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Redundant Synthesis of Cysteinyl-tRNA^{Cys} in *Methanosarcina mazei*[*]

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A subset of methanogenic archaea synthesize the cysteinyl-tRNA^{Cys} (Cys-tRNA^{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^{Cys} (Sep-tRNA^{Cys}) is first synthesized by phosphoseryl-tRNA synthetase (SepRS), and this misacylated intermediate is then converted to Cys-tRNA^{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^{Cys} isoacceptors, further indicating the unusual and complex nature of Cys-tRNA^{Cys} synthesis in this organism. Comparative aminoacylation kinetics by *M. mazei* CysRS and SepRS reveals that each enzyme prefers a distinct tRNA^{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*-adenosylmethionine-dependent formation of ^{m1}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 synthase and the archaeal/eukaryotic SepSecS enzyme. Together, these data suggest that both metabolic routes and all three tRNA^{Cys} species in *M. mazei* play important roles in the cellular physiology of the organism.

Methanosarcina mazei strain Gö1 is a methanogenic archaeon capable of utilizing a broad spectrum of growth substrates, including H_2/CO_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^{Cys} is first aminoacylated with the nonstandard amino acid phosphoserine by phosphoseryl-tRNA synthetase (SepRS).[2] The misacylated Sep-tRNA^{Cys} is then converted to Cys-tRNA^{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^{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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[2] The abbreviations used are: SepRS, phosphoseryl-tRNA synthetase; SepCysS, Sep-tRNA:Cys-tRNA synthase; Sep-tRNA^{Cys}, phosphoseryl-tRNA^{Cys}; CysRS, cysteinyl-tRNA synthetase; DTT, dithiothreitol; PLP, pyridoxal phosphate; βME, β-mercaptoethanol.

The only nonmethanogen known to possess the two-step Cys-tRNA^{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^{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^{Cys} to Cys-tRNA^{Cys}, a complex reminiscent of the transamidosome 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^{Cys} synthesis pathways in *M. mazei*. Both *M. mazei* and *M. barkeri* possess genes encoding three distinct isoacceptors of tRNA^{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^{Cys} species as substrates for either CysRS or the combined SepRS/SepCysS pathway, we show that each enzyme prefers a distinct tRNA^{Cys} isoacceptor or pair of isoacceptors and that the nucleotides determining CysRS *versus* SepRS selectivity among tRNA^{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^{Cys} isoacceptor preferences of SepRS and SepCysS are identical. This supports the notion that tRNA^{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*-adenosylmethione-dependent Trm5 enzyme, consistent with a similar dependence found for SepRS (11, 13). Since *S*-adenosylmethione is synthesized from methionine (14), the tRNA^{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 NdeI 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-β-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 resuspended in a buffer containing 50 mM NaH_2PO_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_2PO_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_2PO_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_2PO_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_2PO_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_2PO_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^{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$_1^{Cys}$ and

$tRNA_3^{Cys}$ genes were as follows: $tRNA_1^{Cys}$, 5′-AATTCCTGCCGTAATACGACTCACTATAGCCAAGGTGGCGGAGCGGTCACGCAATCGC-3′ and 5′-mUmGGAGCCAAGGTCCGGATTTGAACCAGGACTGAATCGCTCTGCTGGCGATTGCGT-3′; $tRNA_3^{Cys}$, 5′-AATTCCTGCAGTAATACGACTCACTATAGCCAAGGTGGCGGAGCGGCTACGCAATCGCCT-3′ and 5′-mUmGGAGCCAAGGTCCGGATTCGAACCGGAATGGTATCGCTCTGCAGGCGATTGC-3′, where 2′-*O*-methyl nucleotides are represented by mU and mG, the underlined portions represent the overlap region, and boldface indicates the T7 RNA polymerase promoter. Oligonucleotides used to construct the $tRNA_2^{Cys}$ template are listed in the accompanying paper (13). To remove unmethylated transcripts, the 16-mer oligodeoxynucleotides complementary to the tRNA sequence flanking nucleotide G37 were as follows: $tRNA_1^{Cys}$, 5′-mUmGmAmAmUmCmGmCTCTGmCmUmGmG-3′; $tRNA_2^{Cys}$ and $tRNA_3^{Cys}$, 5′-mGmGmUmAmUmCmGmCTCTGmCmAmGmG-3′ (where "m" indicates a 2′-*O*-methyl ribose modification). Individual $tRNA^{Cys}$ isoacceptors are depicted in Fig. 1*B*.

Steady-state Aminoacylation Kinetics—SepRS plateau aminoacylation levels and assays to determine steady-state kinetic parameters were carried out using tRNA ^{32}P-labeled at the 3′-internucleotide linkage, using the methodology reported in the accompanying paper (13, 17). Measurements of aminoacylation by CysRS with all $tRNA^{Cys}$ species were performed with a conventional assay that tracks incorporation of ^{35}S-labeled cysteine into tRNA, as described (18). Saturation for tRNA was not observed in any CysRS reaction, limiting the analysis to determination of k_{cat}/K_m. k_{cat}/K_m was derived by fitting initial velocities to the following linear relation,

$$(V/(S))/[E]_t = k_{cat}/K_m \qquad \text{(Eq. 1)}$$

where (v/S) is the slope of the linear fit, and $[E]_t$ is the enzyme concentration. Errors are reported in Tables 1–3 as the mean and S.D. values of at least two measurements.

SepCysS Activity Assay—SepCysS was assayed anaerobically under an atmosphere of 95% N_2, 5% H_2/CO_2 in an anaerobic chamber (Coy Laboratories). All enzymes, buffers, and substrates entering the chamber were first deoxygenated by five cycles of vacuuming followed by purging with argon (19). Anoxic solutions were verified by testing larger volumes with a BBL anaerobic test strip. Anoxic solutions were kept in sealed glass vials and stored at −20 °C until needed.

