Dual Substrate and Reaction Specificity in Mouse Serine Racemase: Identification of High-Affinity Dicarboxylate Substrate and Inhibitors and Analysis of the β-Eliminase Activity†

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ABSTRACT: Mouse serine racemase (mSR) is a pyridoxal 5′-phosphate dependent enzyme that catalyzes the biosynthesis of the *N*-methyl-D-aspartate receptor coagonist D-serine in the brain. Furthermore, mSR catalyzes β-elimination of serine and L-serine-*O*-sulfate into pyruvate. The biological significance of this β-elimination activity and the factors influencing mSR substrate and reaction specificity, which are crucial for prospective inhibitor design, are poorly understood. Using a bacterial expression system and ATP−agarose affinity chromatography, we have generated a pure and active recombinant mSR and investigated its substrate and reaction specificity in vitro by analyzing a systematic series of compounds derived from L-Ser and L-serine-*O*-sulfate. The analysis revealed several competitive inhibitors of serine racemization including glycine (K_I = 1.63 mM), several dicarboxylic acids including malonate (K_I = 0.077 mM), and L-*erythro*-3-hydroxyaspartate (K_I = 0.049 mM). The latter compound represents the most effective inhibitor of SR reported to date. A simple inversion of the β-carbon configuration of the compound yields an excellent β-elimination substrate L-*threo*-3-hydroxyaspartate. Inhibition analysis indicates that racemization and β-elimination activities of mSR reside at the same active site. While the racemization activity is specific to serine, the β-elimination activity has a broader specificity for L-amino acids with a suitable leaving group at the β-carbon and optimal spatial orientation of the α-carboxyl and leaving groups. The possible implications of our observations for inhibitor design, regulation of activity, and function of mSR are discussed.

All known living organisms use L-amino acids as building blocks for ribosomal proteosynthesis. While D-amino acids occur commonly in bacteria, which synthesize and use them, for example, during cell wall peptidoglycan biosynthesis, they were considered not to have a role in higher organisms. However, research during the past decade has consistently revealed significant levels of D-serine in mouse and rat brains (*1*, *2*), and D-serine has been found to act as a coagonist at the "glycine site" of the *N*-methyl-D-aspartate (NMDA)[1] subtype of L-glutamate receptors occurring in the mammalian central nervous system (CNS) (*3*−*5*). NMDA receptor signaling is crucial for many physiological processes in mammals including brain development, learning, and memory (*6*−*8*). Overactivation of NMDA receptors leads to neuronal death and contributes to postischemic brain damage and neuropathologies. Importantly, pharmacological blockers of the glycine site are protective in animal models of stroke (*7*). D-Serine and glycine bind to the regulatory glycine site of NMDA receptors with similar affinity, and their relative importance for physiological coactivation of NMDA receptors is still a matter of debate (reviewed in refs *6* and *7*). Glycine is present in high intracellular concentration in all cells, and its extracellular concentrations in mammalian CNS are buffered by several specific transporters. D-Ser CNS levels are particularly high before and shortly after birth and decline in adulthood (*9*). It is possible that both D-Ser and Gly are physiologically relevant for coactivation of NMDA receptors with a distinct spatiotemporal pattern.

The origin of D-Ser in mammals was unclear until serine racemase, which catalyzes direct conversion of L-Ser to D-Ser, was isolated from rat brain (*10*). Its orthologues displaying similar enzymatic properties were also found in mouse and human (*11*, *12*), and it has been suggested to be responsible for D-Ser biosynthesis in vivo. Mouse serine racemase (mSR) is a unique pyridoxal 5′-phosphate (PLP) enzyme whose activity is dependent on divalent cations and stimulated by ATP and, to a lesser extent, by ADP, GTP, and other nucleoside triphosphates (*13*, *14*). It forms dimers

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[1] Abbreviations: CNS, central nervous system; CI, competitive inhibitor; DAAO, D-amino acid oxidase; DTT, DL-dithiothreitol; LDH, lactate dehydrogenase; Lt3HA, L-*threo*-3-hydroxyaspartate; L-SOS, L-serine *O*-sulfate; mSR, mouse serine racemase; MgATP, equimolar mixture of $MgCl_2$ and ATP; NADH, β-nicotinamide adenine dinucleotide, reduced form; NMDA, *N*-methyl-D-aspartate; PLP, pyridoxal 5′-phosphate; SEM, standard error of the mean.

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and tetramers in solution with a monomer molecular mass of about 37 kDa (*15*, *16*). We and others previously observed that mSR not only racemizes serine but it also converts L-serine and L-serine-*O*-sulfate into pyruvate via its β-elimination activity (*13*, *14*, *16*). The L-serine-*O*-sulfate β-elimination activity of mSR is about 2 orders of magnitude higher than both its serine racemization and β-elimination activities (*17*). However, the factors regulating the reaction specificity of mSR and the physiological significance of its β-elimination activity in vivo remain poorly understood.

In this work we aim to analyze substrate and reaction specificity of mSR and to define the structural features important for the high-affinity binding of a ligand to serine racemase. To this end, we developed a prokaryotic expression system for mSR, employed a novel application of cofactor affinity chromatography, and, using thereby highly purified and active recombinant enzyme, carried out inhibition analysis of a series of systematically varied L-serine and L-serine *O*-sulfate derivatives. Among these, we identified several competitive inhibitors and several β-elimination substrates.

MATERIALS AND METHODS

DNA Cloning and Protein Expression. Our *Escherichia coli* expression system for mSR employs the arabinose-inducible pMPM vector series (*18*). Due to the presence of both *Nco*I and *Eco*RI sites within the mSR coding sequence, these sites could not be used for cloning of the 5′-end of mSR into pMPM-A4. Therefore, the *Nco*I site in pMPM-A4 was mutated to *Nde*I by PCR mutagenesis. The pMPM-A4 vector omega-interposon part was amplified using oligonucleotide primers 5′-GTGAAAGGAGGAATTC-CATATGGCTCTAGAGCTTTATGC-3′ and 5′-CGTTCT-GATTTAATCTGTATCAGGC-3′ and back-inserted into pMPM-A4 via *Eco*RI and *Xho*I restriction sites to yield pMPM-A4-2. The mouse serine racemase gene [GenBank nucleotide accession number AF148321 (*12*)] was PCR-amplified using oligonucleotides 5′-GACCCACATATGT-GTGCTCAGTACTGCATC-3′ and 5′-TCCCCGCGGCTC-GAGTTATTAAACAGAAACCGTCTGGTA-3′ and inserted into pMPM-A4-2 via *Nde*I and *Xho*I restriction sites to yield the expression construct pKS-mSR. The DNA sequence of the insert was verified by automated DNA sequencing using BigDye3.0 (Amersham).

