Assembly stoichiometry of bacterial selenocysteine synthase and SelC (tRNA\textsuperscript{sec})

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1. Introduction

Selenocysteine (Sec – U) is incorporated into selenoproteins and is present in the three different domains of life: Bacteria, Archaea and Eukarya [1]. Sec is encoded by a UGA codon associated to an mRNA-specific structure named SElenoCysteine Insertion Sequence (SECIS), and is dependent of a complex biosynthesis and incorporation pathway [2,3] which involves a specific tRNA (tRNA\textsuperscript{sec} or SelC). In prokaryotes Seryl–tRNA\textsuperscript{sec} is converted to Selenocysteyl–tRNA\textsuperscript{sec} by the homodecameric enzyme Selenocysteine Synthase (SelA, EC 2.9.1.1) [3], a pyridoxal 5\textsuperscript{0}-phosphate (PLP) dependent protein [4,5].

The existence of a SelA homolog in Archaea was demonstrated but no homolog is observed in the Eukarya domain. No apparent biological function was found for the putative SelA from the Archaea Methanococcus jannaschii, which exhibit 30% amino acid sequence identity with Escherichia coli SelA [6]. Moreover the putative Archaea SelA shows a dimeric – rather than the bacterial decameric structure.

The PLP cofactor is covalently linked to E. coli SelA through the lysine residue (K295) [4] and participates in the Ser-Sec conversion reaction in two steps. The first step is the formation of a Schiff’s base between the \(\alpha\)-amino group of the serine residue with the formyl group of PLP, resulting in the dehydration of the residue and the formation of the intermediate Aminoacrylyl-tRNA\textsuperscript{sec} [3]. Selenium is then transferred to the Aminoacrylyl-tRNA\textsuperscript{sec} intermediate from selenophosphate (a product of the selenophosphate synthase enzyme, SelD) resulting in the formation of selenocysteyl–tRNA\textsuperscript{sec} [7–9].

Early gel permeation experiments indicated that one tRNA\textsuperscript{sec} molecule binds to a SelA dimer, resulting in five tRNA\textsuperscript{sec} molecules bound to the SelA decamer [3]. Those samples were then investigated by electron microscopy using negative stain techniques and the stoichiometric composition was determined by scanning transmission electron microscopic mass determination indicating a structure for the SelA–tRNA\textsuperscript{sec} complex with one tRNA\textsuperscript{sec} bound per SelA dimer [10]. Cryo-EM experiments [11] of Moorella thermoacetica SelA confirmed the fivefold symmetry and the dimensions of the SelA protein.

In the present communication an optimized purification protocol for the E. coli SelA allowed the characterization of its oligomeric state in solution by fluorescence anisotropy and electron micros-
copy symmetry-analysis techniques. Our results show clearly that the binary SelA–tRNA\textsuperscript{sec} complex exhibits a molecular ratio of 1:1 rather than a previously proposed 2:1 ratio.

Our new data is of fundamental importance since it corrects the established stoichiometry of five tRNA\textsuperscript{sec} molecules per SelA homodecamer which, in turn, lead to an incorrect assessment of the complex's structure and to an incorrect interpretation of the molecular binding selectivity of the SelA–tRNA\textsuperscript{sec} binary complex. Future functional and structural investigations of the SelA–tRNA\textsuperscript{sec} complex would make no sense without taking the correct number of tRNAs into account.

2. Materials and methods

2.1. Optimization of the production of E. coli SelA protein

To overproduce SelA protein, *Escherichia coli* WL81460 (λ DE3) cells expressing the recombinant SelA gene were induced aerobi-cally in LB medium, as described [12]. In the first desalted fraction, Nycodenz\textsuperscript{®} reagent solution was added to a final concentration of 20% and the mixture was concentrated using Amicon 50000 MW concentrator. In the next step, the sample was submitted to a Superdex 200 HL column (1.6 \times 60 cm size) (GE) equilibrated with potassium phosphate buffer (20 mM, pH 7.5) without PLP and the protein fractions were concentrated using Amicon 100000 MW concentrator. SelA protein concentration was expressed as the total amount of monomeric subunits.

2.2. In vitro transcription of tRNAs

Each gene encoding *E. coli selC* and tRNA\textsuperscript{ser} and *Trypanosoma brucei selC*, were initially amplified by PCR [S1] using primers with 20 bases overlap [S2]. The PCR products purified from 2% agarose gel using Perfect Gel Cleanup kit (Eppendorf) were used in in vitro transcription reaction templates (MEGAscript kit, Ambion, Austin, TX, USA).

