Enhanced transaminase activity of a bifunctional L-aspartate 4-decarboxylase

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Abstract

L-Aspartate 4-decarboxylase (Asd) catalyzes mainly the β -decarboxylation of aspartate and also transamination with α -keto acids. To investigate residues that are critical in directing the reaction pathway, seven point mutations were designed based on the differences between Asd and aminotransferases in conservative amino acid residues. All mutant Asds were purified and characterized. The F204W mutant enhanced aminotransferase activity, and its ratio to β -decarboxylase activity was 3.8-fold. Its K_m values for aspartate and α -ketoglutarate were 1.3 and 0.17 mM, respectively, representing a large increase in the binding affinity with substrates. The K347R mutation did not increase transaminase activity. The D360P mutation decreased transaminase activity and was more specific in catalyzing β -decarboxylation reaction. This is the first study that successfully increased transaminase activity in Asd via site-directed mutagenesis. The modeled protein structure reveals how the residue may involve in reaction specificity, providing insights into comprehending the molecular evolution of this bifunctional enzyme.

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L-Aspartate 4-decarboxylase (EC: 4.1.1.12, Asd) has been found to be a bifunctional enzyme that catalyzes mainly the decarboxylation on carbon-4 of L-aspartate and a very minor part of the transamination reaction [1]. As both Asd and aminotransferase (AT) are pyridoxal 5'-phosphate (PLP)-dependent enzymes, to participate in these two types of reactions, they need α -keto acids, which act as activators in the β -decarboxylation reaction [2] but as substrates in the transamination reaction.

PLP-dependent enzymes play critical roles in the metabolism of amino acids. Members of these enzymes catalyze various reactions with similar structures of the PLP domain [3]. Only delicate structural differences cause their reaction specificities to diverge [4,5]. In the transamination

and 4-decarboxylation reactions of L-aspartate, they share the same quinonoid intermediate. The reaction pathway was diverged in the following steps to perform transamination or β -elimination. An R292K mutation of aspartate aminotransferase (AspAT) from *Escherichia coli* caused a 440-fold decrease in transaminase activity of that of the wild-type enzyme [5]. Afterward, a Y225R/R386A substitution, which enhanced decarboxylase activity and decreased AT activity, was combined with K292R in AspAT to direct the molecular evolution in changing reaction specificity of AspAT into Asd [6]. The only sequence of Asd was from *Pseudomonas dacunhae* in a U.S. patent at that time.

In recent years, more *asd* genes have been released in the databank including two from our previous studies [7,8]. However, the precise residues that dominate reaction specificity are still unclear. In the present study, we designed point mutations according to the sequence alignment between these two enzymes. Seven amino acid residues that are highly conservative in AT but different in Asd were

Abbreviations: Asd, L-aspartate 4-decarboxylase; AT, aminotransferase; PLP, pyridoxal 5'-phosphate; α -KG, α -ketoglutarate; HPLC, high performance liquid chromatography; CD, circular dichroism.

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selected to be replaced, and the effects of these changes on reaction specificity were studied. A partially modeled Asd were used to identify the active site and to explain the function of residues.

Materials and methods

Sequence analysis. Protein sequences were retrieved from protein databases on the website of National Center for Biotechnology Information. AspAT from *E. coli* (eAspAT, Accession No. P00509), pig cytosolic AspAT (cAspAT, Accession No. NP_999092), TyrAT from *E. coli* (eTyrAT, Accession No. AAC77024) and aspartate/tyrosine/aromatic aminotransferase from *Burkholderia fungorum* LB400 (bAroAT, Accession No. ZP_00282851) were selected to represent aminotransferases. Asd from *Alcaligenes faecalis* CCRC 11585 (aAsd, Accession No. AAK58507) and from *Pseudomonas* sp. ATCC 19121 (pAsd, Accession No. AAQ07948) were used. Numbering of amino acid residues mentioned in this study follows cAspAT for aminotransferase and aAsd for aspartate 4-decarboxylase. Clustalx1.83 [9] was used for multiple sequence alignment.

Site-directed mutagenesis and protein purification. Asd mutants were prepared using the QuikChange site-directed mutagenesis kit (Stratagene). These substitutions were PCR-amplified using pES1, containing *asd* from *Pseudomonas* sp. ATCC 19121 [8], as the template and the designed antiparallel primers carrying codons for the required substitutions (Table 1). The product was treated with *DpnI* at 37 °C for 1 h and proliferated in *E. coli* XL1-Blue supercompetent cells. Selected clones were sequenced to verify the required mutations and transformed into *E. coli* BL21(DE3)-pLysS for protein expression. Expression conditions and purification of the His-tagged proteins were performed as described previously [8]. Purified enzyme was analyzed by SDS-PAGE following the method of Laemmli [10] and verified by polyclonal anti-Asd antisera as described previously [8].

