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

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Contributed by Dieter Sölle, June 16, 2014 (sent for review April 24, 2014)

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 synthetase (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-SepCysS-SepCysE structure model suggests that this ternary complex enables substrate channeling of SepCysS. 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 aminated 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 synthetase (SepCysS) with a sulfur donor (Fig. L4) (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 structure consists of an α₀-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 His₆-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. L8). We named this protein “SepCysE” (SepRS/SepCysS pathway enhancer, encoded by scsE). The primary sequence of SepCysE is not related to any

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 multi-enzyme 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.

Author contributions: Y.L., A.N., and D.S. designed research; Y.L., A.N., Y.N., N.A., K.A.F., and M.J.H. performed research; Y.L., A.N., I.T., M.Y., and D.S. analyzed data; and Y.L., A.N., and D.S. wrote the paper.

The authors declare no conflict of interest.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org [PDB ID codes 3WKR (SepCysS-SepCysE) and 3WKS (SepCysS-SepCysE, NTD)].

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10520–10525 | PNAS | July 22, 2014 | vol. 111 | no. 29
SepRS, SepCysS, and SepCysE Assemble into a Stable Ternary Complex with High Affinity for tRNA\textsubscript{Cys}\textsuperscript{\textdagger}. 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 \( M_\text{r} \) of 270,000 and 93,000, respectively (Fig. 2A), consistent with a tetrameric SepRS structure (monomeric \( M_\text{r} = 66,000 \)) (9, 10) and a dimeric SepCysS structure (monomeric \( M_\text{r} = 47,000 \)) (12). They appeared as separate peaks when mixed together (at concentrations up to 30 \( \mu \)M) 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 \( M_\text{r} \) of \sim 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 \( M_\text{r} = 26,000 \)) molecules. Similarly, the mixture of SepCysS and SepCysE (1:1 molar ratio) eluted with an apparent \( M_\text{r} \) of 170,000, matching a complex composed of two SepRS 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 \( M_\text{r} \) of \sim 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\textsubscript{Cys} binding was measured with the \( M. \ jannaschii \) proteins by a filter-binding assay (17). SepRS and SepCysE showed similar affinities for tRNA\textsubscript{Cys} (ascribing to \( K_\text{D} \) values of 0.46 \pm 0.04 and 0.33 \pm 0.04 \( \mu \)M, respectively (Fig. 2D and Fig. S2). This demonstrates that SepCysE by itself binds tRNA\textsubscript{Cys}. On the other hand, SepCysS (at concentrations up to 10 \( \mu \)M) did not show measurable binding to tRNA\textsubscript{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 SepCysS for tRNA\textsubscript{Cys} (Fig. 2D). Furthermore, the SepRS-SepCysE-SepCysS ternary complex exhibited the strongest binding to tRNA\textsubscript{Cys}, with a \( K_\text{D} \) value \sim 17-fold lower than that of SepRS alone (Fig. 2D). This

To confirm the involvement of SepCysE in cysteine biosynthesis, mutagenesis studies were performed in \( M. \ maripaludis \). This archaea has both the direct and indirect pathways for Cys-tRNACys formation (Fig. 1A). However, deletion of \textit{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 \( \Delta \textit{sepS} \Delta \textit{scsE} \) 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.

protein of known function. Furthermore, mass spectrometry analysis also confirmed the presence of SepRS (66\% coverage) in a \sim 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.

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Fig. 1. SepCysE is an essential factor for tRNA-dependent Cys biosynthesis. (A) The direct and indirect pathways for Cys-tRNACys\textsuperscript{\textdagger} formation. The direct pathway acylates tRNA\textsubscript{Cys} with Cys by CySRS. The indirect pathway acylates tRNACys with Sep by SepRS (encoded by \textit{sepS}). Then Sep is converted to Cys by SepCyS3 (encoded by \textit{pscS}) with a sulfur donor. (B) Pull-down of His\textsubscript{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\textsubscript{6}-SepCysS (right lane) were separated by SDS/PAGE, in-gel trypic 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. \( \Delta \textit{sepS} \Delta \textit{scsE} \) mutant with SepRS expressed from a vector; \( \textit{sepS} \Delta \textit{scsE} \). \( \Delta \textit{sepS} \Delta \textit{scsE} \) mutant with both SepRS and SepCysE expressed from a vector; \( \textit{sepS} \Delta \textit{scsE} \). \( \Delta \textit{sepS} \Delta \textit{scsE} \) mutant with SepCysE expressed from a vector; \( \textit{sepS} \Delta \textit{scsE} \). SepCysS and SepCysE double mutant. Data are mean \pm 3 SDs from three replicative cultures.

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

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result demonstrates that formation of the SepRS-SepCysE-SepCysS complex greatly increases the protein affinity for tRNA\(^{\text{Cys}}\).

