Ancient translation factor is essential for tRNA-dependent cysteine biosynthesis in methanogenic archaea

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Methanogenic archaea lack cysteinyl-tRNA synthetase; they synthesize Cys-tRNA and cysteine in a tRNA-dependent manner. Two enzymes are required: Phosphoseryl-tRNA synthetase (SepRS) forms phosphoseryl-tRNA^{Cys} (Sep-tRNA^{Cys}), which is converted to Cys-tRNA^{Cys} by Sep-tRNA:Cys-tRNA synthase (SepCysS). This represents the ancestral pathway of Cys biosynthesis and coding in archaea. Here we report a translation factor, SepCysE, essential for methanococcal Cys biosynthesis; its deletion in *Methanococcus maripaludis* causes Cys auxotrophy. SepCysE acts as a scaffold for SepRS and SepCysS to form a stable high-affinity complex for tRNA^{Cys} causing a 14-fold increase in the initial rate of Cys-tRNA^{Cys} formation. Based on our crystal structure (2.8-Å resolution) of a SepCysS·SepCysE complex, a SepRS·SepCysE·SepCysS structure model suggests that this ternary complex enables substrate channeling of Sep-tRNA^{Cys}. A phylogenetic analysis suggests coevolution of SepCysE with SepRS and SepCysS in the last universal common ancestral state. Our findings suggest that the tRNA-dependent Cys biosynthesis proceeds in a multienzyme complex without release of the intermediate and this mechanism may have facilitated the addition of Cys to the genetic code.

methanogen | aminoacyl-tRNA | protein complex

Translation requires a full set of aminoacyl-tRNAs (aa-tRNAs) with precisely matched amino acids and tRNAs (1). The aa-tRNAs are mainly synthesized via direct attachment of amino acids to their corresponding tRNAs by aa-tRNA synthetases (aaRSs), which specifically recognize the amino acid:tRNA pairs to maintain the fidelity of protein synthesis (2, 3). However, many methanogenic archaea lack the Cys-tRNA synthetase (CysRS) that acylates tRNA^{Cys} with Cys; instead, they rely on an indirect pathway to produce Cys-tRNA^{Cys} (4). In a two-step process, tRNA^{Cys} is initially aminoacylated with *O*-phosphoserine (Sep) by *O*-phosphoseryl-tRNA synthetase (SepRS) to form Sep-tRNA^{Cys}, which is then converted to Cys-tRNA^{Cys} by the tRNA-dependent modifying enzyme Sep-tRNA:Cys-tRNA synthase (SepCysS) with a sulfur donor (Fig. 1*A*) (4).

SepRS and SepCysS always coexist and are confined to certain archaea including most methanogens, methanotrophic archaea, and the *Archaeoglobus* and *Ferroglobus* species. Phylogenetic studies suggest that the SepRS/SepCysS pathway was the ancestral mechanism of Cys biosynthesis and coding in archaea (5). Later on, the bacterial Cys biosynthesis pathway and the class I CysRS were horizontally transferred to some archaeal lineages; this replaced the indirect pathway (5–7). The question of why some archaea preserve the SepRS/SepCysS pathway is unclear. Interestingly, the SepRS/SepCysS containing archaea have higher Cys content in their proteome (~1.3% on average) than other archaea (~0.7% on average), suggesting that the tRNA-dependent Cys biosynthesis correlates with high Cys content in archaea (8).

Both SepRS and SepCysS have been characterized. SepRS is a class II aaRS, and its consists of an α_4-tetramer (9, 10)

that binds two tRNA^{Cys} molecules in the crystal structure (10) and in solution (11). SepCysS has a similar structure to the pyridoxal-5′-phosphate–dependent Cys desulfurases (12). It is a dimer with the active site located near the dimer interface (12). Three conserved Cys residues of the active site are required for sulfur transfer (13, 14). A binary complex of SepRS and SepCysS that may promote reaction efficiency and sequester Sep-tRNA^{Cys} was proposed (15). However, formation of this complex caused both SepRS and SepCysS to dissociate from their oligomeric states into monomers (15), and thus this complex is unlikely to be physiologically active. Furthermore, addition of SepRS to the SepCysS reaction does not improve Cys-tRNA^{Cys} production (16). Here we present a translation factor, SepCysE, essential for tRNA-dependent Cys biosynthesis in methanococci. It forms a ternary complex with SepRS and SepCysS that may enable substrate channeling of Sep-tRNA^{Cys}.

Results

SepCysE Is Essential for tRNA-Dependent Cysteine Biosynthesis in *Methanococcus maripaludis*. To study the physiological interactions of proteins involved in the tRNA-dependent Cys biosynthesis pathway, the His$_6$-tagged SepCysS (MMP1240) was overexpressed in *M. maripaludis* for a pull-down experiment. Metal affinity chromatography followed by mass spectrometry analysis revealed a ~25-kDa protein (MMP1217 identified with 72% coverage) associated with SepCysS (Fig. 1*B*). We named this protein "SepCysE" (SepRS/SepCysS pathway enhancer, encoded by *scsE*). The primary sequence of SepCysE is not related to any

