Synthesis of Cysteinyl-tRNA^{Cys} by a Genome That Lacks the Normal Cysteine-tRNA Synthetase†

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ABSTRACT: Synthesis of cysteinyl-tRNA^{Cys} by cysteine-tRNA synthetase is required for decoding cysteine codons in all known organisms. The genome of the archaeon *Methanococcus jannaschii* lacks the gene for a normal cysteine-tRNA synthetase. The activity of the enzyme, however, was identified recently, and it allowed the purification of the enzyme and cloning of its gene. Sequence analysis of the gene showed that it encodes proline-tRNA synthetase and, thus, raised the possibility of dual activities in a single aminoacyl-tRNA synthetase. Assays of aminoacyl-adenylate synthesis confirmed the ability of the enzyme to activate proline and cysteine and showed that both activities were independent of tRNA. Assays of tRNA aminoacylation established the specific attachment of proline to tRNA^{Pro} and cysteine to tRNA^{Cys}. However, in contrast to a recent report of comparable activities with cysteine and proline, results here indicate that the adenylate synthesis and aminoacylation activities with cysteine are significantly lower than the respective activity with proline. In addition, there is evidence of overlapping amino acid-binding sites and tRNA-binding sites. These considerations, among others, raised the distinct possibility that the *M. jannaschii* proline-tRNA synthetase may recruit additional protein or RNA factors to facilitate the synthesis of cysteinyl-tRNA^{Cys}.

The decoding of genetic information requires aminoacyl-tRNAs that provide each triplet codon with a correct amino acid. Synthesis of aminoacyl-tRNAs is accomplished by aminoacyl-tRNA synthetases (AARSs). There are 20 families of synthetases, one for each amino acid. Members of the same amino acid family catalyze activation of the amino acid with ATP to form an aminoacyl-adenylate (AA-AMP) and then transfer the activated aminoacyl-adenylate to their cognate tRNAs to form the aminoacyl-tRNAs. The conservation of the genetic code demands that the synthesis of aminoacyl-tRNAs be precise. As expected, synthetases of the same amino acid family share strong conserved sequence motifs for activation of amino acid and for aminoacylation of their tRNAs. These sequence motifs are easily identified in databases of all three domains of life, the eubacteria, the eukarya, and the aryrchaea (*1*). In addition, synthetases specific to 10 amino acids also share a conserved catalytic fold for amino acid activation, while synthetases specific to 10 others share another conserved catalytic fold (*1, 2*). The two types of catalytic fold, known as the class I fold and the class II fold, display different structures and stereochemistries of aminoacylation.

While many organisms, including humans, require a set of 20 synthetases and use these enzymes specifically, many others have an unusual arrangement to synthesize aminoacyl-tRNAs. For example, some Gram-positive eubacteria and archaea lack glutaminyl-tRNA synthetase (GlnRS) or asparaginyl-tRNA synthetase (AsnRS) (*3, 4*). The genomes of these organisms do not encode the gene for GlnRS or AsnRS, and cell lysate of these organisms lacks the respective enzyme activities. Instead, synthesis of Gln-tRNA^{Gln} is achieved by glutamate-tRNA synthetase (GluRS), and the synthesis of Asn-tRNA^{Asn} is achieved by aspartate-tRNA synthetase (AspRS) (*3*). The precursors Glu-tRNA^{Gln} and Asp-tRNA^{Asn} are then converted to the correct forms by a transamidase (*4*−*6*).

The genomes of the archaea *Methanococcus jannaschii* and *Methanobacterium thermoautotrophicum* have additional unusual features (*7, 8*). In addition to the lack of genes for GlnRS and AsnRS, they do not encode the LysRS of the same catalytic fold as that of the majority of LysRS enzymes (*9*). Further, these genomes do not encode a normal CysRS that can be identified by searches of homology. While the unusual LysRS of *M. jannaschii* and *M. thermoautotrophicum* is also found in many other methanogens and even in some eubacteria (*9, 10*), the lack of a normal CysRS appears unique to these organisms. For example, each of the related methanogens, *Methanococcus maripaludis* and *Methanosarcina barkeri*, contains a normal CysRS that can functionally substitute the CysRS of *Escherichia coli* (*11*). The genomes of two other hyperthermophilic archaea, *Archaeoglobus*

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fulgidus and *Pyrococcus horikoshii*, encode in each a normal CysRS (*12*−*14*).

