Divergent Adaptation of tRNA Recognition by Methanococcus jannaschii Prolyl-tRNA Synthetase*

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Analysis of prolyl-tRNA synthetase (ProRS) across all three taxonomic domains (Eubacteria, Eucarya, and Archaea) reveals that the sequences are divided into two distinct groups. Recent studies show that Escherichia coli ProRS, a member of the “prokaryotic-like” group, recognizes specific tRNA bases at both the acceptor and anticodon ends, whereas human ProRS, a member of the “eukaryotic-like” group, recognizes nucleotide bases primarily in the anticodon. The archaeal Methanococcus jannaschii ProRS is a member of the eukaryotic-like group, although its tRNAPro* possesses prokaryotic features in the acceptor stem. We show here that, in some respects, recognition of tRNAPro by M. jannaschii ProRS parallels that of human, with a strong emphasis on the anticodon and only weak recognition of the acceptor stem. However, our data also indicate differences in the details of the anticodon recognition between these two eukaryotic-like synthetases. Although the human enzyme places a stronger emphasis on G35, the M. jannaschii enzyme places a stronger emphasis on G36, a feature that is shared by E. coli ProRS. These results, interpreted in the context of an extensive sequence alignment, provide evidence of divergent adaptation by M. jannaschii ProRS; recognition of the tRNA acceptor end is eukaryotic-like, whereas the details of the anticodon recognition are prokaryotic-like. This divergence may be a reflection of the unusual dual function of this enzyme, which catalyzes specific aminoacylation with proline as well as with cysteine.

Recognition of tRNA by aminocetyl-tRNA synthetases provides the basis for decoding genetic information. Through this recognition, each of the 20 families of tRNA is specifically charged with an amino acid by the cognate synthetase, so that the amino acid/anticodon trimucleotide relationship can be used to translate nucleic acid sequences into proteins. Although each tRNA molecule consists of ~76 nucleotides, only a few are important for specific aminoacylation, and these are generally located in the acceptor end and in the anticodon (1). In some cases, the position and identity of a strong recognition element has been maintained through evolution (2–5), whereas there are other examples where specificity determinants have changed to adapt to alterations in the synthetase sequence or structure (6–12). For the latter cases, tRNA-synthetase co-adaptation is necessary to preserve the specificity of recognition and the fidelity of the decoding system through evolution.

In the case of tRNAPro* recognition by prolyl-tRNA synthetase (ProRS), the determinants for the eubacterial Escherichia coli enzyme are located at both the acceptor end and in the anticodon sequence (6). In contrast, those for the human enzyme are located only in the anticodon (13). This shift of determinants reflects a change in enzyme sequence and structure from E. coli to human. Indeed, based on the crystal structure of ProRS of Thermus thermophilus and an extensive sequence alignment of ProRS enzymes from all three taxonomic domains, the E. coli and human synthetases are clearly members of two distinct groups of ProRS (Fig. 1) (13–15). Although both the “prokaryotic-like” and “eukaryotic-like” groups contain three conserved motifs that are characteristic of the class II aminocetyl-tRNA synthetases, specific amino acid sequences that constitute these motifs differ between the two groups. This divergence is particularly striking in the variable loop connecting the β-strands of motif 2 (12). Specific amino acid residues within the motif 2 loop are known to make critical acceptor stem contacts in many class II synthetases including E. coli ProRS (12, 16–20). The prokaryotic-like group of ProRS enzymes differs from the eukaryotic-like group by another major feature. Whereas the prokaryotic enzymes contain an insertion sequence between motif 2 and motif 3, the eukaryotic group lacks this insertion and instead contains a longer C-terminal extension (Fig. 1) (13).

To complement previous studies of E. coli and human ProRS and to determine the relatedness of tRNA recognition by ProRS from all three domains of life, we investigated here ProRS of the archaeon Methanococcus jannaschii. Although this ProRS belongs to the eukaryotic group, its tRNA shares some acceptor stem features with E. coli tRNAPro*. In particular, M. jannaschii tRNAPro contains an A73 discriminator base, which plays an important role in aminoacylation by E. coli ProRS (6). In contrast, human tRNAPro contains a C73, which is not a critical determinant for aminoacylation by the human enzyme (13).

