PH.D. DISSERTATION

Characterization of recombinant human serine racemase

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Contents

Brief Abstract

The pyridoxal-5'-phosphate-dependent enzyme serine racemase (SR) is responsible for the biosynthesis of D-serine in the mammalian central nervous system. D-serine acts as a neurotransmitter and coagonist, together with L-glutamate, of ionotropic *N*-methyl-D-aspartate receptors (NMDARs). Excitotoxic D-serine levels have been implicated in neuropathologies including Alzheimer's disease and amyotrophic lateral sclerosis. SR inhibitors offer a novel and potentially highly specific approach for attenuation of NMDAR-mediated glutamate excitotoxicity and for further study of the pathway. Many of the SR inhibitors described to date are small, naturally occurring compounds, and novel structures capable of influencing SR's activity are highly sought after. Moreover, structural information about this enigmatic enzyme is lacking, and suitable animal models need to be identified for inhibitor studies.

This thesis presents the first published biochemical comparison of mouse and human SR orthologs, validating, at least in part, the use of mouse models in SR research. Additionally, hydroxamic acids are introduced as a novel class of SR inhibitors. While the experimentally determined structure of a mammalian SR remains elusive, random and site-directed mutagenesis experiments in combination with multiple sequence alignment offer insight into structure-function relationships within the enzyme.

Abbreviations

Aβ	amyloid β-peptide
ALS	amyotrophic lateral sclerosis (Lou Gehrig's disease)
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
DAAO	D-amino acid oxidase
DPFC	dorsolateral prefrontal cortex
DS	D-serine
DTT	1,4-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tag
GB1	B1 domain of *Streptococcal* protein G
GSNO	*S*-nitroso-glutathione
GST	glutathione-*S*-transferase
GRIP	glutamate receptor interacting protein
hSR	human serine racemase
LEHA	L-*erythro*-3-hydroxyaspartate
LS	L-serine
LSOS	L-serine-*O*-sulfate
LTHA	L-*threo*-3-hydroxyaspartate
MBP	maltose binding protein
mSR	mouse serine racemase
NMDA	*N*-methyl-D-aspartate
NMDAR	*N*-methyl-D-aspartate receptor
PCA	polymerase cycling assembly
PICK1	protein interacting with kinase C
PLP	pyridoxal 5' phosphate
SR	serine racemase

Chapter 1: Introduction

1.1 A brief history of D-serine and its synthetic enzyme, serine racemase

One of the enigmas of the origin of life is why terrestrial life is based on certain chiral molecules rather than their enantiomers; that is, on L-amino acids, for example, rather than D-amino acids. [1]

L-Amino acids are indispensible for life as the building blocks for ribosomal proteosynthesis and as metabolic intermediates, while their enantiomers have been considered "unnatural" artifacts for most of the history of biochemistry and are seldom mentioned in textbooks. The occurrence of D-amino acids in bacteria, in which they are incorporated into cell wall peptidoglycans, was recognized early on, but until the mid-twentieth century, D-amino acids were not considered to play a role in higher organisms. In 1935, Sir Hans Krebs serendipitously isolated a novel enzyme from kidney. This enzyme was able to break down D-amino acids but not their L-counterparts, and Krebs termed it D-amino acid oxidase (DAAO) [2]. Its discovery was surprising and raised questions about how and why such an enzyme had evolved. One possibility, of course, was that DAAO evolved to metabolize D-amino acids ingested *via* the diet or from intestinal bacteria [3]. It was not until several decades later that modern HPLC techniques revealed significant levels of free, endogenous D-amino acids in mammals, specifically in the brain.

High levels of D-asparate [4], D-alanine [5], and D-serine [6] were identified in mammalian brain about two decades ago. D-Serine (DS) became the subject of considerable research interest when it was revealed to be involved in excitatory neurotransmission *via* interaction with *N*-methyl-D-aspartate receptors (NMDARs) (recently reviewed by [7] and [8]).

NMDARs are tetrameric ligand-gated ion channels formed by a combination of NR1 and NR2 subunits that play a key role in glutamatergic excitatory neurotransmission [9]. The NR2 subunit bears the glutamate binding site. Uniquely, in addition to glutamate, NMDARs require binding of a coagonist, originally thought to be glycine, to the NR1 subunit in order to mediate ion influx [10]. However, the idea that glycine acts as the major coagonist was challenged by findings that DS distribution mirrors NMDAR distribution [11, 12] while Gly distribution does not. Furthermore, DS binds to NMDARs with up to three-fold higher affinity than Gly [13, 14].

Depletion of DS by addition of DAAO [15] or D-serine deaminase [16, 17] results in markedly decreased NMDAR-mediated neurotransmission. Today, it is generally accepted that DS is the primary endogenous coagonist at the strychnine-insensitive "glycine" site of NMDARs, while glycine is an agonist of the inhibitory, strychnine-sensitive glycine receptors localized mainly in the spinal cord and brainstem. Recently, however, the hypothesis that glycine serves as an NR1 subunit agonist at extrasynaptic receptors while DS acts through synaptic NMDA receptors was introduced [18].

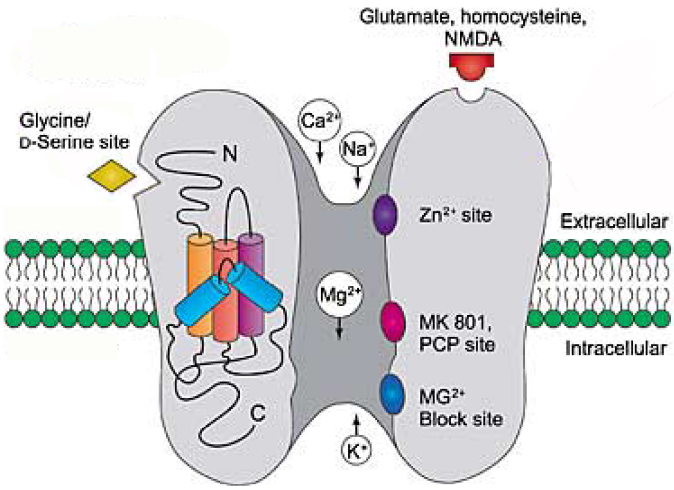


Figure 1. *Cartoon representation of the NMDAR cation channel, with regulatory sites indicated. This figure was adapted from the original shown in [19].*

But what is the source of brain DS? One could think of several metabolic paths, such as synthesis from β-hydroxypyruvate by a D-amino acid aminotransferase or formation from *O*-phosphoserine by an enzyme analogous to *O*-phosphoserine phosphohydrolase. DS could be produced from glycine by a hypothetical analog of serine hydroxymethyltransferase, or it could be produced from L-serine by a serine racemase. This latter proposition was supported by the results of Dunlop and Neidle, who administered radiolabeled L-serine into rat brain and observed passage of the label into D-serine [20].

The first eukaryotic serine racemase was isolated in 1998 from pupae of the silkworm *Bombyx mori*. The authors used an HPLC-based assay to confirm that the isolated enzyme was able to catalyze the interconversion of L- and D-serine, and furthermore, they established that the racemase activity was specific to serine [21]. The following year, Wolosker *et al.* isolated the

first mammalian serine racemase from rat brain [22]. Later in 1999, the cloning of mouse serine racemase (SR) was reported [23]. Mouse SR is comprised of 339 amino acids, with a predicted molecular weight of 36.3 kDa. In 2000, the gene encoding human serine racemase was cloned from a commercially available cDNA library. The gene encompasses 7 exons and localizes to chromosome 17q13.3. Human SR is 340 amino acids in length with a predicted molecular weight of 36.5 kDa and shares 89% sequence identity with its mouse ortholog [24]. A few years later, a group of researchers at Merck independently cloned the human SR gene from a teratocarcinoma/neuron lambda cDNA library and obtained identical results [25].

1.2 The biochemistry of serine racemase

This thesis is primarily concerned with mammalian SR, in particular the human and mouse SR orthologs (hSR and mSR, respectively), although SR orthologs from other species will be mentioned where appropriate.

1.2.1 SR acts as both a racemase and a dehydratase

Two types of amino acid racemases exist: those that are dependent on pyridoxal-5'-phosphate (PLP), the biologically active form of vitamin B6, and PLP-independent racemases. SR purified from rat brain was shown to be a PLP-dependent racemase, as evidenced by its inhibition by hydroxylamine and aminooxyacetic acid, two reagents known to react with PLP and result in its removal from the active site. Furthermore, the absorbance spectrum of rat SR showed peaks at 340 and 420 nm, which is typical for PLP-dependent enzymes [22]. Cloning of mammalian SR genes revealed a consensus sequence for PLP-binding near the N-terminus (see Figure 2).

In all PLP-dependent enzymes, the co-factor is bound to the enzyme by a Schiff base linkage with a lysine residue (K56 in h/mSR). Somewhat surprisingly, PLP seems to be loosely bound to SR. Uo and coworkers observed that when silkworm SR was dialyzed against buffer without added PLP, the enzyme lost 78% of its activity. The activity could be restored by addition of PLP [21]. The same phenomenon was observed with SR isolated from rat brain [22]. Our recombinant mSR and hSR behave similarly; upon dialysis against PLP-free buffer the enzyme readily loses the cofactor (unpublished observations).

Based on its primary amino acid sequence (see Figure 2), SR is predicted to be a member of the fold-type II family of PLP-dependent enzymes. Fold type II enzymes are active as homodimers,

and indeed, recombinant SR forms homodimers in solution, as our group has shown by gel filtration [26]. The pH optimum of mammalian SR is in the alkaline range, from pH 8.0 – 9.0, and the temperature optimum of the enzyme is 37 °C [22].

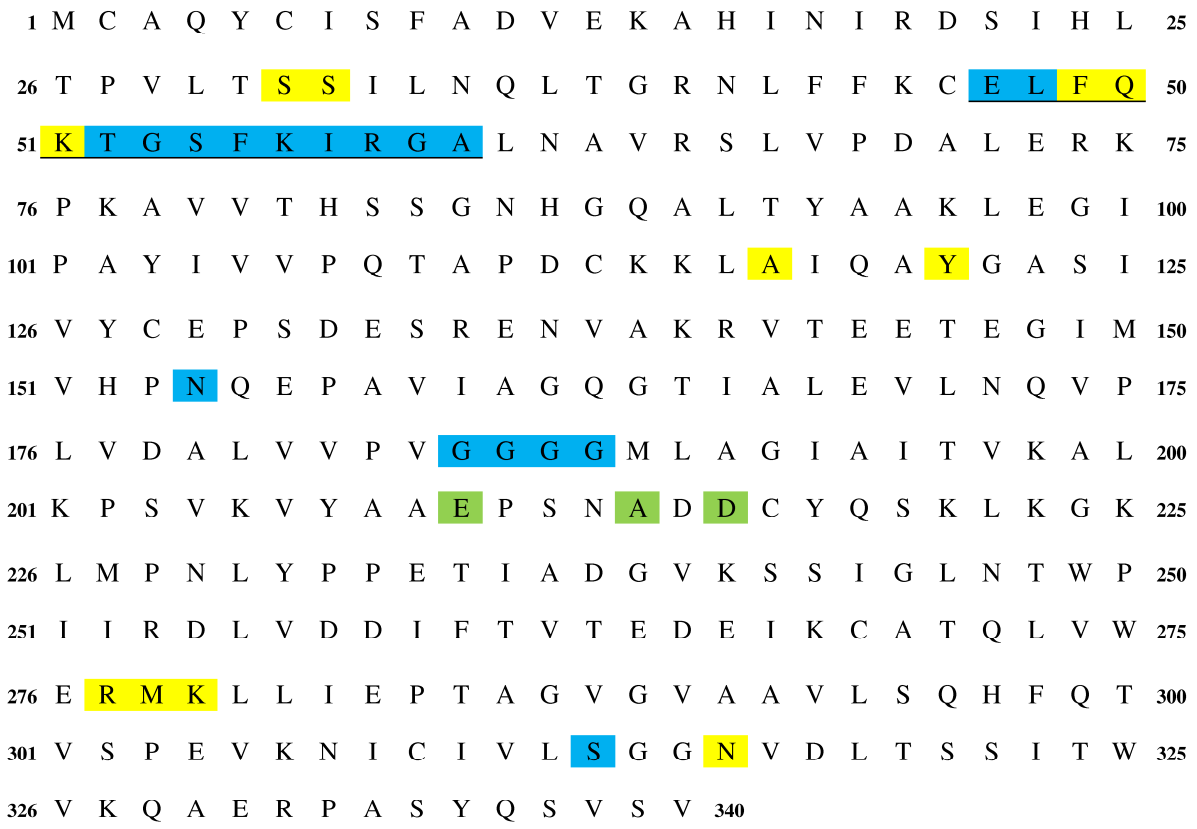


Figure 2. *Human SR amino acid sequence. PLP-binding residues are highlighted in blue, and the PLP-binding consensus sequence (Prosite accession number PS00165) is underlined. Putative metal-coordinating residues are highlighted in green, and putative nucleotide-binding residues are in yellow.*

The racemization activity of mammalian SR (recombinant or purified from rodent brains) *in vitro* is surprisingly low. Even when activity is accessed in the presence of SR cofactors and activators (see Section 1.2.3), the K_M and k_{cat} values for the L-serine (LS) → DS reaction are approximately 4 mM and 45 min^{-1}, respectively. For the opposite reaction, the K_M for DS is 10 – 14 mM, while k_{cat} is approximately 100 min^{-1} (values are taken from [27] and Paper I of this thesis). The high K_M values indicate that the enzyme activity is likely metabolically regulated by local L-(or D-)serine levels. However, the k_{cat} values are almost 400-fold lower than the comparable values for alanine racemases [28]. It is possible that SR is more efficient *in vivo*

than *in vitro* due to interactions with other proteins and/or posttranslational modifications, and these possibilities will be discussed in Section 1.2.4.

