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ORIGINAL ARTICLE

Metal ion dependency of serine racemase from *Dictyostelium discoideum*

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Abstract D-Serine is known to act as an endogenous co-agonist of the *N*-methyl-D-aspartate receptor in the mammalian brain and is endogenously synthesized from L-serine by a pyridoxal 5′-phosphate-dependent enzyme, serine racemase. Though the soil-living mycetozoa *Dictyostelium discoideum* possesses no genes homologous to that of NMDA receptor, it contains genes encoding putative proteins relating to the D-serine metabolism, such as serine racemase, D-amino acid oxidase, and D-serine dehydratase. *D. discoideum* is an attractive target for the elucidation of the unknown functions of D-serine such as a role in cell development. As part of the elucidation of the role of D-serine in *D. discoideum*, we cloned, overexpressed, and examined the properties of the putative serine racemase exhibiting 46% amino acid sequence similarity with the human enzyme. The enzyme is unique in its stimulation by monovalent cations such as Na^+ in addition to Mg^{2+} and Ca^{2+}, which are well-known activators for the mammalian serine racemase. Mg^{2+} or Na^+ binding caused two- to ninefold enhancement of the rates of both racemization and dehydration. The half-maximal activation concentrations of Mg^{2+} and Na^+ were determined to be 1.2 μM and 2.2 mM, respectively. In the L-serine dehydrase reaction, Mg^{2+} and Na^+ enhanced the k_{cat} value without changing the K_m value. Alanine mutation of the residues E207 and D213, which correspond to the Mg^{2+}-binding site of *Schizosaccharomyces pombe* serine racemase, abolished the Mg^{2+}- and Na^+-dependent stimulation. These results suggest that Mg^{2+} and Na^+ share the common metal ion-binding site.

Keywords D-Serine · Serine racemase · *Dictyostelium discoideum* · Metal ion activation

Abbreviations

SR	Serine racemase
DdSR	Serine racemase of *Dictyostelium discoideum*
PLP	Pyridoxal 5′-phosphate
2,4-DNP	2,4-Dinitrophenyl hydrazine

Introduction

In the mammalian brain, D-serine acts as an endogenous co-agonist of the *N*-methyl-D-aspartate (NMDA) receptor and modulates brain function by binding to the "glycine site" of its NR1 subunit (Wolosker et al. 2008). The NMDA receptor, a key excitatory neurotransmitter receptor, is involved in central nervous functions, including memory, synaptic plasticity, and development. The hyper- and hypofunction of NMDA receptor brought by D-serine signaling dysfunction is thought to be involved in diseases, such as schizophrenia,

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Alzheimer's disease, and amyotrophic lateral sclerosis (ALS) (Nishikawa 2005; Sasabe et al. 2007). Reduced levels of D-serine in cerebrospinal fluid and serum were reported in schizophrenia patients. Sasabe et al. (2007) proposed that elevation of D-serine concentration is the key determinant of glutamate toxicity in ALS. Excessive amounts of D-serine are thought to cause glutamate toxicity and lead to motoneuronal cell death. In addition to neurotransmission, D-serine is thought to be related to cell development. D-Serine concentration has been reported to change along with the developmental stage of some tissues (Hamase et al. 2002). D-Serine content reaches 270 nmol/g in the newborn rat cerebellum but rapidly decreases and is almost diminishes in 2-week-old rats. We found that the D-serine concentration in the silkworm *Bombyx mori* transiently increases during specific stages of metamorphosis, including pupation and eclosion (our unpublished results).

D-Serine is synthesized from L-serine by a serine racemase (SR), a member of the fold-type II group of pyridoxal 5′-phosphate (PLP)-dependent enzymes (Wolosker et al. 2008; Yoshimura and Goto 2008). SR is recognized to be responsible for D-serine biosynthesis in mammals because D-serine levels in SR-deficient mice were reported to be ~10% of that of wild-type mice (Basu et al. 2009). In addition to serine racemization, the enzyme catalyzes serine dehydration (α,β-elimination) to yield pyruvate and ammonia (De Miranda et al. 2002). The rate of serine dehydration is comparable to that of serine racemization (Strisovsky et al. 2005).

Because of the physiological importance of D-serine, the regulation of intrinsic D-serine has attracted interest. SR possibly modulates the level of D-serine not only by its synthesis but also by its decomposition. The enzymatic activities of SRs were reported to be modulated by various agents (Kim et al. 2005; Mustafa et al. 2007; Balan et al. 2009). Among them, divalent cations, such as Mg^{2+}, Ca^{2+}, Mn^{2+}, and Mg-adenosine triphosphate (MgATP) are common activators of SRs.

In this work, we carried out enzymological studies of the SR of *Dictyostelium discoideum* (DdSR). *D. discoideum* is a soil-living mycetozoa that is used in developmental studies because it has a unique life cycle consisting of a unicellular ameba phase and a multicellular body at the sporulation stage. *D. discoideum* contains genes that are homologous to those of the serine racemase (De Miranda et al. 2002; Strisovsky et al. 2005; Goto et al. 2009), eukaryotic D-serine dehydratase (DdDSD) (Ito et al. 2008), and D-amino acid oxidase (DdDAO) (Pollegioni et al. 2007), and expresses their cognate mRNA (Dicty Base, http://www.dictybase.org). The presence of a set of D-serine metabolizing enzymes implies that *D. discoideum* synthesizes and metabolizes D-serine, yet it has no genes that are homologous to NMDA receptors. *D. discoideum* is an attractive target for the elucidation of the unknown functions of D-serine such as its role in cell development. In the current work, we studied the properties of DdSR, focusing on the effect of metal ions and nucleotide phosphate. DdSR showed 46, 47, and 38% sequence similarity to that of human, mouse, and *S. pombe* SRs, respectively.

DdSR is a dimer that catalyzes both serine racemization and dehydration. Like other SRs, DdSR is regulated by some divalent metal ions and metal ion-nucleotide complexes such as Mg^{2+} and MgATP, respectively. Unlike other SRs, DdSR is stimulated by Na^{+} in addition to the divalent metal ions. Our data indicate that the Na^{+} and divalent metal ions bind to the same binding site of the enzyme. We also demonstrated that the binding site of the MgATP complex differs from that of the Na^{+} and divalent cations. Binding of MgATP brings conformational change to the enzyme and enhances its catalytic activity.