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Significance

Translation requires aminoacyl-tRNAs that are mainly formed by acylating tRNAs with the corresponding amino acids. Methanogenic archaea synthesize Cys-tRNA in an unusual indirect fashion. They attach a precursor amino acid, phosphoserine, to tRNA^{Cys}, which is then converted to cysteine. This study shows that the indirect Cys-tRNA formation is carried out in a multienzyme complex assembled by a translation factor. Complex formation markedly promotes reaction efficiency. Because the indirect Cys-tRNA formation is the ancestral pathway of Cys biosynthesis in archaea, this complex may represent a remnant of a primordial machinery for Cys coding.
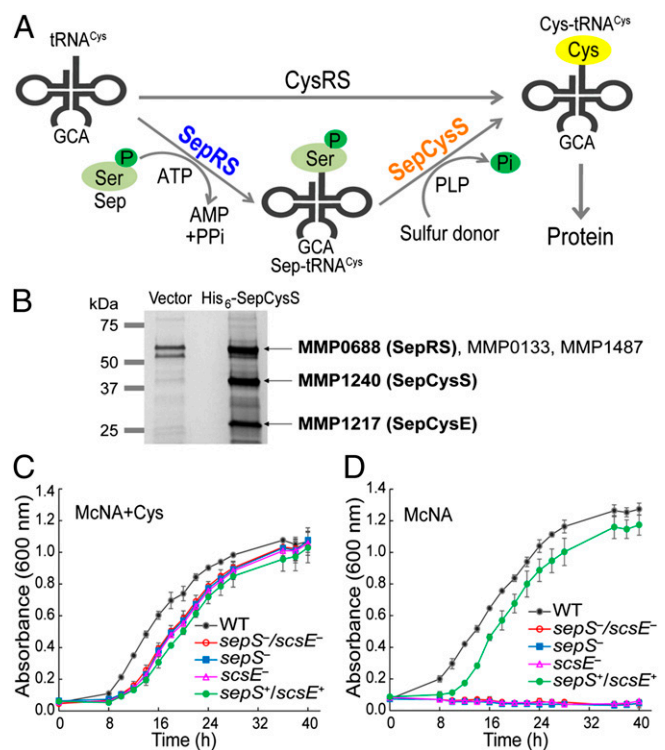
Fig. 1. SepCysE is an essential factor for tRNA-dependent Cys biosynthesis. (A) The direct and indirect pathways for Cys-tRNA^{Cys} formation. The direct pathway acylates tRNA^{Cys} with Cys by CysRS. The indirect pathway acylates tRNA^{Cys} with Sep by SepRS (encoded by *sepS*), and then Sep is converted to Cys by SepCysS (encoded by *pscS*) with a sulfur donor. (B) Pull-down of His$_6$-tagged SepCysS expressed in *M. maripaludis*. Proteins purified from 200 mL cells transformed with the empty vector (left lane) or with the vector for His$_6$-SepCysS expression (right lane) were separated by SDS/PAGE, in-gel tryptic digested, and analyzed by liquid chromatography-tandem mass spectrometry analysis. The locus tag of identified proteins (>60% coverage) only present in the right lane are labeled in bold. (C) Growth of the *M. maripaludis* strains in the defined medium (McNA) with 1 mM L-Cys. (D) Growth of the *M. maripaludis* strains in McNA without Cys. *scsE$^-$*, Δ*sepS*/Δ*scsE* mutant with SepRS expressed from a vector; *sepS$^+$/scsE$^+$*, Δ*sepS*/Δ*scsE* mutant with both SepRS and SepCysE expressed from a vector; *sepS$^-$*, Δ*sepS*/Δ*scsE* mutant with SepCysE expressed from a vector; *sepS$^-$/scsE$^-$*, Δ*sepS*/Δ*scsE* double mutant. Data are mean ± SDs from three replicate cultures.

protein of known function. Furthermore, mass spectrometry analysis also confirmed the presence of SepRS (66% coverage) in a ∼60-kDa protein band (Fig. 1B), which additionally contains two contaminating proteins of similar size (MMP0133 and MMP1487). These results indicate that SepCysS physiologically interacts with SepRS and SepCysE.

To confirm the involvement of SepCysE in cysteine biosynthesis, mutagenesis studies were performed in *M. maripaludis*. This archaeon has both the direct and indirect pathways for Cys-tRNA^{Cys} formation (Fig. 1A). However, deletion of *sepS* (encoding SepRS) causes Cys auxotrophy (4), indicating that the indirect pathway is the primary manner of de novo Cys biosynthesis. In this study we constructed a Δ*sepS*/Δ*scsE* double mutant. As expected, this mutant required Cys in the medium for growth (Fig. 1C); expression of both SepRS and SepCysE from a shuttle vector restored its growth without Cys (Fig. 1D). However, expression of either SepRS or SepCysE alone (confirmed by Western blot; Fig. S1) was unable to rescue Cys auxotrophy (Fig. 1D). These genetic results demonstrate that SepCysE is essential for the tRNA-dependent Cys biosynthesis in methanococci.

SepRS, SepCysS, and SepCysE Assemble into a Stable Ternary Complex with High Affinity for tRNA^{Cys}. The interactions between recombinant *Methanocaldococcus jannaschii* SepRS, SepCysS, and SepCysE were studied by gel filtration chromatography using a Superdex 200 size-exclusion column (GE Healthcare). SepRS and SepCysS eluted with an apparent M_r of 270,000 and 93,000, respectively (Fig. 2A), consistent with a tetrameric SepRS structure (monomeric $M_r = 66,000$) (9, 10) and a dimeric SepCysS structure (monomeric $M_r = 47,000$) (12). They appeared as separate peaks when mixed together (at concentrations up to 30 μM) before loading, suggesting that SepRS and SepCysS cannot form a stable complex under the experimental condition. On the other hand, when SepRS and SepCysE were mixed together (1:1 molar ratio), gel filtration showed a single peak corresponding to an apparent M_r of ∼400,000 (Fig. 2B). This pattern suggests that both proteins fully associated with each other in a complex composed of four SepRS and four SepCysE (monomeric $M_r = 26,000$) molecules. Similarly, the mixture of SepCysS and SepCysE (1:1 molar ratio) eluted with an apparent M_r of 170,000, matching a complex composed of two SepCysS and two SepCysE (Fig. 2B). The mixture of SepRS, SepCysS, and SepCysE (1:1:1 molar ratio) also yielded a single peak with an apparent M_r of ∼600,000 (Fig. 2C), matching a ternary complex composed of four SepRS, four SepCysE, and four SepCysS molecules.