**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\(^{\text{Cys}}\) to a plateau level of ~10% (Fig. 3A). Steady-state kinetics showed that the *K_\text{M* of Sep-tRNACys and *k_\text{cat* of this reaction (Fig. 3B) 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. 3A). The SepRS-SepCysE-SepCysS ternary complex raised the plateau level of Sep-tRNA\(^{\text{Cys}}\) production to 50%, decreased the *K_\text{M* of tRNA\(^{\text{Cys}}\) by about fourfold, and increased the catalytic efficiency (*k_\text{cat}/K_\text{M*)) by ~18-fold compared with SepRS alone (Fig. 3A and B). These results indicate that complex formation improves the substrate binding and turnover of SepRS.

Second, the stability of Sep-tRNA\(^{\text{Cys}}\) 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. 3C). This suggests that Sep-tRNA\(^{\text{Cys}}\) 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. 3C). In contrast, the hydrolysis was irrespective to the presence of SepCysE, SepCysS or elongation factor 1A (EF1A) (Fig. 3C and Fig. S3). These results suggest that SepRS in the SepRS-SepCysE-SepCysS complex can protect Sep-tRNA\(^{\text{Cys}}\) from deacylation before its transfer to SepCysS.

Third, the overall activity of Cys-tRNA\(^{\text{Cys}}\) production was determined with sodium sulfide as a sulfur donor. When SepRS and SepCysS were incubated with Sep, ATP, Na\(_2\)S, and tRNA\(^{\text{Cys}}\), the final product Cys-tRNA\(^{\text{Cys}}\) was only formed up to 4% of the total tRNA\(^{\text{Cys}}\) (Fig. 3 D and E). The intermediate, Sep-tRNA\(^{\text{Cys}}\), was produced as a major product (Fig. 3D), 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\(^{\text{Cys}}\) did not accumulate (Fig. 3D), suggesting that Sep is quickly converted to Cys in the complex. About 28% of the total tRNA\(^{\text{Cys}}\) was converted to Cys-tRNA\(^{\text{Cys}}\), with a 14-fold faster initial velocity (0.84 ± 0.18 molCys-tRNA\(^{\text{Cys}}\)/min·molSepCysS) than that without SepCysE (0.06 ± 0.02 molCys-tRNA\(^{\text{Cys}}\)/min·molSepCysS) (Fig. 3E). These results suggest that complex formation markedly improves the overall production of Cys-tRNA\(^{\text{Cys}}\). Furthermore, the presence of the *M. jannaschii* translation EF1A raised the plateau level of Cys-tRNA\(^{\text{Cys}}\) production to 38% (Fig. 3E), suggesting that cognate Cys-tRNA\(^{\text{Cys}}\) 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* SepCysE-SepCysS complex was determined at 2.6-Å resolution (Table S1). In this structure, SepCysS and SepCysE are arranged in an αβ\(_2\) complex (Fig. 4A), consistent with the stoichiometry determined by gel filtration. The complex exhibits a twofold axis of symmetry through the dimer interface of each protein (Fig. 4A). 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. S4A). This suggests that SepCysS did not undergo large conformational changes upon association with SepCysE. Only the C-terminal part (residues 295–390) moved slightly toward SepCysE (Fig. S4A). This region possibly interacts with the tRNA\(^{\text{Cys}}\) 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\(_{\text{NDT}}\), residues 37–103) is composed of antiparallel helix bundles in direct contact with SepCysS, and its C-terminal domain (SepCysE\(_{\text{CTD}}\), residues 104–213) is disordered in our structure. We then determined the structure of the SepCysS-SepCysE\(_{\text{NDT}}\) complex at 3.0-Å resolution (Table S1). Deletion of SepCysE\(_{\text{CTD}}\) did not cause substantial conformational changes in either SepCysS or SepCysE\(_{\text{NDT}}\) (Fig. S4B). These results suggest that SepCysE\(_{\text{NDT}}\) is sufficient to form a complex with SepCysS, and that SepCysE\(_{\text{CTD}}\) is flexible and may work independently of SepCysE\(_{\text{NDT}}\). Two experiments confirmed the essential role of SepCysE\(_{\text{NDT}}\) 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\(_{\text{NDT}}\) brought down both SepRS and SepCysS, whereas SepCysE\(_{\text{CTD}}\) failed to do so (Fig. 4B). Second, the complex formation with SepCysE\(_{\text{NDT}}\) was analyzed by gel filtration chromatography. SepCysE\(_{\text{NDT}}\) and SepCysS\(_{\text{CTD}}\) eluted as a dimer and monomer, respectively (Fig. 4C). When SepCysE\(_{\text{NDT}}\) was mixed with both SepRS and SepCysS at a 1:1:1 molar ratio, a single peak eluted with an apparent *M* of 500,000 matching a tetramer of SepRS-SepCysE\(_{\text{NDT}}\)-SepCysS (Fig. 4D). On the other hand, SepCysE\(_{\text{CTD}}\) failed to form a complex with either SepRS or SepCysS (Fig. 4E).

