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Methanococcus jannaschii Prolyl-Cysteinyl-tRNA Synthetase Possesses Overlapping Amino Acid Binding Sites†

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ABSTRACT: The protein translation apparatus of *Methanococcus jannaschii* possesses the unusual enzyme prolyl-cysteinyl-tRNA synthetase (ProCysRS), a single enzyme that attaches two different amino acids, proline and cysteine, to their cognate tRNA species. Measurement of the ATP−PP_i exchange reaction revealed that amino acid activation, the first reaction step, differs for the two amino acids. While Pro-AMP can be formed in the absence of tRNA, Cys-AMP synthesis is tRNA-dependent. Studies with purified tRNAs indicate that $tRNA^{Cys}$ promotes cysteine activation. The k_{cat} values of wild-type ProCysRS for tRNA prolylation (0.09 s^{-1}) and cysteinylation (0.02 s^{-1}) demonstrate that both aminoacyl-tRNAs are synthesized with comparable rates, the cysteinyl-tRNA synthetase activity being only 4.5-fold lower than prolyl-tRNA synthetase activity. Kinetic analysis of ProCysRS mutant enzymes, generated by site-directed mutagenesis, shows glutamate at position 103 to be critical for proline binding, and proline at position 100 to be involved in cysteine binding. The proximity in ProCysRS of amino acid residues affecting binding of either cysteine or proline strongly suggests that structural elements of the two amino acid binding sites overlap.

Accurate aminoacylation of transfer RNA (tRNA) by aminoacyl-tRNA synthetases (AARSs)[1] is a crucial step in the faithful translation of messenger RNA (*1*). The presence of 20 AARS proteins, each one specific for a single amino acid, provided a sound basis when one considered the exquisite specificity of amino acid and tRNA discrimination during aminoacyl-tRNA synthesis. This view was challenged by the discovery of an astonishing dual-specificity enzyme, prolyl-cysteinyl-tRNA synthetase (ProCysRS), capable of and required for supplying both Pro-tRNA and Cys-tRNA during protein synthesis in the archaea *Methanococcus jannaschii* (*2, 3*) and *Methanobacterium thermoautotrophicum* (*2, 4*). Immediately this led to the question as to how this dual-specificity enzyme is able to select 2 out of the 20 canonical amino acids and to discriminate accurately between them when acylating $tRNA^{Pro}$ or $tRNA^{Cys}$ (*5*). Like other prolyl-tRNA synthetases (*6*), ProCysRS forms Pro-AMP in the presence of proline and ATP. As tRNA-mediated amino acid recognition by AARSs is well-known (*7, 8*), it was encouraging to see that Cys-AMP synthesis by ProCysRS was dependent on the presence of tRNA (*2*). However, a recent publication on the *M. jannaschii* enzyme reports tRNA-independent Cys-AMP synthesis and implies, in addition to ProCysRS, further cofactor(s) for the formation of Cys-tRNA (*9*). Here we present evidence for tRNA-dependent cysteine activation by *M. jannaschii* wild-type ProCysRS and for overlapping amino acid binding sites on this enzyme.

MATERIALS AND METHODS

Chemicals. [^{35}S]Cysteine (1075 Ci/mmol) and [^{32}P]PP_i (15 Ci/mmol) were from NEN, [^{3}H]proline (104 Ci/mmol) and [^{14}C]proline (248 mCi/mmol) were from Amersham Pharmacia Biotech, and Ni−NTA matrix was from Qiagen. GF/C glass microfiber filters were from Whatman. Nitrocellulose filters (0.45 μm) were from Schleicher & Schuell. Centricon YM-10 was from Amicon. DNA high-fidelity T7 polymerase and inorganic pyrophosphatase (0.2 unit/μL) were from Boehringer Mannheim. The TOPO-TA cloning kit was from Invitrogen. *Epicurian coli* BL21-CodonPlus competent cells were purchased from Stratagene. Oligonucleotide synthesis and DNA sequencing were performed by the Keck Foundation Research Biotechnology Resource Laboratory at Yale University.

tRNA Preparation. All steps were performed at 4 °C. *M. jannaschii* cells (27 g) were suspended in 40 mL of extraction buffer (20 mM Tris-HCl, pH 7.5, 20 mM magnesium acetate) and sonicated. Total nucleic acids were recovered by phenol extraction with 40 mL of acid-buffered phenol (pH 4.6). After agitation (20 min) and centrifugation at 4000*g* (10 min), the aqueous phase was removed and saved. To the remaining organic phase was added 40 mL of extraction buffer, and a

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[1] Abbreviations: for amino acids (AA) and aminoacyl-tRNA synthetases (AARSs), the three-letter code is used, e.g., ProCysRS for prolyl-cysteinyl-tRNA synthetase; AA-tRNA, aminoacyl-tRNA; KF, potassium fluoride; DTT, dithiothreitol.

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second extraction was performed. The pooled aqueous phases were re-extracted with phenol, and the aqueous layer was recovered. DNA was partially removed by precipitation with 20% (v/v) 2-propanol. After centrifugation for 15 min at 4500*g*, the supernatant was adjusted to 60% (v/v) 2-propanol to precipitate tRNAs. The tRNAs were harvested by centrifugation (20 min at 4500*g*), washed, and briefly dried. The pellet was suspended in 5 mL of 200 mM Tris-HCl (pH 8.0) and incubated at 37 °C for 30 min to deacylate AA-tRNA. Total tRNA was then recovered by ethanol precipitation. After centrifugation, the pellet was washed, dried briefly, and resuspended in 10 mL of water. The yield of tRNA was 75 mg.