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1 The abbreviation used is: ProRS, prolyl-tRNA synthetase.
The presence of A73 in *M. jannaschii* tRNA^Pro^ raises the question of whether tRNA recognition by the archaeal ProRS might resemble that of the *E. coli* enzyme by placing a strong emphasis on acceptor stem recognition. On the other hand, the sequence similarity between the *M. jannaschii* and human synthetases (Fig. 1) (13, 21) suggests that acceptor stem recognition might follow that of the human enzyme, which does not recognize position 73 in a base-specific manner.

In addition to aminoacylation of the cognate tRNA^Pro^, *M. jannaschii* ProRS has the novel ability to also catalyze aminoacylation of *M. jannaschii* tRNA^Pro^ with cysteine (22, 23). This dual functional ProRS is unique to *M. jannaschii*, its close relatives *Methanococcus maripaludis* and *Methanobacterium thermoautotrophicum* (22), and to the deep-rooted eukaryote *Giardia lamblia* (21). The specific aminoacylation with both proline and cysteine is expected to make unusual demands on the enzyme structure that might include relocation of determinants required for specific aminoacylation of *M. jannaschii* tRNA^Pro^ compared with those important for the *E. coli* or human enzymes, which lack this dual function. Here, the results of biochemical studies of tRNA^Pro^ recognition by *M. jannaschii* ProRS, together with an extensive sequence analysis, have provided new insights into these questions.

**EXPERIMENTAL PROCEDURES**

**Preparation of *M. jannaschii* tRNA^Pro^**—The wild-type gene for *M. jannaschii* tRNA^Pro^ was constructed by oligonucleotide hybridization and ligated into plasmid vectors pTfMa or pF119 according to published procedures (24). The recombinant plasmids were linearized by restriction enzymes NcoI (pTfMa) or PstI (pF119) and transcribed in *vitro* by T7 RNA polymerase. Point mutations in the gene were introduced by site-directed mutagenesis using the Mutagen (Bio-Rad) or QuickChange (Strategene) kits and the gene sequences were confirmed by dideoxy sequencing. T7 transcripts of the wild-type and mutant genes were purified by denaturing 12% 7M urea polyacrylamide gel electrophoresis.

**Preparation of Recombinant *M. jannaschii* ProRS**—The gene for *M. jannaschii* ProRS, identified from the genomic data base (25), was isolated by polymerase chain reaction amplification of the genomic DNA. The amplified gene was cloned into the pET-19 vector (Novagen) behind the sequence encoding the histidine tag, and errors that arose from the polymerase chain reaction were corrected by site-directed mutagenesis. The sequence of the final cloned gene was confirmed by dideoxy sequencing of the entire open reading frame. Expression of the recombinant gene was achieved in *E. coli* BL21(DE3) grown to an A600 of 0.4–0.6 and induced with 0.1–0.3 mM isopropyl-1-thio-β-D-galactopyranoside for 3–4 h at 37 °C. The recombinant ProRS was soluble and well expressed and was purified using the Co^2+^-chelated Talon resin (CLONTECH) or Ni^2+^-NTA resin (Qiagen) according to the manufacturer’s instructions. The concentration of the purified ProRS was determined based on active-site titration using the adenylate burst assay (26).

**Aminoacylation with Proline**—Proline and ^3^H-labeled proline (100 Ci/mmol) were purchased from Sigma and PerkinElmer Life Sciences, respectively. Assays were carried out at 60 or 65 °C under conditions described previously (23). Aminoacylation activity of tRNA mutants with substitutions in the acceptor end was measured with 2.5 nM ProRS and 1.0–16.0 μM tRNA. Values reported are the average of at least three determinations with an average standard deviation of 23.8%. Aminoacylation activity of tRNA mutants with substitutions in the anticodon was measured using 5 nM ProRS and 0.5 μM of a tRNA transcript, which was below the estimated K_m of the tRNA. Preliminary studies indicated that the K_m for the wild-type transcript was 1.0 μM (under the experimental conditions) and that of mutants containing substitutions in the anticodon was in the range of 3–6 μM. Values reported are the average of at least two determinations, which differed by less than 6.1%.

