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ORIGINAL ARTICLE

Catalytic mechanism of serine racemase from *Dictyostelium discoideum*

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Abstract The eukaryotic serine racemase from *Dictyostelium discoideum* is a fold-type II pyridoxal 5′-phosphate (PLP)-dependent enzyme that catalyzes racemization and dehydration of both isomers of serine. In the present study, the catalytic mechanism and role of the active site residues of the enzyme were examined by site-directed mutagenesis. Mutation of the PLP-binding lysine (K56) to alanine abolished both serine racemase and dehydrase activities. Incubation of D- and L-serine with the resultant mutant enzyme, K56A, resulted in the accumulation of PLP-serine external aldimine, while less amounts of pyruvate, α-aminoacrylate, antipodal serine and quinonoid intermediate were formed. An alanine mutation of Ser81 (S81) located on the opposite side of K56 against the PLP plane converted the enzyme from serine racemase to L-serine dehydrase; S81A showed no racemase activity and had significantly reduced D-serine dehydrase activity, but it completely retained its L-serine dehydrase activity. Water molecule(s) at the active site of the S81A mutant enzyme probably drove D-serine dehydration by abstracting the α-hydrogen in D-serine. Our data suggest that the abstraction and addition of α-hydrogen to L- and D-serine are conducted by K56 and S81 at the *si*- and *re*-sides, respectively, of PLP.

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Abbreviations

SR	Serine racemase
DdSR	Serine racemase of *Dictyostelium discoideum*
SpSR	Serine racemase of *Schizosaccharomyces pombe*
PLP	Pyridoxal 5′-phosphate

Introduction

Eukaryotic serine racemase (SR) is a pyridoxal 5′-phosphate (PLP)-dependent enzyme that catalyzes the racemization of D- and L-serine (Wolosker et al. 1999). In addition to serine racemization, SR catalyzes the dehydration of D- and L-serine to yield pyruvate and ammonia (De Miranda et al. 2002). SR is widely distributed among eukaryotic organisms, including, mammals, plants and fungi. The primary structure of SR shows sequence homology with enzymes in the serine/threonine dehydratase family, and SR is classified into the fold-type II group of PLP-dependent enzymes (Yoshimura and Goto 2008). In the mammalian brain, SR localizes to astrocytic glia cells and neurons, and supplies D-serine, which serves as a physiological co-agonist of the *N*-methyl-D-aspartate (NMDA) receptor, a key component in glutamatergic neurotransmission (Mothet et al. 2000; Kartvelishvily et al. 2006). The SR deletion in mouse shows an approximate 80–90 % decrease in the forebrain D-serine content and showed impaired learning and memory (Inoue et al. 2008; Mori and Inoue 2010). In the mammalian brain, SR is thought to serve as a sole endogenous source of D-serine and as a modulator of brain functions. Dysfunction of the NMDA receptor is reportedly associated with various neurodegenerative

disorders, such as stroke, schizophrenia, Alzheimer's disease, and amyotrophic lateral sclerosis (Nishikawa 2011). SR is regarded as a pharmacological target for these diseases. Elucidation of the structure–function relationship of SR would be valuable for developing mechanism-based inactivators or activators of the enzyme.

To date, crystal structures of a few SRs have been solved. Goto et al. first solved the crystal structures of four kinds of *Schizosaccharomyces pombe* SR (SpSR)—wild-type enzyme (WT), WT bound with an ATP analog, modified enzyme, and the modified enzyme with serine (Goto et al. 2009; Yamauchi et al. 2009). More recently, Smith et al. (2010) published the structures of human and rat enzymes in the presence or absence of malonate, which is an orthosteric inhibitor. These structures revealed that SRs have an overall fold typical of the β-family of PLP enzymes that consists of two domains—a large and a small flexible domain. The cofactor PLP lies in a groove at the domain interface and is linked covalently to a lysine residue (K57 in SpSR and K56 in the human enzyme) to form an internal Schiff base.

The reaction mechanism of PLP-dependent amino acid racemase has been intensively studied with the alanine racemase of a thermopile, *Geobacillus stearothermophilus*. The alanine racemase belongs to the fold-type III PLP enzyme family, and its primary and three-dimensional structures are distinctly different from those of eukaryotic SRs (Yoshimura and Goto 2008; Shaw et al. 1997). The alanine racemase reaction proceeds through a 2-base mechanism, with Lys39 bound to PLP and Tyr265′ lying on the side opposite to Lys39, against the PLP plane. Lys39 abstracts the α-hydrogen of D-alanine resulting in the production of an anionic intermediate that is stabilized by PLP. Re-protonation of the α-carbon occurs via Tyr265′ on the side of the PLP plane opposite to the side where the α-hydrogen abstraction takes place, and L-alanine is produced. The reaction from L-alanine to D-alanine proceeds through the reverse process—the α-hydrogen of L-alanine is abstracted by Tyr265′, and Lys39 donates a proton on the opposite side of PLP to produce D-alanine (Watanabe et al. 2002). Although the eukaryotic SR is structurally different from the alanine racemase, Goto et al. postulated a similar two-base mechanism for the enzyme on the basis of wild-type SpSR models complexed with the substrates L-serine and D-serine. The side chains of the PLP-binding K57 and S82 are located at the *si*- and *re*-face in the PLP plane within a distance of 3.3 and 3.0 Å, respectively, from the α-carbon of the substrate (Goto et al. 2009). In the conversion from L-serine to D-serine, a proton is probably abstracted from the α-carbon of the L-serine moiety by the ε-amino group of K57, which is located on the *si*-face of the PLP-L-serine intermediate. A proton is then donated by S82 to the α-carbon of the resultant anionic intermediate.

We previously cloned, purified, and examined the properties of an SR from the cellular slime mold *Dictyostelium discoideum* (DdSR), which exhibits 38 and 46 % sequence homology with SpSR and human SR. DdSR is a dimer in solution and is activated by Na^+ as well as other activators such as Mg^{2+} and Mg^{2+}ATP (Ito et al. 2012a). This unique stimulation by Na^+ is probably caused by its binding to the divalent cation-binding site because mutation of the divalent metal ion coordinating residues E207 and D213 abolished both Na^+ and Mg^{2+} stimulation.

In the present study, we examined the reaction mechanism and roles of active site residues of DdSR by substitution with alanine, serine, and/or cysteine by site-directed mutagenesis. The K56 residue of DdSR, which corresponds to K57 of SpSR, anchors PLP and is involved in α-proton shuttling on the *si*-face of the PLP plane. The S81 mutation located on the *re*-face of PLP (the opposite side of K56 with respect to the PLP plane) completely lost racemase activity, but its L-serine dehydrase activity was not affected. These data suggest that the serine racemization catalyzed by DdSR proceeds through a two-base mechanism that involves K56 and S81.

