

Pre-transfer Editing by Class II Prolyl-tRNA Synthetase

ROLE OF AMINOACYLATION ACTIVE SITE IN “SELECTIVE RELEASE” OF NONCOGNATE AMINO ACIDS*

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Sanchita Hati^{†1}, Brigitte Ziervogel^{†1}, Julius SternJohn[§], Fai-Chu Wong[‡], Maria C. Nagan[‡], Abbey E. Rosen[‡], Paul G. Siliciano[§], Joseph W. Chihade[¶], and Karin Musier-Forsyth^{‡§2}

From the Departments of [‡]Chemistry and [§]Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minnesota 55455 and the [¶]Department of Chemistry, Carleton College, Northfield, Minnesota 55057

Aminoacyl-tRNA synthetases catalyze the attachment of cognate amino acids to specific tRNA molecules. To prevent potential errors in protein synthesis caused by misactivation of non-cognate amino acids, some synthetases have evolved editing mechanisms to hydrolyze misactivated amino acids (pre-transfer editing) or misacylated tRNAs (post-transfer editing). In the case of post-transfer editing, synthetases employ a separate editing domain that is distinct from the site of amino acid activation, and the mechanism is believed to involve shuttling of the flexible CCA-3' end of the tRNA from the synthetic active site to the site of hydrolysis. The mechanism of pre-transfer editing is less well understood, and in most cases, the exact site of pre-transfer editing has not been conclusively identified. Here, we probe the pre-transfer editing activity of class II prolyl-tRNA synthetases from five species representing all three kingdoms of life. To locate the site of pre-transfer editing, truncation mutants were constructed by deleting the insertion domain characteristic of bacterial prolyl-tRNA synthetase species, which is the site of post-transfer editing, or the N- or C-terminal extension domains of eukaryotic and archaeal enzymes. In addition, the pre-transfer editing mechanism of *Escherichia coli* prolyl-tRNA synthetase was probed in detail. These studies show that a separate editing domain is not required for pre-transfer editing by prolyl-tRNA synthetase. The aminoacylation active site plays a significant role in preserving the fidelity of translation by acting as a filter that selectively releases non-cognate adenylates into solution, while protecting the cognate adenylate from hydrolysis.

Aminoacyl-tRNA synthetases catalyze a two-step reaction resulting in the esterification of specific amino acids onto the 3'-adenosine of cognate tRNA molecules. In the first step, an amino acid is activated in the presence of ATP to form an enzyme-bound aminoacyl-adenylate (aa-AMP)³ intermediate.

In the second step, the activated amino acid is transferred to the 3'-end of the tRNA (1). The fidelity of this process is critical for the faithful translation of genetic information. Each aminoacyl-tRNA synthetase selects and activates its cognate amino acid from a cellular pool of 20 different proteinaceous amino acids. The similar sizes and/or chemical structures of many amino acid side chains make accurate discrimination by synthetases challenging. As a result, misactivation of non-cognate amino acids and misacylation of tRNAs may occur. Such errors, if left uncorrected, ultimately would lead to cell death due to incorporation of the incorrect amino acid during protein synthesis (2–4). To correct these errors, some synthetases have evolved editing or proofreading mechanisms (5). In pre-transfer editing, the non-cognate aa-AMP is hydrolyzed prior to transfer to the tRNA (Fig. 1, *paths 1–3*). Post-transfer editing involves deacylation of a mischarged tRNA (Fig. 1, *path 4*). The pre-transfer editing process may occur in either a tRNA-dependent (Fig. 1, *path 3*) or tRNA-independent (Fig. 1, *paths 1 and 2*) manner.

Two distinct classes of aminoacyl-tRNA synthetases have been identified on the basis of conserved sequence and structural motifs (6). Evidence of hydrolytic editing was first discovered in class I isoleucyl-tRNA synthetase, which hydrolyzes Val-AMP in a tRNA^{Ile}-dependent manner (7), in addition to deacylating Val-tRNA^{Ile} via post-transfer editing (8). Similarly, two other class I enzymes, valyl-tRNA synthetase and leucyl-tRNA synthetase, have been shown to edit misactivated amino acids and mischarged tRNAs (9–17). Editing activity has also been demonstrated for the following class II synthetases: alanyl-tRNA synthetase (18, 19), threonyl-tRNA synthetase (20), prolyl-tRNA synthetase (ProRS) (21, 22), and phenylalanyl-tRNA synthetase (23). To explain post-transfer editing, a “double-sieve” mechanism of editing was proposed (24). In this model, the aminoacylation active site acts as a coarse first sieve, excluding larger noncognate amino acids based upon size, whereas a second active site acts as a fine sieve, editing noncognate amino acids of smaller size than the cognate amino acid. Support for the double-sieve model to explain post-transfer editing has been obtained from biochemical studies (16, 24–26) and x-ray crystallography (10, 27–29). In class I enzymes, the

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¹ Both authors contributed equally to this work.

² 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.

³ The abbreviations used are: aa-AMP, aminoacyl adenylate; ProRS, prolyl-tRNA synthetase; CP1, connective polypeptide 1; INS, insertion domain;

Ec, *Escherichia coli*; Sc, *Saccharomyces cerevisiae*; Mj, *Methanococcus jannaschii*; Mm, *Methanococcus maripaludis*; Mt, *Methanobacterium thermoautotrophicum*; TES, N-tris(hydroxyl-methyl)methyl-2-aminoethanesulfonic acid; L-ASA, 5'-O-[N-(L-alanyl)-sulfamoyl]adenosine; L-PSA, 5'-O-[N-(L-prolyl)-sulfamoyl]adenosine; WT, wild type.

