-turnover experiments by monitoring the pyridoxal chromophore. This procedure also facilitated the need to examine the many possible substrates for this enzyme. The wild-type enzyme was studied in a similar manner, in order to provide a direct comparison. The data so obtained with either enzyme preparation conformed excellently to monophasic pseudo-first-order time courses, indicating further that the contaminating activity must constitute a very small fraction of the total protein.

pH Titration of AATases. The pK_a values of the internal aldimine Schiff base in the wild-type and R292D enzymes were determined by titrating the proteins directly (see Experimental Procedures) at both low (μ = 0.10) and high (μ = 1.0) ionic strength. The data obtained are summarized in Table I. In each case absorption maxima at 350–352 and 427–430 nm, due respectively to the unprotonated and protonated forms of the internal aldimine, were observed with an isosbestic point at 378–381 nm.

Substrate Specificities. The lower specific activity of the R292D enzyme, compared with wild type, toward anionic amino acid substrates and the low activity of both enzymes toward cationic amino acids required the use of high concentrations of these substrates during kinetic studies. Irregular

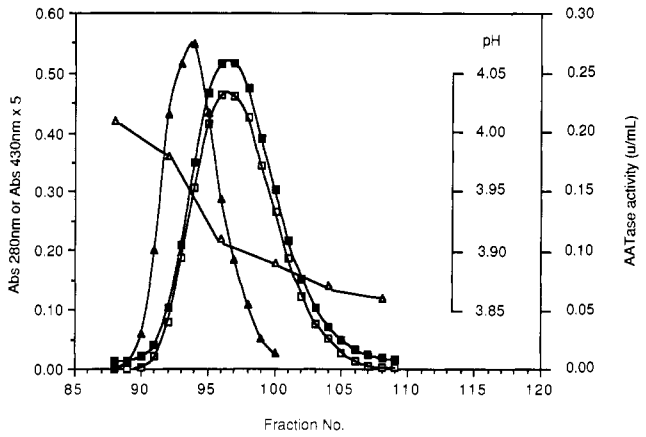

FIGURE 2: Chromatofocusing of 5 mg of purified R292D mutant AATase on Polybuffer exchanger 94 in the pH range 5.50–3.85 was performed as described under Experimental Procedures. Only those fractions from the pH gradient showing significant absorption at 280 nm are shown. Fractions were assayed for MDH-coupled AATase activity (recovery was 106%) (▲), pH (△), and absorption at 280 nm (■) and 430 nm (□).

results, obtained when the substrate contribution to the ionic strength was not taken into consideration, necessitated conducting the kinetic studies at a constant high ionic strength (μ = 1.0 maintained with potassium chloride).

The kinetic properties of the wild-type and R292D enzymes with charged amino acids as substrates are summarized in Table II. The mutation results in a reduction in the specificity constant, k_{cat}/K_M, by over 5 orders of magnitude toward amino acids carrying anionic side chains, whereas this parameter is increased toward all tested amino acids bearing a cationic side chain. The finding that the k_{cat}/K_M values for R292D toward cationic amino acids are greater than those toward anionic amino acids reveals an inversion in substrate charge preference. The kinetics of R292D and wild-type deamination of L-arginine show some indication of saturation at higher concentrations of this substrate (Figure 3). Whether this represents a specific salt effect or true saturation has not been resolved at present. The steady-state values of k_{cat}/K_M for L-aspartate and α-ketoglutarate reported previously (Cronin et al., 1987) are significantly lower than those determined here under single-turnover conditions. This might represent the presence of some inactive enzyme that would specifically affect the steady-state-determined parameters or might perhaps reflect some specific interactions between AATase and the MDH coupling enzyme (M. McLeish, D. A. Julin, and J. F. Kirsch, submitted for publication).

Eukaryotic AATases have a rather broad substrate specificity and have been reported to be particularly active with aromatic amino acids [e.g., Mavrides and Christen (1978)].