Due to the vast mechanistic versatility of PLP-dependent enzymes and the fact that many reaction pathways share common intermediates, it is not unusual for PLP-dependent enzymes to catalyze various side-reactions [29]. In fact, eukaryotic SR acts not only as a racemase but also as an eliminase (see Figure 3).

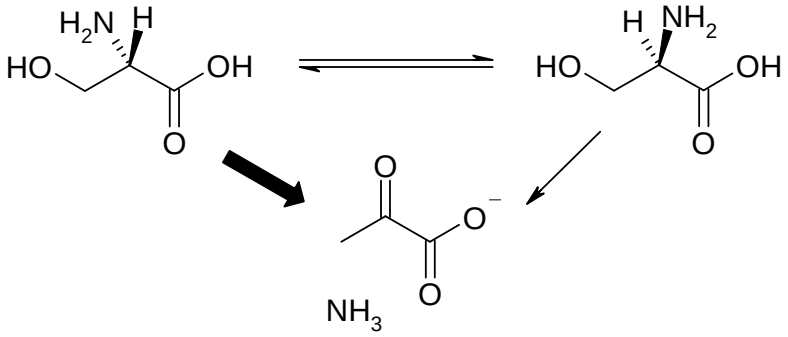


Figure 3*. Reactions catalyzed by mammalian SR. LS and DS are interconverted, and LS undergoes beta-elimination to release pyruvate and ammonia. DS undergoes elimination to a far lesser extent.*

The first evidence that SR can act as an eliminase was reported by Panizzutti *et al.*, who identified the serine analog L-serine-*O*-sulfate (LSOS) as both a non-competitive inhibitor and a substrate of recombinant SR. LSOS has been shown to act as a "suicide substrate" of other PLP-dependent enzymes. LSOS reacts with the enzyme to form an aminoacrylate intermediate, which can then remain bound to the active site and irreversibly inhibit the enzyme or be broken down into pyruvate, sulfate, and ammonia [30]. In the case of SR, most of the LSOS undergoes elimination. The LSOS elimination reaction is roughly 500-fold more efficient than the conversion of LS to DS [31]. In this work, Panizzutti *et al.* also reported that SR was unable to catalyze the β–elimination of its natural substrate LS [31]. Soon after, however, three independent reports characterizing the SR-catalyzed elimination of LS to pyruvate were published within a few months of each other [26, 32, 33]. Working with a mammalian cell line transfected with a vector encoding mSR, De Miranda *et al.* noted that 4 mol of LS were consumed for every mole of DS produced. Furthermore, addition of LS to cells caused a spike in pyruvate levels. When de-proteinized cell extracts were added to the purified enzyme, they were also able to observe formation of pyruvate from LS. They pinpointed ATP and Mg^{2+} as the

cofactors necessary to increase pyruvate production above basal levels (see Section 1.2.3) [32]. Dunlop and Neidle, who worked with SR purified from mouse brain, observed that the elimination of LS to pyruvate proceeds two-fold faster than the conversion of LS to DS [33]. Stříšovský *et al.* reached similar conclusions, showing that recombinant mSR can both racemize serine and convert LS to pyruvate, performing the latter reaction more efficiently. The authors also show that when DS is used as substrate, very little pyruvate is produced, suggesting that the β-elimination activity is specific for LS [26]. However, others have suggested that SR can also accept DS as an elimination substrate as a means of degrading DS in DAAO-poor brain regions [34], and it still remains unclear whether or not SR is able to produce pyruvate directly from DS or if conversion of DS to pyruvate is a two-step process (DS→ LS→ pyruvate).

The substrate specificity for the elimination activity of SR is much broader than for its racemization activity, which is specific to serine. Besides LSOS, LS, and possibly DS, SR also catalyzes the conversion of L-threonine [27, 34], β-chloro-L-alanine, and L-*threo*-3-hydroxyaspartate (LTHA) [27] to the corresponding oxoacids. In fact, the conversion of LTHA to oxaloacetate is the most efficient reaction of mammalian SR characterized to date (k_{cat}/K_M two orders of magnitude higher than for serine racemization) [27]. See Section 1.4.4.1 for an overview of SR's sequence similarity to LTHA dehydratases from bacteria and yeast. LTHA has been shown to act as a blocker of glutamatergic neurotransmission in experimental settings and has been shown to use the aspartic and glutamic acid reuptake transporters [35, 36], although its precise physiological role remains unknown.

1.2.2 Proposed mechanism of action

In all PLP-dependent enzymes, the PLP cofactor forms a Schiff base with an active site lysine residue (Figure 4, **3**) The putative reaction mechanisms of most PLP-dependent enzymes, regardless of reaction specificity, begin with the condensation of PLP and the amino acid substrate to form a Schiff base referred to as the external aldimine (**4**). Next, the alpha hydrogen is abstracted and the resulting carbanion is referred to as the quinoid intermediate (**6**) (for review, see [29]). At this point in the catalytic cycle of SR, there are two possibilities for the fate of the quinoid intermediate. The first is that the alpha hydrogen is replaced from the opposite side, resulting in an inversion of stereochemistry at Cα (**5**), and the newly formed Ser enantiomer is released from the active site. In the second case, the quinoid undergoes dehydration to form an

aminoacrylate aldimine intermediate (**7**). The aminoacrylate is then released from PLP and hydrated to form pyruvate, with concomitant release of ammonia.

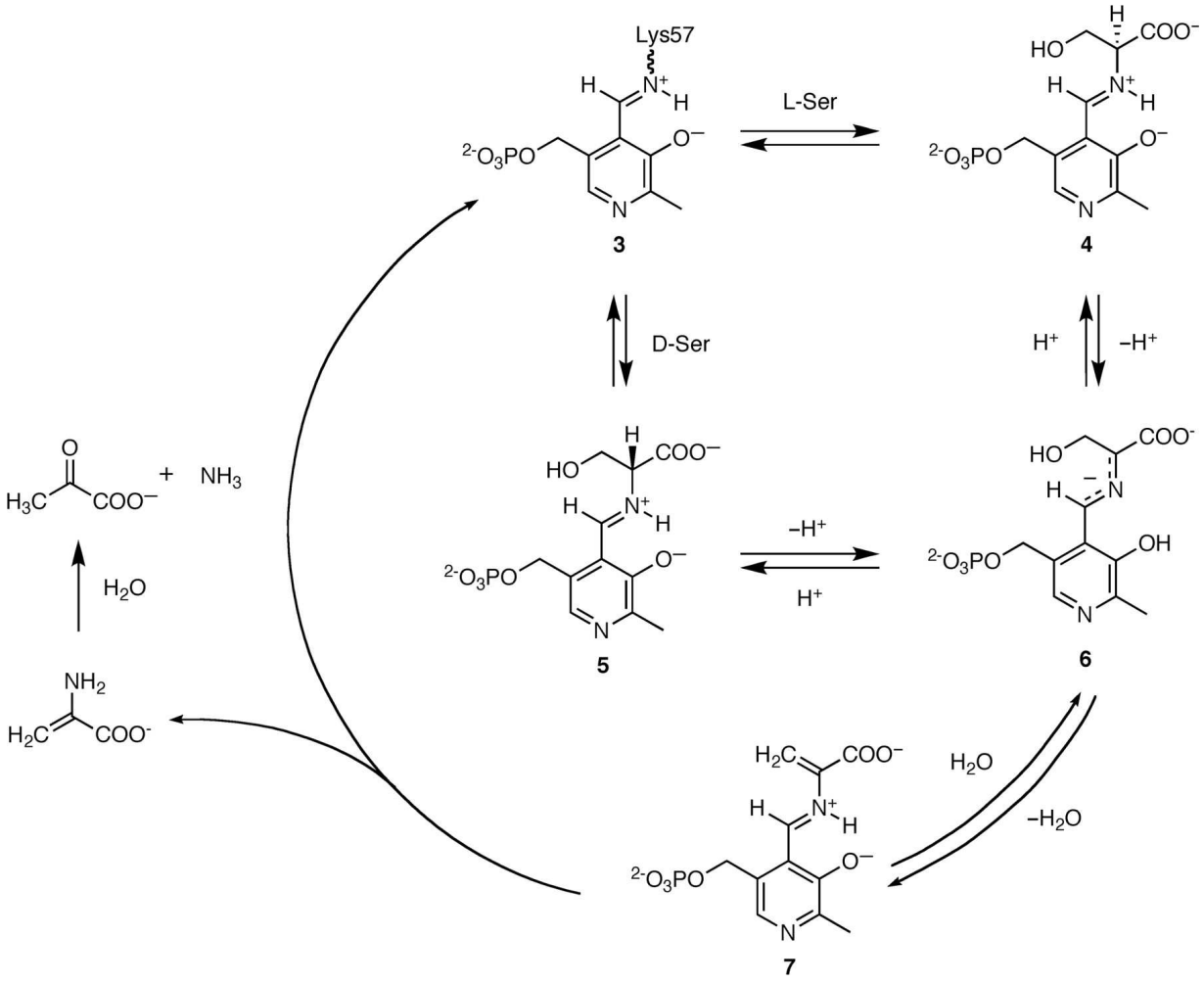


Figure 4. *Proposed SR reaction scheme, taken from [37]. Lys57 (*S. pombe *SR numbering) corresponds to Lys56 of hSR.*

In their 2008 review of amino acid racemases and their recently published study of *S. pombe* SR crystal structures, Yoshimura and Goto proposed a two-base mechanism for SR [28, 38]. In this proposal, the PLP-binding lysine (K56, hSR numbering) catalyzes abstraction of the alpha hydrogen of LS while a serine residue (S84) catalyzes addition of hydrogen to form DS. In the opposite reaction, S84 removes hydrogen from the DS substrate while K56 catalyzes addition of hydrogen to form LS. The authors provide three lines of support for their proposal: 1) biochemical data on mammalian SR show that racemization in the LS to DS direction is slightly different than in the opposite direction and that the elimination reactions are specific for L-

isomers, 2) modeling of the DS external aldimine in the crystal structure of *S. pombe* SR revealed S82 (which corresponds to S84 of hSR) to be a candidate proton shuttle on the *re*-face side of PLP, and 3) site-directed mutagenesis experiments with *S. pombe* SR; the S82A mutant lacked racemase and DS dehydratase activity but retained substantial LS dehydratase activity [38].

However, while it is convenient and aligns well with the available biochemical data, the current proposed mechanism is limited. Based on mutagenic studies, other residues are also directly involved in the reaction specificity of SR. The Q155D mutant of mSR exhibited no elimination activity but was more efficient than wild-type SR at conversion of LS to DS. Mutations in proximal residues, such as H152S, P153S, and N154F, had a similar effect [34].

1.2.3 Key effectors and cofactors

1.2.3.1 Divalent cations

Serine racemase is strongly activated by divalent cations, in particular Mg^{2+}, Mn^{2+}, and Ca^{2+}. Fe^{2+} and Ni^{2+} have negligible effects, and Cu^{2+}, Co^{2+}, and Zn^{2+} markedly decrease the activity of the enzyme [26, 39]. There is some debate about which cation is the physiologically relevant regulator. The intracellular Mg^{2+} concentration in the brain is about 0.6 mM [32, 40], meaning that SR activity would always be upregulated. The release of DS has been shown to be directly related to the cytosolic Ca^{2+} concentration [41], which some researchers see as evidence that Ca^{2+} is the endogenous activator. However, it is important to note that Ca^{2+} concentration is correlated to DS release and not necessarily to DS synthesis.

Addition of EDTA to recombinant SR abolishes the enzymatic activity; however, it does not alter the secondary or quarternary structure of the enzyme, according to circular dichroism measurements and gel filtration experiments, respectively [39]. On the other hand, there is evidence that binding of calcium (or another divalent cation) influences SR's tertiary folding. Changes in the fluorescence spectrum of calcium-saturated SR upon addition of EDTA indicate conformational changes. Furthermore, the thermal denaturation profile of SR is influenced by the presence/absence of calcium [39].

Based on homology models, SR is predicted to coordinate its cation in an octahedral manner, with 3 water molecules, E210, D216, and the carboxyl group of A214 (hSR numbering) participating [42].

1.2.3.2 Nucleotides

Nucleotides were identified as allosteric effectors of SR by Dunlop and Neidle [33]. SR purified from mouse brain was activated by Mg^{2+} (or Mn^{2+} or Ca^{2+}) and DTT. Addition of yeast extract resulted in further upregulation. Nucleotides were found to be the source of this upregulation, with ATP, ADP, and GTP being the most effective. ATP significantly stimulates SR at concentrations as low as 0.1 mM, and maximal activation occurs at 1 mM. Both the serine racemization and elimination activities are upregulated by ATP [33]. The effect is mostly on the K_M, which is reduced 10-fold in the presence of 1 mM ATP, while k_{cat} changes very little, suggesting that ATP activates SR allosterically. ATP is not needed to satisfy an energy requirement; ADP and the non-hydrolyzable ATP analog adenosine 5'-*O*-(3-thiotriphosphate) are equally effective [33], and no hydrolysis of [γ-^{32}P]-ATP was observed [32]. Human embryonic kidney cells expressing mSR were treated with apyrase, which is known to remove the γ and β phosphates from ATP. As expected, DS and pyruvate production were downregulated [32].

