Methanocaldococcus jannaschii prolyl-tRNA Synthetase Charges tRNA\textsuperscript{Pro} with Cysteine\textsuperscript{*}

Received for publication, July 11, 2002, and in revised form, July 16, 2002
Published, JBC Papers in Press, July 18, 2002, DOI 10.1074/jbc.M206929200

Alexandre Ambrogetti, Ivan Ahel, Carla Polycarpo, Shipra Bunjan-Srihari, Bethany Krett, Clarisse Jacquin-Becker, Benfang Ruan, Caroline Köhler, Constantinos Stathopoulos, Uttam L. RajBhandary, and Dieter Söll\textsuperscript{**}

The Journals of Biological Chemistry Vol. 277, No. 38, Issue of September 20, pp. 34749–34754, 2002
Printed in U.S.A.

Methanocaldococcus jannaschii prolyl-tRNA synthetase (ProRS) was previously reported to also catalyze the synthesis of cysteinyl-tRNA\textsuperscript{Cys} (Cys-tRNA\textsuperscript{Cys}) to make up for the absence of the canonical cysteinyl-tRNA synthetase in this organism (Stathopoulos, C., Li, T., Longman, R., Vorhees, U. C., Becker, H., Ibba, M., and Söll, D. (2000) Science 287, 479–482; Lipman, R. S., Sowers, K. R., and Hou, Y. M. (2000) Biochemistry 39, 7792–7798). Here we show by acid urea gel electrophoresis that pure heterologously expressed recombinant \textit{M. jannaschii} ProRS misaminoacylates \textit{M. jannaschii} tRNA\textsuperscript{Pro} with cysteine. The enzyme is unable to aminoclayt purified mature \textit{M. jannaschii} tRNA\textsuperscript{Cys} with cysteine in contrast to facile aminoacylation of the same tRNA with cysteine by \textit{Methanococcus maripaludis} cysteinyl-tRNA synthetase. Although \textit{M. jannaschii} ProRS catalyzes the synthesis of Cys-tRNA\textsuperscript{Cys} readily, the enzyme is unable to edit this misaminoacylated tRNA. We discuss the implications of these results on the in vivo activity of the \textit{M. jannaschii} ProRS and on the nature of the enzyme involved in the synthesis of Cys-tRNA\textsuperscript{Cys} in \textit{M. jannaschii}.

Cysteinyl-tRNA\textsuperscript{Cys} (Cys-tRNA\textsuperscript{Cys}) is an essential component of the translational machinery of all organisms for the incorporation of cysteine into proteins. Interestingly, an analysis of the genome sequences of the methanogenic archaea \textit{Methanocaldococcus jannaschii} (1), \textit{Methanothermobacter thermautotrophicus} (2), and \textit{Methanopyrus kandleri} (3) revealed the absence in these organisms of an open reading frame encoding a canonical cysteinyl-tRNA synthetase (CysRS), the enzyme that synthesizes Cys-tRNA\textsuperscript{Cys} (4, 5). This raised the question of how Cys-tRNA\textsuperscript{Cys} is synthesized in these methanogenic archaea. The reports that purified recombinant \textit{M. jannaschii} prolyl-tRNA synthetase (ProRS) was able to aminoclaylate tRNA\textsuperscript{Cys} with cysteine provided an apparent answer to this question (6–11). This led to the notion of ProRS as an unusual dual specificity enzyme capable of attaching proline to tRNA\textsuperscript{Pro} and cysteine to tRNA\textsuperscript{Cys} (6, 7); thus, the enzyme was named ProCysRS (12). The finding (6, 9) that unmodified tRNA\textsuperscript{Cys}, produced by in vitro transcription of the corresponding tRNA\textsuperscript{Cys} gene, could not be aminoclaylated with cysteine by \textit{M. jannaschii} ProCysRS suggested that nucleotide modifications present in the mature \textit{M. jannaschii} tRNA\textsuperscript{Cys} were essential for correct aminoclaylation with cysteine (11) as shown before for some tRNAs (13–15). However, much of the previous work was done with unfractionated \textit{M. jannaschii} tRNA. Our recent finding that cysteine activation is an inherent property of ProRS enzymes (43) prompted us to undertake an in-depth investigation of the tRNA specificity of archaeal prolyl-tRNA synthetases as exemplified by the \textit{M. jannaschii} enzyme. Here we show that a purified recombinant form of the \textit{M. jannaschii} enzyme, previously called ProCysRS, misaminoacylates tRNA\textsuperscript{Pro} with cysteine but is unable to aminoacylate tRNA\textsuperscript{Cys} with cysteine. These properties of \textit{M. jannaschii} ProRS raise the question of the true in vivo activities of this enzyme. In view of these results, we have renamed the enzyme ProRS in this paper.

