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Biochemical characterization of human serine racemase: substrates, inhibitors and allosteric effectors

Coordinator:
Prof. Andrea Mozzarelli

Tutor:
Prof. Alessio Peracchi

PhD student:
MARIALAURA MARCHETTI

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INTRODUCTION

D-SERINE: ORIGIN, LOCALIZATION AND FUNCTIONS

Biological role of D-amino acids in mammals

Amino acids are among the most important biological molecules, both as components of proteins and in signal transmission. Until recently, it was thought that L-amino acids were accidentally selected during evolution as the only biologically relevant forms and homochirality was considered essential for life because of its role in the stabilization of polypeptides, enzymatic specificity and structural interactions (Mason 1984).

During the twentieth century, several studies suggested the presence of several D-amino acids in microorganisms, such as D-alanine and D-glutamine, in the peptidoglycan of the cell walls (Corrigan 1969). Later, it was demonstrated that bacteria can produce, metabolize and utilize D-amino acids.

In the early nineties, some pioneering studies highlighted the presence of relevant concentrations of free D-amino acids in the mammalian central nervous system (CNS) and peripheral tissues, in particular D-aspartate and D-serine (Hashimoto et al. 1992 A; Schell et al. 1995). However, their functional roles were not clear and in many cases these molecules were assumed to derive from the endogenous microbial flora, from the diet or from the spontaneous racemization of L-amino acids during ageing.

Nowadays, it is known that D-serine and D-aspartate play important, specific roles in neurotransmission and in development and endocrine regulation (Yang et al. 2005; Wolosker 2007; Wolosker et al. 2008; Van Horn et al. 2013). Altered levels of these two amino acids are associated with various pathological conditions and the enzymes involved in their metabolism have been proposed as therapeutic targets (Fuchs et al. 2005).

Localization of D-serine and its functions

D-serine was first identified in the brain of rodents (Hashimoto et al. 1992 A); subsequently, many studies confirmed the presence of large amounts of this amino acid in the cerebral cortex, hippocampus and striatum, limbic forebrain, diencephalon and midbrain, and low levels in the pons, medulla, cerebellum and spinal cord (Hashimoto et

al. 1993 A; Hashimoto et al. 1993 B; Nagata et al. 1994). D-serine concentration in the brain has been shown to vary during development, both in humans and rodents, with the highest levels recorded during embryonic and early postnatal life (Hashimoto et al. 1993 A).

Although D-serine has been detected also in peripheral tissues, its main localization is in the CNS and its distribution strongly correlates with that of the *N*-methyl D-aspartate receptors (NMDARs), in particular NR2A/B subtypes, a very important type of ionotropic glutamate receptors involved in neuro-excitatory transmission (Schell et al. 1997).

NMDA receptors

NMDARs are ionotropic glutamate receptors, highly permeable to Ca^{2+}, which mediate the majority of excitatory neurotransmission in the mammalian CNS. Every NMDAR is a heterotetramer, composed of two GluN1 (also called NR1) subunits, together with either two GluN2 (or NR2) subunits or a combination of GluN3 (or NR3) subunits (Fig. 1) (Al-Hallaq et al. 2002; Schuler et al. 2008). The different subunit assembly is regionally regulated during the development and in different brain areas (Wong et al. 2002). The activation of these receptors requires the simultaneous binding of both an agonist (glutamate or aspartate, on GluN2 subunits) and a co-agonist (on GluN1 or GluN3 subunits). At first, this co-agonist site was thought to be physiologically occupied by glycine, but it was later demonstrated that D-serine binds more tightly and represents the main physiological regulator in many brain areas (Furukawa and Gouaux 2003).

In resting neurons, the NMDAR ion channel is blocked by an Mg^{2+} ion (Fig. 1). The activation is voltage-dependent and after the activation of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPAR), the entry of Na^{+} causes the depolarization of the membrane and the removal of Mg^{2+} block on NMDARs.

NMDARs are associated with synaptic plasticity, long term potentiation (LTP) and neuro-toxicity (Kim et al. 2005; Panatier et al. 2006; Henneberger et al. 2010). The specific deletion of the gene encoding for serine racemase (SR), the enzyme responsible for D-serine synthesis, causes a decrease of more than the 85% of normal forebrain D-serine levels and altered glutamatergic neurotransmission in mouse models. These findings

reinforce the notion that D-serine is a physiological NMDAR co-agonist (Basu et al. 2008; Inoue et al. 2008; Horio et al. 2011).

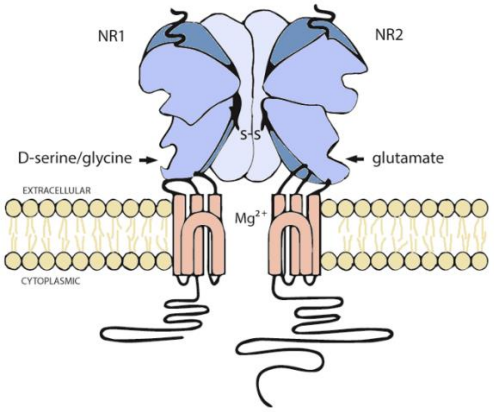


Figure 1: Section of an NMDA receptor.

Origin and brain distribution of D-serine: neurons versus astroglia

Humans can obtain D-serine from food, as a metabolite derived from the resident flora or through *de novo* biosynthesis. The main source of D-serine is L-serine, which can be racemized into its enantiomer by SR. D-serine concentration is consequently related to L-serine levels and reaches on average 0.3 mM, which is about one-third of the intracellular L-serine concentration (Hashimoto et al. 1992 A). The first step in L-serine biosynthesis is performed by 3-phosphoglycerate dehydrogenase (Phgdh), which has an exclusive localization in astrocytes (Yamasaki et al. 2001). In mouse models, deletion of Phgdh results in decreased D-serine levels, showing the importance of astrocytic Phgdh in D-serine generation (Yang et al. 2010).

The mechanisms that control D-serine release are not thoroughly understood yet and its pattern of distribution is not well defined. Moreover, the contribution of astrocytes and neurons to D-serine dynamics are not entirely clear. In the past twenty years, different studies reported discordant results about D-serine distribution and the role played by brain glia and neurons.

D-serine distribution must be analyzed, of course, also with respect to the expression of SR. At first, SR was thought to be predominantly localized in astrocytes. For this reason,

D-serine had been initially classified as a glio-transmitter (Martineau et al. 2006), as confirmed by many experimental evidences. When hippocampal neurons are co-cultured with astrocytes or supplemented with exogenous D-serine, LTP is evoked (Yang et al. 2003). In addition, the blockage of glial metabolism impairs NMDAR-mediated synaptic transmission and LTP induction by reducing extracellular levels of D-serine (Henneberger et al. 2010; Fossat et al. 2012).Other studies demonstrated that SR is localized also in neurons (Wolosker et al. 2008). In the meantime, the employment of new, more sensitive and specific antibodies, immunoisolation techniques and SR-KO mice revealed a more prominent SR expression in neurons than in astroglia (Kartvelishvily et al. 2006; Miya et al. 2008; Balu et al. 2014). Specific deletion of SR gene in neurons leads to lower D-serine levels in forebrain, corresponding to LTP deficits. In contrast, the same deletion in astrocytes results in a minimal variation of SR expression, along with almost unchanged levels of D-serine and NMDAR activity (Benneyworth et al. 2012). The strong reduction in SR expression in mice with neuron-selective SR deletion, however, is not linked to a comparably decreased concentration of D-serine, that could be therefore provided by peripheral sources (Benneyworth et al. 2012).

To date, the predominant localization of D-serine in astrocytes or neurons is still debated (Kartvelishvily et al. 2006; Williams et al. 2006; Martineau et al. 2013; Balu et al. 2014). In agreement with the astrocytic compartmentalization, the hypothesis of a serine shuttle gives a possible explanation about the distribution of D- and L-serine in the brain. According to the serine shuttle model, L-serine would be almost exclusively synthesized in astrocytes from glucose by Phgdh. It would then be exported to neurons, where it would be converted into D-serine by SR. D-serine would be subsequently shuttled back and stored in astrocytes, from which it could be released upon stimulation (Fig. 2) (Wolosker 2011).

The results reported by Balu and coworkers seem to support this hypothesis, even though D-serine would not be shuttled between neurons and astrocytes but rather between neurons (Balu et al. 2014).

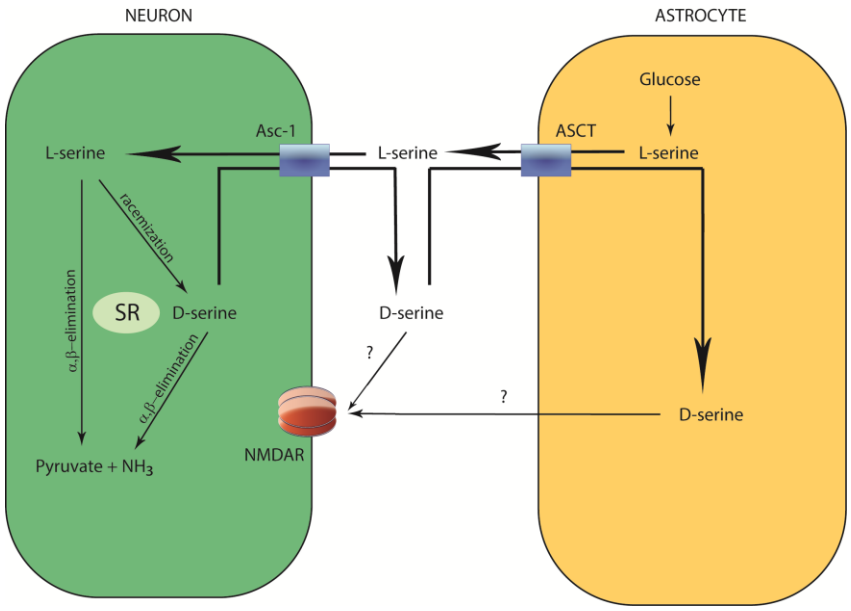


Figure 2: The serine shuttle between neurons and astrocytes (Wolosker 2011).

Neuron-derived D-serine also plays an important role in NMDAR activity, enhancing LTP and synaptic transmission and plasticity (Rosenberg et al. 2013) and controlling dendritic arborization and spine density in pyramidal neurons of somatosensory cortex (Balu and Coyle 2012).

D-serine dynamics is evidently fine-tuned and needs the participation and regulation of both neuronal and astrocytic metabolisms. For this reason, any definitive conclusion cannot be drawn about the role of each source, in relation to the available data. It is possible that spatio-temporal dynamics and metabolic needs could require preferentially the participation of neuronal or glial D-serine sources, depending on adaptive mechanisms at individual or groups of synapses (Martineau et al. 2014). In addition, D-serine metabolism is developmentally regulated (Puyal et al. 2006; Dun et al. 2008). Under these assumptions, a differential onset of glial and neuronal D-serine release during postnatal development can be envisaged. Moreover, many data demonstrate that the astrocytic network is formed only after several weeks after birth (Yang et al. 2013). It can be theorized, therefore, that neuronal D-serine will be available and maintained during postnatal development and adulthood, while glial D-serine would become accessible only after the third postnatal week (Martineau et al. 2014).

HUMAN SERINE RACEMASE

The biosynthesis of D-serine in mammals was clarified in 1999 by Wolosker and colleagues by the identification of SR gene in the rat brain (Wolosker et al. 1999). The following year, the human gene was cloned and the enzyme was functionally characterized (De Miranda et al. 2000).

Human SR (hSR, E.C. 5.1.1.18) is a pyridoxal 5'-phosphate (PLP) dependent enzyme of 37 kDa, belonging to the fold-type II and is active as a homodimer. SR catalyzes the reversible racemization of L-serine into its enantiomer and the degradation of both L- and D-serine to pyruvate and ammonia, by eliminating a water molecule. hSR is mainly expressed in the brain, but recently it has also been discovered away from the CNS, i.e. in keratinocytes (Inoue et al. 2014). Its principal role, however, is linked to the NMDAR neurotransmission, which mediates important events, such as synaptic plasticity, LTP and cell migration (Kim et al. 2005; Panatier et al. 2006; Henneberger et al. 2010).

Structure

There are two hSR crystallographic structures available in the PDB (3L6B and 3L6R), both complexed with malonate (Smith et al. 2010), a competitive inhibitor of the enzyme (Hoffman et al. 2009 A).

hSR is a homodimer and every monomer is composed of 340 residues, organized in a larger and a smaller domain, both characterized by an open α-β architecture. The large domain is formed by 6 β-strands surrounded by 10 α-helices and contains the PLP cofactor, covalently bound to Lys56, and an atom of a divalent cation, namely magnesium or calcium (manganese in the available crystallographic structures). The small domain is made up of 4 β-strands surrounded by 3 α-helices. The two domains are connected by a very flexible hinge region (Fig. 3).

Human and rat SR are about 90% identical in sequence and almost structurally indistinguishable. In addition, there is a 40% sequence identity between *S. pombe* and human and rat SR. By the superimposition of the SR structures of *S. pombe* (spSR, 1V71 and 1WTC), *R. norvegicus* (rSR, 3HMK and 3L6C) and *H. sapiens*, Smith and colleagues could

analyze the domains rearrangements in the holo and malonate-complexed forms (Smith et al. 2010).

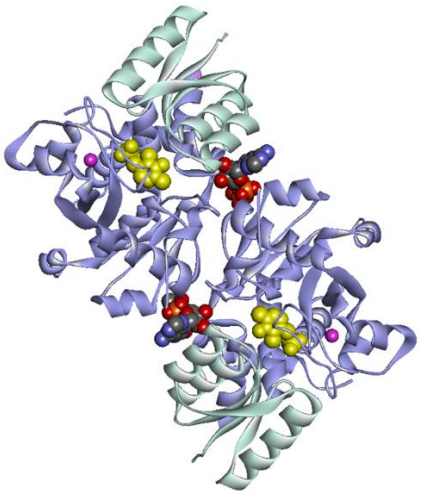


Figure 3. Homodimeric structure of hSR (PDB 3L6B). Every monomer is formed by a larger domain (light violet) and a smaller domain (light green). At the interface between the two monomers there are two adenylylmethylenediphosphonate (AMP-PCP, an ATP analog) molecules. The PLP (yellow) is covalently bound to Lys56 in the active site. Every large domain coordinates an atom of Mn^{2+} (pink).

The presence of the hinge region allows for the movement of the small domain during the closure of the catalytic site. This rearrangement occurs only after recognition of the substrate, along with the concomitant formation of the active site pocket. When the active site is occupied by a ligand, the enzyme shows an open-to-closed conformation shift, as it can be seen from the structural alignment of the rat holo and the complexed human SR (Fig. 4) (Smith et al. 2010).

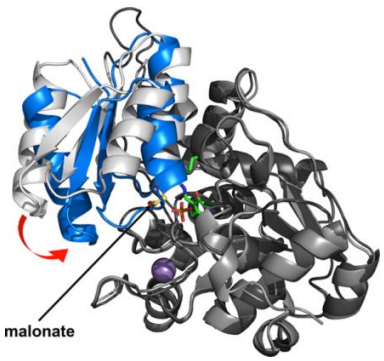


Figure 4: Structural alignment of rat holo (grey) and complexed human (blue) SR (Smith et al. 2010). The red arrow indicates the movement of the small domain from the open (unbound) to the closed (ligand bound) conformation.

At the interface between the two monomers, every dimer can bind two molecules of ATP complexed with Mg^{2+}, which behave as structural cofactors and are not hydrolyzed during catalysis. The phosphate groups are buried inside the dimerization interface, with the adenosyl group facing the external solution (Fig. 3). Until now, there is no crystallographic structure available for the human enzyme complexed with ATP. Recently, Jiraskova-Vanickova and colleagues reported a model for hSR-ATP where two Mg^{2+}-ATP molecules were docked into the hSR dimer, using the spSR-ATP structure as a template (Jiraskova-Vanickova et al. 2011).

Catalysis

Like many other PLP-dependent enzymes, hSR is able to catalyze two distinct reactions on the same substrate: it can racemize L-serine into its enantiomer (and vice versa) or it can eliminate a water molecule from L- or D-serine, producing pyruvate and ammonia. These two reactions are possible thanks to the presence into the active site of the PLP cofactor, which delocalizes the negative charges on the pyridine ring, functioning like an "electron sink" (John 1995).

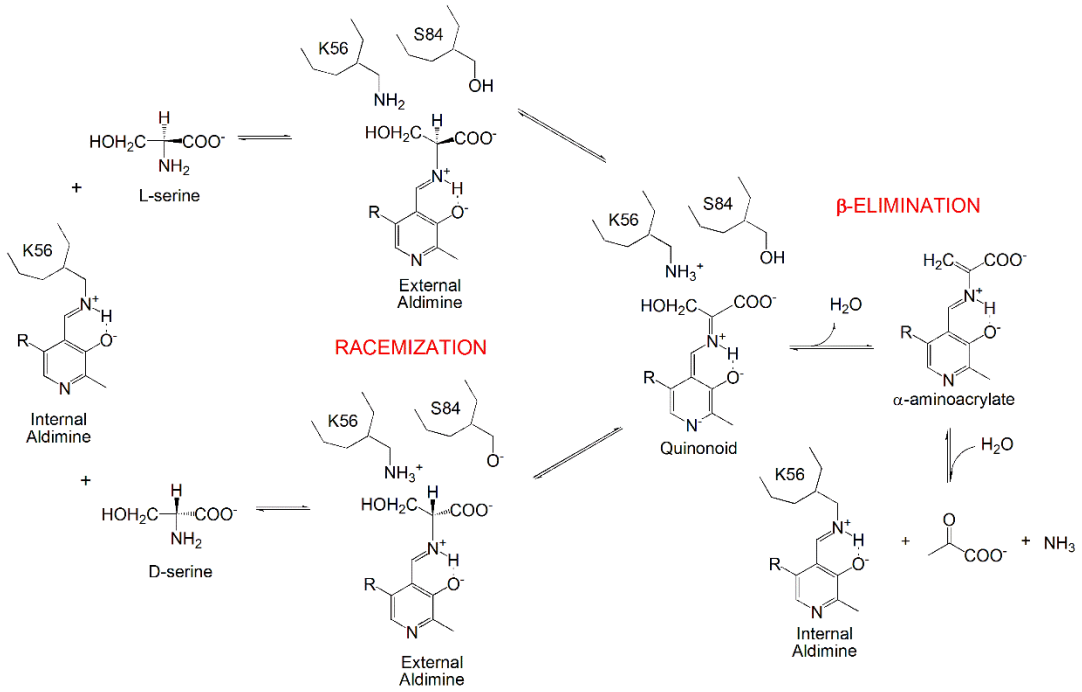
The catalytic mechanism of mammalian SR has never been studied in detail, because of the low purification yields of the recombinant protein. The data available thanks to the crystallographic structures and the studies on the bacterial homologs contributed to the determination of a reaction mechanism (Goto et al. 2009; Smith et al. 2010).

The first steps of the reaction mechanism are common to all PLP-dependent enzymes. In the holo hSR, PLP forms a Schiff base with the ε-amino group of Lys56, called internal aldimine. When the amino acid substrate (either L- or D-serine) enters the active site, the PLP is attacked on its C_4 by an unprotonated α-amino group to form a new Schiff base (external aldimine). Concomitantly, Lys56 is released in an unprotonated state (Scheme 1).

The racemization, the main physiological reaction, proceeds through a two-base mechanism that involves Lys56 and Ser84. These two residues act on the opposite sides of the substrate: in particular, Lys56 deprotonates L-serine, while D-serine is deprotonated by Ser84. The amino acid is then re-protonated on the opposite side by the other residue (L-

serine by Ser84 and D-serine by Lys56, respectively) (Scheme 1). Because of the absence of a protonated nitrogen on the pyridine ring of fold type II enzymes (Griswold and Toney 2011), no formation of a quinonoid intermediate was postulated. Although it has never been isolated for hSR, its existence has been however hypothesized on the basis of studies carried on alanine racemase (Toney 2005).

The β-elimination activity is supposed to be a residual reaction conserved through evolution from a common ancestor of SR and serine dehydratase. This hypothesis is supported by the decreasing catalytic efficiency observed from *Archea* to mammals (Jiraskova-Vanickova et al. 2011). This activity, however, could be more relevant *in vivo* than it has been believed and the ratio between racemization and elimination is regulated by the relative concentrations of ATP and divalent cations (De Miranda et al. 2002).



Scheme 1: Reaction mechanism of hSR.

The β-elimination activity was first observed on β-substituted amino acids by Wolosker and colleagues (Panizzutti et al. 2001), while the β-elimination of serine was described one year later (De Miranda et al. 2002).

The elimination occurs via the hypothetical quinonoid intermediate and proceeds through the protonation of the β-hydroxyl group (by Lys56 or Ser84 on L-serine or D-serine, respectively). A water molecule is subsequently eliminated and the α-aminoacrylate formed spontaneously degrades into pyruvate and ammonia (Scheme 1) (Foltyn et al. 2005).

Wolosker and colleagues proposed that, under physiological conditions, β-elimination of D-serine acts like a natural regulation on neurotransmitter concentrations (Foltyn et al. 2005). This observation could be relevant in particular in the brain areas lacking D-amino acid oxidase (DAAO), an enzyme involved in the catabolism of neutral D-amino acids (Krebs 1935; Pollegioni et al. 2007; Pollegioni and Sacchi 2010). The production of pyruvate from the β-elimination of D-serine is unlikely to have an energetic role because its amount is estimated to be less than 0.1% of that obtained by glycolysis (Dunlop and Neidle 2005).

Regulation of SR

The activity of hSR is finely regulated by many interacting factors, including cations, organic and inorganic molecules and proteins (Fig. 5). The regulation is carried out at different levels and in different ways, and it is still an object of study.

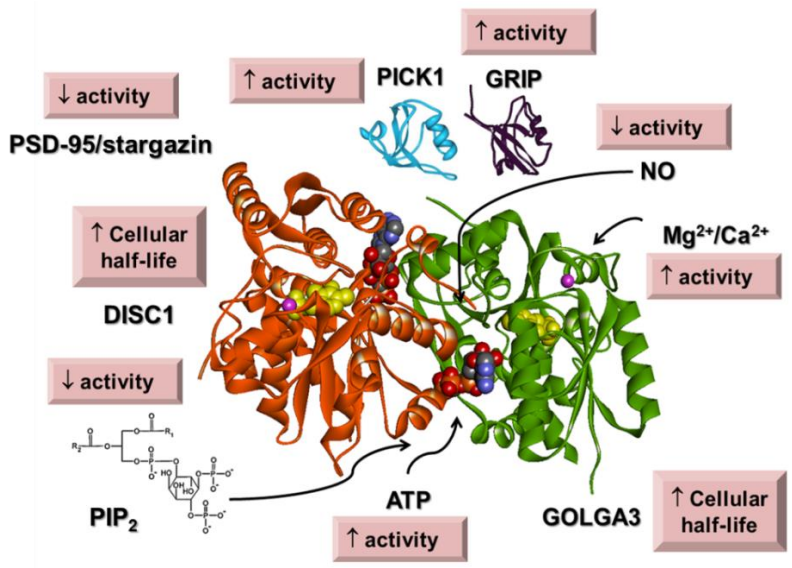


Figure 5. SR regulation by proteins, cations, organic and inorganic molecules and their effect on the enzyme.

ATP and cations

The initial studies on the allosteric regulation were performed on mouse brain SR (Neidle and Dunlop 2002). The first regulators to be discovered were ATP and divalent cations, in particular magnesium and calcium, both exercising a positive effect on the catalysis (De Miranda et al. 2002; Neidle and Dunlop 2002). ATP and Mg^{2+} act synergistically and induce a 5-fold increase in the racemization reaction. Furthermore, there is no detectable production of pyruvate in the absence of cofactors (De Miranda et al. 2002). ATP can strongly activate SR only in the presence of Mg^{2+} because it binds to the protein as Mg^{2+}-ATP complex. The other nucleotide triphosphates, as well as ADP and AMP, can activate the enzyme to lesser extent than ATP (De Miranda et al. 2002; Neidle and Dunlop 2002). ATP and cations probably cause conformational changes that stabilize a more active structure of the enzyme, affecting the K_M but not the k_{cat} (De Miranda et al. 2002; Neidle and Dunlop 2002). Interestingly, it has been found that mouse SR (mSR) is fully activated by ATP and Mg^{2+} at concentrations much lower than those found in the cytosol (De Miranda et al. 2002). Thus, until recently, SR has been thought to be fully saturated (and constitutively activated) by Mg^{2+} and ATP *in vivo.*

The conformational changes induced by calcium binding have been studied on mSR by Cook and colleagues (Cook et al. 2002). In this work, they demonstrate that increased intracellular calcium concentration activates SR in primary astrocytes and raises the levels of extracellular D-serine. The half maximal stimulation observed, however, occurs at higher concentrations than the Ca^{2+} present in resting cells. For this reason, De Miranda et al. propose a more prominent role of Mg^{2+}, since it can stimulate SR at lower concentrations and is more abundant into the cells (De Miranda et al. 2002).

Nitrosylation

The D-serine released from astrocytes enhances, by the activation of NMDAR, the Ca^{2+} uptake and, consequently, the activity of the neural nitric oxide synthase (nNOS) that increases the intracellular levels of NO.

Shoji and colleagues demonstrated that SR activity is decreased by NO in a human glioblastoma cell line (Shoji et al. 2006 A; Shoji et al. 2006 B). Subsequent studies revealed that the target of nNOS nitrosylation is Cys113 (human SR numeration), a residue conserved in the human, mouse and rat structures. Cys113 is situated near the ATP binding-site and its nitrosylation interferes with ATP binding, regulating the activation and causing enzyme inhibition. NO might constitute, therefore, a feedback signal that influences synaptic plasticity (Mustafa et al. 2007).

Interacting proteins

The control of hSR activity is also exerted through the enzyme subcellular localization and the interaction with other proteins. These interactors bind to SR at its N- or C-termini, generally via a repeated PDZ-domain motif, and influence SR catalytic efficiency or its cellular half-life.

Up to now, the interactome of SR has been prevalently investigated in mouse and rat. Many published studies are based on the employment of the two-hybrid screening, which allowed for the identification of interacting proteins.

The first interactor to be discovered was the *glutamate receptor interacting protein 1* (GRIP1), associated to AMPAR in resting cells (Kim et al. 2005). GRIP1 binds to the extreme C-terminal portion of SR and co-localizes with AMPAR and SR in astrocytes. The activation of AMPAR promotes GRIP1 dissociation from the receptor and its binding to SR, which, in turn, stimulates D-serine production (Kim et al. 2005). Baumgart and coworkers found that GRIP1 binding to mSR via its PDZ6 domain causes a conformational change that results in variations in both the K_M and the V_{max} for D-serine synthesis (Baumgart et al. 2007). In this context, AMPAR-GRIP1-SR interactions seem to play an important role in granular cells migration during the first developmental stages (Kim et al. 2005). The interaction of GRIP1 with the human isoform of SR has not yet been demonstrated.

Protein interacting with C kinase 1 (PICK1) is a PDZ-domain containing scaffold involved in AMPAR trafficking, cell morphology and migration (Hanley 2008). Thanks to a two-hybrid screening approach, Fujii and colleagues demonstrated that PICK1 binds to hSR

C-terminal sequence (Fujii et al. 2005), but it is still unclear how this interaction takes place *in vivo*. Subsequently, it was demonstrated that the deletion of PICK1 gene decreased levels of D-serine in the forebrain of neonatal mice. These results suggest the involvement of PICK1 in the modulation of D-serine synthesis (Hikida et al. 2008).

The degradation of SR through the ubiquitin-proteasome system is directly mediated by at least two proteins, GOLGA3 and DISC1. The *Golgin subfamily A member 3* (GOLGA3, also known as GCP170 or golgin-160) was discovered as SR interactor through the use of two-hybrid screening (Dumin et al. 2006). Dumin and colleagues showed that GOLGA3 and SR co-localize in the cytosol, at the perinuclear Golgi region and at the neuronal and glial processes in primary rat cell cultures (Dumin et al. 2006). GOLGA3 binds to the N-terminal region of SR and promotes the accumulation of functional enzyme, decreasing its ubiquitination *in vivo* by about 50% and enhancing its stability (Dumin et al. 2006).

Disrupted-in-schizophrenia-1 (DISC-1) is an interesting protein directly linked to schizophrenia and to other psychiatric disorders and, therefore, considered as a promising potential therapeutic target (Buchanan and Carpenter 1997; Morris et al. 2003; Hikida et al. 2012). Through overexpression and immunoprecipitation techniques, it was demonstrated that wild type DISC-1 binds to hSR (Ma et al. 2013). The deletion of the C-terminal region of the protein exerts a pathogenic action on the wild type form, disrupting its function. Furthermore, the overexpression of mutant DISC-1 results in decreased D-serine levels, that depend on an increased hSR ubiquitination. The functional interaction between DISC-1 and hSR is predominant in glia, although a neuronal role might also be important (Ma et al. 2013).

Very recently, Ma and colleagues demonstrated that hSR binds also to the C-terminal region of stargazin and to the PDZ3 domain of *postsynaptic density protein 95* (PSD-95) (Ma et al. 2014). Stargazin interacts with AMPAR and is involved in the receptor translocation to the cellular membrane. These complexes are retained at the cell surface thanks to the stargazing-PSD-95 binding (Schnell et al. 2002). The interaction with PSD-95 and stargazin associates SR with the membrane and inhibits its catalytic activity. The activation of AMPAR

allows for the dissociation of the complex, leading to enhanced catalytic activity of SR (Ma et al. 2014).

Phosphorylation and translocation to the membrane

Some studies show that SR activity can be also modulated by phosphorylation and by its association with the plasma membrane. So far, these two types of regulation have not been studied on the human isoform of SR. A certain number of experimental evidences, however, are available for mSR and rSR.

In a pioneering study, Dumin and co-workers found that mSR was present both in the cytosolic and in the membrane fraction of cellular homogenates (Dumin et al. 2006). These findings were more deeply investigated in further studies, where mSR was described to be strongly bound to the synaptosomal membrane and largely inactive toward D-serine synthesis (Balan et al. 2009). In neurons, the membrane-binding would be promoted by NMDAR activation, probably in order to avoid D-serine production and to prevent the overactivation of neurotransmission. The binding was proposed to take place thanks to the phosphorylation at Thr227 and palmitoylation (Balan et al. 2009). Afterwards, it was found that both the cytosolic and the membrane-bound mSR are phosphorylated at Thr71, whereas the phosphorylation at Thr227 is specifically detected on the membrane-bound SR. Thr71 is not conserved in the human sequence, and it could have a species-specific role. Its phosphorylation affects the turnover rate of the murine enzyme (Foltyn et al. 2010).

In astrocytes, the association with the membrane is mediated by phosphatidylinositol (4,5)-bisphosphate (PIP_2). PIP_2 competes with the ATP binding and inhibits SR activation. Metabotropic glutamate transmission stimulates phospholipase C that degrades PIP_2, removing SR inhibition and leading to an increased D-serine production and release (Mustafa et al. 2009).

D-SERINE AND NEUROPATHOLOGIES

As mentioned previously, D-serine is necessary for the NMDAR-mediated responses and regulates the LTP in the hippocampus, a region associated with learning and memory (Yang et al. 2003; Yang et al. 2005; Mothet et al. 2006). The D-serine signalling is, therefore, very important in the regulation of NMDAR activity. The NMDAR dysfunctions are linked to several neuropathologies, such as neurodegenerative and neuropsychiatric diseases, characterized by hypo- or overactivation of the glutamatergic transmission.

NMDAR hypofunction is involved in the pathologies of schizophrenia and temporal lobe epilepsy. Schizophrenia is a psychiatric disorder influenced by heritable factors concerning several genes that modulate NMDAR responses (Ross et al. 2006; van Os and Kapur 2009). Schizophrenic patients show a reduction in D-serine levels in the plasma and in the cerebrospinal fluid (Bendikov et al. 2007; Calcia et al. 2012). The NMDAR hypoactivation can have different origins and several studies have identified a considerable number of mutations in D-serine metabolic enzymes as risk factors (i.e. single-nucleotide polymorphism variants of SR, DAAO or their interacting proteins) (Morita et al. 2007; Müller et al. 2011; Caldinelli et al. 2013; Ma et al. 2013)

The temporal lobe epilepsy is frequently associated with D-serine deficiency, that leads to a low saturation of the co-agonist binding site of synaptic and extrasynaptic NMDARs (Klatte et al. 2013).

The excessive stimulation of NMDAR has been, instead, implicated in many acute and chronic neurodegenerative diseases, such as stroke, amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD) and Parkinson's disease (PD). All these disorders are characterized by high levels of D-serine, that cause NMDAR overactivation and excitotoxicity, linked to neuronal death (Katsuki et al. 2004; Wu et al. 2004; Sasabe et al. 2007). Several experimental evidences demonstrate that the specific deletion of SR gene, the enzymatic degradation of D-serine by DAAO or the employment of NMDAR antagonists, namely memantine, ameliorate the clinical pattern (Katsuki et al. 2004; Lipton 2004; Inoue et al. 2008; Hardingham and Bading 2010).

Human serine racemase as a therapeutic target

Acute and chronic neurologic diseases are among the leading causes of death, disability and economic expense in the world. In this context, excitotoxic cell death occurs in many neurodegenerative disorders and is in part due to the NMDAR over-activation and the excessive Ca^{2+} influx. Because of their central role in many serious diseases, NMDARs have been selected as potential drug target for the treatment of these pathologies (Trippier et al. 2013). The research is directed to the discovery of NMDAR antagonists, although NMDAR potentiation is also of interest (Lisman et al. 2008). The NMDAR basal activity is absolutely critical for normal neuronal functionality. For this reason, channel blockers and glutamate site antagonists show generally severe side effects (Gill et al. 2005; Muir 2006). In contrast, it was found memantine as a good inhibitor, because it blocks excessive NMDAR activity without disrupting the normal one (Bormann 1989; Chen et al. 1992; Chen and Lipton 2006). Memantine and its derivatives represent a few effective exceptions targeting NMDARs and demonstrating neuroprotective properties. Memantine is already employed in the treatment of AD (Mount and Downton 2006; Zemek et al. 2014) and also causes benefits to patients with moderate to severe vascular dementia and dementia of PD, although it is not yet approved for such use (Emre et al. 2010; Peng et al. 2013).

The NMDAR activation requires the occupation of two sites by glutamate and a co-agonist, glycine or D-serine. D-serine has been demonstrated to be the principal ligand of NMDAR in many brain areas and its involvement in the receptors activation apparently provides a fail-safe mechanism to prevent over-activation of NMDARs by glutamate (Dixon et al. 2006). With respect to these observations, the research on targets for the treatment of neurological diseases has been extended upstream and downstream of the NMDAR. Among the others, SR, being the enzyme directly responsible for the endogenous D-serine production, can be considered a putative therapeutic target for neuropathic pain treatment (Panizzutti et al. 2001; Strisovsky et al. 2005; Dixon et al. 2006; Jiraskova-Vanickova et al. 2011; Vorlova et al. 2014).

Human serine racemase inhibitors

Despite its potential relevance as a pharmacological target, there is a limited number of studies focusing on the development of SR inhibitors (Panizzutti et al. 2001; Strisovsky et al. 2005; Dixon et al. 2006; Jiraskova-Vanickova et al. 2011; Vorlova et al. 2014).

Campanini and coworkers have reviewed SR known inhibitors, based on their mechanism of action, grouping them into three classes based on their target: i) the PLP with reversible or ii) irreversible modifications, or iii) the active site (Campanini et al. 2013).

Among the molecules reacting reversibly with PLP, the stronger inhibitor is glycine, that exhibits a K_i of about 400 μM for hSR (Hoffman et al. 2009 A; Marchetti et al. 2013). Other L- and D-amino acids were tested and it was found that the presence of amine groups, negative charges or bulky groups did not affect the access into the active site of mSR (Dunlop and Neidle 2005). Amino acids containing sulphydryl groups act as inhibitors as well, reacting with PLP to form thiazolidine derivatives (Panizzutti et al. 2001; Dunlop and Neidle 2005). Another strategy to decrease D-serine synthesis consists in the use of substrates that elicit the β-elimination reaction. L-serine *O*-sulfate (Panizzutti et al. 2001), L-erythro 3-hydroxyaspartate (Hoffman et al. 2009 A), L-*threo*-3-hydroxyaspartate (Jiraskova-Vanickova et al. 2011), β-halogenated alanines (Dunlop and Neidle 2005) are examples of these molecules (Dunlop and Neidle 2005).

A series of hydroxamic and dihydroxamic acids derivatives were assayed as hSR and mSR inhibitors in different works (Panizzutti et al. 2001; Hoffman et al. 2009 B). They generally react irreversibly with the PLP-enzyme linkage, forming an aldoximine. This type of inhibition shows low specificity, as it can involve several PLP-dependent enzymes.

The enzyme active site has been widely studied in order to identify molecules with geometric complementarity that bind tightly. To this purpose, different approaches were followed. The best known competitive inhibitor of hSR is malonate, a small dicarboxylic acid able to make strong ionic bonds with active site residues. It shows a K_D in the micromolar range (33 μM (Strisovsky et al. 2005), 77 μM (Marchetti et al. 2014)) and it is present in the crystallographic structures available for hSR (PDB 3L6B and 3L6R) (Smith et al. 2010). Several malonate derivatives were synthesized and tested, but showed no

notable increase in affinity (Strisovsky et al. 2005; Jiraskova-Vanickova et al. 2011; Vorlova et al. 2014).

Dixon and colleagues screened a large combinatorial library of molecules, employing the one-bead one-compound approach (Dixon et al. 2006). This method allowed for the identification of several peptides showing moderate inhibitory effects and no structural similarities to the substrate L-serine. The selected molecules displayed slow-binding kinetics and the structural motif observed involves histidine and phenylpropionic moieties.

The search of new compounds directed to the allosteric effectors sites, the cation binding site, the reactive Cys113 and the regulator protein binding sites has not been tackled so far. In particular, the allosteric site could be an interesting target for the search of new compounds, because allosteric modulators generally show greater specificity, reduced side effects and lower toxicity (Lu et al. 2014).

References

Al-Hallaq, R. A., B. R. Jarabek, Z. Fu, S. Vicini, B. B. Wolfe and R. P. Yasuda (2002). "Association of NR3A with theN-Methyl-d-aspartate Receptor NR1 and NR2 Subunits." Molecular Pharmacology **62**(5): 1119-1127.

Balan, L., V. N. Foltyn, M. Zehl, E. Dumin, E. Dikopoltsev, D. Knoh, Y. Ohno, A. Kihara, O. N. Jensen, I. S. Radzishevsky and H. Wolosker (2009). "Feedback inactivation of D-serine synthesis by NMDA receptor-elicited translocation of serine racemase to the membrane." PNAS **106**(18): 7589-7594.

Balu, D., S. Takagi, M. Puhl, M. Benneyworth and J. Coyle (2014). "d-Serine and Serine Racemase are Localized to Neurons in the Adult Mouse and Human Forebrain." Cellular and Molecular Neurobiology **34**(3): 419-435.

Balu, D. T. and J. T. Coyle (2012). "Neuronal d-serine regulates dendritic architecture in the somatosensory cortex." Neuroscience Letters **517**(2): 77-81.

Basu, A. C., G. E. Tsai, C. L. Ma, J. T. Ehmsen, A. K. Mustafa, L. Han, Z. I. Jiang, M. A. Benneyworth, M. P. Froimowitz, N. Lange, S. H. Snyder, R. Bergeron and J. T. Coyle (2008). "Targeted disruption of serine racemase affects glutamatergic neurotransmission and behavior." Molecular Psychiatry **14**(7): 719-727.

Baumgart, F., J. M. Mancheño and I. Rodríguez-Crespo (2007). "Insights into the activation of brain serine racemase by the multi-PDZ domain glutamate receptor interacting protein, divalent cations and ATP." FEBS Journal **274**(17): 4561-4571.

