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A synthetic tRNA for EF-Tu mediated selenocysteine incorporation *in vivo* and *in vitro*

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Abstract

Incorporation of selenocysteine (Sec) in bacteria requires a UGA codon that is reassigned to Sec by the Sec-specific elongation factor SelB and a conserved mRNA motif (SECIS element). These requirements severely restrict the engineering of selenoproteins. Earlier a synthetic $tRNA_{Sec}$ was reported that allowed canonical Sec incorporation by EF-Tu; however, serine misincorporation limited its scope. We report a superior $tRNA_{Sec}$ variant ($tRNA_{UTuX}$) that facilitates EF-Tu dependent stoichiometric Sec insertion in response to UAG both *in vivo* in *Escherichia coli* and *in vitro* in a cellfree protein synthesis system. We also demonstrate recoding of several sense codons in a SelB supplemented cell-free system. These advances in Sec incorporation will aid rational design and directed evolution of selenoproteins.

1. Introduction

Organisms pay a high fitness cost for the benefit of endowing proteins with the unique properties of the 21st amino acid, selenocysteine (Sec) [1,2], and have evolved complex biosynthetic and translational mechanisms to incorporate Sec [3,4]. At the interface of Sec synthesis and insertion lies $tRNA^{Sec}$. Initially acylated by seryl-tRNA synthetase (SerRS) to form Ser-$tRNA^{Sec}$, the bacterial enzyme SelA catalyzes the conversion of Ser to Sec in a single step on the tRNA [3]. Once synthesized, selenocysteinyl-tRNA (Sec-$tRNA^{Sec}$) is bound by the specialized Sec-specific elongation factor SelB, which subsequently binds to a highly conserved mRNA motif denoted as Selenocysteine Insertion Sequence (SECIS), facilitating insertion of Sec at a UGA codon [3]. In bacteria, the SECIS sequence is located directly after the suppressed UGA and is thus part of the coding sequence of bacterial genes, making engineering of newly designed selenoproteins very difficult [5–7]. Recently, we

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found in the online version.

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reported construction of a synthetic tRNA (tRNA^{UTu}) that enabled SECIS-independent and EF-Tu-dependent insertion of Sec in *Escherichia coli* [8]. This tRNA^{UTu} combines the aminoacyl acceptor helix of tRNA^{Sec} with the backbone of tRNA^{Ser}, and serves as a substrate for the essential proteins SerRS, SelA, and EF-Tu. By virtue of its interaction with EF-Tu, Sect-RNA^{UTu} circumvents the need for the Sec-specific elongation factor SelB, and more importantly does not require the SECIS mRNA motif. Sec-tRNA^{UTu} therefore participates in canonical translation, allowing versatile sequence-independent production of designed selenoproteins programmed by UAG.

While SelB recognizes only Sec-tRNA^{Sec} [9,10], EF-Tu serves all other aminoacyl-tRNAs (aa-tRNAs). Therefore, if the SelA-dependent conversion of Ser-tRNA^{UTu} to Sec- tRNA^{UTu} is not complete, Ser will be incorporated instead of the desired Sec residue. This was an impediment in the earlier work in which ~30% misincorporation of Ser was observed [8]. We reasoned that by designing an improved tRNA^{UTu} with better substrate properties for SelA, misincorporation could be prevented. Here we report such a tRNA (tRNA^{UTuX}) that allows complete Sec incorporation *in vivo* and *in vitro* in response to UAG.

2. Materials and methods

2.1. In vitro Sec-tRNA formation

To characterize *in vitro* formation of Sec-tRNA, tRNA species were radiolabeled using [α-^{32}P]ATP and the *E. coli* CCA editing enzyme [11]. Ser-tRNA formation by SerRS, selenophosphate production by SelD, and Ser to Sec conversion by SelA was carried out under anoxic conditions as previously described [8]. Conversion rates were determined by autoradiography and quantitation of aminoacyl-AMP after thin layer chromatography of nuclease P1 digests of aminoacyl-tRNA^{UTu} [12]. For use in cell free protein synthesis experiments, Sec-tRNA was phenol-chloroform extracted, ethanol precipitated, and resuspended in RNase free H_2O to desired concentration.

2.2. In vivo tRNA^{UTu} utilization assay

E. coli selA selB fdhF strain MH5 was co-transformed with plasmids pACYC-[*E. coli selA*$^{+}$, *M. jannaschii pstk*] and pGFIB-[tRNA$^{UTu}{}_{am}$], or pGFIB-[tRNA$^{UTuX}{}_{am}$] variants as well as pRSF-[*E. coli serS-fdhF$_{am}$*] and grown on LB medium supplemented with the corresponding antibiotics ampicillin, chloramphenicol, or kanamycin. As a control *E. coli* MH5 was co-transformed with the plasmids pACYC-[*E. coli selA*$^{+}$, *M. jannaschii pstk*], pRSF-[*E. coli serSfdhF$_{op}$*] and pET15b-[*E. coli selB*] to reconstitute the wild-type (WT) Sec formation apparatus using the genomically encoded tRNA^{Sec}. *E. coli* MH5 carrying plasmids pACYC-[*E. coli selA*$^{+}$*M. jannaschii pstk*], pRSF-[*E. coli serS-fdhF$_{am}$*] and pET15b-[*E. coli selB*] served as a second control. Overnight cultures of these clones were plated on LB agar plates supplemented with 10 µM IPTG, 1 µM Na_2MoO_4, 1 µM Na_2SeO_3 and 50 mM sodium formate, as previously described [13,14], and were grown anaerobically at 37°C overnight. Plates were then overlaid with a top agar containing 1 mg/mL benzyl viologen (BV), 250 mM sodium formate, and 25 mM KH_2PO_4 pH 7.0. The appearance of a purple color indicates catalytically active FDH_H, which depends on Sec insertion at position 140.