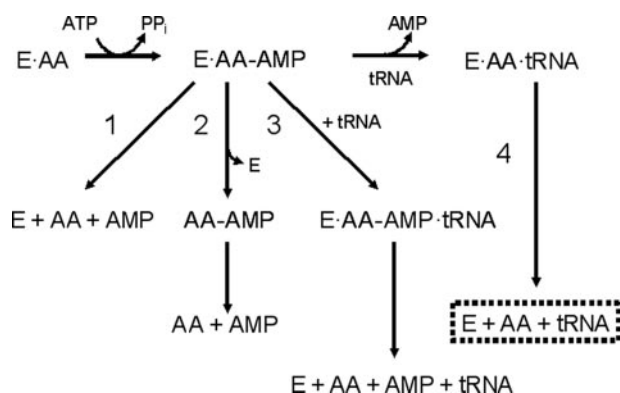


FIGURE 1. Scheme showing proposed pre-transfer (paths 1–3) and post-transfer (path 4) editing pathways.

hydrolytic editing domain is the conserved connective polypeptide 1 (CP1) domain inserted into the aminoacylation active site (13, 16, 25, 26, 30). In class II synthetases, the domains responsible for post-transfer editing are more diverse in their structure and location (19, 20, 23, 31).

Based on available structural and biochemical data, a model for post-transfer editing has been proposed for both class I and II enzymes wherein the flexible CCA-3' end of a misacylated tRNA is translocated from the aminoacylation active site to the hydrolytic editing domain (10, 28, 32–34). By contrast, the site and mechanism of pre-transfer editing are less well understood, especially for class II synthetases. Fluorescence-based assays and mutational analysis of class I synthetases have led to the proposal that misactivated amino acids are translocated from the catalytic site to the editing domain in a tRNA-dependent manner (32, 33, 35, 36). However, based on x-ray crystallography, the two active sites are separated ~ 30 Å, and the mechanism of this translocation remains unclear. Recently, a post-transfer initiated pre-transfer editing model has been postulated for isoleucyl-tRNA synthetase and leucyl-tRNA synthetase, where an initial post-transfer editing step is used to trigger a conformational change to an editing active conformation that can now perform pre-transfer editing in the CP1 domain (36). Support for the role of the CP1 domain in pre-transfer editing also comes from x-ray crystallography studies showing that both pre- and post-transfer editing substrate analogs bind in overlapping sites in the CP1 domain of leucyl-tRNA synthetase and isoleucyl-tRNA synthetase (9, 27, 37, 38). However, recent studies of class I glutamyl-tRNA synthetase, which normally lacks editing function and a spatially separate editing domain, have provided evidence for a pre-transfer editing-like reaction occurring in the synthetic active site (39).

The location of pre-transfer editing in the case of class II synthetases has not been extensively investigated. Class II *Escherichia coli* (Ec) threonyl-tRNA synthetase, which edits misacylated Ser-tRNA^{Thr} via an N-terminal editing domain, appears to lack pre-transfer editing activity (20). On the other hand, Ec alanyl-tRNA synthetase and phenylalanyl-tRNA synthetase edit Gly- and Ser-tRNA^{Ala} and Trp-tRNA^{Phe}, respectively, and also possess a tRNA-dependent pre-transfer editing function (19, 23).

ProRS is another class II synthetase that possesses both pre- and post-transfer hydrolytic editing activities (21, 22). Post-

transfer editing of Ala-tRNA^{Pro} by bacterial ProRS is carried out by a large insertion domain (INS) located between motifs 2 and 3, which make up the aminoacylation active site (31, 40). An N-terminal domain with weak homology to INS also appears to function as a post-transfer editing domain in some lower eukaryotic species (41). Pre-transfer editing of alanyl-adenylate has been observed in ProRSs from all three kingdoms of life, even those that lack an INS domain (22). In the ProRS system, hydrolysis of the misactivated adenylate occurs in the absence of tRNA and tRNA addition does not stimulate the activity (21, 22). Thus, this represents an ideal system to specifically probe the mechanism of pre-transfer editing, because the lack of a requirement for tRNA ensures only pre-transfer editing is being observed. In this work, we investigate ProRS truncation mutants designed to probe the INS domain of bacterial ProRS, as well as the N- and C-terminal extension domains found in lower eukaryotic and archaeal ProRSs, respectively, for their role in amino acid activation, aminoacylation, and pre-transfer editing.