Bendikov, I., C. Nadri, S. Amar, R. Panizzutti, J. De Miranda, H. Wolosker and G. Agam (2007). "A CSF and postmortem brain study of d-serine metabolic parameters in schizophrenia." Schizophrenia Research **90**(1-3): 41-51.

Benneyworth, M., Y. Li, A. Basu, V. Bolshakov and J. Coyle (2012). "Cell Selective Conditional Null Mutations of Serine Racemase Demonstrate a Predominate Localization in Cortical Glutamatergic Neurons." Cellular and Molecular Neurobiology **32**(4): 613-624.

Bormann, J. (1989). "Memantine is a potent blocker of N-methyl-D-aspartate (NMDA) receptor channels." European Journal of Pharmacology **166**(3): 591-592.

Buchanan, R. W. and W. T. Carpenter (1997). "The Neuroanatomies of Schizophrenia." Schizophrenia Bulletin **23**(3): 367-372.

Calcia, M. A., C. Madeira, F. V. Alheira, T. C. S. Silva, F. M. Tannos, C. Vargas-Lopes, N. Goldenstein, M. A. Brasil, S. T. Ferreira and R. Panizzutti (2012). "Plasma levels of D-serine in Brazilian individuals with schizophrenia." Schizophrenia Research **142**(1-3): 83-87.

Caldinelli, L., S. Sacchi, G. Molla, M. Nardini and L. Pollegioni (2013). "Characterization of human DAAO variants potentially related to an increased risk of schizophrenia." Biochimica et Biophysica Acta - Molecular Basis of Disease **1832**(3): 400-410.

Campanini, B., F. Spyrakis, A. Peracchi and A. Mozzarelli (2013). "Serine racemase: a key player in neuron activity and in neuropathologies." Frontiers in Bioscience **18**: 1112-28.

Chen, H.-S. V. and S. A. Lipton (2006). "The chemical biology of clinically tolerated NMDA receptor antagonists." Journal of Neurochemistry **97**(6): 1611-1626.

Chen, H. S., J. W. Pellegrini, S. K. Aggarwal, S. Z. Lei, S. Warach, F. E. Jensen and S. A. Lipton (1992). "Open-channel block of N-methyl-D-aspartate (NMDA) responses by memantine: therapeutic advantage against NMDA receptor-mediated neurotoxicity." The Journal of Neuroscience **12**(11): 4427-4436.

Cook, S. P., I. Galve-Roperh, Ã. MartÌnez del Pozo and I. Rodriguez-Crespo (2002). "Direct Calcium Binding Results in Activation of Brain Serine Racemase." The Journal of Biological Chemistry **277**(31): 27782-27792.

Corrigan, J. J. (1969). "D-Amino Acids in Animals." Science **164**(3876): 142-149.

De Miranda, J., R. Panizzutti, V. N. Foltyn and H. Wolosker (2002). "Cofactors of serine racemase that physiologically stimulate the synthesis of the N-methyl-d-aspartate (NMDA) receptor coagonist d-serine." PNAS **99**(22): 14542-14547.

De Miranda, J., A. Santoro, S. Engelender and H. Wolosker (2000). "Human serine racemase: moleular cloning, genomic organization and functional analysis." Gene **256**(1-2): 183-188.

Dixon, S. M., P. Li, R. Liu, H. Wolosker, K. S. Lam, M. J. Kurth and M. D. Toney (2006). "Slow-Binding Human Serine Racemase Inhibitors from High-Throughput Screening of Combinatorial Libraries." Journal of Medicinal Chemistry **49**(8): 2388-2397.

Dumin, E., I. Bendikov, V. N. Foltyn, Y. Misumi, Y. Ikehara, E. Kartvelishvily and H. Wolosker (2006). "Modulation of D-Serine Levels via Ubiquitin-dependent Proteasomal Degradation of Serine Racemase." The Journal of Biological Chemistry **281**(29): 20291-20302.

Dun, Y., J. Duplantier, P. Roon, P. M. Martin, V. Ganapathy and S. B. Smith (2008). "Serine racemase expression and d-serine content are developmentally regulated in neuronal ganglion cells of the retina." Journal of Neurochemistry **104**(4): 970-978.

Dunlop, D. S. and A. Neidle (2005). "Regulation of serine racemase activity by amino acids." Molecular Brain Research **133**(2): 208-214.

Emre, M., M. Tsolaki, U. Bonuccelli, A. Destée, E. Tolosa, A. Kutzelnigg, A. s. Ceballos-Baumann, S. Zdravkovic, A. Bladstrom and R. Jones (2010). "Memantine for patients with Parkinson's disease dementia or dementia with Lewy bodies: a randomised, double-blind, placebo-controlled trial." The Lancet Neurology **9**(10): 969-977.

Foltyn, V. N., I. Bendikov, J. De Miranda, R. Panizzutti, E. Dumin, M. Shleper, P. Li, M. D. Toney, E. Kartvelishvily and H. Wolosker (2005). "Serine racemase modulates intracellular D-serine levels through an a,b-elimination activity." The Journal of Biological Chemistry **280**(3): 1754-63.

Foltyn, V. N., M. Zehl, E. Dikopoltsev, O. N. Jensen and H. Wolosker (2010). "Phosphorylation of mouse serine racemase regulates D-serine synthesis." FEBS Letters **584**(13): 2937-41.

Fossat, P., F. R. Turpin, S. Sacchi, J. Dulong, T. Shi, J.-M. Rivet, J. V. Sweedler, L. Pollegioni, M. J. Millan, S. H. R. Oliet and J.-P. Mothet (2012). "Glial D-Serine Gates NMDA Receptors at Excitatory Synapses in Prefrontal Cortex." Cerebral Cortex **22**(3): 595-606.

Fuchs, S. A., R. Berger, L. W. J. Klomp and T. J. de Koning (2005). d-Amino acids in the central nervous system in health and disease. Molecular Genetics and Metabolism. **85:** 168-180.

Fujii, K., K. Maeda, T. Hikida, A. K. Mustafa, R. Balkissoon, J. Xia, T. Yamada, Y. Ozeki, R. Kawahara, M. Okawa, R. L. Huganir, H. Ujike, S. H. Snyder and A. Sawa (2005). "Serine racemase binds to PICK1: potential relevance to schizophrenia." Molecular Psychiatry **11**(2): 150-157.

Furukawa, H. and E. Gouaux (2003). "Mechanisms of activation, inhibition and specificity: crystal structures of the NMDA receptor NR1 ligand-binding core." The EMBO Journal **22**(12): 2873-2885.

Gill, S., O. Pulido, D. Small and J. Tauskela (2005). Glutamate Receptor Pharmacology: Lessons Learned from the Last Decade of Stroke Trials. Glutamate Receptors in Peripheral Tissue: Excitatory Transmission Outside the CNS, Springer US**:** 27-45.

Goto, M., T. Yamauchi, N. Kamiya, I. Miyahara, T. Yoshimura, H. Mihara, T. Kurihara, K. Hirotsu and N. Esaki (2009). "Crystal Structure of a Homolog of Mammalian Serine Racemase from Schizosaccharomyces pombe." The Journal of Biological Chemistry **284**(38): 25944-25952.

Griswold, W. R. and M. D. Toney (2011). "Role of the Pyridine Nitrogen in Pyridoxal 5'-Phosphate Catalysis: Activity of Three Classes of PLP Enzymes Reconstituted with Deazapyridoxal 5'-Phosphate." Journal of the American Chemical Society **133**(37): 14823-14830.

Hanley, J. G. (2008). "PICK1: A multi-talented modulator of AMPA receptor trafficking." Pharmacology & Therapeutics **118**(1): 152-160.

Hardingham, G. E. and H. Bading (2010). "Synaptic versus extrasynaptic NMDA receptor signalling: implications for neurodegenerative disorders." Nature Reviews Neuroscience **11**(10): 682-696.

Hashimoto, A., S. Kumashiro, T. Nishikawa, T. Oka, K. Takahashi, T. Mito, S. Takashima, N. Doi, Y. Mizutani, T. Yamazaki, T. Kaneko and E. Ootomo (1993 A). "Embryonic Development and Postnatal Changes in Free d-Aspartate and d-Serine in the Human Prefrontal Cortex." Journal of Neurochemistry **61**(1): 348-351.

Hashimoto, A., T. Nishikawa, T. Hayashi, N. Fujii, K. Harada, T. Oka and K. Takahashi (1992 A). "The presence of free D-serine in rat brain." FEBS Letters **296**(1): 33-36.

Hashimoto, A., T. Nishikawa, T. Oka and K. Takahashi (1993 B). "Endogenous d-Serine in Rat Brain: N-Methyl-d-Aspartate Receptor-Related Distribution and Aging." Journal of Neurochemistry **60**(2): 783-786.

Henneberger, C., T. Papouin, S. H. R. Oliet and D. A. Rusakov (2010). "Long-term potentiation depends on release of d-serine from astrocytes." Nature **463**(7278): 232-236.

Hikida, T., N. J. Gamo and A. Sawa (2012). "DISC1 as a therapeutic target for mental illnesses." Expert Opinion on Therapeutic Targets **16**(12): 1151-1160.

Hikida, T., A. K. Mustafa, K. Maeda, K. Fujii, R. K. Barrow, M. Saleh, R. L. Huganir, S. H. Snyder, K. Hashimoto and A. Sawa (2008). "Modulation of d-Serine Levels in Brains of Mice Lacking PICK1." Biological Psychiatry **63**(10): 997-1000.

Hoffman, H. E., J. Jiraskova, P. Cigler, M. Sanda, J. Schraml and J. Konvalinka (2009 B). "Hydroxamic Acids As a Novel Family of Serine Racemase Inhibitors: Mechanistic Analysis Reveals Different Modes of Interaction with the Pyridoxal-5'-phosphate Cofactor." The Journal of Medicinal Chemistry **52**(19): 6032-6041.

Hoffman, H. E., J. Jiraskova, M. Ingr, M. Zvelebil and J. Konvalinka (2009 A). "Recombinant human serine racemase: Enzymologic characterization and comparison with its mouse ortholog." Protein Expression and Purification **63**(1): 62-67.

Horio, M., M. Kohno, Y. Fujita, T. Ishima, R. Inoue, H. Mori and K. Hashimoto (2011). "Levels of d-serine in the brain and peripheral organs of serine racemase (Srr) knock-out mice." Neurochemistry International **59**(6): 853-859.

Inoue, R., K. Hashimoto, T. Harai and H. Mori (2008). "NMDA- and beta-Amyloid1-42-Induced Neurotoxicity Is Attenuated in Serine Racemase Knock-Out Mice." The Journal of Neuroscience **28**(53): 14486-14491.

Inoue, R., Y. Yoshihisa, Y. Tojo, C. Okamura, Y. Yoshida, J. Kishimoto, X. Luan, M. Watanabe, M. Mizuguchi, Y. Nabeshima, K. Hamase, K. Matsunaga, T. Shimizu and H. Mori (2014). "Localization of Serine Racemase and Its Role in the Skin." Journal of Investigative Dermatology **134**(6): 1618-1626.

Jiraskova-Vanickova, J., R. Ettrich, B. Vorlova, H. E. Hoffman, M. Lepsik, P. Jansa and J. Konvalinka (2011). "Inhibition of Human Serine Racemase, an Emerging Target for Medicinal Chemistry." Current Drug Targets **12**(7): 1037-1055.

John, R. A. (1995). "Pyridoxal phosphate-dependent enzymes." Biochimica et Biophysica Acta - Protein Structure and Molecular Enzymology **1248**(2): 81-96.

Kartvelishvily, E., M. Shleper, L. Balan, E. Dumin and H. Wolosker (2006). "Neuron-derived D-Serine Release Provides a Novel Means to Activate N-Methyl-D-aspartate Receptors." The Journal of Biological Chemistry **281**(20): 14151-14162.

Katsuki, H., M. Nonaka, H. Shirakawa, T. Kume and A. Akaike (2004). "Endogenous d-Serine Is Involved in Induction of Neuronal Death by N-Methyl-d-aspartate and Simulated Ischemia in Rat Cerebrocortical Slices." Journal of Pharmacology and Experimental Therapeutics **311**(2): 836-844.

Kim, P. M., H. Aizawa, P. S. Kim, A. S. Huang, S. R. Wickramasinghe, A. H. Kashani, R. K. Barrow, R. L. Huganir, A. Ghosh and S. H. Snyder (2005). "Serine racemase: Activation by glutamate neurotransmission via glutamate receptor interacting protein and mediation of neuronal migration." PNAS **102**(6): 2105-2110.

Klatte, K., T. Kirschstein, D. Otte, L. Pothmann, L. Muller, T. Tokay, M. Kober, M. Uebachs, A. Zimmer and H. Beck (2013). "Impaired d-Serine-Mediated Cotransmission Mediates Cognitive Dysfunction in Epilepsy." The Journal of Neuroscience **33**(32): 13066-13080.

Krebs, H. (1935). "Metabolism of amino-acids: Deamination of amino-acids." Biochemistry Journal **29**(7): 1620-44.

Lipton, S. (2004). "Failures and successes of NMDA receptor antagonists: Molecular basis for the use of open-channel blockers like memantine in the treatment of acute and chronic neurologic insults." NeuroRX **1**(1): 101-110.

Lisman, J. E., J. T. Coyle, R. W. Green, D. C. Javitt, F. M. Benes, S. Heckers and A. A. Grace (2008). "Circuit-based framework for understanding neurotransmitter and risk gene interactions in schizophrenia." Trends in Neurosciences **31**(5): 234-242.

Lu, S., S. Li and J. Zhang (2014). "Harnessing Allostery: A Novel Approach to Drug Discovery." Medicinal Research Reviews **34**(6): 1242-1285.

Ma, T. M., S. Abazyan, B. Abazyan, J. Nomura, C. Yang, S. Seshadri, A. Sawa, S. H. Snyder and M. V. Pletnikov (2013). "Pathogenic disruption of DISC1-serine racemase binding elicits schizophrenia-like behavior via D-serine depletion." Molecular Psychiatry **18**(5): 557-67.

Ma, T. M., B. D. Paul, C. Fu, S. Hu, H. Zhu, S. Blackshaw, H. Wolosker and S. H. Snyder (2014). "Serine Racemase Regulated by Binding to Stargazin and PSD-95: Potential NMDA-AMPA Glutamate Neurotransmission Cross-talk." The Journal of Biological Chemistry.

Marchetti, M., S. Bruno, B. Campanini, S. Bettati, A. Peracchi and A. Mozzarelli (2014). "Regulation of human serine racemase activity and dynamics by halides, ATP and malonate." Amino Acids **47**(1): 163-173.

Marchetti, M., S. Bruno, B. Campanini, A. Peracchi, N. Mai and A. Mozzarelli (2013). "ATP binding to human serine racemase is cooperative and modulated by glycine." FEBS Journal **280**(22): 5853-5863.

Martineau, M., G. r. Baux and J.-P. Mothet (2006). "d-Serine signalling in the brain: friend and foe." Trends in Neurosciences **29**(8): 481-491.

Martineau, M., V. Parpura and J.-P. Mothet (2014). "Cell-type specific mechanisms of D-serine uptake and release in the brain." Frontiers in Synaptic Neuroscience **6**.

Martineau, M., T. Shi, J. Puyal, A. M. Knolhoff, J. r. m. Dulong, B. Gasnier, J. r. Klingauf, J. V. Sweedler, R. Jahn and J.-P. Mothet (2013). "Storage and Uptake of d-Serine into Astrocytic Synaptic-Like Vesicles Specify Gliotransmission." The Journal of Neuroscience **33**(8): 3413-3423.

Mason, S. F. (1984). "Origins of biomolecular handedness." Nature **311**(5981): 19-23.

Miya, K., R. Inoue, Y. Takata, M. Abe, R. Natsume, K. Sakimura, K. Hongou, T. Miyawaki and H. Mori (2008). "Serine racemase is predominantly localized in neurons in mouse brain." The Journal of Comparative Neurology **510**(6): 641-654.

Morita, Y., H. Ujike, Y. Tanaka, K. Otani, M. Kishimoto, A. Morio, T. Kotaka, Y. Okahisa, M. Matsushita, A. Morikawa, K. Hamase, K. Zaitsu and S. Kuroda (2007). "A Genetic Variant of the Serine Racemase Gene Is Associated with Schizophrenia." Biological Psychiatry **61**(10): 1200-1203.

Morris, J. A., G. Kandpal, L. Ma and C. P. Austin (2003). "DISC1 (Disrupted-In-Schizophrenia 1) is a centrosome-associated protein that interacts with MAP1A, MIPT3, ATF4/5 and NUDEL: regulation and loss of interaction with mutation." Human Molecular Genetics **12**(13): 1591-1608.

Mothet, J. P., E. Rouaud, P. M. Sinet, B. Potier, A. Jouvenceau, P. Dutar, C. Videau, J. Epelbaum and J. M. Billard (2006). "A critical role for the glial-derived neuromodulator d-serine in the age-related deficits of cellular mechanisms of learning and memory." Aging Cell **5**(3): 267-274.

Mount, C. and C. Downton (2006). "Alzheimer disease: progress or profit?" Nature Medicine **12**(7): 780-784.

Muir, K. W. (2006). "Glutamate-based therapeutic approaches: clinical trials with NMDA antagonists." Current Opinion in Pharmacology **6**(1): 53-60.

Müller, D. J., C. C. Zai, T. Shinkai, J. Strauss and J. L. Kennedy (2011). "Association between the DAOA/G72 gene and bipolar disorder and meta-analyses in bipolar disorder and schizophrenia." Bipolar Disorders **13**(2): 198-207.

Mustafa, A. K., M. Kumar, B. Selvakumar, G. P. H. Ho, J. T. Ehmsen, R. K. Barrow, L. M. Amzel and S. H. Snyder (2007). "Nitric oxide S-nitrosylates serine racemase, mediating feedback inhibition of d-serine formation." PNAS **104**(8): 2950-2955.

Mustafa, A. K., D. B. v. Rossum, R. L. Patterson, D. Maag, J. T. Ehmsen, S. K. Gazi, A. Chakraborty, R. K. Barrow, L. M. Amzel and S. H. Snyder (2009). "Glutamatergic regulation of serine racemase via reversal of PIP2 inhibition." PNAS **106**(8): 2921-2926.

Nagata, Y., K. Horiike and T. Maeda (1994). "Distribution of freed-serine in vertebrate brains." Brain Research **634**(2): 291-295.

Neidle, A. and D. Dunlop (2002). "Allosteric Regulation of Mouse Brain Serine Racemase." Neurochemical Research **27**(12): 1719-1724.

Panatier, A., D. T. Theodosis, J.-P. Mothet, B. Touquet, L. Pollegioni, D. A. Poulain and S. p. H. R. Oliet (2006). "Glia-Derived d-Serine Controls NMDA Receptor Activity and Synaptic Memory." Cell **125**(4): 775-784.

Panizzutti, R. r., J. De Miranda, C. t. S. Ribeiro, S. Engelender and H. Wolosker (2001). "A new strategy to decrease N-methyl-d-aspartate (NMDA) receptor coactivation: Inhibition of d-serine synthesis by converting serine racemase into an eliminase." PNAS **98**(9): 5294-5299.

Peng, D., X. Yuan and R. Zhu (2013). "Memantine hydrochloride in the treatment of dementia subtypes." The Journal of Clinical Neuroscience **20**(11): 1482-1485.

Pollegioni, L., L. Piubelli, S. Sacchi, M. S. Pilone and G. Molla (2007). "Physiological functions of D-amino acid oxidases: from yeast to humans." Cellular and Molecular Life Sciences **64**(11): 1373-1394.

Pollegioni, L. and S. Sacchi (2010). "Metabolism of the neuromodulator d-serine." Cellular and Molecular Life Sciences **67**(14): 2387-2404.

Puyal, J., M. Martineau, J.-P. Mothet, M.-T. Nicolas and J. Raymond (2006). "Changes in D-serine levels and localization during postnatal development of the rat vestibular nuclei." The Journal of Comparative Neurology **497**(4): 610-621.

Rosenberg, D., S. Artoul, A. C. Segal, G. Kolodney, I. Radzishevsky, E. Dikopoltsev, V. N. Foltyn, R. Inoue, H. Mori, J.-M. Billard and H. Wolosker (2013). "Neuronal d-Serine and Glycine Release Via the Asc-1 Transporter Regulates NMDA Receptor-Dependent Synaptic Activity." The Journal of Neuroscience **33**(8): 3533-3544.

Ross, C. A., R. L. Margolis, S. A. J. Reading, M. Pletnikov and J. T. Coyle (2006). "Neurobiology of Schizophrenia." Neuron **52**(1): 139-153.

Sasabe, J., T. Chiba, M. Yamada, K. Okamoto, I. Nishimoto, M. Matsuoka and S. Aiso (2007). "d-Serine is a key determinant of glutamate toxicity in amyotrophic lateral sclerosis." The EMBO Journal **26**(18): 4149-4159.

Schell, M. J., R. O. Brady Jr, M. E. Molliver and S. H. Snyder (1997). "d-Serine as a Neuromodulator: Regional and Developmental Localizations in Rat Brain Glia Resemble NMDA Receptors." The Journal of Neuroscience **17**(5): 1604-1615.

Schell, M. J., M. E. Molliver and S. H. Snyder (1995). "D-serine, an endogenous synaptic modulator: localization to astrocytes and glutamate-stimulated release." PNAS **92**(9): 3948-3952.

Schnell, E., M. Sizemore, S. Karimzadegan, L. Chen, D. S. Bredt and R. A. Nicoll (2002). "Direct interactions between PSD-95 and stargazin control synaptic AMPA receptor number." PNAS **99**(21): 13902-13907.

Schuler, T., I. Mesic, C. Madry, I. Bartholomaus and B. Laube (2008). "Formation of NR1/NR2 and NR1/NR3 Heterodimers Constitutes the Initial Step in N-Methyl-D-aspartate Receptor Assembly." The Journal of Biological Chemistry **283**(1): 37-46.

Shoji, K., S. Mariotto, A. R. Ciampa and H. Suzuki (2006 A). "Regulation of serine racemase activity by d-serine and nitric oxide in human glioblastoma cells." Neuroscience Letters **392**(1-2): 75-78.

Shoji, K., S. Mariotto, A. R. Ciampa and H. Suzuki (2006 B). "Mutual regulation between serine and nitric oxide metabolism in human glioblastoma cells." Neuroscience Letters **394**(3): 163-167.

Smith, M. A., V. Mack, A. Ebneth, I. Moraes, B. Felicetti, M. Wood, D. Schonfeld, O. Mather, A. Cesura and J. Barker (2010). "The Structure of Mammalian Serine Racemase: EVIDENCE FOR CONFORMATIONAL CHANGES UPON INHIBITOR BINDING." The Journal of Biological Chemistry **285**(17): 12873-12881.

Strisovsky, K., J. Jiraskova, A. Mikulova, L. Rulisek and J. Konvalinka (2005). "Dual substrate and reaction specificity in mouse serine racemase: identification of high-affinity dicarboxylate substrate and inhibitors and analysis of the beta-eliminase activity." Biochemistry **44**(39): 13091-100.

Toney, M. D. (2005). "Reaction specificity in pyridoxal phosphate enzymes." Archives of Biochemistry and Biophysics **433**(1): 279-287.

Trippier, P. C., K. Jansen Labby, D. D. Hawker, J. J. Mataka and R. B. Silverman (2013). "Target- and Mechanism-Based Therapeutics for Neurodegenerative Diseases: Strength in Numbers." The Journal of Medicinal Chemistry **56**(8): 3121-3147.

Van Horn, M. R., M. Sild and E. S. Ruthazer (2013). "D-serine as a gliotransmitter and its roles in brain development and disease." Frontiers in Cellular Neuroscience **7**.

van Os, J. and S. Kapur (2009). "Schizophrenia." The Lancet **374**(9690): 635-645.

Vorlova, B., D. Nachtigallova, J. Jiraskova-Vanickova, H. Ajani, P. Jansa, J. Rezac, J. Fanfrilk, M. Otyepka, P. Hobza, J. Konvalinka and M. Lepsik (2014). "Malonate-based inhibitors of mammalian serine racemase: Kinetic characterization and structure-based computational study." The European Journal of Medicinal Chemistry **89**(0): 189-197.

Williams, S. M., C. M. Diaz, L. T. Macnab, R. K. P. Sullivan and D. V. Pow (2006). "Immunocytochemical analysis of D-serine distribution in the mammalian brain reveals novel anatomical compartmentalizations in glia and neurons." Glia **53**(4): 401-411.

Wolosker, H. (2007). "NMDA receptor regulation by D-serine: new findings and perspectives." Molecular Neurobiology **36**(2): 152-64.

Wolosker, H. (2011). "Serine racemase and the serine shuttle between neurons and astrocytes." Biochimica et Biophysica Acta - Proteins and Proteomics **1814**(11): 1558-1566.

Wolosker, H., S. Blackshaw and S. H. Snyder (1999). "Serine racemase: A glial enzyme synthesizing d-serine to regulate glutamate-N-methyl-d-aspartate neurotransmission." PNAS **96**(23): 13409-13414.

Wolosker, H., E. Dumin, L. Balan and V. N. Foltyn (2008). "d-Amino acids in the brain: d-serine in neurotransmission and neurodegeneration." FEBS Journal **275**(14): 3514-3526.

Wong, H.-K., X.-B. Liu, M. F. Matos, S. F. Chan, I. Pérez-Otaño, M. Boysen, J. Cui, N. Nakanishi, J. S. Trimmer, E. G. Jones, S. A. Lipton and N. J. Sucher (2002). "Temporal and regional expression of NMDA receptor subunit NR3A in the mammalian brain." The Journal of Comparative Neurology **450**(4): 303-317.

Wu, S.-Z., A. Bodles, M. Porter, W. S. Griffin, A. Basile and S. Barger (2004). "Induction of serine racemase expression and D-serine release from microglia by amyloid beta-peptide." The Journal of Neuroinflammation **1**(1): 2.

Yamasaki, M., K. Yamada, S. Furuya, J. Mitoma, Y. Hirabayashi and M. Watanabe (2001). "3-Phosphoglycerate Dehydrogenase, a Key Enzyme forl-Serine Biosynthesis, Is Preferentially Expressed in the Radial Glia/Astrocyte Lineage and Olfactory Ensheathing Glia in the Mouse Brain." The Journal of Neuroscience **21**(19): 7691-7704.

Yang, J. H., A. Wada, K. Yoshida, Y. Miyoshi, T. Sayano, K. Esaki, M. O. Kinoshita, S. Tomonaga, N. Azuma, M. Watanabe, K. Hamase, K. Zaitsu, T. Machida, A. Messing, S. Itohara, Y. Hirabayashi and S. Furuya (2010). "Brain-specific Phgdh Deletion Reveals a Pivotal Role for l-Serine Biosynthesis in Controlling the Level of d-Serine, an N-methyl-d-aspartate Receptor Co-agonist, in Adult Brain." The Journal of Biological Chemistry **285**(53): 41380-41390.

Yang, S., H. Qiao, L. Wen, W. Zhou and Y. Zhang (2005). "D-Serine enhances impaired long-term potentiation in CA1 subfield of hippocampal slices from aged senescence-accelerated mouse prone/8." Neuroscience Letters **379**(1): 7-12.

Yang, Y., W. Ge, Y. Chen, Z. Zhang, W. Shen, C. Wu, M. Poo and S. Duan (2003). "Contribution of astrocytes to hippocampal long-term potentiation through release of d-serine." PNAS **100**(25): 15194-15199.

Yang, Y., H. Higashimori and L. Morel (2013). "Developmental maturation of astrocytes and pathogenesis of neurodevelopmental disorders." The Journal of Neurodevelopmental Disorders **5**(1): 22.

Zemek, F., L. Drtinova, E. Nepovimova, V. Sepsova, J. Korabecny, J. Klimes and K. Kuca (2014). "Outcomes of Alzheimer's disease therapy with acetylcholinesterase inhibitors and memantine." Expert Opinion on Drug Safety **13**(6): 759-774.

AIMS OF THE RESEARCH

Serine racemase is the PLP-dependent enzyme that catalyzes the conversion of L-serine in D-serine, as well as the formation of pyruvate and ammonia from both L- and D-serine. The relevance of human serine racemase is due to the role played by D-serine as an essential co-agonist, together with glutamate, of NMDA receptors. These receptors are associated with many neurological functions and their abnormally high activity causes neurotoxicity in several neuropathologies, such as Alzheimer, Parkinson and Amyotrophic Lateral Sclerosis. The NMDAR antagonists developed for the treatment of these diseases have exhibited mild to severe adverse effects, suggesting alternative therapeutic strategies based on the regulation of the metabolism of NMDAR agonists. Among these alternative drug targets, serine racemase is considered particularly promising.

Surprisingly, to date, a detailed knowledge of the catalytic activity and regulation of the human enzyme is rather limited. The few published biochemical studies provide a limited insight into its regulatory mechanisms. ATP, magnesium and calcium are known to play pivotal physiological roles affecting serine racemase activity, but their physiological relevance remains unknown. In this framework, our work was aimed at the elucidation of the cross-talk between active and allosteric sites via a thorough characterization of enzyme dynamics, function and regulatory properties. Activity assays and spectroscopic methods were applied to explore the activation by ATP on β-elimination and racemization both in the absence and presence of enzyme inhibitors. Strong cooperativity of ATP binding was observed, as well as a strong effect of ATP on the binding of ligands at the active site, and vice versa. We also investigated the dependence on the activity of molecules present in the intracellular environment: the bivalent ions magnesium and calcium, monovalent ions and crowding agents.

The limited number of works and the scarce results so far obtained in the search of human serine racemase inhibitors point out the difficulty in the design of effective ligands. With the purpose of designing new effective compounds, we applied both i*n silico* screenings and rational drug design approaches. The predicted inhibitors were tested using both activity assay and fluorimetric methods. In spite of intense efforts, no high affinity

inhibitor at the active site was identified, suggesting the exploration of allosteric sites as alternative binding sites for inhibitors.

COOPERATIVE BINDING OF ATP

ATP binding to human serine racemase is cooperative and modulated by glycine

Marialaura Marchetti[1], Stefano Bruno[2], Barbara Campanini[2], Alessio Peracchi[1], Nicole Mai[2] and Andrea Mozzarelli[2,3]

[1] Department of Life Sciences, University of Parma, Italy
[2] Department of Pharmacy, University of Parma, Italy
[3] National Institute of Biostructures and Biosystems, Rome, Italy

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Abstract

The NMDA receptors play a key role in the excitatory neurotransmission and control learning, memory and synaptic plasticity. Their activity is modulated by the agonist glutamate and by the co-agonists D-serine and glycine. In the human brain, D-serine is synthesized from L-serine by the dimeric, pyridoxal 5'-phosphate-dependent enzyme serine racemase, which also degrades L- and D-serine to pyruvate and ammonia. The dependence of L- and D-serine β-elimination and L-serine racemization activities on ATP concentration was characterized and found to be strongly cooperative, with Hill coefficients close to 2 and apparent ATP dissociation constants ranging from 0.22 to 0.41 mM. ATP binding to the holo-enzyme, monitored by the fluorescence changes of the coenzyme, was also determined to be cooperative, with an apparent dissociation constant of 0.24 mM. Glycine, an active site ligand, increases the serine racemase affinity for ATP by about 22-fold, abolishing cooperativity. Conversely, ATP increases 15-fold the non-cooperative glycine binding. These results indicate a cross-talk between allosteric and active sites, leading to the stabilization of two alternative protein conformations endowed with ATP affinities of about 10 μM and 1.8 mM, as evaluated within the Monod, Wyman and Changeux model. Therefore, intracellular ATP and glycine control D-serine homeostasis and, indirectly, NMDA receptor activity. Because hyper- and hypo-activation of NMDA receptors are associated with neuropathologies, the development of allosteric drugs modulating serine racemase activity is a promising therapeutic strategy.

Introduction

The N-methyl D-aspartate receptors (NMDARs) are a sub-type of ionotropic receptors for glutamate, the main excitatory neurotransmitter in the central nervous system of vertebrates. NMDARs play a key role in the excitatory neurotransmission and control learning, memory and synaptic plasticity (Martineau et al. 2006; Wolosker 2007). NMDARs activation requires the binding of the agonist glutamate as well as one of the co-agonists D-serine and glycine (Johnson and Ascher 1987; Boehning and Snyder 2003; Wolosker et al. 2008). Recent studies proposed that D-serine is the main agonist of synaptic NMDARs, whereas glycine is the agonist of extra-synaptic NMDARs (Papouin et al. 2012; Henneberger et al. 2013; Rosenberg et al. 2013). NMDARs are also modulated by antagonists, like kynurenic acid (Kessler et al. 1989) produced by kynurenine aminotransferase (Passera et al. 2011). Hypo-activation of NMDARs is associated with schizophrenia (Labrie et al. 2012; Ma et al. 2013), whereas hyper-activation is associated with several neuropathologies, including Alzheimer's and Parkinson's disease, ischemia (Mustafa et al. 2010), amyotrophic lateral sclerosis and Rett syndrome (Campanini et al. 2013).

D-serine is produced by serine racemase (SR, EC 5.1.1.18) (Wolosker et al. 1999), a pyridoxal 5'-phosphate (PLP)-dependent, homodimeric enzyme localized both in neurons and astrocytes (Ding et al. 2011; Benneyworth et al. 2012; Ito et al. 2013). SR shows a dual activity: the reversible racemization of L-serine to D-serine and the irreversible β–elimination of water from both enantiomers of serine to generate pyruvate and ammonia (De Miranda et al. 2002; Foltyn et al. 2005; Yoshimura and Goto 2008). β–Elimination and racemization take place simultaneously, albeit with different catalytic efficiencies (De Miranda et al. 2002; Hoffman et al. 2009), which dictate the relative amount of the products. Both reactions were proposed to be physiologically relevant, with the former leading to D-serine production and the latter to D-serine breakdown, a route for the control of its homeostasis in brain areas (Foltyn et al. 2005) that lack the main degradative enzyme for D-serine, D-amino acid oxidase (DAAO) (Pollegioni and Sacchi 2010; Sacchi et al. 2012) . Additional complex mechanisms regulate D-serine concentration, including the shuttle of D-serine from neurons to astrocytes and its release in the synaptic

space (Wolosker 2011), and SR modulation by ATP, divalent cations, at least four proteins, and post-translational modifications (Fig. 1) (Foltyn et al. 2010; Jiraskova-Vanickova et al. 2011; Campanini et al. 2013).

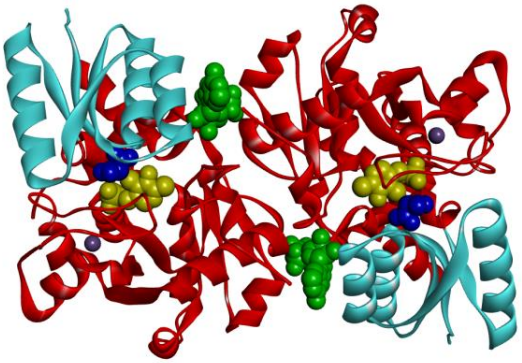

Figure 1. Cartoon representation of a model of human SR complexed with malonate, Mn^{2+} and ATP. The three-dimensional structure of holo human SR in the presence of malonate and Mn^{2+} was determined by Smith et al., 2010 (PDB code 3L6B) (Smith et al. 2010). The large and the small domains are shown in red and cyan, respectively, PLP bound to the active site Lys56 is displayed in yellow space-filling mode, the competitive inhibitor malonate is displayed in blue space-filling mode, and the Mn^{2+} ion is displayed in violet. By overalapping the structure of human SR with the structure of SR from *Schizosaccharomyces pombe* (PDB code 1V71) (Goto et al. 2009), that contains the ATP analog 5'-adenylyl methylenediphosphonate (AMP-PCP), the site of the ATP binding site to human SR is proposed. AMP-PCP is displayed in green space-filling mode.

Despite the pharmacological relevance of SR as the source of the neuromodulator D-serine (Wolosker et al. 2008; Wolosker and Mori 2012), many aspects of its activity and regulation are still unclear. In particular, although the allosteric activation of murine SR by ATP was described more than ten years ago (De Miranda et al. 2002), the molecular details of this regulatory mechanism, which modulates the overall activity of SR and the relative rates of racemization and β-elimination (De Miranda et al. 2002; Neidle and Dunlop 2002; Foltyn et al. 2005), have never been thoroughly characterized. For instance, based on the reported apparent dissociation constant (K_D) of about 10 μM for ATP to the murine enzyme (De Miranda et al. 2002; Mustafa et al. 2007) and an intracellular concentration of ATP ranging from about 1 to 6 mM (Silver and Erecinska 1997; Ainscow et al. 2002), it was assumed that ATP constitutively saturates the two symmetric sites at the dimer interface

(Fig. 1) (Goto et al. 2009), resulting in a permanently fully-activated enzyme. Here, we show that ATP binds to human SR with strong cooperativity, and increases the affinity of the active site ligand glycine. Conversely, glycine stabilizes a protein conformation that binds ATP non-cooperatively and with high affinity, in a cross-talk between allosteric and active sites. Cooperativity evolutionarily developed as a molecular mechanism to maximize the dependence of protein function on the concentration of an effector in a narrow concentration range (Eaton et al. 2007; Cui and Karplus 2008; Peracchi and Mozzarelli 2011; Changeux 2012; Nussinov and Tsai 2013). The cooperative behaviour of hSR opens the possibility of a physiologically relevant regulation of D-serine synthesis by the intracellular levels of ATP and glycine. The pharmacological implications of these novel findings are discussed.

Results and Discussion

Catalytic parameters of hSR for the β–elimination and racemization reactions.

We first measured the catalytic activity of hSR in the β–elimination reaction, using either L- or D-serine as substrates, in the absence and presence of ATP (Tab. 1, and Fig. 2A, B). The K_M for L-serine was determined to be 76 ± 10 mM and 12 ± 1 mM, and the K_M for D-serine 56 ± 12 mM and 144 ± 27 mM, in the absence and presence of ATP, respectively. The k_{cat} for L-serine is 37 ± 4 min^{-1} and 147 ± 3 min^{-1}, and for D-serine 0.9 ± 0.1 min^{-1} and 6.6 ± 0.6 min^{-1}, in the absence and presence of ATP, respectively. Therefore, ATP decreases the K_M for L-serine 6-fold and increases the k_{cat} about 4-fold. The resulting catalytic efficiency (k_{cat}/K_M) for L-serine elimination is 0.49 min^{-1} mM^{-1} and 12.25 min^{-1} mM^{-1}, in the absence and presence of ATP, respectively, indicating a 25-fold ATP-induced activation of the β–elimination (Tab. 1). For D-serine, ATP increases both the K_M and the k_{cat} (Table 1). The resulting catalytic efficiency is 0.016 min^{-1} mM^{-1} and 0.046 min^{-1} mM^{-1}, in the absence and presence of ATP, respectively, indicating an ATP-induced activation of only 2.9-fold for the D-serine β–elimination (Tab. 1).

Reaction	K_M (mM)		k_{cat} (min^{-1})		k_{cat}/K_M ($min^{-1}mM^{-1}$)	
	-ATP	+ATP	-ATP	+ATP	-ATP	+ATP
L-serine β-elimination	76 ± 10	12 ± 1	37 ± 4	147 ± 3	0.49 ± 0.12	12.25 ± 1.28
D-serine β-elimination	56 ± 12	144 ± 27 (204 ± 27)	0.9 ± 0.1	6.6 ± 0.6 (8.7 ± 1)	0.016 ± 0.005	0.046 ± 0.013 (0.042 ± 0.013)
L-serine racemization	48 ± 16	40 ± 15	2.9 ± 0.3	10 ± 1	0.06 ± 0.03	0.25 ± 0.14

Table 1. Catalytic parameters of hSR. Measurements were carried out at 37 °C as described in Materials and Methods. The kinetic parameters were determined by a nonlinear regression fitting of initial reaction rates to the Michaelis–Menten equation. Experimental curves and fittings are reported in Fig. 2. For D-serine β-elimination in the presence of ATP, the catalytic parameters obtained in the absence of NaCl are reported in parenthesis.