The Sep-$tRNA^{Cys}$ substrate needed for the assay of SepCysS was generated in one of two ways. A 2-fold excess of SepRS, usually 30–40 μM, was incubated with 10–20 μM $tRNA^{Cys}$ in a buffer containing 50 mM Tris (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 5 mM ATP, 3 mM phosphoserine, and 5 mM DTT for 8 min. ^{32}P-labeled tRNA sample mixes were prepared as described above for aminoacylation assays. Aliquots were removed for evaluation of plateau aminoacylation levels. After phosphoserylation, the reactions were diluted 2-fold in water, and concentrated sodium acetate (pH 5.0) was then added to a final concentration of 200 mM to acidify the reaction and prevent tRNA deacylation. The reaction was then extracted with phenol/chloroform/isoamyl alcohol, and the phosphoserylated tRNA was recovered by ethanol precipitation. tRNA was then resuspended in the anaerobic chamber in an anoxic solution consisting of 20 mM sodium cacodylate (pH 6.0), 5 mM $MgCl_2$. Aminoacylation levels were requantified by removing a small aliquot and digesting with P1 nuclease followed by TLC separation as described above. The aminoacylated tRNA was then stored in a sealed glass vial at −20 °C until needed.

Alternatively, Sep-$tRNA^{Cys}$ was generated in the same reaction as used for the SepCysS assay, by first performing the aminoacylation reaction with SepRS under anaerobic conditions. Excess SepCysS and sulfur donor are then added to initiate the SepCysS activity assay. In this approach, 20 μM ^{32}P-labeled $tRNA^{Cys}$ was first heated to 85 °C and slowly cooled under anaerobic conditions in the presence of 2 mM $MgCl_2$. Sep-$tRNA^{Cys}$ 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 phosphoserylation reaction was initiated by adding SepRS to 20–30 μM and incubating for 8 min at 37 °C. SepCysS was preincubated at 37 °C in a buffer containing 10 mM NaH_2PO_4 (pH 7.4), 20 mM NaCl, 2 mM $MgCl_2$, 1 mM βME, 100 μM PLP, and 50% glycerol and was then added to concentrations of 0.05–40 μM, followed immediately by sodium sulfide to a concentration of 5 mM. Typical concentrations of Sep-$tRNA^{Cys}$ used in the second reaction, after dilution with reaction components, were 5–10 μM. In reactions where Sep-$tRNA^{Cys}$ 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. Anhydrous sodium sulfide was purchased from Aldrich (product 407410) and was opened and stored in an anaerobic environment. The pH of cysteine and sodium sulfide stock solutions was adjusted to neutrality.

Sep-$tRNA^{Cys}$, Cys-$tRNA^{Cys}$, an intermediate-$tRNA^{Cys}$ (each as 3′-aminoacylated A76), and nonreacted substrate (as unmodified AMP) were separated by TLC on polyethyleneimine-cellulose plates, which were run in 1 M acetic acid with adjustment of the pH to 3.5 using concentrated ammonium hydroxide. These conditions for TLC separation were reoptimized for visualization of the Cys-AMP product and differ from those employed to characterize SepRS reactions alone (13). 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-$tRNA^{Cys}$ preparation were identical.

tRNA Binding to SepCysS—Equilibrium fluorescence titrations were performed at ambient temperature with 0.25 μM *M. mazei* SepCysS in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 5 mM βME. 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, $F_c = F_{obs} \times \text{antilog}((A_{295} + A_{350})/2)$ (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 μM. The K_d values were derived by fitting

