Structure and Catalytic Mechanism of Eukaryotic Selenocysteine Synthase

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In eukaryotes and Archaea, selenocysteine synthase (SecS) converts O-phospho-l-seryl-tRNA[Ser] into selenocysteyl-tRNA[Ser]Sec using selenophosphate as the selenium donor compound. The molecular mechanisms underlying SecS activity are presently unknown. We have delineated a 450-residue core of mouse SecS, which retained full selenocysteyl-tRNA[Ser]Sec synthesis activity, and determined its crystal structure at 1.65 Å resolution. SecS exhibits three domains that place it in the fold type I family of pyridoxal phosphate (PLP)-dependent enzymes. Two SecS monomers interact intimately and together build up two identical active sites around PLP in a Schiff-base linkage with lysine 284. Two SecS dimers further associate to form a homotetramer. The N terminus, which mediates tetramer formation, and a large insertion that remodels the active site set SecS aside from other members of the family. The active site insertion contributes to PLP binding and positions a glutamate next to the PLP, where it could repel substrates with a free α-carboxyl group, suggesting why SecS does not act on free O-phospho-l-serine. Upon soaking crystals in phosphate buffer, a previously disordered loop within the active site insertion contracted to form a phosphate binding site. Residues that are strictly conserved in SecS orthologs but variant in related enzymes coordinate the phosphate and upon mutation disrupt SecS activity. Modeling suggested that the phosphate loop accommodates the γ-phosphate moiety of O-phospho-l-seryl-tRNA[Ser]Sec and, after phosphate elimination, binds selenophosphate to initiate attack on the proposed aminoacryl-tRNA[Ser]Sec intermediate. Based on these results and on the activity profiles of mechanism-based inhibitors, we offer a detailed reaction mechanism for the enzyme.

Organisms that co-translationally incorporate selenocysteine (Sec)2 into proteins in response to UGA codons in mRNA are frequently encountered in all three domains of life, prokaryotes, Archaea, and eukaryotes. The human genome contains 25 known genes encoding selenoproteins (1), several of which have essential functions. Selenoprotein biosynthesis requires two specialized metabolic pathways, the first to synthesize Sec and the second to incorporate Sec into proteins. The benefits of selenoproteins, e.g. their unique redox and catalytic properties, apparently outweigh selenium toxicity and the burden of maintaining intricate Sec synthetic and decoding machineries.

Notwithstanding some common themes, both Sec synthesis and decoding (for reviews, see Refs. 2–4) differ in bacteria and eukaryotes, and archaeal selenoprotein biosynthesis largely follows the eukaryotic schemes (5). In all organisms a special tRNA[Ser]Sec bearing an anticodon complementary to the UGA codon is central to both processes (6, 7). All tRNA[Ser]Sec species exhibit a number of non-canonical features whereby they are exclusively utilized for the Sec synthesis and Sec insertion pathways (8).

For Sec insertion at UGA codons, selenocysteyl-tRNA[Ser]Sec is recognized by a special elongation factor, SelB in bacteria (9) and EFSec in eukaryotes (10, 11), that replaces EF-Tu and EF-1α in escorting the aminoacylated Sec-tRNA[Ser]Sec to the ribosome. Bacterial SelB binds a stem-loop structure located within the coding region of selenoprotein mRNAs and directly downstream of a UGA codon (12). Such stem-loop structures thereby act as Sec insertion sequence elements that recode UGA, which normally signals translational termination. In contrast to bacteria, eukaryotic Sec insertion sequence elements are located in the 3′-untranslated regions of selenoprotein mRNAs (13), alleviating the burden of having to maintain functional secondary structures within the coding region and facilitating insertion of more than one Sec into a single protein chain. Such recognition from a distance requires a special adaptor protein, Sec insertion sequence-binding protein 2 (14). Additional factors appear to be involved in UGA recoding in
higher organisms. For example, SECP43 was recently found to associate with a Sec-tRNA^{Ser}\[Sec]\-EFsec complex in vitro and to enhance the interaction between EFsec and Sec insertion sequence-binding protein 2 in vivo (15). SECP43 also influences the specific post-transcriptional modification of tRNA^{Ser}\[Sec] (16). Furthermore, a role for ribosomal protein L30 in Sec decoding on the ribosome has recently surfaced (17). Sec synthesis invariably takes place on tRNA^{Ser}\[Sec] (6, 7, 18), which is initially aminocoylated with serine by seryl-tRNA^{Ser} synthetase (6, 19). The pathways then diverge in the different domains and recapitulate a trend seen with the decoding systems; i.e. eukaryotes rely on a more complex ensemble of factors. In bacteria, a pyridoxal phosphate (PLP)-dependent enzyme, selenocysteine synthase (SelA), uses selenophosphate (SeP) to directly convert Ser-tRNA^{Ser}\[Sec] into Sec-tRNA^{Ser}\[Sec] enzyme, selenocysteine synthase (SelA), uses selenophosphate (SeP) to directly convert Ser-tRNA^{Ser}\[Sec] into Sec-tRNA^{Ser}\[Sec] (20). However, in eukaryotes Ser-tRNA^{Ser}\[Sec] is first phosphorylated by O-phospho-l-seryl (PSer)-tRNA^{Ser}\[Sec] kinase to produce PSer-tRNA^{Ser}\[Sec] (21). Phosphate apparently is required as a leaving group by eukaryotic Sec synthase (SecS), which subsequently gives rise to Sec-tRNA^{Ser}\[Sec]. In bacteria SeP is provided by selenophosphate synthetase, SelD (22). Eukaryotes harbor two homologs of SelD, selenophosphate synthetases 1 and 2 (23), but only selenophosphate synthetase 2 can synthesize SeP for Sec synthesis (24). The molecular identity of eukaryotic/archaeal SecS has only recently been elucidated. An archaeal open reading frame, annotated as SecS, did not act on Ser-tRNA^{Ser}\[Sec] or PSer-tRNA^{Ser}\[Sec] (25). RNA-mediated interference technology provided the first direct evidence for an essential role of soluble liver antigen/liver and pancreas antigen (SLA/LP) in selenoprotein biosynthesis (16). SLA/LP was originally identified as the target of autoantibodies from patients with a severe form of autoimmune chronic active hepatitis (26, 27). Indeed, SLA/LP interacted with SECP43 and tRNA^{Ser}\[Sec] as detected by co-immunoprecipitation (16, 26, 28). Structural homology modeling predicted that SLA/LP is a PLP-dependent enzyme of the aspartate aminotransferase family (29). Unequivocal evidence that SLA/LP embodied the elusive eukaryotic/archaeal SecS was finally provided independently by two groups (28, 30) who directly demonstrated the conversion of PSer-tRNA^{Ser}\[Sec] to Sec-tRNA^{Ser}\[Sec] by the enzyme. The identification of eukaryotic/archaeal SecS paved the way for understanding the molecular mechanisms underlying its catalytic activity. Xu et al. (28) showed that mouse SecS is able to dephosphorylate PSer-tRNA^{Ser}\[Sec], indicating that aminoacyl-tRNA^{Ser}\[Sec] is a likely intermediate in the reaction. It was also unequivocally established that SecS employs SeP produced by selenophosphate synthetase 2 as the activated selenium donor (24, 28). Apart from these aspects, the enzyme is presently enigmatic. In particular, it is not known (i) how SecS recognizes its two substrates (PSer-tRNA^{Ser}\[Sec] and SeP) and whether binding occurs concomitantly or sequentially, (ii) how SecS differs from related enzymes that recognize low molecular weight amino acid substrates, (iii) whether and how the enzyme discriminates against free PSer, and (iv) which residues participate in PSer to Sec conversion on tRNA^{Ser}\[Sec].

