Analysis of the Substrate-Recognition Mode of Aromatic Amino Acid Aminotransferase by Combined Use of Quasisubstrates and Site-Directed Mutagenesis: Systematic Hydroxy-Group Addition/Deletion Studies to Probe the Enzyme−Substrate Interactions†

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ABSTRACT: *Escherichia coli* aromatic amino acid aminotransferase (ArAT) catalyzes transamination reactions of both dicarboxylic amino acids and aromatic amino acids. Because both reactions are supposed to occur in a single reaction center, whether ArAT provides alternative binding sites for the two different types of substrate side chains has been an intriguing question. This was probed by spectroscopic analysis of the complexes of β-hydroxylated substrates and the wild-type and [Tyr70 → Phe] mutant enzymes. Both L-*erythro*-3-hydroxyaspartate and L-*erythro*-3-phenylserine reacted with the wild-type ArAT to give an absorption maximum at around 500 nm, reflecting the formation of the quinonoid intermediate. When the hydroxy group of Tyr70 of ArAT was deleted by replacement of the residue with phenylalanine, the 500-nm absorption greatly decreased in either of the ArAT−β-hydroxy amino acid complexes, showing the presence of specific interactions, which stabilize the 500-nm absorbing quinonoid intermediates, between the phenolic hydroxy group of Tyr70 and the β-hydroxy groups of the two quasisubstrates. From these results, it was concluded that the conformations of the two quasisubstrates are essentially identical in their enzyme-bound forms. This implies that the phenyl group of the substrate phenylalanine occupies the same region as that occupied by the β-carboxyl group of the substrate aspartate, and the region should be near Arg292, the residue that binds the β-carboxylate group of substrates. The [Arg292 → Ala] or [Arg292 → Leu] mutation increased the K_m values for aromatic amino acids 5−10-fold, and the [Arg292 → Lys] mutation increased these values 10−100-fold, without affecting the k_{cat} values. This shows that the side chain of Arg292 is partially involved in the binding of the aromatic ring of substrates to ArAT.

Aromatic amino acid aminotransferase (ArAT;[1] EC 2.6.1.57) is a pyridoxal 5′-phosphate (PLP)-dependent enzyme which catalyzes the following reversible transamination reactions

$$E_L + \text{phenylalanine} \rightleftarrows E_M + \text{3-phenylpyruvate} \quad (1)$$

$$E_L + \text{tyrosine} \rightleftarrows E_M + \text{3-(4-hydroxyphenyl)pyruvate} \quad (2)$$

$$E_L + \text{tryptophan} \rightleftarrows E_M + \text{3-indolylpyruvate} \quad (3)$$

$$E_L + \text{aspartate} \rightleftarrows E_M + \text{oxalacetate} \quad (4)$$

$$E_L + \text{glutamate} \rightleftarrows E_M + \text{2-oxoglutarate} \quad (5)$$

where E_L and E_M denote the PLP-form and the pyridoxamine 5′-phosphate (PMP)-form of the enzyme, respectively. Combinations of any two of the reactions 1−5 yield a variety of transamination reactions between amino acids and oxo acids. In the shikimic acid pathway, ArAT functions at the final step of phenylalanine and tyrosine biosyntheses by transferring an amino group from aspartate or glutamate to the aromatic oxo acids (combinations of the reactions 1/2 and 4/5). The enzyme in *Escherichia coli* shows 43% amino acid sequence homology with the *E. coli* aspartate aminotransferase (AspAT) and to lesser extents with animal AspATs (Kuramitsu et al., 1985; Fotheringham et al., 1986). The active-site residues of AspAT which had been identified by X-ray crystallographic studies (Arnone et al., 1985; Jansonius et al., 1985; Okamoto et al., 1994) were found to be conserved in the primary structures of ArAT at the corresponding positions (Kuramitsu et al., 1985; Fotheringham et al., 1986). Both ArAT and AspAT are composed of two identical subunits of about 43K and contain one PLP molecule per subunit. The absorption and circular dichroism spectra and their pH dependency were essentially identical between the two enzymes (Hayashi et al., 1993). More intriguing was the finding that ArAT reacts with dicarboxylic amino and oxo acids (reactions 4 and 5) as efficiently as AspAT, the enzyme catalyzing predominantly the reactions 4 and 5 (Powell & Morrison, 1978; Hayashi et al., 1993; Köhler et al., 1994). In AspAT, Arg292[2] is the critical

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[1] Abbreviations: ArAT, aromatic amino acid aminotransferase (EC 2.6.1.57); AspAT, aspartate aminotransferase (EC 2.6.1.1); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); HOAsp, L-*erythro*-3-hydroxyaspartate; HOPhe, L-*erythro*-3-phenylserine; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; WT ArAT (AspAT), wild-type ArAT (AspAT); Y70F ArAT, ArAT in which the residue Tyr70 was replaced by a phenylalanine residue. Other mutant enzymes are named in a similar way.

[2] The amino acid residue is numbered according to the sequence of pig cytosolic aspartate aminotransferase (Ovchinnikov et al., 1973).

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residue that binds the $\beta(\gamma)$-carboxylate group of dicarboxylic substrates. Arg292 is conserved in ArAT, and it is reasonable to assume that ArAT also utilizes this residue as a tool to recognize dicarboxylic substrates. The question to be addressed is then how ArAT can accommodate aromatic substrates. One model assumes that the side chains of dicarboxylic and aromatic substrates bind to alternate pockets (Jäger et al., 1992). Another model allows the aromatic side chain to occupy the same site as the carboxylic side chain, by reorienting the side chain of Arg292 (Seville et al., 1988). Both models have been proposed on the basis of model-building on the framework of the closely related enzyme AspAT, but no experimental data on ArAT itself have been presented to support either of the models.

erythro-3-Phenylserine has been shown to react with ArAT, yielding a prominent absorption at 499 nm which is ascribed to a quinonoid structure (Hayashi et al., 1993). The *threo* diastereoisomer did not yield 499-nm absorption. Thus, the stereochemistry of the reaction of 3-phenylserine with ArAT is identical to that of the reactions of 3-hydroxyaspartate with ArAT (Hayashi et al., 1993) and AspAT (Jenkins, 1979). These stereospecific interactions of the β-hydroxy groups of quasisubstrates with the enzyme protein have important implications for the substrate-binding mode of ArAT. Because the hydroxy group of Tyr70 has been discussed to be involved in stabilizing the quinonoid intermediate formed from L-*erythro*-3-hydroxyaspartate and AspAT (Kirsch et al., 1984; Taylor et al., 1990; Toney & Kirsch, 1991; Hayashi & Kagamiyama, 1995), we can consider that the corresponding residue of ArAT may interact similarly with the two β-hydroxy amino acids. Therefore, in this study we analyzed the reactions of the two β-hydroxy amino acids with both the wild-type and [Tyr70 → Phe] mutant ArATs. Spectroscopy on the complexes of these 3-hydroxy amino acids and ArAT provided strong support to the notion that the conformation of phenylalanine and aspartate, when they are bound to ArAT, are almost identical. This suggested the interaction of the aromatic ring of aromatic substrates with Arg292 of ArAT, which was further analyzed by site-directed mutagenesis on the residue.