The effect of complex formation on tRNA^{Cys} binding was measured with the *M. jannaschii* proteins by a filter-binding assay (17). SepRS and SepCysE showed similar affinities for tRNA^{Cys}, exhibiting K_D values of 0.46 ± 0.04 and 0.33 ± 0.04 μM, respectively (Fig. 2D and Fig. S2). This demonstrates that SepCysE by itself binds tRNA^{Cys}. On the other hand, SepCysS (at concentrations up to 10 μM) did not show measurable binding to tRNA^{Cys} even at a stoichiometry of 2,000 molecules per tRNA. The formation of binary complexes with SepCysE significantly increased the affinities of both SepRS and SepCysE for tRNA^{Cys} (Fig. 2D). Furthermore, the SepRS·SepCysE·SepCysS ternary complex exhibited the strongest binding to tRNA^{Cys}, with a K_D value ∼17-fold lower than that of SepRS alone (Fig. 2D). This
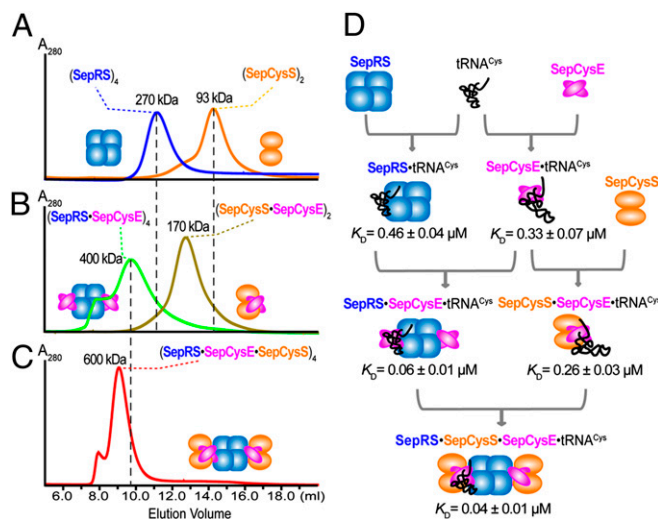

Fig. 2. The *M. jannaschii* SepRS, SepCysS, and SepCysE form a complex with high affinity for tRNA^{Cys}. (A) Gel filtration of SepRS (blue) and SepCysS (orange). (B) Gel filtration of a SepRS and SepCysE mixture (green) and a SepCysS and SepsCysE mixture (brown). (C) Gel filtration of a SepRS, SepCysS, and SepCysE mixture (red). Each protein was present at 30 μM. The shoulders of the major SepRS·SepCysE and SepRS·SepCysE·SepCysS peaks were probably due to protein aggregation under the experimental conditions. (D) Affinity of the *M. jannaschii* proteins for tRNA^{Cys}. K_D of proteins for tRNA^{Cys} binding were determined by protein titration in a filter-binding assay that quantifies free tRNA and protein·tRNA complex. Data are mean ± SDs (n = 3).

result demonstrates that formation of the SepRS·SepCysE·SepCysS complex greatly increases the protein affinity for tRNA^{Cys}.

Complex Formation Accelerates Reaction Rate. The benefit of complex formation on enzymatic reactions was investigated by three assays. First, the Sep acylation assay showed that the *M. jannaschii* SepRS only charged tRNA^{Cys} to a plateau level of ~10% (Fig. 3*A*). Steady-state kinetics showed that the K_M (of tRNA^{Cys}) and k_{cat} of this reaction (Fig. 3*B*) were comparable to the values determined for the *M. maripaludis* SepRS (18). The addition of SepCysS to the SepRS reaction did not change the activity significantly, whereas the addition of SepCysE together with SepCysS markedly improved the activity (Fig. 3*A*). The SepRS·SepCysE·SepCysS ternary complex raised the plateau level of Sep-tRNA^{Cys} production to 50%, decreased the K_M of tRNA^{Cys} by about fourfold, and increased the catalytic efficiency (k_{cat}/K_M) by ~18-fold compared with SepRS alone (Fig. 3 *A* and *B*). These results indicate that complex formation improves the substrate binding and turnover of SepRS.

Second, the stability of Sep-tRNA^{Cys} was measured in a hydrolysis protection assay. Although its half-life at pH 7.5 was ~30 min at 37 °C (Fig. S3), it was only ~5 min at 60 °C (Fig. 3*C*). This suggests that Sep-tRNA^{Cys} is unstable in hyperthermophiles, e.g., *M. jannaschii* with a growth optimum at 85 °C. When SepRS or the SepRS·SepCysE·SepCysS complex was present, no significant hydrolysis was observed for over 30 min (Fig. 3*C*). In contrast, the hydrolysis was irrespective to the presence of SepCysE, SepCysS or elongation factor 1A (EF1A) (Fig. 3*C* and Fig. S3). These results suggest that SepRS in the

SepRS·SepCysE·SepCysS complex can protect Sep-tRNA^{Cys} from deacylation before its transfer to SepCysS.

