Crystal structure of a pyridoxal 5'-phosphate-dependent aspartate racemase derived from the bivalve mollusc *Scapharca broughtonii*

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Aspartate racemase (AspR) is a pyridoxal 5'-phosphate (PLP)-dependent enzyme that is responsible for D-aspartate biosynthesis *in vivo*. To the best of our knowledge, this is the first study to report an X-ray crystal structure of a PLP-dependent AspR, which was resolved at 1.90 Å resolution. The AspR derived from the bivalve mollusc *Scapharca broughtonii* (SbAspR) is a type II PLP-dependent enzyme that is similar to serine racemase (SR) in that SbAspR catalyzes both racemization and dehydration. Structural comparison of SbAspR and SR shows a similar arrangement of the active-site residues and nucleotide-binding site, but a different orientation of the metal-binding site. Superposition of the structures of SbAspR and of rat SR bound to the inhibitor malonate reveals that Arg140 recognizes the β-carboxyl group of the substrate aspartate in SbAspR. It is hypothesized that the aromatic proline interaction between the domains, which favours the closed form of SbAspR, influences the arrangement of Arg140 at the active site.

1. Introduction

Recently developed analytical techniques have enabled the identification of free D-amino acids in diverse living organisms, including humans and other mammals. Among these, D-serine and D-aspartate are attracting attention because of their important biological roles. In mammals, D-serine is predominantly concentrated in forebrain areas such as the hippocampus and cortex (Hashimoto & Oka, 1997), where it acts as an endogenous co-agonist for the glycine site on N-methyl-D-aspartate (NMDA) receptors (Kleckner & Dingledine, 1988). By activating NMDA-type glutamate receptors, D-serine serves as a gliotransmitter and regulates synaptic plasticity, including long-term potentiation and depression (Oliet & Mothet, 2009; Henneberger *et al.*, 2010). D-Serine is synthesized by a pyridoxal 5'-phosphate (PLP)-dependent serine racemase (SR) that catalyzes racemization and dehydration reactions of both the D- and L-serine enantiomers (Wolosker, Blackshaw *et al.*, 1999; Wolosker, Sheth *et al.*, 1999; Stříšovský *et al.*, 2003). D-Aspartate is one of the most abundant free D-amino acids present in the nervous and reproductive systems and plays important physiological roles, including regulating developmental processes, hormone secretion and steroidogenesis (Di Fiore *et al.*, 2014; Katane & Homma, 2011; Ota *et al.*, 2012). Although several lines of evidence indicate that D-aspartate has an endogenous origin (Wolosker *et al.*,

Table 1

Macromolecule-production information.

Source organism	<i>S. broughtonii</i>
DNA source	<i>S. broughtonii</i> cDNA
Forward primer†	5'-TTTCCATATGGCGTCTAAATTCACAA-3'
Reverse primer‡	5'-AATTCTCGAGAATTCCTATTTTGTGTCTTT TTTGGTCC-3'
Cloning vector	pCR4 Blunt-TOPO (Invitrogen)
Expression vector	pET-25b (Novagen)
Expression host	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced	MASKIPQFEV TVTDIKKAYD RISKHILYTP VFSTPTFDRM VGSKAGRQFY FKAENLQKTG SFKARGALNA ILCALEREPS LAGVVTSSG NHGQALAWAS KRAGVKCCVV VPKTAPQVKF DAMENYGAEV VKCEPNPTSR KETCEGLAKS RGYKIYSSD DYDVIAGQGT IALELLQQQP RLDAILVSVS AGGMASGICV YTKNTKSDLK VFLVEPEGKM LEECISKRER LWPNPQFLD TIADGIIQQ CGNKTWPIIL ELPEKEVITV NNDNIVEAMR FVFARMKLVI EAAAGATVAA AMTERFQNFH PEAKKVGII CGGNVDIEKL PWTKKDTK

† The NdeI site for cloning is underlined. ‡ The EcoRI site for cloning is underlined.