2.3. RNA synthesis and fluorescein labeling

For titration assays, tRNA\textsuperscript{sec} from *E. coli* and *T. brucei*, tRNA\textsuperscript{ser} from *E. coli* and a 79 base single-stranded desoxiribonucleotide from part of a *M. jannaschii* gene were 5' end labeled with fluorescein maleimide, using the kit 5’ EndTag\textsuperscript{TM} Nucleic Acid Labeling System (Vector Laboratories, Burlingame, CA, USA). After labeling, the tRNAs or single-stranded DNA (ssDNA) were heated to 80 °C and slowly cooled to 35 °C with the addition of MgCl\textsubscript{2} (20 mM). SelA fluorescein labeling (16 h at 10 °C) was performed with 3 μM SelA and 25 mM fluorescein isothiocyanate – FITC (Invitrogen) followed by a HiTrap (GE) desalting chromatography.

2.4. Fluorescence anisotropy binding assay of SelA–tRNA\textsuperscript{sec} and SelA–nucleic acids interactions

The fluorescence measurements were performed in an ISS-PC spectrofluorimeter (ISS, Champaign, IL, USA). Protein labeling efficiency was calculated as shown in Eq. (1):

$$\text{FITC/SELA (mol/mol)} = (\frac{\text{Abs}_{\text{FITC494nm}}}{\frac{\text{E}_{\text{FITC494nm}}}{\text{E}_{\text{FITC494nm}}}}) / (\frac{\text{Abs}_{\text{Fm280nm}}}{\frac{\text{E}_{\text{Fm280nm}}}{\text{E}_{\text{Fm280nm}}}})$$

using 65000 M\textsuperscript{−1} cm\textsuperscript{−1} and 35 785 M\textsuperscript{−1} cm\textsuperscript{−1} as the extinction coefficient (E) of protein-bound FITC at SelA absorbance (Abs) at 494 nm and at 280 nm, respectively [13]. Fluorescence anisotropy assay of SelA–SelA monomers binding was performed by measurements in “L” geometry, in 20 mM potassium phosphate buffer, at 25 °C. Aliquots of an unlabeled SelA were added to 30 nM fluorescein-labeled SelA for steady-state anisotropy measurements with excitation set to 480 nm and emission recorded through a 515 nm cut-off filter. Anisotropy values and total fluorescence were calculated by the ISS program. In all cases maximal dilution was less than 20%.

Fluorescence anisotropy binding assays of tRNAs or ssDNA to SelA were performed as described previously [13]. Briefly, aliquots of concentrated SelA (400 μM) were sequentially added to 10 nM of fluorescein-labeled tRNAs or ssDNA. SelA–tRNA\textsuperscript{sec} stoichiometry binding assays were performed using 40 μM unlabeled and 10 nM fluorescein-labeled tRNA\textsuperscript{sec} and aliquots of SelA protein were added as described above. For inverse stoichiometry titration assay, 10 μM unlabeled and 30 nM fluorescein-labeled SelA was titrated with tRNA\textsuperscript{sec}. The mixtures were homogenized and equilibrated for 3 min at 25 °C prior to anisotropy measurements. The fitting of the titration curves was performed as described in Ref. [14].

2.5. Transmission electron microscopy

For SelA–tRNA\textsuperscript{sec} complex visualization by negative stain, SelA samples (0.5 mg/ml) in potassium phosphate buffer (20 mM, pH
7.5) were incubated with tRNA^sec in the same buffer for 30 min at 25 °C at a molecular ratio of 10 SelA monomers for 12 tRNA^sec molecules. Holey carbon-coated grids were glow discharged for 25 s at 15 mA using an easiGlow system (PELCO). A 3 μl sample was deposited onto the grid for 30 s, followed by two washing steps (HEPES 10 mM, pH 7.5), staining with 3 μl of 2% uranyl acetate (30 s), blotting and air-drying. Images were recorded at −3 μm defocus at 60000× magnification using a Jeol JEM-2100 operating at 200 kV. For eigenimage analysis, micrographs were recorded on Kodak SO-163 film and digitalized using an ArtixScan F1 (Microtek) scanner. SelA "stack" images were taken using a MultiScan 794 MSC (Gatan). Image analysis was performed using the IMAGIC 4D software package [15]. A total of 7582 particles were picked using a semi-automatic approach, filtered, normalized, centered and subjected to multivariate statistical analysis (MSA) [16,17] to obtain the eigenimages of the dataset and classified to obtain the different characteristic molecular views. The dataset was split in two groups: "top" views (7388 particles) and "side" views (194 particles). "Top" views were rotationally aligned to the second eigenimage of the full dataset and subjected to MSA. The results were classified in 700 classes using the three first eigenimages of "top" views dataset, generating class averages that show a distribution between unbound SelA and tRNA^sec bound states.