Because, differently from previously reported works, our assay buffer solutions contained 150 mM sodium chloride, we evaluated its effect on the rate of D-serine

elimination and found it negligible (Tab. 1). The observed ATP-induced activation of L-serine elimination reaction is similar to that observed previously for the murine enzyme (De Miranda et al. 2002). Overall, the net effect of ATP binding on the β–elimination reaction is a strong stimulation of L-serine degradation with a small effect on D-serine degradation.

We then measured the effect of ATP binding on racemization, using L-serine as substrate (Tab. 1, Fig. 2). In the absence and presence of ATP, the K_M for L-serine is 48 ± 16 mM and 40 ± 15 mM, and the k_{cat} 2.9 ± 0.3 min^{-1} and 10 ± 1 min^{-1}, respectively (Tab. 1). The k_{cat}/K_M is 0.06 min^{-1} mM^{-1} and 0.25 min^{-1} mM^{-1} (Tab. 1). Therefore, ATP leads to a 4-fold activation of L-serine racemization. It is interesting to notice that, in the absence of ATP, the ratio for L-serine β–elimination and racemization efficiency is 8.2, whereas in the presence of ATP is 50, indicating that ATP binding preferentially stimulates L-serine β–elimination rather than racemization. Furthermore, D-serine elimination occurs both in the absence and presence of ATP with a very low efficiency with respect to L-serine β–elimination (29- and 271-fold in the absence and presence of ATP, respectively) casting some doubt on its proposed role in controlling D-serine homeostasis (Foltyn et al. 2005). When D-serine is formed, the rate of its degradation by human SR is very low, and D-serine accumulates. Interestingly, in the absence of ATP, the efficiency of all measured reactions is lower, thus indicating that ATP plays a significant role in controlling D-serine concentration. These findings suggest that the development of compounds that target the ATP allosteric site might be a powerful strategy for SR modulation.

With respect to the only published characterization of the catalytic parameters of hSR in the presence of ATP (Hoffman et al. 2009), we have found a 2-fold lower efficiency for L-serine β–elimination, about 17-fold lower efficiency for D-serine β–elimination, and about 40-fold lower efficiency for L-serine racemization. These differences might be due to differences in enzyme assays, or different purification and stabilization protocols. Significant differences were already observed between the catalytic parameters of murine SR, reported by Hoffman et al. (Hoffman et al. 2009) and Foltyn et al. (Foltyn et al. 2005).

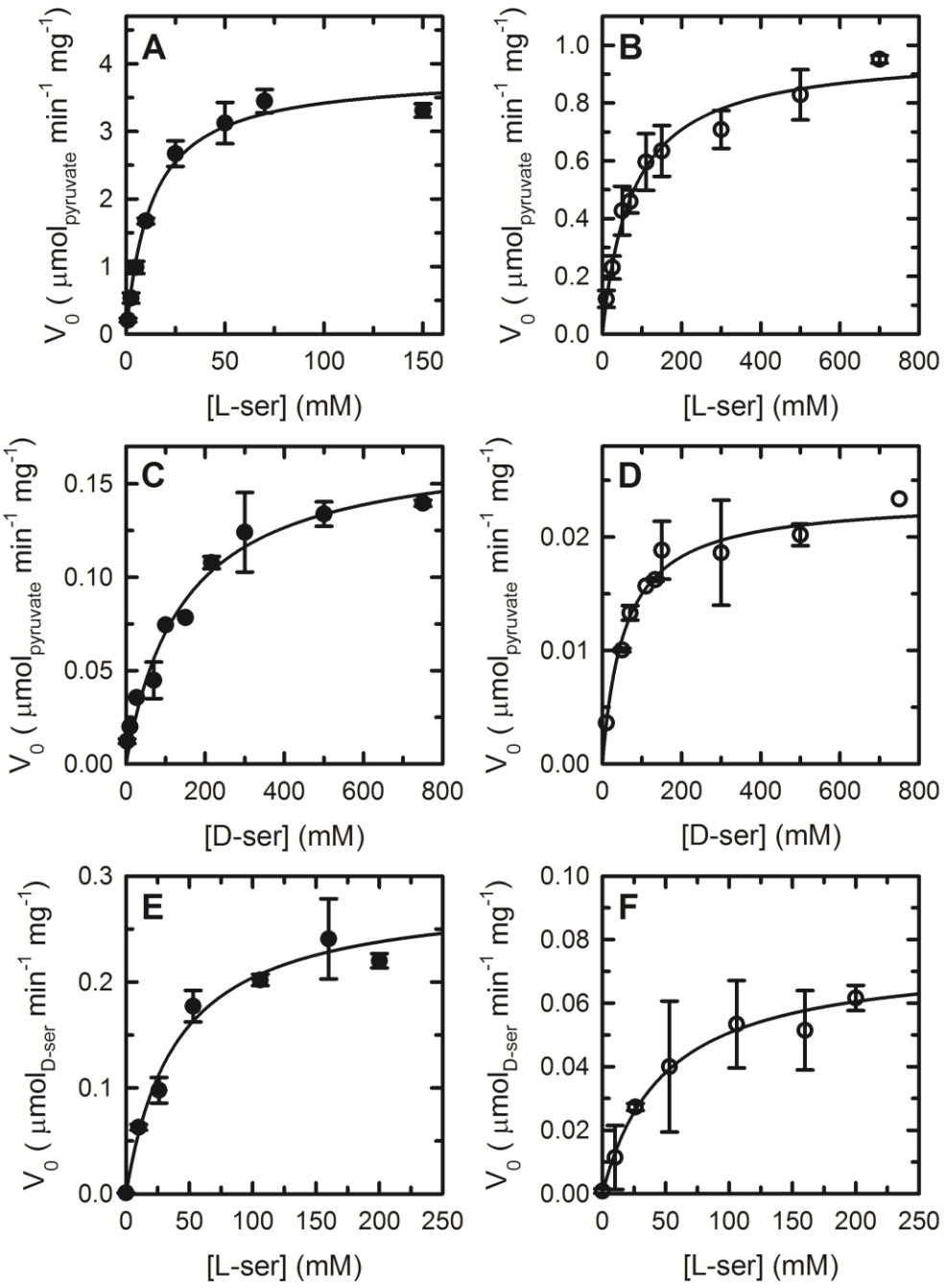

Figure 2. Dependence of the initial rate of β-elimination on L-serine concentration (A, B) and D-serine concentration (C, D) and of racemization on L-serine concentration (E, F), in the absence (A, C, E) and presence of ATP (B, D, F), respectively. The initial rate was determined as reported in Materials and Methods. Data points were fitted to the Michaelis-Menten equation. The calculated K_M, k_{cat} and k_{cat}/K_M values for L- and D-serine are reported in Table 1.

ATP binding affinity to hSR measured in the presence of saturating concentrations of either L- or D-serine.

Because ATP increases hSR catalytic activities, we exploited this effect for determining ATP affinity by monitoring the β–elimination activity at increasing ATP concentrations in the presence of saturating L- or D-serine (Fig. 3A, B) or by monitoring the racemization activity in the presence of saturating L-serine (Fig. 3C). Unexpectedly, the ATP binding curve exhibited a marked sigmoidal shape, indicating positive cooperativity (Fig. 3). The fitting of the titrations to the Hill equation allowed us to determine a cooperative coefficient of 1.7 ± 0.2 for L-serine β–elimination, 1.9 ± 0.2 for D-serine β–elimination, and 2.1 ± 0.7 for L-serine racemization (Tab. 2).

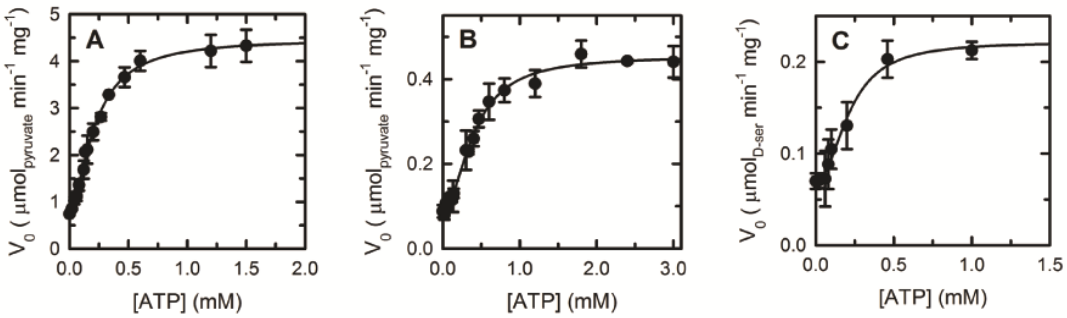

Figure 3. A) Dependence of the L-serine β-elimination activity of hSR on ATP concentration, in the presence of saturating substrate. Experiments were carried out at 37 °C as reported in Materials and Methods, in the presence of 530 mM L-serine. The experimental data were fitted to the Hill equation, yielding a Hill coefficient of 1.7 ± 0.2 and an apparent K_D for ATP of 0.22 ± 0.01 mM. **B) Dependence of the D-serine β-elimination activity of hSR on ATP concentration in the presence of saturating substrate**. Experiments were carried out at 37 °C as reported in Materials and Methods, in the presence of 750 mM D-serine. The experimental data were fitted to a Hill equation, yielding a Hill coefficient of 1.9 ± 0.2 and an apparent K_D for ATP of 0.41 ± 0.03 mM. **C) Dependence of the racemization activity of hSR on ATP concentration in the presence of a saturating concentration of L-Serine**. Experiments were carried out at 37 °C as reported in Materials and Methods, in the presence of 200 mM L-serine. The experimental data were fitted to the Hill equation (see Table 2) yielding a Hill coefficient of 2.1 ± 0.7 and an apparent K_D for ATP of 0.22 ± 0.05 mM.

These Hill coefficients are close or equal to the maximum theoretical value for a protein containing two binding sites, indicating a strong link between the two ATP sites, that are 24 Å away (Fig. 1). The apparent ATP K_D values, determined from the fittings (Fig. 3; Tab. 2), are 0.22 ± 0.01 mM for L-serine β–elimination, 0.41 ± 0.02 mM for D-serine β–elimination, and 0.22 ± 0.05 mM for L-serine racemization (Tab. 2). The cooperative ATP

binding was retained at subsaturating concentrations of L-serine (data not shown) and was not associated to a modulation of the monomer-dimer equilibrium of hSR by ATP, as the specific activity was not dependent on protein concentration in the range 0.5-5 μM (data not shown). The observed cooperativity in ATP binding to SR indicates that the enzyme exists as an equilibrium of at least two conformations endowed with significantly different microscopic dissociation constants for ATP.

Method of binding detection	K_D ATP (mM)	Hill coefficient
Activity assay (L-serine β-elimination)	0.22 ± 0.01	1.7 ± 0.2
Activity assay (D-serine β-elimination)	0.41 ± 0.03	1.9 ± 0.2
Activity assay (L-serine racemization)	0.22 ± 0.05	2.1 ± 0.7
Coenzyme fluorescence (- glycine)	0.26 ± 0.02	1.8 ± 0.2
Coenzyme fluorescence (+ glycine)	0.0049 ± 0.0006	1.02 ± 0.09

Table 2. Binding parameters of ATP to hSR determined by monitoring either L- or D-serine β–elimination, L-serine racemization, and by coenzyme fluorescence changes in the absence and presence of glycine. The dependence of hSR catalytic activity on ATP was carried out as described in Fig. 3 and Materials and Methods, in the presence of either 530 mM L-serine or 750 mM D-serine, and were fitted to the Hill equation. The dependence of coenzyme fluorescence on ATP was carried out as described in Fig. 4-5 and Materials and Methods, in the absence and presence of 50 mM glycine.

ATP binding to hSR detected by fluorescence measurements.

To investigate ATP binding to hSR by directly monitoring changes in protein conformation, we exploited the spectroscopic properties of the bound PLP. The absorption spectrum of hSR exhibited a band centered at 412 nm (Fig. 4A), attributed to the ketoenamine form of the PLP Schiff base with Lys56. When the enzyme was excited at 412 nm, a fluorescence emission band centered at 500 nm was observed (Fig. 4B). PLP emission at 500 nm is a very sensitive probe of the active site conformation both in terms of intensity and wavelength of maximum emission, as previously demonstrated in the investigation of ligand binding to *O*-acetylserine sulfhydrylase (Salsi et al. 2010; Salsi et al. 2010; Spyrakis et al. 2013). Binding of ATP to hSR causes an increase of the emission at 500 nm with a

small blue shift (Fig. 4B). The dependence of fluorescence intensity on ATP concentration was found to be sigmoidal, with a Hill coefficient of 1.8 ± 0.2 and an apparent K_D of 0.26 ± 0.02 mM (Fig. 4B), mirroring the cooperative behaviour observed monitoring enzyme activity (Fig. 3). In addition, the effect of ATP binding on the PLP fluorescence spectrum indicates that ATP induces a conformational change of the active site, with a decrease in its polarity and/or solvent accessibility. Considering that the apparent dissociation constant for ATP determined from fluorimetric measurements in the absence of ligands is close to the values determined by activity assays at saturating concentrations of substrates, it can be concluded that reaction intermediates of neither L- nor D-serine allosterically affect the ATP site.

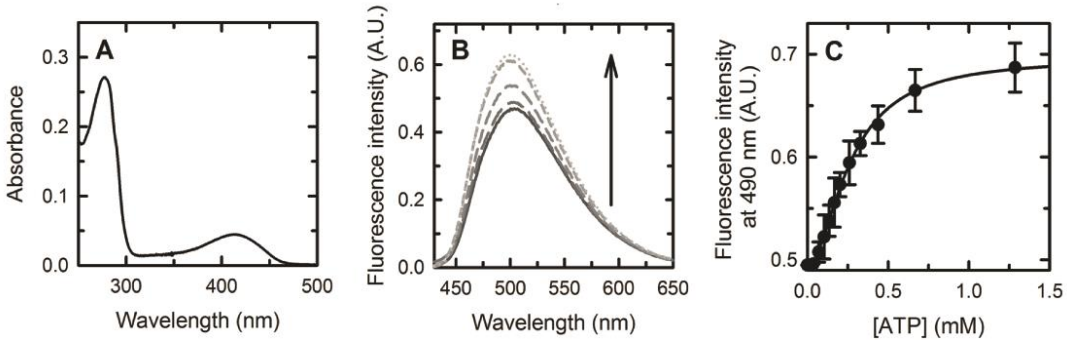

Figure 4. ATP binding to hSR. A) Absorption spectrum of a solution containing 7.4 μM hSR, 50 mM TEA, 150 mM NaCl, 1 mM EDTA, pH 8.0. **B)** Fluorescence emission spectra, upon excitation at 412 nm, of a solution containing 2.7 μM hSR, 50 mM TEA, 150 mM NaCl, 5 mM TCEP, 1 mM $MgCl_2$, pH 8.0 and increasing concentrations of ATP, at 20 °C. **C)** Dependence on ATP concentration of the fluorescence emission intensity at 490 nm upon excitation at 412 nm. The experimental points were fitted to the Hill equation, obtaining a K_D of 0.26 ± 0.02 mM and a Hill coefficient of 1.8 ± 0.2.

To assess whether the allosteric communication between the two ATP sites occurs when the enzyme is involved in a stable complex with a ligand, ATP binding was monitored in the presence of saturating concentrations of glycine, an amino acid capable of forming a reversible Schiff base with SR. Glycine is present in the brain at a concentration that varies significantly depending on localization, from 8–12 μM in the extracellular space (Matsui et al. 1995; Horio et al. 2011) to about 0.6 mM within neuronal cells (Choi et al. 2011), and is released by the same cells responsible for the liberation of D-serine (Rosenberg et al. 2013). The ATP binding curve in the presence of glycine (Fig. 5A) was found to be hyperbolic and

strongly left-shifted with respect to the binding curve for the glycine-free hSR. The calculated K_D was 0.0049 ± 0.0006 mM, indicating a very tight binding. A similar value for ATP affinity was previously reported (De Miranda et al. 2002). Therefore, the presence of glycine stabilizes an hSR conformation that binds ATP with high affinity and non-cooperatively. The novel finding is that ATP binding to the glycine-free and bound forms exhibits remarkable differences, confirming that SR possesses distinct interconverting conformations.

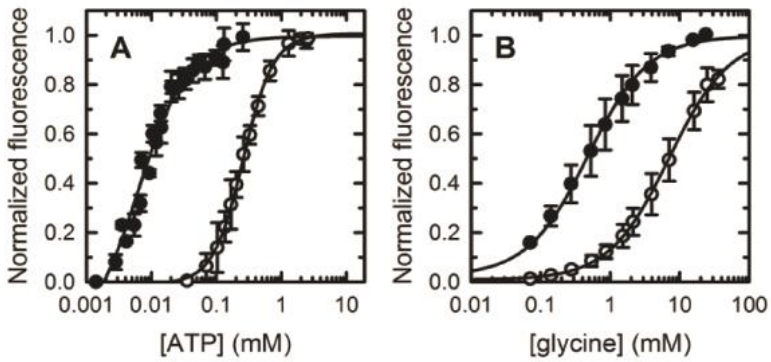

Figure 5. A) Binding of ATP to human SR in the absence and presence of glycine. Fluorescence emission spectra upon excitation at 412 nm of a solution containing 2.7 µM hSR in 50 mM TEA, 150 mM NaCl, 5 mM TCEP, 1 mM $MgCl_2$, pH 8.0, at 20 °C, were collected at increasing ATP concentrations in the absence (open circles, data from Fig. 4C) and in the presence (closed circles) of 50 mM glycine. The dependence of the normalized fluorescence intensity at 490 nm on ATP concentration was fitted to the Hill equation, resulting in the absence of glycine in a Hill coefficient of 1.8 ± 0.2 and K_D of 0.26 ± 0.02 mM. In the presence of glycine data were fitted to the quadratic equation for tight ligand binding (see Materials and Methods) leading to a K_D of 0.0049 ± 0.0006 mM. **B) Binding of glycine to hSR in the absence and presence of ATP.** Fluorescence emission spectra upon excitation at 412 nm were collected at increasing glycine concentrations. Experiments were carried out at 20 °C on a solution containing 2.7 µM hSR in 50 mM TEA, 150 mM NaCl, 5 mM TCEP, 1 mM $MgCl_2$, pH 8.0, in the absence (open circles) and presence (closed circles) of 2 mM ATP. The dependence of the fluorescence emission intensity at 482 nm on glycine concentration was fitted to a binding isotherm, obtaining a K_D for glycine of 0.47 ± 0.03 mM in the presence of ATP and 7.0 ± 0.3 mM in the absence of ATP.

To further evaluate the allosteric effect of ATP binding on the active site, glycine binding was investigated both in the absence and presence of ATP (Fig. 5B). We found that glycine binds non-cooperatively in both cases. A non-cooperative binding was also observed for L- and D-serine, as proved by the pure Michaelis-Menten rate dependence (Fig. 2 and (Foltyn et al. 2005)). However, it is noteworthy that ATP decreased the K_D for glycine about 15-fold, from 7 mM to 0.47 mM. Therefore, ATP-bound SR exhibits a K_D for glycine lower than the intracellular concentration of glycine (0.6 mM, (Choi et al. 2011)),

suggesting a physiological interplay between ATP and glycine that might lead to a complex pattern of SR modulation, that will be further investigated.

Analyzing ATP binding according to the Monod, Wyman, Changeux model.

Within the framework of the Monod, Wyman, Changeux (MWC) model (Monod et al. 1965) applied to ATP binding, glycine-bound SR coincides with a high-affinity, non-cooperative state (R), which is in equilibrium with a low affinity state (T) in the glycine-free or serine-bound SR. Under this assumption, the experimentally determined K_D for ATP in the presence of glycine was assumed to correspond to the dissociation constant of the R state (K_D^R). The direct determination of this dissociation constant at 37 °C by fluorimetric titrations was hampered by the limited stability of SR and ATP. To overcome these limitations, the temperature dependence of the K_D^R was measured by ATP titrations in the presence of glycine at 10, 15 and 20 °C and analyzed as a van't Hoff temperature dependence (Fig. 6).

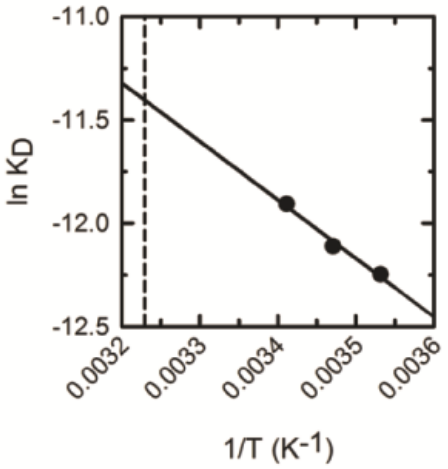

Figure 6. Dependence of binding affinity of ATP in the presence of glycine as a function of temperature.

The extrapolated value of K_D^R for ATP at 37 °C was 0.0115 mM. A global fitting of the activity data reported in Figure 2 using the MWC equation and this fixed K_D^R value allowed for the estimation of a K_D^T for ATP of 1.8 $\pm$ 0.5 mM. This value remarkably falls within the intracellular ATP concentration, that ranges from 1 mM (Ainscow et al. 2002) to about 6

mM (Silver and Erecinska 1997), but locally might be even lower, suggesting that SR is not constantly ATP-saturated. More specifically, this finding indicates that small changes in ATP concentration, as well as glycine concentration, have a strong impact on the distribution between low and high efficiency SR sites.

Conclusions

ATP elicits conformational changes both at the active site (15 Å away) where serine β–elimination and racemization take place and at the symmetric site for ATP (24 Å away) (Fig. 1). Neither L- nor D-serine, within the catalytic cycle, allosterically affect the ATP binding sites or the symmetric active site, whereas glycine binding completely shifts the conformational equilibrium towards the ATP high-affinity state. The structural changes responsible for this complex cross-talk are not known. The yeast homologue of SR crystallized in the absence and presence of the ATP analogue AMP-PCP shows a different relative orientation of the monomers (Goto et al. 2009). It has been suggested that a hydrogen bond network connecting the active site to the ATP-binding site might be responsible for the ATP-dependent modulation of an open/close conformational transition of the active site (Goto et al. 2009). Investigations toward the elucidation of the molecular basis of the communication between allosteric and active sites, exploiting both *in silico* and spectroscopic signals are currently underway in our laboratory. Furthermore, by exploiting the sol-gel method for protein encapsulation (Ellerby et al. 1992), we might be able to trap and fully characterize distinct hSR conformations, as previously carried out for other proteins, including hemoglobin (Bettati and Mozzarelli 1997; Abbruzzetti et al. 2001; Bruno et al. 2001; Shibayama and Saigo 2001; Samuni et al. 2004; Viappiani et al. 2004; Ronda et al. 2006; Ronda et al. 2008), aspartate transcarbamilase (West and Kantrowitz 2003), tryptophan synthase (Pioselli et al. 2005) and green fluorescent protein (Chirico et al. 2002; Campanini et al. 2005). Finally, considering that the intracellular ATP concentration in neurons and astrocytes, where human SR has been detected, is about 1 mM and can vary significantly during metabolic activity and depending on energy supply, our findings suggest that intracellular ATP concentration, as well as the relative concentration of glycine and L-serine, might play a significant role in controlling D-serine homeostasis and, therefore, NMDA receptors activity. In turn, these results support the exploitability of hSR (Amadasi et al. 2007; Wolosker et al. 2008; Conti et al. 2011; Billard 2013; Campanini et al. 2013) as a target for the development of allosteric drugs that, by fine-tuning enzyme activity, might

serve for the treatment of psychiatric disorders and neuropathologies caused by NMDA receptor dysfunctions.

Materials and Methods

Materials

Chemicals were of the best commercial quality available and purchased from Sigma-Aldrich, with the exception of tris(2-carboxyethyl)phosphine (TCEP) from Apollo Scientific, and glycine, NADH and lysozyme from Fluka. Horseradish peroxidase (HRP) was purchased from Sigma-Aldrich. Recombinant DAAO of *Rhodotorula gracilis* was a generous gift from Professor Loredano Pollegioni, University of Insubria, Varese, Italy.

Enzyme preparation

A pET28a-derived plasmid encoding for hexahistidine-fused hSR (Dixon et al. 2006), provided by Prof. Michael Toney, University of California, Davis, CA, USA, was transformed into *E.coli* BL21 CodonPlus (DE3)-RIL cells (Merck-Millipore, Darmstadt, Germany). Cells were grown at 37 °C in LB medium supplemented with kanamycin (50 μg/ml) and chloramphenicol (50 μg/ml). At OD_{600}≈0.5, benzyl alcohol was added to the medium to a final concentration of 10 mM (de Marco et al. 2005) and the growth temperature was lowered to 20 °C. Benzyl alcohol was added because it is known to increase the expression of molecular chaperones in response to a stress condition (de Marco et al. 2005). After 20 minutes, IPTG was added to a final concentration of 10 μM and the culture was further incubated at 20 °C for 20 hours. Cells were harvested by centrifugation and the paste was resuspended in lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 5 mM TCEP, 50 μM PLP, 200 μM PMSF, 200 μM benzamidine, 1.5 μM pepstatine at pH 8.0). Cell walls were then disrupted by treatment with lysozyme (1 mg/ml), followed by sonication. The homogenate was clarified by centrifugation and the supernatant was incubated with a TALON His-Tag Purification Resin (Clontech, CA, USA). After washing the resin with a buffer containing 50 mM sodium phosphate, 150 mM NaCl, 20 mM imidazole, 5 mM TCEP, pH 8.0, the protein was recovered by suspending the resin in a buffer containing 50 mM sodium phosphate, 150 mM NaCl and 250 mM imidazole at pH 8.0. The protein solution was diafiltered three times in Amicon™ stirred cell (Merck-Millipore, Darmstadt, Germany) in 50 mM TEA, 150 mM NaCl, 1 mM EDTA, pH 8. All steps were carried out at 4 °C. The protein solution was concentrated to 64 μM in monomers, supplemented with 5% glycerol, 5 mM

TCEP, flash-frozen and stored at -80 °C in small aliquots. Protein purity was assessed to be 98% by densitometry of Coomassie-blue-stained bands of an SDS-PAGE gel using a Chemidoc gel imaging system (Biorad, CA, USA) (data not shown). The yield of pure SR was 2.5 mg per liter of *E. coli* colture.

Activity Assays

β–elimination reactions - The initial rate of L- or D-serine β-elimination was monitored by coupling the reaction with pyruvate reduction by lactate dehydrogenase and following NADH disappearance at 340 nm (Eisenthal and Danson 1993), using a Varian CARY400 spectrophotometer with a thermostatted cell holder. Within the time course of a measurement (around 10 minutes) it was estimated that less than 0.03 % of the substrate (either L-serine or D-serine) is converted to its enantiomer via racemization. The typical activity assay solution contained 50 mM TEA, 150 mM NaCl, 50 µM PLP, 5 mM DTT, 1 mM $MgCl_2$, 60 U/ml LDH, 300 µM NADH. The dependence of the initial rate on either L- or D-serine concentration was determined in the absence and presence of 1-2 mM ATP.

Racemization reaction - The initial rate of D-serine formation as a function of L-serine concentration was determined via a discontinuous assay based on the oxidation of D-serine by DAAO, that produces hydrogen peroxide (Molla et al. 2012). Hydrogen peroxide-mediated oxidation of *o*-dianisidine by HRP leads to the formation of a chromophoric product that, once treated with sulfuric acid to increase solubility, absorbs at 530 nm. L-serine at different final concentrations was added to a solution containing 50 mM TEA, 150 mM NaCl, 50 µM PLP, 1 mM $MgCl_2$ and 2.3 µM hSR, either in the absence or presence of ATP at concentrations ranging from 0.01 to 2 mM. The reaction mixture was incubated at 37 °C and aliquots were periodically sampled for the determination of D-serine concentration. Because the commercially available L-serine contains a significant amount of D-serine, L-serine solutions were purified prior to use by incubation for 48 hours with 400 U/ml DAAO and 100 U/ml catalase, which were then thermally inactivated. Absolute concentrations of D-serine in the assays were assessed using calibration curves obtained by reacting pure D-serine at known concentrations with DAAO and peroxidase.

Dependence on ATP of L- and D-serine β–elimination and L-serine racemization

The dependence of the initial velocity on ATP concentration was determined either in the presence of saturating L-serine (530 mM) or D-serine (750 mM), corresponding to about a 10-fold excess with respect to the K_M determined in the absence of ATP (Tab. 1). The reactants were pre-equilibrated at 37.0 ± 0.5 °C in the thermostatted cell holder of the spectrophotometer before starting the reaction by adding the enzyme at a final concentration of typically 0.4-0.5 μM. To make sure that dimer dissociation was not affecting activity measurements, the final concentration of hSR was raised up to 5 μM, without observing any change in the catalytic parameters. This demonstrates that the specific enzyme activity did not depend on protein concentration, neither in the absence nor in the presence of ATP.

Absorbance and fluorescence measurements

Absorption spectra were carried out using a Varian CARY400 spectrophotometer, with the cell holder thermostatted at 20.0 ± 0.5 °C. Solutions contained 50 mM TEA, 150 mM NaCl, 1 mM EDTA and 7.4 μM hSR, pH 8.0.

Fluorescence spectra of hSR in the absence and presence of ligands were collected using a FluoroMax-3 fluorometer (HORIBA-Jobin Yvon), with the cell holder thermostatted at 20.0 ± 0.5 °C. Emission spectra upon direct excitation of the cofactor at 412 nm were recorded with slits set for optimal signal-to-noise ratio on a solution containing 2.7 μM enzyme, 50 mM TEA, 150 mM NaCl, 5 mM TCEP, 1 mM $MgCl_2$, pH 8.0. The binding affinity of ligands to hSR was determined by monitoring the increase in fluorescence emission of the coenzyme upon excitation at 412 nm, as previously reported for other PLP-dependent enzymes (Campanini et al. 2005).

Data analysis

To determine the Hill coefficient and the apparent K_D, data points were fitted to the Hill equation:

$$y = y_0 + a * \frac{[L]^n}{{K_D}^n + [L]^n}$$

where *y* is the fluorescence emission intensity or the initial reaction velocity, *[L]* is the ligand concentration (ATP) and *n* the Hill coefficient. y_0 is a horizontal offset and *a* the amplitude.

Given the very high affinity, the determination of the K_D of ATP in the presence of glycine was carried out by fitting the experimental points to a quadratic equation that describes tight binding (Copeland 2000):

$$y = y_0 + a * \frac{[P] + [L] + K_D - \sqrt{([P] + [L] + K_D)^2 - 4 * [P] * [L]}}{2}$$

where *y* is the fluorescence emission intensity, *[P]* is the fixed protein concentration and *[L]* the variable ligand concentration. y_0 is a an horizontal offset and *a* the amplitude.

For the analysis of the data points using a Monod-Wyman and Changeux model (Monod et al. 1965), the following equation for a two-sites protein was applied:

$$y = y_0 + a * \frac{L^{MWC} c\alpha(1 + c\alpha) + \alpha(1 + \alpha)}{(1 + \alpha)^2 + L^{MWC}(1 + c\alpha)^2}$$

where *y* is the fraction of the effect, $L^{MWC} = [T]_0/[R]_0$ is the MWC allosteric constant, i.e. the ratio between the concentration of the two states in the absence of ligand, $c = K_D^R/K_D^T$ is the ratio between the microscopic dissociation constants of the two forms and $\alpha = [L]/K_D^R$ is the normalized concentration of the ligand with respect to K_D^R. y_0 is an horizontal offset and *a* the amplitude. The K_D^R used in the analysis was independently estimated from the binding curves of ATP in the presence of glycine.

The dependence of K_D^R as a function of temperature was determined at 10, 15 and 20 °C and the value at 37 °C was extrapolated using a linear regression using the van't Hoff plot:

$$\ln(K_D) = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R}$$

where *T* is the temperature in °K, ΔH^0 the standard enthalpy, ΔS^0 the standard entropy, *R* the gas constant.

References

Abbruzzetti, S., C. Viappiani, S. Bruno, S. Bettati, M. Bonaccio and A. Mozzarelli (2001). "Functional characterization of heme proteins encapsulated in wet nanoporous silica gels." Journal of Nanoscience and Nanotechnology **1**(4): 407-15.

Ainscow, E. K., S. Mirshamsi, T. Tang, M. L. Ashford and G. A. Rutter (2002). "Dynamic imaging of free cytosolic ATP concentration during fuel sensing by rat hypothalamic neurones: evidence for ATP-independent control of ATP-sensitive K(+) channels." The Journal of Physiology **544**(Pt 2): 429-45.

Amadasi, A., M. Bertoldi, R. Contestabile, S. Bettati, B. Cellini, M. L. di Salvo, C. Borri-Voltattorni, F. Bossa and A. Mozzarelli (2007). "Pyridoxal 5'-phosphate enzymes as targets for therapeutic agents." Current Medicinal Chemistry **14**(12): 1291-324.

Benneyworth, M. A., Y. Li, A. C. Basu, V. Y. Bolshakov and J. T. Coyle (2012). "Cell selective conditional null mutations of serine racemase demonstrate a predominate localization in cortical glutamatergic neurons." Cellular and Molecular Neurobiology **32**(4): 613-24.

Bettati, S. and A. Mozzarelli (1997). "T state hemoglobin binds oxygen noncooperatively with allosteric effects of protons, inositol hexaphosphate, and chloride." The Journal of Biological Chemistry **272**(51): 32050-5.

Billard, J. M. (2013). "Serine racemase as a prime target for age-related memory deficits." European Journal of Neuroscience **37**(12): 1931-8.

Boehning, D. and S. H. Snyder (2003). "Novel neural modulators." Annual Review of Neuroscience **26**: 105-31.

Bruno, S., M. Bonaccio, S. Bettati, C. Rivetti, C. Viappiani, S. Abbruzzetti and A. Mozzarelli (2001). "High and low oxygen affinity conformations of T state hemoglobin." Protein Science **10**(11): 2401-7.

Campanini, B., S. Bologna, F. Cannone, G. Chirico, A. Mozzarelli and S. Bettati (2005). "Unfolding of Green Fluorescent Protein mut2 in wet nanoporous silica gels." Protein Science **14**(5): 1125-33.

Campanini, B., F. Speroni, E. Salsi, P. F. Cook, S. L. Roderick, B. Huang, S. Bettati and A. Mozzarelli (2005). "Interaction of serine acetyltransferase with O-acetylserine sulfhydrylase active site: evidence from fluorescence spectroscopy." Protein Science **14**(8): 2115-24.

Campanini, B., F. Spyrakis, A. Peracchi and A. Mozzarelli (2013). "Serine racemase: a key player in neuron activity and in neuropathologies." Frontiers in Bioscience **18**: 1112-28.

Changeux, J. P. (2012). "Allostery and the Monod-Wyman-Changeux model after 50 years." Annual Review of Biophysics **41**: 103-33.

Chirico, G., F. Cannone, S. Beretta, A. Diaspro, B. Campanini, S. Bettati, R. Ruotolo and A. Mozzarelli (2002). "Dynamics of green fluorescent protein mutant2 in solution, on spin-coated glasses, and encapsulated in wet silica gels." Protein Science **11**(5): 1152-61.

Choi, C., S. K. Ganji, R. J. DeBerardinis, I. E. Dimitrov, J. M. Pascual, R. Bachoo, B. E. Mickey, C. R. Malloy and E. A. Maher (2011). "Measurement of glycine in the human brain in vivo by 1H-MRS at 3 T: application in brain tumors." Magnetic Resonance in Medicine **66**(3): 609-18.

Conti, P., L. Tamborini, A. Pinto, A. Blondel, P. Minoprio, A. Mozzarelli and C. De Micheli (2011). "Drug discovery targeting amino acid racemases." Chemical Reviews **111**(11): 6919-46.

Copeland, R. A. (2000). Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis, Wiley-VCH, Inc.

Cui, Q. and M. Karplus (2008). "Allostery and cooperativity revisited." Protein Science **17**(8): 1295-307.

de Marco, A., L. Vigh, S. Diamant and P. Goloubinoff (2005). "Native folding of aggregation-prone recombinant proteins in Escherichia coli by osmolytes, plasmid- or benzyl alcohol-overexpressed molecular chaperones." Cell Stress Chaperones **10**(4): 329-39.

De Miranda, J., R. Panizzutti, V. N. Foltyn and H. Wolosker (2002). "Cofactors of serine racemase that physiologically stimulate the synthesis of the N-methyl-D-aspartate (NMDA) receptor coagonist D-serine." PNAS **99**(22): 14542-7.

Ding, X., N. Ma, M. Nagahama, K. Yamada and R. Semba (2011). "Localization of D-serine and serine racemase in neurons and neuroglias in mouse brain." Neurological Sciences **32**(2): 263-7.

Dixon, S. M., P. Li, R. Liu, H. Wolosker, K. S. Lam, M. J. Kurth and M. D. Toney (2006). "Slow-binding human serine racemase inhibitors from high-throughput screening of combinatorial libraries." The Journal of Medicinal Chemistry **49**(8): 2388-97.

Eaton, W. A., E. R. Henry, J. Hofrichter, S. Bettati, C. Viappiani and A. Mozzarelli (2007). "Evolution of allosteric models for hemoglobin." IUBMB Life **59**(8-9): 586-99.

Eisenthal, R. and M. Danson, Eds. (1993). "Enzyme Assays.", Oxford Univ. Press.

Ellerby, L. M., C. R. Nishida, F. Nishida, S. A. Yamanaka, B. Dunn, J. S. Valentine and J. I. Zink (1992). "Encapsulation of proteins in transparent porous silicate glasses prepared by the sol-gel method." Science **255**(5048): 1113-5.

Foltyn, V. N., I. Bendikov, J. De Miranda, R. Panizzutti, E. Dumin, M. Shleper, P. Li, M. D. Toney, E. Kartvelishvily and H. Wolosker (2005). "Serine racemase modulates intracellular D-serine levels through an alpha,beta-elimination activity." The Journal of Biological Chemistry **280**(3): 1754-63.

Foltyn, V. N., M. Zehl, E. Dikopoltsev, O. N. Jensen and H. Wolosker (2010). "Phosphorylation of mouse serine racemase regulates D-serine synthesis." FEBS Letters **584**(13): 2937-41.

Goto, M., T. Yamauchi, N. Kamiya, I. Miyahara, T. Yoshimura, H. Mihara, T. Kurihara, K. Hirotsu and N. Esaki (2009). "Crystal structure of a homolog of mammalian serine racemase from Schizosaccharomyces pombe." The Journal of Biological Chemistry **284**(38): 25944-52.

Henneberger, C., L. Bard, C. King, A. Jennings and D. A. Rusakov (2013). "NMDA receptor activation: two targets for two co-agonists." Neurochemical Research **38**(6): 1156-62.

Hoffman, H. E., J. Jiraskova, M. Ingr, M. Zvelebil and J. Konvalinka (2009). "Recombinant human serine racemase: enzymologic characterization and comparison with its mouse ortholog." Protein Expression and Purification **63**(1): 62-7.

Horio, M., M. Kohno, Y. Fujita, T. Ishima, R. Inoue, H. Mori and K. Hashimoto (2011). "Levels of D-serine in the brain and peripheral organs of serine racemase (Srr) knock-out mice." Neurochemistry International **59**(6): 853-9.

Ito, T., M. Maekawa, S. Hayashi, M. Goto, H. Hemmi and T. Yoshimura (2013). "Catalytic mechanism of serine racemase from Dictyostelium discoideum." Amino Acids **44**(3): 1073-84.

Jiraskova-Vanickova, J., R. Ettrich, B. Vorlova, H. E. Hoffman, M. Lepsik, P. Jansa and J. Konvalinka (2011). "Inhibition of human serine racemase, an emerging target for medicinal chemistry." Current Drug Targets **12**(7): 1037-55.