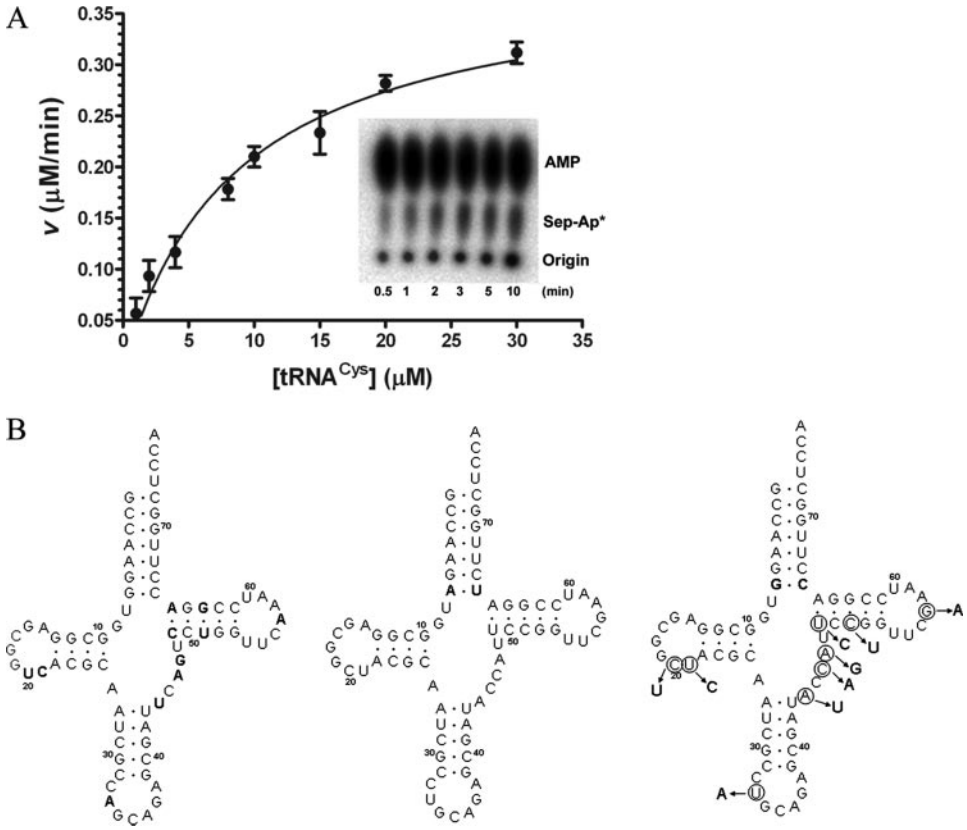

FIGURE 1. *A*, dependence of reaction rate on tRNA concentration for aminoacylation by *M. mazei* SepRS. Replot of $tRNA_1^{Cys}$ concentration *versus* initial velocities determined from time courses. The *inset* shows the imaged TLC plate of a single time course for $tRNA_1^{Cys}$. *B*, the secondary structures of *M. mazei* $tRNA^{Cys}$ isoacceptors $tRNA_1^{Cys}$, $tRNA_2^{Cys}$, and $tRNA_3^{Cys}$ are depicted from *left* to *right*. The 7-66 base pair at the bottom of the acceptor stem, which distinguishes $tRNA_2^{Cys}$ from $tRNA_3^{Cys}$, is shown in *boldface type* on the $tRNA_2^{Cys}$ cloverleaf at the center. Nucleotides distinguishing $tRNA_1^{Cys}$ (*left*) from both $tRNA_2^{Cys}$ and $tRNA_3^{Cys}$ are shown in *boldface type* on the $tRNA_1^{Cys}$ cloverleaf. Mutations of $tRNA_3^{Cys}$ (*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: $tRNA^{Cys}\Delta2$, introduction of A33 from $tRNA_1^{Cys}$ into $tRNA_3^{Cys}$; $tRNA^{Cys}\Delta3$, introduction of A57 from $tRNA_1^{Cys}$ into $tRNA_3^{Cys}$; $tRNA^{Cys}\Delta5$, introduction of both C49 and U51 from $tRNA_1^{Cys}$ into $tRNA_3^{Cys}$; $tRNA^{Cys}\Delta4$, introduction of the five D-loop and variable loop nucleotides U20, C21, U44, A46, and G47 from $tRNA_1^{Cys}$ into $tRNA_3^{Cys}$.

the data to a quadratic binding equation using *Scientist* software.

RESULTS AND DISCUSSION

Differential Utilization of $tRNA^{Cys}$ Isoacceptors by SepRS and CysRS—We chose to study Cys-$tRNA^{Cys}$ 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 $tRNA^{Cys}$ isoacceptors (Fig. 1). In contrast, *M. acetivorans*, the other *Methanosarcina* for which the genome sequence is available, possesses all three enzymes but only one $tRNA^{Cys}$ acceptor that reads both the UGU and UGC codons. Among the other sequenced methanogens, *M. maripaludis* and *Methanospirillum hungatei* possess biosynthetic apparati with identical components to *M. acetivorans*, whereas *Methanothermobacter thermautotrophicus*, *M. jannaschii*, *Methanopyrus kandleri*, and *Methanococcoides burtonii* possess only the two-step indirect pathway with a single $tRNA^{Cys}$ isoacceptor. Only the human intestinal archaea *Methanosphaera stadtmanae* and *Methanobrevibacter smithii* provide examples of methanogens possessing just the canonical CysRS with one $tRNA^{Cys}$ acceptor and lacking the SepRS/SepCysS pathway (22, 23).

Among all sequenced bacteria and archaea, the presence of three $tRNA^{Cys}$ 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 $tRNA^{Cys}$ isoacceptors. In addition to the GCA anticodon, all three *M. mazei* isoacceptors possess the U73 discriminator base typical of $tRNA^{Cys}$ species; these four nucleotides form a key part of the "identity set" for recognition by both CysRS and SepRS (10, 24). $tRNA_2^{Cys}$ and $tRNA_3^{Cys}$ 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, $tRNA_1^{Cys}$ 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 $tRNA^{Cys}$ 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 $tRNA_1^{Cys}$ isoacceptor from *M. barkeri*). $tRNA_1^{Cys}$ also differs from the other two *M. mazei* $tRNA^{Cys}$ 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 $tRNA_1^{Cys}$ 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-$tRNA^{Cys}$ 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 $^{m1}G37$ using recombinant *M. jannaschii* Trm5, and were purified from unmethylated transcripts (see "Experimental Procedures" and Refs. 11 and 13). SepRS aminoacylation

TABLE 1
Determinants of tRNA specificity in CysRS and SepRS

	SepRS				CysRS				
	k_{cat}	K_m	k_{cat}/K_m	pmol/OD	k_{cat}	K_m	k_{cat}/K_m[a]	pmol/OD	Specificity[b]
	s^{-1}	μM	$s^{-1}\ M^{-1}$		s^{-1}	μM	$s^{-1}\ M^{-1}$		
$tRNA_1^{Cys}$[c]	0.12 ± 0.01	6.4 ± 0.2	(2.0 ± 0.3) × 10^4	1250 ± 60	>0.17	>80	2070 ± 33	780 ± 50	9.5
$tRNA_2^{Cys}$[c]	0.51 ± 0.11	6.0 ± 0.8	(8.5 ± 0.9) × 10^4	1360 ± 80	>0.06	>160	380 ± 60	830 ± 70	223
$tRNA_3^{Cys}$[c]	0.58 ± 0.09	7.6 ± 0.3	(8.0 ± 0.1) × 10^4	1300 ± 80	>0.03	>140	229 ± 21	530 ± 40	143
$tRNA^{Cys}\Delta1$[d]	0.23 ± 0.02	13.1 ± 2.8	(1.6 ± 0.3) × 10^4	840 ± 40	>0.16	>90	1700 ± 57	720 ± 40	9.4
$tRNA^{Cys}\Delta2$[e]	0.52 ± 0.10	10.2 ± 1.2	(5.1 ± 0.4) × 10^4	990 ± 70	>0.05	>130	409 ± 83	580 ± 30	125
$tRNA^{Cys}\Delta3$[f]	0.30 ± 0.04	9.9 ± 0.7	(3.0 ± 0.1) × 10^4	660 ± 40	>0.09	>120	752 ± 95	600 ± 40	39.5
$tRNA^{Cys}\Delta4$[g]	0.25 ± 0.03	10.1 ± 1.5	(2.5 ± 0.3) × 10^4	890 ± 50	>0.14	>110	1250 ± 43	480 ± 20	20
$tRNA^{Cys}\Delta5$[h]	ND[i]	ND	ND	140 ± 20	ND	ND	ND	125 ± 15	ND
$tRNA^{Cys}\Delta6$[j]	0.18 ± 0.02	8.6 ± 0.6	(2.1 ± 0.3) × 10^4	900 ± 30	>0.18	>90	1950 ± 44	740 ± 50	10.8