Materials and methods

Materials

PLP, D- and L-serine, nicotinamide adenine dinucleotide (NADH), isopropyl-β-D-thiogalactoside (IPTG), methanol, and lactic dehydrogenase from pig heart were obtained from Wako Pure Chemicals (Osaka, Japan). ATP was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Synthetic oligonucleotide primers were obtained from Fasmac Inc. (Tsukuba, Japan). All other chemicals were of the highest grade commercially available.

Construction and purification of DdSR mutants

The point mutation of DdSR was introduced by PCR with KOD-plus DNA polymerase (Toyobo, Osaka, Japan) using complementary primers pair containing the desired mutation. The K56, S80, S81, H84, K111, R132, and P150 were mutated and obtained the K56A, S80A, S80C, S81A, H84A, K111A, R132A or P150S mutant enzymes. The following primers and their complementary primers were used: 5′-caggaagttttgcaatgcgtggtgc-3′ for K56A, 5′-ggtgttgtaactcatgctagtggtaatcatggtcaagc-3′ for S80A, 5′-ggtgttgtaactcattgtagtggtaatcatggtcaagc-3′ for S80C, 5′-gttgtaactcatagtgctggtaatcatggtcaagc-3′ for S81A, 5′-gtagtggtaatgccggtcaagcattatc-3′ for H84A, 5′-gcaccatcagttgcattgaatgctatttgtgg-3′ for K111A, 5′-gtaaagcaacattggaagcagcagaatcaaatactaaac-3′ for R132A and 5′-cattcttgtaagttaattcattcattcgataatttacaagttatcgc-3′

for P150S. The previously constructed DdSR expression plasmid pDdSR was used as a template (Ito et al. 2012a). Mutation of DNA inserts was verified by DNA sequencing. Each wild-type and mutant plasmid was introduced into the *E. coli* Rosetta 2 (DE3) cells (Novagen, Madison, WI, USA). The wild-type and the mutant enzymes were over-expressed with 0.1 mM IPTG, and purified according to the same method for the wild-type enzyme as described previously (Ito et al. 2012a).

Assay of enzymatic activity

Serine dehydration catalyzed by the wild type and mutant enzymes was assayed by monitoring the pyruvate formation with lactate dehydrogenase and NADH by following the decrease in absorbance at 340 nm according to the previously described protocols (Ito et al. 2008, 2012b). If otherwise the reaction (500 µl) was performed in a mixture containing 50 mM Tris–HCl buffer (pH 8.5), 20 µM PLP, 1 mM ATP, 1 mM $MgCl_2$, 50 mM substrate, and DdSR (0.9–7 µM) at 30 °C for 5 min. The reaction was stopped by boiling for 5 min and the mixture was centrifuged at 21,000×*g* for 5 min. Then, 200 µl of the supernatant was collected and transferred to the 800 µl of reaction solution consisting of 50 mM Tris–HCl buffer (pH 8.5), 3 mM NADH and 10 U lactic dehydrogenase, and incubated at 30 °C for 5 min. Pyruvate formed was calculated from the decrease in the absorbance at 340 nm.

Serine racemase activity was assayed by measuring the amount of each antipodal serine formed from D- or L-serine. The DdSR reaction was performed in a 500 µl mixture containing 50 mM Tris–HCl (pH 8.5), 20 µM PLP, 1 mM ATP, 1 mM $MgCl_2$, 1 mM L-homocysteic acid (L-HCA) as an internal standard, 50 mM substrate, and DdSR at 30 °C for 5 min. The reaction was stopped by boiling for 5 min and the mixture was centrifuged at 21,000×*g* for 5 min. Amino acids were derivatized with BOC-L-Cys-OPA reagent (*t*-butoxycarbonyl-L-cysteine and *O*-phatalaldehide mixture) to produce diastereomeric derivatives which were separated by HPLC using a Cosmosil $5C_{18}$-AR-II column (4.6 × 150 mm, Nacalai tesque, Kyoto, Japan) as described previously (Ito et al. 2012a).

Effect of methanol on the DdSR activity was assayed as follows. The reactions were performed in a mixture containing 50 mM Tris–HCl (pH 8.5), 20 µM PLP, 1 mM ATP, 1 mM $MgCl_2$, 1 mM L-HCA, 10 mM substrate and increasing concentrations (0–40 %) of methanol at 30 °C. The reactions were stopped by boiling for 5 min. The racemase activities catalyzed by WT were determined with HPLC. For the determination of the D-serine dehydrase activity catalyzed by WT, the pyruvate formed was determined with lactic dehydrogenase and NADH as described above. The D-serine dehydrase activity catalyzed by S81A were calculated by the decrease in the amount of the D-serine, which was determined by the above-mentioned HPLC method.

Spectroscopic analysis

UV–visible spectra were obtained with a Shimadzu UV-2450 spectrophotometer (Kyoto, Japan). The spectra were recorded in 50 mM Tris–HCl buffer (pH 8.5) containing 0.5 mM $MgCl_2$, 0.5 mM ATP and 25 mM D- or L-serine in the wavelength range from 250 to 550 nm using 1-cm light pass cell at 25 °C.

Fluorescence spectra of the wild-type and mutant DdSRs (0.22 mg/ml) were measured with a Shimadzu RF5300PC spectrofluorophotometer in 50 mM Tris–HCl buffer (pH 8.5) containing 0.5 mM ATP, 0.5 mM $MgCl_2$ and 25 mM D- or L-serine at 25 °C. Excitation was performed at 330 or 420 nm, and the excitation and emission slit widths were 5 nm. Emission spectra were recorded in the wavelength ranging from 340 to 540 nm (when excited at 340 nm) and from 430 to 630 nm (excited at 420 nm). The data were obtained by the subtraction of the blank (spectra of the sample without enzyme) from the obtained spectra. In the presence of D- or L-serine, the spectra were recorded immediately after mixing of the amino acid.

Circular dichroism studies

Circular dichroism (CD) spectra were collected with a JASCO J720WI CD spectrophotometer with a path length of 0.2 cm. The proteins were dissolved in 50 mM borate–NaOH buffer (pH 8.5) at a final concentration 5.7 µM. The scans were performed from 200 to 260 nm at 50 nm per min at 25 °C. The raw data were converted to molar ellipticity values based on the molecular weight and the concentration of DdSRs.

