Formation of a Ternary Complex for Selenocysteine Biosynthesis in Bacteria*§

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Background: Selenoprotein biosynthesis requires the interaction of tRNA Sec and specific enzymes that drive the synthesis of selenocysteine.

Results: Formation of a molecular complex of selenophosphate synthetase, selenocysteine synthase, and tRNA Sec was identified and characterized.

Conclusion: The ternary complex formation is necessary for selenoprotein synthesis.

Significance: Our findings demonstrate the formation of a ternary complex and provide a possible scenario for selenium metabolism in bacteria.

The synthesis of selenoprotein-containing proteins (seleno-proteins) involves the interaction of selenocysteine synthase (SelA), tRNA (tRNA Sec), selenophosphate synthetase (SelD, SPS), a specific elongation factor (SelB), and a specific mRNA sequence known as selenocysteine insertion sequence (SECIS). Because selenium compounds are highly toxic in the cellular environment, the association of selenium with proteins throughout its metabolism is essential for cell survival. In this study, we demonstrate the interaction of SPS with the SelA-tRNA Sec complex, resulting in a 1.3-MDa ternary complex of 27.0 ± 0.5 nm in diameter and 4.02 ± 0.05 nm in height. To assemble the ternary complex, SPS undergoes a conformational change. We demonstrated that the glycine-rich N-terminal region of SPS is crucial for the SelA-tRNA Sec-PS interaction and selenoprotein biosynthesis, as revealed by functional complementation experiments. Taken together, our results provide new insights into selenoprotein biosynthesis, demonstrating for the first time the formation of the functional ternary SelA-tRNA Sec-PS complex. We propose that this complex is necessary for proper selenocysteine synthesis and may be involved in avoiding the cellular toxicity of selenium compounds.

Selenium has been recognized as an essential trace element for many life forms, although it is toxic at high levels due to its high chemical reactivity of its metabolites (1, 2). Organisms in all three domains of life (bacteria, archaea, and eukarya) synthesize selenocysteine (Sec) as the main form of organic selenium in the cells, which is incorporated into specialized proteins, known as selenoproteins, that are involved in several functions including oxidoreductions, redox signaling, and antioxidant defense (1, 3).

Sec is synthesized on the specific l-serine-aminocylated tRNA (Ser-tRNA Sec) and incorporated into selenoproteins at UGA codons via a complex pathway that works through transient protein–RNA and protein–protein interactions. In bacteria, this pathway requires the specific tRNA Sec (SelC) and an mRNA-specific structure called selenocysteine insertion sequence (SECIS) (1, 3). E. coli tRNA Sec has 8- and 5-bp stems in the acceptor and T arms, respectively, whereas the canonical tRNAs have a 7 + 5 secondary structure. The D arm of E. coli tRNA Sec has a 6-bp stem and a 4-nucleotide loop, whereas the canonical tRNAs have a 3–4-bp D stem and 7–12-nucleotide D loop. In addition, the extra arms of the bacterial tRNA Sec have 5–7-bp stems, in contrast to the 6–9-bp stem observed in E. coli tRNA Sec (4).

Sec biosynthesis is initiated by the conversion of l-seryl-tRNA Sec, aminocylated with serine by seryl-tRNA synthetase (SerRS), to l-selenocysteyl-tRNA Sec in a reaction catalyzed by selenocysteine synthase (E.C. 2.9.1.1., SelA), which is a pyridoxal 5’-phosphate (PLP)-dependent homodecameric enzyme of ~500 kDa (5). The co-factor PLP is covalently linked to the Lys295 amino acid residue in each monomer of E. coli SelA prior to Ser-Sec conversion (5). Therefore, seryl-tRNA Sec is linked to SelA in the cofactor site, resulting in a binary complex consisting of one SelA decamer:10 tRNA Sec (6). Recently, the structures of Aquifex aeolicus SelA and its binary complex SelA-tRNA Sec* have been reported.

1 The abbreviations used are: Sec, selenocysteine; SPS, selenophosphate synthetase; PLP, pyridoxal 5’-phosphate; H/Dex, hydrogen/deuterium exchange; AFM, atomic force microscopy.

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4 This article contains supplemental Tables 1A and 1B.
were resolved by x-ray crystallography, highlighting that the decameric conformation is mandatory to provide the catalytic site for binding the tRNA molecule (4).

