Serine Racemase Modulates Intracellular D-Serine Levels through an α,β-Elimination Activity*

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Mammalian brain contains high levels of D-serine, an endogenous co-agonist of N-methyl D-aspartate type of glutamate receptors. D-Serine is synthesized by serine racemase, a brain enriched enzyme converting L- to D-serine. Degradation of D-serine is achieved by D-amino acid oxidase, but this enzyme is not present in forebrain areas that are highly enriched in D-serine. We now report that serine racemase catalyzes the degradation of cellular D-serine itself, through the α,β-elimination of water. The enzyme also catalyzes water α,β-elimination with L-serine and L- threonine. α,β-Elimination with these substrates is observed both in vitro and in vivo. To investigate further the role of α,β-elimination in regulating cellular D-serine, we generated a serine racemase mutant displaying selective impairment of α,β-elimination activity (Q155D). Levels of D-serine synthesized by the Q155D mutant are several-fold higher than the wild-type both in vitro and in vivo. This suggests that the α,β-elimination reaction limits the achievable D-serine concentration in vivo. Additional mutants in vicinal residues (H152S, P153S, and N154F) similarly altered the partition between the α,β-elimination and racemization reactions. α,β-Elimination also competes with the reverse serine racemase reaction in vivo. Although the formation of L- from D-serine is readily detected in Q155D mutant-expressing cells incubated with physiological D-serine concentrations, reversal with wild-type serine racemase-expressing cells required much higher D-serine concentration. We propose that α,β-elimination provides a novel mechanism for regulating intracellular D-serine levels, especially in brain areas that do not possess D-amino acid oxidase activity. Extracellular D-serine is more stable toward α,β-elimination, likely due to physical separation from serine racemase and its elimination activity.

D-Serine is a D-amino acid that occurs at high levels in the mammalian brain and is an endogenous ligand of the “glycine site” of N-methyl D-aspartate (NMDA) receptors (1–4). NMDA receptors play key roles in excitatory synaptic transmission, plasticity, and learning and memory (5). Overactivation of the NMDA receptor and the resultant influx of calcium into cells is a major culprit in the cell death that occurs following stroke and neurodegenerative diseases. Blockers of the “glycine site” of the receptor are neuroprotective in animal models of stroke (5).

Endogenous D-serine is required for NMDA receptor activation, and its removal markedly decreases NMDA receptor activity (3). In the vertebrate retina, endogenous D-serine may also mediate the light-dependent increase in neuronal activity by activating NMDA receptors (6). More recently, D-serine was suggested to play a role in the long term potentiation of synaptic transmission in the hippocampus, indicating a role of endogenous D-serine in long term synaptic plasticity (7).

D-Serine is synthesized by serine racemase, a pyridoxal phosphate (PLP)-dependent enzyme enriched in the mammalian brain (8, 9). Serine racemase has high sequence homology with the fold-type II group of PLP enzymes, such as serine/threonine dehydratase and D-serine dehydratase (10, 11). In addition to converting L- to D-serine, serine racemase catalyzes the α,β-elimination of water from L-serine to form pyruvate and ammonia (12). The initial rates of racemization and α,β-elimination of L-serine by serine racemase are strongly stimulated by magnesium and ATP, indicating that the complex Mg-ATP is a physiological ligand of the enzyme (12).

In accordance with accepted mechanisms of PLP-catalyzed reactions (13–16), a mechanism for racemization and α,β-elimination catalyzed by serine racemase is depicted in Scheme 1. PLP, bound to the enzyme through an internal aldime with Lys56, reacts with L-serine to give an external aldime intermediate. Subsequent α-proton abstraction forms a resonance stabilized carbanion. Reprotonation of this intermediate on the opposite face of the planar carbanion leads to the formation of the α,β-elimination reaction. Elimination of the β-hydroxyl group from the carbanionic intermediate leads to the formation of the aminoaeryl-PLP intermediate. Subsequent transamination releases the initial aminoaeryl product and regenerates free enzyme. The aminoaeryl released undergoes rapid non-enzymatic hydrolysis to give pyruvate and ammonia.

The termination of signaling by a neurotransmitter in the brain normally requires its re-uptake and metabolism. D-Serine signaling is thought to involve its release from cells to

SR, serine racemase; DMEM, Dulbecco’s modified Eagle’s medium; CMV, cytomegalovirus; GFP, green fluorescent protein; BME, basal medium Eagle.
stimulate NMDA receptors in neurons, but its re-uptake and degradation are not well understood. D-Amino acid oxidase is the only mammalian enzyme known to degrade D-serine. It is constructed by replacing Lys56 with glycine (9) or green fluorescent protein (GFP) cloned into pRK5-ks using Polyfect reagent (Qiagen). Cells were used 24–48 h after transfection.