**Experimental Procedures**

General—Oligonucleotide synthesis and DNA sequencing were performed by the Kecf Foundation Research Biotechnology Resource Laboratory at Yale University. The TOPO-TA cloning kit was from Invitrogen. pET-15b expression vector was purchased from Novagen. Epicurian coli\textsuperscript{BL21-CodonPlus}\textsuperscript{Tm} competent cells were from Stratagene. [\textsuperscript{35S}]Cysteine (1075 Ci/mmol) was from PerkinElmer Life Sciences, and [\textsuperscript{3}H]proline (104 Ci/mmol), [\textsuperscript{14}C]proline (516 cpm/pmol), \textsuperscript{[3H]alanine (52 Ci/mmol), [\textsuperscript{32}P]ATP, and (\textsuperscript{[\textsuperscript{3]}P\textsuperscript{32}]P)ATP (10 Ci/mmol) were from Amersham Biosciences. Nitrocellulose filters (0.45 \textmu m) were from Schleicher & Schuell. Cysteine analogues were from Sigma. Plasmid T7–911 for preparation of the His\textsubscript{6}-tagged recombinant T7 RNA polymerase was a gift from T. Shadr (Albert Einstein College of Medicine, New York, NY).

Isolation of tRNA and tRNA Transcripts—Isolation of T7 RNA polymerase transcripts of tRNA\textsuperscript{Pro} and \textit{M. jannaschii} total tRNA was carried out as described previously (6, 8).

Purification of tRNA\textsuperscript{Pro} and tRNA\textsuperscript{Cys} by Affinity Chromatography—Individual Cys-tRNA\textsuperscript{Cys} and Cys-tRNA\textsuperscript{Cys} samples were purified by affinity chromatography on immobilized \textit{Thermus thermophilus} EF1A-GTP as described previously (16). Unfractionated \textit{M. jannaschii} tRNA was aminoacylated with [\textsuperscript{35S}]Cysteine using either ProRS or \textit{M. maripaludis} CysRS. The resulting Cys-tRNA was separated from the uncharged tRNA by the formation of a ternary complex with \textit{T. thermophilus} EF1A-GTP immobilized on a Ni-NTA-agarose column. After elution of Cys-tRNA and removal of cysteine by decylation (30 min at 37 °C in 0.2 M Tris acetate, pH 9.0), the tRNAs were found to be

*This work was supported in part by the NIGMS, National Institutes of Health Grants GM22854 (to D. S.) and GM17151 (to U. L. R.), grants from the Department of Energy (to D. S.) and the National Aeronautics and Space Administration (to D. S.), and the U. S. Army Research Office Grant DAAD19-199-1-0300 (to U. L. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**To whom correspondence should be addressed: Dept. of Molecular Biophysics and Biochemistry, Yale University, P. O. Box 208114, 266 Whitney Ave., New Haven, CT 06520-8114 and the §Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

\textsuperscript{*} The abbreviations used are: Cys-tRNA \textsuperscript{Cys}, cysteinyl-tRNA \textsuperscript{Cys}; CysRS, cysteinyl-tRNA synthetase; ProRS, prolyl-tRNA synthetase; Ni-NTA, nickel-nitrilotriacetic acid; ProRS\textsubscript{A50}, carboxyl-terminal deletion of \textit{M. jannaschii} ProRS.
highly purified as judged by amino acid acceptor activity (1440 pmol/Am100 for tRNA Pro and 1040 pmol/Am150 for tRNA Pro).