To achieve Ser-Sec conversion, selenium is transferred to the binary complex on its biologically active form, selenophosphate, a product of the reaction catalyzed by the 72.4-kDa dimeric enzyme selenophosphate synthetase (E.C. 2.7.9.3, SelD or SPS), from selenide and ATP (8). Selenophosphate is produced in a two-step reaction, in which selenide is phosphorylated by the ATP γ-phosphate moiety and then ADP is hydrolyzed, releasing selenophosphate, AMP, and orthophosphate (8–11). Selenide originates from selenite reduction, from converted methylated selenium compounds, or through selenium removal from selenoprotein degradation (12).

Because the $K_m$ value of 20 μM for selenide in vitro results in toxic levels of this compound in the cellular environment, it was hypothesized that SPS in vivo obtains selenide from the PLP-dependent NifS-like enzymes CsdB, CSD, and IscS (12). In E. coli, these PLP-donor enzymes act as β-lyases, catalyzing the cleavage of the C–S bond from Cys or the C-Se bond from Sec to Ala and S0 or Se0, respectively (3, 11, 13). However, an interaction between SPS and NifS-like enzymes has not been shown, although a structural basis for the interaction of E. coli CsdB and A. aeolicus SPS was proposed because the molecular surfaces surrounding the active sites of CsdB and SPS exhibit complementarity by molecular docking (10). It is possible that thioredoxin reductase, which is involved in selenite reduction, is also involved in delivering selenide for SPS (3, 13). After selenophosphate is synthesized, it remains bound to NifS-like enzymes CsdB, CSD, and IscS (12). In E. coli, these enzymes catalyze the reaction between selenophosphate and selenide, releasing selenophosphate, AMP, and orthophosphate (8–11). Selenide originates from selenite reduction, from converted methylated selenium compounds, or through selenium removal from selenoprotein degradation (12).

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TCGAGTGGCTGAAGGACTGGCCTCCTAAATCCAG-GTTGGGCCGCCAGCGGTCCGGGACGTGCTGACCTCCTGCTGCTGCTCCGGGTAGGGGCGCTGGACTTCAAA-

Functional Complementation Assay—The functional complementation experiments were conducted according to Sculacchio et al. (16) for N-terminally truncated SPS. Briefly, the E. coli strain WL400 (DE3), which lacks the functional selD gene (7), was transformed with the full-length E. coli selD construct. The isothermal fluorescence anisotropy measurements were performed in an ISS-PC spectrofluorometer (ISS, Champaign, IL). The uncharged tRNASec molecules were fluorescein-labeled and its interaction with SelA was conducted using this methodology (16). The functional comple- mentation experiments were performed using this methodology using the E. coli strain JS1 (DE3), which lacks the functional selD gene, under the same anaerobic conditions (16) for 48 h in 30 °C.

Fluorescence Anisotropy Assay—Fluorescence anisotropy measurements were performed in an ISS-PC spectrofluorimeter (ISS, Champaign, IL). The uncharged tRNA^{Sec} molecules were fluorescein-labeled, and its interaction with SelA was conducted using this methodology (16). Similarly, the SelA and N-terminally truncated SelA complementation experiments were performed using this methodology using the E. coli strain JS1 (DE3), which lacks the functional selD gene, under the same anaerobic conditions (16) for 48 h in 30 °C. The resulting fluorescence anisotropy values were fitted, using the program Origin 8.0, to the Hill equation

\[
\frac{r}{r_0} = \frac{\left(\frac{[S_{\text{monomer}}]}{K_d} + 1\right)}{\left(\frac{[S_{\text{monomer}}]}{K_d} + 1\right)^n} \quad \text{(Eq. 1)}
\]

with \( r_0 \) and \( r_f \) representing the initial and final fluorescence anisotropy measures. \([S_{\text{monomer}}]\) is the titrated SPS concentration in units of monomers. Thus, the apparent dissociation constant \( K_d \) and the Hill constant \( n \) were determined.

Experiments for determination of the stoichiometry of SelA-tRNA^{Sec}-SPS binding were performed using 5000 nm SelA bound to 4990 nm unlabelled tRNA^{Sec} and 10 nm fluorescein-labeled tRNA^{Sec}. The same procedures as described above were used during the SPS titration. Mutant tRNA^{Sec} molecules were also tested for interaction with SelA by fluorescence anisotropy assays, as described previously (6).