Third, the overall activity of Cys-tRNA^{Cys} production was determined with sodium sulfide as a sulfur donor. When SepRS and SepCysS were incubated with Sep, ATP, Na$_2$S, and tRNA^{Cys}, the final product Cys-tRNA^{Cys} was only formed up to 4% of the total tRNA^{Cys} (Fig. 3 *D* and *E*). The intermediate, Sep-tRNA^{Cys}, was produced as a major product (Fig. 3*D*), suggesting that Sep→Cys conversion was a rate-limiting step. However, when the SepRS·SepCysE·SepCysS ternary complex was incubated with the substrates, Sep-tRNA^{Cys} did not accumulate (Fig. 3*D*), suggesting that Sep is quickly converted to Cys in the complex. About 28% of the total tRNA^{Cys} was converted to Cys-tRNA^{Cys}, with a 14-fold faster initial velocity (0.84 ± 0.18 mol$_{Cys-tRNA}$/min·mol$_{SepCysS}^{-1}$) than that without SepCysE (0.06 ± 0.02 mol$_{Cys-tRNA}$/min·mol$_{SepCysS}^{-1}$) (Fig. 3*E*). These results suggest that complex formation markedly improves the overall production of Cys-tRNA^{Cys}. Furthermore, the presence of the *M. jannaschii* translation EF1A raised the plateau level of Cys-tRNA^{Cys} production to 38% (Fig. 3*E*), suggesting that cognate Cys-tRNA^{Cys} is protected by EF1A against deacylation.

The N-Terminal Domain of SepCysE Is Important for Complex Formation. To reveal the structural basis of protein–protein interactions, an X-ray crystal structure of the *M. jannaschii* SepCysS·SepCysE complex was determined at 2.8-Å resolution (Table S1). In this structure, SepCysS and SepCysE are arranged in an $\alpha_2\beta_2$ complex (Fig. 4*A*), consistent with the stoichiometry determined by gel filtration. The complex exhibits a twofold axis of symmetry through the dimer interface of each protein (Fig. 4*A*). The SepCysS structure in this complex is quite similar to that of the *Archaeoglobus fulgidus* SepCysS dimer (12) with an rmsd of 1.1 Å between 688 corresponding Cα atoms (Fig. S4*A*). This suggests that SepCysS did not undergo large conformational changes upon association with SepCysE. Only the C-terminal part of SepCysS (residues 295–396) moved slightly toward SepCysE (Fig. S4*A*). This region possibly interacts with the tRNA^{Cys} acceptor stem (12, 13); thus, its movement may affect tRNA binding.

Made up of 213 aa, SepCysE belongs to the Pfam family DUF2100. There is no structural homolog in the Protein Data Bank; thus it represents a previously unidentified structure. Its N-terminal domain (SepCysE_NTD, residues 37–103) is composed of antiparallel helix bundles in direct contact with SepCysS, and its C-terminal domain (SepCysE_CTD, residues 104–213) is disordered in our structure. We then determined the structure of the SepCysS·SepCysE_NTD complex at 3.0-Å resolution (Table S1). Deletion of SepCysE_CTD did not cause substantial conformational changes in either SepCysS or SepCysE_NTD (Fig. S4*B*). These results suggest that SepCysE_NTD is sufficient to form a complex with SepCysS, and that SepCysE_CTD is flexible and may work independently of SepCysE_NTD.

Two experiments confirmed the essential role of SepCysE_NTD in complex formation. First, the His-tagged N- and, C-terminal domains of SepCysE were separately expressed in *Escherichia coli* together with SepRS or SepCysS for pull-down experiments. SepCysE_NTD brought down both SepRS and SepCysS, whereas SepCysE_CTD failed to do so (Fig. 4*B*). Second, the complex formation with SepCysE_NTD was analyzed by gel filtration chromatography. SepCysE_NTD and SepCysE_CTD eluted as dimer and monomer, respectively (Fig. 4*C*). When SepCysE_NTD was mixed with both SepRS and SepCysS at a 1:1:1 molar ratio, a single peak eluted with an apparent M_r of 500,000 matching a tetramer of SepRS·SepCysE_NTD·SepCysS (Fig. 4*D*). On the other hand, SepCysE_CTD failed to form a complex with either SepRS or SepCysS (Fig. 4*E*).

Although SepCysE_NTD is sufficient to link SepRS and SepCysS into a ternary complex, two lines of evidence suggest that SepCysE_CTD is still required for other functions. First, the NTD or CTD alone did not improve the binding affinity of SepRS for tRNA^{Cys} (Fig. 4*F*). This suggests that full-length
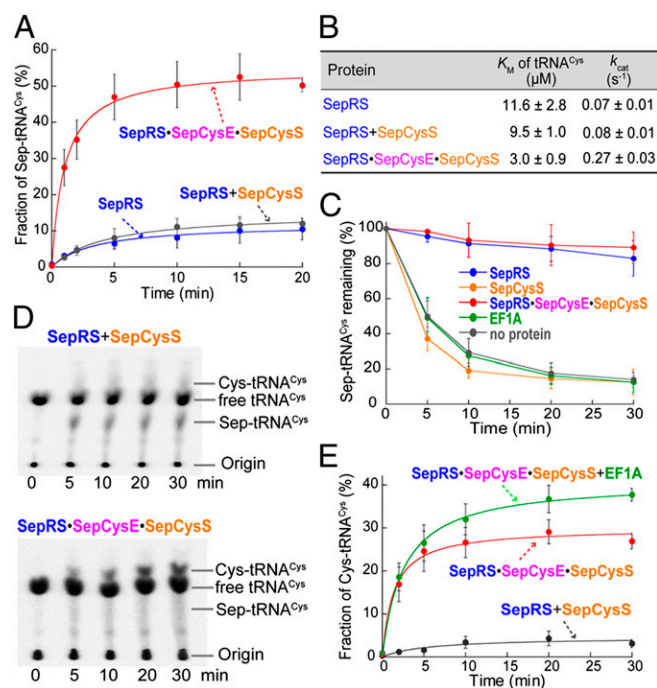

