Divergence of selenocysteine tRNA recognition by archaeal and eukaryotic O-phosphoseryl-tRNA<sub>Sec</sub> kinase

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ABSTRACT

Selenocysteine (Sec) biosynthesis in archaea and eukaryotes requires three steps: serylation of tRNA<sub>Sec</sub> by seryl-tRNA synthetase (SerRS), phosphorylation of Ser-tRNA<sub>Sec</sub> by O-phosphoseryl-tRNA<sub>Sec</sub> kinase (PSTK), and conversion of O-phosphoseryl-tRNA<sub>Sec</sub> (Sep-tRNA<sub>Sec</sub>) by Sep-tRNA:Sec-tRNA synthase (SepSecS) to Sec-tRNA<sub>Sec</sub>. Although SerRS recognizes both tRNA<sub>Sec</sub> and tRNA<sub>Ser</sub> species, PSTK must discriminate Ser-tRNA<sub>Sec</sub> from Ser-tRNA<sub>Ser</sub>. Based on a comparison of the sequences and secondary structures of archaeal tRNA<sub>Sec</sub> and tRNA<sub>Ser</sub>, we introduced mutations into Methanococcus maripaludis tRNA<sub>Sec</sub> to investigate how Methanocaldococcus jannaschii PSTK distinguishes tRNA<sub>Sec</sub> from tRNA<sub>Ser</sub>. Unlike eukaryotic PSTK, the archaeal enzyme was found to recognize the acceptor stem rather than the length and secondary structure of the D-stem. While the D-arm and T-loop provide minor identity elements, the acceptor stem base pairs G2-C71 and C3-G70 in tRNA<sub>Sec</sub> were crucial for discrimination from tRNA<sub>Ser</sub>. Furthermore, the A5-U68 base pair in tRNA<sub>Ser</sub> has some antideterminant properties for PSTK. Transplantation of these identity elements into the tRNA<sub>Ser</sub> UGA scaffold resulted in phosphorylation of the chimeric Ser-tRNA. The chimera was able to stimulate the ATPase activity of PSTK albeit at a lower level than tRNA<sub>Sec</sub>, whereas tRNA<sub>Ser</sub> did not. Additionally, the seryl moiety of Ser-tRNA<sub>Sec</sub> is not required for enzyme recognition, as PSTK efficiently phosphorylated Thr-tRNA<sub>Sec</sub>.

INTRODUCTION

While UGA is typically a stop codon, selenocysteine is co-translationally inserted into proteins in response to in-frame Sec-decoding UGA codons in a limited number of organisms from all three domains of life (1). Sec is formed by a tRNA-dependent transformation of Ser to Sec. In Sec-decoding organisms, tRNA<sub>Sec</sub> is first aminoaicylated with Ser by SerRS (2–4). Bacteria convert Ser to Sec in one step using selenocysteine synthase (Sele) in the presence of the selenium donor selenophosphate (1). Eukaryotes and archaea perform the Ser to Sec conversion using two enzymes: PSTK phosphorylates the serine moiety of Ser-tRNA<sub>Sec</sub> to Sep-tRNA<sub>Sec</sub> (5–8), and SepSecS catalyzes the Sep-tRNA<sub>Sec</sub> to Sec-tRNA<sub>Sec</sub> conversion (9,10).

About four decades ago the presence of a Sep-tRNA was discovered in rooster (11) and rat liver (12) and later in other eukaryotes and archaea (5,13–15). Also established early (14) was the Sep-tRNA synthesis requirement for two enzymes, SerRS and a ‘phosphotransferase activity’. The latter enzyme was purified from bovine liver (7), the human counterpart was characterized with regard to tRNA recognition (8) and finally the protein responsible was identified (5) as PSTK. Detailed characterizations of PSTK activity were subsequently performed on the mouse (5) and Methanocaldococcus jannaschii enzyme (15). PSTK transfers the γ-phosphate from ATP to Ser-tRNA<sub>Sec</sub> yielding Sep-tRNA<sub>Sec</sub> and ADP (5,15). tRNA<sub>Sec</sub> binds to PSTK with high affinity and specifically induces its ATPase activity (15). Although SerRS must be able to recognize and aminoaicylate both tRNA<sub>Ser</sub> and tRNA<sub>Sec</sub>, proper interpretation of the genetic code requires that PSTK differentiate Sep-tRNA<sub>Sec</sub> from Ser-tRNA<sub>Ser</sub>. tRNA<sub>Sec</sub> species from all three domains of life are unusual in both length (>90 nt) and structure. While most tRNAs including tRNA<sub>Ser</sub> are in a 7/5 cloverleaf form (i.e. 7 bp in the acceptor stem and 5 in the T/C arm), bacterial tRNA<sub>Sec</sub> is in an 8/5 form (16), while eukaryal tRNA<sub>Sec</sub> (17,18) and archaeal tRNA<sub>Sec</sub> (17,19) likely exist in a 9/4 clover leaf form. Besides the difference in acceptor stem length at 9 bp in archaeal and eukaryotic tRNA<sub>Sec</sub> versus 7 bp in tRNA<sub>Ser</sub>, several other features of tRNA<sub>Sec</sub> are significantly different.
different from tRNA\textsuperscript{Sec}. Eukaryotic and archaeal tRNA\textsuperscript{Sec} species have 6 or 7 bp D-stems, respectively (17–19); in contrast tRNA\textsuperscript{Ser} has a 3 to 4 bp D-stem. Molecular modeling suggested that a 7 bp D-stem in archaeal tRNA\textsuperscript{Sec} would compensate for the short 4 bp T-stem (5bp in tRNA\textsuperscript{Ser}) thus allowing the normal interaction between the D- and T-loops (19). The sequence, length and orientation of the variable arm of tRNA\textsuperscript{Sec} also vary from those of tRNA\textsuperscript{Ser}.

