Structural Asymmetry of the Terminal Catalytic Complex in Selenocysteine Synthesis*

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Background: SepSecS catalyzes the terminal step of selenocysteine synthesis in a reaction that requires tRNA\textsuperscript{Sec}. Aminoacyl-tRNA synthetases (aaRSs)\textsuperscript{2} couple specific amino acids to their cognate tRNAs and generate aminoacyl-tRNAs (aa-tRNAs), which serve as substrates for translating ribosomes. The ability of aaRS to bind a specific amino acid and set of isoacceptor tRNAs ensures accurate translation of the genetic code (1, 2). Four amino acids (Asn, Gln, Cys, and Sec) can be paired with tRNAs via indirect tRNA-dependent aminoacylation pathways, but only Sec requires such a pathway in all domains of life (3–5). In the first reaction, which is conserved in all organisms, SerRS attaches a serine residue onto tRNA\textsuperscript{Sec}. Although the bacterial enzyme SelA is capable of directly converting Ser-tRNASec to Sec-tRNASec, both archaea and eukaryotes require two enzymatic reactions to complete the conversion. O-Phosphoseryl-tRNA\textsuperscript{Sec} kinase first phosphorylates the seryl group yielding phosphoseryl-tRNA\textsuperscript{Sec}, which is the substrate for the terminal synthetic enzyme, SepSecS. Subsequently, SepSecS substitutes phosphate with selenol in a reaction based on the pyridoxal phosphate (PLP) co-factor (6, 7).

SepSecS is a distinct type fold I PLP-dependent enzyme that acts on a tRNA-based substrate. Its closest homologs are SepCysS, an enzyme essential for biosynthesis of Cys-tRNA\textsuperscript{Cys} in methanogenic archea (8), and SelA, an enzyme responsible for Sec synthesis in bacteria (9). However, SepSecS forms its own branch in the phylogenetic tree of the PLP-dependent enzymes, suggesting that it was present in the last common ancestor (10). In contrast to dimeric SepCysS and decameric SelA, SepSecS forms a stable tetramer in solution, which is held by extensive interactions between two homodimers (6, 10). The tetrameric arrangement was thought to give rise to four structurally and functionally equivalent tRNA binding and active sites, located on the opposite sides of the tetramer. This oligomeric nature of SepSecS is reminiscent of a similar arrangement adopted by a large number of canonical aaRS (reviewed in Ref. 11). With the exception of several monomeric class I enzymes (e.g. LeuRS, IleRS, ValRS, ArgRS, GlnRS, and GluRS) and class II eukaryotic AlaRS, all other aaRS are either dimers or (homo/hetero)tetramers that contain multiple tRNA-recognition and aminoacylation sites. It has been shown that dimeric

Selenocysteine (Sec), the 21\textsuperscript{st} amino acid, is synthesized from a serine precursor in a series of reactions that require selenocysteine tRNA (tRNA\textsuperscript{Sec}). In archaea and eukaryotes, O-phosphoseryl-tRNA\textsuperscript{Sec}-selenocysteinyl-tRNA\textsuperscript{Sec} synthase (SepSecS) catalyzes the terminal synthetic reaction during which the phosphoseryl intermediate is converted into the selenocysteinyl moiety while being attached to tRNA\textsuperscript{Sec}. We have previously shown that only the SepSecS tetramer is capable of binding to and recognizing the distinct fold of tRNA\textsuperscript{Sec}. Because only two of the four tRNA-binding sites were occupied in the crystal form, a question was raised regarding whether the observed arrangement and architecture faithfully recapitulated the physiologically relevant ribonucleoprotein complex important for selenoprotein formation. Herein, we determined the stoichiometry of the human terminal synthetic complex of selenocysteine by using small angle x-ray scattering, multi-angle light scattering, and analytical ultracentrifugation. In addition, we provided the first estimate of the ratio between SepSecS and tRNA\textsuperscript{Sec} in vivo. We show that SepSecS preferentially binds one or two tRNA\textsuperscript{Sec} molecules at a time and that the enzyme is present in large molar excess over the substrate tRNA in vivo. Moreover, we show that in a complex between SepSecS and two tRNAs, one enzyme homodimer plays a role of the noncatalytic unit that positions CCA ends of two tRNA\textsuperscript{Sec} molecules into the active site grooves of the other, catalytic, homodimer. Finally, our results demonstrate that the previously determined crystal structure represents the physiologically and catalytically relevant complex and suggest that allosteric regulation of SepSecS might play an important role in regulation of selenocysteine and selenoprotein synthesis.

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MetRS is capable of generating two moles of methionyl adenylate per enzyme dimer (12) and that AspRS (13) and ThrRS (14) dimers can simultaneously bind two tRNA molecules. In contrast, other oligomeric aARS contain multiple active sites that are not simultaneously chemically equivalent. These enzymes exhibit “half of the sites” activity that is presumably regulated by negative allosterism. The TyrRS dimer binds one tRNA\(^{ Tyr}\) (15) and catalyzes formation of 1 mol of tyrosyl adenylate (16–20), and tetrameric PheRS (15, 21) and SepRS (22) employ two of their four catalytic and tRNA-binding sites at a time. Likewise, class II enzymes, bacterial LysRS-II (23, 24), AspRS (25), and HisRS (26) and the archaeal and eukaryotic PrORs (27, 28) behave according to the “half of the sites” model. The situation is less clear in the case of PyrRS and SerRS. Although crystallized in complex with two molecules of tRNA\(^{Pyl}\) (29), molecular dynamics studies suggested that PyrRS exhibits functional asymmetry in solution (30). On the other hand, the SepRS-tRNA\(^{Sec}\) complex contained one tRNA\(^{ Sec}\) bound to the enzyme dimer in the crystal (31), whereas other studies suggested that the enzyme may cooperatively bind two tRNAs (32).