Fig. 3. Complex formation accelerates reaction rate and protects Sep-tRNA^{Cys} from deacylation. (*A*) Time course of Sep-tRNA^{Cys} formation by SepRS (blue), SepRS+SepCysS (gray), or the SepRS·SepCysE·SepCysS complex (red) determined in the Sep acylation assay. (*B*) Table summarizing steady-state kinetics of Sep-tRNA^{Cys} formation. (*C*) Protection of Sep-tRNA^{Cys} from deacylation. The ^{32}P-labeled Sep-tRNA^{Cys} (10 μM) was incubated at pH 7.5 with 10 μM each protein at 60 °C. (*D*) Representative phosphorimages of time-dependent Cys-tRNA^{Cys} formation by SepRS+SepCysS (*Upper*) and the SepRS·SepCysE·SepCysS complex (*Lower*). (*E*) Time course of Cys-tRNA^{Cys} formation by SepRS+SepCysS (gray), SepRS·SepCysE·SepCysS (red), and SepRS·SepCysE·SepCysS+EF1A (green). All enzymes are recombinant *M. jannaschii* proteins expressed and purified from *E. coli*. Data are mean ± SDs (n = 3).
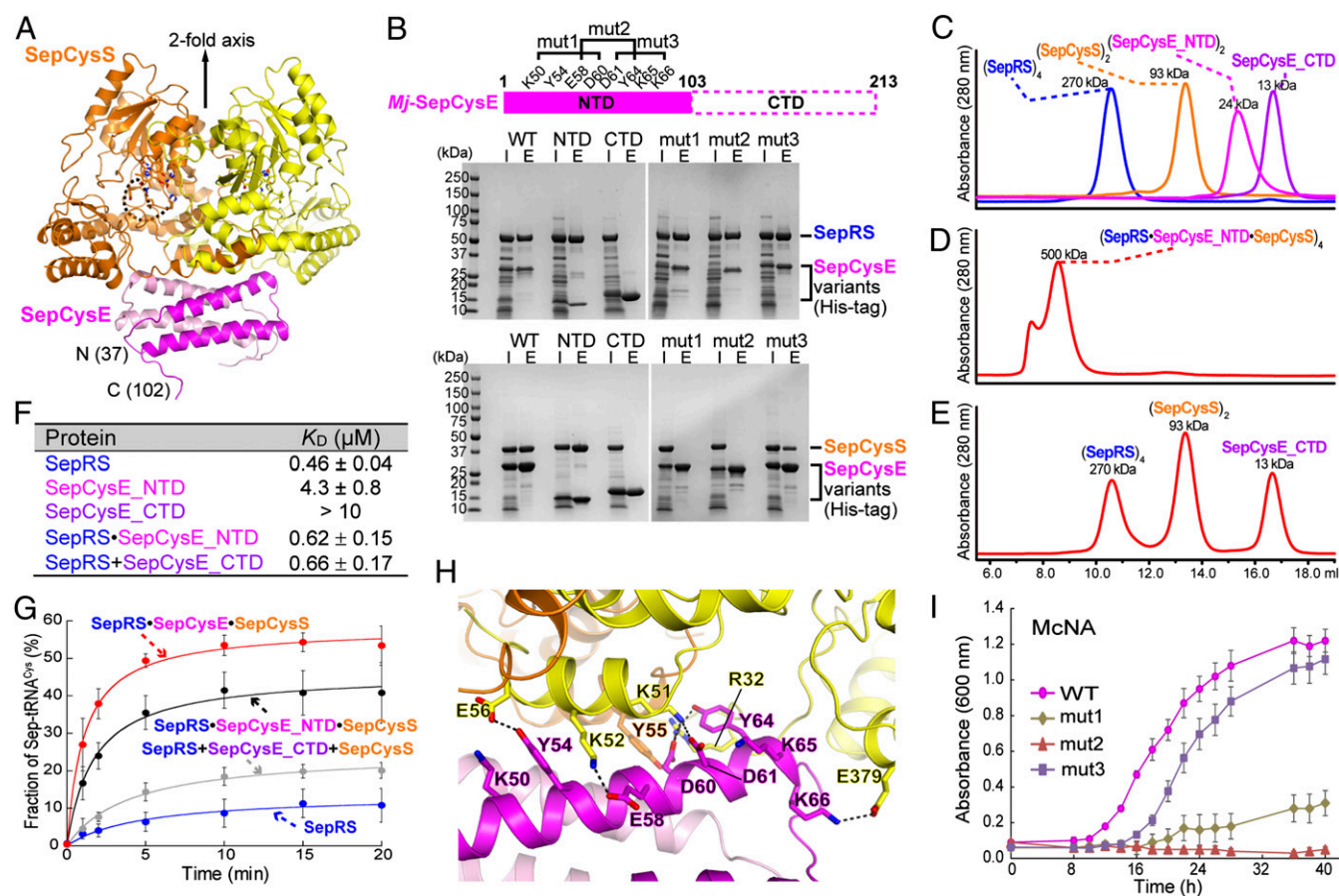
Fig. 4. Crystal structure and characterization of the *M. jannaschii* SepCysS·SepCysE complex. (*A*) Overall structure of a *M. jannaschii* (*Mj*) SepCysS·SepCysE complex. It consists of a SepCysS dimer (orange and yellow) and a SepCysE dimer (magenta and pink) arranged in an $\alpha_2\beta_2$ organization with twofold axis symmetry. The dashed line indicates a disordered loop at the SepCysS active site. (*B*) Pull-down analysis of WT and mutant *Mj*-SepCysE. CTD, *Mj*-SepCysE C-terminal domain (residues 104–213); E, elution from the nickel chromatography purification; I, input of the crude extracts after heat treatment at 70 °C; mut1, the SepCysE Lys50Ala/Tyr54Ala/Glu58Ala/Asp60Ala variant; mut2, the SepCysE Glu58Ala/Asp60Ala/Asp61Ala/Tyr64Ala variant; mut3, the SepCysE Asp61Ala/Tyr64Ala/Lys65Ala/Lys66Ala variant; NTD, *Mj*-SepCysE N-terminal domain (residues 1–103). (*C*) Gel filtration of SepRS (blue), SepCysS (orange), SepCysE_NTD (magenta), and SepCysE_CTD (purple). (*D*) Gel filtration of a SepRS, SepCysS, and SepCysE_NTD mixture. (*E*) Gel filtration of a SepRS, SepCysS, and SepCysE_CTD mixture. Each protein was present at 30 μM. (*F*) Table summarizing the affinity of the *M. jannaschii* proteins for tRNA^{Cys}. K_D of proteins for tRNA^{Cys} binding were determined in a filter-binding assay. Data are mean ± SDs (*n* = 3). (*G*) Time courses of Sep-tRNA^{Cys} formation by *M. jannaschii* SepRS (blue), SepRS·SepCysE·SepCysS (red), SepRS·SepCysE_NTD·SepCysS (black), and SepRS+SepCysE_CTD+SepCysS (gray) determined in the Sep acylation assay. Data are mean ± SDs (*n* = 3). (*H*) Interactions of SepCysS and SepCysE via hydrogen bonds. (*I*) Growth of the *M. maripaludis* Δ*sepRS*/Δ*scsE* strain expressing SepRS together with the SepCysE variants (WT, mut1, mut2, or mut3) in the McNA (Cys-free) medium. Data are mean ± SDs from three replicate cultures.

