Species-specific Differences in Amino Acid Editing by Class II Prolyl-tRNA Synthetase*

Penny J. Beuning† and Karin Musier-Forsyth§
From the Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455

Aminoaacyl-tRNA synthetases are a family of enzymes responsible for ensuring the accuracy of the genetic code by specifically attaching a particular amino acid to their cognate tRNA substrates. Through primary sequence alignments, prolyl-tRNA synthetases (ProRSs) have been divided into two phylogenetically divergent groups. We have been interested in understanding whether the unusual evolutionary pattern of ProRSs corresponds to functional differences as well. Previously, we showed that some features of tRNA recognition and aminoacylation are indeed group-specific. Here, we examine the species-specific differences in another enzymatic activity, namely amino acid editing. Proofreading or editing provides a mechanism by which incorrectly activated amino acids are hydrolyzed and thus prevented from misincorporation into proteins. “Prokaryotic-like” Escherichia coli ProRS has recently been shown to be capable of misactivating alanine and possesses both pretransfer and post-transfer hydrolytic editing activity against this noncognate amino acid. We now find that two ProRSs belonging to the “eukaryotic-like” group exhibit differences in their hydrolytic editing activity. Whereas ProRS from Methanococcus jannaschii is similar to E. coli in its ability to hydrolyze misactivated alanine via both pretransfer and post-transfer editing pathways, human ProRS lacks these activities. These results have implications for the selection or design of antibiotics that specifically target the editing active site of the prokaryotic-like group of ProRSs.

Specific aminoaacylation of tRNAs by aminoaacyl-tRNA synthetases is critical for the accurate translation of the genetic code. This is accomplished in a two-step process. In the first step, the cognate amino acid is activated with ATP to form the aminoaacyl-adenylate. In the second step, the amino acid is transferred to the 3'-end of the cognate tRNA (1). The aminoaacyl-tRNA synthetases must ensure that the relationship between anticodon and amino acid is faithfully maintained (1). It is expected that the selection of the correct amino acid is more error-prone than the selection of the cognate tRNA isoacceptor group, because the amino acid is a much smaller molecule with fewer distinguishing features (2, 3). Misactivation of amino acids can be corrected in several ways. In pretransfer editing, the noncognate aminoaacyl-adenylate is hydrolyzed by the synthetase in an ATP-dependent manner, and in many cases this activity is stimulated by the presence of the cognate tRNA (3). In post-transfer editing, a mischarged tRNA is deacylated in an ATP-independent manner (3). Synthetases may possess one or both types of editing activities and, in some cases, lack an editing function altogether.

In general, amino acid editing has been less well characterized than tRNA recognition. However, there are several well studied editing systems, especially among class I synthetases. For example, isoleucyl-tRNA synthetase (IleRS)1 misactivates valine and hydrolyzes misactivated Val-tRNA as well as mischarged Val-tRNAleu (4–6). In IleRS, a subclass Ia-specific insertion domain, the so-called connective polypeptide 1 (CP1), has been shown to be responsible for post-transfer editing (7–10). The CP1 domain is highly conserved among IleRSs, including those from Escherichia coli, yeast, and humans (7, 11). Accordingly, the editing activity of IleRS is present in all species examined to date (5, 6, 10, 12, 13). Two other class Ia enzymes, ValRS and LeuRS, also possess editing functions (8, 9, 14, 15). The CP1 domain of both of these enzymes has been shown to be responsible for editing threonine in the case of ValRS and isoleucine and methionine in the case of LeuRS (8, 9, 14–16). Similar to IleRS, the CP1 domain of ValRS and LeuRS are highly conserved (9, 15), and editing activity is present in a range of species examined thus far (9, 14, 15, 17–22).

Some class II synthetases have also been shown to edit noncognate amino acids. Recent structural and biochemical work showed that the N-terminal domain of E. coli threonyl-tRNA synthetase (ThrRS) is responsible for editing mischarged Ser-tRNAThr (23). This class II synthetase uses an active site Zn2+ ion to prevent misactivation of isosteric, but hydrophobic, valine (24). However, this Zn2+ ion cannot prevent the activation of serine, whose hydroxyl group is able to bind to the metal ion. Thus, in the absence of the N-terminal editing domain, the misactivated seryl-adenylate is charged onto tRNAThr (24). The N-terminal domain is highly conserved among ThrRSs from all species except archaeabacteria, and to date, ThrRSs from both E. coli and yeast have been shown to edit serine (23, 25). We recently showed that another class II synthetase, E. coli prolyl-tRNA synthetase (ProRS), misactivates noncognate amino acids, including alanine (26). The side chain of alanine is smaller than that of proline and could easily be accommodated in the amino acid binding pocket of ProRS. Indeed, this enzyme possesses both pre- and post-transfer editing activity against alanine, because it has been shown to hydrolyze misactivated Ala-AMP in a tRNA-independent fashion and to rapidly deacetyl a mischarged Ala-tRNA variant (26).