Results

Spectroscopic properties of DdSR

We first analyzed the spectroscopic properties of the wild-type enzyme (WT). Based on our previous finding that DdSR, similar to other SRs, is stimulated by MgATP (Ito et al. 2012a), we obtained all data in the presence of MgATP in Tris–HCl buffer at pH 8.5. In the presence of MgATP, the wild-type DdSR (WT) gave an absorption

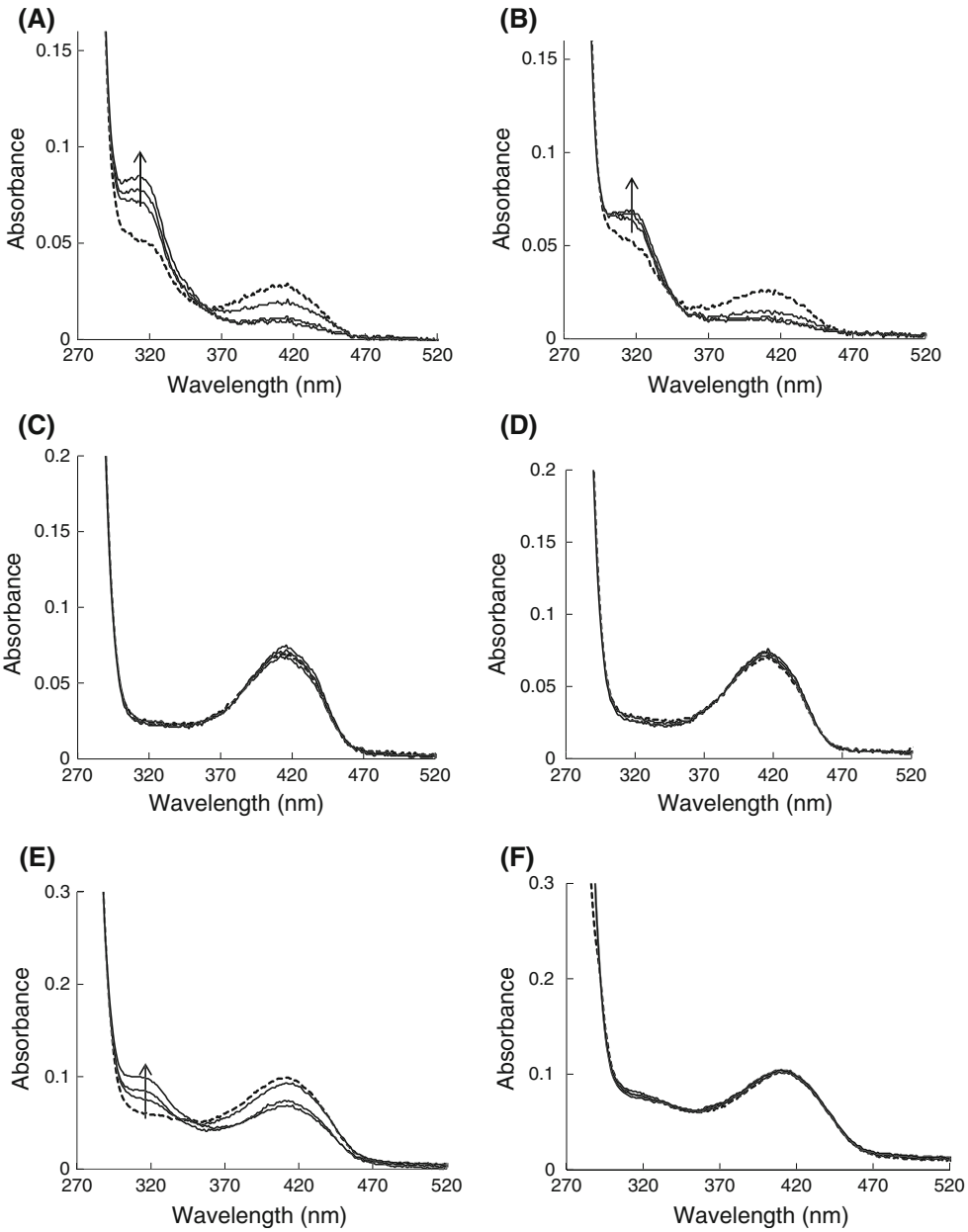

Fig. 1 Spectral properties of DdSRs in the presence of D- or L-serine. UV–visible spectra of WT (**a**, **b** 0.5 mg/mL), K56A (**c**, **d** 0.77 mg/mL) and S81A (**e**, **f** 0.77 mg/mL). In each panel, spectrum in the absence of amino acid is shown as a *dotted line*. Each spectrum was recorded at 25 °C with 50 mM Tris–HCl (pH 8.5) containing 0.5 mM $MgCl_2$ and 0.5 mM ATP. Time-dependent spectral changes caused by the addition of 25 mM L-serine (**a**, **c**, and **e**) and 25 mM D-serine (**b**, **d**, and **f**) are also shown. The 3 spectra in each panel, represented as *solid lines*, are the spectra at 1, 3, and 5 min (*from bottom to top* at 320 nm) after the addition of substrate

spectrum with wavelength maxima at 315 and 416 nm (Fig. 1a, broken line). The absorbance values at 315 and 416 nm are probably attributed to the two forms of the Lys56-PLP internal aldimine—enolimine (unprotonated internal aldimine) and ketoenamine (protonated internal aldimine) tautomers, respectively. Incubation of D- and L-serine with WT resulted in a rapid decrease in the absorbance at 416 nm and a gradual increase in the absorbance at approximately 310 nm (Fig. 1a, b, solid line). The absorbance change at 416 nm indicated a decrease in the levels of the ketoenamine tautomer and/or the external aldimine. On the other hand, the increase in absorbance at 310 nm was partially due to the accumulation of pyruvate formed from D- and L-serine. When excited at 420 nm, WT exhibited an emission fluorescence centered at 495 nm. The peak originated from the ketoenamine tautomer of the internal Schiff base. The addition of 25 mM D- or L-serine caused a rapid peak shift from 495 to 485 nm, which increased the emission intensity (Fig. 2a). An increase in the emission peak indicated the formation of external aldimine (PLP–serine complex) (Vaccari et al. 1996). Each emission peak was decreased in a time-dependent manner (data not shown), reflecting a decrease in the ketoenamine form and the external aldimine tautomers, as observed by UV–visible spectrophotometry (Fig. 1a, b).