The existence of an allosteric ATP binding site makes SR unique among PLP-dependent enzymes. De Miranda *et al.* observed a synergy between the effects of Mg^{2+} and ATP. Both Mg^{2+} and ATP alone are able to increase the basal activity of SR; however, maximal activation is achieved in the presence of both cofactors [32].

1.2.3.3 Reducing agents

Mammalian SR contains a large number of cysteine residues, 7 in mSR and 8 in hSR. During the initial isolation of SR from rat brain, it was observed that oxidized glutathione reduces the enzymatic activity, suggesting that sulfhydryl groups may play a role in SR activity [22]. Additionally, SR is inhibited by thiol-modifying reagents [39] [43]. SR is believed to be localized primarily in the cytosol (see Section 1.3.4), a reducing environment, and it displays the highest activity *in vitro* in the presence of reducing agents.

1.2.4 Posttranslational modifications

Posttranslational modifications do not seem to be essential for serine racemase activity. Numerous *E. coli* based systems for expression of recombinant SR, including our own (Paper I), have successfully yielded active protein [27, 39, 43, 44]. However, in comparison to related enzymes, for example rat liver L-serine dehydratase (EC number 4.3.1.17), the *in vitro* specific activity of SR is about 2-3 orders of magnitude lower. This might imply that SR is upregulated *in vivo* by posttranslational modifications, though nothing conclusive has yet been shown.

1.2.4.1 S-*nitrosylation*

As mentioned in Section 1.2.3.3, serine racemase boasts a multitude of Cys residues, many of which are predicted to occur at the surface of the enzyme based on homology models. Thus, it seems plausible that *S*-nitrosylation of one or more of these residues could modulate the activity of SR. Cook *et al*. tested the racemization activity of recombinant mSR in the presence of (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate, a nitric oxide releasing compound; they observed no *S*-nitrosylation or influence on enzymatic activity [39]. In contrast, Mustafa and coworkers report that mSR is nitrosylated *in vitro* by the NO donor *S*-nitroso-glutathione (GSNO) at C113. They claim that C113 nitrosylation reduces SR activity by hindering the binding of ATP, and they show that activity can be restored by the addition of DTT, which reverses nitrosylation [45]. However, as a recent review of SR biochemistry points out, GSNO is also used for glutathionylation of proteins, and this could be the real effect. Experiments with milder reagents lacking glutathione would be necessary to remove ambiguities and firmly establish that SR can be nitrosylated at C113 [46].

1.2.4.2 Phosphorylation

The demonstrated interaction of SR and PICK1 (see Section 1.2.5.2) may indicate that SR is brought into the proximity of protein kinase C, where it could potentially undergo phosphorylation. The NetPhos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos/) predicts that hSR has 11 potential phosphorylation sites (6 Ser, 3 Thr, 2 Tyr). However, no phospho-SR was observed until very recently, when LC-MS analysis revealed that Thr227 of membrane-associated SR can be phosphorylated [47].

1.2.4.3 Acylation

A certain fraction of neuronal SR is bound tightly to the plasma membrane, and acylation may play a key role in translocation of SR [47]. SR does not possess the amino acid motifs needed for prenylation, isoprenylation, or myristoylation. However, its high number of Cys residues makes it attractive to speculate that SR undergoes *S*-palmitoylation. Indeed, SR undergoes palimitoylation in neuroblastoma cells when overexpressed in the presence of [^{3}H]palmitic acid. However, the acylation is not abolished by hydroxylamine, which specifically cleaves thioesters, but it is sensitive to NaOH, which suggests that SR is palmitoylated at a Ser or Thr residue [47]. While *O*-acylation of mammalian proteins is not unheard of, it is quite rare and has been described for just two proteins (Wnt and ghrelin). [^{3}H]octanoic acid was also incorporated into SR, so it is possible that SR is *O*-acylated *in vivo* by fatty acids of varying lengths [47].

1.2.4.4 Ubiquitination

Addition of the proteasome inhibitors MG132 or lactacystin to primary astrocyte cultures resulted in significantly increased SR levels, suggesting that SR degradation is mediated by the ubiquitin-proteasome system [48]. An *in vitro* ubiquitinylation assay showed that SR undergoes poly-ubiquitinylation in an ATP-dependent manner [48].

1.2.5 Interaction partners

1.2.5.1 Glutamate receptor interacting protein (GRIP)

Glutamate receptor interacting protein (GRIP) is a large protein consisting of seven PSD-95/discs large/ZO-1 (PDZ) domains and two domains of unknown function termed GAP1 and GAP2. GRIP associates with α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors *via* its PDZ4 and PDZ5 domains. GRIP binding may target SR to the Ca^{2+}-permeable AMPA channel. The proposed model involves activation of AMPA receptors by glutamate, leading to receptor phosphorylation and dissociation of bound GRIP. The released GRIP binds to and activates SR, stimulating release of DS, which joins glutamate in stimulating NMDA receptors [49]. AMPA treatment of primary glial cultures elicits a 3-fold increase in the release of DS into the medium [49].

Binding of recombinant GRIP domains PDZ4-PDZ5-PDZ6-GAP2-PDZ7 to recombinant SR causes a very modest increase in SR activity; a 5.8:1 molar ratio of GRIP:SR results in a 2.8-fold increase in specific activity. Both the K_M and k_{cat} for conversion of LS to DS increase in the

presence of GRIP, suggesting that GRIP binding to SR results in structural changes [42]. Interestingly, GRIP binds to mSR and hSR *via* interaction of the PDZ6 domain of GRIP with the four carboxy terminal amino acids of SR (SVSV in hSR, TVSV in mSR) [49]. However, other mammalian SRs, such as rat SR, are truncated at the carboxy terminus (see Section 1.4) and therefore do not possess the GRIP binding motif.

1.2.5.2 Protein interacting with kinase C (PICK1)

Yeast two-hybrid screening revealed that SR interacts with protein interacting with kinase C (PICK1) [50]. Like GRIP, PICK1 is believed to interact with the carboxy terminus of SR *via* a PDZ domain. The coiled-coil domain of PICK1 also appears to be necessary for binding [50]. Hikida *et al*. observed decreased levels of DS in the forebrains of neonatal PICK1-knockout mice, while adult mice did not have altered DS levels, and neither adult nor neonatal knockout mice showed altered SR expression compared to control. Furthermore, based on co-transfection experiments in HEK293 cells, the authors suggest that PICK1 binding upregulates SR activity [51]. A physiological role for the PICK1-SR interaction has not yet been ascertained, though studies suggest there may be some relevance to schizophrenia (see Section 1.5.1).

1.2.5.3 Golgin subfamily A member 3 (Golga3)

An interaction between SR and Golgin subfamily A member 3 (Golga3) was revealed by yeast two-hybrid screens. Unlike PICK1 and GRIP, Golga3 binds to the first 66 N-terminal amino acids of SR. The role of the Golga3-SR interaction seems to be to modulate proteosome-mediated SR degradation. In co-transfection experiments in HEK293 cells, Golga3 was associated with an increase in steady-state SR levels and concomitant increase in DS levels. Golga3 inhibits the polyubiquitination of SR both in cell culture, and pulse-chase experiments show that the presence of Golga3 elicits a three-fold increase in SR half-life [48].

1.3 Distribution of serine racemase

1.3.1 Tissue distribution of serine racemase in human

As of January 2010, the EST profile of hSR (NCBI, Hs.461954) suggests that SR is present in adrenal gland, blood, bone, brain, eye, heart, intestine, kidney, liver, lung, mammary gland, muscle, pharynx, prostate, stomach, testis, thymus, uterus, and vascular tissue. The presence of SR on the protein level has been experimentally confirmed in human brain, liver, heart, and

kidney. Additionally, SR has been observed in rodents in the peripheral nervous system [52] and in sensory organs such as retina [53, 54] and the peripheral vestibular system [55].

1.3.2 SR in brain

Northern blot analysis of human brain samples by De Miranda *et al*. revealed a single transcript of 2.7 kb, which agrees with the results of other groups working with human [25] or rodent [23] [56] brains. SR is widely distributed throughout the brain. The highest levels of serine racemase RNA in human brain were found in the hippocampus and corpus callosum, intermediate levels were found in substantia nigra and caudate, and very low levels were found in amygdala, thalamus, and subthalamic nuclei [24].

On the protein level, Steffek *et al.* observed the presence of SR in all the brain sections they studied by Western blotting – the hippocampus, dorsolateral prefrontal cortex (DPFC), anterior cingulate cortex, superior temporal gyrus, and primary visual cortex [57]. Immunohistochemistry revealed the presence of SR in the DPFC and hippocampus; in both, SR is prevalent in white matter [58]. Additionally, SR was observed in all layers of the cerebellum. In the Purkinje cell layer, SR was found principally in Bergmann glia, with some staining of Purkinje cells. In the white matter, SR was concentrated in "small round cells" presumed to be glia [58].

1.3.3 SR in other tissues

Researchers at Merck identified three SR mRNA transcripts of 1.6, 2.6, and 4.5 kb in heart, skeletal muscle, kidney, and liver, and they postulated three possible splice variants by EST clustering and genome/gene structure analyses. They found no signal in colon, small intestine, spleen, thymus, leukocyte, or lung [25].

Immunohistochemistry was used to further investigate expression in heart and kidney. Expression of SR in kidney was restricted to ducts in the medullary ray, and double label immunohistochemistry experiments with cytokeratin revealed that these were converted tubules. In heart, SR was homogenously expressed in ventricular myocytes and co-expressed with muscle actin. SR was only located on smooth muscle; elastic fibers and components of atrial wall and veins showed no immunoreactivity. The role of SR in kidney and heart has not yet been

determined, but DS may interact with NMDA receptors in these organs. Since DS is nephrotoxic [59], the true role of SR in kidney may be related to DS breakdown rather than synthesis.

1.3.4 Cellular localization of SR

Due to the localization of DS in glia, SR was originally hypothesized to be an astrocytic enzyme [12]. This was initially confirmed by the pioneering work of Wolosker *et al.,* who observed the selective enrichment of SR in astrocytes by immunohistochemistry as well as by comparison of SR expression in astrocyte-enriched *vs.* neuronal cultures [23]. Stevens and coworkers probed the distribution of DS and SR in rodent retinas by immunohistochemistry and found that both were present in Müller glial cells and astrocytes [53]. Wu *et al.* also found SR in glial cells, specifically in Schwann cells and fibroblasts in the peripheral nervous system of rats [52].

The proposal that astrocytes are the sole source of DS is rather incompatible with the classical definition of a neurotransmitter, which should be produced in and released from neurons. In fact, just two years after mammalian SR was discovered, a brief report describing the presence of DS in neurons as well as glial cells of rat brain was published [60]. In 2006, Kartvelishvily *et al.* conducted a detailed analysis of SR protein expression both in neuron and glial cultures and in brain slices and found that SR is expressed in neurons as well as glia. They further show that activation of glutamate receptors induces neuronal DS release [16]. Around the same time, a Japanese group reported that primary neuron cultures contain SR mRNA and protein and that both mRNA and protein levels are higher in neurons than in astrocytes [61] [62]. A follow-up *in situ* hybridization study indicated that SR mRNA is present in neurons and glia of rat retina, optic nerve head, and lamina cribrosa [63]. In 2008, Miya *et al.* conducted immunohistochemical experiments using novel SR knock out mice as controls and found that SR is predomininantly localized in pyramidal neurons in the cerebral cortex and hippocampal CA1 region but not in glial cells [64].

The assumption that SR was solely glial may be, as Kartvelishvily *et al.* suggest, attributable to differences in antibodies and experimental techniques [16] or due to the fact that the proximity of glial processes with neuronal processes complicates interpretation of immunohistochemical data [54]. Today, however, we can be reasonably confident that SR is expressed in both neurons and glia.

SR lacks a transmembrane domain and is believed to be primarily located in the cytosol, which is supported by subcellular fractionation of brain extracts [16, 23], immunofluorescence imaging of cultured Schwann cells [52], and immunocytochemistry of SR-containing neurons, which suggested that SR was confined primarily to the cell body [54]. However, there is evidence that some fraction of cellular SR is membrane bound. Dememes and colleagues observed intense SR immunoreactivity along the membranes of rat vestibular epithelial cells [55]. A recent report indicates that a small fraction of neuronal SR undergoes *O*-acylation and translocation to the membrane [47].

1.3.5 Developmental regulation of SR expression

SR expression levels in cortex and hippocampus of postnatal mice increased gradually from P7 to P56, indicating some sort of developmental regulation [64], though few detailed studies in this area have been conducted. Dun *et al*. studied SR expression in mouse retinas by RT-PCR, *in situ* hybridization, Western blotting, immunohistochemistry, and immunocytochemistry. They found that SR levels are developmentally regulated with high levels during the post-natal period and diminished levels in mature retina (18 week old mice) [54].