Table II: Substrate Specificities of Wild-Type and R292D Mutant Aspartate Aminotransferases toward Charged Amino Acids[a]

amino acid[b]	k_{cat}/K_M (M^{-1} s^{-1}) wild type	k_{cat}/K_M (M^{-1} s^{-1}) R292D	K_M^{app} (M) (wild type)	K_M^{app} (M) (R292D)	selectivity ratio $[k_{cat}/K_M(R292D)]/[k_{cat}/K_M(wild\ type)]$
L-aspartate	34500 ± 1800[c]	0.0695 ± 0.0029	0.00689 ± 0.00081[c]	*d*	(2.01 ± 0.14) × 10^{-6}
L-glutamate	4790 ± 100[c]	0.0102 ± 0.0004	0.30 ± 0.14[c]	*d*	(2.13 ± 0.10) × 10^{-6}
L-arginine	0.0276 ± 0.0008	0.429 ± 0.026	2.6 ± 1.6	0.80 ± 0.30	15.5 ± 1.0
L-ornithine	0.00992 ± 0.00041	0.127 ± 0.007	*d*	*d*	12.8 ± 0.9
L-lysine	0.0183 ± 0.0002	0.156 ± 0.013	*d*	*d*	8.5 ± 0.7

[a] Single-turnover conditions at pH 8.5, 25 °C, and ionic strength 1.0. [b] The concentration ranges of the tested amino acids were 2.5–20 and 25–300 mM for L-aspartate with wild type and R292D, respectively; 2.5–50 and 50–200 mM for L-glutamate with wild type and R292D, respectively; 25–300 and 25–350 mM for L-arginine with wild type and R292D, respectively; and 25–300 mM for L-lysine and 50–300 mM for L-ornithine with both enzymes. Values of K_M^{app} and k_{cat}/K_M were determined as described under Experimental Procedures. [c] Determined by stopped-flow spectrophotometry. [d] Saturation was not apparent within the substrate range used, and the values of k_{cat}/K_M were determined by linear least-squares regression of the data.

Table III: Substrate Specificities of Wild-Type and R292D Mutant Aspartate Aminotransferases toward Aromatic and Other Neutral Amino Acids[a]

amino acid[b]	k_{cat}/K_M (M^{-1} s^{-1}) wild type	k_{cat}/K_M (M^{-1} s^{-1}) R292D	K_M^{app} (M) (wild type)	K_M^{app} (M) (R292D)	selectivity ratio $[k_{cat}/K_M(R292D)]/[k_{cat}/K_M(wild\ type)]$
L-tyrosine[c]	829 ± 49	126 ± 3	0.014 ± 0.004	*d*	0.152 ± 0.010
L-phenylalanine[c]	368 ± 8	79.3 ± 1.8	*d*	*d*	0.215 ± 0.007
L-histidine[e]	17.3 ± 1.2	2.78 ± 0.05	0.021 ± 0.008	0.068 ± 0.007	0.161 ± 0.012
L-alanine	0.606 ± 0.039	0.273 ± 0.011	*d*	*d*	0.450 ± 0.034
L-glutamine	0.362 ± 0.033	0.179 ± 0.016	*d*	*d*	0.494 ± 0.063
L-serine	0.0394 ± 0.0016	0.00424 ± 0.00010	*d*	*d*	0.108 ± 0.005
L-citrulline	1.15 ± 0.03	0.773 ± 0.022	*d*	*d*	0.672 ± 0.026

[a] Single-turnover conditions at pH 8.5, 25 °C, and ionic strength 1.0. [b] The concentration ranges of the tested amino acids were 1–5 mM for L-tyrosine, 2.5–15 mM for L-phenylalanine, 10–50 mM for L-glutamine, 100–500 mM for L-serine, and 10–50 mM for L-citrulline with both enzymes; 1–10 and 5–30 mM for L-histidine with wild type and R292D, respectively, and 25–150 and 25–200 mM for L-alanine with wild type and R292D, respectively. Values of K_M^{app} and k_{cat}/K_M were determined as described under Experimental Procedures. [c] Determined by stopped-flow spectrophotometry. [d] Saturation was not apparent within the substrate range used, and the values of k_{cat}/K_M were determined by linear least-squares regression of the data. [e] L-Histidine is included here as a neutral amino acid since the side-chain imidazole is unchanged at the assay pH.

Table IV: Substrate Specificities of Wild-Type and R292D Mutant Aspartate Aminotransferases toward α-Keto Acids[a]

α-keto acid[b]	k_{cat}/K_M (M^{-1} s^{-1}) wild type	k_{cat}/K_M (M^{-1} s^{-1}) R292D	K_M^{app} (M) (wild type)	selectivity ratio $[k_{cat}/K_M(R292D)]/[k_{cat}/K_M(wild\ type)]$
α-ketoglutarate	23800 ± 600[c]	0.0593 ± 0.0016[d]	0.0334 ± 0.0088[c]	(2.49 ± 0.09) × 10^{-6}
pyruvate	8.06 ± 0.30	3.16 ± 0.06	*e*	0.392 ± 0.016