Determination of protein concentration and enzyme activity. The protein concentration was determined as described by Bradford [11], using the Bio-Rad protein assay with bovine albumin as the standard. Kinetic parameters for 4-decarboxylase activities of wild-type and mutant Asds were estimated in a reaction mixture containing 5 µg purified His-tagged protein, 0.5 mM PLP, and 40 mM α -ketoglutarate (α -KG) in 0.1 M Na acetate buffer, pH 5.0. After preincubation at 37 °C for 40 min, 0–40 mM aspartate was added to start the reaction, which was stopped by boiling after 20 min. For the estimation of AspAT activity, 5 µg purified enzyme was preincubated in 0.3 M Na acetate buffer (pH 5.0) with 0.5 mM PLP and 0.1–20 mM α -KG at 37 °C for 40 min. The reaction was started by adding 40 mM L-aspartate and stopped after 6 min by boiling for 15 min. The reaction mixture was treated with 2% 5-sulfosalicylic acid, cooled at 4 °C for 1 h and centrifuged at 15,000 rpm for 20 min. Amino acid products, alanine for decarboxylation and glutamate for transamination, in the supernatant were derivatized with *o*-phthalaldehyde reagent and then analyzed with a LichroCART RP-18 column (5 µm, 250 × 4.6 mm, Merck) by high performance liquid chromatography as described by Jones and Gilligan [12]. The K_m and V_{max} values were estimated by Lineweaver–

Burk treatment. The values of k_{cat} were estimated with the assumption that the dodecameric form of Asd existed at pH 5.0.

Structural analysis. Circular dichroism (CD) spectra were taken in the range of 190–250 nm at 0.1-nm increments and a 0.25-s acquisition time on a Jasco j-715 CD spectropolarimeter (Japan). Each purified protein sample was moved into 10 mM sodium phosphate buffer (pH 6.0) at a concentration of 2 µM with 0.1 mM PLP and scanned in a 1 mm quartz cuvette three times with 10 mdeg sensitivity. A partially modeled Asd (Wang, Ko, Liou and Lee, unpublished data) using PLP domain of 3TAT as the template was used to examine the structural relationships between mutated residues and the active site.

Results

Sequence comparison and protein identification

Asd sequences from *Pseudomonas* sp. ATCC 19121 and *Alcaligenes faecalis* CCRC 11585 were multiple aligned with aminotransferases to identify the conservative amino acid residues between Asd and AT (Fig. 1). There are 19 conserve residues that are identical to AT in Asd, six of which are in the PLP-binding motif, including the active-site Lys315 residue (numbered 258 in AT) that contributes to the formation of covalent binding with coenzyme. A noteworthy point is that the Y225R/R292K/R386A mutation of AspAT from *E. coli* converts its activity into an aspartate 4-decarboxylase [6]. However, we found that residues Tyr225 and Arg386 were the same in AT with Asd, which were numbered as Tyr289 and Arg497, respectively, in Asd, and the relative residue to Arg292 of AT is Lys347 in Asd. This implies that Lys347 in Asd plays a critical role in controlling the reaction specificity, but the K347R mutation of Asd in our study failed to enhance transaminase activity (Table 2). In this study, His123, Met178, Phe204, His337, Asp360 and Val475 of the Asd were also chosen to be mutated respectively into the residue conserved in aminotransferases according to the alignment shown in Fig. 1. The mutation of the *asd* was confirmed by sequencing and expressed in *E. coli* BL21(DE3)pLysS. The expressed protein was identified with antiserum of the Asd from *Pseudomonas* sp. ATCC 19121 (Fig. 2).

Activity analysis

The enzyme activity of the purified native and mutant Asd was assayed by HPLC analysis of the reaction prod-

Table 1
Sequence of primers used to introduce mutations in Asd

Mutation	Forward	Reverse
H123Y	5'-CGCCGCCTTCTCTGTCACGAGATGGTGG-3'	5'-CCACCATCTCGTACAGGAAGGCGGCG-3'
M178L	5'-GGCACGGCGGCCCTTGGCCTATATCTTC-3'	5'-GAAGATATAGGCCAAGGCCCGCGTGCC-3'
F204W	5'-CATCGGCATGCCGGTGTGGACGCCGTATATC-3'	5'-GATATACGGCGTCCACACCGGCATGCCGATG-3'
H337R	5'-CAACGTGTTTCGACCGTGCACGTGTCGAG-3'	5'-CTGCGACAGTGCAAGGTCGAACACGTTG-3'
K347R	5'-CCCGAGTCCGCTAGGAAGCGCGTGG-3'	5'-CCAGCGCCTTCCTAGCGGACTCGGG-3'
D360P	5'-CTCGCTGCTGCCCGCTGTGCGCTCGCTC-3'	5'-GAGCGAGCGCACAGGGGCGAGCAGCGAG-3'
V475L	5'-CATGCTGTTCCGCCCTGGCGGACGAGACC-3'	5'-GGTCTCGTCCGCCAGCGGGAACAGCATG-3'

Mismatched bases are underlined.