2000), the enzyme responsible for mammalian D-aspartate biosynthesis has not yet been identified. Mouse glutamate-oxaloacetate transaminase 1-like 1 (GOT1L1) has been reported to function as an aspartate racemase (AspR) and to be involved in adult neurogenesis (Kim *et al.*, 2010). In contrast, Matsuda *et al.* (2015) reported that the rat and human homologues of mouse GOT1L1 are not involved in D-aspartate biosynthesis. Furthermore, mammalian SR has been suggested to be responsible for D-aspartate biosynthesis in some organs and/or tissues (Ito *et al.*, 2016). In addition, it has been shown that D-aspartate in the bivalve mollusc *Scapharca broughtonii* is produced by a PLP-dependent AspR (SbAspR) similar to mammalian SR (Shibata *et al.*, 2003). The amino-acid sequence of SbAspR shows approximately 42.7% identity to that of rat SR and 36.6% identity to that of yeast SR. SR is activated by ATP (de Miranda *et al.*, 2002; Neidle & Dunlop, 2002; Ito *et al.*, 2012), whereas the activity of SbAspR is increased and decreased by AMP and ATP, respectively (Shibata *et al.*, 2003). X-ray crystallographic studies have revealed the ATP-binding sites in human, rat and yeast SRs (Goto *et al.*, 2009; Smith *et al.*, 2010). However, the structural mechanism underlying the activation and inhibition of SbAspR by AMP and ATP has not been reported to date. To elucidate the mode of substrate recognition and modulation of SbAspR activity according to nucleotide type, we used X-ray crystallographic analysis to ascertain the structure of recombinant SbAspR.

2. Materials and methods

2.1. Macromolecule production

The cloning and expression of SbAspR in *Escherichia coli* and its purification were performed as described previously (Abe *et al.*, 2006). Briefly, the gene encoding SbAspR was PCR-amplified using *S. broughtonii* cDNA, and NdeI and EcoRI restriction sites were introduced for cloning into

Table 2

Crystallization.

Method	Vapour diffusion, hanging drop
Plate type	24-well plates
Temperature (K)	277
Protein concentration (mg ml ⁻¹)	2.5
Buffer composition of protein solution	10 mM Tris-HCl, 20 µM PLP, 1 mM β-mercaptoethanol pH 8.8
Composition of reservoir solution	27% (w/v) PEG 4000, 50 mM sodium acetate pH 5.0, 200 mM ammonium acetate, 15% (v/v) glycerol
Volume and ratio of drop (µl)	6 (4:2 protein:reservoir)
Volume of reservoir (µl)	100

pET-25b. The resulting construct was transformed into *E. coli* BL21 (DE3) cells for expression. Transformed cells were grown in LB medium containing 100 µg ml⁻¹ ampicillin at 30°C to an absorbance of 0.5 at 600 nm; overexpression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside. After incubation at 30°C for 6 h, the transformants were collected by centrifugation at 5000g for 10 min at 4°C. The cells were then lysed by sonication after resuspending in lysis buffer (5 mM Tris-HCl pH 8.8, 1 mM malonic acid, 1 mM EDTA, 2 mM β-mercaptoethanol, 20 µM PLP). The lysate was applied onto a Blue Sepharose 6 Fast Flow column (GE Healthcare). After washing the column, SbAspR was eluted with buffer incorporating a linear NaCl gradient ranging from 0 to 500 mM. Fractions containing the enzyme were pooled and loaded onto a HiPrep Sephacryl S-100 HR column (GE Healthcare) which had been equilibrated with lysis buffer containing 150 mM NaCl; the enzyme was eluted using the same buffer. Detailed information on the compounds used for macromolecule production is provided in Table 1.

2.2. Crystallization

SbAspR was purified to homogeneity based on the findings from SDS-PAGE. SbAspR was dialyzed three times against 10 mM Tris-HCl, 20 µM PLP, 1 mM β-mercaptoethanol pH 8.8 and concentrated to 2–4 mg ml⁻¹ using cellulose ultrafiltration membranes (Amicon). The hanging drops in the initial crystallization screening consisted of 4 µl protein solution (2.5 mg ml⁻¹) plus 2 µl reservoir solution. Initial crystallization trials were set up using Crystal Screen from Hampton Research and were optimized to 27% (w/v) PEG 4000, 50 mM sodium acetate pH 5.0, 200 mM ammonium acetate, 15% (v/v) glycerol, yielding crystals that grew in one week at 277 K. The crystals were monoclinic, belonging to space group C2, with two subunits per asymmetric unit. Details of the final crystallization process are listed in Table 2.