Johnson, J. W. and P. Ascher (1987). "Glycine potentiates the NMDA response in cultured mouse brain neurons." Nature **325**(6104): 529-31.

Kessler, M., T. Terramani, G. Lynch and M. Baudry (1989). "A glycine site associated with N-methyl-D-aspartic acid receptors: characterization and identification of a new class of antagonists." Journal of Neurochemistry **52**(4): 1319-28.

Labrie, V., A. H. Wong and J. C. Roder (2012). "Contributions of the D-serine pathway to schizophrenia." Neuropharmacology **62**(3): 1484-503.

Ma, T. M., S. Abazyan, B. Abazyan, J. Nomura, C. Yang, S. Seshadri, A. Sawa, S. H. Snyder and M. V. Pletnikov (2013). "Pathogenic disruption of DISC1-serine racemase binding elicits schizophrenia-like behavior via D-serine depletion." Molecular Psychiatry **18**(5): 557-67.

Martineau, M., G. Baux and J. P. Mothet (2006). "D-serine signalling in the brain: friend and foe." Trends in Neurosciences **29**(8): 481-91.

Matsui, T., M. Sekiguchi, A. Hashimoto, U. Tomita, T. Nishikawa and K. Wada (1995). "Functional comparison of D-serine and glycine in rodents: the effect on cloned NMDA receptors and the extracellular concentration." Journal of Neurochemistry **65**(1): 454-8.

Molla, G., L. Piubelli, F. Volonte and M. S. Pilone (2012). "Enzymatic detection of D-amino acids." Methods in Molecular Biology **794**: 273-89.

Monod, J., J. Wyman and J. P. Changeaux (1965). "On the nature of allosteric transitions: a plausible model." The Journal of Molecular Biology **12**: 88-118.

Mustafa, A. K., A. S. Ahmad, E. Zeynalov, S. K. Gazi, G. Sikka, J. T. Ehmsen, R. K. Barrow, J. T. Coyle, S. H. Snyder and S. Dore (2010). "Serine racemase deletion protects against cerebral ischemia and excitotoxicity." The Journal of Neuroscience **30**(4): 1413-6.

Mustafa, A. K., M. Kumar, B. Selvakumar, G. P. Ho, J. T. Ehmsen, R. K. Barrow, L. M. Amzel and S. H. Snyder (2007). "Nitric oxide S-nitrosylates serine racemase, mediating feedback inhibition of D-serine formation." PNAS **104**(8): 2950-5.

Neidle, A. and D. S. Dunlop (2002). "Allosteric regulation of mouse brain serine racemase." Neurochemical Research **27**(12): 1719-24.

Nussinov, R. and C. J. Tsai (2013). "Allostery in disease and in drug discovery." Cell **153**(2): 293-305.

Papouin, T., L. Ladepeche, J. Ruel, S. Sacchi, M. Labasque, M. Hanini, L. Groc, L. Pollegioni, J. P. Mothet and S. H. Oliet (2012). "Synaptic and extrasynaptic NMDA receptors are gated by different endogenous coagonists." Cell **150**(3): 633-46.

Passera, E., B. Campanini, F. Rossi, V. Casazza, M. Rizzi, R. Pellicciari and A. Mozzarelli (2011). "Human kynurenine aminotransferase II--reactivity with substrates and inhibitors." FEBS Journal **278**(11): 1882-900.

Peracchi, A. and A. Mozzarelli (2011). "Exploring and exploiting allostery: Models, evolution, and drug targeting." Biochimica Et Biophysica Acta - Proteins and Proteomics **1814**(8): 922-933.

Pioselli, B., S. Bettati and A. Mozzarelli (2005). "Confinement and crowding effects on tryptophan synthase alpha2beta2 complex." FEBS Letters **579**(10): 2197-202.

Pollegioni, L. and S. Sacchi (2010). "Metabolism of the neuromodulator D-serine." Cellular and Molecular Life Sciences **67**(14): 2387-404.

Ronda, L., S. Bruno, S. Faggiano, S. Bettati and A. Mozzarelli (2008). "Oxygen binding to heme proteins in solution, encapsulated in silica gels, and in the crystalline state." Methods in Enzymology **437**: 311-28.

Ronda, L., S. Bruno, C. Viappiani, S. Abbruzzetti, A. Mozzarelli, K. C. Lowe and S. Bettati (2006). "Circular dichroism spectroscopy of tertiary and quaternary conformations of human hemoglobin entrapped in wet silica gels." Protein Science **15**(8): 1961-7.

Rosenberg, D., S. Artoul, A. C. Segal, G. Kolodney, I. Radzishevsky, E. Dikopoltsev, V. N. Foltyn, R. Inoue, H. Mori, J. M. Billard and H. Wolosker (2013). "Neuronal D-serine and glycine release via the Asc-1 transporter regulates NMDA receptor-dependent synaptic activity." The Journal of Neuroscience **33**(8): 3533-44.

Sacchi, S., L. Caldinelli, P. Cappelletti, L. Pollegioni and G. Molla (2012). "Structure-function relationships in human D-amino acid oxidase." Amino Acids **43**(5): 1833-50.

Salsi, E., A. S. Bayden, F. Spyrakis, A. Amadasi, B. Campanini, S. Bettati, T. Dodatko, P. Cozzini, G. E. Kellogg, P. F. Cook, S. L. Roderick and A. Mozzarelli (2010). "Design of O-acetylserine sulfhydrylase inhibitors by mimicking nature." The Journal of Medicinal Chemistry **53**(1): 345-56.

Salsi, E., B. Campanini, S. Bettati, S. Raboni, S. L. Roderick, P. F. Cook and A. Mozzarelli (2010). "A two-step process controls the formation of the bienzyme cysteine synthase complex." The Journal of Biological Chemistry **285**(17): 12813-22.

Samuni, U., D. Dantsker, L. J. Juszczak, S. Bettati, L. Ronda, A. Mozzarelli and J. M. Friedman (2004). "Spectroscopic and functional characterization of T state hemoglobin conformations encapsulated in silica gels." Biochemistry **43**(43): 13674-82.

Shibayama, N. and S. Saigo (2001). "Direct observation of two distinct affinity conformations in the T state human deoxyhemoglobin." FEBS Letters **492**(1-2): 50-3.

Silver, I. A. and M. Erecinska (1997). "Energetic demands of the Na+/K+ ATPase in mammalian astrocytes." Glia **21**(1): 35-45.

Smith, M. A., V. Mack, A. Ebneth, I. Moraes, B. Felicetti, M. Wood, D. Schonfeld, O. Mather, A. Cesura and J. Barker (2010). "The structure of mammalian serine racemase: evidence for conformational changes upon inhibitor binding." The Journal of Biological Chemistry **285**(17): 12873-81.

Spyrakis, F., P. Felici, A. S. Bayden, E. Salsi, R. Miggiano, G. E. Kellogg, P. Cozzini, P. F. Cook, A. Mozzarelli and B. Campanini (2013). "Fine tuning of the active site modulates specificity in the interaction of O-acetylserine sulfhydrylase isozymes with serine acetyltransferase." Biochimica et Biophysica Acta - Proteins and Proteomics **1834**(1): 169-181.

Viappiani, C., S. Bettati, S. Bruno, L. Ronda, S. Abbruzzetti, A. Mozzarelli and W. A. Eaton (2004). "New insights into allosteric mechanisms from trapping unstable protein conformations in silica gels." PNAS **101**(40): 14414-9.

West, J. M. and E. R. Kantrowitz (2003). "Trapping specific quaternary states of the allosteric enzyme aspartate transcarbamoylase in silica matrix sol-gels." Journal of the American Chemical Society **125**(33): 9924-5.

Wolosker, H. (2007). "NMDA receptor regulation by D-serine: new findings and perspectives." Molecular Neurobiology **36**(2): 152-64.

Wolosker, H. (2011). "Serine racemase and the serine shuttle between neurons and astrocytes." Biochimica et Biophysica Acta **1814**(11): 1558-66.

Wolosker, H., S. Blackshaw and S. H. Snyder (1999). "Serine racemase: a glial enzyme synthesizing D-serine to regulate glutamate-N-methyl-D-aspartate neurotransmission." PNAS **96**(23): 13409-14.

Wolosker, H., E. Dumin, L. Balan and V. N. Foltyn (2008). "D-amino acids in the brain: D-serine in neurotransmission and neurodegeneration." FEBS Journal **275**(14): 3514-26.

Wolosker, H. and H. Mori (2012). "Serine racemase: an unconventional enzyme for an unconventional transmitter." Amino Acids **43**(5): 1895-904.

Yoshimura, T. and M. Goto (2008). "D-amino acids in the brain: structure and function of pyridoxal phosphate-dependent amino acid racemases." FEBS Journal **275**(14): 3527-37.

ACTIVITY REGULATION BY LIGANDS

Regulation of human serine racemase activity and dynamics by halides, ATP and malonate

Marialaura Marchetti[1], Stefano Bruno[2,6], Barbara Campanini[2], Stefano Bettati[3,4,5], Alessio Peracchi[1] and Andrea Mozzarelli[2,4,5]

[1] Department of Life Sciences, University of Parma, Italy
[2] Department of Pharmacy, University of Parma, Italy
[3] Department of Neurosciences, University of Parma, Italy
[4] Biopharmanet-TEC Center, University of Parma, Italy
[5] National Institute of Biostructures and Biosystems, Rome, Italy
[6] Institute of Biosciences and Bioresources, CNR, Naples, Italy

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Abstract

D-serine is a non-proteinogenic amino acid that acts as a co-agonist of the NMDA receptors in the central nervous system. D-serine is produced by human serine racemase (hSR), a homodimeric pyridoxal 5'-phosphate-dependent enzyme that also catalyzes the physiologically relevant β-elimination of both L- and D-serine to pyruvate and ammonia. After improving the protein purification yield and stability, which had so far limited the biochemical characterization of hSR, we found that the catalytic activity is affected by halides, in the order fluoride>chloride>bromide. On the contrary, iodide elicited a complete inhibition, accompanied by a modulation of the tautomeric equilibrium of the internal aldimine. We also investigated the reciprocal effects of ATP and malonate, an inhibitor that reversibly binds at the active site, 20 Å away from the ATP binding site. ATP increased nine-fold the affinity of hSR for malonate and malonate increased 100-fold that of ATP, confirming an allosteric interaction between the two binding sites. To further investigate this allosteric communication, we probed the active site accessibility by quenching of the coenzyme fluorescence in the absence and presence of ATP. We found that ATP stabilizes a closed conformation of the external aldimine Schiff base, suggesting a possible mechanism for ATP-induced hSR activation.

Introduction

D-serine is a co-agonist of the N-methyl D-aspartate receptors (NMDARs) for glutamate, the main excitatory neurotransmitter in the central nervous system of vertebrates. The NMDARs are unique in that they respond to three different amino acid agonists, i.e. glutamate, glycine and D-serine (Traynelis et al. 2010). Human serine racemase (hSR, EC 5.1.1.18) (Campanini et al. 2013; Canu et al. 2014; De Miranda et al. 2002; Foltyn et al. 2005; Foltyn et al. 2010; Hoffman et al. 2009; Wolosker et al. 1999) is a pyridoxal 5'-phosphate (PLP)-dependent, homodimeric enzyme predominantly localized in neurons and astrocytes (Benneyworth et al. 2012; Ding et al. 2011). SR has also been found in cells not involved in glutamatergic neurotransmission, such as keratinocytes (Inoue et al. 2014) and even in organisms where no NMDARs homologs are present (Ito et al. 2013). SR belongs to the fold-type II of PLP-dependent enzymes and is structurally similar to O-acetylserine sulfhydrylase (OASS) and to the β-subunit of tryptophan synthase (TS) (Campanini et al. 2005; Marabotti et al. 2001; Mozzarelli and Bettati 2006; Mozzarelli et al. 2011; Raboni et al. 2009; Salsi et al. 2010; Spyrakis et al. 2013; Tian et al. 2010). SR catalyzes both the reversible racemization of L-serine to D-serine and the irreversible β-elimination of both enantiomers to produce pyruvate and ammonia (De Miranda et al. 2002; Foltyn et al. 2005; Marchetti et al. 2013), a reaction analogous to those catalyzed by other PLP-dependent enzymes (Bruno et al. 2001; Kaiser et al. 2003; Phillips et al. 2002; Toney 2011). Both reactions contribute to D-serine homeostasis, with the former producing and the latter removing D-serine. The brain areas lacking D-amino acid oxidase (Sacchi et al. 2012), the main degradative enzyme for D-amino acids, rely only on SR to control the concentration of D-serine (Foltyn et al. 2005).

A defective NMDARs-mediated neurotransmission is associated with several pathologies. A hypo-activation is linked to schizophrenia and a hyper-activation is linked to Alzheimer's and Parkinson's diseases, ischemia, amyotrophic lateral sclerosis and Rett syndrome (Labrie et al. 2012; Ma et al. 2013; Mustafa et al. 2010). Direct pharmacological targeting of NMDARs was shown to produce side effects that are not compatible with the long-term treatment of these diseases (Lipton 2006; Molla et al. 2006). For this reason, the

indirect pharmacological modulation of NMDARs via the adjustment of D-serine synthesis and degradation by SR has been proposed as a promising strategy (Conti et al. 2011; Jiraskova-Vanickova et al. 2011; Lipton 2006), also considering that PLP-dependent enzymes are emerging as increasingly relevant drug targets (Amadasi et al. 2007; Amori et al. 2012; Spyrakis et al. 2014). This approach requires an in-depth knowledge of the subtle molecular details of SR function and regulation. It was demonstrated that ATP, that binds at two symmetric sites at the subunit interface (Goto et al. 2009), increases hSR activity (De Miranda et al. 2002), resulting in changes of both the overall activity of SR and of the relative rates of racemization and β-elimination (De Miranda et al. 2002; Foltyn et al. 2005; Neidle and Dunlop 2002). We recently showed that ATP binds in a strongly cooperative fashion (Marchetti et al. 2013), indicating a regulation of SR activity within the range of intracellular ATP concentrations (Ainscow et al. 2002; Silver and Erecinska 1997). We also showed that glycine, another co-agonist of NMDARs and an unproductive ligand of SR, increases ATP affinity and abolishes cooperativity, hinting at a physiologically relevant regulation of D-serine synthesis by the intracellular levels of ATP and glycine (Marchetti et al. 2013). SR function is also modulated by divalent cations (De Miranda et al. 2002), S-nitrosylation (Mustafa et al. 2007), phosphorylation and translocation to the membrane (Balan et al. 2009), and by proteins like GRIP1 (Kim et al. 2005), PICK1 (Fujii et al. 2006; Zhuang et al. 2010), Golga3 (Dumin et al. 2006) and DISC1 (Ma et al. 2013).

Some fold type II PLP-dependent enzymes are known to be regulated by cations or anions binding at specific sites (Burkhard et al. 2000; Peracchi et al. 1995). For example, the catalytic activity and the distribution of reaction intermediates of TS are strongly affected by monovalent cations, which bind to a specific site near the active site (Peracchi et al. 1995), whereas the activity of OASS is affected by chloride, which binds at the intersubunit interface (Burkhard et al. 2000). SR from the soil-living mycetozoan *Dictyostelium discoideum*, uniquely among SR homologs, is modulated by Na^+ binding at the same site as Mg^{2+}, with a twofold increase of the k_{cat} and no effect on the K_M (Ito et al. 2013). Here, we explored the conformational and catalytic properties of hSR by assessing its regulation by

monovalent cations, halides and phosphates. We also investigated the interplay between binding of ATP and the noncovalent active site ligand malonate.

Results and Discussion

Human serine racemase stability

The stability of hSR in storage buffer 1 (SB1, see Materials and Methods) was evaluated with microdialysis experiments against 50 different exchange solutions. For each condition, the residual L-serine β-elimination activity was assessed by enzyme assays and the residual soluble fraction was determined by SDS-PAGE. Dialysis against 50 mM TRIS buffer or TEA buffer at pH 8.0 led to an almost complete loss of the enzyme activity and to protein precipitation (Fig. 1). On the contrary, exchange with a buffer containing 50 mM phosphate, a weak chelating agent, led to a significantly higher residual activity and prevented precipitation, with a further improvement when 1 mM EDTA was added (Fig. 1).

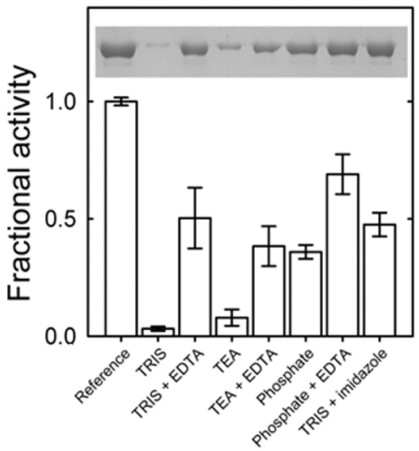


Figure 1. Optimization of buffer composition for hSR activity and stability. L-serine β-elimination activity of hSR after three cycles of dialysis (1.5 hours each) against exchange solutions containing: i) 50 mM TRIS (TRIS), ii) 50 mM TRIS in the presence of 1 mM EDTA (TRIS + EDTA); iii) 50 mM TEA (TEA), iv) 50 mM TEA in the presence of 1 mM EDTA (TEA + EDTA), v) 50 mM sodium phosphate (phosphate), vi) 50 mM phosphate in the presence of 1 mM EDTA (phosphate + EDTA); vii) 50 mM TRIS plus 100 mM imidazole (TRIS+ imidazole). All solutions were at pH 8.0. The activity is expressed as a fraction of the activity of the enzyme before dialysis (Reference), 1.7 $\mu mol_{pyruvate} min^{-1} mg^{-1}$. The assays were carried out in an assay solution (AS, see Materials and Methods).
Inset. SDS-PAGE gel of the supernatant of protein solutions.

These findings suggested that precipitation in TEA and TRIS buffers is associated with the presence of metal ions. Consistently, addition of 1 mM EDTA to the TRIS and TEA buffers prevented loss of activity and precipitation (Fig. 1), leading to an enzyme stable and active for at least 8 hours, at 20 °C (data not shown). This finding explained our earlier observation

that imidazole, a weak metal chelating agent used in Co^{2+}-columns chromatography, stabilized the protein until its concentration dropped below a threshold during the post-chromatography dialysis (unpublished observation). Dialysis experiments showed that protein solubility was strongly dependent on imidazole concentration in the range 20-250 mM, with the lower concentrations resulting in complete protein precipitation (data not shown). At 100 mM concentration, activity was largely retained (Fig. 1).

When hSR was pre-treated with an EDTA-containing solution, dialysis against the same exchange solutions reported in Figure 1, including those not containing EDTA, did not affect the enzyme activity, indicating that, once the heavy metals are removed, the protein is stable irrespective of the final buffer composition (Fig. 2).

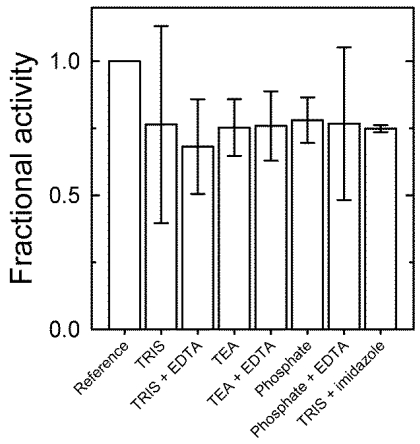


Figure 2. Residual β-elimination activity of hSR stored in the presence of 1 mM EDTA after cycles of dialysis versus solutions containing 50 mM TRIS (TRIS); 50 mM TRIS, 1 mM EDTA (TRIS + EDTA); 50 mM TEA (TEA), 50 mM TEA, 1 mM EDTA (TEA + EDTA), 50 mM sodium phosphate (Phosphate); 50 mM sodium phosphate, 1 mM EDTA (Phosphate + EDTA); 50 mM TRIS plus 100 mM imidazole (TRIS + imidazole). All solutions were at pH 8.0. The residual activity is expressed as a fraction of the activity of the enzyme before dialysis, 1.8 $\mu mol_{pyruvate} min^{-1} mg^{-1}$.

To confirm this hypothesis, $CoCl_2$ at 2 mM concentration was added to an hSR solution pre-treated with EDTA and incubated for 2 hours, resulting in a complete inactivation and precipitation, as determined by enzyme assays and SDS-PAGE. Similar results were obtained in the presence of Ni^{2+} (data not shown). These findings have a very practical implication in the preparation of purified hSR. In previously published protocols (Smith et al. 2010; Wang and Barger 2011), the enzyme was eluted from either Co^{2+} or Ni^{2+} columns

with imidazole and then dialyzed against a TEA buffer in the absence of chelating agents. Alternative protocols were needed to increase yields (Nagayoshi et al. 2005; Nagayoshi et al. 2009). The influence of buffer composition on the activity of hSR, particularly TRIS and phosphate buffers, was already reported (Wang and Barger 2011), and interpreted as a change in oligomer population and an intrinsic higher activity of the enzyme in phosphate buffers. Here we demonstrated that metal chelation stabilizes SR, whereas Co^{2+} (or Ni^{2+}) causes SR precipitation.

We also evaluated the role of PLP on hSR stability and activity. Upon extensive dialysis against a PLP-free solution, less than 15% of hSR was in the apo state, indicating a strong affinity for the coenzyme. An equivalent 15% decrease in activity of the dialyzed enzyme was fully reversed upon addition of free PLP to the assay solution (data not shown).

Based on the stability assays, the original conservation solution SB1 was changed in subsequent experiments by i) eliminating the excess of PLP and $MgCl_2$, and ii) adding the chelating agent EDTA. The resulting storage buffer (SB2) contained 50 mM TEA, 150 mM NaCl, 1 mM EDTA and 5% glycerol, pH 8.0. The modified purification protocol led to higher yields (2.5 mg/l vs 1.0 mg/l of culture), a higher stability and a twofold increase in specific activity. The absence of free PLP in the storage buffer allowed for the spectrofluorimetric characterization of hSR (Marchetti et al. 2013).

Effects of phosphates on hSR activity

To evaluate whether phosphates increase the enzyme activity, as previously suggested (Wang and Barger 2011), possibly partially eliciting the effects of ATP by binding to the same site, either 50 mM phosphate or 50 mM pyrophosphate were added to the assay mixtures containing L-serine at concentrations of either 30 mM, a value close to the K_M, or 300 mM, close to saturation. Phosphate did not elicit any relevant effect, neither in the presence or absence of ATP, in disagreement with reported results on hSR (Wang and Barger 2011). In contrast, 50 mM pyrophosphate completely abolished enzyme activity both in the absence and in the presence of 2 mM ATP (data not shown). To evaluate whether this effect was due to a competition with ATP at the ATP binding site or to

chelation of Mg^{2+}, either ATP or Mg^{2+} were added in large excess to an assay mixture containing 5 mM pyrophosphate. Only supplementation with Mg^{2+} led to a recovery of activity (Fig. 3), indicating that pyrophosphate is likely to act as a chelating agent rather than as a ligand of hSR. AMP was also tested as a ligand at the allosteric site, considering that the ATP/AMP ratio reflects the metabolic state of the cell and might therefore have a physiological relevance. Considering that AMP alone, unlike ATP, only marginally affects the enzyme activity (Neidle and Dunlop 2002), displacement assays were carried out with a ten-fold excess with respect to ATP. AMP did not decrease the activity of hSR, indicating that ATP is not significantly displaced (data not shown).

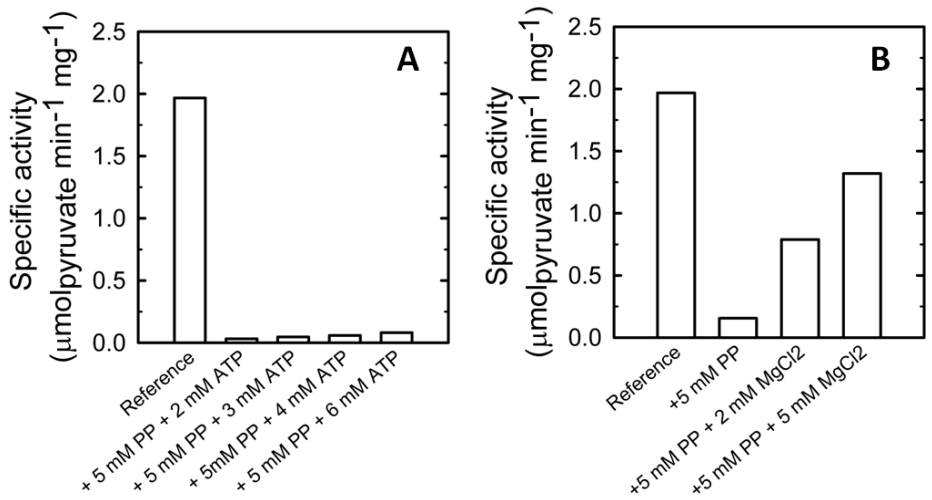


Figure 3. Activity of hSR in the presence of 5 mM sodium pyrophosphate (PP) and in the absence and presence of increasing concentrations of ATP (A) or Mg^{2+} (B). The reference is the assay carried out in the absence of sodium pyrophosphate.

Effects of halides on hSR activity

Considering that the catalytic parameters of several fold type II PLP-dependent enzymes are affected by cations or anions (Peracchi et al. 1995; Burkhard et al. 2000), we investigated the effects of monovalent cations and halides on hSR.

Monovalent cations showed no additional effect with respect to chloride, their counter ion, irrespective of size (Fig. 4).

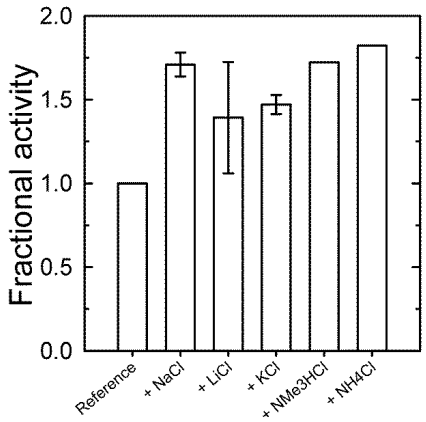


Figure 4. β-Elimination activity of hSR in chloride-free AS with L-serine at 100 mM concentration. Assays were carried out in the absence (reference) and in the presence of 150 mM LiCl, KCl, NMe_3·HCl and NH_4Cl. All activities were normalized to a specific activity measured in the absence of salts of 2.3 $\mu mol_{pyruvate} min^{-1} mg^{-1}$.

Halides, on the other hand, added to a chloride-free assay solution (AS, see Materials and Methods) as sodium salts, exhibited a concentration-dependent effect on hSR activity, without apparently reaching a plateau, in inverse order of ionic radius (Fig. 5A). At 200 mM concentration, fluoride increased the activity almost 3-fold and chloride 2-fold, whereas bromide had almost no effect and iodide abolished the activity completely at concentrations higher than 300 mM (Fig. 5A). Further increases in fluoride concentration were hampered by the lower solubility of its sodium salt in AS. Sodium acetate, which was included to evaluate non-specific ionic strength effects, slightly reduced the activity to the same extent as bromide, indicating that the effects of fluoride and chloride, on the one hand, and iodide, on the other, are not simply associated with ionic strength. The same assays were also carried out in the absence of ATP, with comparable results (data not shown), indicating that halides do not affect ATP binding.

Since chloride is the only physiologically abundant halide, the effects on hSR kinetic parameters were studied in detail (Fig. 5B). At 150 mM concentration, chloride affected both the K_M and the k_{cat}, with a twofold increase of both parameters (from 76.5 $\pm$ 1 to 176 $\pm$ 5 min^{-1} for the k_{cat} and from 15.9 $\pm$ 0.8 to 31.9 $\pm$ 3.6 mM for the K_M). Therefore, the effect of chloride resembles that of 'uncompetitive activators' (e.g. (Wild et al. 1976; Maruyama 1990)), which do not change the catalytic efficiency (k_{cat}/K_M).

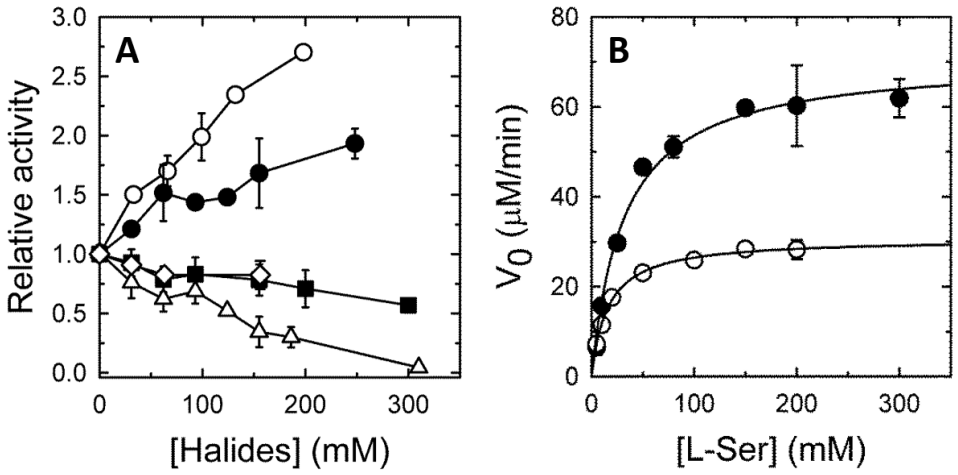


Figure 5. Effect of monovalent ions on β-elimination activity of hSR. A. Dependence of the L-serine β-elimination activity on the concentration of sodium halides: fluoride (open circles), chloride (closed circles), bromide (open diamonds), iodide (open triangles). Sodium acetate (closed squares) was evaluated as a non-halide reference. Reactions were carried out in AS buffer and in the presence of 100 mM L-serine. All values are normalized to the specific activity measured in the absence of halides equal to 2.3 $\mu mol_{pyruvate} min^{-1} mg^{-1}$. **B. Dependence of initial velocity for L-Ser β-elimination on substrate concentration in the presence (closed circles) and absence (open circles) of 150 mM NaCl.** The assays were carried out in a solution containing 50 mM TEA, 2 mM ATP, 50 μM PLP, 5 mM DTT, 1 mM $MgCl_2$, 60 U/ml LDH and 300 μM NADH, pH 8.0, at 37 °C. The lines through the data points are the fitting to the Michaelis-Menten equation with K_M = 15.9 ± 0.8 mM and k_{cat} = 76 ± 1 min^{-1} in the absence of NaCl and K_M = 31.9 ± 3.6 mM and k_{cat} = 176 ± 5 min^{-1} in the presence of NaCl. The catalytic efficiency is 4.77 min^{-1} mM^{-1} and 5.51 min^{-1} mM^{-1} in the absence and presence of NaCl, respectively.

Although it is unlikely that halides at the concentrations we tested could have any physiological relevance, their effect might reveal a conformational equilibrium involving ionic interactions. In the case of haemoglobin, for instance, chloride ions mask positive charges and shift the T-R conformational equilibrium (Perutz et al. 1994). The open-to-closed transition upon active-site ligand binding of hSR (Smith et al. 2010) could be regulated in a similar manner. The larger effect of fluoride with respect to chloride and the negligible effect of bromide might reflect the limited steric accessibility of the anion binding site.

The negative effect of iodide on enzyme activity (Fig. 5A) could not be explained by the mechanism proposed for the smaller halides and was, therefore, further investigated, exploiting PLP and tryptophan residues as absorption and fluorescence probes. Indeed, one molecule of PLP is bound to each hSR monomer and three Trps are present in its primary structure, which can be exploited as conformational probes due to the sensitivity of their

emission spectrum to the microenvironment. Trp249, Trp275 and Trp325 are located in the large domain, with Trp249 buried in the protein matrix and Trp275 and Trp325 more exposed to the solvent (Fig. 6).

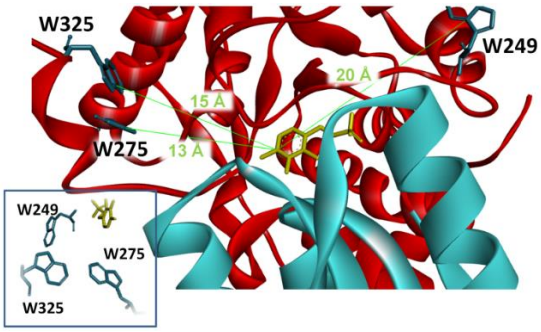


Figure 6. Tryptophan residues in hSR three dimensional structure. Three dimensional structure of hSR (PDB code: 3L6B) showing tryptophan residues and PLP in a ball-and-stick representation. The calculated distances (green lines) between the aromatic residues and PLP are as follows: PLP-W249, 20 Å; PLP-W325, 15 Å; PLP-W275, 13 Å. Inset: the structure was rotated to show the relative orientation of PLP and tryptophans. The figure was prepared using Discovery Studio Visualizer Software (Accelrys Software Inc., San Diego, 2013).

The relative distance between Trps and PLP is comparable to that observed for other fold-type II PLP-dependent enzymes and is compatible with a Förster resonance energy transfer (FRET) from Trps to PLP (Lakowicz 2006). The UV-vis absorption spectrum of hSR shows two bands, at 278 and 412 nm, attributed to the aromatic residues and to the ketoenamine form of the PLP Schiff base with the active site Lys56, respectively (Marchetti et al. 2013) (Figure 7A). Upon excitation of hSR at 298 nm, two emission peaks were observed (Fig. 7A and Fig. 7B).

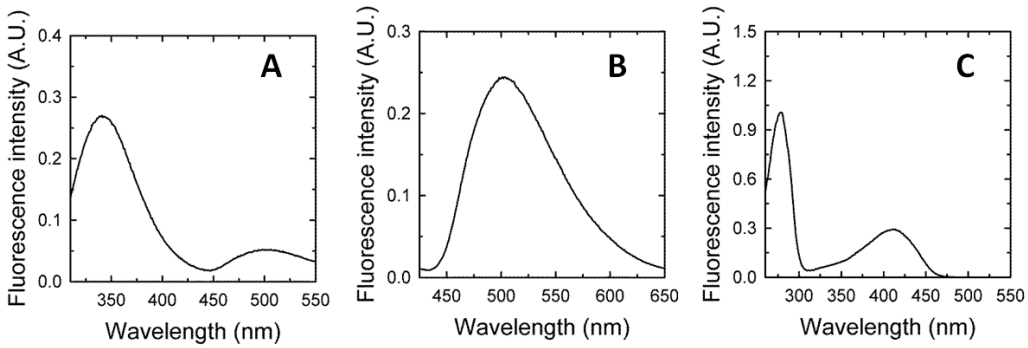


Figure 7. Fluorescence spectra of hSR were recorded in a solution containing 2.4 μM hSR, 50 mM TEA, 150 mM NaCl, 5 mM TCEP, 1 mM $MgCl_2$, pH 8.0 at 20 °C. **A.** Fluorescence emission spectrum upon excitation at 298 nm. **B.** Fluorescence emission spectrum upon excitation at 412 nm. **C.** Fluorescence excitation spectrum for emission at 500 nm. $Slit_{ex}$ was 5 nm and $slit_{em}$ was 5 nm.

The band at 345 nm is due to the direct emission of tryptophan residues that, based on the wavelength of peak maximum, are partially exposed to the solvent. The emission band centred at 500 nm was attributed to the FRET from Trps to PLP by analogy with other PLP-dependent enzymes, such as TS (Strambini et al. 1992), OASS (McClure and Cook 1994; Strambini et al. 1996; Benci et al. 1999; Bettati et al. 2000; Campanini et al. 2003), cystathionine β-synthase (Quazi and Aitken 2009; Lodha et al. 2010), DOPA decarboxylase (Dominici et al. 1997), D-amino acid aminotransferase (Martinez del Pozo et al. 1992). This conclusion was supported by the observation that, upon direct coenzyme excitation at 412 nm, the emission peak was centred at 502 nm (Fig. 7B).

This attribution was confirmed by the excitation spectrum at 500 nm showing peaks at 280 and 412 nm (Fig. 7C). Based on structural (Fig. 6) and spectrofluorimetric data (Fig. 7), Trp249, whose indole ring is almost coplanar to PLP, is likely to be the residue more involved in FRET to the cofactor, whereas the more exposed Trp275 and Trp325 are likely to contribute mainly to the direct emission centred at 345 nm.

Addition of sodium iodide up to 0.29 M, in the same concentration range in which iodide affects enzyme activity (Fig. 5A), brought about a progressive decrease of the absorption band at 412 nm, with a concomitant blue shift and the appearance of a band at 330 nm (Fig. 8A). The presence of an isosbestic point suggests that only two species are involved in the equilibrium. The attribution of absorption peaks at 330 nm has always been controversial in PLP-dependent enzymes biochemistry, due to the high number of PLP intermediates/adducts that absorb at this wavelength. In the case of fold-type II PLP-dependent enzymes, as SR, two alternatives for the attribution have been proposed: the enolimine tautomer of the internal aldimine and a substituted aldamine. The latter has been occasionally described in the literature and is formed by addition of a nucleophile to the imine double bond (Bertoldi et al. 2002; Zhang et al. 2005; Gut et al. 2006). Here, the effect of iodide on hSR spectra is interpreted as an alteration - unique among halides - of the equilibrium between the ketoenamine and enolimine tautomers of PLP, which is very sensitive to the active site polarity (Campanini et al. 2003; Chattopadhyay et al. 2007; Faeder and Hammes 1971; Kallen et al. 1985; Mozzarelli and Bettati 2006; Schnackerz et

al. 1995). A similar effect on the tautomeric equilibrium of PLP was observed for aspartate transaminase (Burridge and Churchich 1974) and attributed to the binding of iodide to a site near the cofactor. Absorption and fluorescence spectra of hSR were also recorded in the absence and presence of either 150 mM NaF or 150 mM NaCl. Unlike with iodide, no effect was observed in either absorption (data not shown) or fluorescence spectra (Fig. 9), indicating that enzyme activation by chloride and fluoride is not associated with a change in the tautomeric equilibrium of PLP.

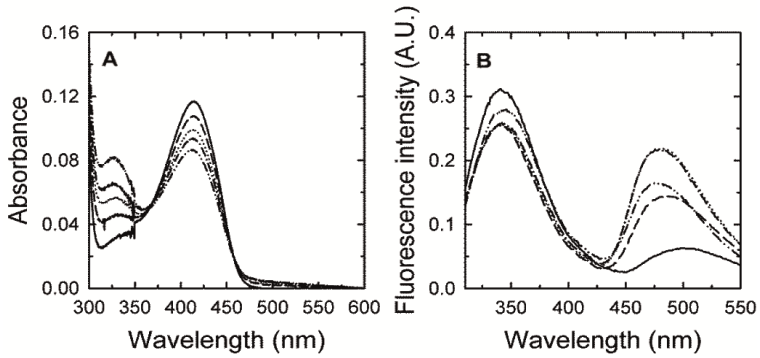


Figure 8. Effect of NaI on hSR spectroscopic properties. A. Absorption spectra of a solution containing 32 µM hSR in 50 mM TEA, 150 mM NaCl, 5 mM TCEP, 1 mM EDTA, 5% glycerol, pH 8.0 at 20 °C in the absence (solid line) and presence of 0.057 (dashed line), 0.126 (dotted line), 0.210 (dash-dotted line) and 0.290 M (dash-dot-dotted line) NaI. **B.** Fluorescence emission spectra of hSR upon excitation at 298 nm ($slit_{ex}$=5 nm, $slit_{em}$=5 nm) in the absence (solid line) and in the presence of 0.064 (dashed line), 0.126 (dotted line), 0.210 (dash-dotted line) and 0.290 M (dash-dot-dotted line) NaI.

