Properties of the *Escherichia coli* rhodanese-like protein SseA: contribution of the active-site residue Ser240 to sulfur donor recognition

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Abstract The product of *Escherichia coli sseA* gene (SseA) was the subject of the present investigation aimed to provide a tool for functional classification of the bacterial proteins of the rhodanese family. *E. coli* SseA contains the motif CGSGVTA around the catalytic cysteine (Cys238). In eukaryotic sulfurtransferases this motif discriminates for 3-mercaptopyruvate:cyanide sulfurtransferase over thiosulfate:cyanide sulfurtransferases (rhodanese). The biochemical characterization of *E. coli* SseA allowed the identification of the first prokaryotic protein with a preference for 3-mercaptopyruvate as donor substrate. Replacement of Ser240 with Ala showed that the presence of a hydrophobic residue did not affect the binding of 3-mercaptopyruvate, but strongly prevented thiosulfate binding. On the contrary, substitution of Ser240 with an ionizable residue (Lys) increased the affinity for thiosulfate.

Key words: Rhodanese-like protein; Substrate selectivity; Structural requirement; *Escherichia coli*

1. Introduction

Sulfurtransferases (EC 2.8.1.x) are a class of enzymes widely distributed among many species that catalyze the transfer of a sulfane sulfur atom from a donor molecule to a thiophilic acceptor substrate [1,2]. Bovine rhodanese (thiosulfate:cyanide sulfurtransferase (TST)) (EC 2.8.1.1) is the best characterized enzyme [3–5], and it represents the reference structure for developing patterns for the rhodanese family. Sequencing of several bacterial genomes has allowed the identification of many genes encoding proteins containing conserved regions referred to as 'rhodanese signatures', therefore named 'rhodanese-related sulfurtransferases'. These signatures are related to the two domains of the bovine rhodanese: the non-catalytic, N-terminus domain, and the C-terminus domain of the enzyme which contains the active-site cysteine residue. The rhodanese domain is observed in a single arrangement, coupled to differently structured domains, or in a tandem, in putative sulfurtransferases. The *Escherichia coli sseA* gene [6] is considered as a reference for '*sseA* Cluster of Orthologous Groups' (COG 2897, http://www.ncbi.nlm.nih.gov/COG) containing genes encoding proteins (to date 19) with both 'rhodanese signatures'. In spite of the wide presence of gene products referred to as putative sulfurtransferases, the knowledge on structure/function linkage of these prokaryotic proteins is still very limited. A structural feature common to the eukaryotic sulfurtransferases, for which the catalytic behavior in the presence of thiosulfate or 3-mercaptopyruvate was demonstrated [7–10], is an active-site cysteine residue that promotes the formation of a persulfide intermediate during the catalytic cycle [1]. The conserved Cys is surrounded by polar and apolar residues that are deemed determinant for substrate specificity [3,4,8], the stretch around the catalytic cysteine is CRKGVTA for TSTs (rhodaneses) and CGS(T)GVTA for 3-mercaptopyruvate:cyanide sulfurtransferases (MSTs), including the recently characterized plant MSTs [11–13]. None of the prokaryotic putative sulfurtransferases contains the sequence stretch CRKGVTA, and *Bacillus halodurans* BH1708, *Deinococcus radiodurans* DR2531, *Vibrio cholerae* VCA620, *Pseudomonas aeruginosa* PA1292, and *E. coli* SseA are the only proteins containing the stretch CGSGVTA.

The characterization at the molecular and biochemical levels of *E. coli* SseA allowed the identification of the first prokaryotic enzyme with preference for 3-mercaptopyruvate over thiosulfate as sulfur donor. With the aim of exploring the role of residues surrounding the catalytic cysteine in sulfur donor selectivity for sulfurtransferase reaction, site-specific mutants at Ser240 (Ser240Ala and Ser240Lys SseAs) were produced, and functionally characterized.

2. Materials and methods

2.1. Cloning of sseA

The *sseA* gene (accession number: P31142) was amplified via PCR from the *E. coli* strain 71-18 genomic DNA by using MD1 and MD2 primers (Table 1) containing respectively a *Nde*I and an *Eco*RI restriction site. The 875 bp PCR product obtained was cloned into expression vector pET28a (Novagen), downstream the coding region for a six histidine tag, and sequenced to check fidelity of the PCR reaction. The resulting plasmid, containing the *sseA* open reading frame, was named pPW9710.