Fig. 1. Multiple alignment of L-aspartate 4-decarboxylase and related aminotransferases. The boxed conserved region was predicted as the aminotransferase class-I PLP attachment site. The triangular mark represents the active site Lys that binds PLP covalently during catalysis. Black boxes show the three sites that Graber et al. mutated in AspAT [6], Tyr225, and Arg386 in AT, corresponding to Tyr289 and Arg497 in Asd, respectively. Arrows indicate residues mutated in this study. Accession numbers and abbreviations of these sequences are as described in Materials and methods.

Table 2

Aspartate 4-decarboxylase and aminotransferase activities of the purified wild-type and mutant Asds

Enzyme	4-Decarboxylation		Aminotransferase		Activity ratio (Asd/AT)
	Specific activity (U/mg)	Relative activity (%)	Specific activity (U/mg)	Relative activity (%)	
Wild type	128.8 ± 4.4	100	0.060 ± 0.010	100	2145.9
H123Y	103.1 ± 22.3	79.6 ± 14.6	0.066 ± 0.021	106.8 ± 16.4	1562.4
M178L	100.9 ± 26.2	77.7 ± 17.7	0.061 ± 0.016	100.2 ± 10.0	1653.2
F204W	111.5 ± 5.1	86.8 ± 6.9	0.197 ± 0.020	331.9 ± 21.8	566.0
H337R	98.0 ± 20.7	75.6 ± 13.5	0.059 ± 0.018	96.0 ± 13.2	1660.5
K347R	125.8 ± 10.7	97.5 ± 5.0	0.054 ± 0.011	89.9 ± 2.6	2329.0
D360P	131.2 ± 11.9	101.7 ± 5.8	0.032 ± 0.008	53.2 ± 5.2	4100.1
V475L	118.3 ± 6.5	91.8 ± 1.9	0.062 ± 0.016	101.4 ± 9.4	1907.3

One unit of enzyme activity is defined as the formation of 1 μmol of alanine or glutamate per min at 37 °C for Asd or AT, respectively.

Fig. 2. (A) SDS-PAGE and (B) Western analysis of the expressed wild-type and mutant Asds. Lanes 1–8 and 9–16 were cell lysates and purified proteins, respectively. Lanes 1 and 9, wild-type Asd; lanes 2 and 10, H123 mutant; lanes 3 and 11, M178L mutant; lanes 4 and 12, F204W mutant; lanes 5 and 13, H337R mutant; lanes 6 and 14, K346R mutant; lanes 7 and 15, D360P mutant; lanes 8 and 16, V475L mutant.

Table 3
Estimates of the kinetic constants of Asds

Enzyme	4-Decarboxylation ^a			Aminotransferation ^b		
	$K_m(\text{Asp})$ (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)	$K_m(\alpha\text{-KG})$ (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)
Wild type	27.1	1519.9	56.1	0.33	69.2	217.7
H123Y	26.2	1169.1	44.7	—	—	—
M178L	33.8	1688.8	49.9	—	—	—
F204W	1.3	135.5	101.5	0.17	81.8	468.2
H337R	27.3	1334.1	48.9	—	—	—
K347R	27.1	1193.8	44.1	0.34	86.8	292.0
D360P	18.4	1106.9	60.0	0.43	61.5	140.6
V475L	27.4	1315.8	48.0	—	—	—

^a This is one representative of two independent experiments. Values were determined in 0.1 M Na acetate, pH 5.0 at 37 °C for 20 min and 5 μ g purified protein by Lineweaver–Burk analysis of the rate of alanine produced.

^b Shown is the average value of two independent experiments. Values were determined in 0.3 M Na acetate buffer (pH 5.0) containing 0.5 mM PLP, 40 mM L-Asp and 0.1–20 mM α -KG at 37 °C for 6 min and 5 μ g protein by Lineweaver–Burk analysis of the rate of glutamate produced. —, not determined.

Fig. 3. Circular dichroism spectra of the wild-type and mutant Asds.

ucts, alanine for 4-decarboxylation and glutamate for transformation. The F204W mutant protein catalyzed transamination between L-aspartate and α -KG, resulting in a 3.8-fold increase in the ratio of aminotransferase to decarboxylase activity, which resulted from the 3-fold transaminase activity increase and 13.2% decarboxylase activity decrease. Another special mutation, D360P, which decreased the transaminase activity by half, was the cause behind the two times increase in the ratio of decarboxylase to aminotransferase activity. This D360P mutation made Asd a more efficient 4-decarboxylase. Other mutations, H123Y, M178L, H337R, and V475L, did not result in obvious alteration in reaction activity or catalytic specificity.