In addition to probing the site of pre-transfer editing, we also explore the mechanism of tRNA-independent pre-transfer editing by Ec ProRS in more detail. Any mechanism that involves formation of a non-cognate adenylate, followed by preferential removal of that adenylate from the reaction pathway leading to aminoacylated tRNA, can be considered pre-transfer editing. However, there are multiple possible mechanisms to achieve selectivity after the adenylate has been synthesized. One mechanism, which we will term “selective hydrolysis,” involves the transport of the adenylate to a distinct active site in which the hydrolysis of non-cognate adenylates, but not cognate adenylates, is catalyzed. Alternatively, enzymatic hydrolysis of non-cognate adenylates may occur in the aminoacylation active site. In a third possible mechanism, which we will term “selective release,” non-cognate adenylates are bound to the enzymatic active site considerably less strongly than cognate adenylates, leading to the release of the non-cognate products into solution, where the intrinsic instability of mixed anhydride functionality leads to rapid, non-enzymatic, hydrolysis (Fig. 1, path 2). When the rate of adenylate release is faster than the rate of aminoacyl transfer, this path has been referred to as “kinetic proofreading” (42).

To distinguish between enzyme-catalyzed selective hydrolysis and selective release of Ala-AMP, a thin-layer chromatography (TLC)-based assay was employed to directly measure the rate of AMP formation by Ec ProRS. A separate “chase” assay was used to release bound adenylate from the active site of the enzyme to measure the rate of non-catalytic adenylate hydrolysis in solution. The results of these experiments support a critical role for the aminoacylation active site in selective release of noncognate adenylates by Ec ProRS.

EXPERIMENTAL PROCEDURES

Materials—All amino acids were purchased from Sigma and were of the highest quality available (>99% pure). ³H-Labeled proline and alanine were purchased from Amersham Biosciences. Primers for site-directed mutagenesis and PCR were purchased from Integrated DNA Technologies, Inc. (IDT).

Pre-transfer Editing by Class II ProRS

Enzyme Preparation—Overexpression and purification of histidine-tagged Ec wild-type (WT) ProRS was performed as described earlier (43). Two constructs encoding Ec ProRS lacking the INS domain were constructed using Excite PCR-based site-directed mutagenesis (Stratagene). The 163 residues of the INS domain (amino acids 232–394) were replaced with either an 8-residue Gly₆Ser₂ linker or a 16-residue Gly₁₂Ser₄ linker by PCR amplification of the full-length plasmid pCS-M1S (44). The following primers were used for the 8-residue linker: 5'-AGAACCGCCGCCACCAGAACCGCCCGCTGCATAGTCAGAGGTGTCGGAGAAGAC-3' and 5'-TCTGGTGGCGGCGGTTCTGGTGGCCCAAGCCCGGATGGCCAGGGTAGGCTGCTG-3'. The underlined region encodes the linker residues and a similar strategy was used to construct the 16-residue linker. The PCR product was first digested with DpnI to remove the parental plasmid followed by blunt end ligation. The ligated product was then transformed into Ec XL1-blue supercompetent cells (Stratagene), plated on Luria-Bertani agar supplemented with 100 μg/ml of ampicillin, and incubated at 37 °C overnight. Results of cloning were confirmed by automated DNA sequencing (Microchemical Facility, University of Minnesota). Plasmids encoding both constructs were transformed into Ec SG13009 (pREP4) (Qiagen) competent cells and protein expression was induced with 0.1 mM isopropyl β-D-thiogalactoside for 20 h at room temperature. Histidine-tagged protein was purified using a Co²⁺-chelated Talon resin (Clontech) according to the manufacturer's protocol. The desired proteins (designated EcΔINS Gly₆Ser₂ and EcΔINS Gly₁₂Ser₄) were eluted with 25 mM imidazole, concentrated, and stored as described earlier (43).

Plasmid pM445Mj-P encoding the WT *Methanococcus jannaschii* (Mj) *proS* gene and plasmid pM446Mtht-P encoding WT *Methanobacterium thermoautotrophicum* (Mt) ProRS were provided by Professor Kiyotaka Shiba (Japanese Foundation for Cancer Research, Tokyo, Japan). Each WT *proS* gene was cloned into the pKS583 plasmid (45), which contains a histidine-tagged sequence. Mt ProRS was overexpressed and purified according to previously described procedures (46). Site-directed mutagenesis to incorporate a stop codon at position 374 of Mj ProRS (to generate MjΔC81) was performed with the QuikChange kit (Stratagene). The presence of the desired mutation and the absence of undesired changes at other positions were confirmed by sequencing the entire gene. Plasmids encoding histidine-tagged WT Mj ProRS and MjΔC81 (pPRS374) were transformed into BL21(DE3)pLysE cells and colonies were selected by growth on chloramphenicol and ampicillin media. Purification of WT Mj ProRS was performed as described earlier (46). MjΔC81 was purified using the Talon batch method. Briefly, Co²⁺-chelated Talon resin (Clontech) was prepared according to the manufacturer's protocol and equilibrated for 20 min in sonication buffer (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM β-mercaptoethanol, saturated 2-propanol solution of phenylmethanesulfonyl fluoride to a final concentration of 0.75 mg/ml). The resin was then centrifuged at 3,000 × *g* for 5 min and the supernatant was removed. Supernatant from the sonicated cells was added to the prepared resin and allowed to equilibrate with shaking (<100 rpm) for 20–30 min. The resin was washed with 10 bed volumes of son-

ication buffer, followed by 20 bed volumes of wash buffer (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM β-mercaptoethanol, 10 mM imidazole). The desired protein was then eluted with 10 1-ml aliquots of wash buffer containing 80 mM imidazole. Peak fractions containing MjΔC81 were detected by SDS-PAGE.