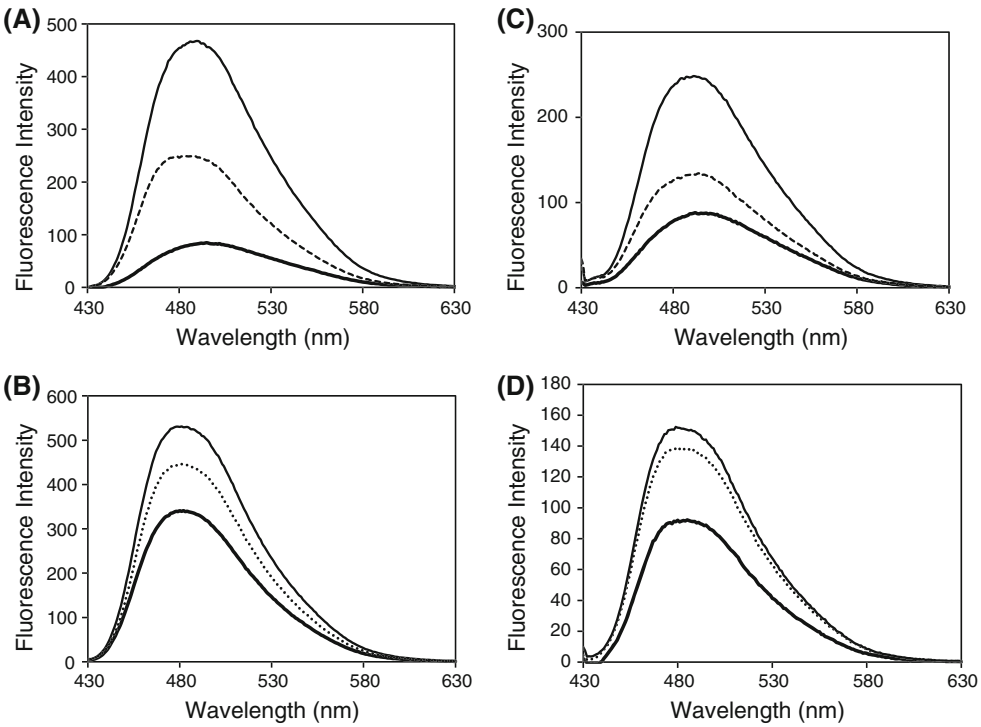

Fig. 2 Emission spectra of WT (**a**), untreated K56A (**b**), S81A (**c**) and reconstituted K56A (**d**) in the absence and presence of D- or L-serine. Emission spectra upon excitation at 420 nm of WT (0.1 mg/mL), S81A (0.1 mg/mL), and K56A (0.03 mg/mL) were recorded at 25 °C in a 50 mM Tris–HCl (pH 8.5) containing 0.5 mM $MgCl_2$ and 0.5 mM ATP. The spectra were recorded in the absence of serine (*bold line*), presence of 25 mM L-serine (*thin line*), and presence of 25 mM D-serine (*dotted line*). The spectra of WT and S81A were recorded immediately after amino acid mixing. The spectra of untreated and reconstituted-K56 were taken 6 min after the addition of amino acid

Mutation of the active-site residues of DdSR

To study the reaction mechanism of DdSR, we mutated one of each of the seven active-site residues—K56, S80, S81 H84, K111, R132, and P150—that are highly conserved among SRs and thus obtained K56A, S80A, S80C, S81A, H84A, K111A, R132A and P150S mutant DdSRs. Each mutant residue corresponds to the following residues in SpSR, whose three-dimensional structure has been determined: K57, S81, S82, H85, K112, R133, and P151. WT and mutant DdSRs were overexpressed in *Escherichia coli* cells and purified according to a previously described protocol (Ito et al. 2012a).

Effects of the K56 mutation

Based on the structure of SpSR bound to L-serine, Goto et al. suggested that the side chain amine nitrogen of the PLP-binding K57 corresponding to K56 of DdSR is located within 3.3 Å of the substrate Cα, and it probably catalyzes the abstraction and addition of the α-hydrogen of the external Schiff base formed from L-serine and PLP (Fig. 3) (Goto et al. 2009).

Although we expected that the K56A mutant enzyme would lose its ability to bind to PLP, its absorption spectrum exhibited two peaks at approximately 280 and 416 nm (Fig. 1c, broken line and Fig. 4, line 1). The absorption maximum at 416 nm is characteristic of the Schiff base, but not of the free PLP aldehyde, which absorbs at 325 and 388 nm at neutral pH. The 416-nm absorption peak is possibly derived from the Schiff base between PLP and a free amino acid, or PLP and some residue of the enzyme other than K56.

Extensive dialysis of K56A with dialysis buffer (50 mM borate–NaOH pH 8.5, 200 mM NaCl, 1 mM hydroxylamine and 1 % glycerol) for 16 h resulted in the enzyme exhibiting a single peak at 280 nm (Fig. 4, line 2), indicating the apo-enzyme formation. Further dialysis of the apo-K56A with the buffer containing PLP produced the holo-K56A (reconstituted K56A). The reconstituted K56A absorbed at 280 nm and showed a broad absorption band at approximately 320 and 400 nm (Fig. 4, line 3).

Incubation with D- and L-serine resulted in no absorption spectral changes with K56A (Fig. 1c, d). An increase in the absorption at approximately 320 nm would have been observed if the enzyme had catalyzed the dehydration of substrate. The absence of the absorption maxima at the 470 and 500 nm indicated the absence of the α-aminoacrylate and quinonoid intermediates, respectively. When excited at 420 nm, K56A exhibited a strong emission peak at 481 nm (Fig. 2b). When estimated at 490 nm, the fluorescence intensity (per μg protein) of K56A was approximately eightfold higher than that of the WT. Unlike WT, the addition of D- or L-serine to K56A did not cause immediate changes in the fluorescence spectra (data not shown). However, prolonged incubation of D- or L-serine with K56A led to a gradual increase in the emission intensity without changing the λ_{max} (Fig. 2b). These observations

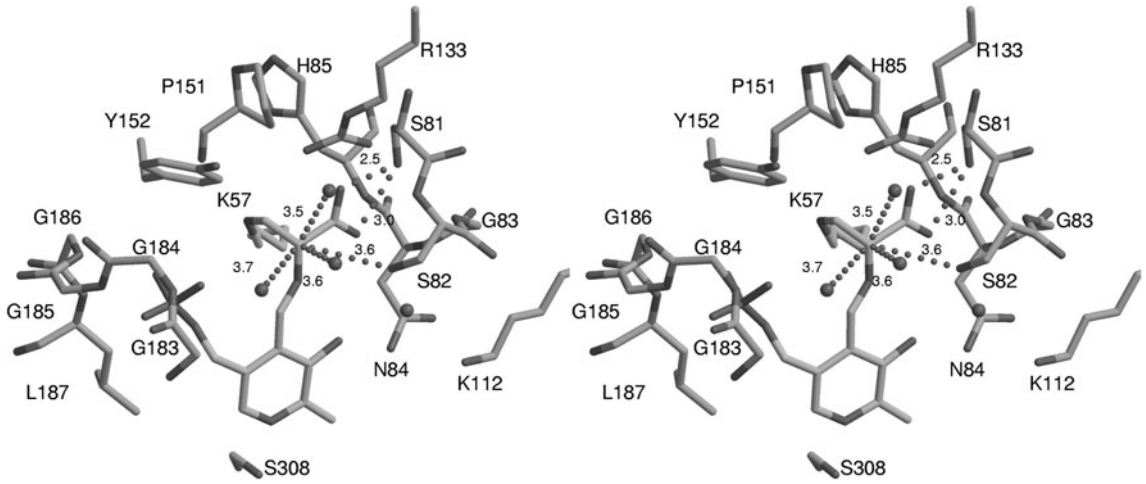

Fig. 3 Active site of modified SpSR with substrate serine (SpSRm). The active site of modified SpSR (Goto et al. 2009) containing 4 water molecules is depicted as stick model. The Schiff base lysine residue (K57) is converted to lysino-D-alanyl residue. Distances between S82-OH and α-carbon of substrate, water molecule and the α-carbon, and S81-OH and carboxy oxygen atoms of substrate are indicated. The K57, S81, S82, H85, K112, R133, and P151 of SpSR correspond to the K56, S80, S81, H84, K111, R132, and P150 of DdSR, respectively. Structural figure was generated using the Molscript (Kraulis 1991)

