BBA - Proteins and Proteomics xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

BBA - Proteins and Proteomics

journal homepage: www.elsevier.com/locate/bbapap

Human serine racemase is nitrosylated at multiple sites

Francesco Marchesani[a], Stefano Bruno[a,*], Gianluca Paredi[a], Samanta Raboni[a], Barbara Campanini[a], Andrea Mozzarelli[a,b]

[a] Dipartimento di Scienze degli Alimenti e del Farmaco, Università di Parma, Parma, Italy
[b] Istituto di Biofisica, Consiglio Nazionale delle Ricerche, Pisa, Italy

ARTICLE INFO

Keywords:
Serine racemase
S-nitrosylation
Enzyme regulation
D-Amino acids
Allosteric regulation

ABSTRACT

Serine racemase is a pyridoxal 5′-phosphate dependent enzyme responsible for the synthesis of D-serine, a neuromodulator of the NMDA receptors. Its activity is modulated by several ligands, including ATP, divalent cations and protein interactors. The murine orthologue is inhibited by S-nitrosylation at Cys113, a residue adjacent to the ATP binding site. We found that the time course of inhibition of human serine racemase by S-nitrosylation is markedly biphasic, with a fast phase associated with the reaction of Cys113. Unlike the murine enzyme, two additional cysteine residues, Cys269, unique to the human orthologue, and Cys128 were also recognized as S-nitrosylation sites through mass spectrometry and site-directed mutagenesis. The effect of S-nitrosylation on the fluorescence of tryptophan residues and on that of the pyridoxal phosphate cofactor indicated that S-nitrosylation produces a partial interruption of the cross-talk between the ATP binding site and the active site. Overall, it appears that the inhibition results from a conformational change rather than the direct displacement of ATP.

1. Introduction

Human serine racemase (EC 5.1.1.18, hSR) [1–7] is a pyridoxal 5′-phosphate (PLP)-dependent enzyme that catalyses the reversible racemization of L-serine to D-serine – a co-agonist of the NMDA glutamatergic receptors - and the irreversible conversion of both L- and D-serine to pyruvate and ammonia [2,3,8]. The two reactions contribute to D-serine homeostasis along with that catalysed by D-amino acid oxidase (DAAO), the main degradative enzyme for D-amino acids [9]. SR is mostly localized in neurons [10,11], but it is also found in cells not involved in glutamatergic neurotransmission, such as keratinocytes [12] and even in organisms where no NMDARs homologs are present [13].

Several allosteric effectors and protein interactors regulate SR activity. ATP binds in a strongly cooperative fashion at two symmetry-related sites, producing a 10-fold increase in activity [8,14]. Glycine, another co-agonist of NMDA receptors, is a competitive inhibitor and stabilizes a conformation that binds ATP with a 100-fold higher affinity [8]. SR activity is also modulated by divalent cations [2,15], NADH [16,17], halides [18], phosphorylation and translocation to the membrane [19,20], and by proteins like GRIP1 [21], PICK-1 [22,23], Golga3 [24], stargazin [25], GAPDH [17] and DISC1 [26]. Moreover, S-nitrosylation of Cys113, adjacent to the ATP binding site, results in a 10-fold reduction in the activity of the murine orthologue, a regulation mechanism that was interpreted as a feedback control of NMDA activation [27].

S-nitrosylation was recognized as a reversible mechanism of allosteric regulation in several proteins, affecting enzyme activity, protein-protein interaction, receptor activation and channel gating [28,29]. S-nitrosylation can be produced by the direct reaction of cysteine residues with the signaling molecule NO followed by oxidation [28], or it can be the result of transnitrosylation by S-nitrosylated peptides, particularly *S*-nitrosoglutathione (GSNO) [30], or S-nitrosylated proteins, some of which are emerging as universal donors of nitroso groups [31]. The comparison of the sequences and structures of S-nitrosylated proteins has led to the proposal of various possible consensus motifs, with a cysteine-flanking acid-base dyad indicated as a possible mechanism in the selective stabilization of nitrosothiols [32]. More recently, it was suggested that S-nitrosylated proteins share a revised acid-base motif involved in the recognition by nitrosylases [33]. Despite the analysis