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2.3. Cell-free selenoprotein synthesis

Cell-free *in vitro* translation experiments were conducted using the PURExpress *in* vitro Protein Synthesis Kit (E6800S) or PURExpress RF123 kit (E6850S, New England Biolabs Inc.), as noted. Reactions were prepared according to the manufacturer's instructions inside an anaerobic chamber, and were supplemented with 1 μM sodium molybdate, 40U RNasin Plus RNase Inhibitor (Promega), and 250 ng of *fdhF* mutants at position 140 cloned into PURE vector. Reactions were normalized against expressed dihydrofolate reductase (DHFR) as a negative control, a protein that did not exhibit measureable reduction of BV.

For translation mediated by $tRNA^{Sec}_{am}$, $tRNA^{Sec}_{op}$, $tRNA^{Sec}_{CGU}$, $tRNA^{Sec}_{GCC}$, $tRNA^{Sec}_{CCU}$, and $tRNA^{Sec}_{UCG}$, PURExpress kit reactions were supplemented with 12 μM Sec-$tRNA^{SelC}_{am}$, 12 μM SelB, and were allowed to proceed for two hr at 37°C. For expression mediated by $tRNA^{UTu}_{am}$, $tRNA^{UTuX}_{am}$, or $tRNA^{SecUX}_{am}$, PURExpress RF123 kit reactions were prepared in the absence of RF1, supplemented with 70 μM Sec-tRNA, 67 μM EF-Tu, and were allowed to proceed for five hr at 37°C. Plasmid containing *fdhF* with corresponding cognate codon at position 140 were also added to each reaction. Following protein synthesis, 0.7 mg/ml BV and 7 mM sodium formate were added to the reaction mixture to a final volume of 30 μl. FDH_H activity was monitored over time via absorbance at 578 nm measuring using a Nanodrop 2000 (Thermo-Scientific NanoDrop 2000 UV-Vis Spectrophotometer). As a negative control, a reaction was prepared with 12 μM Ser-$tRNA^{Sec}_{am}$ instead of SectRNA^{Sec}_{am}; absence of BV reduction activity was confirmed following this reaction.

3. Results

The co-crystal structure of decameric *Aquifex aeolicus* SelA protein in complex with ten *Thermus tengcongensis* $tRNA^{Sec}$ molecules provided molecular insight into the enzyme's substrate recognition and coordination, illustrating the mechanism by which SelA discriminates between $tRNA^{Ser}$ and $tRNA^{Sec}$ [15]. This information was used to rationally engineer additional $tRNA^{UTu}$ variants to act as ideal substrates for SelA that would increase the yield of Sec insertion.

Using site-directed mutagenesis, we incrementally changed the sequence of the original $tRNA^{UTu}$ to more closely resemble the features of $tRNA^{Sec}$ that contribute to binding of SelA. These modifications comprised both single and combined exchanges as well as insertions and deletions of nucleotides in various regions of the tRNA. However, they left the critical $tRNA^{UTu}$ features that (i) provide thermodynamic binding specificity for EF-Tu [16] and (ii) contribute to the incompatibility between $tRNA^{Sec}$ and EF-Tu [17] (Fig. 1A). A total of 29 tRNA variants were produced and subsequently tested for their capacity to mediate Sec insertion, using *E. coli* FDH_H as a reporter protein (Fig. 2). A natural selenoenzyme, FDH_H contains an essential Sec residue at position 140, and catalyzes the electron transfer from formate onto the artificial electron acceptor benzyl viologen under anaerobic conditions. We used the FDH_H-mediated BV reduction as a sensitive colorimetric *in vivo* reporter system for functional Sec insertion, as reduced BV adopts a dark purple color [18] (Fig. 1B). Consequently, the *E. coli selA selB fdhF* triple deletion strain MH5 was complemented with a vector encoding SelA, PSTK, FDH_{H140am} and each $tRNA^{UTu}_{am}$

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variant, subsequently grown in the presence of formate and BV [8]. To serve as a positive control, FDH_{H140op} expressed with the genomic WT $tRNA^{Sec}{}_{op}$ was also grown (Fig. 1B). Using this growth assay, a tRNA variant capable of producing active FDH_H with the same apparent BV reduction activity as the WT was identified from among the 29 $tRNA^{UTu}$ variants (Fig. 1B, Fig. 2), notably producing visibly more reduced BV than the original synthetic variant. This improved tRNA was named $tRNA^{UTuX}{}_{am}$; it differs from the original $tRNA^{UTu}$ in 11 positions (Fig. 1A, Fig. 2, Fig. S1). To validate the observed BV color change, $tRNA^{UTuX}{}_{am}$ was then characterized in a set of *in vitro* experiments.

While the modifications introduced in $tRNA^{UTuX}$ focused on better interaction with SelA, it was a prerequisite to retain robust Ser-$tRNA^{UTuX}$ formation by SerRS. Serylation assays of $tRNA^{UTuX}$, $tRNA^{Sec}$, and the original $tRNA^{UTu}$ did not reveal any significant differences among the three tRNA species (Table S1), as the K_M (3.5 μM), k_{cat} (0.42min^{-1}), and k_{cat}/K_M (0.12 μM^{-1}min^{-1}) of $tRNA^{UTuX}$ were found to be very close to those of $tRNA^{Sec}$ and $tRNA^{UTu}$. Subsequently, conversion of Ser-$tRNA^{UTuX}$ to Sec-tRNA by SelA was examined (Fig. 1C). In contrast to the original $tRNA^{UTu}$, which showed about ~50% SelA-dependent conversion to Sec-tRNA, Ser-$tRNA^{UTuX}$ and WT Ser-$tRNA^{Sec}$ were very similar and yielded ~90% Sec formation (after 20 min). Tighter binding of SelA was further confirmed by RNase protection in the presence of excess SelA protein, revealing that within 20 min twice the amount of Ser-$tRNA^{UTu}$ was digested by nuclease P1 than Ser-$tRNA^{UTuX}$ or Ser-$tRNA^{Sec}$ (Fig. S2).

