Redundant Synthesis of Cysteinyl-tRNA\textsuperscript{Cys} in Methanosarcina mazei*

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A subset of methanogenic archaea synthesize the cysteinyl-tRNA\textsuperscript{Cys} (Cys-tRNA\textsuperscript{Cys}) needed for protein synthesis using both a canonical cysteinyl-tRNA synthetase (CysRS) as well as a set of two enzymes that operate via a separate indirect pathway. In the indirect route, phosphoseryl-tRNA\textsuperscript{Cys} (Sep-tRNA\textsuperscript{Cys}) is first synthesized by phosphoseryl-tRNA synthetase (SepRS), and this misacylated intermediate is then converted to Cys-tRNA\textsuperscript{Cys} by Sep-tRNA:Cys-tRNA synthase (SepCysS) via a pyridoxal phosphate-dependent mechanism. Here, we explore the function of all three enzymes in the mesophilic methanogen Methanosarcina mazei. The genome of M. mazei also features three distinct tRNA\textsuperscript{Cys} isoacceptors, further indicating the unusual and complex nature of Cys-tRNA\textsuperscript{Cys} synthesis in this organism. Comparative aminoacylation kinetics by M. mazei CysRS and SepRS reveals that each enzyme prefers a distinct tRNA\textsuperscript{Cys} isoacceptor or pair of isoacceptors. Recognition determinants distinguishing the tRNAs are shown to reside in the globular core of the molecule. Both enzymes also require the S-adenosylmethioninedependent formation of m\textsuperscript{15}G37 in the anticodon loop for efficient aminoacylation. We further report a new, highly sensitive assay to measure the activity of SepCysS under anaerobic conditions. With this approach, we demonstrate that SepCysS functions as a multiple-turnover catalyst with kinetic behavior similar to bacterial selenocysteine synthetase and the archaeal/eukaryotic SepSecS enzyme. Together, these data suggest that both metabolic routes and all three tRNA\textsuperscript{Cys} species in M. mazei play important roles in the cellular physiology of the organism.

Methanosarcina mazei strain Go1 is a methanogenic archaeon capable of utilizing a broad spectrum of growth substrates, including H\textsubscript{2}/CO\textsubscript{2}, acetate, methanol, and methylamines (1). The capacity of M. mazei to adapt to changing environmental conditions is a hallmark of the Methanosarcina. Organisms belonging to this genus coexist with fermenting bacteria in freshwater and marine sediments, where their capacity to metabolically transform bacterially produced acetate into methane forms a closed carbon cycle that helps to determine atmospheric levels of this greenhouse gas (2). In some marine environments, the Methanosarcina also flourish together with methanotrophs and sulfate-reducing bacteria, where they participate in a microbial community that generates very high sulfate reduction rates via anaerobic methane oxidation (3). Comparative genomic studies of M. mazei, Methanosarcina barkeri, and Methanosarcina acetivorans C2A, the three Methanosarcina for which complete sequences are available, show that the 4.1-megabase M. mazei genome is the smallest so far known in the genus and may approximate the ancestral state (1, 4, 5). Of the 3371 M. mazei open reading frames identified, 1043 find their closest homolog in the bacterial domain (1), suggesting that horizontal gene transfer has played an important evolutionary role in the development of the organism.

The distinguishing methanogenesis pathway, present in all methanogens, produces methane as a catabolic product while also generating an electrochemical gradient that is harnessed to synthesize ATP (5, 6). Methanogens also possess many subsidiary metabolic activities that function to directly support methane production, including those involved in cofactor biosynthesis and in energy transduction through proton and sodium gradients (6). Interestingly, genomic data reveal a further correlation; all but two methanogens for which full sequence information is available also possess a unique two-step pathway by which cysteine is incorporated into proteins (7). In this pathway, tRNA\textsuperscript{Cys} is first aminoacylated with the nonstandard amino acid phosphoserine by phosphoseryl-tRNA synthetase (SepRS). The misacylated Sep-tRNA\textsuperscript{Cys} is then converted to Cys-tRNA\textsuperscript{Cys} through the action of the PLP-dependent enzyme SepCysS. For some methanogens, which lack a canonical cysteinyl-tRNA synthetase (CysRS), this indirect route is the sole pathway to synthesis of Cys-tRNA\textsuperscript{Cys}. However, other methanogens, including all three Methanosarcina for which genome sequences are available, possess both pathways. Comparative phylogenetic studies have shown that both the direct CysRS route and the indirect two-step pathway were in existence at the time of the last universal common ancestor (8). The coincident phylogenetic distributions of SepRS/SepCysS and the enzymes of methanogenesis also suggest the existence of a linkage between primary energy production and protein synthesis in contemporary methanogens, although the nature of and biological rationale for such a linkage have so far been obscure (8).

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‡§1 The abbreviations used are: SepRS, phosphoseryl-tRNA synthetase; SepCysS, Sep-tRNA:Cys-tRNA synthase; Sep-tRNA\textsuperscript{Cys}, phosphoseryl-tRNA\textsuperscript{Cys}; CysRS, cysteinyl-tRNA synthetase; DTT, dithiothreitol; PLP, pyridoxal phosphate; βME, β-mercaptoethanol.

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The only nonmethanogen known to possess the two-step Cys-tRNA\(^{\text{Cys}}\) biosynthetic pathway is the hyperthermophilic sulfate-reducing archaeon *Archaeoglobus fulgidus*, a closely related archaeon that retains nearly all of the methanogenesis enzymes and cofactors needed for the hydrogenotrophic pathway, with the exception of the essential methyl-CoM reductase (9).

Recently, it was reported that SepRS and CysRS from *Methanococcus maripaludis* share major identity elements for heterologous aminoacylation of an unmodified, in vitro transcript corresponding to the single tRNA\(^{\text{Cys}}\) species present in the *M. jannaschii* genome (10). Consistent with other studies in which a common tRNA is recognized by distinct tRNA synthetases, these data were interpreted to support a general hypothesis that aminoacyl-tRNA synthetases evolved in the context of preestablished tRNA identity. Other work on the *M. jannaschii* pathway has shown that SepRS and SepCysS form a stable binary complex that assists in conversion of the intermediate Sep-tRNA\(^{\text{Cys}}\) to Cys-tRNA\(^{\text{Cys}}\), a complex reminiscent of the trans-amidosome particle that facilitates prokaryotic tRNA-dependent asparagine biosynthesis (11, 12).

