

Chapter 21

Aspartate Racemase: Function, Structure, and Reaction Mechanism

Masafumi Yohda

Abstract Aspartate racemases distribute and function to produce D-aspartate in eubacteria, archaea, invertebrates, and vertebrates. The aspartate racemases of eubacteria and hyperthermophilic archaea are pyridoxal 5'-phosphate (PLP) independent, and two conserved cysteine residues constitute the catalytic center. The crystal structure of the aspartate racemase of hyperthermophilic archaeon was determined. Based on this structure, the detailed reaction mechanism of the pyridoxal 5'-phosphate-independent aspartate racemase was studied by characterizing mutants and molecular dynamics simulations. However, it is still unclear how the catalytic cysteine residue can abstract a proton from the α -carbon. The aspartate in hyperthermophilic archaea is highly racemized, but the physiological role of aspartate racemase and D-aspartate in hyperthermophilic archaea is unknown. The aspartate racemases in invertebrates and vertebrates are PLP dependent. The aspartate racemases from invertebrates, bivalves, and *Aplysia californica* are homologous to serine racemases, but it has taken many years to identify the aspartate racemase responsible for the synthesis of D-Asp in mammals due to the lack of other amino acid racemases. The gene for the mammalian aspartate racemase was obtained *via* its homology with glutamate-oxaloacetate transaminase. Further studies on aspartate racemase will promote research on the mysterious functions of D-Asp in various organisms.

Keywords Aspartate racemase • PLP independent • D-Aspartate

21.1 Introduction

Aspartate racemase activity was first reported in *Lactobacillus fermenti* (Johnston and Diven 1969). In 1972, the incorporation of D-aspartate into a peptidoglycan in lactic acid bacteria (Staudenbauer and Strominger 1972) and the partial purification of aspartate racemase from *Enterococcus faecalis* (formerly *Streptococcus faecalis*)

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were reported (Lamont et al. 1972). However, a detailed characterization of aspartate racemase, including the kinetics and cofactor dependency, was not described, most likely due to the low purification yield. I became involved in the study for aspartate racemase when I was working for the Central Research Center of Asahi Glass Co. Ltd. Our group has purified aspartate racemase and cloned the gene for it from *Streptococcus thermophilus* (Okada et al. 1991; Yohda et al. 1991). This was the first purified and cloned aspartate racemase. The subsequent amino acid sequencing and biochemical analysis showed that it is pyridoxal 5'-phosphate (PLP) independent. Since the project on the application of aspartate racemase was terminated and I have retired from Asahi Glass Co. Ltd., no further study has been performed on the enzyme.

I encountered aspartate racemase again while working for RIKEN. It is a very curious story. When I was attending the annual meeting of the Japanese Society for Molecular Biology in Kobe, my old friend, Professor Toshiko Ohta, showed me a slide for the sequence of a gene from a hyperthermophilic archaeon. I was very much astonished to see that it was highly homologous to *Streptococcus* aspartate racemase. Since then, I have performed various studies to reveal the reaction mechanism of PLP-independent aspartate racemases (Yohda et al. 1996; Matsumoto et al. 1999; Liu et al. 2002a, b; Yoshida et al. 2006; Ohtaki et al. 2008).

D-Aspartates also exist in invertebrates and vertebrates as well. Aspartate racemases that are involved in the synthesis of D-aspartate have recently been identified and were found to be PLP dependent. Professors Yamada and Kera have performed excellent studies on D-aspartate and aspartate racemase in the blood shell *Scapharca broughtonii* (Shibata et al. 2003a, b; Abe et al. 2006; Yamada et al. 2006). Although D-aspartate exists in various mammalian organs and is especially abundant in the developing brain, the search for the enzyme responsible for the formation of D-aspartate has been *in vain*. The difficulty is due to the small quantity in the cell and also the lack of sequence homology with known amino acid racemases. Finally, Professor Snyder's group succeeded in cloning aspartate racemase *via* an ingenious speculation (Kim et al. 2010). In this article, I will review the studies on both types of aspartate racemases.

21.2 PLP-Independent Aspartate Racemases

21.2.1 Aspartate Racemases from Lactic Acid Bacteria

Okada et al. examined the distribution of aspartate racemases in lactic acid bacteria (Okada et al. 1991). They purified aspartate racemase from *Streptococcus thermophilus* IAM1198 (StAspR) to homogeneity with a 3400-fold purification. The purified StAspR is likely to be a homodimer with a molecular mass of 28 kDa. StAspR is specific to aspartate and does not catalyze the racemization of alanine and glutamate, and the addition of pyridoxal 5'-phosphate has no effect on its

activity. On the contrary, the addition of SH-protecting reagents, 2-mercaptoethanol, or dithiothreitol activates StAspR, suggesting the involvement of cysteine residues in the catalysis. The gene encoding StAspR was cloned from the *S. thermophilus* genome using a probe designed from the N-terminal amino acid sequence (Yohda et al. 1991). The gene contains an open reading frame of 729 nucleotides coding for 243 amino acids. The calculated molecular mass of 27,945 Da agrees well with the apparent molecular mass. The recombinant StAspR was expressed in *Escherichia coli* and was used for the study of the detailed reaction mechanism (Yamauchi et al. 1992). StAspR does not contain PLP or other cofactors such as FAD, NAD⁺, and metal ions. Neither carbonyl reagents, such as hydroxylamine, nor sodium borohydride affects StAspR. On the contrary, StAspR is strongly inhibited by iodoacetamide and other thiol reagents. In addition to aspartate, cysteate and cysteine sulfinic acid are good substrates for StAspR. The K_m values for L- and D-aspartate are 35 and 8.7 mM, respectively. StAspR catalyzes the exchange of the α -hydrogen from the substrate with the solvent's hydrogen. The racemization of L-aspartate in D₂O shows an overshoot in the optical rotation of aspartate before the substrate is fully racemized. This shows that the removal of the α -hydrogen from the substrate is at least partially rate limiting. When L- or D-aspartate is incubated with aspartate racemase in tritiated water, the tritium is preferentially incorporated into the product enantiomer. The results strongly suggest that aspartate racemase functions *via* a two-base mechanism, similar to other PLP-independent amino acid racemases, glutamate racemases and proline racemases. In this mechanism, an α -hydrogen from the substrate amino acid is abstracted on one face as a proton, while a proton is incorporated on the other face. Thus, two conserved cysteine residues are thought to be involved in the reaction of these amino acid racemases.