3. Results

3.1. SelA supramolecular assembly

Solution binding isotherms using fluorescence anisotropy spectroscopy were performed to investigate the SelA assembly process in the absence of tRNA^sec. Fluorescein-labeled SelA (30 nM) was titrated with increasing amount of non-labeled SelA and the fluorescence anisotropy was monitored. Representative data of binding isotherms are shown in Fig. 1a. At low SelA concentration, the SelA fluorescein anisotropy is of about 40 mA. Increasing protein concentration results in accompanying increase in fluorescein anisotropy from the conjugated SelA. No clear convergence for a saturating plateau at SelA concentrations up to 10 μM was observed. Further increase in protein concentration was not possible due to technical limitations. Negative stain images of SelA samples

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Fig. 2. Binding of SelA to tRNA^sec and tRNA^ser from *E. coli*, tRNA^sec from *T. brucei* and a non-specific DNA. (a) Binding isotherms for tRNA^sec* (○) and tRNA^ser* from *E. coli* (○), tRNA^sec* from *T. brucei* (▲) and non-specific DNA (△) in 20 mM of potassium phosphate buffer, pH 7.5. Inset: log scale. (b) tRNA^sec* from *E. coli* binding isotherms with 20 mM of potassium phosphate buffer, pH 7.5 (○), 100 mM of NaCl and 20 mM of MgCl2 (●), 300 mM of NaCl and 50 mM of MgCl2 (▲). Inset: log scale. (c) tRNA^ser* from *E. coli* binding isotherms in same conditions as for tRNA^sec*. Inset: log scale.
(10 μM monomer concentration) reveal a polydisperse system with the putative decamers, often associated into supramolecular stacks or side-by-side assemblies (Fig. 1b). This observation supports the fluorescence anisotropy isotherms interpretation of fluorescein-labeled SelA, as a combined process of formation of decamers followed by their association into larger supramolecular assemblies of decamers.

3.2. Specificity of SelA–tRNAs interaction

In order to investigate the mechanisms of SelA binding to tRNAs, we performed fluorescence anisotropy binding assays with SelA and fluorescein-labeled tRNAs. E. coli tRNA^sec^ and tRNA^ser^, T. brucei (a Kynetoplastidae eukaryote) tRNA^sec^ (Fig. S2) and a 79 nucleotide long single-stranded DNA (ssDNA, as control) at 10 nM concentration were titrated with increasing amounts of unlabeled SelA in separate experiments. The fluorescence anisotropy increased progressively as a function of SelA concentration, for all tRNAs tested (Fig. 2a). Both E. coli tRNA^sec^ and tRNA^ser^ show no apparent saturation in the binding curves up to 18 μM (Fig. 2b and c, respectively) and the SelA–tRNA binding was not affected by the solution ionic strength (Na^+^ and Mg^2+^).

The binding isotherms of SelA to tRNA^sec^ from both E. coli and T. brucei also displayed non-saturating binding curves up to 18 μM of SelA. In contrast to what was observed for SelA–tRNA^sec^, the binding curve for SelA–tRNA^ser^ (Fig. 2a) shows a clear saturation plateau and a K_d of about 2.5 μM. The homodecamer complex binding to the ssDNA control showed a reduced affinity up to 20 μM. These data indicate the selective nature of the SelA binding to tRNA^sec^.