Recombinant mSR was expressed in *E. coli* MC1061 (*19*) using nutrient-rich Turbo medium (Athena Enzyme Systems, Baltimore, MD). Briefly, calcium chloride competent bacteria were transformed by pKS-mSR, plated onto LB agar plates containing ampicilin (100 μg/mL), and incubated at 37 °C for 18 h. The resulting colonies were washed off the plates and inoculated into the prewarmed growth medium containing ampicilin (100 μg/mL) to an OD_{600} of ~0.05. Cultures were grown at 37 °C, 300 rpm, in an orbital shaker (New Brunswick Scientific). Intensive aeration of the culture during the expression experiment was required for high mSR expression. When the culture density reached $OD_{600} = 0.8$, mSR expression was induced by the addition of 1 mM L-arabinose (Sigma). After 5 h of induction bacteria were harvested by centrifugation (10000*g*, 10 min, 4 °C) and stored at −70 °C.

Purification of Recombinant Mouse Serine Racemase. Pellets of induced bacteria were suspended in QA buffer [20 mM triethanolamine hydrochloride−NaOH, pH 7.0, 1 mM $MgCl_2$, 20 μM pyridoxal 5′-phosphate, 0.1 mM DL-dithiothreitol, and 0.02% (w/v) NaN_3] using a glass Dounce homogenizer at 3 mL/g of cell paste, supplemented with 1 mM phenylmethanesulfonyl fluoride and the Complete EDTA-free protease inhibitor mix (Roche Molecular Biochemicals), and incubated with 0.2 mg/mL chicken egg lysozyme and 0.05% (w/v) sodium deoxycholate for 30 min at room temperature. After the addition of 10 mM $MgCl_2$ and DNase I (Roche Molecular Biochemicals) to 10 μg/mL and 30 min incubation at room temperature, the cell suspension was sonicated on ice 3 × 20 s with 20 s intervals between cycles. The resulting crude lysate was centrifuged at 20000*g* for 30 min at 4 °C. The supernatant was loaded onto a phenyl-Sepharose FastFlow (Pharmacia) column preequilibrated in QA buffer. After being washed with three column volumes of QA buffer, mSR was eluted with 50% (v/v) ethylene glycol in QA buffer, pH 7.0. The eluate was immediately diluted with an equal volume of QA buffer and applied to a Q-Sepharose FastFlow (Pharmacia) column preequilibrated in QA buffer. After being washed with five column volumes of QA buffer, mSR was eluted in a linear salt gradient generated by QB buffer [20 mM triethanolamine hydrochloride−NaOH, pH 7.0, 0.5 M NaCl, 1 mM $MgCl_2$, 20 μM pyridoxal 5′-phosphate, 0.1 mM DL-dithiothreitol, and 0.02% (w/v) NaN_3] at approximately 220−320 mM sodium chloride. The mSR-containing fractions (whose intensively yellow color is an indication of bound pyridoxal 5′-phosphate) were concentrated 10-fold using Centriprep concentrators (Millipore), diluted with QA buffer, reconcentrated 10-fold, and applied to the ATP−agarose (Sigma) column preequilibrated in QA buffer. The bound mSR was eluted by 2 column volumes of 2 mM ATP in QA buffer, concentrated to approximately 0.5 mg/mL as determined by the Bio-Rad protein assay (Bio-Rad Laboratories GmbH, Munich, Germany), dialyzed three times against 200 volumes of QA buffer at 4 °C, and stored at −70 °C. Concentration of pure mSR was determined by quantitative amino acid analysis. The enzyme sample was hydrolyzed for 20 h in vacuo with 6 M HCl containing 3% phenol, dried, and analyzed using a Biochrom 20 amino acid analyzer (Pharmacia Biotech) with ninhydrin detection of eluted amino acids.

Activity Assays. Enzymatic activities of mSR were analyzed in a pH 8.0 reaction buffer containing 100 mM HEPES−NaOH, 10 μM pyridoxal 5′-phosphate, 1 mM $MgCl_2$, 5 mM DL-dithiothreitol, and 1 mM ATP. Reactions typically proceeded for 30 min at 37 °C. As mSR is unstable below pH 5 and easily precipitates (unpublished observations), the reactions were stopped by the addition of 20 μL of 1.875 M $HClO_4$ to 100 μL of the reaction mixture. After centrifugation (15000*g*, 10 min, room temperature), the solution was neutralized by the addition of 20 μL of 1.875 M KOH and incubated on ice for 10 min, and the $KClO_4$ precipitate was separated by centrifugation. For analysis of amino acid racemization, 10 μL of 5 mM glycine was added as an internal standard to the neutralized supernatant, and the amino acid mixture was derivatized by 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide [Marfey's reagent (*20*, *21*)] and resolved using reversed-phase HPLC as described (*16*).

Formation of 2-oxo acids was determined either by spectrophotometric assay of NADH depletion accompanying

their reduction to 2-hydroxy acids by lactate dehydrogenase and NADH (*14*) or by derivatization of reaction mixtures with 2,4-dinitrophenylhydrazine (*13*). The derivatization of 50 μL of the reaction mixture by 50 μL of the dinitrophenylhydrazine solution was performed in the presence of 900 μL of 50% (v/v) methanol at room temperature for 10 min. Dinitrophenylhydrazones of α-oxo acids were resolved using reversed-phase HPLC on Zorbax Extend C_{18} (Agilent Technologies) in a gradient of 40−85% methanol in 50 mM tetrabutylammonium hydroxide adjusted to pH 4.3 by ultrapure acetic acid and detected by UV absorption at 366 nm (*22*). In this system, 2-oxo acids yielded stable patterns of more derivatives, and their quantification was based on the sum of the resulting peaks.

Enzyme Kinetics. The steady-state initial velocities of enzyme reactions were determined by end point measurement of product. The dependence of initial velocities on initial substrate concentration was fitted into the Michaelis−Menten equation by nonlinear regression using GraFit program, version 5 (Erithacus Software Ltd., Surrey, U.K.).

Computational Details. All calculations were carried out with Turbomole 5.6 software (*23*). The geometry optimizations were performed using a density-functional (DFT) (*24*, *25*), TZVP basis set (*26*). The calculations were expedited by the resolution-of-identity (RI-J) approximation (*27*, *28*) and carried out in a dielectric continuum ($\epsilon = 80$, i.e., representing water), using the conductor-like screening model (COSMO) (*29*−*31*).

RESULTS

Preparation of Recombinant mSR: ATP Affinity Chromatography. To study substrate specificity and inhibition of mouse serine racemase (mSR), we have constructed an *E. coli* expression system using arabinose-inducible expression vector pMPM_A4Ω (*18*). Recombinant mSR was expressed in *E. coli* as described in Materials and Methods. After cell lysis, mSR was isolated from the soluble fraction of the cell lysate using hydrophobic and anion-exchange chromatography to yield an mSR-enriched fraction (Figure 1A, lane QS). This preparation was purified to homogeneity using ATP affinity chromatography that we developed on the basis of the observation that ATP binds and activates mSR with a half-maximal activatory concentration ($[MgATP]_{0.5}$) of 3−4 μM without being hydrolyzed (*13*, *14*). We tested mSR binding to three commercially available ATP−agarose supports, which differ in the site of attachment of the ATP moiety to the agarose support. The C8-linked ATP−agarose (Figure 1B) was by far most efficient, followed by the N6-linked one, whereas the 2′,3′-OH-linked ATP−agarose did not bind mSR at all (data not shown). The binding of mSR to the C8-linked ATP−agarose was inhibited by 0.5 M NaCl, and it was therefore essential for the efficiency of the ATP affinity chromatography to keep the ionic strength below 100 mM. Specific elution of mSR from the affinity column was achieved by elution with 2 mM ATP. Thereby, purified mSR was homogeneous on an overloaded SDS−PAGE (Figure 1A, lane AA). The overall yield was about 2 mg of pure mSR/L of bacterial culture.