Assay of Serine and Threonine in Cells—HEK 293-transfected cells were incubated in 6-well plates with 1 ml of DMEM medium. To measure L-and D-amino acid levels, a 0.1-ml aliquot of culture medium was removed, and the samples were processed for HPLC as described previously (18). Briefly, samples were first deproteinized by addition of trichloroacetic acid at 5% final concentration. The suspension was centrifuged at 20,000 × g for 5 min, and the supernatant was analyzed by HPLC after removal of trichloroacetic acid by four extractions with water-saturated diethyl ether. To calculate specific synthesis of D-serine, the values of contaminating D-serine in DMEM (about 6 μM contaminating D-serine) were subtracted from the values obtained after addition of 10 μM L-serine. For intracellular amino acid determination, cells were washed twice with cold PBS, followed by addition of 5% trichloroacetic acid to extract free amino acids. The suspension was centrifuged at 20,000 × g for 5 min, and the supernatant was analyzed after removal of trichloroacetic acid by three extractions with water-saturated diethyl ether.

Assay of Keto Acids—To monitor pyruvate and 2-ketobutyrate, generated from L-serine and L-threonine α,β-elimination, respectively, a 0.1-ml aliquot of culture medium was removed and boiled for 5 min to inactivate endogenous lactate dehydrogenase activity. The samples were centrifuged at 20,000 × g for 10 min to remove precipitated protein, and the supernatant was analyzed by monitoring the decrease in NADH (0.2 mM) absorbance at 340 nm, because the keto acids are reduced by added lactate dehydrogenase (1 μg/ml) (19).

Recombinant Serine Racemase—Mouse full-length serine racemase DNA was subcloned into pET 28a+, which encodes a 6×-histidine tag and introduced into BL21 codon plus bacteria (Strategene). Expression of serine racemase was induced by 0.5 mM isopyrrol 1-thio-β-D-galactopyranoside (Sigma) for 12 h at 30 °C. Cells were collected by centrifugation and disrupted by sonication in medium containing 20 mM Tris-HCl (pH 7.4), 15 μM PLP, 10 mM imidazole, and 400 mM NaCl. After addition of 1% Triton X-100, the suspension was cleared by centrifugation (40,000 × g for 15 min), and serine racemase was purified from the supernatant by binding to Talon resin (Clontech) or nickel-nitriolactiaceic acid-agarose (Qiagen) according to the manufacturers’ instructions. About 1–2 mg of protein was obtained for every liter of bacterial culture. The activity could be easily checked by adding 20 μl of enzyme bound to the resin to the assay medium. The enzyme bound to beads was active, and it displayed the same kinetic characteristics of the purified enzyme when eluted with imidazole and subjected to dialysis. In some experiments, recombinant serine racemase was purified from mammalian cells as described before (20), with identical results. Recombinant enzyme was of high purity as revealed by SDS-PAGE (data not shown).

Site-directed Mutagenesis—Point mutants of serine racemase were obtained by PCR with Pfu turbo polymerase (Stratagene) using complementary primers containing the desired base changes. After 22 cycles, the PCR product was digested with DPN I and transformed into XL1-Blue Escherichia coli. The mutants were verified by double-strand DNA sequence.

In Vitro Activity Assays—Reaction media contained 40 mM Tris-HCl (pH 7.4), 15 μM PLP, and the different test substrates in a final reaction volume of 50–70 μl. The reaction was started by addition of recombinant SR (0.02–0.05 mg/ml final concentration) and stopped after 1–2 h
Serine Racemase Modulates Intracellular D-Serine

RESULTS

Serine racemase has been shown previously to possess two distinct catalytic activities: (i) the synthesis of D-serine and (ii) the α,β-elimination of water from L-serine to give pyruvate and ammonia (12, 26) (Scheme 1). We now demonstrate that serine racemase utilizes other substrates for the α,β-elimination activity.

We examined cells transfected with serine racemase for utilization of L-threonine added to the culture medium. Transfection of serine racemase is associated with a large decrease in medium L-threonine when compared with control cells transfected with green fluorescent protein (GFP) or with a catalytically inactive mutant of the enzyme (K56G) (Fig. 1). The decrease in L-threonine levels is comparable to that observed with L-serine (Fig. 1, A and B). L-Threonine disappearance is accompanied by increases in keto acid concentration in the culture medium (Fig. 1, C and D). In contrast to the production of D-serine by racemization of L-serine, D-serine synthesis from L-threonine is not detectable, either in intact cells or with recombinant enzyme (Fig. 1, E and F, and data not shown).