The gene encoding *Saccharomyces cerevisiae* (Sc) ProRS was cloned via PCR amplification of genomic DNA isolated from bakers' yeast using *Pwo* polymerase (Roche) and PCR primers, 5'-CGCGCATGCCTGCCTGTTTCGGAAAGCGTTT-3' and 5'-CGCAAGCTTCTAATAAGAACGACCGAACAT-3'. The PCR product was gel purified, digested with SphI and HindIII, and ligated into the histidine-tagged protein expression vector pQE30 (Qiagen) to construct plasmid pJS550. A truncated Sc ProRS mutant lacking the N-terminal 183 residues (ScΔ183) was constructed similarly using primers 5'-CGCGCATGCCTTATTGGTATCACCGTAGAC-3' and 5'-CGCAAGCTTCTAATAAGAACGACCGAACAT-3'. Histidine-tagged WT Sc ProRS and ScΔ183 proteins were purified as described previously (43).

The genomic sequence of *Methanococcus maripaludis* (Mm) was obtained from Professor John Leigh at the University of Washington. PCR amplification of the Mm *proS* gene from Mm cell paste (provided by Professor Jim Brown, North Carolina State University) was carried out using *Taq* polymerase and the following PCR primers: 5'-CGCGGATCCGCGGAATTTTC-GGAATGGTAC-3' and 5'-CGCGTCGACTTAGTATGTTCTGGCAACTGAAAT-3'. The PCR product was digested with BamHI and SalI and ligated into pCR2.1TOPO cloning vector (Invitrogen). The results of cloning were confirmed by automated DNA sequencing and the product was then digested with BamHI and SalI and directionally cloned into the histidine-tagged protein expression vector pQE30 (Qiagen), resulting in plasmid pJS860. The plasmid was transformed into Ec SG13009 (pREP4) (Qiagen) competent cells and Mm ProRS was overexpressed and purified using a Co²⁺-chelated Talon resin (Clontech). The desired protein was eluted with 0.1 M imidazole and stored as described previously (43).

RNA Preparation—Ec tRNA^{Pro} was prepared by *in vitro* transcription using BstNI-linearized plasmid as described previously (44). Plasmids encoding WT Mj tRNA^{Pro} and the U70-tRNA^{Pro} mutant were linearized using FokI prior to *in vitro* transcription (22, 43). A plasmid encoding the Mm tRNA^{Pro} gene was constructed by incorporating three changes (deletion of U20b; mutation of G45A and A57G) into the plasmid encoding WT Mj tRNA^{Pro} using the QuikChange multisite-directed mutagenesis kit (Stratagene). The resulting plasmid encoding Mm tRNA^{Pro} contains a FokI site within the target sequence so the template for *in vitro* transcription was generated via PCR using primers 5'-CCTGCAGTAATACGACTCACTATAGGG-3' and 5'-TGGTGGGCCTGCCAGATTTCGAAC-3', and *Turbo* polymerase (Stratagene). The gene encoding Sc tRNA^{Pro} was obtained by PCR of genomic DNA isolated from bakers' yeast using primers 5'-CGGATTTCTCCTGCAGTAATACGACTCACTATAGGGCGTGTGGTCTAGTGG-3' and 5'-CGCGGATCCTGGGGGCGAGCTGGGAATTG-3'. The PCR products were digested with BamHI and EcoRI and ligated into plasmid pFVal119 (47). An intron present within the tRNA^{Pro} gene was removed using Transformer site-directed mutagene-

sis (Clontech) to generate pJS607t. The plasmid was linearized using FokI and tRNA was transcribed using T7 RNA polymerase. The WT coding sequence was confirmed by DNA sequencing.

Circular Dichroism (CD) Spectroscopy—CD spectra of WT and truncated Ec ProRS were recorded using a J-715 spectrophotometer (Jasco) and 0.5 mg/ml protein in 50 mM sodium sulfate and 10 mM TES (pH 7.5), at room temperature.

Enzyme Assays—Enzyme concentrations for all proteins were determined initially using the Bio-Rad Protein Assay Kit (Bio-Rad) and then by active site titration using the adenylate burst assay (48).

ATP-PP_i Exchange Assays—The ATP-PP_i exchange reaction was performed according to the published method (21, 49). The concentrations of proline ranged from 0.05 to 2 mM for WT Ec ProRS and 2 to 25 mM for EcΔINS Gly₁₂Ser₄. The alanine concentrations ranged from 200 to 800 mM, whereas the *cis*-4-hydroxy-proline concentration varied from 25 to 500 mM. The enzyme concentrations used for proline activation were 1 nM for WT Ec ProRS and 10 nM for EcΔINS Gly₁₂Ser₄. The enzyme concentrations used for alanine activation were 20 nM for WT Ec ProRS and 0.5–1.7 μM for EcΔINS Gly₁₂Ser₄. For *cis*-4-hydroxyproline, 10 nM WT Ec ProRS and 200 nM EcΔINS Gly₁₂Ser₄ were used. Assays were performed at 37 °C and kinetic parameters were determined from Lineweaver-Burk plots and represent the average of at least two determinations.