The lack of a normal CysRS from *M. jannaschii* and *M. thermoautotrophicum* does not mean the absence of cysteine from their protein synthesis. Indeed, many of their enzymes use cysteine in iron−sulfur complexes that are important for electron transport (such as ferredoxins) in the pathway of methanogenesis. For example, the key enzymes for production of methane, such as methyl transferase and methyl-CoM reductase, contain multiple Fe−S complexes, where the iron is bonded to the sulfur of cysteine and to inorganic sulfide. Clearly, the presence of cysteine is essential for *M. jannaschii* and *M. thermoautotrophicum*, and the elucidation of the mechanism for introducing cysteine into their proteins is of central importance.

We recently showed that the cell lysate of *M. jannaschii* contained the aminoacylation activity of CysRS (*15*). This activity is specific with cysteine, is dependent on ATP and Mg^{2+}, and catalyzes the attachment of cysteine onto *M. jannaschii* $tRNA^{Cys}$. The only unusual feature of this activity is that it requires tRNA modifications for aminoacylation, whereas all other characterized CysRS enzymes do not (*15*). We report here purification of this activity from the cell lysate of *M. jannaschii* and cloning of the gene that encodes this activity. Analysis of the gene and biochemical studies show that this activity is carried out by the enzyme ProRS that is responsible for synthesis of Pro-$tRNA^{Pro}$. A recent report also identified ProRS as the enzyme for synthesis of Cys-$tRNA^{Cys}$ (*16*). However, in contrast to that report, we showed that the activity of *M. jannaschii* ProRS to synthesize cysteinyl-AMP is independent of tRNA and that the activity of aminoacylation with cysteine is significantly lower than with proline. The lower levels of aminoacylation with cysteine, together with the lack of apparent domains in *M. jannaschii* ProRS that may specify the CysRS activity, raise the possibility of the recruitment of other protein or RNA factors to assist *M. jannaschii* ProRS with its synthesis of Cys-$tRNA^{Cys}$. These considerations further highlight the unusual arrangement of the *M. jannaschii* genome to organize its machinery of protein synthesis.

MATERIALS AND METHODS

Cell Strain and Growth. M. jannaschii strain JAL-1 was obtained from the Oregon Collection of Methanogens (OCM 168 = DSM 2661). Media were prepared anaerobically with the addition of a stock of $Na_2S(H_2O)_9$ (2.5% w/v) after sterilization (*17*). Inoculating cells were pressurized and cultures were grown at 83 °C with a H_2-CO_2 (80:20) flow rate of 0.2 vvm at 1.7×10^5 Pa. Cells from a 250 L fermentor were harvested and stored in liquid nitrogen.

Enzyme Purification and Sequence Analysis. Purification of the enzyme responsible for synthesis of Cys-$tRNA^{Cys}$ was based on the aminoacylation activity of *M. jannaschii* $tRNA^{Cys}$ (*15*). S100 [in 10 mM Tris-HCl, pH 7.4, 20 mM β-Me (β-mercaptoethanol), 1 mM EDTA, 4 mM $MgCl_2$, and 15% glycerol] was passed through a DEAE Sepharose column with the same buffer as that of S100 and was eluted with a gradient of 0−0.5 M NaCl. Active fractions were passed through a MonoQ FPLC column (in 20 mM Bis-Tris, pH 6.0, 50 mM NaCl, 20 mM β-Me, 1 mM EDTA, 4 mM $MgCl_2$, and 10% glycerol) and eluted with a gradient to 0.5 M NaCl. Active fractions were passed through a hydoxyapatite column (in 10 mM Na_3PO_4, pH 6.8, 10 mM β-Me, 1 mM EDTA, 4 mM $MgCl_2$, and 10% glycerol) and eluted with a gradient of 0−0.5 M Na_3PO_4 buffer, pH 6.0. Active fractions were passed through a blue Sepharose CL-6B column (in 20 mM Tris-HCl, pH 7.0, 10 mM NaH_2PO_4, 10 mM β-Me, 1 mM EDTA, 4 mM $MgCl_2$, and 10% glycerol) and eluted with a gradient of 0−1.0 M NaCl. The final active fraction (in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, and 50% glycerol) contained two major proteins. Both proteins were removed from an SDS−PAGE gel and subjected to sequence analysis, and the identified sequences were searched against the *M. jannaschii* genome by the Harvard Microchemistry Facility (Cambridge, MA).