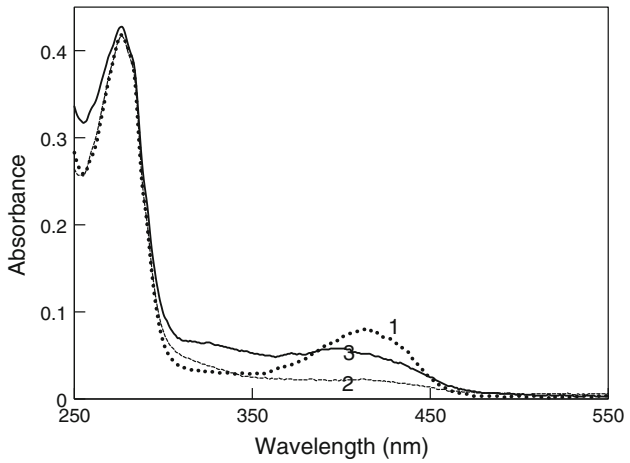

Fig. 4 UV–visible spectra of K56A. UV–vis spectra of untreated-K56A (*line 1*), apo-K56A (*line 2*) and reconstituted-K56A (*line 3*) in 50 mM Tris–HCl pH 8.5 were shown. Each spectrum was taken for a solution containing 0.9 mg/ml protein

probably reflect the accumulation of PLP-serine Schiff base. Accumulation of the external aldimine with K56A suggests that K56 functions as a residue that abstracts the α-hydrogen from the Schiff base intermediate.

We also examined the catalytic activities of the untreated and reconstituted K56A. As shown in Table 1, neither untreated nor reconstituted K56A exhibited racemase activity with D- or L-serine. The mutant enzymes did not show serine dehydrase activity with either D- or L-serine. K56A showed no apparent difference from WT in the circular dichroism (CD) spectrum (Fig. 5) and the elution profile generated using gel-filtration chromatography (data not shown), suggesting that the mutation did not cause any gross conformational alternations.

In some PLP enzymes, addition of a small primary amine such as methylamine or ethylamine recovers the activity of the mutant enzyme following a mutation of the lysine residue at the active site (Watanabe et al. 1999). We added 1, 25, or 1,000 mM of methylamine or ethylamine to the reaction mixture with K56A, however, we did not detect any racemase or dehydrase activities with D- or L-serine (data not shown).

Role of S81 for abstraction/donation of the α-hydrogen of D-serine

The active site structure of SpDR strongly suggests that S81 of DdSR is located on the *re*-face of PLP, which is the opposite side of the catalytic lysine (K56) across the PLP plane (Fig. 3). Furthermore, its hydroxyl group is oriented toward the α-proton of the substrate moiety in the PLP-D-serine Schiff base. The structure also suggests that S81 is a catalytic base for the abstraction and donation of the α-hydrogen of D-serine.

The CD spectrum of S81A was identical to that of WT in the far-UV region (between 200 and 260 nm), suggesting that the mutation did not result in gross changes in the structure of the enzyme (Fig. 5). The S81A mutant enzyme showed absorption maxima at 280 and 412 nm (Fig. 1e, dotted line). The addition of 25 mM L-serine to S81A resulted in a decrease in the absorbance at 412 nm, with a concomitant increase in the absorbance at 320 nm (Fig. 1e). In contrast, addition of D-serine resulted in almost no spectral change in the S81A mutant enzyme

Table 1 Relative activities of mutant enzymes

	Racemase		Dehydrase	
	(L- to D-Ser)	(D- to L-Ser)	(L-Ser to Pyr)	(D-Ser to Pyr)
WT	100	100	100	100
K56A	ND	ND	ND	ND
S80A	ND	ND	ND	ND
S80C	83.9	133	54.5	131
S81A	ND	ND	95.9	3.3
H84A	35.3	9.1	61.1	16.4
K111A	2.6	2.1	5.4	2.3
R132A	58.2	54.2	40.0	49.3
P150S	396	250	29.4	73.6

The serine dehydrase activities were obtained by lactic dehydrogenase coupling assay method. The racemase activities were determined by HPLC. The data are represented as percentages of activity, with the activity of WT being assumed to be 100 %

ND not determined

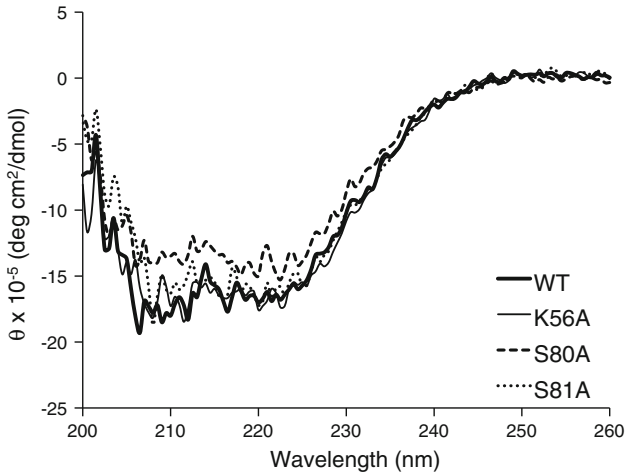

Fig. 5 CD spectra of the wild type and mutant DdSRs. The far-UV CD spectra of WT DdSR (*bold line*), K56A (*thin line*), S80A (*bold broken line*), and S81A (*thin dotted line*) were recorded as described in "Materials and methods"

(Fig. 1f). When excited at 420 nm, the S81A mutant enzyme displayed an emission spectrum with a major peak at λ_{max} of 495 nm (Fig. 2c, bold line). The fluorescence emission spectrum, including its intensity, was almost identical to that of WT. The emission band was blue-shifted by 5–490 nm, and the intensity increased by the addition of L-serine as observed with WT (Fig. 2c). In addition, D-serine induced a blue shift of the spectrum (λ_{max} 490 nm) and increased the emission intensity (Fig. 2c). These results suggest that S81A forms an external Schiff base upon addition of both D- and L-serine.

