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A tRNA-dependent cysteine biosynthesis enzyme recognizes the selenocysteine-specific tRNA in *Escherichia coli*

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ABSTRACT

The essential methanogen enzyme Sep-tRNA:Cys-tRNA synthase (SepCysS) converts *O*-phosphoseryl-tRNA^{Cys} (Sep-tRNA^{Cys}) into Cys-tRNA^{Cys} in the presence of a sulfur donor. Likewise, Sep-tRNA:Sec-tRNA synthase converts *O*-phosphoseryl-tRNA^{Sec} (Sep-tRNA^{Sec}) to selenocysteinyl-tRNA^{Sec} (Sec-tRNA^{Sec}) using a selenium donor. While the Sep moiety of the aminoacyl-tRNA substrates is the same in both reactions, tRNA^{Cys} and tRNA^{Sec} differ greatly in sequence and structure. In an *Escherichia coli* genetic approach that tests for formate dehydrogenase activity in the absence of selenium donor we show that Sep-tRNA^{Sec} is a substrate for SepCysS. Since Sec and Cys are the only active site amino acids known to sustain FDH activity, we conclude that SepCysS converts Sep-tRNA^{Sec} to Cys-tRNA^{Sec}, and that Sep is crucial for SepCysS recognition.

1. Introduction

The tRNA-dependent amino acid modification reactions provide aminoacyl-tRNAs (aa-tRNAs) for at least four amino acids [1]. The enzymes involved in this process must recognize the amino acid of the aa-tRNA as well as a part of the tRNA. Because of the importance of these reactions for protein synthesis, and their possible application for making unnatural aa-tRNA species, it is desirable to know what part(s) of the tRNA is recognized by these enzymes. There is a reasonable amount of information on the tRNA-dependent amidotransferases, which are responsible for -glutaminyl- and asparaginyl-tRNA formation, and also Sep-tRNA:Sec-tRNA synthase (SepSecS), the enzyme that forms Sec-tRNA from Sep-tRNA [2,3].

SepSecS appears to be highly specific for Sep-tRNA^{Sec} based on the crystal structure of the SepSecS:tRNA^{Sec} binary complex. Its unique homotetrameric quaternary state not only interacts with the Sep moiety but also specifically recognizes tRNA^{Sec} by measuring the 13 bp long acceptor/TΨC helix (Fig. 1). The shorter 12 bp acceptor/TΨC helix of canonical tRNAs precludes them from being substrates for SepSecS since the tip of their acceptor arm cannot reach the active site of the enzyme [4]. SepSecS uses selenophosphate as the selenium donor, but cannot differentiate it from its sulfur-containing analog thiophosphate and forms Cys-tRNA^{Sec} in vitro [4]. This non-discriminative nature of SepSecS towards thiophosphate and selenophosphate is compensated in vivo by the highly specific activity of selenophosphate synthetase (SelD), which discriminates against sulfide and only forms selenophosphate in the presence of ATP [5]. Furthermore, the long acceptor/TΨC helix precludes binding of Sec-tRNA^{Sec} to elongation factor Tu (EF-Tu) and serves as the distinct feature that the selenocysteine (Sec)-specific elongation factor SelB (SelB) is recognizing [6,7], which ensures accurate Sec incorporation in response to Sec UGA codons.

Similar information on substrate specificity is lacking for Sep-tRNA:Cys-tRNA synthase (SepCysS), the essential enzyme in methanogenic tRNA-dependent cysteine biosynthesis. SepCysS converts Sep-tRNA^{Cys} to Cys-tRNA^{Cys} in the presence of a sulfur donor [8,9]. Cys-tRNA^{Cys}, the product of this tRNA-dependent pathway, is either used for protein synthesis or it provides free cysteine for other biosynthetic pathways via its deacylation [8,9].