Preparation of M. jannaschii Total tRNA. Total *M. jannaschii* tRNA was isolated from frozen cells by modification of a previous method (*18*). Frozen cells (5 g) were suspended in 10 mL of 50 mM sodium acetate and 10 mM magnesium acetate and were extracted with phenol. The released tRNA was ethanol-precipitated, and rRNA was removed by precipitation in 1 M NaCl, while DNA was removed by precipitation with 35% isopropyl alcohol. The tRNA was then precipitated in 50% isopropyl alcohol and loaded on a 10% denaturing PAGE. The tRNA in the gel was identified by UV, eluted and precipitated, and assayed by aminoacylation to determine the fractions of $tRNA^{Cys}$ and $tRNA^{Pro}$. Both tRNAs were 1−2% of the total tRNA. Separation of $tRNA^{Cys}$ or $tRNA^{Pro}$ by periodate inactivation of all other tRNAs was performed as described (*19*).

Aminoacylation Assays. The activity of aminoacylation with cysteine was measured by the acid-precipitable counts of ^{35}S-cysteinyl-$tRNA^{Cys}$ at 65 °C as described (*15*). The *M. jannaschii* total tRNA was used as the tRNA substrate. The activity of aminoacylation with proline was measured by the acid-precipitable counts of ^{3}H-prolyl-$tRNA^{Pro}$ as described (*20*), except at 65 °C. The T7 transcript of *M. jannaschii* $tRNA^{Pro}$ was used as the tRNA substrate.

ATP−PP_i Exchange Assays. Assays for the ATP−PP_i exchange activity were conducted at 65 °C using ^{32}P−PP_i (NEN; 10^6 cpm/μmol) as described (*21*). The concentration of the amino acid (either cysteine or proline) was 0.5 mM and that of the enzyme was 0.126−1.26 μM. Inhibition by prolinamide (Sigma) was performed with the addition of the inhibitor at desired concentrations to an assay mixture.

RESULTS

Purification of the Enzyme Responsible for the Synthesis of Cys-$tRNA^{Cys}$. We used the aminoacylation assay to identify and purify the enzyme responsible for the synthesis of Cys-$tRNA^{Cys}$. The assay contained ^{35}S-cysteine, and the aminoacylation activity was previously detected as ^{35}S-Cys-$tRNA^{Cys}$ in the S100 cell lysate of *M. jannaschii* by gel analysis (*15*). It was also detected as acid-precipitable counts of ^{35}S-Cys-$tRNA^{Cys}$ in the fraction that was retained by DEAE sepharose. Because the activity was detected only with $tRNA^{Cys}$ from total *M. jannaschii* tRNA, but not with the unmodified transcript of $tRNA^{Cys}$, we concluded that it required modifications in $tRNA^{Cys}$ (*15*). We therefore used total *M. jannaschii* tRNA as the substrate throughout purification of the enzyme. After sequential elutions from DEAE Sepharose, MonoQ, hydroxyapatite, and blue Sepharose

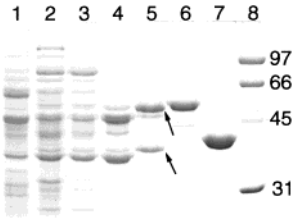

FIGURE 1: SDS−PAGE gel analysis of purification of *M. jannaschii* CysRS. Lane 1, S100 extract of *M. jannaschii* (20 μg); lanes 2−5, active eluates (10 μg each) from DEAE Sepharose, MonoQ, hydroxyapatite, and blue Sepharose CL-6B, respectively; lanes 6−7, cloned and expressed recombinant *M. jannaschii* His_6-ProRS (7 μg) and Mj1338-His_6 (5 μg), respectively; lane 8, molecular weight markers. Arrows point to the two major species in the active fraction of blue Sepharose CL-6B (lane 5).

CL-6B, we obtained an active fraction that showed only two major bands on an SDS−PAGE (Figure 1). One band corresponded to a molecular weight of 53 kDa, while the other corresponded to a molecular weight of 38 kDa. We isolated both bands from the SDS−PAGE and subjected them to sequence analysis.