Materials and methods

Materials

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

Cloning and expression of the DdSR

The serine racemase genes of *D. discoideum* were amplified by KOD-plus DNA polymerase (TOYOBO, Tokyo, Japan), using the following primers: DdSR-FW, 5′-cac catggaacccatggcaacagtaac-3′ and DdSR-RV, 5′-aatttgga tcctcaatgatgatgatgatgatttaaaatctttgaaattgaac-3′. The EST clone of the serine racemase (DDB0230209, ID of Dictybase) obtained from Japan National Bio Resource Project (NBRP-nenkin) was used as a template to amplify the target gene. The resultant PCR product was treated with *Nco*I and *Bam*HI followed by ligation with pET-16b vector (Novagen, Madison, WI, USA) and introduced into *Escherichia coli* Top10 cells (Invitrogen, Gaithersburg, MD, USA). The resultant expression vector pDdSR was designed to express DdSR with a C-terminal 6-histidine tag. The sequence of the inserted gene was confirmed by DNA sequencing using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Construction of DdSR mutants

Mutations of the E207 and D213 residues of DdSR to alanine residue (the resultant mutant enzymes were named E207A and D213A, respectively) were carried out by PCR using the following synthetic primers and their complimentary oligonucleotides: 5′-gtatttgctgctgcaccattaggtgctgatg-3′ for E207A and 5′-ccattaggtgctgatgctacttatcgttcac-3′ for D213A with KOD-plus DNA polymerase and a pDdSR as a template. The reaction mixture was then treated with *Dpn*I to degrade the parental plasmid and the *E. coli* Top10 cells were transformed with the resultant mutated plasmids. Sequences of DNA inserts of the plasmid constructs encoding the mutant enzymes were verified by DNA sequencing.

Expression and purification of the enzymes

For the expression of DdSR, *E. coli* Rosetta II (DE3) cells (Novagen, Madison, WI, USA) harboring plasmids were grown in LB medium containing 100 μg/mL of ampicillin and 30 μg/mL of chloramphenicol at 30°C. The protein expression was achieved by the addition of isopropyl-*β*-D-thiogalactoside (IPTG) to the medium at the mid-log phase to a final concentration of 0.1 mM and the cells were harvested after 13-h incubation. The cells were suspended in a binding buffer (20 mM Tris–HCl, 500 mM NaCl, and 20 mM imidazole, pH 7.9) and sonicated and centrifuged at 20,000×*g* for 30 min. The supernatant was applied to a Ni^{2+}-affinity column equilibrated with the binding buffer. The column was washed with washing buffer (20 mM Tris–HCl buffer containing 500 mM NaCl and 80 mM imidazole, pH 7.9) and the DdSR was eluted with elution buffer (20 mM Tris–HCl buffer containing 500 mM NaCl and 500 mM imidazole, pH 7.9). The eluted protein was passed through an Econo-Pac 10DG desalting column (Bio-Rad, Hercules, CA, USA) with 10 mM Tris–HCl buffer (pH 8.5) containing 20 μM PLP and stored with 10% glycerol at −80°C until use. The N-terminal amino acid sequence of the purified enzyme was confirmed with an Applied Biosystems Procise HT. The concentration of protein was determined by measuring the absorbance at 280 nm using the molar absorption coefficient of 15,950 $M^{-1}cm^{-1}$.

Assay of enzyme activities

Serine dehydrase activity was assayed as follows. The reaction mixture (1 mL) contained 50 mM buffer (Tris–HCl or borate–NaOH buffer, pH 8.5), 20 μM PLP, 50 mM D- or L-serine, 0.3 mM NADH, 10 units of lactic dehydrogenase, and 10–370 μg of DdSR. The reaction was carried out at 30°C and the pyruvate released from the substrate was assayed spectrophotometrically at 340 nm derived from the NADH oxidation coupled with the conversion of pyruvate to lactate (Ito et al. 2007, 2008). Pyruvate was also assayed using the 2,4-dinitrophenyl hydrazine (2,4-DNP) method as described previously (Ito et al. 2007, 2008).

Serine racemase activity was assayed by measuring the amount of each antipodal serine formed from D- or L-serine. Enantioselective determination of the serine was performed with a high performance liquid chromatography (HPLC) after the serine was derivatized to a fluorescent diastereomer. The reaction was performed at 30°C for 30 min in a reaction mixture (200 μL) consisting of 50 mM buffer (Tris–HCl or borate–NaOH buffer, pH 8.5), 20 μM PLP, 50 mM substrate, and 3.5 μM of DdSR. The reaction was stopped by the addition of 200 μL of 10% trichloroacetic acid (TCA) to the reaction mixture followed by incubation of the mixture at 4°C for 15 min. After three times extraction with water-saturated diethyl ether, the aqueous layer was collected. The solution was diluted appropriately and a 10 μL portion was mixed with 190 μL of borate–NaOH buffer (pH 9.0) and 50 μL of BOC-L-Cys-OPA, a mixture of 5 mg of *t*-butoxycarbonyl-L-cysteine and 5 mg *o*-phthalaldehyde mixture in 500 μL of methanol. Separation and quantification of the derivatized D- and L-serine were performed as described previously (Hashimoto et al. 1992).

Gel-filtration chromatography

To estimate the molecular weight of DdSR, the purified enzyme (2.5 mg) was subjected to gel-permeation chromatography with a HiLoad 16/60 Superdex 200 pg column (GE-Healthcare, Waukesha, WI, USA) equipped with FPLC system (Pharmacia, Uppsala, Sweden). DdSR was eluted with 10 mM borate–NaOH (pH 8.5), 20 μM PLP, 200 mM NaCl, and 1 mM EDTA at a flow rate of 1 mL/min at 4°C. The column was calibrated with catalase (molecular mass, 232 kDa), aldolase (158 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), and chymotrypsin (25 kDa).

Spectrophotometric measurements

The UV–visible spectrum of the enzyme (22 μM) was recorded in 10 mM borate buffer (pH 8.5) with a Shimadzu UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan). Fluorescence spectra were taken with a HITACHI F-4500 fluorescence spectrophotometer (HITACHI, Hitachi, Japan) at 4°C using 5.0 nm of excitation and emission bandwidths at a protein concentration of 0.1 mg/mL in 50 mM Tris–HCl buffer (pH 8.5). The excitation wavelength was 295 nm.