MATERIALS AND METHODS

Chemicals. L-*erythro*-3-Phenylserine was synthesized as described by Jones (1979). L-*erythro*-3-Hydroxyaspartic acid was synthesized by the method of Jenkins (1979). The wild-type and mutant ArATs were prepared as described previously (Hayashi et al., 1993; Iwasaki et al., 1994). For preparation of mutant *tyrB* genes, site-directed mutagenesis was performed on the single-stranded pUC118-*tyrB* using the Sculptor IVM system (Amersham, U.K.) as described previously (Iwasaki et al., 1994). The oligonucleotides used are G-CCC-TTC-CAT-GGG-TAA-GAA-AAG-CGC-CAT-G for Tyr70 → Phe; GTA-GTT-GCG-GGC-AAC-TGT-TGC for Arg292 → Ala; GTA-GTT-GCG-GAG-AAC-TGT-TGC for Arg292 → Leu; and A-GTA-GTT-GCG-GTT-AAC-TGT-TGC for Arg292 → Lys (underlines indicate mismatches).

Determination of Protein Concentration. The concentration of the ArAT subunit in solution was determined spectrophotometrically. The apparent molar extinction coefficients used were $\epsilon_M = 5.3 \times 10^4$ M^{-1} cm^{-1} for the PLP-form enzyme and $\epsilon_M = 5.2 \times 10^4$ M^{-1} cm^{-1} for the PMP-form enzyme at 280 nm (Hayashi et al., 1993).

Spectrophotometric Measurements. Absorption spectra were measured using a Hitachi spectrophotometer U-3300. The buffer solution for the absorption measurements contained 50 mM PIPES-NaOH, 50 mM HEPES-NaOH, or 50 mM sodium borate as buffer component(s) and 0.1 M KCl. Protein concentrations were generally $(1-2) \times 10^{-5}$ M. The pK_a values of the PLP−Lys258 aldimine of WT and mutant ArATs were obtained from the pH dependency of the apparent molar absorptivity values at 358 and 430 nm corresponding to the deprotonated and the protonated forms, respectively, of the aldimine (Hayashi et al., 1993). The SD values of pK_a were less than 0.05 for all enzymes.

Kinetic Analysis. Stopped-flow spectrophotometry was performed using Applied Photophysics SX.17MV. The dead time for this system was generally 2.0 ms under a pressure of 500 kPa. Single-turnover reactions of the PLP-form of the enzyme with amino acids, or those of the PMP-form of the enzyme with oxo acids, were analyzed as described by Kuramitsu et al. (1990). The reactions of HOPhe and HOAsp with WT or Y70F ArAT were analyzed similarly to those of HOAsp with AspAT (Hayashi & Kagamiyama, 1995). The exponential absorption changes were analyzed with the program provided with the SX.17MV stopped-flow spectrophotometer. The apparent rate constants obtained were then analyzed for their dependency on the substrate concentration, and kinetic parameters were obtained by nonlinear regression with the software Igor Pro (ver. 2.04, WaveMetrics, Lake Oswego, OR).

RESULTS AND DISCUSSION

Quinonoid Structures Produced by the β-Hydroxy Groups of Quasisubstrates. Both L-*erythro*-3-phenylserine (HOPhe) and L-*erythro*-3-hydroxyaspartate (HOAsp) react with ArAT to yield intense ($\epsilon_{app} > 20\,000$ M^{-1} cm^{-1}) quinonoid absorption bands at 499 and 496 nm, respectively, with smaller absorption bands at 330 nm (Hayashi et al., 1993; Figure 1A,C). The reactions of the two quasisubstrates with ArAT were analyzed using a stopped-flow spectrophotometer and were found to proceed in the same manner as the reaction of HOAsp with AspAT (Hayashi & Kagamiyama, 1995). Thus, in each reaction, there was a rapid increase followed by a slow decrease in absorbance at around 500 nm. The biphasic absorption changes could be analyzed by assuming the following scheme (Hayashi & Kagamiyama, 1995):

$$E_L + S \overset{K_S}{\rightleftharpoons} ES_1 \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} ES_2 \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} ES_3 \qquad (6)$$

Here S denotes the β-hydroxy amino acid, ES_1 the external aldimine, and ES_2 the quinonoid intermediate. ES_3 is a species absorbing primarily at 330 nm and was considered to be the carbinolamine intermediate in the reaction of AspAT with HOAsp. K_S, k_{+2}, k_{-2}, k_{+3}, and k_{-3} denote the dissociation constant and the rate constants for the indicated reaction steps. The fast phase of the absorption changes corresponds to the rapid formation of ES_2, and the slow phase represents the slow equilibrium between ES_2 and ES_3. The apparent rate constants, k_{fast} and k_{slow}, for the exponential changes during the fast and slow phases, respectively, were

