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[ARG292 → VAL] OR [ARG292 → LEU] MUTATION ENHANCES THE REACTIVITY OF ESCHERICHIA COLI ASPARTATE AMINOTRANSFERASE WITH AROMATIC AMINO ACIDS

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SUMMARY Arg292 of E. coli aspartate aminotransferase was substituted with valine or leucine by site-directed mutagenesis. In comparison with the wild-type enzyme, either of the mutant enzymes showed a decrease by over 5 orders of magnitude of k_{cat}/K_m values for aspartate and glutamate. This supports the contention that Arg292 is important for determining the specificity of this enzyme for dicarboxylic substrates. In contrast, mutant enzymes displayed a 5- to 10-fold increase in k_{cat}/K_m values for aromatic amino acids as substrates. Thus, introduction of an uncharged, hydrophobic side chain into position 292 leads to a striking alteration in substrate specificity of this enzyme, thereby improving catalytic efficiency toward aromatic amino acids.

A recent X-ray crystallographic study on E. coli aspartate aminotransferase [L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1.] (AspAT) (1) revealed that the structure of the subunit is closely similar to those of pig cytosolic (2) and chicken mitochondrial (3,4) AspATs. Most of the active site residues identified by X-ray crystallography of animal enzymes (2-4) are conserved in the E. coli enzyme (5). Of the residues forming the active site of AspAT, Arg292 is involved in substrate binding by forming hydrogen bond(s) with substrate ω-carboxyl group (2,4), and has been believed to be critical for the recognition of aspartate and glutamate as substrates. Cronin and Kirsch (6) described that replacement of Arg292 of E. coli AspAT by

Abbreviations: AspAT, aspartate aminotransferase; Bicine, N,N-bis(2-hydroxyethyl)glycine; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid.
Amino acid residues are numbered according to the sequence of cytosolic AspAT from pig (4).

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an aspartyl residue produced inversion of the substrate charge specificity. In an attempt to examine the role of the side chain of the amino acid residue at position 292 in determining preference for the side-chain structure of a substrate, we found that [Arg292 → Val] and [Arg292 → Leu] mutant enzymes showed a remarkably increased reactivity toward aromatic amino acid substrates. In this communication, we report the properties of these mutant enzymes.

MATERIALS AND METHODS

Chemicals Good buffers (MES, HEPES, Bicine) were obtained from Dojin Laboratories, Kumamoto. Oligonucleotides were synthesized with a Beckman System I plus DNA synthesizer and purified by reversed-phase high performance liquid chromatography on a Hitachi gel #3063 C18 column (Hitachi Ltd., Tokyo). All other chemicals used were the same as previously described (7).

Bacterial Strains and Phage or Plasmids E. coli TY103 strain was made from JM103 (Δ(lac proAB), thi, strA, supE, endA, sbcB15, hsdR4, [F', traD36, proAB, LacI^{q}ZΔM15]) (8) by incorporating kanamycin, chloramphenicol, and tetracyclin resistant genes into aspC (9), tyrB (10), and recA genes, respectively. The resulting strain no longer produced active AspAT. Details of this will be described elsewhere. The phage and plasmid used were M13mp18 (11) and pUC (12), respectively.

Medium E. coli was grown in YT medium containing 0.5 % yeast extract (Nacalai Tesque Co., Kyoto, Japan), 0.8% Tryptone (Difco Lab., Detroit), and 0.25% NaCl.

Site-directed Mutagenesis of Aspartate Aminotransferase The aspC gene was excised from pKDHE19 (13) with EcoRI and HindIII, and recloned into M13mp18 phage. A mixture of 24-mer oligonucleotides having a sequence of A-GCG(Ala)-GCG(Ala)-ATT(Ile)-NNN(Xxx)-GCT(Ala)-AAC(Asn)-TAC(Tyr)-TC was used for mutagenesis according to the method of Taylor et al. (14) using an Amersham oligonucleotide-directed in vitro mutagenesis system. Individual mutant clones were investigated for their sequences by the method of Sanger et al. (15). The [Arg292 → Val] and [Arg292 → Leu] mutants were verified by DNA sequencing of the entire coding frame. After preparing the replicative form of the recombinant phages, the mutant aspC genes were excised with EcoRI and HindIII, and were transferred into the EcoRI/HindIII site of the pUC19 vector. The resulting plasmids overproduced the mutated AspATs in E. coli TY103 and the mutated enzymes were purified, as in the case for wild type AspAT (13). The mutant enzymes thus obtained showed identical mobility as the wild-type enzyme on sodium dodecyl sulfate / polyacrylamide gel electrophoresis. The amino-terminal sequences of the mutant enzymes were the same as that of wild-type enzyme.