In a short communication, human SR was identified by MALDI MS and MS/MS from a single spot on a two-dimensional gel of perireticular nucleus proteins. The perireticular nucleus is a transient structure present in developing human brain. The presence of SR in this structure may indicate that SR plays a role in the correct formation of corticothalamic and thalamocortical connections [65].

1.4 Human serine racemase orthologs and homologs

Although this thesis is primarily concerned with the biochemistry of recombinant human and mouse serine racemase, several other eukaryotic SRs have been identified and characterized, and these merit brief mention. It is important to note that eukaryotic SRs are quite distinct from prokaryotic alanine and serine racemases, which will not be discussed in detail. Recently, however, a serine/threonine racemase with 26% sequence identity to human SR was isolated from *Pyrobaculum islandicum*, an anaerobic hyperthermophilic archaeon [66].

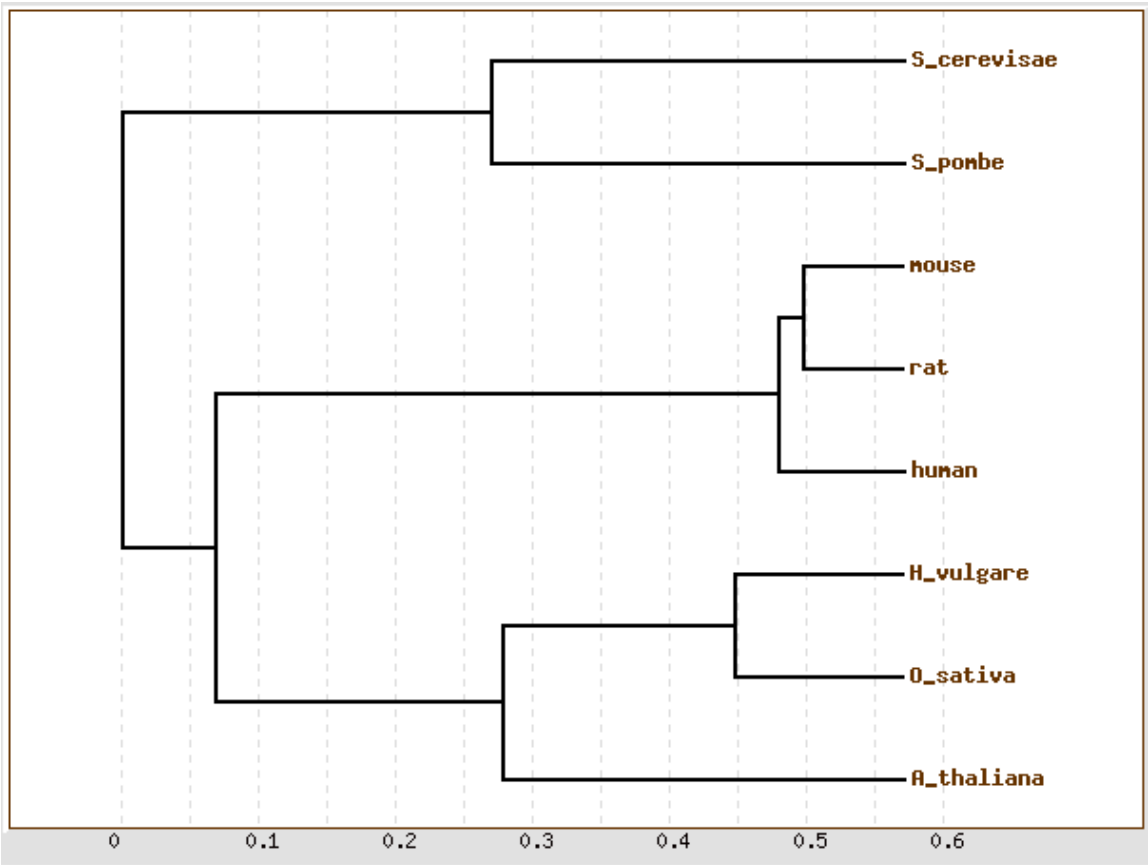


Figure 5. *Phylogenetic tree relating the SR orthologs described in Section 1.4 produced with the TreeTop webserver (http://www.genebee.msu.su/services/phtree_reduced.html). The "cluster algorithm," in which the notion of distance between groups of sequences is used for setting the branching order, was employed.*

1.4.1 SR from *Rattus norvegicus*

Rat brain SR was the first mammalian SR to be isolated [22], and its gene was subsequently cloned [56]. Rat SR is 96% identical to mSR and 92% identical to hSR based on primary sequence alignments. However, rat SR is truncated at the carboxy terminus and consists of only 333 residues due to a nonsense mutation at the 334th codon. If the nucleotide sequence beyond the 334th codon would be translated, rat SR would have a carboxy terminus similar to the mouse and human enzymes. This deletion may be relevant, however, since the C-terminal residues of mouse and human SR have been implicated in protein-protein interactions (see Section 1.2.5).

1.4.2 Other mammalian serine racemases

The gene sequences of several other putative mammalian SRs have been deposited in databases. SR from cow (*Bos taurus*, GenBank accession number AAI26701) is 334 amino acid residues in

length and 85% identical to hSR. Like rat SR, it is truncated at the C-terminus. Two mRNA isoforms resulting from transcription of the SR gene in dog (*Canis familiaris*) have been described. The protein product resulting from isoform 1 (NCBI accession number XP_548320) consists of 339 amino acids and is 88% identical to hSR.

Multiple mRNA isoforms have also been identified for SRs from chimpanzee (*Pan troglodytes*) and rhesus monkey (*Macaca mulatta*). The protein products resulting from translation of chimp isoform 3 (NCBI accession number XP_001156186) and rhesus monkey isoform 2 (XP_001087203) boast 99% and 96% identity to hSR, respectively. The other isoforms result in protein products (XP_001156128, XP_001156080, XP_001087081) that are significantly shorter at the C-terminus (by approximately 35-65 amino acids) than the putative full-length SRs. Very little biochemical work has been done with these SR orthologs, although in one study rhesus monkey brain rather than human brain was used to map SR expression patterns [25].

1.4.3 SR from plants

The role of DS in plants has not yet been determined, but plant growth is inhibited by 0.5 mM DS, leading to speculation that plant SR may be primarily involved in DS breakdown rather than synthesis. Notably, all of the plant SRs described below exhibit an elimination activity that is roughly 20-fold higher than the racemization activity. Preferential expression of SR in the meristem of *Arabidopsis thaliana* suggests that the enzyme may play a role in supplying energy *via* pyruvate production [67].

1.4.3.1 SR from the dicot Arabidopsis thaliana

A gene encoding SR was isolated from the plant *Arabidopsis thaliana*, and the enzyme was recombinantly expressed in *E. coli*. SR from *A. thaliana* is a PLP-dependent SR with 46% and 45% sequence identity with hSR and mSR, respectively. Like the mammalian SRs, *A. thaliana* SR catalyzes both racemization and dehydration reactions and is activated by divalent cations, particularly Mg^{2+}, Mn^{2+}, and Ca^{2+}. In contrast to mammalian SR, it is not activated by ATP [68].

1.4.3.2 SR from monocot plants: barley and rice

SR from barley, *Hordeum vulgare* L., is a 337 kDa enzyme that shares 46% and 45% sequence identity with hSR and mSR, respectively. SR from rice, *Oryza sativa*, is similar to the barley enzyme. SRs from barley and rice catalyze conversion of LS to both DS and pyruvate and are

activated by divalent cations such as Mg^{2+}, Mn^{2+}, and Ca^{2+} [69]. Since rice SR contains four tryptophan residues, fluorescence quenching with acrylamide was performed in the presence and absence of Mg^{2+}. Changes in the fluorescence profile suggest that the enzyme undergoes conformational change upon magnesium binding [70]. Unlike mammalian SR and like SR from *A. thaliana*, barley SR is not activated by ATP [69]. However, the racemase activity of rice SR decreased with addition of ATP, while the dehydratase activity was up-regulated [70]. Phylogenetic analysis shows that these SR orthologs are distinct from mammalian SRs as well as from *A. thaliana* SR [69].

1.4.4 SR from yeast

1.4.4.1 Saccharomyces cerevisae

A putative serine racemase gene was cloned from the yeast *Saccharomyces cerevisae*. Overall, the primary amino acid sequence was 40% identical to mSR, and the N-terminus showed significant similarity to L-*threo*-3-hydroxyaspartate (LTHA) dehydratase from *Pseudomonas* sp. T62. Like mammalian SR, the enzyme is activated by divalent cations and is PLP-dependent. However, the yeast serine racemase displayed only a weak racemase activity, about 10% the activity of purified rat brain SR, and an efficient dehydratase activity that was specific toward L-*threo*-3-hydroxyaspartate. Thus, this putative serine racemase ortholog might be better characterized as a 3-hydroxyaspartate dehydratase [71].

In fact, the gene encoding LTHA dehydratase was recently cloned from the soil bacterium *Pseudomonas* sp. T62, and the enzyme was recombinantly expressed in *E. coli* [72]. The enzyme shares high levels of sequence identity with yeast SRs, particularly SR from fission yeast (64%). However, the recombinant enzyme did not display any detectable serine or aspartate racemase activity, and its dehydratase activity was specific for LTHA. The enzymatic activity was inhibited by EDTA and activated by Mg^{2+}, Mn^{2+}, and Ca^{2+}. AMP and ADP modestly increased the activity, while ATP and GDP acted as inhibitors. L-*erythro*-3-hydroxyaspartate was identified as a potent competitive inhibitor (K_i = 0.20 mM), while DS inhibited the enzyme non-competitively.

1.4.4.2 Schizosaccharomyces pombe

SR from the fission yeast *Schizosaccharoymces pombe* is perhaps the most important ortholog to mention, since it is the only eukaryotic serine racemase to date whose structure has been solved experimentally by X-ray crystallography, as was mentioned in Section 1.2.2. Two structures (PDB accession codes 1v71 and 1wtc, unliganded and complexed with the non-hydrolyzable ATP analog AMPPCP, respectively) were deposited in the PDB by Goto and coworkers in 2005. However, no publication was released until 2008, when Yoshimura and Goto mentioned the structures in their review on amino acid racemases [28]. The following year, Yoshimura, Goto, and associates published a third structure of *S. pombe* SR (PDB code 2zpu, modified SR with a catalytic lysinoalanyl residue) and a partial biochemical analysis of recombinant *S. pombe* SR [37]. Most recently, the same group published a comparison of the various structures and introduced a new structure – modified *S. pombe* SR in complex with serine (2zr8) [38]. *S. pombe* SR is a member of the fold-type II family of PLP-dependent enzymes, also known as the tryptophan synthase family, and all other eukaryotic SRs are predicted to be members of this family based on primary sequence alignments. The crystal structures of *S. pombe* SR have been used as templates for homology models of mammalian SR by numerous groups, including ourselves (unpublished data).

Recombinant *S. pombe* SR exhibits racemization and dehydration activities that are slightly enhanced by addition of Mg^{2+} and ATP. The absorbance spectrum of the enzyme reveals peaks at 280 nm and 410 nm; the latter peak is characteristic of a Schiff base with PLP. However, when the enzyme was incubated with LS for a prolonged period of time, the peak at 410 nm decreased and a new peak appeared at 315 nm. MS analysis of sodium-borohydride reduced enzyme suggested that *S. pombe* SR was modified with dehydroalanine derived from serine. About 97% of the enzyme was modified, while the specific activities for racemization and elimination were 54% and 68%, respectively, of those of the unmodified enzyme. X-ray crystallography (PDB structure 2zpu) supported the notion that the PLP-binding lysine had been converted to a lysine-D-alanyl residue forming a Schiff base with PLP. The alpha amino group of the D-alanyl moiety can probably serve as a catalytic base like the ε-amino group of Lys57 of unmodified *S. pombe* SR (see Section 1.2.2). The authors claim that mSR may undergo a similar modification [28, 37].

Potential active-site modifications notwithstanding, comparison of the *S. pombe* SR structures offers valuable insight into structure-function relationships within SR. Comparison of structures with and without bound serine reveals that, like aspartate aminotransferases, SR has an "open" and "closed" form. *S. pombe* SR consists of two domains: a large domain consisting of an open twisted beta sheet flanked by nine alpha helices and a small domain consisting of a three-stranded parallel beta sheet surrounded by four alpha helices. Upon ligand binding, the small domain shifts approximately 20° in order to enclose the ligand in the active site, which is formed along the domain interface [38]. The crystal structures also allow identification of PLP, Mg^{2+}, and ATP binding sites. The majority of the PLP and Mg^{2+} binding residues are conserved or similar in hSR, and eleven of the 14 identified ATP binding residues are conserved (see Figure 2) [38]. Lastly, the *S. pombe* SR crystal structures provide valuable insight into the SR reaction mechanism, as discussed Section 1.2.2.