Hydrogen/Deuterium Exchange Analyzed by Mass Spectrometry (H/DEx-MS)—We used hydrogen/deuterium exchange coupled with mass spectrometry to map the surfaces of SelA and SPS following the formation of the SelA-tRNA^{Sec} binary complex and the SelA-tRNA^{Sec}-SPS ternary complex. The various samples (SelA, SelA-tRNA^{Sec}, SPS, tRNA^{Sec}, and SelA-tRNA^{Sec}-SPS) were prepared using a published protocol (17). Briefly, the samples were labeled by diluting the sample to a final concentration of D_{2}O of ~90%. At each time point analyzed, aliquots (20 μl) were taken out of the exchange tube and quenched by mixing the solution with a 1:1 ratio of the quenching buffer (D_{2}O, 100 mM sodium phosphate, pH 2.5) and cooled to 0 °C to slow down the H/D exchange. These sample aliquots were digested for 5 min at 0 °C after the addition of 1 μl of a precooled pepsin solution (1 mg/ml in 5% (v/v) formic acid) and were injected directly to the mass spectrometer using a flow of 80 μl/min. The MS experiments were performed with an electrospray ionization triple quadrupole instrument, model Quatro II (Micromass UK), using the same procedures described by Figueira et al. (17). The spectral data were acquired and monitored using the MassLynx software (Micromass); the spectra deconvolution of the intact protein samples was performed with the program Transform (Waters). The theoretical digest was performed using the MS-Digest web server, and the error at each data point was determined to be 0.3 Da (based on multiple measurements).

Molecular Modeling of E. coli SelA Decamer—The structural model of E. coli SelA decamer was obtained using the I-TASSER server (18) that joins multiple threading alignments to rounds of iterative structural assembly simulations for protein structure modeling.

FTIR Spectroscopy—Infrared spectra of protein solutions were collected in a Nicolet Nexus 670 FTIR spectrometer equipped with a DTGS KBr detector, corresponding to 512 scans at a resolution of 2 cm\(^{-1}\) over the wavenumber range 4000–400 cm\(^{-1}\) at 25 °C. During data acquisition, the spectrometer was continuously purged with nitrogen. The buffer spectrum was subtracted digitally from the sample spectrum. The second derivative was used to identify the peak positions of the major components of the amide I band on the original (non-smoothed) protein vibrational spectra. To estimate the secondary structure content, Gaussian curve fitting was performed in the region of 1500–1700 cm\(^{-1}\) using GRAMS/386 software package (Galactic Industries). For FTIR analyses, SelA and SPS were prepared isolated in solution but also in the combinations 1SPS:1SelA, 1SelA:1tRNA^{Sec}, and 1SelA:1tRNA^{Sec}:1SPS (molar ratios in monomer units). Difference infrared spectra
were used to monitor the initial and the final state of SelA after SelA-tRNA^Sec complex formation obtained by spectrum subtraction of the complex with the isolated samples. The final state of SPS after SelA-tRNA^Sec-SPS complex formation was assessed by subtracting the experimental FTIR signal for the ternary complex, previously subtracted by the FTIR signal of the binary complex, to the SPS spectrum. The combination 1SelA:1SPS was also analyzed.

Atomic Force Microscopy (AFM)—To analyze the external dimensions, 1 μl of each sample, at 0.5 mg/ml, was incubated in binding buffer without PLP for 40 min at 25°C, deposited on a mica square (10 × 10 mm), and dried at room temperature for 3 h. This mica square was fixed in a metal base and analyzed in a Bruker Digital Instruments Nanoscope IIIA atomic force microscope (LNNano, CNPEM) using the non-contact mode and silicon tip of 1-nm diameter with 256 lines of scanning (19). The n-Surf 1.0 beta software was used to analyze the images and determine the dimensions of the ternary complex.

Results

SPS Interacts with SelA-tRNA^Sec Binary Complex—To test the hypothesis that SelA-tRNA^Sec interacts with SPS, we isothermally titrated 500 nm SelA-tRNA^Sec binary complex fluorescein labeled with increasing amounts of dimeric SPS in the absence of their substrates. Fluorescence anisotropy of labeled tRNA^Sec, covalently bound to SelA, progressively increased as a function of free SPS concentration (Fig. 1A) up to 5000 nm. Thereafter, no further change in fluorescence anisotropy was observed, indicating the saturation of the interaction sites at the inflection point of the curve. This pattern is consistent with a SelA-tRNA^Sec binary complex composed of 10 SelA monomers of 50.6 kDa each (resulting in a 506-kDa decamer) bound to 10 tRNA^Sec molecules of 31 kDa each (contributing with 310 kDa to the complex) interacting with 5 dimers of SPS of 72.4 kDa (contributing with 362 kDa to the complex), forming the predicted ternary SelA-tRNA^Sec-SPS complex of ~1.3 MDa, maintained by surface contact between each component.