The mutation of S81 to the alanyl residue completely abolished the racemase activity, but its L-serine dehydrase activity was fully retained (Table 1). The K_m and k_{cat} values of S81A for L-serine dehydration were 47.5 mM and 68.9 min^{-1}, respectively. The k_{cat}/K_m value was 1.45 min^{-1} mM^{-1}, which is approximately one-fourth that of WT (5.86 min^{-1} mM^{-1}, Ito et al. 2012a). Conversion of SR to L-serine dehydratase by the S81 mutation is compatible with the expected role of S81; i.e., S81 serves as a catalytic residue for the abstraction and addition of the α-hydrogen of D-serine. However, unexpectedly, the S81A mutant catalyzed D-serine dehydration very slowly (at approximately 3.3 % of the rate of the reaction catalyzed by WT), with K_m and k_{cat} values of 97.5 mM and 1.22 min^{-1} (Table 1), respectively.

Attribution of water to D-serine dehydration catalyzed by S81A

The weak D-serine dehydrase activity of S81A raised the question of whether S81 really catalyzes abstraction (and donation) of the Cα-H of D-serine. One possible explanation is that DdSR possesses another mechanism for α-proton abstraction of D-serine in addition to S81.

Aspartate aminotransferase from chicken has been found to very slowly racemize some amino acids, such as L-Glu, L-Asp, L-Phe, L-Tyr, and L-Ala in the presence of the cognate oxo-acid despite the absence of ionizable catalytic bases. The reaction rate decreased by the diffusion of water molecules of the active site when methanol was added to the reaction mixture, whereas the canonical aminotransferase activity of the enzyme remained unchanged (Kochhar and Christen 1992). These phenomena can be explained by assuming that the water molecule in the active site is responsible for the racemization. The water molecule probably donates a proton to Cα of the anionic intermediate of PLP–amino acid complex on the opposite side of the PLP plane where α-hydrogen abstraction occurs. Like the aspartate aminotransferase, the SpSR and DdSR lack the residues capable of hydrogen transfer on the *re*-side of PLP other than S81. However, in the active site of SpSR, 3 water molecules exist within a distance of 3.6 Å from the substrate Cα atom (Fig. 3). Therefore, we cannot rule out the possibility that a water molecule is responsible for D-serine dehydration catalyzed by S81A DdSR.

We then examined the contribution of water molecule(s) to D-serine dehydrase activity catalyzed by S81A. Dehydration of L-serine was slightly increased rather than decreased by the addition of methanol to the reaction mixture (up to 40 % v/v), confirming that S81A is active under these conditions. In contrast, the rate of D-serine dehydration catalyzed by S81A decreased with the increasing concentration of methanol. The catalytic activity was suppressed to 38 % of that under purely aqueous conditions, when 40 % of solvent water was replaced by methanol (Fig. 6). In contrast, almost no effects on

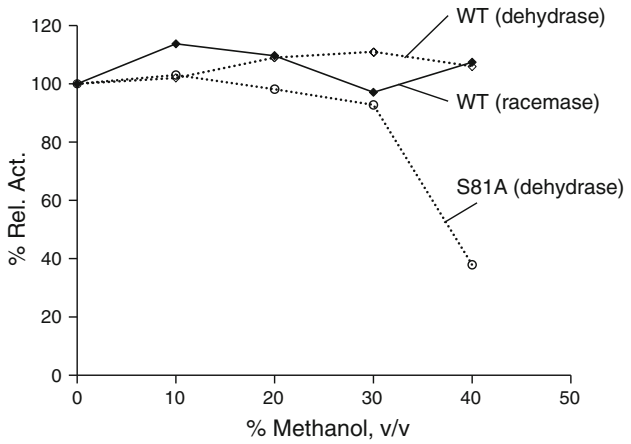

Fig. 6 Effect of methanol on D-serine racemization and dehydration catalyzed by WT and S81A. Serine racemization and dehydration catalyzed by WT (1.9 μM) and S81A (7.4 μM) were performed at 30 °C in the 500 μL of reaction mixture consisting of 50 mM Tris–HCl (pH 8.5), 20 μM PLP, 1 mM ATP, 1 mM $MgCl_2$, 10 mM D-serine, 1 mM L-homocysteic acid (L-HCA) as an internal standard, and indicated concentrations of methanol. The reactions with WT and S81A enzymes were terminated by boiling for 5 min after incubation for 5 min and 4 h, respectively. The racemase (*filled diamonds* for WT) and dehydrase (*open diamonds* for WT, and *open circles* for S81A) activity were determined by analyzing the rates of L-serine formation and D-serine consumption using HPLC. The rates in the absence of methanol were regarded as 100 %

dehydration and racemization were observed when methanol was added to the WT reaction (Fig. 6), indicating that the water molecule is unlikely to participant in the WT reaction. These results, taken together with those from the crystallographic data of SRs, provide evidence for the assignment of S81 as the sole catalytic base for α-hydrogen transfer of PLP-D-serine external aldimine. The small activity of D-serine dehydration catalyzed by S81A is probably attributed to the water molecule(s).

Effect of mutating S80, H84, K111, R132, and P150

As shown in Table 1, substitution of alanine for S80 resulted in complete loss of all enzyme activity. In the closed form of SpSR structure, a loop consisting of ^{81}Ser-^{82}Ser-^{83}Gly-^{84}Asn-^{85}His recognizes the substrate serine, and the hydroxyl group of S81, which corresponds to S80 of DdSR, coordinates with the carboxyl group of the substrate at a distance of 2.5 and 3.0 Å, respectively (Fig. 3). S80 of DdSR probably plays a role in substrate binding by interacting with the carboxyl group of the substrate. To confirm this, the S80 mutant enzyme was incubated with or without the substrate and subjected to spectral analysis. S80A exhibited absorbance maxima at 412 nm, but no change in the absorbance was observed with D- and L-serine (data not shown). When excited at 420 nm, S80A showed an emission spectral peak at approximately 492 nm. The fluorescence emission spectrum was almost identical to that of WT. However, no emission spectral changes were observed upon the addition of D- or L-serine, suggesting that S80A lacks the ability to form a PLP-serine Schiff base (data not shown). These observations suggest that S80 is essential for substrate binding. In contrast, substitution of S80 by cysteine (the resultant enzyme was S80C) caused little difference in the associated rates of serine racemase and dehydrase reactions compared with the WT. As shown in Table 1, the rates of L-serine racemization, L-serine dehydration, D-serine racemization, and D-serine dehydration catalyzed by S80C were 83.9, 54.5, 133, and 131 % of those of WT, respectively. The SH-group can be fully substituted for an OH-group at position 80.