1.5 Serine racemase in neuropathological conditions

1.5.1 SR and DS in schizophrenia: the NMDAR hypofunction hypothesis

Clinical observations that phencyclidine and ketamine block the NMDAR ion channel and lead to schizophrenic-like symptoms eventually led to the "NMDAR hypofunction hypothesis" of schizophrenia, which has been reviewed by Marino and Conn [73] and more recently by Krivoy and colleagues [74]. Since DS, which is produced by SR, is a co-agonist of NMDARs, it stands to reason that DS and/or SR may be aberrantly regulated in schizophrenia. Indeed, non-clozapine treated schizophrenic patients who were administered DS showed significant improvement in positive, negative, and cognitive symptoms. In contrast, administration of glycine or D-cycloserine, another NMDA receptor agonist, improved negative and some cognitive symptoms but did not improve the positive symptoms [75].

In fact, several research groups agree that schizophrenic patients lack DS. Hashimoto and coworkers observed that schizophrenics have higher serum levels of LS and lower levels of DS relative to age and sex-matched controls [76]. Bendikov *et al.* reported lower concentrations of DS in the cerebrospinal fluid of schizophrenics, as well as a decreased DS/LS ratio [77]. These results suggest that SR may be involved, although it is also possible that the observed decreases are due to DAAO overexpression or overactivation. In fact, many studies have been conducted on DAAO, its putative activator G72, and their potential relevance to schizophrenia. However,

these studies have not yet led to a conclusive link between DAAO upregulation and schizophrenia, and a detailed review of this area of research is beyond the scope of this thesis.

To date, many research groups have attempted to clarify the relationship between SR and schizophrenia, if indeed any such relationship exists. However, the data gleaned from their work remains inconclusive, and in some cases contradictory, which can perhaps be explained in part by the many different schizophrenic subtypes, different diagnostic criteria, *etc.*

1.5.1.1 Altered SR expression in schizophrenia

Steffek and coworkers conducted a Western blot analysis of normal and schizophrenic brains, and they found that SR levels remained unchanged in DPFC, anterior cingulated cortex, superior temporal gyrus, and primary visual cortex, while SR was slightly overexpressed in schizophrenic hippocampus relative to the control [57]. Using the same commercially available antibody, a second group observed an increase in SR expression in schizophrenia in the DPFC but not in the cerebellum [58]. A third group observed that SR protein levels in both the frontal cortex and the hippocampus of post-mortem schizophrenic brains were marginally but significantly lower than in control samples. Furthermore, they observed a reduction in the SR/DAAO ratio in schizophrenic hippocampus [77].

1.5.1.2 Polymorphism in the 5' promoter region of the SR gene

The SR gene is localized to chromosome 17, which has not been linked to or associated with schizophrenia [78]. However, SR interacts with PICK1 (Section 1.2.5.2). The PICK1 gene is localized to chromosome 22q13.1, within a region with well-documented links to schizophrenia. PICK1 SNPs have been shown to be associated with the disorganized schizophrenia type [50].

The SR gene has four 5' untranslated exons, giving rise to four SR mRNA isoforms (GenBank accession numbers AY743705, AY743706, AY743707, and AY743708) [78]. Mutation screening of these isoforms revealed four single nucleotide polymorphisms (SNPs), two of which (IVS1a + 465G>C and IVS1b + 15G>A) were considered to be genetically informative with minor allele frequencies ≥ 0.1. The authors analyzed serum serine levels and genotypes in the same individuals. However, they failed to detect any significant correlation between SNPs and DS levels. The authors conclude that polymorphisms in the 5' region of the SR gene do not play a significant role in susceptibility to schizophrenia in Japanese populations, although they cannot

rule out the possibility that a subset of schizophrenics are biologically affected by the SR gene [78]. These conclusions were supported by the work of a second group, who found that the 5'-G/C SNP is not a major risk factor for schizophrenia in a Russian population [79]. A German group found no association between the SR gene and schizophrenia [80].

However, a more recent report suggests that the 1VS1a + 465C allele is prevalent in schizophrenics of the paranoid subtype. In a reporter assay, the IVS1a +465C allele showed reduced promoter activity compared to the IVS1a + 465G allele, suggesting that the IVS1a + 465C allele may be a risk factor for paranoid schizophrenia [81].

1.5.2 Alzheimer's disease

In 2003, Hashimoto and coworkers reported that Alzheimer's disease patients have higher LS and lower DS levels than age-matched controls, and they suggest that this is a result of low expression or inactivation of SR [82]. However, more recent efforts have indicated that overactivation, not underactivation, of SR expression and/or activity contributes to the physiopathology of Alzheimer's disease.

Amyloid β-peptide (Aβ) is the major constituent of so-called amyloid plaques in the brains of Alzheimer's disease patients, and it is capable of eliciting a release of Glu from microglia. Wu *et al.* investigated whether or not Aβ has a similar effect on the NMDA coagonist DS, and they found that Aβ indeed causes an increase in DS levels but not glycine levels in primary microglial cultures. Furthermore, they observed an increase in SR mRNA after activation with Aβ, and a promoter-reporter assay suggests that SR is transcriptionally induced by Aβ. Finally, they probed several human brain samples and found increased levels of SR mRNA in Alzheimer's disease patients relative to age-matched controls [83].

Recently, Inoue *et al.* showed that Aβ induced neurotoxicity is significantly reduced in SR knock-out mice, providing the most compelling evidence so far for utilizing SR inhibition as a novel strategy for treatment of Alzheimer's disease [84].

1.5.3 Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS, Lou Gehrig's disease) is characterized by massive motoneuronal death, a principal cause of which is glutamate-mediated excitotoxicity. D-serine levels in the spinal cords of ALS mice increase with disease progression, and this increase is

associated with glial activation. Accordingly, SR expression is upregulated as the disease progresses, but DAAO levels remain unchanged. Addition of phenazine methosulfate, an SR inhibitor, to primary cultured spinal cord cells from ALS mice significantly alleviated NMDA toxicity [85].

1.5.4 Knock out mouse studies

In addition to the results of Inoue *et al*. [84] mentioned in Section 1.5.2, a second group also recently published generation of a SR knockout mouse [86]. In the brains of both knock outs, DS could be observed at levels around 10% of the wild-type, suggesting that there are additional sources of DS other than SR. Nevertheless, the knock out mice showed altered glutamatergic neurotransmission as well as subtle behavioral abnormalities such as hyperactivity and impaired spatial memory in knock out males and elevated anxiety in the female knock outs [86].

1.6 Inhibitors of serine racemase

Treatment of patients with high-affinity NMDAR blockers often results in undesirable side effects such as hallucinations. In recent years, there has been a movement toward development of low-affinity NMDAR blockers that are both therapeutically effective and better tolerated by patients [87, 88]. In a similar manner, SR inhibitors might offer a more "gentle" approach to decrease NMDAR overactivation.

Despite SR's promise as a target for treatment of various neuropathies (outlined in Section 1.5), very few potent and specific inhibitors have been identified. In general, PLP-dependent enzymes are less than ideal candidates for drug discovery. With very few exceptions all PLP-dependent enzymes share a common transition state, the so-called external aldimine (see Section 1.2.2), rendering the identification of specific inhibitors a particular challenge. As a result of this common transition state, many PLP-dependent enzymes are capable of catalyzing side-reactions. As discussed in Section 1.2.1, SR catalyzes both racemization and deamination of serine, as well as slow deamination of threonine and other amino acid analogues such as L-*threo*-3-hydroxyaspartate and L-serine-*O*-sulfate. The architecture of the SR active site is likely related to those of the serine and threonine dehydratases, other key enzymes in the human body, further complicating identification of SR-specific inhibitors. However, important successes have been realized in the field of PLP enzymes as therapeutic targets, as recently reviewed in [89], and the discovery of a potent, specific SR inhibitor with therapeutic potential remains a realistic dream.

1.6.1 Amino and carboxylic acid inhibitors

The majority of SR inhibitor development has focused on analogs of L-serine and other amino acids. One of the earliest reports on the effect of amino acids on SR activity stated that L-lysine and L- and D-cysteine act as potent SR inhibitors [31]. However, several groups have since noted that lysine is not inhibitory [27, 39, 90], and there is some disagreement about the inhibition of SR by cysteine. Cook *et al.* found that, out of a panel of LS analogs, only L-Cys, L-cycloserine, and LSOS showed inhibition [39]. They used absorbance spectroscopy to confirm that L-Cys and L-cycloserine act by reacting with the PLP-cofactor. In contrast, our group has found that L-Cys does not inhibit SR [27] and that inhibition by L-cycloserine is very modest (unpublished results). However, we measured the effect of these compounds under highly reducing conditions (5 mM DTT), which activate the enzyme according to our results, while Cook *et al.* did not; this might account for the observed differences.

In 2005, Dunlop and Neidle studied the effect of various amino acids analogs on the activity of SR purified from mouse brain. According to the results of their screening (1 mM LS, 2 mM test compound), they identified several criteria that an inhibitor or non-natural substrate must meet: L-configuration at the α-carbon, a three carbon chain, a free amino group, and a free carboxyl group. Additionally, an electron withdrawing substituent on C3 seems to be required, as neither L- or D-alanine, β-alanine, nor L-serine amide have a significant effect on racemase activity. Furthermore, a substituent on C2 (α-methyl-D,L-serine) or on the hydroxyl group of serine (*O*-methyl-D,L-serine) also prevents competition with LS. Additionally, Dunlop and Neidle identified a few key exceptions to this set of criteria. Glycine proved to be a competitive SR inhibitor with K_i of 0.15 mM. L-Aspartic acid also competitively inhibits SR with a K_i of 1.9 mM, close to the intracellular concentration of L-Asp in brain. The related compounds L-homocysteic acid, L-Asn, and oxaloacetic acid also inhibited SR [90].

Dunlop and Neidle's results are in agreement with the results of our group, which were also published in 2005. Working with purified recombinant mSR, Stříšovský and colleagues screened a panel of compounds derived from LS and LSOS for SR inhibition. Their experimental set-up (5 mM LS and 5 mM test compound) was designed to reveal ligands whose affinity for SR is comparable to or greater than that of LS. They identified several low millimolar or sub-millimolar competitive inhibitors of SR, including Gly, L-cysteine-*S*-sulfate, L-Asn, L-*erythro*-3-hydroxyaspartate (LEHA), meso-tartrate, dihydroxyfumarate, maleate, and

malonate. In fact, LEHA and malonate are two of the most potent competitive SR inhibitors identified to date, with K_i values for mSR of 49 µM and 71 µM, respectively. Stříšovský *et al*. concluded that the most effective SR inhibitors have two carboxylate moieties separated by 2.6 – 3.2 Å. The presence of an α-amino group and a suitable leaving group at C3 increases the affinity of the ligand for mSR [27].

1.6.2 Peptidic inhibitors

In 2006, Dixon *et al.* described a unique one-bead one-compound combinatorial approach for identification of short (~3 amino acids) peptidic hSR inhibitors. One exciting aspect of the study is that the inhibitors are not structurally related to serine. A less exciting aspect of the study is the fact that the majority of the positive "hits" were insoluble [43]. Of the 25 inhibitory peptides identified, only 10 proved to be sufficiently soluble for further analysis. Dixon *et al.* claim that the inhibitors are competitive, but the kinetic data they show is not very convincing in this regard. Furthermore, the peptides identified are not very potent inhibitors; the two most effective have K_i values of 320 ± 70 µM and 610 ± 120 µM.

Future SR inhibitor development will likely focus on small molecules, particularly amino and carboxylic acids and their analogs (such as the compounds described in Paper II). An experimentally determined 3D structure of mammalian SR would be a helpful resource for the rational design of a specific, high affinity SR inhibitor. Identification of novel structures capable of inhibiting SR is especially relevant, since most of the small molecule inhibitors identified to date are naturally abundant compounds present in a variety of cell types.

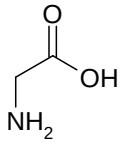
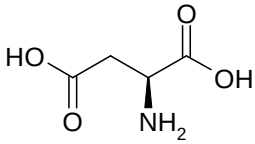
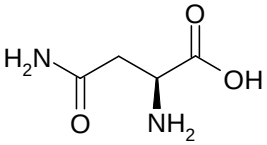
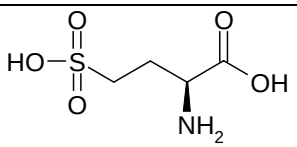
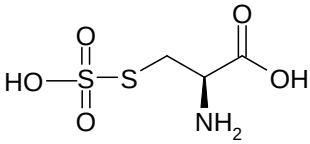
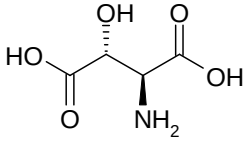
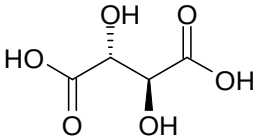
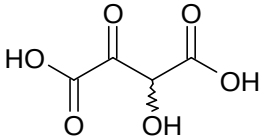
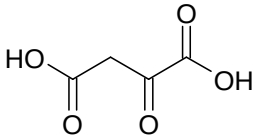
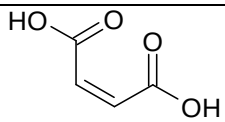
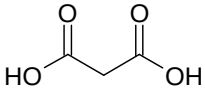
Compound name	Structure	K_i for mSR
Glycine		1.64 ± 0.03 mM [27] 0.15 mM [90]
L-aspartic acid		1.9 mM [90]
L-asparagine		1.13 ± 0.03 mM [27]
L-homocysteic acid		n.d.
L-cysteine-*S*-sulfate		0.64 ± 0.14 mM [27]
L-*erythro*-3-hydroxyaspartate		43 ± 7 µM [27]
Meso-tartrate		0.66 ± 0.1 mM [27]
dihydroxyfumarate		0.69 ± 0.05 mM [27]
Oxalacetic acid		n.d.
Maleate		0.55 ± 0.12 mM [27]
Malonate		71 ± 16 µM [27]

Table 1. *Selected small molecule SR inhibitors and their associated inhibitory constants corresponding to a competitive mode of action. n.d. = not determined*

Chapter 2: Results

2.1 Aims of the project

There were three major aims for this project. The first was to establish a system for recombinant expression and purification of human serine racemase and to compare recombinant human and mouse SR in terms of their kinetic parameters and inhibitor-sensitivity (Paper I). The second aim was to identify potent, specific SR inhibitors for use in tissue culture and animal model experiments, and we introduce aliphatic hydroxamic acids as SR inhibitors in Paper II. The third aim for the project was the experimental determination of the 3D structure of human SR. This goal has yet to be realized; however, in Paper III we describe a method for the generation and screening of random hSR mutants, which allowed us to glean some important information about the structure-function relationships within the enzyme.