Here we report on a combined structural and biochemical analysis of a mammalian SecS which illuminates the above questions. We determined high resolution crystal structures of SecS from mouse (mmuSecS) in which we find that the active site is constructed around a Lys-284-bound PLP cofactor at the interface of two protomers of a close dimer. A SecS-specific N terminus leads to association of two dimers into a homotetramer, which has possible relevance for PSer-tRNA^{Ser}\[Sec] positioning. The active site is complemented by a SecS-specific loop, which is disordered in the absence of ligands but contracts in the presence of a substrate-mimicking phosphate moiety. A glutamate neighboring the PLP cofactor is ideally positioned to deter substrates bearing a free α-carboxyl group. In line with the structural results, we show that changes of phosphate-coordinating residues, which are solely conserved in SecS orthologs, lead to reduced activity of the enzyme and that SecS does not act on free PSer. Using in addition the response of SecS toward mechanism-based inhibitors, we propose a detailed catalytic mechanism.

**EXPERIMENTAL PROCEDURES**

**Protein Production and Site-directed Mutagenesis** —A DNA fragment encoding the full-length secS gene from mouse (GenBank™ accession number NM_172490) was amplified and cloned into pETM-13 vector to allow the expression of a C-terminal His\_\_\_\_\_\_tagged protein. The insert was verified by DNA sequencing. The resulting plasmid was termed pETM-13-secS.

Rosetta2(DE3) cells were transformed with the pETM-13-secS expression construct. Overproduction of the target protein was carried out at 289 K using auto-inducing medium (31). Cells were harvested when the maximum culture density was reached and resuspended in buffer A (50 mM HEPES-NaOH, pH 7.5, 500 mM NaCl, 20 mM imidazole, 2 mM β-mercaptoethanol). 1 mg of lysozyme and 3 μl of Dnase (1 mg/ml) were added per gram of wet cells, and the mixture was incubated for 30 min on ice with stirring. After completing cell rupture by sonication and removing cell debris by centrifugation, soluble fusion protein was captured on a nickel-nitrilotriacetic acid-Sepharose column (Qiagen), washed with buffer B (50 mM HEPES-NaOH, pH 7.5, 1 mM NaCl, 20 mM imidazole, 2 mM β-mercaptoethanol), and eluted with a linear gradient of imidazole (20 to 250 mM) in buffer A. Fractions containing mmuSecS were pooled, concentrated, and further purified by gel filtration on a Superdex-200 HiLoad 26/60 column (GE Healthcare) equilibrated with buffer C (10 mM HEPES-NaOH, pH 7.5, 500 mM NaCl, 2 mM dithiothreitol). Purified target protein was concentrated to 14 mg/ml using Vivaspin 15 concentrators (30,000 MWCO; Sartorius Vivascience), separated into aliquots, flash-frozen in liquid nitrogen, and stored at 193 K until use. Under the above protocol we obtained ~1.5 mg of mmuSecS per liter of bacterial culture at a purity of greater than 95% as estimated by SDS-PAGE analysis (see Fig. 1A).

Mutants of mmuSecS_{Arg-313} and mmuSecS_{Gln-105} were generated in pETM-13-secS via the QuikChange protocol (Stratagene). The mutations were verified by DNA sequencing. All mutants of mmuSecS were purified in the same way as the wild type protein.

**Limited Proteolysis and Analytical Gel Filtration Analysis** —50 μl of mmuSecS (14 mg/ml) were incubated with 3 μg of elastase at 277 K for 80 min. The major band on an SDS gel
involving PSer-tRNA[Ser]Sec preparation.
tRNA[Ser]Sec was gel-purified.
cription mixture was incubated for 2 h at 310 K, and synthetic
was in-gel-digested with trypsin, and fragments were analyzed
by matrix-assisted laser desorption ionization-mass spectros-
tomy system (GE Healthcare).

In Vitro Transcription and Filter Binding Assay—The tem-
plate for in vitro transcription was prepared as described (21).
Uniformly 32P-labeled tRNA[Ser]Sec was transcribed in 50 μl of
transcription buffer (30 mm HEPES-NaOH, pH 7.5, 200 mm
NaCl, 5 mm MgCl2, 1 mm dithiothreitol, 0.5 mg/ml total E. coli tRNA).
10–μl aliquots of the reaction mixtures were loaded on a
Protran BA 83 nitrocellulose membrane (Whatman) and washed with 50 ml of buffer C. For the detection of
RNA-protein complexes, the membrane was exposed to a
PhosphorImager screen overnight, which was then scanned
using a Typhoon 8600 (GE Healthcare).

SecS Activity Assay—Synthetic tRNA[Ser]Sec was used in all
reactions for assaying SecS activity. Synthetic tRNA[Ser]Sec was
aminoacylated with serine by seryl-tRNA[Ser]Sec synthetase, the seryl
moiety was phosphorylated (21), and the PSer-tRNA[Ser]Sec was
isolated (28). The extent of serylation of tRNA[Ser]Sec in the
presence of seryl-tRNA[Ser] synthetase, serine, and other reaction
components was 80–90%, and subsequent phosphoryla-
tion in the presence of PSer-tRNA[Ser]Sec kinase, ATP, and
other reaction components reached 100% (Refs. 21 and 28 and
references therein). Because less than 20% of the
seryl-tRNA[Ser]Sec was deacylated during their preparation and use (Ref. 21 and
references therein), the relative amounts of tRNA[Ser]Sec, seryl-
tRNA[Ser]Sec, and PSer-tRNA[Ser]Sec used in reactions with SecS generating Sec-tRNA[Ser]Sec can be estimated to be ~45:0:55,
respectively, wherein an ~20% deacylation occurs at both steps
involving PSer-tRNA[Ser]Sec preparation.

The activities of mmuSecSelast and of the mmuSecS point
mutants relative to that of the full-length wild type mmuSecS were
determined. Thioredoxin (Trx) was used as a negative control.
Sec synthetic reactions were carried out as described
originating from elastase treatment (mmuSecSelast, see Fig. 1A)
was in-gel-digested with trypsin, and fragments were analyzed by
matrix-assisted laser desorption ionization-mass spectros-
copy as described (32).
Analytical gel filtration was conducted on a Superdex-200 PC
3.2/30 size exclusion column (2.4-ml gel bed, column dimen-
sions 3.2 × 300 mm) on a SMART fast protein liquid chroma-
tography system (GE Healthcare). Escherichia coli SelA (a
decamer of ~506 kDa), Saccharomyces cerevisiae cystathionine
γ-lyase (a tetramer of ~170 kDa), and Methanocaldococcus
jannaschii SelA-like protein (MJ0158; a dimer of ~84 kDa)
served as size standards.

In Vitro Transcription and Filter Binding Assay—The template for in vitro transcription was prepared as described (21).
Uniformly 32P-labeled tRNA[Ser]Sec was transcribed in 50 μl of
transcription buffer (30 mm HEPES-NaOH, pH 7.5, 200 mm
NaCl, 5 mm MgCl2, 1 mm dithiothreitol, 0.5 mg/ml total E. coli tRNA). 10–μl aliquots of the reaction mixtures were loaded on a
Protran BA 83 nitrocellulose membrane (Whatman) and washed with 50 ml of buffer C. For the detection of
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The activities of mmuSecSelast and of the mmuSecS point
mutants relative to that of the full-length wild type mmuSecS were
determined. Thioredoxin (Trx) was used as a negative control.
Sec synthetic reactions were carried out as described
(28). The selenium donor, SeP, was generated from chemically
synthesized [(CH3)3SiO]3PSe (28), which was a generous gift of
Dr. Richard Glass, and 0.2 mM SeP was used in all Sec synthesis
assays.

