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ORIGINAL ARTICLE

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

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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 (PLP)-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 ninefold 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.

Keywords D-Serine · Serine racemase · Pyridoxal 5′-phosphate · Allostery · Neuropathologies

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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 NMDA receptors are unique in that they respond to three different amino acid agonists, i.e., glutamate, glycine and D-serine (Traynelis et al. 2010). Serine racemase (SR, EC 5.1.1.18) (Campanini et al. 2013; Canu et al. 2014; De Miranda et al. 2002; Foltyn et al. 2005, 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 human serine racemase (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 NMDA receptors 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), PICK-1 (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 k_{cat} and no effect on 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.

Materials and methods

Materials

Chemicals were of the best commercial quality available and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

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 tris(2-carboxyethyl)phosphine (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 $\varepsilon_{388} = 6{,}600\ M^{-1}\,cm^{-1}$. 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 h at 4 °C against exchange solutions in homemade devices based on 12,000 MW cut-off membranes. The exchange solutions varied by (1) type and concentration of buffering agents (tris(hydroxymethyl)aminomethane (TRIS), TEA, and sodium phosphate); (2) presence or absence of chelating agents (EDTA); (3) presence or absence of reducing agents (TCEP or DTT); (4) 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 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, sodium chloride, lithium chloride, potassium chloride, trimethylamine hydrochloride or ammonium chloride was added to a sodium chloride-free AS, at a fixed final concentration of 150 mM. To assess the activity dependence on anions, sodium fluoride, sodium chloride, sodium bromide, sodium iodide, or sodium acetate was 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

hSR fluorescence emission spectra 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, 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 (Campanini et al. 2005; Schiaretti et al. 2004; Schnackerz et al. 1995). 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 mM 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):

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

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.

Results and discussion

hSR stability

The stability of hSR in SB1 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). 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 h 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

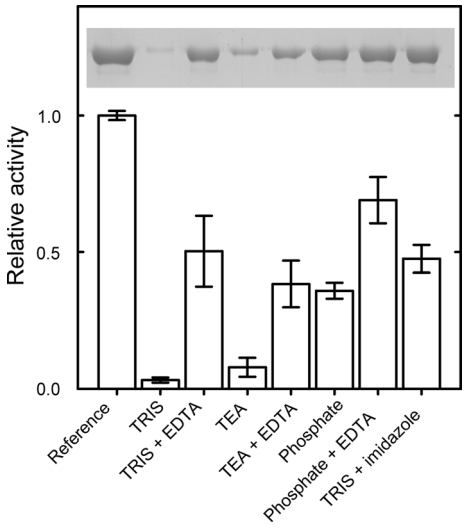

Fig. 1 Optimization of buffer composition for hSR activity and stability. L-Serine β-elimination activity of hSR after three cycles of dialysis (1.5 h each) against exchange solutions containing: (i) 50 mM TRIS (TRIS), (ii) 50 mM TRIS in the presence of 1 mM EDTA (TRIS + EDTA); (iii) 50 mM TEA (TEA), (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 μmol $_{\text{pyruvate}}$ min^{-1} mg^{-1}. The assays were carried out in AS buffer. *Inset* SDS-PAGE gel of the supernatant of protein solutions

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 Fig. 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 (Online resource 1, Fig. S1). To confirm this hypothesis, $CoCl_2$ at 2 mM concentration was added to hSR solution pre-treated with EDTA and incubated for 2 h, 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, 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 (1) eliminating the excess of PLP and $MgCl_2$, and (2) 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 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 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 was 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 nor in the 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+} was added in large excess to an assay mixture containing 5 mM pyrophosphate. Only supplementation with Mg^{2+} led to a recovery of activity (Online resource 1, Fig. S2), 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 tenfold excess with respect to ATP. AMP did not decrease the activity of

hSR, indicating that ATP is not significantly displaced (data not shown).

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 (Burkhard et al. 2000; Peracchi et al. 1995), 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 (Online resource 1, Fig. S3). Halides, on the other hand, added to a chloride-free AS as sodium salts, exhibited a concentration-dependent effect on hSR activity, without apparently reaching a plateau, in inverse order of ionic radius (Fig. 2a). At 200 mM concentration, fluoride increased the activity almost threefold and chloride twofold, whereas bromide had almost no effect and iodide abolished the activity completely at concentrations higher than 300 mM (Fig. 2a). Further increases in fluoride concentration were hampered by the lower solubility of its sodium salt in AS. Sodium acetate was included to evaluate nonspecific ionic strength effects. It slightly reduced the activity to the same extent as bromide, indicating that the effects of fluoride, chloride and iodide are not simply associated with ionic strength. The same assays were also carried out in the absence of ATP, with comparable results (Online resource 1, Fig. S4), indicating that halides do not affect ATP binding.