[a] Single-turnover conditions at pH 8.5, 25 °C, and ionic strength 1.0. [b] The concentration ranges of the tested α-keto acids were 3.33–30 and 2.5–120 mM for α-ketoglutarate with wild type and R292D, respectively, and 2.5–20 and 5–50 mM for pyruvate with wild type and R292D, respectively. Values of K_M^{app} and k_{cat}/K_M were determined as described under Experimental Procedures. [c] Determined by stopped-flow spectrophotometry. [d] Substrate activation of the R292D enzyme by α-ketoglutarate is manifest at concentrations above 10 mM. The value of k_{cat}/K_M was determined by a linear least-squares regression of the data obtained at low levels of α-ketoglutarate. [e] Within the ranges used, neither substrate exhibits saturation kinetics with the R292D enzyme, nor does pyruvate with wild-type AATase. Values of k_{cat}/K_M were determined by a linear least-squares regression of the data.

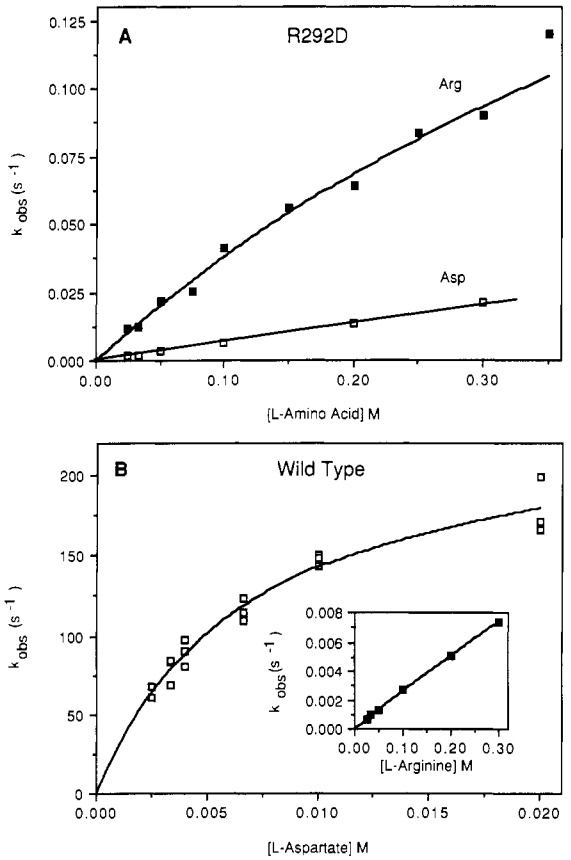

FIGURE 3: Effect of L-aspartate and L-arginine concentration on k_{obsd} for the reactions with wild-type and R292D AATases. Conditions: pH 8.50, 25 °C, ionic strength 1.0. (A) R292D AATase kinetics determined by conventional spectrophotometry. (B) Wild-type AATase rates were determined by stopped-flow spectrophotometry. (Inset) Effect of arginine concentration on k_{obsd} for wild-type AATase studied as described in (A).

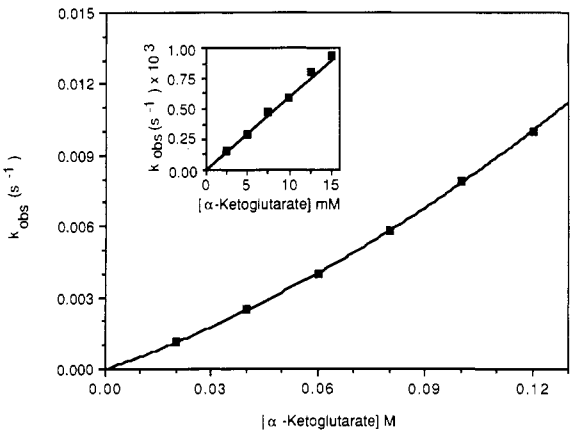

FIGURE 4: k_{obsd} versus α-ketoglutarate concentration for the reaction with R292D AATase. Conditions: pH 8.50, 25 °C, ionic strength 1.0. (Inset) Data obtained at low concentrations of α-ketoglutarate.

Kondo et al. (1987), however, have recently reported that only the *E. coli* enzyme is a significant catalyst of aromatic amino acid transamination. In order to examine the effect of the mutagenesis on this aspect of the *E. coli* AATase, the activities of both wild-type and mutant enzymes toward a number of neutral amino acids were studied. These data are given in Table III. In contrast to the results obtained with charged amino acids, the activity (in terms of k_{cat}/K_M) of the R292D enzyme toward amino acids having aromatic or other neutral side chains is depressed by only 2–9-fold in comparison to wild type.