To determine the yield of Sec insertion by $tRNA^{UTuX}$ we next measured the specific activity of purified FDH_H. Using FDH_{H140op} produced by WT $tRNA^{Sec}$ as a standard, the specific activity of FDH_{H140am} synthesized in the presence of either $tRNA^{UTuX}{}_{am}$ or $tRNA^{UTu}{}_{am}$ was measured for comparison. FDH_H made with $tRNA^{UTu}{}_{am}$ had ~70% of the specific activity of the enzyme made with WT $tRNA^{Sec}$, and $tRNA^{UTuX}{}_{am}$ produced an enzyme with activity equivalent to the WT (Fig. 3A).

A similar analysis was then performed using the small *E. coli* redox protein glutaredoxin, Grx1, which contains two Cys residues (positions 11 and 14). These residues were mutated to amber11/Ser14 and Cys11/Ser14 to generate Grx1 variants containing a single Cys or Sec residue at position 11 [8]. $Grx1_{amber11/Ser14}$ was then expressed in the presence of either $tRNA^{UTuX}{}_{am}$ or $tRNA^{UTu}{}_{am}$ and purified while $Grx1_{Cys11/Ser14}$ served as a control. DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] reacts with Cys and Sec residues, while it has no affinity for Ser [19]. This results in a visible color change, which can be quantified spectroscopically. This reaction was used to determine the Sec insertion ratio in correlation to $Grx1_{Cys11/Ser14}$. In agreement with the results obtained FDH_H (Fig. 3A), DTNB treatment of the $Grx1_{amber11/Ser14}$ gene product synthesized with $tRNA^{UTuX}{}_{am}$ gave the same colorimetric signal intensity as $Grx1_{Cys11/Ser14}$; this indicated stoichiometric Sec insertion. In contrast, the $Grx1_{amber11/Ser14}$ gene product made by the original $tRNA^{UTu}{}_{am}$ [8] had a weaker signal, exhibiting only 70% of the WT intensity. This is in line with the earlier finding of 30% misincorporation of Ser [8] (Fig. 3B). These results were confirmed by intact mass FT-ICR mass spectrometry (Fig. 3C). Peaks at masses of 11,018.33 and 11,040.31 Da were observed, which correspond to the calculated masses for a Grx1-Sec11-GSH

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(11,019.43) and a Grx1-Sec11-GSH/Na^+ adduct (11,040.39), respectively. No mass peaks that coincide with a Grx1-Ser11 species were detected.

In vitro protein synthesis has previously been very successful in synthesizing proteins containing nonstandard amino acids [20,21]. Sec insertion has also been observed in eukaryotic cell free systems [22,23], detected as read-though products of a luciferase reporter protein. We sought to test the capacity of $tRNA^{Sec}$ and the synthetic $tRNA^{UTu}$ to synthesize a natural selenoenzyme by *in vitro* translation. For this we used the PURExpress *in vitro* translation system (NEB). To achieve Sec insertion, Sec-$tRNA^{Sec}{}_{am}$ was prepared biochemically and added to the reaction. However, as Sec-$tRNA^{Sec}$ must compete with canonical tRNA for EF-Tu binding, we anticipated a large quantity would be required to mediate translation. Additionally, while translation of *E. coli* FDH_H enabled sensitive colorimetric detection of Sec insertion, the large size of the protein and presence of both molybdenum and iron-sulfur cofactors meant an extended elongation time would likely be required.

Translation reactions were run under anoxic conditions, using a plasmid containing the $fdhF_{140am}$ gene under a T7 promoter to facilitate transcription to FDH_{H140am} mRNA. To eliminate competition for the UAG140 codon, the translation system lacked release factor 1 (PURExpress RF123 kit, NEB) while including RF2 and RF3, and was supplemented with sodium molybdate, RNase inhibitor, and elongation factor SelB. Using Sec-$tRNA^{Sec}$, active FDH_H was successfully produced after the standard 2 hr reaction time, yet these conditions produced active selenoenzyme only with wild-type Sec-$tRNA^{Sec}$. However, with the addition of an excess of EF-Tu, elevated levels of Sec-tRNA, and a long (5 hr) incubation time, all three synthetic tRNA variants ($tRNA^{UTu}{}_{am}$, $tRNA^{UTuX}{}_{am}$, and the recently reported $tRNA^{SecUX}{}_{am}$ [24]) were found to give active FDH_H protein (Fig. 4A). Using the specific activity calculated for WT FDH_H, the *in vitro* yield of active protein under the respective optimal conditions for each tRNA was roughly 34.7 ng using $tRNA^{Sec}{}_{am}$, 47.1 ng using $tRNA^{SecUX}$, 78.7 ng using $tRNA^{UTu}$, and 83.6 ng using $tRNA^{UTuX}$. Thus, each synthetic tRNA produced FDH_H activity similar to both one another and WT $tRNA^{Sec}$.