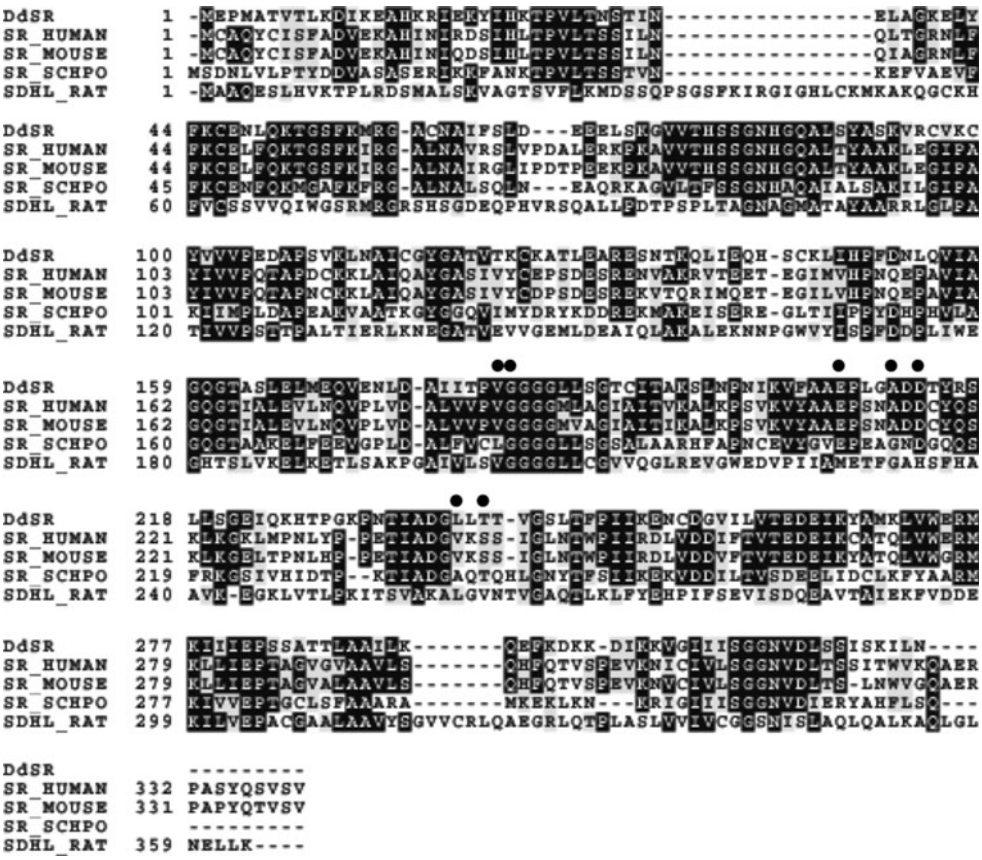

Fig. 1 Alignment of the deduced amino acid sequences of serine racemases (SR) and rat L-serine dehydratase. The deduced amino acid sequences of the *D. discoideum* SR (DdSR), human SR (SR_HUMAN), mouse SR (SR_MOUSE), *S. pombe* SR (SR_SCHPO), and the rat L-serine dehydratase (SDHL_RAT) were aligned using the Clustal W program. The *numbers on the left* are the residue numbers of each amino acid sequence. The Mg^{2+} and K^{+} binding residues of the yeast SR and rat L-serine dehydratase, respectively, are shown by *closed circles*. In the yeast SR, Leu182, Gly183, Ala237, and Thr239 were indirectly involved in the Mg^{2+} binding (Goto et al. 2009)

Results

Cloning and expression of DdSR

To examine the roles of D-serine and its metabolism in *D. discoideum*, we constructed an *E. coli* expression system of the putative serine racemase of *D. discoideum* (DdSR). DdSR showed 46, 47, and 38% sequence similarity to human, mouse, and *S. pombe* SRs, respectively (Fig. 1). The recombinant protein with an additional C-terminal 6-histidine tag was successfully obtained in the soluble fraction of the *E. coli* cell lysate and purified to homogeneity using Ni^{2+}-chelating column chromatography. DdSR showed a single protein band on SDS-PAGE gel with an estimated molecular mass of 35 kDa (Fig. 2a) and exhibited absorption maxima at around 280 and 420 nm (Fig. 2b, solid line). The peak at around 420 nm was decreased by the reduction with $NaBH_4$ with a concomitant increase at around 320 nm (Fig. 2b, dotted line), which is a well-known phenomena that occurs upon reduction of the Schiff base between PLP and an ε-amino group of the lysyl residue of the enzyme.

Enzymatic characterization of DdSR

We compared the enzymatic properties of DdSR to those of other SRs. The molecular mass of DdSR was deduced to be 74.4 kDa by gel-permeation chromatography (Fig. 2c). The calculated molecular mass of DdSR is 35 kDa, suggesting that the enzyme exists as a dimer in solution. As shown in Fig. 3, the L-serine dehydrase and racemase activities of DdSR increased as the buffer pH increased. Similar pH dependency was obtained in Britton–Robinson buffer system (pH 6–10.5) (Supplementary Fig. 1). The enzyme was stable within the pH range of 6–10.5 (data not shown). The reasons for the low catalytic activities in Tris–HCl and potassium phosphate buffers (KPB) are discussed later. We used buffers (borate or Tris) of pH 8.5 during the course of the study.

The steady-state kinetic parameters of racemization and dehydration (α,β-elimination) catalyzed by DdSR for both L-serine and D-serine as substrates were obtained in borate buffer and are shown in Supplementary Table 1. The K_m and k_{cat} values in the serine racemization were determined to be 30.4 mM and 87.4 min^{-1} for L-serine and 34.0 mM and 69.5 min^{-1} for D-serine. The k_{cat}/K_m values of serine racemization were similar in both directions. In the dehydrase reaction, the K_m and k_{cat} values for L-serine were 40.1 mM and 57.9 min^{-1}, while those for D-serine were 20.7 mM and 13.2 min^{-1}, respectively. These values were obtained in the absence of known activators for SRs, such as metal ions and nucleotides.