The crystal structure of the human SepSecS-tRNA\(^{ Sec}\) binary complex revealed that only two tRNA binding sites located on the same recognition surface in the enzyme were occupied and that both tRNAs interacted with the enzyme in a cross-dimer fashion (6). Intriguingly, in that crystal form, one SepSecS homodimer acted as a noncatalytic unit that served to position the CCA ends of both tRNAs toward the active sites located in the other, catalytic homodimer (6). However, the access to the other two tRNA binding sites was hindered by crystal contacts, and it remained unclear whether the observed architecture was a consequence of the crystal packing or whether it represented the biologically relevant ribonucleoprotein complex. Also, it remained unknown whether SepSecS is capable of simultaneously binding and acting on multiple tRNA substrates in solution.

Here, we present a detailed biophysical analysis of the complexes formed between holo SepSecS and tRNA\(^{ Sec}\) under conditions with varying molar excess of tRNA. Our results reveal the stoichiometry, architecture, and arrangement of the SepSecS-tRNA\(^{ Sec}\) binary complex in solution. We show that the terminal catalytic complex of selenocysteine adopts the same structure in solution as in the crystal. Finally, the observed structural and functional asymmetry suggests that the activity of SepSecS, and thus selenocysteine and selenoprotein synthesis, may be allosterically regulated.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of SepSecS—**The human SepSecS was expressed in *Escherichia coli* and purified as previously described (6). Briefly, the cells were harvested by centrifugation at 5,000 rpm for 15 min. The cell pellet was resuspended in 20 mM Tris, pH 8.0, 300 mM NaCl, 10% (v/v) glycerol, 1 mM \(\beta\)-mercaptoethanol, 10 \(\mu\)M PLP, and 0.5 mM tris(2-carboxyethyl)phosphine. The purified protein was flash frozen in liquid nitrogen and stored at –80 °C.

**In Vitro Transcription and Purification of tRNA\(^{ Sec}\)—**The human tRNA\(^{ Sec}\) was cloned and expressed as described (6). The tRNA\(^{ Sec}\) gene was amplified by PCR, and RNA was synthesized by *in vitro* T7 RNA polymerase run-off transcription (33). The transcription reaction was performed at 37 °C for 3 h in 40 mM Tris, pH 8.1, 22 mM MgCl\(_2\), 1 mM DTT, 1 mM spermidine, 0.01% Triton X-100, 50 \(\mu\)g/ml BSA, 10 mM GMP, 2 mM nucleotides (ATP, GTP, CTP, and UTP), the PCR product of the tRNA\(^{ Sec}\) gene (20 ng/\(\mu\)l), and 3 mM T7 RNA polymerase. The cleared reaction was loaded on to a Resource-Q column (GE Healthcare) and tRNA\(^{ Sec}\) was purified using a linear gradient of NaCl (0.2–1.0 M) in 20 mM Tris, pH 8.1. Following elution, the human tRNA\(^{ Sec}\) was further purified on a S200 Superdex size exclusion column (GE Healthcare) equilibrated with 20 mM Tris, pH 8.1, and 150 mM NaCl. The eluted tRNA\(^{ Sec}\) was flash frozen in liquid nitrogen and stored at –80 °C.

**Analytical Ultracentrifugation (AUC)—**In all samples tRNA\(^{ Sec}\) was held constant at 23 ng/\(\mu\)l, and varying amounts of holo SepSecS were added to give the final molar ratio of 2:1, 4:1, 6:1, and 8:1 with tRNA\(^{ Sec}\) in excess (all ratios are expressed in terms of tRNA\(^{ Sec}\) molecules: SepSecS tetramer). SepSecS and tRNA\(^{ Sec}\) alone were tested at a concentration of 1.5 mg/ml and 280 \(\mu\)g/ml, respectively. Sedimentation velocity experiments were conducted using the Beckman XLA-70 at the Keck Facility of Northwestern University (Evanston, IL). Centrifugation was carried out at 35,000 rpm at 20 °C with water as reference. Absorbance was measured at 260 nm in intensity mode until complete sedimentation of the sample occurred. Data processing was completed using the Ultrascan III software package and the LIMS III database (34–36). The Ultrascan software was used to obtain a diffusion-corrected integral distribution of sedimentation coefficients \(S(s)\), which were corrected to that of water at +20 °C, which is indicated by \(S_{20,w}\) values. The Ultrascan software was used to calculate both the solvent densities and partial specific volumes of samples and to perform the van Holde-Weiszheit analysis (37, 38).

