Crystal structure of maize serine racemase with pyridoxal 5'-phosphate

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Serine racemase (SR) is a pyridoxal 5'-phosphate (PLP)-dependent enzyme that is responsible for D-serine biosynthesis *in vivo*. The first X-ray crystal structure of maize SR was determined to 2.1 Å resolution and PLP binding was confirmed in solution by UV–Vis absorption spectrometry. Maize SR belongs to the type II PLP-dependent enzymes and differs from the SR of a vancomycin-resistant bacterium. The PLP is bound to each monomer by forming a Schiff base with Lys67. Structural comparison with rat and fission yeast SRs reveals a similar arrangement of active-site residues but a different orientation of the C-terminal helix.

1. Introduction

Serine racemase (SR; EC 5.1.1.18), an enzyme that requires pyridoxal 5'-phosphate (PLP) as a cofactor, catalyzes the interconversion of D-serine and its enantiomer L-serine. The human and rat SRs have been well characterized. In mammals, D-serine, an endogenous agonist of N-methyl-D-aspartate receptors (NMDARs) for glutamate, is essential for the activity of NMDARs during normal neurotransmission (Wolosker & Mori, 2012). Mammalian SRs also catalyze the irreversible dehydration reaction of D- or L-serine to generate pyruvate and ammonia (Fig. 1). Thus, D-serine is synthesized by racemization and removed by dehydration. Crystal structures of SRs from the fission yeast *Schizosaccharomyces pombe*, rat and human reveal that eukaryotic SRs form a dimer. SRs belong to the type II PLP-dependent enzymes and are strikingly similar to serine/threonine dehydratases (Goto *et al.*, 2009; Smith *et al.*, 2010). Each SR monomer contains a large domain and a small domain that are linked by a flexible loop. The structure of fission yeast SR in complex with the ATP analogue AMP-PCP shows that 14 residues are involved in ATP binding (Goto *et al.*, 2009). The racemase activity of human SR is increased by ATP (Marchetti *et al.*, 2013, 2015).

In some vancomycin-resistant bacteria, a unique membrane-bound protein VanT has been identified as a serine/alanine racemase. VanT is composed of two domains, with the N-terminal domain bound to the membrane and probably involved in the uptake of L-serine. The C-terminal domain is located in the cytoplasm and is homologous to bacterial alanine racemase (Abadía Patiño *et al.*, 2002; Berthet *et al.*, 2015). The cytoplasmic domain of VanT from *Enterococcus gallinarum* has been reported to have preferential racemase activity for L-serine but not for L-alanine (Arias *et al.*, 1999).

The crystal structure of the C-terminal domain of VanT from *E. faecalis* revealed that this domain is a member of the type III PLP-dependent enzymes and evolved from an ancient alanine racemase (Meziane-Cherif *et al.*, 2015).

In plants, D-serine regulates the glutamate receptor-like channel between male gametophyte and pistil tissue, similar to amino-acid-mediated communication in mammalian nervous systems (Michard *et al.*, 2011). The SRs from *Arabidopsis thaliana*, barley and rice display several different features from their mammalian homologues (Fujitani *et al.*, 2006, 2007; Gogami *et al.*, 2009). ATP has no effect on the racemase activity of barley SR (Fujitani *et al.*, 2007). It decreases the racemase activity but increases the dehydratase activity of rice SR (Gogami *et al.*, 2009). From an amino-acid sequence comparison, a maize (*Zea mays* L.) SR homologue (ZmSR) has been identified. It could be responsible for synthesizing the D-serine identified in maize (Herrero *et al.*, 2007) and decomposing externally supplied D-serine, which inhibits plant growth (Erikson *et al.*, 2005). To characterize the molecular basis for D-serine homeostasis in maize, we report the crystal structure of ZmSR determined to 2.1 Å resolution. It displays a similar overall fold to other reported eukaryotic SRs and a similar arrangement of active-site residues, but a slightly different C-terminal helix orientation.

2. Materials and methods

2.1. Expression and purification of ZmSR

As shown in Table 1, the plasmid for expressing ZmSR with a C-terminal His₆ tag was constructed; the nontagged ZmSR shares about 92% amino-acid sequence identity with rice SR (Fujitani *et al.*, 2007; Gogami *et al.*, 2009). After induction at 301 K for 12 h, the *Escherichia coli* cells were collected, resuspended in 50 ml buffer A (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 10% glycerol pH 8.0), lysed by sonication and centrifuged (28 000g, 30 min). The supernatant was loaded onto 3 ml nickel–nitrilotriacetic acid (Ni–NTA) resin (Qiagen, Germany) packed in a 15 ml column and pre-equilibrated with three column volumes of buffer A. The column was washed with the same volume of buffer A

Table 1
Macromolecule-production information.