We also examined the effects of the mutation of H84, K111, R132, and P150, which are thought to be located around PLP (see Fig. 3). Relative activities of the mutant enzymes were compared with those of WT (Tables 1, 2). No significant differences in the rates of racemization and dehydration reactions were observed with these mutant enzymes. The rates of L-serine racemization (L- to D-serine), D-serine racemization (D- to L-serine), L-serine dehydration, and D-serine dehydration catalyzed by H84A were 35.3, 9.1, 61.1, and 16.4 % of WT, respectively. The replacement of R132 with alanine (R132 corresponds to R133 of SpSR and R135 of human SR) caused a slight decrease in the enzyme's catalytic activities. The rates of L-serine racemization, D-serine racemization, L-serine dehydration, and D-serine dehydration catalyzed by R132A were 58.2, 54.2, 54.0, and 49.3 % of WT. Although R133 is regarded as a key residue for recognition of the substrate in modified spSR (Goto et al. 2009), and R135 of human SR is suggested to stabilize the transition state to enhance the reaction rate (Smith et al. 2010), no significant changes were observed in the activities as a result of the mutation of the corresponding residue (R132) of DdSR. Mutation of K111 brought about a more marked decrease in the catalytic activities. The catalytic efficiencies of L-serine

Table 2 Kinetic parameters of D- and L-serine dehydration catalyzed by WT and S81A

Reaction	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1}/mM)
L-Ser dehydrase			
(WT)	6.7 ± 1.10	39.3 ± 1.50	5.86[(1)]
(S81A)	47.5 ± 1.81	68.9 ± 1.17	1.45
D-Ser dehydrase			
(WT)	22.4 ± 3.82	4.31 ± 0.25	0.19
(S81A)	97.5 ± 11.3	1.22 ± 0.08	0.01

(1) The values are from Ito et al. (2012a)

racemization, D-serine racemization, L-serine dehydration, and D-serine dehydration catalyzed by K111A were 2.6, 2.1, 5.4, and 2.3 % of those of WT, respectively. The rates of L- and D-serine racemization increased following the mutation of P150 to serine. The P150S mutation altered the partition between racemization and dehydration. This mutant exhibited a 250 and 396 % increase in the rates of L- and D-serine racemization, respectively, whereas the rates of L- and D-serine dehydration were 29.4 and 73.6 % relative to those of WT. A similar effect was observed in P153S mouse SR (Foltyn et al. 2005). Because these mutants showed significant catalytic activities, H84, K111, R132, and P150 were not essential for either racemization or dehydration of D- and L-serine.

Discussion

As determined from the structures of SpSR and human SR, K56 is the PLP-binding lysine residue of DdSR. However, purified K56A exhibited an absorption peak at 416 nm, which suggests the presence of an Schiff base of PLP. Similar Schiff base formation has been reported with some PLP-dependent enzymes lacking the PLP-binding lysine residue. The K87T $\alpha_2\beta_2$-tryotophan synthase (Miles et al. 1989) and K42A *O*-acetylserine sulfhydrylase (Rege et al. 1996) contained PLP, thereby forming an Schiff base with the free serine, and methionine or leucine, respectively. Our data suggest that the K56A DdSR contains an Schiff base of PLP with a free amino acid or some residue of DdSR. Upon addition of L-serine, the untreated- and reconstituted-K56A gradually formed the external aldimine, but D-serine and pyruvate were not produced. Absorption spectra of the enzyme indicated the absence of the formation of α-aminoacrylate or quinonoid intermediate with L-serine. These results suggest that K56 is dispensable for the external aldimine formation, but is involved in the α-proton abstraction from the L-serine moiety of the PLP-L-serine external aldimine. This finding is compatible with the expected role of the active-site lysine, as suggested by crystallography studies of SpSR and human SR: its ε-amino group is situated on the *si*-face of PLP and implicated in abstracting and donating the α-hydrogen of L-serine.

According to the active-site structures of SpSR and human SR, the hydroxyl side chain of S81 of DdSR is probably located close to the α-carbon of substrate on the *re*-face of PLP. S81 is the candidate catalytic base for α-hydrogen transfer of D-serine. S81A displayed impaired serine racemase and D-serine dehydrase activities, whereas it exhibited a functional L-serine dehydrase activity. S81 is not required for L-serine dehydration. Similar results were recently reported with the S82A mutant of SpSR. Although the S82A SpSR catalyzed L-serine dehydration at a similar rate to that of the wild-type SpSR ($K_m = 45$ mM, $V_{max} = 450$ nmol min^{-1} mg for S82A and $K_m = 36$ mM, $V_{max} = 870$ nmol min^{-1} mg for WT), it abolished both racemase and D-serine dehydrase activity (Goto et al. 2009). Unlike SpSR, S81A of DdSR shows a little D-serine dehydration activity, which probably occurs via a mechanism different from WT. With S81A, the α-hydrogen of D-serine is probably abstracted by a water molecule because addition of a high concentration of methanol decreased the rate of D-serine dehydration without any loss in L-serine dehydration. Neither the serine racemase nor D-serine dehydrase activity of WT were affected by the addition of methanol, indicating that the water molecule-mediated catalysis hardly occurs with WT. These observations confirm that the Ser81 residue is responsible for the abstraction/donation of the α-hydrogen of D-serine-PLP complex at the *re*-side of PLP.

The implication of Ser81 in D-serine dehydration is reminiscent of L-serine dehydratase. The L-serine dehydratase from rat liver (LSDH) exhibits 27 % sequence identity with the primary structure and a similar quaternary structure with SRs (Yamada et al. 2003). In spite of the similarity, LSDH catalyzes neither serine racemization nor D-serine dehydration. Comparison of the active site between SR and LSDH showed a critical difference at position 81. Ser81 of DdSR is located in the ^{80}Ser-^{81}**Ser**-^{82}Gly-^{83}Asn-^{84}His segment (the so-called asparagine loop), which is highly conserved among SRs. The segment is also conserved among LSDHs, but the residue corresponding to S81 of DdSR is replaced by Ala (A65 in the rat enzyme). A similar segment, ^{167}Ser-^{168}**Thr**-^{169}Gly-^{170}Asn, is also found in the *E. coli* D-serine dehydratase belonging to the same PLP-dependent enzyme family of SRs, fold-type II. According to the active site model of the *E. coli* D-serine dehydratases, the threonine residue (Thr168, underlined) is located in the corresponding position of DdSR S81 and is suggested to abstract the α-proton of D-serine (Urusova et al. 2012). Presence of the residue with a hydroxyl group at this position is probably critical for its reactivity toward D-serine.

Site-directed mutagenesis studies suggest that the serine racemase reaction catalyzed by DdSR proceeds through a two-base mechanism with K56 and S81 in a manner similar to the bacterial alanine racemase (Fig. 7). In contrast to racemization, L-serine dehydration can be achieved using one catalytic residue, as exemplified by the observation that S81A completely catalyzes L-serine dehydration. Interestingly, we found that K56A did not catalyze D-serine dehydration, suggesting that K56 is also essential for D-serine dehydration. K56 is most probably involved in the release of an α-aminoacrylate from the α-aminoacrylate intermediate (Fig. 7, IV) through transaldimination.