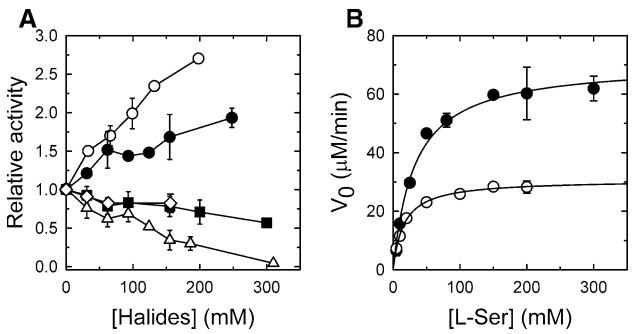

Fig. 2 Effect of monovalent ions on β-elimination activity of hSR. **a** Dependence of the L-serine β-elimination activity on the concentration of sodium halides: fluoride (*open circles*), chloride (*closed circles*), bromide (*open diamonds*), iodide (*open triangles*). Sodium acetate (*closed squares*) 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 μmol $_{pyruvate}$ $min^{-1}mg^{-1}$. **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 $K_M = 15.9 \pm 0.8$ mM and $k_{cat} = 76 \pm 1$ min^{-1} in the absence of NaCl and $K_M = 31.9 \pm 3.6$ mM and $k_{cat} = 176 \pm 5$ min^{-1} in the presence of NaCl. The catalytic efficiency is 4.77 and 5.51 min^{-1} mM^{-1} in the absence and presence of NaCl, respectively

Since chloride is the only physiologically abundant halide, the effects on hSR kinetic parameters were studied in detail (Fig. 2b). At 150 mM concentration, chloride affected both K_M and k_{cat}, with a twofold increase of both parameters (from 76.5 ± 1 to 176 ± 5 min^{-1} for k_{cat} and from 15.9 ± 0.8 to 31.9 ± 3.6 mM for K_M). Therefore, the effect of chloride resembles that of 'uncompetitive activators' (e.g., Maruyama 1990; Wild et al. 1976), which do not change the catalytic efficiency (k_{cat}/K_M).

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 hemoglobin, 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. 2a) 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. 3). 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) (Fig. 4a). Upon excitation of hSR at 298 nm, two emission peaks were observed (Fig. S5A and Fig. 4b). 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 (Benci et al. 1999; Bettati et al. 2000;

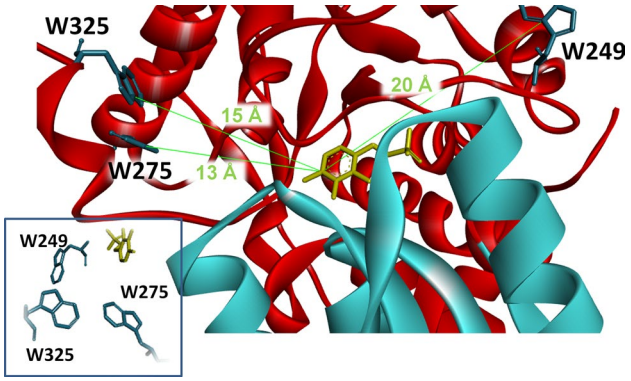

Fig. 3 Tryptophan residues in hSR three-dimensional structure. Three-dimensional structure of hSR (PDB code: 3L6B) showing tryptophan residues and PLP in a ball-and-stick representation. The calculated distances (*green lines*) between the aromatic residues and PLP are as follows: PLP-W249, 20 Å; PLP-W325, 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, USA 2013)

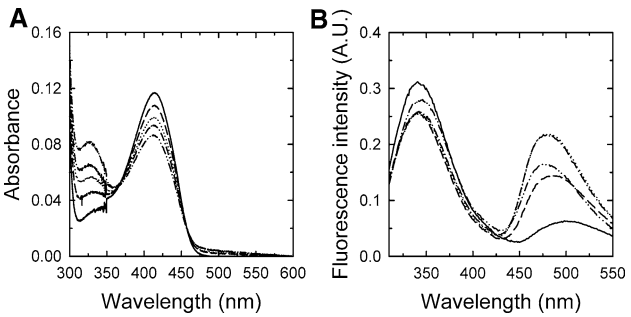

Fig. 4 Effect of NaI on hSR spectroscopic properties. **a** Absorption spectra of a solution containing 32 μM hSR in 50 mM TEA, 150 mM NaCl, 5 mM TCEP, 1 mM EDTA, 5 % glycerol, pH 8.0 at 20 °C in the absence (*solid line*) and presence of 0.057 (*dashed line*), 0.126 (*dotted line*), 0.210 (*dash-dotted line*) and 0.290 M (*dash-dot-dotted line*) NaI. **b** Fluorescence emission spectra of hSR upon excitation at 298 nm ($slits_{ex}$ = 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

Campanini et al. 2003; McClure and Cook 1994; Strambini et al. 1996), cystathionine β-synthase (Lodha et al. 2010; Quazi and Aitken 2009), DOPA decarboxylase (Dominici et al. 1997), D-aminoacid 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. S5B). This attribution was confirmed by the excitation spectrum at 500 nm showing peaks at 280 and 412 nm (Fig. S5C). Based on structural (Fig. 3) and spectrofluorimetric data (Fig. S5A), 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. 2), 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. 4a). 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 aldimine. The latter has been occasionally described in the literature and is formed by the 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 (Online resource 1, Fig. S6), indicating that enzyme activation by chloride and fluoride is not associated with a change in the tautomeric equilibrium of PLP.