Previous work with HeLa cell extracts (8) demonstrated that the length and secondary structure of the D-stem of human tRNA\textsuperscript{Sec} are the major determinants for serine phosphorylation by a kinase activity (in the following called ‘human PSTK’). Here we present the basis of tRNA\textsuperscript{Sec} discrimination from tRNA\textsuperscript{Ser} by an archaeal PSTK from \textit{M. jannaschii}. The G2-C71 and C3-G70 base pairs within the acceptor stem of \textit{Methanococcus maripaludis} tRNA\textsuperscript{Sec} are the major identity elements for tRNA-dependent serine phosphorylation by archaeal PSTK.

\section*{MATERIALS AND METHODS}

\subsection*{Materials and reagents}

All oligonucleotide synthesis and DNA sequencing were carried out by the Keck Foundation Biotechnology Research Laboratory at Yale University. L-[U-\textsuperscript{14}C]Ser (163 mCi/mmole) was from Amersham Biosciences and [\textsuperscript{32}P]-ATP (3000 Ci/mmole) was from GE Healthcare. L-[\textsuperscript{1-14}C]Thr (50–60 mCi/mmole) was from American Radiolabeled Chemicals.

\subsection*{Expression and purification of enzymes}

Since heterologous overexpression of the \textit{M. maripaludis} PSTK gene in \textit{Escherichia coli} was not successful, \textit{M. jannaschii} PSTK-His\textsubscript{6} (MJ1538) in pET20b (Novagen) was overproduced and purified as described (15). \textit{M. maripaludis} SerRS was overproduced and purified as described (2).

\subsection*{Cloning, purification, transcription and \textsuperscript{32}P-labeling of tRNAs}

All tRNAs were cloned into pUC19, expressed in \textit{E. coli} DH5\textsubscript{z}, transcribed by T7 RNA polymerase, gel purified and folded as described previously (15). Refolded transcript was \textsuperscript{32}P-labeled on the 3’ terminus using the \textit{E. coli} CCA-adding enzyme and [\textsuperscript{32}P]-ATP as before (15). After phenol/chloroform extraction the reaction was carried out over a Sephadex G25 Microspin column (Amersham Biosciences) to remove excess ATP.

\subsection*{Serylation and phosphorylation of tRNA\textsuperscript{Sec}}

These assays were carried out in 1 x PSTK buffer [50 mM HEPES-KOH (pH 7.5), 10 mM MgCl\textsubscript{2}, 20 mM KCl, 1 mM DTT] with 1 mM L-Ser (Sigma), 5 mM ATP, 600 mM \textit{M. maripaludis} SerRS, 50 mM \textit{M. jannaschii} PSTK and 1 \mu M \textsuperscript{32}P-labeled transcript for 45 min at 37°C. Aliquots (2 \mu l) of each reaction were quenched on ice with 3 \mu l of 100 mM sodium citrate (pH 5.0) and 0.66 mg/ml nuclease P1 (Sigma) and incubated at room temperature for 35 min (20,21). Assays that included 100 \mu M [\textsuperscript{14}C]-Ser were carried out in 1 x PSTK buffer with 1 \mu M tRNA\textsuperscript{Sec} transcript, 5 mM ATP, 1.2 \mu M SerRS and 200 nM PSTK for 45 min at 37°C. The reactions were stopped by phenol/chloroform extraction and purified over a G25 column to remove unincorporated [\textsuperscript{14}C]-Ser, and aliquots (2 \mu l) of each reaction were quenched and digested as above. To separate Sep-AMP, Ser-AMP and AMP, 1 \mu l of quenched, digested sample was spotted on glass polyethyleneimine (PEI) cellulose 20 cm x 20 cm thin layer chromatography (TLC) plates (EMD) and developed for 75 min in 100 mM ammonium acetate and 5% acetic acid. The plates were exposed on an imaging plate (FujiFilms), scanned on a Molecular Dynamics Storm 860 PhosphorImager, and quantified using ImageQuant software.

\subsection*{Preparation of seryl-tRNA}

tRNA\textsuperscript{Sec}, chimera and tRNA\textsuperscript{Ser} \textsuperscript{32}P-labeled transcripts were each aminoacylated in 1 x PSTK buffer with 1 mM L-Ser (Sigma), 5 mM ATP, 3 \mu M \textit{M. maripaludis} SerRS and 5 \mu M \textsuperscript{32}P-labeled transcript as described previously (15). Reactions were incubated at 37°C for 1 h followed by phenol/chloroform extraction, ethanol precipitation and resuspension in water. The samples were passed over Sephadex G25 Microspin columns (Amersham Biosciences) equilibrated with water. To check aminoacylation levels, 2 \mu l aliquots were removed at the end of the reactions, quenched on ice with 3 \mu l of 100 mM sodium citrate (pH 5.0) and 0.66 mg/ml nuclease P1 (Sigma), and analyzed as described above.