As reported previously, the racemization and dehydration catalyzed by SRs from mammals and *S. pombe* are

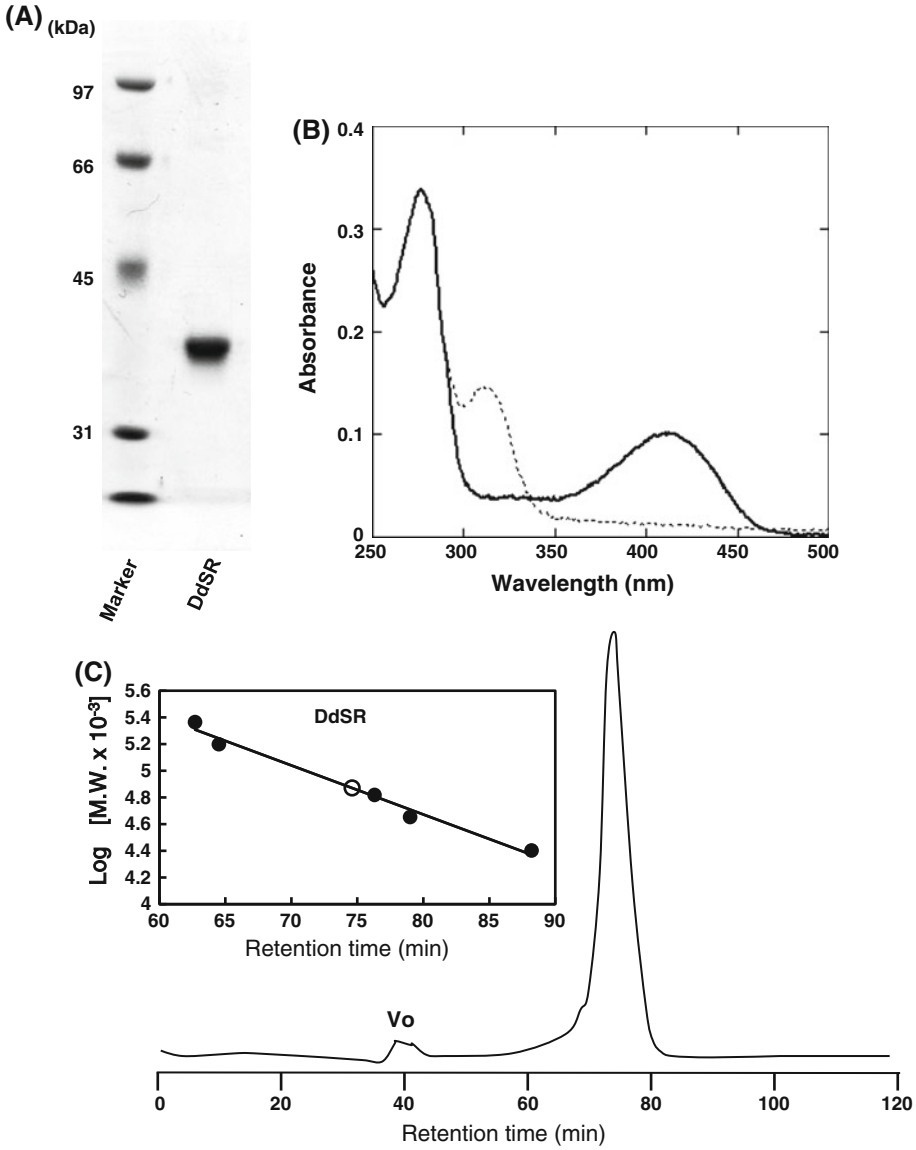

Fig. 2 **a** SDS-PAGE analysis of DdSR. The purified DdSR (2.4 μg) was analyzed by 10% SDS-PAGE gel and stained with coomassie blue. **b** Absorption spectra of DdSR. The *solid line* represents the purified enzyme (22 μM). The *dotted line* illustrates the spectrum of the enzyme mixed with 0.5 mM $NaBH_4$ at 5 min after mixing. The measurements were taken in a 1-cm light pass cell at 25°C in 10 mM borate–NaOH buffer (pH 8.5). **c** Gel-filtration chromatography of DdSR. Elution profile of DdSR is shown. The *inset figure* shows the calibration curve obtained with a set of molecular mass standard

stimulated by the addition of Mg^{2+} or MgATP. In addition to Mg^{2+}, other divalent cations such as Ca^{2+} and Mn^{2+} also act as SR activators. Mg^{2+} and MgATP enhance the mouse enzyme by five- to tenfold in racemization and dehydration, and the addition of EDTA abolishes the enzyme activities (De Miranda et al. 2002). In the presence of MgATP, the K_m and k_{cat} values of the mouse enzyme in the racemization with L-serine as a substrate were reported to be 3.8 mM and 45.5 min^{-1}, while those with D-serine were 14.5 mM and 113 min^{-1}, respectively. Under the same conditions, the K_m and k_{cat} values in the L-serine dehydration were 4.0 mM and 81.3 min^{-1} and those in the D-serine dehydration were 3.2 mM and 8.8 min^{-1} (Strisovsky et al. 2005). The k_{cat} values in both racemization and dehydration were similar to those of DdSR obtained without the use of Mg^{2+} or MgATP. In contrast, the K_m values of DdSR were about one order of magnitude higher than those of the mouse SR.

Unlike the mouse and yeast SRs, DdSR was inhibited by Mg^{2+}, Ca^{2+}, and MgATP under the assay conditions using borate–NaOH buffer (Fig. 4a). Presence of 1 mM Mg^{2+} and Ca^{2+} decreased the racemase and dehydrase activities by around 50%. Addition of free ATP and EDTA showed neither stimulatory nor inhibitory effects. Treatment of the enzyme with EDTA also had no effect on the enzyme when it was assayed in the borate–NaOH buffer (data not shown).

