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Magnesium and calcium ions differentially affect human serine racemase activity and modulate its quaternary equilibrium toward a tetrameric form

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ABSTRACT

Serine racemase is the pyridoxal 5′-phosphate dependent enzyme that catalyzes both production and catabolism of D-serine, a co-agonist of the NMDA glutamate receptors. Mg^{2+}, or, alternatively, Ca^{2+}, activate human serine racemase by binding both at a specific site and – as ATP-metal complexes – at a distinct ATP binding site. We show that Mg^{2+} and Ca^{2+} bind at the metal binding site with a 4.5-fold difference in affinity, producing a similar thermal stabilization and partially shifting the dimer-tetramer equilibrium in favour of the latter. The ATP-Ca^{2+} complex produces a 2-fold lower maximal activation in comparison to the ATP-Mg^{2+} complex and exhibits a 3-fold higher EC_{50}. The co-presence of ATP and metals further stabilizes the tetramer. In consideration of the cellular concentrations of Mg^{2+} and Ca^{2+}, even taking into account the fluctuations of the latter, these results point to Mg^{2+} as the sole physiologically relevant ligand both at the metal binding site and at the ATP binding site. The stabilization of the tetramer by both metals and ATP-metal complexes suggests a quaternary activation mechanism mediated by 5′-phosphonucleotides similar to that observed in the distantly related prokaryotic threonine deaminases. This allosteric mechanism has never been observed before in mammalian fold type II pyridoxal 5′-phosphate dependent enzymes.

1. Introduction

Mammalian serine racemase (SR, EC 5.1.1.18) [1] is a neuron-localized [2] pyridoxal 5′-phosphate (PLP)-dependent enzyme that catalyzes the reversible racemization of L- to D-serine and the irreversible conversion of both to pyruvate and ammonia by β-elimination [3,4]. D-serine is a co-agonist of the *N*-methyl D-aspartate receptors for glutamate (NMDARs) [5,6] and its altered levels in the central nervous system have been associated to schizophrenia, among other conditions [7]. Because of the relevance of D-serine in several neuropathologies, the catalytic and allosteric properties of SR have been investigated in depth, particularly in search of druggable sites [8–13].

ATP was the first described allosteric effector of SR [3] and produces a strong activation, especially of the β-elimination reaction [3,4,14], in a cooperative fashion [15]. Crystallographic data on *Schizosaccharomyces pombe* SR (SpSR) co-crystallized with the ATP analog phosphomethylphosphonic acid adenylate ester (AMP-PCP) have provided structural details on the ATP binding site, which is located at the dimer interface [16]. Mg^{2+} binds to ATP and forms interactions with the beta and gamma phosphate groups and with a number of water molecules (Fig. 1). From the only available crystal structure with AMP-PCP bound, it appears that Mg^{2+} does not form any direct interactions with amino acid side chains [16].

Mg^{2+} or, alternatively, Ca^{2+}, were identified as positive allosteric effectors and both bind at the same metal-binding site, distinct from that of ATP [3,17] (Fig. 1). This site was recognized crystallographically and involves residues Glu210, Asp216 and Ala214 (human SR numbering), about 10 Å apart from the active site and 25 Å apart from the ATP-binding site [11,16,18]. The metal-binding site is conserved in mammalian, plant and yeast homologs [19] but not in SR from the hyperthermophilic archaeon *Pyrobaculum islandicum*, which is not affected by ATP nor by

Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; hSR, human serine racemase; AMw, apparent molecular weight; TEA, triethanolamine; LDH, lactate dehydrogenase.

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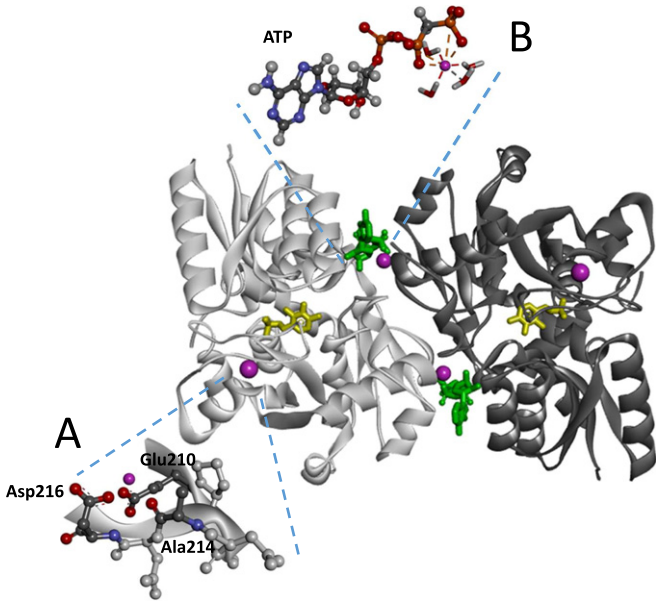

Fig. 1. Metal binding sites of hSR. Model of human SR indicating the positions of Mg^{2+}, ATP-Mg^{2+} and pyridoxal 5′-phosphate, represented in purple, green and yellow, respectively. The model is based on PDB entry 3L6B, reported as a homodimer, which binds Mg^{2+} at the metal binding site but not ATP. The ATP binding site was approximately defined by overlapping the structure of hSR with that from *Schizosaccharomyces pombe* (PDB 1WTC, reported as a dimer), in complex with the ATP analog AMP-PCP. Insets: (A) detail of the metal-binding site; (B) details of the ATP-Mg^{2+} (Ca^{2+}) binding sites. Whereas the molecular model of inset A is derived from a crystallographic structure [16], the model of inset B is only inferred from the superimposition of hSR and SpSR.

divalent cations [20]. On the other hand, the activity of SR racemase from the soil-living mycetozoa *Dictyostelium discoideum* is not only modulated by Mg^{2+}, but also by Na^{+}, which binds at the same site as Mg^{2+} [21].