**Tryptophan Fluorescence Quenching—**Tryptophan fluorescence was recorded on a P1T Quantamaster spectrophuorimeter equipped with double monochromators for both excitation and emission. Slits of 1.0 and 4.0 nm were used for excitation and emission, respectively, with an integration time of 2 s. Experiments were carried out at +25 °C, with a 1-cm optical path, and in 20 mM Tris, pH 8.0, 150 mM NaCl, and 5% glycerol. Tryptophan fluorescence was excited at 295 nm, and the emission spectrum was recorded from 310 to 420 nm. The emission wavelength of 336 nm was chosen for data collection. SepSecS (62.5 nM; concentration expressed in terms of tetramers) was titrated with 5–3,000 nM tRNA\(^{ Sec}\). Inner filter effects were avoided by using low concentrations of protein and tRNA. Samples were corrected for dilution, and \(K_D\) values were calculated using the modified Stern-Volmer equation to take into account the heterogeneous quenching of the tryptophans in SepSecS.
Cells from ten 100-mm dishes were lysed in a total of 3 ml of Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% Nonidet P-40) by passage through a 27-gauge syringe needle five times. The protein amounts indicated in Fig. 5 were resolved by 12% SDS-PAGE and blotted to nitrocellulose. The blot was then incubated with a 1:1,000 dilution of an affinity-isolated polyclonal anti-SepSecS antibody (Sigma-Aldrich) overnight at +4 °C. The blot was then rinsed and probed with a 1:50,000 dilution of anti-rabbit HRP-conjugated IgG. HRP activity was detected using PicoWest detection reagents (Pierce) and captured on a Carestream RS4000 imager. Band intensity was quantified using the Carestream Molecular Imaging software. The standard used for SepSecS quantification was the pure His-tagged SepSecS. A portion of the cell lysate (0.3 ml) was used for acid-phenol RNA purification, which enriches tRNA content (47). RNAs were resolved on 6% acid urea mini-gels, blotted to charged nylon using a semi-dry electrobinder (Bio-Rad) at 400 mA for 1 h at +6 °C. Using UltraHyb Oligo (Invitrogen), the blot was then hybridized with 10⁶ cpm/ml of [33P] end-labeled oligonucleotide complementary to the tRNAsec anticodon loop (CAGTCTAGGTGTTGAAGCTGCACC) and rinsed according to the manufacturer’s protocol. Signal was detected and quantitated using a Typhoon PhosphorImager (GE Healthcare). The standard for tRNAsec quantitation was in vitro transcribed human tRNAsec.

RESULTS

SepSecS Interacts and Forms a Stable Complex with tRNAsec in Solution—Despite containing four tRNA-binding sites, the SepSecS tetramer was shown to bind only two tRNAsec molecules (6). The detailed analysis of the crystal structure revealed that two tRNAs interacted with tRNA-binding sites located on opposite recognition surfaces. Also, the occupancy of binding sites was 50%, suggesting that the enzyme might effectively bind only one tRNA at a time. Because access to the remaining two tRNA binding sites was hindered by crystal contacts, the exact stoichiometry between SepSecS and tRNAsec could not be determined. As the first step toward establishing the stoichiometry of the SepSecS-tRNAsec complex in solution, we employed AUC. Because we have previously demonstrated that SepSecS binds unacylated tRNAsec and full-length mimics of O-phosphoseryl-tRNAsec in the same manner (48), only unacylated tRNAsec was used in this study. More importantly, we determined, by tryptophan fluorescence quenching experiments (22), that SepSecS binds unacylated tRNAsec with the Kd of 77.9 ± 8.9 nm, which suggests that a stable, high-affinity binary complex forms in solution.

The samples containing free tRNAsec, holo SepSecS, and different molar ratios between tRNAsec and SepSecS (e.g. 2:1, 4:1, 6:1, and 8:1) were subjected to AUC analysis. It is important to note that molar ratios are expressed in terms of the number of tRNA molecules per SepSecS tetramer used in the experiment and that all S values are expressed in terms of the standard solvent of water at 20 °C (S20,w). Our results show that tRNAsec sediments at 4.3 S, whereas SepSecS sediments at 8.88 and 9.44 S (Fig. 1). In case of samples containing both tRNAsec and SepSecS, the sigmoidal shape of the van Holde-Weischet curves suggests a stable interaction between SepSecS and tRNAsec. This indicates that the SepSecS-tRNAsec complex forms in vivo.
solution (Fig. 1A). Furthermore, in addition to free tRNA\textsuperscript{Sec}, these samples contain several larger species that sediment at values >10 S (Fig. 1 and Tables 1 and 2). The most dominant species in SepSecS-tRNA\textsuperscript{Sec} samples sediments at 10.7–11.3 S (11 S). Interestingly, once the molar ratio between tRNA\textsuperscript{Sec} and SepSecS (tetramer) exceeds 2:1, both the size and relative abundance of the dominant particle remain constant regardless of the further increases in the tRNA\textsuperscript{Sec} molar excess (Table 1). In agreement with that observation, the analysis of van Holde-Weischet curves shows that an increase in the molar excess of tRNA\textsuperscript{Sec} yields a larger fraction of free tRNA\textsuperscript{Sec} and not an increase in the binary complex abundance (Fig. 1). For instance, whereas free tRNA\textsuperscript{Sec} comprises less than 10% of the 2:1 sample, its fraction increases to ~40% in the 4:1 sample and peaks at ~70% in the 8:1 sample (Fig. 1A). The G(s) distribution shows the same pattern: the height of free tRNA\textsuperscript{Sec} peak correlates with an increase in molar excess of tRNA over SepSecS (Fig. 1B). Moreover, we have observed even larger species that sediment at 12.6–12.9 S (13 S) in samples containing 6:1 and 8:1 molar excess of tRNA\textsuperscript{Sec} over the SepSecS tetramer (Tables 1 and 2). The relative frequency of the 13 S particle is on average ~3.5-fold lower than that of the 11 S particle, and its fraction is in inverse correlation with the molar excess of tRNA\textsuperscript{Sec} (Table 2). Thus, our AUC results show that SepSecS and tRNA\textsuperscript{Sec} interact and form a stable complex in solution, that the dominant binary complex sediments at 11 S, and that the molar excess of tRNA\textsuperscript{Sec} over the enzyme tetramer beyond the 2:1 molar ratio does not have an effect on the complex size and abundance.

