

Mouse brain serine racemase catalyzes specific elimination of L-serine to pyruvate

Kvido Strišovský^{a,b}, Jana Jirásková^{a,b}, Cyril Bařinka^{a,b}, Pavel Majer^c, Camilo Rojas^c,
Barbara S. Slusher^c, Jan Konvalinka^{a,b,*}

^aInstitute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo n.2, Praha 6, 166 10, Czech Republic

^bDepartment of Biochemistry, Faculty of Natural Science, Charles University, Hlavova 2030, Praha 2, Czech Republic

^cGuilford Pharmaceuticals Inc., 6610 Tributary Street, Baltimore, MD 21224, USA

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Abstract D-Serine was previously identified in mammalian brain and was shown to be a co-agonist at the ‘glycine’ site of the N-methyl-D-aspartate (NMDA)-type receptors. Racemization of serine is catalyzed by serine racemase, a pyridoxal 5'-phosphate-dependent enzyme expressed mainly in brain and liver. NMDA receptor overactivation has been implicated in a number of pathological conditions and inhibitors of serine racemase are thus potentially interesting targets for therapy. We expressed recombinant mouse serine racemase in insect cells and purified it to near homogeneity. The enzyme is a non-covalent homodimer in solution and requires divalent cations Mg²⁺, Ca²⁺ or Mn²⁺ for activity but not for dimerization. In addition to the racemization it also catalyzes specific elimination of L-Ser to pyruvate. D-Serine is eliminated much less efficiently. Both L-serine racemization and elimination activities of serine racemase are of comparable magnitude, display alkaline pH optimum and are negligible below pH 6.5.

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Key words: Serine racemase; Pyridoxal phosphate; Pyruvate; D-Serine; Glutamate receptor agonist

1. Introduction

D-Serine has been identified in rat and mouse brain [1,2], shown to be co-localized with N-methyl-D-aspartate (NMDA) receptor immunoreactivity [3,4] and to co-activate at the ‘glycine’ site of the NMDA receptor [3,5–7]. D-Serine has been localized into the protoplasmic astrocytes which enwrap nerve terminals in the areas rich in NMDA receptors [3].

The source of D-serine remained elusive until Wolosker et al. purified L-Ser racemization activity from rat brain homogenates, and cloned the cDNA for mouse serine racemase (mSR) [8,9]. These authors showed that mSR catalyzes serine racemization both in vivo [8] and in vitro [9]. Serine racemase is expressed in liver and brain where it is localized mostly in

the cytoplasm of astrocytes [8]. The human counterpart of mSR has been cloned recently [10]. Interestingly, putative homologs of serine racemase occur not only in mammals but also in yeast *Saccharomyces cerevisiae* and plant *Arabidopsis thaliana* [8]. In mammals, NMDA receptor overactivation has been implicated in neuronal ischemic injury following cerebral stroke [11,12] and its hypofunction has been connected with schizophrenia [13,14].

Very recently, Chumakov et al. identified a new human gene that might be associated with schizophrenia [15]. The gene product was shown to activate D-serine amino oxidase implicating thus the importance of tight regulation of D-serine metabolism. Hence, inhibitors or activators of serine racemase as the biosynthetic enzyme for D-serine are of potential therapeutic interest. In search for potential serine racemase inhibitors, Panizzutti et al. identified an artificial substrate, L-Ser-O-sulfate, which is not racemized by serine racemase but is rather eliminated to pyruvate, ammonia and sulfate. This reaction is much more efficient than serine racemization by the enzyme [16].

We set out to establish an efficient system for recombinant expression of mSR for enzymologic analysis and future crystallization and X-ray structure determination of the enzyme. While investigating the in vitro substrate and reaction specificity of mSR, we identified its previously unknown enzymatic activity, elimination of L-serine to pyruvate. In this paper we provide enzymatic characterization of the enzyme and analyze this novel activity in detail.