2.2. Construction of sseA mutants

Site-directed substitution of codon TCT (Ser240) with codon GCG (Ala) and codon AAG (Lys) was achieved in two steps. An *Ava*I restriction site was initially introduced by silent substitution of codon TCT with codon TCG. Plasmid pPW9710 which contains the *sseA* gene was used as template in the PCR reactions. Sequence of the primers used in the PCR reaction is shown in Table 1. The 958 bp region of the gene upstream the TCT codon for Ser was amplified by using the forward primer G02 and the mutagenic reverse primer G04 which introduces the *Ava*I site. The rest of the gene (173 bp) was amplified by using a reverse primer G05 and the forward mutagenic primer G01 which contains the *Ava*I site. The PCR products obtained

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were digested respectively with *Nco*I/*Ava*I and *Eco*RI/*Ava*I and inserted in pET28a, thus giving rise to the full length wild-type *sseA* gene which contained the *Ava*I site (plasmid named pMG1). Fidelity of PCR and cloning step were checked by DNA sequencing.

Substitution of codon TCG with codon GCG and AAG was obtained in a second step by using the 'QuickChange™ Site-Directed Mutagenesis kit' (Stratagene). Plasmid pMG1 was used as template with the mutagenic primers M08 and M05 for introduction of AAG codon and G09 and G07 for the introduction of GCG codon. After transformation, mutant plasmids were identified by restriction analysis with *Ava*I. The presence of mutation and accuracy of PCR were checked by DNA sequencing. The mutated plasmids were named pMG2 and pMG3 for Ser240Lys and Ser240Ala, respectively.

2.3. Overexpression and purification of the His-tagged proteins

Overexpression of wild-type, Ser240Lys and Ser240Ala SseAs was obtained by addition of 1 mM isopropyl-thio-β-D-galactoside to 500 ml BL21(DE3) mid-log cultures ($OD_{600} = 0.600$) containing pPW9710, pMG2 and pMG3, respectively. After 4 h induction cells were harvested by centrifugation, and resuspended in 50 mM Tris–HCl buffer (pH 8.0) containing 0.3 M NaCl. Cell disruption was carried out by incubation with 0.3 mg/ml lysozyme and sonication. SseAs were purified by chromatography on a Ni-NTA agarose column (gel volume, 8 ml). Homogeneous His-tagged proteins were eluted by addition of 60 mM imidazole, for activity assays and spectroscopic measurements they were dialyzed against 50 mM Tris–HCl buffer (pH 7.6).

2.4. Enzyme assays

The discontinuous method that quantitates the product thiocyanate, based on the absorption of the ferric–thiocyanate complex at 460 nm, was used to determine either TST (rhodanese) [14] or MST [15] activities. The assays lasted 1 or 2 min, and one unit of enzyme is defined as the amount of enzyme that produces 1 μmol thiocyanate/min at 37°C. The protein concentration was determined by dye binding colorimetric assay [16].

2.5. Spectroscopic measurements

Fluorescence measurements were performed using an LS50 luminescence spectrometer (Perkin Elmer, UK) equipped with a thermostated (20°C) stirred cell holder. The excitation wavelength was 280 nm in all the fluorescence experiments, slit widths for excitation and emission were 5 and 3 nm, respectively. Emission spectra were scanned from 300 to 400 nm 1 min after reagent addition, and the samples were continually stirred. In the titration experiments the fluorescence intensities observed at 336 nm (F_{obs}) are presented as $\Delta F(\%)$ defined as:

$$\Delta F(\%) = -\frac{F_{obs} - F_0}{F_0} \times 100$$

where F_0 is the original fluorescence intensity of SseAs.

The experimental data of the titration curves of SseA and its mutated forms with either 3-mercaptopyruvate or thiosulfate were fitted using the following cooperative binding equation:

$$\Delta F(\%) = \frac{\Delta F_{sat} \times L^n}{K_d + L^n} \quad (1)$$

Table 1
Sequences of primers used in this study

Primer	Sequence
MD1	5′-TGGAGATGCaTATGTCCAAC-3′
MD2	5′-CCgAATTCGGCGGTTTTTTATTGC-3′
G02	5′-CCAGCAACCGCACCTGTGGCG-3′
G04	5′-GCCGTTACACC**c**GAGCCGCAGC-3′
G01	5′-GCTGCGGCTC**g**GGTGTAACGG-3′
G05	5′-CTCGAATTCGGCGGTTTTTTATTGCC-3′
M08	5′-CGTCAGCTGCGGC**aa**GGGTGTAACGGC-3′
M05	5′-GCCGTTACACCC**tt**GCCGCAGCTGACG-3′
G09	5′-CGTCAGCTGCGGC**gc**GGGTGTAACGGC-3′
G07	5′-GCCGTTACACCC**gc**GCCGCAGCTGACG-3′

The nucleotides changed for introducing restriction sites are shown in lowercase, those for amino acid substitutions in bold-typed lowercase.