Preparation of Periodate-Oxidized tRNAs from M. jannaschii. Total tRNA (1.2 mg) was aminoacylated as described previously (*2*) in a 1 mL reaction mixture at 70 °C in the presence of either [^{14}C]proline or [^{35}S]cysteine using 30 μg of ProCysRS. When the charging plateau was reached, 10% (v/v) 3 M sodium acetate (pH 5.2) was added. AA-tRNAs were then extracted with acid-buffered phenol (pH 4.6), followed by a chloroform extraction and ethanol precipitation. To protect thiolated bases from oxidation, the dried pellets of AA-tRNAs were suspended in 10 mL of a 25 mM sodium phosphate buffer (pH 6.8) containing 0.3 mM sodium dinitrobenzoate (*10*). After a 20 min room-temperature incubation, the AA-tRNAs were recovered by ethanol precipitation, harvested, and dried. The AA-tRNAs were suspended in 5 mL of 50 mM sodium acetate buffer (pH 5.0) containing 4 mM sodium periodate and incubated 20 min at room temperature and in the absence of light. After addition of KCl to make the solution 0.2 M, the periodate precipitated and was removed. Traces of remaining periodate were eliminated by addition of 0.2 mL of ethylene glycol to the supernatant and overnight dialysis against 5 mM sodium acetate buffer (pH 5.0). After ethanol precipitation, AA-tRNAs were recovered by centrifugation, dried, and deacylated by incubation in 2 mL of a 1.8 M Tris-HCl (pH 8.0) solution during 30 min at 37 °C. The ethanol-precipitated tRNAs were dried and suspended in 0.1 M DTT (2 mL), and regeneration of the thiolated bases was achieved by a 4 h incubation on ice. After ethanol precipitation, the tRNAs were dried and suspended in 0.5 mL of sterile water. Between 0.3 and 0.4 mg of tRNA was recovered and stored at −20 °C until use.

Purification of M. jannaschii tRNA^{Cys} and tRNA^{Pro} by Affinity Column Chromatography on Nickel−Agarose-Immobilized EF-Tu. Total *M. jannaschii* tRNA (1 mg) was aminoacylated at saturating substrate concentrations (see following section) either with [^{3}H]proline or with [^{35}S]-cysteine. Once aminoacylation plateaus were reached, the mixture was supplemented with 10% (v/v) 3 M sodium acetate (pH 5.2), and AA-tRNAs were extracted with acid-buffered phenol, followed by a chloroform extraction and ethanol precipitation. The dried pellets were suspended in 400 μL of sterile water and applied onto a 1.5 mL column of nickel−agarose-immobilized GTP-activated EF-Tu (5 mg). All chromatographic steps were performed as described previously (*11*). Fractions (2−3 mL) containing Cys-tRNA^{Cys} or Pro-tRNA^{Pro} were pooled, concentrated by centrifugation on Centricon YM-10 (final volume of 0.4 mL), supplemented with glycogen, and ethanol-precipitated. The dried pellets were suspended in 30 μL of 1.8 M Tris-HCl (pH 8.0), and deacylation was achieved by incubation for 30 min at 37 °C. After ethanol precipitation, tRNA was recovered by centrifugation, washed, dried, and suspended in 10 μL of sterile water. This procedure allowed recovery of approximately 20 μg of pure tRNA^{Cys} and 10 μg of pure tRNA^{Pro} with a yield of 50% and 12.5%, respectively.

Enzyme Preparation. All steps were performed at 4 °C, and all buffers contained 10% (v/v) glycerol, 5 mM 2-mercaptoethanol, and 1 mM benzamidine. The cell paste resulting from 1.5 L of culture of the different ProCysRS-overexpressing wild-type and mutant strains was suspended in 5 mL of sonication buffer (50 mM Tris-HCl, pH 8.0, containing 500 mM NaCl, 5 mM $MgCl_2$, 0.1% Triton X-100, and 10 μg/mL lysozyme). The cells were disrupted by 10 cycles of 30 s sonication at 50 V, and the lysate was centrifuged twice for 30 min at 14000*g* and for 1 h at 100000*g*. The resulting S-100 extract was applied to a 2 mL Ni-NTA−agarose column equilibrated with the sonication buffer. After washing the column with 20 mL of sonication buffer containing imidazole (30 mM), elution of the ProCysRS was achieved with 10 mL of the same buffer but containing 750 mM imidazole. The enzyme-containing fractions were pooled and dialyzed during 12 h against 50 mM HEPES−KOH (pH 7.0), 50 mM KCl, and 15 mM $MgCl_2$. Traces of contaminating proteins were eliminated by a 20 min flocculation step at 70 °C and 20 min centrifugation at 14000*g*. The pure protein solution was then concentrated on PEG 15000, dialyzed against 50 mM HEPES−KOH (pH 7.0), 50 mM KCl, 15 mM $MgCl_2$, and 50% (v/v) glycerol, and stored at −20 °C. Active site titration shows that 60% of the molecules are active in this enzyme preparation.