The observed difference in the fluorescence anisotropy initial values shown in Fig. 1, A and B, for the binary complex was larger than would typically be expected from instrument variation. It may be related to the variation of local viscosity due to the initial binary complex sample concentration being 10 times higher for the stoichiometry measurement experiment when compared with the binding measurement experiment.

H/DEx-MS Reveals the Binding Interfaces, and Fluorescence Anisotropy Spectroscopy Indicates tRNA^Sec Contact Regions—H/DEx-MS followed by peptide mapping allowed the specific identification of solvent-accessible exchange sites in the dimeric SPS, the homodecameric SelA, the SelA-tRNA^Sec binary complex, and the SelA-tRNA^Sec-SPS ternary complex. Because SPS binding to SelA-tRNA^Sec disturbs secondary structure elements of both proteins of the binary complex, altering the solvent accessibility of the contact regions, binding interfaces could be mapped by comparing the rates of H/D exchange on proteins in the bound and unbound states (17, 20).

Overall, 41 peptides (including those with overlapping sequences), covering 57% of the SelA primary structure, were identified by tandem MS/MS, as shown by the coverage map (Fig. 2A). The region from Ala14 to Arg17, the SelA N-terminal domain, and regions Ala104–Thr117, Asp146–Cys148, and Ile304–
Lys\textsuperscript{321}, show small percentages of deuterium incorporation, even after 30 min of deuterium exposure. Thus, these amino acid residues were hidden within the protein structure, as surface contacts in \textit{E. coli} SelA decamer in solution, as observed in the crystallographic structure of the homologous \textit{A. aeolicus} SelA (4).

Following SelA-tRNA\textsuperscript{Sec} covalent binding, we detected 41 peptides (including those with overlapping sequences), covering 63\% of the SelA amino acid sequence (Fig. 2A). Characterization of the solvent accessibility of the N-terminal domain shows that regions Leu\textsuperscript{27}–Gly\textsuperscript{31} and Leu\textsuperscript{40}–Ile\textsuperscript{51} are hidden after SelA-tRNA\textsuperscript{Sec} binding (Fig. 2B). These regions were recently observed to interact with tRNA\textsuperscript{Sec} D-loop in the crystallographic structure of \textit{A. aeolicus} SelA-tRNA\textsuperscript{Sec} (4). Other regions, including fragment Leu\textsuperscript{137}–Ala\textsuperscript{154} and the amino acid residues near the active site (Lys\textsuperscript{295}), also have low incorporation of deuterium even after 30 min of exposure (Fig. 2, B and C and \textit{supplemental Tables 1A and 1B}). These regions must be non-covalent SelA-tRNA\textsuperscript{Sec} contacts on the surface of SelA.

In addition, evaluation of the effect of stereo chemical block in tRNA\textsuperscript{Sec} interaction with SelA by qualitative fluorescence anisotropy spectroscopy assays showed a decrease in SelA-tRNA\textsuperscript{Sec} observed binding when the acceptor arm, D-loop, and variable arm were mutated for the corresponding \textit{E. coli} tRNA\textsuperscript{Sec} region, highlighting the importance of these regions in SelA-tRNA\textsuperscript{Sec} specific interaction (Fig. 3, A–G). As a negative control, we titrated fluorescein-labeled single-stranded DNA (Fig. 3G). The interaction pattern of SelA-(mutant) tRNA\textsuperscript{Sec} binding is similar to that previously observed by Manzine \textit{et al.} (6) and does not present a saturation plateau because decameric SelA can stack side-by-side and one on top of each other.

The anticodon and TΨC arms variations did not affect the SelA-tRNA\textsuperscript{Sec} interaction (Fig. 3, A and B, respectively); however, the substitution of the D-loop by a fragment from \textit{E. coli} tRNA\textsuperscript{Sec} D-loop caused a decrease in the binary complex interaction (Fig. 3C). These results highlight the D-loop as responsible for the specificity of SelA-tRNA\textsuperscript{Sec} recognition, which corroborates with the SelA-tRNA\textsuperscript{Sec} binary complex crystallographic structure from \textit{A. aeolicus} (Protein Data Bank (PDB) ID 3W1K (4)). Based on amino acid sequence alignment between \textit{E. coli} and \textit{A. aeolicus} SelA (data not shown) and \textit{A. aeolicus} SelA-tRNA\textsuperscript{Sec} structure analysis (4), we identified by H/D-Ex MS the \textit{E. coli} SelA Leu\textsuperscript{27}–Gly\textsuperscript{31} and Leu\textsuperscript{40}–Ile\textsuperscript{51} regions as interaction points to \textit{E. coli} tRNA\textsuperscript{Sec} D-loop and TΨC arms (Fig. 2, A and B).