SepCysE is required for improved tRNA binding. Second, the addition of either NTD or CTD moderately increased the Sep acylation activity, but to a lower level than full-length SepCysE (Fig. 4*G*). This suggests that the NTD and CTD are both required for maximal enhancement of aminoacylation activity.

Conserved Residues Coordinate Interactions of SepCysS and SepCysE. The SepCysS·SepCysE protein–protein interface revealed a basic region of SepCysS to interact with an acidic region of SepCysE (Fig. S4*C*), forming a hydrogen-bond network (Fig. 4*H*). Chiefly, the negatively charged, highly conserved Glu58, Asp60, and Asp61 residues in SepCysE form H bonds with the conserved SepCysS residues Arg32, Lys51, and Lys52 (Fig. S5). Two experiments confirmed the importance of these residues for complex formation. First, three His-tagged SepCysE variants (mut1:Lys50Ala/Tyr54Ala/Glu58Ala/Asp60Ala, mut2:Glu58Ala/Asp60Ala/Asp61Ala/Tyr64Ala, and mut3:Asp61Ala/Tyr64Ala/Lys65Ala/Lys66Ala) were expressed in *E. coli*, and their ability to form a complex with SepRS or SepCysS was examined by pull-down experiments (Fig. 4*B*). Although all three variants associated with SepRS, SepCysE mut1, and SepCysE mut2 did not bring down SepCysS, this indicates that SepCysE residues

Glu58, Asp60, and Asp61 are important for interaction with SepCysS but dispensable for interaction with SepRS. Second, expression of SepCysE mut1 or SepCysE mut2 together with SepRS in the *M. maripaludis* Δ*sepRS*/Δ*scsE* mutant strain did not rescue Cys auxotrophy, whereas coexpression of SepCysE mut3 with SepRS exhibited near WT growth (Fig. 4*I*); this indicates that Glu58, Asp60, and Asp61 of SepCysE are necessary for Cys-tRNA^{Cys} production by the indirect pathway.

Discussion

SepCysE Is an Essential Translation Factor for tRNA-Dependent Cys-tRNA^{Cys} Formation. Taken together, our data demonstrate that SepCysE is an essential component of the tRNA-dependent Cys-tRNA^{Cys} biosynthetic pathway that links Cys biosynthesis with translation in methanococci. SepCysE acts as a scaffold that facilitates formation of a ~600-kDa tetrameric SepRS·SepCysE·SepCysS complex. This leads to (*i*) an increase in affinity of SepRS and SepCysS for tRNA^{Cys}, (*ii*) enhanced aminoacylation rate, (*iii*) protection of the labile Sep-tRNA^{Cys} especially at elevated temperatures, and (*iv*) improved overall efficiency of Cys-tRNA^{Cys} production.