The abbreviations used are: IleRS, isoleucyl-tRNA synthetase; CP1, connective polypeptide 1; ValRS, valyl-tRNA synthetase; LeuRS, leucyl-tRNA synthetase; ThrRS, threonyl-tRNA synthetase; ProRS, prolyl-tRNA synthetase; PheRS, phenylalanyl-tRNA synthetase.

* This work was funded by Grant GM49928 from the National Institutes of Health. 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.

† Present address: Biology Dept., 68-653, Massachusetts Inst. of Technology, 77 Massachusetts Ave., Cambridge, MA 02139.
‡ To whom correspondence should be addressed: Dept. of Chemistry, University of Minnesota, 207 Pleasant St. SE, Minneapolis, MN 55455. Tel.: 612-624-0286; Fax: 612-626-7541; E-mail: musier@chem.umn.edu.

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The family of ProRSs can be sorted into two phylogenetically distinct groups based on their primary structures (27, 28). The “prokaryotic-like” group, consisting of prokaryotic and putative eukaryotic mitochondrial enzymes, is characterized by a large insertion domain (185 amino acids in the case of E. coli ProRS) between motifs 2 and 3. In contrast, the “eukaryotic-like” group, consisting of ProRSs from eukarya, archaea, and a few bacteria, lacks the prokaryotic insertion domain but instead has a C-terminal extension that is missing in the prokaryotic-like group. Thus, the sorting of ProRSs into two distinct groups does not conform to the canonical phylogenetic division of bacterial and archaeal/eukaryal synthetases (28). A recent structure-based phylogeny of ProRSs reinforced the notion that there are major differences between the canonical universal tree and ProRS (29). In particular, a mixture of ProRS types is found even among closely related species of eubacteria, and lateral transfer within a canonical three-kingdom structure cannot account for the unusual positions of eubacterial and eukaryotic sequences.

Using representative ProRSs from all three domains of life, we previously demonstrated species-specific differences in tRNA recognition (28, 30, 31). In particular, whereas anticondogen recognition is important for ProRSs from both the prokaryotic-like and eukaryotic-like groups, base-specific acceptor stem recognition is critical only for the prokaryotic-like E. coli enzyme (32). In contrast, base-specific acceptor stem discrimination is not an important feature of human ProRS, and similarly, the eukaryotic-like Methanococcus jannaschii ProRS has only modest acceptor stem recognition (31). Thus, the segregation of ProRSs into two evolutionarily divergent groups is reflected in differences in tRNA acceptor stem recognition. Although the editing function of a prokaryotic ProRS has been described (26), it is unknown whether this function is conserved throughout evolution. The prokaryotic-like insertion domain is similar in size to the CP1 editing domain present in class I IleRS and is a good candidate for an editing domain. However, this domain is missing in the eukaryotic-like group, and the unique C-terminal extension present only in the latter group bears little resemblance to the prokaryotic insertion. Thus, in this work, we chose to study both the human and the archaeabacterial M. jannaschii ProRSs as representative eukaryotic-like enzymes. We show that only the archaeabacterial synthetase, like the E. coli enzyme, possesses hydrolytic editing activity. Thus, the division of ProRSs into two distinct groups is not strictly correlated with the presence or the absence of amino acid editing functions.

EXPERIMENTAL PROCEDURES

Materials—All amino acids were purchased from Sigma and were of the highest quality commercially available. Creation of mutant tRNA genes was accomplished using the QuikChange site-directed mutagenesis kit (Stratagene). RNA was transcribed from BstN1-linearized plasmid using T7 RNA polymerase as described (28, 33). Transcripts were gel purified on denaturing 12% polyacrylamide-TBE gels, eluted, and desalted using published conditions (34). Bulk native E. coli tRNA was purchased from Sigma. Bulk native tRNA from M. jannaschii was a gift from Prof. Ya-Ming Hou (Thomas Jefferson University). Overexpression and purification of histidine-tagged E. coli, human, and M. jannaschii ProRS from E. coli cells were performed as described previously (31, 35).