Abbreviations: ACN, acetonitrile; ATP, adenosine 5′-triphosphate; CTP, citidine 5′-triphosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; GSNO, *S*-nitrosoglutathione; GTP, guanosine 5′-triphosphate; HCCA, α-cyano-4-hydroxycinnamic acid; HRP, horseradish peroxidase; hSR, human serine racemase; LDH, lactate dehydrogenase; MMTS, methyl methanethiosulfonate; NADH, nicotinamide adenine dinucleotide; NMDA, *N*-methyl-D-aspartate; PLP, pyridoxal 5′-phosphate; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCEP, *tris*(2-carboxyethyl)phosphine; TEA, triethanolamine; TFA, trifluoroacetic acid

* Corresponding author.
E-mail address: stefano.bruno@unipr.it (S. Bruno).

https://doi.org/10.1016/j.bbapap.2018.01.009
Received 30 November 2017; Received in revised form 18 January 2018; Accepted 29 January 2018

of > 1000 S-nitrosylated proteins, there is still no agreement on the structural features that make only a small number of cysteine residues prone to S-nitrosylation. In the present work, we have characterized the reactivity of GSNO with human SR, which has eight potential nitrosylation sites in its sequence.

2. Methods

2.1. Materials

Chemicals were of the best commercial quality available and were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. *Tris*(2-carboxyethyl)phosphine (TCEP) was purchased from Apollo Scientific (Br edbury, UK); Biotin-HPDP and avidin conjugated with horseradish peroxidase were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Protein expression and purification

Recombinant hSR was expressed as a hexa-His tagged fusion protein encoded in a pET28a-derived plasmid [34] and was expressed in *E. coli* BL21 CodonPlus (DE3)-RIL cells (Merck-Millipore, Darmstadt, Germany), as previously described [8]. D318N and C113S mutants were expressed and purified using the same protocol.

2.3. Site-directed mutagenesis

Point mutations C113S and D318N were introduced directly in the pET28a-derived expression vector by the QuikChange site-directed mutagenesis protocol (Stratagene, San Diego, CA, USA) using the primers 5′-gtattgcaagttttt**act**gtctggagctgtctggg-3′ and 5′-cccagacagctccagac**agt**aaaaaacttgcaatac-3′ for C113S and 5′gctcagtggtggaaacgtt**aac**ttaacctcctccataacttggg3′ and 5′cccaagttatggaggaggttaa**gtt**aacgtttcc accactgagc-3′ for D318N. The codon bearing the mutations is shown in bold. In the case of the D318 mutation, a restriction site was also introduced (underlined) to allow for a faster identification of the recombinants carrying the mutation. All resulting constructs were confirmed by automated sequencing and transformed into *E. coli* BL21 CodonPlus (DE3)-RIL cells.

2.4. S-nitrosylation

S-nitrosylation of hSR was produced by incubation with nitrosylglutathione (GSNO) at concentrations ranging from 2 to 500 μM for up to 6 h depending on the experiments, at 25 °C, in a buffered solution containing 50 mM triethanolamine (TEA), 150 mM NaCl, 6 mM adenosine triphosphate (ATP), 6 mM $MgCl_2$, 50 μM pyridoxal 5′-phosphate (PLP), pH 8.0. hSR was added at concentrations of 0.5 μM for the enzyme assays, 4 μM for fluorescence measurements or 11 μM for mass spectrometry experiments. For time-dependent experiments, aliquots of the incubation mixtures were assayed for residual activity by adding 50 or 500 mM L-serine, 300 μM NADH and 60 U/mL lactate dehydrogenase (LDH).

2.5. Activity assays

Activity assays for L-serine β-elimination [3,18] were carried out in a solution containing 50 mM TEA, 2 mM ATP, 500 mM L-Ser, 50 μM PLP, 2 mM $MgCl_2$, 150 mM NaCl, 60 U/mL lactate dehydrogenase and 300 μM NADH, at pH 8.0. The concentrations of L-serine or ATP were modified in some experiments, as specified. For the determination of the enzyme parameters, the reaction was triggered by addition of hSR at a final concentration of 0.3–0.5 μM. All reactions were carried out at 37 °C. GSNO was maintained in the assay mixtures at the same concentration as in the incubation mixtures to avoid dilution. Lactate dehydrogenase (LDH), used as the coupled enzyme, was preliminary shown not to be affected by GSNO. For long incubations, a control sample of enzyme in the absence of GSNO was periodically tested for stability and used for correction, when needed.