The results of the sequence analysis were used to search for gene sequences in the genome of *M. jannaschii*. The sequence of the 53 kDa protein matched the predicted sequence of another *M. jannaschii* synthetase, which was ProRS. The sequence of the 38 kDa protein matched the predicted sequence of Mj1338 ORF, which encodes a protein related to members of the family of methylene-tetrahydromethanopterin dehydrogenases. Analysis of each step of the purification (Table 1) indicated approximately a 500-fold purification of the end products. This suggested that each of the end products accounted for 0.2% of the total cellular proteins. For an aminoacyl-tRNA synthetase, such as ProRS, the cellular fraction of 0.2% was reasonable. We thus obtained the gene for ProRS from Dr. K. Shiba (unpublished) and the gene for Mj1338 by using PCR to amplify from the genome of *M. jannaschii*. ProRS was expressed as a recombinant enzyme with an N-terminal His tag, whereas Mj1338 was expressed with a C-terminal His tag. We purified both recombinant proteins from *E. coli* through a metal chelate column.

Activation of Cysteine. We assayed *M. jannaschii* ProRS and Mj1338 for their ability to activate cysteine. Only ProRS showed the activity (Figure 2a), and this activity was similar to that of the purified enzyme isolated after the blue Sepharose step (Figure 1, lane 5). Thus, the recombinant ProRS recapitulated the activity of the native enzyme. Addition of Mj1338 to the recombinant ProRS did not enhance the activity further. This suggests that ProRS was solely responsible for activation of cysteine with ATP to synthesize Cys-adenylate (Cys-AMP). The ProRS enzyme was also active with the synthesis of Pro-AMP, as expected. This provided evidence of two adenylate synthesis activities in one synthetase. Both activities were detected in the absence of tRNA. To ensure that the detected activities, particularly the cysteine activity, were not dependent on a trace amount of RNA that might have copurified with the recombinant enzyme, we treated the enzyme with RNase A at 37 °C for 15 min to remove any residual RNA. We then tested the RNase A-treated ProRS again for its ability to synthesize Cys-AMP and Pro-AMP. Both activities were fully retained (Figure 2b). Control experiments indicated that RNase A was active under the conditions tested. Incubation of RNase A with 20 μg of tRNA eliminated essentially all of the tRNA. These experiments confirmed that the detected syntheses of both Cys-AMP and Pro-AMP were independent of RNA.

Comparison of the activity for synthesis of Pro-AMP and that for Cys-AMP, however, showed a difference (Figure 2a). While synthesis of Pro-AMP was achieved with an initial rate of 132.3 pmol/s/pmol enzyme, the synthesis of Cys-AMP was achieved with an initial rate of 0.132 pmol/s/pmol enzyme (Figure 2a,b). The initial rate of synthesis of Pro-AMP was measured under conditions that led to the plateau of the reaction (Figure 2b), and as such, it is an approximation of k_{cat}. The value of 132.3 s^{-1} as k_{cat} is comparable to that of k_{cat} of 142 s^{-1} of *E. coli* HisRS, for example (*22*). Thus, while the rate of synthesis of Pro-AMP is normal for *M. jannaschii* ProRS, that of Cys-AMP is 1000-fold slower. The 1000-fold difference in rates was consistent whether the enzyme was used alone or was incubated with the recombinant Mj1338 or with RNase A.

We tested if the addition of an RNA, such as a tRNA, to the adenylate synthesis reaction would stimulate the activity for cysteine or for proline. For synthesis of Cys-AMP, we showed that none of the tRNAs tested at 1.0 mg/mL stimulated the activity. These tRNAs included the total tRNA isolated from *M. jannaschii*, the transcript of *M. jannaschii* $tRNA^{Cys}$, the transcript of *M. jannaschii* $tRNA^{Lys}$, and the transcript of *M. jannaschii* $tRNA^{Pro}$ (not shown). Likewise, we saw no effect on the synthesis of Pro-AMP by the total tRNA isolated from *M. jannaschii* or by the transcript of *M. jannaschii* $tRNA^{Pro}$ (at 1.0 mg/mL) (not shown). As a control, we showed that the *E. coli* CysRS enzyme also did not respond to the addition of the transcript of *E. coli* $tRNA^{Cys}$ for the synthesis of Cys-AMP. Thus, in all cases, the adenylate synthesis for Cys-AMP or for Pro-AMP was not stimulated by tRNA. The lack of an effect of tRNA on adenylate synthesis was reasonable, given that the rate of adenylate synthesis was much faster than that for tRNA aminoacylation. For example, the *M. jannaschii* ProRS enzyme synthesized Cys-AMP at a rate of 0.132 pmol/s/pmol enzyme (see above), while it synthesized Cys-$tRNA^{Cys}$ at a rate of 0.0055 pmol/s/pmol enzyme (see below). Because the rate of tRNA aminoacylation was much slower, we expected that the addition of tRNA to the adenylate synthesis reaction would have little effect.