Here, we sought to address distinct questions pertaining to the contemporary function of apparently redundant Cys-tRNA\(^{\text{Cys}}\) synthesis pathways in *M. mazei*. Both *M. mazei* and *M. barkeri* possess genes encoding three distinct isoacceptors of tRNA\(^{\text{Cys}}\), a feature not known in other bacteria or archaea and suggestive that the machinery for incorporation of cysteine into proteins in these organisms may interact with other cellular components (1, 4, 8). By examining aminoacylation of the three wild-type as well as a set of hybrid tRNA\(^{\text{Cys}}\) species as substrates for either CysRS or the combined SepRS/SepCysS pathway, we show that each enzyme prefers a distinct tRNA\(^{\text{Cys}}\) isoacceptor or pair of isoacceptors and that the nucleotides determining CysRS versus SepRS selectivity among tRNA\(^{\text{Cys}}\) species are located in the globular core of the tRNA. Through the development and application of a new anaerobic assay for the quantitative analysis of SepCysS kinetics, we also provide evidence that the tRNA\(^{\text{Cys}}\) isoacceptor preferences of SepRS and SepCysS are identical. This supports the notion that tRNA\(^{\text{Cys}}\) acceptors functioning in the two-step pathway could be efficiently shuttled between these two enzymes. Together, these findings suggest that both pathways may play an important role in the cellular physiology. We also show that the aminoacylation efficiency of *M. mazei* CysRS is very significantly enhanced by the addition of a methyl group to the N1 position of G37 by the S-adenosylmethionine-dependent Trm5 enzyme, consistent with a similar dependence found for SepRS (11, 13). Since S-adenosylmethionine is synthesized from methionine (14), the tRNA\(^{\text{Cys}}\) methylation requirement for aminoacylation by both CysRS and SepRS links protein synthesis with sulfur metabolism in this organism.

**Experimental Procedures**

**Expression, Purification, and Quaternary Structure Determination of M. mazei CysRS and SepCysS—** *M. mazei* genomic DNA was purchased from the American Type Culture Collection and used as template for PCR amplification of the CysRS and SepCysS open reading frames. For SepCysS, the resulting DNA fragments were digested with Ndel and XhoI and inserted into pet22b+ for expression of His-tagged proteins in *Escherichia coli* Rosetta2(DE3) pLysS cells. Cultures were grown aerobically at 37 °C in LB medium supplemented with 100 μg/ml ampicillin, 34 μg/ml chloramphenicol, and 0.01% pyridoxine. When the cultures reached an \(A_{600}\) of 0.5, expression of His-tagged protein was induced by adding isopropyl-\(\beta\)-D-thiogalactoside to a final concentration of 0.6 mM. Cultures were then grown for a further 5 h at 37 °C prior to harvesting.

Cells expressing SepCysS were suspended in a buffer containing 50 mM NaH\(_2\)PO\(_4\) (pH 8.0), 20 mM NaCl, 3 mM DTT, 20 μM PLP, and 10% glycerol and were disrupted by a French press. Under aerobic conditions, the clarified lysate was applied to a 30-ml DEAE column preequilibrated with 50 mM NaH\(_2\)PO\(_4\) (pH 8.0), 20 mM NaCl, 3 mM DTT, and 20 μM PLP. SepCysS was eluted with a gradient from 20–500 mM NaCl, and the pooled fractions were dialyzed into a solution containing 50 mM NaH\(_2\)PO\(_4\) (pH 8.0), 150 mM NaCl, 5 mM βME, and 20 μM PLP. The enzyme was then applied to a Superdex 200 gel filtration column preequilibrated with 50 mM NaH\(_2\)PO\(_4\) (pH 8.0), 150 mM NaCl, 5 mM βME, and 20 μM PLP. Fractions containing SepCysS were then directly applied to a Ni\(^{2+}\)-nitrilotriacetic acid column equilibrated with 50 mM NaH\(_2\)PO\(_4\) (pH 8.0), 300 mM NaCl, 5 mM βME, and 20 μM PLP. After washing of the column in this buffer, the enzyme was then eluted by the addition of 150 mM imidazole. The enzyme was then dialyzed into a solution containing 10 mM NaH\(_2\)PO\(_4\) (pH 7.4), 20 mM NaCl, 2 mM MgCl\(_2\), 1 mM βME, 100 μM PLP, and 50% glycerol and stored at −20 °C. His-tagged SepCysS was recovered at better than 95% purity as judged by SDS-polyacrylamide gel electrophoresis, at a yield of 2 mg of protein/liter of culture. The PLP content of SepCysS was verified using a colorimetric assay in which the titration of PLP released upon boiling is monitored (15).

Construction of the recombinant DNA vector, induction of enzyme expression, and purification of *M. mazei* CysRS were accomplished by procedures identical to those described for *M. mazei* SepRS (13). The yield of highly purified CysRS was ~5 mg of protein/liter of culture.

The oligomeric structures of CysRS and SepCysS were determined by analytic size exclusion chromatography, using conditions and molecular mass markers identical to those reported in the accompanying paper (13). The 473-amino acid *M. mazei* CysRS enzyme (calculated \(M_r = 53,861\)) exhibits 40.2% sequence identity with the monomeric *E. coli* CysRS and migrates as a monomer, as expected. *M. mazei* SepCysS belongs to the PLP-dependent superfamily of enzymes, which typically function as dimers with one molecule of PLP bound in each active site (16). As expected, the 386-amino acid enzyme (predicted \(M_r = 42,324\) Da) elutes at a position corresponding to a molecular mass of 84 kDa, consistent with dimer formation (13).