[a] k_{cat}/K_m values were determined from initial velocities using the relation, $v = k_{cat}/K_m[E][S]$.
[b] Specificity for each tRNA is determined as the ratio, k_{cat}/K_m(SEPRS)/k_{cat}/K_m(CYSRS).
[c] Structures of the *M. mazei* isoacceptors $tRNA_1^{Cys}$, $tRNA_2^{Cys}$, and $tRNA_3^{Cys}$ are shown in Fig. 1*B*.
[d] The A33U mutation in $tRNA_1^{Cys}$.
[e] The U33A mutation in $tRNA_3^{Cys}$.
[f] The G57A mutation in $tRNA_3^{Cys}$.
[g] The C20U/U21C/A44U/C46A/A47G mutations in $tRNA_3^{Cys}$.
[h] The U49C/C51U mutations in $tRNA_3^{Cys}$.
[i] ND, not determined.
[j] The C20U/U21C/A44U/C46A/A47G/G57A mutations in $tRNA_3^{Cys}$.

kinetics was measured as described (13) (Fig. 1*A*), whereas CysRS aminoacylation kinetics was measured using a filter binding assay in which [^{35}S]cysteine is attached to tRNA (18). Preliminary plateau aminoacylation assays showed that SepRS aminoacylates each transcript to ~1300 pmol/OD (78–85% aminoacylation), whereas CysRS aminoacylates the transcripts to lower levels (33–52% aminoacylation) (Table 1). As observed for SepRS (11, 13), plateau levels for aminoacylation of all three $tRNA^{Cys}$ species by *M. mazei* CysRS were very low in the absence of methylation by Trm5 (Fig. 2).

Steady-state parameters for phosphoserylation and cysteinylation of the three isoacceptors revealed that each enzyme possesses distinct preferences. SepRS preferentially aminoacylates $tRNA_2^{Cys}$ and $tRNA_3^{Cys}$ by a factor of 4–5-fold as compared with $tRNA_1^{Cys}$, with the selectivity manifested almost entirely in k_{cat} (Table 1). In contrast, CysRS aminoacylates $tRNA_1^{Cys}$ 5–9-fold more efficiently than the other isoacceptors. We also find that SepRS is the better functioning enzyme for all isoacceptors; phosphoserylation is favored over cysteinylation by 140–220-fold for $tRNA_2^{Cys}$ and $tRNA_3^{Cys}$ and by 9.5-fold for $tRNA_1^{Cys}$. The k_{cat} of 0.5–0.6 s^{-1} toward preferred tRNAs by SepRS is comparable with many other tRNA synthetases, including the *M. jannaschii* SepRS (11), and is ~5-fold greater than reported for phosphoserylation of fully unmodified *M. jannaschii* $tRNA^{Cys}$ by *M. maripaludis* SepRS (10). The primary defect in aminoacylation by *M. mazei* CysRS is the inability to observe tRNA saturation at concentrations up to 100 μM, limiting analyses to the determination of k_{cat}/K_m. However, it is also notable that the unusual structure of $tRNA_1^{Cys}$ that apparently produces antideterminants for phosphoserylation nonetheless operates to provide positive recognition features for cysteinylation that are not present in $tRNA_2^{Cys}$ or $tRNA_3^{Cys}$. 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 $tRNA^{Cys}$ isoacceptor preferences of SepRS and CysRS, we constructed a series of tRNA mutants in which structural elements of $tRNA_1^{Cys}$ were systematically transplanted into $tRNA_3^{Cys}$ and evaluated the effectiveness of the mutants as CysRS and SepRS

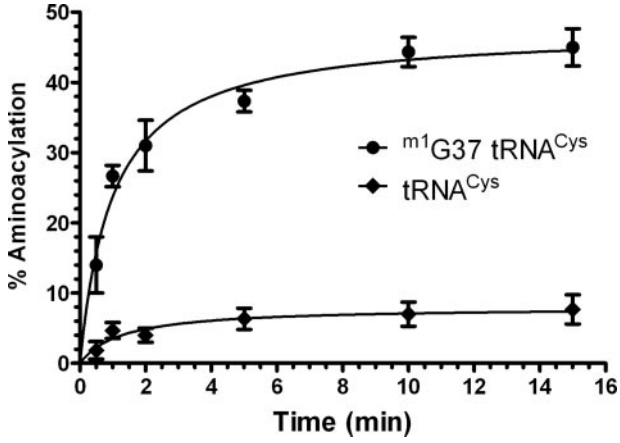

FIGURE 2. **Time course for plateau cysteinylation by *M. mazei* CysRS in the absence or presence of ^{m1}G37 in $tRNA_2^{Cys}$.** The effect of methylating $tRNA_1^{Cys}$ and $tRNA_3^{Cys}$ is nearly identical (not shown).

substrates by steady-state aminoacylation (Fig. 1 and Table 1). As observed for the three wild-type isoacceptors, all of the tRNA mutants are aminoacylated by SepRS more efficiently than by CysRS. A33 is not a distinguishing determinant, because introduction of this nucleotide into $tRNA_3^{Cys}$ does not significantly alter the relative preferences of SepRS and CysRS for that substrate ($tRNA^{Cys}\Delta2$; Table 1). (In a separate experiment, mutation of A33 to U33 in the $tRNA_1^{Cys}$ framework also does not shift enzyme preferences ($tRNA^{Cys}\Delta1$; Table 1)). Incorporation of A57 into $tRNA_3^{Cys}$ ($tRNA^{Cys}\Delta3$), however, both decreases the efficiency of phosphoserylation and increases the efficiency of cysteinylation; the SepRS/CysRS preference of this mutant is decreased from about 140- to 40-fold. Incorporation into $tRNA_3^{Cys}$ of the $tRNA_1^{Cys}$ 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 ($tRNA^{Cys}\Delta4$; Table 1). Finally, combining the $tRNA^{Cys}\Delta3$ and $tRNA^{Cys}\Delta4$ substitutions generates a mutant that fully recapitulates the SepRS/CysRS preferences of $tRNA_1^{Cys}$ ($tRNA^{Cys}\Delta6$; Table 1). Therefore,