Wild type and Mutant ProRS and CysRS Gene Constructs and Purification of Enzymes—The M. jannaschii proS gene and the M. maripaludis cysS gene previously cloned into PET15b expression vector (6, 17) were used. A 3-`terminal deletion of M. jannaschii ProRS (named ProRS 550) was constructed by the removal of 50 carboxy-terminal amino acids. The PCR product was cloned into TOPO-TA vector, sequenced, and finally subcloned into PET15b vector for overexpression. The His6-tagged M. jannaschii ProRS and ProRS 550 as well as His6-tagged M. maripaludis CysRS (17) were obtained by overexpression in Escherichia coli and purified by Ni-NTA chromatography. These enzymes are referred to as recombinant enzymes, because they are derived by heterologous expression of cloned genes. Protein concentration was measured by the Bradford assay (Bio-Rad) using bovine serum albumin as standard. The enzyme preparations were >95% pure as judged by SDS-PAGE followed by staining with Coomassie Brilliant Blue.

Aminoacylation of tRNA—The standard reaction mixture contained 50 mM HEPES-KOH, pH 7.0, 50 mM KCl, 15 mM MgCl2, 5 mM dithiothreitol, 10 mM ATP, 50 mM [35S]Cysteine, 50 mM [3H]Proline or 500 mM [3H]alanine, 1 mg/ml unfraccionated M. jannaschii tRNA (unless specifically otherwise indicated, and either M. jannaschii ProRS or M. maripaludis CysRS to a final volume of 120 ml. The reactions were incubated at 50 °C for the time specified in the figure.

Active Site Titration of M. jannaschii ProRS—For active site titration (18), the enzyme preparations were incubated in 100 ml Tris-HCl, pH 7.5, 50 mM KCl, 50 mM MgCl2 with inorganic pyrophosphatase (1 mg/ml) (Roche Molecular Biochemicals), ATP (final concentration 4 mM), and [3H]Proline (final concentration 19 mM) in a volume of 140 ml at 50 °C. Samples (30 ml) were taken at various time points.

Acid Urea Gel Electrophoresis of tRNA and Aminocytic-tRNA—This method (19) allows the separation of charged from uncharged tRNA attributed to a difference in electrophoretic mobility between the two species. Hybridization of a sequence-specific probe permits the determination of the identity of the aminoacylated tRNA on the gel. Unfractionated M. jannaschii tRNA was aminoacylated either with proline (by M. jannaschii ProRS) or cysteine (by M. jannaschii ProRS or M. maripaludis CysRS) as described previously (6, 19). After phenol extraction and ethanol precipitation, the aminoacyl-tRNAs were dissolved in 10 mM sodium acetate, pH 4.5, 1 mM Na-EDTA at a final concentration of 7 M urea and run at 4 °C, 600 V in 0.1 M sodium acetate, pH 5.0, for 48 h. The portion of the gel containing the tRNAs was electroblotted onto a Hybond N membrane (Amersham Biosciences) using a Hoefer Electroblot apparatus at 10 V for 15 min and then at 30 V for 2 h with 10 mM Tris acetate, pH 8.0, 5 mM sodium acetate, and 0.5 mM Na-EDTA as transfer buffer. The membranes were then baked at 95 °C for 2 h. The tRNAs were detected by hybridization to a 5-`-32P-labeled oligodeoxyribonucleotide probe. The probes were complementary to nucleotides 1–21 and 26–51 of tRNA Pro and tRNA Pro, respectively.