Abbreviations: aa-tRNA, aminoacyl-tRNA; EF-Tu, elongation factor Tu; FDH$_H$, formate dehydrogenase H; FDH$_N$, formate dehydrogenase N; IPTG, isopropyl-D-thiogalactoside; PLP, pyridoxal phosphate; PSTK, phosphoseryl-tRNA^{Sec} kinase; Sec, selenocysteine; SelA, selenocysteine synthase; SelB, elongation factor SelB; SelD, selenophosphate synthetase; Sep, *O*-phosphoserine; SepCysS, Sep-tRNA:Cys-tRNA synthase; SepSecS, Sep-tRNA:Sec-tRNA synthase

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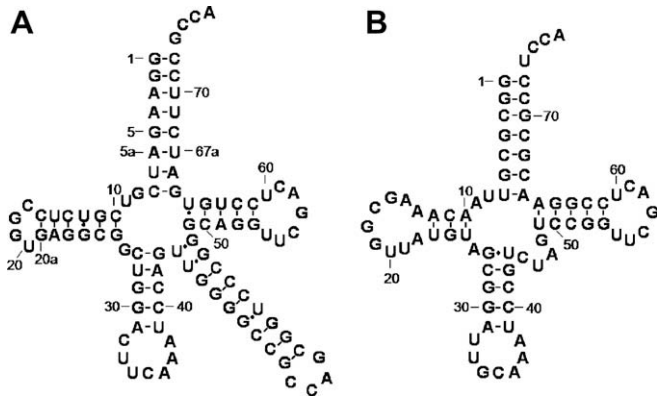

Fig. 1. Secondary structures of $tRNA^{Sec}$ and $tRNA^{Cys}$. The cloverleaf structures of *E. coli* $tRNA^{Sec}$ (A) and $tRNA^{Cys}$ (B) are shown.

SepCysS resembles SepSecS in several aspects. Both enzymes use phosphoserylated tRNAs as substrates and catalyze amino acid conversions by a pyridoxal phosphate (PLP)-dependent mechanism [4,10,11]. The reactions start with the formation of a Schiff base between the *O*-phosphoserine (Sep) moiety of the Sep-tRNA and PLP. This ultimately leads to release of Sep's phosphate group and formation of a PLP-bound dehydroalanyl-tRNA intermediate. Nucleophilic attack of this intermediate by the incoming sulfur or selenium atom yields an oxidized form of Cys- or Sec-tRNA respectively that is subsequently reduced and released from the enzyme. In contrast to SepSecS, little is known about the substrate specificity of SepCysS besides that it can use multiple sulfur donors in vitro such as sulfide, thiophosphate and cysteine [11]. Given the apparent similarity of SepCysS and SepSecS catalysis, the different structures of $tRNA^{Sec}$ and $tRNA^{Cys}$ (Fig. 1), and the established in vivo functional assay for $tRNA^{Sec}$ utilizing formate dehydrogenase [4,10,12,13], we decided to test in vivo whether Sep-$tRNA^{Sec}$ is a substrate for SepCysS.

2. Materials and methods

2.1. General

DNA sequencing was performed by the Keck Foundation Biotechnology Resource Laboratory at Yale University. [^{75}Se]selenite was purchased from the University of Missouri Research Reactor Facility (Columbia, MO).

2.2. Plasmids for in vivo complementation

The plasmids were constructed as described before [12]. Specifically, the *O*-phosphoseryl-$tRNA^{Sec}$ kinase (PSTK) gene (*pstK*) from *Methanocaldococcus jannaschii* and *selD* from *Escherichia coli* were cloned into the pACYC vectors individually. *E. coli selA*, *M. jannaschii SepSecS* and SepCysS genes (encoded by *spcS* and *pscS*, respectively) were cloned into pET15b vectors individually.

2.3. Construction of the E. coli ΔselA ΔselD double deletion strain

Construction of the *ΔselA ΔselD* double deletion strain was carried out according to a published method [14]. The KmR cassette, which is disrupting *selA* in *E. coli* strain JS1, was excised by FLP recombinase-mediated homologous recombination between the FRT sites flanking the KmR cassette, upon transformation of the JS1 strain with plasmid pCP20 [14]. In the resulting strain, the *selD* gene was then disrupted by a FRT-KmR cassette as previously described [14], thus yielding strain MH1.