**Sequence Analysis**—Multiple sequence alignments were performed using the PILEUP program provided by the Genetics Computer Group (Madison, WI). The value of 2.600 was used for the gap creation penalty. Sequences used for alignments were obtained from ProRS sequences in public data bases.

**RESULTS AND DISCUSSION**

*M. jannaschii* ProRS is a Eukaryotic-like Enzyme—Multiple sequence alignments of ProRS, including members from all three domains of life, have divided the enzymes into two distinct groups (13, 14). *M. jannaschii* ProRS is a member of the eukaryotic-like group (Fig. 1), which also includes all eukaryotic enzymes, some eubacterial enzymes (such as mycoplasma and spirochetes), and *T. thermophilus* ProRS. The x-ray crystal structure of the *T. thermophilus* enzyme is known and is shown in Fig. 1. The prokaryotic-like group of ProRS includes the majority of the eubacterial enzymes as well as the mitochondrial enzymes of eucarya. Although there is no known three-
dimensional structure of a ProRS from this group, an analysis of the alignments, in addition to the crystal structure of T. thermophilus ProRS, reveal clear structural differences between the two groups. Enzymes of the eukaryotic-like group have an extra C-terminal domain of about 80 residues beyond the anticodon-binding domain. In the structure of T. thermophilus ProRS, this domain binds one atom of zinc, and its extreme C terminus folds back into the conserved catalytic core (14). In contrast, the prokaryotic enzymes have a large insertion domain (about 180 residues) of unknown function between motif 2 and motif 3 (Fig. 1).

Recognition of tRNA by M. jannaschii ProRS—The UGG isoacceptor was used for studies of tRNA recognition by M. jannaschii ProRS (Fig. 2). This isoacceptor contains three G:C base pairs at the extreme acceptor end and an A73 discriminator base, all of which are conserved in the GGG isoacceptor for tRNAPro (not shown). The features of the acceptor stem in the two known isoacceptors of M. jannaschii tRNAPro thus are notable for the presence of both C72 and A73. This combination is distinct from that found in the corresponding positions of E. coli and human tRNAPro (5, 6) (Fig. 4 below). Specifically, the acceptor stem of all eubacterial proline tRNAs contains C72 and A73, both of which are important for aminoacylation (6). In contrast, the acceptor stem of all eukaryotic proline tRNAs contains C72 and C73, which play only a minor role in aminoacylation (6). Substitution with any other nucleotide has a small effect on aminoacylation by the human enzyme (13).

As shown in Fig. 2, substitution of A73 in M. jannaschii tRNAPro by any of the other three nucleotides has a small effect (about 10-fold) on $k_{cat}/K_m$. The G73 substitution has the most severe effect (12.2-fold), whereas the C73 or U73 substitution has a smaller effect (2-fold and 6-fold, respectively). The relative decreases in activity are also shown in Fig. 3A, where the initial rates of aminoacylation of the C72, U73, and G73 mutants relative to that of the wild-type are compared. The effects at A73 are minor compared with those previously observed in the E. coli system. In E. coli tRNAPro, substitution of A73 with G had a greater than 100-fold effect on aminoacylation, and substitution with the two pyrimidine nucleotides U73 and C73 resulted in 30–40-fold decreases in $k_{cat}/K_m$, respectively (6). Thus, A73 is much less significant for M. jannaschii tRNA recognition than it is for the E. coli tRNA. The weak contribution of A73 to recognition by M. jannaschii ProRS, however, is comparable with that of C73 in the human system. Substitution of C73 in the human tRNA with A or G only had an ∼2-fold effect (13).