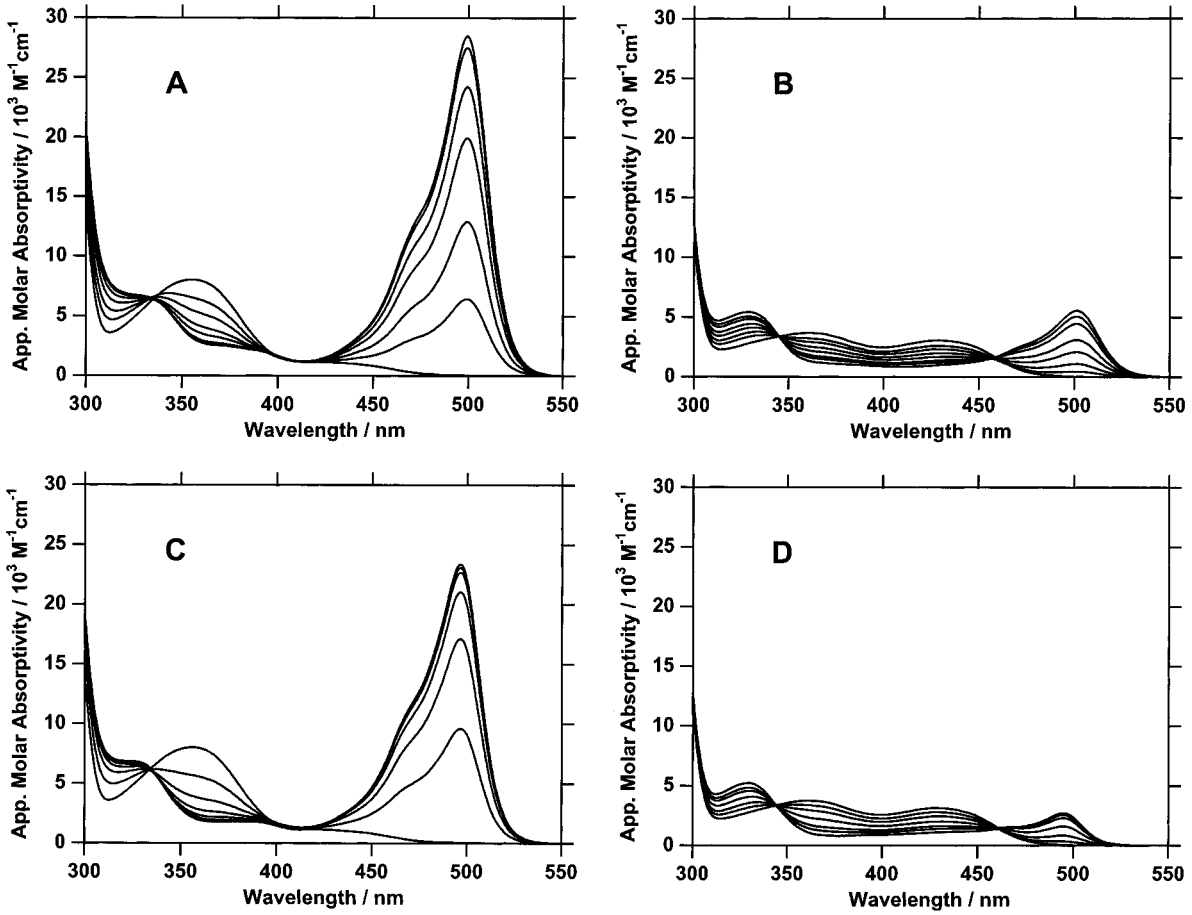

FIGURE 1: Absorption spectra of WT (A and C) and Y70F (B and D) ArATs in the presence of various concentrations of HOPhe or HOAsp. The spectra were taken at 25 °C in 50 mM HEPES-NaOH buffer, pH 8.0, containing 0.1 M KCl, with 20 μM enzymes. In panels A and B, the concentrations of HOPhe added are (from bottom to top of the 500-nm absorption) 0, 0.15, 0.39, 0.95, 1.8, 3.1, and 5.2 mM. In panel B, the spectrum with 13 mM HOPhe is also shown. In panels C and D, the concentrations of HOAsp added are (from bottom to top of the 500-nm absorption) 0, 0.050, 0.15, 0.39, 0.95, 1.8, and 10 mM. In each panel, a further addition of the quasisubstrate did not essentially alter the spectrum.

Table 1: Parameters for the Reactions of WT and Y70F ArATs with L-*erythro*-3-Hydroxy Amino Acids[a]

	K_S[b]	k_{+2}[c]	k_{-2}[c]	k_{+3}[c]	k_{-3}[c]
HOPhe + WT	13 ± 0.7	270 ± 5	9.6 ± 1.9	0.071 ± 0.001	0.098 ± 0.001
HOPhe + Y70F	14 ± 0.6	78 ± 0.5	27 ± 0.6		
HO-Asp + WT	1.6 ± 0.1	660 ± 10	17 ± 4	1.1 ± 0.05	1.2 ± 0.05
HO-Asp + Y70F	1.1 ± 0.2	490 ± 18	94 ± 15		

[a] The reactions were followed in an Applied Photophysics SX.17MV stopped-flow spectrophotometer by monitoring the change in absorbance at 499 nm (for the reaction with HOPhe) and 496 nm (for the reaction with HOAsp) in 50 mM HEPES-NaOH buffer, pH 8.0, containing 0.1 M KCl at 25 °C. WT ArAT and Y70F ArAT (each 20 μM) were reacted with a variety of concentrations of HOPhe or HOAsp, and the apparent rate constants were obtained for the fast phase (k_{fast}) and the slow phase (k_{slow}) of absorption changes. The kinetic parameters were obtained by fitting the data to eqs 7 and 8. For the reactions of WT ArAT with HOPhe and HOAsp, dissociation of ES_3 into E_M plus an oxo acid is negligible at enzyme concentrations of above 20 μM which we used here (Hayashi & Kagamiyama, 1995). However, the dissociation cannot be neglected for the corresponding reactions of Y70F ArAT because of the increased free energy level of ES_3 caused by the Y70F mutation. Therefore, only K_S, k_{+2}, and k_{-2} could be determined accurately for these reactions. [b] In mM. [c] In s^{-1}.

well fitted to the following equations (Hammes & Schimmel, 1966, 1970; Hayashi & Kagamiyama, 1995):

$$k_{fast} = k_{+2}\frac{[S]}{K_S + [S]} + k_{-2} \quad (7)$$

$$k_{slow} = k_{+3}\frac{k_{+2}}{k_{+2} + k_{-2}}\frac{[S]}{\frac{k_{-2}}{k_{+2} + k_{-2}}K_S + [S]} + k_{-3} \quad (8)$$

The kinetic parameters were obtained by analyzing the concentration dependence of the apparent rate constants, k_{fast} and k_{slow}, and are summarized in Table 1. Using these parameters and the changes in the apparent molar absorptivity at λ_{max}, the ϵ values of the ES_2 species were calculated to be 47 000 ± 100 M^{-1} cm^{-1} (499 nm) for the HOPhe−ArAT complex and 43 000 ± 100 M^{-1} cm^{-1} (496 nm) for the HOAsp−ArAT complex [see eq 8 in Hayashi & Kagamiyama (1995)]. These values are similar to those of the ES_2 species of the HOAsp−AspAT complex (45 300 M^{-1} cm^{-1} at 494 nm; Hayashi & Kagamiyama, 1995) and the quinonoid intermediates of amino acid complexes of glycine hydroxymethyltransferase (41 000 M^{-1} cm^{-1}; Ulevitch & Kallen, 1977) and tryptophan indole lyase (46 900 M^{-1} cm^{-1}; Metzler et al., 1991). The coincidence of the values supports the idea that the kinetically identified species ES_2 in the

reactions of ArAT with HOPhe and HOAsp are the quinonoid intermediates.