Analysis of intracellular levels of L-threonine confirmed that the amino acid is a serine racemase substrate. A specific decrease in L-threonine is observed both at low (0.6 mM) and high (7 mM) concentrations of L-threonine in the medium (Fig. 1G). Even in the presence of a 10-fold higher L-serine concentration in the medium (7 mM L-serine), a decrease in cellular L-threonine is observed (Fig. 1G).

To verify the characteristics of L-threonine α,β-elimination, we checked its properties in vitro. Unlike other PLP-dependent enzymes, serine racemase cannot use L-threonine as a substrate. When we added L-threonine to HEK 293 cells expressing serine racemase, we did not observe any decrease in intracellular L-threonine levels (Fig. 2A). These results suggest that the enzyme selectively eliminates L-threonine when it is present at high concentrations in the medium.

Immunocytochemistry for D-Serine—Intracellular D-serine levels were revealed with an antibody against glutaraldehyde-conjugated D-serine (Mobitec, Germany). Briefly, cells were fixed with 0.5% sodium borohydride, cells were blocked and permeabilized with 5% normal goat serum, 0.2% Triton X-100 in PBS. The staining was revealed by ABC Elite kit (Vector laboratories) using D-amino acid oxidase as peroxidase substrate.

Primary Astrocyte and Slice Cultures—Primary astrocyte cultures were prepared from cerebral cortex of P0–P2 Sprague-Dawley rats as described (23). Cells were cultured in 6-well plates in BME plus 10% fetal bovine serum for 2 weeks before use. Synthesis of D-serine from the cultures was monitored by HPLC as described (12). Cortico-striatal organotypic slices were cut using a McIlwain tissue chopper and cultured in Millicell inserts (Millipore), as described (24). The slices were cultured in minimum essential medium-Hepes medium supplemented with 20% horse serum for 7–12 days prior to determination of D-serine.

Lentivirus Production—Mouse full-length serine racemase gene was subcloned into pTRK108 lentiviral vector (a gift from Dr. T. Kafri, University of North Carolina, Chapel Hill, NC) containing CMV promoter. The virus was produced by calcium-mediated co-transfection of the lentiviral vector (5 μg), packaging vector pCMV-dR8.74 (3 μg), and vesicular stomatitis virus coat envelope pMD2G (2 μg) (a gift from Prof. D. Trono, University of Geneva) in HEK 293 cells grown in 10-mm diameter dishes (25). Control virus consisted of green fluorescent protein (GFP) under the control of CMV promoter. Viral stocks were produced by concentrating the viral particles present in the culture medium by centrifugation at 120,000 g for 2 h. The pellet was suspended in a small volume of BME culture medium and stored at −70 °C until use.

To infect primary astrocyte cultures, viral stocks were added to cultured cells grown in 24-well plates, and the transfection efficiency was determined after 4 days with fluorescent microscopy (for GFP) or immunocytochemistry (for serine racemase). Preparations exhibiting virtually 100% transfection efficiency were employed for experiments of D-serine metabolism.

FIG. 1. α,β-Elimination of L-threonine and L-serine in intact cells. HEK 293 cells were transfected with green fluorescent protein (GFP), an inactive mutant of SR (K56G), or mouse serine racemase (WT). A and B, levels of L-threonine (A) or L-serine (B) in media of SR-WT transfected cultures. Amino acid levels were determined 24 h after addition of 7 mM L-threonine (A) or L-serine (B) to media. C and D, keto acid produced in culture media was analyzed 24 h after addition of either 7 mM L-threonine (C) or L-serine (D), E and F, synthesis of D-amino acids in SR-transfected cells. No D-threonine synthesis was detected when media contained 7 mM L-threonine (E). Supplementation of 7 mM L-serine in culture media elicited D-serine synthesis (F). G, depletion of intracellular levels of L-threonine in SR-WT transfected cells but not in K56G mutant-transfected cells. Media contained 0.6 mM L-threonine, 7 mM L-threonine, or 7 mM L-serine. The results represent the average ± S.E. of three independent transfection experiments. * different from control at p < 0.050 (Student’s t test).
Serine Racemase Modulates Intracellular D-Serine