Aminoacylation of tRNA. The standard reaction mixture (100 μL) contained 50 mM HEPES−KOH (pH 7.0), 50 mM KCl, 15 mM $MgCl_2$, 5 mM DTT, 10 mM ATP, 20 μM [^{35}S]-cysteine (1075 Ci/mmol) or [^{3}H]proline (104 Ci/mmol), 1 mg/mL *M. jannaschii* unfractionated tRNA, and 20−150 μg/mL purified recombinant ProCysRS. Reactions were performed at 70 °C, and the radioactive AA-tRNAs synthesized after 1−60 min were determined in 20 μL aliquots as described (*2*). K_M values were determined from double-reciprocal plots using limiting concentrations of the variable substrates (1−500 μM [^{35}S]cysteine or [^{3}H]proline; 0.01−1 mM ATP) and saturating concentrations (10−100 × K_M) of the fixed substrates. ProCysRS concentration was 0.002−2 μM, and tRNA was 1 mg/mL. Values of k_{cat} were determined by initial rate measurements using saturating substrate concentrations (300 μM [^{35}S]cysteine or [^{3}H]proline, 600 μM ATP), 3 mg/mL *M. jannaschii* total tRNA, and 50−150 nM ProCysRS.

ATP−[^{32}P]PP$_i$ Exchange. The reaction mixture of 200 mL contained 100 mM HEPES-Na (pH 7.2), 10 mM $MgCl_2$, 50 mM KCl, 40 mM DTT, 2 mM KF, ATP either fixed at 2 mM or varying from 0.04 to 3 mM for K_M determination, L-proline or L-cysteine either fixed at 2 mM or varying from 0.02 to 2 mM for K_M determinations, 2 mM [^{32}P]PP$_i$ (1.6 cpm/pmol), when indicated 5 μM tRNA^{Cys} or 10 μM tRNA^{Pro} in *M. jannaschii* unfractionated tRNA, and 0.15−0.5 μM ProCysRS. After various incubation times at 70 °C, the [^{32}P]-ATP present in 40 μL aliquots of the reaction mixture was specifically adsorbed on acid-washed Norit [200 μL of a 1% (w/v) Norit in 0.4 M sodium pyrophosphate solution with

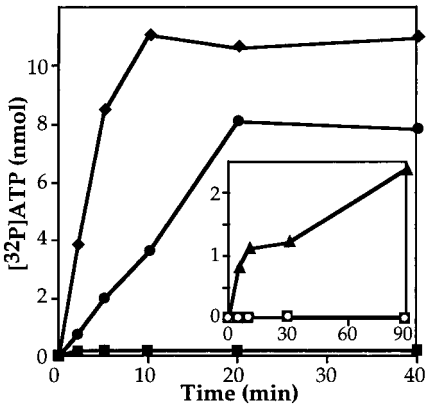

FIGURE 1: Cys-AMP and Pro-AMP synthesis by *M. jannaschii* wild-type ProCysRS as measured in the ATP−PPi exchange reaction. The assay conditions are described under Materials and Methods. Pro-AMP (◆) was measured in the presence of 2 mM proline and 365 nM enzyme. Cys-AMP was measured in the presence (●) or absence (■) of unfractionated *M. jannaschii* tRNA (3 μg/μL); the cysteine concentration was 2 mM (●) or 20 mM (■). Inset: Cys-AMP formation in the presence of 2 mM cysteine with pure tRNA samples and 0.36 μM *M. jannaschii* ProCysRS. *M. jannaschii* $tRNA^{Cys}$, 2 μM (▲); *M. jannaschii* $tRNA^{Pro}$, 4 μM (□); *E. coli* $tRNA^{Lys}$, 3.2 μM (○).

15% (v/v) perchloric acid], rinsed with 15 mL of water on Whatman GF/C fiber glass filter disks, dried, and liquid-scintillation-counted. The k_{cat} values were determined at 70 °C in the presence of saturating ATP and amino acid concentrations (10−100 × K_M), 5−10 μM each $tRNA^{Cys}$ and $tRNA^{Pro}$ in *M. jannaschii* total tRNA, respectively, and 0.15−0.5 μM *M. jannaschii* ProCysRS.

Active Site Titration. Formation of Pro∼AMP:ProCysRS complexes took place in 50 μL of standard aminoacylation mixture containing 0.5 unit of inorganic pyrophosphatase and 680 μM [^{14}C]proline (100 cpm/pmol), in the presence of varying concentrations of ProCysRS (1−10 μM). After 10 and 20 min incubation at 30 °C, aliquots of 20 μL were spotted onto nitrocellulose filters, filtered, and washed 4 times with 5 mL of 50 mM BisTris-HCl (pH 6.0), 10 mM $MgCl_2$. The filters were dried and liquid-scintillation-counted.

Site-Directed Mutagenesis of the M. jannaschii proS Gene. Oligonucleotide primers (20−25 nt in length) were designed for creation of the mutant *M. jannaschii proS* genes by PCR. After amplification, the genes were cloned into the TOPO-TA vector, and the mutant character of the genes was confirmed by DNA sequencing. The genes carrying the mutations were subcloned into the pET15b expression vector and used to transform *E. coli* BL21-CodonPlus competent cells.