Replacement of the 3-`terminal Adenosine of tRNAs by 2-Deoxyadenosine or 3-`Deoxyadenosine—Replacement of the terminal adenosine of M. jannaschii tRNA Pro (A31 = 12.5 units) by 2-deoxyadenosine, 3-`deoxyadenosine, or adenosine was performed as described previously (20–22) and consisted of the following steps: periodate oxidation (50 mM sodium acetate, pH 6.5, 15 mM sodium periodate) for 2 h in the dark at 37 °C followed by the removal of excess periodate (addition of 0.2% glucose at 25 °C for 30 min in the dark), β-elimination (250 mM l-lysine, pH 9.0, for 4 h at 25 °C), dephosphorylation (50 mM Tris-HCl, pH 9.0, 1 mM MgCl2, 25 units of bacterial alkaline phosphatase for 90 min at 37 °C), and reconstruction of the 3-`terminal residue (20 mM 2-`-dATP, 3-`-dATP, or ATP in presence of yeast tRNA CCA-nucleotidyltransferase in 100 mM Tris-HCl, pH 9.0, 50 mM KCl, 25 mM MgCl2, 2 mM dithiothreitol, and 0.1 mg/ml bovine serum albumin). tRNAs were extracted with phenol/chloroform (1:1), precipitated with ethanol in the presence of glyogen (American Scientific), and resuspended in sterile water. The same procedure was followed to isolate the 2-`deoxyadenosine-containing tRNAs starting from unfraccionated M. jannaschii tRNA. The tRNAs reconstructed with ATP had similar charging levels compared with the original tRNA Pro transcript (proline charging, 300 pmol/A145) or the unfraccionated tRNA (cysteine charging, 20 pmol/A260).

Assays for Post-transfer Editing by M. jannaschii ProRS—Mischarged Cys-tRNA Pro and Ala-tRNA Pro were generated by the charging of unfraccionated M. jannaschii tRNA with M. jannaschii ProRS at 50 °C in 15 min in standard reaction buffer (23) with 50 mM [35S]cysteine and 500 mM [3H]alanine, respectively. After phenol and chloroform extraction, aminoacyl-tRNA was precipitated with ethanol and the precipitate was washed and dried. Deacetylation assays were performed as described previously (24) at 37 °C in the presence of 4.5 mM enzyme (M. jannaschii ProRS, M. maripaludis CysRS) for 2 h. No enzyme or 1.2 M NaOH was used as negative and positive controls, respectively.

Inhibition of Aminoacylation by Cysteine Analogues—Ten different cysteine analogues were tested as inhibitors using standard aminoacylation conditions: [35S]Cysteine (50 μM), inhibitor (1 mM), M. jannaschii ProRS (300 nM), and total M. jannaschii tRNA (1 mg/ml) for 20 min at 50 °C. The t-cysteine sample contains <0.3% L-cysteine.

Activation of Cysteine Analogues in ATP-PP Exchange—ATP-PP exchange reactions were performed as described previously (6). Reactions were conducted in 50 mM HEPES-NaOH, pH 7.2, 10 mM MgCl2, 50 mM potassium fluoride, 5 mM dithiothreitol, 2 mM KF, 2 mM ATP, 2 mM 3PP, (2 ppm/pmol), 1 μM M. jannaschii ProRS, 2 mM L-cysteine, or α-cysteine, homocysteine or L-cysteine methyl ester (200 mM each) in a final volume of 300 μl at 50 °C. [32P]ATP formation was followed by specific absorption on acid-washed Norit (200 μl of a 1% suspension (v/v) of Norit in a solution of 0.4 mM sodium pyrophosphate solution containing 15% (v/v) perchloric acid), filtration on Whatman GF/C filters followed by washing with 15 ml of water and 5 ml of ethanol.