Fig. 2. Kinetic characteristics of L-threonine and L-serine α,β-elimination. Reaction medium contained 40 mM Tris-HCl (pH 7.4), 15 μM PLP, and 10 mM of either L-serine or L-threonine and different concentrations of cofactors. A, free Mg²⁺ stimulated L-serine, but not L-threonine α,β-elimination. B, Mg-ATP dependence for L-serine and L-threonine α,β-elimination. Free Mg²⁺ concentration was fixed at 100 μM by using a Mg-EDTA buffer as described under “Experimental Procedures.” C, pH dependence of L-threonine α,β-elimination. The results of A and B represent the average ± S.E. of three to four replicate experiments, and C is a representative result of three experiments done with three different enzyme preparations.

enzymes, serine racemase activity displays a unique dependence on ATP, which acts as an activator (12). In the absence of ATP, no L-threonine α,β-elimination was observed (Fig. 2A). By contrast, partial activation of L-serine α,β-elimination was achieved by increasing free magnesium concentration alone (Fig. 2A). Mg-ATP greatly stimulated L-threonine α,β-elimination with generation of 2-oxobutyrate (Fig. 2B). The concentration of Mg-ATP required for half-maximal stimulation of L-threonine utilization was somewhat higher than that required for L-serine (Fig. 2B). Hydrolysis of ATP was not required for enzyme activation and non-hydrolyzable analogs of ATP stimulated threonine α,β-elimination as well (data not shown). L-Threonine utilization was optimal at alkaline pH (Fig. 2C). The Michaelis constant for L-threonine was in the millimolar range (Kₘ = 55 mM), higher than for L-serine (Kₘ = 10 mM), which suggests a lower affinity for the former (Table I). The efficiency of L-threonine α,β-elimination calculated by the kₗ/Kₘ ratio was 3.3 lower than that with L-serine as substrate (Table I). Ammonia generated from the aminoaclrylate intermediate, formed by the α,β-elimination of water from L-threonine, was also detected (data not shown). D-Threonine (up to 100 mM) was not used as substrate by serine racemase, indicating a higher specificity for the L-form.

The utilization of L-threonine by serine racemase indicates that the α,β-elimination activity displays a broader specificity than previously assumed. This prompted us to investigate α,β-elimination of additional substrates. Although considered a reaction product, we found that D-serine was strongly eliminated by serine racemase in an ATP-dependent manner, generating pyruvate and ammonia (Table I). The kₗ for D-serine α,β-elimination was about one-fourth of that observed with L-serine in the presence of ATP, compared with one-tenth of L-serine α,β-elimination in the absence of ATP. The Kₘ for D-serine in the α,β-elimination reaction was similar to that of L-serine, and the relative efficiency of D-serine α,β-elimination in the presence of ATP was 3.6 lower than that of L-serine α,β-elimination as estimated by the kₗ/Kₘ ratio (Table I).

The α,β-elimination of D-serine by serine racemase is in apparent contradiction with its role in D-serine biosynthesis. Therefore, we sought to investigate in more detail the kinetic characteristics of D-serine α,β-elimination both with recombinant enzyme and in intact cells.

Previous measurements of the initial rate of D-serine synthesis in vitro were carried out using a large excess of L-serine (10 mM (8, 12, 27)). This may have masked D-serine α,β-elimination. When present at physiological-like concentration (1 mM), L-serine was rapidly consumed by the combined racemization and α,β-elimination activities of serine racemase (3A). Under this condition, D-serine was unstable; its levels fell as the concentration of L-serine decreased, reflecting its α,β-elimination (Fig. 3B). Because EDTA impairs α,β-elimination (12), we examined its effects on the stability of D- and L-serine. Consistent with a previous report (12), the initial rate of D-serine synthesis in the presence of EDTA was much slower than with Mg²⁺ and ATP. In the presence of EDTA, however, synthesized D-serine was stable because of the lower α,β-elimination activity. As a result, D-serine reached higher values at longer reaction times when α,β-elimination was impaired by EDTA (Fig. 3B). A similar stabilization promoted by EDTA was observed for L-serine levels (Fig. 3A). Production of pyruvate inversely correlated with the decrease in L-serine levels (Fig. 3C).

Fig. 3B suggests that α,β-elimination of D-serine may limit its accumulation. Thus, we next examined if serine racemase is able to maintain the same ratio of L-to D-serine in vitro as that found in vivo (3:1 (18)). When 1 mM L-serine and 0.3 mM D-serine were present from the start of the reaction, levels of both L- and D-serine decreased over time in the presence of the cofactors Mg²⁺ and ATP (Fig. 3D). The degradation of D-serine observed in Fig. 3D by the racemase was not due to consumption of the precursor L-serine. Even when levels of L-serine were only reduced by 20–30%, a decrease in D-serine was observed (Fig. 3D). Addition of 1 mM L-serine did not significantly slow down D-serine α,β-elimination by serine racemase, whereas EDTA effectively blocked it (Fig. 3E). As expected, levels of pyruvate inversely correlated with the decrease in L- and D-serine levels (Fig. 3F).