Na^+ acts as a DdSR activator

X-ray crystallographic studies revealed the Mg^{2+} and MgATP-binding site of the human, rat, and yeast SRs

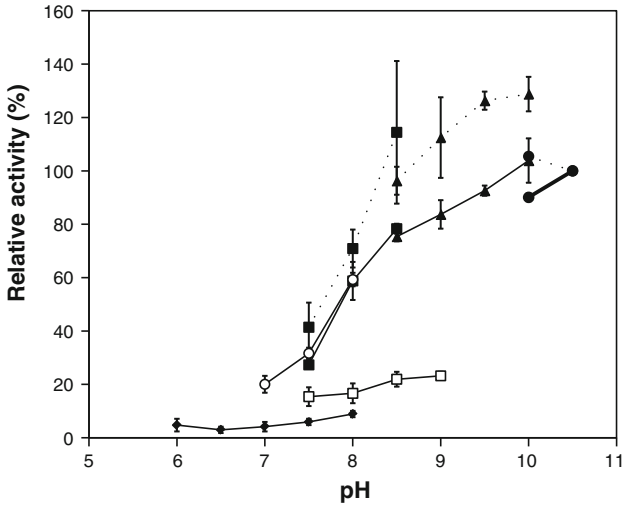

Fig. 3 The pH dependency of DdSR. The effect of pH on the L-serine dehydration (*continuous line*) and racemization (*dotted line*) catalyzed by DdSR was studied using the following buffers: potassium phosphate (pH 6–8), HEPES–NaOH (pH 7–8), Tricine–NaOH (pH 7.5–8.5), Tris–HCl (pH 7.5–9), borate–NaOH (pH 8.5–10), or CAPS–NaOH (pH 10–11). The L-serine dehydrase reaction (100 µl) was performed with each 50 mM buffer, 20 µM PLP, 50 mM L-serine, and 0.8 µM of DdSR at 30°C for 30 min. The pyruvate formed was assayed with 2,4-dinitrophenyl hydrazine (2,4-DNP). Racemase activities were assayed with HPLC. *Error bars* indicate standard deviation (±SD) from the average of three independent measurements

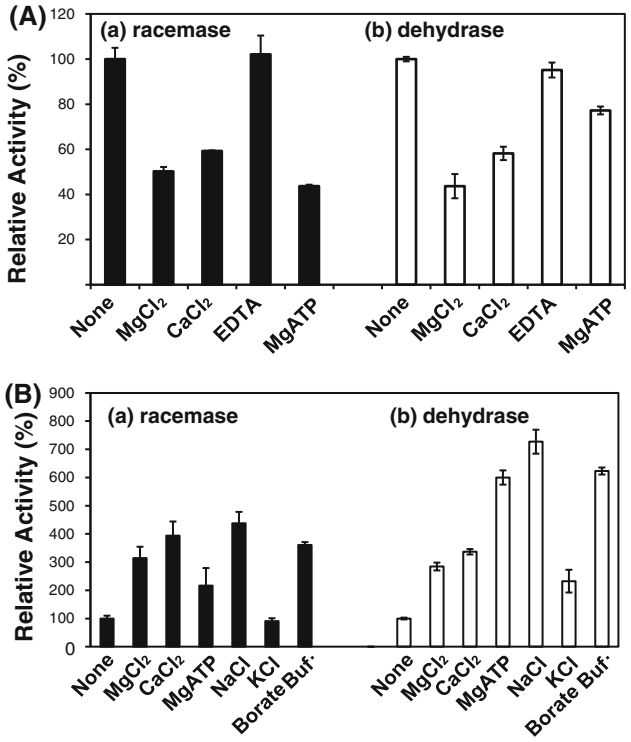

Fig. 4 The effect of metal ions and MgATP on DdSR activity. Racemization with L-serine as a substrate and L-serine dehydration catalyzed by DdSR were assayed in borate–NaOH buffer (**a**) or Tris–HCl buffer (**b**) with the indicated additives (1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM EDTA, 1 mM MgATP, 20 mM NaCl, or 20 mM KCl). The reaction mixture (**a**) consisting of 100 µL of 50 mM borate–NaOH buffer (pH 8.5), 20 µM PLP, 50 mM L-serine, and each 1 mM additive was incubated at 30°C for 30 min. The assay for (**b**) was carried out under the same conditions as (**a**), except that Tris–HCl buffer (pH 8.5) was used instead of borate–NaOH buffer. Racemase activities were assayed with HPLC. Dehydratase activities were determined with 0.3 µM of DdSR using a 2,4-DNP assay according to Ito et al. (2007, 2008). The data are represented as percentages of activity, and the values without additives were assumed to be 100%. The mean values of at least three independent experiments are represented. *Error bars* represent ±SD from the measurements

(Goto et al. 2009; Smith et al. 2010). The structure of the *S. pombe* enzyme bound with an ATP analog, 5′-adenylyl (β, γ-methylene)diphosphonate (AMP-PCP), reveals that the SR binds to Mg^{2+}. One Mg^{2+} binds with the nucleotide complex (Mg-AMP-PCP) at a groove formed between the domain and the subunit interface, which changes the relative orientation of the two subunits. The other Mg^{2+} locates beside the glycine-rich region and interacts with Glu208, Asp214, Gly212, and three water molecules. The three water molecules are supported by the backbone carbonyl oxygens of Leu182, Gly183, Ala237, and Thr239 (Goto et al. 2009). As shown in Fig. 1, the residues in the Mg^{2+}-binding site are well conserved in most of the SRs including DdSR. It is, therefore, interesting that the divalent cations had no activation effects on DdSR.

We further studied the effect of divalent cations on DdSR and found that the enzyme was activated by Mg^{2+} when the enzyme reaction was performed in Tris–HCl buffer instead of borate–NaOH buffer. In Tris–HCl buffer, the serine racemase activity (L- to D-serine) was stimulated about three- to fourfold by the addition of 1 mM Mg^{2+} or Ca^{2+} (Fig. 4b). Likewise, L-serine dehydrase activity was also stimulated about threefold by the addition of 1 mM Mg^{2+} or Ca^{2+} in Tris–HCl buffer (Fig. 4b). The dehydration of D-serine was enhanced about fourfold by the addition of 1 mM Mg^{2+} (data not shown). MgATP also stimulated DdSR in Tris–HCl buffer: L-serine dehydrase, D-serine dehydrase, and serine racemase activities were activated about 6-, 2-, and 2.5-fold, respectively (Fig. 4b).

