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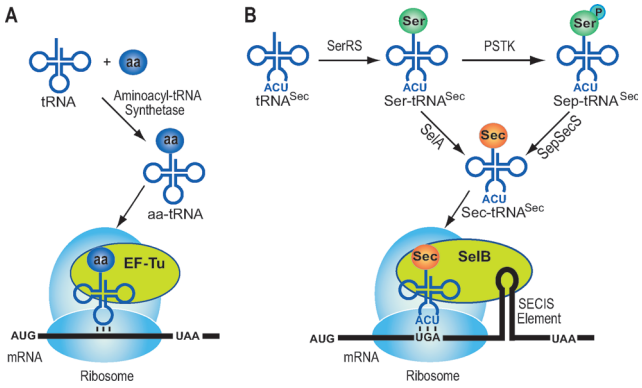
Protein Engineering

Rewiring Translation for Elongation Factor Tu-Dependent Selenocysteine Incorporation**

*Caroline Aldag, Markus J. Bröcker, Michael J. Hohn, Laure Prat, Gifty Hammond, Abigail Plummer, and Dieter Söll**

Selenium is an essential micronutrient for animals.[1] Humans contain 25 presumably essential selenoproteins[2] in which selenium is found in the form of selenocysteine (Sec).[3] In this 21st genetically encoded amino acid[4] the thiol moiety of Cys is replaced by a selenol group. In all Sec-decoding organisms, Sec biosynthesis (Scheme 1 B) starts with the acylation of $tRNA^{Sec}$ by seryl-tRNA synthetase (SerRS) to form Ser-$tRNA^{Sec}$ (reviewed in Ref. [5]). In bacteria, conversion of Ser-$tRNA^{Sec}$ to Sec-$tRNA^{Sec}$ is achieved by selenocysteine synthase (SelA; reviewed in Ref. [4]). In contrast, archaea and eukaryotes employ an additional phosphorylation step. *O*-phosphoseryl-$tRNA^{Sec}$ kinase (PSTK) phosphorylates the tRNA-bound Ser moiety of Ser-$tRNA^{Sec}$ to form *O*-phosphoseryl-$tRNA^{Sec}$ (Sep-$tRNA^{Sec}$),[6] the substrate for SepSecS that catalyzes the tRNA-dependent Sep to Sec conversion.[7] The selenium donor for both SelA and SepSecS is selenophosphate (reviewed in Refs. [4, 7b]).

During selenoprotein synthesis, Sec is co-translationally incorporated by a re-programmed UGA stop codon. A specialized elongation factor (SelB in bacteria) and an RNA structural signal (SECIS element) located within the bacterial open reading frame (ORF) sequence are required for unambiguous Stop to Sec recoding.[4] EF-Tu does not recognize Sec-$tRNA^{Sec}$ and also discriminates against Ser-$tRNA^{Sec}$.[4]



Scheme 1. Aminoacyl-tRNA formation and first steps of protein synthesis. A) Canonical amino acids: aa-tRNA gets delivered by EF-Tu to the ribosome. B) Selenocysteine gets formed while bound to tRNA; Sec-tRNA transfer to the ribosome and accurate codon recognition are achieved by SelB (Sec-specific elongation factor) and the SECIS element (RNA structure within the open reading frame of bacterial selenoprotein mRNAs).

Selenium and sulfur are in the same group of elements in the periodic table and share certain properties (e.g., size, electronegativity, major oxidation states); yet, Cys and Sec are distinguished by different electrode potentials,[8] nucleophilicity (Cys < Sec),[9] and side-chain pK_a (8.3 for Cys versus 5.2 for Sec).[10] Thus, selenoproteins have unique properties.[11] Sec is frequently found in the active sites of enzymes, endowing these proteins (e.g., redox enzymes) with superior catalytic activities. Sec to Cys replacements in selenoenzymes may lead to 10 to 1000-fold activity loss (reviewed in Ref. [11b]). Although disulfides occur frequently in proteins to increase stability or provide redox functions, diselenides are much less frequent.[12] The occurrence of diselenides in proteins has exciting biological and biomedical significance, because they are more stable than disulfides[13] and sometimes even resistant to reduction by DTT.[12]