RESULTS

M. jannaschii ProRS Aminoacylates tRNA Pro with Cysteine—Acid urea gel electrophoresis has been a reliable tool to determine the aminoacylation specificity of aminoacyl-tRNA synthetases (19). The method is based on the electrophoretic separation of tRNA and aminoacyl-tRNA and subsequent hybridization with a sequence-specific oligonucleotide to determine the identity of the aminoacylated tRNA. To identify which of the M. jannaschii tRNAs were being aminoacylated by the recombinant M. jannaschii ProRS, we aminoacylated unfraccionated M. jannaschii tRNAs with the ProRS in the presence of proline or cysteine. Following acid urea gel electrophoresis, the tRNAs that were aminoacylated were identified by Northern blot analysis using probes specific for tRNA Pro (Fig. 1A) or tRNA Cys (Fig. 1B). A positive control for aminoacylation of tRNA Pro, recombinant M. maripaludis CysRS was used in a separate aminoacylation experiment. Figure 1A shows the results. M. jannaschii ProRS charges proline onto tRNA Pro (Fig. 1A, lane 6) but not onto tRNA Cys (Fig. 1B, lane 10). M. jannaschii ProRS also aminoacylates tRNA Pro essentially quantitatively with cysteine (Fig. 1B, lane 12). The tRNA Cys in the unfraccionated tRNA preparation used is fully active, because it could not be charged with cysteine by M. jannaschii ProRS and M. maripaludis CysRS after acid-urea gel separation. The tRNAs were probed with [3P]-labeled oligonucleotides complementary to M. jannaschii tRNA Pro (A) and tRNA Cys (B). Bands indicated by arrows 1, 2, and 3 correspond to uncharged tRNA Pro, Cys-tRNA Pro, and Pro-tRNA Pro, respectively.
aschii tRNAPro and tRNA Cys isolated by EF1A affinity and confirmed by aminoacylation of purified preparations of M. jan
unfractionated M. jannaschii synthesis of Cys-tRNA Pro (M. maripaludis M. jannaschii tRNAPro (M. maripaludis CysRS). As expected, all of the aminoacyl-tRNAs could be deacylated to tRNA by treatment with base (Fig. 1). The tRNAs were modified (see “Experimental Procedures”) at the 3’ termini as follows: tRNA lacking the 3’-terminal adenosine (.), terminus reconstructed with 3’-deoxyadenosine (○), 2’-deoxyadenosine (□, and adenosine (□).

Site of amino acid attachment (2’ or 3’) in M. jannaschii Pro-tRNAPro (A) and Cys-tRNAPro (B). Time course of aminoacylation of different tRNAPro species with proline (A) and cysteine (B) using M. jannaschii ProRS. The percent of aminoacylation of the different tRNAPro species is compared with that of the original M. jannaschii tRNAPro transcript and unfractionated M. jannaschii tRNA. The tRNAs were modified (see “Experimental Procedures”) at the 3’ termini as follows: tRNA lacking the 3’-terminal adenosine (.), terminus reconstructed with 3’-deoxyadenosine (○), 2’-deoxyadenosine (□, and adenosine (□).

Site of Aminoacylation of the tRNAPro with Proline and Cysteine—tRNAPro molecules in which the terminal adenosine was replaced by 2’- or 3’-deoxyadenosines were used as substrates for the M. jannaschii ProRS to identify the site of attachment of the amino acid in Pro-tRNAPro and Cys-tRNAPro. The results (Fig. 3) show that both for aminoacylation with proline (Fig. 3A) and with cysteine (Fig. 3B), the tRNAPro transcript with the 2’-deoxyA at the 3’-end is a better substrate than the transcript with the 3’-deoxyA. Thus, the site of attachment of the amino acid is primarily the 3’-hydroxyl group. There is also amino attachment on the 2’-hydroxyl group, in line with the earlier data on E. coli ProRS (20–22). This conclusion agrees with the fact that class II aminoacyl-tRNA synthetases transfer the amino acid onto the 3’-hydroxyl group of the terminal adenosine of tRNA, whereas the class I enzymes transfer it to the 2’-hydroxyl group (25, 26).