Automated N-terminal Edman degradation analysis revealed that Met^1 and Cys^2 were removed from the recombinant mSR by *E. coli* enzymes, presumably by methionine aminopeptidase, which has been shown to effectively remove the second amino acid after the initiator methionine if the third amino acid is small (Gly, Ala, Ser) (*32*). MALDI mass spectrometry of dialyzed pure mSR yielded a single molecular mass peak at 36121.2 Da. This value corresponds well to the predicted molecular mass of the Ala^3 to Val^{339} fragment of mSR, 36123.6 Da, showing that no C-terminal proteolytic cleavage took place in *E. coli*. Aggregation state analysis by gel permeation chromatography and analytical ultracentrifugation demonstrated that the enzyme forms dimers and tetramers in solution (data not shown), as found previously for both the native mSR (*13*) and a recombinant mSR from different expression systems (*15*). The minor N-terminal trimming of our recombinant mSR thus did not significantly affect its oligomerization properties.

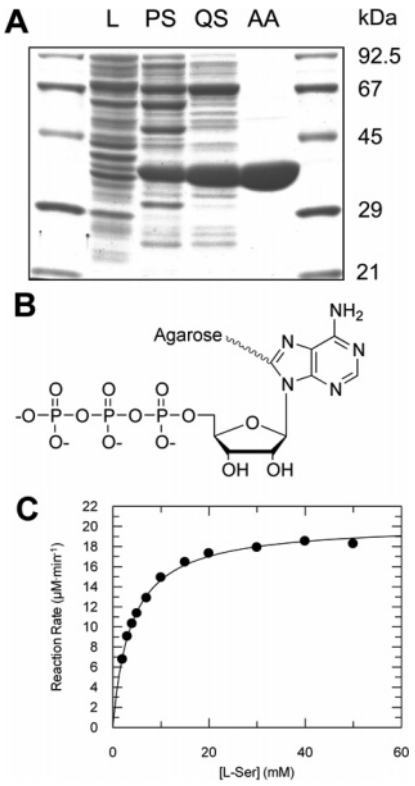


FIGURE 1: Purification and characterization of recombinant mouse serine racemase. (A) Coomassie-stained SDS−PAGE gel showing the purification procedure after each stage: L, soluble fraction of bacterial lysate; PS, pooled fractions after phenyl-Sepharose; QS, pooled fractions after Q-Sepharose; AA, final pure enzyme after ATP−agarose. Each lane contains 10 μg of protein. Molecular mass standards are shown in the side lanes. (B) Attachment of the ATP moiety to the Sepharose support used for the affinity chromatography in this study. (C) Characterization of enzymatic activity of purified recombinant mSR. A representative hyperbolic plot showing the dependence of the initial racemization rate on substrate (L-Ser) concentration at pH 8.0, 10 μM PLP, 1 mM MgATP, and 5 mM DTT. The steady-state kinetic constants were calculated by nonlinear fitting of these data into the Michaelis−Menten equation.

Kinetic Characterization of the Recombinant mSR. To compare the enzymatic properties of our purified recombinant mSR to those of the native enzyme and recombinant enzymes produced in other systems, we analyzed its enzymatic activity using the previously described substrates L-Ser, D-Ser, and L-Ser-*O*-sulfate (*11*, *17*). The analysis was carried out under 1 mM MgATP, which might best reflect the in vivo conditions in glial cells (*33*), and at pH 8.0. The pure recombinant mSR was stable following at least four freeze−

Table 1: Kinetic Characterization of Recombinant mSR[a]

reaction	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1}·mM^{-1})
L-Ser → D-Ser	3.8 ± 0.1	45.5 ± 0.5	12.0 ± 0.4
D-Ser → L-Ser	14.5 ± 1.1	113 ± 3	7.8 ± 0.6
L-Ser → pyruvate	4.0 ± 0.5	81.3 ± 2.8	20.3 ± 2.6
D-Ser → pyruvate	3.2 ± 0.3	8.8 ± 0.2	2.7 ± 0.4
L-SOS → pyruvate	0.49 ± 0.05	967 ± 17	1973 ± 205

[a] Measurements were carried out at pH 8.0, 10 μM PLP, 5 mM DTT, and 1 mM MgATP at 37 °C as described under Materials and Methods. The kinetic parameters of known mSR substrates undergoing racemization or β-elimination were determined by nonlinear regression fitting of initial reaction rates into the Michaelis−Menten equation. Values are represented as the mean ± SEM.

thaw cycles, and it preserved full activity during 5 h incubation in the assay buffer at 37 °C (data not shown). On the basis of these observations and the application of a bioaffinity purification step, we assumed that the purified mSR preparation contained only active species, and we considered its analytical concentration as determined by amino acid analysis identical to the concentration of active mSR needed for k_{cat} calculation. A representative Michaelis−Menten kinetics hyperbolic plot showing dependence of the initial reaction rates of L-Ser racemization on the initial L-Ser concentration is depicted in Figure 1C. Likewise, we have determined the steady-state kinetic parameters for racemization and β-elimination of L-Ser, D-Ser, and L-Ser-*O*-sulfate (Table 1). Racemization was assayed by chemical modification of the reaction mixture by *N*-α-(2,4-dinitro-5-fluorophenyl)-L-alanine amide (Marfey's reagent) and HPLC separation of the resulting diastereoisomers. Pyruvate concentration was determined either by spectrophotometric assay of NADH depletion accompanying the reduction of pyruvate to lactate by lactate dehydrogenase (LDH) and NADH (L-Ser-*O*-sulfate, L-Ser) or by dinitrophenylhydrazine derivatization and HPLC analysis (D-Ser, L-Ser).

It is evident that L-Ser displays nearly equal K_M values for both racemization and β-elimination. In contrast, the K_M values for D-Ser racemization and β-elimination are significantly different, and the k_{cat} of D-Ser β-elimination is an order of magnitude lower than that of its racemization. In particular, the K_M value of D-Ser β-elimination is comparable to the K_M values for L-Ser racemization and β-elimination, while the K_M value for D-Ser racemization is about four times higher. While L-Ser-*O*-sulfate is effectively β-eliminated by mSR, its racemization was not observed. The substrate selectivity constant (k_{cat}/K_M) for L-Ser-*O*-sulfate β-elimination is about 2 orders of magnitude higher than those of any reaction involving serine, and the apparent affinity of L-Ser-*O*-sulfate for the enzyme (K_M) is 1 order of magnitude higher than that of serine. Therefore, we asked which substrate features are responsible for its high affinity for the enzyme active site, which substrate features determine the reaction specificity of mSR, and whether the racemization and β-elimination activites reside in a single active site or separate active sites within mSR oligomers.