\subsection*{Phosphorylation of seryl-tRNA}

These assays were carried out in 1 x PSTK buffer with 1 \mu M \textsuperscript{32}P-labeled Ser-tRNA transcript, 5 mM ATP and 50 nM PSTK at 37°C. Reaction mixes were preincubated at 37°C and started by addition of enzyme. At each time point, 2 \mu l aliquots were taken and treated as described above.

\subsection*{ATPase activity measurement}

ATPase activity was determined by measuring the amount of [\textsuperscript{\alpha-\textsuperscript{32}P}]ATP converted to [\textsuperscript{\alpha-\textsuperscript{32}P}]ADP as described before with modifications (15). These assays were carried out in a 12 \mu l reaction volume including 1 x PSTK buffer with 130 mM cold ATP, 100 nM [\textsuperscript{\alpha-\textsuperscript{32}P}]ATP and 1 \mu M enzyme at 37°C for 30 min. Unless noted otherwise, 1 \mu M unlabeled tRNA (tRNA\textsuperscript{Sec}, G2-C71-C-G tRNA\textsuperscript{Sec} chimera tRNA or tRNA\textsuperscript{Ser}) was included. At six time points, 0.75 \mu l aliquots were taken from each reaction and quenched by the addition of 9.25 \mu l ice-cold 55 mM EDTA. One microliter of each reaction mixture was spotted on PEI cellulose TLC plates (EMD) and developed in 1 M LiCl for 60 to 75 min. After separation, the [\textsuperscript{\alpha-\textsuperscript{32}P}]ATP and [\textsuperscript{\alpha-\textsuperscript{32}P}]ADP spots were quantified by PhosphorImager using ImageQuant software.

\subsection*{Secondary structure alignment of archaeal tRNAs}

Archaeal tRNA\textsuperscript{Sec} and tRNA\textsuperscript{Ser} sequences were acquired from The Institute for Genomic Research, USCS.
Archaeal Genome Browser (22) and from the Microbial Genomes at the Joint Genome Institute. The secondary structures were determined by Aragorn 1.1 (23) and aligned manually by secondary structure.

Phosphorylation of threonyl-tRNA\textsuperscript{Sec}

These assays were carried out in 1 × PSTK buffer with 5 mM ATP, 2.5 mM threonine (Thr) (Fluka, 99.5% purity), 1 μM \textsuperscript{32}P-labeled tRNA\textsuperscript{Sec} transcript, 1.2 μM SerRS and with or without 200 nM PSTK for 45 min at 37°C. The reactions were quenched, digested and analyzed by TLC as stated above.

RESULTS

A survey of tRNA identity elements of archaeal PSTK

We decided to undertake a preliminary survey for potential tRNA identity elements for phosphorylation by \textit{M. jannaschii} PSTK. Our assay conditions (see Materials and Methods section and Table 1) guarantee achievement of plateau levels of Ser-tRNA\textsuperscript{Sec} formation, while phosphorylation may or may not reach plateau values for all the different mutants. Such an approach will reveal major elements, but will miss some less important ones. Previous work established that Ser-tRNA\textsuperscript{Sec} but not Ser-tRNA\textsuperscript{Ser} is a substrate for phosphorylation by PSTK (7,8,12–15). Thus, the identity elements for phosphorylation must lie within tRNA\textsuperscript{Sec} (Figure 1A) and be absent from tRNA\textsuperscript{Ser} (Figure 1B), while any antideterminants would be found only in tRNA\textsuperscript{Ser}. Based on a comparison of the sequences and secondary structures of archaeal tRNA\textsuperscript{Sec} and tRNA\textsuperscript{Ser}, we designed transplantation mutants of \textit{M. maripaludis} tRNA\textsuperscript{Sec} by replacing the acceptor stem + T-arm, D-arm, anticodon, anticodon stem–loop, variable arm, T-arm or T-loop of \textit{M. maripaludis} tRNA\textsuperscript{Ser}UGA with those of \textit{M. maripaludis} tRNA\textsuperscript{Sec}. Additionally, we made point mutations to tRNA\textsuperscript{Sec} to localize the nucleotides recognized by PSTK. While the mutations did not overlap with previously identified identity elements for the archaeal \textit{Methanosarcina barkeri} SerRS (the long variable arm, G1-C72, the discriminator base G73 and the anticodon stem base-pair G30-C40) (24) some of the mutants did not serylate efficiently (Table 1). Generally, tRNA\textsuperscript{Sec} mutants that did not serylate efficiently would decayslrate during purification of Ser-tRNA, leaving little charged tRNA to test for phosphorylation by PSTK. To circumvent this issue we tested the mutants in an activity assay similar to what was described previously (15), in which tRNA was serylated with SerRS and phosphorylated by PSTK in the same reaction. Thus, mutants of tRNA\textsuperscript{Sec} that serylated poorly were phosphorylated by PSTK without a purification of the Ser-tRNA\textsuperscript{Sec} prior to phosphorylation by PSTK. A similar approach for tRNA mutants that serylated inefficiently was implemented in a study of human PSTK in partially purified HeLa cell extracts (8). In our assay, after serylation by purified \textit{M. maripaludis} SerRS and phosphorylation by purified \textit{M. jannaschii} PSTK, the tRNA\textsuperscript{Sec} mutants were digested with nuclease P1, and the Sep-[\textsuperscript{32}P]AMP, [\textsuperscript{32}P]AMP and Ser-[\textsuperscript{32}P]AMP products were separated by TLC. The amounts of aminocylated (serylated plus phosphorylated) and phosphorylated products were determined by PhosphorImager analysis (Table 1). Additionally, a filter-binding assay (15) was used to determine the affinity of PSTK for each tRNA mutant (Supplementary Table 1). We should note that in this study we used the transcript of the \textit{M. maripaludis} tRNA\textsuperscript{Sec} for our identity studies; SerRS serylated both \textit{M. jannaschii} tRNA\textsuperscript{Sec} and \textit{M. maripaludis} tRNA\textsuperscript{Sec} similarly (Table 1), but the \textit{M. jannaschii} Ser-tRNA\textsuperscript{Sec} deacylated during purification. Additionally, PSTK from \textit{M. jannaschii} was used because we were able to obtain sufficient quantities of purified and active enzyme.