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

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For further details and experimental methods, please refer to the original paper. The image Caption 3 refers to the following content:

**Fig. 3.** Complex formation accelerates reaction rate and protects Sep-tRNA\(^{\text{Cys}}\) from deacylation. (A) Time course of Sep-tRNA\(^{\text{Cys}}\) 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\(^{\text{Cys}}\) formation. (C) Protection of Sep-tRNA\(^{\text{Cys}}\) from deacylation. The \(32^P\)-labeled Sep-tRNA\(^{\text{Cys}}\) (10 µM) was incubated at pH 7.5 with 10 µM each protein at 60 °C. (D) Representative phosphorimages of time-dependent Cys-tRNA\(^{\text{Cys}}\) formation by SepRS-SepCysS (Upper) and the SepRS-SepCysE-SepCysS complex (Lower). (E) Time course of Cys-tRNA\(^{\text{Cys}}\) 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).
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. 4G). This suggests that the NTD and CTD are both required for maximal enhancement of aminocacylation 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. 4C), forming a hydrogen-bond network (Fig. 4H). Chiefly, the negatively charged, highly conserved Glu58, Asp60, and Asp61 residues in SepCysE form 11 bonds with the conserved SepCysS residues Arg32, Lys51, and Lys52 (Fig. 5S). 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 M. jannaschii ΔsepCysE mut1 or SepCysE mut2 together with SepRS in the McNA (Cys-free) medium. Data are mean ± SDs (n = 3). Second, expression of SepCysE mut1 or SepCysE mut2 together with SepRS in the M. maripaludis ΔsepRS/ΔscsE mutant strain did not rescue Cys auxotrophy, whereas coexpression of SepCysE mut3 with SepRS exhibited near WT growth (Fig. 4I); this indicates that Glu58, Asp60, and Asp61 of SepCysE are necessary for Cys-tRNA<sup>Cys</sup><sup>+</sup> production by the indirect pathway.

Discussion

**SepCysE Is an Essential Translation Factor for tRNA-Dependent Cys-tRNA<sup>Cys</sup> Formation.** Taken together, our data demonstrate that SepCysE is an essential component of the tRNA-dependent Cys-tRNA<sup>Cys</sup><sup>+</sup> biosynthetic pathway that links Cys biosynthesis with tRNA<sup>Cys</sup> production 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<sup>Cys</sup><sup>+</sup>, (ii) enhanced aminocacylation rate, (iii) protection of the labile Sep-tRNA<sup>Cys</sup><sup>+</sup> especially at elevated temperatures, and (iv) improved overall efficiency of Cys-tRNA<sup>Cys</sup><sup>+</sup> production.
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\(^{35}\) structure (10) (Fig. S4). In this model, SepRS and SepCysS bind to opposite sides of tRNA\(^{35}\), 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\(^{35}\) 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\(^{35}\) species between the different enzymes in the complex.

Accordingly, we propose the following scheme of tRNA-dependent Cys biosynthesis (Fig. 5B). First, SepCysE assembles SepRS and SepCysS into a ternary complex that recognizes tRNA\(^{35}\). SepRS mainly interacts with the anticodon loop and acceptor stem of tRNA\(^{35}\) (10), whereas SepCysE may interact with other parts of tRNA\(^{35}\). Next, the 3′ CCA terminus of tRNA\(^{35}\) 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 tRNACys with a sulfur donor, the complex releases Cys-tRNACys 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\(^{35}\) biosynthesis in methanogens, many bacteria and archaea lack asparaginyl-tRNA synthetase (AsnRS) and/or glutaminyl-tRNA synthetase (GlnRS) 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 Gln (25), respectively; 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 Hpo1001 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 GlntRNA\(^{Gln}\) 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, SepCysE, and SepCysS 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 Methanopyrus kandleri and Methanobrevibacter \(\times\) novacutii, and Methanopyrales orders but missing in class II (the Methanoarchaea) order (37). On the other hand, SepCysE is conserved in class I methanogens (the Methanobacteriales, Methanococcales, and Methanopyrales orders) but missing in class II (the Methanosarcinales order) and class III (the Methanorhabdaceae 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). 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 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. S7B), 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
Cys biosynthetic pathway as well as CyRS through horizontal gene transfer (5–7). Biochemical studies have shown that the bacterial Cys biosynthesis enzymes are active in some Methanococccaceae 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 RNA-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 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

The affinity of proteins for tRNA 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 a filter-binding 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) N2 and 5% (vol/vol) H2. The assay used 1 mM Na2S as the sulfur donor and was carried out as described (16) with modification (SI Materials and Methods).

Crytallization and Structure Determination

The crystals of both SepCysE-SepCysS and SepCysE-SepCysS-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 (SepCysE-SepCysS) and 3WKS (SepCysE-SepCysS-NTD).