Recognition of Cysteine by ProRS—Because M. jannaschii ProRS synthesizes Cys-tRNAPro and because misaminoacyla-
tion of tRNA<sub>Pro</sub> with cysteine in vitro appears to be a general feature of ProRSs (43), it was of interest to investigate the recognition of cysteine analogues by the enzyme. Two complementary methods were used. First, the ability of non-radioactive cysteine analogues to inhibit Cys-tRNA<sub>Pro</sub> formation was evaluated. As seen in Table I, compounds with an altered carboxyl group (cysteamine, L-cysteine methyl ester, and S-methyl-L-cysteine), which did not inhibit aminoacylation, N-Acetyl-L-cysteine, D-cysteine, and DL-homocysteine were also found to be inhibitors. Second, the question of whether the inhibitors could be activated by M. jannaschii ProRS was investigated (Fig. 4). It was found that D-cysteine and DL-homocysteine were substrates in the ATP-PP<sub>i</sub> exchange reaction. These results suggest that the sulphydryl group and to a lesser extent the SH group with the amino group of cysteine are recognized by M. jannaschii ProRS. E. coli CysRS, co-crystallized with cysteine, shows a clear interaction of the amino group with the enzyme and the SH group with a protein-bound zinc atom (5).

**Does ProRS Decaylate Cys-tRNA<sub>Pro</sub> by Editing?**—Editing of the misacylated Ala-tRNA<sub>Pro</sub> by E. coli ProRS was shown to be highly efficient and ascribed to the existence of an insertion domain in the bacterial-type ProRSs compared with the archaeal ProRS (27). The archaeal type ProRS does not contain this insertion sequence but has an extension at the carboxyl terminus. Thus, the latter region could be a possible editing domain. Hydrolysis of a mutant Ala-tRNA<sub>Pro</sub> by M. jannaschii ProRS, although not as efficient as by the bacterial ProRS, was recently reported (23). Because this editing function might be extended to the hydrolysis of other mischarged tRNAs, we investigated whether the deletion of the carboxyl terminus of M. jannaschii ProRS would affect either the formation or the hydrolysis of Cys-tRNA<sub>Pro</sub> and Ala-tRNA<sub>Pro</sub>. We reasoned that the deletion of a possible editing domain would lead to an increased mischarging of cysteine and alanine onto unfractionated M. jannaschii tRNA.

First, we checked the ability of the M. jannaschii ProRSΔ50 enzyme (lacking the 50 carboxyl-terminal amino acids) to charge proline, cysteine, or alanine onto M. jannaschii tRNA<sub>Pro</sub>. There was no difference between the wild-type and the truncated ProRS in an initial velocity of aminoacylation for proline, although the mutant enzyme was less active for cysteine (~3-fold) and alanine (~10-fold) (Fig. 5). Second, we checked the editing activity of the wild-type ProRS or the ProRSΔ50 enzyme toward Cys-tRNA<sub>Pro</sub> (Fig. 6A) or Ala-tRNA<sub>Pro</sub> (Fig. 6B) in post-transfer editing experiments. There was no measurable decaylation of either of the substrates. Thus, under our experimental conditions, we could not show in M. jannaschii ProRS an editing mechanism for mature Cys-tRNA<sub>Pro</sub> or Ala-tRNA<sub>Pro</sub>. Whether the difference between our results and those of Beuning and Musier-Forsyth (23) is because of their use of a mutant Ala-tRNA<sub>Pro</sub> for the deacylation experiments is not known.