Specificity Mapping of Active Site Ligand Binding to mSR. To analyze the substrate and reaction specificity of serine racemase, we studied a panel of systematically modified compounds derived from the two known substrates, L-Ser and L-Ser-*O*-sulfate, and quantified their ability to interfere with L-Ser racemization. The structures of selected compounds were grouped into three subsets. Group I compounds can be described as L-Ser derivatives in which individual functional groups such as −OH, $-CH_2-OH$, −COOH, and $-NH_2$ were removed or replaced to yield different derivatives. Likewise, group II compounds represent L-Ser-*O*-sulfate derivatives with the $-OSO_3H$ group replaced by its structural analogues. Finally, group III compounds represent structural and configurational variations of the group II skeleton devoid of the α-amino group.

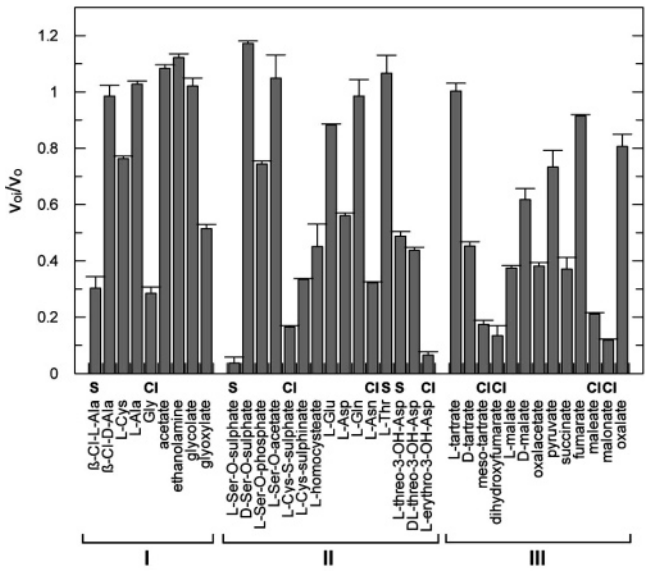


FIGURE 2: Mapping the structural requirements for compound interaction with mSR. Influence of selected compounds on mSR-catalyzed L-Ser racemization. Both L-Ser and the tested compounds were 5 mM. Group I is comprised of serine derivatives, group II includes derivatives and analogues of L-Ser-*O*-sulfate, and group III represents variations of the group II skeleton devoid of the α-amino group. The compounds found to act as mSR substrates or competitive inhibitors are denoted S and CI, respectively.

All compounds were first prescreened for inhibition of L-Ser racemization, as we assumed that those interacting with the active site of mSR, whether inhibitors or substrates, will inhibit L-Ser racemization in proportion to the magnitude of their binding affinity. The tested compounds and L-Ser were equimolar at 5 mM, which is slightly higher than the K_M of L-Ser (3.8 mM in the conditions used, Table 1). In this setup, the assay is sensitive to inhibitors with an affinity comparable to or better than that of L-Ser. The primary results of the ligand prescreening are summarized in Figure 2.

A few compounds (acetate, ethanolamine, and D-Ser-*O*-phosphate) activated mSR by 10−15%, but this effect was considered weak and was not analyzed further. The compounds that inhibited L-Ser racemization by a factor of 3 or more relative to the control, which would correspond to a K_I of ∼1−2 mM in the case of competitive inhibition (as estimated from the experimental setup, kinetic parameters of L-Ser racemization, and a competitive inhibition model), were further analyzed for their inhibition mechanism and inhibitory constants.

Using Lineweaver−Burk analysis, glycine (Figure 3A), L-Cys-*S*-sulfate, L-asparagine, L-*erythro*-3-hydroxyaspartate (Figure 3A), *meso*-tartrate, dihydroxyfumarate, maleate, and malonate were found to be competitive inhibitors of L-Ser racemization (denoted CI in Figure 2), and their K_I values are summarized in Table 2.