A57 together with the five nucleotides U20/C21/U44/A46/G47 represents the distinguishing identity set (Fig. 1*B*). Interestingly, the unusual T-stem base pairs in $tRNA_1^{Cys}$ appear not to play a role in modulating the SepRS/CysRS preference. Independent introduction of these two base pairs into $tRNA_3^{Cys}$ produced a mutant that could not be efficiently aminoacylated by either enzyme ($tRNA^{Cys}\Delta5$; Table 1). This suggests that other structural elements in $tRNA_1^{Cys}$ 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 $tRNA^{Cys}$ identity elements for SepRS (10). $tRNA_2^{Cys}$ and $tRNA_3^{Cys}$ of *M. mazei* each possess all six of these nucleotides, whereas $tRNA_1^{Cys}$ is missing C20 and A47. Further, the crystal structure of the *A. fulgidus* SepRS·$tRNA^{Cys}$ 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 $tRNA^{Cys}$ K_m values of 6–8 μM measured toward SepRS are some 10-fold higher than typically observed for highly efficient cognate aminoacylation by tRNA synthetases, and the equivalent values toward CysRS are more than 100-fold higher. It is notable that the addition of the ^{m1}G37 modification, which substantially improves both initial velocities of aminoacylation and aminoacylation plateau 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-$tRNA^{Cys}$ formation by SepRS, CysRS, or both enzymes. A positive tRNA modification requirement for efficient aminoacylation 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 aminoacylation 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 ^{m1}G37 modification functions as a negative determinant to ensure aminoacylation specificity of yeast $tRNA^{Asp}$; 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 aminoacylation efficiency *in vitro*.

Embedded metabolic linkages among macromolecules responsible for synthesis of Cys-$tRNA^{Cys}$, 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-$tRNA^{Cys}$ 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-$tRNA^{Cys}$ 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 $tRNA^{Cys}$ isoacceptors among the known *Methanosarcina*. *M. acetivorans*, which retains only duplicate copies of a single $tRNA^{Cys}$ gene that is highly similar to $tRNA_2^{Cys}$ and $tRNA_3^{Cys}$ 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 $tRNA^{Cys}$; alternatively, the organism might use a weakly related *O*-phosphoserine sulfhydrylase 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 $tRNA_1^{Cys}$ 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 $tRNA_1^{Cys}$ to a function other than protein synthesis would suggest a further linkage between Cys-$tRNA^{Cys}$ synthesis and other aspects of metabolism. Although $tRNA_1^{Cys}$ is clearly an unusual tRNA, its selective aminoacylation 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 $tRNA^{Cys}$ Isoacceptor Substrate Preference of SepCysS—Rigorous characterization of the enzymatic activity of SepCysS, which catalyzes the conversion of Sep-$tRNA^{Cys}$ to Cys-$tRNA^{Cys}$, 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