2. Materials and methods

2.1. Expression and purification of serine racemase

mSR was obtained from recombinant baculovirus-infected Sf21 cells from ATG Laboratories Inc., Minnesota, USA. Briefly, serine racemase gene (GenBank accession number AF148321) was cloned into pBacPAK9 baculovirus expression vector (Clontech, Palo Alto, CA, USA) and expressed in *Spodoptera frugiperda* Sf21 cells. Cells were harvested by centrifugation and stored at –70°C for further use. In order to isolate soluble cytoplasmic fraction, cell pellets were resuspended in lysis buffer (50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) pH 7.2, 10% (v/v) glycerol, 1 mM MgCl₂, 0.1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 0.05% (w/v) polyethyleneglycol 8000, 50 mM NaCl and 50 μM pyridoxal 5'-phosphate (PLP)) and lysed by three 20 s bursts of sonication. After centrifugation (15 000 × g, 30 min, 4°C), the resulting supernatant was purified by ion exchange chromatography using Q-Sepharose Fast Flow (pH 7.2, lysis buffer, elution by NaCl gradient) and gel filtration chromatography using Superdex 200HR (lysis buffer+150 mM NaCl). For the purpose of molecular mass determination

*Corresponding author.

E-mail addresses: kvido.strisovsky@uochb.cas.cz (K. Strišovský),
konval@uochb.cas.cz (J. Konvalinka).

Abbreviations: mSR, mouse serine racemase; PLP, pyridoxal 5'-phosphate; NADH, nicotinamide adenine dinucleotide reduced form; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid; EDTA, ethylenediamine tetraacetic acid

of native serine racemase, the gel filtration column was calibrated by aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen A (25 kDa). Protein concentration was quantitated using the Bio-Rad protein assay (Bio-Rad Laboratories GmbH, Munich, Germany).

2.2. Activity assays

Serine was incubated in the presence of mSR for a defined time, the reaction was stopped by precipitation of protein by 5% (w/v) trichloroacetic acid which was subsequently removed by extraction into water-saturated diethyl ether. To quantify substrate racemization, the extracted reaction mixture was derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent, [17]) and the resulting diastereoisomers were resolved on a Zorbax Extend C₁₈ (4.6×250 mm, particle size 5 µm) reversed phase high-performance liquid chromatography (HPLC) column (Agilent Technologies, USA) in an isocratic solvent system composed of 100 mM sodium acetate pH 4.4 and methanol (65:35) and detected by ultraviolet (UV) absorption at 340 nm [18]. Derivatized pure L- and D-Ser were used as calibration standards.

Formation of pyruvate in Ser racemization reactions was detected by end-point analysis using lactate dehydrogenase-catalyzed reduction of pyruvate to lactate by nicotinamide adenine dinucleotide reduced form (NADH) and subsequent spectrophotometric determination of NADH at 340 nm. The reaction mixtures were, after protein precipitation by 5% (w/v) trichloroacetic acid and diethyl ether extraction, mixed with the reaction buffer to yield 50 mM triethanolamine pH 7.5, 200 µM NADH and 2.5 U lactate dehydrogenase in a final volume of 500 µl and incubated at 37°C in a thermostated cuvette holder. The reaction proceeded to completion within 2 min. The calibration curve was made with 0–130 µM pyruvate and was linear in this concentration range.

Kinetic constants for simple Michaelis–Menten kinetics were determined by non-linear regression using GraFit program, version 5 (Erihtacus Software Ltd., Surrey, UK).

3. Results

3.1. Expression and purification of recombinant serine racemase

Expression of mSR in baculovirus-infected insect cells re-

sulted in overproduction of active enzyme which was further purified by a combination of ion exchange and gel filtration chromatography (data not shown). The resulting protein preparation was 90% pure with overall activity yield of 30% and protein yield of approximately 100 µg of mSR per 100 mg of wet Sf21 cell paste (about 2×10^7 cells). Using densitometry of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels and purified enzyme as a mass standard we estimate that mSR accounts for about 10% of soluble cellular protein in the producing Sf21 cells.