**DISCUSSION**

Based on the nature of the final aminoacyl-tRNA products, aminoacyl-tRNA synthetases may be divided into three groups. The first group, which comprises the majority of these enzymes, attaches the cognate amino acid to the cognate set of isoacceptor RNAs. These enzymes correct any misacytation of the amino acid or any misacylation (mischarging) of the tRNA by pre-transfer and/or post-transfer editing mechanisms (28). Another group consisting of the non-discriminating aspartyl-, glutamyl-, and seryl-tRNA synthetases recognizes the cognate amino acid yet charges it onto two different sets of tRNAs. These non-discriminating AspRS and GluRS enzymes provide the crucial first step, the production of mischarged tRNA, in the transamination pathway that leads to formation of Asn-tRNA and Gln-tRNA (29, 30). SerRS similarly is involved in the formation of selenocysteinyl-tRNA (31). Lastly, the archaeal ProRS described in this study may belong to a third type as it charges two canonical amino acids onto the same set of tRNAs, generating a correctly charged and a mischarged aminoacyl-tRNA. Mutant aminoacyl-tRNA synthetases with a compromised editing function (a valyl-tRNA synthetase forming Cys-tRNA<sub>Val</sub> and a leucyl-tRNA synthetase forming Ile-tRNA<sub>Leu</sub>) are known to do the same (32–34) and may also be placed in the third group.

How does the archaeal prolyl-tRNA synthetase recognize two canonical amino acids? Cysteine is well recognized by the M. jannaschii ProRS, which is significantly more mischarging than the ProRS enzymes of many other organisms (43). Earlier biochemical experiments showed that in M. jannaschii ProRS, the binding sites for proline and cysteine are overlapping in that the mutation of amino acid 100 greatly affected CysRS activity, whereas the mutation of amino acid 103 greatly affected ProRS activity (8). The crystal structure of M. jannaschii ProRS is consistent with this conclusion and shows that the proline binding pocket of this enzyme readily accommodates cysteine. Further support is derived from suggestions based on a modeling experiment that the archaeal-type T. thermophilus ProRS could also bind cysteine (35). Proline and cysteine have similar molecular volumes, although they have different shapes. This particular feature may explain the lack of speci-

---

TABLE I

| Compound                   | Inhibition % |
|----------------------------|--------------|
| Cysteamine                 | 70           |
| L-cysteine methyl ester    | 60           |
| L-cysteine ethyl ester     | 60           |
| d-cysteine                 | 50           |
| D,L-homocysteine           | 45           |
| N-acetyl L-cysteine        | 25           |
| L-sulfinic acid            | 0            |
| L-cysteic acid             | 0            |
| S-methyl L-cysteine        | 0            |
| Homocysteine thiolactone   | 0            |

*See “Experimental Procedures” for conditions.*
ficity of *M. jannaschii* ProRS for the two amino acids. Also, these two amino acids may be too similar to be distinguished by a double sieve editing mechanism (36). CysRS also must face the challenge of discriminating proline. The crystal structure of *E. coli* CysRS (5) shows that this enzyme has developed a different strategy to ensure accuracy of aminoacylation. The enzyme selects cysteine specifically by coordination of the sulfhydryl group with a zinc atom. As this mechanism ensures better specificity, the enzyme can dispense with an editing domain (5). A metal ion (Zn$^{2+}$/H11001)-based mechanism is also used by threonyl-tRNA synthetase to discriminate against the isosteric valine (37).

An alternative mechanism to increase discrimination for the cognate amino acid in aminoacylation of tRNA Pro was suggested for *T. thermophilus* ProRS (38). Based on the crystal structures of *T. thermophilus* ProRS complexed with tRNA Pro, proline, ATP, and prolyl-adenylate, it was proposed that the binding and activation of proline triggers structural changes in the active site that promote the correct binding of the 3’-end of tRNA$^{Pro}$ at the active site. Such an induced fit mechanism in which binding or activation of the cognate amino acid results in the enzyme adopting a fully ordered active site conformation necessary for aminoacylation also seems to be used by some other class IIa enzymes, e.g. *E. coli* histidyl-tRNA synthetase (39). In *T. thermophilus* ProRS, Ala-206 is one of the amino acids in the “proline binding loop” close to the active site, which undergoes rearrangement upon the activation of proline. This rearrangement results in the formation of a hydrogen bond between the main chain of Ala-206 and His-83 in the “ordering loop.”