RESULTS

Cysteine Activation. M. jannaschii ProCysRS activates proline in the absence of tRNA while it requires tRNA for cysteine activation (*2*). As there is some disagreement on this point (*9*), we resequenced our *M. jannaschii proS* clone and ascertained that it had the correct (MJ1238) sequence. The overexpressed pure ProCysRS enzyme was not able to activate cysteine under the conditions (2 mM Cys) used earlier (Figure 1); even cysteine concentrations at up to 20 mM as well as increasing amounts of enzyme did not bring about Cys-AMP formation in the absence of tRNA. In addition, a 10-fold excess of dithiothreitol to prevent disulfide formation of the cysteine thiol group did not change the results (data not shown). However, in the presence of unfractionated *M. jannaschii* tRNA, cysteine-dependent ATP−PP_i exchange took place easily (Figure 1). To determine the nature of the tRNA species required for cysteine activation, we purified by EF-Tu affinity chromatography *M. jannaschii* $tRNA^{Cys}$ and $tRNA^{Pro}$. Only pure homologous $tRNA^{Cys}$ induced Cys-AMP synthesis, while *M. jannaschii* $tRNA^{Pro}$ or *E. coli* $tRNA^{Lys}$ could not do so (Figure 1, inset), suggesting that $tRNA^{Cys}$ is the tRNA mediating cysteine recognition. This provides a plausible mechanism of discrimination between the two amino acids 'cognate' to this dual-specificity synthetase.

Table 1: Kinetic Constants of *M. jannaschii* ProCysRS in ATP−PP_i Exchange and tRNA Aminoacylation[a]

		ProRS activity		CysRS activity	
		ATP−PP_i exchange[b]	tRNA amino-acylation	ATP−PP_i exchange[c]	tRNA amino-acylation
amino acid	K_M (μM)	285	27	90	22
ATP	K_M (μM)	465	49	390	60
	k_{cat} (s^{-1})	4.8	0.09	0.04	0.02

[a] The conditions are described under Materials and Methods. [b] ATP−PP_i exchange was conducted in the absence of tRNA. [c] ATP−PP_i exchange was conducted in the presence of tRNA.

Kinetic Parameters for tRNA Prolylation and Cysteinylation by Wild-Type ProCysRS. The kinetic constants (Table 1) were determined (see Materials and Methods) in both the aminoacylation and ATP−PP_i exchange reactions for the CysRS and ProRS activities of the enzyme. The K_M values for proline and cysteine in the activation reaction, 285 and 90 μM, respectively, are in the range commonly observed for *E. coli* ProRS [300 μM (*6*)] and for *E. coli* and *Paracoccus denitrificans* CysRSs [50 μM (*12*) and 12.5 μM (*13*)]. In the aminoacylation reaction, K_M values of 27 and 22 μM were determined for proline and cysteine, respectively. Again, these values are consistent with those determined for CysRS enzymes from bacteria and from rabbit liver [11 μM (*14*)]. The K_M values for ATP in ATP−PP_i exchange are similar for both ProRS and CysRS activities (465 and 390 μM, respectively) and are 9.5 and 6.5 times higher than the K_M values obtained for proline and cysteine in the tRNA aminoacylation assay, a fact also seen in other AARSs (*15*). As far as catalytic constants are concerned, the k_{cat} of Pro-AMP formation (4.8 s^{-1}) is 120 times higher than the k_{cat} of Cys-AMP formation. However, the k_{cat} of Pro-tRNA synthesis (0.09 s^{-1}) is only 4.5 times higher than the value for Cys-tRNA formation (0.02 s^{-1}); thus, both aminoacyl-tRNAs are formed with comparable rates and in the general range observed for aminoacyl-tRNA synthetases (*16*, *17*).

Are Additional Factors Needed for Optimal CysRS Activity? It was suggested that Cys-tRNA formation by *M. jannaschii* ProCysRS may require additional components for efficient catalysis (*9*). We therefore compared the rates for Pro-tRNA and Cys-tRNA formation in a *M. jannaschii* S-100 cell extract (Figure 2). Under optimal substrate concentrations, the rate of Pro-tRNA synthesis is about 3−5 times higher than that of Cys-tRNA formation. This is similar to the properties of the pure enzyme where the k_{cat} for Pro-tRNA formation is 4.5 times higher than that for Cys-tRNA

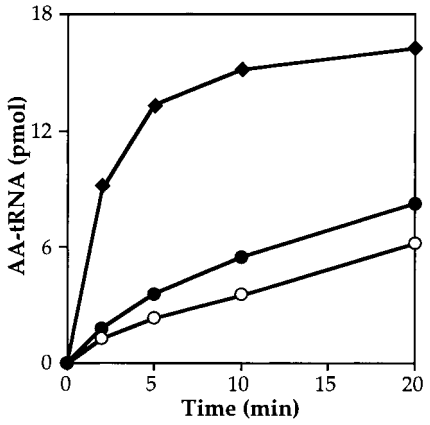

FIGURE 2: Effect of pyrophosphatase on ProCysRS activity in a *M. jannaschii* S-100 cell-free extract. Pro-tRNA (◆) formation was measured in the absence of inorganic pyrophosphatase, and Cys-tRNA formation was measured in the presence (○) or absence (●) of inorganic pyrophosphatase (10 units/mL). The reaction contained 250 μM [^{3}H]proline or [^{35}S]cysteine, 1.2 μg/μL unfractionated *M. jannaschii* tRNA, and 3.2 μg/mL *M. jannaschii* S-100.

formation (see above). Inorganic pyrophosphate is known to inhibit the activities of some AARSs (*18*); consequently, the addition of pyrophosphatase increases aminoacylation efficiency (*19*). Therefore, we investigated the effect of pyrophosphatase on the rate as well as on the plateau of Cys-tRNA formation. However, addition of yeast inorganic pyrophosphatase does not increase the rate or plateau of cysteine charging by ProCysRS either in S-100 extracts (Figure 2) or by the pure enzyme (data not shown). The efficiency of in vitro Cys-tRNA formation by the *M. jannaschii* and the *Giardia lamblia* ProCysRS proteins (*20*), and the fact that the cloned *proS* gene from *M. jannaschii* (*2*) and *Giardia lamblia* (*20*) complements a CysRS-deficient *E. coli* strain (*21*), demonstrates that viable Cys-tRNA synthesis can be performed by ProCysRS alone. Nevertheless, the possibility of a factor that enhances the observed efficient CysRS activity can never be ruled out with certainty.