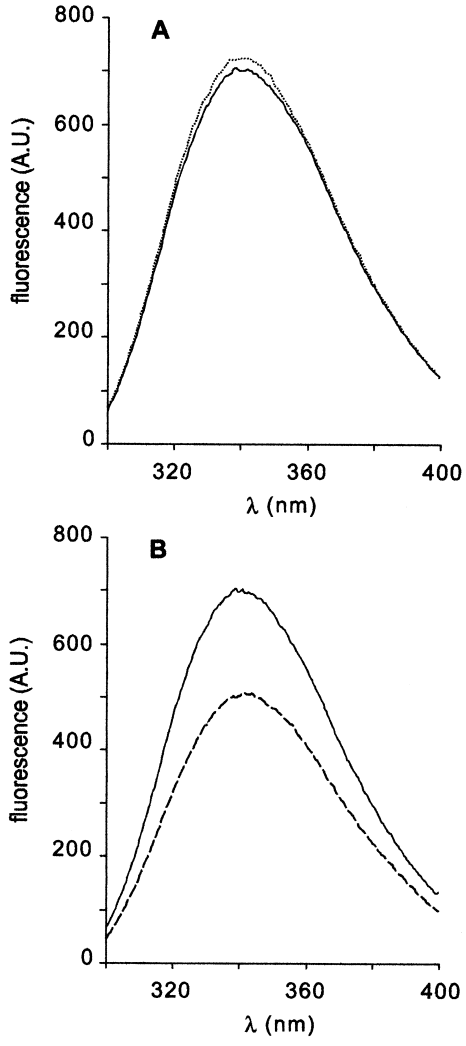

Fig. 1. Fluorescence spectra of SseA. (A) Fluorescence spectra of 6 μM purified SseA in 50 mM Tris–HCl buffer, pH 7.6 (full line), and after addition of 2-fold molar excess of KCN (dotted line). (B) Fluorescence spectra of 6 μM purified SseA in 50 mM Tris–HCl buffer, pH 7.6 (full lines), and after addition of 2-fold molar excess of 3-mercaptopyruvate (dashed lines). The observed fluorescence intensities were corrected for dilutions due to KCN or 3-mercaptopyruvate addition. A.U., fluorescence arbitrary units.

where L was the titrant concentration, and ΔF_{sat} was the ΔF at saturating titrant concentration.

Far UV circular dichroism (CD) spectra were recorded at 25°C in a Jasco (Great Dunmow, UK) J-810 spectropolarimeter at a scan rate of 5 nm/min. Data were collected at 0.1 nm intervals in 0.01 cm quartz cells, and the spectra were corrected for the buffer baseline. Molar ellipticity values [Θ] are expressed in ° cm^2 $dmol^{-1}$.

3. Results

3.1. Sulfur donor selectivity of E. coli SseA

The overexpressed SseA protein was purified from the extract in one fast chromatography step taking advantage of the inserted histidine tag. Activity measurements in the presence of either 3-mercaptopyruvate or thiosulfate (Table 2, wild-type) clearly indicated that 3-mercaptopyruvate was the best donor substrate for this prokaryotic sulfurtransferase for which MST activity was 130-fold higher than TST activity.

In rhodanese a key event in sulfur transfer catalysis [1,2] is

Table 2
Sulfurtransferase activities of the purified SseAs in the presence of either 3-mercaptopyruvate (A, MST) or thiosulfate (B, TST) as sulfur donor

SseA protein	k_{cat} (min^{-1})		
	A	B	Ratio MST/TST
Wild-type	27 800 ± 100	210 ± 10	130
Ser240Lys	2 900 ± 100	120 ± 20	24
Ser240Ala	6 500 ± 100	40 ± 5	160

the formation of the covalent enzyme–sulfur intermediate, which can be detected by measuring the intrinsic fluorescence quenching of the enzyme due to energy transfer between the persulfide and the initially excited tryptophan residues [17,18]. The chemical mechanism of MST catalysis is, on the other hand, not well known because the mammalian enzyme is unstable and rather difficult to purify highly, but the formation of a persulfide at the catalytic cysteine is considered an accepted intermediate step also for MST [19,20]. The fluorescence spectrum of purified wild-type SseA did not change following the addition of cyanide (Fig. 1A), the nucleophilic acceptor that could remove the sulfane sulfur in the case of its presence on the catalytic cysteine forming a persulfide. This spectroscopic evidence was in agreement with the finding that the single sulfhydryl group of *E. coli* SseA (Cys238) was accessible to 5,5′-dithio-bis(2-nitrobenzoic acid) titration (data not shown). The original intrinsic fluorescence was quenched by addition of 3-mercaptopyruvate, and no further quenching was detected when the substrate concentration reached the value of 3-mercaptopyruvate/enzyme = 1/1 mol/mol (Fig. 1B).