Spectrophotometric Measurements The absorption and circular dichroic (CD) spectra of the wild-type and mutant AspATs were measured using a Hitachi spectrophotometer (model 320) and a Jasco spectropolarimeter (model J-500), respectively, at 25 °C. A buffer solution (MES, HEPES, or Bicine at 50 mM) of ionic strength 0.1, maintained with KCl, was used to adjust the pH of the enzyme solution.

Protein Concentration The protein concentrations of the wild-type and mutant AspATs were determined based on a molar absorption coefficient of 5.1×10^4 $M^{-1} \cdot cm^{-1}$ at 280 nm (7).

Stopped-flow Kinetic Studies The reaction of enzymes with the substrates was studied at pH 8.0, 25 °C, by using a stopped-flow apparatus (Union Giken, RA-1300). The apparatus had a dead time of 1.5 ms under our operating conditions (5 Kg/cm^2 N_2 gas-pressure). A data-processing unit (Union Giken, RA-451) was used for fitting the theoretical curves. The enzyme concentration in the reaction mixture was about 5 μM.

RESULTS AND DISCUSSION

The CD spectra of mutant AspATs in the 200-250 nm region were indistinguishable from that of the wild-type enzyme, thereby suggesting the lack of gross conformational change upon these mutations. The CD spectra within the visible region, which provide information on the state of the coenzyme bound to the enzyme, were essentially the same between the wild-type and mutant enzymes (Fig. 1). Both the wild-type and mutant enzymes showed two absorption bands at 360 and 430 nm. The absorbance at 430 nm increased with decreasing pH, and is attributed to protonation of the aldimine bond formed between pyridoxal 5'-phosphate and Lys258 (16). The pK_a values of the internal aldimine bond can thus be determined by spectrophotometric titrations of the enzymes. The pK_a values for [Arg292 → Val] and [Arg292 → Leu] mutant enzymes were 6.98 and 6.95, respectively. These values are slightly higher than the corresponding value of 6.75 for the wild-type enzyme. This small pK_a shift may result from replacement of the positively-charged side chain by an uncharged one at position 292.

The rate of reaction of purified wild-type and mutant enzymes with acidic and aromatic amino acid substrates was determined by monitoring either the decrease in absorbance at 360 nm or the increase in absorbance at 330 nm under single turnover conditions (6). At pH 8.0, both the wild-type and mutant enzymes (pyridoxal form) show an absorption maximum at 360 nm. Upon addition of tested amino acids, the absorption maximum shifted from 360 nm to 330 nm, due to the conversion of the coenzyme pyridoxal 5'-phosphate into pyridoxamine 5'-phosphate. Both the decrease in absorbance at 360 nm and the increase in absorbance at 330 nm followed a pseudo-first-order kinetics. The pseudo-

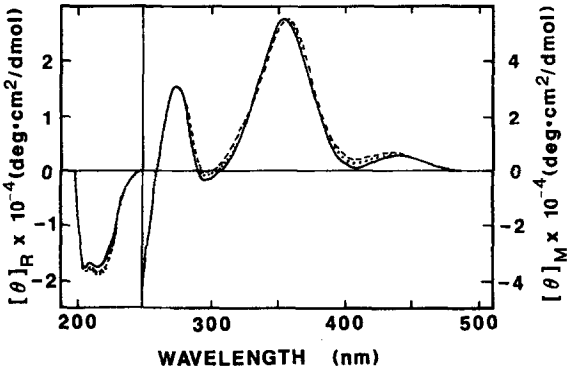

Fig. 1 Circular dichroic spectra of wild-type (———) and [Arg292 → Val] (- - - -) and [Arg292 → Leu] (• • • •) mutant enzymes. The spectra in the region from 200 to 250 nm were obtained with 40 μg/ml enzymes in 20 mM potassium phosphate buffer (pH 8.0). The spectra in the region above 250 nm were obtained with each enzyme (1.0 mg/ml) in 50 mM HEPES buffer (pH 8.0). All the solutions were adjusted to μ = 0.1 with KCl. $[\theta]_R$, mean residue ellipticity; $[\theta]_M$, molar ellipticity.

first-order rate constants (k_{app}) thus obtained were plotted against the concentrations of amino acids. Several examples of such plots are shown in Fig. 2. The reaction of the wild-type enzyme with aspartate or glutamate showed saturation kinetics. However, the value of k_{app} showed linear dependence on substrate concentration in all the other reactions studied. Thus, the specificity constant k_{cat}/K_m was obtained from the slope of the line. These values are summarized in Table 1.