Mutants were then selectively chosen to test their kinetic parameters in 4-decarboxylation and transamination reaction. Table 3 shows that H123Y, M178L, K347R, and V475L mutants exhibit decarboxylase activity similar to that of the native Asd; meanwhile, F204W and D360P

mutants were found to increase by 20- and 1.5-fold the binding affinity with L-aspartate. In the transamination reaction, 0.1–20 mM α -KG was added to the reaction mixture in the presence of 40 mM L-aspartate to examine the binding affinity of α -KG with enzyme. In Table 3, a 2-fold increase in K_m value can be found in F204W, which makes the F204W mutant catalyze the transamination reaction more efficiently than wild-type Asd. This tendency is consistent with its reverse mutation, W140F mutated on AspAT, as reported in a previous study [13], which showed K_m increasing and catalytic efficiency (k_{cat}/K_m) decreasing in the W140F mutant. Furthermore, the D360P mutation showed less affinity in binding α -KG (Table 3), which led to a 35% decrease in the catalytic efficiency in the transamination reaction and was more catalytically specific to the 4-decarboxylation reaction.

Structural view

The circular dichroic spectra of Asd mutants did not show obvious changes at the secondary structure of proteins upon mutations (Fig. 3 and Table 4), which suggests that the differences in activity were influenced by these site-directed mutations.

Some residues selected to be mutated are shown in a partially modeled Asd (Fig. 4A) from *Pseudomonas* sp. ATCC 19121. Three mutated amino acid residues, His123, Asp360, and Val475, are excluded from the predicted modeled region of the PLP domain. H337 is appar-

Table 4
Results of CD spectral analysis

Protein	α_R	α_D	Total α	β_R	β_D	Total β	Turn	Unordered	NRMSD
Wild type	0.39	0.21	0.60	0.04	0.04	0.08	0.11	0.21	0.009
F204W	0.38	0.19	0.57	0.09	0.07	0.16	0.11	0.18	0.011
K347R	0.44	0.20	0.64	0.06	0.04	0.10	0.14	0.14	0.008
D360P	0.33	0.19	0.52	0.05	0.04	0.09	0.15	0.25	0.02

α_R , regular α -helix; α_D , distorted α -helix; β_R , regular β -strand; β_D , distorted β -strand; NRMSD, normalized root mean square deviation. The CD spectra were analyzed by CDSSTR method at Dichroweb [14].

Fig. 4. Structural view of the active site of the modeled Asd from *Pseudomonas* sp. ATCC 19121. (A) Two subunits of Asd are shown as solid ribbons in blue and cyan; those of AT are shown as threads in dark and light green. The residues that were mutated are shown as stick models and colored by element. Also shown are the PLP and maleate (an analog of aspartate) from the AT structure. (B) Two residues in the active site are colored according to the protein chain to which they belong. The Trp that substituted Phe204 of Asd is likely to form a hydrogen bond by the indole N atom with the carboxyl group of aspartate. Trp140 and Arg386 of AT and Arg292* from the counter subunit are shown as lines. These two arginines are involved in hydrogen bonding with α -carboxyl and β -carboxyl group of the substrate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

ently remote from the catalytic site; meanwhile, K347, F204, and M178 are located in the catalytic center of the modeled Asd. As regards the F204W mutation of Asd (Fig. 4B), when an indole ring of Trp was introduced into

this position, the π - π interaction between the pyridine ring of PLP and the indole ring of Trp might shift the electron distribution and cause some rotational movement of PLP during catalysis, since the indole ring of Trp140 is important for stacking with the pyridine ring of PLP [13].

Discussion

It is noteworthy that Graber et al. made a three point mutation to convert AspAT into Asd [6]. The only difference in the three amino acid residues between these two enzymes is Arg292 in AspAT (Lys347 in Asd). A change in reaction specificity is expected if a K347R mutation is made in Asd. Our findings, however, go against this assumption. The K347R mutant exhibited 4-decarboxylase and aminotransferase activity similar to the native Asd (Tables 2 and 3), although Vacca et al. found that the replacement of Arg292 of AspAT into Lys decreased transaminase activity to 0.22% [5]. It appears that the reverse is not true in Asd.

On the other hand, the present study shows that an F204W mutation of Asd enhanced AT/Asd activity ratio 3.8-fold and decreased the K_m value for aspartate 20-fold. It is suggested that this large increase in the binding affinity is due to a hydrogen bonding between the β -carboxyl group of aspartate and the N-1 atom of Trp. In conclusion, Phe204 in Asd is endowed with a substantial importance in catalyzing 4-decarboxylation reaction of L-aspartate, which might be explained by the π - π interaction between the pyridine ring of PLP and the indole ring of Trp, an interaction that might affect the electron distribution at the active site. A more precise prediction will be made through the use of Asd crystallography in the near future.

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