Without divalent cations, both serine racemase and dehydrase activities of DdSR in the Tris–HCl buffer were less than one-fourth of those in the borate–NaOH buffer (Figs. 3, 4b). As shown in Fig. 3, DdSR activities in the potassium phosphate and Tris–HCl buffers were lower than those in the HEPES, Tricine, borate, and CAPS buffers. Because the buffers giving higher activities contained NaOH as a pH adjuster, we speculated that Na^+ activates DdSR. As shown in Fig. 4b, 20 mM Na^+ enhanced serine racemase activity by fourfold in the Tris–HCl buffer. L-Serine and D-serine dehydrase activities were also enhanced by seven- and fourfold, respectively, by the addition of 20 mM NaCl. In all cases, the enhanced activities were comparable to the DdSR activities in

borate–NaOH buffer (Fig. 4b). The borate–NaOH buffer used in the DdSR assay (50 mM, pH 8.5) was calculated to contain 11.2 mM Na^+. In addition to Na^+, Li^+ partly stimulates the enzymatic activity (data not shown). On the other hand, K^+ did not elicit enzymatic activation (Figs. 3, 4b). K^+ probably does not bind to the enzyme because the reaction in the borate–NaOH buffer was neither inhibited nor activated by the addition of 100 mM KCl (data not shown). This is probably because the ionic radius of K^+ (138 pm) is much larger than that of Na^+ (102 pm). The difference between the activation effects by NaCl and that by KCl rule out the possibility that Cl^- is an effector molecule. These results indicate that DdSR is activated by monovalent and divalent cations. We concluded that the high reactivity in borate buffer is derived from the Na^+ in the buffer.

To examine the effective concentration of these metal ions, ratios of the activation of L-serine dehydration were plotted against the concentrations of Na^+ (Supplementary Fig. 2A), Mg^{2+}, and Ca^{2+} (Supplementary Fig 2B). The half-maximal activation concentration of Na^+, Mg^{2+}, and Ca^{2+} determined from the plots was estimated to be 2.2 mM, 1.2 μM, and 1.2 μM, respectively. The half-maximal activation concentrations of Mg^{2+} and Ca^{2+} were about three orders of magnitude lower than that of Na^+. In contrast, the maximum activation ratio with Na^+ was about two to three times higher than that with Mg^{2+} and Ca^{2+}.

Effect of Na^+, Mg^{2+}, and MgATP on DdSR catalysis and structure

We examined the tryptophan fluorescence of DdSR to investigate whether the metal ions and MgATP affect enzyme structure. In the presence of Na^+ or Mg^{2+}, no changes were observed in the fluorescence emission spectrum at around 340 nm when the enzyme was excited at 295 nm. This suggests that these metal ions induced no significant structural changes. On the other hand, MgATP caused the fluorescent spectral change and probably brought about the DdSR conformational change (Supplementary Fig. 3).

The kinetic parameters of DdSR in L-serine dehydration were obtained with Tris–HCl buffer in the presence of these activators and summarized in Table 1. The K_m and k_{cat} values for L-serine with no metal ions, 20 mM Na^+, and 1 mM Mg^{2+} were 53.0 mM and 7.4 min^{-1}, 42.3 mM and 62.5 min^{-1}, and 36.9 mM and 13.7 min^{-1}, respectively. Mg^{2+} and Na^+ ions greatly increase the k_{cat} values without significantly changing the K_m values. In contrast, MgATP affected the K_m value: in the presence of 1 mM MgATP, the K_m and k_{cat} values in the L-serine dehydration were determined to be 6.7 mM and 39.3 min^{-1}, respectively; in the presence of MgATP, the catalytic efficiency (k_{cat}/K_m) of L-serine dehydration was stimulated about 40-fold. Change in the K_m value supports the speculation that MgATP causes the conformational change of DdSR (Supplementary Fig. 3). With the *S. pombe* SR, MgATP has been reported to cause conformational change: Mg-AMP-PCP changed the relative orientation between the two subunits without altering subunit conformation (Goto et al. 2009).

Table 1 Effect of Na^+, Mg^{2+} and MgATP on the kinetic parameters

	Additive	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1}/mM)
Wild type	(None)	53.0 ± 6.6	7.4 ± 0.4	0.14
	(Na^+)	42.3 ± 3.3	62.5 ± 2.1	1.48
	(Mg^{2+})	36.9 ± 4.4	13.7 ± 0.7	0.37
	(MgATP)	6.7 ± 1.1	39.3 ± 1.5	5.86
E207A	(None)	47.4 ± 3.2	9.5 ± 0.3	0.20
	(MgATP)	24.0 ± 0.4	64.6 ± 0.38	2.69

The rates of L-serine dehydration were determined by lactic dehydrogenase coupling assay method with 50 mM Tris–HCl (pH 8.5), 20 μM PLP, 0.3 mM NADH, 2, 5, 20, 50, or 100 mM substrate, 10 U lactic dehydrogenase, 0.5–3.2 μM DdSR at 30°C for 5 min, and the indicated additive. The additives used were 20 mM NaCl, 1 mM $MgCl_2$, or 1 mM MgATP. The K_m and k_{cat} values were determined by fitting data points to the Michaelis–Menten equation using the Kaleida Graph program

Mg^{2+} and Na^+ are recognized by an identical site

We found that the activation effect on L-serine dehydration by 20 mM Na^+ was partially canceled by the presence of 1 mM Mg^{2+} (Supplementary Fig. 4). L-Serine dehydrase activity with 200 mM Na^+ and 1 mM Mg^{2+} was nearly the same as that with 20 mM Na^+ and no Mg^{2+}. These results suggest that Na^+ competes with Mg^{2+} in DdSR activation and that the cations share a binding site. As described previously, amino acid residues consisting of the Mg^{2+}-binding site of the *S. pombe* SR (Glu208, Asp214) are conserved in DdSR. We mutated the corresponding residues of DdSR, Glu207, and Asp213 to alanine residues. As shown in Table 2, the E207A and D213A mutant enzymes retained their racemase and dehydrase activities, but the mutant enzymes were insensitive to the monovalent and divalent metal ions (Fig. 5a, b). Neither Mg^{2+} nor Ca^{2+} showed any inhibitory or stimulatory effects on the E207A and D213A mutant enzymes in the Tris–HCl or borate–NaOH buffers. These results suggest that both residues are required for the divalent metal binding. E207 and D213 are also important for Na^+-dependent activation. The stimulatory effect of Na^+ was completely abolished in the racemase and dehydrase reactions catalyzed by the E207A mutant enzyme (Fig. 5a). D213 mutation greatly weakened the Na^+ stimulatory effect. Even with 100 mM NaCl, D213A-catalyzing L-serine