**Preparation and Methylation of tRNAs—** To prepare tRNA for kinetic analysis, the three *M. mazei* tRNA\(^{\text{Cys}}\) genes were each transcribed from a synthetic duplex DNA template, methylated at the N1 position of G37, purified, and refolded using procedures identical to those described in the accompanying paper (13). The oligonucleotides used for the tRNA\(^{\text{Cys}}\) and
tRNA\textsubscript{Cys} genes were as follows: tRNA\textsubscript{1Cys}, 5’-AATTCCTGCGT-GAATACGCCTCAGCCAAGGTGCGGAGCGGT-CGATGGGACCAAGGTGCGGAGCGGT-CGATGGGACCAAGGTGCGGAGCGGT-CGATGGGACCAAGGTGCGGAGCGGT-CGATGGGACCAAGGTGCGGAGCGGT-CGATGGGACCAAGGTGCGGAGCGGT-CGATGGGACCAAGGTGCGGAGCGGT-CGATGGGACCAAGGTGCGGAGCGGT-CGATGGGACCAAGGTGCGGAGCGGT-CGATGGGACCAAGGTGCGGAGCGGT-CGATGGGACCAAGGTGCGGAGCGGT-CGATGGGACCAAGGTGCGGAGCGGT-CGATGGGACCAAGGTGCGGAGCGGT-CGATGGGACCAAGGTGCGGAGCGGT-CGATGGGACCAAGGTGCGGAGCGGT-

**Phosphoseryl Cysteine Synthetase (SepCysS) Assay**

- SepCysS and sulfur donor are then added to initiate the SepCysS activity assay. In this approach, 20 \( \mu \)M \( ^{32} \)P-labeled tRNA\textsubscript{Cys} was first heated to 85°C and slowly cooled under anaerobic conditions in the presence of 2 mM MgCl\(_2\). Sep-tRNA\textsubscript{Cys} was then synthesized in a buffer consisting of 50 mM Tris (pH 7.5), 20 mM KCl, 10 mM MgCl\(_2\), 5 mM ATP, 3 mM phosphoserine, and 5 mM DTT. The phosphoseryl reaction was initiated by adding SepRS to 20–30 \( \mu \)M and incubating for 8 min at 37°C. SepCysS was preincubated at 37°C in a buffer containing 10 mM NaH\(_2\)PO\(_4\) (pH 7.4), 20 mM NaCl, 2 mM MgCl\(_2\), 1 mM BME, 100 \( \mu \)M PLP, and 50% glycerol and was then added to concentrations of 0.05–40 \( \mu \)M, followed immediately by sodium sulfide to a concentration of 5 mM. Typical concentrations of Sep-tRNA\textsubscript{Cys} used in the second reaction, after dilution with reaction components, were 5–10 \( \mu \)M. In reactions where Sep-tRNA\textsubscript{Cys} was generated outside of the anaerobic chamber, it was first preincubated in the chamber at 37°C with 50 mM Tris (pH 7.5), 20 mM KCl, 10 mM MgCl\(_2\), and 5 mM DTT for several minutes before the addition of SepCysS and sodium sulfide.

**Phosphorimaging Analysis**

Quantitation was done by phosphorimaging analysis, as described previously (17). Time courses from 0.5 to 60 min were fit to linear or sigmoidal equations. Results using either method of Sep-tRNA\textsubscript{Cys} preparation were identical.

**tRNA Binding to SepCysS**

Equilibrium fluorescence titrations were performed at ambient temperature with 0.25 \( \mu \)M \( M. \textit{mazei} \) SepCysS in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 5 mM BME. Tryptophan fluorescence was excited at 295 nm, and the emission was monitored from 300 to 400 nm. An emission wavelength of 350 nm was used to quantitate binding after correction for dilution and for intrinsic quenching due to RNA absorbance at 295 nm, according to the formula, \( F_0 = F_{oobs} \times \text{antilog}(A_{295} + A_{350}/2) \) (20). Control solutions of bovine serum albumin or of tryptophan showed no fluorescence response to tRNA. tRNA was titrated in the concentration range of 0.05–10 \( \mu \)M. The \( K_d \) values were derived by fitting...
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FIGURE 1. A, dependence of reaction rate on tRNA concentration for aminoaaclylation by M. mazei SepRS. Replot of tRNA\(^{\text{Cys}}\) concentration versus initial velocities determined from time courses. The inset shows the imaged TLC plate of a single time course for tRNA\(^{\text{Cys}}\). B, the secondary structures of M. mazei tRNA\(^{\text{Cys}}\) isoacceptors tRNA\(^{\text{Cys}1}\), tRNA\(^{\text{Cys}2}\), and tRNA\(^{\text{Cys}3}\) are depicted from left to right. The 7-66 base pair at the bottom of the acceptor stem, which distinguishes tRNA\(^{\text{Cys}1}\) from tRNA\(^{\text{Cys}2}\), is shown in boldface type on the tRNA\(^{\text{Cys}2}\) cloverleaf at the center. Nucleotides distinguishing tRNA\(^{\text{Cys}1}\) (left) from both tRNA\(^{\text{Cys}2}\) and tRNA\(^{\text{Cys}3}\) are shown in boldface type on the tRNA\(^{\text{Cys}2}\) cloverleaf. Mutations of tRNA\(^{\text{Cys}1}\) (right) made in this study are circled. The correspondence between the nucleotides and the mutations described under “Results and Discussion” and in Table 1 is as follows: tRNA\(^{\text{Cys}2}\)Δ2, introduction of A33 from tRNA\(^{\text{Cys}2}\) into tRNA\(^{\text{Cys}2}\); tRNA\(^{\text{Cys}3}\)Δ3, introduction of A57 from tRNA\(^{\text{Cys}3}\) into tRNA\(^{\text{Cys}3}\); tRNA\(^{\text{Cys}5}\)Δ5, introduction of both C49 and U51 from tRNA\(^{\text{Cys}5}\) into tRNA\(^{\text{Cys}5}\); tRNA\(^{\text{Cys}4}\)Δ4, introduction of the five D-loop and variable loop nucleotides U20, C21, U44, A46, and G47 from tRNA\(^{\text{Cys}4}\) into tRNA\(^{\text{Cys}4}\).

RESULTS AND DISCUSSION

Differential Utilization of tRNA\(^{\text{Cys}}\) Isoacceptors by SepRS and CysRS—We chose to study Cys-tRNA\(^{\text{Cys}}\) formation in M. mazei because genetic techniques have been developed in this organism (21) and because its biosynthetic apparatus is expanded to its known limit in M. mazei and M. barkeri, suggesting the possibility that the macromolecules involved may be regulated in unusual ways or may perform physiological roles unrelated to protein synthesis. Both of these Methanosarcina possess all three enzymes corresponding to the two distinct pathways, CysRS, SepRS, and SepCysS, as well as three tRNA\(^{\text{Cys}}\) isoacceptors (Fig. 1). In contrast, M. acetivorans, the other Methanosarcina for which the genome sequence is available, possesses all three enzymes but only one tRNA\(^{\text{Cys}}\) acceptor that reads both the UGU and UGC codons. Among the other sequenced methanogens, M. maripaludis and Methanospirillum hungatei possess biosynthetic apparatus with identical components to M. acetivorans, whereas Methanomethobacter thermautotrophicus, M. jannaschii, Methanopyrus kandleri, and Methanococcoides burtonii possess only the two-step indirect pathway with a single tRNA\(^{\text{Cys}}\) isoacceptor. Only the human intestinal archaea Methanosphaera stadtmanae and Methanobrevibacter smithii provide examples of methanogens possessing just the canonical CysRS with one tRNA\(^{\text{Cys}}\) acceptor and lacking the SepRS/SepCysS pathway (22, 23).