21.2.2 Aspartate Racemases from Hyperthermophilic Archaea

As murein (peptidoglycan) is the only cell wall polymer that forms rigid cell walls in eubacteria, almost all eubacteria have D-amino acids and amino acid racemases. Archaea have a variety of cell wall and cell envelope polymers. Many archaea (including all crenarchaeotes, euryarchaeotes, halophiles, and methanogens) have an outer envelope (or S-layer) composed of hexagonally or tetragonally arranged proteins or glycoproteins that are easily disintegrated by mechanical shearing or detergents. In methanogens, the structure of this polymer is similar to that of murein, and it is referred to as "pseudomurein." Pseudomurein differs from murein in several respects. In particular, no D-amino acids are present in pseudomurein. Thus, there have been only a few reports on D-amino acids or amino acid racemases in archaea (Nagata et al. 1998).

The existence of aspartate racemase and D-aspartate in hyperthermophilic archaea was revealed by accident. While examining the roles of heat shock proteins in the thermotolerance of hyperthermophiles, Professor Toshiko Ohta (Emeritus Professor of Tsukuba University) intended to clone the genes encoding heat shock proteins from hyperthermophilic archaea. She attempted to obtain a DNA fragment from the *hsp70* by PCR amplification from the genomic DNAs of several hyperthermophilic archaea using a set of primers designed from the consensus sequences of *hsp70s*. Among those tested, a fragment of approximately 400 bp was amplified from the genomic DNA of *Desulfurococcus* strain SY. However, the sequence of the amplified DNA was not homologous to other *hsp70s*, but rather, it was similar to *StAspR*. Next, we examined whether aspartate racemase really exists in hyperthermophilic archaea (Yohda et al. 1996). The full-length gene was cloned from the genome of *D. SY*. It contains a 705-bp open reading frame, encoding a 235-residue polypeptide with a molecular mass of 25,977 Da. It shares considerable homology (35.2% identity and 63.1% similarity in the amino acid sequence) with *StAspR*. The amino acid sequence is also homologous to those of glutamate racemases from *Bacillus sphaericus*, *E. coli*, *Lactobacillus brevis*, *Lactobacillus fermenti*, and *Pediococcus pentosaceus*. The homology scores are 28.2, 24.0, 21.6, 27.6, and 27.7%, respectively.

The putative hyperthermophilic aspartate racemase was expressed in *E. coli*. The recombinant protein exhibited amino acid racemase activity, which was highly specific for aspartate and increased proportionally with the temperature from 37 to 90 °C. Therefore, the protein was identified as the first hyperthermophilic archaeal amino acid racemase. Aspartate racemase activity was also detected in the crude extract of *D. SY*.

Matsumoto et al. investigated the occurrence of free D-amino acids and aspartate racemases in several hyperthermophilic archaea (Matsumoto et al. 1999). The aspartate in all hyperthermophilic archaea is highly racemized. The ratio of D-aspartate to total aspartate is in the range of 43.0–49.1%. The crude extracts of the hyperthermophiles exhibit aspartate racemase activity at 70 °C, and their homologous aspartate racemase genes have been identified by PCR. The D-enantiomers of other amino acids (alanine, leucine, phenylalanine, and lysine) in *Thermococcus* strains have also been detected. Although the presence of D-aspartate in some hyperthermophilic archaea has been proven, their function is unclear.

21.2.3 The Structure and Reaction Mechanism of PLP-Independent Aspartate Racemase

21.2.3.1 The Structure of Aspartate Racemase from *Pyrococcus horikoshii* OT3

Liu et al. determined the three-dimensional structure of aspartate racemase from the hyperthermophilic archaeon *P. horikoshii* OT3 (PhAspR) at a 1.9-Å resolution

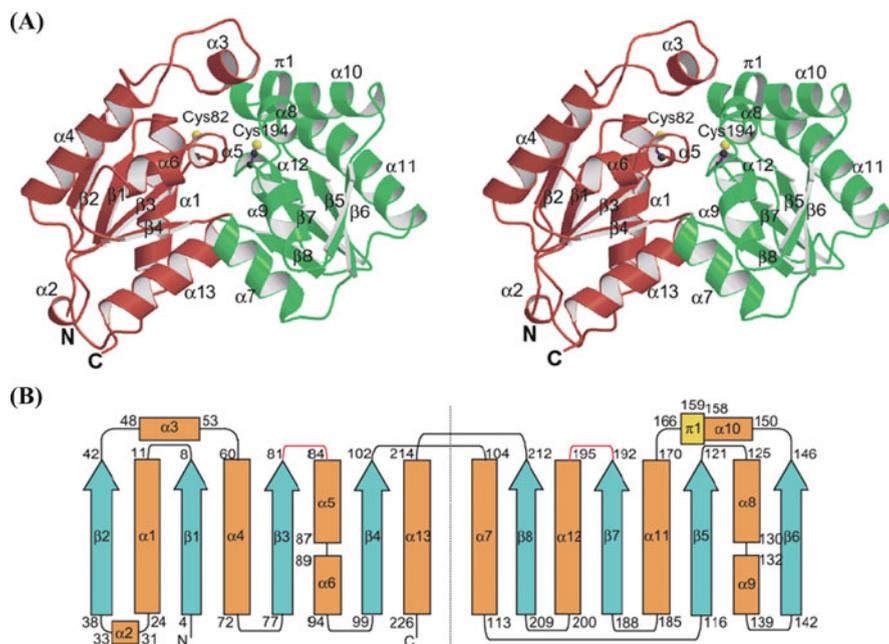


Fig. 21.1 (a) A stereoview of the ribbon diagram of the overall monomer structure of *P. horikoshii* OT3 AspR. The N- and C-terminal domains are colored red and green, respectively. All of the helices and strands, represented as coils and arrows, are labeled sequentially. The positions of Cys82 and Cys194 are labeled, and their side chains are depicted with ball-and-stick models. (b) A topology diagram of the secondary structural elements of *P. horikoshii* OT3 AspR. The α -helices are represented by orange bars, the π -helix by a fat yellow bar, and the β -strands by blue arrows. The two loops containing the two active-site cysteine residues are colored red (Reproduced from Figure 1 of Liu et al. 2002a)

using X-ray crystallography and refined it to a crystallographic R factor of 19.4% (R_{free} of 22.2%) (Liu et al. 2002a) (Fig. 21.1). PhAspR forms a stable dimeric structure *via* a strong three-layered inter-subunit interaction. The subunit consists of two structurally homologous α/β -domains, each containing a four-stranded parallel β -sheet flanked by six α -helices. Two strictly conserved cysteine residues (Cys82 and Cys194) are located on both sides of a cleft between the two domains. The spatial arrangement of these two cysteine residues supports the “two-base” mechanism. The previous hypothesis that the active site of aspartate racemase is located at the dimeric interface is clearly incorrect. The structure revealed a unique pseudo-mirror symmetry in the spatial arrangement of the residues around the active site, which may explain the molecular recognition mechanism of the mirror-symmetric aspartate enantiomers by the non-mirror-symmetric aspartate racemase.