3.2. Serine racemase catalyzes specific elimination of L-serine to pyruvate

In the activity tests, Sf21 cells expressing β-galactosidase were used as a negative control. In our HPLC-based activity assay, based on derivatization of α-amino groups of the detected amino acids, in the reactions containing serine racemase the total amount of L- and D-Ser decreased in time as opposed to control reactions containing β-galactosidase (data not shown). We hypothesized that it might be due to a side reaction catalyzed by serine racemase which causes loss of the α-amino group. The generalized scheme of possible reaction mechanisms of PLP-dependent enzyme-catalyzed reactions with amino acids [19] suggests that serine could alternatively be reacted directly either to pyruvate or 3'-hydroxypyruvate (Fig. 1).

We have decided to detect possible formation of pyruvate by use of its reduction with NADH catalyzed by lactate dehydrogenase and spectrophotometric detection of the decrease in NADH concentration at 340 nm. Lysates of Sf21 cells expressing mSR or β-galactosidase (negative control) were incubated with 10 mM L- or D-Ser and the content of racemization products and total serine was determined by derivatization with Marfey's reagent and HPLC. Whereas zero or background levels of pyruvate were detected in the negative

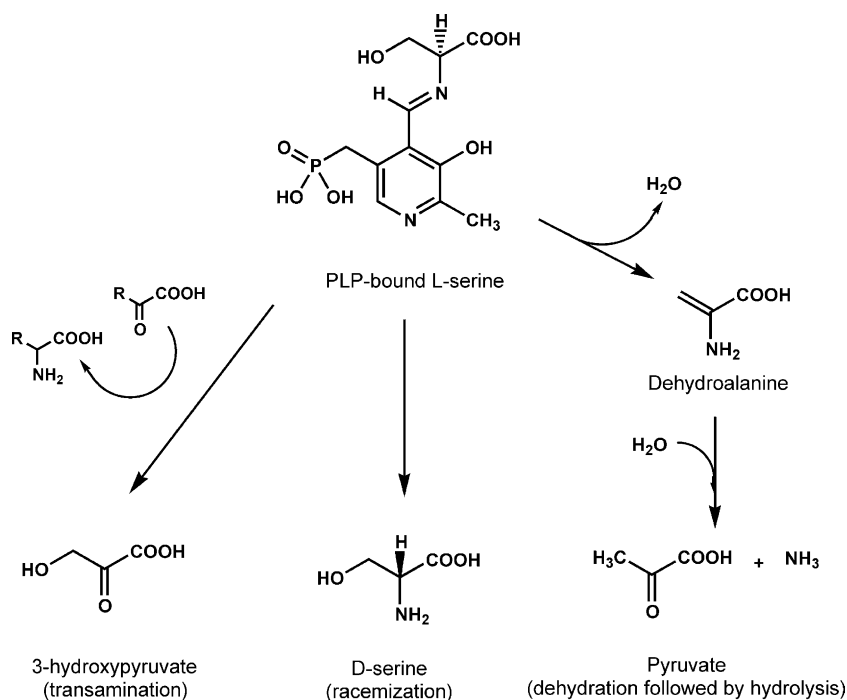


Fig. 1. Potentially relevant pyridoxal phosphate-dependent reaction pathways of serine catalyzed by serine racemase.