These Sec-dependent properties indicate that inclusion of Sec in proteins could be useful in designing proteins for various applications (e.g., X-ray crystallography, PET studies, protein folding, NMR spectroscopy, electron paramagnetic resonance spectroscopy).[14] There are currently several strategies to produce selenoproteins. First, the *E. coli* Sec insertion machinery can be exploited for heterologous overexpression of selenoproteins,[15] but its use is severely limited by sequence constraints of the SECIS sequence, which inhibits facile site-directed replacement of any amino acid with Sec. Mammalian Sec insertion appears to be somewhat easier.[16] Second, solid-phase chemical synthesis of Sec-

[*] C. Aldag,[+] M. J. Bröcker,[+] M. J. Hohn,[+] L. Prat,[+] G. Hammond, A. Plummer, D. Söll
Department of Molecular Biophysics and Biochemistry, and Chemistry, Yale University
New Haven, CT 06520 (USA)
E-mail: dieter.soll@yale.edu

[+] These authors contributed equally to this work.

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containing peptides[17] as well as site-specific chemical modification of existing proteins (e.g., Ref. [18]) are limited by constraints on the sequence context. Finally, use of a Cys-auxotrophic *E. coli* strain randomly replaces up to 80% of all Cys residues in a protein with Sec.[19] Consequently, there is a need to develop a more versatile site-directed Sec insertion system.

Herein, we describe a system for general selenoprotein synthesis that allows site-specific insertion of Sec into any desired position of a protein. The system is based on a synthetic $tRNA^{UTu}$ (namely a tRNA for Sec (U) that is recognized by EF-Tu) that must be a substrate for SerRS and for SelA to be converted into Sec-$tRNA^{UTu}$. To avoid the need for SelB and the SECIS element, this Sec-tRNA was also designed to be carried by EF-Tu to the ribosome, where the tRNA anticodon decodes the desired mRNA codon. Thus, $tRNA^{UTu}$ should participate in protein synthesis like any canonical tRNA species (Scheme 1A).

We expected that our $tRNA^{UTu}$ design would result in some chimera of the *E. coli* $tRNA^{Ser}$ and $tRNA^{Sec}$ (Figure 1).

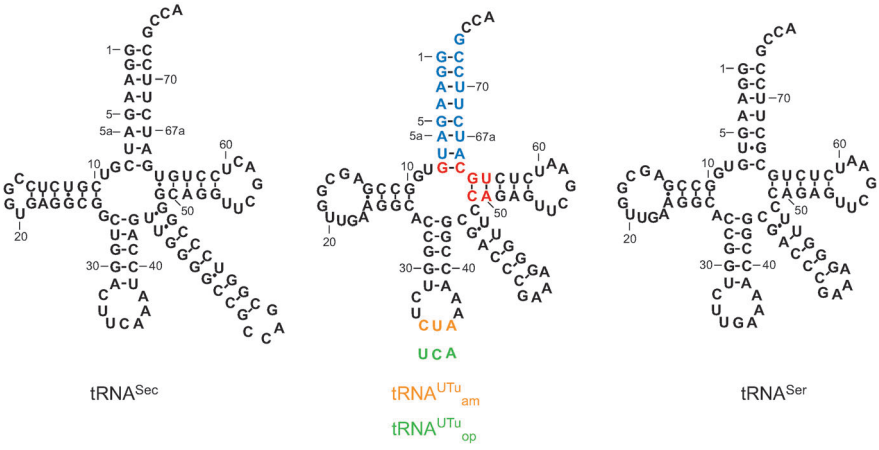

Figure 1. Secondary structure of *E. coli* $tRNA^{Ser}$ and $tRNA^{Sec}$, and $tRNA^{UTu}$. *E. coli* $tRNA^{Ser}$ is the major scaffold of $tRNA^{UTu}$, the acceptor stem originates from $tRNA^{Sec}$ (blue), and recognition elements for EF-Tu were retained from $tRNA^{Ser}$ (red). The amber anticodon CUA (orange) is $tRNA^{UTu}_{am}$, while the opal anticodon UCA (green) defines $tRNA^{UTu}_{op}$.