Iodide is a commonly used quencher of Trp and PLP fluorescence (vide infra). However, in the case of hSR, NaI did not behave simply as a quencher of hSR fluorescence emission, but interfered with the conformation of the active site and the tautomeric equilibrium of the cofactor. As a matter of fact, NaI affects the fluorescence emission intensity of the cofactor upon energy transfer and causes a blue shift of the band from 502 to 475 nm (Fig. 8B). The effect was more pronounced in the presence of ATP (data not shown). The increase of the emission intensity at 502 nm due to the energy transfer from Trps to PLP is mainly associated with the increase in the concentration of enolimine tautomer (see Fig. 8A) that is efficiently excited at 345 nm. The subsequent decrease in emission intensity and the blue-shift at 475 nm can be due to a reorientation of the cofactor with respect to Trps, to quenching by NaI of PLP emission or both. In conclusion, absorption and fluorescence emission spectra indicate that iodide induces a change in the tautomeric

equilibrium of the cofactor and, probably, also in the conformation of the active site, stabilizing a less active enzyme form.

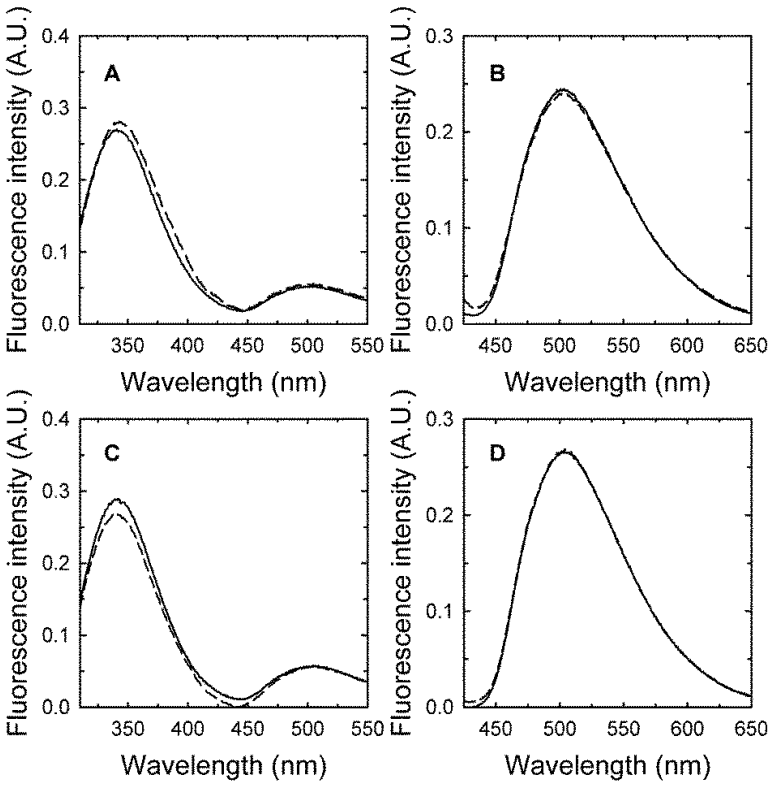


Figure 9. Effect of fluoride and chloride on the fluorescence emission spectra of hSR. Fluorescence emission spectra, upon excitation at 298 nm (panels A and C) and at 412 nm (panels B and D) of a solution containing 2.4 µM hSR in 50 mM TEA, 5 mM TCEP, 1 mM $MgCl_2$, pH 8.0 at 20 °C either in the absence (solid line) and presence (dashed line) of 150 mM fluoride (panels A and B) or 150 mM chloride (panel C and D). $Slit_{ex}$ was 5 nm and $slit_{em}$ was 5 nm.

Active site accessibility by fluorescence quenching

The complex dynamic landscape of hSR was further explored by fluorescence quenching, in order to gain insight into the accessibility of the active site. We inspected the ability of three commonly used quenchers, acrylamide, caesium and iodide, to quench PLP emission. Caesium chloride was ineffective as a quencher, as previously observed for other PLP-dependent enzymes (Rust et al. 2001), likely due to charge repulsion at the active site entrance. Indeed, Cs^+ is able to quench fluorescence emission of free PLP and model compounds simulating PLP covalently bound to a protein (Kossekova et al. 1996; Rust et al.

2001). Acrylamide was only effective at quenching fluorescence emission upon excitation at 330 nm, but the low emission signal hampered the retrieval of meaningful quantitative data (data not shown). On the contrary, iodide was effective at quenching coenzyme fluorescence of both the internal aldimine and the external aldimine with glycine, either in the absence or presence of ATP. The complex effect of iodide on the emission spectrum of hSR internal aldimine (Fig. 8B) did not allow to retrieve meaningful quantitative quenching constants. On the contrary, the behaviour of hSR external aldimine with glycine is less complex (Fig. 10), with NaI causing a decrease of the emission intensity at 483 nm, both upon excitation at 298 nm (data not shown) and upon direct excitation at 412 nm (Fig. 10A), without any shift in the emission maximum.

The Stern-Volmer plot for iodide quenching of hSR external aldimine in the presence and absence of ATP (Figure 10B) shows that the active site is significantly less accessible to the quencher in the presence of ATP (K_{sv}= 1.77 ± 0.09 M^{-1}) than in its absence (K_{sv}= 5.91 ± 0.01 M^{-1}). Although the calculation of the bimolecular quenching constant, that is the true measure of the fluorophore accessibility, requires the knowledge of fluorescence lifetime, K_{sv} (the slope of the Stern-Volmer plot) is generally interpreted as an estimate of fluorophore accessibility (Eftink and Ghiron 1981). The stabilization of a closed conformation in the external aldimine form might have the function of increasing substrate affinity and correctly orienting active site residues for catalysis. This finding is in keeping with the tighter binding of glycine caused by ATP binding (Marchetti et al. 2013).

At NaI concentrations higher than 300 mM, the Stern-Volmer plot deviates from linearity, suggesting the release of the coenzyme. This behaviour was also observed for the internal aldimine form of the enzyme (data not shown) and was confirmed by dialyzing a sample of hSR exposed to 0.58 M iodide and observing that the band of the cofactor fully disappeared (data not shown). An alternative interpretation for the upward curvature observed in the Stern-Volmer plot would involve a static quenching component (Eftink and Ghiron 1981). However, fitting with the equation for mixed static and dynamic quenching (Materials and methods, Equation 2) was not satisfactory, giving K_{sv} values lower than 0.1.

In addition, the linearization of quenching data, that should prove the coexistence of static and dynamic quenching (Lakowicz 2006), failed.

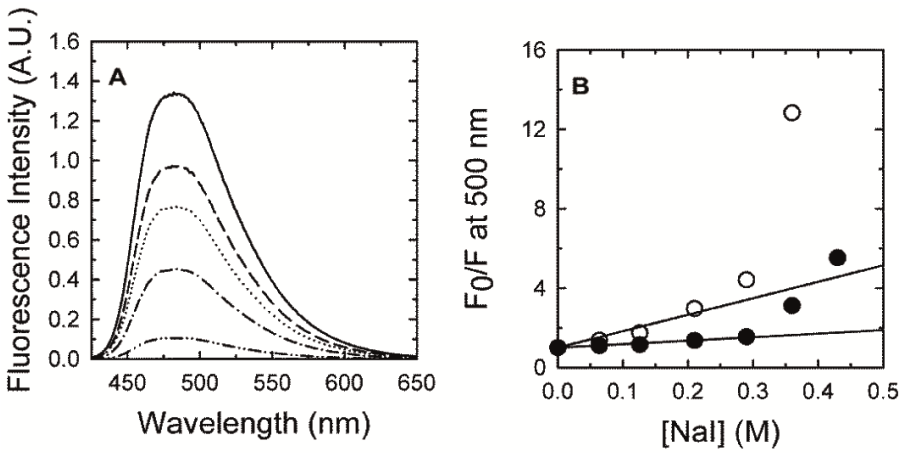


Figure 10. Fluorescence quenching of hSR-bound PLP. Spectra of a solution containing 2.4 µM hSR, 50 mM TEA, 150 mM NaCl, 5 mM TCEP, 1 mM $MgCl_2$, pH 8.0, at 20 °C. **A.** Fluorescence emission spectra of hSR external aldimine with glycine (final concentration 50 mM) upon excitation at 298 nm ($slit_{ex}$=4 nm, $slit_{em}$=4 nm) in the absence (solid line) and presence of 0.064 (dashed line), 0.210 (dotted line), 0.360 (dash-dotted line) and 0.760 M (dash-dot-dotted line) NaI. **B.** Stern-Volmer plot of quenching of hSR external aldimine by NaI in the absence (open circles) and presence (closed circles) of 2 mM ATP. Lines represent the fitting of data points from 0 to 0.21 M NaI (0.29 M in the case of ATP-bound hSR) to the Stern-Volmer equation (Equation 1, Materials and Methods) with Ksv= 5.91 ± 0.01 M-1 and 1.77 ± 0.09 M-1 in the absence and presence of ATP, respectively.

Effects of malonate on ATP binding

It has been shown that a covalent dead-end ligand of hSR, glycine, allosterically affects the binding constants for ATP (Marchetti et al. 2013). In order to assess the role of the covalent bond in eliciting the conformational switch between a low ATP affinity form and a high ATP affinity form, we investigated the binding of malonate, a reversible competitive inhibitor (Strisovsky et al. 2005) for which the three dimensional structure of the complex with hSR is available (Smith et al. 2010).

While Trp emission was unaffected by malonate binding, coenzyme emission upon either FRET from Trps or direct excitation at 412 nm was strongly enhanced (Fig. 11A, B). Furthermore, the peak wavelength of the coenzyme emission was slightly blue-shifted to 498 nm.

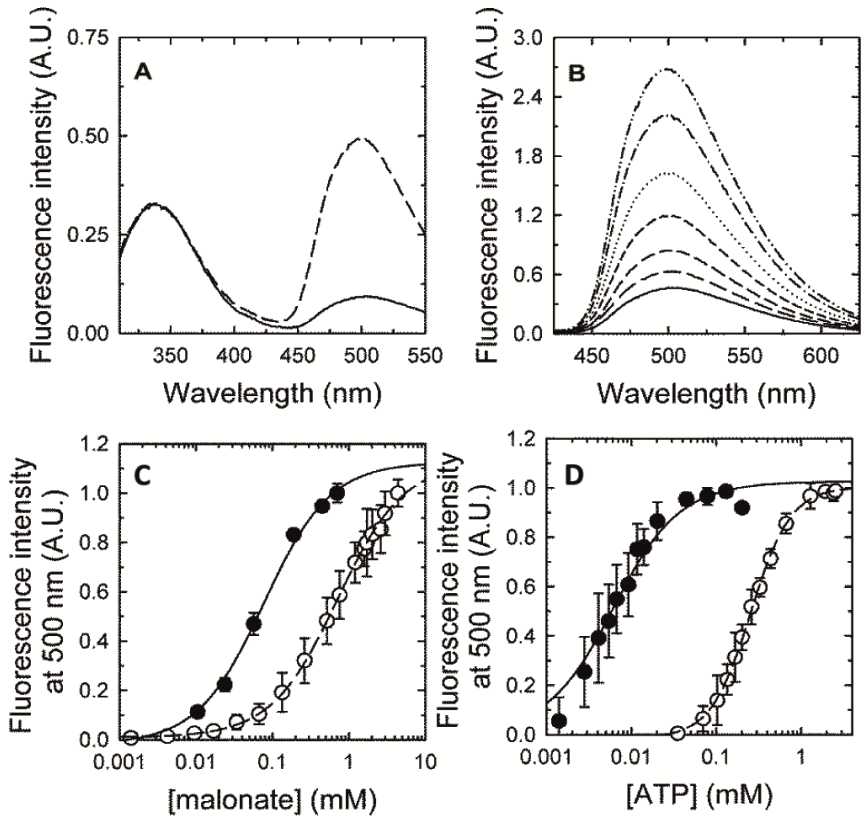


Figure 11. Cross-talk between the active site and the ATP-binding site probed by fluorescence emission spectroscopy. Spectra of a solution containing 2.4 μM hSR, 50 mM TEA, 150 mM NaCl, 5 mM TCEP, 1 mM $MgCl_2$, pH 8.0, at 20 °C. **A.** Fluorescence emission spectra of hSR upon excitation at 298 nm ($slit_{ex}$=5 nm, $slit_{em}$=5 nm) in the absence (solid line) and presence (dashed line) of 4.4 mM malonate. **B.** Fluorescence emission spectra of hSR upon excitation at 412 nm ($slit_{ex}$=5 nm, $slit_{em}$=5 nm) in the absence (solid line) and presence of increasing concentrations of malonate, ranging from 68 μM to 4.4 mM. **C.** Fluorescence emission intensity at 500 nm upon excitation at 412 nm of hSR as a function of malonate concentration in the absence (open circles) and presence (closed circles) of 2 mM ATP. The dependence of the fluorescence emission intensity at 500 nm on malonate concentration was fitted to a binding isotherm, obtaining a K_D for malonate of 77 $\pm$ 9 μM and 710 $\pm$ 33 μM in the presence and absence of ATP, respectively. **D.** Fluorescence emission intensity at 500 nm upon excitation at 412 nm of hSR as a function of ATP concentration in the absence (open circles) and presence (closed circles) of 7.1 mM malonate. The dependence of the fluorescence emission intensity at 500 nm on ATP concentration in the presence of malonate was fitted to the equation for tight binding (Equation 3, Materials and Methods), obtaining a K_D for ATP of 5.0 $\pm$ 0.6 μM, and in the absence of malonate to a binding isotherm, obtaining a K_D for ATP of 255 $\pm$ 4 μM.

These findings suggest that malonate causes a conformational change in the enzyme active site that enhances the coenzyme fluorescence emission yield and leads to a decrease in the microenvironment polarity. A similar effect was observed for glycine binding to hSR (Marchetti et al. 2013) and ligand binding to OASS (Campanini et al. 2005; Spyrakis et al. 2013). The conformational change is likely localized at the active site because tryptophan

emission is not affected. The dependence of the emission intensity on malonate concentration allowed determining a dissociation constant of 710 ± 33 μM (Fig. 11C). However, when the dependence of coenzyme emission on malonate was carried out in the presence of ATP, the dissociation constants was found to be tenfold lower, 77 ± 9 μM (Fig. 11D), in good agreement with previous studies under similar experimental conditions (Hoffman et al. 2009). The enhanced coenzyme fluorescence emission and the ATP-dependent binding affinity of malonate parallel the results previously obtained with glycine, that however, unlike malonate, is a covalent inhibitor forming an external aldimine (Marchetti et al. 2013). This finding indicates that the ATP-induced conformational change is sensed also by a non-covalent ligand and, more importantly, that the ligation state of the active site affects ATP affinity.

Conclusions

Human serine racemase, a potential drug target for the modulation of glutamatergic neurotransmission, exhibits complex conformational dynamics, with several allosteric effectors capable of altering its catalytic properties. Here, we showed that also halides smaller than bromide, i.e. fluoride and chloride, bring about a significant effect on catalysis, possibly because they are involved in ionic interactions that stabilize a more active enzyme conformation by masking positive charges and favoring the open-to-closed conformational transition. Iodide, on the other hand, showed a unique negative effect on hSR activity, associated with a drastic change in the tautomeric equilibrium of the internal aldimine. The conformational space of serine racemase was further explored by showing that malonate, a non-covalent ligand of the enzyme, exerts allosteric effects on the ATP binding site. ATP, reciprocally, modulates malonate binding to the catalytic site. The structural details of this cross-talk have been evaluated by means of fluorescence spectroscopy, revealing that ATP activates the enzyme mainly by stabilizing a closed form of the external aldimine. The interplay between active site and ATP allosteric site has relevant consequences on the medicinal chemistry of hSR, suggesting that modulation of hSR activity by allosteric effectors might be exploited for the control of D-serine homeostasis.

Materials and methods

Materials

Chemicals were of the best commercial quality available and were purchased from Sigma-Aldrich (St. Louis, MO, USA), with the exception of tris(2-carboxyethyl)phosphine (TCEP) from Apollo Scientific.

Enzyme preparation

Recombinant hSR was expressed as a hexa-His tagged fusion protein encoded in a pET28a-derived plasmid (Dixon et al. 2006) transformed into *E. coli* BL21 CodonPlus (DE3)-RIL cells (Merck-Millipore, Darmstadt, Germany), as previously described (Marchetti et al. 2013). Purification was carried out using a TALON® His-Tag Purification Resin (Clontech, CA, USA). The protein was concentrated in a storage buffer (SB1) containing 50 mM triethanolamine (TEA), 150 mM NaCl, 1 mM $MgCl_2$, 5% glycerol, 50 µM PLP, 5 mM TCEP, pH 8.0. Small aliquots were flash-frozen and thawed before use. After assessing the influence of different storage solutions on protein stability, SB1 was replaced with SB2, containing 50 mM TEA, 150 mM NaCl, 1 mM EDTA, 5% glycerol, pH 8.0, for subsequent preparations. The PLP content was determined by releasing the coenzyme in 0.1 M NaOH, using the ε_{388} = 6600 $M^{-1}cm^{-1}$. The extinction coefficient measured for hSR was determined to be 34,140 $M^{-1}cm^{-1}$ at 278 nm and 5,990 $M^{-1}cm^{-1}$ at 412 nm. Protein concentration is expressed as monomers.

Activity assays

Activity assays for the β-elimination of L-serine (Foltyn et al. 2005; Marchetti et al. 2013) were carried out in an assay solution (AS) containing 50 mM TEA, 2 mM ATP, 50 µM PLP, 5 mM DTT, 1 mM $MgCl_2$, 150 mM NaCl, 60 U/ml LDH and 300 µM NADH, pH 8.0, unless otherwise specified. The reaction was typically started by adding hSR at a concentration of 0.3-0.5 µM. All reactions were carried out at 37 °C.

Protein stability in different buffer solutions

To evaluate hSR stability, 20 µl aliquots of the stock protein solution in SB1 were extensively dialyzed for 4 hours at 4 °C against exchange solutions in home-made devices

based on 12000 MW cut-off membranes. The exchange solutions varied by i) type and concentration of buffering agents (tris(hydroxymethyl)aminomethane (TRIS), TEA, and sodium phosphate); ii) presence or absence of chelating agents (EDTA); iii) presence or absence of reducing agents (TCEP or DTT); iv) presence or absence of PLP. At the end of dialysis, the residual β-eliminase activity of the hSR solution was tested in activity assays with a L-serine concentration of 100 mM, which is nearly saturating for hSR (Marchetti et al. 2013). On the same samples, the amount of soluble protein was determined by centrifugation, followed by SDS-PAGE, to ascertain whether the activity loss was associated with either protein inactivation or protein precipitation. All experiments were carried out at least in duplicate.

Ion dependence

To assess the dependence of hSR activity on monovalent cations, either sodium chloride, lithium chloride, potassium chloride, trimethylamine hydrochloride or ammonium chloride were added to a sodium chloride-free AS, at a fixed final concentration of 150 mM. To assess the activity dependence on anions, either sodium fluoride, sodium chloride, sodium bromide, sodium iodide, or sodium acetate were added to a sodium chloride-free AS at final concentrations ranging from 10 mM to 300 mM. The effect of sodium phosphate and sodium pyrophosphate on enzyme activity was measured at final concentrations of 5 and 50 mM in AS. To evaluate a possible competition between ATP and AMP, ATP concentration in AS was reduced to 133 µM, close to the K_D for hSR (Marchetti et al. 2013), and AMP was added at concentrations ranging from 133 µM to 13 mM.

Fluorescence measurements

Fluorescence emission spectra of hSR were collected using a FluoroMax-3 fluorometer (HORIBA-Jobin Yvon, NJ, USA) equipped with a cell holder thermostatted at 20.0 ± 0.5 °C. Solutions contained 2.4-4 µM hSR in buffer F (50 mM TEA, 150 mM NaCl, 5 mM TCEP and 1 mM $MgCl_2$, pH 8.0). Slit width was chosen to optimize the signal-to-noise ratio and to prevent cofactor bleaching. Spectra were corrected for buffer contribution. The binding affinity of ligands to hSR was determined by monitoring the increase in fluorescence emission of the coenzyme upon excitation at 412 nm, as previously reported for other PLP-

dependent enzymes (Schnackerz et al. 1995; Schiaretti et al. 2004; Campanini et al. 2005). Accessibility of the cofactor was assessed by fluorescence quenching. CsCl, NaI and acrylamide solutions were prepared in 50 mM TEA buffer, 5 mM TCEP, 1 mM $MgCl_2$, 150 NaCl, pH 8.0. $Na_2S_2O_3$ at 0.01 mM concentration was added to NaI solutions to prevent iodide oxidation. Quenching data were analyzed according to the Stern-Volmer equation (Eftink and Ghiron 1981):

(1) $$\frac{F_0}{F} = 1 + K_{SV} \cdot [Q]$$

where F_0 is the fluorescence intensity in the absence of the quencher, F is the fluorescence at each given quencher concentration, K_{SV} is the Stern-Volmer constant and [Q] is the concentration of the quencher.

Mixed static and dynamic quenching is described by Equation 2:

(2) $$\frac{F_0}{F} = (1 + K_{SV} \cdot [Q]) \cdot e^{V \cdot [Q]}$$

where F_0, F, K_{SV} and Q are as described in equation 1 and V is a static quenching constant that represents an active volume element surrounding the excited fluorophore (Eftink and Ghiron 1981).

The dependence of the fluorescence emission intensity on ATP concentration in the presence of malonate was fitted to the equation for tight binding (Copeland 2000).

(3) $$y = y_0 + a * \frac{[P]+[L]+K_D-\sqrt{([P]+[L]+K_D)^2-4*[P]*[L]}}{2}$$

where y is the fluorescence emission intensity, *[P]* is the fixed protein concentration and *[L]* the variable ligand concentration. y_0 is a an horizontal offset and a the amplitude.

References

Ainscow, E. K., S. Mirshamsi, T. Tang, M. L. Ashford and G. A. Rutter (2002). "Dynamic imaging of free cytosolic ATP concentration during fuel sensing by rat hypothalamic neurones: evidence for ATP-independent control of ATP-sensitive K(+) channels." Journal of Physiology **544**(Pt 2): 429-45.

Amadasi, A., M. Bertoldi, R. Contestabile, S. Bettati, B. Cellini, M. L. di Salvo, C. Borri-Voltattorni, F. Bossa and A. Mozzarelli (2007). "Pyridoxal 5'-phosphate enzymes as targets for therapeutic agents." Current Medicinal Chemistry **14**(12): 1291-324.

Amori, L., S. Katkevica, A. Bruno, B. Campanini, P. Felici, A. Mozzarelli and G. Costantino (2012). "Design and synthesis of trans-2-substituted-cyclopropane-1-carboxylic acids as the first non-natural small molecule inhibitors of O-acetylserine sulfhydrylase." Medicinal Chemistry Communication **3**(9): 1111-1116.

Balan, L., V. N. Foltyn, M. Zehl, E. Dumin, E. Dikopoltsev, D. Knoh, Y. Ohno, A. Kihara, O. N. Jensen, I. S. Radzishevsky and H. Wolosker (2009). "Feedback inactivation of D-serine synthesis by NMDA receptor-elicited translocation of serine racemase to the membrane." PNAS **106**(18): 7589-94.

Benci, S., S. Vaccari, A. Mozzarelli and P. F. Cook (1999). "Time-resolved fluorescence of O-acetylserine sulfhydrylase." Biochimica et Biophysica Acta **1429**(2): 317-30.

Benneyworth, M. A., Y. Li, A. C. Basu, V. Y. Bolshakov and J. T. Coyle (2012). "Cell selective conditional null mutations of serine racemase demonstrate a predominate localization in cortical glutamatergic neurons." Cellular and Molecular Neurobiology **32**(4): 613-24.

Bertoldi, M., B. Cellini, T. Clausen and C. B. Voltattorni (2002). "Spectroscopic and Kinetic Analyses Reveal the Pyridoxal 5'-Phosphate Binding Mode and the Catalytic Features of Treponema denticola Cystalysin" Biochemistry **41**(29): 9153-9164.

Bettati, S., S. Benci, B. Campanini, S. Raboni, G. Chirico, S. Beretta, K. D. Schnackerz, T. L. Hazlett, E. Gratton and A. Mozzarelli (2000). "Role of pyridoxal 5'-phosphate in the structural stabilization of O-acetylserine sulfhydrylase." The Journal of Biological Chemistry **275**(51): 40244-51.

Bruno, S., F. Schiaretti, P. Burkhard, J. P. Kraus, M. Janosik and A. Mozzarelli (2001). "Functional properties of the active core of human cystathionine beta-synthase crystals." The Journal of Biological Chemistry **276**(1): 16-9.

Burkhard, P., C. H. Tai, J. N. Jansonius and P. F. Cook (2000). "Identification of an allosteric anion-binding site on O-acetylserine sulfhydrylase: structure of the enzyme with chloride bound." The Journal of Molecular Biology **303**(2): 279-86.

Burridge, N. and J. E. Churchich (1974). "Effects of potassium iodide on aspartate aminotransferase." European Journal of Biochemistry **41**(3): 533-8.

Campanini, B., S. Raboni, S. Vaccari, L. Zhang, P. F. Cook, T. L. Hazlett, A. Mozzarelli and S. Bettati (2003). "Surface-exposed tryptophan residues are essential for O-acetylserine sulfhydrylase structure, function, and stability." The Journal of Biological Chemistry **278**(39): 37511-9.

Campanini, B., F. Speroni, E. Salsi, P. F. Cook, S. L. Roderick, B. Huang, S. Bettati and A. Mozzarelli (2005). "Interaction of serine acetyltransferase with O-acetylserine sulfhydrylase active site: evidence from fluorescence spectroscopy." Protein Science **14**(8): 2115-24.

Campanini, B., F. Spyrakis, A. Peracchi and A. Mozzarelli (2013). "Serine racemase: a key player in neuron activity and in neuropathologies." Frontiers in Bioscience **18**: 1112-28.

Canu, N., M. T. Ciotti and L. Pollegioni (2014). "Serine racemase: a key player in apoptosis and necrosis." Frontiers in Synaptic Neuroscience **6**.

Chattopadhyay, A., M. Meier, S. Ivaninskii, P. Burkhard, F. Speroni, B. Campanini, S. Bettati, A. Mozzarelli, W. M. Rabeh, L. Li and P. F. Cook (2007). "Structure, mechanism, and conformational dynamics of O-acetylserine sulfhydrylase from Salmonella typhimurium: comparison of A and B isozymes." Biochemistry **46**(28): 8315-30.

Conti, P., L. Tamborini, A. Pinto, A. Blondel, P. Minoprio, A. Mozzarelli and C. De Micheli (2011). "Drug discovery targeting amino acid racemases." Chemical Reviews (Washington, D. C.) **111**(11): 6919-46.

De Miranda, J., R. Panizzutti, V. N. Foltyn and H. Wolosker (2002). "Cofactors of serine racemase that physiologically stimulate the synthesis of the N-methyl-d-aspartate (NMDA) receptor coagonist d-serine." PNAS **99**(22): 14542-14547.

Ding, X., N. Ma, M. Nagahama, K. Yamada and R. Semba (2011). "Localization of D-serine and serine racemase in neurons and neuroglias in mouse brain." Neurological Science **32**(2): 263-7.

Dixon, S. M., P. Li, R. Liu, H. Wolosker, K. S. Lam, M. J. Kurth and M. D. Toney (2006). "Slow-binding human serine racemase inhibitors from high-throughput screening of combinatorial libraries." The Journal of Medicinal Chemistry **49**(8): 2388-97.

Dominici, P., P. S. Moore, S. Castellani, M. Bertoldi and C. B. Voltattorni (1997). "Mutation of cysteine 111 in Dopa decarboxylase leads to active site perturbation." Protein Science **6**(9): 2007-15.

Dumin, E., I. Bendikov, V. N. Foltyn, Y. Misumi, Y. Ikehara, E. Kartvelishvily and H. Wolosker (2006). "Modulation of D-serine levels via ubiquitin-dependent proteasomal degradation of serine racemase." The Journal of Biological Chemistry **281**(29): 20291-302.

Eftink, M. R. and C. A. Ghiron (1981). "Fluorescence quenching studies with proteins." Analytical Biochemistry **114**(2): 199-227.

Faeder, E. J. and G. G. Hammes (1971). "Kinetic studies of tryptophan synthetase. Interaction of L-serine, indole, and tryptophan with the native enzyme." Biochemistry **10**(6): 1041-5.

Foltyn, V. N., I. Bendikov, J. De Miranda, R. Panizzutti, E. Dumin, M. Shleper, P. Li, M. D. Toney, E. Kartvelishvily and H. Wolosker (2005). "Serine racemase modulates intracellular D-serine levels through an alpha,beta-elimination activity." Journal of Biological Chemistry **280**(3): 1754-63.

Foltyn, V. N., M. Zehl, E. Dikopoltsev, O. N. Jensen and H. Wolosker (2010). "Phosphorylation of mouse serine racemase regulates D-serine synthesis." FEBS Letters **584**(13): 2937-41.

Fujii, K., K. Maeda, T. Hikida, A. K. Mustafa, R. Balkissoon, J. Xia, T. Yamada, Y. Ozeki, R. Kawahara, M. Okawa, R. L. Huganir, H. Ujike, S. H. Snyder and A. Sawa (2006). "Serine racemase binds to PICK1: potential relevance to schizophrenia." Molecular Psychiatry **11**(2): 150-7.

Goto, M., T. Yamauchi, N. Kamiya, I. Miyahara, T. Yoshimura, H. Mihara, T. Kurihara, K. Hirotsu and N. Esaki (2009). "Crystal structure of a homolog of mammalian serine racemase from Schizosaccharomyces pombe." The Journal of Biological Chemistry **284**(38): 25944-52.

Gut, H., E. Pennacchietti, R. A. John, F. Bossa, G. Capitani, D. De Biase and M. G. Grutter (2006). "Escherichia coli acid resistance: pH-sensing, activation by chloride and autoinhibition in GadB." The EMBO Journal **25**(11): 2643-2651.

Hoffman, H. E., J. Jiraskova, M. Ingr, M. Zvelebil and J. Konvalinka (2009). "Recombinant human serine racemase: enzymologic characterization and comparison with its mouse ortholog." Protein Expression and Purification **63**(1): 62-7.

Inoue, R., Y. Yoshihisa, Y. Tojo, C. Okamura, Y. Yoshida, J. Kishimoto, X. Luan, M. Watanabe, M. Mizuguchi, Y. Nabeshima, K. Hamase, K. Matsunaga, T. Shimizu and H. Mori (2014). "Localization of Serine Racemase and Its Role in the Skin." Journal of Investigative Dermatology.

Ito, T., M. Maekawa, S. Hayashi, M. Goto, H. Hemmi and T. Yoshimura (2013). "Catalytic mechanism of serine racemase from Dictyostelium discoideum." Amino Acids **44**(3): 1073-84.

Jiraskova-Vanickova, J., R. Ettrich, B. Vorlova, H. E. Hoffman, M. Lepsik, P. Jansa and J. Konvalinka (2011). "Inhibition of human serine racemase, an emerging target for medicinal chemistry." Current Drug Targets **12**(7): 1037-55.

Kaiser, J. T., S. Bruno, T. Clausen, R. Huber, F. Schiaretti, A. Mozzarelli and D. Kessler (2003). "Snapshots of the cystine lyase C-DES during catalysis. Studies in solution and in the crystalline state." The Journal of Biological Chemistry **278**(1): 357-65.

Kallen, R. G., T. Korpela, A. E. Martell, Y. Matsushima, C. M. Metzler, D. E. Metzler, Y. V. Morozow, I. M. Ralston, F. A. Savin and Y. M. Torchinsky (1985). "Chemical and spectroscopic properties of pyridoxal and pyridoxamine phosphate." Transaminases. P. C. a. D. E. Metzler. New York, USA, John Wiley and Sons**:** 37–108.

Kim, P. M., H. Aizawa, P. S. Kim, A. S. Huang, S. R. Wickramasinghe, A. H. Kashani, R. K. Barrow, R. L. Huganir, A. Ghosh and S. H. Snyder (2005). "Serine racemase: activation by glutamate neurotransmission via glutamate receptor interacting protein and mediation of neuronal migration." PNAS **102**(6): 2105-10.

Kossekova, G., M. Miteva and B. Atanasov (1996). "Characterization of pyridoxal phosphate as an optical label for measuring electrostatic potentials in proteins." Journal of Photochemistry and Photobiology B **32**(1-2): 71-9.

Labrie, V., A. H. Wong and J. C. Roder (2012). "Contributions of the D-serine pathway to schizophrenia." Neuropharmacology **62**(3): 1484-503.

Lakowicz, J. R. (2006). "Principles of Fluorescence Spectroscopy". New York, USA, Plenum Press.

Lipton, S. A. (2006). "Paradigm shift in neuroprotection by NMDA receptor blockade: memantine and beyond." Nature Reviews: Drug Discovery **5**(2): 160-70.

Lodha, P. H., E. M. Hopwood, A. L. Manders and S. M. Aitken (2010). "Residue N84 of yeast cystathionine beta-synthase is a determinant of reaction specificity." Biochimica et Biophysica Acta **1804**(7): 1424-31.

Ma, T. M., S. Abazyan, B. Abazyan, J. Nomura, C. Yang, S. Seshadri, A. Sawa, S. H. Snyder and M. V. Pletnikov (2013). "Pathogenic disruption of DISC1-serine racemase binding elicits schizophrenia-like behavior via D-serine depletion." Molecular Psychiatry **18**(5): 557-67.

Marabotti, A., D. De Biase, A. Tramonti, S. Bettati and A. Mozzarelli (2001). "Allosteric communication of tryptophan synthase. Functional and regulatory properties of the beta S178P mutant." The Journal of Biological Chemistry **276**(21): 17747-53.

Marchetti, M., S. Bruno, B. Campanini, A. Peracchi, N. Mai and A. Mozzarelli (2013). "ATP binding to human serine racemase is cooperative and modulated by glycine." FEBS Journal **280**(22): 5853-5863.

Martinez del Pozo, A., T. Yoshimura, M. B. Bhatia, S. Futaki, J. M. Manning, D. Ringe and K. Soda (1992). "Inactivation of dimeric D-amino acid transaminase by a normal substrate through formation of an unproductive coenzyme adduct in one subunit." Biochemistry **31**(26): 6018-23.

Maruyama, K. (1990). "Activation of Pseudomonas ochraceae 4-hydroxy-4-methyl-2-oxoglutarate aldolase by inorganic phosphate." Journal of Biochemistry **108**(2): 334-40.

McClure, G. D., Jr. and P. F. Cook (1994). "Product binding to the alpha-carboxyl subsite results in a conformational change at the active site of O-acetylserine sulfhydrylase-A: evidence from fluorescence spectroscopy." Biochemistry **33**(7): 1674-83.

Molla, G., S. Sacchi, M. Bernasconi, M. S. Pilone, K. Fukui and L. Polegioni (2006). "Characterization of human D-amino acid oxidase." FEBS Letters **580**(9): 2358-64.

Mozzarelli, A. and S. Bettati (2006). "Exploring the pyridoxal 5'-phosphate-dependent enzymes." The Chemical Record **6**(5): 275-87.

Mozzarelli, A., S. Bettati, B. Campanini, E. Salsi, S. Raboni, R. Singh, F. Spyrakis, V. P. Kumar and P. F. Cook (2011). "The multifaceted pyridoxal 5'-phosphate-dependent O-acetylserine sulfhydrylase." Biochimica et Biophysica Acta **1814**(11): 1497-510.

Mustafa, A. K., A. S. Ahmad, E. Zeynalov, S. K. Gazi, G. Sikka, J. T. Ehmsen, R. K. Barrow, J. T. Coyle, S. H. Snyder and S. Dore (2010). "Serine racemase deletion protects against cerebral ischemia and excitotoxicity." Journal of Neuroscience **30**(4): 1413-6.

Mustafa, A. K., M. Kumar, B. Selvakumar, G. P. Ho, J. T. Ehmsen, R. K. Barrow, L. M. Amzel and S. H. Snyder (2007). "Nitric oxide S-nitrosylates serine racemase, mediating feedback inhibition of D-serine formation." PNAS **104**(8): 2950-5.

Nagayoshi, C., M. Ishibashi, Y. Kita, M. Matsuoka, I. Nishimoto and M. Tokunaga (2005). "Expression, refolding and characterization of human brain serine racemase in Escherichia coli with N-terminal His-tag." Protein and Peptides Letter **12**(5): 487-90.

Nagayoshi, C., M. Ishibashi and M. Tokunaga (2009). "Purification and characterization of human brain serine racemase expressed in moderately halophilic bacteria." Protein and Peptide Letters **16**(2): 201-6.

Neidle, A. and D. S. Dunlop (2002). "Allosteric regulation of mouse brain serine racemase." Neurochemical Research **27**(12): 1719-24.

Peracchi, A., A. Mozzarelli and G. L. Rossi (1995). "Monovalent Cations Affect Dynamic and Functional-Properties of the Tryptophan Synthase Alpha(2)Beta(2) Complex." Biochemistry **34**(29): 9459-9465.

Perutz, M. F., D. T. Shih and D. Williamson (1994). "The chloride effect in human haemoglobin. A new kind of allosteric mechanism." The Journal of Molecular Biology **239**(4): 555-60.

Phillips, R. S., T. V. Demidkina, L. N. Zakomirdina, S. Bruno, L. Ronda and A. Mozzarelli (2002). "Crystals of tryptophan indole-lyase and tyrosine phenol-lyase form stable quinonoid complexes." The Journal of Biological Chemistry **277**(24): 21592-7.

Quazi, F. and S. M. Aitken (2009). "Characterization of the S289A,D mutants of yeast cystathionine beta-synthase." Biochimica et Biophysica Acta **1794**(6): 892-7.

Raboni, S., S. Bettati and A. Mozzarelli (2009). "Tryptophan synthase: a mine for enzymologists." Cellular and Molecular Life Sciences **66**(14): 2391-403.

Rust, E., D. L. Martin and C. H. Chen (2001). "Cofactor and tryptophan accessibility and unfolding of brain glutamate decarboxylase." Archives of Biochemistry and Biophysics **392**(2): 333-40.

Sacchi, S., L. Caldinelli, P. Cappelletti, L. Pollegioni and G. Molla (2012). "Structure-function relationships in human D-amino acid oxidase." Amino Acids **43**(5): 1833-50.

Salsi, E., A. S. Bayden, F. Spyrakis, A. Amadasi, B. Campanini, S. Bettati, T. Dodatko, P. Cozzini, G. E. Kellogg, P. F. Cook, S. L. Roderick and A. Mozzarelli (2010). "Design of O-acetylserine sulfhydrylase inhibitors by mimicking nature." The Journal of Medicinal Chemistry **53**(1): 345-56.

Schiaretti, F., S. Bettati, C. Viappiani and A. Mozzarelli (2004). "pH dependence of tryptophan synthase catalytic mechanism: I. The first stage, the beta-elimination reaction." The Journal of Biological Chemistry **279**(28): 29572-82.

Schnackerz, K. D., C. H. Tai, J. W. Simmons, 3rd, T. M. Jacobson, G. S. Rao and P. F. Cook (1995). "Identification and spectral characterization of the external aldimine of the O-acetylserine sulfhydrylase reaction." Biochemistry **34**(38): 12152-60.

Silver, I. A. and M. Erecinska (1997). "Energetic demands of the Na+/K+ ATPase in mammalian astrocytes." Glia **21**(1): 35-45.

Smith, M. A., V. Mack, A. Ebneth, I. Moraes, B. Felicetti, M. Wood, D. Schonfeld, O. Mather, A. Cesura and J. Barker (2010). "The Structure of Mammalian Serine Racemase: EVIDENCE FOR CONFORMATIONAL CHANGES UPON INHIBITOR BINDING." The Journal of Biological Chemistry **285**(17): 12873-12881.

Spyrakis, F., B. Cellini, S. Bruno, P. Benedetti, E. Carosati, G. Cruciani, F. Micheli, A. Felici, P. Cozzini, G. E. Kellogg, C. B. Voltattorni and A. Mozzarelli (2014). "Targeting Cystalysin, a Virulence Factor of Treponema denticola-Supported Periodontitis." ChemMedChem.