We investigated the binding site for cysteine and for proline (Figure 2c). We tested to see if the proline analogue prolinamide was an inhibitor for both adenylate synthesis activities. The analogue prolinamide retains the proline side chain but replaces the carboxylate group of proline with an amide group. It was used to probe the binding site for the proline side chain. If the proline-binding site completely or partially overlapped with the cysteine-binding site, we expected that the prolinamide analogue would inhibit both activities. Incubation of prolinamide over a range of concentrations in the activation assay showed inhibition of both activities (Figure 2c). Specifically, prolinamide at concentrations of 15 mM or higher almost eliminated the activity for synthesis of Pro-AMP, except for the 3−4% residual activity. Prolinamide at the same concentrations eliminated 65−70% of the activity for synthesis of Cys-AMP. Because the effective inhibitory concentrations of prolinamide were the

Table 1: Purification Scheme of *M. jannaschii* CysRS

	activity (pmol/min)	protein concn (mg/mL)	total protein (mg)	total activity (pmol/min)	specific activity (pmol/min/mg)	fold purification
S100	2.15	40.033	1281.06	17179	13.41	1.0
DEAE pool	1.50	2.720	125.12	17224	137.66	10.3
MonoQ pool	4.58	2.276	64.87	32627	503.00	37.5
hydroxyapatite pool	1.19	0.189	14.18	22234	1568.53	117.0
blue Sepharose pool	18.92	0.697	2.44	16558	6787.46	506.1

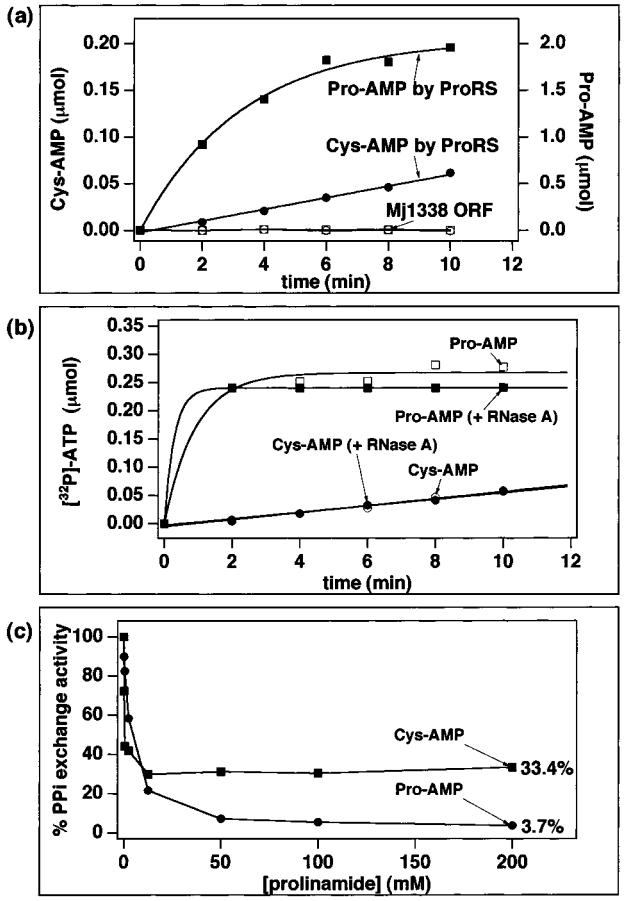

FIGURE 2: PP_i−ATP exchange assay for synthesis of Cys-AMP and Pro-AMP: (a) by *M. jannaschii* His_6-ProRS (0.126 μM) and Mj1338-His_6 (1.26 μM) and (b) by *M. jannaschii* His_6-ProRS (1.26 μM) with or without RNase A (1.26 μM). (c) Inhibition of the ability of *M. jannaschii* His_6-ProRS (1.26 μM) to synthesize Cys-AMP and Pro-AMP by prolinamide. The concentration of proline and cysteine was 0.5 mM each, while the concentrations of prolinamide were 0, 15, 50, 100, and 200 mM, respectively.