The structural homology and functional similarity between the two domains suggest that this enzyme evolved from an ancestral domain *via* gene duplication and gene fusion. Liu et al. expressed only the C-terminal domain of PhAspR (SC-domain) and determined its three-dimensional structure by X-ray

crystallography (Liu et al. 2002b). The SC-domain structure has the same folding pattern as that of the C-domain in the intact PhAspR, containing a four-stranded parallel β -sheet flanked by six α -helices from two sides. In both cases, all of the secondary structural elements, including the π 1 helix as well as the C- and N-termini, showed no significant structural differences. A superimposition of the SC-domain and C-domain revealed a root-mean-square deviation of the C α atoms of 0.50 Å, implying that the domain structure is highly stable. The high structural stability of this domain supports the existence of the ancestral domain. When compared with other amino acid racemases, we suggest that gene duplication and gene fusion are conventional ways in the evolution of PLP-independent amino acid racemases.

21.2.3.2 The Catalytic Center of PLP-Independent Aspartate Racemase

The mutation of either Cys82 or Cys194 to alanine (C82A, C194A) markedly reduced the reaction rate but only marginally affected the K_m constant (Yoshida et al. 2006) (Table 21.1). These mutants do not show any specificity in either reaction direction, suggesting cooperation between the two cysteine residues. As the transitional state of proton transfer between two atoms is essentially a hydrogen bond interaction, the proton transfer in the process of aspartate racemization ($-S^- \cdot H-C\alpha \cdot H-S-$) will proceed through the approximate distance of two hydrogen bonds. Thus, the “cooperation distance” between the γ -sulfur atoms of the two active cysteine residues could be assigned as approximately 8.0 Å, which corresponds to twice the sulfur-involved hydrogen bond distance. However, the distance between the two γ -sulfur atoms of Cys82 and Cys194 is 9.6 Å, which is beyond this cooperation distance. At such a large distance, cooperation between the two cysteine residues is likely difficult, although the two-base mechanism could be supported by the pseudosymmetrical arrangement of these residues at the well-conserved active site.

This contradiction appears to be dissolved by the conformational change. One possibility is the so-called hinge motion between the two domains. As PhAspR is hyperthermophilic and functions at high temperatures, the distance between the side chains of the two cysteine residues may be reduced by the motion. To test this hypothesis, molecular dynamics simulations of PhAspR were performed in a wide temperature range from 300 to 425 K, and the temperature dependence of the intramolecular motion was investigated. The time evolution of the distance between the two catalytic γ -sulfur atoms at 300, 375, 400, and 425 K was calculated. Although the average distance remained over 10 Å up to 375 K, a noticeable change in the time evolution of the distance was observed in the simulations over 400 K. During the simulation time range from 500 to 1000 ps at 425 K, the distance between the two γ -sulfur atoms decreased from 10 to 6 Å and then increased back up to 10 Å. At 680 ps, the two γ -sulfur atoms approached each other at a distance of

Table 21.1 Kinetic parameters of mutant PhAspRs

	L → D direction			D → L direction		
	K_m (mM)	k_{cat} (min ⁻¹)	k_{cat}/K_m	K_m (mM)	k_{cat} (min ⁻¹)	k_{cat}/K_m
WT	1.3×10^{-1}	1.9×10^4	1.5×10^5	3.1×10^{-1}	2.6×10^4	8.4×10^4
C82A	1.8	4.0×10^{-1}	2.2×10^{-1}	3.6	1.6×10^{-1}	4.5×10^{-2}
C194A	1.3	9.3×10^{-1}	7.3×10^{-1}	4.5×10^{-1}	1.4×10^{-1}	3.0×10^{-1}
R48I	3.0×10^2	1.0×10^2	3.5×10^{-1}	4.0×10	6.8	1.7×10^{-1}
R48K	2.3×10	5.5×10^2	2.4×10	3.3	5.8×10	1.8×10
R48A	N.D.	N.D.	N.D.	1.5×10^2	1.1×10	7.7×10^{-2}
K164L	4.3	2.8×10	6.6	5.4	2.6×10	4.9
K164R	1.0×10^2	4.1×10	4.1×10^{-1}	3.1×10	1.1×10	3.4×10^{-1}
K164A	2.3×10^2	1.0×10	4.6×10^{-2}	8.7×10	2.9	3.3×10^{-2}
N83G	3.9	2.4×10^4	6.1×10^3	1.8	7.8×10^3	4.3×10^3
T84A	8.2×10^{-2}	9.0×10^2	1.1×10^4	2.6×10^{-1}	1.6×10^3	6.0×10^3
T124A	1.8×10^{-1}	1.8×10^3	9.7×10^3	3.2×10^{-1}	1.5×10^3	4.7×10^3
T127A	1.4×10^{-1}	3.5×10^3	2.4×10^4	1.6×10^{-1}	3.5×10^3	2.2×10^4

Reproduced from Table 21.1 of Yoshida et al. 2006

k_{cat} and K_m values of mutant PhAspRs for L- and D-aspartate at 70 °C are shown

N.D. not determined

5.8 Å. This distance is much smaller than the predicted cooperation distance of 8.0 Å.

Another possibility is the substrate-induced conformational change. Ohtaki et al. determined the crystal structure of an inactive mutant PhAspR complexed with citric acid (Cit) at a resolution of 2.0 Å (Ohtaki et al. 2008). Cit contains the substrate analogue moieties of both L- and D-aspartate and exhibits a low competitive inhibition activity against PhAspR. In the structure, Cit binds to the catalytic site of PhAspR, which induces a conformational change to close the active site. The distance between the thiolates was estimated to be 7.4 Å, suggesting a conformational change in PhAspR following the substrate binding.