The deletion of the variable arm or its substitution by the \textit{E. coli} tRNA\textsuperscript{Sec} variable arm (Fig. 3, D and E, respectively) and the acceptor arm reduction from 8+5 to 7+5 (Fig. 3F) caused a marked decrease in the anisotropy values. The 8+5 folding is a key difference to other tRNAs and must be an important SelA recognition point that was not identified based on structural analysis (4).

\textbf{FIGURE 2}. \textit{E. coli} SelA and SPS H/D exchange mapped by mass spectrometry. A, deuterium incorporation of SelA after $t_1 = 5$ min, $t_2 = 10$ min, $t_3 = 15$ min, and $t_4 = 30$ min. SelA-tRNA\textsuperscript{Sec} binary complex after $t_1^* = 10$ min and $t_2^* = 30$ min deuterium incorporation. SelA-tRNA\textsuperscript{Sec}, after $t_1^{**} = 30$ min upon deuterium incorporation. B, deuterium incorporation of SPS after $t_1 = 5$ min, $t_2 = 10$ min, $t_3 = 15$ min, and $t_4 = 30$ min. SPS-SelA-tRNA\textsuperscript{Sec}, after $t_1^{**} = 30$ min of deuterium incorporation. C, the \textit{E. coli} SelA structural model generated by I-TASSER server (18) was colored to indicate the deuterium-incorporating regions before and after SelA-tRNA\textsuperscript{Sec} binary complex binding. \textit{Blue to red colors indicate low (blue) to high (red) accessibility to deuterium incorporation. Pink cylinders indicate β-strand regions, and yellow arrows indicate α-helix regions predicted by the PSIPRED server (28) for SelA and observed in \textit{E. coli} SPS crystallographic structure (site 1). Blue bars indicate confidence in secondary structure prediction (site 2).
Mapping the surface interactions of SelA to form the ternary complex shows that the N-terminal region (Glu46–Arg52) of SelA and two small loops (Glu67–Asp69 and Ala111–Thr117) have low deuterium incorporation when compared with SelA in the binary complex (Fig. 2B). We believe that these are the most important SelA-SPS interaction regions.

For SPS, 41 peptides were identified, covering 68.7% of the primary structure. Amino acid residues Leu136–Asp143, Ser239–Gly245, and Pro271–His283 presented low rates of deuterium incorporation even after 30 min of exposure. These regions are hidden within the protein and either are near or participate in the SPS dimerization interface (Fig. 2, B and D). The SPS N-terminal loop showed a high deuterium incorporation rate after 5 min of exposure, indicating that it is a flexible region.

It is worth noting that within 30 min, 68.7% of the amide hydrogen atoms in SPS were replaced with deuterium, whereas only 62.5% were replaced in the presence of SelA-tRNA\textsuperscript{Sec} binary complex, indicating that some amide protons were protected from deuterium exchange upon ternary complex formation. The SPS N-terminal flexible loop (Met1–Thr9) is hidden from H/D exchange after the interaction of SPS with the binary complex, resulting in lower deuterium incorporation. Two other loop regions (Leu43–Val54 and Met71–Pro72) and an α-helix region (Glu120–Cys129) that are near the catalytic site of the dimeric enzyme become inaccessible to the solvent after the interaction (Fig. 2, B and D). Our data identify the regions of molecular contact between the various components of the ternary complex and indicate that the regions near the active sites are crucial to the interaction between SPS and SelA-tRNA\textsuperscript{Sec} to form a ternary complex.