Among all sequenced bacteria and archaea, the presence of three tRNA\(^{\text{Cys}}\) isoacceptors is found only in M. mazei and M. barkeri. Indeed, analysis of genomic data shows that only 10 bacterial or archaeal species possess even two tRNA\(^{\text{Cys}}\) isoacceptors. In addition to the GCA wobble, all three M. mazei isoacceptors possess the U73 discriminator base typical of tRNA\(^{\text{Cys}}\) species; these four nucleotides form a key part of the “identity set” for recognition by both CysRS and SepRS (10, 24). tRNA\(^{\text{Cys}2}\) and tRNA\(^{\text{Cys}3}\) are of canonical structure and are highly similar to each other, being distinguished only by the base pair at position 7-66 at the bottom of the acceptor stem (Fig. 1). However, tRNA\(^{\text{Cys}1}\) possesses several unusual features: the presence of a rare purine at position 33 (found in only four other known tRNAs, two of which are also tRNA\(^{\text{Cys}}\) acceptors from methanogens), a GU wobble pair at position 51-63 of the T-stem, and an A65-C49 mismatch at the inside of the T-stem (found in only one other known tRNA, the homologous tRNA\(^{\text{Cys}2}\) isoacceptor from M. barkeri). tRNA\(^{\text{Cys}1}\) also differs from the other two M. mazei tRNA\(^{\text{Cys}}\) isoacceptors in featuring A57 in the T-loop as well as sequence differences at positions 20, 21, 44, 46, and 47 in the D-loop and variable loops. The last five differences probably produce distinct base triples and/or stacking interactions in the augmented D-stem region of the globular core. Despite the unusual structure, analysis of the genomic DNA surrounding tRNA\(^{\text{Cys}1}\) reveals the presence of both the A-box and B-box transcriptional initiation motifs (25). Therefore, this unusual tRNA may well be expressed in vivo. Resequencing of the genomic DNA in this region also revealed no errors in the deposited sequence (data not shown).

We sought to explore the biological rationale for the existence of redundancy in the machinery for Cys-tRNA\(^{\text{Cys}}\) synthesis by determining the efficiency by which each of the three tRNAs is processed by the two pathways. The three tRNAs were synthesized by in vitro transcription, were each methylated at position m\(^{1}\)G37 using recombinant M. jannaschii Trm5, and were purified from unmethylated transcripts (see “Experimental Procedures” and Refs. 11 and 13). SepRS aminoacylation
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| TABLE 1 Determinants of tRNA specificity in CysRS and SepRS |
|------------------------------------------------------------|
| **SepRS** | **CysRS** |
| kcat | Km | kcat/Km | pmol/OD | kcat | Km | kcat/Km | pmol/OD | Specificity |
| tRNA3Cys<sup>c</sup> | 0.12 ± 0.01 | 6.4 ± 0.2 | (2.0 ± 0.3) × 10<sup>4</sup> | 1250 ± 60 | >0.17 | >80 | 2070 ± 33 | 780 ± 50 | 9.5 |
| tRNA5Cys<sup>c</sup> | 0.51 ± 0.11 | 6.2 ± 0.3 | (8.5 ± 0.9) × 10<sup>4</sup> | 1360 ± 80 | >0.06 | >160 | 380 ± 60 | 830 ± 70 | 223 |
| tRNA3Cys<sup>j</sup> | 0.38 ± 0.09 | 7.6 ± 0.3 | (8.0 ± 0.1) × 10<sup>4</sup> | 1300 ± 80 | >0.03 | >140 | 229 ± 21 | 530 ± 40 | 143 |
| tRNA3Cys<sup>k</sup> | 0.23 ± 0.02 | 13.1 ± 2.8 | (1.6 ± 0.3) × 10<sup>4</sup> | 840 ± 40 | >0.16 | >90 | 1700 ± 57 | 720 ± 40 | 9.4 |
| tRNA3Cys<sup>j</sup> | 0.52 ± 0.10 | 10.2 ± 1.2 | (5.1 ± 0.4) × 10<sup>4</sup> | 990 ± 70 | >0.05 | >130 | 409 ± 83 | 580 ± 30 | 125 |
| tRNA5Cys<sup>j</sup> | 0.30 ± 0.04 | 9.9 ± 0.7 | (3.0 ± 0.1) × 10<sup>4</sup> | 660 ± 40 | >0.09 | >120 | 752 ± 95 | 680 ± 40 | 39.5 |
| tRNA5Cys<sup>i</sup> | 0.25 ± 0.03 | 10.1 ± 1.5 | (2.5 ± 0.1) × 10<sup>4</sup> | 900 ± 50 | >0.14 | >110 | 1250 ± 43 | 480 ± 20 | 20 |
| tRNA5Cys<sup>j</sup> | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| tRNA5Cys<sup>j</sup> | 0.18 ± 0.02 | 8.6 ± 0.6 | (2.1 ± 0.3) × 10<sup>4</sup> | 900 ± 30 | >0.18 | >90 | 1590 ± 44 | 740 ± 50 | 10.8 |

<sup>a</sup> kcat/Km values were determined from initial velocities using the relation, v = kcat/Km[E][S].
<sup>b</sup> Specificity for each tRNA is determined as the ratio, kcat/Km(SEPRS)/kcat/Km(CYSRS).
<sup>c</sup> Structures of the M. mazei isoacceptors tRNA<sub>Cys</sub>, tRNA<sub>Cys</sub>, and tRNA<sub>Cys</sub> are shown in Fig. 1B.
<sup>d</sup> The A33U mutation in tRNA<sub>Cys</sub>.
<sup>e</sup> The U33A mutation in tRNA<sub>Cys</sub>.
<sup>f</sup> The C20U/U21C/A44U/C46A/A47G mutations in tRNA<sub>Cys</sub>.
<sup>g</sup> The U94C/C51U mutations in tRNA<sub>Cys</sub>.
<sup>h</sup> ND, not determined.
<sup>i</sup> The C20U/U21C/A44U/C46A/A47G/G57A mutations in tRNA<sub>Cys</sub>.