However, it is still unclear how the catalytic cysteine residues can abstract protons from an α-carbon.

21.2.3.3 The Study of the Reaction Mechanism by the Characterization of Mutants and Molecular Dynamics Simulations

Yoshida et al. examined the molecular mechanism of PhAspR by mutational analyses and molecular dynamics simulations (Yoshida et al. 2006) (Fig. 21.2, Table 21.1). The two putative catalytic cysteine residues and the surrounding amino acid sequences (Cys-Asn-Thr and Gly-Cys-Thr) are highly conserved among all PLP-independent aspartate and glutamate racemases. In addition to these conserved fragments, Arg48 and Lys164 are strictly conserved in all AspRs. In addition, Thr124 and Thr127 are also conserved in both AspRs and GluRs. Arg48 and

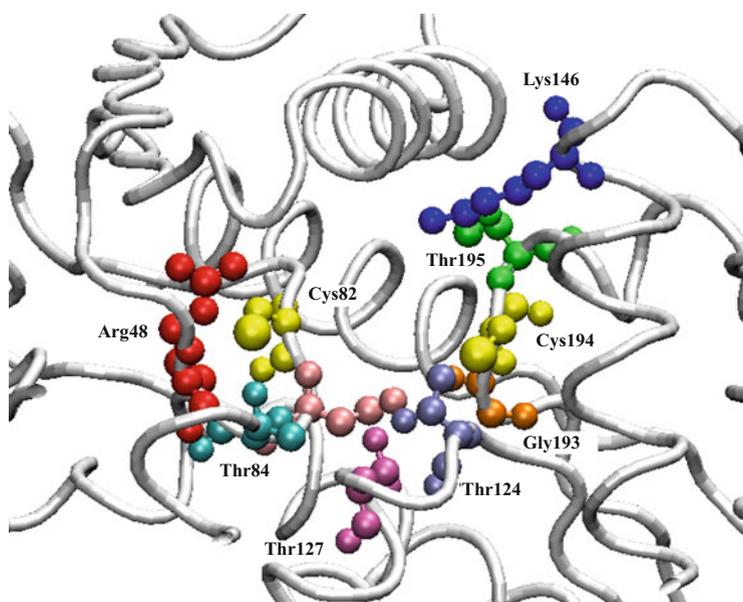


Fig. 21.2 The structure of the catalytic site of PhAspR. The backbone is drawn as a tube model, and the important putative catalytic amino acid residues (Arg48, Cys82, Asn83, Thr84, Thr124, Thr127, Lys164, Gly193, Cys194, and Thr195) are shown as ball-and-stick models. The *arrow* designates the distance between the two catalytic cysteine residues (Reproduced from Figure 1 of Yoshida et al. 2006)

Thr84 are located near Cys82, and Lys164 and Thr124 are located near Cys194. The spatial arrangement of these residues is quasi-mirror symmetrical. The residues of Arg48/Lys164, with a positively charged long side chain, can ionically interact with the side-chain carboxylate of L-/D-Asp via its guanidinium/ammonium group, and Thr84/Thr124 can form a hydrogen bond with the amino acid. Arg48 was replaced with alanine, isoleucine, or lysine, and Lys164 was replaced with alanine, leucine, or arginine. All of the mutants showed a significant decrease in the k_{cat} compared with the wild-type enzyme, but the effects were relatively small when compared with the mutations of the catalytic cysteine residues. Therefore, both Arg48 and Lys164 are important but not indispensable for catalysis. In comparison with other mutants, R48K exhibits relatively strong catalytic activity as well as substrate affinity, suggesting that the positively charged residue of R48 contributes to both the substrate binding and the reaction rate of PhAspR. K164A has the weakest activity among the K164 mutants, and K164L shows a significantly lower K_m than the other mutants of R48 and K164. This result suggests that not only the charge but also the size of the side chain at the 164th position is related to the binding of both aspartate enantiomers to PhAspR. Other mutations (N83G, T84A, T124A, and T127A) also affect the activity. However, the effects are marginal compared to the mutations at Cys82, Cys194, Arg48, and Lys164. Thus, Asn83, Thr84, Thr124, and Thr127 are not essential for catalysis.

Molecular dynamics simulations of PhAspR also provided important insights into the roles of the amino acid residues at the catalytic site and also the activation mechanism of a hyperthermophilic aspartate racemase at high temperatures. As described above, at high temperatures, the γ -sulfur atoms of the cysteine residues oscillate to periodically become in closer proximity than the predicted cooperative distance. The conformation of Tyr160, which is located at the entrance of the cleft and inhibits the entry of a substrate, changes periodically to open the entrance at 375 K. The opening of the gate is likely to be induced by the motion of the adjacent amino acid, Lys164. The entrance of an aspartate molecule was also observed in the molecular dynamics simulations, driven by the force of the electrostatic interaction with Arg48, Lys164, and Asp47 (Fig. 21.3).

21.3 PLP-Dependent Aspartate Racemases

21.3.1 *PLP-Dependent Aspartate Racemases in Invertebrates and Archaea*

A high concentration of D-aspartate exists in the tissues of the blood shell *Scapharca broughtonii*, and aspartate racemase activity was detected in the extracts of its foot muscle and mantle. Shibata et al. purified aspartate racemase (SbAspR) from the foot muscle to homogeneity (Shibata et al. 2003b). The apparent molecular mass shown by SDS-PAGE is 39 kDa, and it appears at 51–63 kDa in a gel filtration. The absorption spectrum and the effects of aminooxyacetate or NaBH₄ show that SbAspR is dependent on pyridoxal 5'-phosphate. SbAspR is highly specific to aspartate and does not racemize alanine, serine, and glutamate. Curiously, its activity is modulated by nucleotides. The activity increases with purine nucleoside monophosphates, while purine nucleoside triphosphates decrease the activity. AMP increases the activity as high as sevenfold, while ATP at saturating concentrations decreases the activity to 7%. These results suggest the possibility that SbAspR may be involved in energy metabolism.