2.2 Publications included in the thesis

I. Hoffman, H.E.; Jirásková, J.; Ingr, M.; Zvelebil, M.; Konvalinka, J., Recombinant human serine racemase: enzymologic characterization and comparison with its mouse ortholog. *Protein Expr Purif.* 2009, 63(1), 62-67.

II. Hoffman, H.E.; Jirásková, J.; Cígler, P.; Šanda, M.; Schraml, J.; Konvalinka, J., Hydroxamic acids as a novel family of serine racemase inhibitors: mechanistic analysis reveals different modes of interaction with the pyridoxal-5'-phosphate cofactor. *J Med Chem.*2009. 52(19), 6032-6041.

III. Hoffman, H.E.; Jirásková, J.; Zvelebil, M.; Konvalinka, J., Random mutagenesis of human serine racemase reveals residues important for the enzymatic activity. *Collect Czech Chem Commun.* 2010. *In press.*

2.3 Paper I: Background and comments

The aim of this work was twofold: 1) to establish a system for recombinant expression and purification of human SR in our lab and 2) to compare the activity and inhibitor-sensitivity of hSR and its mouse ortholog. Human and mouse SR share 89% primary sequence identity (see Figure 1 of the paper), and both orthologs are widely and often interchangeably used in SR research. Paper I includes the synthesis of an hSR gene variant, the expression and purification of recombinant hSR, and the first published biochemical comparison of the human and mouse SR orthologs.

2.3.1 Synthesis of the hSR gene

Gene synthesis can offer several advantages for recombinant protein expression, *e.g.* overcoming the limitations of codon usage bias, adjustment of GC content to control the transcription rate or the function of a gene, insertion of protease cleavage sites, and removal or addition of restriction enzyme recognition sites. Based on the previous work of our lab with mSR [27] and on the work of other groups with hSR [43, 44], we chose *Escherichia coli* as the expression host. Due to the challenges inherent in expressing human proteins in a bacterial host, we decided to synthesize a variant of the hSR gene with codon usage optimized for heterologous expression in *E. coli*. The synthesis of the hSR gene was performed by Dr. Marek Ingr at the Department of Biochemistry, Faculty of Natural Sciences, Charles University. Dr. Ingr used polymerase cycling assembly (PCA) in order to assemble two pieces of the hSR gene variant, called synthons. PCA uses the same technology as normal PCR, but in PCA, multiple oligonucleotides are designed with overlaps between adjacent sequences. During the thermal cycles, the oligonucleotides anneal to complementary fragments and are then filled in by the polymerase. Of course, this produces a number of short, incomplete fragments as well as the desired synthetic gene fragment. Therefore, a second PCR cycle is performed using outside primers in order to amplify the fragment of interest.

2.3.2 Recombinant expression of hSR in *E. coli*

The protocol developed by our group for purification of mSR [27] is effective but somewhat laborious, and yields are modest (~2mg/L of culture). Therefore, I cloned the hSR gene into a variety of vectors and analyzed its expression with various purification tags and solubility-enhancing partners as summarized in Table 2.

Tag or fusion partner	Vector	Promoter	notes
none	pET41a	T7	hSR in inclusion bodies
none	pMPMA4	*ara*BAD	Most hSR in the soluble fraction
C-terminal GST	pKS78	T7	hSR in inclusion bodies
N-terminal GST	pET41a	T7	Most hSR in inclusion bodies
N-terminal MBP	pET22b	T7	Most hSR in inclusion bodies
N-terminal MBP	pMPMA4	*ara*BAD	Most hSR in inclusion bodies
C-terminal poly-His	pMPMA4	*ara*BAD	hSR somewhat soluble, easy 1-step purification
N-terminal poly-His	pETM-series	T7	Most hSR in inclusion bodies
N-terminal thioredoxin	pETM-series	T7	Most hSR in inclusion bodies
N-terminal GB1	pETM-series	T7	Most hSR in inclusion bodies

Table 2. *List of fusion partners and purification tags used in hSR solubility and purification screens. Constructs used in downstream applications are highlighted in blue.*

Glutathione-*S*-transferase (GST) [91], maltose binding protein (MBP) [92], and the B1 domain of *Streptococcal* protein G (GB1) [93] have been reported to enhance the solubility of the proteins to which they are fused, and thioredoxin has been reported to enhance both the solubility and "crystallizability" of its fusion partner [94]. However, the GST, MBP, GB1, and thioredoxin hSR fusions were all less soluble than untagged hSR. SR with a C-terminal His-tag worked rather well; solubility was decreased but not critically compromised and purification could be accomplished in just one chromatographic step.

For this work, however, we chose to express untagged hSR from the arabinose-inducible pMPMA4 vector, which our group previously used for expression of mSR, and to conduct a similar purification [27]. The key advantage of our purification scheme is the ATP-agarose step. As described in Section 1.2.3.2, ATP is an allosteric activator of SR, and this step therefore ensures that only correctly folded, biologically active SR is isolated (see also [27]).

2.3.3 Activity assay

Once purified recombinant hSR had been obtained, activity measurements were performed. Both the racemization and elimination activities of the enzyme were accessed with HPLC-based activity assays, according to protocols previously established in our lab [26, 27]. LS and DS in

the reaction mixtures were derivatized with Marfey's reagent **(**1-fluoro-2-4-dinitrophenyl-5-L-alanine amide). The resulting diastereomers were resolved on a Zorbax Extend C_{18} reversed phase HPLC column (4.6×250 mm, particle size 5 μm, Agilent Technologies, USA) mounted on an Alliance 2795 HPLC system (Waters Co., Milford, MA, USA). An isocratic solvent system composed of 50 mM sodium acetate pH 4.4 and methanol (65:35) was used, and the derivatives were detected by absorption at 340 nm. Our group has also developed a capillary zonal electrophoresis (CZE) method for resolution of LS and DS [95]. Similar to the HPLC method, the CZE method involves a derivatization step, in this case with *O*-phthaldialdehyde. The CZE method and HPLC method suffer from the same disadvantage, namely a high error rate due to pipetting errors and the imperfect reproducibility of the derivatization reactions. Thus, in both cases, multiple replicates of each reaction must be performed in order to obtain reasonable data. There were several reasons for our choice of the HPLC method for the hSR activity measurements. Most importantly, our activity and inhibition assays for mSR were conducted using this method, facilitating the comparison. Practical reasons were also a consideration – the HPLC is freely available, whereas there is often a longer wait to run CZE samples, and the capillary becomes obstructed and needs to be changed after 200-300 runs.

For the elimination reactions, we determined the pyrvate content in the reaction mixtures using an HPLC based assay. The column and instrumentation used was the same as above; however, a different derivatization procedure and mobile phase were employed for resolution and detection of pyruvate. Reaction mixtures were derivatized with 2,4-dinitrophenylhydrazine in 50 % methanol/water. The derivatives were resolved using a gradient of 40 – 85 % methanol in 50 mM tetrabutylammonium hydroxide, pH 4.3, and detected at 366 nm.[27]

2.3.4 Determination of catalytic constants and inhibitor-sensitivity

The first task of this paper was to compare the catalytic activities of mouse and human SR. As noted in Section 1.2.1, SR catalyzes not only the interconversion of LS and DS, but also their elimination. Thus, using the activity assays described above, we measured the initial velocity of five SR-catalyzed reactions (LS → DS, DS → LS, LS → pyruvate, DS → pyruvate, LSOS → pyruvate) at a variety of substrate concentrations. SR follows Michaelis Menten kinetics, and we therefore employed the nonlinear fit template offered in the GraFit software package [96] to determine the catalytic constants K_M, the substrate concentration at which the reaction rate

reaches half its maximal value, and k_{cat}, the "turnover number," or the number of reactions catalyzed per time unit. In almost all cases, the constants were very similar for mouse and human serine racemase. The only noteworthy difference was the K_M values for DS elimination. One might expect that the observed K_M values for DS racemization and elimination would be similar, since both reactions are thought to occur at the same active site. Indeed, this is the case for hSR. However, in the case of mSR, the observed K_M for DS elimination is significantly lower than the observed K_M for DS racemization. In fact, the observed K_M for DS elimination is similar to the K_M for LS racemization and elimination. From these results, we concluded that hSR and its mouse ortholog behave similarly *in vitro* in general, though there may be minor differences in their manner of DS processing.

We next investigated whether or not the orthologs were similarly sensitive to a selection of SR inhibitors. For the reasons outlined in Section 1.5, SR inhibitors could be useful tools for investigation and treatment of a variety of neuropathologies. However, most of the inhibitor studies performed to date have been carried out with the mouse ortholog. We selected three well-characterized competitive mouse SR inhibitors (LEHA, malonate, glycine) and tested them for inhibition of hSR. All three compounds inhibited hSR with a competitive mechanism of action. In all cases, the K_i values for hSR were slightly lower than for mSR, but the order of inhibition potency was the same (LEHA was most potent, followed by malonate, then glycine). Thus, we concluded that mouse and human SR share similar inhibitor-sensitivity, providing a partial rationale for the use of mouse models in SR inhibitor development.

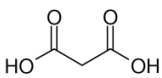
During the course of this work, Dr. Marketa Zvelebil prepared homology models of both human and mouse SR. Though we did not describe this work in detail here, the models support the experimental data. The majority of residue differences seem to occur at the surface of the enzyme, while all the putative substrate and inhibitor binding residues are conserved in both orthologs. Based on these models, we noted one difference between the orthologs – in the channel leading up to the active site residue 231 is a His in mSR and a Tyr in hSR. We generated the Y231H hSR mutant in order to see if this change had any effect on the inhibitor potency. We found that it did not have an appreciable effect.

My contribution: I subcloned the hSR gene from pUC19_hSR into the pMPM expression plasmid, expressed and purified recombinant hSR, performed all the hSR activity and inhibition assays, and wrote the paper.

2.4 Paper II: Background and comments

Paper II evolved as a result of our efforts to identify SR inhibitors more potent than malonate and LEHA. In particular, we hoped to identify a compound with sub-micromolar potency that could be applied to study SR in animal models or tissue cultures. Toward this end, we screened a wide variety of malonate analogs. The first series of compounds was a commercially available set of compounds of the form HOOC-R-COOH. We screened the compounds in the same manner described in Paper II – monitoring of the racemization reaction in the presence of 5 mM substrate (L-serine) and 5 mM test compound.

Parent Compound: Malonic acid



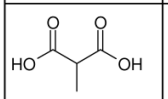
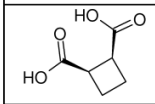
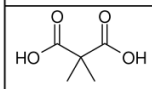
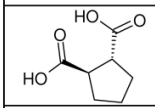
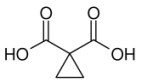
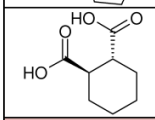
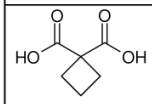
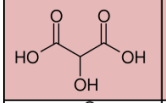
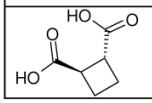
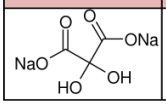
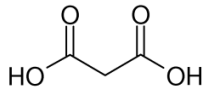
Compound	V_{oi}/V_o	Compound	V_{oi}/V_o
	0.52		0.77
	0.8		1.11
	0.9		1.1
	1.01		0.11
	1.05		0.23

Table 3. *Malonate analogs of the form HOOC-R-COOH screened for inhibition of mSR. The compounds were evaluated in terms of the initial velocity of the inhibited reaction compared to the initial velocity of the uninhibited reaction. The most successful inhibitor from this series, tartronic acid, is highlighted pink.*

Most of the compounds tested did not inhibit or were modest inhibitors. The most promising compound from this series was tartronic acid (Table 3, highlighted), which inhibited mouse SR in a competitive manner with a K_i of 24 ± 6 μM. This does not represent a very significant improvement over malonic acid, which inhibits mSR with a K_i of 70 μM and hSR with a K_i of 33

μM. We therefore abandoned the idea of introducing aliphatic modifications at C2 and focused instead on screening malonate analogs of the form XC-CH_2-CX.