Dephosphorylation Activity of mmuSecS—Dephosphoryla-
tion of PSer-tRNA[Ser]Sec was carried out as described (28) with
the following modifications. In the inhibition assays, mmuSecS was incubated with different concentrations of propargylgly-
ceine (PG) or trifluoroalanine (F3-Ala) for 3 min at room tem-
perature in a 10-μl volume of buffer (28), 100 ng of 32P-labeled
and 1.0 μg of cold PSer-tRNA[Ser]Sec were added to bring the
total volume to 20 μl, and the reaction was incubated at 310 K
for 15 min. To assess the activity of mmuSecS on free PSer, a stock solution of 32P-PSer was prepared by deacylating 0.5 μg of
32P-PSer-tRNA[Ser]Sec in 25 μl at pH 9.0 and 315 K for 1 h. 5 μl
of 32P-PSer were added to each of the mmuSecS reactions and
then incubated for 15 min at 310 K. 10 units of alkaline
phosphatase were used to dephosphorylate 32P-PSer, and the
resulting phosphate was used as a positive control. Reactions
were prepared for chromatography and chromatographed as
given (28).

Crystallographic Procedures—For crystallization, 1.5 μl of
mmuSecSelast (14 mg/ml in buffer C) were mixed with an equal
volume of reservoir solution (11% v/v ethylene glycol without
other buffer components) in a 24-well Cryschem plate (Ham-
pton Research) immediately after elastase treatment. Crystals
were grown by sitting-drop vapor diffusion at 293 K. They
appeared overnight and continued to grow for the next 2 days.
For data collection, crystals of mmuSecSelast were shock-frozen
in a 100 K nitrogen stream (Oxford Cryosystems) after transfer
into a cryo-protecting buffer (100 mM HEPES-NaOH, pH 7.5,
250 mM NaCl, 1 mM dithiothreitol, 35% (v/v) ethylene glycol).
All data were recorded on a Bruker-Nonius FR591 rotating
anode generator producing CuKa X-radiation (λ = 1.54179 Å)
at 45 kV and 100 mA equipped with Osmic mirrors and a
MAR345 image plate (MARResearch). Data were processed with
the HKL package (33) (Table 1).

The structure of mmuSecSelast was solved via a single iso-
 morphous replacement with anomalous scattering strategy.
Crystals were soaked for 30 s in cryo-protecting buffer sup-
plemented with 0.5 M sodium iodide and immediately shock-
 frozen in a 100 K nitrogen stream. Friedel pairs were kept sep-
 arate during data reduction (Table 1). The iodide-soaked
crystals proved isomorphous to the native crystals. 42 iodide
positions were found by using SHELXD (34) and used for initial
phase calculations (Table 1). Solvent flattening with SHELXE
clearly indicated the correct hand of the heavy atom substructure
(Table 1) and yielded a high quality experimental electron
density map (supplemental Fig. S1).

424 of the 438 residues located in the final structure were
positioned in the first round of automatic model building with
ARP/wARP (35). The structure was completed by manual
model building and automatic refinement with Refmac5 (36).
Water molecules were automatically placed with ARP/wARP.
One solute species was identified as a chloride ion based on the
presence of 250 mM NaCl in the crystallization buffer and resid-
ual positive electron density after placement of a water mole-
cule. Another solvent molecule was interpreted as an ethylene
glycol molecule originating from the crystallization or cryo-protecting buffer. TLS refinement (37) was conducted to model differential global anisotropic displacements of the three domains of mmuSecSelast. During all stages of refinement, a randomly selected set of 5% of the reflections was used for cross-validation (Table 1). The iodide-soaked crystal structure was refined by the same strategy including the 42 iodide ions located by SHELXD. Additional, lower occupancy iodide positions were found in an anomalous difference Fourier map, obtained with phases calculated from the final refined native structure and the anomalous differences measured for the iodide data set.

For monitoring of phosphate binding, crystals were soaked for 1 min in cryo-protecting buffer supplemented with 0.5 M sodium phosphate, pH 7.5. Similarly, crystals could be derivatized with sulfate (not shown). Data were collected as described, and the structure of a phosphate-soaked crystal was solved by molecular replacement with MOLREP (38) using the structure coordinates of the native protein as a search model while omitting the solvent structure, the PLP cofactor, and alternative side chain conformations. Model building and refinement were conducted as described for the native protein (Table 1). Coordinates and structure factors have been submitted to the Protein Data Bank.

RESULTS

Limited Proteolysis Delineates a SecS Core Fully Active in PSer-tRNA[Sec][Sec] to Sec-tRNA[Sec][Sec] Conversion—We expressed, purified, and crystallized full-length SecS from mouse (mmuSecS), but the crystals diffracted only to ~6 Å resolution. To explore the possibility that flexible regions hindered generation of well ordered crystals, mmuSecS (~55 kDa) was digested with various proteases. Elastase gave rise to a stable fragment of about 49 kDa (Fig. 1A). Tryptic mass spectroscopic fingerprinting showed that the elastase-resistant fragment encompassed residues 19–468 (not shown). Thus, the protease removed most of the C-terminal portion that carries the main SLA/LP antigenic epitope (26, 27), also lacking in protein in the reactions are indicated. We next tested the Sec synthesis activity of mmuSecSelast using a paper chromatographic assay (28). After removal of elastase by gel filtration chromatography, mmuSecSelast was incubated with buffer containing SeP and PSer-tRNA[Sec][Sec] (obtained by phosphorylation of Ser-tRNA[Sec][Sec] by PSer-tRNA[Sec][Sec]) (21, 28). As positive and negative controls, mmuSecS and Trx, respectively, were substituted for mmuSecSelast. The efficiency at which mmuSecSelast converted PSer-tRNA[Sec][Sec] to Sec-tRNA[Sec][Sec] was indistinguishable from that of the full-length enzyme (Fig. 1D). The above results demonstrate that mmuSecSelast structurally and functionally closely resembles the full-length enzyme and constitutes a suitable platform on which to explore the structure-activity relationships of SecS-dependent Sec biosynthesis.

SecS Is a Member of the Fold Type I Family of PLP-dependent Enzymes with Distinct Structural Elements—mmuSecSelast crystallized readily after the addition of 11% (v/v) ethylene glycol at room temperature. The crystals diffracted to high resolution on a rotating anode x-ray generator and could be derivatized by quick-soaking in 0.5 M sodium iodide for structure solution by single isomorphous replacement with anomalous scattering (Table 1; Fig. S1). Refinement converged at R/Rfree factors of 16.8/19.8% (Table 1). The only amino acids lacking well defined electron density were residues 19–22 at the N terminus, residue 468 at the C terminus, and residues 98–104, constituting a flexible loop.

FIGURE 1. Characterization of mmuSecSelast. A, SDS-PAGE of mmuSecS (lane 1) and mmuSecSelast (lane 2). M, molecular weight markers. B, analytical gel filtration analysis of mmuSecS (red) and mmuSecSelast (gold), E. coli SelA (ecoSelA) (black, ~506 kDa), S. cerevisiae cystathionine γ-lyase (sceCGL; blue, ~170 kDa), and MJ0158 (green, ~84 kDa) served as molecular mass markers. Peaks containing the respective proteins (arrows) were verified by monitoring the absorbance at 240 nm and by SDS-PAGE analysis (not shown). The high molecular weight peak (left) in the full-length mmuSecS run represents a contaminating nucleic acid fraction. C, filter binding assay showing similar affinity of mmuSecS (top panel) and mmuSecSelast (bottom panel) for decylated tRNA[Ser][Sec]. Experiments were conducted in the presence of unlabeled competitor tRNA and, thus, represent specific affinities. Concentrations of protein in the reactions are indicated. D, conversion of [3H]PSer-tRNA[Sec][Sec] to [3H]Sec-tRNA[Sec][Sec] by mmuSecS (red) and mmuSecSelast (gold and blue, which are duplicates, mmuSecSelast 1 and mmuSecSelast 2). Trx (black) served as a negative control. Details are given under “Experimental Procedures.”
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Structural homology searches (39) suggested that mmu-SecSe last exhibits significant similarity to the fold type I family of PLP-dependent enzymes (also referred to as the aspartate aminotransferase family) (40). Where appropriate, we will compare the structure of mmuSecSe last to those of Archaeoglobus fulgidus PSer-cysteine synthase (afuPSerCysS) (41), members of the NifS family of Cys/Sec lyases (42–46), and the cystine C-S lyase C-DES from Synechocystis (synC-DES) (47). A quantitative comparison with these proteins is given in Table 2. These enzymes act or can act on related substrates and may share some catalytic properties with SecS. In particular, PSerCysS from methanogenic Archaea affords a precedence for the tRNA-based amino acid synthesis via a PSer-tRNA intermediate. In these organisms the sole pathway for cysteine biosynthesis is via PSer-tRNA Cys, obtained by direct aminocoylation of tRNA Cys with PSer by PSer-tRNA Cys synthetase, and subsequent PSer-tRNA Cys to Cys-tRNA Cys conversion by PSerCysS (48).