Functionally, the activation by divalent cations was first proposed as a Ca^{2+} sensing system [22], although it was later objected that the alternative ligand Mg^{2+} has a much higher intracellular concentration (Table 1) [23] and would be, therefore, a more likely physiological ligand. Considering the excitability of neurons and the highly variable Ca^{2+} transient concentration within the excitation cycle (Table 1), the activation by Ca^{2+} was not ruled out in specific cellular compartments or under specific cellular conditions [19]. Herein, we investigated in detail the differential binding of Ca^{2+} and Mg^{2+} to hSR, both as ligands of the metal-binding site and of ATP. Free-metal concentrations were evaluated through a dedicated software [24].

Although the available SR crystal structures were all reported as homodimers [11,16,18], there are conflicting data about the quaternary state in solution. Early gel filtration experiments on murine SR (mSR) led to the estimation of a molecular weight apparently intermediate between a monomer and a dimer [14]. Two distinct populations of dimers and tetramers were also described for mSR [22]. More recently, it was observed that purified human SR (hSR) can exist either as a monomer or a dimer, depending on the elution conditions, with phosphate solutions stabilizing the dimer and Tris solutions stabilizing the monomer [25]. Here, to address these inconsistencies, and following our previous observation that the binding of Mg^{2+} and ATP-Mg^{2+} induces tertiary conformational changes [15], we evaluated the effect of ATP, Ca^{2+} and Mg^{2+} on the quaternary structure of hSR.

The relevance of the quaternary state of hSR stems from reports that both the activity and the quaternary equilibrium of SR distantly related prokaryotic homologs, threonine deaminases, long considered model proteins in allostery studies, are also affected by phosphonucleotides [18,26,27]. Indeed, the biodegradative threonine deaminases (tdcBs) of *E. coli* and *Salmonella typhimurium*, 99% identical in sequence to each other but only 36% identical to hSR, are positively regulated by AMP [26,28], whereas threonine deaminase from *Clostridium tetanomorphum*, only 44% identical to tdcBs and 36% identical to hSR, is upregulated by ADP [29]. For both *S. typhimurium* and *E. coli* tdcBs, it was suggested that AMP activation results from the stabilization of a tetrameric form [18], although tetramerization was shown to be necessary but not sufficient for *E. coli* TdcB activation, hinting at concomitant tertiary conformational changes [27]. Here, we investigated if a quaternary equilibrium similar to that of threonine deaminases is present also for hSR, in search for an evolutionarily conserved allosteric mechanism within fold type II PLP-dependent enzymes.

Table 1
Concentrations of ATP, free Ca^{2+} and free Mg^{2+} in the cytosol of neurons.

Condition	$[Ca^{2+}]_{free}$	$[Mg^{2+}]_{free}$	[ATP]
At rest	~100 nM [39]	400–600 μM [23]	~1–5 mM [37,38]
Activated Microdomains	500–1000 nM [39] ~100 μM [40,41]		

2. Materials and methods

2.1. Chemicals and 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. Recombinant D-amino acid oxidase (DAAO) of *Rhodotorula gracilis* was a generous gift from Professor Loredano Pollegioni, University of Insubria, Varese, Italy. The plasmid for His-tagged TEV protease expression and the plasmid containing His-tagged thioredoxin were both generously provided by Professor Christopher S. Hayes, UC Santa Barbara, Santa Barbara, CA, USA.

2.2. Enzyme preparation

To remove the polyhistidine tag, a potential metal chelator, the gene encoding for hSR in a pET-28-derived plasmid [15,30] (provided by Michael Toney, Department of Chemistry, University of California, Davis, CA, USA) was amplified by PCR using the primers 5′-TTTGGATCCGAGAATCTATATTTTCAATCTGGTACTTGTGCTCAGTACTGCATCTC-3′ and 5′-CCCCTCGAGCTAAACAGAAACAGACTGATAGGAAGCTGGCC-3′. The construct was subcloned into a pET21b-derived expression vector [31,32], in frame with the gene encoding for His-tagged thioredoxin (Trx) at the N-terminus. A proteolysis site for the TEV protease [33] is encoded between Trx and hSR for the removal of the His-TrxA tag. The fusion protein was expressed in BL21 (DE3)-RIL cells (Agilent technologies), which were lysed by lysozyme incubation (45 min at 4 °C), followed by sonication in a buffer containing 50 mM Na_2HPO_4, 150 mM NaCl, 4 mM TCEP, 50 μM PLP, 0.2 mM PMSF, 0.2 mM benzamidine, 1.5 μM pepstatine, pH 8.0. The fusion protein was purified using a TALON® resin (Clontech, CA, USA) and incubated with His-tagged TEV protease (60 μg per mg of protein) for 3 h at 4 °C to remove the His-tagged thioredoxin fragment. After dialysis to remove imidazole, the uncleaved protein and the His-tagged TEV protease were removed by further incubation with the TALON® resin. The cleaved protein was supplemented of 400 μM EDTA to remove all residual metal ions from the Co^{2+} resin, which were shown to promote protein precipitation [34] and then extensively dialyzed against a buffer containing 50 mM TEA, 150 mM NaCl, at pH 8.0. Finally, hSR was concentrated to 33.6 μM and flash-frozen in 20 μl aliquots. Purity and cleavage yields were assessed using a Chemidoc MP system (Biorad).