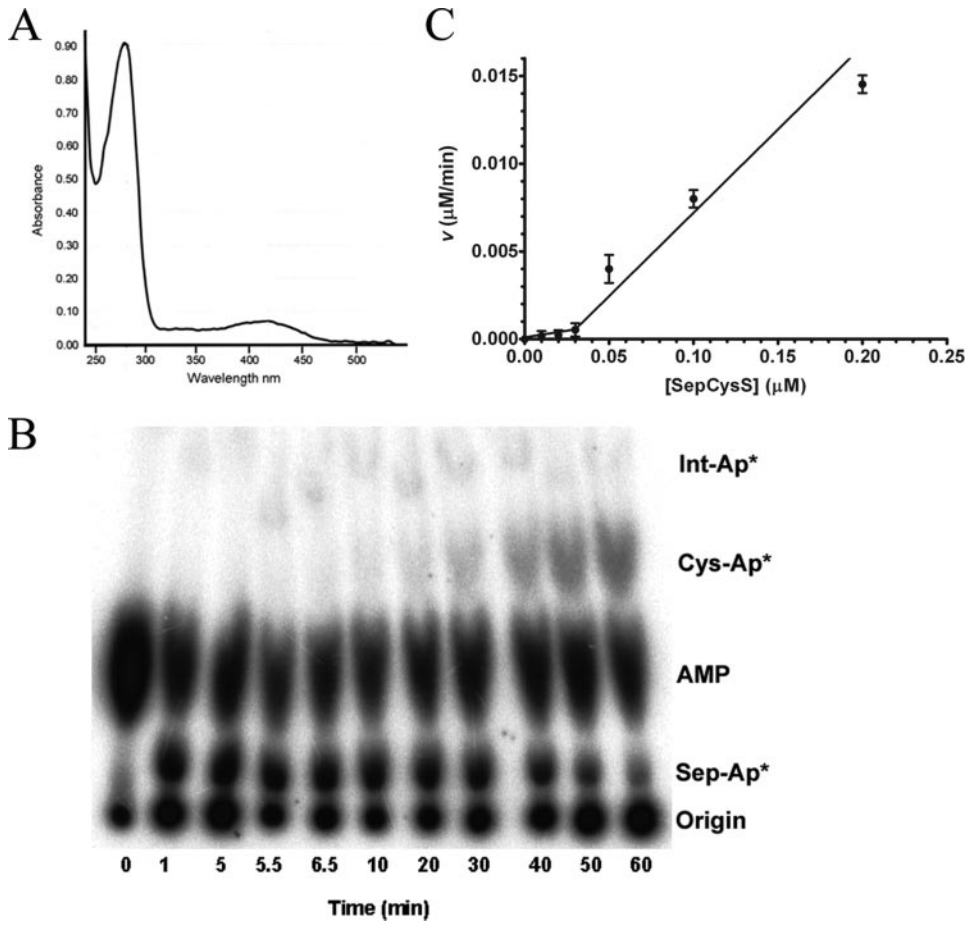

FIGURE 3. *A*, UV-visible absorbance spectrum of SepCysS revealing the presence of bound PLP in the overexpressed recombinant enzyme. *B*, imaged TLC plate showing time-dependent formation of Cys-tRNA^{Cys} catalyzed by SepCysS. Conditions were as follows: 50 mM Tris (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 5 mM ATP, 3 mM phosphoserine, 5 mM DTT, 10 μM tRNA^{Cys}, and 20 μM SepRS. After 5 min, Cys-tRNA^{Cys} formation was initiated by the addition of SepCysS to 20 μM and sodium sulfide to 5 mM. The percentage conversion to Cys-tRNA^{Cys} is determined by the ratio of the intensities for Sep-AMP and Cys-AMP and converted to molarity by multiplying by the concentration of Sep-tRNA^{Cys} determined from the plateau aminoacylation value. *C*, plot of the velocity of Cys-tRNA^{Cys} synthesis catalyzed by SepCysS *versus* enzyme concentration under steady-state conditions. No activity is observed at enzyme concentrations below 50 nM. A constant $tRNA_2^{Cys}$ concentration of 5 μM was used.

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. 3*A*) (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 aldimine linkage to Lys^{209} in both subunits (44).

We next considered how best to quantitate SepCysS activity. The only reported functional assay monitors the PLP-dependent transformation of ^{14}C-labeled phosphoserine attached to tRNA^{Cys} into ^{14}C-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 ^{32}P-labeled Sep-tRNA^{Cys} with the radiolabel positioned at the 3′-internucleotide linkage of the tRNA, can be extended to monitor the conversion of Sep-tRNA^{Cys} to Cys-tRNA^{Cys} (11, 13, 45). After the SepCysS reaction using ^{32}P-labeled Sep-tRNA^{Cys} 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. 3*B*). 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-tRNA^{Cys} may become deacylated in the processing steps between reactions.

We generated the Sep-tRNA^{Cys} 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-tRNA^{Cys} 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-tRNA^{Cys} 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-tRNA^{Cys} 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-tRNA^{Cys}. 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 ^{m1}G37 modification. Whether the added methyl group influences the activity of SepCysS is not known, because in the absence of methylation, the levels of Sep-tRNA^{Cys} that

TABLE 2
Alternative sulfur donors used by SepCysS

	Conversion to Cys-tRNA^{Cys}
	%
HS^-	72.1 ± 9.5
Thiophosphate	52.3 ± 4.5
Cysteine	55.4 ± 5.0

TABLE 3
tRNA discrimination by SepCysS

	Percentage conversion to Cys-tRNA^{Cys}	K_d	k_{cat}/K_m[a]
	%	μM	$min^{-1}\ \mu M^{-1}$
Sep-tRNA$_1^{Cys}$	52.2 ± 9.5	1.1 ± 0.2	0.009 ± 0.002
Sep-tRNA$_2^{Cys}$	75.3 ± 4.5	0.4 ± 0.15	0.014 ± 0.003
Sep-tRNA$_3^{Cys}$	72.2 ± 5.0	0.55 ± 0.18	0.012 ± 0.002
tRNA$^{Cys}\Delta 2$	72.0 ± 8.0	ND[b]	ND
tRNA$^{Cys}\Delta 4$	50.0 ± 6.0	ND	ND

[a] k_{cat}/K_m values were determined from initial velocities using the relation $v = k_{cat}/K_m[E][S]$.
[b] 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$_2^{Cys}$ as substrate, in the presence of 5 mM sodium sulfide, 5 mM thiophosphate, 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^{Cys} to Cys-tRNA^{Cys}) providing higher activity than either thiophosphate or cysteine (52–55% conversion; Table 2). Only sodium sulfide yields the correct Cys-tRNA^{Cys} 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^{Cys} isoacceptors, a 5 μM concentration of each Sep-tRNA^{Cys} 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^{Cys} was reproducibly higher for tRNA$_2^{Cys}$ and tRNA$_3^{Cys}$ (72–75%) as compared with tRNA$_1^{Cys}$ (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$^{Cys}\Delta 2$ and tRNA$^{Cys}\Delta 4$ further confirmed the SepCysS preference. tRNA$^{Cys}\Delta 2$, which is strongly preferred by SepRS, is also a good substrate for SepCysS, whereas tRNA$^{Cys}\Delta 4$, 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_d for tRNA$_2^{Cys}$ and tRNA$_3^{Cys}$ are 2–3-fold lower than K_d for tRNA$_1^{Cys}$ (Table 3).