Parent Compound: Malonic acid



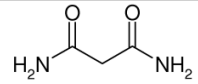
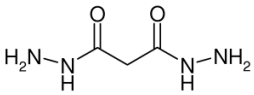
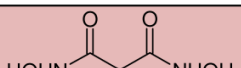
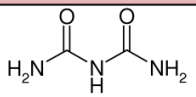
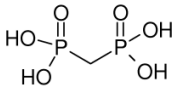
Compound	V_{oi}/V_o
	1.1 ± 0.3
	0.60 ± 0.12
	0.01 ± 0.001
	0.87± 0.06
	0.74 ± 0.08

Table 4. *Malonate analogs of the form XC-CH_2-CX screened for inhibition of mSR. The compounds were evaluated in terms of the initial velocity of the inhibited reaction compared to the initial velocity of the uninhibited reaction. The most successful inhibitor from this series, malonodihydroxamic acid, is highlighted pink.*

This strategy led us to consider hydroxamic acids as a novel class of SR inhibitors – racemization activity was almost completely abolished in the presence of 5 mM malonodihydroxamic acid (highlighted). Due to the reputation of hydroxamic acids as potent metal chelators, we first checked that the mechanism of action was not chelation of the functionally important Mg^{2+} ion. However, the inhibition of SR by these compounds was not diminished even with a large molar excess of $MgCl_2$ over the test compound.

2.4.1 Inhibition screens and enzyme kinetics analysis

We therefore expanded the test set of hydroxamic acids, including a variety of aliphatic dihydroxamic acids as well as hydroxamic acid analogs of amino acids (Figure 1 of the paper). The inhibition screening data revealed that some hydroxamic acids were potent SR inhibitors, while structurally related molecules

had a negligible effect on SR activity. Hydroxamic acid analogs of the known SR inhibitors malonic acid, succinic acid, and L-aspartic acid were particularly effective inhibitors. Determination of the mechanism of inhibition showed that succinodihydroxamic acid and L-aspartic acid β-hydroxamate acted as competitive inhibitors with low micromolar K_i values. In fact, succinodihydroxamic acid appeared to be the most potent competitive SR inhibitor characterized to date, with a K_i value of 3.6 μM. Surprisingly, unlike its dicarboxylic acid analog malonic acid, malonodihydroxamic acid acted in a non-competitive manner.

2.4.2 Specificity study

In order to determine the specificity of the hydroxamic acids for SR, we tested selected compounds for inhibition of a small panel of structurally and functionally diverse PLP-dependent enzymes. Both of the dihydroxamic acids we chose (malono- and succino-) had an inhibitory effect on all the enzymes tested; in contrast, malonic acid was specific for SR. This led us to hypothesize that dihydroxamic acids are somehow reactive with PLP. Indeed, follow-up experiments confirmed this notion. When the reaction buffer was supplemented with increasing amounts of PLP, we observed quenching of the inhibition.

Unlike malono- and succinodihydroxamic acid, L-aspartic acid β-hydroxmate showed some specificity, acting as an effective inhibitor of SR and its close homolog serine dehydratatase but not of the more distantly related alanine racemase. The compound acted as a weak substrate of transaminase, the natural substrate of which is L-aspartic acid.

The specificity screening indicated that, due to their general reactivity with PLP-dependent enzymes, the dihydroxamic acids are not ideal for tissue culture or *in vivo* applications. However, L-aspartic acid β-hydroxamate, with SR selectivity and low micromolar potency, could be a useful lead molecule for further inhibitor development.

2.4.3 Solution stability and reactivity studies

We decided to investigate the apparent PLP-reactivity of hydroxamic acids in detail, as this property of hydroxamic acids had not been previously reported. Since PLP contains a chromophore, we first focused on analysis of buffered aqueous solutions of PLP in the absence and presence of hydroxamic acids by UV/vis spectrophotometry. This analysis showed that a reaction indeed takes place in the presence of the inhibitory dihydroxamic acids. The spectral shift that occurs in the presence of these compounds is identical to the spectral shift that occurs when hydroxylamine (NH_2OH) is added to PLP. The reaction of hydroxylamine and PLP is well-known and results in formation of a PLP-aldoxime. The similar spectral shifts observed in the reaction of PLP and dihyroxamic acids may indicate that similar or identical

products are formed. Addition of L-aspartic acid β-hydroxamate to PLP resulted in a unique spectrum, with a shift that we hypothesized might correspond to a PLP-amino-acid aldimine.

In order to support the results of the UV/vis assay, we employed ESI-MS and NMR analysis of the reaction mixtures. In the case of the dihydroxamic acids, application of these methods allowed us to unambiguously identify the reaction products as the *syn*- and *anti*-aldoxime shown in Scheme 1 of the paper. In the case of L-aspartic acid β-hydroxamate, we were not able to reach clear-cut conclusions. However, we did collect strong support in favor of the aldimine hypothesis from both the MS and NMR data.

The final question that remained was why some dihydroxamic acids react with PLP to form aldoximes, while structurally related compounds do not. Hydrolysis of some dihydroxamic acids to release hydroxylamine, which can then react with PLP to form aldoxime species, was an attractive hypothesis, especially since it has been shown that oxalo-, malono-, and succinodihydroxamic acid exhibit strikingly different hydrolytic stabilities in acid [97]. We therefore conducted hydrolysis experiments in buffered aqueous solutions (pH 8.0 or 7.4), and we found that, indeed, the inhibitory dihydroxamic acids undergo hydrolysis to release hydroxylamine. Based on the available data, intramolecular condensation resulting in hydroxylamine release is an attractive hypothesis. The non-inhibitory oxalo-, adipo-, and suberodihydroxamic acids would form sterically unfavored 3-, 7- and 9-membered rings, respectively. On the other hand, the inhibitory compounds succino- and glutarodihydroxamic acid would form 5- and 6-membered rings, respectively, which are favored conformations. Intramolecular condensation of malonodihydroxamic acid would result in formation of a 4-membered ring, which is not an especially favored conformation, so it is possible that this compound undergoes hydrolysis *via* a different mechanism. The hydrolysis experiments were supported by MS analysis of 1-2 day old inhibitor solutions. In the case of the inhibitory compounds, significant signals corresponding to the respective monohydroxamic acids were visible, as well as signals that might correspond to cyclic species (monohydroxamic acids minus a water molecule; however, these signals could also be an artifact of ionization). In the case of non-inhibitory compounds, the only significant signal observed was that corresponding to the dihydroxamic acid.

My contribution: I participated in the initial screening, and I conducted the specificity study, UV/vis spectrophotometry, and hydrolysis experiments.

2.5 Paper III: Background and Comments

A decade after the discovery of mammalian serine racemase, a three-dimensional structure of the enzyme remains elusive. The 3D structure of mammalian SR would be a valuable tool for increasing understanding of SR catalysis, structure-function relationships within the enzyme, and possible regulatory mechanisms as well as for the design of active-site-directed ligands. Our group has been involved in attempts to crystallize the mouse and human SR orthologs for several years without any great success (see Chapter 3). Random mutagenesis in combination with multiple sequence alignment offers an attractive, fairly straightforward way to identify structurally and catalytically important residues.

2.5.1 Random mutagenesis and screening

A large-scale random mutagenesis experiment requires an easy method for purification and analysis of mutants. The three-step purification strategy outlined in Paper I is less than ideal for this purpose, so we chose to work with the C-terminally His-tagged hSR construct (see Table 2), which allows for easy, 1-step purification. We aimed for a mutation rate of 1-4 amino acid changes per construct. Based on a recent review of mutagenesis strategies [98], we elected to use error-prone PCR (EP PCR) to introduce random mutations into the SR gene; and we used two different EP PCR protocols to ensure a high level of diversity. The mutated hSR genes were ligated into the pMPM expression vector in order to produce a plasmid library.

The plasmid library was transformed into an expression strain, and the resulting colonies were screened by dot blot (see Figure 1 of the paper), which allowed us to select soluble, well-expressing hSR variants. The corresponding bacterial colonies were picked and used to inoculate small quantities of auto-inducing medium. The use of auto-inducing medium [99] offered two advantages. First, the cultures could be inoculated in the evening and harvested the next morning, saving time and allowing for quick processing of the samples. Second, the cultures grew to a very high density (OD_{600} of 10 – 15 at the time of harvest). Thus, despite the rather low expression yield of His-tagged hSR, a single culture grown in a 50 mL falcon tube was generally sufficient to obtain enough protein for activity trials. SR was partially purified by immobilized metal affinity chromatography. Successfully purified mutants were evaluated for their ability to racemize and eliminate LS. When adjusted to account for the quantity and purity

of the mutant in question, the activity results gave us a rough estimate of how the mutant's ability to process LS compared to that of the wild-type (see Table 1 of the paper).

The results obtained from the first twenty-seven distinct mutants characterized showed us that in some cases SR is tolerant of multiple mutations. Furthermore, most of the sites of mutation were predicted to occur at the enzyme surface, which suggested to us that we might be able to use random mutagenesis to identify "crystallizable" SR variants. For the present study, we chose to carefully characterize three of the randomly generated mutants – C217S, K221E, and S84G/P111L.

2.5.2 Biochemical characterization of the mutants

The C217S and K221E mutants were both essentially inactive, and C217 and K221 are located in proximity to the Mg^{2+} binding site. Since SR is activated by the presence of Mg^{2+}, we hypothesized that C217 and K221 may participate directly or indirectly in metal binding. Interestingly, C217 and K221 are not widely conserved among eukaryotic SRs. In contrast, both S84 and P111 are strongly conserved, and furthermore, recent work on *S. pombe* SR implicates S84 in catalysis [38]. These three mutants were expressed and purified on a large scale, and characterized in terms of thermal stability and activity. Under the conditions used, wild-type hSR was mildly thermally stabilized by $MgCl_2$ and ATP and was greatly destabilized by addition of the metal chelator EDTA. The wild-type enzyme was dramatically stabilized by DTT, and both its racemization and elimination activities were strongly activated in the presence of DTT. S84G/P111L exhibited a thermal stability profile analogous to that of wild-type but showed very low activity. LS elimination activity could be partially recovered by addition of DTT, but racemization activity could not. In contrast, the thermal stability data showed that C217S and K221E hSR were deficient in Mg^{2+} binding. These variants also showed extremely low activity levels and low sensitivity to DTT.

To complement the biochemical data, we constructed a multiple sequence alignment of eukaryotic serine racemases. While S84 and P111 are conserved throughout the family, C217 and K221 are conserved only in mammalian serine racemases, while the corresponding residues in plant and yeast SRs vary greatly. Our ongoing homology modeling studies are briefly referred to in the paper, and these studies support a key catalytic role for S84 and an important structural role for P111. A structural reason underlying the impact of the mutations at C217 and

K221 is less clear but is probably related to altered intramolecular interactions in the vicinity of the metal-binding site.

My contribution: I performed the error-prone PCR and cloning and developed the screening assay. I did most of the screening, large-scale expression, and purification work, and I participated in gel filtration analysis of the mutants. I did all of the thermal stability and activity measurements.

Chapter 3: Discussion

SR is an intriguing enzyme, with potential involvement in a variety of neuropathological processes. Since the discovery of eukaryotic SR just over a decade ago, substantial progress has been made toward understanding the enzymatic activity, tissue distribution, and physiological role of this enigmatic enzyme. However, researchers interested in exploiting the therapeutic potential of SR still have a long road ahead of them. In Paper I of this thesis, we showed that recombinant forms of mouse and human SR exhibit comparable catalytic parameters (K_M and k_{cat}) and inhibitor-sensitivity, validating, at least in part, the use of mouse models for discovery and characterization of human SR inhibitors. In Paper II, we identified a class of hydroxamic acids as novel inhibitors of PLP-dependent enzymes. In particular, one of these compounds, L-aspartic acid β-hydroxamate, was selective for SR and its homolog serine dehydratase and showed a competitive mechanism of inhibition with a low micromolar K_i. This compound could serve as a lead molecule for further SR inhibitor development. In Paper III, we used random mutagenesis as a tool to gain insight into structurally and functionally significant residues. Additionally, we constructed a homology model of human SR, which can be a useful tool for increasing our understanding of SR and its regulation and for the design of novel ligands.

Still, though, the SR field is missing a few key discoveries. Specific inhibitors with sub-micromolar potency are needed in order to conduct experiments *in vivo* and in tissue culture. In order to design these specific and potent active-site ligands, it would be useful to have an experimentally determined three-dimensional structure of mammalian SR. However, such a structure remains stubbornly elusive, despite the work of multiple groups. Our group has been engaged in attempts to crystallize recombinant mouse and human SR orthologs for several years. Since attempts to crystallize the wild-type enzymes repeatedly failed, we generated several series of hSR mutants in hopes of finding an enzyme variant with an enhanced ability to crystallize.