Table 2 Effect of mutation of putative Mg^{2+}-binding residues, E207 and D213

	Wild type		E207A		D213A	
	Sa[a]	Ratio[b]	Sa	Ratio	Sa	Ratio
Racemase activity						
L-Serine to D-serine	0.77	100	0.09	11.4	0.16	21.4
D-Serine to L-serine	0.56	100	0.09	15.7	0.37	66.5
Dehydrase activity						
L-Serine to pyruvate	1.26	100	0.13	10.2	0.47	37.0
D-Serine to pyruvate	0.36	100	0.03	8.6	0.15	42.5

The serine dehydrase activities were obtained by lactic dehydrogenase coupling assay method in the presence of 50 mM Borate–NaOH (pH 8.5), 20 μM PLP, 0.3 mM NADH, 50 mM substrate, 10 U lactic dehydrogenase, and 0.6–8.1 μM DdSR at 30°C. For the assay of racemase activity, 3.1–16 μM DdSR was incubated at 30°C for 30 min in the presence of 50 mM Borate–NaOH (pH 8.5), 20 μM PLP, and 50 mM substrate. After the incubation, the resultant amino acids were determined by HPLC as described in "Materials and methods". The values represented are normalized of three independent experiments

[a] The unit of Sa (specific activity) was μmol/min/mg

[b] Ratio of the specific activity in each reaction catalyzed by the mutant enzyme to that catalyzed by the wild-type enzyme

dehydration and racemization were enhanced only by 1.2- and 2.2-fold, respectively (data not shown).

On the other hand, fivefold activation by MgATP was found in the E207A-catalyzing L-serine dehydrase activity. The L-serine dehydrase activity of D213A was stimulated 1.5-fold by MgATP. ATP alone did not activate the mutant enzymes, suggesting that the complexed MgATP is a ligand of the enzyme. The kinetic parameters of E207A in the presence or absence of MgATP are shown in Table 1. Although E207A was substantially activated by MgATP, the presence of MgATP caused a relatively small change in the K_m value of E207A mutant compared to the corresponding value of the wild-type enzyme. The tryptophan fluorescence of E207A was not significantly altered by the addition of MgATP (data not shown). This implies that the binding of Mg^{2+} to the metal ion-binding site constituted by E207 and D213 might be important for MgATP-mediated dynamic structural rearrangement.

Discussion

D. discoideum contains a set of D-serine metabolizing enzymes, DdSR, DdDSD, and DdDAO. We have cloned and expressed all the genes of these enzymes in the *E. coli* cells, purified the gene products, and confirmed their activities. The existence of the set of D-serine metabolizing enzymes implies that *D. discoideum* synthesizes and metabolizes D-serine in vivo. As described above, D-serine serves as a co-agonist of the NMDA receptor in mammals;

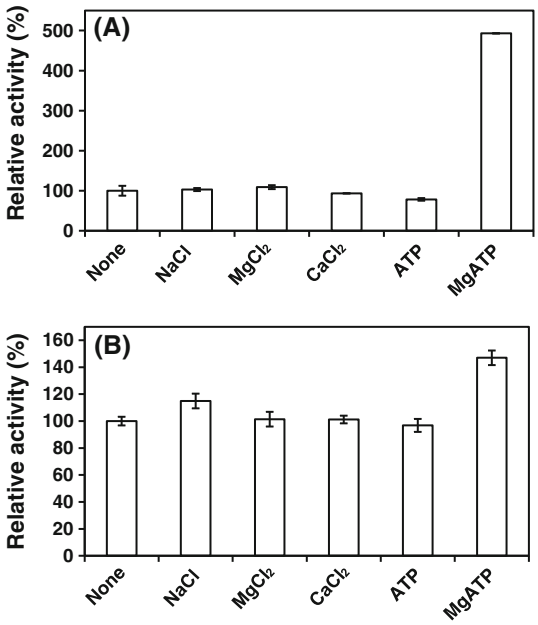

Fig. 5 Effects of metal ions and MgATP on the E207A and D213A mutant enzymes. The L-serine dehydratase activities of E207A (**a**) and D213A (**b**) were determined in the presence of 20 mM NaCl, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM ATP, or 1 mM MgATP. The reaction (100 μl) was performed in 50 mM Tris–HCl (pH 8.5) buffer containing 20 μM PLP, 50 mM L-serine, each additive, and mutant proteins (1.6 μM of E207A or 1.5 μM of D213A) at 30°C for 30 min. The pyruvate formed was determined using the 2,4-DNP method. *Error bars* indicate ±SD ($n = 3$)

however, *D. discoideum* contains no genes that are homologous to those of the NMDA receptors. *D. discoideum* is one of the attractive targets for elucidating the D-serine functions of low eukaryotic organisms.

Regulation of SR and D-serine in mammals has attracted interest because of its physiological importance. Activity of the mammalian SRs is regulated by various factors, such as divalent cations, MgATP (De Miranda et al. 2002), *S*-nitrosylation (Mustafa et al. 2007), phosphorylation, and/or translocation (Kim et al. 2005; Balan et al. 2009). In this work, we studied the properties of DdSR, focusing on its activation by metal ions and MgATP. DdSR is activated by monovalent cations, such as Na^+ and Li^+ and divalent cations, such as Mg^{2+} and Ca^{2+}, and MgATP. The metal ions alone affect the k_{cat} value of the enzyme without drastic changes in the K_m value. MgATP causes structural change and affects the enzyme's K_m and k_{cat} values. To the best of our knowledge, DdSR is the first example of serine racemase stimulated by Na^+. Our results suggest that monovalent and divalent cations share a binding site, which is also the unique aspect of DdSR.