β-Hydroxy Groups of HOPhe and HOAsp Specifically Interact with Tyr70 of ArAT. Although HOPhe and HOAsp produce intense quinonoid absorption at around 500 nm on the reaction with ArAT, the *threo* isomers of HOPhe and HOAsp did not essentially show absorption at around 500 nm (Hayashi et al., 1993). This indicates that when the β-hydroxy amino acids are in the *erythro* configuration, the β-hydroxy group specifically interacts with active site residues to stabilize the quinonoid intermediate. In the previous study on the reaction of HOAsp with AspAT (Hayashi & Kagamiyama, 1995), it was proposed that a hydrogen-bonding network is formed between the β-hydroxy group of HOAsp, the phenolic hydroxy group of Tyr70, the phosphate group of PLP, and the protonated amino group of Lys258. The Lys258 amino group is protonated only in the quinonoid and the carbinolamine intermediates, and in these intermediates the entire hydrogen-bonding network is stabilized by mutual compensation of the positive charge of the amino group of Lys258 and the negative charge of the phosphate group of PLP. Similarly to the quinonoid absorption of the HOAsp−AspAT complex, the quinonoid absorption bands of the HOPhe−ArAT and HOAsp−ArAT complexes are greatly decreased on Y70F mutation of the enzyme (Figure 1B,D). This suggests that, also in ArAT, the Y70F mutation destabilizes the quinonoid intermediates formed from β-hydroxy amino acids and the enzyme. However, because the quinonoid intermediate (ES_2) of the β-hydroxy amino acid−ArAT complexes are expected to be in equilibrium with the carbinolamine intermediate (ES_3), the decreased quinonoid absorption bands (Figure 1B,D) can be accounted for by alterations in the equilibrium between ES_2 and ES_3. Moreover, Y70F ArAT showed anomalous spectroscopic behavior; it has significant absorption at 430 nm even at pH 8.0, where most of the active site PLP−Lys258 Schiff base of the wild-type enzyme exists as an unprotonated form absorbing at 358 nm (Figure 1), although Y70F AspAT showed spectroscopic properties essentially identical to those of WT AspAT (Toney & Kirsch, 1991). Therefore, more careful and quantitative analyses are required to discuss the involvement of Tyr70 in the stabilization of the quinonoid intermediates produced by β-hydroxy amino acids.

The pH dependency of the spectra of Y70F ArAT showed that the pK_a value of the PLP−Lys258 Schiff base is 7.50 (data not shown), whereas that of WT ArAT is 6.65 (Hayashi et al., 1993). The reason for this anomalously high pK_a value is yet to be clarified. The protonated form of the PLP−Lys258 aldimine of ArAT is an unreactive species that does not bind the substrate amino acids (Iwasaki et al., 1994). As a consequence, a shift in the aldimine pK_a directly affects the substrate binding step, although it does not directly affect the following chemical steps once the amino acids are bound to the enzyme. The fast-phase reactions of Y70F ArAT with HOPhe and HOAsp were analyzed similarly to those of WT ArAT (Table 1). Addition of the β-hydroxy group to phenylalanine or aspartate altered the binding of these amino acids to both WT and Y70F ArATs, as indicated by the K_m values for phenylalanine and aspartate and K_S values for HOPhe and HOAsp (Tables 1 and 2). These effects, produced by the hydroxy-group addition/deletion events in the enzyme and substrates, should affect the free energy level of the quinonoid intermediate (ES_2) relative to that of the free enzyme plus (quasi)substrate (E + S). Therefore, it is more appropriate to focus on the free energy difference between the quinonoid intermediate (ES_2) and the external aldimine (ES_1), in order to discuss the stability of the quinonoid intermediates, because this energy difference is not affected by alterations in the (quasi)substrate-binding step.

Table 2: Kinetic Parameters for the Half-Reactions of Y70F ArAT[a]

substrate	k_{cat} (s^{-1})	K_m (mM)
phenylalanine	620 (1300)	1.7 (1.0)
phenylpyruvate	380 (490)	0.16 (0.025)
aspartate	43 (290)	6.6 (5.0)
oxalacetate	590 (830)	0.40 (0.082)

[a] The reactions were followed in an Applied Photophysics SX.17MV stopped-flow spectrophotometer by monitoring the change in absorbance at 360 nm in 50 mM HEPES-NaOH buffer, pH 8.0, containing 0.1 M KCl at 25 °C. Amino acids and oxo acids were reacted with the PLP-form and the PMP-form of Y70F ArAT, respectively. The data were analyzed as described previously (Hayashi et al., 1993). The SD values of the kinetic parameters were less than 10% of the fitted values. The values in parentheses are the corresponding values of WT ArAT (Hayashi et al., 1993).

The values of the free energy difference between the quinonoid intermediates (ES_2) and the external aldimines (ES_1) are obtained in the same way as those for the reactions of AspAT with HOAsp and aspartate (Hayashi & Kagamiyama, 1995). Thus, for the reactions of WT and Y70F ArATs with HOPhe or HOAsp, the $[ES_2]_{eq}/[ES_1]_{eq}$ values were obtained from the kinetic parameters, k_{+2} and k_{-2} (Table 1). For the reactions with phenylalanine or aspartate, the values were obtained from the equilibrium concentrations of ES_2 and ES_1 in the presence of saturating amounts of amino and oxo acids. Under these conditions, the enzyme species that exist in the solutions are ES_1, ES_2, and ES_3. The amounts of ES_2 are estimated from the absorbance at λ_{max} near 500 nm (Figure 2). Together with the values of the kinetic parameters for the reaction of WT and Y70F ArATs with amino and oxo acids, the amounts of ES_1 are then calculated. The $[ES_2]_{eq}/[ES_1]_{eq}$ values were 2.7/27 (WT) and 2.4/37 (Y70F) in the presence of phenylalanine and phenylpyruvate and 0.74/74 (WT) and 0.84/92 (Y70F) in the presence of aspartate and oxalacetate (the number indicates the percentage of each species in the total enzyme species). Then, using the relationship $G_{ES_2} - G_{ES_1} = RT \ln([ES_1]_{eq}/[ES_2]_{eq})$, the values of the free energy difference were calculated and are shown in Figure 3.