The unusual D-arm provides a minor identity element for archaeal PSTK

The length and structure but not sequence of the D-stem of human tRNA\textsuperscript{Sec} were shown to be the major identity elements for serine phosphorylation by human PSTK (8), but our investigation revealed the D-arm to be a minor identity element for archaeal PSTK. The D-arm of tRNA\textsuperscript{Sec} likely has a 7-bp stem and a 4 nt loop, whereas in the isoacceptors of tRNA\textsuperscript{Sec} the stem and loop are 3 to 4 bp and 9 to 13 nt, respectively (Figure 1A and B). Replacing the D-arm of tRNA\textsuperscript{Sec} with the D-arm from tRNA\textsuperscript{Ser} resulted in a mutant (D-arm, Table 1) that serylated poorly (1.4%), which made assaying for phosphorylation difficult. The severe effects propagated by the D-arm mutant were possibly due to disruption of tertiary structure. More specific mutations of the D-arm disrupted 1 bp (U16-A20a or C15-G20b) or 2 bp (U16-A20a and C15-G20b) in the 7-bp D-stem: U16A, U16G, C15G, U16A/C15G, A20aU and A20aC. Disrupting 1 bp of the D-stem by mutation of U16 to A or G or A20aU had moderate effects on the relative phosphorylation efficiency of the mutant tRNAs compared to wild-type, while the C15G and A20aC mutations had little effect (Table 1). Compensatory mutations that retained or closed the 7-bp stem retained or restored phosphorylation by PSTK (A20aG, A20aU/U16A, Table 1). The relative phosphorylation efficiency of the U16A/C15G double mutant (disrupted 2 bp in the D-stem) was similar to the single U16 mutants, showing no added effect due to a shorter 5-bp D-stem (Table 1). Although the affinity of PSTK was about 10-fold lower for most of the D-stem mutants, the U16A/C15G double mutant did cause a 28.5-fold decrease over wild-type tRNA\textsuperscript{Sec}, which is comparable to PSTK recognition of tRNA\textsuperscript{Ser} (Supplementary Table 1). Thus, the D-stem of tRNA\textsuperscript{Sec} does provide a minor identity element for PSTK recognition and phosphorylation but is not the signal for phosphorylation by archaeal PSTK.

PSTK recognition of the acceptor stem of archaeal tRNA\textsuperscript{Sec} is essential for phosphorylation

The acceptor stems of archaeal tRNA\textsuperscript{Sec} and tRNA\textsuperscript{Ser} from Sec-decoding archaea differ in both length and sequence (Figure 1A and B and Supplementary Figure 1), which probably affects the secondary and tertiary structures of these tRNAs. The L-form tertiary structure...
of tRNA has two helical domains; domain I consists of the anticodon stem and the D-stem, and domain II consists of the T-stem and the acceptor stem (25–27). Domain II has 13 bp (9-bp acceptor stem and 4-bp T-stem) in archaean tRNA<sup>Sec</sup> and 12 bp (7-bp acceptor stem and 5-bp T-stem) in tRNA<sup>Ser</sup> (Figure 1A and B). Thus to maintain the domain II structure as that of tRNA<sup>Ser</sup>, we transplanted both the acceptor stem and T-arm from tRNA<sup>Ser</sup> to tRNA<sup>Sec</sup>, resulting in a 12-bp domain II. The mutant did not serylate efficiently (20.1%), and the relative phosphorylation efficiency was poor (2.1%) (acceptor stem + T arm, Table 1), suggesting that elements within the acceptor stem were required for phosphorylation by archaean PSTK.

To determine whether the length of the acceptor stem was an important identity element for PSTK recognition of tRNA<sup>Sec</sup>. tRNA<sup>Sec</sup> mutants with deletions of base pairs within the acceptor stem were analyzed: Δ5a-67b,
Deletion of 5a-67b or 5b-67a did not influence phosphorylation efficiency (Table 1). Deletion of both 5a-67b and 5b-67a resulted in mutant tRNA Sec that serylated poorly, similarly to the acceptor stem + T arm mutant (8.9% and 20.1%, respectively). Unlike the acceptor stem + T arm mutant, the relative phosphorylation efficiency was higher at 54.9% of the wild-type tRNASec level (Table 1). This may suggest that the length of the acceptor stem plays a role in phosphorylation. Additionally, mutant tRNA Sec with a G2-C71 deletion serylated efficiently (73.4%) but its relative phosphorylation efficiency was only 1.3% (Table 1), signifying a possible sequence-specific interaction with PSTK.