ProCysRS Mutants Suggest Overlapping Amino Acid Binding Sites. A Clustal X alignment of 22 bacterial, 3 eukaryal, and 12 archaeal ProRS sequences facilitated the identification of the 3 characteristic motifs of this class II AARS. Since residues preceding motif 2 in class II enzymes are usually involved in amino acid binding, we examined this region in more detail. As can be seen (Figure 3), all archaeal and some bacterial/eukaryal ProRS proteins have a strictly conserved sequence, RPTSE (positions 99−103 in the *M. jannaschii* protein). As a first attempt, these residues were mutated to alanine, and those conserved only in the archaeal ProRS sequences were mutated to the most frequent amino acid present at the same position in the bacterial and eukaryal enzymes. Of the mutants made, we selected two informative examples, P100A and E103A, for further study. The corresponding genes were overexpressed and the mutant proteins purified. Thermostability of the mutant enzymes was unaltered, since initial velocities for tRNA cysteinylation or prolylation were unchanged after a 20 min heat treatment at 70 °C (data not shown). A direct comparison of their tRNA cysteinylation and prolylation activities was made using the same concentrations (250 nM) of mutant and wild-type enzyme and substrate concentrations that were optimal for measuring $V_{i,max}$ with the wild-type enzyme. Compared to

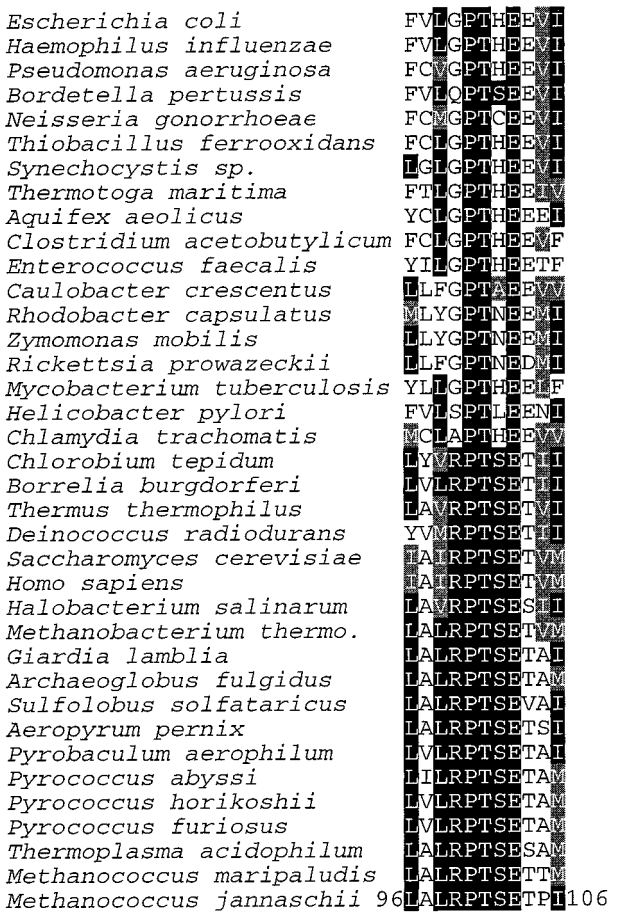

FIGURE 3: Alignment of the amino acid sequences of 37 ProRS enzymes. The region of interest for the mutant enzymes (P100A, E103A) is shown. The full-length protein sequences were aligned with CLUSTAL X (V1.84). Black shading denotes at least 50% amino acid identity, while gray signifies at least 50% homology.

wild-type ProCysRS, the P100A enzyme retained all its prolylation efficiency while it lost 90% of its cysteinylation capability (Figure 4A). Conversely, enzyme E103A does not show any detectable ProRS activity but still possesses 5% of the wild-type's CysRS activity (Figure 4A). To ascertain that the mutant enzymes are indeed affected in their amino acid binding abilities, we performed the same comparative study with the ATP−PP$_i$ exchange reaction. The results (Figure 4B) are identical to those of the aminoacylation reaction. Therefore, it is probable that these mutational changes affect the structure of the amino acid binding sites.