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These advantages of complex formation are supported by a SepRS·SepCysE·SepCysS model obtained by docking the SepCysS·SepCysE complex onto the *A. fulgidus* SepRS·tRNA^{Cys} structure (10) (Fig. 5*A*). In this model, SepRS and SepCysS bind to opposite sides of tRNA^{Cys}; this allows the movement of tRNA between the two enzymes without dissociation from the complex. The active sites of SepRS and SepCysS are about 70 Å apart, a distance close enough for the phosphoserylated 3′ CCA terminus of tRNA^{Cys} to flip between these two active sites. Such a mechanism is used by the mischarged 3′ tRNA termini to move from the aminoacylation site to the editing domain in certain aaRSs (19, 20). Thus, this structural model suggests that the SepRS·SepCysE·SepCysS ternary complex facilitates substrate channeling (21–23) of the unstable Sep-tRNA^{Cys} species between the different enzymes in the complex.

Accordingly, we propose the following scheme of tRNA-dependent Cys biosynthesis (Fig. 5*B*). First, SepCysE assembles SepRS and SepCysS into a ternary complex that recognizes tRNA^{Cys}. SepRS mainly interacts with the anticodon loop and acceptor stem of tRNA^{Cys} (10), whereas SepCysE may interact with other parts of tRNA^{Cys}. Next, the 3′ CCA terminus of tRNA^{Cys} is phosphoserylated at the active site of SepRS and sequentially transferred to the active site of SepCysS. Finally, after conversion of Sep to Cys on tRNA^{Cys} with a sulfur donor, the complex releases Cys-tRNA^{Cys} that is immediately captured by EF1A and delivered to the ribosome for protein synthesis.

Complex Formation Is a Common Feature of tRNA-Dependent Amino Acid Modification. Similar to the indirect Cys-tRNA^{Cys} biosynthesis in methanogens, many bacteria and archaea lack asparaginyl-tRNA synthetase (AsnRS) and/or glutaminyl-tRNA synthetase (GlnRS) and rely on indirect pathways for asparagine and glutamine biosynthesis and aa-tRNA formation. In these processes, the nondiscriminating aaRSs (ND-aaRSs) charge tRNA^{Asn} and tRNA^{Gln} with Asp (24) and Glu (25), respectively; then they are converted to Asn-tRNA^{Asn} (24, 26) and Gln-tRNA^{Gln} (27), respectively, by amidotransferases (AdTs). The ND-aaRSs and AdTs can form complexes called transamidosomes (28), which could allow channeling of the mischarged aa-tRNAs from the ND-aaRSs to the AdTs without dissociation from the complexes (29, 30). Several different types of transamidosome and regulatory features have been discovered (31). (*i*) The Asn transamidosome in *Thermus thermophilus* requires tRNA^{Asn} as a scaffold for formation and is stable over the sequential reactions (28, 29). It enhances the aminoacylation activity, protects the mischarged Asp-tRNA^{Asn} from deacylation, and increases the overall production of Asn-tRNA^{Asn} (28). (*ii*) The Gln and Asn transamidosomes in *Helicobacter pylori* are transiently formed in the presence of tRNA^{Gln} and tRNA^{Asn}, respectively (32, 33). A protein component Hp0100 can facilitate the formation of a stable Asn transamidosome in *H. pylori* (34). (*iii*) The Gln transamidosome in *Methanothermobacter thermautotrophicus* is tRNA independent and unstable through Gln-tRNA^{Gln} formation (35). It has no major effect on the kinetics of aminoacylation or transamidation (35, 36). These varied transamidosomes are probably adaptations to different organisms' metabolism. Presumably, complex formation in indirect aa-tRNA biosynthetic pathways is selected to (*i*) improve reaction efficiency via substrate channeling and (*ii*) safeguard the genetic code against mistranslation by sequestering the mischarged aa-tRNAs. Hence, it will be interesting to investigate whether such a mechanism also applies to the tRNA-dependent Sec-tRNA^{Sec} formation.

SepRS, SepCysS, and SepCysE Coevolved. The recruitment of the SepRS·SepCysE·SepCysS ternary complex for tRNA-dependent Cys biosynthesis raises the question of whether these three proteins coevolved. SepRS and SepCysS are conserved in all methanogens except the *Methanobrevibacter* and *Methanosphaera* species. They are ancient enzymes that were possibly already present at the time of the last universal common ancestral state (5, 37). On the other hand, SepCysE is conserved in class I methanogens (the Methanobacteriales, Methanococcales, and Methanopyrales orders) but missing in class II (the Methanomicrobiales order) and class III (the Methanosarcinales order) methanogens (Fig. S6). Two lines of evidence support that SepCysE is as ancient as SepRS and SepCysS. First, the sequence-based phylogenetic pattern of SepCysE homologs is consistent with that of the ribosomal RNA and methanogenesis genes (Fig. S7*A*). Therefore, SepCysE is at least as old as the class I methanogen lineage, which evolved before other methanogens and may have appeared as the ancestral Euryarchaeota (38). Second, the ratios of evolutionary distances (RED) analysis suggests that SepRS, SepCysS, and SepCysE share a common evolutionary history. In RED analysis, the evolutionary distances (E_d) of an experimental gene are plotted against a set of control genes, and a linear plot would indicate the same evolutionary history of the experimental gene and the control genes (6, 39). The SepCysE E_d values correlate with those of SepRS and SepCysS, and the E_d of all three of these genes correlate with the average E_d of the control genes (the ribosomal protein L2P, leucyl-tRNA synthetase, and protein translocase SecY) (Fig. S7*B*), which are believed to have primarily undergone vertical evolution (6). This suggests that SepRS, SepCysS, and SepCysE were all vertically inherited and coevolved with the organismal lineages. A recent structure-based phylogenetic analysis suggests that SepRS/SepCysS predated the tRNA-independent pathway and arose as the first Cys biosynthetic route (40). In this scenario, the SepRS·SepCysE·SepCysS complex probably represents a remnant of an ancient translation apparatus that facilitated the addition Cys to the genetic code.