ATP-PP2 Exchange Assay—The ATP-PP2 exchange reaction was performed according to the published conditions (35). For E. coli and human ProRS, the concentration of proline ranged from 0.05–2 mM, whereas with M. jannaschii ProRS the proline range was 0.001–0.5 mM. The alanine concentrations for all three enzymes ranged from 25 to 500 mM. The concentrations of cysteine assayed ranged from 10 to 100 mM. M. jannaschii ProRS concentrations were 1 nM for activation of proline and cysteine, and 61.5 nM for activation of other noncognate amino acids. Human ProRS concentrations were 1 nM for proline and 20 nM for noncognate amino acids. Assays with the E. coli and human enzymes were performed at 37 °C, whereas those with M. jannaschii ProRS were carried out at 60 °C. Kinetic parameters were determined from Lineweaver-Burk plots and represent the average of at least three determinations.

ATP Hydrolisis Assay—ATP hydrolisis reactions were performed according to the published procedure (26). Those reactions with tRNA contained 3–10 μM cognate tRNA. The reactions were initiated with enzyme to give the following final concentrations of ProRS: 0.9 μM (human), 0.5 μM (M. jannaschii), and 1–2 μM (E. coli). The assays with E. coli and human ProRS were performed either at room temperature or at 37 °C, whereas those with M. jannaschii ProRS were carried out at 60 °C.

Aminoacylation Assays—Aminoacylation assays were performed at room temperature according to published conditions (36). When charged tRNA was isolated for deacylation assays, all of the amino acid present in the reaction was from a tritiated stock (55 Ci/mmol Ala or 90 Ci/mmol Pro). In some cases, inorganic pyrophosphatase (2 units/ml) was added to increase the extent of mischarging. The assays were first performed to determine the required time to reach plateau levels of aminoacylation. At the desired time, acetic acid was added to 1% final concentration to quench the reaction. The charged tRNA was purified by repeated phenol/CHCl3/isooamyl alcohol (50:48:2) extractions, followed by ethanol precipitation. Phenol was equilibrated against diethylylglycolcarbonate-treated water. Charged tRNA was quantified by scintillation counting and stored at −20 °C in 50 mM Tris-HCl, pH 7.8.

Mischarging assays were carried out using the standard conditions, with the exception of the enzyme and tRNA concentrations. In these experiments, 5.0 μM tRNA was used along with the following concentrations of ProRS: 4.4 μM (M. jannaschii), 2.0 μM (human), and 5.3 μM (E. coli). Assays with E. coli and human ProRS were performed at room temperature, whereas assays with M. jannaschii ProRS were carried out at 60 °C.

Deacylation Assays—The deacylation reactions were performed as described (26) and contained 0.1–1.0 μM charged tRNA in a final reaction volume of 70 μl. The reactions were carried out at 24 °C and were initiated with human ProRS (2.0 μM) or with NaOH (0.12 N) as a positive control. Negative controls were identical to these reactions except with enzyme omitted. The reactions with M. jannaschii ProRS (4.4 μM) were performed at 37 °C. At each time point, 10-μl aliquots were removed, and the reactions were quenched on Whatman 3MM filter pads, which had been presoaked with 5% trichloroacetic acid. The pads were immediately dropped into ice-cold 5% trichloroacetic acid and washed as described for aminoacylation assays (36).

RESULTS

Activation of Noncognate Amino Acids—All 20 amino acids were tested in the ATP-PP2 exchange reaction for activation by E. coli, M. jannaschii, and human ProRS. All three enzymes activate select noncognate amino acids to varying extents. We previously found that E. coli ProRS activates alanine, with significantly decreased kcat and elevated Km. The overall reduction in efficiency of activation of alanine relative to proline by the E. coli enzyme is −10-fold. Similarly, human and M. jannaschii ProRSs activate alanine, albeit with −10-fold reduced efficiency relative to cognate proline (Table I). Therefore, based solely on the relative catalytic efficiency (kcat/Km) of alanine activation, both eukaryotic-like ProRSs examined in this study are approximately 1 order of magnitude more accurate in the initial selection of cognate amino acid than E. coli ProRS. All three enzymes activate other amino acids, most notably glycine, to a measurable extent (data not shown).