Although in nature selenocysteine is encoded by UGA, we have previously shown that many *E. coli* codons can be reassigned to Sec if the WT UGA codon preceding the SECIS element is replaced by a sense codon [10]. Using our cell-free selenoprotein synthesis system, we investigated the capacity to *in vitro* recode sense codons in FDH_H to Sec. Mutant genes encoding $fdhF_{140}$GGC, $fdhF_{140}$CGA, $fdhF_{140}$AGG, $fdhF_{140}$ACG, and WT $fdhF_{140}$op were paired with their respective cognate $tRNA^{Sec}$ mutants, and expressed *in vitro* using in the presence of SelB. While several of these codons did not produce active FDH_H *in vivo*, all variants tested were found to yield active enzyme *in vitro* (Fig. 4B) with estimated yields of 5.3 ng using $tRNA^{Sec}{}_{GCC}$ ($fdhF_{140}$GGC), 10.9 ng using $tRNA^{Sec}{}_{UCG}$ ($fdhF_{140}$CGA), 18.4 ng using $tRNA^{Sec}{}_{CCU}$ ($fdhF_{140}$AGG), 2.6 ng using $tRNA^{Sec}{}_{CGU}$ ($fdhF_{140}$ACG) and 12.8 ng using WT $tRNA^{Sec}{}_{op}$ ($fdhF_{140}$op).

In light of the *in vitro* recoding capacity of codons AGG and CGA, we reinvestigated the activity of these *fdhF* variants *in vivo*. Translation of $fdhF_{140}$AGG and $fdhF_{140}$CGA mRNA, isolation of FDH_H, and determination of specific activity showed recoding levels of AGG

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and CGA to be 65% and 46%, respectively. These results are incorporated in the data shown in Fig. S3.

4. Discussion

The major improvement of $tRNA^{UTuX}$ over $tRNA^{UTu}$ is seen in its ability to be an almost WT $tRNA^{Sec}$-like substrate for SelA ensuring optimal Ser to Sec conversion (Fig. S2). At the same time, $tRNA^{UTuX}$ is a better SerRS substrate than $tRNA^{Sec}$ (Table S1). These properties make $tRNA^{UTuX}$ our best $tRNA^{UTu}$ molecule for selenoprotein production.

Based on similar criteria for elongation and release factor recognition/rejection a different tRNA molecule for canonical Sec insertion was recently selected [24]. As its design began with the WT $tRNA^{Sec}$ scaffold, $tRNA^{SecUX}$ and $tRNA^{UTuX}$ are of different sequence in the D-arm, anticodon stem, variable arm, and T-arm; however, the two tRNAs contain the same critical structural parameters for EF-Tu binding [17]. This demonstrates that similar recognition and biological properties can be achieved by different nucleic acid landscapes.

While cell free synthesis of selenoproteins has previously been reported in partially purified components of the eukaryotic pathway (including purified Sec-$tRNA^{Sec}$) [22], we established an *in vitro* translation system for selenoproteins using commercially available bacterial components. Since protein synthesis quality controls for EF-Tu and SelB are relaxed in this PURExpress translation system, it was foreseen that protein yields would be low. Yet the availability of a sensitive color assay (benzyl-viologen reduction [18]) encouraged us to synthesize FDH_H, a selenoprotein whose enzyme activity depends on the presence of Sec, an iron sulfur cluster, and molybdenum as cofactor. Our strategy was successful; now the task at hand is to optimize protein production. Addition or co-expression of SelA and SelD (and selenite) may increase Sec-$tRNA^{UTuX}$ yield leading to additional rounds of protein synthesis. In view of the extensive efforts to optimize bacterial cell-free expression systems [25] and the success of generating sufficient Sec-tRNA *in vitro*, we anticipate this approach to lead to the *in vitro* generation of additional selenoproteins in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Arner ES. Selenoproteins-What unique properties can arise with selenocysteine in place of cysteine? Exp. Cell Res. 2010; 316:1296–1303. [PubMed: 20206159]