Source organism	Maize (<i>Z. mays</i> L.)
DNA source	Leaf cDNA
Forward primer†	5'-GCGGATCCATATGGGAAGTAAAGACGGCAC-3'
Reverse primer‡	5'-GTGCTCGAGGTGTTTGTACATAGACTGCCAG-3'
Cloning vector	pUC18-T vector
Expression vector	pet-22-ZmSR
Expression host	<i>E. coli</i> BL21(DE3)
Complete amino-acid sequence of the construct produced§	MGSKDGTGDISEAQGYAADIDSIREAQARIAPYV-HRTPVMSSTSIDAMVGKKLFFKCECFQKAGAF-KIRGASNSIFALDDEQVSKGVVTHSSGNHAA-VALAAKLRGIPAHVIPRNAPASKVENVKCYG-GHIWSDASIESREYVSKRVQEETGAVLIHPI-NSKYTISGQGTVSLELLEQVPEIDTIIVPISG-GGLISGVALAAKAINPSIRILAAEPKGADDSA-QSKAAGKIITLPSTNTIADGLRAFLGDLTPV-VRLVDVIVVDDTAIVDAMKMCYEILKVAVE-PSGAIGLAAALSDEFKQSSAWHESSKIGIIVS-GGNVDLGTLWQSMYKLEHHHHHH

† The NdeI site is underlined. ‡ The XhoI site is underlined. § The two extra amino-acid residues and the C-terminal His₆ tag are underlined.

containing an additional 20 mM imidazole pH 8.0 and the bound ZmSR protein was eluted with buffer A containing an additional 250 mM imidazole pH 8.0. The purified protein was concentrated by ultrafiltration with an Amicon Ultra-15 Centrifugal Filter Unit (10 kDa molecular-mass cutoff, Millipore), exchanged with buffer B (20 mM Tris–HCl, 100 mM NaCl, 10% glycerol pH 8.0) and analyzed by 15% SDS–PAGE. The gel was stained with Coomassie Brilliant Blue R-250. Protein concentration was determined by the Bradford method using bovine serum albumin as a standard. The purified ZmSR was aliquoted and stored at 203 K.

2.2. Determination of the oligomeric state

A Superdex HR200 column (10 × 300 mm) on an FPLC system (GE Healthcare, Amersham, England) was pre-equilibrated with 100 ml buffer B. The ZmSR that had been purified using an Ni–NTA column was diluted to a final concentration of 1 mg ml^{−1} in buffer B and eluted from the Superdex HR200 column at room temperature at a flow rate of 0.6 ml min^{−1} using buffer B. The elution volume of each marker protein (GE Healthcare) was also recorded.

2.3. Absorption spectra of the purified enzyme

Ultraviolet–visible absorption spectra for purified ZmSR (1 mg ml^{−1}) before and after dialysis against 500 volumes of buffer C (20 mM Tris–HCl, 1 mM DTT, 10 μM PLP, 10 mM sodium borohydride pH 7.5) were recorded at room temperature using a U-2900 spectrometer (Hitachi, Japan).

2.4. Crystallization, data collection and processing

The crystallization of ZmSR is reported in Table 2. Crystals were soaked in cryoprotectant composed of the mother liquor supplemented with 20% glycerol for 30 s, mounted on nylon loops and flash-cooled in a nitrogen-gas stream at 95 K prior to data collection.

Figure 1
The reversible racemization and irreversible dehydration reactions catalyzed by eukaryotic serine racemase.

Table 2
Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	24-well
Temperature (K)	277
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	20 mM Tris-HCl, 100 mM NaCl, 10% glycerol pH 8.0
Composition of reservoir solution	0.2 M calcium acetate, 0.1 M sodium cacodylate pH 6.5, 18% polyethylene glycol 8000
Volume and ratio of drop	1 µl; 1:1
Volume of reservoir (µl)	200

A native ZmSR data set was collected on BL17U at Shanghai Synchrotron Radiation Facility with a wavelength of 0.979 Å at 100 K. Diffraction images for ZmSR crystals were processed using *HKL-2000* (Otwinowski & Minor, 1997). The crystal belonged to space group *C222*₁, with unit-cell parameters $a = 50.99$, $b = 204.02$, $c = 211.85$ Å, $\alpha = \beta = \gamma = 90^\circ$. The collected and processed data are presented in Table 3. The data set was 98.9% complete to 2.1 Å resolution, with an R_{merge} of 7.5%.