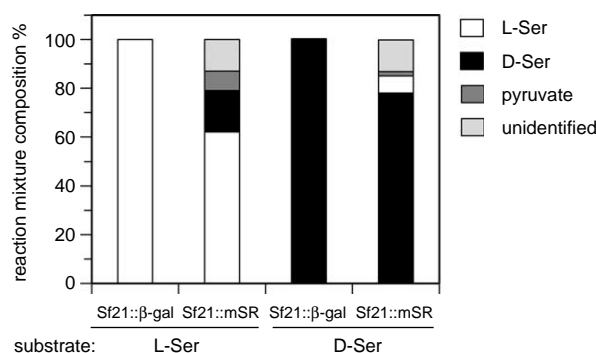


Fig. 2. Serine racemase catalyzes specific conversion of L-Ser to pyruvate and other, uncharacterized product(s). The negative control (Sf21::β-gal) shows the absence of a non-specific activity. Lysate from Sf21 cells expressing serine racemase (Sf21::mSR) or β-galactosidase (negative control, Sf21::β-gal) was reacted with 10 mM L- or D-Ser at pH 8.0, 20 μM PLP and 1 mM MgCl₂ at 37°C for 3 h. Samples were analyzed for L/D-Ser content by modification with Marfey's reagent and HPLC and for pyruvate content by coupled reaction with NADH and lactate dehydrogenase as described in Section 2. □, L-Ser; ■, D-Ser; ▒, pyruvate; ░, unidentified product(s).

control (Sf21 cells expressing β-galactosidase), confirming the absence of any non-specific activity, significant amounts of pyruvate were formed in reactions containing serine racemase (Fig. 2). Strikingly, about five times more pyruvate is formed from L-Ser than from D-Ser. Presumably, the pyruvate formed when D-Ser is the substrate originates from the L-Ser formed by D-Ser racemization since the observed extent of racemization of D-Ser is much lower than that of L-Ser. Mass balance of the reaction suggests that in addition to the racemization product and pyruvate, other yet unidentified reaction product(s) is formed.

Taken together, serine is both racemized and eliminated to pyruvate specifically by serine racemase. L-Ser is converted to pyruvate more efficiently than D-Ser by serine racemase and both enantiomers are converted to other unidentified side products.

3.3. Serine racemase activity requires presence of divalent metal ions

Purification of mSR according to the published original protocol [9] resulted in a complete loss of racemization activity. Further analyses revealed that the inactivation was due to the presence of ethylenediamine tetraacetic acid (EDTA) (data not shown), an inhibitor of serine racemase [20]. Therefore, the L-Ser racemization and elimination activities of mSR were determined in the presence of 1 mM Mg²⁺, Ca²⁺, Mn²⁺, Fe²⁺, Co²⁺, Ni²⁺, Cu²⁺, or Zn²⁺ (Fig. 3A). Whereas 1 mM Mg²⁺ and Mn²⁺ slightly increase the racemization and elimination activity, Ca²⁺, Fe²⁺ and Ni²⁺ were slightly inhibitory and Cu²⁺, Co²⁺ and Zn²⁺ inhibited both activities of mSR significantly.

We examined which of the positively tested cations (Mg²⁺, Ca²⁺, Mn²⁺, Fe²⁺ and Ni²⁺) are able to reactivate the EDTA-inactivated mSR (Fig. 3B). Highest reactivation was achieved by 1 mM Mg²⁺, which recovered 87% of racemization and 107% of elimination activity. Calcium and manganese were less effective, recovering 67 and 50% of racemization and 92 and 70% of elimination activity, respectively, and Fe²⁺ and Ni²⁺ could reactivate serine racemase only to less than 25%.

Only Mg²⁺, Ca²⁺ and Mn²⁺ can thus bind serine racemase and activate it significantly. Our findings are similar to those of Cook et al. [20], with the difference that we observe highest activation effect with Mg²⁺ whereas Cook et al. found Ca²⁺ being the most efficient activator. Based on the above results, we included 1 mM MgCl₂ in all buffers throughout all purification steps and kinetic measurements.