Cysteine was also activated quite well by all three enzymes, a phenomenon that has been observed in other systems (37). Based on initial rates of cysteine activation, we estimated that the ProRSs examined here activate cysteine with the following relative kcat/Km: 0.23 (E. coli), 0.026 (M. jannaschii), and 0.064 (human). These numbers are relative to proline activation by the respective ProRS. Thus, the reduction in catalytic efficiency for cysteine activation by these three enzymes, assayed under the conditions described under “Experimental Procedures,” range from only −4-fold for the E. coli enzyme to −38-fold for the M. jannaschii enzyme. That M. jannaschii ProRS can activate cysteine either in a tRNA-independent (38) or tRNA-dependent fashon (39) has been previously noted. All kinetic
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TABLE I

| ProRS species | Amino acid | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ | $k_{cat}/K_m$ (relative)$^b$ |
|---------------|------------|----------|--------|--------------|----------------------------|
| E. coli       | Proline$^b$ | 70 ± 25  | 0.25 ± 0.035 | 280           | 1 | 4.6 × 10^{-5} |
|               | Alanine$^b$ | 1.7 ± 0.56 | 140 ± 65    | 0.013         | 1 | 5.3 × 10^{-6} |
| M. jannaschii | Proline     | 210 ± 44 | 0.012 ± 0.0043 | 12000        | 1 | 3.2 × 10^{-6} |
|               | Alanine     | 2.0 ± 0.14 | 31 ± 20    | 0.063         | 1 | 2.9 × 10^{-6} |
| Human         | Proline$^c$ | 80 ± 20   | 0.18 ± 0.05 | 440           | 1 | 3.2 × 10^{-6} |
|               | Alanine     | 0.11 ± 0.09 | 79 ± 10    | 0.00014       | 1 | 2.2 × 10^{-6} |

$^a$ $k_{cat}/K_m$ is relative to proline in each set, which was set at 1.0. Individual kinetic parameters are based on the average of three determinations.

$^b$ As reported in Ref. 26.

$^c$ As reported in Ref. 35.

Parameters for amino acid activation reported here were determined in the absence of tRNA.

Mischarging of tRNA$^{Pro}$ with Alanine—Attempts to mischarge wild-type E. coli tRNA$^{Pro}$ with alanine using E. coli ProRS were unsuccessful (Fig. 1) (26). In fact, we estimate that aminoacylation with alanine is at least 3.2 × 10^5-fold reduced compared with charging with cognate proline. This may be due to efficient pre- and post-transfer editing of alanine by E. coli ProRS (26). Human ProRS is able to mischarge alanine onto tRNA$^{Pro}$ detectably but at a level that is over 2.9 × 10^4-fold reduced compared with cognate charging. Interestingly, M. jannaschii ProRS mischarges tRNA$^{Pro}$ with alanine at a rate that is only 6,800-fold reduced relative to charging with proline (Fig. 1). We also detected mischarging with cysteine of a tRNA$^{Pro}$ transcript with M. jannaschii ProRS (Table II). The latter activity is examined in more detail in a separate report.$^2$

Pretransfer Editing—ATP hydrolysis is diagnostic of pre-transfer hydrolytic editing (3). Similar to the E. coli enzyme (26), M. jannaschii ProRS stimulates ATP hydrolysis activity with the noncognate amino acid alanine (Fig. 2A) but, as expected, not in the presence of proline. As we previously determined for the E. coli enzyme, M. jannaschii ProRS also stimulates ATP hydrolysis in the presence of the proline analogs cis- and trans-4-hydroxyproline. Only a very low level of ATP hydrolysis is observed in the presence of the four-membered ring analog of proline, azetidine-4-carboxylic acid, similar to the result obtained with E. coli ProRS (Fig. 2A). Thus, the M. jannaschii enzyme parallels the E. coli enzyme in its pretransfer editing activity.

M. jannaschii ProRS has been reported to possess dual specificity, activating cysteine and aminoacylating tRNA$^{Cys}$, in addition to its normal proline-specific activity (38, 39). In accordance with this dual specificity and in agreement with a recent independent report,$^2$ we do not detect significant pretransfer editing against cysteine by M. jannaschii ProRS (Fig. 2A).