The quinonoid intermediate is stabilized by 14 and 20 kJ mol^{-1} by the addition of the β-hydroxy group (to form the *erythro* configuration) to phenylalanine and aspartate, respectively (comparison of "E−OH S" and "E−OH HO−S" in Figure 3). These values can be compared to the stabilization energy of 19 kJ mol^{-1} brought about by the β-hydroxy group of HOAsp in the quinonoid intermediate of the HOAsp−AspAT complex (Hayashi & Kagamiyama, 1995). When the hydroxy group of Tyr70 was removed (i.e., changing "E−OH" to "E" in Figure 3) the quinonoid intermediates produced from HOPhe and HOAsp were destabilized by 5.6 and 5.0 kJ mol^{-1}, respectively (comparison of "E HO−S" and "E−OH HO−S" in Figure 3), whereas the energy levels of the quinonoid intermediates produced from normal substrates were essentially unchanged (comparison of "E S" and "E−OH S" in Figure 3). These results indicate that the phenolic hydroxy group of Tyr70 does not stabilize the quinonoid intermediate in the reaction with

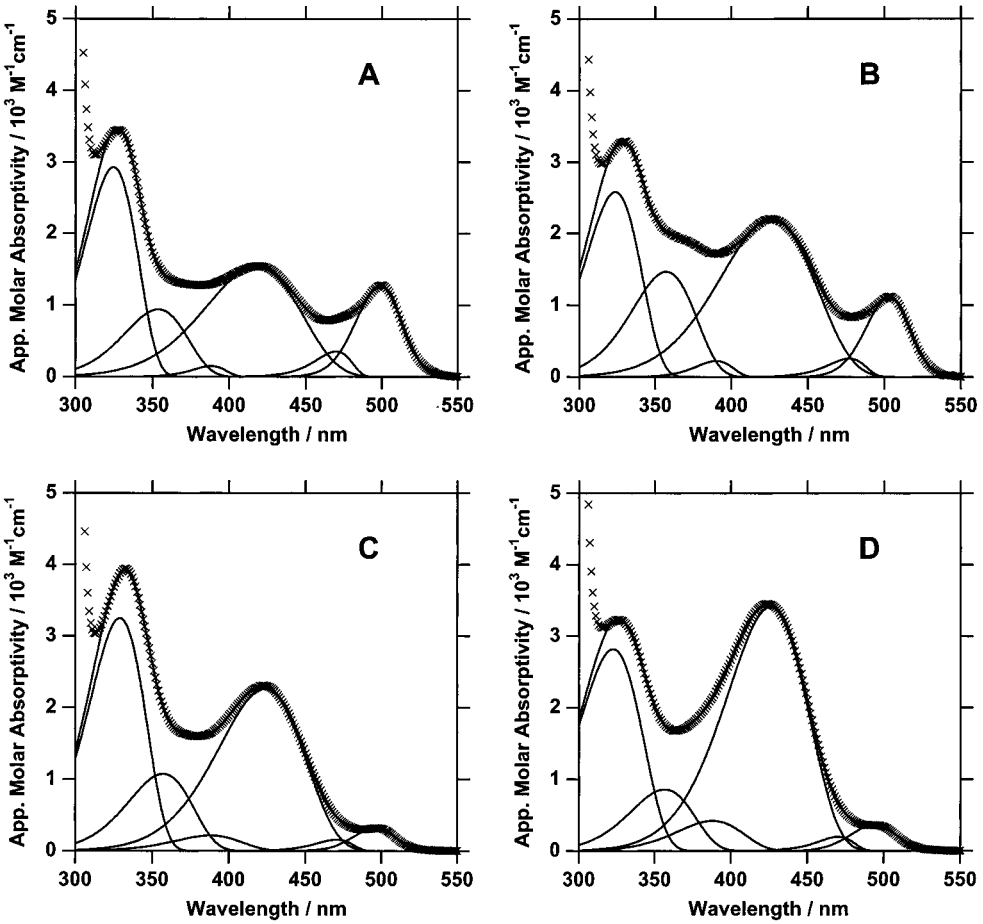

FIGURE 2: Absorption spectra of WT (A and C) and Y70F (B and D) ArATs in the presence of 20 mM phenylalanine and 5 mM phenylpyruvate (A and B) or 80 mM aspartate and 10 mM oxalacetate (C and D). The spectra were taken in 50 mM HEPES-NaOH buffer, pH 8.0, containing 0.1 M KCl, at 25 °C, with 20 μM enzymes. The spectra between 320 and 550 nm have been fitted with six lognormal distribution curves, which are denoted by solid lines, using PeakFit (ver. 3.18, Jandel Scientific, San Rafael, CA). The summation curves pass close to the experimental points ($\times$). The rightmost band in each panel represents the quinonoid intermediate, and the value of the height was used to calculate the abundance of the species. These values are 2.7% (A), 2.4% (B), 0.74% (C), and 0.84% (D) of the total enzyme molecules and are calculated using the molar absorptivity values of 47 000 M^{-1} cm^{-1} (determined for the quinonoid structures produced from HOPhe and ArAT) at 499 nm for A and B, and 43 000 M^{-1} cm^{-1} (determined for the quinonoid structures produced from HOAsp and ArAT) at 496 nm for C and D. This is because the true molar absorptivity values for the quinonoid absorption in this figure cannot be obtained by kinetic analysis of the reactions of WT and Y70F ArAT with normal substrates, as has been observed for the relevant reactions in AspAT (Hayashi & Kagamiyama, 1995).

normal substrates aspartate and phenylalanine, but its interaction with the β-hydroxy groups of HOPhe and HOAsp causes stabilization of the quinonoid intermediate by 5.6 and 5.0 kJ mol^{-1}, respectively. The remaining 8.4 kJ mol^{-1} (HOPhe) and 15 kJ mol^{-1} (HOAsp) of the stabilization energies of the quinonoid intermediates are considered to be obtained through interactions of the β-hydroxy group with other residues in the active site. The similarity of the reactions to the reaction of HOAsp with AspAT suggests that the residue is most probably the protonated ϵ-amino group of Lys258 (Hayashi & Kagamiyama, 1995). These discussions are in agreement with the model shown in Figure 4, which was drawn according to the model of the quinonoid structure of the HOAsp−AspAT complex (Hayashi & Kagamiyama, 1995). In this model, both the hydrogen bond between the β-hydroxy group of HOPhe or HOAsp and Tyr70 and that between the β-hydroxy group and the amino group of Lys258 stabilize the quinonoid intermediate.