In comparison to the wild-type enzyme, k_{cat}/K_m values for aspartate and glutamate was decreased by over 5 orders of magnitude, whereas the corresponding values for aromatic amino acids increased about 10-fold upon mutation. The dramatic decrease in k_{cat}/K_m for acidic amino acids is consistent with the hypothesis that Arg292 may determine the substrate specificity for dicarboxylic amino acids, as deduced from the crystallographic finding that Arg292 forms stable hydrogen bonds with the distal carboxylate group of dicarboxylic substrate analogues (2,4). A recent study on the [Arg292 → Asp] mutant AspAT by Cronin and Kirsch (6) showed a 5 x 10^5-fold reduction in k_{cat}/K_m for aspartate by the mutation. A similar degree of reduction in catalytic efficiency was observed upon mutation to leucine or

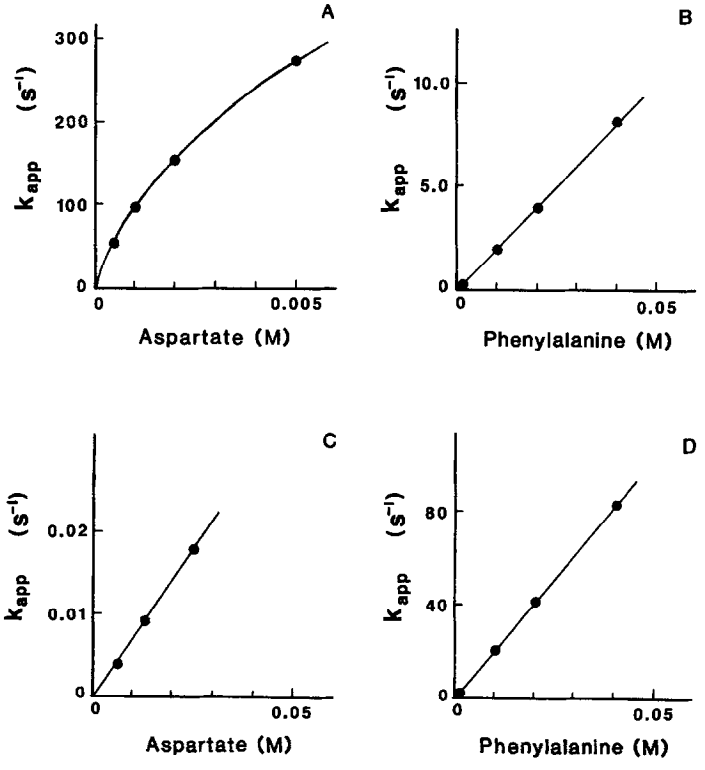

Fig. 2 Plots of the pseudo-first-order rate constants (k_{app}) versus concentration of the substrates. A: Wild-type AspAT with aspartate. B: Wild-type AspAT with phenylalanine. C: [Arg292 → Val] mutant AspAT with aspartate. D: [Arg292 → Val] mutant AspAT with phenylalanine.

Table I. Comparison of k_{cat}/K_m values of wild-type and mutant AspATs for acidic and aromatic amino acids[a]

Substrate[b]	k_{cat}/K_m (M^{-1} s^{-1})		
	Wild-type	[Arg292 → Val]	[Arg292 → Leu]
Aspartate	131000[c]	0.720	0.825
Glutamate	18000[c]	0.051	0.103
Phenylalanine	198	2110[c]	2090[c]
Tyrosine	687	3840[c]	4000[c]
Tryptophan	877	10100[c]	7840[c]

[a]The reaction was performed in 50 mM HEPES buffer, pH 8.0, which was adjusted to μ = 0.1 with KCl, at 25°C. [b]Substrate concentration ranges were 0.5-5 mM (wild-type) and 7.5-25 mM (mutants) for aspartate, 5.0-50 mM for glutamate, 2.0-40 mM for phenylalanine, 0.1-1.0 mM for tyrosine, and 1.0-10 mM for tryptophan. [c]Determined by stopped-flow kinetics.

valine. This suggests that the loss of activity of the [Arg292 → Asp] enzyme toward acidic amino acids may result from loss in an attractive force between Arg292 and the distal carboxylate rather than from generation of electrostatic repulsion between Asp292 and the distal carboxylate.

Wild-type E. coli AspAT shows a fairly high activity toward aromatic amino acids as substrates, when compared to eukaryotic enzymes (17). Although enhancement of reactivity with aromatic amino acids by mutation of Arg292 to Val or Leu was only 5-10 fold , as indicated by the increase in k_{cat}/K_m values, the value of the [Arg292 → Val] mutant enzyme particularly for tryptophan was of the same order of magnitude as the value of the wild-type enzyme for glutamate, which is a natural substrate. The precise mechanism of this enhancement is not yet clear at the present. Both elimination of the charge and reduction in the size of the side chain of the residue at position 292 may generate a favorable microenvironment for accomodating aromatic amino acids as substrates. Since k_{cat}/K_m values of [Arg292 → Glu], [Arg292 → Asp], and [Arg292 → Lys] mutants for aromatic amino acids were almost the same as those of the wild-type enzyme (Hayashi et al., unpublished results), the presence of a charged side-chain at position 292 is considered unfavorable for accomodating aromatic amino acids. Replacement of Arg292 by other neutral amino acids, including alanine and isoleucine, is currently being underway in our laboratory.

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