Making mutations throughout the tRNA sequence revealed that only tRNA variants with changes in the acceptor helix or the tRNA core were active SelA substrates. The $tRNA^{Sec}$ acceptor helix has an additional 8th base pair (bp); this longer acceptor helix is important for $tRNA^{Sec}$ recognition by *E. coli* SelA, and it precludes $tRNA^{Sec}$ from being recognized by EF-Tu.[20] However, $tRNA^{Ser}$ is a regular substrate for EF-Tu but not for SelA.[21] Thus, some elements of EF-Tu and SelA recognition could be mutually exclusive. $tRNA^{Ser}$ and other canonical tRNAs contain three bp (49–65, 50–64, 51–63; highlighted in red in Figure 1) that provide the thermodynamic binding specificity for EF-Tu.[22] Different bases are found in those locations in $tRNA^{Sec}$ which appear to contribute to the incompatibility between $tRNA^{Sec}$ and EF-Tu.[23] Our final $tRNA^{UTu}$ design (Figure 1) included anticodons that recognize the stop codons opal UGA ($tRNA^{UTu}_{op}$) and amber UAG ($tRNA^{UTu}_{am}$).

Given the major challenge of designing a tRNA required to be an acceptable substrate for three major *E. coli* proteins, we first used in vivo experiments as tests of our designs. We tested $tRNA^{UTu}$ variants in vivo for selenoprotein synthesis in three experiments. When grown anaerobically, *E. coli* produces formate dehydrogenase H (FDH_H) containing an essential Sec at position 140; its activity in the presence of formate enables the cells to reduce benzyl viologen (BV) to generate a purple color.[24] An *E. coli* Δ*selA*, Δ*selB*, Δ*fdhF* strain was transformed with the appropriate combinations of plasmid-encoded *selA*, *selB*, and either an amber (*fdhF*$_{UAG140}$, $tRNA^{UTu}_{am}$) or opal (*fdhF*$_{UGA140}$, $tRNA^{UTu}_{op}$) reporter system for Sec insertion (Figure 2A). The data show that $tRNA^{UTu}_{am}$ when co-expressed with SelA successfully read the amber codon of *fdhF*$_{am}$ (Figure 2A4). The BV color was almost as intense as in the positive control (Figure 2A2), in which *E. coli* $tRNA^{Sec}$ in combination with the homologous Sec insertion machinery (SelA, SelB, and SECIS) translates the opal codon in *fdhF*$_{op}$ to produce active FDH_H. Furthermore, no unspecific translation of *fdhF*$_{am}$ by *E. coli* $tRNA^{Sec}$ is observed (Figure 2A3). Translation of *fdhF*$_{op}$ by $tRNA^{UTu}_{op}$ seems to be less efficient than of *fdhF*$_{am}$ by $tRNA^{UTu}_{am}$ judged by the much slower appearance of colored cells (Figure 2A1).

The second experiment demonstrated direct Sec insertion into FDH_H. We incubated the culture described in Figure 2A1 with [^{75}Se]selenite and ran the extracted protein on SDS-PAGE (Figure 2B). The appearance of a radioactive 80 kDa band corresponds to ^{75}Se-labeled FDH_H. The second radioactive band (approximately 40 kDA) is an FDH_H degradation product as described earlier.[7a]

Third, we functionally replaced Cys in an enzyme active site with Sec. Thymidylate synthase (ThyA) catalyzes the conversion of deoxyuridylate to thymidylate, and its *thyA* gene, in contrast to *fdhF*, is devoid of a SECIS element. An *E. coli* Δ*thyA* strain that carries a deletion of the endogenous *thyA* gene is thymine auxotrophic. The enzyme's active-site residue at position 146 is Cys. Replacement of this residue with Ser reduces activity 5000-fold.[25] To check whether Cys146 can be replaced by Sec, we transformed an *E. coli* Δ*thyA*, Δ*selA*, Δ*selB* strain with the requisite plasmids and checked for growth on minimal medium in the presence and absence of thymine (Figure 2C). As expected, complementation with *thyA*$^+$ and with *thyA*$_{146am}$/$tRNA^{UTu}_{am}$ restored prototrophic growth, while *thyA*$_{146Ser}$ or empty vector did not. Thus, $tRNA^{UTu}_{am}$ permitted insertion of Sec, which takes on the function of the active-site Cys. Taken together, the three experiments above established the suitability of $tRNA^{UTu}$ for UAG-directed Sec insertion.