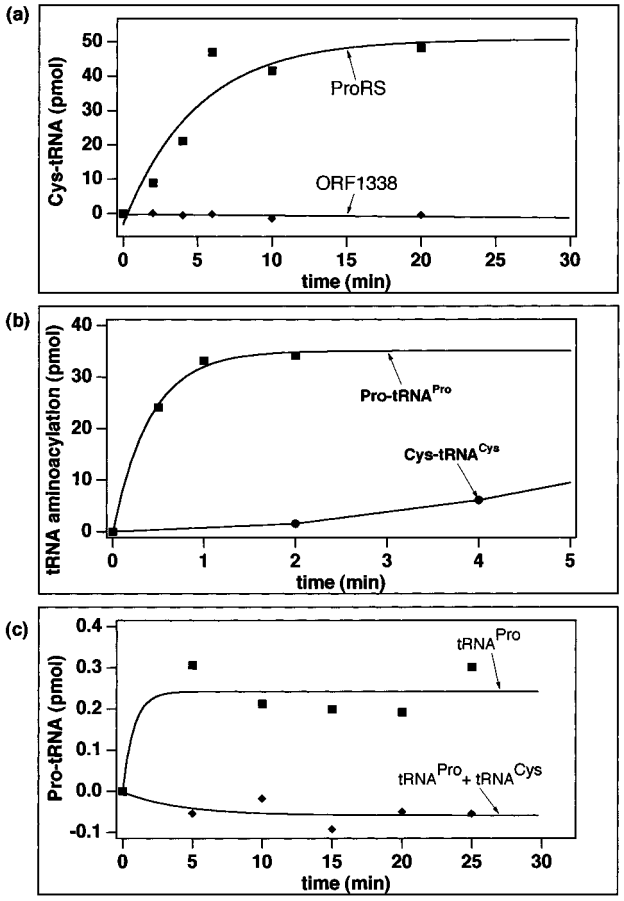

FIGURE 3: (a) Aminoacylation of *M. jannaschii* $tRNA^{Cys}$ (2.0 μM) for synthesis of Cys-$tRNA^{Cys}$ by *M. jannaschii* His_6-ProRS and Mj1338-His_6 (3.8 μM each). (b) Comparison of the rate of aminoacylation of *M. jannaschii* native $tRNA^{Pro}$ with proline and that of *M. jannaschii* native $tRNA^{Cys}$ with cysteine by *M. jannaschii* His_6-ProRS (0.126 μM), using total *M. jannaschii* tRNA as the substrate. (c) Aminoacylation of *M. jannaschii* $tRNA^{Pro}$ (57 nM in the total tRNA) by *M. jannaschii* His_6-ProRS (1 nM). This aminoacylation was prevented by a 20-fold excess of *M. jannaschii* native $tRNA^{Cys}$ isolated by periodate inactivation of all other non-cysteine-tRNAs.

same for both activities, this suggested that the two binding sites overlapped. The complete inhibition of Pro-AMP synthesis versus 70% inhibition of Cys-AMP synthesis suggested an overlap of 70% of the two sites in function. Control experiments confirmed that the inhibition of synthesis of Cys-AMP by prolinamide was due to the ability of the analogue to specifically bind to *M. jannaschii* ProRS but was not due to nonspecific interaction. Incubation of prolinamide with *E. coli* CysRS had little effect on the synthesis of Cys-AMP by the *E. coli* enzyme. This was done at the concentration of prolinamide (200 mM) that completely inhibited *M. jannaschii* ProRS.

The inhibition by prolinamide was detected at cysteine and proline concentrations of 0.5 mM, respectively. We have not determined the inhibition by prolinamide over a range of cysteine and proline concentrations. As such, we could not conclude if the two binding sites were competitive, noncompetitive, or uncompetitive.

Aminoacylation with Cysteine. We assayed *M. jannaschii* ProRS and Mj1338 protein for their ability to catalyze tRNA aminoacylation with cysteine. Using the *M. jannaschii* total tRNA as the substrate, we showed that the recombinant ProRS was active, whereas Mj1338 was inactive (Figure 3a). Addition of Mj1338 to ProRS increased the activity somewhat, but this enhancement was minor and was less than 5% (not shown). Thus, the single ProRS was largely or completely responsible for the aminoacylation activity with cysteine. We also confirmed the aminoacylation activity with

proline for ProRS. We observed similar activities using $tRNA^{Pro}$ in either *M. jannaschii* total tRNA or that which was made by in vitro transcription (not shown). This suggests that, in contrast to aminoacylation with cysteine, the aminoacylation activity with proline does not require tRNA modifications. We primarily used the native $tRNA^{Pro}$ in *M. jannaschii* tRNA for aminoacylation in this study.