2.5. Structure determination and refinement

The crystal structure of ZmSR was solved by molecular replacement using *MrBUMP* from *CCP4* (Winn *et al.*, 2011). The initial model was based on rat SR (PDB entry 3hmk; Smith *et al.*, 2010). The presence of three monomers was assumed in the asymmetric unit, as suggested by the Matthews coefficient V_M of 2.50 Å³ Da⁻¹ (Matthews, 1968). After several rounds of model building and refinement using *Coot* (Emsley & Cowtan, 2004) and refinement using *REFMAC5* (Murshudov *et al.*, 2011) the electron density was improved,

Table 3
Data collection and processing.

Diffraction source	BL17U, SSRF
Wavelength (Å)	0.9793
Temperature (K)	100
Detector	CCD
Crystal-to-detector distance (mm)	250
Rotation range per image (°)	1
Total rotation range (°)	220
Exposure time per image (s)	1
Space group	<i>C222</i> ₁
a , b , c (Å)	50.99, 204.02, 211.85
α , β , γ (°)	90, 90, 90
Mosaicity (°)	0.44
Resolution range (Å)	50.0–2.10 (2.14–2.10)
Total No. of reflections	354295 (64847)
No. of unique reflections	64417 (11580)
Completeness (%)	98.9 (100)
Multiplicity	5.5 (5.6)
$\langle I/\sigma(I) \rangle$	17.82 (4.68)
R_{rim}	0.085 (0.415)
Overall B factor from Wilson plot (Å ²)	24.2

and waters and the PLP cofactor were then built into the structure. The final structure had an R factor of 19.72% and an R_{free} of 22.87%. The root-mean-square deviations (r.m.s.d.s) of bond lengths and angles were 0.0082 Å and 1.304°, respectively. Intermonomer interactions in the structure were analyzed using the *Protein Interfaces, Surfaces and Assemblies* service (*PISA*) at the European Bioinformatics Institute (http://www.ebi.ac.uk/pdbe/prot_int/pistart.html; Krissinel & Henrick, 2007). Comparisons with other structures were performed with the respective deposited PDB files. The final model was validated using *PROCHECK* (Laskowski *et al.*, 1993). The resultant Ramachandran plot indicates that 97.8% of the residues are in the most favoured regions and 2.2% are in additionally allowed regions. Structure-determination and

Figure 2

(a) Ribbon structure of the ZmSR monomer. (b) The ZmSR dimer in the asymmetric unit. All colour images were generated in *PyMOL* (<http://www.pymol.org>). The two domains in one monomer or two monomers in one dimer are indicated in different colours.

refinement statistics are summarized in Table 4. The structure of ZmSR has been deposited in the PDB with the accession code 5cvc.

3. Results and discussion

3.1. Overall structure

The structure of ZmSR monomer *A* includes amino acids 19–338. Residues 1–18 and the C-terminal His₆ tag were not visible in the electron-density map. The ZmSR monomer comprises two domains (Fig. 2*a*). The large domain (residues 1–66 and 163–338) folds into a twisted α/β architecture containing nine α -helices and five β -sheets. It is referred to as the catalytic domain, in which one PLP molecule is bound. The small domain (67–162) consists of four α -helices and a three-stranded β -sheet. The two domains are linked by a flexible loop region comprised of residues 85–89 and 154–157.

There are three ZmSR molecules in the asymmetric unit. The buried surface between molecules *A* and *C* is about 1444 Å², while that between molecules *A* and *B* is about 482 Å². There is no intermolecular contact between molecules *B* and *C* (Supplementary Fig. S1). The purified ZmSR has a molecular mass of 35 kDa as shown by SDS–PAGE and displays a single peak on size-exclusion chromatography, with a holoenzyme molecular mass of 82 kDa (Supplementary Fig. S2), suggesting that the purified protein is a homodimer. Therefore, molecules *A* and *C* were treated as a dimer (Fig. 2*b*). Two molecules *B* from adjacent asymmetric units form another dimer with a buried surface of 1255 Å². Several positively and negatively charged residues are located on the protein surface and are responsible for the obvious charge polarity of the ZmSR monomer (Fig. 3).

Table 4

Structure determination and refinement.