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2. Beld J, Woycechowsky KJ, Hilvert D. Selenoglutathione: efficient oxidative protein folding by a diselenide. Biochemistry. 2007; 46:5382–5390. [PubMed: 17419591]
3. Böck, A.; Thanbichler, M.; Rother, M.; Resch, A. Selenocysteine. In: Ibba, M.; Francklyn, CS.; Cusack, S., editors. Aminoacyl-tRNA Synthetases. Georgetown, TX: Landes Bioscience; 2005. p. 320-327.
4. Yoshizawa S, Böck A. The many levels of control on bacterial selenoprotein synthesis. Biochim. Biophys. Acta. 2009; 1790:1404–1414. [PubMed: 19328835]
5. Aldag C, Gromov IA, Garcia-Rubio I, von Koenig K, Schlichting I, Jaun B, Hilvert D. Probing the role of the proximal heme ligand in cytochrome P450cam by recombinant incorporation of selenocysteine. Proc. Natl. Acad. Sci. U. S. A. 2009; 106:5481–5486. [PubMed: 19293375]
6. Arner ES. Recombinant expression of mammalian selenocysteine-containing thioredoxin reductase and other selenoproteins in *Escherichia coli*. Methods Enzymol. 2002; 347:226–235. [PubMed: 11898411]
7. Xu J, Croitoru V, Rutishauser D, Cheng Q, Arner ES. Wobble decoding by the *Escherichia coli* selenocysteine insertion machinery. Nucleic Acids Res. 2013; 41:9800–9811. [PubMed: 23982514]
8. Aldag C, Bröcker MJ, Hohn MJ, Prat L, Hammond G, Plummer A, Söll D. Rewiring translation for elongation factor Tu-dependent selenocysteine incorporation. Angew. Chem. Int. Ed. Engl. 2013; 52:1441–1445. [PubMed: 23193031]
9. Paleskava A, Konevega AL, Rodnina MV. Thermodynamic and kinetic framework of selenocysteyl-$tRNA^{Sec}$ recognition by elongation factor SelB. J. Biol. Chem. 2010; 285:3014–3020. [PubMed: 19940162]
10. Bröcker MJ, Ho JM, Church GM, Söll D, O'Donoghue P. Recoding the genetic code with selenocysteine. Angew. Chem. Int. Ed. Engl. 2014; 53:319–323. [PubMed: 24511637]
11. Sherrer RL, Ho JM, Söll D. Divergence of selenocysteine tRNA recognition by archaeal and eukaryotic O-phosphoseryl-$tRNA^{Sec}$ kinase. Nucleic Acids Res. 2008; 36:1871–1880. [PubMed: 18267971]
12. Sheppard K, Akochy PM, Söll D. Assays for transfer RNA-dependent amino acid biosynthesis. Methods. 2008; 44:139–145. [PubMed: 18241795]
13. Yuan J, Palioura S, Salazar JC, Su D, O'Donoghue P, Hohn MJ, Cardoso AM, Whitman WB, Söll D. RNA-dependent conversion of phosphoserine forms selenocysteine in eukaryotes and archaea. Proc. Natl. Acad. Sci. U. S. A. 2006; 103:18923–18927. [PubMed: 17142313]
14. Araiso Y, Palioura S, Ishitani R, Sherrer RL, O'Donoghue P, Yuan J, Oshikane H, Domae N, Defranco J, Söll D, Nureki O. Structural insights into RNA-dependent eukaryal and archaeal selenocysteine formation. Nucleic Acids Res. 2008; 36:1187–1199. [PubMed: 18158303]
15. Itoh Y, Bröcker MJ, Sekine S, Hammond G, Suetsugu S, Söll D, Yokoyama S. Decameric SelA:$tRNA^{Sec}$ ring structure reveals mechanism of bacterial selenocysteine formation. Science. 2013; 340:75–78. [PubMed: 23559248]
16. Schrader JM, Chapman SJ, Uhlenbeck OC. Understanding the sequence specificity of tRNA binding to elongation factor Tu using tRNA mutagenesis. J. Mol. Biol. 2009; 386:1255–1264. [PubMed: 19452597]
17. Rudinger J, Hillenbrandt R, Sprinzl M, Giege R. Antideterminants present in $minihelix^{Sec}$ hinder its recognition by prokaryotic elongation factor Tu. EMBO J. 1996; 15:650–657. [PubMed: 8599948]
18. Lacourciere GM, Levine RL, Stadtman TC. Direct detection of potential selenium delivery proteins by using an *Escherichia coli* strain unable to incorporate selenium from selenite into proteins. Proc. Natl. Acad. Sci. U. S. A. 2002; 99:9150–9153. [PubMed: 12084818]
19. Ellman GL. Tissue sulfhydryl groups. Arch. Biochem. Biophys. 1959; 82:70–77. [PubMed: 13650640]
20. Kang TJ, Suga H. Ribosomal synthesis of nonstandard peptides. Biochem. Cell. Biol. 2008; 86:92–99. [PubMed: 18443622]
21. Seebeck FP, Ricardo A, Szostak JW. Artificial lantipeptides from in vitro translations. Chem. Commun. (Camb.). 2011; 47:6141–6143. [PubMed: 21528125]

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22. Gupta N, DeMong LW, Banda S, Copeland PR. Reconstitution of selenocysteine incorporation reveals intrinsic regulation by SECIS elements. J. Mol. Biol. 2013; 425:2415–2422. [PubMed: 23624110]
23. Mehta A, Rebsch CM, Kinzy SA, Fletcher JE, Copeland PR. Efficiency of mammalian selenocysteine incorporation. J. Biol. Chem. 2004; 279:37852–37859. [PubMed: 15229221]
24. Thyer R, Robotham SA, Brodbelt JS, Ellington AD. Evolving $tRNA^{Sec}$ for efficient canonical incorporation of selenocysteine. J. Am. Chem. Soc. 2015; 137:46–49. [PubMed: 25521771]
25. Jewett MC, Calhoun KA, Voloshin A, Wuu JJ, Swartz JR. An integrated cell-free metabolic platform for protein production and synthetic biology. Mol. Syst. Biol. 2008; 4:220. [PubMed: 18854819]

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Highlights

- A chimaera of $tRNA^{Ser}$ and $tRNA^{Sec}$, $tRNA^{UTuX}$, binds EF-Tu to insert Sec at UAG codons
- $tRNA^{UTuX}$ was used for complete, high fidelity Sec insertion
- We show *in vitro* selenoprotein synthesis, compatible with wild-type and synthetic tRNA
- Sense codons were recoded *in vitro* in the presence of SelB
- Formate dehydrogenase activity demonstrated *in vitro* selenoenzyme synthesis