2.4. Complementation test using the benzyl viologen assay

The *E. coli* JS1 and MH1 strains were transformed with genes as indicated in the figure legends and tested for formate dehydrogenase H (FDH_H) activity by the benzyl viologen assay as described before [4,10,12,13,15]. The transformants were grown anaerobically on glucose-minimum medium agar plates at 30 °C for 24–48 h in the presence of 0.01 mM isopropyl-D-thiogalactoside (IPTG). After they were removed from the anaerobic jar, the plates were immediately overlaid with 0.75% top agar (containing 1 mg/ml benzyl viologen, 0.25 M sodium formate and 25 mM KH_2PO_4, pH 7.0).

2.5. Complementation test using the McConkey nitrate assay

E. coli JS1 transformants were plated on McConkey nitrate plates (40 g/l McConkey agar base, 20 g/l KNO_3, 1 g/l glycerol, 0.5 g/l sodium formate and 0.1 g/l glucose) [16] and grown anaerobically at 37 °C for 24 h.

2.6. Metabolic labeling with radioactive selenium

The procedure was carried out as described [12]. Overnight cultures of JS1 transformants were diluted (1:50) in 5 ml of TGYEP medium (0.5% glucose, 1% tryptone, 0.5% yeast extract, 1.2% K_2HPO_4, 0.3% KH_2PO_4, 0.1% formate, 1 μM Na_2MoO_4, pH 6.5) [17] supplemented with 1 μCi [^{75}Se]selenite and 0.05 mM IPTG, and grown under anaerobic conditions at 37 °C for 24 h. The cells were harvested and the cell lysates analyzed by SDS–PAGE followed by autoradiography. Various conditions were used to increase the sensitivity of the assay including a higher amount of [^{75}Se]selenite (10 μCi), higher concentration of IPTG (up to 0.5 mM) and longer anaerobic growth time (up to 48 h).

3. Results

3.1. The $tRNA^{Sec}$ is a substrate for SepCysS

To test whether SepCysS can utilize Sep-$tRNA^{Sec}$ as a substrate in vivo, we transformed the *E. coli ΔselA* strain JS1 with the SepCysS gene (*pscS*) in the presence or absence of the PSTK gene (*pstK*). Transformants were then tested for the activities of two selenoproteins, FDH_H and formate dehydrogenase N (FDH_N) [18]. Active FDH_H can use formate to reduce benzyl viologen which results in purple colored colonies [15]. Transformants grown anaerobically on minimal medium plates supplemented with selenite were layered with top agar containing formate and benzyl viologen. Complementation of the *selA* deletion was observed when strain JS1 was either cotransformed with the PSTK and SepSecS genes, or with *E. coli selA* (Fig. 2, left). Cells cotransformed with SepCysS and PSTK genes also turned purple, however to a lesser extent, suggesting a reduced FDH_H activity (Fig. 2, left). Transformation of the SepCysS gene or PSTK gene alone did not restore FDH_H activity in the *E. coli* JS1 strain (Fig. 2, left).

To further confirm these results we tested the transformed JS1 strains for the activity of another selenoprotein, FDH_N, which uses nitrate as an electron acceptor when formate is oxidized. Transformants were grown anaerobically on McConkey plates containing nitrate and a pH indicator. Cells containing active FDH_N consume nitrate, resulting in an increase of the pH shown as yellow colored colonies, whereas cells with inactive FDH_N remain acidic and form red colonies [16]. Our results (Fig. 2, right) are in agreement with the data from the benzyl viologen assays and confirm that FDH formation in the *E. coli* JS1 strain can be restored by the simultaneous presence of PSTK and SepCysS. This suggests that in *E. coli* SepCysS