ACKNOWLEDGMENTS. We thank Drs. William B. Whitman, Jiqiang Ling, Xiao-Long Zhou, and Patrick D’Oleughoe for insightful discussions. For assistance with data collection, we thank the Photon Factory beam line staff. This work was supported by National Institute of General Medical Sciences Grant GM22854 (to D.S.); Department of Energy Office of Basic Energy Sciences Grant DE-FG02-89ER20231 (to D.S. for the genetic aspects of this study); and the Platform for Drug Discovery, Informatics, and Structural Life Science from the Ministry of Education, Culture, Sports, Science and Technology, Japan (T.I.). A.N. was a Japan Society for the Promotion of Science Postdoctoral Fellow for Research Abroad.
Methanococcus maripaludis

The affinity of sla pac (4). The re-

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gene sepS host proteins were denatured at 70 °C for

+ Δ

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cassette was removed by transforming

that was constructed for expression of N-ter-

Δ

(MJ1481) gene with a C-

(MJ1678) genes with an N-terminal His

cells per

tRNA

pscS

Rosetta 2(DE3) strain (Nova-

was generated in the markerless

M. maripaludis

for 30 min at 4° C, and

dna5

for

5 mL culture. Before inoculation, 3 mM sodium sulfide was

5 mL McC (rich medium) or McNA (minimal

was cloned into the vector pMEV2 (6) and transformed into the

strain. The genotype of the

(M. maripaludis

with a puromycin resistance (hpt

was obtained by transformation of S761 with pMEV2-

mmp0688-mmp1217 (Clontech). Proteins bound to the column were eluted with 10 mL

membrane and an underlying Hybond-N

supporting Information

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SI Materials and Methods

Media and Culture Conditions for Methanococcus maripaludis Growth. M. maripaludis was grown at 37 °C in 28-mL aluminum

seal tubes with 5 mL McC (rich medium) or McNA (minimal

medium, 10 mM sodium acetate, 1 mM l-Ala) (1) reduced with 3 mM 2-mercaptoethanesulfonate. Tubes were pressurized with

275 kPa of H₂:CO₂ (4:1, vol/vol). The 1.5-L cultures were grown in 2-L bottles with 0.2 M sodium formate buffered with 0.2 M
glycylglycine (pH 7.0). Purumycin (2.5 µg/mL) and neomycin

(0.5 mg/mL in plates and 1 mg/mL in broth) were added when needed. When comparing the growth of the WT and mutants,

antibiotics were omitted and the inoculum size was ~10⁴ cells per

5 mL culture. Before inoculation, 3 mM sodium sulfide was

added as the sulfur source.

Mutagenesis of M. maripaludis. The replacement of the sepS gene

(mmp0688), encoding O-phosphoseryl-tRNA synthetase (SepRS),

with a puromycin resistance (pac) cassette (2) was generated in the

M. maripaludis Δhpt strain Mm900 (3) by homologous re-

combination. Then the pac cassette was removed by transforming the ΔsepS:pac strain with the plasmid pHLP, which encodes the

Fp recombinase under the control of the hmvA promoter (4). Transformants were grown for 5–10 consecutive passages and

screened by PCR for the removal of pac (4). The re-

placement of the sscE gene (mmp1217), encoding the SepRSPac-tRNA:Cs-tRNA synthase (SepCysS) pathway enhancer

(SepCysE), with pac was generated in the markerless ΔsepS

strain. The genotype of the ΔsepS/ΔsscE:pac strain (S761) was confirmed by Southern hybridization. The sscE

strain (S762) was obtained by transformation of S761 with pMEV2-mmp0688 that was constructed for expression of N-terminal Hisc-tagged

SepRS under the control of the hmvA promoter (4). The expression of Hisc-tagged SepRS and

SepCysE under the control of the

hmvA promoter, respectively. The expression of Hisc-tagged SepRS and

SepCysE was confirmed by Western blot using an HRP-con-

jugated anti-His antibody (Thermo Fisher). The mutations of

mmp0688-mmp1217 (mut1), E58A/D60A/D61A/Y64A (mut2), and D61A/Y64A/K65A/

MMP1217

—

mut3)—were constructed with the plasmid pMEV2-

mmp0688-mmp1217 using a QuikChange mutagenesis kit (Agilent Technologies) and then transformed into S761.

Pull-Down Assay and Protein Identification by Mass Spectrometry. The N-terminal Hisc-tagged pscS (mmp1240), encoding SepCysS,

was cloned into the vector pMEV2 (6) and transformed into the M. maripaludis WT strain S2 using the PEG method (7). The metal

affinity purification was performed in an anaerobic chamber with an atmosphere of 95% (vol/vol) N₂ and 5% (vol/vol) H₂. Cells

harvested from 1.5 L cultures were resuspended in 10 mL binding buffer [20 mM sodium 2-[4-(2-hydroxyethyl)piperezin-1-yl]ethanesulfonic acid (Hepes), 0.1 M NaCl, 5 mM MgCl₂, 20 mM imidazole, pH 7.5], and then disrupted by twice freezing (~80 °C) and thawing. DNA and RNA were digested with 10 U of Benzonase Nuclease (Sigma). The cell lysate was centrifuged at 100,000 × g for 30 min at 4 °C, and the supernatant was applied to 1 mL TALON Metal Affinity Resin (Clontech). Proteins bound to the column were eluted with 10 mL

equilution buffer [20 mM sodium Hepes (pH 7.5), 0.1 M NaCl, 5 mM

MgCl₂, 0.2 M imidazole]. The purified proteins were separated by SDS/PAGE and stained with silver. The protein bands were excised, in-gel digested with trypsin, and analyzed by liquid chromatogra-

phy mass spectrometry at the Keck Mass Spectrometry and Proteomics Resource Laboratory (Yale University).