The cDNA clone for SbAspR was cloned and sequenced (Abe et al. 2006). It contains an open reading frame of 1017 bp encoding a protein of 338 amino acids with a molecular mass of 37.1 kDa, which is in good agreement with the apparent molecular mass of the native protein, at 39 kDa. The deduced amino acid sequence contains a highly conserved PLP-binding motif. Thus, this confirms that this enzyme is the first eukaryotic and PLP-dependent aspartate racemase. SbAspR shares significant amino acid sequence homologies with mammalian serine racemase, which is also PLP dependent, and the highest identity is 44–43%. SbAspR is also homologous to other PLP-dependent enzymes, such as threonine dehydratases from various sources, with a 39–33% identity (Fig. 21.4). On the contrary, no microbial aspartates or glutamate racemases show any significant identity because they are PLP-independent enzymes.

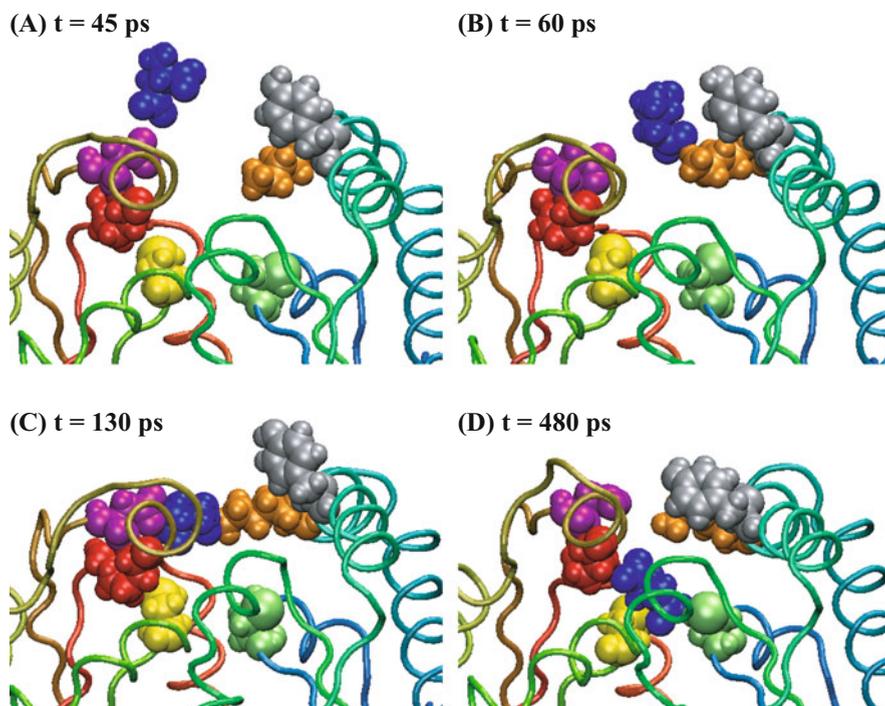


Fig. 21.3 The docking process of L-aspartic acid into the catalytic site of PhAspR. Schematic drawings for the position of the L-aspartic acid and the conformation of the catalytic site of PhAspR at 45 ps (a), 60 ps (b), 130 ps (c), and 480 ps (d) during the docking MD simulation are shown. The backbone is shown as tube model. L-Aspartic acid (blue) and the important residues around the catalytic site, Asp47 (purple), Arg48 (red), Lys164 (orange), Tyr160 (gray), Cys82 (yellow), and Cys194 (green), are drawn as *van der Waals* model (Reproduced from Fig. 9 of Yoshida et al. 2006)

SbAspR was expressed in *E. coli* and purified to homogeneity (Abe et al. 2006). The recombinant SbAspR displayed essentially identical properties with the native protein, including the possession of a bound PLP and its sensitivity to AMP and ATP. The recombinant SbAspR exhibited relatively low dehydratase activity toward *L-threo*-3-hydroxyaspartate to produce oxaloacetate. This activity may represent only a side reaction often observed in PLP-dependent enzymes due to the chemistry of PLP. However, it is also possible that the activity somehow reflects the structural homology with threonine dehydratases as well as serine racemase, which also shows dehydratase activity.

Significant levels of D-Asp are present in the cerebral ganglion of the F- and C-clusters of the invertebrate *A. californica*, and D-Asp appears to be involved in cell-cell communication in this system. Wang et al. identified a gene encoding an amino acid racemase from *A. californica* (DAR1) (Wang et al. 2011). DAR1 converts aspartate and serine to their other chiral forms in a PLP-dependent manner. DAR1 has a predicted length of 325 amino acids and is 55 % identical to SbAspR