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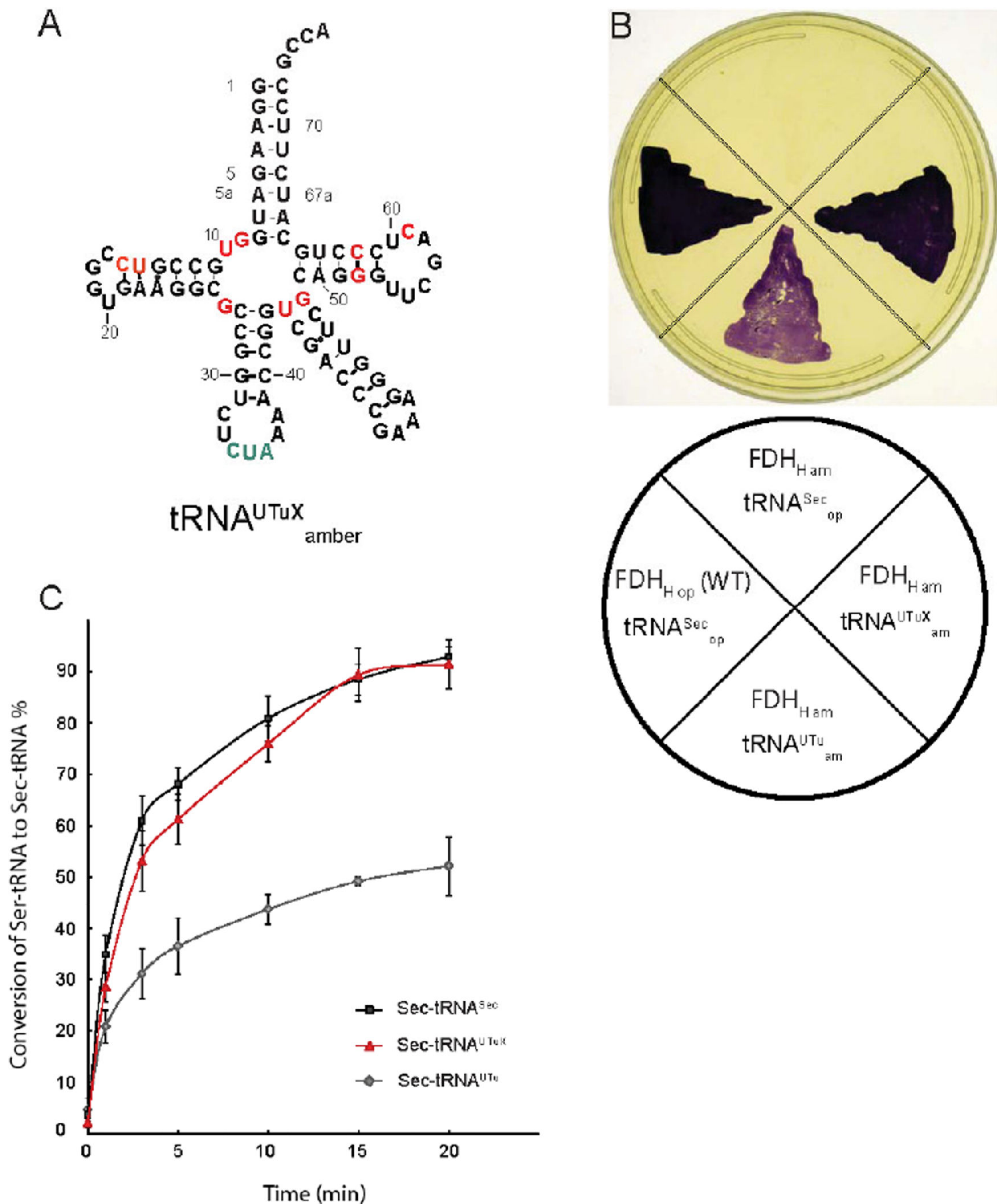

Fig. 1.

(A) Secondary structure of $tRNA^{UTuX}$. Nucleotides that were changed from the original $tRNA^{UTu}$ are highlighted in red, the amber anticodon is depicted in green. (B) $tRNA^{UTuX}$ mediates functional Sec insertion in FDH_H. An *E. coli* *selA* *selB* *fdhf* triple deletion strain was separately complemented with *E. coli* SelA, *M. jannaschii* PSTK alongside $tRNA^{Sec}{}_{op}$, *E. coli* SelB, and WT FDH_{Hop}; $tRNA^{UTu}{}_{am}$ and FDH_{Ham}; $tRNA^{UTuX}{}_{am}$ and FDH_{Ham}; and a negative control with $tRNA^{Sec}{}_{op}$, *E. coli* SelB, and FDH_{H140am}. FDH_H activity was assessed by appearance of the purple colored reduced BV. (C) *In vitro*

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conversion of Ser-tRNA^{Sec}, Sert-RNA^{UTu}, and Ser-tRNA^{UTuX} by SelA. 5 μM SelD, Reactions were pre-incubated with 1 mM Na_2SeO_3 and 5 mM ATP at pH 7.2 under anaerobic conditions at 37°C for 30 min and then supplemented with 1 μM SelA and 10 μM of [α^{32}-P] radiolabeled Ser-tRNA species for up to 20 min. Aliquots of 1.5 μL were taken at different time points, digested with nuclease P1, and spotted onto cellulose thin layer chromatography plates. After developing, plates were analyzed by autoradiography. While approximately 50% of Ser-tRNA^{UTu} was converted, both tRNA^{Sec} and tRNA^{UTuX} support nearly full conversion to Sec-tRNA over a course of 20 min.