mmuSecSe last can be divided into three domains (Fig. 3, A and B). Domain 1 (blue scaffold in Fig. 3, A and B) is a composite of residues 23–130 and 313–330 and is purely α-helical (encompassing helices α1–α4 and α12). The N terminus (residues 23–44; element I in Fig. 3, A and B) differs from that of other fold type I enzymes (Fig. 3, C and D). In mmuSecSe last, helix α1 is positioned at the protein surface, running approximately perpendicular to the scaffolding helices of the domain. Domain 1 also exhibits a long insertion between helices α2 and α4 (residues 62–108; element II in Fig. 3, A and B), which encompasses two loops separated by helix α3. The corresponding element in other enzymes of the fold type I family is significantly shorter (Fig. 3, C and D). Seven residues within the second loop of the insertion (residues 98–104) are disordered due to intrinsic flexibility (bordered by spheres in Fig. 3A). Both the non-canonical N terminus and the unique insertion are conserved among SecS orthologs (Fig. 2) and, therefore, are expected to confer unique functions on the enzyme.

Domain 2 of SecS is the largest module of the protein (residues 131–312; cyan scaffold in Fig. 3, A and B). It comprises a α/β/α sandwich fold encompassing a seven-stranded β-sheet (β1–β9–β8–β7–β6–β2–β3) characteristic of the fold type I family. The β-sheet is parallel except for strand β9 (Fig. 3, A and B). A short helix (α10) between strands β8 and β9 carries a PLP cofactor in Schiff-base linkage to Lys-284 (Figs. 3A and 4). Helices α7, α8, and α9 line the β-sheet at the convex outside, and helices α5, α6, and α11 lie at the concave inside. A short β-hairpin (β4 and β5) is inserted between strand β3 and helix α7. Domain 2 is connected to the second part of domain 1 by a short loop (residues 311–314; element III in Fig. 3, A and B).

Domain 3 of SecS (residues 331–467; steel blue scaffold in Fig. 3, A and B) exhibits an α/β sandwich fold with three almost parallel helices (α13, α14, and α15) on the outside covering an antiparallel three-stranded β-sheet (β10–β14–β11), which in turn rests on top of domain 2. A long loop (residues 408–430; element IV in Fig. 3, A and B) with a β-hairpin (β12–β13) at the tip is inserted between strands β11 and β14. The three-stranded β-sheet and the β-hairpin of the loop are at approximately right angles and encircle part of domain 2 (Fig. 3A).

Strands β11 and β12 thereby form one rim of an active site funnel leading from the surface to the PLP (Fig. 3A). In the NifS-like enzymes, the analog of the long domain 3 loop (element IV) is often disordered (43) and bears a conserved Cys that can be charged in the active site with elemental sulfur. The resulting persulfide is thought to donate a S0 building block for iron-sulfur cluster biosynthesis. Element IV of mmuSecSe last does not contain a Cys.

Cross-strutting via the N Nermius Leads to Homotetramers That Exhibit Surface Properties Suitable for Binding PSer-tRNA Ser —mmuSecSe last crystals contained one protein molecule per asymmetric unit. Consistent with the gel filtration analysis, the orthorhombic crystal symmetry gave rise to tetramers in which the protomers are related by three orthogonal 2-fold axes (see Fig. 5A, left). Within a tetramer, two pairs of monomers (Mol I/II and Mol III/IV; Fig. 5A, left) interact intimately, burying 7343 Å² of combined surface area upon association. Two of these close dimers further associate into tetramers via less extensive interactions between Mol I and Mol III, viz. Mol II/Mol IV (1891 Å² combined surface area buried in each contact), and between Mol I and Mol IV, viz. Mol II/III (276 Å² combined surface area buried in each contact; Fig. 5A, left). The tetramers are held together by the formation of a short antiparallel coiled-coil between the α2 helices of Mol I and III (Mol II and IV), which is cross-strutted by helices α1 (Fig. 5B). In contrast, afuPSerCysS, NifS relatives, or synC-DES lack the surface-exposed N terminus (elements I in Fig. 3, C and D). Consistently, all these latter proteins exist as dimers.

Fig. 5C shows the electrostatic potential mapped to the surface of a mmuSecSe last tetramer. Large patches of positive charge (blue) are visible, consistent with the overall basic pl of 8.3 calculated for the protein. In particular, the funnel leading to the active site is strongly positively charged. Therefore, the surface properties of mmuSecSe last appear to be designed to contact the sugar-phosphate backbone of tRNA Ser at multiple positions. Consistent with this view, we observed avid binding of anions to mmuSecSe last. After soaking with NaCl, we located 62 iodide ions per protomer bound to the surface of mmuSecSe last (Fig. 5D). One of these positions was always occupied by a chloride ion in structures not treated with iodide (not shown).

The PLP Cofactor Is Tightly Anchored by Non-canonical Contacts to Both Protomers of a Close Dimer—A close dimer exhibits its two identical active sites at the protomer interfaces pinpointed by a PLP cofactor (Fig. 4A). Because the PLP was refined at full occupancy, leaving no residual difference density, all four subunits of a tetramer bear a cofactor. This situation is different from afuPSerCysS, where only one of two potential active sites in a dimer was equipped with PLP (41). We refer to the PLP attached to Lys-284 of a reference molecule and the surrounding active site as “cis”; the PLP attached to the opposite protomer and its surrounding active site are referred to as “trans.”

In mmuSecSe last, both monomers of a close dimer contribute side chains for PLP binding in an active site (Fig. 4A). Apart from the covalent linkage to Lys-284, PLP is additionally bound via multiple hydrogen bonds and electrostatic and van der Waals interactions in cis. These interactions
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TABLE 1
Crystallographic data

|                    | Native          | Iodide          | Phosphate        |
|--------------------|-----------------|-----------------|------------------|
| Data collection    |                 |                 |                  |
| Space group        |                 |                 |                  |
| Unit cell parameters (a, b, c; Å) |                 |                 |                  |
| Resolution (Å)     | 30.0-1.65 (1.74-1.65)* | 30.0-2.25 (2.38-2.25)* | 30.0-1.85 (1.95-1.85) |
| Reflections        |                 |                 |                  |
| Unique             | 68,502 (9,938)  | 52,394 (7,738)  | 49,649 (7,322)   |
| Completeness (%)   | 97.3 (95.3)     | 98.3 (97.0)     | 99.3 (99.2)      |
| Redundancy         | 4.7 (4.5)       | 4.7 (4.7)       | 3.5 (3.1)        |
| R(i/o)             | 20.5 (2.1)      | 13.4 (3.8)      | 16.3 (1.90)      |
| Rfree(i/o)         | 0.045 (53.9)    | 0.093 (35.4)    | 0.046 (54.2)     |