SbAspR	1	MASKIPQFEVVTDIKKAYDRISKHILYTFVETSTPTFDRMVCSKAGRCYFKAEENLQKIG
Human_SR	1	...MCAQYCI ⁶³ SFADVEKAHINIRD ⁶³ IHLTPVLTSSILNQT...RNLFFKCELFQKIG
Mouse_SR	1	...MCAQYCI ⁶³ SFADVEKAHINIQD ⁶³ IHLTPVLTSSILNQT...RNLFFKCELFQKIG
Rat_SR	1	...MCAQYCI ⁶³ SFADVEKAHLNIQD ⁶³ VHLTPVLTSSILNQT...RNLFFKCELFQKIG
Eco_TDH	1	.MADSQPLSGAPEGAEYI ⁶³ RAVIRAFVYEAAQV ⁶³ PLQKMEKLSRSSRLDNVILV ⁶³ RDRC ⁶³ PVH
63		
SbAspR	61	SFKIRGALNAIRLCA ⁶¹ TER..E ⁶¹ PSLAGVV ⁶¹ HSSGNHGOAL ⁶¹ AWA ⁶¹ SKRAGV ⁶¹ KCCVVV ⁶¹ PKA ⁶¹ CV
Human_SR	54	SFKIRGALNAVRS ⁵⁴ LV ⁵⁴ EDAL ⁵⁴ ERKPKAVV ⁵⁴ HSSGNHGOAL ⁵⁴ YAAKLEGIPAYIVV ⁵⁴ QTA ⁵⁴ ADC
Mouse_SR	54	SFKIRGALNAIRGL ⁵⁴ IP ⁵⁴ TP ⁵⁴ EPKPKAVV ⁵⁴ HSSGNHGOAL ⁵⁴ YAAKLEGIPAYIVV ⁵⁴ QTA ⁵⁴ ADC
Rat_SR	54	SFKIRGALNAIRGL ⁵⁴ IP ⁵⁴ TP ⁵⁴ EPKPKAVV ⁵⁴ HSSGNHGOAL ⁵⁴ YAAKLEGIPAYIVV ⁵⁴ QTA ⁵⁴ ADC
Eco_TDH	60	SFKLRGAYAMMAG ⁶⁰ ITEE...Q ⁶⁰ AHGVI ⁶⁰ ASA ⁶⁰ GNHAQGV ⁶⁰ ESSARL ⁶⁰ GVKAL ⁶⁰ IVM ⁶⁰ PTATADI
119		
SbAspR	119	RFDAMENYGA ¹¹⁹ IVY ¹¹⁹ CEP ¹¹⁹ NPT ¹¹⁹ RKETCEGLAKSRGYKY ¹¹⁹ ISSSD ¹¹⁹ DYD ¹¹⁹ VIA ¹¹⁹ OGG ¹¹⁹ I ¹¹⁹ ALELLOQ
Human_SR	114	KKLAI ¹¹⁴ QAYGA ¹¹⁴ IVY ¹¹⁴ CEP ¹¹⁴ DE ¹¹⁴ RENVAKRV ¹¹⁴ TET ¹¹⁴ EGIMV ¹¹⁴ HP ¹¹⁴ NOEP ¹¹⁴ AVIAGOG ¹¹⁴ I ¹¹⁴ ALEVLNO
Mouse_SR	114	KKLAI ¹¹⁴ QAYGA ¹¹⁴ IVY ¹¹⁴ CEP ¹¹⁴ DE ¹¹⁴ REKVTORIM ¹¹⁴ QET ¹¹⁴ EGIMV ¹¹⁴ HP ¹¹⁴ NOEP ¹¹⁴ AVIAGOG ¹¹⁴ I ¹¹⁴ ALEVLNO
Rat_SR	114	KKLAI ¹¹⁴ QAYGA ¹¹⁴ IVY ¹¹⁴ SEP ¹¹⁴ DE ¹¹⁴ RENVAKR ¹¹⁴ II ¹¹⁴ QET ¹¹⁴ EGIMV ¹¹⁴ HP ¹¹⁴ NOEP ¹¹⁴ AVIAGOG ¹¹⁴ I ¹¹⁴ ALEVLNO
Eco_TDH	117	KVD ¹¹⁷ AVRGG ¹¹⁷ EG ¹¹⁷ EVLL ¹¹⁷ HGANF ¹¹⁷ LEAKAK ¹¹⁷ IELS ¹¹⁷ QOQ ¹¹⁷ FTW ¹¹⁷ PP ¹¹⁷ DH ¹¹⁷ AVIAGOG ¹¹⁷ I ¹¹⁷ ALELLOQ
179		
SbAspR	179	QFDLDA ¹⁷⁹ ILVSV ¹⁷⁹ SAGCMAS ¹⁷⁹ ICVY ¹⁷⁹ TKNT ¹⁷⁹ SDLKV ¹⁷⁹ LV ¹⁷⁹ PEGKMLE ¹⁷⁹ FCISK ¹⁷⁹ RER...T ¹⁷⁹ W ¹⁷⁹ NP
Human_SR	174	VFLVDALV ¹⁷⁴ VGGG ¹⁷⁴ GLAGI ¹⁷⁴ IT ¹⁷⁴ VKALK ¹⁷⁴ PSVKVYAA ¹⁷⁴ EPSN...ADD ¹⁷⁴ CY ¹⁷⁴ SKL ¹⁷⁴ KGL ¹⁷⁴ MPN ¹⁷⁴ .L
Mouse_SR	174	VFLVDALV ¹⁷⁴ VGGG ¹⁷⁴ VAGI ¹⁷⁴ IT ¹⁷⁴ TKALK ¹⁷⁴ PSVKVYAA ¹⁷⁴ EPSN...ADD ¹⁷⁴ CY ¹⁷⁴ SKL ¹⁷⁴ KGL ¹⁷⁴ MPN ¹⁷⁴ .L
Rat_SR	174	VFLVDALV ¹⁷⁴ VGGG ¹⁷⁴ VAGI ¹⁷⁴ IT ¹⁷⁴ TKALK ¹⁷⁴ PSVKVYAA ¹⁷⁴ EPSN...ADD ¹⁷⁴ CY ¹⁷⁴ SKL ¹⁷⁴ KGL ¹⁷⁴ MPN ¹⁷⁴ .L
Eco_TDH	177	DAH ¹⁷⁷ LRV ¹⁷⁷ FV ¹⁷⁷ VGGG ¹⁷⁷ LAGV ¹⁷⁷ AVL ¹⁷⁷ IK ¹⁷⁷ OL ¹⁷⁷ MP ¹⁷⁷ Q ¹⁷⁷ KVI ¹⁷⁷ VE ¹⁷⁷ AED...S ¹⁷⁷ ACL ¹⁷⁷ KAA ¹⁷⁷ L ¹⁷⁷ DAGHPVD ¹⁷⁷ .L
237		
SbAspR	237	QFLD ²³⁷ IADG ²³⁷ ILQCC ²³⁷ NK ²³⁷ WF ²³⁷ IIL ²³⁷ ELPE ²³⁷ KEV ²³⁷ LV ²³⁷ VND ²³⁷ NIVE ²³⁷ MR ²³⁷ VF ²³⁷ ARM ²³⁷ KLVI ²³⁷ EAA ²³⁷ AGA
Human_SR	231	Y ²³¹ PEPE ²³¹ IADG ²³¹ VK ²³¹ SS ²³¹ I ²³¹ GLN ²³¹ WF ²³¹ IIR ²³¹ DLV ²³¹ .D ²³¹ VF ²³¹ VE ²³¹ EIK ²³¹ Y ²³¹ AT ²³¹ LV ²³¹ WR ²³¹ M ²³¹ KL ²³¹ LIE ²³¹ PTAGV
Mouse_SR	231	H ²³¹ PEPE ²³¹ IADG ²³¹ VK ²³¹ SS ²³¹ I ²³¹ GLN ²³¹ WF ²³¹ IIR ²³¹ DLV ²³¹ .D ²³¹ VF ²³¹ VE ²³¹ EIK ²³¹ Y ²³¹ AT ²³¹ LV ²³¹ WR ²³¹ M ²³¹ KL ²³¹ LIE ²³¹ PTAGV
Rat_SR	231	H ²³¹ PEPE ²³¹ IADG ²³¹ VK ²³¹ SS ²³¹ I ²³¹ GLN ²³¹ WF ²³¹ IIR ²³¹ DLV ²³¹ .D ²³¹ VF ²³¹ VE ²³¹ EIK ²³¹ Y ²³¹ AT ²³¹ LV ²³¹ WR ²³¹ M ²³¹ KL ²³¹ LIE ²³¹ PTAGV
Eco_TDH	233	PRVGLF ²³³ AE ²³³ GVAV ²³³ KR ²³³ IC ²³³ DE ²³³ ER ²³³ LC ²³³ Q ²³³ Y ²³³ LD ²³³ .D ²³³ I ²³³ LV ²³³ SD ²³³ AL ²³³ IC ²³³ AK ²³³ ML ²³³ FD ²³³ VR ²³³ AV ²³³ EP ²³³ .S ²³³ CA
297		
SbAspR	297	TVA ²⁹⁷ AM ²⁹⁷ DR ²⁹⁷ FQ ²⁹⁷ NF ²⁹⁷ H ²⁹⁷ EAK ²⁹⁷ KV ²⁹⁷ C ²⁹⁷ ITL ²⁹⁷ CG ²⁹⁷ NVD ²⁹⁷ IEK ²⁹⁷ .T ²⁹⁷ FW ²⁹⁷ KK ²⁹⁷ DK ²⁹⁷
Human_SR	289	GVA ²⁸⁹ AVL ²⁸⁹ QH ²⁸⁹ FQ ²⁸⁹ VS ²⁸⁹ PE ²⁸⁹ VKN ²⁸⁹ IC ²⁸⁹ IVL ²⁸⁹ SG ²⁸⁹ NVD ²⁸⁹ L ²⁸⁹ SS ²⁸⁹ IT ²⁸⁹ W ²⁸⁹ Q ²⁸⁹ AER ²⁸⁹ PS ²⁸⁹ YS ²⁸⁹ QV ²⁸⁹ SV ²⁸⁹
Mouse_SR	289	ALA ²⁸⁹ AVL ²⁸⁹ QH ²⁸⁹ FQ ²⁸⁹ VS ²⁸⁹ PE ²⁸⁹ VKN ²⁸⁹ IC ²⁸⁹ IVL ²⁸⁹ SG ²⁸⁹ NVD ²⁸⁹ L ²⁸⁹ SS ²⁸⁹ IT ²⁸⁹ W ²⁸⁹ Q ²⁸⁹ AER ²⁸⁹ PS ²⁸⁹ PY ²⁸⁹ QT ²⁸⁹ VS ²⁸⁹
Rat_SR	289	GLA ²⁸⁹ AVL ²⁸⁹ QH ²⁸⁹ FQ ²⁸⁹ VS ²⁸⁹ PE ²⁸⁹ VKN ²⁸⁹ IC ²⁸⁹ IVL ²⁸⁹ SG ²⁸⁹ NVD ²⁸⁹ L ²⁸⁹ SS ²⁸⁹ IT ²⁸⁹ W ²⁸⁹ Q ²⁸⁹ AER ²⁸⁹ PS ²⁸⁹
Eco_TDH	291	LAL ²⁹¹ GM ²⁹¹ KY ²⁹¹ I ²⁹¹ AL ²⁹¹ HN ²⁹¹ IR ²⁹¹ GER ²⁹¹ LA ²⁹¹ HIL ²⁹¹ GAN ²⁹¹ VNF ²⁹¹ HG ²⁹¹ .T ²⁹¹ RY ²⁹¹ V ²⁹¹ SER ²⁹¹ CEL ²⁹¹ EQ ²⁹¹ REAL ²⁹¹ AV ²⁹¹ TI ²⁹¹ PEEK