In spite of the overall similarity in the mode of reaction with ArAT between HOPhe and HOAsp, there were some distinct differences. First, the quinonoid stabilization energy provided by the interaction of the β-hydroxy group of HOAsp with the ϵ-amino group of Lys258, as calculated above, is larger than that of HOPhe by 6.6 kJ mol^{-1}. Second, although the addition of the β-hydroxy group to aspartate increased slightly the affinity of the amino acid for ArAT, the addition of the β-hydroxy group to phenylalanine decreased the affinity of the amino acid (Tables 1 and 2). In the model of the quinonoid intermediate of HOAsp with ArAT, the β-hydroxy group lies in the plane of the β-carboxylate group, and the distance between $O^{\gamma 1}$ of the β-hydroxy group and $O^{\delta 1}$ of the β-carboxylate group of HOAsp is 2.7 Å (Figure 4A). Therefore, the β-carboxylate group of HOAsp can be involved in the hydrogen bonding network that stabilizes the quinonoid intermediate. If the negative charge of the β-carboxylate group stabilizes the overall network by electrostatic interaction with the positive charge of the amino group of Lys258, we can expect a larger stabilization energy provided by the β-carboxylate group of HOAsp. Furthermore, this $O^{\gamma 1}-O^{\delta 1}$ interaction may also contribute to increasing the affinity of HOAsp for ArAT by enforcing the $C^{\beta}-C^{\gamma}$ torsion angle of HOAsp preferable for the binding to the active site of ArAT. The β-hydroxy group of HOPhe is supposed to have an adverse effect. The unfavorable interaction between the β-hydroxy group and the phenyl ring would destabilize the conformation of HOPhe shown in

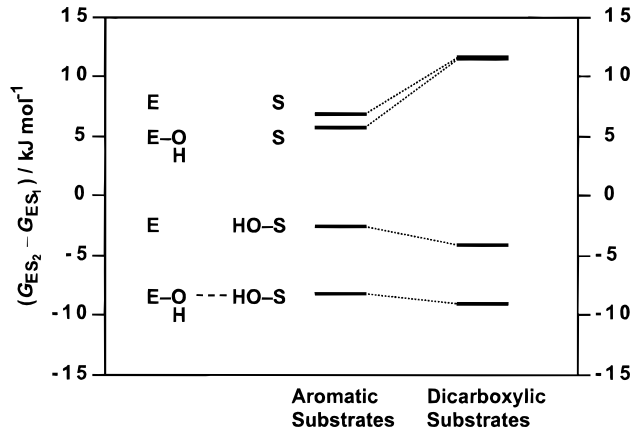

FIGURE 3: Free energy levels of the quinonoid intermediates (ES_2) formed from the reactions of WT and Y70F ArAT with aromatic substrates (phenylalanine and HOPhe) and dicarboxylic substrates (aspartate and HOAsp). The free energy levels of the external aldimine (ES_1) for these reactions are taken as zero, and the values relative to these free energy levels are shown. For the reactions with HOPhe and HOAsp, the values are calculated from the kinetic parameters (k_{+2} and k_{-2}) shown in Table 1. For the reactions with phenylalanine and aspartate, the free energy levels are calculated using the k_{cat} values (forward and reverse) of the half-reactions (Table 2) and the populations of the quinonoid intermediates obtained from Figure 2. The method of calculations is described in Hayashi and Kagamiyama (1995). Beside the free energy levels are shown the enzyme−(quasi)substrate interactions schematically. E−OH, WT ArAT; E, Y70F ArAT; HO−S, HOPhe or HOAsp; S, phenylalanine or aspartate.

Figure 4B. A similar interaction is expected in the Michaelis or the external aldimine complex, and this would decrease the affinity of HOPhe for ArAT compared with that of phenylalanine. Therefore, the quantitative differences in the kinetic (or thermodynamic) parameters between the HOPhe−ArAT and the HOAsp−ArAT reactions can also be understood within the framework of the model shown in Figure 4. In the model, the structures of HOPhe and HOAsp are almost superimposable. Because it is reasonable to assume that the phenyl group and the carboxylate group of the normal substrates phenylalanine and aspartate are recognized by ArAT in the same way as those of HOPhe and HOAsp, we can expect that both the side chain of the substrate phenylalanine and that of the substrate aspartate are located near Arg292 when they are bound to ArAT. Thus the role of Arg292 in the recognition of the two sets of substrates, the one aromatic substrates and the other dicarboxylic substrates, is the next focus of this study.

Arg292 Is Responsible for the Recognition of Both the Carboxylic and Aromatic Side Chains of Substrates. In order to determine the commitment of Arg292 to the recognition of dicarboxylic and aromatic substrates, the effect of mutation of the residue on the kinetic parameters for these substrates was studied (Table 3). All three mutant enzymes, R292A, R292L, and R292K ArATs, reacted with aspartate very slowly. The apparent rate constants for the half reactions showed no saturation of aspartate concentration, showing that the K_m value for aspartate is greatly increased by these mutations. The k_{cat}/K_m values for aspartate of R292A and R292L ArATs were lower by 10^5-fold, and that of R292K ArAT was lower by 10^4-fold, than that of WT ArAT. This clearly indicates that the guanidinium group of Arg292 is indispensable for the recognition of aspartate as the substrate, and even the positively charged ammonium group of a lysine residue only partially substitutes for the function of the guanidinium group. X-ray crystallographic studies of AspAT complexed with 2-methylaspartate show a bifurcated hydrogen bond connecting the guanidinium group of Arg292 and the β-carboxylate group, in addition to that connecting Arg386 and the α-carboxylate group (Arnone et al., 1985; Jansonius et al., 1985; Okamoto et al., 1994). For the interaction of the α-carboxylate group of aspartate and the guanidinium group of Arg386 of AspAT, a lysine residue could not substitute for the function of the arginine residue (Inoue et al., 1989), and this was considered to be due to the inability of a lysine residue to form the strong bifurcated hydrogen bond with a carboxylate group. Therefore, it is reasonable to consider that, like the reaction of aspartate with