Phasing

| Resolution (Å)     | 30.0-2.0        |                  |                  |
| Heavy atom sites   | 42              |                  |                  |
| Correlation coefficient<sub>c</sub> | SHELXD Cc<sub>calc</sub>/Cc<sub>weak</sub> | 50.86/37.73 |                  |
| SHExLE Cc<sub>calc</sub>/Cc<sub>weak</sub> | 35.52 |                  |                  |
| CCoverall          | 62.69/26.92     |                  |                  |
| FOM<sup>d</sup>    | 0.582           |                  |                  |

Refinement

| Resolution (Å)     | 20.0-1.65 (1.69-1.65) | 30.0-2.25 (2.31-2.25) | 20.0-1.85 (1.99-1.85) |
| Reflections        |                 |                 |                  |
| Number             | 65,002 (4,662)  | 26,104 (1,839)  | 47,049 (3,157)   |
| Completeness (%)   | 97.3 (95.0)     | 98.0 (96.3)     | 98.8 (93.0)      |
| Test set (%)       | 5.1             | 5.0             | 5.1              |
| R<sub>c</sub>      | 0.168 (0.390)   | 0.183 (0.199)   | 0.166 (0.341)    |
| R<sub>free</sub>   | 0.198 (0.401)   | 0.237 (0.303)   | 0.202 (0.372)    |
| ESU (Å)            | 0.061           | 0.129           | 0.077            |

| Contents of asymmetric unit |                  |                  |                  |
| Protein molecules/residues/atoms | 1/433/3647 |                  |                  |
| Water oxygens          | 723             | 264             | 509              |
| PO<sub>4</sub><sup>-</sup>/Cl<sup>-</sup> | -/1/- |                  |                  |
| Mean B-factors (Å<sup>2</sup>) |                  |                  |                  |
| Wilson                 | 20.0            | 32.2            | 23.9             |
| Protein               | 15.1            | 26.9            | 21.0             |
| Ions                  | 38.4            | 28.7            | 38.2             |
| Lons                  | 16.5            | 45.9            | 45.9             |
| Ramachandran Plot<sup>f</sup> |                  |                  |                  |
| Favored               | 97.9 (424/433)  | 97.7 (421/431)  | 97.7 (431/441)   |
| Allowed               | 1.9 (8/433)     | 2.1 (9/433)     | 2.3 (10/441)     |
| Outliers              | 0.2 (1/433)     | 0.2 (1/433)     | 0.0              |
| r.m.s.d. target geometry |                  |                  |                  |
| Bond lengths (Å)      | 0.012           | 0.010           | 0.010            |
| Bond angles (*)       | 1.34            | 1.22            | 1.19             |
| r.m.s.d. B-factors (Å<sup>2</sup>) |                  |                  |                  |
| Main chain bonds      | 0.726           | 0.423           | 0.563            |
| Main chain angles     | 1.122           | 0.787           | 0.894            |
| Side chain bonds      | 2.005           | 1.402           | 1.604            |
| Side chain angles     | 3.152           | 2.318           | 2.540            |

<sup>a</sup> Data for the highest resolution shell in parentheses.
<sup>b</sup> R<sub>free</sub>(i/o) = \( \sum_{i,j} R_i(\|I_{\text{obs}}(hkl) - \langle I(\text{obs})\rangle\|) / \sum_{i,j} \langle I(\text{obs})\rangle \) for \( n \) independent reflections and \( i \) observations of a given reflection; \( \langle I(\text{obs})\rangle \), average intensity of the \( i \) observations.
<sup>c</sup> CC = \( \sum_{h} E_{\text{calc}}(h) - \sum_{h} E_{\text{calc}}(h) \), \( \sum_{h} E_{\text{calc}}(h) - \sum_{h} E_{\text{calc}}(h) \), \( \sum_{h} E_{\text{calc}}(h) - \sum_{h} E_{\text{calc}}(h) \), \( \sum_{h} E_{\text{calc}}(h) - \sum_{h} E_{\text{calc}}(h) \).
<sup>d</sup> Figure of merit (FOM) = \( |F(hkli)_{\text{calc}}| / |F(hkli)_{\text{obs}}| \), \( F(hkli)_{\text{calc}} = \sum P(\alpha) F(\alpha)(hkl) \), \( w \), weight (see SHExLE software for full definitions).
<sup>e</sup> Calculated with MolProbity (58).

FIGURE 2. Structure-based multiple sequence alignment of SecS and enzymes of the fold type I family. Structure-based multiple sequence alignment generated by the 3DCoffee-option of Tcoffee (57) and shaded by BoxShade. Sequences are numbered at the beginning of each line. The darker background represents higher conservation. The background of the PLP lysines is in green; the background of residues whose side chains contact PLP and of residues contacting the P1 phosphate is in red. P-loop residues undergoing a disorder-order transition upon phosphate binding are boxed in red. Residues contacting the P2 phosphate are indicated by a red bar. Secondary structure elements are color-coded by domain; blue, domain 1; cyan, domain 2; steel blue, domain 3. Secondary structure elements belonging to elements I–IV are in red, and helix \( \alpha 10 \) (bearing the PLP cofactor) is in gold. hsa, Homo sapiens; mmu, Mus musculus; dme, Drosophila melanogaster; cel, Caenorhabditis elegans; mma, Methanococcus maripaludis; mja, M. jannaschii; afu, A. fulgidus; syn, Synchocystis; eco, E. coli; tma, T. maritima.

exclusively involve residues from domain 2. The PLP phosphate group is positioned over the N terminus of helix \( a 5 \), interacting favorably with the helix macro-dipole and engaging in hydrogen bonds to the backbone amides of Thr-144 and Gly-145. The pyridine nitrogen maintains hydrogen bonds to the side chains of Cys-175 and Asn-252. Asn-252 is at variance with the vast majority of fold type I enzymes, in which an Asp at the equivalent position is the only strictly conserved residue apart from the PLP-bound Lys (40). An Asn is expected to support the electron sink character of the pyridine ring less than an Asp, possibly demanding a good leaving group such as a phosphate on the \( \beta \)-carbon. Interest-
ingly, in afaPSerCysS, in which phosphate is also the leaving group, the pyridine nitrogen is again bound to an Asn (Fig. 2) (41). The PLP pyridine ring of mmuSecSelast is sandwiched between the side chains of Gln-172 and Ala-254 on the re and si faces, respectively. Archaeal SecS enzymes feature a His in place of Gln-172 (Fig. 2). A similar His in a NifS-like protein from *Thermotoga maritima* has been discussed as a tunable acid-base catalyst in the reaction mechanism (43). Thus, the enzymatic mechanisms of archaeal and eukaryotic SecS may differ in detail.

Interactions with PLP in trans (Fig. 4, A and B) involve residues from the SecS-specific insertion in domain 1 (element II) and from the short element III connecting domain 2 and the second part of domain 1 (Fig. 3, A and B). Both elements primarily interact with the PLP phosphate. Arg-75 (originating from the first loop of element II) is deposited on the phosphate side of the PLP pyridine ring where its side chain can engage in two charged interactions with anionic phosphate oxygens. Arg-75 is appropriately positioned by a double salt bridge interaction with Asp-283 from the cis protomer (Fig. 4, A and B). The preceding residue of element II, Glu-74, comes to lie on the opposite side of the pyridine ring and is connected via water molecules to the C3 hydroxyl group of PLP and the nitrogen of the Schiff base. It is kept in place by van der Waals contacts to Tyr-255 of the cis protomer (Fig. 4, A and B). In addition, the backbone nitrogen of Arg-313 (from element III) hydrogen bonds to an anionic phosphate oxygen of the PLP.

In other fold type I PLP-dependent enzymes, element III is often significantly longer than in SecS (Fig. 3, C and D) and provides additional residues for binding the PLP phosphate. In contrast, the region corresponding to element II in afaPSerCysS is much shorter than in SecS (21 versus 47 residues) and is completely disordered in the structure (41), failing to provide stable PLP anchoring (Fig. 3, C). In syncC-DES, the equivalents of helices α2 and α4 are longer and place a very short element II, which is suspended between them, remote from the trans PLP and close to domain 3.