Purification of SepRS, SepCysS, and SepCysE for Gel Filtration and Biochemical Assays. The Methanococcalesococcus jannaschii sepS

(MJ1660) and pscS (MJ1678) genes with an N-terminal Hisc-tag were cloned into the pRSF and pET15b (Novagen) vectors, respectively. The M. jannaschii sscE (MJ1481) gene with a C-
terminal Hisc-tag was cloned into the pDCH vector that is derived from the pCDF-Duet vector (Novagen).

For protein expression, the expression vectors were transformed into the Escherichia coli Rosetta 2(DE3) strain (Novagen). The transformants were grown in 1 L LB medium at 37 °C with shaking until they reached an absorbance at 600 nm of

0.6 to ~0.8. Then 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added to induce overnight production at 25 °C. For

anaerobic protein purification, the harvested E. coli cells were transferred into the anaerobic chamber [atmosphere of 95% (vol/vol) N₂ and 5% (vol/vol) H₂] and resuspended in 10 mL

binding buffer [50 mM sodium Hepes (pH 7.5), 0.3 M NaCl, 5 mM MgCl₂, 20 mM imidazole]. Cells were disrupted by soni-
cation in the presence of 0.5 mg/mL lysozyme and 0.1 mg/mL

DNase I. E. coli host proteins were denatured at 70 °C for

30 min, and then the cell lysate was centrifuged at 100,000 × g for

30 min at 4 °C. All of the following processes were carried out at

temperature. The clarified supernatant was applied to 1 mL TALON Metal Affinity Resin (Clontech) equilibrated with the

binding buffer. Proteins bound to the column were eluted

with the elution buffer [50 mM sodium Hepes (pH 7.5), 0.3 M

NaCl, 5 mM MgCl₂, 0.2 M imidazole] and dialyzed against the

storage buffer [50 mM sodium Hepes (pH 7.5), 0.3 M NaCl, 5 mM

MgCl₂, 20% (vol/vol) glycerol]. The purified proteins were stored at ~80 °C until use.

Analytical Gel Filtration. The protein samples (10 µM of each

protein subunit in 200 µL) were loaded onto a 24-mL Superdex

200 10/300 GL column (GE Healthcare) equilibrated with the

running buffer [50 mM sodium Hepes (pH 7.5), 0.3 M NaCl, 5 mM

MgCl₂]. The protein complexes were separated by fast protein liquid chromatography at a flow rate of 0.5 mL/min. Pooled fractions were concentrated by ultrafiltration before SDS/PAGE analysis.

Preparation of Labeled tRNA⁵⁹S. The M. jannaschii tRNA⁵⁹S sub-

strate was synthesized by in vitro T7-RNAP run-off transcription as described (8). Before use, the tRNA transcripts were folded

by heating at 80 °C for 5 min, cooling down slowly to 45 °C, and

further cooling on ice in the presence of 5 mM MgCl₂. Refolded transcript was [³²P]-labeled on the 3′ terminus using the E. coli

CCA-adding enzyme and [α-³²P]ATP as described (9).

Measurement of Protein Binding Affinity to tRNA⁵⁹S. The affinity of proteins for tRNA⁵⁹S was measured by the filter-binding assay

(10), in which 5 mM [³²P]-labeled M. jannaschii tRNA⁵⁹S was in-

cubated at room temperature for 30 min with the M. jannaschii proteins ranging from 0.5 nM to 10 µM in 25 µL binding buffer

[50 mM sodium Hepes (pH 7.5), 0.3 M NaCl, 5 mM MgCl₂]. Then the reaction mixtures were filtered through a nitrocellulose

membrane and an underlying Hybond-N⁺ membrane (GE
Healthcare) with a 96-well vacuum manifold (Hybri-dot 96; Whatman Biometra). Aliquots of 7 μL from each binding reaction were spotted in triplicate and washed with 200 μL binding buffer at room temperature. The protein-tRNA complexes (bound to the nitrocellulose membrane) and the free tRNA Cys (bound to the Hybond membrane) were then exposed to imaging plates (Fuji Films), which were scanned on a Molecular Dynamics Storm 860 Phosphorimager and quantified using the ImageQuant software. The dissociation constants were calculated by fitting the data to the equation, [protein-tRNA complex] = ([total tRNA] × [protein])/(Kd + [protein]) using KaleidaGraph 4.0 (Synergy Software).