2.6. Fluorescence measurements

hSR fluorescence spectra in the absence and presence of ligands were collected using a FluoroMax-3 fluorometer (HORIBA-Jobin Yvon), thermostated at 20.0 ± 0.5 °C, in a solution containing 4 μM hSR, 50 mM TEA, 150 mM NaCl, 10 mM $MgCl_2$, pH 8.0. The fluorescence of Trp residues was selectively excited at 298 nm. The fluorescence of the pyridoxal 5′-phosphate was excited at 445 nm, to avoid any inner filter effect from GSNO, which has an absorption peak centred at about 334 nm. Slits were set for optimal signal-to-noise ratio.

2.7. Identification of nitrosylated cysteines by mass spectrometry

Cysteine residues forming stable S-nitroso adducts upon incubation with GSNO were identified using a differential alkylation-based strategy [35] coupled with mass spectrometry. Purified hSR was first reacted with GSNO at 500 μM concentration for 6 h, and then with 170 mM methyl methanethiosulfonate (MMTS) for 30 min at 50 °C under vigorous stirring to block non-nitrosylated cysteine residues. The protein was precipitated by addition of five volumes of cold acetone followed by centrifugation. The pellet was extensively washed with cold acetone to remove residual MMTS and then resuspended in a solution containing 50 mM TEA, 2 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS), pH 8.0. To reduce nitrosylated cysteines and to label nascent thiols, sodium ascorbate and iodoacetamide were added to final concentrations of 1.6 mM and 85 mM, respectively. The control sample underwent the same procedure with the exception of GSNO incubation.

For peptide digestion after conjugation with MMTS and iodoacetamide, 5 μg of protein samples were run in a 12% SDS-PAGE gel in the absence of reducing agents. The gel was extensively washed with water and stained with colloidal Coomassie stain (Bio-Rad, CA, USA). The bands corresponding to hSR were excised, incubated with a solution containing 50% ethanol and 10% acetic acid until fully destained, washed twice with a buffered solution containing 25 mM ammonium bicarbonate and pure acetonitrile (ACN) 1:1 for 20 min and finally incubated with pure ACN for 5 min to reach complete dehydration. After removal of ACN, a solution containing trypsin in a 25 mM ammonium bicarbonate solution, pH 7.4, was added for gel rehydration. In-gel digestion was performed at 37 °C for 16 h. The reaction of trypsin was stopped by addition of ACN: 0.1% trifluoroacetic acid (TFA) 1:1. Peptides were extracted by incubating the gel fragment with ACN: 0.1% TFA 1:1 twice for 20 min at 37 °C, before complete drying using a vacuum concentrator and resuspension with ACN:TFA 0.1% 1:1 before mass spectrometry experiments.

Mass spectrometry on digested peptides was carried out using either a 4800 Plus MALDI TOF/TOF (AbSciex) or an LTQ Orbitrap (Thermo Fisher Scientific) mass spectrometer. In MALDI experiments, the peptide mixture were analysed using the dried droplet method. Briefly, 1 μL of 10 mg/mL α-cyano-4-hydroxycinnamic acid (HCCA) in ACN:TFA 0.1% 1:1 was mixed with 1 μL of sample and spotted onto a MALDI plate. Spectra were acquired in m/z 500–4000 range mediating 500 laser shots for each spectrum. In the LTQ Orbitrap experiments, the peptide mixture was separated in a Phenomenex Aeris™ PEPTIDE 3.6 μm XB-C18 (150 mm × 2.1 mm) reverse-phase column, developed in a 0.2% formic acid/water-0.2% formic acid/acetonitrile gradient (200 μL/min). Peptide identification from LTQ-Orbitrap experiments was carried out using the software PEAKS Studio (version 8.5, Bioinformatics Solutions, Waterloo, Canada), set to a precursor mass tolerance of 10 ppm, a fragment mass error tolerance of 0.1 Da and a −10lgP of 25. For the recognition of nitrosylation sites, at least three independent experiments were carried out.