We also determined the efficiency and accuracy of the current system and which of the components (SerRS, SelA,

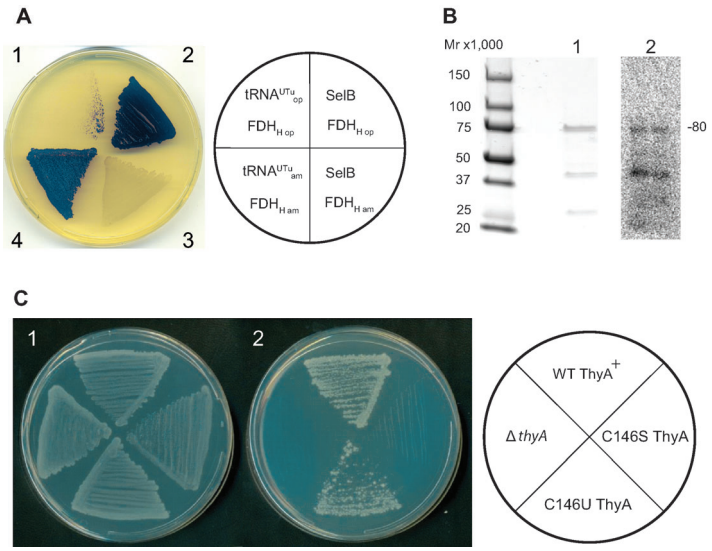

Figure 2. A) tRNA^{UTu} mediates functional Sec incorporation in FDH$_H$. An *E. coli* Δ*selA*, Δ*selB*, Δ*fdhF* deletion strain was complemented with *E. coli* SelA, *M. jannaschii* PSTK, and 1) tRNA$^{UTu}_{op}$ and FDH$_{H op}$; 4) tRNA$^{UTu}_{am}$ and FDH$_{H am}$. Controls lacked 2) tRNA$^{UTu}_{op}$ or 3) tRNA$^{UTu}_{am}$, and tested FDH$_{H op}$ and FDH$_{H am}$ with genomic *E. coli* tRNA^{Sec} and recombinant SelB. FDH$_H$ activity was visualized by the purple color in a benzyl viologen assay. B) ^{75}Se incorporation into *E. coli* FDH$_H$. The *E. coli* culture described in (A4) was grown in the presence of [^{75}Se]selenite. 6xHis-tagged FDH$_H$ protein was purified and analyzed by SDS-PAGE (lane 1) followed by autoradiography (lane 2); FDH$_H$ corresponds to the protein band of approximately 80 kDa. C) ThyA C146U restores thymine prototrophy. An *E. coli* Δ*selA*, Δ*selB*, Δ*thyA* deletion strain was complemented with wild-type *thyA*$^+$, *thyA*$_{146Sec}$ or *thyA*$_{146am}$ plasmids along with tRNA$^{UTu}_{am}$ and SelA. All clones showed growth on M9 minimal medium supplemented with thymine (1) while only *thyA*$_{146am}$ (expressing C146U ThyA) was able to reconstitute the wild-type phenotype (ThyA$^+$) on M9 minimal medium in the absence of thymine (2).

EF-Tu, or tRNA^{UTu}) are limiting to the reaction. Serylation kinetics with pure *E. coli* SerRS (Supporting Information, Table S1) revealed that tRNA$^{UTu}_{am}$ is as good a substrate as tRNA^{Sec} and tRNA$^{Ser}_{UCA}$; thus serylation of tRNA^{UTu} is no impediment. Given that Sec-tRNA^{UTu} is formed from a Ser-tRNA^{UTu} intermediate, it is possible that some Ser misincorporation may occur. This was of no concern in the cases of ThyA and FDH$_H$, which are inactive when Ser is present in place of Sec.