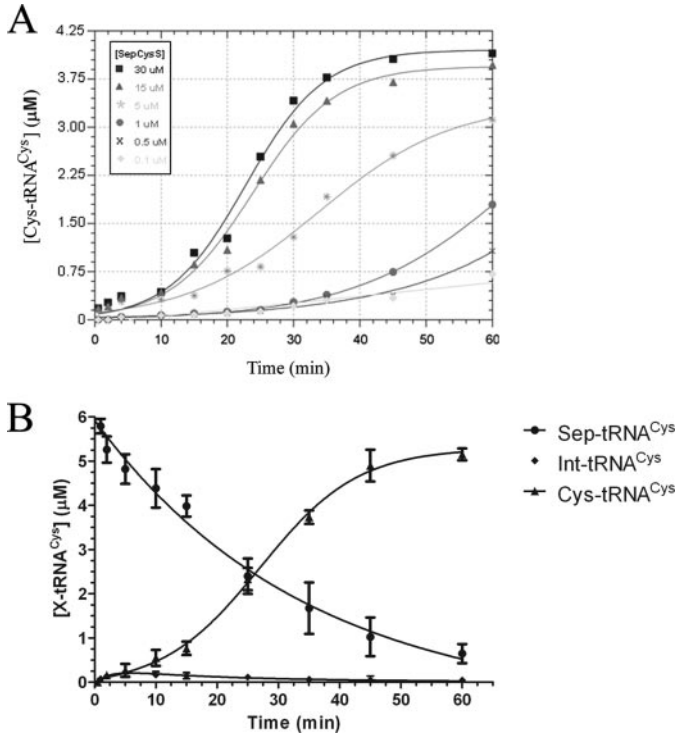

FIGURE 4. *A*, time courses of Cys-tRNA^{Cys} synthesis. Sigmoidal behavior is observed under conditions of enzyme excess. The conditions used were 0.10–30 μM SepRS as indicated by the different *symbols*, 5 mM sodium sulfide, and 5 μM Sep-tRNA^{Cys}. Error bars are omitted for clarity but are similar to those depicted in Fig. 3*C*. *B*, plot of the decrease in Sep-tRNA^{Cys} concentration together with Cys-tRNA^{Cys} formation. Quantitation of spots on the imaged TLC plate resulting in concentration of each species present was plotted against time. The conditions were as follows: 50 mM Tris (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 5 mM ATP, 3 mM phosphoserine, 5 mM DTT, 6 μM Sep-tRNA^{Cys}, and 20 μM SepCysS. A small amount of an intermediate species is observed that forms quickly and then disappears as Cys-tRNA^{Cys} formation increases.

To further characterize the properties of *M. mazei* SepCysS, we investigated the kinetics of converting Sep-tRNA^{Cys} to Cys-tRNA^{Cys}. Under steady-state conditions at 1–50 nM SepCysS, we observed no reaction when Sep-tRNA^{Cys} substrate levels were maintained below 1 μM. However, product formation was detected at 50 nM SepCysS and 5 μM Sep-tRNA^{Cys}. At this substrate concentration, reaction velocities then were found to increase with enzyme concentration (Fig. 3*C*). It is likely that this behavior arises from dissociation of the SepCysS dimer into monomers, with K_d 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^{Cys} saturation was observed under steady-state conditions of substrate excess ($[S]/[E] > 20$) and with Sep-tRNA^{Cys} concentrations up to 15 μM. However, the initial velocities allow estimation of k_{cat}/K_m at 0.009 – 0.014 $min^{-1}\ \mu M^{-1}$, 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$_2^{Cys}$ and tRNA$_3^{Cys}$ by SepCysS matches the preference of SepRS and suggests that these two species may be ded-

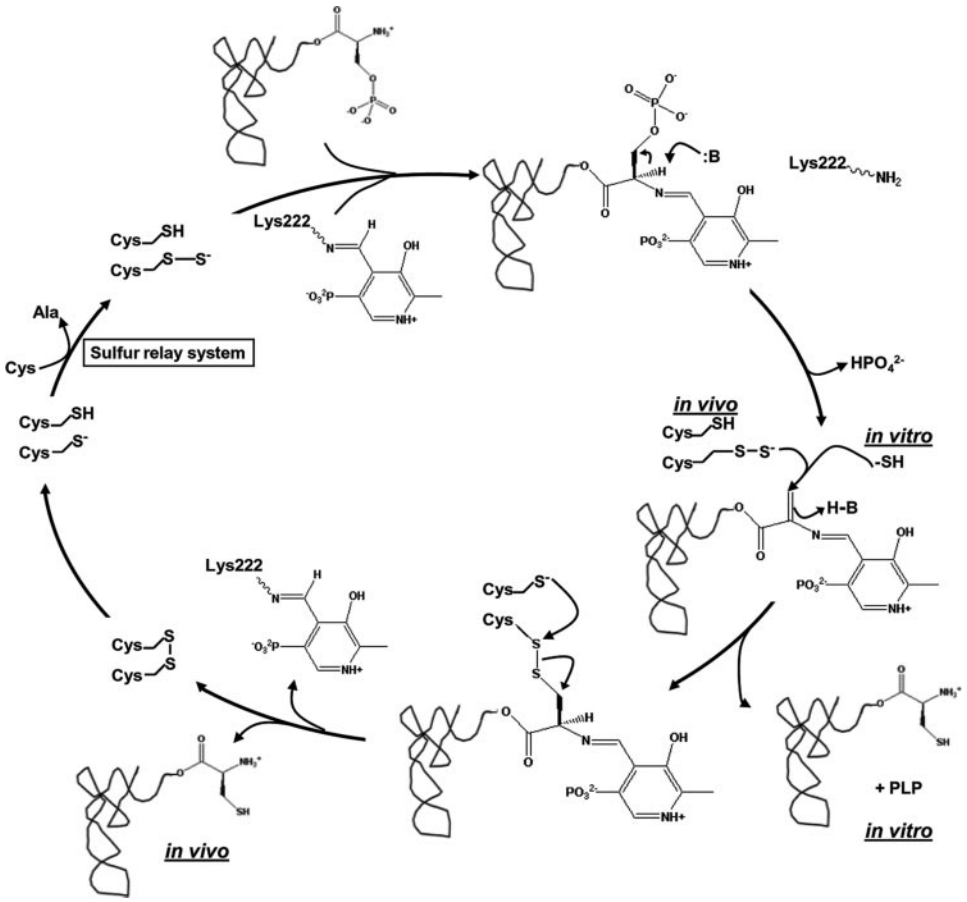

FIGURE 5. **Proposed mechanism for the synthesis of Cys-tRNA^{Cys} by SepCysS.** The mechanism is based upon a proposed sulfur relay system and on similarity to Sec-tRNA$^{[Ser]Sec}$ biosynthesis. The movement of electrons into PLP has been omitted. The inclusion of bisulfide as the sulfur donor indicates how the enzyme functions in this *in vitro* study (*bottom right*; the PLP generated would reform the internal aldimine with Lys^{222}). The identification of Lys^{222} as the likely side chain that ligates PLP is based on a structure-based sequence alignment with *A. fulgidus* SepCysS.