TABLE 1 Corrected S values for SepSecS-tRNA\textsuperscript{Sec} complexes

| Molar ratio (tRNA\textsuperscript{Sec}-SepSecS tetramer) | S\textsubscript{20,w} Fraction | \(\times 10^{-23}\) | % |
|-------------------------------------------------|---------------------------------|----------------|---|
| 2:1                                             | 11.3 ± 2.39 × 10^{-2}           | 17             | |
| 4:1                                             | 10.7 ± 3.42 × 10^{-2}           | 8              | |
| 6:1                                             | 11.3 ± 2.07 × 10^{-2}           | 18.05          | |
|                                                 | 12.6 ± 1.92 × 10^{-2}           | 6.45           | |
| 8:1                                             | 10.7 ± 0.797 × 10^{-2}          | 16.52          | |
|                                                 | 12.9 ± 3.55 × 10^{-2}           | 4.72           | |

TABLE 2 Corrected S values for all samples analyzed by AUC

| Molar ratio (tRNA\textsuperscript{Sec}-SepSecS tetramer) | S\textsubscript{20,w} Fraction | \(\times 10^{-23}\) | % |
|-------------------------------------------------|---------------------------------|----------------|---|
| 0:1 (free tRNA\textsuperscript{Sec})            | 4.30 ± 0                         | 100            | |
| 0.1 (holo SepSecS)                              | 8.88 ± 1.18 × 10^{-2}           | 45.94          | |
|                                                 | 9.44 ± 1.18 × 10^{-2}           | 54.04          | |
|                                                 | 1.19 ± 1.16 × 10^{-2}           | 10.37          | |
|                                                 | 2.62 ± 13.5 × 10^{-2}           | 2.51           | |
|                                                 | 4.10 ± 23.7 × 10^{-2}           | 13.52          | |
| 2:1                                             | 6.31 ± 18.5 × 10^{-2}           | 4.53           | |
|                                                 | 8.90 ± 3.56 × 10^{-2}           | 0              | |
|                                                 | 9.70 ± 1.29 × 10^{-2}           | 51.83          | |
|                                                 | 11.3 ± 3.39 × 10^{-2}           | 17             | |
|                                                 | 1.34 ± 1.10 × 10^{-2}           | 16.92          | |
| 4:1                                             | 5.86 ± 0.703 × 10^{-2}          | 75.15          | |
|                                                 | 10.7 ± 3.42 × 10^{-2}           | 8.01           | |
|                                                 | 2.00 ± 1.51 × 10^{-2}           | 4.98           | |
|                                                 | 2.50 ± 5.89 × 10^{-2}           | 1.53           | |
|                                                 | 3.96 ± 0.353 × 10^{-2}          | 59.98          | |
| 6:1                                             | 6.11 ± 6.81 × 10^{-2}           | 5.11           | |
|                                                 | 9.74 ± 3.02 × 10^{-2}           | 3.87           | |
|                                                 | 11.3 ± 2.07 × 10^{-2}           | 18.05          | |
|                                                 | 12.6 ± 1.92 × 10^{-2}           | 6.65           | |
|                                                 | 2.06 ± 4.35 × 10^{-2}           | 7.85           | |
|                                                 | 3.98 ± 0.465 × 10^{-2}          | 65.89          | |
| 8:1                                             | 5.79 ± 7.54 × 10^{-2}           | 5.03           | |
|                                                 | 10.7 ± 79.7 × 10^{-2}           | 16.52          | |
|                                                 | 12.9 ± 3.55 × 10^{-2}           | 4.72           | |
The SepSecS Tetramer Preferentially Binds One or Two tRNA<sup>Sec</sup> Molecules at a Time—The AUC results have shown that SepSecS and tRNA<sup>Sec</sup> interact and form a stable binary complex in solution. In addition, those measurements have revealed that adding an excess of tRNA<sup>Sec</sup> beyond the 2:1 molar ratio over SepSecS tetramer did not substantially affect the size and relative abundance of the dominant, 11 S species. However, we could not determine from those measurements the number of tRNAs bound to the enzyme tetramer. To determine the molar mass of each macromolecular species in these samples and to subsequently infer from that mass the composition of each of the macromolecular species, we have subjected a series of samples to multi-angle light scattering coupled with size exclusion chromatography (SEC-MALS) analysis. The SEC-MALS results show that the SepSecS tetramer preferentially binds one or two tRNA<sup>Sec</sup> molecules at a time.

We analyzed samples of free unacylated tRNA<sup>Sec</sup>, holo SepSecS, and mixtures containing tRNA<sup>Sec</sup> and SepSecS tetramers in 2:1, 4:1, 6:1, and 8:1 molar ratios. Our results show that holo SepSecS and free tRNA<sup>Sec</sup> separate well on a SEC column; holo SepSecS elutes at ~28 min, and tRNA<sup>Sec</sup> elutes at ~36 min (compare blue and red lines in Fig. 2A). Further, the binary complex between SepSecS and tRNA<sup>Sec</sup> is stable, and it elutes at ~26.5 min (Fig. 2A). As in the AUC experiments, an increase in molar excess of tRNA<sup>Sec</sup> over SepSecS beyond the 2:1 molar ratio does not have an apparent effect on the size of the binary complex, but it rather increases the fraction of unbound tRNA<sup>Sec</sup> (light blue and purple lines in Fig. 2A).