Archaeal tRNA Sec has invariant G2-C71 and C3-G70 base pairs and a well-conserved C5-G68 base pair in the acceptor stem (Figure 1A) (C5-A68 in Methanococcus voltae and G5-C68 in Methanopyrus kandleri, Supplementary Figure 1). We mutated each of these base pairs to determine their importance for PSTK recognition. Mutation of the invariant tRNA Sec G2-C71 bp was detrimental to phosphorylation with a C2-G71 mutation producing the most severe decrease in relative phosphorylation efficiency at 96.3% below wild-type level (Table 1); interestingly, C2-G71 is highly conserved in all archaeal tRNA Sec but invariant in tRNA Ser from Sec-decoding archaea (Supplementary Figure 1). Mutation of the invariant C3-G70 base pair of archaeal tRNA Sec to G3-C70 had a severe effect on phosphorylation with an 87.5% decrease in the relative efficiency, while mutation to A3-U70 had a moderate decrease (24.1%) (Table 1). G3-C70 and A3-C70 are the most common base pairs found at this position in tRNA Ser from Sec-decoding archaea although the isoacceptors of M. kandleri tRNASer have a conserved C3-G70 bp (Supplementary Figure 1). It should be noted that the genome of M. kandleri has an accelerated rate of evolution among the archaea (28). Mutation of C3-G70 to U3-A70 or U3-G70 had little to no influence on the relative phosphorylation efficiency (Table 1). Mutation of the well-conserved C5-G68 base pair of tRNA Sec to G5-C68, U5-A68, or U5-G68 had a minimal effect on phosphorylation while mutation to A5-U68, the most common base pair at that position in tRNA Sec from Sec-decoding archaea, caused a 54.5% decrease in phosphorylation over wild-type tRNA Sec (Table 1). Additionally, Ser-tRNASec from M. kandleri, which has a G5-C68 base pair, phosphorylates as efficiently as M. maripaludis Ser-tRNASec (Table 1). These data demonstrate that A5-U68 in tRNASer has some antideterminant value for PSTK recognition.

The anticodon stem–loop and long variable arm are not identity elements for phosphorylation by PSTK

The anticodon stem–loop is highly conserved in archaeal tRNA Sec compared to the anticodon stem–loops of tRNA Ser isoacceptors (with the anticodons UGA, GGA and GCU) found in Sec-decoding archaea (Figure 1 and Supplementary Figure 1). Neither a mutation of the anticodon of tRNA Sec from UCA to one of a tRNA Ser isoacceptor, UGA (anticodon UCA to UGA), nor

\[ \Delta 5b-67a, \Delta 5a-67b/\Delta 5b-67a \] and \[ \Delta G2-C71. \] Deletion of 5a-67b or 5b-67a did not influence phosphorylation efficiency (Table 1). Deletion of both 5a-67b and 5b-67a resulted in mutant tRNA Sec that serylated poorly, similarly to the acceptor stem + T arm mutant (8.9% and 20.1%, respectively). Unlike the acceptor stem + T arm mutant, the relative phosphorylation efficiency was higher at 54.9% of the wild-type tRNA Sec level (Table 1). This may suggest that the length of the acceptor stem plays a role in phosphorylation. Additionally, mutant tRNA Sec with a G2-C71 deletion serylated efficiently (73.4%) but its relative phosphorylation efficiency was only 1.3% (Table 1), signifying a possible sequence-specific interaction with PSTK.

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replacing the entire anticodon stem–loop of tRNA^Sec with that of tRNA^Ser_{UGA} (antidodon stem–loop) had a negative effect on the relative phosphorylation efficiency by PSTK (Table 1).

The variable arms of tRNA^Sec and tRNA^Ser differ in sequence, length and orientation (Figure 1A and B and Supplementary Figure 1). Replacement of the variable arm of tRNA^Sec with that of tRNA^Ser_{UGA} did not influence the relative phosphorylation efficiency (variable arm, Table 1).

The T-loop of tRNA^Sec is a minor identity element for phosphorylation

The structure of the T-arm of archaean tRNA^Sec is quite unusual in that the T-stem has 4 bp while most tRNAs, including tRNA^Ser, have 5-bp T-stems (Figure 1A and B and Supplementary Figure 1). Unlike the T-stems of archaean tRNA^Sec isoacceptors, the sequences of the T-stems of archaean tRNA^Sec found to date are invariant (Figure 1A and B and Supplementary Figure 1). Replacement of the T-arm of tRNA^Sec with that of tRNA^Ser_{UGA} resulted in a 31.1% decrease in relative phosphorylation efficiency compared to wild-type tRNA^Sec (T-arm, Table 1) and a 29.1-fold decrease in the affinity of PSTK for the mutant tRNA (Supplementary Table 1). The T-arm mutation also had a significant effect on serylation, serylating at 37.9% as compared to 80.3% for wild-type tRNA^Sec (Table 1), possibly due to disruption of the tertiary structure. Mutation of the first 2 bp of the T-stem from G50-C64 to C-G and G51-C63 to C-G had little effect on the relative phosphorylation efficiency (G50-C64 to C-G/G51-C63 to C-G, Table 1).