Comparison of aminoacylation activities with proline and cysteine showed that the activity with proline was significantly higher than that with cysteine. Using the total tRNA as the substrate, we showed that the initial rate for aminoacylation with proline was 0.20 pmol/s/pmol enzyme while that with cysteine was 0.0055 pmol/s/pmol enzyme (Figure 3b). As aminoacylation with proline reached the plateau of the reaction, the initial rate was an approximation of k_{cat}. The k_{cat} of 0.2 s^{-1} for aminoacylation with proline is in the range of k_{cat} of 1.0 s^{-1} for synthetases in general. Thus, while the rate for aminoacylation with proline was normal, that with cysteine was not. The initial rate of aminoacylation with cysteine was 40-fold below the rate with proline.

We investigated the binding sites for $tRNA^{Pro}$ and $tRNA^{Cys}$ on ProRS. Aminoacylation of *M. jannaschii* total tRNA with proline protected the native $tRNA^{Pro}$ from periodate oxidation. The protected tRNA, after deacylation, could be reaminoacylated with proline but not with cysteine (Figure 3c). The same principle was used to separate *M. jannaschii* $tRNA^{Cys}$ from all other tRNAs and to demonstrate that this tRNA was specific to cysteine. These observations showed the specificity of each native tRNA. The periodate inactivation of tRNAs that were not aminoacylated with proline or cysteine also provided a means to generate the native $tRNA^{Pro}$ or $tRNA^{Cys}$ with natural modifications. This allowed determination of the relationship of the two tRNA-binding sites. The use of the native tRNA was necessary, as recent studies showed that the transcript of $tRNA^{Cys}$ does not bind to *M. jannaschii* ProRS (unpublished results). Here, the addition of the native $tRNA^{Cys}$ (purified through periodate inactivation of non-cysteine-specific tRNA) to the native $tRNA^{Pro}$ inhibited aminoacylation of $tRNA^{Pro}$ with proline (Figure 3c). The amount of $tRNA^{Cys}$ was in 20-fold excess to that of $tRNA^{Pro}$; this was to partially compensate for the 40-fold lower activity of aminoacylation of $tRNA^{Cys}$. Thus, under the conditions tested, the ability of $tRNA^{Cys}$ to inhibit $tRNA^{Pro}$ for aminoacylation with proline suggested at least a partial overlap of the two tRNA-binding sites.

DISCUSSION

The identification of *M. jannaschii* ProRS as the enzyme that catalyzes the synthesis of Cys-$tRNA^{Cys}$ suggests a novel design of synthetase specificities and catalytic properties. Normally, each synthetase is specific to only one amino acid. Although some synthetases occasionally activate an incorrect amino acid, they remove the incorrect amino acid and maintain the specificity of aminoacyl-tRNAs. For example, while *E. coli* IleRS synthesizes the correct Ile-AMP, it also synthesizes the incorrect Val-AMP. This enzyme has an editing activity to hydrolyze Val-AMP at a site that is distinct from the synthetic site (*23*). The separation of the editing site from the synthetic site demonstrates the specificity of each site (*24*, *25*). Herein, the *M. jannaschii* ProRS was shown to have an overlapping site for proline and cysteine and an overlapping site for $tRNA^{Pro}$ and $tRNA^{Cys}$. While the specificity of aminoacylation is maintained for each tRNA, the mechanism that underlies this specificity while using overlapping sites remains to be explained. Insights from studies of the mechanism should illuminate novel features of the design principle of *M. jannaschii* ProRS.

A recent report also identified ProRS as the enzyme for CysRS in *M. jannaschii* (*16*). Results of our study differ from those of that study primarily in two respects. First, in contrast to the reported tRNA-dependent activation of cysteine by *M. jannaschii* ProRS, we showed that this activation was tRNA-independent. We detected the activity of the synthesis of Cys-AMP without adding tRNA, and we showed that this activity remained intact even after the synthetase was treated with RNase A. In addition, we showed that this activity was not stimulated by tRNA, regardless if a tRNA was isolated with natural modifications or was prepared by T7 transcription. The ability to activate cysteine independent of tRNA is common to all other known CysRS enzymes in the database (*26*). Second, in contrast to the reported levels of comparable activities, we showed that the activity for synthesis of Cys-AMP was below the activity of Pro-AMP by 1000-fold and that the activity of aminoacylation with cysteine was below the activity with proline by 40-fold. This comparison raises the possibility that, because the synthesis of Cys-$tRNA^{Cys}$ is slow relative to that of other aminoacyl-tRNAs, the decoding of cysteine codons might be rate-limiting and as such might severely impair the synthesis of enzymes required for the central metabolism of *M. jannaschii*. One way to minimize the effect of the rate-limiting step for the synthesis of Cys-$tRNA^{Cys}$ would be to have an extremely high cellular concentration of cysteine. However, in eubacterial organisms where cellular amino acids have been measured, the level of cysteine is normally at the low end, below that of proline by 2−3-fold.