The MALs data were used to determine a fraction of SepSecS bound to one, two, three, or four tRNAs at a time. First, we determined that the molecular masses of tRNA<sup>Sec</sup> and holo SepSecS are 30.3 and 225.7 kDa, respectively. The experimentally determined values were used to calculate molecular masses of SepSecS complexed with one (256 kDa), two (286.4 kDa), three (316.7 kDa), and four tRNA<sup>Sec</sup> molecules (347 kDa). Next, we obtained molecular masses for every part of the binary complex peak and compared these values with the calculated molecular masses for each complex. Based on the comparison, each section in the peak was assigned to a specific binary complex species, and the resulting distribution was plotted as a bar chart (Fig. 2B). The boundary between the neighboring complex species was defined as the midpoint between the corresponding molecular masses (e.g. the boundary between SepSecS complexed with one and two tRNAs was set at 271.3 kDa, which is a midpoint between 256 and 286.4 kDa). The area of the complex peaks with molecular masses between 211 and 241 kDa were assigned as holo SepSecS, and those with values above 361.9 kDa were considered as aggregates. Our analysis shows that, irrespective of the molar ratio used in the experiment, the binary complexes containing one or two tRNA<sup>Sec</sup> molecules constitute 97% of the complex sample (Fig. 2B). We determined that samples with 0.25:1, 0.5:1, and 1:1 molar ratios contain almost exclusively SepSecS in complex with one tRNA<sup>Sec</sup>; the fraction of this complex is in range from 93 to 96% (data not shown). In the sample containing 2:1 molar excess of tRNA<sup>Sec</sup> over the SepSecS tetramer, the fraction of the complex with one and two tRNA<sup>Sec</sup> is 66 and 32%, respectively (orange bars in Fig. 2B). Interestingly, further increase in the molar excess of tRNA<sup>Sec</sup> increased the relative concentration of SepSecS with one tRNA<sup>Sec</sup> bound. For instance, in the 8:1 sample, the fractions of SepSecS bound to one and two tRNAs are ~80 and 20%, respectively. Thus, our analysis reveals that SepSecS can simultaneously bind either one or two tRNA<sup>Sec</sup> molecules (Fig. 2B).

The Binary SepSecS-tRNA<sup>Sec</sup> Complex Is the Same in Solution as in the Crystal—We have previously shown that only the SepSecS tetramer can bind tRNA<sup>Sec</sup> and that each tRNA molecule interacts with the enzyme in a cross-dimer fashion (6).
However, it remained unclear whether the architecture captured in the crystal represented the physiologically relevant complex found in solution. To provide an answer to this question, we analyzed the binary complex by SAXS. Samples of free unacylated tRNAsec, holo SepSecS, and mixtures of tRNAsec and SepSecS in 2:1, 4:1, and 6:1 molar ratios were run on a SEC column coupled to the SAXS sample cell (SEC-SAXS). SAXS data collection was initiated after the void volume was eluted from the column. The in-line SEC-SAXS setup was used to separate the binary complex from free SepSecS and tRNAsec, to significantly reduce radiation damage, and to establish whether concentration-dependent events take place by analyzing a single sample over a range of concentrations. Inspection of the Guinier region revealed the absence of aggregation and/or formation of higher order multimers in all samples. Further, comparative analysis of the pair-distance distribution function, $P(r)$, clearly shows that peaks of holo SepSecS and SepSecS-tRNAsec contain distinct macromolecular species (Fig. 3A). The shape of the curve and the size of the peak remained practically identical in three independently measured binary complex samples (Fig. 3A). Also, addition of tRNAsec causes the radius of gyration ($R_g$) to increase from 43.15 Å in holo SepSecS to $\sim 49$ Å in the binary complex sample (Table 3). In agreement with these observations, the SAXS envelope derived from the binary complex peak scattering is enlarged and of different shape when compared with the holo SepSecS envelope (Fig. 3B). Taken together, these results suggest that a stable SepSecS-tRNAsec complex is formed in solution.

Next, we asked whether the number of bound tRNA molecules could be estimated and whether the complex architecture could be determined using SAXS data. The theoretical calculations for holo SepSecS and SepSecS complexed with one, two, three, or four tRNAs predict a steady increase in $R_g$ value from 41.81 to 55.04 Å with each additional tRNA bound (Table 4). However, the experimentally obtained $R_g$ value is constant at $\sim 49$ Å regardless of the molar excess of tRNAsec used (Table 3). Because the theoretical $R_g$ of SepSecS complexed with one and two tRNAsec is almost identical (49.2 and 51.6 Å, respectively) and similar to the experimentally determined $R_g$ for the binary complex ($\sim 49$ Å), it is likely that SepSecS binds either one or
two tRNAs at a time in solution. Further, we determined the complex architecture by comparing the SAXS envelope with the x-ray crystal structure. The SAXS envelope was derived from the binary complex peak that contained a 6-fold molar excess of tRNA<sup>Sec</sup> over SepSecS (Fig. 3, B and C). The superimposition shows that the complex arrangement observed in the crystal structure agrees well with the envelope (Fig. 3C). This is further corroborated by excellent agreement between the experimental and theoretical scattering curves (Fig. 3D). The theoretical scattering curve was calculated using the existing crystal structure of the complex. In contrast, complexes in which two bound tRNA molecules were arranged differently than in the crystal structure did not fit into the envelope (Fig. 4, A and B). Also, the superimposition of the complex between SepSecS and four tRNA<sup>Sec</sup> revealed poor agreement with the SAXS density (Fig. 4C). Our results, therefore, strongly argue that the SepSecS tetramer predominantly binds up to two tRNA<sup>Sec</sup> molecules at a time. Also, the complex with two tRNAs has a particular architecture in which a noncatalytic SepSecS homodimer binds and posi-

### Table 3

**Experimental \( R_g \) values for holo SepSecS and SepSecS-tRNA<sup>Sec</sup> samples**

The experimental radius of gyration (\( R_g \)) was calculated with PRIMUS (40, 41) using the Guinier plot obtained from the SAXS data collected for each sample.