The Mg^{2+}-binding site of DdSR is conserved in other SRs. Thus, it is interesting whether other SRs that conserve the similar metal ion-binding site are also activated by monovalent cations. We have examined the Na^+

dependency of the mouse SR. The L-serine dehydrase activity of the mouse SR was stimulated to 2.4- and 10-fold by the addition of 1 mM Mg^{2+} and 1 mM MgATP, respectively (Supplementary Fig. 5). These results were consistent with those reported previously (De Miranda et al. 2002; Strisovsky et al. 2005). On the other hand, addition of 100 mM NaCl enhanced the enzymatic activity by only 1.2-fold (Supplementary Fig. 5). The mouse SR is activated mainly by Mg^{2+} and MgATP, but not by Na^{+}.

The mechanism of the DdSR activation by monovalent cations is interesting. Mg^{2+} in *S. pombe* SR is octahedrally coordinated with Glu208, Asp214, Gly212, and three water molecules lined by Leu182, Gly183, Ala237, and Thr239. The two carboxyl groups of acidic amino acids are assumed to be one reason for the high affinity of Mg^{2+}. We constructed 3D structure models of DdSR by using a Swiss-Model homology modeling server (http://www.expasy.org/swissmod/SWISS-MODEL.html) (Fig. 6). In the DdSR model structure, the Thr214 side chain, which is located next to Asp213 and is unique for DdSR, intrudes into the metal ion-binding site. The existence of Thr seemingly expands the space slightly among the glycine-rich region, Glu207, and Asp213 compared with that of the Mg^{2+}-binding site of the *S. pombe* SR. The widened space probably enables the binding of larger cations.

The *E. coli* D-serine dehydratase and rat L-serine dehydratase belonging to the fold-type II of PLP enzyme probably bear similar primary and quaternary structures as those of SRs and have been reported to be activated by K^{+} (Kojiro et al. 1989; Yamada et al. 2003). The *Z* scores (strength of structural similarity) between the *S. pombe* SR and the rat L-serine dehydratase were calculated to be 34.4 with 24% sequence identity. The Mg^{2+} of yeast SR and the K^{+} of rat L-serine dehydratase locate with almost identical geometry in each structure (Goto et al. 2009; Yamada et al. 2003). In the rat L-serine dehydratase, the K^{+} is located close to the tetra glycine loop and coordinates with the six oxygen atoms of Gly168, Ala198, Leu223, Val225, Glu194, and Ser200. These residues are well conserved in both *S. pombe* SR and DdSR in all of the amino acids except for Ser200, the rat L-serine dehydrase of which is replaced by an aspartate residue (Asp214) in the *S. pombe* SR (Goto et al. 2009; Yamada et al. 2003). DdSR have unique threonine residues (Thr214) next to the corresponding aspartate residue (Asp213). It might be possible that in the monovalent cation binding of DdSR, Thr214, not Asp213, participates in the binding. If this is the case, we can explain why the D213A mutant enzyme was partially activated by Na^{+} but not by the divalent cations.

Although DdSR showed the highest k_{cat} value in the presence of Na^{+}, Na^{+} is probably not the principal regulator of DdSR. We found the half-maximal activation concentration of Mg^{2+} is about 1800-fold lower than that of Na^{+}. In vivo, Mg^{2+} is most likely the physiological relevant cation of DdSR because its concentration in the *D. discoideum* cells is reported to be about 3.5 mM (Padh and Brenner 1984). The concentration is enough to fully activate DdSR.

As shown in Fig. 3, the DdSR activity became higher at higher pH. The other SRs, such as mouse and *Arabidopsis thaliana* enzymes, show their maximum activity at around pH 8–9. The physiological significance of the high pH preference of DdSR is currently unclear because the intracellular pH of *D. discoideum* is reported to be at around pH 7.1 (Aerts et al. 1985). Because the alkaline pH enhances both serine racemization and dehydration, the solvent pH is assumed to affect the common catalytic steps between serine racemization and dehydration. We believe that the higher pH is at least beneficial for the formation of the substrate-PLP Schiff base (external aldimine), as

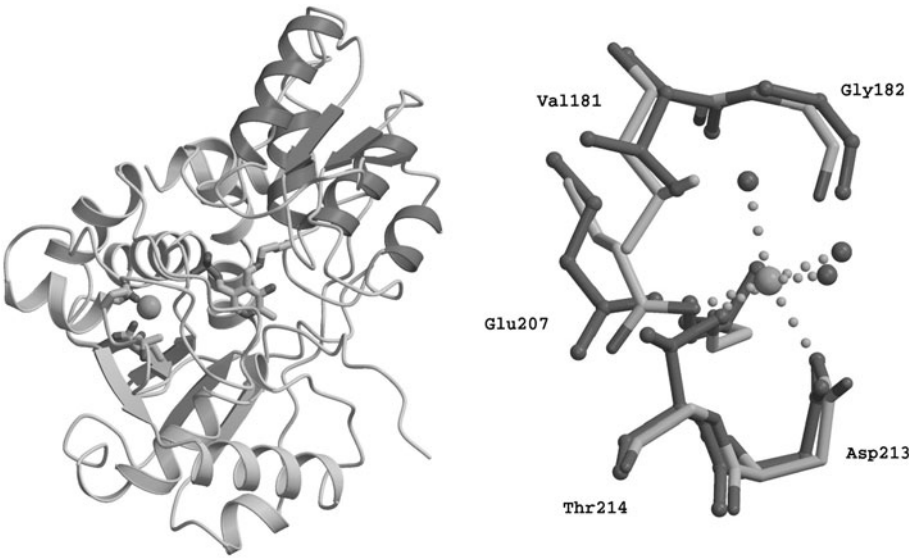

Fig. 6 Model structure of DdSR. *Left* The estimated tertiary structure of DdSR with putative metal ion-binding residues. *Right* Superposition of the putative metal ion-binding sites of *S. pombe* SR and DdSR. These structures were generated with Swiss-Model homology modeling server. The residues of *S. pombe* SR and DdSR are shown in *gray* and *black*, respectively. The Mg^{2+} and oxygen atoms of water molecules of *S. pombe* SR are represented in *gray* and *black balls*, respectively. The *numbers* are for the residues of DdSR. These two structural figures were drawn with MolScript (Kraulis 1991)

alkaline conditions are probably more advantageous than neutral conditions for the substrate nitrogen to react with the carbonyl carbon of PLP upon the formation of the external Schiff base.

To elucidate the physiological roles and reaction mechanisms of DdSR, construction of the DdSR gene-deletion mutant of *D. discoideum* and the X-ray crystallography of the enzyme are now under way.

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