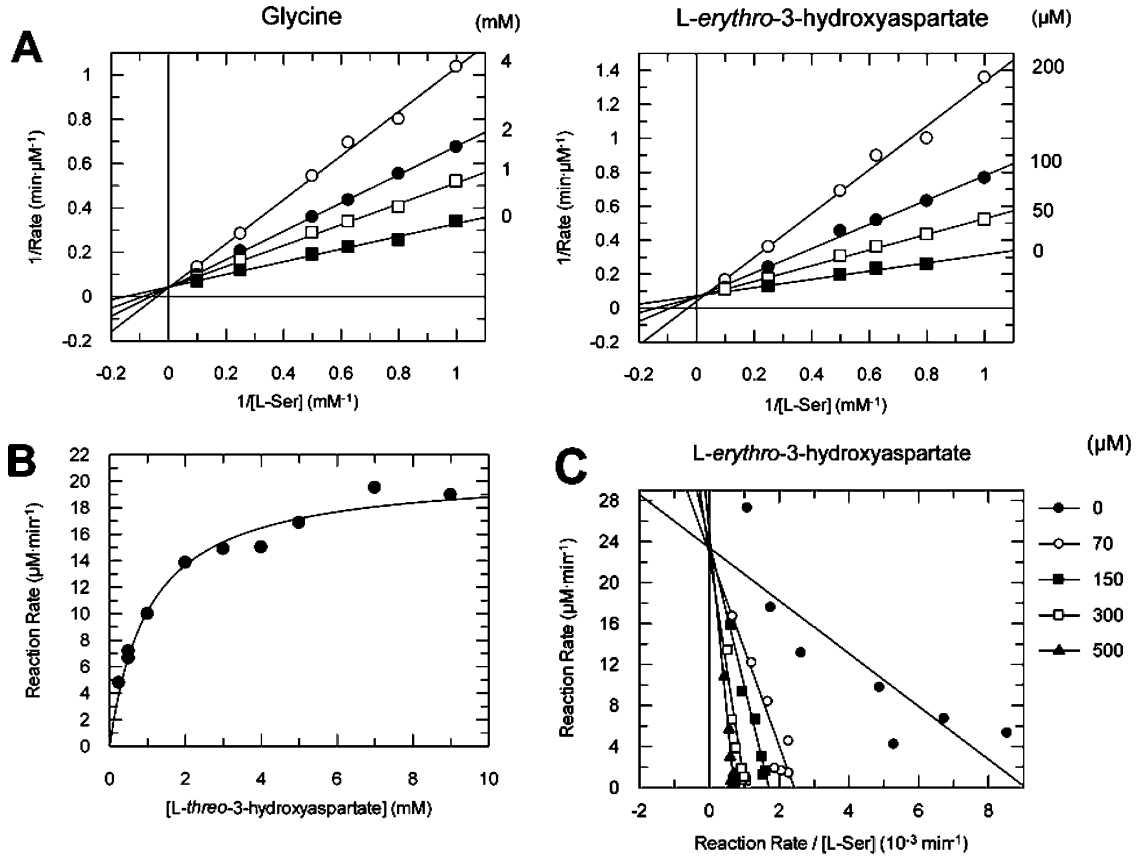


FIGURE 3: Compounds inhibiting L-Ser racemization by a factor of 3 or more relative to the control were analyzed by for their mode of inhibition and their inhibitory constants. (A) The Lineweaver−Burk plot for glycine and L-*erythro*-3-hydroxyaspartate is shown as an example. (B) The steady-state kinetic parameters of the compounds acting as mSR substrates were determined by nonlinear regression from hyperbolic plots. A representative plot for L-*threo*-3-hydroxyaspartate is shown. Kinetic constants are summarized in Table 3. (C) L-Ser racemization and elimination occur at the same active site. The Eadie−Hofstee plot shows that L-*erythro*-3-hydroxyaspartate is a competitive inhibitor of L-Ser elimination. Measurements were carried out at pH 8.0, 10 μM PLP, 1 mM MgATP, and 5 mM DTT.

Other inhibiting compounds shown in Figure 2 are expected to be weaker but competitive as well because they are all structurally similar to the competitive inhibitors listed in Table 2. Some of the interacting compounds shown in Figure 2 are in fact substrates of the enzyme. Their inhibitory activities are only indicative and cannot be directly compared since the compounds are being eliminated during the analysis and their concentration does not remain constant. The kinetic constants of those compounds are summarized in Table 3 (see below).

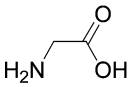
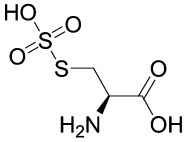
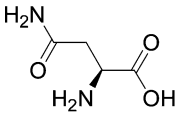
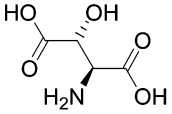
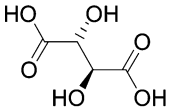
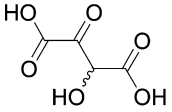
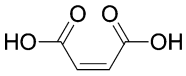
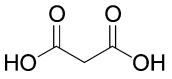
A Minimal Ligand: Glycine Is a Competitive Inhibitor of mSR. The analysis of L-Ser derivatives (group I compounds, Figure 2) shows that the replacement of the hydroxyl group from the β-carbon of L-Ser by the electronegative −Cl atom preserves binding to mSR, but only in the case of the L-isomer. Its replacement by a less electronegative and less polar −SH group to yield L-Cys diminishes the affinity of the compound to mSR. L-Cys does not interact with mSR under these conditions. Furthermore, when the β-hydroxyl group of L-Ser is removed to yield L-Ala, the inhibition of mSR is virtually lost. However, removal of the β-carbon of the inert L-Ala results in competitively inhibiting Gly with a K_I value of 1.64 mM (Table 2). The removal of the α-amino group of glycine, its replacement by a hydroxyl, or removal of its carboxyl group prevents binding of the resulting acetate, glycolate, and ethanolamine, respectively, whereas replacement of the α-amino group by an oxo group, as in glyoxylate, preserves some binding. It is intriguing that the smallest and ubiquitous amino acid glycine binds mSR with such a relatively high affinity. Assuming that substrate binding to a PLP-dependent enzyme is faster than any subsequent catalytic steps (*34*), we can approximate the L-Ser substrate dissociation constant K_S by its Michaelis constant K_M (*35*, *36*). Under this assumption, glycine would have about 2.4-fold higher affinity for mSR than L-Ser.

L-erythro- and L-threo-3-Hydroxyaspartates: A High-Affinity mSR Inhibitor and an Efficient Substrate Differing in a Single Carbon Configuration. For group II, all substitutions to the terminal $-O-SO_3H$ group of L-Ser-*O*-sulfate decrease the inhibitory properties of the compounds (Figure 2), and the D-isomers of the studied L-Ser-*O*-sulfate analogues do not inhibit L-Ser racemization under these conditions at all. The cysteine analogues of L-Ser-*O*-sulfate, L-Cys-*S*-sulfate and its derivatives L-cysteinesulfinate and L-homocysteate, are stronger inhibitors of mSR than those L-Ser-*O*-sulfate derivatives in which the whole terminal sulfo group is substituted by a phospho, acetyl, or a carboxymethyl group to yield L-Ser-*O*-phosphate, L-Ser-*O*-acetate, and L-Glu, respectively. Interestingly, L-Cys-*S*-sulfate is not a substrate for elimination by mSR.

Although L-Glu inhibits mSR weakly, its one methylene shorter analogues, L-Asp and L-Asn, are significantly stronger inhibitors.

The addition of a β-hydroxyl group to L-Asp (to partially mimic the natural substrate L-Ser) yields hydroxyaspartate, a molecule with two chiral centers and four diastereoisomers.

Table 2: Competitive Inhibition of mSR[a]

Compound		K_I (mM)
	glycine	1.64 ± 0.03
	L-Cys-S-sulfate	0.64 ± 0.14
	L-asparagine	1.13 ± 0.03
	L-*erythro*-3-hydroxyaspartate	**0.043 ± 0.007**
	*meso*-tartrate	0.66 ± 0.1
	dihydroxyfumarate	0.69 ± 0.05
	maleate	0.55 ± 0.12
	malonate	**0.071 ± 0.016**

[a] The compounds inhibiting L-Ser racemization by a factor of 3 or more relative to control (Figure 2) were subjected to Lineweaver–Burk plot analysis, and all were found to be competitive. Measurements were carried out at pH 8.0, 10 μM PLP, 5 mM DTT, and 1 mM MgATP at 37 °C. Inhibitory constants are given as the mean ± SEM.

While L-*threo*-3-hydroxyaspartate (2*S*,3*S*) inhibits L-Ser racemization moderately and D-*threo*-3-hydroxyaspartate (2*R*,3*R*) shows no significant synergistic effect in our assay (Figure 2), the L-*erythro* isomer of 3-hydroxyaspartate (2*S*,3*R*) is the strongest competitive inhibitor of L-Ser racemization among all analyzed compounds (K_I = 0.049 mM, Figure 3A and Table 2) and the strongest competitive inhibitor of mSR described so far. Its affinity to mSR is approximately 30 times higher than that of glycine and about 80 times higher than the affinity of the natural substrate L-Ser (compare Tables 2 and 1). The fact that L-*erythro*-3-hydroxyaspartate is a stereoisomer of an efficient β-elimination substrate and it is a competitive inhibitor of both L-Ser racemization and elimination (Figure 3C) suggests that the racemization and β-elimination occur at the same active site.