As found for the anionic amino acids, the k_{cat}/K_M value (Table IV) toward the natural α-keto acid, α-ketoglutarate, is reduced by over 5 orders of magnitude in the R292D enzyme. However, the activity toward the uncharged α-keto acid, pyruvic acid, is relatively unaffected. Apparent substrate activation is observed at elevated levels of α-ketoglutarate

concentration (Figure 4). The k_{cat}/K_M value given in Table IV was calculated only from the data obtained at low substrate concentrations (Figure 4 inset). Substrate activation of the wild-type enzyme was not observed at the lower range of α-ketoglutarate concentrations studied.

The standard ping-pong kinetic mechanism that describes the AATase reaction is

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} F + P \quad (2a)$$

$$F + B \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} FB \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + Q \quad (2b)$$

The values of k_{cat}, K_M^A, K_M^B, k_{cat}/K_M^A, and k_{cat}/K_M^B, which are readily derivable from the velocity expression given by Segel (1975), are

$$k_{cat} = k_2k_4/(k_2 + k_4) \quad (3)$$

$$K_M^A = k_4(k_2 + k_{-1})/k_1(k_2 + k_4) \quad (4)$$

$$K_M^B = k_2(k_{-3} + k_4)/k_3(k_2 + k_4) \quad (5)$$

$$k_{cat}/K_M^A = k_1k_2/(k_{-1} + k_2) \quad (6)$$

$$k_{cat}/K_M^B = k_3k_4/(k_{-3} + k_4) \quad (7)$$

The dependence of the single-turnover pseudo-first-order rate constant for the first half-reaction as a function of substrate concentration is given by

$$k_{obsd} = k_2[A]/(K_M^{app} + [A]) \quad (8)$$

where $K_M^{app} = (k_2 + k_{-1})/k_1$. Therefore, the value of k_{cat}/K_M determined by steady-state methods equals the k_2/K_M^{app} value derived from single-turnover experiments. Stopped-flow studies on the reaction of the wild-type enzyme with the natural substrates L-aspartate, α-ketoglutarate, and L-glutamate provided values of 238 ± 17, 790 ± 190, and 1450 ± 663 s^{-1}, respectively, for the maximal single-turnover rate constants and the K_M^{app} values shown in Tables II and IV. A steady-state analysis under similar conditions using the MDH-coupled assay gave k_{cat} = 139 ± 7 s^{-1} and K_M values toward L-aspartate and α-ketoglutarate equal to 7.54 ± 0.50 and 9.38 ± 0.68 mM, respectively. The calculated value of the steady-state parameter, k_{cat}, from the respective half-reaction rate constants using eq 3 is 183 ± 14 s^{-1}, which is in reasonable agreement with the observed value. A small discrepancy may reflect the presence of some inactive enzyme in the steady-state measurements or failure to estimate accurately the enzyme concentration.

Chemical Modification of Wild-Type AATase. The behavior of R292D toward the natural anionic substrates is in quantitative agreement with the results obtained from arginine modification studies of both the pig heart cytosolic enzyme by 1,2-cyclohexanedione (Gilbert & O'Leary, 1975) and the chicken mitochondrial enzyme by phenylglyoxal (Sandmeier & Christen, 1982), namely, a reduction of 5 orders of magnitude in activity. In the latter study, Arg-292 was implicated as being the residue whose modification resulted in the loss of AATase activity. It was reported in both studies that the modification has little effect on the rate of alanine turnover. Thus, by performing an identical modification of the *E. coli* wild-type enzyme, it was hypothesized that the relative importance of the introduction of the anionic Asp-292, as opposed to the removal of the cationic Arg-292, to the cationic transaminase activity of R292D could be probed. Treatment of the *E. coli* AATase with 1,2-cyclohexanedione or phenylglyoxal, under conditions identical with those reported for the above AATases, inactivates the *E. coli* enzyme with respect to L-aspartate transamination with rate constants of 0.030 and 0.23 M^{-1} s^{-1}, respectively. However, in contrast with the results obtained with these reagents acting on the eukaryotic enzymes, the *E. coli* enzyme, which had been inactivated by over 95% toward reaction with L-aspartate, exhibited no significant activity toward L-alanine. A very slow similar single-turnover rate with L-aspartate, L-arginine, and L-alanine was observed for the 1,2-cyclohexanedione-inhibited enzyme, suggesting a slow rate-determining dissociation of the enzyme-inhibitor complex followed by a relatively rapid reaction with the amino acid.