3.4. Serine racemase is a non-covalent homodimer

Since most PLP-dependent enzymes are oligomers [19], we

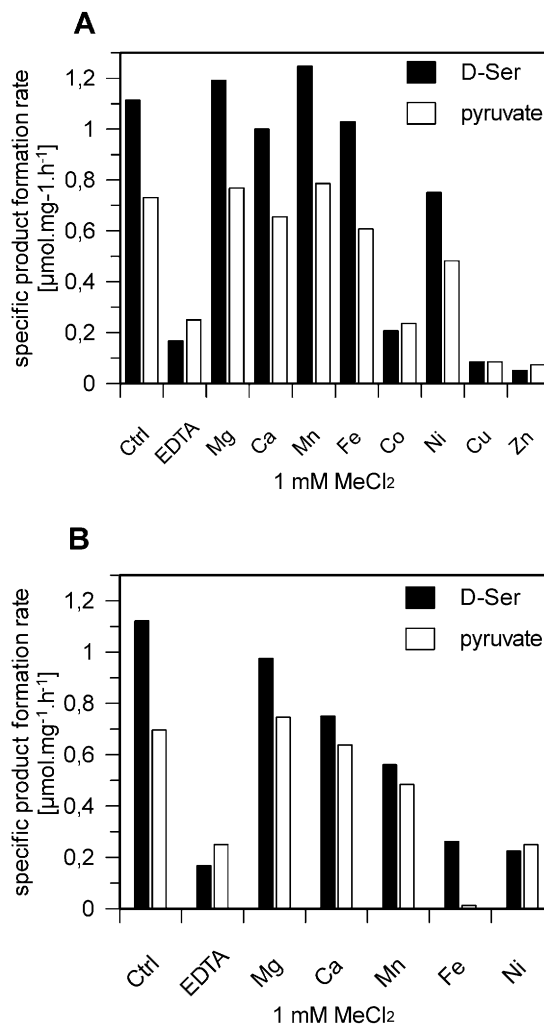


Fig. 3. A: Influence of divalent metal cations on mSR activities. Serine racemase was incubated for 30 min in the presence of 1 mM individual metal chlorides and subsequently, enzyme activity was determined at pH 8.0, 10 mM L-Ser, 20 μM PLP and 1 mM respective metal chloride (37°C, 2 h). B: Reactivation of serine racemase by divalent metal cations after its inactivation by EDTA. Serine racemase was inactivated by 30 min incubation in the presence of 5 mM EDTA. Thereafter, solutions of individual metal chlorides were added to 6 mM concentration and enzyme activity was determined as in A. The amounts of D-Ser were quantified by HPLC after derivatization with Marfey's reagent and pyruvate content was determined by coupled reaction with NADH and lactate dehydrogenase as described in Section 2. In all cases, each data point represents the average of three determinations with the maximum relative difference of 10% between the individual values within the triplicates. The columns named 'Ctrl' refer to mSR activities prior to EDTA treatment without addition of exogenous divalent metal ions and the columns named 'EDTA' refer to mSR activities after the EDTA treatment. ■, D-Ser; □, pyruvate.

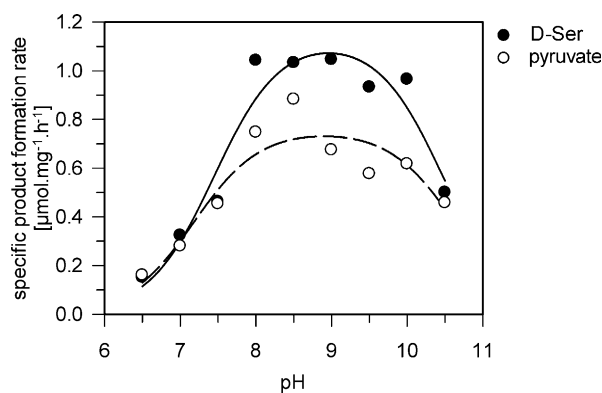


Fig. 4. The pH dependence of L-Ser racemization and elimination. Serine racemase was incubated for 2 h at 37°C with 10 mM L-Ser, 20 μ M PLP and 1 mM MgCl_2 at the indicated pH. The D-serine content was quantified by HPLC after derivatization with Marfey's reagent and pyruvate content was determined by coupled reaction with NADH and lactate dehydrogenase as described in Section 2. Each data point represents the average of three determinations with the maximum relative difference of 10% between the individual values within the triplicates. ●, D-Ser; ○, pyruvate.

analyzed the aggregation state of serine racemase by gel permeation chromatography (data not shown). Serine racemase elutes from the Superdex 200HR gel filtration column as a prominent peak (97% total peak area) of about 78 kDa (data not shown) which corresponds to a dimer composed of 37 kDa protomers. This finding is in agreement with the findings of Cook et al. [20]. The inactivation of mSR by chelation of divalent cations by EDTA does not have significant influence on the dimer stability. The contact between the two protomers of mSR is mediated by non-covalent bonds since pure serine racemase migrates as a single band of 37 kDa on both reducing and non-reducing SDS-PAGE (data not shown).