To compensate for the slower rate of aminoacylation with cysteine, *M. jannaschii* ProRS may recruit additional protein or RNA factors to enhance its CysRS activity. There are three additional reasons that suggest the involvement of outside factors. First, because of the overlapping substrate-binding sites, this raises the concern of specificity. Although the *M. jannaschii* ProRS is shown as specific in vitro, the situation under the dynamics of a cellular environment may be different. The additional protein or RNA factors may bind to the enzyme to form a better binding site for cysteine or for $tRNA^{Cys}$ and, thus, to improve specificity. Second, the amino acid sequence of *M. jannaschii* ProRS shares an overall high degree of homology to sequences of other ProRSs in the database, including enzymes that are specific for proline (*20*). The high homology suggests that the sequence of *M. jannaschii* ProRS alone, both within and outside of the catalytic site, cannot provide novel folds or domains that specify the CysRS activity. Other factors must be recruited to act in trans. Third, *M. jannaschii* ProRS is a class II synthetase, which normally approaches its tRNA from the opposite direction than that of class I synthetases (*27*). However, *M. jannaschii* $tRNA^{Cys}$ shares common features with cysteine-specific tRNAs that are recognized by the normal CysRS, which is class I (*28*, *29*). These features include the conserved U73 in the acceptor stem (*26*, *30*). Thus, the design of the class II ProRS may not appropriately match the design of tRNA elements in the acceptor stem,

and this mismatch may require additional factors to transform into the productive form for catalysis.

We have identified Mj1338 in the purification of ProRS. Although Mj1338 did not enhance the CysRS activity, this might be due to the loss of its cofactor. The cofactor of Mj1338 is methanopterin, which was lost during protein purification (unpublished) and was not present in the preparation of the recombinant Mj1338 from *E. coli*. Whether the native Mj1338, bound with its cofactor, plays a role in the CysRS activity of *M. jannaschii* ProRS remains to be determined. However, examples of synthetases to recruit additional protein factors are known. For example, *Saccharomyces cerevisiae* MetRS and GluRS recruit the Arc1p protein to enhance their ability to interact with their tRNAs to improve the efficiency of aminoacylation (*31, 32*). The domains in Arc1p that are responsible for interactions with MetRS and GluRS and with the two tRNAs have now been identified (*31*). In higher eukarya, ranging from mammals to insects, nine aminoacyl-tRNA synthetases form a complex that also includes three protein factors (*33*). Interestingly, ProRS is a member of the complex (*34*). The three protein factors in the complex generally mediate the association of the complex with other components of the protein synthesis machinery (*35*).

Alternatively, RNAs may act as the cofactor. Most notably, the binding of tRNA to GlnRS, GluRS, and ArgRS enables these synthetases to activate their amino acids and to optimize their specificity (*36*). Also, the presence of the correct tRNA enhances the error-proof activity of IleRS (*37*). Although we showed here that tRNA does not enhance the adenylate synthesis activity of *M. jannaschii* ProRS, other small RNAs are possible. These RNAs may not be easily retained during protein purification if the enzyme−cofactor interaction is only transient.

The genome of *M. thermoautotrophicum* appears to have a similar organization of aminoacyl-tRNA synthetases (*16*). It also encodes a ProRS that carries out the CysRS activity, although the levels of Cys-tRNA^{Cys} have not been characterized or compared. A distinct feature of the *M. jannaschii* or *M. thermoautotrophicum* ProRS is that it is a synthetase with two specificities. This is unlike the ProRS of higher eukarya, where the sequence of ProRS is joined with the sequence of GluRS in a single polypeptide (*38*). The multitude of surprises of *M. jannaschii* ProRS was thus not expected, and the molecular basis for using ProRS, but not other synthetases, as the surrogate for CysRS is not understood. The operation of the genome of *M. jannaschii* or that of *M. thermoautotrophicum* thus contains previously unrecognized networks of aminoacyl-tRNA synthetases, both among themselves and possibly with other cofactors. The delineation of these networks should shed light on the evolution of the modern day decoding machinery.

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