Pre-transfer Editing Assays—ATP hydrolysis reactions to monitor pre-transfer editing were carried out as described previously (21). The alanine concentration used was 500 mM and the proline concentration varied depending on the species as follows: 10 mM (Mm and Sc), 10 or 100 mM (Ec) and 500 mM (Mj and Mt). *Cis*- and *trans*-4-hydroxyproline concentrations were 500 mM. The reactions were initiated with enzyme to give the following final concentrations of ProRS: 2 μM (Mt and Mm), 2–10 μM (WT Ec and EcΔINS Gly₁₂Ser₄), 0.5 μM (Mj), 1 μM (MjΔC81), and 4 μM (Sc). Assays were conducted at 24 °C with Ec and Mm ProRS, 30 °C with Sc ProRS, and 60 °C with Mj and Mt enzymes. The reactions in the presence of tRNA were performed with 5–10 μM final concentration of cognate tRNA.

Aminoacylation Assays—Aminoacylation reactions were performed under standard conditions (50) with 0.5–25 μM tRNA^{Pro} and 22.7 μM [³H]proline. The enzyme concentrations used were 5 nM (WT Mj ProRS), 50 nM (MjΔC81), 100 nM (WT Ec ProRS, EcΔINS Gly₁₂Ser₄), WT Sc ProRS, ScΔN183, and Mm ProRS), and 500 nM (EcΔINS Gly₆Ser₂).

TLC Assay to Monitor AMP Formation by Ec ProRS—AMP formation was measured in reaction mixtures containing either 150 or 250 μM ATP, 1 mM proline or 500 mM alanine, 100 mM Tris-HCl (pH 7.0), 10 mM potassium fluoride, 10 mM 2-mercaptoethanol, 10 mM MgCl₂, 0.06 μg/μl pyrophosphatase, and 0.25 μM α-³²P]ATP (Amersham Biosciences). Reactions were incubated at 37 °C and initiated by addition of 0.5 μM Ec ProRS. Aliquots (2.0 μl) were quenched in 8.0 μl of 200 mM sodium acetate (pH 5.0). Quenched aliquots (1.0 μl each) were spotted in duplicate on polyethyleneimine cellulose plates (Sigma) pre-washed with water. Separation of aa-³²P]AMP, ³²P]AMP, and ³²P]ATP was performed by developing TLC plates in 0.1 M ammonium acetate, 5% acetic acid (51). Plates were visualized

by phosphorimaging and data were analyzed using Bio-Rad Molecular Imager FX software. The positions corresponding to elution of Ala-AMP and free AMP were confirmed by using chemically synthesized Ala-AMP prepared as described (52) and commercially available AMP (Sigma). All data points were corrected for a small background rate of ATP hydrolysis in the absence of ProRS. The concentration of aa-AMP and AMP in each separated band was determined by spotting a known concentration of [³²P]ATP on each TLC plate. The volume density for the known [³²P]ATP spot was then compared with the volume densities for [³²P]AMP and aa-³²P]AMP bands. Rate constants were obtained from graphs of aa-³²P]AMP and ³²P]AMP formation plotted against time. To check the effect of cognate tRNA on the rate of AMP formation, some reactions were performed in the presence of 15 μM tRNA^{Pro}. All reactions were performed in triplicate.

Non-enzymatic Hydrolysis of Adenylates—The rate of non-enzymatic adenylate hydrolysis was measured by performing a chase assay wherein a non-hydrolyzable adenylate analog, 5'-O-[N-(L-prolyl)-sulfamoyl]adenosine (L-PSA, RNA-Tec, Leuven, Belgium), was added to the reaction mixtures following initiation of Pro-AMP and Ala-AMP synthesis as described above. This adenylate analog was previously shown to be a potent inhibitor of Ec ProRS (49), and when added in vast excess to the reaction mixtures, L-PSA is expected to release cognate and non-cognate aminoacyl-adenylate from the synthetic active site of the enzyme. For the chase experiments, which were adapted from Perona and co-workers (39), 0.5 μM ProRS was first incubated with 1 mM proline or 500 mM alanine, 150 or 250 μM ATP, and 0.25 μM α-³²P]ATP for 10 min at 37 °C. Following the addition of L-PSA (1 mM–10 mM), hydrolysis activity was quenched at various time points (0.5 to 10 min) by mixing 2 μl of the reaction mixture with 8 μl of 200 mM sodium acetate (pH 5.0). TLC separation was then performed and reactions were quantified as described above. Rate constants for non-enzymatic hydrolysis were obtained by plotting the concentration of aa-³²P]AMP against time and fitting the data to a first-order decay curve. All reactions were performed in triplicate.

Determination of K_i for L-ASA—The ATP-PP_i exchange reaction was used to determine the inhibition constant (K_i) for 5'-O-[N-(L-alanyl)-sulfamoyl]adenosine (L-ASA, RNA-Tec, Leuven, Belgium). ATP-PP_i exchange reactions were performed essentially as described above. For the inhibition studies, 1 nM ProRS, L-ASA (25 to 100 nM), and variable amounts of proline (0.1 to 2 mM) were incubated in 144 mM Tris-HCl, pH 8.0, and 0.2 mg/ml bovine serum albumin for 10 min at 37 °C. Reactions were initiated with addition of 2.4 mM ATP, which had been pre-equilibrated for 10 min at 37 °C. Aliquots (50 μl) were quenched in 500 μl of stop solution containing 11% HClO₄, 0.3 M PP_i, and 1% activated charcoal at 4 °C, and vortexed. The quenched solutions were then vacuum filtered on glass filter paper circles and washed with 7 ml of 1% HClO₄, 25 mM PP_i, followed by 7 ml of water. The filter paper pads were counted in 5 ml of scintillation fluor. Specific activity was measured by counting 10 μl of the reaction mixture in 5 ml of scintillation fluor. Initial reaction rates were determined from plots of [³²P]ATP formation