Measurement of Sep-tRNA Cys Formation. The activity of Sep-tRNA Cys formation was measured by Wolfson assay (11), in which 200 nM of the M. jannaschii enzymes (SepRS, SepCysS, or SepRS-SepCysE-SepCysS) were incubated with 10 μM 32P-labeled tRNA Cys, 1 mM O-phosphoserine, 10 mM ATP, and 27 μg/mL pyrophosphatase (Roche) in 20 μL SepRS buffer [50 mM sodium Hepes (pH 7.5), 20 mM KCl, 10 mM MgCl₂] at 60 °C for 20 min. Time points were taken by removing 2-μL aliquots from the reaction followed by quenching with 3 μL of 0.66-mg/mL pcele (Sigma) in the 250 mM sodium citrate buffer (pH 4.5). Digestion was carried out for 30 min at room temperature. Then 1-μL aliquots were spotted on the polyethylenimine-cellulose thin-layer chromatography (TLC) plates (EMD Chemicals) and developed in the running buffer containing 1 M ammonium acetate (pH 3.5) (12) for up to 120 min. The separated radioactive spots for AMP and Sep-AMP (derived from free tRNA Cys and Sep-tRNA Cys, respectively) were then exposed to imaging plates (Fuji Films), which were scanned on a Molecular Dynamics Storm 860 Phosphorimager and quantified using the ImageQuant software. The relative positions of AMP and Sep-AMP were compared with those reported (12) under the same TLC separation condition. Kinetics were determined by varying tRNA concentrations from 0.5 to 20 μM in a 30-s reaction. Kinetic constants were derived from plotting the initial velocity versus [tRNA] and fitting to the Michaelis-Menten curve using KaleidaGraph 4.0 (Synergy Software).

Sep-tRNA Cys Hydrolysis Protection Assay. The 32P-labeled tRNA Cys was acylated with Sep by the M. jannaschii SepRS-SepCysE-SepCysS complex and then purified by phenol/chloroform extraction. Hydrolysis of 10 μM Sep-tRNA Cys in the SepRS buffer [50 mM sodium Hepes (pH 7.5), 20 mM KCl, 10 mM MgCl₂] was monitored at 60 °C in the presence of 10 μM M. jannaschii SepRS, SepCysS, SepCysE, SepCysS-SepCysE, SepRS-SepCysS-SepCysS, or elongation factor 1A (EF1A). EF1A activity was activated as described (13) before addition to the protection assay solution. Time course and TLC procedures were the same as the Sep-tRNA Cys formation assay.

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) N₂ and 5% (vol/vol) H₂. The M. jannaschii SepCysS (40 μM) was preincubated with 100 μM pyridoxal-5’-phosphate and 4 mM Na₂S in 5 μL SepRS buffer at 60 °C for 10 min. Then this mixture was supplemented with 10 μM 32P-labeled tRNA Cys, 10 μM SepRS or SepRS-SepCysE, 1 mM O-phosphoserine, 10 mM ATP, and 27 μg/mL pyrophosphatase (Roche) to a final volume of 20 μL in the SepRS buffer. In variation, 10 μM M. jannaschii EF1A was added to the reaction after activation as described (13). Reactions were carried out at 60 °C for 30 min. Time course and TLC procedures were the same as the Sep-tRNA Cys formation assay. The relative positions of AMP, Sep-AMP, and Cys-AMP (derived from free tRNA Cys, Sep-tRNA Cys, and Cys-tRNA Cys, respectively) were compared with those reported (12) under the same TLC separation condition.

Preparation of the SepCysS-SepCysE Complex for Crystallization. The gene encoding the M. jannaschii SepCysS (MJ1481) was cloned into the pDCH vector derived from the pCDF-Duet vector (Novagen). The gene encoding the M. jannaschii SepCysS (MJ1678) with an N-terminal His₆-tag was cloned into the PET15b vector (Novagen). The E. coli strain B834 (DE3)-pRARE2 (Novagen) was cotransformed with these two plasmids. For protein expression, the transformants were grown in LB medium containing 50 μg/mL streptomycin, 100 μg/mL ampicillin, and 34 μg/mL chloramphenicol at 37 °C to an OD₆₀₀ of 0.6. IPTG was then added to a final concentration of 0.5 mM to induce protein expression overnight at 25 °C. Then the cells were harvested by centrifugation at 4,000 × g for 15 min at 4 °C and disrupted by sonication in the binding buffer [50 mM sodium Hepes (pH 7.5), 300 mM NaCl, 5 mM MgCl₂] with 0.5 mg/mL lysozyme and 0.1 mg/mL DNase I. The homogenate was heat treated at 75 °C for 30 min to denature E. coli host proteins, and then clarified by centrifugation at 40,000 × g for 30 min at 4 °C. The following steps were carried out at room temperature. The clarified supernatant was loaded onto a HiTrap HP column (GE Healthcare) prequilibrium with the binding buffer. The column was washed with the binding buffer plus 20 mM imidazole, and proteins were eluted with a linear gradient of 20–250 mM imidazole. Pooled fractions were loaded onto a HiTrap Heparin column (GE Healthcare) and then eluted with a linear gradient from 300 mM to 1 M NaCl. The protein fractions were then loaded onto a HiLoad 26/60 Superdex 200 prep grade column (GE Healthcare) equilibrated with the buffering containing 20 mM sodium Hepes (pH 7.5) and 300 mM NaCl. Pooled fractions were concentrated by ultrafiltration to a final concentration of 10 mg/mL.