3.3. Stoichiometry of SelA–tRNA^sec^ interaction

The binary complex SelA–tRNA^sec^ binding stoichiometry was determined by fluorescence anisotropy binding assays using fluorescein-labeled and unlabeled tRNA^sec^ at a higher concentration than the apparent K_d determined from Fig. 2a. A progressive increase in tRNA^sec^ fluorescence anisotropy was observed as a function of the addition of SelA (Fig. 3a). At a SelA–tRNA^sec^ ratio of 1:1 we observed a change in the tRNA^sec^ dependant anisotropy as a function of SelA concentration as shown by the inflection in Fig. 3a, indicated by a dashed line. The previously proposed ratio of 2:1 is indicated in the same figure by a dotted line. This pattern is compatible with a change in binding mechanism, most likely from a specific to a non-specific binding mode, indicating a specific interaction of 1 SelA monomer to 1 tRNA^sec^ molecule.

The reverse stoichiometric binding assay, using unlabeled and fluorescein-labeled SelA, showed high initial anisotropy values that are consistent with the existence of both the decamers and supramolecular oligomers, as observed in Fig. 1. A steep decrease in SelA anisotropy is observed as a function of increasing concentration of tRNA^sec^ (Fig. 3b), reaching a minimum value at 10 μM of tRNA^sec^ and a plateau in the fluorescein anisotropy. Upon addition of the tRNA^sec^, we believe that the SelA decamers detach from the supramolecular-stack structures, due to a higher affinity to tRNA^sec^, forming the binary complex. This assay shows a sharp inflection at a SelA–tRNA^sec^ ratio of 1:1, indicating the binding stoichiometry of 10 tRNA to a decamer of SelA. This inflection is indicated as a dashed line in Fig. 3b.

3.4. Electron Microscopy (EM) characterization of the SelA–tRNA^sec^ assembly

The binary complex stoichiometry (prepared with saturating concentrations of tRNA^sec^) was investigated by Electron Microscopy (EM) Symmetry Analysis [16]. The first ten eigenimages of the centered-particles dataset are shown in Fig. 4a. The first eigenimage (Fig. 4a, 1) is equivalent to the sum of all particles; the second and third eigenimages (Fig. 4a, 2–3) together show that the main symmetry component of the dataset is fivefold (these eigenimages relate to each other as a sine and a cosine function along circles). At the same time, these eigenimages are not just fivefold symmetric but also exhibit mirror symmetry (5 m), indicative of a projection image along the fivefold axis of a D5 pointgroup symmetry structure, rather than that of a C5 structure. The sixth eigenimage (Fig. 4a, 6), a rotationally symmetric white ring, may be related to the size variation of the molecule when the tRNAs are present. The seventh and eighth eigenimages (Fig. 4a, 7–8) are associated with the side view images of the complex, which views are relatively rare within the dataset.

Fig. 4b shows the first five eigenimages of the top-views dataset after rotationally aligning the images with respect to the second

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Fig. 3. Stoichiometric solution binding assay ofSelA to tRNA^sec^. (a) Fluorescence anisotropy binding assay. Absolute values are shown for the interaction of unlabeled (40 μM) and fluorescein maleimide-labeled (10 nM) tRNA^sec^ with the addition of SelA protein in potassium phosphate buffer, pH 7.5. , o and • are binding curves of independent experiments. SelA–tRNA^sec^ ratio of 1:1 and 2:1 are indicated by dashed and dotted lines respectively. Inset: normalized values. (b) Reverse stoichiometric binding assay. Anisotropy absolute values are shown for the interaction of unlabeled (10 μM) and fluorescein-labeled (30 nM) SelA monomer in potassium phosphate buffer (20 mM, pH 7.5) with the addition of tRNA^sec^. • and o are binding curves of independent experiments. SelA–tRNA^sec^ ratio of 1:1 is indicated by a dashed line. Inset: normalized values.
The first eigenimage (Fig. 4b, 1) represents the sum of all rotationally aligned images in the dataset and exhibits a fivefold symmetric structure [10] plus mirror symmetry, indicating the presence of an overall D5 pointgroup symmetry. Eigenimages 2 and 3 of Fig. 4b also show fivefold symmetry, and mirror symmetry, also indicating D5 group symmetry for the molecule. Eigenimage 3 (Fig. 4b) shows 10 strong intensities localized at the periphery of the decamers. This eigenimage discriminates between structures with and without the 10 tRNAsec molecules bound. The dataset is a mixture of a population of SelA–tRNAsec complexes and of SelA homodecamers. Different class-averages of these two subpopulations were obtained after classification of the top views using the three first eigenvectors shown in Fig. 4b–d shows five different class-averages for the SelA homodecamer and the binary complex respectively. Extra mass in the perimeter of the SelA–tRNAsec complex can be observed compared to the SelA homodecamer.