The T-loop size is conserved between archaean tRNA^Sec and tRNA^Ser but the sequences are variant in two positions, 57 and 59 (Figure 1A and B and Supplementary Figure 1). A57 is invariant in the isoacceptors of tRNA^Ser from Sec-decoding archaean, whereas G57 is found in most archaean tRNA^Sec. U59 is invariant in archaean tRNA^Sec, while A59 is highly conserved in archaean tRNA^Ser. Transplanting the T-loop from tRNA^Ser_{UGA} onto tRNA^Sec which mutates G57A and U59A, causes a 41.1% decrease in the relative phosphorylation efficiency and a decrease in serylation to 44.9% compared to wild-type tRNA^Sec (T-loop, Table 1); these results are similar to replacement of the entire T-arm with that of tRNA^Ser_{UGA}, suggesting that G57 and U59 might be responsible. Nevertheless, the single mutations G57A or U59A serylated efficiently and had minimal effects on the relative phosphorylation efficiency (Table 1). Consequently, although neither single mutation had a significant effect, the collective mutation of G57 and U59 in the T-loop mutant had a moderate effect on serylation, phosphorylation and binding (T-loop, Table 1 and Supplementary Table 1).

Transplantation of acceptor stem base pairs G2-C71, C3-G70 and C5-G68 into tRNA^Ser allows robust phosphorylation by PSTK

Although mutation of G2-C71 in tRNA^Sec resulted in a mutant that only phosphorylated at 3.7% of the wild-type level, transplantation of G2-C71 alone into the tRNA^Ser_{UGA} backbone was not sufficient to confer phosphorylation onto this mutant (transplant 1, Table 1). The archaean tRNA^Sec identity elements found at base pairs G2-C71 and C3-G70 were transplanted into the tRNA^Ser_{UGA} backbone, and the negative determinant A5-U68 was mutated to G5-C68 as found in tRNA^Sec (Figure 1C). PSTK phosphorylated the chimera with a relative phosphorylation efficiency of 68% compared to wild-type tRNA^Sec (chimera, Table 1). Furthermore, the affinity of PSTK for the chimeric tRNA was higher than that for tRNA^Ser_{UGA} (Supplementary Table 1). Phosphorylation of the chimeric Ser-tRNA^Sec by PSTK demonstrated the admirable substrate qualities of the transplanted tRNA^Ser species (Figure 2). Neither mutation of the D-arm in the tRNA chimera to add base pairs to the D-stem (transplant 2, Table 1), thus making a smaller D-loop as seen for tRNA^Sec, nor addition of an additional base pair to the acceptor stem of the chimera to lengthen it from 7 to 8 bp improved phosphorylation (transplant 3, Table 1). Thus, the major identity elements for phosphorylation by archaean PSTK are found in the acceptor stem at base pairs G2-C71 and C3-G70.

Chimeric tRNA induces the ATPase activity of PSTK

The ATPase activity of PSTK is specifically induced by tRNA^Sec and there was minimal ATPase activity of the enzyme in the presence of tRNA^Ser or in the absence of tRNA (15). When the ATPase activity of PSTK was tested in the presence of the G2-C71 to C-G tRNA^Sec mutant, which was poorly phosphorylated (Table 1), the little ATPase activity detectable was similar to the ATPase activity in the presence of tRNA^Ser (Figure 3). Yet, the chimeric tRNA (G2-C71, C3-G70, and C5-G68 of tRNA^Sec transplanted into tRNA^Ser_{UGA}) (Figure 1C) significantly induced the ATPase activity of PSTK (Figure 3), further demonstrating the importance of these acceptor stem base pairs in phosphorylation by PSTK.

Divergence in tRNA^Sec acceptor stems from Archaea, Eukarya and Bacteria

Alignment of archaean and eukaryal tRNA^Sec by secondary structure (Figure 4A) revealed insightful differences in the acceptor stems in light of the described identity elements for serine phosphorylation of M. maripaludis tRNA^Sec by M. jannaschii PSTK and human tRNA^Sec by human PSTK (8). As established by the phosphotransferase assay with M. jannaschii PSTK and M. maripaludis tRNA^Sec, G2-C71 is a major identity element for phosphorylation (Table 1) and is invariant in archaean tRNA^Sec (Figures 1A and 4A). Eukaryal tRNA^Sec, however, has a highly conserved C2-G71 bp at this position (Figure 4A and Supplementary Figure 2); only 2 out of 57 eukaryal tRNA^Sec genes analyzed were variant at this position; tRNA^Sec from the diatom Thallassiosira pseudonana has a U2-A71 bp (29). A BLAST search against the genome of the marsupial Monodelphis domestica identified a probable tRNA^Sec (by secondary structure and sequence conservation), which has a U2-G71 bp at this position (Supplementary Figure 2). Archaean tRNA^Sec is invariant
at bp C3-G70, and 84% of eukaryal tRNA Sec genes analyzed also have a C3-G70 bp (Figure 4A and Supplementary Figure 2). Of the remaining 16% of eukaryal tRNA Sec, only 3.5% have a base pair (G3-C70) that caused poor phosphorylation in the context of archaeal tRNA Sec by archaeal PSTK (Table 1). The A5-U68 with negative determinant properties in archaeal tRNA Ser is found in 4% of eukaryal tRNA Sec genes (Supplementary Figure 2). Although human tRNA Sec has a C2-G71 bp rather than the major identity element G2-C71 found in archaeal tRNA Sec, it retains the C3-G70 bp in the acceptor stem (Figure 4A), as well as other similar structural features such as the unusual D-arm, and archaeal PSTK is able to weakly phosphorylate human tRNA Sec (Table 1). Thus, eukaryal tRNA Sec has diverged at a critical identity element, G2-C71, for serine phosphorylation by archaeal PSTK.