**FTIR Spectroscopy Suggests SPS Conformational Changes**—Structural changes due to SPS binding to the SelA-tRNA\textsuperscript{Sec} binary complex were investigated via FTIR spectroscopy because the amide I region (1600–1700 cm\(^{-1}\)) of the FTIR spectra is sensitive to changes in the protein secondary structure (21–24). SPS and SelA amide I bands were resolved into seven bands each. The bands appearing at 1628 and 1676 cm\(^{-1}\) are attributed to the low- and high-frequency components of
that SPS consists of 43.1% $\beta$-sheet, whereas the band centered at 1665 and 1640 cm$^{-1}$ (Fig. 4, A and B, respectively) can be assigned to turns and unordered structures, respectively (22–24). These results indicate that SPS does not have a significant secondary structure change upon SelA-tRNASec interaction when we analyzed the difference spectrum between SelA-tRNASec binary complex and the circular dichroism spectrum deconvolution of SelA (15), respectively which corroborates the crystallographic structure of E. coli SPS PDB ID 3U0O (10) and the circular dichroism spectrum deconvolution of SelA (15), respectively which corroborates the crystallographic structure (PDB.ID 3WCN) (27).

We observed that the amide I absorption band of SelA did not change upon SelA-tRNA$^{Sec}$ interaction when we analyzed the difference spectrum between SelA-tRNA$^{Sec}$ binary complex and SelA, which implies that SelA does not have a significant secondary structure variation upon tRNA$^{Sec}$ binding. Additionally, concerning the ternary complex formation, we propose that the most significant secondary structure change is more likely to be in SPS.

Indeed, there is an evident shift in the amide I absorption band of SPS (Fig. 4C) upon its binding to the SelA-tRNA$^{Sec}$ binary complex when compared with the SPS sample, indicating that SPS undergoes a conformational change to form the ternary complex. Such a shift was not observed in the absence of tRNA$^{Sec}$, implying that the SelA-SPS interaction is dependent on previous tRNA$^{Sec}$ interaction with SelA.

To further analyze the change in the secondary structure of SPS after its interaction with the SelA-tRNA$^{Sec}$ binary complex, we obtained a difference spectrum by subtracting the spectrum of free SPS from that of the bound protein, which was previously subtracted by the contribution of SelA-tRNA$^{Sec}$ (Fig. 4D). The result shows a large negative band of $\sim$1653 cm$^{-1}$ and a positive band in the 1640–1620 cm$^{-1}$ range. This pattern can be due to the loss of an $\alpha$-helical component, as first described by Trewehlla et al. (24), indicating that a structural element in the $\alpha$-helix configuration in SPS loses conformation to enable the formation of the ternary complex SelA-tRNA$^{Sec}$-SPS.

**Functional Assay Reveals That Selenoprotein Synthesis in E. coli Is Dependent on N-terminal Regions of SPS and SelA—** Because the H/D change experiment strongly suggested the participation of the SPS N-terminal loop in the SelA-tRNA$^{Sec}$-SPS complex assembly, we investigated the potential role of this region in selenoprotein biosynthesis. Previous in situ limited proteolysis experiments with chymotrypsin protease removed the first 11 residues of E. coli SPS (D11-SPS) (data not shown), but the catalytic residues Cys$^{17}$ and Lys$^{56}$ were preserved. Fluorescence anisotropy of SelA-tRNASec is not altered with D11-SPS titration, indicating a lack of specific interaction between D11-SPS and the binary complex (Fig. 5A). Functional complementation assays in E. coli strain WL400, which lacks the SPS gene, transformed with D11-SPS, were unable to restore the selenoprotein biosynthesis (Fig. 5B), despite the presence of the known catalytic residues. The positive control WL400 transformed with the E. coli SPS gene (Fig. 5C) developed the purple color characteristic of selenoprotein biosynthesis. We also investigated whether this region is required for assembly of the SelA-tRNA$^{Sec}$-SPS complex. SPS multiple sequence alignment analysis revealed three highly conserved residues (Leu$^{8}$, Thr$^{9}$, and Tyr$^{11}$) in the SPS N-terminal sequence; however, the biological significance of these residues has not yet been investigated. Together, these results suggest that the SPS N-terminal region is essential to SelA-tRNA$^{Sec}$-SPS complex assembly and that its deletion impairs selenoprotein biosynthesis.

Additionally, because H/D exchange experiments showed that the N-terminal domain of SelA is part of its decamerization interface, we also tested its requirement in Sec synthesis in a functional complementation assay in the E. coli strain JS1, which lacks the sela gene. N-terminally truncated SelA was unable to restore Sec synthesis (Fig. 5D) as seen in the positive control E. coli SelA (Fig. 5E). It is worth noting that Methanocaldococcus jannaschii SelA, which lacks an equivalent N-terminal domain but shares 30% amino acid sequence identity with E. coli SelA, is organized as a non-functional dimer and does not interact with tRNA$^{Sec}$ (25).