Spyrakis, F., P. Felici, A. S. Bayden, E. Salsi, R. Miggiano, G. E. Kellogg, P. Cozzini, P. F. Cook, A. Mozzarelli and B. Campanini (2013). "Fine tuning of the active site modulates specificity in the interaction of O-acetylserine sulfhydrylase isozymes with serine acetyltransferase." Biochimica et Biophysica Acta **1834**(1): 169-81.

Strambini, G. B., P. Cioni and P. F. Cook (1996). "Tryptophan luminescence as a probe of enzyme conformation along the O-acetylserine sulfhydrylase reaction pathway." Biochemistry **35**(25): 8392-400.

Strambini, G. B., P. Cioni, A. Peracchi and A. Mozzarelli (1992). "Conformational-Changes and Subunit Communication in Tryptophan Synthase - Effect of Substrates and Substrate-Analogs." Biochemistry **31**(33): 7535-7542.

Strisovsky, K., J. Jiraskova, A. Mikulova, L. Rulisek and J. Konvalinka (2005). "Dual substrate and reaction specificity in mouse serine racemase: identification of high-affinity dicarboxylate substrate and inhibitors and analysis of the beta-eliminase activity." Biochemistry **44**(39): 13091-100.

Tian, H., R. Guan, E. Salsi, B. Campanini, S. Bettati, V. P. Kumar, W. E. Karsten, A. Mozzarelli and P. F. Cook (2010). "Identification of the structural determinants for the stability of substrate and aminoacrylate external Schiff bases in O-acetylserine sulfhydrylase-A." Biochemistry **49**(29): 6093-103.

Toney, M. D. (2011). "Pyridoxal phosphate enzymology." Biochimica et Biophysica Acta **1814**(11): 1405-6.

Traynelis, S. F., L. P. Wollmuth, C. J. McBain, F. S. Menniti, K. M. Vance, K. K. Ogden, K. B. Hansen, H. Yuan, S. J. Myers and R. Dingledine (2010). "Glutamate receptor ion channels: structure, regulation, and function." Pharmacological Reviews **62**(3): 405-96.

Wang, W. and S. W. Barger (2011). "Roles of quaternary structure and cysteine residues in the activity of human serine racemase." BMC Biochemistry **12**: 63.

Wild, J. R., W. L. Belser and G. A. O'Donovan (1976). "Unique aspects of the regulation of the aspartate transcarbamylase of Serratia marcescens." Journal of Bacteriology **128**(3): 766-75.

Wolosker, H., S. Blackshaw and S. H. Snyder (1999). "Serine racemase: a glial enzyme synthesizing D-serine to regulate glutamate-N-methyl-D-aspartate neurotransmission." PNAS **96**(23): 13409-14.

Zhang, J., A. V. Cheltsov and G. C. Ferreira (2005). "Conversion of 5-aminolevulinate synthase into a more active enzyme by linking the two subunits: Spectroscopic and kinetic properties." Protein Science **14**(5): 1190-1200.

Zhuang, Z., B. Yang, M. H. Theus, J. T. Sick, J. R. Bethea, T. J. Sick and D. J. Liebl (2010). "EphrinBs regulate D-serine synthesis and release in astrocytes." Journal of Neuroscience **30**(47): 16015-24.

INHIBITION STUDIES:

RATIONAL DRUG DESIGN

Identification and screening of novel malonate-based human serine racemase inhibitors

The computational studies and the compound synthesis presented in the following work were performed by Prof. Gabriele Costantino and its research group (Department of Pharmacy, University of Parma). The design and synthesis details about the compounds are discussed in the doctoral dissertation "*Design and Synthesis of Human Serine Racemase inhibitors, a challenge to modulate NMDAR dysregulation and neurodegeneration induced"* (C. Pecchini, 2014, University of Parma).

Abstract

D-serine, an important modulator of N-methyl-D-aspartate receptors (NMDARs), is synthesized by serine racemase (SR), a pyridoxal 5'-phosphate (PLP)-dependent enzyme. NMDARs-mediated neurotoxicity is involved many neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and in cerebral ischemia. Therefore, the inhibition of SR could represent a possible novel strategy to treat several pathological conditions. Here we considered the enzymatic conformational ensemble to develop a new series of SR competitive inhibitors based on cyclopropane scaffold and on malonate core modifications. Our results can provide useful indications for the development of novel SR inhibitors.

Introduction

In the last twenty years, D-serine has been discovered as the main endogenous co-agonist on N-methyl-D-aspartate receptors (NMDARs) in mammalian central nervous system (Furukawa and Gouaux 2003; Shleper et al. 2005; Panatier et al. 2006). NMDARs dysregulation is often linked to severe neurological diseases, such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and neuropathic pain (Van Horn et al. 2013). Based on these observations, the modulation of D-serine metabolism has been envisaged as a possible mean to regulate NMDARs activation (Lipton 2006; Conti et al. 2011; Jiraskova-Vanickova et al. 2011). In particular, the research on a rational target for the development of new drugs focused the attention on serine racemase (SR, E.C. 5.1.1.18, (Wolosker et al. 1999)), the enzyme responsible of D-serine synthesis (Panizzutti et al. 2001; Strisovsky et al. 2005; Dixon et al. 2006). Drugs aimed at modulating its activity must be very specific and able to cross the blood-brain barrier. Until now, only a few competitive inhibitors have been identified, demonstrating that the development of specific and potent human SR (hSR) inhibitors is very challenging (Jiraskova-Vanickova et al. 2011). The low recombinant protein purification yields and stability, the low enzymatic reaction rate and the wide conformational flexibility of the enzyme complicate the search of new inhibitors (Campanini et al. 2013).

The most potent hSR inhibitor identified so far is malonate, a dicarboxylic acid that binds competitively to the active site (Strisovsky et al. 2005; Hoffman et al. 2009; Smith et al. 2010). The small dimensions and the carboxylate groups establishing strong ionic interaction with the active site, make this molecule able to enter and bind relatively strongly to the catalytic pocket (Fig. 1A). Smith and colleagues crystallized the SR-malonate complex and demonstrated, through comparison with the unliganded structure, that binding to the active site promotes a series of rearrangements towards a closed conformation (Fig. 1B) (Smith et al. 2010). Before the present study, a computational investigation was carried out by means of a targeted molecular dynamics (TMD) simulation (Bruno et al. 2011). The aim of that work was to simulate the open-to-closed

conformational transition of SR and, through clustering analysis, to identify the most relevant enzyme conformations to be used for docking/virtual screening purposes.

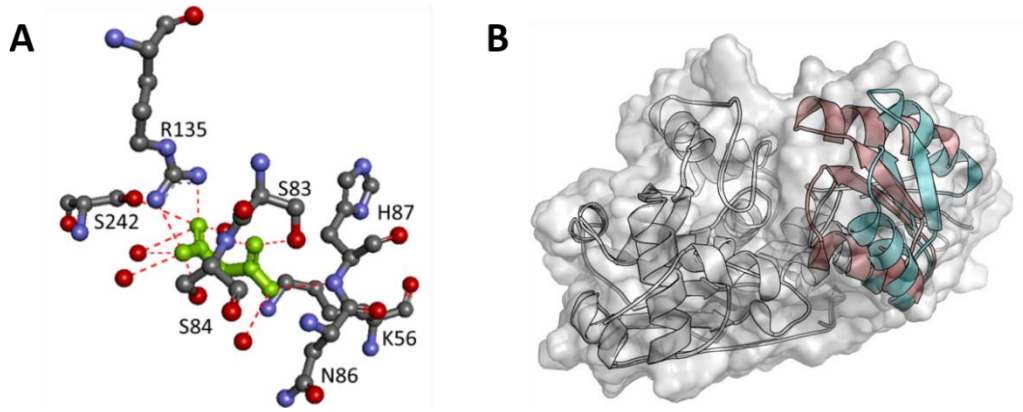


Figure 1. A. Malonate binding to hSR active site. The hydrogen bonds formed between malonate (green structure) and the surrounding residues and water molecules (red spheres) are shown by red dashed lines. One carboxylate interacts with the backbone nitrogens of Ser84, Asn86 and His87, with the side chain of Ser83, the side chain nitrogen of Lys56 and a water molecule; the other one forms a salt bridge interaction with Arg135 and contacts Ser84, Ser242 and three water molecules. **B. Superimposition of the open and the closed SR conformations.** The major rearrangements involve the small domain (cyan and red open and closed conformation, respectively) that bends over the binding site closing the access to the pocket.

From the simulation results, it seemed that the open-to-closed rearrangement is a dynamic process not characterized by discrete states. This evidence enormously complicates any attempt to rationally design drugs based on docking studies (Bruno et al. 2011). In the present research, malonate was chosen as the reference compound and its structure was revised introducing various types of modifications. The investigation implied the synthesis of several compounds that were tested on hSR through *in vitro* assays, exploiting the elimination activity of the enzyme and the fluorescence properties of PLP cofactor, leading to an improved structure-activity relationship (SAR) study.

Results and discussion

Mammalian SR is a PLP-dependent homodimeric enzyme belonging to the fold type II family (Wolosker et al. 1999; De Miranda et al. 2000; Foltyn et al. 2005; Hoffman et al. 2009; Campanini et al. 2013). Every monomer is composed of a large domain and a smaller, flexible one that rearranges as a consequence of substrate binding to close the active site (Smith et al. 2010). Ligand recognition leads to important conformational changes to establish a proper interaction. The limited number of enzyme conformations captured by X-ray crystallography cannot delineate all the features of this structural rearrangement. The TDM study (Bruno et al. 2011), simulating the open-to-closed SR transition, allowed for the generation of six representative structures, covering the whole transition from the open to the malonate-complexed closed forms. These selected structures were used for docking a small library of known SR inhibitors and closely related inactive analogs. The inclusion of protein flexibility in the computational simulation showed better results than the docking on individual crystallographic structures. Malonate was chosen as the reference structure and template for the development of new molecules. Its structure can be easily modified and shows good physiochemical parameters such as solubility and lipophilicity, in accordance with the drug-likeness required characteristics.

The research aimed to discover novel hSR competitive inhibitors and it was pursued following two approaches: i) the constrain of the structure, to favour a stronger interaction at the target binding site (compounds belonging to Group A, Tab. 1); ii) the modification of the malonate core, in order to define which substituents could be tolerated (compounds belonging to Group B, Tab. 2).

Design of substituted cyclopropanes

We reasoned that cyclopropane-1,2-dicarboxylic structure could mimic the malonate conformation, specifically in the two carboxylic extremities. In addition, maleic acid, reported as a moderate hSR inhibitor (K_i=0.55±0.12 mM, (Strisovsky et al. 2005)), well superimposes with both molecules (Fig. 2). In agreement with these observations, a small library of compounds enclosing the malonate structure in the rigid conformation of

cyclopropane was synthesized. Moreover, it was considered that a more rigid structure could have led to a stronger interaction with the active site (Tab. 1).

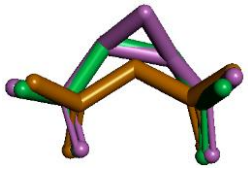


Figure 2. Superimposition of malonate (orange), maleate (green) and the cyclopropane nucleus (purple). The three molecules show a high degree of superimposition.

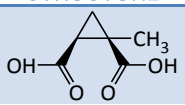
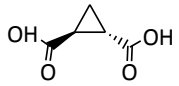
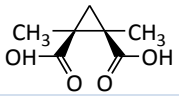
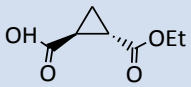
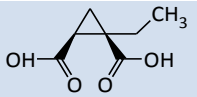
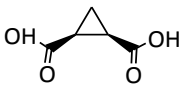
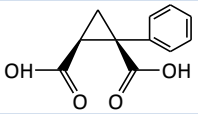
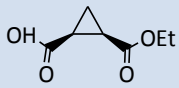
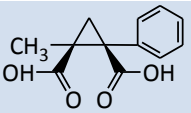
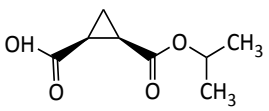
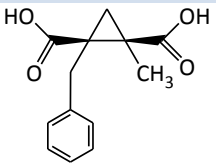
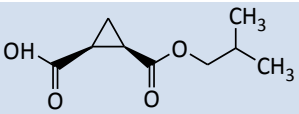
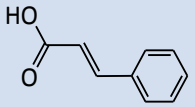
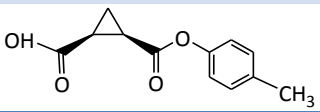
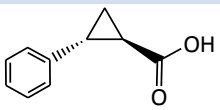
COMPOUND	STRUCTURE	COMPOUND	STRUCTURE
MALEIC ACID		8 A	
1 A		9 A	
2 A		10 A	
3 A		11 A	
4 A		12 A	
5 A		13 A	
6 A		14 A	
7 A		15 A	

Table 1. Group A compounds. The molecules are designed adding different substituents on the cyclopropane-1,2-dicarboxylic scaffold

Cis and *trans* conformation were investigated, in order to identify which isomer was better tolerated. It was also checked whether the introduction of bulkier and/or less polar substituents would be compatible with the binding to the active site structure (Fig. 3).

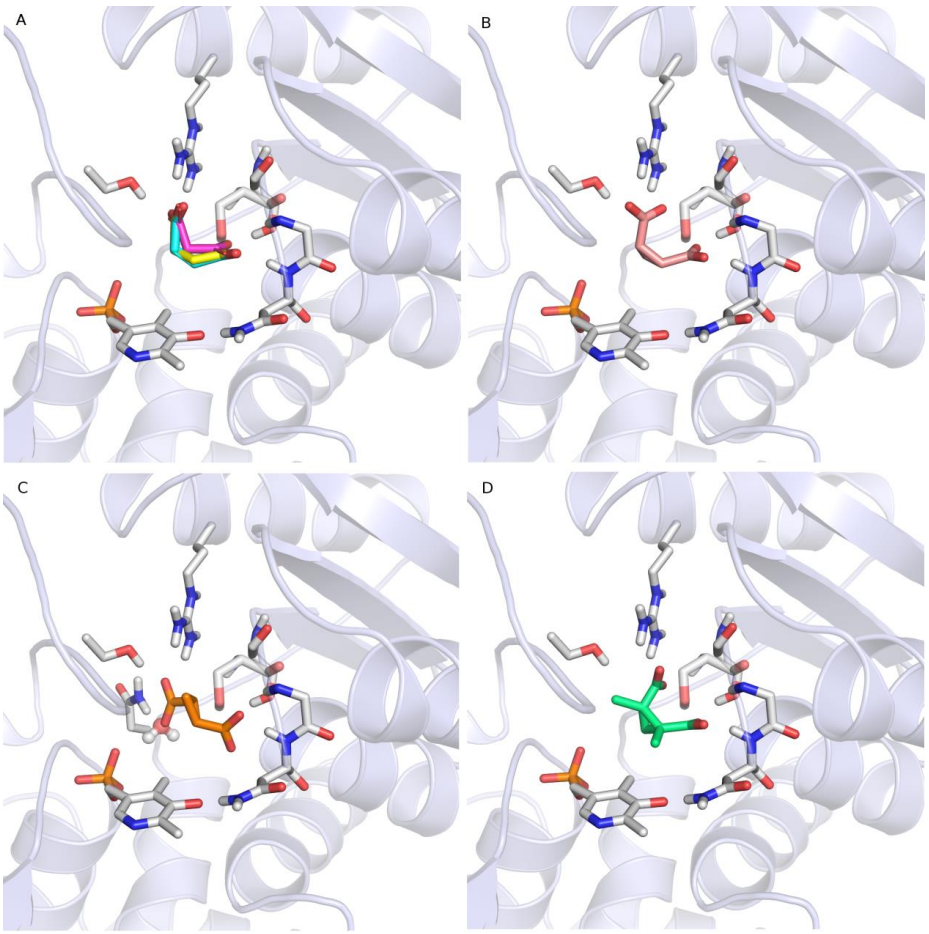


Figure 3. A. Superimposition of the docking poses of malonic acid (magenta), succinic acid (yellow) and maleic acid (cyan); **B.** Docking pose of compound 3 A; **C.** Binding mode of compound 1 A; **D.** Docking pose of compound 9 A.

Design of malonate derivatives

Along with the previous compounds, another series of molecules was conceived, considering a set of substituted malonates and also some bioisosteres. The purpose of this investigation was to refine the SAR for malonate derivatives and to identify the accepted modifications to yield a better inhibitor.

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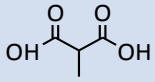
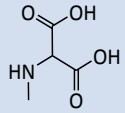
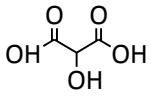
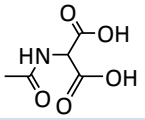
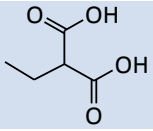
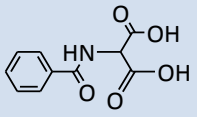
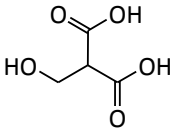
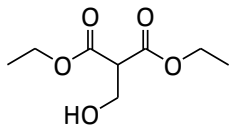
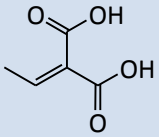
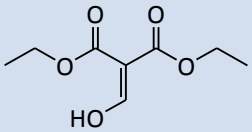
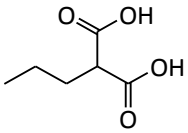
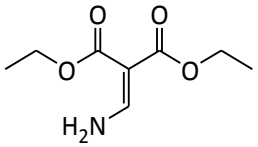
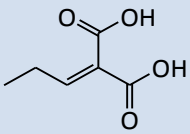
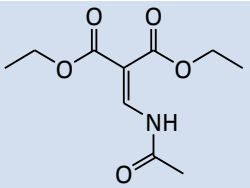
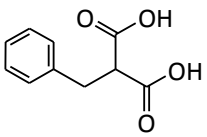
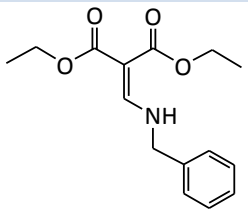
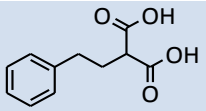
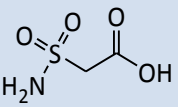
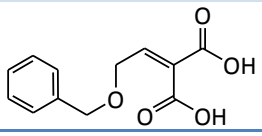
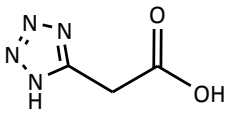
1 B		11 B	
2 B		12 B	
3 B		13 B	
4 B		14 B	
5 B		15 B	
6 B		16 B	
7 B		17 B	
8 B		18 B	
9 B		19 B	
10 B		20 B	

Table 2. Group B compounds. The molecules are obtained by adding different substituent groups on the malonate structure. Modifications were added on the α-carbon and by esterification of the two carboxyl groups. Compounds 19 B and 20 B are bioisosteres of malonate.

The designed molecules show small polar and apolar groups on the α-carbon, bulky groups and groups with different electrochemical characteristics as substituents.

Compound library screening

Before the evaluation of the compounds activity, some considerations were made about the screening criteria and the *in vitro* assays were optimized. The goal of the compound library screening is not to identify drugs, but rather to find hits of the target enzyme in order to define lead compounds suitable for drug optimization (Copeland 2005). Because of the large number of molecules to be tested, the screening process must be standardized. For this reason all the compounds were prepared in stocks with the same concentration, i.e. 100 mM, which was considered as a good compromise in terms of solubility and volumes needed in the assays setup. Drug-like inhibitors often have a limited solubility, so we decided to solubilize all the molecules in the same aprotic solvent, in particular 100% dimethyl sulfoxide (DMSO). The tolerance for this solvent varies between different enzymes and, therefore, we first evaluated how the DMSO concentration affected the catalytic activity of hSR. A DMSO titration was performed, to define the maximum concentration tolerated and to analyze the enzymatic behaviour. hSR is able to perform two distinct catalytic activities, racemization and β-elimination, on the same substrate L-serine (Strisovsky et al. 2005). Since the two reactions are known to share the same enzymatic active site, it is possible to study hSR activity by analyzing either reaction. In this case, β-elimination reaction was chosen as the simplest activity to be monitored. The dependence was carried out at two different substrate concentrations, in particular at the K_M value and at saturating concentrations of substrate. We found that, at L-serine concentrations close to the K_M, the hSR initial activity increases at increasing DMSO concentrations (see Appendix). When the same analysis was performed at saturating L-serine concentration, we noticed that the V_{max} values were almost unaltered, leading to the conclusion that DMSO affected the catalytic efficiency but not the k_{cat} of hSR. The activity differences at subsaturating L-serine concentrations in the absence and in the presence of DMSO required the determination of the catalytic parameters in the presence of the

solvent. The activity dependence on L-serine concentration was carried out both in the absence and in the presence of 5% DMSO (v/v), considering it as a reasonable percentage of solvent for the screening. In the presence of 5% DMSO, the K_M of hSR was about a half with respect to that calculated in the absence of solvent (data not shown). The tolerance test was also performed on static fluorescence, another mean to determine the compound binding to hSR active site, in order to verify the enzyme stability. Protein solution spectra were recorded at different time points after hSR dilution in a buffer solution containing 5% DMSO and the signal was found to be stable (data not shown).

We decided to proceed to an initial screening of all the molecules, testing their effect on enzymatic activity. Activity assays were carried out at the physiological temperature of 37 °C. The compounds effectiveness was evaluated comparing the initial enzymatic rate in the presence and in the absence of the potential inhibitor. In order to consider the effect of the solvent, DMSO was added in the reference assay at the same concentration present in the inhibition test. An 80% residual activity was taken as a threshold above which the inhibitory effect could not be reliably calculated.

For each compound, the corresponding inhibition constant (K_i) value was determined with the equation for competitive inhibition (see Materials and Methods). For the compounds showing the most interesting activities, the dissociation constant (K_D) was determined by fluorescence measurements (Fig. 4). With this purpose, it is possible to exploit the fluorescence emission properties of the PLP cofactor, by measuring its increase in fluorescence emission at 500 nm upon direct excitation at 412 nm (Campanini et al. 2005 B; Marchetti et al. 2013; Spyrakis et al. 2013 A; Marchetti et al. 2014). Measurements were carried out at 20 °C, a temperature that combines protein stability, good fluorescence signal intensity and short times for the equilibrium of protein-ligand complex formation. The dependence of the fluorescence emission on ligand concentration was then fitted by a binding isotherm, in order to calculate the compound K_D (Fig. 4 and see also Materials and Methods).

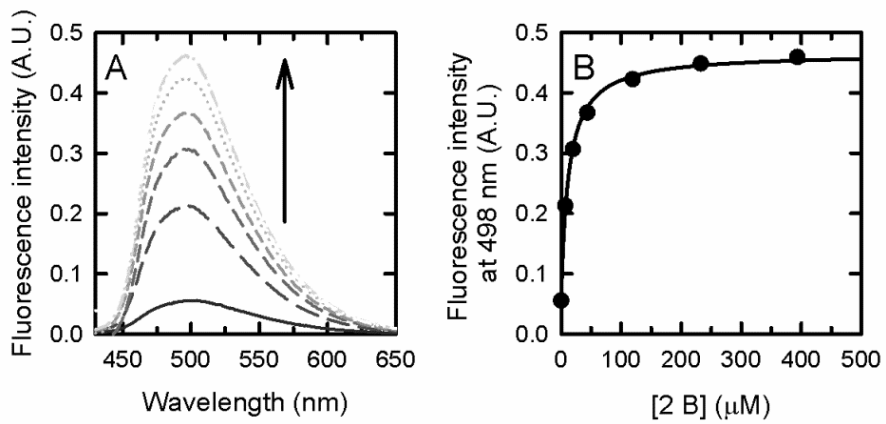


Figure 4. Example of K_D determination by fluorimetric titration. A. Fluorescence emission spectra, upon excitation at 412 nm, of a solution containing 2.4 μM hSR, 50 mM TEA, 150 mM NaCl, 5 mM TCEP, 1 mM $MgCl_2$, 5% DMSO (v/v), pH 8.0, and increasing concentration of 2 B, at 20 °C. **B**. Dependence on 2 B concentration of the fluorescence emission intensity at 498 nm upon excitation at 412 nm. The experimental points were fitted by a binding isotherm equation, yielding a K_D of 12.0±0.6 μM.

Malonate and maleate were also tested on hSR as a control. The control assays values were in agreement with the results already reported in literature (Strisovsky et al. 2005; Smith et al. 2010; Jiraskova-Vanickova et al. 2011), confirming the experimental reliability of the test. The results for the most representative molecules are reported in Table 3.

COMPOUND	ACTIVITY K_I (μM)	FLUORESCENCE K_D (μM)
Malonate	66	15.0±0.6
Maleate	600	352±14
3 A	1300	1000±100
8 A	2700	>5000
9 A	3500	>5000
5 A	3900	2500±500
1 A	3800	>5000
12 A	>5000	>5000
11 B	70	32.9±2.7
2 B	90	12.0±0.6
1 B	620	432±18
20 B	3800	>3000
8 B	>5000	≥2500
19 B	>5000	>2500
12 B	>5000	>5000

Table 3. Screening results of some compounds listed in Tables 1 and 2. The K_I and the K_D values, measured by activity and fluorescence binding assays, were obtained in the presence of 5% DMSO. Compounds are divided in three sections (controls, group A and group B) and are reported in order of potency.

In the case of competitive inhibition, the K_i calculated by the activity assay and the K_D calculated by fluorimetric titrations should be identical. Here, small difference between the two values could be due to the different temperature used for the two assays.

Substituted cyclopropanes

In the cyclopropane derivatives group, compound 3 A was the only one that demonstrated an affinity for hSR around 1 mM and for this reason it was further investigated. Its IC_{50} value was calculated in the presence of 10% DMSO (see Materials and Methods) and it was found to be 3.3±0.4 mM, leading to the conclusion that the structural constrain imposed in the cyclopropanic structure is detrimental for activity. This result was almost puzzling because from the computational studies these compounds were fitting the binding cavity almost perfectly (Fig.4). Therefore the molecular modeling results were analyzed again in light of the outcome of the experimental assays. We saw that, despite the good docking score similar to that of malonate and compound 3 A, compound 1 A was completely ineffective. To understand the reason of its inactivity, the binding mode of compound 1 A was compared with the original crystal structure. In the crystallographic complex a water molecule is located approximately where the carboxyl group of this *trans* derivative is accommodated and it is conserved in almost all the crystal structures (also in the open enzyme form), establishing hydrogen bonds with Asn154 and the phosphate group of PLP. We reasoned that the inhibitor carboxyl group in this position could have displaced this water molecule, enforcing the interaction with enzyme, but experimental tests did not confirm our hypothesis. Subsequent docking experiments in the presence of this crystallographic water molecule do not produce any meaningful pose for 1 A, therefore it is likely that it resulted inactive because it is not able to displace the water molecule.

Concerning the other derivatives, from our study we also understood that introducing geometrical constraints to the small known inhibitors of SR is detrimental for the activity. In fact, maleic acid, the closest to our compounds among known inhibitors, was reported to have a K_i of 550 µM (Strisovsky et al. 2005), whereas the K_i of the most potent of our derivatives, 3 A, is only 1 mM.

Malonate derivatives

In the case of malonate analogues, despite the weak activity of the tested molecules, a few SAR clues can be described. Compounds 2 B and 11 B were found to show the best activity, with an affinity comparable to malonate. We calculated the IC_{50} value for 11 B and it was found to be 0.125 ± 0.025 mM. This might be consistent with a stronger interaction at the target binding site with polar amino acids, warranted by the nature of hydrogen bond donor/acceptor of the hydroxyl and aminic groups. When a more lipophilic group such as methyl, unable to establish hydrogen bonds, replaces the hydroxyl group, the activity decreased and is completely lost when the substituent group, beside lipophilic, is also particularly bulky.

Since it seems that a hydrogen bond acceptor is necessary as substituent on C_α, an amidic functionality was inserted. However the compound resulted to be inactive, as well as for the bulkier derivatives (compound 12 B). Finally it is clear that the carboxylic moieties must have an extremely specific interaction with the target binding site, as the substitution of the carboxylic acid with its isosteres led to a loss of compound activity (compounds 19 B and 20 B).

Conclusions

The cyclopropane derivatives resulted less potent than malonate in our tests. The most active cyclopropanic compound was 3 A, which showed a K_D of about 1 mM. The synthetic efforts aimed to rationally expand the series led only to compounds with very low affinity for the target. Bulky groups, as well as small functional groups, or polar atoms or more lipophilic substituents, led only to higher K_D. Moreover, it seems evident that constrained molecules are consistently less effective as hSR inhibitors. The conformation imposed by the cyclopropanic structure, therefore, is not compatible with the conformation adopted by the two carboxylic groups. In addition, our findings highlighted the extremely specific interaction of the carboxyl groups also by demonstrating the loss of activity of the malonate isosteres. As demonstrated with malonate derivatives, the insertion of an oxygen or nitrogen atom on C_α that allows for the formation of additional hydrogen bonds ensures an inhibitory activity comparable to that of malonate. This activity is lost when the substituent is bulkier or apolar. This might be consistent with a stronger interaction with polar amino acids in the active site.

While this study was in progress, three other works aiming to identify novel potential SR inhibitors were published (Harty et al. 2014; Mori et al. 2014; Vorlova et al. 2014). The studies were performed following different approaches: Harty et al. designed some substrate analogs as potential inhibitors (Harty et al. 2014), whereas in the paper of Mori et al., novel potential inhibitors of SR are reported derived from an *in silico* ligand based Virtual Screening (Mori et al. 2014). Finally, the study of Vorlova et al. aimed at the analysis of malonate-based inhibitors using docking, analysis of hydration and semiempirical quantum-mechanical calculations (Vorlova et al. 2014). Also in these studies the activity of the identified compounds is in the high micromolar/low millimolar range, confirming the complexity of finding potent inhibitors for this enzyme.

In the present study, we first verified the importance of considering the enzyme conformational ensemble to obtain a more truthful description of the molecules that can bind to SR active site. In conclusion, novel hSR inhibitors were identified based on cyclopropane scaffold starting from an *in silico* screening, followed by chemical synthesis

of the compounds and *in vitro* enzyme assay. With this approach some valuable information was obtained in terms of structural requirements for hSR binding and inhibition that will be used for further development of novel SR inhibitors for the study and treatment of neurodegenerative disorders.

Materials and methods

Materials

Chemicals were of the best commercial quality available and purchased from Sigma-Aldrich, with the exception of NADH from Fluka and of tris(2-carboxyethyl)phosphine (TCEP) from Apollo Scientific.

Compounds preparation

The tested compounds were developed and synthetized by the research group of Prof. Gabriele Costantino (Department of Pharmacy, University of Parma, Italy). The compounds, provided as powders, were dissolved in a volume of 100% DMSO in order to have 100 mM stock solutions. Solutions were stored at -20 °C.

Enzyme preparation

Recombinant hSR was expressed as a hexa-His tagged fusion protein encoded in a pET28a-derived plasmid (Dixon et al. 2006), provided by Prof. Michael Toney (University of California, Davis, CA, USA), in *E. coli* BL21 CodonPlus (DE3)-RIL cells (Merck-Millipore, Darmstadt, Germany). The expression and purification were performed as previously described (Marchetti et al. 2013). The protein was concentrated in a storage buffer, containing 50 mM TEA, 150 mM NaCl, 1 mM EDTA, 5% glycerol, pH 8.0. Small aliquots were flash-frozen and thawed before use. Protein concentration is expressed as monomers.

Activity assays and compounds screening

Activity assays for the β-elimination of L-serine (Foltyn et al. 2005; Marchetti et al. 2013) were carried out in an assay solution containing 50 mM TEA, 2 mM ATP, 50 μM PLP, 5 mM DTT, 1 mM $MgCl_2$, 150 mM NaCl, 60 U/ml LDH and 300 μM NADH, pH 8.0. The reaction kinetics was measured following the NADH disappearance at 340 nm (Eisenthal and Danson 1993), using a Varian CARY400 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA), with the cell holder thermostatted at 37 °C, and was typically started by adding hSR at a concentration of 0.4-0.5 μM.

Initial screening – initially, all the compounds were tested at least in duplicate at a single fixed concentration of 0.3-0.6 mM. The substrate L-serine was added at different

concentrations (typically equal to the K_M or half K_M), depending on the range of sensitivity required. The inhibition effect was evaluated with respect to the enzyme activity registered in the absence of inhibitors, but in the presence of a concentration of DMSO equal to that present in the inhibition assay. The apparent K_i of every compound was calculated with the same Equation:

$$v_0 = \frac{[S] * V_{max}}{[S] + K_M(1 + \frac{K_i}{[I]})}$$

that describes the enzyme activity in the presence of a competitive inhibitor (Copeland 2000). v_0 is the initial rate in the presence of the competitive inhibitor, *[S]* and *[I]* are the concentrations in the assay of substrate and inhibitor, respectively, and K_i is the inhibition constant.

The influence of a compound on the activity was considered significant only when the percentage of residual activity was equal to or lower than 80%.

IC_{50} determination – IC_{50} values were obtained in the presence of an L-serine concentration close to hSR K_M and in the presence of 10% DMSO. Although the compounds were conceived as competitive inhibitors, this substrate concentration ensures the balance between the opposing effects of substrate concentration on calculated IC_{50} for competitive and uncompetitive inhibition mechanisms. Moreover, it is likely to reflect the physiological conditions of many enzymes (Copeland 2005). The percentage of DMSO is related to the compound concentrations needed to reach the saturation of the curve. Fractional rates were fitted with the Equation:

$$IC_{50} = \frac{[I]}{\frac{v_0}{v_i} - 1}$$

where v_0 is the initial rate in the absence of the competitive inhibitor, v_i is the initial rate in the presence of the competitive inhibitor and *[I]* is the concentration of inhibitor in the assay.

Fluorescence binding

The affinity of the compounds for hSR in the absence of substrate was measured exploiting the fluorescence emission properties of the PLP cofactor at 500 nm upon excitation at 412 nm. Measurements were collected using a Spex Fluoromax-2 fluorometer (HORIBA Jobin-Yvon, North Edison, NJ, USA) with the cell holder thermostatted at 20.0 ± 0.5 °C. The assay temperature was chosen since it assures a better stability of hSR during the titration which lasts approximately one hour. The solution contained 2.4 μM hSR in 50 mM TEA, 150 mM NaCl, 5 mM TCEP, 2 mM ATP, 1 mM $MgCl_2$ and 5% DMSO, pH 8.0. The dependence of the fluorescence emission intensity of the cofactor on ligand concentration was fitted by a binding isotherm to calculate the dissociation constant:

$$I = \frac{[L]}{K_D + [L]}$$

where *I* is the fluorescence intensity, *[L]* the ligand concentration and K_D the dissociation constant of the protein-ligand complex.

References

Bruno, A., L. Amori and G. Costantino (2011). "Addressing the Conformational Flexibility of Serine Racemase by Combining Targeted Molecular Dynamics, Conformational Sampling and Docking Studies." Molecular Informatics **30**(4): 317-328.

Campanini, B., F. Speroni, E. Salsi, P. F. Cook, S. L. Roderick, B. Huang, S. Bettati and A. Mozzarelli (2005 B). "Interaction of serine acetyltransferase with O-acetylserine sulfhydrylase active site: evidence from fluorescence spectroscopy." Protein Science **14**(8): 2115-24.

Campanini, B., F. Spyrakis, A. Peracchi and A. Mozzarelli (2013). "Serine racemase: a key player in neuron activity and in neuropathologies." Frontiers in Bioscience **18**: 1112-28.

Conti, P., L. Tamborini, A. Pinto, A. Blondel, P. Minoprio, A. Mozzarelli and C. De Micheli (2011). "Drug discovery targeting amino acid racemases." Chemical Reviews (Washington, D. C.) **111**(11): 6919-46.

Copeland, R. A., Ed. (2000). "Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis", Wiley-VCH, Inc.

Copeland, R. A., Ed. (2005). "Evaluation of Enzyme Inhibitors in Drug Discovery", John Wiley & Sons.

De Miranda, J., A. Santoro, S. Engelender and H. Wolosker (2000). "Human serine racemase: moleular cloning, genomic organization and functional analysis." Gene **256**(1-2): 183-188.

Dixon, S. M., P. Li, R. Liu, H. Wolosker, K. S. Lam, M. J. Kurth and M. D. Toney (2006). "Slow-Binding Human Serine Racemase Inhibitors from High-Throughput Screening of Combinatorial Libraries." Journal of Medicinal Chemistry **49**(8): 2388-2397.

Eisenthal, R. and M. Danson, Eds. (1993). "Enzyme Assays", Oxford Univ. Press.

Foltyn, V. N., I. Bendikov, J. De Miranda, R. Panizzutti, E. Dumin, M. Shleper, P. Li, M. D. Toney, E. Kartvelishvily and H. Wolosker (2005). "Serine racemase modulates intracellular D-serine levels through an alpha,beta-elimination activity." Journal of Biological Chemistry **280**(3): 1754-63.

Furukawa, H. and E. Gouaux (2003). "Mechanisms of activation, inhibition and specificity: crystal structures of the NMDA receptor NR1 ligand-binding core." The EMBO Journal **22**(12): 2873-2885.

Harty, M., M. Nagar, L. Atkinson, C. M. LeGay, D. J. Derksen and S. L. Bearne (2014). "Inhibition of serine and proline racemases by substrate-product analogues." Bioorganic & Medicinal Chemistry Letters **24**(1): 390-393.

Hoffman, H. E., J. Jiraskova, M. Ingr, M. Zvelebil and J. Konvalinka (2009 A). "Recombinant human serine racemase: Enzymologic characterization and comparison with its mouse ortholog." Protein Expression and Purification **63**(1): 62-67.

Jiraskova-Vanickova, J., R. Ettrich, B. Vorlova, H. E. Hoffman, M. Lepsik, P. Jansa and J. Konvalinka (2011). "Inhibition of Human Serine Racemase, an Emerging Target for Medicinal Chemistry." Current Drug Targets **12**(7): 1037-1055.

Lipton, S. A. (2006). "Paradigm shift in neuroprotection by NMDA receptor blockade: Memantine and beyond." Nature Reviews Drug Discovery **5**(2): 160-170.

Marchetti, M., S. Bruno, B. Campanini, S. Bettati, A. Peracchi and A. Mozzarelli (2014). "Regulation of human serine racemase activity and dynamics by halides, ATP and malonate." Amino Acids **47**(1): 163-173.

Marchetti, M., S. Bruno, B. Campanini, A. Peracchi, N. Mai and A. Mozzarelli (2013). "ATP binding to human serine racemase is cooperative and modulated by glycine." FEBS Journal **280**(22): 5853-63.

Mori, H., R. Wada, J. Li, T. Ishimoto, M. Mizuguchi, T. Obita, H. Gouda, S. Hirono and N. Toyooka (2014). "In silico and pharmacological screenings identify novel serine racemase inhibitors." Bioorganic & Medicinal Chemistry Letters **24**(16): 3732-3735.

Panatier, A., D. T. Theodosis, J.-P. Mothet, B. Touquet, L. Pollegioni, D. A. Poulain and S. p. H. R. Oliet (2006). "Glia-Derived d-Serine Controls NMDA Receptor Activity and Synaptic Memory." Cell **125**(4): 775-784.

Panizzutti, R. r., J. De Miranda, C. t. S. Ribeiro, S. Engelender and H. Wolosker (2001). "A new strategy to decrease N-methyl-d-aspartate (NMDA) receptor coactivation: Inhibition of d-serine synthesis by converting serine racemase into an eliminase." PNAS **98**(9): 5294-5299.

Shleper, M., E. Kartvelishvily and H. Wolosker (2005). "D-Serine Is the Dominant Endogenous Coagonist for NMDA Receptor Neurotoxicity in Organotypic Hippocampal Slices." The Journal of Neuroscience **25**(41): 9413-9417.