3.5. Kinetic parameters of serine racemization and elimination by serine racemase

We set out to characterize kinetics of the newly discovered serine elimination activity of serine racemase and compare it to the kinetics of serine racemization. We measured the dependence of initial reaction velocity on substrate concentration for L- and D-Ser in pH 8.0, 1 mM MgCl_2 and 20 μ M PLP. The corresponding parameters for Michaelis–Menten kinetics are summarized in Table 1. The Michaelis constant and the specific activity (V_{max}) of racemization of L-Ser ($K_M = 30$ mM and $V_{\text{max}} = 6.6$ $\mu\text{mol mg}^{-1} \text{h}^{-1}$) and D-Ser ($K_M = 49$ mM and $V_{\text{max}} = 7.9$ $\mu\text{mol mg}^{-1} \text{h}^{-1}$) are consistent with the published values [9,20]. Specific activity (V_{max}) of

7 $\mu\text{mol mg}^{-1} \text{h}^{-1}$ and active enzyme species molecular mass of 74 kDa (dimer) yields a conversion rate of 1 molecule of substrate racemized by 1 molecule of enzyme in about 7 s. Interestingly, serine elimination to pyruvate follows Michaelis–Menten kinetics with the K_M values about twice higher (L-Ser) or similar (D-Ser) to those of racemization. The specific activity (V_{max}) of elimination is comparable to the specific activity (V_{max}) of racemization for L-Ser but is about 20-fold lower for D-Ser, again pointing out the specificity of eliminase activity towards L-Ser.

Both L-serine racemization and elimination activities of serine racemase are pH dependent and of comparable magnitude. The maximum activity window for both activities is rather broad, from about pH 8.0 to 9.5 (Fig. 4). Both activities are negligible below pH 6.5 and their alkaline pH optimum is typical for PLP-dependent amino acid racemases [19].

4. Discussion

The closest enzymologically characterized functional analogs of mammalian serine racemases are bacterial and fungal amino acid racemases. Recombinant alanine racemases from *Mycobacterium tuberculosis* and *Mycobacterium avium* racemize alanine with V_{max} of about 30 and 60 $\mu\text{mol mg}^{-1} \text{h}^{-1}$, respectively and K_M values of about 1 and 0.5 mM, respectively [21] and alanine racemase from *Schizosaccharomyces pombe* racemizes alanine with K_M of 5.0 mM and V_{max} of 670 $\mu\text{mol mg}^{-1} \text{h}^{-1}$ [22]. The maximal racemization velocities of L-Ser for native mSR isolated from mouse brain [9] or recombinant mSR expressed in *Escherichia coli* [20] are 5 and 6.72 $\mu\text{mol mg}^{-1} \text{h}^{-1}$, respectively. The maximal velocity of racemization of the natural substrate of mSR is thus about one to two orders of magnitude lower than the maximal racemization velocities of natural substrates of mycobacterial alanine racemases and a eukaryotic alanine racemase, respectively.

We found that apart from serine racemization, mSR catalyzes specific elimination of L-Ser to pyruvate. Both activities are comparable in their kinetic parameters. It is intriguing why a biosynthetic enzyme producing neurotransmitter D-Ser would also catalyze an apparently useless parallel reaction producing pyruvate. In the original report of Wolosker et al. [9], the authors found no evidence for any other than racemization activity of serine racemase. However, serine racemase has been shown to catalyze elimination of an artificial substrate, L-Ser-O-sulfate, to pyruvate [16]. Several PLP-dependent enzymes have been shown to catalyze more than one reaction type with a single substrate, with one reaction being typically at least two orders of magnitude more effective than