2.3. Data collection and processing

Data collection was performed at cryogenic temperatures. The X-ray diffraction data sets for SbAspR were collected at 100 K and a resolution of 1.90 Å using an X-ray beam with a wavelength of 1.00000 Å and an ADSC Quantum 210 CCD detector on the AR-NW12 beamline at KEK Photon Factory, Tsukuba, Japan. The diffraction data were processed using

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Beamline AR-NW12A, Photon Factory
Wavelength (Å)	1.00000
Temperature (K)	100
Detector	ADSC Quantum 210 CCD
Crystal-to-detector distance (mm)	189.60
Rotation range per image (°)	0.5
Total rotation range (°)	200
Exposure time per image (s)	3.0
Space group	C2
<i>a</i> , <i>b</i> , <i>c</i> (Å)	94.18, 80.71, 87.99
α , β , γ (°)	90, 116.22, 90
Mosaicity (°)	0.21
Resolution range (Å)	46.84–1.90 (2.00–1.90)
Total No. of reflections	186532
No. of unique reflections	46230
Completeness (%)	99.2 (95.1)
Multiplicity	4.0 (3.2)
$\langle I/\sigma(I) \rangle$	17.9 (2.8)
$R_{\text{r.i.m.}}^{\dagger}$	0.049 (0.454)
Overall <i>B</i> factor from Wilson plot (Å ²)	30.0

$\dagger$ Estimated $R_{\text{r.i.m.}} = R_{\text{merge}}[N/(N-1)]^{1/2}$, where *N* is the data multiplicity

programs from the *CCP4* package (Winn *et al.*, 2011) such as *XDS* (Kabsch, 2010), *POINTLESS* (Evans, 2006) and *SCALA* (Evans, 2006). The data-collection and processing statistics are summarized in Table 3.

2.4. Structure solution and refinement

The structure of SbAspR was solved by molecular replacement using *AMoRe* (Navaza, 1994). The structure of *Schizosaccharomyces pombe* SR (PDB entry 1v71; Goto *et al.*, 2009) was used as a search model. Modelling of the polypeptide chain was performed using *Coot* (Emsley *et al.*, 2010), and the structure was refined using *REFMAC* v.5.8.0131 (Murshudov *et al.*, 2011). The *R* factor was estimated to be 20.5% at a resolution of 1.90 Å, with an R_{free} of 25.2%. The

Table 4

Structure determination and refinement.

Values in parentheses are for the outer shell.

Resolution range (Å)	50.00–1.90 (1.949–1.900)
σ Cutoff	0
No. of reflections, working set	44006
No. of reflections, test set	2224
Final <i>R</i> factor	0.205 (0.327)
Final R_{free}	0.252 (0.357)
No. of non-H atoms	
Protein	4933
Ligand	30
Water	206
Total	5169
R.m.s. deviations	
Bonds (Å)	0.0096
Angles (°)	1.3740
Average <i>B</i> factors (Å ²)	
Protein	37.58
Ligand	25.30
Water	37.97
Ramachandran plot	
Most favoured (%)	95.9
Allowed (%)	4.1

resulting model of SbAspR comprised 328 residues (Pro6–Thr333) out of 338 residues for subunit *A*, 324 residues (Pro6–Glu134, Thr138–Leu147 and Lys149–Thr333) for subunit *B* and two PLP cofactors, with 168 water molecules. The final geometry was assessed using *RAMPAGE* (Lovell *et al.*, 2003) and the final refinement statistics for all structures are provided in Table 4. Identification of structurally related proteins in the PDB was performed using *PDBFold* (Krisinel & Henrick, 2004). Graphical representations were designed using the *CCP4mg* package (McNicholas *et al.*, 2011). The refined coordinates and experimental structure factors have been deposited in the PDB as entry 5ybw. Structure-determination and refinement statistics are summarized in Table 4.