Smith, M. A., V. Mack, A. Ebneth, I. Moraes, B. Felicetti, M. Wood, D. Schonfeld, O. Mather, A. Cesura and J. Barker (2010). "The Structure of Mammalian Serine Racemase: EVIDENCE FOR CONFORMATIONAL CHANGES UPON INHIBITOR BINDING." The Journal of Biological Chemistry **285**(17): 12873-12881.

Spyrakis, F., P. Felici, A. S. Bayden, E. Salsi, R. Miggiano, G. E. Kellogg, P. Cozzini, P. F. Cook, A. Mozzarelli and B. Campanini (2013 A). "Fine tuning of the active site modulates specificity in the interaction of O-acetylserine sulfhydrylase isozymes with serine acetyltransferase." Biochimica et Biophysica Acta - Proteins and Proteomics **1834**(1): 169-181.

Strisovsky, K., J. Jiraskova, A. Mikulova, L. Rulisek and J. Konvalinka (2005). "Dual substrate and reaction specificity in mouse serine racemase: identification of high-affinity dicarboxylate substrate and inhibitors and analysis of the beta-eliminase activity." Biochemistry **44**(39): 13091-100.

Van Horn, M. R., M. Sild and E. S. Ruthazer (2013). "D-serine as a gliotransmitter and its roles in brain development and disease." Frontiers in Cellular Neuroscience **7**.

Vorlova, B., D. Nachtigallova, J. Jiraskova-Vanickova, H. Ajani, P. Jansa, J. Rezac, J. Fanfrilk, M. Otyepka, P. Hobza, J. Konvalinka and M. Lepsik (2014). "Malonate-based inhibitors of mammalian serine racemase: Kinetic characterization and structure-based computational study." European Journal of Medicinal Chemistry **89**(0): 189-197.

Wolosker, H., S. Blackshaw and S. H. Snyder (1999). "Serine racemase: A glial enzyme synthesizing d-serine to regulate glutamate-N-methyl-d-aspartate neurotransmission." PNAS **96**(23): 13409-13414.

INHIBITION STUDIES:

STRUCTURE-BASED VIRTUAL SCREENING

Application of structure-based virtual screening for the development of novel human serine racemase inhibitors

The Virtual Screening studies presented in the following work were performed by Prof. Pietro Cozzini, Dott.ssa Francesca Spyrakis and Dott. Luca Dellafiora (Department of Food Sciences, University of Parma).

Abstract

In recent years, many neurodegenerative diseases have been found to be related to the dysfunction of N-methyl-D-aspartate receptors (NMDARs)-mediated neurotransmission. Drugs that directly point to regulate the receptors activity evoke strong responses and very often show severe side effects. For this reason, research efforts are aimed at the modulation of enzymes that produce co-agonists or antagonists in order to operate a milder regulation of neurotransmission. Since in many brain areas D-serine binding is necessary for the complete activation of NMDARs, serine racemase (SR) - the enzyme responsible for D-serine synthesis - has been proposed as an alternative potential therapeutic target. The limited results obtained in the identification of new SR specific inhibitors, however, demonstrate that this enzyme is a difficult target. In this context, the help of computational approaches might reduce the experimental efforts

In this work, a combination of virtual screening and *in vitro* analysis was applied to obtain information on the interaction between potential inhibitors and SR active site.

Introduction

N-methyl-D-aspartate receptors (NMDARs) are the principal responsible of excitatory neurotransmission in mammalian central nervous system. Their activity is strictly related to many important functions, like excitatory neurotransmission, long term potentiation, synaptic plasticity, learning and movement coordination (Mothet et al. 2000; Martineau et al. 2006; Panatier et al. 2006; Niemann et al. 2007). The dysfunction of NMDARs activity regulation very often correlates with severe neuropathologies, such as ischemia, neuropathic pain, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and schizophrenia (Gonzalez et al. 2014; Paul and de Belleroche 2014). As a consequence, the development of drugs able to modulate NMDARs activity assumes a central role in the treatment of several neurodegenerative diseases. As a matter of fact, in a majority of cases molecules that directly regulate receptors activity were demonstrated to evoke severe side effects (Lipton 2004; Chen and Lipton 2006). Among the others, only memantine showed good results, achieving approval for the treatment of moderate to severe Alzheimer's disease (Lipton 2004; Peng et al. 2013) and also receiving the indication for the treatment of patients with moderate to severe vascular dementia and Parkinson's disease (Aarsland et al. 2009; Peng et al. 2013).

In light of the difficulties in the search of NMDAR-targeting drugs, the indirect regulation of the receptors can be considered as an alternative approach. For this reason, in recent years the investigation of new pharmacological targets has been broadened upstream and downstream of the NMDARs regulation pathway. In this context serine racemase (SR), the enzyme responsible for D-serine synthesis, is considered as a good candidate target. Despite its relevance, however, the limited number of studies involving the search of new SR inhibitors and the poor results ensuing from such studies indicate that this enzyme is a hard target. Published works on SR inhibitors were carried out by different approaches (Strisovsky et al. 2005; Dixon et al. 2006; Hoffman et al. 2009 B; Jiraskova-Vanickova et al. 2011). More recent studies have exploited computational methods (Mori et al. 2014; Vorlova et al. 2014).

Computational screening have assumed a crucial role in drug discovery, and the application of approaches such as ligand- or structure-based virtual screening (VS) are widely used in many investigations aimed at identifying novel protein ligands (Ripphausen et al. 2010; Lavecchia 2014).

Although until now the application of computational methods on SR has not led to the identification of molecules with significant inhibitory activity, *in silico* investigations may help to define important structural requirements for the interaction of ligands with the target.

In this work, a commercial library of molecules was screened against SR, leading to the identification of eight compounds with putative inhibitory activity. Although the activity assays showed that these compounds exhibited low inhibitory activity, the study allowed for the definition of steric and chemical features for active inhibitors. These findings may help in the development of new active molecules able to bind to the active site of hSR.

Results and discussion

A VS study requires the selection of a three-dimensional model, with structural and dynamic properties mimicking the conformational plasticity of the enzyme. The structure of human SR complexed with malonate (hSR, PDB code 3L6B, 1.5 Å) was chosen as a reference for the closed conformation. In order to have a model for the open conformation, the apo rat structure (PDB code 3HMK, 2.1 Å) was selected (Smith et al. 2010). The human and the rat sequences show a high degree of identity (89.4%) and differ only at fifteen positions (Fig. 1). These mutations are distant from the active site and, taken into account that the two structures show a very similar architecture of the substrate binding site (Fig. 2), 3HMK was chosen as a model for the human open conformation.

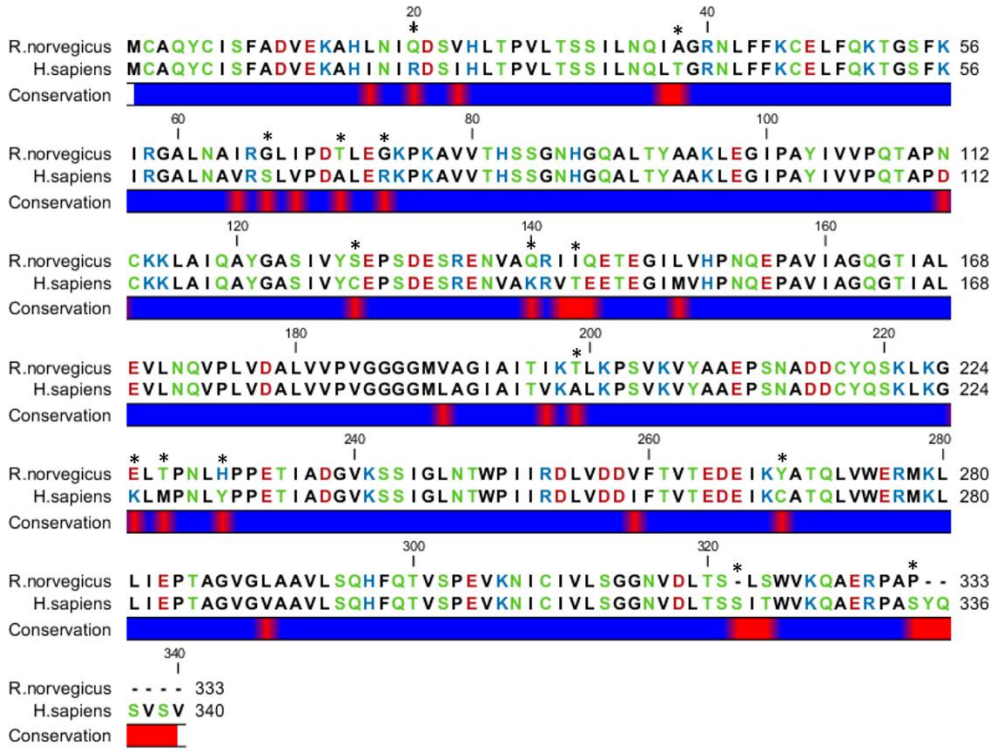


Figure 6. Alignment of human and rat SR sequences. The two sequences are 89.4 % identical. The consensus bar shows the conserved (blue) and the different (red) residues between the two sequences. Some mutations are conservative. The differences involve only fifteen positions: Q20R, A39T, G66S, T71A, G74R, S128C, Q140K, I143T, T199A, E225K, T227M, H231Y, Y269C, P334S and the insertion/deletion of S322 (CLC Sequence Viewer 6, CLC Bio, Qiagen).

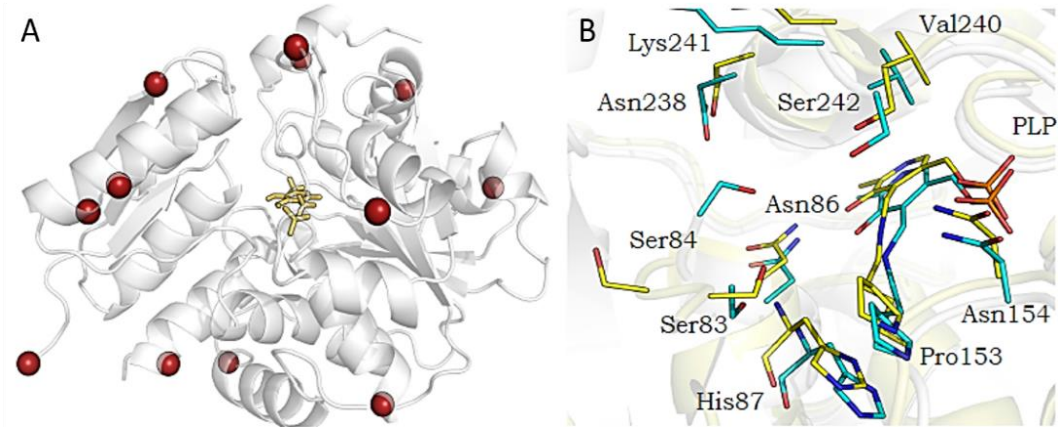


Figure 7. A. Three dimensional structure of hSR (PDB code 3L6B). Mutations are represented as red spheres and PLP in yellow sticks. **B.** Superimposition of human (3L6B, cyan) and rat (3HMK, yellow) SR active sites.

The holo and apo architectures mainly differ from each other for the organization of the small sub-domain (Fig. 3). This local rearrangement determines some differences in shape, architecture and pharmacophoric fingerprint of the binding pocket. In order to better evaluate the interaction between ligands and the protein, both architectures have been taken into account and two pocket conformations have been analyzed with the GRID molecular interaction fields (MIFs) (Goodford 1985; Carosati et al. 2004), calculated by using hydrophobic, H-bond acceptor, and H-bond donor probes. In the holo form, the binding site is well-defined (Fig. 4A). The MIFs calculation showed the overall polar characteristics of the pocket environment (Fig. 4C). Conversely, in the apo form the pocket is open and significantly exposed, thus losing defined boundaries in the upper part (Fig. 4B). Thus, the open conformation seems to be suitable for larger ligands with respect to the closed pocket. In addition, the MIFs calculation revealed the presence of hydrophobic patches in the upper portion of the pocket (Fig. 4D). Taken together these results revealed significant differences between the two conformation of the pocket and, therefore, the use of both models in the VS was worthwhile.

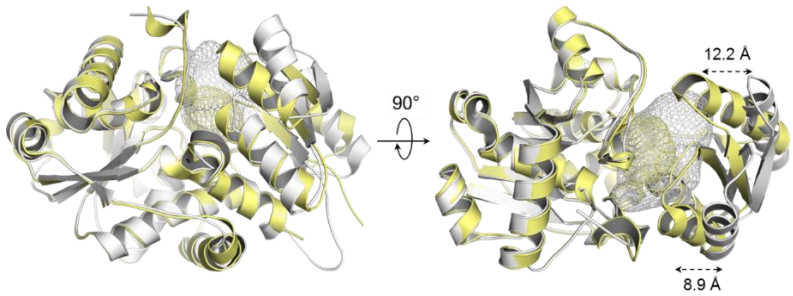


Figure 8. Superimposition of the ribbon-tube representations of holo (yellow) and apo (white) structures of serine racemase. The shapes of the closed (yellow) and open (white) binding pockets are represented in mesh. Dashed arrows indicate the extent of the reorganization at the level of the small sub-domain.

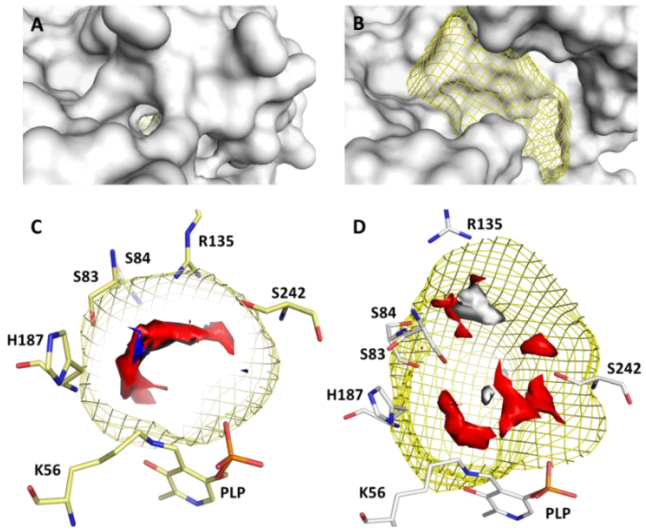


Figure 9. The anatomy of the closed and open binding sites. The shape of the binding sites is represented in yellow mesh while lining amino acids are represented in sticks. **A and B.** Surface details of the holo (A) and apo (B) forms of serine racemase. In the holo form, the binding pocket is closed whereas in the apo structure the pocket is open and exposed to the solvent. **C and D.** The white, red and blue contours represent the favorable binding site regions for placing, respectively, hydrophobic, hydrogen-bond-acceptor, and hydrogen-bond-donor groups. **C.** The pharmacophore fingerprint of the closed pocket (holo form). **D.** The pharmacophore fingerprint of the open pocket (apo form).

The two models were employed for the Structure Based VS (SBVS, see Materials and Methods). Only molecules with calculated LogP ≤ 1 were chosen from the entire SPECS database, containing more than 300000 compounds. The resulting set of 11937 molecules underwent SBVS within both holo and apo forms of SR by using the FLAP software. The shapes of two pockets, generating the respective MIFs, formed the templates. Ligands were ranked in accordance to the FLAP distance from template (Cross et al. 2012), that is an index representing the degree of complementarity between ligands and MIFs templates.

The top 2.5% molecules were visually inspected and the most interesting molecules, in accordance to the similarity with templates, were docked in the corresponding model and re-scored. On the basis of i) the computed binding architecture, ii) the overlapping with the pockets MIFs and iii) the HINT score, 8 compounds were selected for experimental trials (Tab. 1).

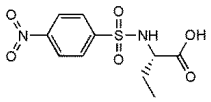
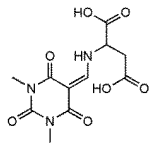
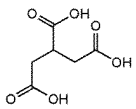
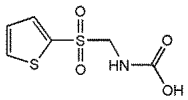
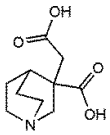
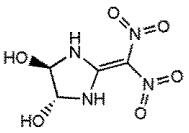
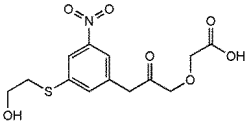
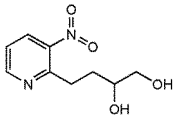
COMPOUND	SPECS CODE	STRUCTURE	HINT SCORE	MODEL
1	AG-205/07908015		600	Open
2	AG-670/36765032		1200	Open
3	AI-942/42301799		3000	Closed
4	AQ-390/42133048		1900	Open
5	AE-641/00361044		1300	Open
6	AG-690/36720008		600	Open
7	AK-968/41924790		2200	Open
8	AI-240/31702043		1700	Open

Table 2. Putative SR inhibitors identified by *in silico* screening.

The experimental screening was carried out by evaluating the initial β-elimination rate on L-serine in the presence and in the absence of the potential inhibitor. For each compound the apparent inhibition constant (K_i) was calculated with the equation for competitive inhibition (see Materials and Methods).

From the screening results, the most active compounds were AG-205, AG-670, AI-942 and AQ-390. Because of the limited quantities of compounds provided from SPECS, it was not possible to determine the IC_{50} values, but only estimated values that were around 2 mM for all the four compounds (Equation 2, Materials and Methods).

Subsequently, the enzyme affinity for these molecules was explored with fluorescence binding titrations in the presence of 5% DMSO. No apparent binding was detected for any compound (data not shown). This may be due to the ability of these molecules to bind to the open form of hSR, without promoting any structural rearrangement that would affect the fluorescence emission of PLP. Because of its similarity to malonate (Hoffman et al. 2009 A; Smith et al. 2010) compound AI-942 was the only one predicted to interact with the closed conformation of hSR. Also in this case, however, we did not record any significant influence on the PLP emission spectra, probably because the K_D was too high to be determined under our experimental conditions.

Conclusions

Overall, on the basis of our computational results, some structural features shared by active ligands can be inferred. First, with the exception of AI-942 that resembles the mode of interaction of the well-known inhibitor malonate, the other molecules, AQ-390, AG-205 and AG-670, were predicted to interact with the open conformation of SR. For this reason, a mechanism involving the stabilization of a conformation resembling the apo form of the enzyme is proposed. In light of the experimental results, the computational analysis reveals that active ligands show complementarity to the open pocket both in terms of shape and physicochemical characteristics. Specifically, active ligands have to be buried within the cleft. Furthermore it seems to be required the presence of at least one carboxyl group to interact with Ser83 (as in the case of AQ-390 and AG-205, Fig. 5).

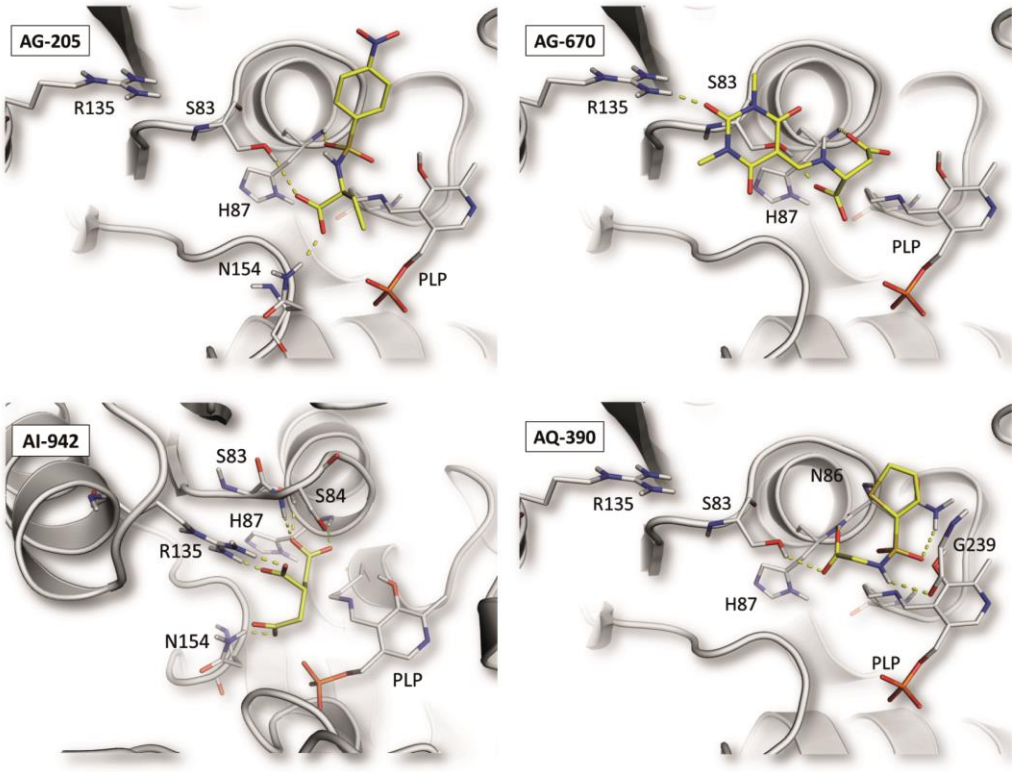


Figure 10. Docking poses of compounds AG-205, AG-670, AI-942 and AQ-390. All the four compounds were found to be active and, with the exception of AI-942, were predicted to interact with the open conformation of SR. The dotted lines represent interactions of the compounds under examination (yellow sticks) with the protein residues and PLP (white sticks) are represented.

In the absence of such contacts, even if the shape match is satisfied, the interaction is prevented (as in the case of AI-240, data not shown). A slightly different pattern of interaction was found for AG-670 since it did not sink within the surface groove. Rather, it protruded beyond the groove simultaneously interacting with Ser83 and Arg135. A similar mode of interaction was also observed for the inactive compound AK-968 that, however, does not interact with Ser83 (data not shown). This may explain at least in part the inactivity of such compound and suggests that the interaction with Ser83 may be a mandatory contact. Whit respect to the inactive molecules AG-690 and AE-641, although they share groups able to interact with Ser83 (including carboxyl groups, as in the case of AE-641), they did not satisfy shape requirements and, in addition, they were much more exposed to the solvent without being able to interact with Arg135. Thus, the presence of the simultaneous interaction with Ser83 and Arg135 seems to be indispensable to stabilize molecules not buried within the active site.

Materials and Methods

Molecular modeling

The models for the holo and apo forms of the protein were retrieved from the Protein Data Bank (http://www.rcsb.org) (PDB codes: 3L6B and 3HMK, respectively (Smith et al. 2010)). The latter structure is the rat ortholog but, in the lack of human apo structures, it can be used as a good model for the human enzyme due to the high sequence identity (almost 90%) and the conserved amino acid composition and architecture of the binding site. Protein structures and ligands were processed by using the software Sybyl, version 8.1 (www.tripos.com). All atoms were checked for atom- and bond-type assignments. Amino- and carboxyl-terminal groups were set as protonated and deprotonated, respectively. Hydrogen atoms were computationally added to the protein and energy-minimized using the Powell algorithm with a coverage gradient of ≤0.5 kcal mol^{-1} $Å^{-}1$ and a maximum of 1500 cycles.

Structure-Based Virtual Screening

The Specs database (http://www.specs.net) was chosen as the library for VS. This archive belongs to the ZINC database (http://www.zinc.docking.org) (Irwin and Shoichet 2004) and provides commercially available molecules with an acceptable purity, as stated in previous works (Carosati et al. 2006; Brincat et al. 2011; Carosati et al. 2012; Spyrakis et al. 2013 A). The database contains more than 300000 molecules with significant chemical and geometric diversity. The entire dataset was downloaded and preliminary filtered according to the calculated LogP values. We filtered molecules having a LogP ≤ 1 (11937 molecules) in order to ensure a sufficient solubility of compounds thus facilitating the experimental management.

The SBVS was performed with FLAP software (*Fingerprint for Ligand And Protein*) (Baroni et al. 2007), developed and licensed by Molecular Discovery Ltd. (http://www.moldiscovery.com). FLAP approach (Cross et al. 2012) is based on molecular interaction fields calculated by GRID algorithm (Goodford 1985) and has been successfully applied in previous studies (Muratore et al. 2012; Spyrakis et al. 2013 A; Spyrakis et al. 2013 B). FLAP allows the fast selection of the most interesting candidates with chemical and

structural complementarity with the receptor binding site (structure-based VS) and/or ligands with known activity (ligand-based VS) (Baroni et al. 2007; Spyrakis et al. 2013 A). In the present work, the structure-based VS was performed by using both the holo and apo protein structures. Compounds were ranked according to the distance from the template (Cross et al. 2012) and the most interesting molecules underwent a docking simulation within the respective protein model. We paid attention to exclude compounds containing free primary amines, in order to avoid the formation of Schiff bases with PLP.

Docking simulations and re-scoring procedures.

The docking simulations of all compounds were performed with the program GOLD version 5.0 (CCDC; Cambridge, U.K.; http://www.ccd.cam.ac.uk). For each GOLD docking search, a maximum number of 100000 operations were performed on a population of 100 individuals with a selection pressure of 1.1. Operator weights for crossover, mutation, and migration were set to 95, 95, and 10, respectively. The number of islands was set to 5 and the niche to 2. The hydrogen bond distance was set to 2.5 Å and the vdW linear cutoff to 4.0. Ligand flexibility options “flip pyramidal N”, “flip amide bonds”, and “flip ring corners” were allowed. All crystallographic waters and ligands were removed and 50 poses for each compound were generated and re-scored by HINT. It was taken into account only the best HINT scored pose for each ligand. The software HINT (Hydrophatic INTeraction) (Kellogg and Abraham 2000) was used as the re-scoring function on the basis of previous studies attesting the higher reliability of HINT scoring with respect to other scoring functions, and its successful use in the search of ligands for other targets, as well as in the estimation of ligand binding free energies (Cozzini et al. 2002; Fornabaio et al. 2003; Fornabaio et al. 2004; Marabotti et al. 2008; Salsi et al. 2010 A; Cozzini and Dellafiora 2012; Dellafiora et al. 2013; Dellafiora et al. 2014)

Materials

Chemicals were of the best commercial quality available and purchased from Sigma-Aldrich, with the exception of NADH from Fluka. Tested compounds were purchased from SPECS (http://www.specs.net). The compounds, available as powders, were dissolved in a

volume of 100% DMSO in order to have 100 mM stock solutions. Solutions were stored at -20 °C.

Enzyme preparation

Recombinant hSR was expressed as a hexa-His tagged fusion protein encoded in a pET28a-derived plasmid (Dixon et al. 2006), provided by Prof. Michael Toney (University of California, Davis, CA, USA), in *E. coli* BL21 CodonPlus (DE3)-RIL cells (Merck-Millipore, Darmstadt, Germany). The expression and purification were performed as previously described (Marchetti et al. 2013). The protein was concentrated in a storage buffer, containing 50 mM TEA, 150 mM NaCl, 1 mM EDTA, 5% glycerol, pH 8.0. Small aliquots were flash-frozen and thawed before use. Protein concentration is expressed as monomers.

Activity assays and compounds screening

Activity assays for the β-elimination of L-serine (Foltyn et al. 2005; Marchetti et al. 2013) were carried out in an assay solution containing 50 mM TEA, 2 mM ATP, 50 µM PLP, 5 mM DTT, 1 mM $MgCl_2$, 150 mM NaCl, 60 U/ml LDH and 300 µM NADH, pH 8.0. The reaction kinetics was measured following the NADH disappearance at 340 nm (Eisenthal and Danson 1993), using a Varian CARY219 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA), with the cell holder thermostatted at 37 °C. Reactions were typically started by the addition of hSR at a final concentration of 0.6-1.1 µM.

The substrate L-serine was added at a concentration equal to the K_M, with the exception of AG-690, that was tested in the presence of a substrate concentration equal to one seventh of the K_M. The K_i of the compounds were calculated by applying the Equation:

(1) $$v_0 = \frac{[S] * V_{max}}{[S] + K_M(1+\frac{K_i}{[I]})}$$

that describes the enzyme activity in the presence of a competitive inhibitor (Copeland 2000). v_0 is the initial rate in the presence of the competitive inhibitor, *[S]* and *[I]* are the concentrations in the assay of substrate and inhibitor, respectively, and K_i is the inhibition constant.

Compounds were tested at 5 mM but some compounds, namely AG-690, AK-968 and AI-240, were tested at concentrations lower than 5 mM, due to their strong absorption at 340 nm. In particular, compound AG-690 was tested at 1.2 mM, while compounds AK-968 and AI-240 at 2.8 mM. Moreover, it was only possible to test AI-942 at the maximum concentration of 2.8 mM, because of the limited quantities provided by SPECS. For each compound, the assays in the presence of the highest concentration of inhibitor were carried out in the presence of 5% DMSO (v/v), in order to have homogeneous data in terms of effect of the solvent.

Based on the experimental conditions needed in the different assays setup, the range of sensitivity was determined (Tab. 2).

COMPOUND	TEST CONCENTRATION (mM)	RANGE OF SENSITIVITY (mM)
AG-205	5	$0.08 \leq K_i \leq 10$
AG-670	5	$0.08 \leq K_i \leq 10$
AI-942	2.8	$0.05 \leq K_i \leq 6$
AQ-390	5	$0.08 \leq K_i \leq 10$
AE-641	5	$0.08 \leq K_i \leq 10$
AG-690	1.2	$0.08 \leq K_i \leq 4.2$ *
AK-968	2.8	$0.03 \leq K_i \leq 3.8$
AI-240	2.8	$0.03 \leq K_i \leq 3.8$

Table 3. Concentrations of potential inhibitor and range of sensitivity of the activity tests for the evaluation of inhibitory activity.*Because of its strong absorption, to ensure a range of sensitivity comparable to other compounds, it was necessary to test AG-690 in the presence of a low concentration of L-serine.

The inhibition was evaluated with respect to the enzyme activity measured in the absence of inhibitors. Reference activity was calculated in the presence of a concentration of DMSO equal to that present in the inhibition assay. The apparent K_i of every compound was calculated with the equation (1). The influence of a compound on the activity was considered significant only when the percentage of residual activity was equal to or lower than 80%.

The estimate of the theoretical IC_{50} values were obtained by applying the equation:

(2) $$IC_{50} = \frac{[I]}{\frac{v_0}{v_i} - 1}$$

where v_0 is the initial rate in the absence of the competitive inhibitor, v_i is the initial rate in the presence of the competitive inhibitor and *[I]* is the concentration of inhibitor in the assay.

Fluorescence binding

The affinity of the compounds for hSR in the absence of substrate was measured exploiting the fluorescence emission properties of the PLP cofactor at 500 nm upon excitation at 412 nm. Measurements were collected using a Spex Fluoromax-2 fluorometer (HORIBA Jobin-Yvon, North Edison, NJ, USA) with the cell holder thermostatted at 20.0 ± 0.5 °C. The assay temperature was set at 20 °C since it assures a good stability of hSR during the titration which lasts approximately one hour. The solution contained 2.4 µM hSR in 50 mM TEA, 150 mM NaCl, 5 mM TCEP, 2 mM ATP, 1 mM $MgCl_2$ and 5% DMSO, pH 8.0. The dependence of the fluorescence emission intensity of the cofactor on ligand concentration was fitted by a binding isotherm to calculate the dissociation constant:

(3) $$y = y_0 + \frac{a*[L]}{b+[L]}$$

where *y* is the fluorescence intensity, y_0 is the initial fluorescence intensity, *[L]* the ligand concentration, *a* is the amplitude of the transition, and *b* represent the dissociation constant of the protein-ligand complex.

References

Aarsland, D., C. Ballard, Z. Walker, F. Bostrom, G. Alves, K. Kossakowski, I. Leroi, F. Pozo-Rodriguez, L. Minthon and E. Londos (2009). "Memantine in patients with Parkinson's disease dementia or dementia with Lewy bodies: a double-blind, placebo-controlled, multicentre trial." The Lancet Neurology **8**(7): 613-618.

Baroni, M., G. Cruciani, S. Sciabola, F. Perruccio and J. S. Mason (2007). "A Common Reference Framework for Analyzing/Comparing Proteins and Ligands. Fingerprints for Ligands And Proteins (FLAP): Theory and Application." Journal of Chemical Information and Modeling **47**(2): 279-294.

Brincat, J. P., E. Carosati, S. Sabatini, G. Manfroni, A. Fravolini, J. L. Raygada, D. Patel, G. W. Kaatz and G. Cruciani (2011). "Discovery of Novel Inhibitors of the NorA Multidrug Transporter of Staphylococcus aureus." The Journal of Medicinal Chemistry **54**(1): 354-365.

Carosati, E., G. Cruciani, A. Chiarini, R. Budriesi, P. Ioan, R. Spisani, D. Spinelli, B. Cosimelli, F. Fusi, M. Frosini, R. Matucci, F. Gasparrini, A. Ciogli, P. J. Stephens and F. J. Devlin (2006). "Calcium Channel Antagonists Discovered by a Multidisciplinary Approach." The Journal of Medicinal Chemistry **49**(17): 5206-5216.

Carosati, E., S. Sciabola and G. Cruciani (2004). "Hydrogen Bonding Interactions of Covalently Bonded Fluorine Atoms: From Crystallographic Data to a New Angular Function in the GRID Force Field." The Journal of Medicinal Chemistry **47**(21): 5114-5125.

Carosati, E., A. Tochowicz, G. Marverti, G. Guaitoli, P. Benedetti, S. Ferrari, R. M. Stroud, J. Finer-Moore, R. Luciani, D. Farina, G. Cruciani and M. P. Costi (2012). "Inhibitor of Ovarian Cancer Cells Growth by Virtual Screening: A New Thiazole Derivative Targeting Human Thymidylate Synthase." TheJournal of Medicinal Chemistry **55**(22): 10272-10276.

Chen, H.-S. V. and S. A. Lipton (2006). "The chemical biology of clinically tolerated NMDA receptor antagonists." Journal of Neurochemistry **97**(6): 1611-1626.

Copeland, R. A., Ed. (2000). "Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis", Wiley-VCH, Inc.

Cozzini, P. and L. Dellafiora (2012). "In silico approach to evaluate molecular interaction between mycotoxins and the estrogen receptors ligand binding domain: A case study on zearalenone and its metabolites." Toxicology Letters **214**(1): 81-85.

Cozzini, P., M. Fornabaio, A. Marabotti, D. J. Abraham, G. E. Kellogg and A. Mozzarelli (2002). "Simple, Intuitive Calculations of Free Energy of Binding for Protein-Ligand Complexes. 1. Models without Explicit Constrained Water." The Journal of Medicinal Chemistry **45**(12): 2469-2483.

Cross, S., F. Ortuso, M. Baroni, G. Costa, S. Distinto, F. Moraca, S. Alcaro and G. Cruciani (2012). "GRID-Based Three-Dimensional Pharmacophores II: PharmBench, a Benchmark Data Set for Evaluating Pharmacophore Elucidation Methods." Journal of Chemical Information and Modeling **52**(10): 2599-2608.

Dellafiora, L., P. Mena, P. Cozzini, F. Brighenti and D. Del Rio (2013). "Modelling the possible bioactivity of ellagitannin-derived metabolites. In silico tools to evaluate their potential xenoestrogenic behavior." Food & Function **4**(10): 1442-1451.

Dellafiora, L., P. Mena, D. Del Rio and P. Cozzini (2014). "Modeling the Effect of Phase II Conjugations on Topoisomerase I Poisoning: Pilot Study with Luteolin and Quercetin." Journal of Agricultural and Food Chemistry **62**(25): 5881-5886.

Dixon, S. M., P. Li, R. Liu, H. Wolosker, K. S. Lam, M. J. Kurth and M. D. Toney (2006). "Slow-Binding Human Serine Racemase Inhibitors from High-Throughput Screening of Combinatorial Libraries." Journal of Medicinal Chemistry **49**(8): 2388-2397.

Eisenthal, R. and M. Danson, Eds. (1993). "Enzyme Assays", Oxford Univ. Press.

Foltyn, V. N., I. Bendikov, J. De Miranda, R. Panizzutti, E. Dumin, M. Shleper, P. Li, M. D. Toney, E. Kartvelishvily and H. Wolosker (2005). "Serine racemase modulates intracellular D-serine levels through an a,b-elimination activity." The Journal of Biological Chemistry **280**(3): 1754-63.

Fornabaio, M., P. Cozzini, A. Mozzarelli, D. J. Abraham and G. E. Kellogg (2003). "Simple, Intuitive Calculations of Free Energy of Binding for Protein-Ligand Complexes. 2. Computational Titration and pH Effects in Molecular Models of Neuraminidase-Inhibitor Complexes." The Journal of Medicinal Chemistry **46**(21): 4487-4500.

Fornabaio, M., F. Spyrakis, A. Mozzarelli, P. Cozzini, D. J. Abraham and G. E. Kellogg (2004). "Simple, Intuitive Calculations of Free Energy of Binding for Protein-Ligand Complexes. 3. The Free Energy Contribution of Structural Water Molecules in HIV-1 Protease Complexes." The Journal of Medicinal Chemistry **47**(18): 4507-4516.

Gonzalez, J., J. C. Jurado-Coronel, M. F. Avila, A. Sabogal, F. Capani and G. E. Barreto (2014). "NMDARs in neurological diseases: a potential therapeutic target." International Journal of Neuroscience published online.

Goodford, P. J. (1985). "A computational procedure for determining energetically favorable binding sites on biologically important macromolecules." The Journal of Medicinal Chemistry **28**(7): 849-857.

Hoffman, H. E., J. Jiraskova, P. Cigler, M. Sanda, J. Schraml and J. Konvalinka (2009 B). "Hydroxamic Acids As a Novel Family of Serine Racemase Inhibitors: Mechanistic Analysis Reveals Different Modes of Interaction with the Pyridoxal-5'-phosphate Cofactor." The Journal of Medicinal Chemistry **52**(19): 6032-6041.

Hoffman, H. E., J. Jiraskova, M. Ingr, M. Zvelebil and J. Konvalinka (2009 A). "Recombinant human serine racemase: Enzymologic characterization and comparison with its mouse ortholog." Protein Expression and Purification **63**(1): 62-67.

Irwin, J. J. and B. K. Shoichet (2004). "ZINC - A Free Database of Commercially Available Compounds for Virtual Screening." Journal of Chemical Information and Modeling **45**(1): 177-182.

Jiraskova-Vanickova, J., R. Ettrich, B. Vorlova, H. E. Hoffman, M. Lepsik, P. Jansa and J. Konvalinka (2011). "Inhibition of Human Serine Racemase, an Emerging Target for Medicinal Chemistry." Current Drug Targets **12**(7): 1037-1055.

Kellogg, E. G. and D. J. Abraham (2000). "Hydrophobicity: is LogPo/w more than the sum of its parts?" European Journal of Medicinal Chemistry **35**(7-8): 651-661.

Lavecchia, A. (2014). "Machine-learning approaches in drug discovery: methods and applications." Drug Discovery Today (in press).

Lipton, S. (2004). "Failures and successes of NMDA receptor antagonists: Molecular basis for the use of open-channel blockers like memantine in the treatment of acute and chronic neurologic insults." NeuroRX **1**(1): 101-110.

Marabotti, A., F. Spyrakis, A. Facchiano, P. Cozzini, S. Alberti, G. E. Kellogg and A. Mozzarelli (2008). "Energy-based prediction of amino acid-nucleotide base recognition." Journal of Computational Chemistry **29**(12): 1955-1969.

Marchetti, M., S. Bruno, B. Campanini, A. Peracchi, N. Mai and A. Mozzarelli (2013). "ATP binding to human serine racemase is cooperative and modulated by glycine." FEBS Journal **280**(22): 5853-5863.

Martineau, M., G. Baux and J. P. Mothet (2006). "D-serine signalling in the brain: friend and foe." Trends in Neurosciences **29**(8): 481-91.

Mori, H., R. Wada, J. Li, T. Ishimoto, M. Mizuguchi, T. Obita, H. Gouda, S. Hirono and N. Toyooka (2014). "In silico and pharmacological screenings identify novel serine racemase inhibitors." Bioorganic & Medicinal Chemistry Letters **24**(16): 3732-3735.