The U49C/C51U mutations in tRNA<sub>3</sub>Cys.

Cys and tRNA<sub>3</sub>Cys are shown in Fig. 1.

The U49C/C51U mutations in tRNA<sub>Cys</sub>.

kcat of 0.5–0.6 s<sup>-1</sup> for tRNA<sub>1</sub>Cys<sup>c</sup> and tRNA<sub>1</sub>Cys<sup>j</sup>.

By comparison, Table 1 shows that SepRS is the better functioning enzyme for all isoacceptors; however, both decreases the efficiency of phosphoseryltransferase. Incorporation into tRNA<sub>3</sub>Cys of A57 into tRNA<sub>3</sub>Cys and by 9.5-fold for tRNA<sub>1</sub>Cys<sup>c</sup>.

This suggests the operation of an evolutionary selection by which the parallel direct pathway is retained by the organism.

To examine the structural basis for the tRNA<sub>Cys</sub> isoacceptor preferences of SepRS and CysRS, we constructed a series of tRNA mutants in which structural elements of tRNA<sub>Cys</sub> were systematically transplanted into tRNA<sub>Cys</sub> and evaluated the effectiveness of the mutants as CysRS and SepRS substrates by steady-state aminocacylation (Fig. 1 and Table 1). As observed for the three wild-type isoacceptors, all of the tRNA mutants are aminocacylated by SepRS more efficiently than by CysRS. A33 is not a distinguishing determinant, because introduction of this nucleotide into tRNA<sub>3</sub>Cys<sup>c</sup> does not significantly alter the relative preferences of SepRS and CysRS for that substrate (tRNA<sub>Cys</sub><sup>j</sup>/tRNA<sub>Cys</sub><sup>i</sup>; Table 1). (In a separate experiment, mutation of A33 to U33 in the tRNA<sub>Cys</sub> framework also does not shift enzyme preferences (tRNA<sub>Cys</sub><sup>j</sup>/tRNA<sub>Cys</sub><sup>i</sup>; Table 1)). Incorporation of A57 into tRNA<sub>Cys</sub> (tRNA<sub>Cys</sub><sup>j</sup>), however, both decreases the efficiency of phosphoseryltransferase and increases the efficiency of cysteinylation; the SepRS/CysRS preference of this mutant is decreased from about 140- to 40-fold. Incorporation into tRNA<sub>Cys</sub> of the tRNA<sub>Cys</sub> nucleotides in the augmented D-stem/variable loop region (C20U/U21C/A44U/C46A/A47G) has qualitatively similar but more marked effects, so that the SepRS/CysRS preference is decreased to 20-fold (tRNA<sub>Cys</sub><sup>j</sup>/tRNA<sub>Cys</sub><sup>i</sup>; Table 1). Finally, combining the tRNA<sub>Cys</sub><sup>j</sup> and tRNA<sub>Cys</sub><sup>j</sup> substitutions generates a mutant that fully recapitulates the SepRS/CysRS preferences of tRNA<sub>Cys</sub> (tRNA<sub>Cys</sub><sup>j</sup>6; Table 1). Therefore,

FIGURE 2. Time course for plateau cysteinylation by M. mazei CysRS in the absence or presence of m<sup>1</sup>G37 in tRNA<sub>Cys</sub><sup>j</sup>. The effect of methylating tRNA<sub>Cys</sub><sup>j</sup> and tRNA<sub>Cys</sub><sup>i</sup> is nearly identical (not shown).
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A57 together with the five nucleotides U20/C21/U44/A46/G47 represents the distinguishing identity set (Fig. 1B). Interestingly, the unusual T-stem base pairs in tRNA$^{\text{Cys}}_{\text{1}}$ appear not to play a role in modulating the SepRS/CysRS preference. Independent introduction of these two base pairs into tRNA$^{\text{Cys}}_{\text{5}}$ produced a mutant that could not be efficiently aminocylated by either enzyme (tRNA$^{\text{Cys}}_{\text{Delta5}}$; Table 1). This suggests that other structural elements in tRNA$^{\text{Cys}}_{\text{1}}$ are required to maintain the structural stability of its unusual core domain.

These data are consistent with studies of other SepRS enzymes. Work in the M. maripaludis system indicates that the G6/C67 base pair, C20, G37, A47, and A59 are putative tRNA$^{\text{Cys}}$ identity elements for SepRS (10). tRNA$^{\text{Cys}}_{\text{2}}$ and tRNA$^{\text{Cys}}_{\text{5}}$ of M. mazei each possess all six of these nucleotides, whereas tRNA$^{\text{Cys}}_{\text{1}}$ is missing C20 and A47. Further, the crystal structure of the A. fulgidus SepRS:tRNA$^{\text{Cys}}$ complex shows that U33 does not contact the enzyme (26), consistent with the ineffectiveness of this nucleotide in modulating the SepRS/CysRS preferences. The importance of T-loop, D-loop, and variable loop nucleotides shown in this work, together with similar findings from studies of the M. maripaludis enzyme, suggest that SepRS makes important interactions with the globular tRNA core. Because the A. fulgidus SepRS crystallized as an unproductive complex in which interactions are made only with the tRNA anticodon (26), further structural analyses will be required to provide a reliable basis for these observations.

The tRNA$^{\text{Cys}}$ $K_m$ values of 6–8 μM measured toward SepRS are some 10-fold higher than typically observed for highly efficient cognate aminocylases by tRNA synthetases, and the equivalent values toward CysRS are more than 100-fold higher. It is notable that the addition of them$^1$G$^37$ modification, which equivalent values toward CysRS are more than 100-fold higher. It is notable that the addition of them$^1$G$^37$ modification, which is missing C20 and A47. Further, the crystal structure of the A. fulgidus SepRS:tRNA$^{\text{Cys}}$ complex shows that U33 does not contact the enzyme (26), consistent with the ineffectiveness of this nucleotide in modulating the SepRS/CysRS preferences. The importance of T-loop, D-loop, and variable loop nucleotides shown in this work, together with similar findings from studies of the M. maripaludis enzyme, suggest that SepRS makes important interactions with the globular tRNA core. Because the A. fulgidus SepRS crystallized as an unproductive complex in which interactions are made only with the tRNA anticodon (26), further structural analyses will be required to provide a reliable basis for these observations.