As a SelA in vitro experiment showed that Ser-tRNA$^{UTu}_{am}$ is less efficiently converted into Sec-tRNA than the natural substrate for SelA, Ser-tRNA^{Ser} (Figure S1), we included PSTK (the eukaryotic kinase that converts Ser-tRNA^{Sec} into Sep-tRNA^{Sec}) in our reaction mixtures; in this way we expected PSTK to decrease the amount of Ser-tRNA^{UTu} by converting it into Sep-tRNA^{UTu} (Figure S2). This aminoacyl-tRNA does not bind to EF-Tu[26] and thus is not transported to the ribosome, although it is a likely substrate for SelA-dependent conversion into Sec-tRNA^{UTu} (Figure S2B, lanes 2,3).[7c] Ser incorporation may also be due to preferential binding of EF-Tu to Ser-tRNA^{UTu} compared to Sec-tRNA^{UTu}. However, thiol-sepharose could separate a Sec-containing protein from a mixture with its Ser homologue; therefore we incorporated tRNA^{UTu}-mediated Sec into a bacterial and a human test protein.

Glutaredoxins are glutathione-dependent reductases that regulate the cellular redox state.[27] *E. coli* glutaredoxin (Grx1) is a small (85 amino acids) redox-active disulfide (C11/C14)-containing monomeric protein with glutathione-disulfide oxidoreductase activity.[28] Grx1 can be easily purified by thiol-sepharose chromatography[29] and has been extensively studied.[27] Partial chemical synthesis afforded the glutathione peroxidase mimic selenoglutaredoxin, a Sec-containing Grx1 variant (C11U/C14S), which was analyzed for its ability to catalyze thiol–disulfide exchange reactions, as well as for its peroxidase activity.[30] This selenoprotein showed superior catalytic properties compared to its Cys homologue (C11/C14S).[30] Thus, we decided to biosynthesize selenoglutaredoxin by tRNA^{UTu}-mediated overexpression in *E. coli* followed by purification on activated thiol-sepharose,[29] to compare its properties with those described earlier.[30] In this way, we obtained the Grx1 variants C11U/C14S and C11S/C14S in pure form in about 50% yield (Figure S3). Incorporation of Sec at position 11 was confirmed by mass spectroscopy (Figure 3, Figure S4). This analysis also showed the selenoglutaredoxin to be free of the C11S/C14S Grx1 variant.

Varying the conditions of protein expression, such as gene dosage or time of selenoprotein induction (see text in the Supporting Information and Figure S5), increased selenoprotein yield to about 65%, while the remainder of the protein mixture was the Ser homologue. In certain cases (e.g., Grx1), thiol-sepharose chromatography could effectively separate the mixture.

Comparison of our selenoglutaredoxin with its Cys (C11/C14S) and Ser (C11S/C14S) homologues was undertaken using two standard assays. Like wild-type Grx1, C14S Grx1 efficiently reduces glutathionyl mixed disulfides;[31] this is measured by the thiol–disulfide interchange between β-hydroxyethyldisulfide and glutathione.[28] Our selenoglutaredoxin exhibited improved activity over that of the Cys homologue C14S Grx1 (90 versus 47 units mg^{-1}; Figure 4A). These values agree well with previously pub-

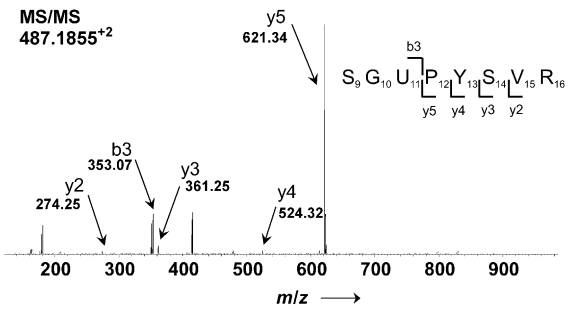

Figure 3. Mass spectroscopic confirmation of Sec incorporation at position 11 in pure C11U/C14S Grx1. Shown is the MS/MS spectrum of the trypsin-digested Sec-containing fragment S$_9$G$_{10}$U$_{11}$P$_{12}$Y$_{13}$S$_{14}$V$_{15}$R$_{16}$. Fragments observed in the second mass spectrometric analysis of this peptide are labeled b3, y2, y3, y4, and y5. *m/z* describes the mass-to-charge ratio.