| Molar ratio (tRNA<sup>Sec</sup>:SepSecS tetramer) | Experimental \( R_g \) Å |
|-------------------------------------------------|-------------------------|
| 0:1 (holo SepSecS)                              | 43.15 ± 0.00            |
| 2:1                                             | 48.45 ± 0.36            |
| 4:1                                             | 49.39 ± 0.11            |
| 6:1                                             | 48.54 ± 0.00            |

### Table 4

**Theoretical \( R_g \) values for holo SepSecS and SepSecS-tRNA<sup>Sec</sup> complexes**

The theoretical \( R_g \) values were calculated in CRYSOIL (45). The crystal structures of holo SepSecS, SepSecS complexed with two tRNA<sup>Sec</sup> molecules, and models of SepSecS in complex with one, three, or four tRNAs were used for calculations.

| Sample                      | Theoretical \( R_g \) Å |
|-----------------------------|-------------------------|
| holo SepSecS                | 41.8                    |
| SepSecS-tRNA<sup>Sec</sup>  | 49.2                    |
| SepSecS-(tRNA<sup>Sec</sup>)<sub>2</sub> | 51.6                    |
| SepSecS-(tRNA<sup>Sec</sup>)<sub>3</sub> | 53.4                    |
| SepSecS-(tRNA<sup>Sec</sup>)<sub>4</sub> | 55.0                    |

**FIGURE 4.** The SepSecS-tRNA<sup>Sec</sup> complexes of different architecture from the one observed in the crystal do not fit into the SAXS envelope of the binary complex. A and B, complexes in which two tRNAs (green) are bound to different SepSecS homodimers (shades of red and blue) were superimposed into the SAXS envelope (beige). The complex shown in A contains two tRNAs bound to the opposite sides of the tetramer, whereas in the complex shown in B, both tRNAs are bound to the same side of the tetramer. In both instances, one tRNA does not fit into the envelope. C, our analysis also shows that a complex between SepSecS and four tRNA<sup>Sec</sup> molecules does not fit into the SAXS envelope of the binary complex. In this case, only the enzyme fits into the envelope, whereas all tRNAs are positioned partially outside the map. The surface representation of the model used in the fitting is shown to orient the reader. The data analysis and structural comparison were done as described in the text.
Stoichiometry of the Human SepSecS-tRNA^{Sec} Binary Complex

tions tRNAs toward the active site crevices in the catalytic homodimer. Finally, these results show that the architecture of the SepSecS-tRNA^{Sec} complex is the same in solution as in the crystal.

SepSecS Is Present in Abundance over tRNA^{Sec} in Vivo—Biophysical analyses of the SepSecS-tRNA^{Sec} complex presented herein and elsewhere (6) were done under in vitro conditions. Although the main conclusions of these studies are largely consistent, the question could be raised of whether the findings are relevant to a physiological situation at all. We therefore sought to estimate what would be the stoichiometry of the binary complex in vivo. In particular, we determined the molar ratio of SepSecS and tRNA^{Sec} in selenium supplemented human cells. Our results show that for three SepSecS tetramers there is approximately one tRNA^{Sec}, suggesting that the binary complex observed in biophysical studies in the presence of tRNA excess recapitulates the actual complex formed in live cells.

To estimate the ratio between SepSecS and tRNA^{Sec} in live cells, we performed quantitative Northern and Western blots on lysates from human hepatoma cell line HepG2. Total tRNA from selenium supplemented HepG2 cells and defined amounts of in vitro transcribed tRNA^{Sec} were analyzed by denaturing acid-urea gel electrophoresis. The RNA was electroblotted and probed with a 32P-labeled oligonucleotide probe complementary to the tRNA^{Sec} anticodon loop (Fig. 5A). The signal from samples containing in vitro transcribed tRNA^{Sec} was used to calculate a standard curve from which the quantity of tRNA^{Sec} in the cell lysate was determined. A similar approach was used to estimate the SepSecS levels in HepG2 cells. Varying amounts of the recombinant purified His-tagged SepSecS were analyzed by Western blot, and the resulting standard curve was used to estimate the amount of SepSecS in HepG2 whole cell lysates (Fig. 5B). The ~55-kDa band in the cell lysate, which is recognized by the commercial anti-SepSecS antibody, was used for quantitation as it corresponds to the expected size of endogenous untagged human SepSecS. The antibody also recognized several bands of lower molecular weight. We performed antigen competition experiments and found that all three of the lower molecular weight bands disappeared when purified SepSecS was preincubated with the antibody (data not shown). These lower molecular weight bands may represent yet unreported SepSecS species that result from alternative splicing. Although warranted, further characterization of these species was beyond the scope of this study. Finally, the quantitative analysis of our data from at least five independent experiments for each macromolecular species indicates ~13:1 molar ratio between the SepSecS monomer and tRNA^{Sec} (Fig. 5C). Thus, the ratio between the SepSecS tetramer and tRNA^{Sec} in vivo is ~3.25:1. Because of the significant molar excess of holo SepSecS over tRNA^{Sec} under physiological conditions, we propose that SepSecS bound to one tRNA^{Sec} represents a dominant catalytic complex in vivo.