Human ProRS was also tested for its ability to stimulate ATP hydrolysis in the presence of noncognate amino acids. With the exception of very weak activity in the presence of cysteine, no significant stimulation was observed with any of the amino acids tested (Fig. 2B). In particular, in contrast to the results obtained with the E. coli and M. jannaschii ProRSs, alanine is not edited by the human enzyme.

As we had previously reported for E. coli ProRS (26), ATP hydrolysis activity was not stimulated upon the addition of the respective tRNA transcripts to the assays with either human or M. jannaschii ProRS. To test the effect of tRNA modifications, we also assayed ATP hydrolysis activity in the presence of bulk native M. jannaschii or E. coli tRNA. Once again, no stimulation was observed with either the human or M. jannaschii synthetases. These results are in accordance with those obtained with E. coli ProRS (26).

$^2$ R. S. A. Lipman, J. Wang, and Y.-M. Hou, submitted for publication.

Post-transfer Editing of Mischarged tRNA$^{Pro}$—We next examined species-specific differences in post-transfer editing. We previously showed that E. coli ProRS efficiently deacylates an Ala-tRNA$^{Pro}$ variant but does not deacylate Pro-tRNA$^{Pro}$ (26). Because misacylated tRNA$^{Pro}$ is difficult to prepare in good yield, we carried out these post-transfer assays using tRNA$^{Pro}$ variants containing acceptor stem mutations that would facilitate aminoacylation with alanine. Incorporation of only one point mutation, C$^{77} \rightarrow$ U$^{77}$, was sufficient to convert M. jannaschii tRNA$^{Pro}$ into an excellent substrate for E. coli AlaRS (Fig. 3A). This variant contains the critical G$^{55}$:U$^{70}$ base pair and all other major recognition elements for alanine aminoacylation (33, 42–44). Two mutations were incorporated into the acceptor stem of human tRNA$^{Pro}$, C$^{77} \rightarrow$ A$^{77}$ and C$^{70} \rightarrow$ U$^{70}$, to convert this tRNA into a substrate for E. coli AlaRS (Fig. 3B). To ensure that these acceptor stem mutations would not interfere with binding to the respective ProRS, we assayed them for aminoacylation activity. The effect of these changes in the tRNAs on aminoacylation with proline in either the human or the M. jannaschii system was less than 4-fold. Using these tRNA$^{Pro}$ variants, it was possible to isolate large amounts of the respective mischarged [3H]Ala-tRNA$^{Pro}$.

We were unable to detect deacylation of the Ala-tRNA$^{Pro}$ variant by human ProRS, even at enzyme concentrations as high as 2 μM (Fig. 3D). As expected, cognate Pro-tRNA$^{Pro}$ was not deacylated by the human enzyme either (data not shown). In contrast, M. jannaschii ProRS hydrolyzes the Ala-tRNA$^{Pro}$ variant used in this study (Fig. 3C). The rate of deacylation of a mischarged E. coli Ala-tRNA$^{Pro}$ variant by E. coli ProRS is shown for comparison (Fig. 3C). As expected, the M. jannaschii enzyme does not deacylate cognate Pro-tRNA$^{Pro}$ above the in-
In vivo and in vitro hydrolysis activity of M. jannaschii ProRS. Shown are graphs of the ATP hydrolysis activity of M. jannaschii (Mj) ProRS (0.5 μM) at 60 °C (A) or human ProRS (0.9 μM) at 24 °C (B) in the presence of 500 mM alanine (Ala, ■), 250 mM trans-4-hydroxyproline (trans, ▲), 250 mM cis-4-hydroxyproline (cis, ▼), 500 mM cysteine (Cys, ●), 250 mM azetidine-4-carboxylic acid (azet, □), or 2 mM proline (Pro, ○).

FIG. 2. ATP hydrolysis activity is stimulated in the presence of noncognate amino acids with M. jannaschii ProRS but not with human ProRS. Shown are graphs of the ATP hydrolysis activity of M. jannaschii (Mj) ProRS (0.5 μM) at 60 °C (A) or human ProRS (0.9 μM) at 24 °C (B) in the presence of 500 mM alanine (Ala, ■), 250 mM trans-4-hydroxyproline (trans, ▲), 250 mM cis-4-hydroxyproline (cis, ▼), 500 mM cysteine (Cys, ●), 250 mM azetidine-4-carboxylic acid (azet, □), or 2 mM proline (Pro, ○).