**SelA-tRNA$^{Sec}$ Binary Complex Dimensions Are Compatible with SPS Interaction—** Engelhardt et al. (26) were the first to visualize, in 1992, the decamers of SelA and SelA-tRNA$^{Sec}$ by transmission electron microscopy of negative stained samples. Manzine et al. (6) determined the stoichiometry of the binary complex (SelA-tRNA$^{Sec}$) as 1SelA$_{decamer}$:10tRNA$^{Sec}$. This sto
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FIGURE 6. Dimension analysis of SelA, SelA-tRNA\textsuperscript{Sec}, and SelA-tRNA\textsuperscript{Sec}-SPS complex by AFM. The samples were analyzed using low concentrations, 0.5 mg/ml, dried in mica grids. A, SelA; B, SelA-tRNA\textsuperscript{Sec}; C, SelA-tRNA\textsuperscript{Sec}-SPS. Grids were observed using a NanoScope III atomic force microscope (Digital Instruments) and analyzed using n-Sur 1.0 beta software (n-Surf).

Discussion

Sec biosynthesis in \textit{E. coli} requires 10 molecules of Ser-tRNA\textsuperscript{Sec} covalently bound to homodecameric SelA to catalyze the conversion of Ser to Sec (5). The SelA-tRNA\textsuperscript{Sec} binary complex can thus be interpreted as a reservoir of cellular tRNA\textsuperscript{Sec}.

It was observed by H/DEx-MS presented here that the N-terminal region of SelA is required for SelA oligomerization, as it becomes hidden from the surface of homodecamers exposed to solvent. Therefore, homodecamerization, and consequently, the Ser-Sec conversion and selenoprotein biosynthesis, is dependent on the N-terminal region (or N terminus), as we observed by functional complementation with the N-terminally truncated \textit{E. coli} SelA. Similar results from \textit{A. aeolicus} SelA N-terminal mutants (27) and the non-functional dimeric \textit{M. jannaschii} SelA (25), which do not interact with tRNA\textsuperscript{Sec}, strengthen our findings.

A Schiff base is formed between the \(\alpha\)-amino group of the Ser residue with the formyl group of PLP following SelA-tRNA\textsuperscript{Sec} interaction, resulting in the synthesis of the intermediate aminoacyl-tRNA\textsuperscript{Sec} upon dehydration of the amino acid residue (5). FTIR experiments show that SelA does not undergo a secondary structure change upon its interaction with tRNA\textsuperscript{Sec} as was also observed in the crystallographic structure of \textit{A. aeolicus} SelA-tRNA\textsuperscript{Sec} complex (4), and fluorescence anisotropy spectroscopy with tRNA\textsuperscript{Sec} mutants has shown that this interaction is dependent on the tRNA\textsuperscript{Sec} acceptor arm, D-loop, and variable arm.

In addition to the D-loop arm (4) as the recognition point of tRNA\textsuperscript{Sec} to SelA, we observed that the difference in the acceptor arm pairing number (8 to 7) is essential for tRNA\textsuperscript{Sec} affinity to \textit{E. coli} SelA. Selenium is transferred to the aminoacyl-tRNA\textsuperscript{Sec} intermediate complex in the form of selenophosphate, a product of dimeric SPS selenide water dikinase catalytic activity (7, 10), to form Sec-tRNA\textsuperscript{Sec}. The SPS dimerization interface is composed of the \(\beta\)-sheet domain of each monomer, a common structural characteristic of the PurM protein superfamily (7). This dimerization domain was confirmed by our H/DEx-MS experiments. In addition, consistent with the SPS crystallographic structures (PDB ID 3U00) that were previously described, the glycine-rich N-terminal region of SPS was observed to be flexible in solution, showing high levels of deuterium exchange even after low deuterium exposure time. This flexibility allows the formation of the SPS active site on its “closed” form, upon ATP binding, releasing the catalysis product in its “open” form (7, 10).