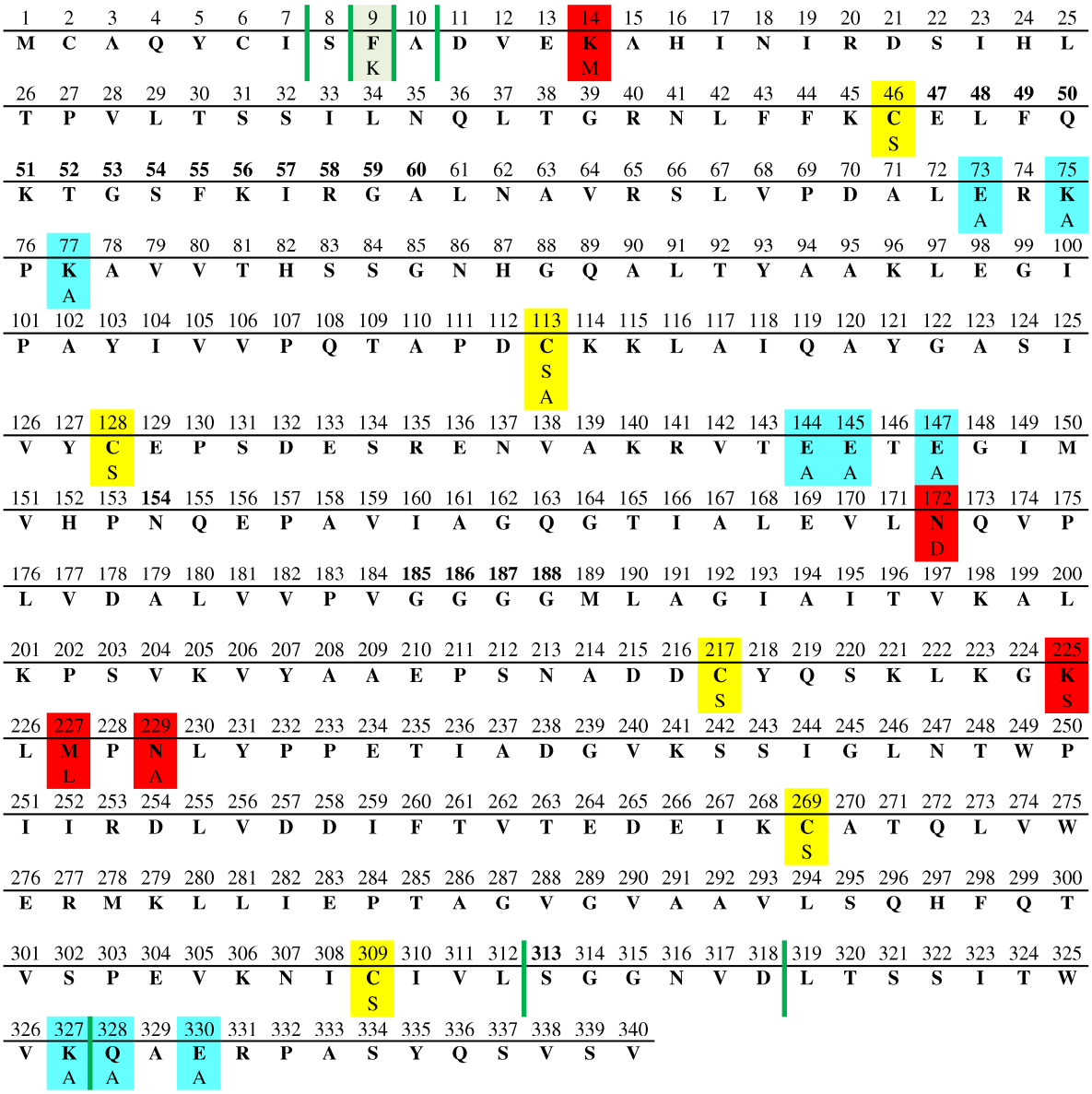


Figure 6. *Primary sequence of hSR with mutation sites indicated. Series 1 mutations are highlighted yellow, series 2 are highlighted blue, and series 3 truncations are indicated in green. Mutations based on random mutagenesis experiments and sequence comparison are highlighted red.*

In the first series of mutants, we individually exchanged six of the eight Cys residues in hSR to Ser or Ala. The soluble expression levels of the mutants were checked, and where appropriate, the racemization activity of partially purified samples (after ammonium sulfate precipitation) was accessed. The C46S mutant had a soluble expression level about 3-fold lower than the wild-type, and furthermore, several degradation products were visible on a Western blot of the lysate.

C113S and C113A hSR expressed well, but were roughly 5-fold less active than wild-type hSR. C128S hSR showed wild-type-like expression and activity levels. This mutant was scaled up and purified (ammonium sulfate precipitation, Q-sepharose, ATP-agarose). 1.5 mg of purified protein were obtained, and these were screened for crystal formation with Sigma Kit #1. No crystals were obtained. The C217S mutant had a soluble expression level comparable to that of the wild-type and was essentially inactive; this mutation was revisited in Paper III. C269S hSR showed WT-like expression levels and activity. I did not work with it further for crystallization purposes since C269 is not conserved in mSR; however, this mutation could be interesting in combination with other mutations. The C309S mutant had extremely low expression levels – at least 5-fold lower than WT, and I did not work with it further due to low expression level.

The second series of mutants consisted of three mutants that were rationally designed according to the results of submitting the hSR sequence to the Surface Entropy Reduction Server (http://www.doe-mbi.ucla.edu/Services/SER) [100]. The SER server suggests mutation candidates that are likely to enhance a protein's crystallizability based on the approach described by Derewenda [101]. Derewenda argues that some proteins contain "entropic shields" composed of long, flexible polar side chains that impede the biomolecule's ability to form intermolecular contacts and render the proteins recalcitrant to crystallization. The SER approach involves replacing high entropy amino acids predicted to occur at the surface of a molecule, usually lysines or glutamates, with low entropy residues such as alanine.

The E73A/K75A/K77A triple mutant showed expression levels near wild-type, but a significant amount of SR in the lysate was degraded. According to our homology model, these residues are located in a flexible, unstructured loop, which makes them good candidates for mutation. However, due to the degradation problem I did not work further with this mutant. The E114A/E145A/E147A mutant also expressed well and was active. According to the homology model, these residues are located in an alpha helix, which does not make them ideal candidates for SER, since ideal candidates should be in an unstructured, surface-exposed loop region. Nevertheless, I attempted to purify the mutant; however, purification of the untagged construct was unsuccessful, as the protein precipitated on the ion-exchange column at a variety of pHs. However, purification of E144A/E145A/E147A hSR with a C-terminal polyhistidine tag was successful, and crystal screening of the His-tagged enzyme was conducted at the National Cancer

Institute (Frederick, Maryland, USA) in autumn 2009. Unfortunately, no promising conditions were identified as a result of the screening. The third SER-inspired mutant was also interesting. K327A/Q328A/E330A expressed well, but almost all of the protein was degraded – on Western blot, only a tiny band was visible at the expected molecular weight, while most was truncated (to around 33-35 kDa). The partially purified preparation did exhibit SR activity. The truncated portion was subject to N-terminal sequencing and had the same N-terminus as the wild-type, suggesting that the C-terminus is truncated. We attempted to characterize the truncated species by mass spectrometry, but reliable data could not be obtained after several attempts. However, the idea of obtaining a soluble, active truncated form of hSR led us to mutant series 3.

Since the N- and C-termini of SR are predicted to be flexible, they might negatively influence the protein's solubility/crystallizability. So, for series 3, several truncated constructs were prepared. Using pMPM_hSR or pMPM_hSR6His as a template, the appropriate sequence was amplified with primers including *Nde*I and *Xho*I sites and then cloned into pMPM. Plasmids were transformed into *E. coli* MC1061 cells and induced with 1 mM arabinose, and soluble expression levels were evaluated by Western blot.

Construct **9-327 (F9K)**, which starts at residue 9 of hSR, ends at residue 327, and contains the F9K mutation, was designed based on predictions that the first secondary structural element of SR (putatively an alpha helix) begins somewhere between residues 9 - 11. The ninth residue of hSR is Phe. Since proteins starting with Phe are easily degraded in *E. coli* (according to the N-end rule), residue 9 was mutated to Lys. An AAA codon immediately following the initiation codon ATG has been correlated with high expression levels of recombinant proteins in *E. coli* [102]. K327 is predicted to be the last residue of the last secondary structural element, an alpha helix. Asp318 is the last residue that is homologous with the *S. pombe* enzyme; the rest of hSR shares no homology with the yeast ortholog. Thus, we also attempted to express **9-318 (F9K)** hSR. Leu312 is predicted to be the last residue of the last beta-sheet of hSR, and construct **9-312 (F9K)**, which lacks the terminal alpha helix, was also screened for SR expression. Since different methods of secondary structure prediction indicated that the first secondary structural element of hSR may start at either residue 9, 10, or 11, we also screened constructs **10-327, 10-318**, **10-312**, **11-327**, **11-318**, and **11-312.** Unfortunately, all of these double-truncated (N- and

C-terminus) mutants were either insoluble or exhibited compromised solubility. Therefore, we did not attempt large-scale expression or purification of any of these constructs.

Rather, we cloned a series of constructs with truncations at the N- or C-terminus (series 4). The following constructs based on series 3 were tested: **9-340 (F9K), 10-340, 11-340, 1-327**, **1-318**, and **1-312**. Additionally, hSR **8-340** was generated; the first 7 amino acid residues were deleted based on the fact that the corresponding residues do not appear in the crystal structure of *S. pombe* SR and are therefore probably quite unstructured. However, like the double-truncated mutants of series 3, the single-truncated mutants of series 4 all exhibited extremely low soluble expression levels compared to the wild-type.

In addition to expressing hSR mutants in *E. coli*, we also attempted expression of the wild-type enzyme in Schneider's S2 cells. While some SR was present in the growth media, expression levels were extremely low – so low, in fact, that our trial expression did not even yield enough SR for activity trials.

In Paper III, we describe the generation of random hSR mutants, with most mutations occurring at the enzyme surface, and we suggest that such mutants might have an enhanced ability to crystallize in comparison to the wild-type. Based on comparison of our hSR homology model with the crystal structure of *S. pombe* SR, the only eukaryotic SR that has been crystallized to date, we chose the random mutant K14M/N172D/M227L for crystallization at the NCI in Frederick in late 2009. However, the screening did not result in any crystals. Further comparative analysis of the hSR model and the SpSR crystal structure led us to add two more mutations to the K14M/N172D/M227L mutant. The human and *S. pombe* SR sequences differ greatly in a large, flexible loop region that participates in crystal contacts in the *S. pombe* structure. We attempted to make this region of hSR more similar to that of SpSR by adding two new mutations – K225S and N229A – to the K14M/N171D/M227L background. The K14M/N171D/K225S/M227L/N229A mutant was also screened at NCI, and it formed crystals in 10 different conditions. Unfortunately, 9 of these turned out to be salt crystals. However, one condition seemed promising; 0.1 M HEPES pH 7.5 with 20% (v/v) Jeffamine M-600 yielded thin, rod-like protein crystals. These crystals did not diffract to an appreciable extent, and attempts to optimize these conditions are currently in progress.

The three-dimensional structure of a mammalian SR would undeniably be a useful tool for furthering our understanding of the SR reaction and control mechanisms and for the design of specific ligands for *in vivo* applications. Experimental structure determination will undoubtedly be a major future focus in the SR field. It's important to remember, though, that a biochemical understanding of SR function is only one small part of the puzzle surrounding the role of D-serine in the human body. D-serine is degraded by D-amino acid oxidase, and the expression and activity levels of this enzyme are thought to work in concert with SR to regulate D-serine levels in the body. Furthermore, the respective contributions of D-serine and glycine to NMDAR signaling have yet to be unraveled, a task that is complicated by the multitude of NMDAR subtypes and their diverse trafficking, pharmacology, and developmental expression patterns. Additionally, the role of D-serine, SR, and DAAO outside of the CNS remains an open area of investigation. Investigation of D-serine and its synthetic and degradatory enzymes in healthy and diseased individuals will pave the way for greater insight into how we view glutamatergic signaling pathways and will perhaps open the door for new methods of therapeutic intervention.

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Prohlášení

Prohlašuji, že jsem tuto práci ani její podstatnou část nepředložila k získání jiného nebo stejného akademického titulu.

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Hillary Elizabeth Hoffman

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My contribution to the publications included in the thesis:

Paper I

I subcloned the hSR gene from pUC19_hSR into the pMPM expression plasmid, expressed and purified recombinant hSR, performed all the hSR activity and inhibition assays, and wrote the paper.

Paper II

I participated in the initial screening, and I conducted the specificity study, UV/vis spectrophotometry, and hydrolysis experiments.

Paper III

I performed the error-prone PCR and cloning and developed the screening assay. I did most of the screening, large-scale expression, and purification work, and I participated in gel filtration analysis of the mutants. I did all of the thermal stability and activity measurements.

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Jan Konvalinka, Ph.D.

Supervisor

Appendix: Reprints of the publications described in the thesis

The following section contains reprints of three publications:

Paper I:

Hoffman, H.E.; Jirásková, J.; Ingr, M.; Zvelebil, M.; Konvalinka, J., Recombinant human serine racemase: enzymologic characterization and comparison with its mouse ortholog. *Protein Expr Purif.* 2009, *63*(1), 62-67.

Paper II:

Hoffman, H.E.; Jirásková, J.; Cígler, P.; Šanda, M.; Schraml, J.; Konvalinka, J., Hydroxamic acids as a novel family of serine racemase inhibitors: mechanistic analysis reveals different modes of interaction with the pyridoxal-5'-phosphate cofactor. *J Med Chem.* 2009, 52(19), 6032-6041.

Paper III:

Hoffman, H.E.; Jirásková, J.; Zvelebil, M.; Konvalinka, J., Random mutagenesis of human serine racemase reveals residues important for the enzymatic activity. *Collect Czech Chem Commun.* 2010. *In press.*