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. 4b). 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. 4a) 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.

Active site accessibility by fluorescence quenching

The complex dynamic landscape of hSR was further explored by fluorescence quenching, 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. 4b) did not allow retrieving meaningful quantitative quenching constants. On the contrary, the behaviour of hSR external aldimine with glycine is less complex (Fig. 5), 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. 5a), 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 (Fig. 5b) shows that the active site is significantly less accessible to the quencher in the presence of ATP ($K_{sv} = 1.77 \pm 0.09\ M^{-1}$) than in its absence ($K_{sv} = 5.91 \pm 0.01\ M^{-1}$). 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

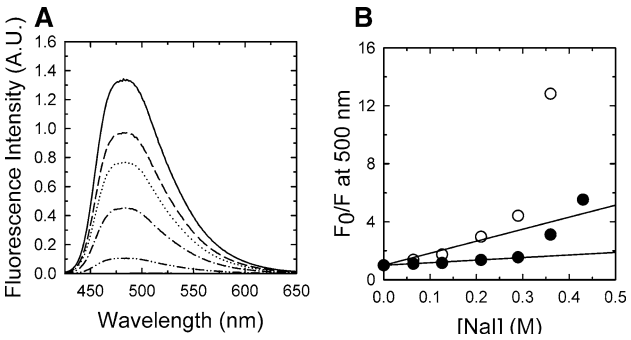

Fig. 5 Fluorescence quenching of hSR-bound PLP. Spectra of a solution containing 2.4 μM hSR, 50 mM TEA, 150 mM NaCl, 5 mM TCEP, 1 mM $MgCl_2$, pH 8.0, at 20 °C. **a** Fluorescence emission spectra of hSR external aldimine with glycine (final concentration 50 mM) upon excitation at 298 nm ($slits_{ex} = 4$ 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 (Eq. 1 in "Materials and methods") with $K_{sv} = 5.91 \pm 0.01$ and $1.77 \pm 0.09\ M^{-1}$ in the absence and presence of ATP, respectively

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 (Online resources 1, Eq. S1) 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.

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). 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. Malonate is a noncovalent 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. 6a, b). Furthermore, the peak wavelength of the coenzyme emission was slightly blue shifted to 498 nm.

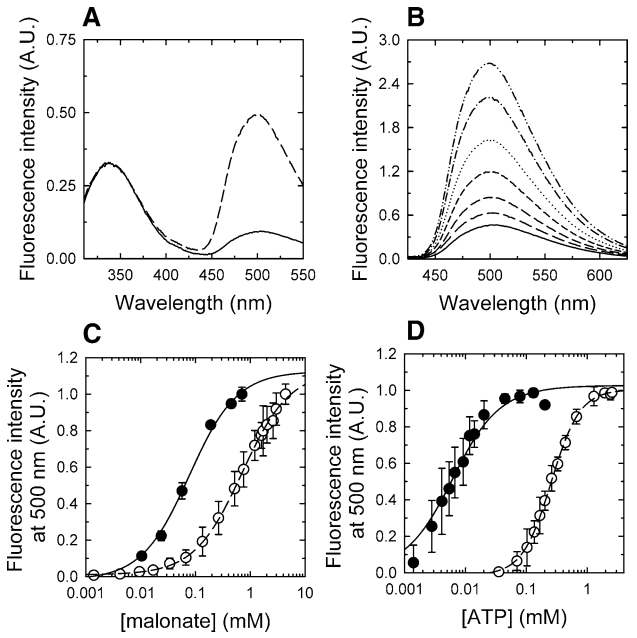

Fig. 6 Cross-talk between the active site and the ATP-binding site probed by fluorescence emission spectroscopy. Spectra of a solution containing 2.4 μM human SR, 50 mM TEA, 150 mM NaCl, 5 mM TCEP, 1 mM $MgCl_2$, pH 8.0, at 20 °C. **a** Fluorescence emission spectra of hSR upon excitation at 298 nm ($slits_{ex}$ = 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 ($slits_{ex}$ = 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 ± 9 μM and 710 ± 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 (Online resource 1, Eq. S2), obtaining a K_D for ATP of 5.0 ± 0.6 μM, and in the absence of malonate to a binding isotherm, obtaining a K_D for ATP of 255 ± 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. 6c). 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. 6d), 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 noncovalent 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 noncovalent 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.

Conflict of interest No conflicts of interest declared by any of the authors.

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