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**TABLE 2**

| PDB code | Reference | Sequence identity (%) | Matching Cα atoms | r.m.s.d. (Å) |
|-----------|-----------|-----------------------|-------------------|-------------|
| afaPSerCysS | 2E7J | 41 | 10.9 | 257 | 2.8 |
| tmaNifS-like Protein | 1EG5 | 43 | 13.1 | 283 | 2.8 |
| synC-DES | 1ELU | 47 | 11.7 | 266 | 3.0 |

*Structures were superimposed by secondary structure matching (59).*
of the other protomer (Fig. 3D). Thus, in other fold type I enzymes, element II serves to reinforce dimerization but does not contribute directly to the active sites. Instead, in *afuPSerCysS*, NifS relatives and *synC-DES* a portion of the N terminus is positioned between domains 1 and 3 and harbors residues, which in some cases contact the cis PLP (Fig. 3, C and D). In *mmuSecS* last, the first loop of the long element II replaces this N-terminal part in trans (Fig. 3A).

In contrast to the situation within a close dimer, there is no cross-communication at the active sites between molecules belonging to the two different close dimers of a tetramer. This situation suggests that the close dimers are sufficient to provide the chemical microenvironment required for catalysis.

**Binding of Phosphate Triggers Disorder-Order Transition in an Active Site Loop**—Substrates of SecS contain a number of phosphates or phosphate-related groups, such as the phosphodiester backbone of tRNA[Ser]Sec, the γ-phosphate of the PSer moiety, and SeP. We reasoned that phosphate could mimic binding of either of these groups at the active site of SecS and determined the crystal structure of *mmuSecS* last after soaking crystals for 30 s in 0.5 M phosphate buffer. Strikingly, we observed that a phosphate (P1 in Fig. 6) is cradled in the second loop of the domain 1 insertion (residues 98–104 of element II; green in Fig. 6), which was previously disordered. Upon phosphate binding, this loop contracts and covers part of the trans active site (Fig. 6, A and B). Arg-313 originating from the loop that connects domain 2 to the second part of domain 1 (element III in Fig. 3A and B) forms the base of the P1 phosphate binding site. To bind the P1 phosphate, Arg-313 and Gln-105 (neighboring the previously disordered element) are profoundly repositioned (Fig. 6, C and D). In addition, the P1 phosphate interacts directly as well as via a water bridge with the side chain of Arg-97 and with the side chain and backbone of...
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A

ecoCGS

B

C

D

E

mmuSecS

elast

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Ser-98, both of which were disordered in the absence of phosphate (Fig. 6, C and D).

A poly-dentate anion appears to be required to engage in the observed interactions and elicit the structural transition. In agreement with this notion, a similar disorder-order transition was observed upon soaking with sulfate ions (not shown), whereas mono-dentate anions did not evoke any such change. For example, 250 mM NaCl were present in all crystallization and soaking experiments, and we did not observe any conformational changes in the structure soaked with an additional 0.5 M sodium iodide (Table 1). We, therefore, refer to the loop between Gly-96 and Pro-106 of element II as the “phosphate loop” (P-loop).

Upon binding of the P1 phosphate, the P-loop closes off part of the active site of mmmuSecSelast (Fig. 6, A and B). It is likely that active site closure accompanies catalysis and may serve, e.g. to locally exclude bulk water. Therefore, the 3′-end of tRNA[Ser] most likely gains access to the PLP cofactor from the side opposite the P-loop (arrow in Fig. 6B).

We observed a second phosphate binding site remote from the active site (P2 in Fig. 6B). This phosphate location could indicate a site of contact to the tRNA[Ser]phosphodiester backbone. However, in contrast to the P-loop and Arg-313 (see below), residues contacting the P2 phosphate (Arg-199, His-368) are not conserved in SecS orthologs (Fig. 2). It is possible that the binding of the P2 phosphate is not functional and restricted to mouse and a few other SecSs.

**Phosphate Binding Is Mediated by Conserved and SecS-specific Residues That Are Essential for Selenocysteine Synthesis**—

The P-loop is a highly conserved element of eukaryotic and archaeal SecS, which is lacking from related enzymes with a different function (Fig. 2). In particular, Arg-97, Ser-98, and Gln-105 from the P-loop as well as Arg-313 from element III, which directly contact the P1 phosphate, are strictly conserved among SecS enzymes but not beyond. These observations are consistent with the idea that the P-loop carries out an essential function that is specific for SecSs.

To directly probe the importance of residues contacting the P1 phosphate, we generated mutant mmmuSecS proteins in which Gln-105 was changed to Glu (mmmuSecS[Q105E]) or Arg-313 was changed to Ser or Gln (mmmuSecS[R313S], mmmuSecS[R313E]). All mutants were expressed as soluble proteins in E. coli, migrated as tetraders in gel filtration, and exhibited a PLP complement comparable with that of the wild type protein, as indicated by their absorption maxima at 334 nm (ketimine form) and 418 nm (aldimine form; not shown). We tested the mutants for their ability to convert PSer-tRNA[Ser]Secto and dephosphorylate free PSer with SecS. Indeed, the enzyme proved to be completely unreactive with respect to the free amino acid (Fig. 7B).

We inspected the active site of mmmuSecSelast for possible filtering devices. Typically, PLP-dependent enzymes of the fold type I deploy a positively charged Arg in the neighborhood of the PLP to bind the negatively charged α-carboxylate of an amino acid substrate. In the absence of a substrate, a sulfate or phosphate group often binds at an equivalent position as the PLP to its catalytic competence. Based on these observations, we suggest that the P1 phosphate mimics binding of a substrate or of a functional portion of a substrate.

The P-loop Could Serve as a Binding Site for the Pser γ-Phosphate and SeP—We scrutinized the possibility that the γ-phosphate of Pser-tRNA[Ser] could be bound by the P-loop. To this end we modeled the structure of an external aldime comprising PLP in a Schiff-base linkage to a Pser esterified at the α-carboxylate. For modeling, we superimposed the structure of Pser-aminotransferase in complex with the substrate mimic α-methyl-l-glutamate (49) onto the mmmuSecSelast-phosphate structure. We replaced the α-methyl-l-glutamate moiety with a α-carboxyl ester of Pser, retained all side chain conformations as observed in the mmmuSecSelast structure in complex with phosphate, and allowed the PLP moiety to adopt a slightly more inclined orientation (Fig. 6E). Even without adjustments of the protein matrix, the γ-phosphate of the Pser ester could be accommodated approximately at the P1 phosphate position. These results suggest that the P-loop could serve to bind the γ-phosphate of Pser-tRNA[Ser].

The similarity of phosphate and SeP suggests that the P1 phosphate could also mimic binding of the co-substrate SeP to the P-loop. Binding of the Pser moiety and SeP to the P-loop could occur sequentially and is not mutually exclusive (see “Discussion”). In contrast, we did not manage to fit a phosphate from the backbone of tRNA[Ser] without clashes in the position of the P1 phosphate at the P-loop, consistent with our above suggestion that the tRNA[Ser] 3′-end approaches the active site distal to the P-loop (Fig. 6B).

SecS Discriminates against Free O-Phospho-l-serine—Free Pser is produced, for example, as an intermediate in the biosynthesis of serine by transamination from 3-phosphohydroxy- pyruvate. Therefore, SecS should be safeguarded against using free Pser as a substrate. We tested this notion by attempting to dephosphorylate free Pser with mmmuSecS. Indeed, the enzyme proved to be completely unreactive with respect to the free amino acid (Fig. 7B).