The tRNA$^{\text{Cys}}$ $K_m$ values of 6–8 μM measured toward SepRS are some 10-fold higher than typically observed for highly efficient cognate aminocylases by tRNA synthetases, and the equivalent values toward CysRS are more than 100-fold higher. It is notable that the addition of the m$^1$G37 modification, which substantially improves both initial velocities of aminocylates on and aminocylase plateaus values for both enzymes (11, 13) (Fig. 2), still produces such weak apparent complementarity in the enzyme-tRNA interfaces. It appears quite possible, therefore, that further base or ribose modification may be required for optimal Cys-tRNA$^{\text{Cys}}$ formation by SepRS, CysRS, or both enzymes. A positive tRNA modification requirement for efficient aminocylates has been observed previously in only a small number of cases, primarily involving the wobble nucleotide at the 5’-position of the anticodon in the isoleucyl, glutamyl, and glutaminyl systems (27–31). The only previous observation of a positive tRNA methylation requirement for aminocylates is the need for 1-methyladenosine at position 9 of nematode mitochondrial tRNAs lacking the T-arm (32). In these cases, however, the methyl group clearly plays a compensating structural role in repairing the tRNA core and may not be directly important for interaction with the tRNA synthetase. It is also of interest to note that the m$^1$G37 modification functions as a negative determinant to ensure aminocylates specificity of yeast tRNA$^{\text{Asp}}$; when G37 is unmodified in that tRNA, it is significantly misacylated by arginyl-tRNA synthetase (33). We know of no tRNA-synthetase system, however, in which modification at two separate nucleotides has been shown to be required for full aminocylate efficiency in vitro.

Embedded metabolic linkages among macromolecules responsible for synthesis of Cys-tRNA$^{\text{Cys}}$, for methanogenesis, and for other aspects of sulfur metabolism could help to explain why the presence of the canonical CysRS has not resulted in elimination of the SepRS/SepCysS pathway in methanogens and the related A. fulgidus, as it has in all other archaea (8). The unusually high number of Fe-S cluster proteins in methanogens may provide one rationale for the existence of unique linkages in these organisms (34). We suggest that the S-adenosylmethione-dependent methylation requirement for Cys-tRNA$^{\text{Cys}}$ synthesis shown here also points to such linkages, since S-adenosylmethione is synthesized intracellularly from methionine (14). Further, free cysteine is known to be a source of metabolic sulfur for biosynthesis in bacteria (35). Hence, the finding that deletion of the SepRS gene in M. maripaludis leads to cysteine auxotrophy suggested a clear connection between the SepRS/SepCysS pathway and cysteine biosynthesis (7). Finally, the finding that SepCysS remains essential in the context of this SepRS deletion (where Cys-tRNA$^{\text{Cys}}$ is provided by CysRS), suggests a further independent role for that enzyme (36).

No correlation is evident between the pathway of cysteine biosynthesis and the number of tRNA$^{\text{Cys}}$ isoacceptors among the known Methanosarcina. M. acetivorans, which retains only duplicate copies of a single tRNA$^{\text{Cys}}$ gene that is highly similar to tRNA$^{\text{Cys}}_{\text{2}}$ and tRNA$^{\text{Cys}}_{\text{5}}$ of M. mazei, possesses both the bacterial and mammalian enzymes needed for cysteine biosynthesis. However, M. barkeri, which similarly possesses redundant cysteine biosynthesis pathways, contains all three tRNAs. By contrast, M. mazei lacks clear homologs for either the O-acetylserine sulfhydrylase or the cystathionine β-synthase enzymes needed for the bacterial and mammalian pathways, respectively (37). Cysteine may be synthesized in M. mazei entirely by means of deacylating tRNA$^{\text{Cys}}$; alternatively, the organism might use a weakly related O-phosphoserine sulfhydrilase to synthesize cysteine from phosphoserine and sulfide ions (38, 39); this enzyme exists in some archaea and provides an alternative to the bacterial pathway from O-acetylserine.

Despite the inconclusive results from these genome comparisons, it is of interest to note that the presence of A33 in the unusual tRNA$^{\text{Cys}}_5$ species may render it a less efficient substrate for the ribosome, because U33 is required for the anticodon loop to adopt the conserved U-turn structure. Several studies have indicated that tRNAs lacking the highly conserved U33 bind the ribosome with lower efficiency (40, 41), although it has also been shown that U33 is not essential for tRNAs to perform in eukaryotic in vitro suppression assays (42). The dedication of tRNA$^{\text{Cys}}_5$ to a function other than protein synthesis would suggest a further linkage between Cys-tRNA$^{\text{Cys}}$ synthesis and other aspects of metabolism. Although tRNA$^{\text{Cys}}_5$ is clearly an unusual tRNA, its selective aminocylates by CysRS, capacity to be methylated by Trm5, conservation in M. barkeri, and retention of transcription activation signals all suggest a true functional role.

Sulfur Donor and tRNA$^{\text{Cys}}$ Isoacceptor Substrate Preference of SepCysS—Rigorous characterization of the enzymatic activity of SepCysS, which catalyzes the conversion of Sep-tRNA$^{\text{Cys}}$ to Cys-tRNA$^{\text{Cys}}$, is challenging because of the need to prepare the misacylated substrate and because the assay requires anaerobic conditions. To first characterize the ligand-bound state of the M. mazei enzyme purified from recombinant E. coli cells, we
monitored the UV-visible absorbance of an extensively dialyzed sample. Distinct peaks were observed at 230, 280, and 415 nm; the latter peak corresponds to the lysine-bound ketoenamine form of the cofactor (Fig. 3A) (43). Colorimetric analysis of SepCysS reveals that the molar ratio of PLP to enzyme subunits is 1.2:1, indicating that both active sites bind the cofactor (data not shown) (13). Consistent with this analysis, the crystal structure of A. fulgidus SepCysS showed PLP bound in a covalent aldime linkage to Lys209 in both subunits (44).

We next considered how best to quantitate SepCysS activity. The only reported functional assay monitors the PLP-dependent transformation of 14C-labeled phosphoserine attached to tRNA^Cys into 14C-labeled cysteine (7). Because this assay is insensitive and requires the use of commercially obtained radiolabel at prohibitive cost, we sought to develop an alternative that would be cost-effective, highly sensitive, and hence more suitable for detailed investigation of enzymatic properties. We found that the assay reported here and elsewhere for SepRS function, which generates 32P-labeled Sep-tRNACys with the radiolabel positioned at the 3'-internucleotide linkage of the tRNA, can be extended to monitor the conversion of Sep-tRNACys to Cys-tRNACys (11, 13, 45). After the SepCysS reaction using 32P-labeled Sep-tRNACys as substrate, the tRNA is hydrolyzed to its constituent nucleotides, and Sep-Ap* (substrate) is separated from Cys-Ap* (product) by thin layer chromatography (Fig. 3B). Separate SepRS and CysRS reactions are used as controls to identify the observed spots. The assay allows for straightforward determination of the fraction of substrate tRNA that is phosphoserylated, to control for the possibility that the input Sep-tRNACys may become deacetylated in the processing steps between reactions.