Fig. 21.4 A multiple alignment of the amino acid sequence of SbAspR with the human, mouse, and rat serine racemase (SR) homologues and *E. coli* biosynthetic threonine dehydratase (*Eco_TDH*). The putative PLP-binding lysine residue is Lys63 of SbAspR. A PLP-binding motif is indicated by the underline. The dotted underline indicates the tetraglycine loop (Modified from Figure 2 of Abe et al. 2006 JB)

and 41% identical to mammalian serine racemase. However, it is only 14% identical to the recently reported mammalian aspartate racemase. Using the recombinant DAR1, its activity toward serine and aspartate was confirmed. DAR1 is the first characterized eukaryotic racemase that can catalyze the racemization of two substrates. DAR1 exists as a homodimer, similar to mouse SerR and SbAspR. ATP and MgCl₂ promote the racemase activity of DAR1, and it has been proposed that ATP and MgCl₂ exert allosteric regulatory effects on mouse SerR by lowering the enzyme K_m .

In the acidothermophilic archaeon *Thermoplasma acidophilum*, the proportion of D-aspartate to total aspartate was as high as 39.7% (Long et al. 2001). Crude extracts of *T. acidophilum* showed aspartate-specific racemase activity. The activity

was very low in the absence of PLP and was insensitive to an SH-modifying reagent (Long et al. 2001). Although the genome of *T. acidophilum* has been completely sequenced, no aspartate racemase genes of either type were identified. Thus, the high levels of D-aspartate may be produced by a new type of PLP-dependent aspartate racemase in *T. acidophilum*.

21.3.2 Mammalian Aspartate Racemase

D-Aspartate exists in various mammalian organs and is especially abundant in the developing brain. However, the enzyme involved in the formation of D-aspartate was unknown until 2010. The search for the aspartate racemase gene in genome sequences by looking for homology to serine racemase or bacterial aspartate racemases was *in vain*.

Kim et al. were the first to identify and clone mammalian aspartate racemase, which co-localized with D-aspartate in the brain and neuroendocrine tissues (Kim et al. 2010). They were inspired by the previous findings that glutamate-oxaloacetate transaminase (GOT) from *E. coli* can generate small amounts of D-aspartate in the process of transaminating L-aspartate to L-glutamate, and its aspartate racemizing activity is enhanced by the double mutations of Trp140 to His and Arg292 to Lys. Kim et al. discovered a gene encoding a protein homologous to the mutant GOT. The gene was found to encode a protein that is more closely related to cytosolic and mitochondrial GOT than to serine racemase or serine dehydratase (Fig. 21.5).