Because one SelA\textsubscript{decamer} molecule and 10 tRNA\textsuperscript{Sec} molecules form a covalently bound binary complex (6), we analyzed the interaction of SPS with the SelA-tRNA\textsuperscript{Sec} complex. Using fluorescein-labeled tRNA\textsuperscript{Sec}, we observed an increase in fluorescence anisotropy following SPS isothermal titration, revealing a specific binding leading to the formation of the ternary complex, with a stoichiometric ratio of one SelA decamer covalently bound to 10 tRNA\textsuperscript{Sec} molecules interacting with five SPS dimers. The SelA-tRNA\textsuperscript{Sec}-SPS interaction dissociation constant of 610 ± 79 nm is consistent with the expected values for biomolecular transient interactions. Hill’s plot (Fig. 1A) indicates a positive cooperativity, with \(n = 2.1 ± 0.4\), for the formation of the ternary complex. Based on this observation, we propose that trapping the selenium compounds in the SelA-tRNA\textsuperscript{Sec}-SPS complex would be an efficient mechanism to avoid the high cellular toxicity posed by free selenium. Additional experiments are necessary to verify this hypothesis.

The SelA-tRNA\textsuperscript{Sec}-SPS interaction is dependent on stereochemical recognition, involving the structural accommodation of one molecule to the other. Remarkably, the height of the
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**FIGURE 7. Proposed sequence of events in the synthesis of selenocysteine.** The SelA homodecamer (light blue) is 210 and 49 Å in maximum distance and height, respectively. This complex interacts with 10 tRNA^{Sec} molecules, resulting in the binary complex SelA-tRNA^{Sec} of −220 and −36 Å, respectively. This variation in height allows the interaction with SPS, forming a ternary complex, with dimensions of −270 and 40 Å, respectively, which can interact with five SPS dimers. The sequence of events reveals the requirement of a conformational change in tRNA^{Sec} to allow the SPS binding.

SelA-tRNA^{Sec} complex is consistent with the SPS dimer size in the interaction region above 3 nm. However, FTIR analysis has indicated a modification in the α-helix segment of *E. coli* SPS only upon binding to the SelA-tRNA^{Sec} complex. We suggest that this conformational change corresponds to the region from Glu^{159} to Val^{161} (α-helix 4) of *E. coli* SPS. Although no information about H/D exchange was obtained for this α-helix, analysis of the *E. coli* SPS crystal structure (7) indicates that it is in the middle of the linker between the aminomidazole ribonucleotide synthetase (AIRS)-related and C-terminal domains, which is consistent with the SelA-tRNA^{Sec}-SPS interaction. Regions close to the active sites of SPS were observed to be hidden from H/D exchange, and we conclude that these regions may be the interaction points that enable the formation of the ternary complex. Our analysis suggests that the formation of the ternary complex occurs via SPS opening its active sites to deliver selenophosphate to the active site of SelA. Furthermore, this represents a probable sequence of events in the synthesis of selenoproteins, with SelA binding to tRNA^{Sec} prior to SPS (Fig. 7).

A flexible conformation of SPS is certainly required to facilitate its interaction with the SelA-tRNA^{Sec} complex, as observed for the NifS-like-SPS interaction (7, 12). In fact, the glycine-rich N-terminal region of SPS is hidden from the solvent after SelA-tRNA^{Sec}-SPS formation, as observed by H/DEx-MS, and SPS with an N-terminal truncation does not interact with SelA-tRNA^{Sec}, as shown by fluorescence anisotropy spectroscopy experiments. *In vivo* studies of Δ11-SPS show that it does not complement SPS function in the SPS-deficient *E. coli* strain WlA400. These data show that SelA-tRNA^{Sec}-SPS complex formation is essential for selenoprotein biosynthesis in *E. coli* and that it follows a sequence of events, *i.e.* SelA interacts with tRNA^{Sec} and undergoes tertiary structure rearrangements allowing the interaction with SPS, without changing its secondary structures (Fig. 7).

As previously noted by Yoshizawa and Böck (3), a second level of fidelity control in selenoprotein pathway, in addition to UGA stop-codon recognition, is the discrimination of Sec from its isosteric form Cys (3). Although widely studied, selenophosphate formation from selenide and ATP in a reaction catalyzed by SPS is not completely understood, and the structural basis for the substrate specificity has not yet been solved (3, 7). Our results provide new insights into the biosynthesis of selenoproteins, for the first time demonstrating the functional macromolecular assembly of the SelA-tRNA^{Sec}-SPS. The significance of this finding centers on the ability of this complex to enable selenium delivery to Sec biosynthesis in the presence of tRNA^{Sec}. We propose that once the ternary complex is formed, selenophosphate can be transferred from SPS to SelA active sites and to the tRNA^{Sec}, concealing the toxic selenium compounds from the cytoplasm. Further investigation awaits to address this hypothesis.

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