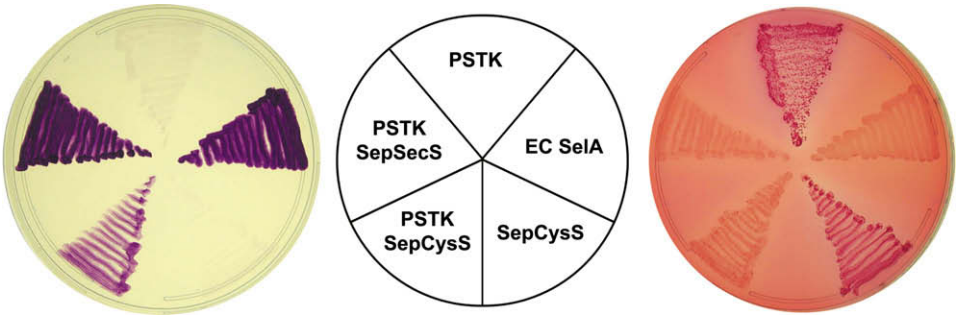

Fig. 2. SepCysS and PSTK complement an *E. coli ΔselA* deletion. The indicated proteins (middle) complement the loss of selenocysteine synthase (SelA) in the *E. coli ΔselA* deletion strain JS1. Activity of the selenoproteins FDH_H and FDH_N was tested with the benzyl viologen assay (left) and the McConkey nitrate plate assay (right), respectively.

recognizes Sep-tRNA^{Sec} as a substrate as do SepSecS and selenocysteine synthase (SelA) [19].

3.2. SepCysS does not form Sec-tRNA^{Sec}

The observation that the JS1 strain transformed with SepCysS and PSTK shows less FDH_H activity compared to the SepSecS and PSTK complemented strain can be explained in two ways: (i) the SepCysS/PSTK complemented strain produces less selenoprotein, or (ii) SepCysS forms Cys-tRNA^{Sec}, leading to cysteine incorporation at the Sec codon generating the sulfur homolog of FDH_H. It is known that a Sec to Cys mutation in FDH_H causes a 110-fold decrease in k_{cat} compared to the wild type Sec-containing enzyme [20], and that Cys and Sec are the only amino acids that confer activity to FDH_H.

To distinguish between these two possibilities, we carried out [^{75}Se] in vivo labeling experiments. Transformed JS1 strains were grown anaerobically in the presence of formate and [^{75}Se]-selenite. Total cell lysates, prepared from equal amounts of cells, were separated by SDS–PAGE. Radioactively labeled selenoprotein was detected by autoradiography. Our results (Fig. 3) show that [^{75}Se] labeled proteins only occur in the *ΔselA* JS1 strain transformed either with *E. coli selA* or with both PSTK and SepSecS genes. Comparison of lanes 5 and 6 in Fig. 3 shows that under our experimental in vivo conditions SepCysS is unable to use the Se-donor selenophosphate as substrate. This is in contrast to SepSecS which accepts both thiophosphate and selenophosphate as substrates [4]. The currently available crystal structures of SepCysS [21] and SepSecS [4,10,22] do not offer any insight on this difference in substrate selection.

No [^{75}Se] labeled proteins are detectable in transformants with PSTK and SepCysS genes, despite various efforts to increase the assay sensitivity (see Section 2 for the conditions tested). This suggests that explanation (ii) is correct, and thus SepCysS forms Cys-tRNA^{Sec} in *E. coli*. To further confirm this result, we performed transformation assays in cells devoid of the selenium donor selenophosphate.

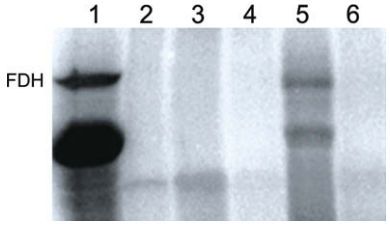

Fig. 3. Metabolic labeling of transformed *ΔselA* strains with ^{75}Se. The *E. coli ΔselA* strain JS1 was complemented with *E. coli selA* (lane 1), empty vector control (lane 2), *M. jannaschii spcS* (coding for SepSecS, lane 3), *M. jannaschii pscS* (coding for SepCysS, lane 4), *M. jannaschii spcS* and *pstK* genes (lane 5), and *M. jannaschii pscS* and *pstK* (lane 6). Two major bands were observed in the positive control lane 1. Based on the molecular weight marker, the upper band corresponds to FDH_H. The lower band is likely a degradation product of FDH_H, the sole selenoprotein in *E. coli* in the indicated growth conditions.