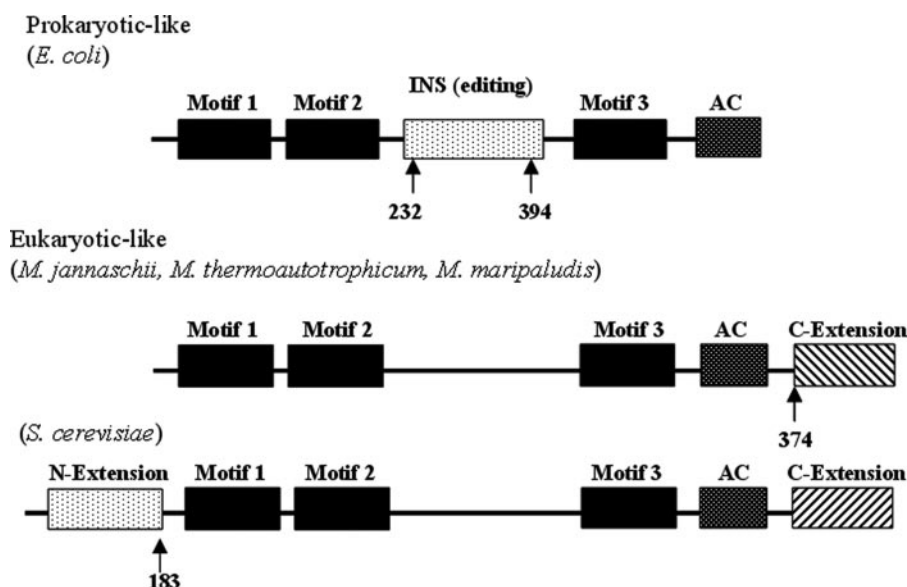


FIGURE 2. Schematic representation of prokaryotic-like and eukaryotic-like ProRSs. Numbering in the insertion domain of prokaryotic-like ProRS indicates the sites of truncation to generate Ec Δ INS Gly₆Ser₂ and Ec Δ INS Gly₁₂Ser₄. Numbering in the C and N terminus of eukaryotic-like ProRSs indicates sites of truncation to generate Mj Δ C81 and Sc Δ N183 ProRSs, respectively. AC indicates the anticodon binding domain.

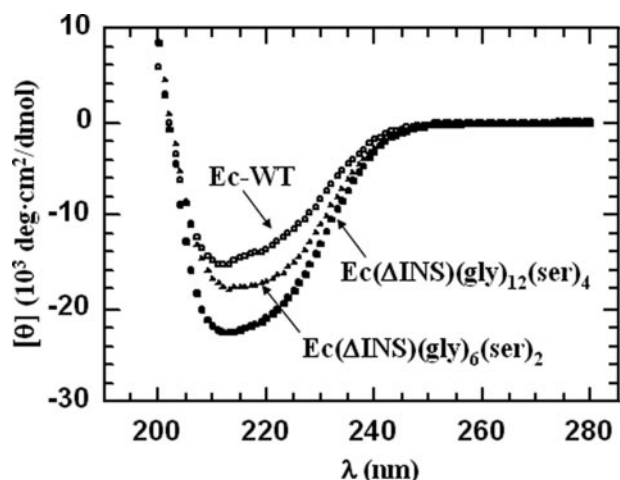


FIGURE 3. CD spectra of Ec WT and truncated ProRSs. CD spectra of Ec WT ProRS (□), Ec Δ INS Gly₆Ser₂ (▲), and Ec Δ INS Gly₁₂Ser₄ (■) were measured as described under "Experimental Procedures" using a 0.1-cm path length cuvette.

TABLE 1
Kinetic parameters for activation of amino acids by WT and truncated Ec ProRS

| Ec ProRS | Amino acid | k_{cat} | K_m | k_{cat}/K_m | k_{cat}/K_m |
|---------------------------|----------------------|------------------|-------------|----------------------|------------------------|
| | | s^{-1} | mM | $s^{-1} mM^{-1}$ | relative |
| WT | Proline ^a | 70 ± 25 | 0.25 ± 0.04 | 280 | 1 |
| | Alanine ^a | 1.7 ± 0.56 | 140 ± 65 | 0.01 | 3.6 × 10 ⁻⁵ |
| | Cis ^b | 21 ± 1.1 | 55 ± 10 | 0.38 | 1.4 × 10 ⁻³ |
| | Trans ^b | 15 ± 1.2 | 237 ± 7 | 0.06 | 2.1 × 10 ⁻⁴ |
| Δ INS ^c | Proline | 12 ± 2.6 | 50 ± 14.4 | 0.24 | 0.9 × 10 ⁻³ |
| | Cis | 0.056 ± 0.01 | 53 ± 1.3 | 0.0011 | 3.8 × 10 ⁻⁵ |

^a As reported in Ref. 22.

^b Cis and trans refer to cis-4-hydroxyproline and trans-4-hydroxyproline, respectively.