The instability of D-serine in vitro prompted us to verify the extent to which D-serine α,β-elimination occurs in intact cells. Thus, intracellular levels of D-serine were monitored in cells cultured in media containing D-serine, but with reduced L-serine concentration to avoid D-serine synthesis. Transfection of wild-type serine racemase was associated with a drastic decrease in D-serine immunoreactivity when compared with the inactive mutant K566G (Fig. 4A). Any cross-reactivity with L-serine was blocked by preincubating the antibody with L-serine-glutaraldehyde conjugate. When the antibody was preabsorbed with excess D-serine-glutaraldehyde conjugate, no immunoreactivity was observed (Fig. 4A, inset). HPLC analysis confirmed the decrease in intracellular D-serine in wild-type
serine racemase-transfected cells, reflecting intracellular αβ-elimination of d-serine (Fig. 4B).

To verify whether d-serine αβ-elimination also occurs in glial cells, we infected primary astrocyte cultures with lentivirus harboring the gene of serine racemase or GFP control and checked the total d-serine in the culture. The rate of d-serine consumption was higher in serine racemase infected astrocytes when compared with GFP control, reflecting the αβ-elimination of d-serine by the enzyme (Fig. 4C).

To verify how the αβ-elimination affects d-serine synthesis by serine racemase, we generated a point mutant (Q155D) that displays impaired αβ-elimination activity against L-serine, D-serine, and L-threonine (Fig. 5 and Table I). Production of pyruvate from L-serine in vitro by the mutant Q155D was several times lower than the wild-type, with little change in the K_m (Table I and Fig. 5A). Analysis of d-serine and L-threonine αβ-elimination also indicated an impairment of αβ-elimination toward these substrates promoted by the Q155D mutation, as revealed by a large decrease in k_cat (Table I). The k_cat/K_m ratio of αβ-elimination of L-serine by the mutant Q155D in the presence of ATP was 4.6-fold lower than the wild-type (Table I).

Conversely, racemization activity of the mutant Q155D was increased several-fold when compared with wild-type enzyme, with no change in K_m (Fig. 5B and Table II). As a result, the ratio of αβ-elimination versus racemization with the Q155D mutant was 15 times lower than the wild-type enzyme (0.25 versus 3.7, Table III).

ATP was found to affect moderately the partition of αβ-elimination and racemization reactions of L-serine, favoring the former. Addition of ATP increased the k_cat/K_m of αβ-elimination by 4.5, whereas the k_cat/K_m of racemization was increased by 2-fold (compare Tables I and II). The activity of mutant Q155D was also strongly stimulated by ATP (Tables I and II). The concentration of ATP required for half-maximal stimulation of Q155D activity was unchanged when compared with wild type enzyme (data not shown).

The pH profile of αβ-elimination and racemization of L-serine were similar and not significantly altered by the mutation Q155D (Fig. 5, compare C and D). Both the wild-type enzyme and the Q155D mutant displayed optimal activities at alkaline pH. Stimulation of activity at alkaline pH values was more prominent in the absence of ATP, but similar for αβ-
Serine Racemase Modulates Intracellular d-Serine

elimination and racemization reactions. One exception was the α,β-elimination of L-serine with Q155D mutant in the absence of ATP, which was very low and did not increase at alkaline pH values. The results imply that the changes in α,β-elimination and racemization reactions by the mutation Q155D are not due to changes in pH profile of the enzyme but to changes in partitioning of the resonance-stabilized carbanion intermediate between reprotonation on Cα and elimination of the β-hydroxyl group (Scheme 1)

The Q155D mutation lies in a conserved region of the racemase with previously unassigned function (amino acids 152–165) that is predicted to be a flexible random coil loop by using secondary structure analysis software (Protean, DNASTar, Inc.). The closest homologue whose structure has been determined is the biosynthetic threonine dehydratase enzyme from E. coli (PDB entry 1T1D), to which serine racemase has significant homology at the amino acid level and for predicted secondary structure. The corresponding loop in threonine dehydratase (residues 154–159, Fig. 5E) connects the catalytic subdomains N1 and N2, lying within 10 Å from the pyridoxal 5'-phosphate (28). An autotrophic mutant at this loop (Pro156→Ser) exhibited a decrease in threonine dehydratase activity, although they render this region of serine racemase to be even more similar to rat serine dehydratase (Fig. 5E, SDHL). None of the mutations changed the Km for either α,β-elimination or racemization, nor did they change the concentration of ATP required for half-maximal stimulation of serine racemase activity (data not shown).