We reasoned that if these mutational changes affect the enzyme's amino acid binding site(s) we should see this reflected in the results of amino acid inhibition on tRNA charging. Cysteinylation by ProCysRS is inhibited by proline while cysteine interferes with prolylation (*2*). Thus, if as inferred from the results above (Figure 4A) enzyme P100A is weakened in cysteine binding, its prolylation activity should then be less affected by cysteine inhibition compared to wild-type. Likewise mutant E103A is no longer able to bind proline but still has the ability to bind cysteine; thus, a large excess of proline should not readily inhibit the enzyme's cysteinylation activity. Our expectation was borne out (Figure 5A), a 40-fold excess of proline over cysteine inhibits the CysRS activity of wild-type ProRS by over 80% whereas that of mutant E103A is unaltered. The ProRS activity of mutant P100A is barely inhibited by a 40-fold

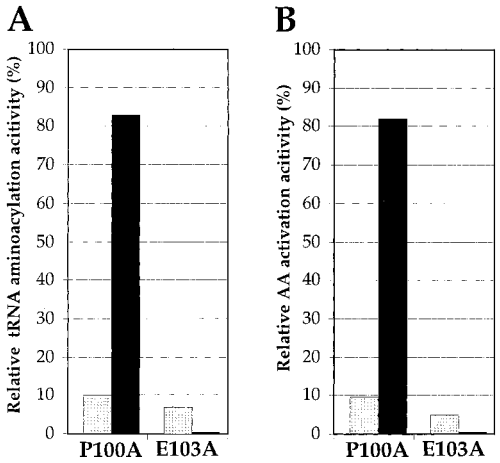

FIGURE 4: Bar graph representation of relative $V_{i,max}$ values of CysRS and ProRS activities of mutant ProCysRS enzymes determined in the aminoacylation and ATP−PP_i exchange reactions. Cys-tRNA (gray bars) and Pro-tRNA (black bars) formation (A) by P100A and E103A, and Cys-AMP (gray bars) and Pro-AMP (black bars) formation (B) by P100A and E103A. 100% activity corresponds to 1.2 pmol of Cys-tRNA or 12.5 pmol of Pro-tRNA, and 0.25 nmol of Cys-AMP or 5.5 nmol of Pro-AMP formed per minute. The assay conditions were those described under Materials and Methods for the wild-type enzyme; concentrations of wild-type and mutant enzyme were 250 nM; of ATP, 2 mM in ATP−PP_i exchange and 10 mM in aminoacylation; and of Cys or Pro, 3 mM in ATP−PP_i exchange and 250 μM in aminoacylation.

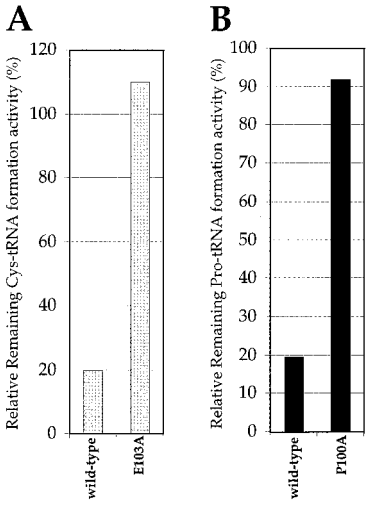

FIGURE 5: Inhibition study of [^{35}S]Cys-tRNA or [^{3}H]Pro-tRNA formation by wild-type or mutant ProCysRS, with an excess of unlabeled proline or cysteine. Aminoacylations were performed with wild-type and E103A mutant enzymes in the presence of 20 μM [^{35}S]cysteine and with or without a 40-fold excess of proline (A); or conversely with wild-type and P100A mutant enzymes in the presence of 20 μM [^{3}H]proline and with or without a 40-fold excess of cysteine (B). Reaction conditions allowed initial velocities to be measured. The remaining relative activities, represented as histograms, are expressed as percentages of the ratio between the initial velocity obtained with the excess of inhibitor over the initial velocity obtained without inhibitor. Wild-type and mutant enzyme concentrations were 250 nM.

excess of cysteine while the wild-type enzyme's ProRS activity is reduced to 20% (Figure 5B). These results show that the ProCysRS mutant enzymes E103A and P100A have lower affinities for proline and cysteine, respectively, than the wild-type ProCysRS. Thus, Glu103 in *M. jannaschii* ProCysRS is essential for proline recognition and Pro100 for cysteine binding. The fact that mutational changes in single amino acids located only three positions apart affect either proline or cysteine recognition indicates that structural elements of their binding sites are overlapping.

DISCUSSION

The surprising existence of a dual-specificity AARS, the archaeal genre ProRS (*22*), responsible for both Cys-tRNA and Pro-tRNA synthesis, raises questions about how one AARS correctly specifies two amino acids. Since this enzyme recognizes two different tRNAs and two different amino acids and prevents the formation of a mischarged Cys-$tRNA^{Pro}$ or Pro-$tRNA^{Cys}$ species, it will be an excellent experimental model for unraveling the mechanism underlying this exquisite substrate discrimination. As a first step, we investigated the activation of the cognate proline and cysteine and attempted to determine whether the amino acid binding sites (or sites for generating an allosteric signal) overlap. Site-directed mutagenesis of a selected region, suspected to be involved in defining the amino acid binding site(s), gave rise to mutant ProCysRS enzymes with drastic changes in their amino acid recognition. The single Glu103→Ala103 mutation resulted not only in the complete loss of ProRS activity but also in alteration of CysRS activity. Glu103 may be part of the proline binding site in *M. jannaschii* ProCysRS, as the crystal structure of *Thermus thermophilus* ProRS indicates that this conserved amino acid (Figure 3) is directly involved in binding the imino group of proline (M. Tukalo, personal communication). In contrast, the Pro100→Ala100 mutation resulted in a 10-fold decrease of CysRS activity, but it did not change ProRS activity. Further clear support that these mutants (P100A and E103A) are indeed affected in their ability to bind either of the two amino acids derives from an inhibition study. Mutant P100A is only altered in its ability to form Cys-AMP, presumably because cysteine binding is reduced. The fact that its ProRS activity is now insensitive to cysteine inhibition supports this assumption. The reverse can be seen from the data with mutant E103A, which is affected in proline binding. The close proximity (positions 100 and 103 in *M. jannaschii* ProCysRS) of these amino acids strongly suggests that structural elements of the proline and cysteine binding sites are overlapping.