Discussion

Expression of AATase. Initially, host cell cultures were induced with IPTG at an A_{650} = 0.9 to derepress the *tac* promoter, as described previously for the wild-type and K258A AATases (Malcolm & Kirsch, 1985; Smith et al., 1986). However, it was found in the present study that the yield of either enzyme, wild type or R292D, may be increased by over 2-fold when cells are grown to stationary phase and that IPTG induction is not required. Although the reason for this is unclear, the finding that IPTG does not appear to alter the expression of the R292D mutant (as judged by SDS–polyacrylamide gel electrophoresis of whole-cell homogenates; C. N. Cronin and J. F. Kirsch, unpublished results) suggests that either the *tac* promoter may not be repressed in the absence of inducer or that an operational constitutive promoter has been cloned together with the *aspC* gene. The original strategy for placing the *aspC* gene under the control of the *tac* promoter was to allow high expression of AATase during the exponential growth phase of the host strain (Amann et al., 1983). Although this feature of the expression system appears to be inoperative, the yield of AATase from cells grown to stationary phase is typically about 10% of total cell protein.

As described under Results, a small amount of an endogenous enzyme exhibiting AATase activity copurifies with the R292D mutant. However, when the MDH-coupled AATase activity in whole-cell homogenates of DB120 (host strain) containing the pKK177-3 plasmid [see Malcolm and Kirsch (1985)] minus the *aspC* gene insert was examined, only 4% of the activity present in the R292D homogenate was found (C. N. Cronin and J. F. Kirsch, unpublished results). This finding suggests that the presence of the plasmid-encoded *aspC* gene is required not only for the expression of the R292D enzyme but also for that of the contaminating AATase activity. A possible model to account for this observation proposes the formation of a more active dimer between one R292D subunit and some otherwise inactive genomic product. Both the *aspC* and *tyrB* gene products were inactivated in strain DB120 by chemical mutagenesis [strain DB120 (Malcolm & Kirsch, 1985) was derived from strain DG70 (Gelfand & Steinberg, 1977)] and not by gene deletion. Thus, the possibility for intersubunit complementation between a damaged chromosomal gene product and an R292D subunit exists [see Wente and Schachman (1987) for an example for intersubunit complementation].

Aldimine pK_a. The large upward shift in the pK_a of the active-site Schiff base following the binding of dicarboxylate substrates and inhibitors to the PLP form of AATase has been reported by several investigators [e.g., Velick and Vavra (1962), Jenkins and D'Ari (1966), Haddad et al. (1977), and Eichele et al. (1978)]. This is a result of placing the two negatively charged carboxylate groups between the Schiff base and the positively charged guanidinium groups of Arg-292 and Arg-386 (Mehler & Eichele, 1984). Therefore, replacement of the guanidinium moiety of Arg-292 with the β-carboxylate group of Asp-292, as in the R292D enzyme, should similarly

shift the pK_a to a higher value. E. L. Mehler (personal communication) has calculated a ΔpK_a of 0.18–0.24 for this mutation. The observed ΔpK_a values are 0.45 ± 0.05 at $\mu = 0.10$ and 0.28 ± 0.06 at $\mu = 1.0$ (Table I). The substantial ionic strength effect on the pK_a values is indicative of significant screening of the charged groups by added salt [cf. Jenkins (1980)]. The difference between the observed and calculated values is not large and may reflect (1) the uncertainties in scaling of the value for the microscopic dielectric constant [see Mehler and Eichele (1984)]; (2) the minor changes seen in the R292D versus wild-type enzyme structures (D. Ringe, personal communication), which may also account for the slightly reduced values of k_{cat}/K_M observed for the reactions of the R292D enzyme with neutral amino acids in comparison to wild type (see below); or (3) some, as yet, unidentified significant differences in the active-site geometry between the eukaryotic and prokaryotic enzymes.

The isoelectric point of *E. coli* AATase is 4.2 (Yagi et al., 1985). The incorporation of aspartate in place of arginine in the R292D enzyme introduces a charge difference of −2, resulting in a lower apparent isoelectric point of 3.9 during chromatofocusing on Polybuffer exchanger 94 (Figure 2). A difference has also been noted in isoelectric focusing experiments (J. M. Goldberg and J. F. Kirsch, unpublished results).