The acceptor stem of bacterial tRNA Sec consists of 8 bp (16) rather than 9 bp as found in archaeal and eukaryal tRNA Sec. Acceptor stems from 50 divergent Sec-decoding bacterial species were analyzed, and the G2-C71 bp is highly conserved if not invariant (Figure 4B). The third base pair in the acceptor stem, however, was 75% conserved as an A3-U70, 21% as a G3-C70 bp and 4% as a U3-A70 or C3-G70 bp. Most bacterial tRNA Sec analyzed did not have the antideterminant for archaeal PSTK, A5-U68. E. coli tRNA Sec has G2-C71, A3-U70 and G5-C68 bp in the acceptor stem (Figure 4B) and is phosphorylated weakly at a relative phosphorylation efficiency of 13.9% compared to wild-type M. maripaludis.

![Figure 2](https://academic.oup.com/nar/article-abstract/36/6/1871/2410201)

**Figure 2.** In vitro conversion of Ser-tRNA to Sep-tRNA by M. jannaschii PSTK. One micromolar [32P]-labeled Ser-tRNA Sec (70.8% serylated), chimera Ser-tRNA (64.5% serylated) or Ser-tRNA Ser (63.4% serylated) was incubated with 50 nM PSTK at 37°C for 15 min. Aliquots of the reactions were quenched with 100 mM sodium citrate, pH 5.0 and digested with 0.66 mg/ml nuclease P1 for 35 min at room temperature. Samples were then spotted onto a PEI-cellulose TLC plate and developed in 100 mM ammonium acetate, 5% acetic acid for 75 min. Following quantification of the intensities of Ser-[32P]AMP, [32P]AMP and Sep-[32P]AMP using ImageQuant, the fraction (%) of aminoacyl-tRNA formed at each time point was calculated by dividing the intensity of the Sep-[32P]AMP and Ser-[32P]AMP spots by the total intensity and the fraction (%) of Sep-tRNA Sec formed at each time point was calculated by dividing the intensity of the Sep-[32P]AMP spot by the total intensity. The phosphorylation efficiency was determined by dividing the percent Sep-tRNA by the percent aminoacyl-tRNA. Error bars represent the standard deviation of three separate experiments.
tRNA^Sec^ (Table 1). Although *E. coli* tRNA^Sec^ has the major identity element for phosphorylation by archaeal PSTK, G2-C71, other features such as the A3-U70 bp, the C59 in the T-loop, the 8/5 cloverleaf arrangement (8-bp acceptor stem and 5-bp T-stem), and other possible differences in tertiary structure could contribute to the poor phosphorylation and binding by archaeal PSTK (Table 1 and Supplementary Table 1).

Amino acid recognition by PSTK

It was observed earlier that *M. barkeri* SerRS misactivates threonine (30) and inefficiently forms Thr-tRNA^Sec^ (I. Weygand-Durasevic, personal communication). Given that *M. jannaschii* PSTK has a similar affinity for tRNA^Sec^ as Ser-tRNA^Sec^ and thus does not seem to recognize the serine moiety on the tRNA (15), we considered whether PSTK could phosphorylate Thr-tRNA^Sec^. *M. maripaludis* [32P]tRNA^Sec^ was mischarged with Thr by *M. maripaludis* SerRS, producing Thr-tRNA^Sec^ (Figure 5, lane 1). Thr-tRNA^Sec^ was subsequently phosphorylated with *M. jannaschii* PSTK (Figure 5, lane 2) and digested with nuclease P1. The products, [32P]AMP, Thr-[32P]AMP and phosphothreonyl-[32P]AMP were separated by TLC on PEI cellulose. While aminoacylation with Thr was poor (only 3.7%), PSTK converted 94.4% of the Thr to phosphothreonine, which migrated similarly to phosphoserine (Figure 5, compare lanes 2 and 4). This corresponds well to the 96.3% conversion of Ser to Sep by PSTK (Figure 5, lane 4). The experiment was performed with [14C]Thr to confirm the results (Supplementary Figure 3). Again, [14C]Thr-tRNA^Sec^ was phosphorylated by PSTK (Supplementary Figure 3, compare lanes 2 and 4). The results provide further evidence that PSTK primarily recognizes the tRNA and not the amino acid, if any, that is attached to the 3’ end of the tRNA.

**DISCUSSION**

The pathway for selenocysteine formation differs in bacteria from that present in eukaryotes and archaea; however, tRNA^Sec^ remains a common factor for selenocysteine formation among the three. The tRNA-dependent conversion of Ser to Sec in eukaryotes and archaea requires that the unusual tRNA^Sec^ be recognized initially by enzymes that interact with many tRNAs such as processing and modifying enzymes and the CCA-adding enzyme prior to ‘mischarging’ with Ser by SerRS, phosphorylation of the resulting Ser-tRNA^Sec^ by PSTK and conversion of Sep-tRNA^Sec^ to Sec-tRNA^Sec^ by SepSecS. Subsequently, the tRNA^Sec^-specific elongation factor SelB must recognize Sec-tRNA^Sec^ specifically for selenoprotein synthesis (31–33). While we have speculated that a complex of the enzymes involved in selenocysteine biosynthesis might exist (15) as has been suggested and shown for the tRNA-dependent amidotransferases (34,35), it is not known which components of the pathway might exist in a complex or whether the enzymes might compete for the tRNA^Sec^ substrate. Yet, PSTK provides the first line of defense in maintaining the fidelity of genetic code translation by binding with high affinity to Ser-tRNA^Sec^ (7,15). This sequesters mischarged tRNA from use in translation and discriminates Ser-tRNA^Sec^ from Ser-tRNA^Ser^.