icated to the indirect pathway. This may represent an adaptation that serves to maximize the efficiency of Cys-tRNA^{Cys} synthesis *in vivo*. However, the SepCysS selectivity is not large and is also not detected at the level of k_{cat}/K_m. Further, our work in assay development showed that the presence of SepRS in the SepCysS reactions does not affect either the rate or plateau level of Sep-tRNA^{Cys} conversion under these experimental conditions. Nevertheless, work on the homologous *M. jannaschii* pathway showed that SepRS and SepCysS from that organism do form a binary complex that binds tRNA^{Cys} and that catalyzes the SepRS reaction with 2-fold higher k_{cat} than SepRS alone (11). Thus, a modest enhancement in substrate throughput may be a common characteristic of the two-step indirect pathway in methanogens. In addition, a further important role for the formation of the SepRS-SepCysS-tRNA^{Cys} ternary complex is to sequester the misacylated Sep-tRNA^{Cys} from EF-1α, thus preventing misincorporation of phosphoserine into cellular proteins (11).

Mechanism of SepCysS—The kinetic experiments performed in the presence of a molar excess of enzyme showed that the required period of incubation to achieve maximal conversion of Sep-tRNA^{Cys} to Cys-tRNA^{Cys} by SepCysS is quite long (Fig. 3*B*). However, 10–40-min time courses were required for 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-tRNA^{Sec} to Sec-tRNA^{Sec} by bacterial selenocysteine synthase when thiophosphate is employed as sulfur donor in that enzyme (47).

To further examine the kinetics of SepCysS, we measured substrate conversion while systematically varying the concentration of enzyme. At 5 μM Sep-tRNA^{Cys} and at SepCysS concentrations above 200 nM, product formation exhibits an upward slope after reactions proceed for ~30 min (Fig. 4*A*). 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-tRNA^{Cys} 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-tRNA^{Cys} formation begins to rapidly increase (Figs. 3*B* and 4*B*). It appears that formation of this intermediate is rate-limiting under single-turnover conditions.

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-tRNA^{Cys} to Cys-tRNA^{Cys} is ~500-fold lower than k_{cat}/K_m for phosphoserylation of tRNA$_2^{Cys}$ and tRNA$_3^{Cys}$ 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-tRNA^{Cys} 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^{Cys}, which would correspond to the inter-

mediate observed on TLC as aminoacrylyl-A76 (Figs. 3*B*, 4*B*, and 5). In our *in vitro* reactions, HS^- 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^{51}, Cys^{54}, and Cys^{260} 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 reaction rate here by properly positioning the sulfur for transfer in relation to the bound Sep-$tRNA^{Cys}$. Interestingly, SepCysS itself is strongly homologous to the cysteine desulfurases CsdB, NifS, and IscS, suggesting that it may function as both a sulfur recipient to form Cys-$tRNA^{Cys}$ and a sulfur donor in other, as yet unknown, reactions (8, 49). In this regard, the essentiality of the enzyme in the context of a cell deleted for SepRS may be relevant (36). The identity of the enzyme that may convert one of the SepCysS active site cysteines to a persulfide is also unknown at this time; by analogy to pathways such as the sulfuration of tRNA, it is possible that the route of sulfur delivery to SepCysS involves transfer through multiple proteins (50). After the sulfur transfer, the resulting enzyme-bound disulfide would then be reduced by reaction with another cysteine residue, releasing PLP and Cys-$tRNA^{Cys}$ (Fig. 5). This mechanism predicts essentiality for at least two of the three conserved active site cysteines. The sulfur relay system would then regenerate persulfided SepCysS for the next catalytic cycle.

Formation of Cys-$tRNA^{Cys}$ by SepCysS probably also has many elements in common with the formation of selenocysteyl-$tRNA^{Sec}$ in archaea and eukaryotes. In these domains, serylation of $tRNA^{[Ser]Sec}$ by SerRS is followed by a phosphorylation reaction leading to formation of a phosphoserylated tRNA: Sep-$tRNA^{[Ser]Sec}$. This intermediate is then converted to Sec-$tRNA^{Sec}$ by another PLP-dependent enzyme designated alternatively as selenocysteine synthase (46, 51) or SepSecS (52). This enzyme uses selenophosphate as a selenide donor and is weakly homologous to SepCysS (46). Kinetic analysis of the selenocysteine synthase reaction has shown that, as proposed for SepCysS and demonstrated for bacterial selenocysteine synthase (48), generation of Sec-$tRNA^{[Ser]Sec}$ from Sep-$tRNA^{[Ser]Sec}$ also occurs in conjunction with an intermediate that is likely to be aminoacrylyl-$tRNA^{[Ser]Sec}$ (46). Interestingly, selenocysteine synthase can also utilize phosphoseryl-$tRNA^{[Ser]Sec}$ as a substrate (46), suggesting that comparative studies among all three enzymes may be a productive approach to acquiring detailed insight into the mechanisms of these remarkable RNA-dependent amino acid modification reactions.

Perspectives—The selectivities of CysRS and SepRS for distinct $tRNA^{Cys}$ isoacceptors clearly suggest that both synthetic routes and all three tRNAs have physiological significance. The detailed nature of how the tRNAs are partitioned between pathways *in vivo*, however, and the extent to which one or more of them may play other metabolic roles remain as open questions for future research. In many simpler tRNA-synthetase systems, *in vitro* reconstitution by expression of a single enzyme, together with use of unmodified tRNA transcripts, suffices to recapitulate most biochemical properties of interest. By contrast, the Cys-$tRNA^{Cys}$ biosynthetic system in *M. mazei* illustrates how the full reconstitution of activities is likely to require multiple tRNA modifications as well as essential cofactor proteins. Identification of additional $tRNA^{Cys}$-modifying enzymes and 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 archaebacteria in the biogeochemical processes determining global climate change offers strong impetus for these experiments.

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