3. Results and discussion

3.1. Overall structure

The crystallographic refinement parameters (Table 4), final $2F_o - F_c$ maps and conformational analysis using *RAMPAGE* indicated that the structure of SbAspR had been determined with acceptable statistics. The crystallographic asymmetric unit of SbAspR contained two subunits forming a biological dimer, as determined using gel filtration with a Sephacryl S-100 column (Abe *et al.*, 2006). Using *PISA* (Krisinel & Henrick, 2007), the surface area of the subunit interface was estimated to be 902 Å² of the 12 521 Å² subunit surface area, predicting that SbAspR is homodimeric in solution. The structure of SbAspR showed an overall fold typical of fold-type II PLP-dependent enzymes (Fig. 1). The negative value of the electrostatic potential (coloured blue in Fig. 1) indicated that the binding pocket for the activity modulator of SbAspR is located at the subunit interface in the same manner as for yeast SR. The subunit comprised two domains (Fig. 2): a catalytic large domain (Ile5–Lys63 and Ser157–Thr333) consisting of a five-stranded β -sheet and nine α -helices, and a

Figure 1

Crystal structure of dimeric SbAspR. The two subunits are coloured differently (blue, chain *A*; green, chain *B*). The molecular surface is shaded according to the electrostatic potential.

small domain (Ala64–Ile156) consisting of a three-stranded β -sheet and four α -helices.

3.2. Structural comparison with other related enzymes

PDBFold was used to search the PDB for enzymes that possess subunit structures similar to that of SbAspR (Krissinel

& Henrick, 2004). The highest Z-scores were calculated to be 14.1 with 42% sequence identity for rat SR in complex with the potent inhibitor malonate (PDB entry 3l6c; Smith *et al.*, 2010), 14.1 with 43% sequence identity for human SR in complex with malonate (PDB entry 3l6b; Smith *et al.*, 2010), 12.9 with 31% sequence identity for *Salmonella typhimurium* threonine deaminase (PDB entry 2gn0; Simanshu *et al.*, 2006),

Figure 2

(a) Superposition of the C α atoms of the subunits of SbAspR (green), yeast SR (yellow; PDB entry 2zpu) and rat SR (red; PDB entry 3l6c). (b) Diagrammatic representation of the SbAspR subunit. The two domains are coloured differently (green, large domain; red, small domain). The cofactor PLP is located at the interface between the large and small domains. (c) Superposition of the nucleotide-binding sites of yeast SR (grey; PDB entry 1wtc) and SbAspR (green, subunit A; blue, subunit B). The structure of yeast SR is bound to the ATP analogue 5'-adenylyl(β,γ -methylene)diphosphonate (AMP-PCP). The AMP-PCP molecule from the yeast SR structure is shown as a stick model in yellow. The side-chain atoms of the residues for nucleotide binding are also shown as sticks. (d) Superposition of the metal-binding site of rat SR (grey; PDB entry 3l6c) and the corresponding position in SbAspR (green). The residue numbers of rat SR and SbAspR are labelled in grey and black, respectively.