3.2. Mutagenic analysis of E. coli SseA

Ser240 was the target of the mutagenic analysis here described, since the amino acid at position +2 with respect to the catalytic cysteine (Cys238 in SseA) was found to clearly discriminate sulfur donor selectivity in vertebrate sulfurtransferases [8–10,19]. The designed substitutions were Ser240Lys and Ser240Ala, and substitution of Ser240 with either Lys or Ala did not significantly alter the SseA fold, as assessed by far UV CD measurements (data not shown).

The determined k_{cat} figures (Table 2) indicated that MST activity of both SseA mutant forms significantly decreased, as compared to wild-type, and no increase of TST activity was detected. The MST/TST ratio of Ser240Lys SseA (Table 2, third column) was significantly lower than that of both wild-type and Ser240Ala SseAs, thus suggesting that substitution of Ser240 with Lys could selectively affect the affinity for thiosulfate. The catalytic efficiency (k_{cat}/K_m) is the parameter generally used to assess the catalytic power of enzymes, including sulfurtransferases and their mutated forms [9–13]. Since, in our opinion, there are a number of problems associated with an accurate calculation of K_m for the donor substrates by the colorimetric assay currently in use for sulfurtransferase enzymes [14,15], the fluorescence approach that allowed the detection of persulfide-containing SseA (Fig. 1B) was chosen to investigate whether the designed mutations could modulate the substrate binding affinity of SseA.

Table 3
Ligand binding properties of wild-type and mutant forms of SseA

Sulfur donor	K_d (μM)		
	Wild-type	Ser240Lys	Ser240Ala
3-Mercaptopyruvate	5 ± 0.2	16 ± 0.8	18 ± 0.8
Thiosulfate	256 ± 20	34 ± 3	not determinable

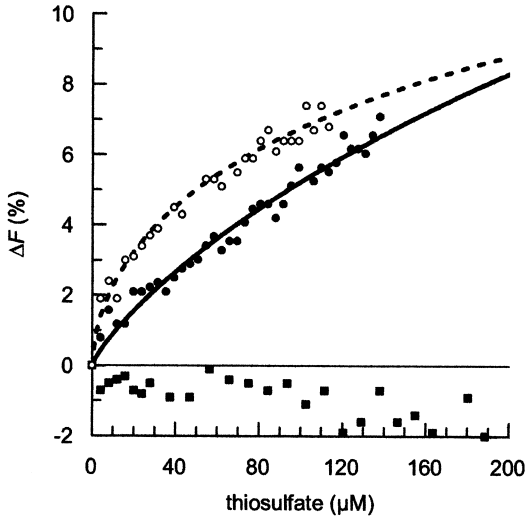

Fig. 2. Fluorescence changes of wild-type (filled circles), Ser240Lys (empty circles), and Ser240Ala (filled squares) SseAs as a function of thiosulfate concentration. Proteins were 6 μM in 50 mM Tris–HCl pH 7.6, further experimental details are in Section 2. The titration curves of wild-type (full line) and Ser240Lys (dotted line) SseAs represent the best fit obtained from experimental data using the binding Eq. 1.

The ability of different sulfur donors to generate the sulfur-substituted form of SseA was, however, monitored by quantitating the intrinsic fluorescence changes as a function of stoichiometric addition of the donor substrates (3-mercaptopyruvate or thiosulfate). The fluorescence spectra of both Ser240Lys and Ser240Ala SseAs recorded before and after cyanide addition were identical (not shown), thus providing evidence that no sulfur-substituted form was present even in the mutated SseAs. Sequential addition of either 3-mercaptopyruvate or thiosulfate caused intrinsic fluorescence quenching, but did not affect the shape of the spectra that were recorded until no further fluorescence quenching was detected. The experimental data were analyzed by plotting the decrease of fluorescence versus the concentration of the titrant (X axis), and the data points were fitted to the binding model Eq. 1, resulting in different binding curves. The curves with thiosulfate as titrant are shown as example in Fig. 2, and the K_d figures obtained in the presence of both the potential sulfur donors for all the SseAs are listed in Table 3. In these analyses the number of binding sites per molecule of enzyme (n) resulted to range from 0.6 to 1.4. Only the results obtained by titrating Ser240Ala SseA with thiosulfate (Fig. 2, filled squares) did not allow binding analysis. The high MST and low TST activities of wild-type SseA are in agreement with the calculated K_d values for 3-mercaptopyruvate and thiosulfate, thus indicating that formation of the sulfur-substituted enzyme intermediate is the rate-limiting step for catalysis. The ability of 3-mercaptopyruvate to act as sulfur donor of SseA decreased when Ser240 was mutated, but comparable K_d figures were found for both Ser240Lys and Ser240Ala SseAs. The selected substitutions (Ser240Lys and Ser240Ala) resulted much more discriminating for thiosulfate binding: thiosulfate