DISCUSSION

Accurate protein translation is of vital importance for the integrity of cellular processes and for organism survival (1). Perhaps the most important factor involved in maintaining efficient translation is the ability of aaRS to correctly pair each genetically encoded amino acid with their cognate tRNA(s) (2, 11). However, a specific aaRS that would pair Sec and tRNA^{Sec} never evolved. Instead, in all domains of life, a set of enzymes has evolved to catalyze the formation of Sec in an indirect aminocacylation pathway. In bacteria, SerRS and SelA catalyze Sec synthesis, whereas in archaea and eukaryotes, SerRS, O-phosphoseryl-tRNA^{Sec} kinase, and SepSecS are required. SepSecS, a member of the type fold I family of PLP-dependent enzymes, catalyzes the terminal synthetic reaction of Sec. The structural and biochemical studies have shown that SepSecS forms a stable tetramer, that its catalytic mechanism is likely based on the PLP co-factor, and that the tetrameric structure is important...
for tRNA recognition (6). We have previously shown that the SepSecS tetramer binds only two tRNA^{Sec} molecules in the crystal (6). Because the remaining binding sites were hindered by crystal contacts, a question was raised regarding whether the crystallized complex faithfully recapitulated the physiologically relevant catalytic complex and whether SepSecS could simultaneously bind more than two substrate tRNAs. Herein, we determined the stoichiometry and architecture of the SepSecS-tRNA^{Sec} binary complex in solution by employing the methods of AUC, MALs, and SAXS. In addition, we assessed for the first time the molar ratio between SepSecS and tRNA^{Sec} in live cells and ascertained the significance of the results obtained under in vitro conditions.

Our tryptophan fluorescence quenching experiments have shown that SepSecS binds unacylated tRNA^{Sec} with high affinity (78 nM) and that solution-based methods can be employed to study the binary complex. The AUC data revealed that tRNA^{Sec} and SepSecS interact and form a stable complex in solution and that complex formation is not greatly affected by an abundance of tRNA. The SepSecS holoenzyme and free tRNA^{Sec} sediment at ~9 and 4 S, respectively, and the shape of the corresponding van Holde-Weischet curves suggests that neither form self-aggregates under the experimental conditions (Fig. 1A). In samples containing mixtures of tRNA^{Sec} and SepSecS, the simultaneous appearance of the 11 S species and the disappearance of the 9 S species were observed. These results suggest that SepSecS forms a stable complex with tRNA^{Sec} and that the 11 S species represents a binary complex. Interestingly, when there are two tRNAs present for every SepSecS tetramer, the majority of tRNA^{Sec} is bound to the enzyme, whereas further addition of tRNA^{Sec} (i.e. 4:1, 6:1, and 8:1 molar ratios) increases the fraction of free tRNA^{Sec} and not that of the complex. This argues against the concentration-dependent interaction and suggests that SepSecS could bind up to two tRNAs at a time. Interestingly, both 6:1 and 8:1 samples contain a species that sediments at 13 S (Tables 1 and 2) and that could represent SepSecS in complex with more tRNAs than the number found in the 11 S binary complex. Our measurements show that the relative abundance of 13 S is ~3.5-fold lesser than that of 11 S. Based on the AUC data, we determined that SepSecS and tRNA^{Sec} form a complex in solution and that adding a large molar excess of tRNA^{Sec} to SepSecS does not have a major effect on the sedimentation coefficient of the presumed 11 and 13 S complex species.

Samples similar to those analyzed by AUC were subjected to SEC-MALS analysis with the aim to determine the exact molecular mass of each macromolecular species present in the binary complex sample and to determine how many tRNA molecules are bound to the enzyme. It is clear from the SEC elution profile that the SepSecS-tRNA^{Sec} complex elutes prior to holo SepSecS and tRNA^{Sec} (Fig. 2A). In agreement with the AUC data, we observed two major complex species: the SepSecS tetramer in complex with one tRNA, which appears with the frequency of ~60–80%, and a complex with two tRNAs that constitutes ~20–35% of the complex sample. Given that a similar difference in abundance was observed between the 11 and 13 S particles, we propose that the 11 and 13 S species represent a complex of SepSecS with one and two tRNA^{Sec} molecules bound, respectively. Further, we show that the simultaneous binding of three or four tRNAs occurs with the frequency of less than 3% and that these binding events are insensitive to tRNA concentration. Intriguingly, the fraction of the complex with two tRNAs appears to be decreasing with an increase in molar excess of tRNA^{Sec}. In simplistic terms, the result could be a consequence of the partial overlap between peaks containing SepSecS complexed with one and two tRNAs. As a consequence, the fraction of the particular complex could only be estimated to be within a certain range, but the precise value would be difficult to determine. Alternatively, the presence of the trend argues that it is plausible that the binding of tRNA^{Sec} to SepSecS could be regulated by positive and negative allostery at low and high substrate concentrations, respectively. Further analyses by kinetic assays would be instrumental in delineating between the two scenarios. In conclusion, the SEC-MALS results clearly demonstrate that SepSecS preferentially binds up to two tRNAs at a time in solution. However, the ability of SepSecS to bind and presumably act on more than two tRNAs could be important when the demand for selenoprotein synthesis is increased. Further studies to determine whether SepSecS is capable of simultaneously acting on four tRNA^{Sec} molecules and whether such activity is regulated are warranted.