The vector for expression of the N-terminal domain of SepCysE (SepCysE_NTD, 1–103 aa) was constructed from the pDCH-SepCysE vector using a QuikChange mutagenesis kit (Agilent Technologies). The SepCysS-SepCysE_NTD complex was obtained with the same procedure as the SepCysS-SepCysE complex.

Crystallization and X-Ray Diffraction Data Collection. The crystals of both the SepCysS-SepCysE and the SepCysS-SepCysE_NTD complexes were obtained by a sitting-drop vapor-diffusion method. The crystals of the SepCysS-SepCysE complex were obtained with a solution containing 180 mM ammonium citrate (pH 6.8) and 16% (wt/vol) PEG3350 at 20 °C. Then they were soaked into a reservoir solution containing 10% (vol/vol) glycerol. The diffraction dataset was collected on the beam line BL1A at the Photon Factory (Tsukuba, Japan) at −173 °C. The crystals of the SepCysS-SepCysE_NTD complex were obtained with a solution containing 100 mM succinic acid and 8% (wt/vol) PEG3350 at 20 °C. Then they were soaked into a reservoir solution containing 20% (vol/vol) glycerol. The diffraction dataset was collected on the beam line AR-NE3A at the Photon Factory (Tsukuba, Japan) at −173 °C. All data were processed using XDS (14). The crystal of SepCysS-SepCysE was monohedrally twinned with the twin operator (h, h−k, −l), and the twin fraction was estimated to be 45% by Britton analysis using the program phenix.xtriage (15).

Structure Determination and Refinement. The SepCysS-SepCysE complex structure was solved by molecular replacement using Phaser (16) with the Archaeoglobus fulgidus SepCysS [Protein Data Bank (PDB) ID code 2E7J (17)] as a searching model. The SepCysS-SepCysE model was rebuilt using PHENIX AutoBuild (18) and LAFIRE (19) and modified manually using Coot (20). Structure refinement was performed using phenix.refine (21) and autoBUSTER (22). The twin operator (h, h−k, −l) was applied during every round of refinement and the twin fraction was refined. The Rwork and Rfree factors were converged to...
22.4% and 24.3%, respectively. The SepCysS-SepCysE_NTD structure was solved by molecular replacement using Phaser with the SepCysS-SepCysE structure as a searching model. After several cycles of refinement by phenix.refine and autoBUSTER and manual fitting by Coot, the Rwork and Rfree factors were converged to 18.9% and 24.0%, respectively. Data collection and refinement statistics are summarized in Table S1. All figures were prepared with PyMOL (The PyMOL Molecular Graphics System, Version 1.5.0.4).

Pull-Down Assays of Recombinant Proteins Expressed in *E. coli*. The expression vectors of SepCysE variants (with C-terminal His-tag), SepRS (tag-free), and SepCysS (tag-free) were constructed using a QuikChange mutagenesis kit (Agilent Technologies). The *E. coli* B834 (DE3)-pRARE2 strain was cotransformed with the vector pDCH-SepCysE together with pET15b-SepCysS or pET15b-SepRS. Cells were grown in 1 L LB medium and disrupted by sonication as described above. The homogenate was heat-treated at 70°C for 30 min to denature *E. coli* host proteins, and then clarified by centrifugation at 40,000 x g for 30 min at 4°C. The clarified supernatant was loaded onto 200 μL Ni-NTA resin (Qiagen) preequilibrated with the binding buffer plus 20 mM imidazole. After washing with 3 mL binding buffer plus 50 mM imidazole, the proteins were eluted with 500 mM imidazole and analyzed by SDS/PAGE.

Phylogeny and Bioinformatics. Protein homologs were identified using BLASTp searches (https://img.jgi.doe.gov/cgi-bin/w/main.cgi) against selected genomes. Sequence alignments were generated using the ClustalX 2.1 program (23). The sequence-based phylogenetic tree was constructed with MEGAS (24) using the Minimum Evolution algorithm. Bootstrap analysis was performed with 1,000 replicates. For comparisons of ratios of evolutionary distances (RED) (25), the evolutionary distances (Ea) were calculated with MEGAS using the PAM-250 matrix with its default settings.

Fig. S1. The expression of His-tagged SepRS and SepCysE in *M. maripaludis* mutant strains detected by Western blot. Whole-cell lysates (from 5 mL culture) were separated by SDS/PAGE, transferred to a PVDF membrane, and incubated with the anti-His antibody conjugated to HRP.
Fig. S2. Representative curves of the binding affinity of the *M. jannaschii* proteins for tRNA\textsuperscript{Cys} determined in a filter-binding assay.