A previous study detailed how a human PSTK discriminates human Ser-tRNA^Sec^ from Ser-tRNA^Ser^ by virtue of the atypical D-stem structure of tRNA^Sec^ (8). While archaeal tRNA^Sec^ retains a D-stem structure similar to that of eukaryal tRNA^Sec^, mutations that open the last two base pairs in the D-stem of *M. maripaludis* tRNA^Sec^-leading to the formation of a 5-bp rather than a 7-bp stem—did not result in a complete loss of serine phosphorylation by *M. jannaschii* PSTK as occurred when similar mutations were made to human tRNA^Sec^ (phosphorylation by human PSTK) (8). Some mutations to the D-stem of *M. maripaludis* tRNA^Sec^ (U16A, U16G, U16A/C15G and A20aU) caused a moderate decrease in phosphorylation but others (C15G and A20aC) did not, which brings into question whether a 7-bp stem has an effect on phosphorylation by archaeal PSTK. The intact 7-bp D-stem per se may not be recognized for phosphorylation; these data might suggest a sequence-specific interaction of archaeal PSTK with U16, but the
compensatory mutation A20aU/U16A that keeps the D-stem intact phosphorylated efficiently even with a mutation of U16. Perhaps mutations of A20aU or of U16 perturb the tertiary structure of tRNA\textsubscript{Sec}, causing the decrease in phosphorylation efficiency by PSTK.

The T-loop of tRNA\textsubscript{Sec} also provides a minor identity element for archaeal PSTK phosphorylation. The combined mutation of G57A and U59A in the T-loop, which resulted in a T-loop identical to that found in tRNA\textsubscript{Ser} from Sec-decoding archaea, caused a moderate decrease in phosphorylation that was not completely attributable to a single mutation of either G57 or U59. Conceivably these mutations, specifically that of U59, could disrupt a tertiary interaction of the D- and T-loops between U17 and U59. A novel tertiary interaction was reported for \textit{E. coli} tRNA\textsubscript{Sec} between C16 (located at the same position as U17 in archaeal tRNA\textsubscript{Sec}) in the D-loop and C59 (U59 in archaeal tRNA\textsubscript{Sec}) in the T-loop (16) although a similar interaction was not found between U16 (located at the same position as U17 in archaeal tRNA\textsubscript{Sec}) and U59 in a study of eukaryotic-type \textit{Xenopus laevis} tRNA\textsubscript{Sec} (18). If archaeal tRNA\textsubscript{Sec} is able to form interactions common to all known tRNA crystal structures between the D-loop and T-loop (25–27) as was supported by molecular modeling of the archaeal-type \textit{M. jannaschii} tRNA\textsubscript{Sec} (19), an alternative explanation is possible; nucleotide 57 stacks with other purines in an intercalation of bases from both the D- and T-loops, and nucleotide 59 stacks to the tertiary base pairs 15–48, playing a crucial role in fixing the juxtaposition of domains I (anticodon stem and D-stem) and II (T-stem and acceptor stem) (19). Although nucleotide 57 is typically conserved as a purine and nucleotide 59 can be any nucleotide, the invariant nature of U57 and highly conserved G59 may be essential for tertiary interactions in archaeal tRNA\textsubscript{Sec}. A future structural study of archaeal tRNA\textsubscript{Sec} would be helpful in making these determinations.

Despite the fact that at present we cannot conclusively state that the length of the acceptor stem is involved in phosphorylation of Ser-tRNA\textsubscript{Sec} by archaeal PSTK, our data definitively revealed that critical identity elements are found within the acceptor stem. This finding is quite unlike what was shown for eukaryotic-type PSTK and tRNA\textsubscript{Sec} where neither the sequence nor length of the acceptor stem was important for phosphorylation (8). Mutagenesis of \textit{M. maripaludis} tRNA\textsubscript{Sec} at the G2-C71 and C3-G70 bp demonstrated their importance for phosphorylation (8), and additionally, the tRNA-dependent amidotransferase GatCAB from bacteria and archaea evolved to recognize different elements within tRNA\textsubscript{Asp} (37,38). While the length and structure of the D-stem of human tRNA\textsubscript{Sec} were essential for phosphorylation by human PSTK (8), the unusual D-stem of archaeal tRNA\textsubscript{Sec} only moderately affected phosphorylation by archaeal PSTK, possibly by means of maintenance of proper tertiary structure. On the contrary, invariant base pairs within the acceptor stem of archaeal tRNA\textsubscript{Sec} were vital for phosphorylation by archaeal PSTK. Moreover, the invariant G2-C71 of archaeal tRNA\textsubscript{Sec} was found to be highly conserved as C2-G71 in eukaryotic tRNA\textsubscript{Sec}, which resulted in minimal phosphorylation by archaeal PSTK in the context of archaeal tRNA\textsubscript{Sec}. This distinction between archaeal and eukaryotic tRNA\textsubscript{Sec} corresponds to a deep evolutionary divide between archaeal-type and eukaryotic-type PSTKs (15). While tRNA\textsubscript{Sec} transverses interactions with a number of enzymes in the selenocysteine biosynthesis pathway, that distinction may indicate co-evolution of archaeal and eukaryotic tRNA\textsubscript{Sec} with their respective PSTKs.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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