The enzyme is significantly less active in aminoacylation (45), but at which the aminoacyl ester bond is stable throughout the duration of the assay. Thus, although we estimate that the rate of deacylation of Ala-tRNAPro observed for the M. jannaschii enzyme is ~34-fold lower than that of E. coli ProRS assayed under similar conditions (Fig. 3C), this difference may be due to the suboptimal conditions of the assay for M. jannaschii ProRS.

### DISCUSSION

Specificity of aminoacylation by the aminoacyl-tRNA synthetases is required for faithful decoding of genetic information and the survival of all organisms. This may be achieved by high initial accuracy in amino acid selection and activation or by editing of misactivated amino acids. Editing can be achieved by a pretransfer route in which the incorrectly formed aminoacyl-adenylate is hydrolyzed or by a post-transfer route in which the mischarged tRNA is deacylated (3). Many synthetases with proofreading activity have been shown to act through a combination of these paths.

Although editing functions are often conserved throughout evolution, there are several reported examples of species-specific differences in the types of editing activities displayed by a particular synthetase. For example, archaeabacterial and eukaryotic class II PheRSs exhibit proofreading against noncognate natural amino acids primarily via a pretransfer mechanism (46, 47). In contrast, PheRS from E. coli seems to proofread mainly via a post-transfer mechanism (47, 48). Similarly, editing by class I yeast LeuRS is predominated by a pretransfer mechanism, whereas E. coli LeuRS exhibits only post-transfer editing (14, 15, 22, 49). Class II ThrRS is another example of a synthetase that displays evolutionary divergence in its editing functions. Yeast ThrRS exhibits both pre- and post-transfer editing of noncognate amino acids, including serine (25). Although no pretransfer editing was detected for serine with E. coli ThrRS, it does display efficient post-transfer editing against Ser-tRNA^Ser^ (23, 24). The N-terminal editing domain is well conserved in ThrRS across all species except archaeabacteria (23), which may therefore be predicted to lack editing activity.

The question of species-specific differences in amino acid editing is of particular interest in the class II ProRS system, because of the unusual nature of the two groupings of ProRS, which do not follow the canonical division into bacterial and archaeal/eukaryal types of synthetases (27–29). Moreover, we previously demonstrated that species-specific differences in tRNA^Pro^ acceptor stem recognition are correlated with the division into two distinct groups (28, 30, 31). The two groupings of ProRS are separated not only by differences in their aminoacylation catalytic sites but also in their pattern of insertion/extension domains. Thus, in addition to group-specific differences in cognate tRNA recognition by ProRS, we expected that we might find differences in alternate functions such as hydrolytic editing activity, which are often attributed to such domains (7–10).
Table II summarizes the amino acid activation, mischarging, and editing activities for noncognate amino acids alanine and cysteine for the three species of ProRS examined here. Despite the fact the cysteine is activated quite well by all three enzymes, only very weak stimulation of ATPase activity is observed in the presence of cysteine with both human and *M. jannaschii* ProRS (Fig. 2). Thus, cysteine does not appear to be edited by either pre- or post-transfer pathways in the cases examined here. Alanine is also activated to some extent by all three enzymes (Table II). We previously showed that *E. coli* ProRS possesses pretransfer editing activity against alanine. Here, we show that *M. jannaschii* ProRS also edits alanine by a pretransfer pathway (Fig. 2A). This is one of only a few reports of pretransfer editing that is not dependent on or stimulated by the presence of the cognate tRNA (3). A comparison of the initial rates of ATPase activity in the presence of noncognate alanine suggests that the rate of pretransfer editing by *M. jannaschii* ProRS is comparable with that of the *E. coli* enzyme. In contrast, the human enzyme weakly activates alanine but lacks this editing activity (Table II). Some nonproteinaceous amino acids, such as cis- and trans-4-hydroxyproline are also misactivated and subsequently hydrolyzed by *E. coli* (26) and *M. jannaschii* ProRS (Fig. 2A). The adenylate of the four-membered ring analog of proline, azetidine-4-carboxylic acid, does not appear to be edited by *E. coli* or *M. jannaschii* ProRS. This compound is toxic to *E. coli* and has been shown to be incorporated into proteins in place of proline; however, its effects on *M. jannaschii* are unknown (50, 51).