To verify the extent to which α,β-elimination affects d-serine synthesis in vivo, HEK 293 cells were transfected with the Q155D mutant. The mutation doubled the production of d-serine in cells (Fig. 6A). Like that observed in vitro, α,β-elimination of L-serine and L-threonine in cells was decreased to a large extent with the Q155D mutant (Fig. 6B and C). Western blot analysis demonstrated that the levels of expression of wild-type serine racemase and Q155D mutant were identical in the experiments (data not shown).

Because the mutant Q155D displayed approximately a 4-fold increase in the efficiency of conversion of d- into L-serine (k_cat/Km of 0.73 compared with 0.21 mM⁻¹ min⁻¹, Table II), we used it as a tool to evaluate the effects of α,β-elimination in the reversibility of the serine racemase catalytic cycle in intact
FIG. 5. Decreased α,β-elimination and increased racemization in vitro by Q155D serine racemase mutation. A and B, decrease in L-serine α,β-elimination (A) and stimulation of L-serine racemization (B) by Q155D mutation. Reaction medium contained 40 mM Tris-HCl (pH 7.4), 15 μM PLP, 0.1 mg/ml of recombinant serine racemase, 1 mM MgCl₂, 1 mM ATP, and different L-serine concentrations. C and D, pH profile of L-serine α,β-elimination (● and ○) and racemization (□ and ■) in the presence of 10 mM L-serine and either in the absence (□ and ■) or in the presence of 1 mM ATP (● and ○). C, wild-type serine racemase. D, Q155D mutant. Note that the scales of the left and right axes in C and D are different. E, alignment of amino acids 151–185 of mouse serine racemase (SRR) with serine/threonine dehydratase homologues in rat (SDH1) and biothetic (THD1) and catalytic (THD2) serine/threonine dehydratases from Saccharomyces cerevisiae, Salmonella typhimurium, and Haemophilus influenzae. Residues in bold represent the amino acids chosen for site-directed mutagenesis. Shaded areas correspond to homologous residues. The results are representative of replicate experiments carried out with three different enzyme preparations.

TABLE II

Kinetic parameters of racemization

The reaction was carried out as in Table I. Standard errors of three to six experiments are given in parenthesis.

|       | kcat | Km (µM) | kcat/Km | kcat/Km | kcat/Km |
|-------|------|---------|---------|---------|---------|
|       | ATP  | +ATP    | ATP  | +ATP    | ATP  | +ATP    |
|       | WT   | Q155D   | WT   | Q155D   | WT   | Q155D   |
| L-Serine | 1.2 (0.10) | 2.8 (0.27) | 2.5 (0.3) | 14 (2) | 9.0 (0.5) | 10 (2) | 9.0 (0.8) | 10.5 (1.7) | 0.13 | 0.28 | 0.27 | 1.33 |
| d-Serine | 0.22 (0.04) | 0.9 (0.2) | 1.7 (0.2) | 6.7 (0.8) | 8.0 (1.1) | 9.2 (0.9) | 8.2 (0.9) | 9.2 (0.7) | 0.028 | 0.1 | 0.21 | 0.73 |

TABLE III

α,β-Elimination/racemization ratio of different mutants

| Mutant | kcat/Km | kcat/Km | kcat/Km |
|--------|---------|---------|---------|
| WT     | 3.70 (0.6) | 3.70 (0.6) | 3.70 (0.6) |
| H152S  | 1.40 (0.1) | 1.40 (0.1) | 1.40 (0.1) |
| P153S  | 0.24 (0.05) | 0.24 (0.05) | 0.24 (0.05) |
| Q155D  | 0.25 (0.04) | 0.25 (0.04) | 0.25 (0.04) |
| N154F  | 0.33 (0.03) | 0.33 (0.03) | 0.33 (0.03) |

* kcat(Km)=kcat(Km) for α,β-elimination of L-serine and kcat(Km) = kcat(Km) for racemization of L-serine. Other conditions were as described in Table I. Standard errors are given in parenthesis.

Serine Racemase Modulates Intracellular d-Serine

Cells. Transfected cells were incubated with media containing 0.3 mM d-serine, but no added L-serine, and back synthesis of L-serine was monitored. Transfection of the wild-type enzyme promoted a decrease in L-serine to levels one-third of the control inactive enzyme (K56G) as well as a decrease in D-serine levels (Fig. 6, D and E). This indicated that in intact cells expressing wild-type serine racemase most of D-serine is eliminated instead of being converted to L-serine. Conversely, the α,β-elimination-deficient mutant Q155D displayed a robust increase in L-serine, both in the intracellular compartment (Fig. 6D) and in the cultured medium (Fig. 6E), caused by racemization of d-serine. Similar reversal with the wild-type enzyme was only observed when cells were incubated with supraphysiological values of d-serine (5 mM) (data not shown).