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The α-carboxylate binding Arg originates in cis from the β-sheet in domain 3. An equivalent Arg is also strictly conserved in SecS orthologs (Arg-404; Fig. 4B). However, in *mmuSecS*<sup>clast</sup>, its side chain is turned away from the PLP. Arg-404 interacts instead by a cation-π interaction with Phe227 (another residue strictly conserved only in the SecS orthologs; Fig. 2). It is additionally fixed by hydrogen bonds to the side chain of Asn-435 and the backbone carbonyl of Met-423 and by water-mediated hydrogen bonds to the backbone carbonyl of Ala-228 and to side chains of His-425 and Tyr-433 (Fig. 4B). The side chain of Arg-404 is thereby stably tugged away, since it does not change orientation in the presence of even 0.5 mM phosphate (as seen in our phosphate-soaked structure), suggesting that Arg-404 is not involved in binding of a α-carboxylate.

As pointed out above, *mmuSecS*<sup>clast</sup> harbors Glu-74 in trans next to the PLP moiety (Fig. 4, A and B). Interestingly, Glu-74 occupies the same spatial position as the equivalent of Arg-404 in other fold type I enzymes (Fig. 4, B and C). This observation and our model of an external aldimine of *mmuSecS* with a PSer ester (Fig. 6E) demonstrate that Glu-74 would strongly disfavor productive placement of a substrate with a free (negatively charged) α-carboxylate. In PSer-tRNA<sup>[Ser][Sec]</sup>, however, the α-carboxylate of PSer is esterified to the 2'- or 3'-hydroxyl group of the 3'-terminal adenosine and does not carry a negative charge. Therefore, we expect that Glu-74 acts as a substrate filter by repelling compounds with a negatively charged α-carboxylate.

**The Inhibition Profile of SecS Resembles That of β-Lyases**—To gain additional insight into the reaction mechanism of SecS, we tested whether the dephosphorylation activity of *mmuSecS* is inhibited by the mechanism-based inhibitors PG and F<sub>3</sub>-Ala. PG preferentially inhibits PLP-dependent enzymes such as cystathionine γ-lyase, which mediate lyase reactions at the α-carbon (50). In contrast, F<sub>3</sub>-Ala preferentially inhibits enzymes such as cystathionine β-lyase, which mediate replacement of a substituent at the β-carbon (51). Strikingly, *mmuSecS* is unaffected by up to 25 mM PG (more than 12,500-fold excess over *mmuSecS* active sites; Fig. 7C). In contrast, partial enzyme inhibition was detected in the presence of 5 mM F<sub>3</sub>-Ala and above (Fig. 7C). These inhibition profiles are in agreement with SecS catalyzing a β-replacement reaction with a high specificity. Furthermore, the data suggest that the lytic half-reaction of SecS
follows the cystathionine β-lyase scheme, strongly supporting aminoacyl-tRNA\textsuperscript{Ser}\textsubscript{Sec} as an intermediate (28).

Although our data do not allow us to derive exact inhibition constants, we note that compared with \textit{E. coli} cystathionine β-lyase (51), the inhibition of \textit{mmu}SecS by F\textsubscript{3}-Ala is weak. For example, in a comparable setup, the halftime for inactivation of \textit{E. coli} cystathionine β-lyase by 1 mM F\textsubscript{3}-Ala was less than 2 min (52), whereas \textit{mmu}SecS is not measurably affected under these conditions (Fig. 7C). This observation is consistent with the suggested substrate discrimination by Glu-74, since F\textsubscript{3}-Ala exhibits a free α-carboxylate and is, therefore, expected to be discouraged from forming an external aldimine.

**DISCUSSION**

SecS Orthologs Constitute a Unique Subclass of Fold Type I PLP-dependent Enzymes—We have presented structural and functional analyses of a mammalian SecS demonstrating how SecS orthologs are set aside from other PLP-dependent enzymes. Although \textit{mmu}Sec\textsubscript{Selast} is composed of three domains whose structural scaffolds exhibit high similarity to the fold type I family of PLP-dependent enzymes (Fig. 3), distinguishing structural and functional characteristics are conferred by remodeled elements both within and outside of these conserved scaffolds (labeled I–IV in Fig. 3). These novel elements clearly define SecS orthologs as a special subclass of the family.

Of paramount importance for the function of SecS are two motifs that are unique to and highly conserved in SecS orthologs. First, a special N terminus (element I) serves as a tetramerization device. It reinforces the interaction between two close dimers by cross-strutting (Fig. 5, A and B). Neither \textit{afu}\textit{PSerCysS} nor NifS relatives nor \textit{synC}-DES exhibit a comparable element, and all of these enzymes form dimers. As further detailed below, we suggest that tetramerization could be crucial for proper PSA-tRNA\textsuperscript{Ser}\textsubscript{Sec} positioning. Second, a long insertion between helices α\textsubscript{2} and α\textsubscript{4} of domain 1 (element II) is involved in catalysis by SecS. Via a first loop, element II provides residues Glu-74 and Arg-75, which anchor the PLP cofactor in trans. It thereby positions the negatively charged Glu-74 ideally to act as a substrate filter. Via a second loop it dispatches the P-loop to the trans active site, which mediates binding of SecS-specific substrates.

Evidently, the evolution of the unique N terminus and of the long domain 1 insertion went hand in hand. Placing the N terminus at the outside of the protein, where it can engage in tetramer formation, liberated a binding site between domains 1 and 3 as seen, e.g., in \textit{afu}\textit{PSerCysS}, NifS relatives, and \textit{synC}-DES proteins (Fig. 3). The first loop of the domain 1 insertion evolved to take advantage of the liberated binding site between domains 1 and 3 in trans. It thereby became ideally positioned to contribute residues to the trans active site (Fig. 3A).

**tRNA Selection Strategy of SecS**—Our results highlight a number of features that enable SecS to specifically recognize its substrates. The highly positively charged surface of \textit{mmu}Sec\textsubscript{Selast} is apparently designed to interact at multiple sites with the phosphodiester backbone of tRNA\textsuperscript{Ser}\textsubscript{Sec}. This expected mode of interaction is supported by our observations of numerous anion (iodide) binding sites (Fig. 5D), binding of a chloride ion in untreated crystals (not shown), and binding of the P2 phosphate ion distal to the active sites in phosphate-soaked crystals (Fig. 6B). tRNA\textsuperscript{Ser}\textsubscript{Sec} exhibits a number of unique structural characteristics compared with canonical tRNAs, such as an elongated (9 + 4 base pairs) helical stack between the acceptor stem and the \textit{TΨC} stem and an unusually long variable arm (8, 53). Multiple latching points on the surface of SecS would allow the enzyme to recognize these global structural features and discriminate against other tRNAs, which are in vast excess in the cell. Thus, the tRNA selection strategy of SecS may resemble that of bacterial SelA, which very inefficiently converts Ser-tRNA\textsuperscript{Ser}\textsubscript{Sec} mutants with a shortened, canonical acceptor stem (54).