^c Δ INS refers to the Ec Δ INS Gly₁₂Ser₄ construct.

against time. Lineweaver-Burk plots were generated and replots of the Lineweaver-Burk plot slopes against L-ASA concentration gave a straight line with an x intercept of $-K_i$ (53).

RESULTS

Wild-type and Truncated ProRS Constructs—ProRSs are subdivided into two phylogenetically distinct groups based on sequence homology and their primary structures: "eukaryotic-like" and "prokaryotic-like" (44, 54). Most prokaryotic-like ProRSs, including the Ec enzyme, contain an alanine-specific post-transfer editing domain (INS) between class II consensus motifs 2 and 3 (40). Eukaryotic-like ProRSs lack this insertion, but instead have C- and/or N-terminal extension domains of unknown function. Fig. 2 shows a schematic diagram of the domain architecture of prokaryotic-like and eukaryotic-like ProRS enzymes examined in this work. Two Ec ProRS mutants were constructed by deleting 163 amino acid residues (residues 232–394) of the

INS domain and inserting either an 8- or 16-residue Gly/Ser spacer peptide (Fig. 2, top). To determine whether the internal truncation mutants, Ec Δ INS Gly₆Ser₂ and Ec Δ INS Gly₁₂Ser₄, were properly folded, we compared the CD spectra of these mutants with that of the WT enzyme. As shown in Fig. 3, the CD spectra of the truncated variants are similar to WT, suggesting that their global fold is similar.

The archaeal enzymes from Mj, Mt, and Mm studied here are characterized by an ~70-residue C-terminal extension with no resemblance to the prokaryotic INS domain. Also, the C-terminal domain of these archaeal ProRSs are closely related (~30% sequence identity), but display much lower homology to the C-terminal extensions found in eukaryotic ProRS. To test the role of the C-terminal domain of Mj ProRS, a truncation mutant was constructed by inserting a stop codon at residue 374 located at the beginning of the extension to generate Mj Δ C81 (Fig. 2, middle).

Finally, Sc ProRS is a eukaryotic-like enzyme containing an N-terminal extension with weak homology to the prokaryotic-like INS domain. To probe the role of the N-terminal domain of Sc ProRS, a truncation mutant, Sc Δ N183, was constructed by deleting 183 residues from the N terminus (Fig. 2, bottom).

Activation of Proline and Alanine—Using the ATP-PP_i exchange reaction, we found that Ec Δ INS Gly₁₂Ser₄ ProRS activates proline, but with a 6-fold lower k_{cat} and significantly elevated K_m (200-fold) relative to the WT Ec enzyme (Table 1). Thus, the overall decrease in proline activation efficiency relative to WT is ~1200-fold. Alanine activation by this internal truncation mutant was also significantly reduced compared with the WT enzyme (Fig. 4). The low level of activity did not allow kinetic parameters to be determined in this case.

In contrast to the low levels of amino acid activation achieved by the variant containing a 16-amino acid linker peptide, the ability of the Ec Δ INS Gly₆Ser₂ mutant to activate proline and

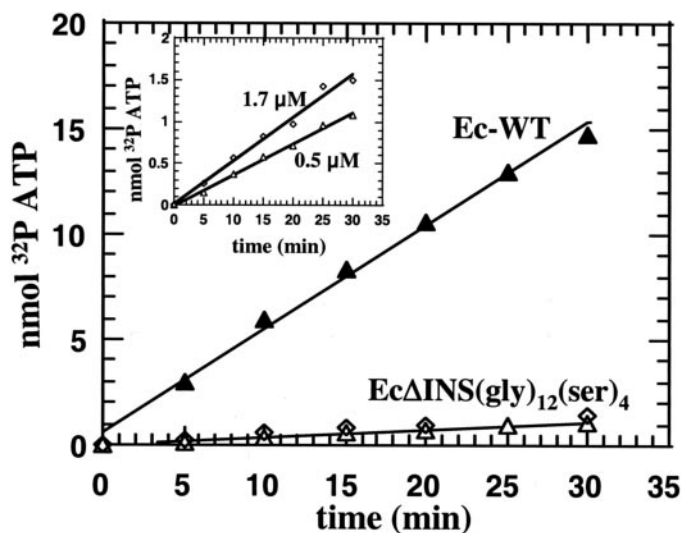


FIGURE 4. Alanine activation by Ec WT and truncated ProRSs. Alanine activation was monitored using the ATP-PP_i exchange reaction carried out at 37 °C using 1 nM WT Ec ProRS (▲) and 0.5 (Δ) or 1.7 μM (○) EcΔINS Gly₁₂Ser₄. The inset expands the reaction time course for EcΔINS Gly₁₂Ser₄.

alanine was completely abolished (data not shown). Addition of cognate tRNA failed to stimulate amino acid activation (data not shown). Taken together, these results suggest that although the global fold of the truncated proteins appears to be similar to the WT enzyme (Fig. 3), deletion of the insertion domain has a profound effect on the amino acid activation reaction.

Ec ProRS is also able to activate *cis*- and *trans*-4-hydroxyproline (21) and the kinetic parameters for the WT enzyme are shown in Table 1. Although the activity of the EcΔINS Gly₁₂Ser₄ variant in the presence of the *trans* analog was too low to measure k_{cat} and K_m , kinetic parameters for the *cis* analog were determined. Interestingly, whereas the k_{cat} was severely reduced (~400-fold), the K_m was similar to that of the WT enzyme (Table 1).