mSR Is Competitively Inhibited by Three- and Four-Carbon Dicarboxylates. To explore in detail the conformational requirements of a dicarboxylate ligand for binding to mSR, we analyzed a systematic series of dicarboxylic acids for their ability to inhibit mSR (Figure 2, group III). These compounds lack the α-amino group and as such are not substrates of mSR in racemization or β-elimination reactions. It seems that if hydroxyl substituents are present at carbons 2 and 3, the (*R*,*S*) isomer of the compound exhibits the highest affinity for mSR. This is documented by the three isomers of tartaric acid. While L-tartrate (*R*,*R*), which is isosteric with D-*threo*-3-hydroxyaspartate, virtually does not inhibit mSR, D-tartrate (*S*,*S*), an isostere of L-*threo*-3-hydroxyaspartate, is a medium-strength inhibitor in our assay (Figure 2) and *meso*-tartrate (*R*,*S*), which sterically corresponds to *erythro*-3-hydroxyaspartate, is the highest affinity inhibitor of the tartrate series (K_I = 0.66 mM; see Table 2). Dihydroxyfumarate, which exists in aqueous solution as 2(*RS*)-hydroxy-3-oxo-1,4-butanedioic acid and is stereochemically related to *meso*-tartrate, shows strong competitive inhibition (K_I = 0.69 mM). It must be mentioned that the replacement of the α-amino group in L-*erythro*-3-hydroxyaspartate (K_I = 0.049 mM) by an isosteric hydroxyl, as occurs in *meso*-tartrate, reduces the affinity for mSR by an order of magnitude (K_I = 0.66 mM). The presence of only a single hydroxy or amino substituent at carbon 2 does not seem to improve binding to the active site of mSR, since succinate inhibits mSR to the same extent as L-malate and oxalacetate and even more potently than D-malate, L-Asn, or L-Asp (Figure 2).

Next, we analyzed a series of conformationally restricted dicarboxylates. Interestingly, while fumarate (*trans*-butenedioic acid) is a very weak inihibitor of mSR, maleate (*cis*-butenedioic acid) is a strong competitive inhibitor (K_I = 0.55 mM) and malonate (propanedioic acid) has an even higher affinity than maleate (K_I = 0.071 mM). The fact that oxalate inhibits mSR even more weakly than fumarate demonstrates that although chelation of magnesium ions by dicarboxylates might contribute to mSR inhibition, it is definitely not the main factor, since oxalate, the strongest chelator (*36*), is the least efficient inhibitor of the series. We propose that the mutual spatial orientation of the two carboxyl groups in a dicarboxylate contributes more to mSR binding affinity than do the functional amino and hydroxy groups at the carbon backbone of the compound.

mSR β-Elimination Activity Is Specific to L-Amino Acids with an Electronegative β-Carbon Substituent. While several compounds from the analyzed set were found to be competitive inhibitors of L-Ser racemization, only the compounds having an α-amino group in the L-configuration and a suitable leaving group at their β-carbon could act as mSR substrates (Figure 2). Whereas β-Cl-L-Ala, L-Thr, and L-*threo*-3-hydroxyaspartate are efficiently β-eliminated to pyruvate, 2-oxobutyrate, and oxalacetate, respectively (see Table 3), only threonine undergoes epimerization (L-Thr to D-*allo*-Thr and D-Thr to L-*allo*-Thr). However, the initial rates of L-Thr racemization are less than 10% of those of its β-elimination, which prohibits accurate determination of steady-state kinetic parameters of the former process. The D-isomers of the studied amino acids were not converted to the corresponding 2-oxo acids at a measurable rate with the exception of D-Ser.

Comparison of the kinetic parameters for β-elimination of β-Cl-L-Ala, L-Thr, and L-*threo*-3-hydroxyaspartate (Table 3) with those of the L-Ser and L-Ser-*O*-sulfate β-elimination reactions (Table 1) suggests that the k_{cat} of the β-elimination reaction increases in proportion to the stability of the anion that is released from the β-carbon and the electrophilicity

Table 3: Kinetic Characterization of Newly Identified mSR β-Elimination Substrates[a]

reaction	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1}·mM^{-1})
β-Cl-L-Ala → pyruvate	1.6 ± 0.4	155 ± 10	97 ± 13
Lt3HA → oxalacetate	1.0 ± 0.1	1860 ± 60	1788 ± 214
L-Thr → 2-oxobutyrate	48 ± 5	627 ± 25	13.1 ± 1.5

[a] Measurements were carried out at pH 8.0, 10 μM PLP, 5 mM DTT, and 1 mM MgATP at 37 °C. The 2-oxo acid product formation was detected spectrophotometrically as the decrease in absorbance at 340 nm caused by LDH-catalyzed NADH oxidation upon the 2-oxo acid reduction into 2-hydroxy acid as described under Materials and Methods. The steady-state kinetic parameters were determined by nonlinear regression fitting of initial reaction rates into the Michaelis–Menten equation. Values are given as the mean ± SEM.

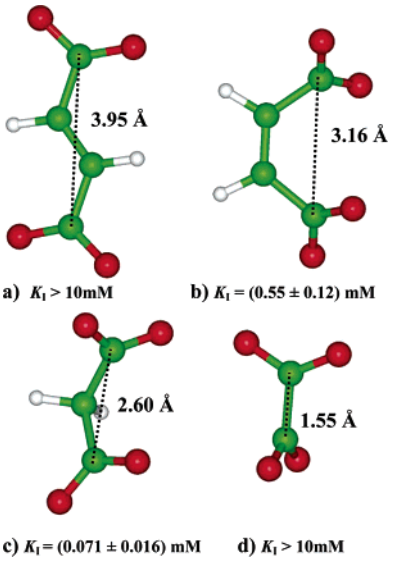


FIGURE 4: Equilibrium geometries of the conformationally restricted dicarboxylate inhibitors of mSR: (a) fumarate, (b) maleate, (c) malonate, and (d) oxalate. All distances are in angstroms. The corresponding K_I values are given.

of the β-carbon (k_{cat} for L-Ser < β-Cl-L-Ala < L-Ser-*O*-sulfate < L-*threo*-3-hydroxyaspartate). This correlation between k_{cat} values and β-carbon electrophilicity/leaving group stability might reflect the thermodynamics of the catalyzed chemical reaction. The specificity of the enzyme reaction is further influenced by the proper alignment of the substrate carbon backbone with the enzyme active site (compare k_{cat} for L-Ser < L-Thr < L-*threo*-3-hydroxyaspartate in Table 3). L-*threo*-3-Hydroxyaspartate conversion to oxalacetate has the highest k_{cat} of all substrates analyzed: about 23 times higher than that of L-Ser to pyruvate and two times higher than that of L-Ser-*O*-sulfate to pyruvate elimination. Interestingly, a simple switch of the β-hydroxyl configuration in L-3-hydroxyaspartate transforms the β-elimination substrate L-*threo*-3-hydroxyaspartate into L-*erythro*-3-hydroxyaspartate, the strongest competitive inhibitor described to date.