*E. coli* ProRS was also previously shown to be capable of rapid post-transfer deacylation of a mischarged Ala-tRNA<sub>Pro</sub> variant (26). Here, we report that human ProRS lacks this activity (Fig. 3D and Table II). The archaean synthetase appears to possess post-transfer editing activity, although deacylation is weaker for the *M. jannaschii* enzyme than for *E. coli* ProRS (Fig. 3C and Table II). Although this may simply be the result of the nonoptimal assay temperature as discussed earlier, it may also reflect an inherently weaker post-transfer activity for the archaean enzyme. This latter possibility is consistent with the observation that we can detect significant mischarging of alanine onto a tRNA<sub>Pro</sub> transcript by *M. jannaschii* ProRS (Fig. 1 and Table II). Similarly, cysteine is also mischarged onto a tRNA<sub>Pro</sub> transcript by *M. jannaschii* ProRS. Interestingly, the accuracy of aminoacylation of *M. jannaschii* tRNA<sub>Pro</sub>, with proline depends on post-transcriptional base modifications (38, 39, 41).

*M. jannaschii* and human ProRSs have an ~10-fold higher initial specificity in the activation of amino acids than *E. coli* ProRS, yet our in vitro data suggest that *M. jannaschii* ProRS possesses editing activities similar to *E. coli* ProRS, whereas the human enzyme appears to lack significant proofreading activity (Table II). In the case of *E. coli* ProRS, the specificity of amino acid discrimination in vitro is quite high (1 misactivation in 23,000), but when the relative in vivo concentrations of proline and alanine are taken into account, the “effective discrimination factor” is only about 1200 (26, 52). Editing is predicted to be required in vivo at error rates of about 1 in 3300 or more 2; so on this basis, *E. coli* ProRS would be expected to require editing activity to maintain specificity.

We find no reports of such a dramatic difference in the in vivo concentrations of alanine versus proline in human tissues. The largest reported difference in a mammalian system is a 10-fold higher concentration of alanine than proline in rat leg muscle.
There is 31% identity between the alignments of the three species. There are seven residues strictly conserved among all three sequences that align in all three species are boxed and shaded.

The proximity of the extension domain to the aminoacylation active site in T. thermophilus ProRS makes it a good candidate for an editing domain in M. jannaschii, and mutagenesis studies in the context of M. jannaschii ProRS are currently underway to test this hypothesis.

At 185 amino acids in E. coli ProRS, the unique prokaryotic-species specific insertion (Fig. 4A) is large enough to constitute an independently folded domain. Indeed, a recent crystal structure of Haemophilus influenzae YbaK, a protein with unknown function but with significant homology to the prokaryotic-like ProRS insertion, shows that this protein contains a putative small ligand binding pocket (55). Recent alignment-guided mutagenesis studies support the hypothesis that the large insertion domain in E. coli ProRS is important for its editing activity. That the human enzyme does not require editing activity to maintain specificity, whereas E. coli ProRS apparently does, suggests that the prokaryotic-like insertion domain may be an attractive target for novel antibiotic development (56). Inhibiting the editing reaction would increase mischarging, which may in turn result in an error catastrophe and cell death (37). An alternate strategy for antibiotic development may induce hydrolytic editing of the cognate substrate, resulting in a lack of availability of a particular charged tRNA, which would also presumably result in cell death (49).

The lack of amino acid proofreading activity in human ProRS may reflect the longer evolutionary history of higher eukaryotic synthetases, during which time they could have evolved higher amino acid specificity and consequently may have lost their proofreading capabilities. The finding that the existence or lack of editing activity is not strictly correlated with the two groupings of ProRS is in accord with the unusual nature of ProRS phylogeny (29–27) and may be related to the capability of the M. jannaschii enzyme to aminoacylate accurately both tRNA\textsuperscript{Pro} with proline and tRNA\textsuperscript{Cys} with cysteine (38–41). There is no convincing evidence for editing of cysteine by the ProRS examined in this work (Table II). Nevertheless, the dual function M. jannaschii enzyme may require one or both of these activities in vivo.

Acknowledgments—We thank Prof. Ya-Ming Hou and Dr. Richard Lipman (Thomas Jefferson University, Philadelphia, PA) for the gift of native M. jannaschii tRNAs and for stimulating discussions. We also thank Prof. Hou and Dr. Lipman and Dr. JinLing Wang for critical reading of the manuscript prior to submission and Dr. Brian Burke for providing materials and for helpful discussions.

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3 P. Beuning, F. Wong, and K. Musier-Forsyth, manuscript in preparation.
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