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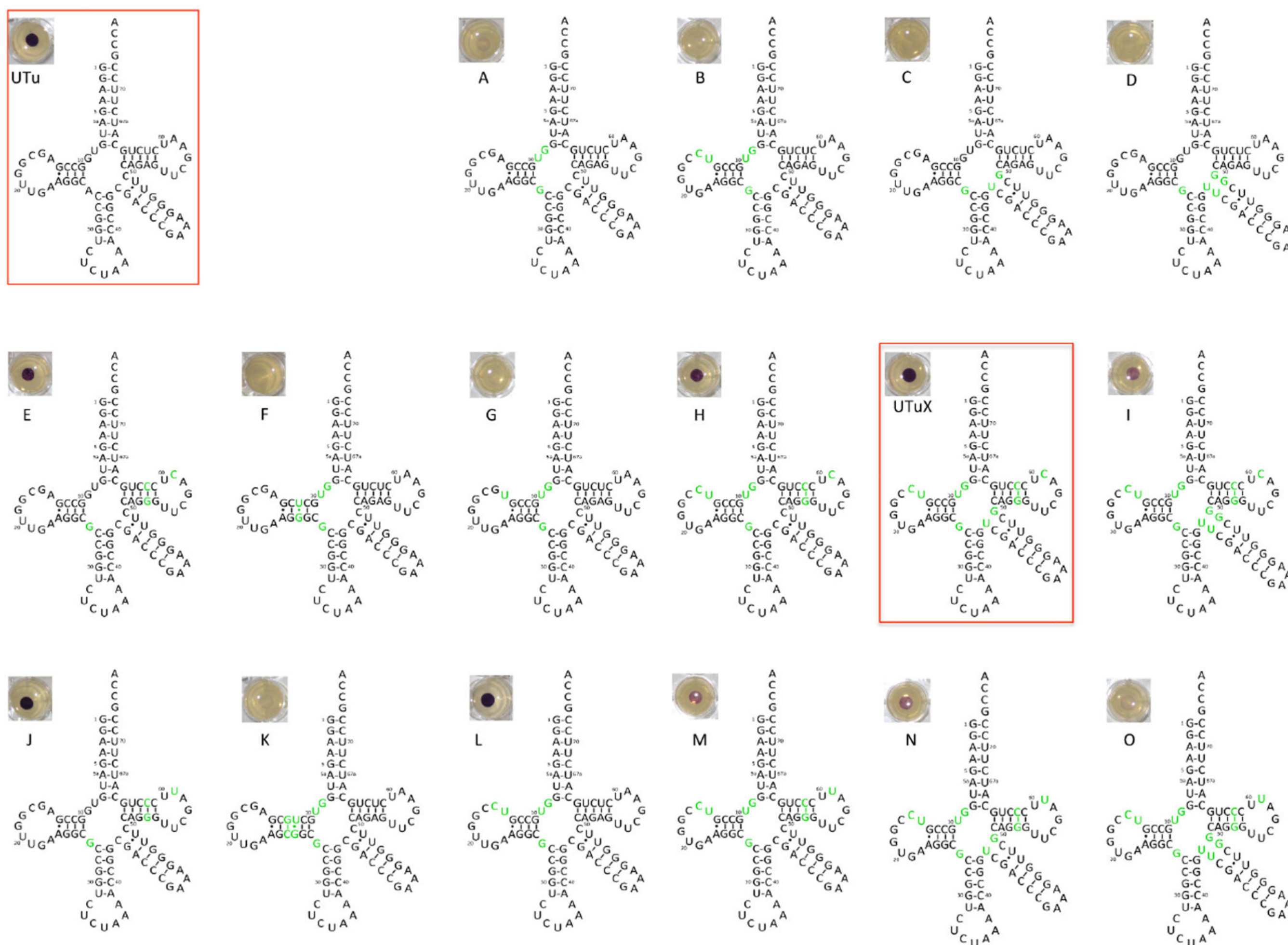

Fig. 2.

Activity of rationally desgined $tRNA^{UTu}$ variants. For each of sixteen variants, mutations relative to $tRNA^{UTu}$ are depicted in green, with the original variant shown in the upper left. Each variant as well as E. coli SelB and SelA was used to complement FDH_{Ham} in *E. coli* *selA* *selB* *fdhf*,. FDH_{H} activity was assessed by appearance of the purple colored reduced BV, with the activity mediated by each variant shown adjacent to it.

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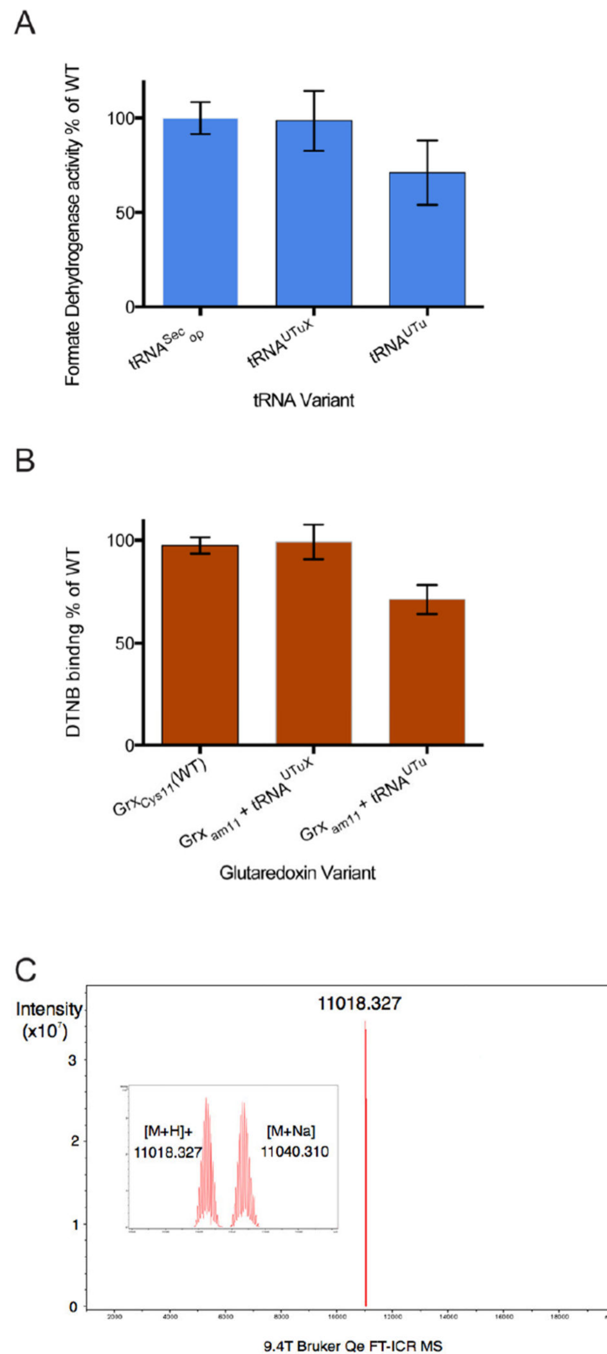

Fig. 3.