We generated the Sep-tRNACys substrate in two ways. First, tRNA^Cys was phosphoserylated to 30–85% plateau levels in an aerobic environment at 37 °C and then isolated by acidic phenol/chloroform extraction and ethanol precipitation. The Sep-tRNACys was then reconstituted in an anaerobic environment, in a buffer consisting of 10 mM sodium cacodylate (pH 6.0) and 2 mM MgCl$_2$. Alternatively, tRNA^Cys transcripts were lyophilized, transported into the anaerobic environment, and subjected to consecutive SepRS and SepCysS reactions without recovering the Sep-tRNACys intermediate. SepCysS converted identical proportions of substrate to product with either method of substrate preparation. Consequently, most experiments were performed using the latter approach, since it obviated the need to isolate Sep-tRNACys and provided continuous assay capability requiring only supplementation of the SepRS reaction with PLP-bound enzyme and sulfur donor. We also verified that both the proportion of overall substrate conversion to product by SepCysS and the rates of conversion were independent of the initial plateau level of phosphoserylation, in the range of 30–85%. Many SepCysS reactions were performed using tRNA^Cys at 40–50% phosphoserylation levels, solely for reasons of economy and efficiency in generating the Sep-tRNACys. All SepCysS reactions were performed under strictly anoxic conditions, using reagents made anoxic through cycles of vacuum followed by purging with oxygen-free argon gas.

All reactions also utilized tRNA^Cys transcripts modified to carry the m1G37 modification. Whether the added methyl group influences the activity of SepCysS is not known, because in the absence of methylation, the levels of Sep-tRNACys that
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TABLE 2
Alternative sulfur donors used by SepCysS

| Conversion to Cys-tRNA<sup>Cys</sup> | %  | μmol | min⁻¹ μmol⁻¹ |
|-----------------------------------|----|------|--------------|
| HS                                | 72.1 ± 9.5 | 1.1 ± 0.2 | 0.009 ± 0.002 |
| Thio phosphate                     | 52.3 ± 4.5 | 0.4 ± 0.15 | 0.014 ± 0.003 |
| Cysteine                          | 55.4 ± 5.0 | ND      | ND           |

TABLE 3
tRNA discrimination by SepCysS

| Percentage conversion to Cys-tRNA<sup>Cys</sup> | K<sub>d</sub> | k<sub>cat</sub>/K<sub>d</sub> |
|------------------------------------------------|------------|-----------------|
| Sep-tRNA<sup>Cys</sup>                           | 52.2 ± 9.5 | 1.1 ± 0.2       |
| Sep-tRNA<sup>Cys</sup>                           | 72.2 ± 5.0 | 0.55 ± 0.18     |
| tRNA<sup>Cys</sup>Δ2                             | 72.0 ± 8.0 | ND              |
| tRNA<sup>Cys</sup>Δ4                             | 50.0 ± 6.0 | ND              |

<sup>a</sup> k<sub>cat</sub>/K<sub>d</sub> values were determined from initial velocities using the relation v = k<sub>cat</sub>/K<sub>d</sub> [E][S].
<sup>b</sup> ND, not determined.

can be synthesized by SepRS are too low to permit measurement of sulfide transfer.

Initial characterization of SepCysS was performed under conditions of molar enzyme excess over tRNA. Previously, sodium sulfide was used as the sulfur donor in SepCysS reactions (7). To explore the sulfur donor requirement more thoroughly, we carried out reactions using Sep-tRNA<sup>Cys</sup> as substrate, in the presence of 5 mM sodium sulfide, 5 mM thio phosphate, or 5 mM cysteine. Higher concentrations of any of these reagents did not increase the overall conversion to product. Each compound functioned as a sulfur donor, with sodium sulfide (72% end point conversion of Sep-tRNA<sup>Cys</sup> to Cys-tRNA<sup>Cys</sup>) providing higher activity than either thio phosphate or cysteine (52–55% conversion; Table 2). Only sodium sulfide yields the correct Cys-tRNA<sup>Cys</sup> product; the capacity of the enzyme to utilize the other donors demonstrates the presence of some structural adaptability in the active site.

To evaluate whether SepCysS exhibits substrate preference among the three M. mazei tRNA<sup>Cys</sup> isoacceptors, a 5 μM concentration of each Sep-tRNA<sup>Cys</sup> species was incubated with 30 μM SepCysS and 5 mM sodium sulfide, and the reactions were allowed to proceed to completion over 1 h. The percentage conversion to Cys-tRNA<sup>Cys</sup> was reproducibly higher for tRNA<sup>Cys</sup>Δ2 and tRNA<sup>Cys</sup>Δ4 (72–75%) as compared with tRNA<sup>Cys</sup> (53%) (Table 3). By this measure, then, SepCysS exhibits the same tRNA substrate preference observed for SepRS (Table 1). Measurement of the SepCysS substrate conversion efficiencies for tRNA mutants tRNA<sup>Cys</sup>Δ2 and tRNA<sup>Cys</sup>Δ4 further confirmed the SepCysS preference. tRNA<sup>Cys</sup>Δ2, which is strongly preferred by SepRS, is also a good substrate for SepCysS, whereas tRNA<sup>Cys</sup>Δ4, which is shifted substantially toward CysRS preference, is a poorer SepCysS substrate (Tables 1 and 3). Part of the SepCysS preference appears to arise from differences in binding affinity to the tRNA body; measurements of equilibrium dissociation constants for SepCysS interaction with each native unacylated isoacceptor, by fluorescence quenching (20), showed that K<sub>d</sub> for tRNA<sup>Cys</sup>Δ2 and tRNA<sup>Cys</sup>Δ4 are 2–3-fold lower than K<sub>d</sub> for tRNA<sup>Cys</sup>Δ2 (Table 3).