12.3 with 37% sequence identity for *Thermus thermophilus* HB8 threonine deaminase (PDB entry 1ve5; RIKEN Structural Genomics/Proteomics Initiative, unpublished work) and 12.0 with 36% sequence identity for *S. pombe* SR (PDB entry 2zpu; Yamauchi *et al.*, 2009). This indicated that the overall fold type of SbAspR was similar to that of the PLP-dependent fold-type II enzymes, containing a large and a small domain, and was almost the same as those of SRs from rat and human (Fig. 2a). Ligand-induced movement of the small domain to close the active site has been observed in PLP-dependent fold-type II enzymes such as threonine synthase (Omi *et al.*, 2003) and SR (Goto *et al.*, 2009), with a rotation of the small domains of 25 and 20°, respectively. The secondary-structure matching of SbAspR onto the open and closed forms of the SRs (PDB entries 1v71 and 2zpu) reveals r.m.s.d.s of 1.68 and 1.43 Å for 300 C α atoms, respectively, indicating that SbAspR is similar to the closed form of SR. The average *B*-factor values of the main-chain atoms in the SbAspR large and small domains were 33.2 and 39.4 Å² for subunit *A* and 33.1 and 55.3 Å² for subunit *B*, indicating that the small domain of SbAspR has sufficient flexibility to make open–closed conformational changes. The driving force for the formation of the closed form of SbAspR is thought to be the aromatic proline interaction between Pro137 and Phe238 (Fig. 2b). Since these residues are not conserved in SR, this interaction is considered to be a mechanism that is unique to SbAspR. Structural comparison of SbAspR with yeast SR bound to an ATP analogue reveals differences in the residues involved in recognition of the γ -phosphate of ATP (Fig. 2c). The hydrophilic Asn25 and Ser33 residues in yeast SR are substituted by the hydrophobic Leu27 and Pro35, respectively, in SbAspR, suggesting that the affinity of SbAspR for ATP is lower than that of yeast SR. The nature of the inhibition by ATP and activation by AMP in SbAspR (Shibata *et al.*, 2003) is thought to involve these residues; however, three-dimensional structures of SbAspR complexed with these adenine nucleotides will be necessary to elucidate the detailed mechanism. Although divalent cations are required for the activity of mammalian SR (Cook *et al.*, 2002; de Miranda *et al.*, 2002), the activity of SbAspR is not inhibited by EDTA (Abe *et al.*, 2006). Comparison of the

divalent cation-binding site of rat SR with the corresponding site of SbAspR revealed that the amino group of the side chain of the Lys219 residue in SbAspR acts as a metal cation (Fig. 2d). This finding reaffirmed the importance of positive charge at this site for catalytic activity.

3.3. Active-site structure and substrate recognition

The active-site structure of SbAspR is shown in Fig. 3. The active site was located at the domain interface of one subunit in the SbAspR dimer. The cofactor PLP formed a typical Schiff-base bond with the NZ atom of the catalytic Lys63. The pyridine ring of PLP was sandwiched between Gly245 and Phe62 from the *re*-face and *si*-face sides, respectively. The N1 atom of the PLP pyridine ring formed a neutral hydrogen bond to the thiol group of Cys321, as seen in rat serine dehydrogenase (Smith *et al.*, 2010). The amide group of Asn91 formed a hydrogen bond to the O3' atom of PLP, which plays a role in fine-tuning the electronic state of the PLP–Schiff base conjugate (Inoue *et al.*, 1991). The so-called tetraglycine loop (Ser190–Ala191–Gly192–Gly193–Met194) formed a binding site for the PLP phosphate group. The asparagine loop (Ser88–Ser89–Gly90–Asn91–His92) acts as the recognition site for the main-chain carboxyl group of the substrate D- and L-aspartate. A similar arrangement of the active-site residues has been identified in rat and *S. pombe* SR and in *E. coli* D-serine dehydratase (PDB entry 3ss7; Goto *et al.*, 2009; Urusova *et al.*, 2011), suggesting that the α -carboxylate of the substrate aspartate is recognized through the same mechanism. Superposition of the active-site residues of rat SR and SbAspR shows that the β -turn consisting of His152–Pro153–Asn154 in rat SR is replaced by Ser157–Ser158–Ser159 in SbAspR. It was also found that Arg140 of SbAspR moves away from the active site, unlike Arg135 in rat SR, which is involved in the binding of the substrate analogue malonate (Fig. 3). Owing to the steric effects of the aromatic proline interaction between the small and large domains, it is presumed that the upper part of the small domain containing Arg140 does not get any closer to the active site. Superposition of L-aspartate on the malonic acid molecules binding to rat SR showed that the β -carboxyl

Figure 3

Stereoview of a superposition of the active sites of SbAspR (green) and rat SR (grey; PDB entry 3l6c). The active-site residues and PLP, malonate and L-aspartate (cyan) molecules are depicted as stick models.

group of the L-aspartate is in a suitable position to form a salt bridge with Arg140. In other words, the positioning of Arg140 would be a special mechanism for the substrate-recognition mechanism of SbAspR. If this theory is correct, the difference between the β -turns is hypothesized to be involved in the recognition of the hydroxyl group of the substrate serine.

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