Mothet, J. P., A. T. Parent, H. Wolosker, R. O. Brady, Jr., D. J. Linden, C. D. Ferris, M. A. Rogawski and S. H. Snyder (2000). "D-serine is an endogenous ligand for the glycine site of the N-methyl-D- aspartate receptor." PNAS **97**: 4926 - 4931.

Muratore, G., B. Mercorelli, L. Goracci, G. Cruciani, P. Digard, G. Palù and A. Loregian (2012). "Human Cytomegalovirus Inhibitor AL18 Also Possesses Activity against Influenza A and B Viruses." Antimicrobial Agents and Chemotherapy **56**(11): 6009-6013.

Niemann, S., H. Kanki, Y. Fukui, K. Takao, M. Fukaya, M. N. Hynynen, M. J. Churchill, J. M. Shefner, R. T. Bronson, R. H. Brown, M. Watanabe, T. Miyakawa, S. Itohara and Y. Hayashi (2007). "Genetic ablation of NMDA receptor subunit NR3B in mouse reveals motoneuronal and nonmotoneuronal phenotypes." European Journal of Neuroscience **26**(6): 1407-1420.

Panatier, A., D. T. Theodosis, J.-P. Mothet, B. Touquet, L. Pollegioni, D. A. Poulain and S. p. H. R. Oliet (2006). "Glia-Derived d-Serine Controls NMDA Receptor Activity and Synaptic Memory." Cell **125**(4): 775-784.

Paul, P. and J. de Belleroche (2014). "The role of D-serine and glycine as co-agonists of NMDA receptors in motor neurone degeneration and amyotrophic lateral sclerosis (ALS)." Frontiers in Synaptic Neuroscience **6**.

Peng, D., X. Yuan and R. Zhu (2013). "Memantine hydrochloride in the treatment of dementia subtypes." The Journal of Clinical Neuroscience **20**(11): 1482-1485.

Ripphausen, P., B. Nisius, L. Peltason and J. r. Bajorath (2010). "Quo Vadis, Virtual Screening? A Comprehensive Survey of Prospective Applications." The Journal of Medicinal Chemistry **53**(24): 8461-8467.

Salsi, E., A. S. Bayden, F. Spyrakis, A. Amadasi, B. Campanini, S. Bettati, T. Dodatko, P. Cozzini, G. E. Kellogg, P. F. Cook, S. L. Roderick and A. Mozzarelli (2010 A). "Design of O-acetylserine sulfhydrylase inhibitors by mimicking nature." The Journal of Medicinal Chemistry **53**(1): 345-56.

Smith, M. A., V. Mack, A. Ebneth, I. Moraes, B. Felicetti, M. Wood, D. Schonfeld, O. Mather, A. Cesura and J. Barker (2010). "The Structure of Mammalian Serine Racemase:

EVIDENCE FOR CONFORMATIONAL CHANGES UPON INHIBITOR BINDING." The Journal of Biological Chemistry **285**(17): 12873-12881.

Spyrakis, F., P. Felici, A. S. Bayden, E. Salsi, R. Miggiano, G. E. Kellogg, P. Cozzini, P. F. Cook, A. Mozzarelli and B. Campanini (2013 A). "Fine tuning of the active site modulates specificity in the interaction of O-acetylserine sulfhydrylase isozymes with serine acetyltransferase." Biochimica et Biophysica Acta - Proteins and Proteomics **1834**(1): 169-181.

Spyrakis, F., R. Singh, P. Cozzini, B. Campanini, E. Salsi, P. Felici, S. Raboni, P. Benedetti, G. Cruciani, G. E. Kellogg, P. F. Cook and A. Mozzarelli (2013 B). "Isozyme-Specific Ligands for O-acetylserine sulfhydrylase, a Novel Antibiotic Target." PLoS One **8**(10).

Strisovsky, K., J. Jiraskova, A. Mikulova, L. Rulisek and J. Konvalinka (2005). "Dual substrate and reaction specificity in mouse serine racemase: identification of high-affinity dicarboxylate substrate and inhibitors and analysis of the beta-eliminase activity." Biochemistry **44**(39): 13091-100.

Vorlova, B., D. Nachtigallova, J. Jiraskova-Vanickova, H. Ajani, P. Jansa, J. Rezac, J. Fanfrilk, M. Otyepka, P. Hobza, J. Konvalinka and M. Lepsik (2014). "Malonate-based inhibitors of mammalian serine racemase: Kinetic characterization and structure-based computational study." European Journal of Medicinal Chemistry **89**(0): 189-197.

CROWDING AGENTS

Dependence of hSR β-eliminase activity on crowding agents

Introduction

The cytosol is a crowded environment where proteins, carbohydrates and nucleic acids are normally present at high concentrations (Ellis 2001). The cytosol of *E. coli* cells, for example, contains a concentration of macromolecules of about 200-400 mg/ml (Zimmerman and Trach 1991), which strongly reduce the solvent availability for diffusion processes. For this reason, intracellular crowding can affect the behaviour of proteins and macromolecules in the cell with respect to *in vitro* tests (Minton 2006).

To mimic the intracellular environment *in vitro*, the crowding effect on enzymatic activity can be achieved by adding macromolecules known as crowding agents. The most common crowding agents used in biochemical studies are ficoll (that mimics a globular structure) and dextran (that mimics a rod-shaped structure). In this study, the effect of the viscosity-increasing agents glycerol and DMSO was also investigated.

Results and discussion

In order to evaluate the effect of crowding agents on hSR activity, the reaction rates at a fixed L-serine concentration were measured in the assay buffer (AS, see Materials and Methods) in the presence of either glycerol, DMSO, ficoll 70 or dextran 40, at a concentration of 200 mg/ml, that nicely mimics the total mass density of cytosol (Cayley and Record 2003; Konopka et al. 2006) (Fig. 1). As only glycerol and DMSO resulted in an increase in activity, titrations at increasing concentrations of the two agents were made (Fig. 2). Michaelis-Menten dependencies were then measured at fixed concentrations of either glycerol or DMSO at 200 mg/ml concentrations (data not shown). The estimated K_Ms in the presence of 20% glycerol and DMSO were determined to be 3-fold and 6-fold lower, respectively, with respect to that measured in the same AS solution without viscosity-increasing agents. The V_{max} did not change in either condition (data not shown).

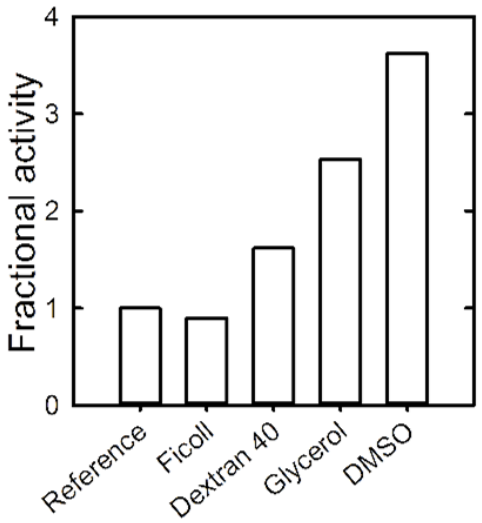


Figure 1. Dependence of hSR activity on the concentration of crowding agents. All crowding agents were at a concentration of 200 mg/ml. The reaction rates were measured at a single concentration of 10 mM L-serine and normalized with respect to the rate measured in the absence of any crowding agent (Reference in the graph).

The effect exerted by co-solutes, at the same concentration, on solution viscosity depends on their molecular weight. The bigger the solute, the bigger is the effect on macroviscosity. However, as highlighted by Hagen (J. Hagen 2010), microviscosity, rather

than macroviscosity, affects ligand diffusion into and out of the active site and protein dynamics. As a consequence, keeping the macroviscosity constant, small co-solutes, like glycerol or DMSO, are expected to affect catalytic parameters more than large polymers (Blacklow et al. 1988).

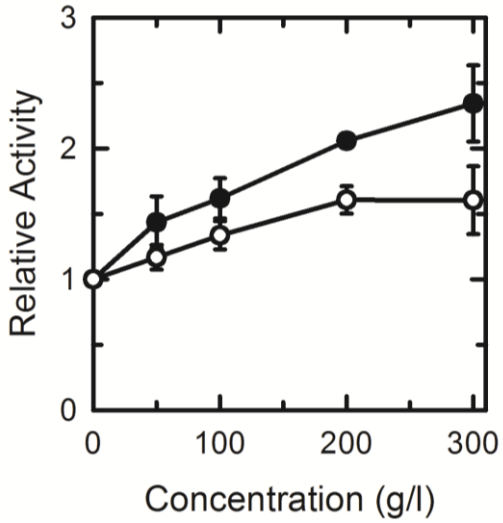


Figure 2. Dependence of hSR activity on the concentration of the viscosity-increasing agents, glycerol (open circles) and DMSO (closed cyrcles). The reaction rate was measured at a single concentration of 33 mM L-serine, a value close to the K_M, and normalized with respect to the rate measured in the absence of either glycerol or DMSO.

The effect of DMSO on the β-elimination catalytic parameters, i.e. the decrease in the K_M, was further confirmed by malonate binding to hSR measured by fluorimetric titrations in the presence and absence of DMSO. Malonate is a known reversible inhibitor of hSR and its affinity to the enzyme is strongly affected by the presence of DMSO at 50 and 100 mg/ml (5% and 10 %) (Fig. 3). The K_D decreased from 77 ± 9 μM in the absence of DMSO to 9.4 ± 0.4 μM at 100 mg/ml DMSO.

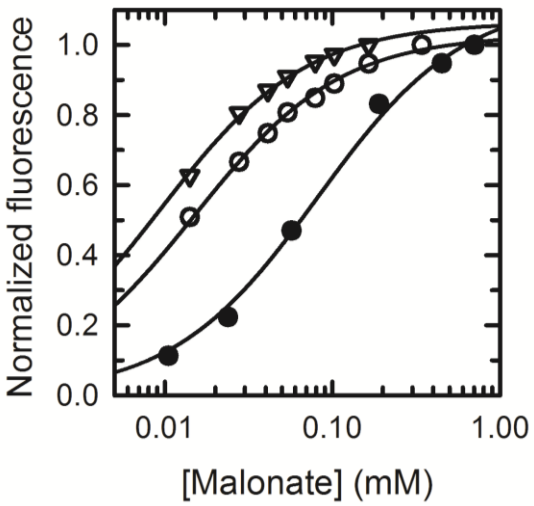


Figure 3. Malonate binding to hSR in the absence and in the presence of DMSO. Fluorescence emission spectra upon excitation at 412 nm of a solution containing 2.7 µM hSR in 50 mM TEA, 150 mM NaCl, 5 mM TCEP, 1 mM $MgCl_2$, pH 8.0, at 20 °C, were collected at increasing concentrations of malonate in the absence (closed circles) and in the presence of 5% (open circles) and 10% (open triangles) of DMSO (v/v). The dependences of the normalized fluorescence intensity at 500 nm on malonate concentration were fitted to a binding isotherm. The resulting K_Ds in the absence and in the presence of DMSO 5% and 10% were of 77 ± 9 µM, 15 ± 0.6 µM and 9.4 ± 0.4 µM respectively.

Materials and Methods

Materials

Chemicals were of the best commercial quality available and purchased from Sigma-Aldrich, with the exception of tris(2-carboxyethyl)phosphine (TCEP) from Apollo Scientific and NADH from Fluka.

Crowding and agent solutions

Ficoll 70 and dextran 40 were dissolved O/N under agitation at 4 °C in 100 mM TEA pH 8.0 at the final concentration of 500 mg/ml. Glycerol was diluted to 50% (w/v) in water and DMSO was used as 100% solution.

Activity assay

The initial rate of L-serine β-elimination was monitored by coupling the reaction with pyruvate reduction by lactate dehydrogenase and following NADH disappearance at 340 nm (Eisenthal and Danson 1993), using a Varian CARY400 spectrophotometer with a thermostatted cell holder. The typical activity assay solution contained 50 mM TEA, 150 mM NaCl, 50 µM PLP, 5 mM DTT, 1 mM $MgCl_2$, 60 U/ml LDH, 300 µM NADH.

The effect on the initial rate of 200 mg/ml crowding and viscosity-increasing agents was determined in the presence of 10 mM L-serine. The dependences of the initial rate in the presence of increasing concentration of glycerol and DMSO were determined in the presence of 30 mM L-serine, a concentration close to the K_M. Data were normalized for the activity recorded in the absence of viscosity-increasing agents.

Fluorescence measurements

Fluorescence spectra of hSR were collected using a FluoroMax-3 fluorometer (HORIBA-Jobin Yvon), with the cell holder thermostatted at 20.0 ± 0.5 °C. Emission spectra upon direct excitation of the cofactor at 412 nm were recorded with slits set for optimal signal-to-noise ratio on a solution containing 2.7 µM enzyme, 50 mM TEA, 150 mM NaCl, 5 mM TCEP, 1 mM $MgCl_2$, pH 8.0 and in the absence and in the presence of 5% and 10% DMSO (v/v). The binding affinity of malonate to hSR was determined by monitoring the

increase in fluorescence emission of the coenzyme upon excitation at 412 nm, as previously reported for other PLP-dependent enzymes (Campanini et al. 2005).

The dependence of the fluorescence emission intensity of the cofactor on ligand concentration was fitted to a binding isotherm to calculate the dissociation constant:

$$I = \frac{[L]}{K_D + [L]}$$

where *I* is the fluorescence intensity, *[L]* the ligand concentration and K_D the dissociation constant of the protein-ligand complex.

References

Blacklow, S. C., R. T. Raines, W. A. Lim, P. D. Zamore and J. R. Knowles (1988). "Triosephosphate isomerase catalysis is diffusion controlled. Appendix: Analysis of triose phosphate equilibria in aqueous solution by 31P NMR." Biochemistry **27**(4): 1158-1167.

Campanini, B., F. Speroni, E. Salsi, P. F. Cook, S. L. Roderick, B. Huang, S. Bettati and A. Mozzarelli (2005). "Interaction of serine acetyltransferase with O-acetylserine sulfhydrylase active site: evidence from fluorescence spectroscopy." Protein Sci **14**(8): 2115-24.

Cayley, S. and M. T. Record (2003). "Roles of Cytoplasmic Osmolytes, Water, and Crowding in the Response of Escherichia coli to Osmotic Stress: Biophysical Basis of Osmoprotection by Glycine Betaine." Biochemistry **42**(43): 12596-12609.

Eisenthal, R. and M. Danson, Eds. (1993). Enzyme Assays, Oxford Univ. Press.

Ellis, R. J. (2001). "Macromolecular crowding: obvious but underappreciated." Trends in Biochemical Sciences **26**(10): 597-604.

Iotti, S., C. Frassineti, L. Alderighi, A. Sabatini, A. Vacca and B. Barbiroli (1996). "In vivo assessment of free magnesium concentration in human brain by 31P MRS. A new calibration curve based on a mathematical algorithm." NMR in Biomedicine **9**(1): 24-32.

J. Hagen, S. (2010). "Solvent Viscosity and Friction in Protein Folding Dynamics." Current Protein and Peptide Science **11**(5): 385-395.

Konopka, M. C., I. A. Shkel, S. Cayley, M. T. Record and J. C. Weisshaar (2006). Crowding and Confinement Effects on Protein Diffusion in Vivo. **188:** 6115-6123.

Minton, A. P. (2006). "How can biochemical reactions within cells differ from those in test tubes?" Journal of Cell Science **119**(14): 2863-2869.

Zimmerman, S. B. and S. O. Trach (1991). "Estimation of macromolecule concentrations and excluded volume effects for the cytoplasm of Escherichia coli." Journal of Molecular Biology **222**(3): 599-620.

DIVALENT CATIONS

Dependence of hSR β-eliminase activity on divalent cations

Introduction

Magnesium (Mg^{2+}) is the fourth most abundant cation in the organism and the second in the intracellular environment, with concentrations ranging from 3 mM in the cerebral tissues to 8 mM in the skeletal muscles (Iotti et al. 2000; Iotti and Malucelli 2008). Magnesium is generally present in the cell as Mg^{2+}-ATP complex and the concentration of free magnesium is about ten-fold lower than its total concentration. The concentration of free Mg^{2+} in the brain *in vivo* was estimated to be about 0.18 mM (Iotti et al. 1996), without significant changes with respect to age and gender. Fluctuations in the intracellular ATP concentration influence the presence of free magnesium inside the cell (Lüthi et al. 1999).

Pathological conditions like ischaemia, hypoxia and epilepsy cause depletion of intracellular ATP. The consequent increase in the concentration of free magnesium alters the activity of several proteins. In addition, the intracellular concentration of free Mg^{2+} is depleted in patients affected by Parkinson's and Alzheimer's diseases (Barbiroli et al. 1996; Andrasi et al. 2000), while it is increased in schizophrenic patients (Hinsberger et al. 1997).

Calcium (Ca^{2+}) is another important regulator of cellular functions and is involved in signal transduction processes. The variations in calcium concentration allow for the modulation of signal transmission in functions like muscular contraction, neurotransmission and cellular proliferation. The intracellular concentration of calcium is finely regulated by the presence of channels and organelles. In resting cells, calcium is mainly sequestered into the endoplasmic reticulum and in mitochondria. Its cytoplasmic concentration is about 100 nM, 10000 fold lower than the extracellular concentration. The intracellular concentration of calcium can vary very rapidly upon different incoming stimuli. Alterations in the regulation of the concentration lead to important cellular damages and cell death (Trump and Berezesky 1995).

Divalent cations and ATP were the first SR modulators to be discovered. They produce an activation on catalysis. Because of its abundance in the intracellular environment, magnesium is thought to be the principal cation bound to hSR.

The sequence analysis shows that the cation binding site (Fig. 3) is highly conserved in different species, with Glu210 and Asp216 unvaried in all the organisms considered. Ala214

is conserved in mammals and plants, while is substituted with a glycine in the microorganism sequences. The conservation of a proline residue, whose features are usually associated with structural rigidity, at position 211 defines the structure of the binding site (Fig. 4).

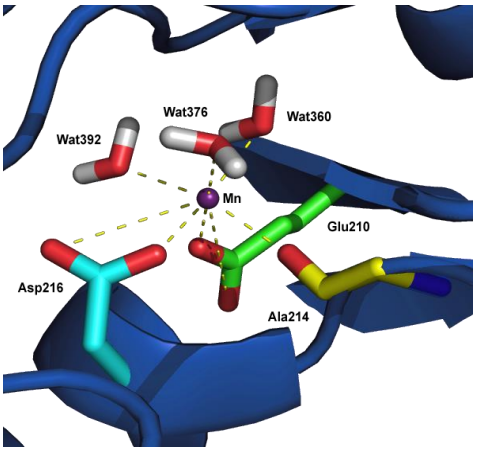


Figure 3. The cation binding-site on hSR structure. The cation (Mn^{2+} in the crystal structure) is coordinated by E210, A214, D216 and three water molecules (PDB code: 3L6B).

In light of these considerations, it is interesting to determine the quantitative effect of Mg^{2+} and Ca^{2+} on hSR activity. The definition of the respective dissociation constants might reveal a relevant physiological regulation linked to the fluctuation of divalent cations *in vivo*.

Results and discussion

The gene of the recombinant hSR used for the previous characterization studies of the enzyme has a His-tag on the N-terminus that can interfere with the analysis on divalent cations. The pET vector has a sequence coding for a thrombin cleavage site, but the stability conditions of the enzyme are not compatible with those necessary for the protease cleavage. For this reason, we cloned the gene in a new system and we are working on the improvement of the purification protocol. Moreover, the analysis is further complicated by the fact that Mg^{2+} binds to the protein on a second binding site as Mg^{2+}-ATP. However, some preliminary observations can be also inferred on His-tag hSR.

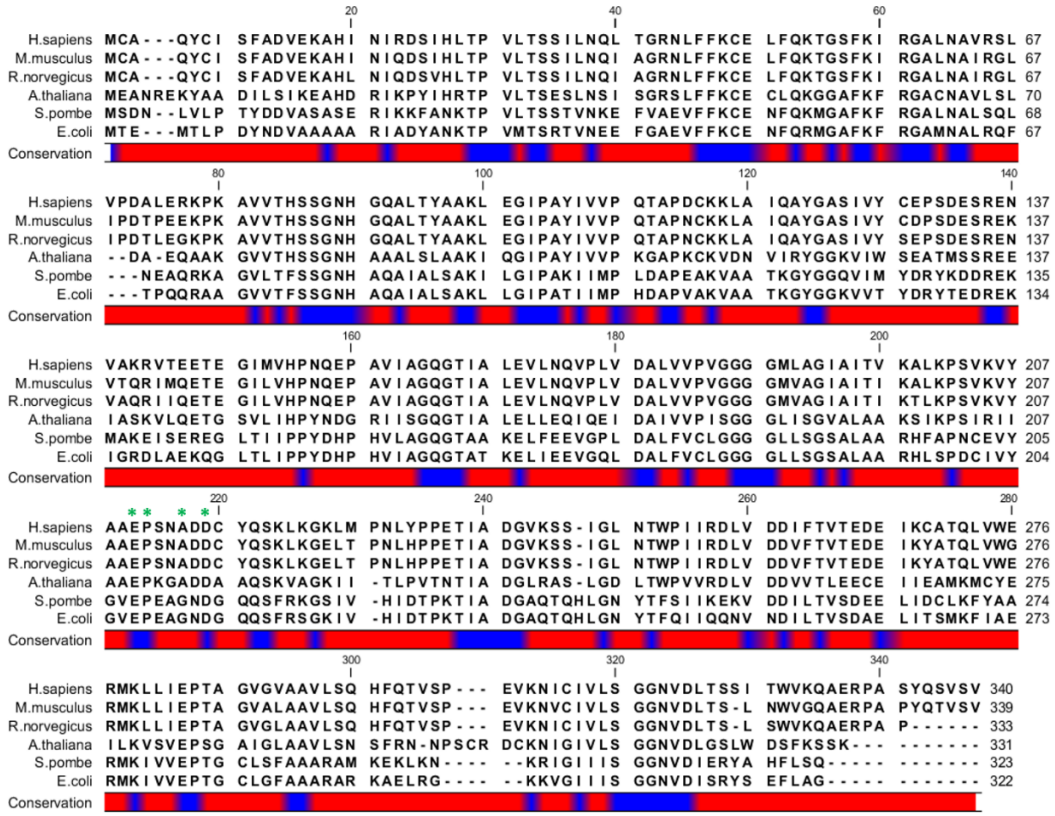


Figure 4. Alignement of SR sequences of different species. The conserved residues of the divalent cation binding site are E210, P211, A214 and D216 (human numeration) and are marked with green asterisks. The consensus bar shows the degree of conservation of the sequences. The conserved regions are represented in blue (CLC Sequence Viewer 6, CLC Bio, Qiagen).

The assay optimization was led monitoring the dependence of hSR activity on Mg^{2+} in the absence of ATP, considered as the simplest condition. Comparing the activity recorded in the absence of divalent cations and in the absence and in the presence of an excess of EDTA, we noticed different hSR initial rates, meaning that in solution were present traces of cations able to activate the enzyme (Fig. 5). Then, we first determined the minimal concentration of EDTA necessary in the activity assay mix in order to eliminate the contaminant cations. With this aim, it was necessary to eliminate the excess of EDTA present in the hSR storage buffer and dialyze the protein against a 50 nM EDTA-containing buffer. The EDTA was not entirely eliminated in order to chelate eventual heavy metals present in solution and avoid the hSR precipitation (Marchetti et al. 2014). The new storage buffer was further diluted in the assay solution, leading to a negligible concentration of EDTA.

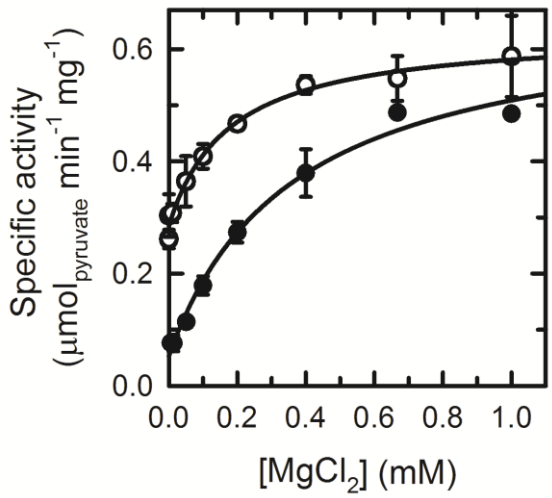


Figure 5. β-Elimination activity of hSR in the absence of ATP and in the absence (open circles) and in the presence (closed circles) of 200 µM EDTA. The specific activity in the absence of EDTA and magnesium is higher than the same activity registered in the presence of 200 µM EDTA, indicating that the contaminant cations present in solution can activate hSR. The dependences of the initial rates on the concentration of $MgCl_2$ were fitted to a binding isotherm.

The minimal concentration of EDTA to chelate the contaminant cations was determined with the registration of the activity in the presence of different concentration of an Mg^{2+}-EDTA buffer (data not shown). In the presence of 200 µM Mg^{2+}-EDTA, hSR activity is comparable to that recorded in the absence of magnesium and in the presence

of an excess of EDTA (data not shown). For this reason, all the subsequent experiments were performed in these conditions.

The association equilibrium of the Mg^{2+}-EDTA complex, and therefore the concentration of free magnesium, is related to the respective concentration of the two components and to the pH and temperature of the solution. In order to determine the effective concentration of free Mg^{2+} able to bind to hSR, we applied the program MaxChelator (see Materials and Methods) that is able to calculate the concentration of free cations in a chelating agents-containing solution at fixed pH and temperature (Schoenmakers et al. 1992).

We performed preliminary titrations of hSR activity at increasing concentrations of magnesium and calcium in the absence and in the presence of ATP, but the complex framework previously described does not allow for the discussion of defined data. In spite of that, the titrations of elimination activity at increasing concentration of ATP in the absence and in the presence of saturating cations can be more easily analyzed. First of all, the titrations of ATP in the absence and in the presence of saturating Mg^{2+} retain positive cooperativity (data not shown), indicating that is not an artefact due to the binding of Mg^{2+}-ATP complex to the protein and confirming the data previously obtained (Marchetti et al. 2013). The comparison of ATP K_{DS} in the presence of saturating concentrations of calcium and magnesium does not reveal significant differences.

We tried also to evaluate the effect of cations on hSR fluorescence spectra, but the instability of the protein in the absence of magnesium hampered the measurements.

In the near future the same experiments and the titrations of the cations will be performed on hSR without His-tag. It will be also interesting to produce hSR with mutation at the ATP and cation binding site, in order to evaluate the separate effects of the two effectors.

Materials and Methods

Materials

Chemicals were of the best commercial quality available and purchased from Sigma-Aldrich, with the exception of tris(2-carboxyethyl)phosphine (TCEP) from Apollo Scientific and NADH from Fluka.

Enzyme preparation

Recombinant hSR was expressed as a hexa-His tagged fusion protein encoded in a pET28a-derived plasmid (Dixon et al. 2006), provided by Prof. Michael Toney (University of California, Davis, CA, USA), transformed into *E. coli* BL21 CodonPlus (DE3)-RIL cells (Merck-Millipore, Darmstadt, Germany), as previously described (Marchetti et al. 2013). Purification was carried out using a TALON® His-Tag Purification Resin (Clontech, CA, USA). The protein was concentrated in a storage buffer (SB), containing 50 mM TEA, 150 mM NaCl, 1 mM EDTA, 5% glycerol, pH 8.0. Small aliquots were flash-frozen and thawed before use. Protein concentration is expressed as monomers.

Protein dialysis

In order to eliminate the excess of EDTA, stock protein was extensively dialyzed against an exchange solution (ES) containing 50 mM TEA, 150 mM NaCl and 50 nM EDTA, pH 8.0. The dialysis was performed for 6 hours at 4°C against ES buffer in home-made devices with 12000 MW cut-off membranes. At the end of dialysis, the protein solution was centrifuged and the final concentration was determined by an absorption spectrum, considering an extinction coefficient of 34,140 $M^{-1}cm^{-1}$ at 278 nm, using a Varian CARY400 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The dialyzed protein solution was then flash-frozen and stored at -80°C in small aliquots.

Activity Assays

Activity assays for the β-elimination of L-serine (Foltyn et al. 2005; Marchetti et al. 2013) were carried out in an assay solution (AS) containing 50 mM TEA, 2 mM ATP, 50 µM PLP, 5 mM DTT, 1 mM $MgCl_2$, 150 mM NaCl, 60 U/ml LDH and 300 µM NADH, pH 8.0, unless

otherwise specified. The reaction was typically started by adding hSR at a concentration of 0.6-0.9 μM. All reactions were carried out at 37°C.

Dependence on divalent cations - To assess the dependence of hSR activity on divalent cations, either magnesium chloride or calcium chloride were added to a magnesium chloride-free AS, at concentrations varying from 0 to 2 mM, in the presence of fixed concentrations of 200 μM EDTA and 530 mM L-serine, in the presence and in the absence of 2 mM ATP (disodium salt). Because of the presence of two chelating agents in the AS solution, EDTA and ATP, real free Mg^{2+} and Ca^{2+} concentrations were calculated using the MAXCHELATOR program WinMaxc32 (http://maxchelator.stanford.edu/). With this program it is possible to calculate the free cations concentration in a solution at fixed temperature and pH values, in the presence of one or more chelating agents. The reactants were pre-equilibrated at 37.0 ± 0.5 °C in the thermostatted cell holder of the spectrophotometer before starting the reaction by adding the enzyme at a final concentration of typically 0.6-0.7 μM.

Dependence on ATP - The dependence of the initial velocity on ATP concentration was determined with β-elimination assays in the presence of saturating L-serine (530 mM), corresponding to about a 10-fold excess with respect to the K_M determined in the absence of ATP (Marchetti et al., 2013). The ATP was supplied as disodium salt. The reactants were pre-equilibrated at 37.0 ± 0.5 °C in the thermostatted cell holder of the spectrophotometer before starting the reaction by adding the enzyme at a final concentration of typically about 0.4 μM or 0.9 μM, in the presence and in the absence of divalent cations, respectively. The dependence in the absence of divalent cations was carried out by adding a fixed concentration of 200 μM EDTA to avoid the unspecific activation of the protein by contaminating cations.

Data analysis

Dependence on ATP. To determine the Hill coefficient and the apparent K_D, data points were fitted to the Hill equation:

$$y = y_0 + a * \frac{[L]^n}{{K_D}^n + [L]^n}$$

where *y* is the fluorescence emission intensity or the initial reaction velocity, *[L]* is the ligand concentration (ATP) and *n* the Hill coefficient. y_0 is a horizontal offset and *a* the amplitude.

References

Andrasi, E., S. Igar, Z. Molnar and S. Makò (2000). "Disturbances of magnesium concentrations in various brain areas in Alzheimer's disease." Magnesium Research **13**(3): 189-196.

Barbiroli, B., P. Martinelli, A. Patuelli, R. Lodi, S. Iotti, P. Cortelli and P. Montagna (1996). "Phosphorus magnetic resonance spectroscopy in multiple system atrophy and Parkinson's disease." Movement Disorders **14**(3): 430-435.

Dixon, S. M., P. Li, R. Liu, H. Wolosker, K. S. Lam, M. J. Kurth and M. D. Toney (2006). "Slow-binding human serine racemase inhibitors from high-throughput screening of combinatorial libraries." J Med Chem **49**(8): 2388-97.

Foltyn, V. N., I. Bendikov, J. De Miranda, R. Panizzutti, E. Dumin, M. Shleper, P. Li, M. D. Toney, E. Kartvelishvily and H. Wolosker (2005). "Serine racemase modulates intracellular D-serine levels through an alpha,beta-elimination activity." Journal of Biological Chemistry **280**(3): 1754-63.

Hinsberger, A. D., P. C. Williamson, T. J. Carr, J. A. Stanley, D. J. Drost, M. Densmore, G. C. MacFabe and D. G. Montemurro (1997). "Magnetic resonance imaging volumetric and phosphorus 31 magnetic resonance spectroscopy measurements in schizophrenia." Journal of psychiatry & neuroscience : JPN **22**(2): 111-117.

Iotti, S., C. Frassineti, L. Alderighi, A. Sabatini, A. Vacca and B. Barbiroli (1996). "In vivo assessment of free magnesium concentration in human brain by 31P MRS. A new calibration curve based on a mathematical algorithm." NMR in Biomedicine **9**(1): 24-32.

Iotti, S., C. Frassineti, L. Alderighi, A. Sabatini, A. Vacca and B. Barbiroli (2000). "In vivo 31P-MRS assessment of cytosolic [Mg2+] in the human skeletal muscle in different metabolic conditions." Magnetic Resonance Imaging **18**(5): 607-614.

Iotti, S. and E. Malucelli (2008). "In vivo assessment of Mg2+ in human brain and skeletal muscle by 31P-MRS." Magnesium Research **21**(3): 157-162.

Lüthi, D., D. Günzel and J. A. S. McGuigan (1999). "Mg-Atp Binding: its Modification by Spermine, the Relevance to Cytosolic Mg2+ Buffering, Changes in the Intracellular Ionized Mg2+ Concentration and the Estimation of Mg2+ by 31P-NMR." Experimental Physiology **84**(2): 231-252.

Marchetti, M., S. Bruno, B. Campanini, S. Bettati, A. Peracchi and A. Mozzarelli (2014). "Regulation of human serine racemase activity and dynamics by halides, ATP and malonate." Amino Acids **47**(1): 163-173.

Marchetti, M., S. Bruno, B. Campanini, A. Peracchi, N. Mai and A. Mozzarelli (2013). "ATP binding to human serine racemase is cooperative and modulated by glycine." FEBS Journal **280**(22): 5853-63.

Schoenmakers, T. J., G. J. Visser, G. Flik and A. P. Theuvenet (1992). "CHELATOR: an improved method for computing metal ion concentrations in physiological solutions." Biotechniques **12**(6): 870-874, 876-879.

Trump, B. F. and I. K. Berezesky (1995). "Calcium-mediated cell injury and cell death." The FASEB Journal **9**(2): 219-28.

CONCLUSIONS

The present biochemical study confirms that the regulation of hSR is produced by an extended and fine-tuned allosteric network. In particular, it was found that the ATP sites are allosterically interconnected to each other and to the catalytic pocket. The binding of ATP stimulates the closure of the active site, probably promoting a tighter interaction with the substrate and the correct orientation of residues involved in catalysis. Moreover, chloride seems to form ionic interactions that stabilize specific active conformations of the enzyme. These effects between the different binding sites of hSR might have physiological implications because all effectors, ATP and glycine, act at concentrations close to the intracellular levels. In this framework, glycine homeostasis and the energetic conditions of the cell probably play a key role in D-serine production *in vivo* and, in a broader context, in the control of N-methyl-D-aspartate receptors-mediated responses.

The research on hSR inhibitors, with the aim to regulate the enzymatic activity in pathological conditions, requires more efforts, given the lack of success in identifying strong inhibitors, in spite of the different strategies that have been pursued. The results of the computational screening and structure-activity relationship analysis, nevertheless, have provided indications on the structural and chemical requirements of potential inhibitors. Based on our findings, however, it is possible to explore a new class of inhibitors targeting the hSR allosteric sites, instead of its catalytic pocket.

In the near future, it will be interesting to further investigate other aspects of hSR regulation that remain still unclear and not in-depth studied. Among these, divalent cations-mediated regulation, the involvement of cysteine residues in hSR activity, the interactions with phospholipids and regulating proteins will be analyzed. All these data together will likely result in a more complete overview of the tuning of hSR activity *in vivo*.

OTHER RESEARCH ACTIVITIES

Human aspartate racemase

Human aspartate racemase (hDR) is a cerebral enzyme responsible for the synthesis of D-aspartate, a putative modulator of adult neurogenesis in men and rodents (D'Aniello et al. 1996; Nagata et al. 1999). In recent years, Kim and colleagues identified the gene coding for the mouse DR (mDR) isoform and characterized the recombinant enzyme (Kim et al. 2011). To date, the activity of hDR has not been biochemically characterized and many aspects are still unclear.

The studies undertaken in our laboratory aimed to the production of the soluble recombinant protein, in order to study its biochemical and biophysical properties. Based on the data published about mDR, the human isoform was identified. The synthetic gene optimized for the *E. coli* codon usage (Mr. Gene, Nuremberg, Germany) was cloned into a pET vector, verified by sequencing and transformed in a bacterial expression strain. Several expression and purification tests were carried out in different conditions but it was not possible to obtain hDR in the soluble form. The presence of hDR in the inclusion bodies was confirmed by denaturant purification and mass spectrometry. The refolding experiments did not allow to obtain the soluble protein with the correct folding.

A very recent work highlighted the impossibility to repeat the results of Kim and colleagues. The soluble enzyme was obtained only when purified with a GST-tag (Tanaka-Hayashi et al. 2014). The biochemical characterization, however, is still not complete and in the future it would be possible to clone the hDR gene in another expression system, as showed by Tanaka-Hayashi and colleagues, to proceed with the characterization studies.

Amino deoxychorismate lyase of *Plasmodium falciparum*

Plasmodium falciparum is one of the etiologic agents of malaria, causing the most severe form of this disease. Several drugs were developed against malaria, but many cases of resistance are arising. For this reason, it is very important to find novel therapies.

The amino deoxychorismate lyase (ADCL) is a PLP-dependent enzyme involved in the synthesis of p-aminobenzoate (pABA), a substrate for the synthesis of tetrahydrofolic acid. The ADCL of *P. falciparum* was identified only few years ago by bioinformatics analysis and partially characterized (Magnani et al. 2013). PfADCL is able to synthesize PABA from amino deoxychorismate and, with low efficiency, to transaminate some D-amino acids in the same catalytic site (Magnani et al. 2013). Because of the absence of its pathway in the human organism, this enzyme is a potential interesting target for the development of novel drugs against malaria.

In order to improve the biochemical characterization of PfADCL, the recombinant enzyme was expressed in bacterial cells and purified. The catalytic efficiency of the transamination on different substrates was determined, in particular D-glutamate and D-aspartate. Moreover, the enantiospecific production of D-aspartate from D-glutamate was investigated. Some potential competitive inhibitors of the physiological lyasic reaction were studied. With this aim, the synthesis of pABA was monitored in the absence and in the presence of putative competitive inhibitors, chosen among the substrates of the transaminasic reaction and compounds similar to the amino deoxychorismate, i.e. dicarboxylic acid like adipate, succinate and glutarate.

These findings improve the knowledge on PfADCL activity regulation, that needs however to be further investigated. In the future, it would be also interesting to test novel synthetic molecules in order to define lead compounds for the search of specific drugs against malaria.

References

D'Aniello, A., A. D. Cosmo, C. D. Cristo, L. Annunziato, L. Petrucelli and G. Fisher (1996). "Involvement of D-Aspartic acid in the synthesis of testosterone in rat testes." Life Sciences **59**(2): 97-104.

Kim, P. M., X. Duan, A. S. Huang, C. Y. Liu, G.-l. Ming, H. Song and S. H. Snyder (2011). "Aspartate racemase, generating neuronal D-aspartate, regulates adult neurogenesis." PNAS **107:** 3175-3179.

Magnani, G., M. Lomazzi and A. Peracchi (2013). "Completing the folate biosynthesis pathway in Plasmodium falciparum: p-aminobenzoate is produced by a highly divergent promiscuous aminodeoxychorismate lyase." Biochemical Journal **455**(2): 149-155.

Nagata, Y., H. Homma, J.-A. Lee and K. Imai (1999). "D-Aspartate stimulation of testosterone synthesis in rat Leydig cells." FEBS Letters **444**(2-3): 160-164.

Tanaka-Hayashi, A., S. Hayashi, R. Inoue, T. Ito, K. Konno, T. Yoshida, M. Watanabe, T. Yoshimura and H. Mori (2014). "Is d-aspartate produced by glutamic-oxaloacetic transaminase-1 like 1 (Got1l1): a putative aspartate racemase?" Amino Acids **47**(1): 79-86.