Because serine racemase continuously degrades d-serine, we wondered if there are cellular mechanisms in place to enhance the stability of d-serine and allow its accumulation in vivo. We found that a large fraction of d-serine produced by the cells was exported to the extracellular medium, in which d-serine may be more stable. In HEK293 cells transfected with serine racemase, about 96% of total synthesized d-serine was present in culture medium (Fig. 7A). Likewise, about 98% of synthesized d-serine was found in the medium of primary astrocyte cultures or in a more physiological preparation consisting of cultured cortico-striatal slices (Fig. 7A). This raised the possibility that compartmentalization of d-serine between the intra- and extra-
with very little remaining after 24 h (Fig. 7B). This was accom-
mpanied by a decrease in intracellular D-serine (0.3 mM) and L-threonine in the brain are about 1, 0.3, and 0.6 mM (18, 30), much below the \( K_m \) values for \( \alpha,\beta \)-elimination and racemization (Tables I and II). The 5- to 6-fold higher \( K_m \) of serine racemase for L-threonine versus D-serine (Table I) raises the doubt that threonine may not be a relevant substrate at physiological concentrations. However, due to its higher \( k_{cat} \) when compared with L-serine (Table I), the efficiency of L-threonine \( \alpha,\beta \)-elimination is in the same range as for D-serine \( \alpha,\beta \)-elimination, with \( k_{cat}/K_m \) about three times lower than for L-serine (Table I). This explains the significant L-threonine \( \alpha,\beta \)-elimination seen by transfecting serine racemase in HEK293 cells (Fig. 1). The amount of keto acid (2-oxobutyrate) accumulated in the culture medium was lower than the observed decrease in L-threonine levels. This may be explained by the cellular oxidation of 2-oxobutyrate through either the mitochondrial branched-chain oxoacid dehydrogenase complex or pyruvate dehydrogenase (31).

Brain D-serine exhibits a half-life of about 16 h (27), but its degradative pathway is not clear. The only enzyme known to degrade D-serine in mammals is D-amino acid oxidase, which occurs in the cerebellum and brainstem. This enzyme, however, occurs only at very low levels in the forebrain areas such as the cerebral cortex and hippocampus, where high levels of D-serine are quite constant from 3- to 86-week-old rats (32). Moreover, mutant mice possessing inactive D-amino acid oxidase enzyme exhibit large increases in D-serine in the cerebellum and brainstem, but do not display increased D-serine in forebrain areas (33). This strongly suggests that D-serine degradation is not mediated by D-amino acid oxidase in the forebrain.

The results presented here imply that the ability of serine racemase to eliminate D-serine may be important to limiting D-serine levels in brain areas where D-amino acid oxidase is poorly expressed. Fig. 8 summarizes the proposed model for removing and further degrading extracellular D-serine in the forebrain. Upon its synthesis and release from the cells, D-serine stimulates NMDA receptors at the co-agonist site. This process could be terminated by D-serine re-uptake into the cells. Our results suggest that serine racemase can degrade D-serine taken up into the cells through its \( \alpha,\beta \)-elimination activity, generating pyruvate and ammonia. Accordingly, primary astrocyte cultures overexpressing serine racemase by lentivirus infection have increased D-serine consumption by \( \alpha,\beta \)-elimination (Fig. 4C). This might constitute a mechanism for regulating intracellular D-serine concentration upon D-serine re-uptake (Fig. 8). One caveat of such a model is that the uptake is key as a mode of synaptic inactivation. Glial cells take up D-serine in vivo (34), and several transporter candidates have been identified for D-serine, both in astrocytes and in neurons. These include Na\(^{+}\)-dependent (23, 35) and Na\(^{+}\)-independent (36, 37) neutral amino acid transporters. However, these transporters mediate only slow or non-selective uptake of D-serine. Alternatively, part of D-serine can leave the brain by efflux transport across the blood-brain barrier and later be excreted in the urine (Fig. 8). This could account for the high levels of D-serine in the urine of rodents and humans (38), but the existence of such pathway remains to be determined.