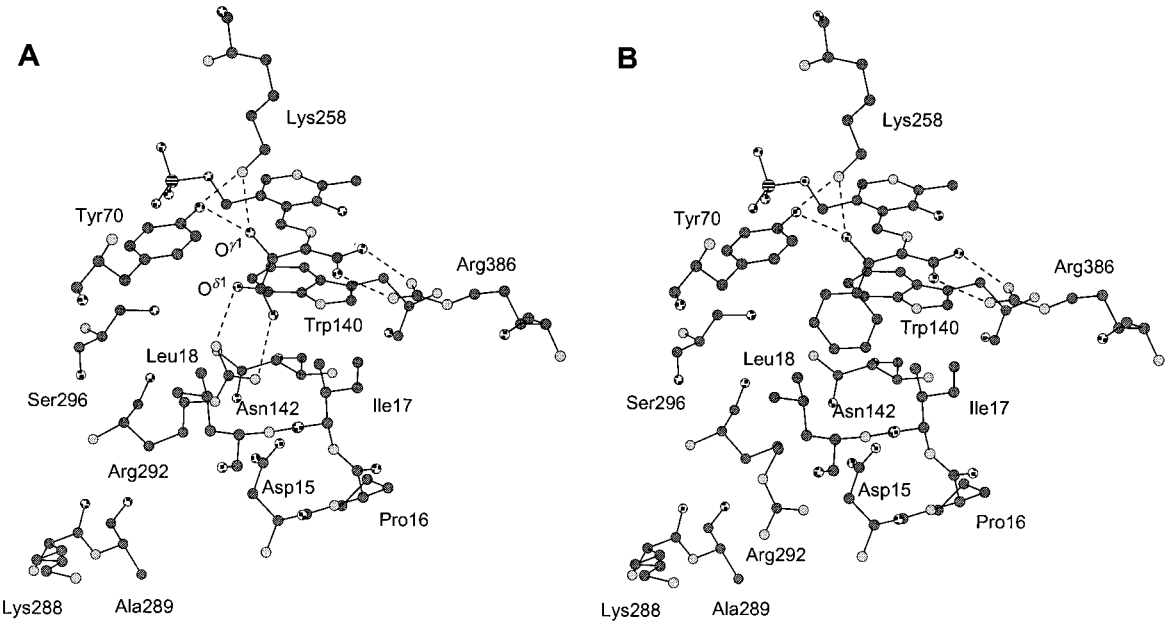

FIGURE 4: Proposed active-site structures of the HOAsp−ArAT (A) and HOPhe−ArAT (B) complexes, which were drawn according to the CHARMm-minimized model structure of the HOAsp−AspAT complex (Hayashi & Kagamiyama, 1995). In B, the torsion angles of the side chain of Arg292 were changed manually to avoid contact with the phenyl group of HOPhe and other residues. As the result, the side chain of Arg292 points outward, with its guanidinium group being able to interact with the solvent. Important hydrogen bonds are shown with dashed lines.

Table 3: Kinetic Parameters and the Aldimine pK_a of WT and Arg292 Mutant ArATs[a]

	WT[b]	R292A	R292L	R292K
aspartate				
k_{cat}[e]	290	ns[c]	ns	ns
K_m[f]	5.0	ns	ns	ns
k_{cat}/K_m[g]	58 000	0.13	0.17	0.97
phenylalanine				
k_{cat}[e]	1200	1300	1200	1400
K_m[f]	1.0	6.7	12	100
k_{cat}/K_m[g]	1 200 000	190 000	100 000	14 000
tyrosine				
k_{cat}[e]	500	500[d]	650[d]	710[d]
K_m[f]	0.83	3.8[d]	2.4[d]	9.0[d]
k_{cat}/K_m[g]	600 000	130 000	270 000	79 000
tryptophan				
k_{cat}[e]	350	270	230	320
K_m[f]	0.68	5.9	6.3	39
k_{cat}/K_m[g]	510 000	46 000	37 000	8200
aldimine pK_a	6.65	6.75	6.75	6.47

[a] The kinetic parameters were obtained from the single-turnover reactions in 50 mM HEPES-NaOH buffer, pH 8.0, containing 0.1 M KCl at 25 °C. The pK_a values of the PLP−Lys258 aldimine were obtained in solutions containing 50 mM buffer component(s) and 0.1 M KCl at 25 °C (see Materials and Methods). [b] Taken from Hayashi et al. (1993). [c] ns, saturation was not observed at the aspartate concentration of 50 mM. [d] Owing to the low solubility of tyrosine (up to 2 mM), the values of the parameters of the mutant ArATs for tyrosine are less certain than those of the other parameters. The SD values of k_{cat} and K_m are 89 s^{-1} and 0.9 mM for R292A, 63 s^{-1} and 0.3 mM for R292L, and 200 s^{-1} and 2.8 mM for R292K. The SD values of the other kinetic parameters were less than 10% of the fitted values. [e] In s^{-1}. [f] In mM. [g] In M^{-1} s^{-1}.

AspAT, the β-carboxylate group of the substrate aspartate is recognized by ArAT through the guanidinium−carboxylate hydrogen bond between the substrate and Arg292.

The k_{cat} values of R292A, and R292L ArATs for aromatic amino acids are almost equal to those of the wild-type enzyme, but the K_m values were increased by about 5−10-fold by these mutations. This indicates that the side chain of Arg292 is at least partially involved in the recognition of the aromatic ring of the substrates. The side chain of a leucine residue resembles the aliphatic portion of the side chain of an arginine residue, although the former has an extra methyl group. Therefore, the increased K_m values of R292L ArAT for aromatic substrates suggest the involvement of the guanidinium group of Arg292 in the recognition of aromatic substrates. Then it is of great interest to examine the effect of replacement of Arg292 by a lysine residue, which has a positive charge similar in position to that of an arginine residue. Like the R292A and R292L mutation, the R292K mutation did not essentially alter the k_{cat} values toward aromatic substrates. However, R292K mutation increased the K_m values for phenylalanine, tyrosine, and tryptophan by 100-, 11-, and 57-fold, respectively; the increases were much more prominent than those caused by R292A or R292L mutation (Table 3). The pK_a value of the aldimine formed between PLP and Lys258 was decreased by almost 0.2 pH unit upon R292K mutation, whereas R292A or R292L mutation increased the value by 0.1 pH unit, reflecting the removal of the positive charge of the guanidinium group (Table 3). This indicates that the ammonium group of Lys292 lies closer to the Lys258−PLP aldimine than the guanidinium group of Arg292 and suggests that the ammonium group undergoes unfavorable interaction with the aromatic ring of substrates. Although both an arginine and a lysine residue has a positive charge at almost the same distance from the polypeptide main chain, only the guanidinium group (of arginine) can maintain the proper conformation of the side chain of the residue 292 suitable for accommodating the aromatic ring of the substrates. This advantage of the guanidinium group over the ammonium group may be ascribed to the ability of the guanidinium group to form a larger number of hydrogen bonds with other groups and/or solvent water molecules than the ammonium group (for the structure of a probable interaction of Arg292 with solvent, refer to Figure 4B).