Resolution range (Å)	50.0–2.1
Completeness (%)	98.9 (100)
σ Cutoff	None
No. of reflections, working set	61516
No. of reflections, test set	3283
Final R_{cryst} (%)	19.72
Final R_{free} (%)	22.87
Cruickshank DPI	0.1994
No. of non-H atoms	
Protein	7032
Ion	3
Ligand	45
Water	196
Total	7276
R.m.s. deviations	
Bonds (Å)	0.0082
Angles (°)	1.304
Average B factors (Å ²)	
Protein	33.356
Ion	29.9
Ligand	26.3
Water	34.6
Ramachandran plot	
Most favoured (%)	97.8
Allowed (%)	2.2

3.2. Structural comparison with other related enzymes

The amino-acid sequence of ZmSR shows about 44% identity to that of rat SR and 38% identity to that of fission yeast SR. Secondary-structure assignments were produced using *ESPrpt* (Gouet *et al.*, 2003). As detailed in Fig. 4(*a*), the helices and strands are named $\alpha 1$ – $\alpha 16$ and $\beta 1$ – $\beta 10$, respectively. Helices adopting 3_{10} conformations are labelled $\eta 1$ and $\eta 2$. Each ZmSR monomer comprises 18 helices (16 α -helices and two 3_{10} -helices) and ten β -sheets, as shown in the topological diagram (Fig. 4*b*).

Figure 3

(*a*) The molecular surface is coloured (blue, positive; red, negative) according to the electrostatic potential of the ZmSR monomer. (*b*) The molecular surface is rotated horizontally by 180°.

Structural comparison of ZmSR with non-ligand-bound rat and fission yeast SRs (PDB entries 3hmk and 1v71; Smith *et al.*, 2010; M. Goto, unpublished work) reveals an r.m.s.d. of 1.7 and 1.8 Å for 322 C α atoms, respectively. α 15 in ZmSR is two

residues longer than that in rat SR and three residues longer than that in fission yeast SR, and the loop between α 4 and α 5 in ZmSR is three residues shorter than that in rat SR. α 16 in ZmSR is six residues longer than that in rat SR and fission

Figure 4

(a) Structure-based sequence alignment of ZmSR with the rat and fission yeast SR homologues. Secondary-structure elements of ZmSR are shown above the sequences. The image was produced using *ESPrpt* (<http://esprpt.ibcp.fr/ESPrpt/ESPrpt/>). Sequence alignment was performed using *ClustalW2* (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). (b) Topological diagram of ZmSR. Helices and strands are shown as cylinders and arrows, respectively. The first and last residues in each secondary-structure element are numbered.

yeast SR. The orientation of $\alpha 16$ in ZmSR is significantly different from that in the rat and fission yeast homologues (Fig. 5).

3.3. The active site

The purified ZmSR displayed absorption maxima at 280 and 415 nm. After dialysis against sodium borohydride, the absorption at 415 nm disappeared and an increase in the absorbance at 330 nm was observed (Supplementary Fig. S3),

indicating that ZmSR is bound by the PLP cofactor in solution. In the $2F_o - F_c$ electron-density map (Fig. 6a), PLP forms a typical Schiff-base bond to the imine N atom of Lys67 in ZmSR. The pyridine ring of PLP is sandwiched by Phe66 and Gly246 from the *si* and *re* faces, respectively. The side chain of Ser322 is hydrogen-bonded to the pyridium N atom of PLP, and Asn94 stabilizes the 3' oxygen of PLP by hydrogen bonding. The phosphate group of PLP is coordinated by the triglycine loop (Gly194–Gly196) in ZmSR, which is a tetraglycine loop in mammalian and fission yeast SRs, although

Figure 5
(a) Superposition of C^α atoms for the SR monomers from maize (blue), rat (yellow) and fission yeast (purple). (b) Superposition of C^α atoms visualized as a ribbon representation.

Figure 6
(a) $2F_o - F_c$ electron-density map of the active site contoured at 1.0σ with an isomesh map ($1.6 \text{ \AA}$ curve). The main-chain and side-chain atoms of the active-site residues are shown as sticks. C, O, N and S atoms are indicated in green, red, blue and yellow, respectively. The cofactor PLP is shown as a stick model. (b) Superposition of the active-site residues of SR from maize (green), rat (yellow) and fission yeast (purple). For ZmSR, a hybrid view is shown with residues from monomer A. The PLP molecules from each structure are also shown as stick models.

three of the four glycine residues are involved in binding PLP. The asparagine (Asn94) loop resides on the O3' side of PLP and acts as the recognition site for the substrate carboxylate in the closed form (Goto *et al.*, 2009). Superposition of the active-site residues of SRs from maize and rat shows that the residues in the asparagine loop are conserved between the mammalian and plant enzymes (Fig. 6*b*).

In this work, we have determined the first crystal structure of a plant SR and have compared the structure with its rat and fission yeast homologues. The overall structure of ZmSR is different from that of the SR from the vancomycin-resistant bacterium *E. faecalis*, but is similar to those of eukaryotic SRs. Structural comparison of ZmSR with rat and fission yeast homologues further reveals their similar arrangement of active-site residues, the binding mode of PLP and a different orientation of the ZmSR C-terminal helix. These structural observations will facilitate the further understanding of the racemase and dehydratase activities of this important enzyme in both mammals and plants.

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