Position 72 of M. jannaschii tRNAPro also has a minor role in aminoacylation (Fig. 2). Substitution with any other nucleotide has a less than 10-fold effect on $k_{cat}/K_m$. The U substitution results in the largest decrease (5.3-fold), followed by the G substitution (2.2-fold). The A substitution is well tolerated with only a 1.4-fold decrease in $k_{cat}/K_m$. Because of the small effects observed at position 72, additional mutations involving position 1 that would maintain a 1:72 base pair in the acceptor stem were not investigated. In contrast, position 72 is an important specificity determinant for E. coli tRNAPro (6). Substitution of G72 in E. coli tRNAPro with either A or U reduces $k_{cat}/K_m$ by more than 150-fold, whereas substitution with C72 has a ∼30-fold effect. Similar to the M. jannaschii system, substitution of C72 in human tRNAPro does not negatively impact aminoacylation by the human enzyme (13).

We next tested mutations in all three anticodon positions of
**M. jannaschii** tRNA$^{\text{Pro}}$ for their effect on aminoacylation catalytic efficiency. As expected, substitution of U34 with A or G had no effect on aminoacylation, whereas substitution with C resulted in only a 1.3-fold decrease (Fig. 2). The absence of an effect at position 34 is in accordance with results previously obtained in the *E. coli* and human tRNAs.

The anticodon nucleotide G35 of *M. jannaschii* tRNA$^{\text{Pro}}$ is a minor determinant for aminoacylation. Substitution of G35 with A has the largest effect (9-fold), followed by the C substitution (8-fold), and the U substitution (1.5-fold) (Fig. 2). The magnitude of these effects is similar to those observed in the *E. coli* tRNA but is minor compared with those in the human tRNA. In the latter case, the substitution of G35 with any other nucleotides results in a large decrease in catalytic efficiency (120–460-fold) (13).

Of all the positions tested in this study, the most important specificity determinant in *M. jannaschii* tRNA$^{\text{Pro}}$ is G36. Substitution with C alone reduces the $k_{\text{cat}}/K_m$ of aminoacylation by 250-fold, and substitution with A results in a nearly 40-fold decrease (Fig. 2). The exception is the U substitution, which by 250-fold, and substitution with A results in a nearly 40-fold decrease (Fig. 2). The size of an arrow indicates the relative importance of the element as follows. A large arrow indicates that at least one mutation tested had a large (>100-fold) effect, and a large arrow indicates that all three mutations had a >100-fold effect on the $k_{\text{cat}}/K_m$ of aminoacylation.

![Motif 2](image)
Recognition of tRNA by M. jannaschii Prolyl-tRNA Synthetase

Anticodon Binding Domain

Fig. 6. Alignment of the anticodon-binding region of representative ProRS sequences, where the color scheme is similar to that in Fig. 5. Residues of identity between the two groups are shaded, whereas residues predicted to be involved in binding G35 and G36 within each group are shaded and boxed (14, 15). Arrows and cylinders indicate β-sheets and α-helices, respectively.

anticodon) to the eukaryotic systems (primarily anticodon) prompted a closer examination of sequences in the motif 2 loop of the respective synthetases. Specific sequences within the motif 2 loop of many class II synthetases, including E. coli ProRS, have been identified as important for recognition of the acceptor stem. In particular, the RPR sequence, conserved in all eubacterial ProRS enzymes, is critical for aminoacylation by E. coli ProRS (12). Substitution of the first R in this sequence (R144) reduces kcat/Km of aminoacylation more than 1000-fold but does not affect amino acid activation, indicating a role exclusively in tRNA recognition. Cross-linking experiments also confirmed that this R is proximal to the critical G72 nucleotide of the E. coli tRNAPro acceptor stem. In contrast, mutation of the corresponding position in the human enzyme (K1084) had no effect on human tRNAPro aminoacylation, a result that is in accordance with the lack of base-specific acceptor stem recognition in the eukaryotic system (12).