To further characterize the properties of M. mazei SepCysS, we investigated the kinetics of converting Sep-tRNA<sup>Cys</sup> to Cys-tRNA<sup>Cys</sup>. Under steady-state conditions at 1–50 nM SepCysS, we observed no reaction when Sep-tRNA<sup>Cys</sup> substrate levels were maintained below 1 μM. However, product formation was detected at 50 nM SepCysS and 5 μM Sep-tRNA<sup>Cys</sup>. At this substrate concentration, reaction velocities then were found to increase with enzyme concentration (Fig. 3C). It is likely that this behavior arises from dissociation of the SepCysS dimer into monomers, with K<sub>d</sub> for subunit association in the range of 10⁻⁷ M. This interpretation is consistent with the crystal structure of A. fulgidus SepCysS, which shows that each active site is composed of amino acids from both subunits, suggesting that monomers do not retain activity (44). No Sep-tRNA<sup>Cys</sup> saturation was observed under steady-state conditions of substrate excess ([S]/[E] > 20) and with Sep-tRNA<sup>Cys</sup> concentrations up to 15 μM. However, the initial velocities allow estimation of k<sub>cat</sub>/K<sub>m</sub> at 0.009–0.014 min⁻¹ μmol⁻¹, with no significant differences measured among the three isoacceptors (Table 3). Levels of product formation substantially exceed the amount of enzyme present, demonstrating unambiguously that SepCysS is able to function as a multiple-turnover catalyst.

The higher substrate conversion efficiency and binding affinities for tRNA<sup>Cys</sup>Δ2 and tRNA<sup>Cys</sup>Δ4 by SepCysS matches the preference of SepRS and suggests that these two species may be ded-
The identification of Lys222 as the likely side chain that ligates PLP is based on a structure-based sequence alignment with A. fulgidus SepCysS.

FIGURE 5. Proposed mechanism for the synthesis of Cys-tRNACys by SepCysS. The mechanism is based upon a proposed sulfur relay system and on similarity to Sec-tRNA[Ser]Sec biosynthesis. The movement of electrons into PLP has been omitted. The inclusion of bisulfide as the sulfur donor indicates how the enzyme functions in this in vitro study (bottom right; the PLP generated would reform the internal aldimine with Lys222). The identification of Lys222 as the likely side chain that ligates PLP is based on a structure-based sequence alignment with A. fulgidus SepCysS.

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To further examine the kinetics of SepCysS, we measured substrate conversion while systematically varying the concentration of enzyme. At 5 μM Sep-tRNACys and at SepCysS concentrations above 200 nM, product formation exhibits an upward slope after reactions proceed for ~30 min (Fig. 4A). Further, at equimolar E/S ratios and under conditions of enzyme molar excess, the reaction progress curve is sigmoidal. For example, at 10 μM Sep-tRNACys and 15–30 μM SepCysS, a distinct lag phase is followed by a rapid increase in product formation, with maximal levels reached at 50–60 min. During the slow lag phase, an intermediate that migrates farthest on the TLC plate is observed to first accumulate and then to disappear as Cys-tRNACys forms.

The distinct lag phase suggests a rate-limiting step associated with the mechanistic step in which the exogenous sulfur reacts (Fig. 5). Further, the measured $k_{cat}/K_m$ for conversion of Sep-tRNACys to Cys-tRNACys is ~500-fold lower than $k_{cat}/K_m$ for phosphoseryl-ylation of tRNA2Cys and tRNA3Cys by SepRS (Tables 1 and 3). Thus, the rates by which the substrate is processed by each enzyme appear to be greatly mismatched; this would produce a highly inefficient pathway if present in vivo. Both of these observations suggest that the sulfur donors employed in these in vitro experiments are not likely to be used in vivo, so that a major experimental challenge associated with elucidating the true pathway of sulfur delivery to the active site remains.

By analogy with the mechanism proposed for the PLP-dependent conversion of seryl-tRNA[Sec] to selenocysteyl-tRNA[Sec] by bacterial selenocysteine synthase (48), we suggest that binding of Sep-tRNACys to SepCysS is followed by formation of a Schiff base between the α-amino group of the phosphoserine residue and the formyl group of pyridoxal 5-phosphate, resulting in elimination of the phosphate group and formation of an aminoacrylyl-tRNA[Sec], which would correspond to the inter-

Maximal substrate conversion by eukaryotic selenocysteine synthase, another PLP-dependent enzyme that acts on aminoacylated tRNA (46). The $k_{cat}/K_m$ for the M. mazei SepCysS reaction with bisulfide as sulfur donor is also very similar to that reported for the analogous conversion of Ser-tRNASec to Sec-tRNASec by bacterial selenocysteine synthase when thiophosphate is employed as sulfur donor in that enzyme (47).
mediate observed on TLC as aminoacryl-tRNA\textsubscript{A76} (Figs. 3B, 4B, and 5). In our in vitro reactions, HS\textsuperscript{−} or another donor then adds slowly to the double bond, in the rate-limiting step, to generate product (Fig. 5).

Use of the natural donor selenophosphate provides a 330-fold rate enhancement to selenocysteine synthase as compared with thiophosphate (47); such an improvement, if conferred to SepCysS via use of the proper sulfur donor, would suffice to nearly match the catalytic efficiency of SepRS (Tables 1 and 3). It is most likely that the true sulfur donor to tRNA is a persulfide group formed from one or more of the three conserved cysteines in the active site (Cys\textsuperscript{51}, Cys\textsuperscript{52}, and Cys\textsuperscript{280} in the M. mazei enzyme) (44). The persulfide group provides sulfur for both cofactor and nucleotide biosynthesis in a wide variety of biological systems (49) and could presumably function to increase the catalytic efficiency of SepRS (Tables 1 and 3).

Interestingly, SepCysS itself is strongly homologous to the cysteine desulfurases CsdB, NifS, and IscS, suggesting that it may function as both a sulfur donor and receptor (52). This enzyme uses selenophosphate as a selenide donor (46), alternatively as selenocysteine synthase (46, 51) or SepSecS (3, 4). Identification of additional tRNA\textsuperscript{Cys}-modifying enzymes of the enzyme that delivers sulfur to SepCysS may suffice for a definitive understanding of tRNA selectivities in CysRS, SepRS, and SepCysS. Such identifications may provide clues that, together with genetic, biochemical, and bioinformatic studies, could elucidate novel metabolic relationships connecting the protein synthesis machinery with sulfur and energy metabolism in methanogens. The crucial role of these archaeabacteria in the biogeochemical processes determining global climate change offers strong impetus for these experiments.

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