Although the above mechanism can explain the recognition of aromatic substrates involving Arg292, there are still unsolved problems. In particular, it is yet to be known why ArAT recognizes aromatic substrates far more efficiently than AspAT, while the residues directly interacting with the substrates are completely conserved among ArAT and AspAT. In AspAT, the R292L mutation increased the k_{cat}/K_m values of the enzyme toward aromatic amino acids by almost 10-fold (Hayashi et al., 1989). Although the K_m values for these reactions have not been obtained, the adverse effects of R292L mutation on ArAT and AspAT suggest differences between the two enzymes in the binding mode of the aromatic substrates and/or movement of the Arg292 side chain upon aromatic-substrate binding. The weak activities of AspAT toward aromatic amino acids can be explained mostly by the hydrophobic interactions between the enzyme and the amino acids, without taking into account the ring aromaticity of aromatic amino acids (Kuramitsu et al., 1990). The slope of the plots of the $\Delta G_T^{\ddagger}$ values for transamination of a variety of amino acids as a function of accessible surface area showed that the active site of ArAT is 1.4 times more hydrophobic than that of AspAT, and this in part accounts for the high activity of ArAT toward aromatic amino acids (Hayashi et al., 1993). However, the $\Delta G_T^{\ddagger}$ values for aromatic amino acids were considerably lower (by about 20 kJ mol^{-1}) than the values estimated by extrapolating the slope of the plots for nonaromatic neutral amino acids to the region for aromatic amino acid substrates (Hayashi et al., 1993). This suggests that, in addition to the hydrophobicity of the active site of ArAT, the aromaticity of the side chain of the aromatic substrates contributes to the binding of these substrates to the ArAT active site. With regard to this, it is interesting to consider the possibility that the guanidinium group directly interacts with the aromatic ring of substrates. The amino−aromatic interactions have been well documented in protein side chains and in model compounds (Burley & Petsko, 1986; Levitt & Pertuz, 1988; Rodman et al., 1993). Recent statistical analysis of high-resolution crystal structures of non-homologous proteins indicated that the arginine side chain is more favorable for interacting with the aromatic rings of phenylalanine and tyrosine than the lysine side chain because the guanidinium group of an arginine residue can maintain the hydrogen bonds with other groups by forming a stacked conformation with aromatic rings (Mitchell et al., 1994). These findings are consistent with the notion that the K_m values for aromatic substrates are increased, either by removing the guanidinium group (R292A and R292L mutations) that interacts with the aromatic ring or by replacing the guanidinium group with an ammonium group (R292K mutation). However, the stacked geometry of Arg292 and the aromatic ring of the substrates was found to be unfavorable when the structure of the ArAT−aromatic amino acid complex was modeled on the closed form of AspAT (Figure 4B), for either the

side chain of Trp140 or that of Leu18 will interfere with the guanidinium group of Arg292 in the stacked geometry. Leu18 is located just after the N-terminal α-helix of AspAT and belongs to the small domain, which on substrate binding moves toward the large domain to form the closed structure of AspAT. If we assume that ArAT allows flexible movement in the region around Leu18, we can expect a different way of side-chain packing around the substrate aromatic ring, which will make possible the stacked arginine−aromatic interactions. A difference in the N-terminal region between ArAT and AspAT may then explain the high efficiency of ArAT toward aromatic substrates.

Recently, Onuffer et al. (1995) showed that AspAT designed to increase the hydrophobicity of the active site by mutating six residues located near the active site to those of ArAT has increased activity toward aromatic amino acids without loss of AspAT activity. This hexamutant AspAT did bind phenylpropionate, an phenylalanine analog known to bind tightly to ArAT (Hayashi et al., 1993; Iwasaki et al., 1994), and X-ray crystallographic analysis of the complex showed that the analog's phenyl group displaces the side chain of Arg292 (Malashkevich et al., 1995). The resultant position of the phenyl group is similar to that shown in Figure 4B, and the guanidinium group of Arg292 locates just at the entrance of the active site and forms several hydrogen bonds with solvent water molecules. If we consider this hexamutant AspAT as the model for ArAT, we can expect that the aliphatic portion of the ArAT Arg292 side chain remains in the active site and forms a part of the wall of the active site, excluding water molecules which otherwise interfere with the aromatic ring of the substrates when they bind to the active site of ArAT. This structure, analogous to that shown in Figure 4B, does not support the "arginine−aromatic interaction" mechanism discussed above. However, these findings are observed on the protein scaffold of AspAT, and do not rule out the possibility that multiple side-chain differences between ArAT and AspAT and the resultant alterations in the backbone structure can cause ArAT to allow some specific interactions between Arg292 and the aromatic ring of the substrates. It is even possible to relate the lack of the aromatic−guanidinium interaction to the relatively low catalytic efficiency of the hexamutant AspAT toward aromatic substrates (10-fold lower k_{cat}/K_m values compared to those of ArAT). X-ray crystallographic studies on ArAT itself, although it resists all efforts at crystallization to date, is still required to elucidate the mechanism of the substrate recognition of ArAT.

Conclusions. The present study showed that the systematic hydroxy-group addition/deletion studies on both the substrates and the enzyme protein, and the resultant artificial formation/removal of hydrogen bonds between the enzyme protein and reaction intermediates, clearly located the site of ArAT that accommodates the aromatic side chain of aromatic substrates. The site overlaps that occupied by the distal carboxylate group of dicarboxylic substrates. Mutagenesis studies on Arg292 showed that Arg292 is both the binding residue for the distal carboxylate group of dicarboxylic substrates and the residue involved in the recognition of the aromatic side chain of aromatic substrates.

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