3.3. SepCysS forms Cys-tRNA^{Sec} in E. coli

The *E. coli selD* gene encodes SelD, the enzyme that forms selenophosphate using selenide and ATP. Selenophosphate is the activated selenium donor required for Sec-tRNA^{Sec} synthesis [23]. Deletion of *selD* abolishes selenoprotein formation but does not affect sulfur metabolism since SelD is specific for selenide [24,25]. To further confirm that transformation of the *ΔselA* strain JS1 by the SepCysS and PSTK genes does not generate Sec synthesized from selenophosphate, we constructed the *E. coli ΔselA ΔselD* double deletion strain MH1. This strain was then transformed with the SepCysS/PSTK or SepSecS/PSTK genes, and the resulting transformants were tested for FDH_H activity with the benzyl viologen assay. Our results show that the SepSecS and PSTK genes are no longer able to restore FDH_H activity in the *ΔselA ΔselD* double deletion strain MH1 (Fig. 4). However, the SepCysS and PSTK genes retain their ability to restore FDH_H activity albeit somewhat weaker compared to the positive control (MH1 strain transformed with *E. coli selA* and *selD*). These results prove that the observed FDH_H activity is due to a Cys active site residue and not due to a Sec one. Deletion of SelD in the MH1 strain precludes SepSecS from acting onto Sep-tRNA^{Sec} since SelD is the only enzyme responsible for selenophosphate formation. On the other hand, SepCysS is still able to use an available sulfur donor and convert Sep-tRNA^{Sec} to Cys-tRNA^{Sec}. Although we do not show direct evidence for the presence of the sulfur homolog of FDH_H, our findings are in agreement with previous reports showing that, other than Sec in the catalytic site of FDH_H, only Cys can retain partial activity [20,26]. Taken together, these data imply that, in the presence of PSTK, SepCysS forms Cys-tRNA^{Sec} in *E. coli*.

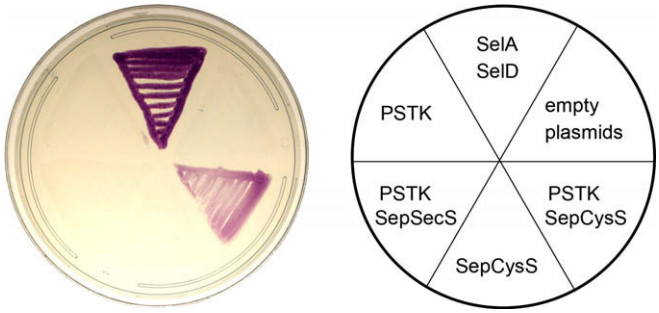

Fig. 4. SepCysS restores FDH_H activity in an *E. coli ΔselA ΔselD* strain. The indicated proteins (right) complement the loss of SelA and SelD in the *E. coli* strain MH1. Activity of the selenoprotein FDH_H is tested with benzyl viologen assay.

4. Discussion

4.1. aa-tRNA recognition by SepCysS

Our results indicate that SepCysS can convert Sep-tRNA^{Sec} to Cys-tRNA^{Sec} in *E. coli*. Its physiological function in methanogens is to convert Sep-tRNA^{Cys} to Cys-tRNA^{Cys}. Thus, SepCysS is the first enzyme involved in tRNA-dependent amino acid transformations shown to have the ability to act on two distinct tRNAs, tRNA^{Cys} and tRNA^{Sec}. Clearly, SepCysS must specifically recognize the Sep moiety attached to either tRNA^{Cys} or tRNA^{Sec}. Indeed, SepCysS alone does not complement the *E. coli ΔselA* strain JS1 suggesting that Ser-tRNA^{Sec} is not a substrate for SepCysS. This is not surprising given that precise recognition of the phosphate moiety of Sep is also the mode by which SepSecS discriminates against Ser-tRNA^{Sec} [4]. The highly divergent sequences and structures of tRNA^{Cys} and tRNA^{Sec} imply that SepCysS does not rely heavily on tRNA identity for activity and strengthens the notion that Sep recognition is the main binding force of Sep-tRNA to the enzyme.