Our data show a tRNA requirement, probably for $tRNA^{Cys}$, in the formation of Cys-AMP by the wild-type *M. jannaschii* ProCysRS. The same tRNA requirement is also seen for the *Giardia lamblia* ProCysRS enzyme (*20*). Recently, Lipman, Sowers, and Hou (*9*) published data on *M. jannaschii* ProCysRS somewhat at a variance with our earlier (*2*) and present results. They described a CysRS ATP−PP_i exchange activity able to proceed in the absence of tRNA, and a CysRS aminoacylation activity (relative to ProRS) about 10-fold lower than what we described above. Whether these apparent discrepancies arise from differences in experimental procedure could not be assessed as detailed experimental protocols were not given, either in the primary work (*23*) or in the publications referred to therein (*9*). Nevertheless, the reported data (*9*) provide some indications of possible sources of these discrepancies. The kinetic parameters of Cys-tRNA formation (Figure 3A, ref *9*) were determined outside the Michaelis−Menten range at an enzyme:substrate ratio of 1.9:1 (compared to 1:100 here), suggesting low enzyme activity. Furthermore, the results on tRNA dependence of the CysRS ATP−PP_i exchange activity are furnished as "data not shown" or

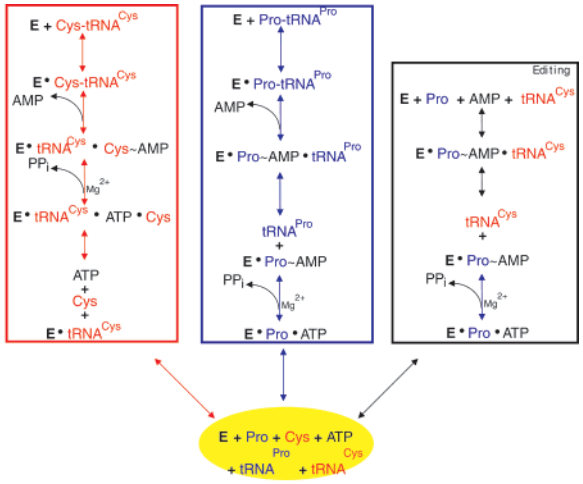

FIGURE 6: Schematic representation of the enzymatic mechanism of *M. jannaschii* ProCysRS. ProCysRS is abbreviated E, and the dots represent complexes between the enzyme and any of the substrates or intermediates of the reaction. The red and blue boxes outline the steps leading to Cys-tRNA^{Cys} and Pro-tRNA^{Pro} formation, respectively. The steps outlined in the black box represent the putative, suggested editing mechanism that explains the lack of Pro-tRNA^{Cys} release by *M. jannaschii* ProCysRS. The data in this paper provide support for the reactions in the left and center panels. The proposed editing reactions need to be investigated.

presented in a figure (Figure 2A, ref *9*) whose individual curves have very different or missing scales. Judging by their two reports (*9*, *23*), our *M. jannaschii* tRNA preparations are 14- or 3-fold better with respect to tRNA^{Cys} levels. Perhaps critically, no characterization of the clone that provided the source of the enzyme is given. In light of our findings that single nucleotide changes in the *proS* gene can 'eliminate' one or the other activity of ProCysRS, sequence determination of the clone used by Lipman et al. (*9*) is essential to guard against the inadvertent introduction of detrimental point mutations.

The rate of cysteine activation (0.04 s^{-1}) is almost equal to the overall reaction rate (0.02 s^{-1}), suggesting that activation is the rate-limiting step in Cys-tRNA formation. Once tRNA^{Cys} is bound, only cysteine can be activated and is immediately transferred to the tRNA. On the other hand, in the absence of tRNA, only proline can be activated, but its transfer onto tRNA^{Pro} is achieved with a 50-fold lower rate. Since the activation rate of proline is unaltered by the presence of tRNA (data not shown), selection of tRNA^{Pro} is critical and limits the prolylation reaction. It is still unknown whether tRNA^{Cys} can bind to the enzyme:Pro∼AMP complex. If this were the case, an editing mechanism (*24*−*29*) must exist that prevents synthesis and release of Pro-tRNA^{Cys} since we were unable to isolate this mischarged tRNA species. Existence of such an editing mechanism is suggested by the fact that when total *M. jannaschii* tRNA was charged with proline and subsequently oxidized by sodium periodate, no tRNA cysteinylation of the treated tRNA was observed after a deacylation step. If Pro-tRNA^{Cys} had been formed initially, this charged tRNA would have been protected against periodate oxidation that abolishes the acceptor capacity of any uncharged tRNA species. After deacylation, this tRNA would have been recharged to Cys-tRNA^{Cys}, since periodate treatment of protected tRNA^{Cys} does not affect the acceptor capacity of this tRNA (*2*).