In addition to the stoichiometry, we sought to determine the architecture and arrangement of the SepSecS-tRNA^{Sec} complex in solution. The previously published crystal structure revealed SepSecS in complex with two tRNA^{Sec} molecules that interacted with the tetrameric enzyme in a cross-dimer fashion; one SepSecS dimer acted as a noncatalytic platform that oriented the CCA ends of both tRNAs toward the active site grooves in the other, catalytic dimer. To test the accuracy of the tRNA arrangement in the crystal structure, we determined the architecture of the complex in solution by SAXS. We utilized a setup in which a size exclusion column was directly coupled to the SAXS sample cell. The advantage of this system was that each species in the sample could be analyzed by SAXS as long as it eluted from the column separately from the other components in the sample. Our results show pronounced differences in the corresponding P(r) distribution curves (Fig. 3A), which suggest that the size and shape of the binary complex and holo SepSecS are different. The increase in R_g value from ~43 Å for holo SepSecS to ~49 Å in the binary complex samples supports that the binary complex is larger than holo SepSecS. Also, the visual inspection of the corresponding SAXS envelopes revealed that the binary complex has a different shape than holo SepSecS (Fig. 3B). Given that the theoretical R_g values of SepSecS in complex with one or two tRNA^{Sec} are almost indistinguishable (49.2 and 51.6 Å, respectively) and close to the experimental R_g value for the binary complex (~49 Å), we concluded that SepSecS most likely binds either one or two tRNAs at a time in solution. However, these calculations could not establish the architecture of the tetramer in complex with two tRNAs. The structural comparison between the experimental SAXS envelope calculated from the binary complex peak(s) and the three-dimensional models representing all plausible combinations between SepSecS and two tRNAs (Figs. 3C and A and B) revealed that only the arrangement observed in the crystal agreed with the
SAXS envelope (Fig. 3C). However, it is evident that the SAXS envelope of the binary complex is not completely symmetrical; the “top” envelope extension is smaller in size than the one on the “bottom” (Fig. 3C). Because the envelope is a result of averaging over the entire complex peak, it could well be that these slight differences are due to partial occupancy of one of the bound tRNAs, in this case the “top” molecule. Alternatively, a somewhat incomplete density may be a consequence of the disordered anticodon loops, which was also observed in the crystal (6). In either case, the SAXS envelope clearly shows that SepSecS binds two tRNA\textsuperscript{Sec} molecules in the same cross-dimer fashion in solution as in the crystal.

The biophysical characterization of the complex was completed under \textit{in vitro} conditions in which the molar ratio between tRNA\textsuperscript{Sec} and SepSecS was artificially adjusted. In live cells, however, we estimated that for every tRNA\textsuperscript{Sec} molecule there are \(\sim 3.25\) SepSecS tetramers, suggesting that the binary complex most likely contains one tRNA\textsuperscript{Sec} bound. Indeed, when a sample with a similar molar ratio was analyzed by AUC and SEC-MALS, we found that the SepSecS tetramer is in complex with only one tRNA\textsuperscript{Sec} molecule (data not shown). It is important to note that the molar ratio determined in HepG2 cells is an estimate applicable to that cell type and that more studies are needed to establish the general physiological ratio between SepSecS and tRNA\textsuperscript{Sec} in human cells. Taken together, our results argue that SepSecS binds and acts on tRNA\textsuperscript{Sec} in substoichiometric ratio. This is in contrast to the homodecameric SelA, the bacterial homolog of SepSecS, which employs all sites to bind 10 tRNA\textsuperscript{Sec} molecules at a time.

The structural asymmetry of SepSecS described in this study is, however, not unprecedented. A number of oligomeric aaRS have previously been shown to exhibit activity of half of the sites despite containing multiple, seemingly chemically equivalent active and tRNA-binding sites (15–28, 30). The mode of tRNA binding described herein argues that SepSecS may follow the sequential model of allosteric regulation (49). In this model (Fig. 6), the binding of the first tRNA molecule to a particular dimer (site 1, dimer 1) may facilitate binding of the second tRNA to a site located on the opposite side of the same dimer (site 1’, dimer 1). Concurrently, both catalytic sites on dimer 1 would be inaccessible through slight, yet undefined, structural changes. As a consequence, the binding affinity of tRNA to sites 2 and 2’ in the second dimer (dimer 2) would be significantly reduced, and the complex would contain up to two tRNA molecules bound. Alternatively, the binding of tRNA to sites 1 and 1’ could negatively regulate binding to sites 2 and 2’ and vice versa. For instance, the binding of tRNA to site 1 may cause a conformational change that would reduce binding affinity of tRNA to site 2 in the neighboring dimer (Fig. 4B). Also, the negative charge repulsion from the tRNA bound to site 1 could hinder the binding of tRNA to site 2’ (Fig. 4A). In contrast, neither the local structural changes nor the strong negative charge of tRNA would have any effect on the tRNA binding to a site located on the same dimer and on the opposite side of the tetramer (i.e. site 1’, dimer 1). In either scenario, in the complex between SepSecS and two tRNAs, one dimer seems to be a noncatalytic, docking platform, whereas the other one plays the role of the catalytic unit.
However, it is not clear why and whether the structural asymmetry observed is needed or required by SepSecS. Perhaps one reason could be that the negative charge repulsion is minimal when two tRNAs are bound to the opposite sides of the same homodimer. In any other arrangement, either acceptor or anticodon stems of the neighboring tRNAs would be in proximity and could destabilize the binary complex. Another explanation could be that binding of one or two tRNAs induces an as yet unidentified, conformational change that permits formation of multienzyme complexes (with SerRS and/or O-phosphoseryl-tRNA\textsuperscript{Sec} kinase) that could employ a substrate channeling mechanism for selenocysteine formation. Further kinetic and structural studies are needed to fully understand whether the structural diversity and protein engineering of the SepSecS-t\textsuperscript{Sec} complex is present at the functional level and to establish whether such asymmetry plays a regulatory role in selenocysteine and selenoprotein synthesis. This knowledge could be of importance for understanding how recently identified mutations in SepSecS that cause neurological disorders (50, 51) may affect the structure and function of the enzyme, selenocysteine and selenoprotein synthesis, and the downstream cellular processes.

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