All known tRNA^{Cys} species adopt the canonical 12 bp acceptor/TΨC helix, while tRNA^{Sec} folds into the distinct 13 bp acceptor/TΨC conformation that is crucial for recognition by SepSecS and most likely by all Sec-specific enzymes [4,27]. Thus, unlike its importance for SepSecS recognition, the unique structure of tRNA^{Sec} does not appear to be an anti-determinant for SepCysS. We can speculate that apart from the Sep moiety SepCysS is also recognizing the tip of the acceptor stem of *E. coli* tRNA^{Sec}. In fact, though *M. jannaschii* tRNA^{Cys} and *E. coli* tRNA^{Sec} have different discriminator bases (U73 and G73, respectively), they do share a common first bp (G1-C72) at their acceptor stems. The benzyl viologen complementation assay has been proven very powerful in characterizing at least two of the enzymes (PSTK and SepSecS) involved in archaeal and eukaryal Sec biosynthesis [4,10,13]. In both cases, in vivo complementation results always correlated with in vitro results by purified enzymes, attesting to the capability of the benzyl viologen assay to render reliable data. Given the present lack of knowledge regarding the nature of the sulfur donor in the SepCysS reaction [9,11], the benzyl viologen assay may be useful for an in vivo study of the tRNA^{Cys} and tRNA^{Sec} recognition by SepCysS.

4.2. aa-tRNA specificity of SelB

Cys-tRNA^{Sec} formed by SepCysS in *E. coli* is incorporated during protein synthesis in response to the in frame UGA codon in the gene encoding formate dehydrogenase. Given the inability of EF-Tu to bind to tRNA^{Sec} [6,28], we conclude that the specialized SelB is transferring Cys-tRNA^{Sec} to the translation apparatus. SelB is known to discriminate against unacylated tRNA^{Sec} and Ser-tRNA^{Sec} both in vivo [7] and in vitro [29,30]. SelB binds Sec-tRNA^{Sec} one thousand times (K_d 0.2 pM) tighter than tRNA^{Sec} or Ser-tRNA^{Sec} (K_d 0.5 μM) in vitro [30] and it does not deliver Ser-tRNA^{Sec} to the ribosome in vivo [7]. The higher affinity of SelB for Sec-tRNA^{Sec} is attributed to the amino acid binding pocket of SelB that is specifically designed to bind Sec [7]. Our results suggest that Cys can be bound tightly enough in the active site of SelB to allow for Cys-tRNA^{Sec} delivery to the ribosome. Thus, SelB adds to the list of enzymes that cannot entirely distinguish sulfur from selenium, such as SepSecS which uses thiophosphate in vitro to form Cys-tRNA^{Sec} [4,10] and CysRS which can acylate Sec onto tRNA^{Cys} [31,32].

4.3. Amino acid ambiguity for UGA decoding in methanogens

The physiological significance of Cys-tRNA^{Sec} in organisms that possess both SepCysS and SepSecS is unclear at this point. In fact, Cys-tRNA^{Sec} would only be formed if SepCysS successfully competes with SepSecS for binding to Sep-tRNA^{Sec}. This would in turn mean that the UGA codon is ambiguous in methanogens as it would encode for both Cys and Sec during translation of the same open reading frame. In such a case, methanogenic selenoproteins would also be expressed with a Cys residue in place of Sec. This may be advantageous in cases of selenium deficiency in the environment since the cysteine homologs of most known selenoproteins are active albeit to a lesser extent than their selenium-containing counterparts [20,33,34]. Further investigation of the SepCysS activity towards Sep-tRNA^{Sec} in Sec decoding archaea will shed light on the physiological importance of Cys-tRNA^{Sec} and the in vivo role of the UGA codon in these organisms.

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