A proposal for an enzymatic mechanism for Pro-tRNA and Cys-tRNA synthesis by ProCysRS is shown in Figure 6. In its ligand-free form, the active site is configured to allow only binding of proline (see left panel). Whether formation of Pro∼AMP induces a conformational change hindering tRNA^{Cys} binding or whether an editing mechanism (right panel) preventing release of Pro-tRNA^{Cys} exists, remains to be determined. Binding of tRNA^{Cys} triggers an induced fit of the active site for only cysteine binding and activation that is immediately transferred onto tRNA^{Cys}. Periodate oxidation of tRNA^{Cys} shows that the tRNA molecule's acceptor function is not required for cysteine activation (data not shown), suggesting that anticodon binding is probably the initiating event for rearrangement of the active site for cysteine binding and activation.

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REFERENCES

1. Söll, D., and Ibba, M. (2000) *Annu. Rev. Biochem. 69*, 617−650.
2. Stathopoulos, C., Li, T., Longman, R., Vothknecht, U. C., Becker, H. D., Ibba, M., and Söll, D. (2000) *Science 287*, 479−482.
3. Bult, C. J., White, O., Olsen, G. J., Zhou, L., Fleischmann, R. D., Sutton, G. G., Blake, J. A., FitzGerald, L. M., Clayton, R. A., Gocayne, J. D., Kerlavage, A. R., Dougherty, B. A., Tomb, J. F., Adams, M. D., Reich, C. I., Overbeek, R., Kirkness, E. F., Weinstock, K. G., Merrick, J. M., Glodek, A., Scott, J. L., Geoghagen, N. S. M., and Venter, J. C. (1996) *Science 273*, 1058−1073.
4. Smith, D. R., Doucette-Stamm, L. A., Deloughery, C., Lee, H., Dubois, J., Aldredge, T., Bashirzadeh, R., Blakely, D., Cook, R., Gilbert, K., Harrison, D., Hoang, L., Keagle, P., Lumm, W., Pothier, B., Qiu, D., Spadafora, R., Vicaire, R., Wang, Y., Wierzbowski, J., Gibson, R., Jiwani, N., Caruso, A., Bush, D., Reeve, J. N., et al. (1997) *J. Bacteriol. 179*, 7135−7155.
5. Yarus, M. (2000) *Science 287*, 440−441.
6. Stehlin, C., Heacock, D. H., Liu, H., and Musier-Forsyth, K. (1997) *Biochemistry 36*, 2932−2938.
7. Schimmel, P., and Schmidt, E. (1995) *Trends Biochem. Sci. 20*, 1−2.
8. Hong, K. W., Ibba, M., Weygand-Durasevic, I., Rogers, M. J., Thomann, H. U., and Söll, D. (1996) *EMBO J. 15*, 1983−1991.
9. Lipman, R. S. A., Sowers, K. R., and Hou, Y.-M. (2000) *Biochemistry 39*, 7792−7798.
10. Becker, H. D. (1998) Ph.D. Thesis, University Louis Pasteur, Strasbourg.
11. Becker, H. D., and Kern, D. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 12832−12837.
12. Fersht, A. R., and Dingwall, C. (1979) *Biochemistry 18*, 1245−1249.
13. Burnell, J. N., and Whatley, F. R. (1976) *Biochim. Biophys. Acta 481*, 266−278.
14. Motorin, Y., and Waller, J.-P. (1998) *Biochimie 80*, 579−590.
15. Becker, H. D., Reinbolt, J., Kreutzer, R., Giegé, R., and Kern, D. (1997) *Biochemistry 36*, 8785−8797.
16. Lui, M., Chakraburtty, K., and Mehler, A. H. (1978) *J. Biol. Chem. 253*, 8061−8064.
17. Lovgren, T. N. E., Pastuszyn, A., and Loftfield, R. B. (1976) *Biochemistry 15*, 2533−2540.

18. Dignam, J. D., and Deutscher, M. P. (1979) *Biochemistry 18*, 3165−3170.
19. Khvorova, A., Motorin, Y., and Wolfson, A. (1999) *Nucleic Acids Res. 27*, 4451−4456.
20. Bunjun, S., Stathopoulos, C., Graham, D., Min, B., Kitabatake, M., Wang, A. L., Wang, C. C., Vivarès, C. P., Weiss, L. M., and Söll, D. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 12997−13002.
21. Bohman, K., and Isaksson, L. A. (1979) *Mol. Gen. Genet. 176*, 53−55.
22. Woese, C. R., Olsen, G. J., Ibba, M., and Söll, D. (2000) *Microbiol. Mol. Biol. Rev. 64*, 202−236.
23. Hamann, C. S., Sowers, K. R., Lipman, R. S. A., and Hou, Y.-M. (1999) *J. Bacteriol. 181*, 5880−5884.
24. Baldwin, A. N., and Berg, P. (1966) *J. Biol. Chem. 241*, 839−845.
25. Fersht, A. R. (1977) *Biochemistry 16*, 1025−1030.
26. Fersht, A. R., and Dingwall, C. (1979) *Biochemistry 18*, 2627−2631.
27. Fersht, A. R., and Kaethner, M. M. (1976) *Biochemistry 15*, 3342−3346.
28. Lin, L., Hale, P. H., and Schimmel, P. (1996) *Nature 384*, 33−34.
29. Schmidt, E., and Schimmel, P. (1994) *Science 264*, 265−267.

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