When cloned and expressed, the putative aspartate racemase (DR), a 45.5-kDa protein, generates substantial D-aspartate but only one-fifth as much L-glutamate and very little D-glutamate. Like serine racemase and SbAspR, DR is PLP dependent. PLP binds to Lys249, and DR is inhibited by aminooxyacetic acid, an inhibitor of PLP-dependent enzymes. The K_m of the recombinant DR for L-aspartate is 3.1 mM, the V_{max} is 0.46 mmol/mg/min, the optimum pH is 7.5, and the optimum temperature is 37 °C. The importance of Lys136, the presumed proton donor, is highlighted by its mutation to tryptophan, which virtually abolishes the racemase activity. DR is expressed most abundantly in the brain, heart, and testes, with somewhat lower levels in the adrenal glands and negligible expression in the liver, lung, and kidney.

The depletion of DR by short hairpin RNA in the newborn neurons of the adult hippocampus elicits profound defects in the dendritic development and survival of the newborn neurons. Because D-aspartate is a potential endogenous ligand for NMDA receptors, the loss of which elicits a phenotype resembling DR depletion, D-aspartate may function as a modulator of adult neurogenesis.

DR	1MTSLSVRDVFTQKLEGGSLKLYRQDGYFSKIL
GOT-1	1MAPPSVFAQVQAPFVLFKLEADFRDPPDERKV
GOT-2	1	MALLHSSRILSGMAAAFHPGLAAAASARASVWTHVEMGPPDFILGVYEAFFKRDITNSKKM
DAR1	34	FLAYKVCMTPEGHFVWSLVVHKTRIQIDDPDLDVEYLLVGLKSFQSSLELLELCKHSE
GOT-1	35	NLGVGAYRDESQPWVLFVVRKVEQKINDNINHEYLEILGLAEFRSCASRLVLGDNLSL
GOT-2	61	NLGVGAYRDNCKPYVLFVSRKAEQIQAKNLDKEYLELGGLAEEFCKASAEILALGKNE
DAR1	94	ATAEKRVGGVHIVCESGAFOLGAQFLKTRRNNVKI...VCTIVSCQKRECCGLIEQDMGFI
GOT-1	95	AIRENRVGGVOSLGGTGALRIGADFLGRWYNGIDNKNTPIYVSSPWFENHNAVESAAGFK
GOT-2	120	VLRSGRFVTVQIISCTGALRVGASFLQRFKFSRD...VFLPKPSWGNFTPIERDAGMQ
DAR1	150	.VVEYSIWNSDLCSDFMFVEVLCHIIVGSLVIG...NIDCKFTQNOWTRFMSLIK
GOT-1	155	DIRPYCYWDEKRLDLOGFLNDLENAPFESHVVLHACAHNPTCTDPPPEQWQIADVMO
GOT-2	176	.LQGYRYDPKTCDFSGALEDDISKIPEQSULLLHACAHNPTCVDPRPEQWRBIASVVK
DAR1	205	SKQIFPFDPICQLSLGDLEEDTKILQYFVLCLEFFCSQSLKKNFGIYDEGVCIIVVA
GOT-1	215	RRLELFPFDSAYOGFASGDLEKDAWAIRYFVSECFLELCAQSSKNGFLYNERVGNLIVV
GOT-2	235	KKNLFAFPDMAYOGFASGDGDKDAWAIVRFIEQGINVCLQSSYAKNMGLYGERVCAIVV
DAR1	265	ALSNQHLICVLSQFMDYVQALWGNPPATGARITISILCNPALEGEWKQSLKGVVNNMLI
GOT-1	275	GKESDSVLRVLSQMEKIVRITWSPNPPAOGARIVAATLSDLELFKEWGNVKTMDRILIM
GOT-2	295	CKLAEAEAKRVESQPKILIRPLYSNPPNLNGARITAAITLSDLELRQWLECEVKCMADRILIM
DAR1	325	KEKVKRKLRLLETPGSWDHIFRCSCTHCVLGLTYQVEFLVKKKHIYLPKSRINFTCTN
GOT-1	335	RELRLARLEAKDPTGWSHIDQICMFSFTGINPKQVEYLVNKKHIYLLPGRINMCGLTN
GOT-2	355	RLQIVSNLRKEGSSHNNQHIIDQICMFCFTGLKPEQVEVRLTKREFSVYMTDRGISVAGVPL
DAR1	385	ARNIDYITQSEIHEAVMLTEG
GOT-1	395	KNLDYVATEIHEAVTKIQ.
GOT-2	415	SGNVGYLAHALEHOVTK....

Fig. 21.5 An amino acid alignment of DAR1, mouse cytoplasmic glutamate-oxaloacetate transaminase (GOT-1), and mouse mitochondrial glutamate-oxaloacetate transaminase (GOT-2). Arg377 is a conserved amino acid residue that recognizes the carboxyl group of the substrate, amino acids, and α -keto acids. Both GOT-1 and GOT-2 have a tryptophan at position 167, whereas DR contains a lysine, which can donate protons for racemization. GOT-1 and GOT-2 also have an arginine at position 319, whereas DR has a glutamine, which allows the access of water molecules (Modified from Fig. S1 of Kim et al. 2010 PNAS S1)

21.4 Conclusion

Aspartate racemases are divided into PLP-independent and PLP-dependent aspartate racemases. The aspartate racemases of eubacteria and hyperthermophilic archaea are PLP independent. Their catalytic sites are constituted by two conserved cysteine residues. However, it is still unknown how the catalytic cysteine residues can abstract a proton from the α -carbon. The aspartate racemases of invertebrates

and vertebrates are PLP dependent, and those of invertebrates, bivalves, and *A. californica* are homologous to serine racemases. It took many years to identify the aspartate racemase responsible for the synthesis of D-Asp in mammals due to the lack of homology with other amino acid racemases. The gene for the mammalian aspartate racemase was finally obtained *via* its homology with glutamate-oxaloacetate transaminase. Although the existence of an aspartate racemase in the acidothermophilic archaeon *T. acidophilus* was shown, it had not been identified until now.

More than 40 years have passed since the first discovery of aspartate racemase. The study of aspartate racemase has been relatively slow, reflecting the difficulty of the research on this enzyme. As we have identified almost all of the different types of aspartate racemases, I hope the study on the physiological function of aspartate racemases and D-aspartate will continue to progress.

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