Assessment of $tRNA^{UTuX}$-mediated Sec insertion fidelity. (A) Sec-dependent *in vitro* BV reduction of recombinant purified FDH_H variants. 100nM each of WT FDH_{H140op} and FDH_{H140am} produced with $tRNA^{UTu}$ and $tRNA^{UTuX}$ were assayed in the linear range of the reaction for 5 min. Relative specific activity of FDH_H variants expressing using $tRNA^{Sec}{}_{op}$, $tRNA^{UTu}$, and $tRNA^{UTuX}$ were determined. Compared to WT $tRNA^{Sec}{}_{op}$, synthetic variants $tRNA^{UTuX}$ and $tRNA^{UTu}$ mediated Sec insertion into FDH_{H140am} allowed 98.5 ± 15.8% and 71.1 ± 17.0% BV reduction activity, respectively. (B) Sec incorporation into recombinant *E.*

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coli Grx1 variants as determined spectroscopically by assaying DTNB (Ellman's reagent) binding. Relative to WT $tRNA^{Sec}_{op}$, expression of $Grx1_{C11am}$ with $tRNA^{UTuX}$ and $tRNA^{UTu}$ resulted in 99.2 ± 8.4% and 71.2 ± 7.1% DTNB coupling, respectively. (C) Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry of purified $Grx1_{C11am}$. Mass peaks of 11,018.33 and 11,040,31 m/z were observed, corresponding to the masses of a $Grx1_{C11U}$ glutathione adduct (11,019.38), and a glutathione plus Na+ adduct (11,041.34). The unit m/z describes the mass-to-charge ratio.

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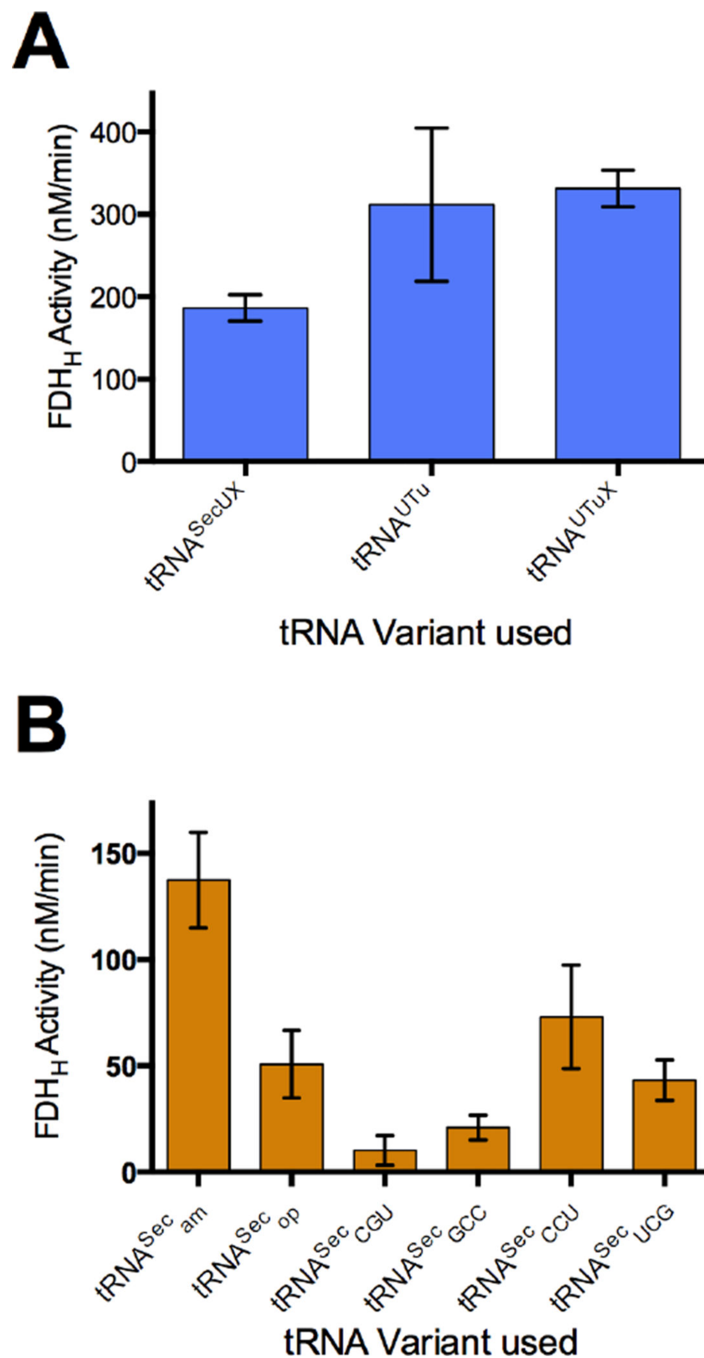

Fig. 4.

Activity of FDH_H produced through cell-free protein synthesis. Cell-free translation of selenoprotein FDH_H was mediated by different Sec-tRNA variants and cognate *fdhF*$_{140}$ mutants under optimized conditions (see Materials and Methods), and FDH_{H140am} activity was monitored through reduction of BV at 578 nm. (A) Activity of $tRNA^{Utu}{}_{am}$, $tRNA^{UTuX}{}_{am}$, and $tRNA^{SecUX}{}_{am}$-mediated FDH_{H140am} translation in the absence of release factor 1 (while in the presence of RF2 and RF3) was found to be comparable for each variant. (B) Translation of FDH_H mutants with cognate tRNA variants $tRNA^{Sec}{}_{am}$,

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$tRNA^{Sec}_{op}$, $tRNA^{Sec}_{CGU}$, $tRNA^{Sec}_{GCC}$, $tRNA^{Sec}_{CCU}$, and $tRNA^{Sec}_{UCG}$ in the presence of SelB demonstrates that each sense codon is capable of recoding with selenocysteine *in vitro*. Absence of activity was confirmed in a negative control reaction prepared with Ser-$tRNA^{Sec}_{am}$ in place of Sec-$tRNA^{Sec}_{am}$, and in a separate negative control prepared with plasmid encoding dihydrofolate reductase (*DHFR*) in place of $fdhF_{am}$.