If SepCysE coevolved with SepRS and SepCysS, and is essential for efficient tRNA-dependent Cys biosynthesis, one may ask why only class I methanogens preserved it. We propose two thoughts: (*i*) Class II and III methanogens acquired the bacterial
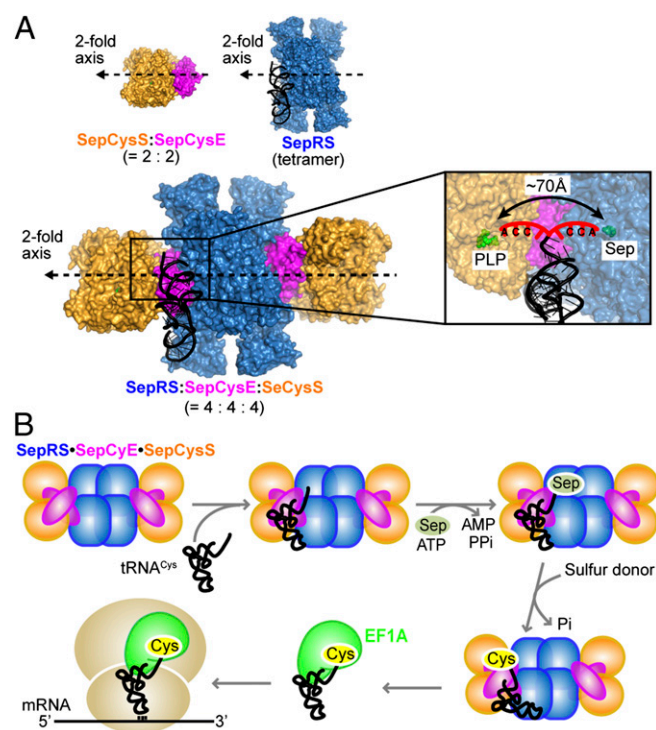

Fig. 5. Proposed scheme of tRNA^{Cys} substrate channeling. (*A*) Docking model of SepRS·SepCysE·SepCysS in complex with tRNA^{Cys}. The SepCysS·SepCysE complex (PDB ID code 3WKR) is docked onto the *A. fulgidus* SepRS·tRNA^{Cys} complex (PDB ID code 2DU3) (10) by overlapping the symmetric axis of these two complexes. SepRS is in blue, SepCysS is in orange, SepCysE is in magenta, and tRNA^{Cys} is in black with the discriminator base and the 3′ CCA terminus highlighted in red (*Inset*). (*B*) Proposed scheme of Cys-tRNA^{Cys} formation for translation in methanococci.

Cys biosynthetic pathway as well as CysRS through horizontal gene transfer (5–7). Biochemical studies have shown that the bacterial Cys biosynthesis enzymes are active in some *Methanosarcina* species (41), suggesting that they have redundant pathways for Cys biosynthesis. On the other hand, class I methanogens lack a recognizable bacterial pathway for Cys biosynthesis (Fig. S6), and therefore need to maintain a highly efficient tRNA-dependent Cys biosynthesis pathway. (*ii*) Class I methanogens have many extreme thermophiles and hyperthermophiles, and the mesophily of some methanococci is a recent adaptation (42), whereas class II and III methanogens are mostly mesophiles. Because Sep-tRNA^{Cys} is unstable at high temperatures and the formation of a SepRS·SepCysE·SepCysS complex affords protection from hydrolysis, class I methanogens—which include thermophiles—may have higher selective pressure to retain SepCysE for complex assembly.

Materials and Methods

Measurement of Protein Binding Affinity to tRNA^{Cys}. The affinity of proteins for tRNA^{Cys} was measured by a filter-binding assay (17) with modification (*SI Materials and Methods*).

Measurement of Sep-tRNA^{Cys} Formation. The activity of Sep-tRNA^{Cys} formation was measured by Wolfson assay (43) with modification (*SI Materials and Methods*). Kinetic constants were derived from plotting the initial velocity

versus [tRNA]. The plots were fitted to the Michaelis–Menten curve using KaleidaGraph 4.0 (Synergy Software).

Measurement of Cys-tRNA^{Cys} Formation. All steps of the Cys-tRNA^{Cys} formation were performed in an anaerobic chamber with an atmosphere of 95% (vol/vol) N$_2$ and 5% (vol/vol) H$_2$. The assay used 1 mM Na$_2$S as the sulfur donor and was carried out as described (16) with modification (*SI Materials and Methods*).

Crystallization and Structure Determination. The crystals of both the Sep-CysS·SepCysE and SepCysS·SepCysE_NTD complexes were obtained by sitting-drop vapor-diffusion method. Both structures were solved by molecular replacement. Additional details are in *SI Materials and Methods*. Atomic coordinates have been deposited in the Protein Data Bank (PDB) under the PDB ID codes 3WKR (SepCysS·SepCysE) and 3WKS (SepCysS·SepCysE_NTD).

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