Fig. S3. Deacylation of Sep-tRNA\textsuperscript{Cys}. The \(^{32}\)P-labeled Sep-tRNA\textsuperscript{Cys} (10 \(\mu\)M) was incubated at pH 7.5 at temperatures indicated. Each protein was present at 10 \(\mu\)M when included. Data are mean ± SDs (\(n = 3\)). RT, room temperature.
Fig. S4. Overlay of crystal structures and interactions between SepCysS and SepCysE. (A) The Mj-SepCysS dimer in the SepCysS-SepCysE complex (in orange; PDB ID code 3WKR) is superimposed on the A. fulgidus SepCysS dimer [in cyan; PDB ID code 2E7J (17)]. The active site of SepCysS is shown in the Inset. (B) The SepCysS dimer (orange) and the SepCysE dimer (magenta) in the SepCysS-SepCysE complex (PDB ID code 3WKR) are superimposed on the SepCysS dimer (cyan) and the SepCysE_NTD dimer (green) in the SepCysS-SepCysE_NTD complex (PDB ID code 3WKS). (C) Surface electrostatic potential of the SepCysS-SepCysE interface (red, −10kT/e; blue, 10kT/e).

Fig. S5. Sequence alignment of SepCysS and SepCysE homologs. The hydrophilic residues located at the SepCysS-SepCysE interface are labeled with stars. Dashed lines indicate hydrogen bonds. The full species names are listed in Fig. S6.

Fig. S6. Phylogenetic distribution of Cys biosynthesis genes in methanogenic archaea. The 16S rRNA phylogenetic tree was constructed with the Minimum Evolution algorithm using MEGA5 (1). Bootstrap analysis was performed with 1,000 replicates, and values >70% are labeled on the nodes. The magenta, green, and blue branches represent class I, II, and III methanogens, respectively. The gray and white boxes indicate the presence and absence of the Cys biosynthesis genes, respectively. CGL, cystathionine γ-lyase; cysK, O-acetylserine (thiol)-lyase-A; cysS, cysteinyl-tRNA synthetase.

Fig. S7. Phylogeny of SepCysE. (A) The sequence-based phylogenetic tree of the SepCysE homologs from Methanococcales (green), Methanobacteriales (purple), and Methanopyrales (dark red) was constructed with the Minimum Evolution algorithm using MEGA5 (1). Bootstrap analysis was performed with 1,000 replicates, and values $>70\%$ are labeled on the nodes. (B) The RED plot of SepRS (blue), SepCysS (orange), and SepCysE (magenta) homologs. The control $E_d$ are the mean $E_d$ of the genes encoding the large ribosomal subunit protein 2 (L2P), leucyl-tRNA synthetase (LeuRS), and the protein translocase SecY.

### Table S1. Data collection and refinement statistics

<table>
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<th>SepCysS·SepCysE</th>
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</tr>
<tr>
<td>Completeness, %</td>
<td>95.0 (94.6)</td>
<td>99.7 (98.6)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>4.1 (3.2)</td>
<td>7.3 (7.3)</td>
</tr>
<tr>
<td><strong>Refinement statistics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of reflections</td>
<td>304,705</td>
<td>22,584</td>
</tr>
<tr>
<td>$R_{\text{work}}$/ $R_{\text{free}}$, %</td>
<td>22.44/24.25</td>
<td>18.89/23.96</td>
</tr>
<tr>
<td>Twin fraction, %</td>
<td>44.9 ($h$, $-h$, $-k$, $-l$)</td>
<td>—</td>
</tr>
<tr>
<td>$R_{\text{twin,obs}}$, $R_{\text{twin,calc}}$, %†</td>
<td>4.4, 29.7</td>
<td>—</td>
</tr>
<tr>
<td>No. of protein atoms</td>
<td>13,917</td>
<td>7,032</td>
</tr>
<tr>
<td>No. of water atoms</td>
<td>113</td>
<td>23</td>
</tr>
<tr>
<td>Average $B$ factor of protein, Å$^2$</td>
<td>99.90</td>
<td>50.20</td>
</tr>
<tr>
<td>Average $B$ factor of water, Å$^2$</td>
<td>52.80</td>
<td>32.70</td>
</tr>
<tr>
<td>rmsd of bond lengths, Å</td>
<td>0.002</td>
<td>0.002</td>
</tr>
<tr>
<td>rmsd of bond angles, °</td>
<td>0.53</td>
<td>0.65</td>
</tr>
<tr>
<td>Ramachandran favored, %</td>
<td>95.4</td>
<td>95.7</td>
</tr>
<tr>
<td>Ramachandran allowed, %</td>
<td>4.3</td>
<td>3.7</td>
</tr>
<tr>
<td>Ramachandran outliers, %</td>
<td>0.35</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Each dataset was collected from a single crystal. Values in parentheses are for highest-resolution shell.

* $R_{\text{meas}}$ is the redundancy-independent $R_{\text{sym}}$.

† $R_{\text{twin}} = \Sigma |I(h) - (\Sigma_{\text{twin}} I(h))/\Sigma |I(h)| + (\Sigma_{\text{twin}} |I(h)|)$, where $S_{\text{twin}}$ is the twin operator (1).