Our unexpected finding that *M. jannaschii* ProRS misaminoacylates native tRNA$^{Pro}$ in *vivo* raises the question of whether this also occurs in *vivo* and, if so, whether any Cys-tRNA$^{Pro}$ that is formed is edited. Because proline and cysteine inhibit the aminoacylation of tRNA with each other by ProRS (6), the relative affinity of these amino acids for ProRS, their intracellular concentrations, their rates of aminoacyl adenylate formation, and rates of aminoacylation of tRNAs will all be important determinants for the relative amounts of Pro-tRNA Pro and Cys-tRNA Pro formed in *vivo*. In *vivo* studies with the *M. jannaschii* ProRS indicate that $k_{cat}/K_m$ for proline activation is 107-fold higher than in cysteine activation (43). Unfortunately, no data are available on the intracellular concentration of these amino acids in *M. jannaschii* (or in any other
formation of Cys-tRNA Cys (7). Attempts in identifying such a possibility is a recently described ProRS of this organism, similar to that described in this paper has been carried out with the ProRS of E. coli, that allows it to aminoacylate tRNA Cys with cysteine cannot, therefore, be ruled out. Alternatively, other RNA and/or protein factors along with M. jannaschii ProRS may be involved in the formation of Cys-tRNA Cys (7). Attempts in identifying such components by rescue of an E. coli cysS knock-out strain have not been successful. Additional possibility is a recently described M. jannaschii protein that has the ability to aminoacylate tRNA with cysteine (41) or an unusual CysRS, which still remains to be discovered. In view of the finding in this paper that M. jannaschii ProRS can misaminoacylate tRNA Cys with cysteine, assays for CysRS activity in vitro must now go beyond relying on the incorporation of radioactive cysteine into unfraccionated tRNA. Such assays will have to ensure that cysteine is being incorporated into tRNA Cys and not into other tRNAs. This will require the use of either purified M. jannaschii tRNA Cys or, as described in here and elsewhere (42), acid urea gel electrophoresis followed by Northern blot hybridization to identify the tRNA that is aminoacylated.

Acknowledgments—We thank M. Ibba, S. Kamtekar, and D. Kennedy for critical discussions.

REFERENCES

1. Buit, 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., Overbeck, R., Kirkness, E. F., Wang, J., Sowers, K. R., and Hou, Y. M. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 14292–14297.

2. Cusack, B., Berthet-Colominas, C., Hartlein, M., Nassar, N., and Leberman, R. (1998) Nature 347, 249–255.

3. Cusack, B., Berthet-Colominas, C., Hartlein, M., Nassar, N., and Leberman, R. (1998) Nature 347, 249–255.

4. Farabaugh, A. R., and Dingwall, C. (1979) Biochemistry 18, 2627–2631.

5. Dock-Bregeon, A., Sankaranarayanan, R., Rompy, P., Cailliet, J., Springer, M., Rees, B., Franklyn, C. S., Ehrehmann, C., and Moros, D. (2000) Cell 103, 877–884.

6. Edelhoch, H., and■Nakanon, unpublished results.

7. Fersht, A. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10869–10873.

8. Fahrae, C., Farrow, M. A., Mukhopadhyay, B., de Crécy-Lagard, V., Schimmel, P., and Marle`re, P. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 7286–7287.

9. Yaremchuk, A., Tukador, M., Grotli, M., and Cusack, S. (2001) J. Mol. Biol. 309, 989–1002.

10. Yaremchuk, A., Tukador, M., Grotli, M., and Cusack, S. (2001) J. Mol. Biol. 309, 989–1002.