Table 1
Reaction kinetics of racemization and elimination by serine racemase

Reaction	Substrate		D-Ser	
	L-Ser			
	K_M (mM)	V_{max} ($\mu\text{mol mg}^{-1} \text{h}^{-1}$)	K_M (mM)	V_{max} ($\mu\text{mol mg}^{-1} \text{h}^{-1}$)
Racemization	30 \pm 5	6.6 \pm 0.3	49 \pm 9	7.9 \pm 0.4
Elimination to pyruvate	75 \pm 14	4.6 \pm 0.4	75 \pm 27	0.26 \pm 0.04

The reaction buffer contained 50 mM HEPES pH 8.0, 20 μ M PLP, 1 mM MgCl_2 and the respective substrate at varying concentrations. Reactions proceeded for 1 h at 37°C. Serine content was quantified by HPLC after derivatization with Marfey's reagent and pyruvate content was determined by coupled reaction with NADH and lactate dehydrogenase as described in Section 2.

others [19,23]. Interestingly, the kinetic parameters of L-Ser-*O*-sulfate elimination are at the level of the 'main', physiologically relevant activities of *E. coli* serine hydroxymethyltransferase and L-threonine aldolase, whereas serine racemization and elimination activity of serine racemase are at the level of the described side activities of the two above-mentioned bacterial enzymes [23]. Of course, in the elimination reaction, sulfate is a much better leaving group than water and hence it is not wholly surprising that L-Ser-*O*-sulfate is converted to pyruvate faster than L-Ser. Nevertheless, it must be noted that there are enzymes in nature that catalyze conversion of L-Ser to pyruvate and ammonia very efficiently, e.g. the rat liver L-serine ammonia-lyase (EC number 4.3.1.17). This enzyme has a specific activity of $16\,698\ \mu\text{mol mg}^{-1}\ \text{h}^{-1}$ [24] which is about three orders of magnitude higher than that of mSR. These comparisons might suggest that either the serine racemase activity in vivo might be upregulated by another cofactor or covalent modification of the enzyme-like phosphorylation or, alternatively, that the true activity and physiological function of serine racemase are different from the production of D-Ser. The fact that serine racemase is expressed also in mouse liver apart from the brain [8] might suggest the latter possibility, since the production of D-Ser in liver is irrelevant due to intrinsically high levels of D-amino acid oxidase which will oxidize D-Ser to pyruvate [25]. Further research in this area is necessary to elucidate the specificity and regulation of activity of serine racemase in vivo.

After the submission of this manuscript, a paper was published by de Miranda et al. [26] that largely confirms our data at the cellular and protein level. In apparent contradiction to their previous results [9], the authors now also observe the production of pyruvate upon the enzymatic racemization of serine both using recombinant serine racemase and in mammalian cells transfected by a plasmid coding for serine racemase. Moreover, the authors identified ATP as an activator of serine racemase.

In summary, mSR was expressed in high yield in a baculovirus system and purified therefrom. We have shown that mSR is a dimeric enzyme which requires for its activity but not for its dimerization either Mg^{2+} , Ca^{2+} , or Mn^{2+} . It catalyzes racemization of L-Ser with specific activity of $6.6 \pm 0.3\ \mu\text{mol mg}^{-1}\ \text{h}^{-1}$ and, additionally, conversion of L-Ser to pyruvate with specific activity of $4.6 \pm 0.4\ \mu\text{mol mg}^{-1}\ \text{h}^{-1}$, measured at pH 8.0 and substrate saturation. In contrast, specific activity of D-Ser conversion to pyruvate is only $0.26 \pm 0.04\ \mu\text{mol mg}^{-1}\ \text{h}^{-1}$ whereas the specific activity of D-Ser racemization is $7.9 \pm 0.4\ \mu\text{mol mg}^{-1}\ \text{h}^{-1}$. Hence, the elimination activity of mSR is specific for the L-enantiomer of serine. Both L-Ser racemization and elimination activities display similar pH dependence with the optimum in the alkaline region.

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