4. Discussion

The selC gene coding for tRNAsec (SelC) was identified as one of four genes whose products are required for the formation of selenoproteins in E. coli [18]. During selenocysteine synthesis and incorporation of the selenocysteine pathway, tRNAsec is aminoacylated with L-serine by Seryl–tRNAsec synthetase [2] and the Seryl–tRNAsec serves as substrate for selenocysteine synthase (SelA), a pyridoxal 5'-phosphate dependent enzyme that catalyzes the conversion of Seryl–tRNAsec to Selenocysteil–tRNAsec [19].

The tRNAsec molecule shows structural differences compared to other tRNAs [20] in regions previously considered invariant. These differences are the presence of 8 bp in the acceptor arm, a purine residue at position 8, a pair purine–pyrimidine at positions 11/24, pyrimidines at positions 14 and 15, and the UGA-decoding UCA anticodon [21]. In our study, we analyzed the formation of the decameric structure of SelA and its interaction with tRNAsec using the E. coli tRNAsec and T. brucei tRNAsec as control RNAs.

SelA expression in E. coli WL81460 (DE3) strain resulted in a tRNAsec-free SelA protein, that would otherwise incur in the interference in binding experiments by having endogenous tRNAsec bound to SelA [12]. This approach resulted in a significant improvement in SelA yield, purity and stability, maintaining the biophysical and biochemical characteristics of this macromolecule. SelA fluorescence anisotropy assays performed to verify the oligomerization process did not show a plateau region in the titration curve. Contrary to expectation, the fluorescence anisotropy values tend to increase without reaching a plateau. Transmission electron microscopy images of SelA showed that the decamer organizes in supramolecular structures as stacks or side by side assemblies, which explains the fluorescence anisotropy results. Since these supramolecular structures are present at low protein concentrations, the determination of SelA decamer dissociation constants by fluorescence anisotropy technique is not possible. Consequently, we inferred that the SelA decamer’s dissociation constant would be in the picomolar range, which are not detectable by fluorescence anisotropy under the conditions employed.

Fluorescence anisotropy analysis of SelA–tRNA interaction has presented interesting results. First, the variation of the buffer ionic strength did not affect the interaction of SelA with either E. coli tRNAsec or tRNAser indicating that the protein–tRNA interaction is specific and not due to ionic interaction alone. In vivo studies from
Baron and coworkers, 1990 [22] demonstrated that the tRNA<sup>sec</sup> acceptor arm length of 8 bp is important for UGA decoding and that a reduction of the variable arm length caused a decrease in Sec incorporation. According to our studies, the maintenance of base pairing of the acceptor arm proved to be essential for the proper interaction of tRNA with SelA and shortening the length of the variable arm (tRNA<sup>sec</sup> T. brucei) actually promoted a decrease in the binding affinity in relation to the specific ligand tRNA<sup>sec</sup>. The profile obtained for SelA–tRNA<sup>sec</sup> interaction suggest a different mechanism for this interaction based on the tRNA<sup>sec</sup> binding affinity with SelA. From these results we propose that tRNA<sup>sec</sup> interacts exclusively with the free SelA decamers resulting in the early stabilization of the fluorescence anisotropy values.

Our electron microscopic top-views of the binary complex show additional material in its peripheral region, compared to the SelA homodecamer. Similar results had been obtained by Engelhardt et al. [10]. Our eigenimage analysis also yielded 10 strong intensities localized at the periphery of the decamers. That extra material is thus the likely location of tRNAs in the binary complex. Since symmetry analysis yielded a D5 pointgroup symmetry, both for the SelA homodecamer, and for the binary complex, the binary complex must also have a stoichiometric ratio of 1:1 of SelA monomers and tRNA<sup>sec</sup>. Our combined data of EM symmetry analysis and direct and reverse fluorescence anisotropy titration showed a stoichiometric ratio of one SelA decamer to ten tRNA<sup>sec</sup> molecules. The results also revealed the presence of supramolecular stack-structures in the samples. A correct structural and functional interpretation of the SelA homodecamer, and for the binary complex, the binary complex must also have a stoichiometric composition. For example, crystallization attempts aiming at achieving a 2:1 stoichiometry, may be hampered due to sample heterogeneity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jfbslet.2013.02.014.

References