The effects exerted by the mutations in the amino acids 152–155 of serine racemase (Table III) are related to changes in the partition ratios for \( \alpha,\beta \)-elimination versus racemization (Scheme I). The results implicate this region as a possible determinant of the reaction specificity of serine racemase. Conceivably, these mutations might alter the conformation of the enzyme and affect the microenvironment of the active site. We found that ATP also had moderate effects on the partitioning between \( \alpha,\beta \)-elimination and racemization of L-serine, favoring the former reaction. ATP also stimulates the efficiency of L-serine \( \alpha,\beta \)-elimination more than 10-fold (Table I). In vivo magnesium and ATP levels are in the millimolar range,

![Fig. 6. \( \alpha,\beta \)-Elimination limits cellular D-serine and reversal of the racemase.](http://example.org/fig6)

**Serine Racemase Modulates Intracellular D-Serine**

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**FIG. 6.** \( \alpha,\beta \)-Elimination limits cellular D-serine and reversal of the racemase. When indicated, HEK 293 cells were transfected with inactive mutant of serine racemase (K56G) wild-type (WT) or Q155D mutant. Amino acid levels were determined 48 h after addition of substrates to culture media, which consisted of BME plus 10% fetal bovine serum. A, stimulation of D-serine synthesis by Q155D mutation. D-serine produced was monitored after addition of 7 mM L-serine to culture media. B, Q155D mutant display decreased L-serine \( \alpha,\beta \)-elimination calculated by subtracting the amount of D-serine synthesized from the total decrease in medium L-serine. C, Q155D mutant display decreased L-threonine \( \alpha,\beta \)-elimination calculated by the decrease in total L-threonine (7 mM) added to culture media. D and E, reversal of serine racemase reaction with Q155D mutant. Culture media of HEK 293 transfected cells were supplemented with 300 \( \mu \)M D-serine and intracellular (D) or medium (E) L-serine was monitored by HPLC analysis. The results represent the average \( \pm \) S.E. of three independent transfection experiments.
whereas the $K_m$ of the serine racemase for Mg-ATP lies in the low micromolar range (12). This suggests that serine racemase activity in the presence of Mg-ATP better reflects the in vivo situation. Failure to include ATP in the enzyme assays explains previous failure or underestimation of the α,β-elimination activity catalyzed by serine racemase (8, 17).

As depicted in Fig. 8, reversal of serine racemase could play a role in degrading excess d-serine. The results with the α,β-elimination-deficient mutant, however, suggest that α,β-elimination effectively competes with the reversal of serine racemase in vivo at physiological d-serine concentrations. This is supported by the experiments in which wild-type enzyme failed to accumulate l-serine from d-serine in cells incubated with 0.3 mM d-serine, whereas robust generation of l-serine was observed with the α,β-elimination-deficient mutant (Fig. 6). Thus, degradation of d-serine does not occur by its racemization to l-serine. The reverse racemization reaction would be less effective than d-serine α,β-elimination in decreasing cellular d-serine.

Extracellular levels of d-serine in the brain are higher than many common amino acids (39). It has been shown that release of d-serine from astrocytes in vitro is elicited by stimulation of AMPA/kainate receptors or by small neutral amino acids (2, 23). Export of d-serine to the extracellular medium might be a mechanism to increase its stability by avoiding the intrinsic d-serine α,β-elimination by serine racemase. This would explain the accumulation of d-serine in the extracellular milieu, despite the serine racemase α,β-elimination reaction. Experiments measuring the l- and d-serine half-life using transfected HEK 293 cells confirmed the stability of extracellular versus intracellular d-serine (Fig. 7). This implies that changes in the degree of d-serine compartmentalization and in the gradient across the membrane (e.g. by increased or decreased cellular transport of d-serine) may affect d-serine metabolism. Thus, increased transport of d-serine into the cells should favor its degradation by the α,β-elimination activity of serine racemase.

Our results have implications for the design of enzyme inhibitors and activators. Serine racemase inhibitors will be useful for conditions in which overstimulation of NMDA receptors takes place, such as stroke and neurodegenerative diseases. Conceivably, inhibitors of both racemization and α,β-elimination activities of serine racemase will block d-serine synthesis, but removal of preformed d-serine will be slow due to the absence of d-amino acid oxidase activity in the forebrain. On the other hand, ligands that stimulate selectively d-serine α,β-elimination will be more effective in decreasing brain d-serine.

D-Serine administration has been shown to ameliorate the negative symptoms of schizophrenia (40). Direct d-serine administration may be problematic due to its accumulation in several tissues and nephrotoxic effects (41). Our results point to the feasibility of designing serine racemase ligands to stimulate l-serine racemization while impairing α,β-elimination. Such compounds will be useful in increasing brain d-serine specifically in the places where the racemase is normally expressed.

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Serine Racemase Modulates Intracellular D-Serine Levels through an $\alpha,\beta$-Elimination Activity

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