An extensive sequence alignment of the motif 2 loop of ProRS from 54 species, consisting of diverse members of the prokaryotic-like and eukaryotic-like groups, was performed. A representative subset of the aligned sequences is presented in Fig. 5. Although the RPRXG motif is strictly conserved within the prokaryotic-like enzymes, it is absent from the eukaryotic-like group and is replaced by the less conserved KHXXP motif. For example, M. jannaschii ProRS has the sequence KHTRP, whereas the corresponding sequence in the human enzyme is KHQFP. Although the function of the KHXXP motif is not entirely clear, mutagenesis of individual amino acids within this sequence of the human enzyme had little effect on aminoacylation (12). Based on the relatively minor role of tRNAPro acceptor stem recognition by M. jannaschii ProRS (Fig. 1), a similar result is predicted for this system, and experiments to test this hypothesis are under way. Thus, the shift of tRNA specificity determinants from the acceptor end in E. coli tRNAPro to the anticodon end in the human tRNA is accompanied by changes in protein motifs that recognize the acceptor end. The M. jannaschii enzyme is segregated with the human enzyme in the sequence alignment, and this segregation is consistent with the weak of tRNA acceptor end recognition.

In contrast to acceptor stem recognition, the pattern of anticodon recognition by M. jannaschii ProRS is more similar to that of the E. coli enzyme than that of the human enzyme. Both the M. jannaschii and E. coli enzymes emphasize G36 more than G35, whereas the opposite holds true for the human enzyme. However, despite functional similarity with the E. coli enzyme, sequence motifs in the anticodon-binding domain of the M. jannaschii enzyme more closely resemble those of the human synthetase (Fig. 6). For example, the M. jannaschii and human enzymes share the conserved RXE motif, where R is position 336 and 1294 in M. jannaschii and human ProRS, respectively. This residue is believed to contact G36 in the tRNA anticodon, as predicted by the co-crystal structure of T. thermophilus ProRS complexed with the anticodon domain of tRNAPro (15). The E. coli enzyme instead has the sequence TIV where T is at position 531. Also, although the M. jannaschii and human enzymes share the conserved EXXXR/KD motif (E338 in M. jannaschii ProRS and E1296 in human ProRS), which is believed to contact G35 in the anticodon, the E. coli enzyme has the VXXXRN motif (V535 in E. coli ProRS). Thus, although the M. jannaschii enzyme has remained segregated with the human enzyme in its anticodon-binding sequence, the details of anticodon recognition appear to have diverged.

The functional divergence in anticodon recognition by M. jannaschii ProRS, despite the sequence similarity with the eukaryotic-like group, may be because of the requirement of the archaeal enzyme to also recognize and specifically aminoacylate tRNAPro with cysteine. The anticodon of M. jannaschii tRNAPro is GCA, which is significantly different from the (U/G)(G) anticodon of tRNAPro. The lack of strong recognition at position 35, which is opposite from that of the human enzyme (Fig. 4), may be important for its unusual dual recognition capability. The molecular basis for how M. jannaschii ProRS recognizes tRNAPro is currently being investigated.

In summary, the investigation of tRNA recognition by the archaeal M. jannaschii ProRS and comparison of this recognition with that of the E. coli and human enzymes have provided evidence of divergent adaptation between the tRNA and synthetase. The archaeal enzyme parallels the human enzyme with regard to acceptor stem recognition, in agreement with their sequence similarity in the motif 2 loop region. In particular, the minor role of A73 in recognition by M. jannaschii ProRS is similar to the lack of recognition of C73 by the human enzyme. These results are in contrast to the strong recognition of this discriminator base nucleotide (A73), as well as G72, by the E. coli enzyme. With regard to recognition of the anticodon, the archaeal enzyme has diverged from the human enzyme, despite their sequence similarity. One manifestation of the divergence is the shift of focus from G35 to G36. The diminished role of G35 for the archaeal enzyme may reflect its dual function in recognition of both tRNAPro and tRNAProC.

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