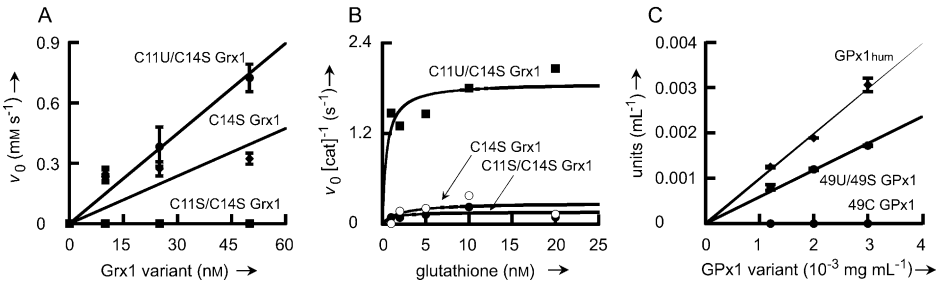

Figure 4. Characterization of Grx1 and GPx1 mutants. A) Glutathione disulfide oxidoreductase activity of Grx1 variants: pure C11U/C14S Grx1, C11S/C14S Grx1, and C11/C14S Grx1 were tested for disulfide oxidoreductase activity. NADPH consumption was followed at 340 nm as a function of Grx1 concentration. B) Peroxidase activity of Grx1 variants: pure C11U/C14S Grx1, C11S/C14S Grx1, and C11/C14S Grx1 were tested for peroxidase activity. NADPH consumption was monitored at 340 nm as a function of reduced glutathione concentration. C) GPx1 peroxidase activity: peroxidase activity of recombinant Sec-containing 49U/49S GPx1 and Cys-containing 49C GPx1 was compared to $GPx1_{hum}$ from human erythrocytes. GPx1 activity was determined with the Sigma cellular activity assay kit. Experiments shown in (A) and (C) were performed in triplicate, and error bars indicate the standard error of the mean.

selenoprotein synthesis from the SECIS/SelB-dependent codon reading; our tRNA design efforts establish that Sec can be adapted to "normal" mRNA translation as if it were a canonical amino acid. Our system has general utility in protein engineering, molecular biology, and disease research.

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lished data.[30] No activity above background could be detected for the Ser homologue C11S/C14S Grx1.

Selenoglutaredoxin functions inefficiently as a glutathione peroxidase (GPx),[30] catalyzing the reduction of peroxides to the corresponding alcohols using glutathione.[32] Consequently, we also examined the GPx activity of our selenoglutaredoxin and its Cys and Ser homologues. C11U/C14S Grx1 showed modest peroxidase activity (approximately $2\times10^{-3}\,s^{-1}$), in accordance with published data.[30] Both, C11S/C14S Grx1 and C14S Grx1 were significantly less active (Figure 4B).

Human glutathione peroxidase (GPx1), a key component of the mammalian defense against oxidative stress, is a 217 amino acid selenoprotein with an essential Sec residue in its active site (position 49); replacement of Sec with Cys leads to 1000-fold activity loss, while Ser substitution renders the enzyme inactive.[33] Synthesis of eukaryotic selenoproteins (e.g., GPx1) relies on a SECIS element in the 3'-untranslated region of the mRNA.[16a] Not surprisingly, human Sec-containing GPx1 has not been expressed in *E. coli*. Therefore, we attempted to produce human GPx1 by $tRNA^{UTu}$-mediated expression in *E. coli*. This yielded a mixture of two GPx1 variants, Sec- and Ser-containing GPx1 (49U/49S) in approximately equal amounts, as deduced from integration of the peaks of the mass spectra (Figure S6). The glutathione peroxidase activity of the protein mixture was six units mg^{-1}, which is similar to ten units mg^{-1} determined for a commercially available pure human GPx1 preparation. The Cys homologue U49C GPx1 was not active under these conditions (Figure 4C).

The data presented herein reveal the success of $tRNA^{UTu}$-mediated site-specific Sec incorporation as exemplified by four selenoproteins, three of bacterial and one of human origin. The remaining challenge is to achieve complete conversion of $tRNA^{UTu}$-bound Ser into Sec. Structural knowledge of tRNA complexes with SelA and with EF-Tu could inspire further rewiring of translation to facilitate optimal EF-Tu-dependent Sec incorporation. We successfully uncoupled

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