The \textit{mmu}Sec\textsubscript{Selast} active site environments are built up entirely by residues originating from the two protomers of a close dimer. Why then does \textit{mmu}SecS form tetramers? One possibility is that the tetramer provides an effective binding platform for the large tRNA\textsuperscript{Ser}\textsubscript{Sec} molecule. Although we have no direct evidence for the mode of PSer-tRNA\textsuperscript{Ser}\textsubscript{Sec} binding to \textit{mmu}SecS, portions of tRNA\textsuperscript{Ser}\textsubscript{Sec} could extend beyond the borders of the molecule to whose active site its 3′-end is bound. To illustrate the relative sizes of the molecules and how the SecS tetramer could serve as a binding platform for tRNA\textsuperscript{Ser}\textsubscript{Sec}, we generated a hypothetical docking model (Fig. 5E). A model of tRNA\textsuperscript{Ser}\textsubscript{Sec} (as derived in Hubert \textit{et al.} (53)) was positioned on the \textit{mmu}Sec\textsubscript{Selast} tetramer with the 3′-end approaching the active site of one monomer distal to the P-loop, leaving the P-loop available for accommodation of the PSer moiety. The body of tRNA\textsuperscript{Ser}\textsubscript{Sec} was then adjusted by rotation about the 3′-terminal nucleotide to avoid clashes with the protein. Significantly, the unique mode of tetramerization provides the \textit{mmu}SecS tetramer with a unique, elongated shape (shown by the characteristic distances between the active sites;...
Structure and Mechanism of Eukaryotic Selenocysteine Synthase

Mechanisms for Substrate Binding and Differentiation in the Active Site—Our structural analyses have shown that the long, conserved domain 1 insertion (element II) of mmuSecS does not merely serve to support PLP anchoring in trans. Part of this insertion, which we refer to as the P-loop, can undergo disorder-order transitions coupled to binding of poly-dentate ions such as phosphate or sulfate. We interpret this observation as direct evidence for the mode of substrate binding by mmuSecS. Our molecular modeling suggests that the P1 phosphate could resemble the binding of the γ-phosphate of the PSer moiety of PSer-tRNA^Sec (Fig. 6E). Evidently, SeP could also bind to the P-loop in a similar fashion as the P1 phosphate. In contrast, we could not position the tRNA^Ser binding by one dimer to position its 3’-end appropriately in an active site of a neighboring dimer. A similar principle may underlie the decameric organization of bacterial SelA. Further experiments are required to test these ideas.

In addition, we suggest that SecS has exploited the domain 1 insertion to install a filtering mechanism that allows it to exclude free PSer and other free amino acids from its active site. Glu-74, positioned strategically next to the trans PLP (Fig. 4, A and B), adopts a similar spatial position as the side chain of an Arg originating from the domain 3 β-sheet in related enzymes, which typically serves as an α-carboxylate recognition device (Fig. 4C). In SecS, an equivalent Arg is present, but it is turned away via interactions with other conserved residues (Fig. 4B), e.g. to engage in alternative interactions with the tRNA^Ser portion. We suspect that Glu-74 repels negatively charged carboxyl groups of free amino acids. Neutral ester moieties as in PSer-tRNA^Ser are presumably allowed to productively approach the PLP internal aldimine. Our finding that SecS does not convert free PSer supports the role of Glu-74 as a substrate discriminator. Similar to the P-loop, the elements constituting the putative substrate filter are highly conserved among SecS orthologs but not beyond. Thus, SecS has acquired specialized functional modules for substrate binding and differentiation.

γ-Phosphate binding at the P-loop provides a facile explanation for the observation that SecS binds PSer-tRNA^Ser preferentially over non-aminoacylated tRNA^Ser (28). However, presently we can only speculate why Ser-tRNA^Ser is bound with least efficiency (28). One clue is provided by the observations that the P-loop tends to bind substrate mimics such as phosphate or sulfate and that PSer-tRNA^Sec, but not Ser-tRNA^Ser, can compete efficiently with these molecules. The unloaded 3’-end of tRNA^Ser may fit next to the contracted P-loop without having to compete for binding at that place. Another possibility is that γ-phosphate binding at the P-loop leads to proper accommodation of the PSer moiety and of the tRNA^Ser 3’-end in the active site, whereas Ser at the 3’-end may engage in alternative interactions, which could be mutually exclusive with proper fitting of tRNA^Ser. Clarification of

Fig. 5A). Other arrangements, such as the stubbier form of E. coli cystathionine γ-synthase (Fig. 5A) (55), generate other relative dispositions of active sites. Thus, SecS may have evolved as a distinctly shaped tetramer to support efficient PSer-tRNA^Ser binding by one dimer to position its 3’-end appropriately in an active site of a neighboring dimer. A similar principle may underlie the decameric organization of bacterial SelA. Further experiments are required to test these ideas.

**FIGURE 7. Functional analysis of mmuSecS.** A, mutational analysis of mmuSecS. Conversion of [3H]PSer-tRNA^Sec to [3H]Sec-tRNA^Sec by mmuSecS (red), mmuSecS/S105E (blue), mmuSecS/S313E (light green), and mmuSecS/S313E (light green). Trx (black) served as a negative control. B, dephosphorylation activity of mmuSecS. Dephosphorylation of [32P]PSer-tRNA^Sec (lanes 1–4) and [32P]PSer (lanes 5–9) in the presence of varying amounts of mmuSecS is shown. Trx was used as a negative control (lanes 1 and 5), and alkaline phosphatase (AP) was used as a positive control for the generation of free [32P]P (lane 9). Details are given under "Experimental Procedures." C, inhibition studies of mmuSecS. [32P]PSer-tRNA^Sec dephosphorylation with mmuSecS (lane 1) in the presence of the mechanism-based inhibitors PG (lanes 2–5) and F-Ala (lanes 6–9); Trx (lane 10) served as a negative control. Details of reactions and monitoring conditions are given under "Experimental Procedures."
these issues may require elucidation of a SecS-tRNA[Sec]Sec complex structure.

Structure-based Reaction Mechanism—NiFS-like enzymes mobilize sulfur for iron-sulfur cluster biosynthesis via a protein-bound persulfide using a conserved cysteine that lies in a long loop of domain 3 (element IV in Fig. 3, A and B) (43). synC-DES employs a related strategy by generating an external cysteine persulfide via cystine C-S cleavage, which remains non-covalently fixed at the active site (47). Furthermore, it has been discussed that PSerCysS could also employ a persulfide mechanism (41). Because NiFS can support selenide delivery (56), SecS could function in an analogous fashion by using a perselenide intermediate. However, although SecS exhibits a domain 3 loop analogous to the persulfide loop of NiFS enzymes, no cysteine that could serve as attachment site for selenium is present in that loop. The only cysteine that is conserved in eukaryotes in the active site cavity is Cys-226 (Fig. 2). However, its sulfur atom is still more than 7 Å away from any atom of the PLP and remote from the modeled substrate. In addition, this residue is not conserved in archaeal SecS (Fig. 2). Furthermore, in light of the observation that SeP delivered by selenophosphate synthetase 2 is the active selenium donor of SecS (24, 28), internal or external perselenide production (via SeP) appears to be an off-pathway reaction. Under these circumstances it is unlikely that SecS functions via an intermediate perselenide moiety.

Instead, a mechanism that is consistent with all our findings evokes direct selenide delivery by SeP (Fig. 8). As suggested by the inhibition results, we based the first part of the scheme on the E. coli cystathionine β-lyase mechanism (51). In our work-
ing model the process is initiated by PSer-tRNA[Sec][Sec] binding and positioning of the γ-phosphate at the P-loop (Fig. 8, I). Lys-284 is expected to be deprotonated after liberation upon and positioning of the phosphate (Fig. 8, III and IV). Next, the liberated phosphate is exchanged for SeP at the P-loop (Fig. 8, IV), which would then be ideally situated to donate Se²⁻ to the β-carbon of the aminocysteine moiety (Fig. 8, IV and V). We suggest that attack of the aminocysteinyl-tRNA[Sec][Sec] intermediate by SeP involves concomitant attack by a water molecule on SeP, again conceivably that Lys-284 directly activates the water molecule without an intervening general base. Evoking a similar scenario, we presently envision sequential binding and conversion of the aldime.

Details of the above model are still in the dark. For example, the function could involve residues from the P-loop/Arg-313 (for SecSelast) and the identity of the thionine/H₉₂⁵₃ for the precursor used in generating selenophosphate, and Arizona, for the precursor used in generating selenophosphate, and.

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[ SUPPLEMENT Figure S1 follows. ]
SUPPLEMENT

Figure S1: Electron density. Experimental SIRAS electron density (blue mesh) at 2.0 Å resolution after solvent flattening contoured at the 1σ level. A. Density covering one molecule of a tetramer. The other three protomers are indicated as light gray ribbons. B. SIRAS density of a α-helical element with the final model indicated as yellow ball-and-sticks.