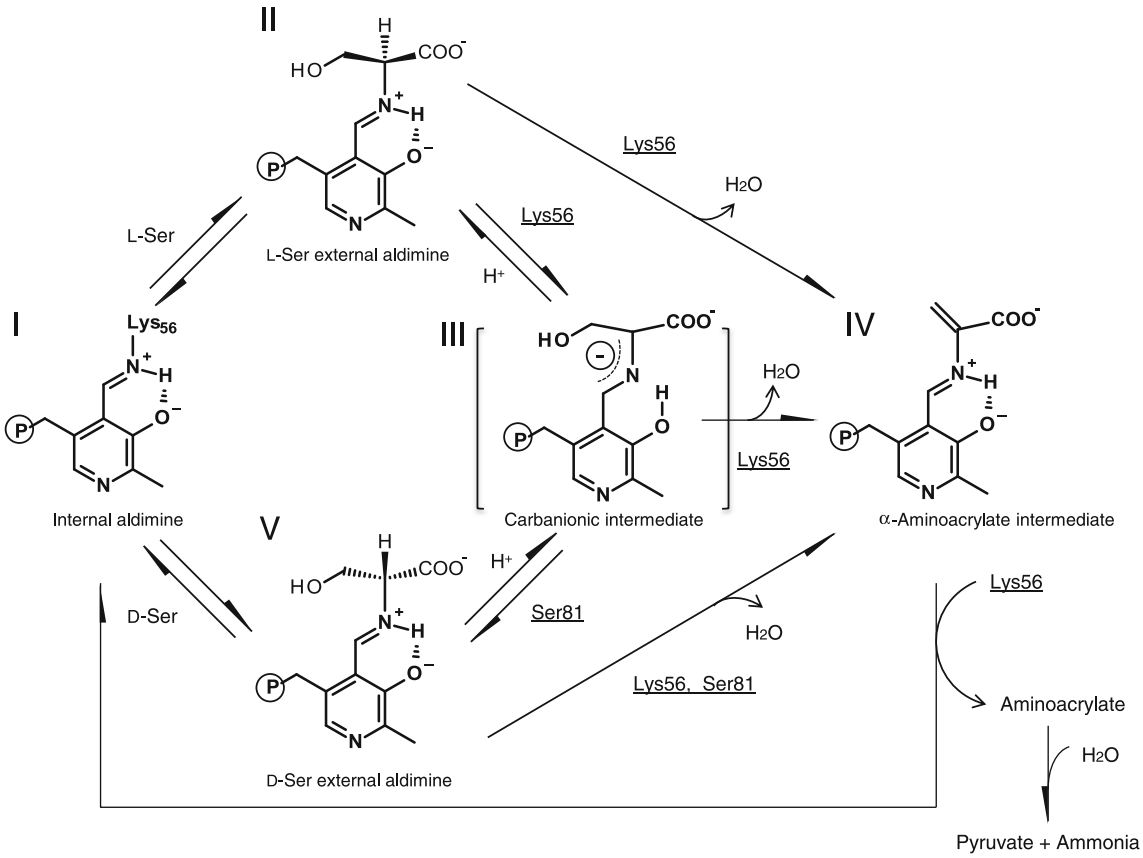

Fig. 7 Proposed reaction mechanism of DdSR. The cofactor PLP forms Schiff base with Lys56 (Internal aldimine, I). DdSR is expected to maintain the N1 pyridine nitrogen in the unprotonated state through the interaction with S308. K56 and S81 are likely to be the acid–base catalyst that shuttle proton to the L-Ser (II) and D-Ser external aldimine (V), respectively, in a two-base mechanism for serine racemization. L-Serine dehydration (II → III → IV) can be achieved using one catalytic residue (K56). D-Serine dehydration (V → III → IV) requires two residues, S81 and K56. It is possible that α-hydrogen abstraction and hydroxyl group elimination proceed in a concerted fashion (II → IV or V → IV) in the serine dehydration. Racemization of serine without formation of the distinct carbanionic intermediate can be also possible, if the α-hydrogen abstraction and addition proceed concertedly (Watanabe et al. 2002)

However, lack of the transaldimination is not the reason for the inability of the D-serine dehydration by K56A, because K56A did not accumulate the detectable amount of α-aminoacrylate intermediate (IV) [or carbanionic intermediate (III)] with D-serine, but accumulated a PLP-D-serine external aldimine (V). These observations suggest that formation of the α-aminoacrylate intermediate (IV) from D-serine external aldimine (V) requires K56. To examine whether K56A catalyzes α-hydrogen abstraction from D-serine, we incubated K56A with [α-^{1}H] D-serine in D_2O and monitored the α-hydrogen–deuterium exchange by ^{1}H NMR. As a result, we found no α-hydrogen–deuterium exchange with D-serine (data not shown). This suggests that K56A catalyzed no α-hydrogen abstraction from D-serine or that the abstracted hydrogen fully returned to Cα without exchanging with the solvent deuterium. If the former possibility holds true, the absence of the α-hydrogen abstraction denies the formation of the carbanionic intermediate (Fig. 7, III). This can be explained by assuming that the α-hydrogen abstraction occurs concertedly with the elimination of the β-hydroxyl group. These speculations are compatible with that of Griswold and Toney (2011): Although racemization and dehydration (β-elimination) reaction start with the proton abstraction at Cα, unlike racemization, β-elimination can occur through a concerted mechanism (E_2-type mechanism) without forming of a distinct carbanionic intermediate and such an E_2-type mechanism of the β-elimination reaction has been proposed in some PLP-dependent enzymes such as rat L-serine dehydratase (Yamada et al. 2003), *Salmonella typhimurium O*-acetylserine sulfhydrylase (Cook 2003), *Saccharomyces cerevisiae* D-serine dehydratase (Ito et al. 2012b) and *G. stearothermophilus* alanine racemase (in α,β-elimination of β-Cl-D-alanine, Griswold and Toney (2011). One of the possible mechanism for the concerted reaction of α-hydrogen abstraction and hydroxyl group elimination catalyzed by DdSR is as follows: The abstracted α-hydrogen is transferred to the β-hydroxyl group, which is

then eliminated as a H_2O, a more efficient leaving group than OH^-. An amino acid racemase should posses some hydrogen relay system between the catalytic residues such as K56 and S81 of DdSR, if not, the racemization reaction would stop after single turnover. It is thus possible that the α-hydrogen is abstracted from D-serine by S81 and relayed to the β-hydroxyl group of D-serine via K56. We also cannot rule out the possibility that dehydration of D-serine occurs after its conversion to L-serine through the racemization.

An understanding the reason behind the inability of K56 to catalyze D-serine dehydration will help the elucidation of the mechanism of bifunctionality of SRs catalyzing both racemization and dehydration.

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