Qualitative Structural Characteristics of mSR Ligands. To explore the stereochemical relationships between the individual mSR ligands, we used quantum chemical calculations to obtain equilibrium geometries for the series of conformationally restricted dicarboxylates (Figure 4) as well as for mSR substrates L-serine, L-serine-*O*-sulfate, L-*threo*-3-hydroxyaspartate, and L-threonine and the inhibitor L-*erythro*-3-hydroxyaspartate (Figure 5). All of the reported optimized

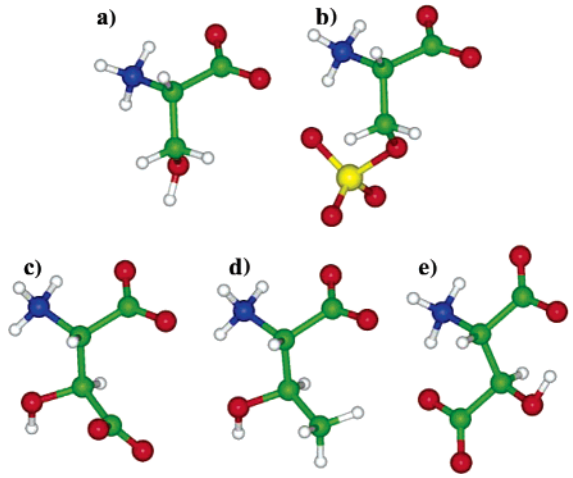


FIGURE 5: Equilibrium geometries of mSR amino acid substrates: (a) L-serine, (b) L-Ser-*O*-sulfate, (c) L-*threo*-3-hydroxyaspartate, (d) L-threonine, and inhibitor, (e) L-*erythro*-3-hydroxyaspartate.

structures correspond to the protonation states at pH 7, i.e., carboxylates deprotonated and amino group protonated (NH_3^+). The equilibrium geometries correspond to an aqueous environment, as they were optimized in the dielectric cavity with $\epsilon_r = 80$, which is a necessary prerequisite for obtaining the stable zwitterionic forms [such structures tend to neutralize in in vacuo calculations, forming (NH_2, COOH) tautomers]. Although the minimized structures reflect the lowest energy conformations of free compounds in water and do not take into account the interaction with the enzyme, some valid observations can be made.

From the structural analysis of the studied conformationally restricted dicarboxylates, it is evident that for efficient binding to mSR the optimal distance between carboxyl carbons is represented by malonate (2.60 Å) and maleate (3.16 Å) (compare Table 2 and Figure 4). Other dicarboxylates can adopt their conformation accordingly, e.g., succinate (compare Figure 2), but it will always be at certain energetic cost unless the molecule is inherently structurally constrained in a favorable way, as is the case of maleate and malonate.

A high degree of similarity between L-*threo*-3-hydroxyaspartate (2*S*,3*S*) and L-threonine (2*S*,3*R*) can be seen in the equilibrium conformations of mSR substrates (Figure 5). Except for the presence of a carboxylate group in L-*threo*-3-hydroxyaspartate, the structures are virtually identical, which can explain why they are both mSR substrates. However, the presence of the second carboxylate in L-*threo*-3-hydroxyaspartate (in place of a methyl group in L-Thr) probably ensures a higher affinity for mSR (compare K_M's in Table 2). Interestingly, the equilibrium conformation of the inhibitor L-*erythro*-3-hydroxyaspartate (2*S*,3*R*) significantly differs from that of its *threo* isomer. In the former, both of its carboxylates participate in two strong hydrogen bonds: one interacts with the OH group and the other with the NH_3^+ group. In contrast to L-*threo*-3-hydroxyaspartate (2*S*,3*S*), all three atoms (X–H···Y) in the two strong intramolecular hydrogen bonds of L-*erythro*-3-hydroxyaspartate (2*S*,3*R*) are in their optimal positions. As a consequence, it is thermodynamically more stable by 33 kJ/mol and has more restricted conformational flexibility (or higher rigidity) than the substrate, L-*threo*-3-hydroxyaspartate (2*S*,3*S*). The lower thermochemical stability of L-*threo*-3-hydroxyaspartate (2*S*,3*S*) can be considered as a favorable

factor for the β-elimination reaction. Since β-elimination of both isomers would result in optically inactive compounds, the excess internal energy of approximately 30 kJ/mol in favor of (2*S*,3*S*) isomers can be used as the driving force for the enzymatic reaction.

DISCUSSION

Recent findings suggest that mouse serine racemase is a bifunctional enzyme that produces both D-Ser and pyruvate from L-Ser in vivo, the latter activity being higher than the former (*13*, *14*, *16*, *33*). The biological significance of this phenomenon and its relevance for the physiological role of mSR are unclear. To obtain a better insight into mSR catalysis and its regulation, we generated recombinant mouse serine racemase of high purity and specific activity and investigated in detail its substrate and reaction specificity in vitro.

While the K_M value for L-Ser racemization of our recombinant mSR corresponds well to values obtained by other researchers using recombinant mSR from different sources, its k_{cat} value is about 20-fold higher than for purified recombinant mSR produced in bacteria or mammalian cells (*33*). This difference could be attributed partly to application of our newly developed ATP affinity chromatography that enriched the mSR preparation in the species of native fold and intact ATP binding site and partly to different assay conditions used in the study of Foltyn et al. (*33*), which was carried out in the absence of reducing agents and at neutral pH. In those conditions mSR is much less active than at pH 8.0 and in the presence of SH-reducing agents, the environment in which our measurements were conducted (*10*, *13*, *16*). Furthermore, since mSR activity is stimulated by ATP (*13*, *14*), we conducted all of our kinetic experiments in the presence of saturating concentrations of MgATP, as this may closely resemble physiological conditions (*33*).

The substrate and inhibitor specificity analysis in this report shows that glycine, which lacks any side chain, is a competitive inhibitor of mSR with approximately 2-fold higher affinity to mSR than the natural substrate L-serine (Tables 1 and 2). Furthermore, several dicarboxylates, which lack an α-amino group, are even stronger competitive inhibitors than glycine itself. Both findings are consistent with a recent report of Dunlop and Neidle (*38*), who found that glycine, L-aspartate, L-asparagine, and oxalacetate competitively inhibit L-Ser racemization by native mSR isolated from mouse brain. Our work extends these observations by showing that oxalacetate is the product of L-*threo*-3-hydroxyaspartate β-elimination and that three- to four-carbon dicarboxylates are moderate to strong mSR ligands, depending on the spatial distance of the two carboxyl groups and their conformational flexibility (Figure 2, Table 2). In previous work by others, the inhibition of mSR by glycine, L-aspartate, and asparagine was not observed, while strong inhibition by L-lysine and cysteine was reported (*15*, *17*). In contrast, we reproducibly observed only weak inhibition by L-cysteine (Figure 2) and no inhibition by L-lysine (not shown), which is, however, consistent with recent reports using native mSR isolated from brain (*13*, *38*). Glycine inhibition might be important for mSR activity regulation in vivo since glycine is found in astrocytes at relatively high concentrations of about 3−6 mM (*39*, *40*), which would be sufficient to significantly inhibit serine racemase activity. A possible solution to this apparent incongruity would be that serine racemase and glycine show different compartmentation within the astrocyte cytosol.