binding was, indeed, completely abolished when the hydrophobic amino acid Ala replaced Ser, in agreement with the results of bovine rhodanese [8]. Replacement of Ser240 with the ionizable amino acid Lys clearly favored thiosulfate binding, showing a K_d of the mutant Ser240Lys seven times lower than that of wild-type SseA. Taken together, these findings indicated that both for the prokaryotic *E. coli* SseA and for vertebrate sulfurtransferases [8–10] cationic side chains are crucial for thiosulfate binding, and less influent for 3-mercaptopyruvate binding.

4. Discussion

The characterization at the molecular and biochemical levels of the product of *sseA* gene of *E. coli* allowed the identification of the first prokaryotic protein of the rhodanese-like family, with a preference for 3-mercaptopyruvate over thiosulfate as a substrate. Even if MST activity was found widely distributed in prokaryotes and eukaryotes [21–23], the only MST enzymes for which molecular characterization is available are that from rat liver [9,10], and the isozymes from *Arabidopsis thaliana* [11–13]. The phylogenetic relation between eukaryotic TSTs and MSTs was claimed [9], and the sequence stretch around the catalytic cysteine is considered a key motif for the preference of 3-mercaptopyruvate or thiosulfate as sulfur donor substrates [9,10,19]. Classification of the prokaryotic proteins as TST or MST on the basis of the above criterion is rather difficult, since even for the few enzymes biochemically characterized (GlpE, RhdA) the sequence stretch around the conserved catalytic cysteine is heterogeneous [24–26]. The structural similarity (i.e. the presence of rhodanese domain in different organization) for which an increasing number of gene products are classified in 'family Rhodanese' (accession number: PF00581, http://www.sanger.ac.uk/Software/Pfam/index.shtml) is not sufficient to infer functional similarity, as revealed by the studies on *Azotobacter vinelandii* RhdA [25,26]. SseA, on the other hand, represents the reference protein of COG 2897 and it is one of the few prokaryotic proteins containing the motif CGSGVTA that in eukaryotic sulfurtransferases [9–13,19] discriminates for MSTs over TSTs. The preference for 3-mercaptopyruvate over thiosulfate as donor substrate of *E. coli* SseA corroborates the critical role of the amino acid residues just behind the catalytic cysteine for substrate selection. Interestingly, none of the prokaryotic proteins listed in the Rhodanese family contains the typical rhodanese module CRKGVTA. The present study proved that in SseA a sulfur-substituted intermediate can be formed with either 3-mercaptopyruvate or thiosulfate as sulfur donors, but interaction with thiosulfate was not favored. The pioneering work of Luo and Horowitz [8] identified the lysine residue (at the position +2 with respect to the catalytic cysteine) as key amino acid for binding and catalysis of thiosulfate in bovine rhodanese. The mutagenic analysis of Ser240 of SseA provided clear evidence that the presence of a hydrophobic residue (Ala) did not affect the binding of 3-mercaptopyruvate, but strongly prevented thiosulfate binding. On the contrary, the presence of a positively charged residue (Lys) significantly increased the affinity for thiosulfate. As already suggested for rat MST [10,19], the interaction with 3-mercaptopyruvate should be favored by other ionizable residues likely close to the catalytic cysteine in the three-dimensional (3D) structure of the catalytic pocket, and the conserved Arg residues (Arg187 and Arg196 in rat MST, Arg178 and Arg187 in SseA) could be good candidates for interacting with the carbonyl group of 3-mercaptopyruvate. Since no 3D structure of MSTs is to date available, the mechanism of 3-mercaptopyruvate binding and catalysis remains speculative. The preference for 3-mercaptopyruvate over thiosulfate as sulfur donor, inferred by the in vitro activity behaviors of SseA, and the findings obtained by mutagenic analysis of Ser240, confirmed that the sequence CGSGVTA can be taken as discriminative motif for MST also in prokaryotic enzymes. The physiological function(s) of MSTs and TSTs in organisms is still a matter of debate, and, although SseA has now been identified as MST, its physiological function and possible association with sulfur metabolism in *E. coli* remain to be elucidated.

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