Substrate Specificities. The nearly millionfold reduction in the activity of the R292D enzyme toward the natural anionic amino and dianionic keto acids provides strong support for the hypothesis that Arg-292 is the residue primarily responsible for binding the side-chain carboxylate of the natural substrates. This contention is supported by the observation that the activity of R292D toward aromatic and other uncharged amino acids and pyruvate is reduced by only 2–9-fold in comparison to that of wild type. This latter finding argues also that, apart from the desired alteration, the remainder of the R292D molecule essentially retains the structural integrity of the wild-type enzyme, in agreement with the preliminary X-ray findings (D. Ringe, personal communication). The contribution of Arg-292 to catalysis of the natural anionic substrates was calculated according to eq 9 to be −7.0 to −7.1 kcal mol^{-1}. This range

$$\Delta G = RT \ln [(k_{cat}/K_M)_{R292D}/(k_{cat}/K_M)_{WT}] \quad (9)$$

has been corrected for the average decrease in the activity toward the eight neutral substrates tested (ΔG = −0.7 kcal mol^{-1}). Fersht et al. (1985) have used site-directed mutagenesis to probe the contribution of certain types of hydrogen bonds to substrate specificity and have reorted values of 0.5–1.5 and ~4 kcal mol^{-1} for hydrogen bonds between an uncharged enzyme side chain and an uncharged or charged substrate group, respectively. The larger effect of −7.0 to −7.1 kcal mol^{-1} observed for the AATase mutation is a measure of both the loss of the hydrogen bond involved in the salt bridge between the substrate carboxylate and Arg-292 and of some additional contribution due to electrostatic repulsion between Asp-292 and the anionic substrates. The latter factor may perhaps be estimated from the relative rate constants for R292D acting on Gln versus Glu as substrates that favor the former by a factor of 18, or −1.6 kcal/mol.

Additional evidence for the role of Arg-292 in the wild-type enzyme comes from the finding that the activity of R292D toward substrates with cationic side chains is increased significantly and exclusively in comparison to wild type. This result could, at first, be interpreted in terms of a specific favorable electrostatic interaction between the side chains of cationic amino acids and Asp-292. The catalytic improvement in the R292D enzyme over wild type with respect to turnover of cationic amino acids, corrected for the loss toward the neutral substrates, is in the range 1.9–2.3 kcal mol^{-1}. However, the observed differences in the substrate charge preferences for each enzyme do not begin to approach the maximum that might have been expected from the symmetrical nature of the mutagenesis experiment; i.e., it might have been anticipated that the turnover rate of arginine by R292D would be similar, quantitatively, to that of aspartate by wild-type enzyme. Thus, although it is clear that a single amino acid change has qualitatively achieved the production of the desired cationic amino acid transaminase activity, this single change is insufficient to generate an enzyme that would be of much practical use. The asymmetric nature of the results leads to the question of whether the observed differences in cationic amino acid turnover between wild type and R292D reflect the removal of an unfavorable charge interaction or the removal of such an interaction together with the introduction of a favorable one, i.e., a salt bridge between Asp-292 and the positively charged side chain of cationic substrates. The finding that the activity of the R292D enzyme with L-citrulline, an uncharged isosteric analogue of L-arginine, as well as with L-alanine and L-glutamine is comparable to the activity toward L-arginine suggests that the positively charged guanidinium group of the latter substrate is not a factor in its rate of turnover and, thus, that the better activity of R292D toward cationic amino acids reflects predominantly the removal of the unfavorable interaction.

Therefore, the question remains as to why the R292D enzyme is not a better cationic amino acid transaminase. One possible reason stems from the finding that in the unliganded chicken mitochondrial enzyme Arg-292 appears to form a salt bridge with Asp-15 (Jansonius et al., 1985). The latter residue is also present in mouse (Obaru et al., 1986) and pig (Barra et al., 1980) mitochondrial AATases and also in the *E. coli* enzyme (Kondo et al., 1984; Fotheringham et al., 1986). The role of Asp-15 in the cytosolic enzyme may be performed by Glu-141, since this molecule has a valine at position 15 (Doonan et al., 1975), whereas position 141 is neutral in the other enzymes listed. Therefore, the loss of such an interaction in the unliganded R292D enzyme might be expected to lead to some catalytically unfavorable flexibility around Asp-292. The full expression of the negative charge at position 292 is muted somewhat by a hydrogen bond formed between the β-carboxylate and the carboxamido moiety of Asn-142 (D. Ringe, personal communication). Second-site mutation studies will be directed both to attempt to improve R292D as a cationic amino acid transaminase and to gain a quantitative understanding of the reasons for the significant muting of the primary mutation.

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