Our analyses suggest that the β-elimination activity of mSR is specific to L-amino acids containing an electronegative substituent at the β-carbon and that it is always higher than the racemization activity toward a given substrate. Whereas we observe efficient β-elimination of L-Ser, β-Cl-L-Ala, L-Thr, L-Ser-*O*-sulfate, and L-*threo*-3-hydroxyaspartate, the D-isomers of these amino acids were not β-eliminated with the exception of D-Ser. However, its β-elimination is 7.5-fold less efficient than that of L-Ser (k_{cat}/K_M, Table 1), and it is the only D-isomer of the above-mentioned amino acids that shows comparable racemization and β-elimination rates. Furthermore, both racemization and β-elimination activities are competitively inhibited by L-*erythro*-3-hydroxyaspartate (Figure 3A,C). It is therefore conceivable that the low level of pyruvate formed from D-Ser might originate from β-elimination of L-Ser produced from D-Ser by its racemization. This model would still be consistent with the conclusion of Foltyn et al., who recently reported that serine racemase decreases D-Ser levels in vivo by D-Ser β-elimination to pyruvate (*33*).

There are several examples of pyridoxal 5′-phosphate (PLP) dependent enzymes that act on amino acids and display ambiguous reaction specificities. In the PLP-dependent enzymes the common initial reaction intermediate, the external aldimine, may undergo isomerization and destabilization at different sites, which results in different reaction specificities (*34*, *41*). For example, α-amino acid decarboxylases can catalyze transaminations (*42*), aspartate aminotransferase racemization (*43*), tryptophan synthase has been shown to catalyze racemization and transamination (*44*), and glycine hydroxymethyltransferase catalyzes α-decarboxylation (*45*), transamination, and racemization (*46*) as side reactions. However, the side reactions of PLP-dependent enzymes are typically about 1−2 or more orders of magnitude slower than the "main" reaction. This is not the case with mSR, with which the β-elimination activity toward all known substrates is either comparable to (L-Ser) or higher than the racemization activity. The high β-elimination activity of mSR corresponds to its significant sequence similarity to serine/threonine dehydratases and less significant similarity to other racemases (*12*). Proteins showing sequence similarity to mSR also occur in organisms that do not even have NMDA receptors such as *Saccharomyces cerevisiae* or *Arabidopsis thaliana*. Interestingly, the mSR homologue from *S. cerevisiae* was shown to have an L-*threo*-3-hydroxyaspartate dehydratase activity but failed to racemize serine (*47*).

It is not clear what the physiological significance of mSR β-elimination activity is. It is likely of no importance for energy metabolism, as glycolysis produces approximately 1000 times more pyruvate than could be produced by mSR [based on the observed turnover of D-Ser in brain (*38*)]. It has been recently suggested that the β-eliminase activity of mSR could regulate intracellular D-Ser levels in vivo by balancing the rates of its synthesis from L-Ser and degradation into pyruvate (*33*). Considering that D-Ser β-elimination activity of mSR is about 1 order of magnitude lower than L-Ser β-elimination activity in our experiments, appreciable D-Ser degradation, be it through direct β-elimination of D-Ser

or through its racemization and subsequent β-elimination of L-Ser (the racemization product), could only be observed if the D-Ser concentration locally approaches or exceeds that of L-Ser. This model again implies the necessity of a particular compartmentation of mSR within the cytosol. It is intriguing that mSR is expressed also in liver (*12*), where no D-Ser is needed for neurotransmission and where high levels of D-amino acid oxidase (DAAO) activity are present (*48*). Conceivably, the role of the mSR in liver might be degradatory, relying on its β-elimination activity of D-Ser or other amino acids related to L-*threo*-3-hydroxyaspartate.

The role of hydroxyaspartates in vivo remains unknown. There are reports based on amino acid analysis and thin-layer chromatography that describe high concentrations of hydroxyaspartate in the cerebrospinal fluid of human patients, but the exact stereochemistry of the compound was not determined (*49*, *50*). An enzymatically active isolate from rabbit skeletal muscle that catalyzes the synthesis of L-*erythro*-3-hydroxyaspartate from dihydroxyfumarate and L-glutamate (*51*) has been reported. L-*threo*-3-Hydroxyaspartate has been used in neurobiology as a competitive inhibitor of L-glutamate high-affinity transporters (*52*). When exogenously added to neocortical minislices, it can accumulate within the L-Asp and L-Glu secretion pool and be secreted in a potassium- and calcium-dependent manner (*53*). It has been observed that in vivo a local administration of L-*threo*-3-hydroxyaspartate together with malonate induces rigidity in rats and damages neurons in some parts of the brain (*54*). Exogenously added L-*threo*-3-hydroxyaspartate apparently inhibits the inward transport of L-Glu from extracellular space and thus causes glutamate excitotoxicity and subsequent damage to adjacent neurons. We cannot exclude the presence of endogenous L-*threo*-3-hydroxyaspartate in vivo. If that was the case, it would be attractive to speculate that glutamate signaling in the CNS might be modulated by this endogenous glutamate transport inhibitor. Serine racemase would then have a dual role in this scheme: producing a coactivator of NMDA receptors, D-serine, and downregulating the levels of L-*threo*-3-hydroxyaspartate, an inhibitor of glutamate retrotransport, to prevent glutamate excitotoxicity.

Our inhibitor analysis reveals that the most important structural feature for binding to mSR is the presence of two adjacent carboxylates with optimal C−C distances of 2.6−3.2 Å. The presence of an α-amino group and a β-hydroxyl or another suitable leaving group at the β-carbon further increases the affinity of the ligand for mSR and is necessary to make a compound a serine racemase substrate. Interestingly, inversion of configuration at the β-carbon transforms the most efficient β-elimination substrate, L-*threo*-3-hydroxyaspartate (Table 3), into the most potent competitive inhibitor of SR, the L-*erythro*-3-hydroxyaspartate (Table 2). It has to be pointed out that malonate is almost as potent an inhibitor as L-*erythro*-3-hydroxyaspartate even though it does not have an α-amino group.

These structural considerations are only qualitative, and the interactions of the compounds with the enzyme may thermodynamically compensate for the internal stabilization effects predicted by quantum chemical calculations. We propose that binding to mSR constrains the position of the two carboxylate groups of a hydroxyaspartate diastereoisomer in a close proximity (as seen in maleate and malonate) and the α-amino group becomes fixed in the external aldimine formed with PLP. As a consequence, the L-*threo* and L-*erythro* isomers of 3-hydroxyaspartate have their β-hydroxyl pointing in different directions when bound to the enzyme, and only the L-*threo*-3-hydroxyaspartate β-hydroxyl is suitably juxtapositioned to an active site residue to enable catalysis. This model could have significant implications for prospective mSR inhibitor design.

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