The eukaryotic-like truncation mutants shown in Fig. 2 were also tested for their ability to activate cognate proline. The amino acid activation efficiency was reduced ~25-fold for MjΔC81 and ~3.5-fold for ScΔN183 compared with their WT counterparts, suggesting that the extension domains play a significant but less important role in adenylate formation relative to the prokaryotic INS domain.

Aminoacylation of tRNA^{Pro}—We next determined the effect of deleting the insertion/extension domains on aminoacylation activity. As expected based on the lack of amino acid activation activity, aminoacylation by EcΔINS Gly₆Ser₂ ProRS was also not detected. Surprisingly, the overall charging efficiency of EcΔINS Gly₁₂Ser₄ was enhanced 4-fold relative to the WT enzyme (Table 2). The kinetic parameters for aminoacylation by WT Ec ProRS were measured to be $k_{\text{cat}} = 0.239 \pm 0.016 \text{ s}^{-1}$ and $K_m(\text{tRNA}^{\text{Pro}}) = 14.14 \pm 2.63 \mu\text{M}$, whereas those of EcΔINS Gly₁₂Ser₄ were k_{cat} of $0.0091 \pm 0.001 \text{ s}^{-1}$ and $K_m(\text{tRNA}^{\text{Pro}}) = 0.131 \pm 0.009 \mu\text{M}$. These results suggest that in the presence of an optimal length linker, the INS domain is dispensable for aminoacylation. On the other hand, deletion of the C-terminal extension of Mj ProRS results in an ~6-fold reduction in aminoacylation efficiency (Table 2). The strictly conserved tyrosine at the C terminus of the eukaryotic-like ProRS has been shown

TABLE 2

Relative aminoacylation efficiencies of WT and truncated ProRS variants

Values reported are the average of two or three experiments with <25% difference between trials. k_{cat}/K_m is given relative to WT for each species, which was set at 1.0.

| ProRS | k_{cat}/K_m (relative) |
|---------|---------------------------------|
| Ec WT | 1.0 |
| EcΔINS | 4.04 |
| Mj-WT | 1.0 |
| MjΔC81 | 0.18 |
| Sc WT | 1.0 |
| ScΔN183 | 0.29 |

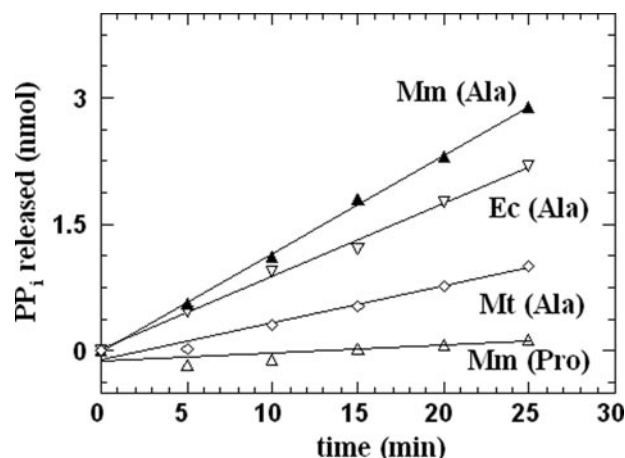


FIGURE 5. Pre-transfer editing activity of WT Ec, Mt, and Mm ProRSs. Plot showing stimulation of ATP hydrolysis in the presence of non-cognate alanine (500 mM) but not in the presence of proline (10 mM) for the indicated enzymes. The enzyme concentrations used were 2 μM for Ec (▽), Mm (Ala) (▲), Pro (Δ), and Mt (◇) ProRS.

to interact with and stabilize the aminoacylation active site (55). Thus, the modest decrease in aminoacylation activity may be due to loss of these stabilizing interactions. Deletion of the N-terminal extension domain of Sc ProRS has an even smaller effect, reducing aminoacylation efficiency ~3-fold relative to the WT enzyme (Table 2).

Pre-transfer Editing Activity—Stimulation of ATP hydrolysis is considered indicative of pre-transfer editing because noncognate amino acids that are hydrolytically edited are repeatedly reactivated by the synthetase, consuming ATP in each cycle (5). In contrast, the cognate adenylate is bound to the synthetase until the transfer reaction and is not expected to significantly stimulate ATP hydrolysis. We previously showed that Ec and Mj ProRSs possess tRNA-independent pre-transfer editing against alanine (21, 49). Here, we tested the pre-transfer editing activity of two additional archaeal ProRSs. For both Mt and Mm ProRS, ATP hydrolysis is stimulated in the presence of alanine but not in the presence of cognate proline, suggesting that these enzymes also possess pre-transfer editing activity (Fig. 5). Moreover, stimulation of ATP hydrolysis occurs in the absence of cognate tRNA and addition of tRNA does not stimulate editing further (data not shown). Similarly, WT Sc ProRS carries out tRNA-independent pre-transfer editing in the presence of alanine but not proline (Fig. 6A).

To probe the role of the INS domain in pre-transfer editing of prokaryotic-like ProRS, we tested the ATP hydrolysis activity of EcΔINS Gly₁₂Ser₄ in the presence of alanine and proline ana-

Pre-transfer Editing by Class II ProRS

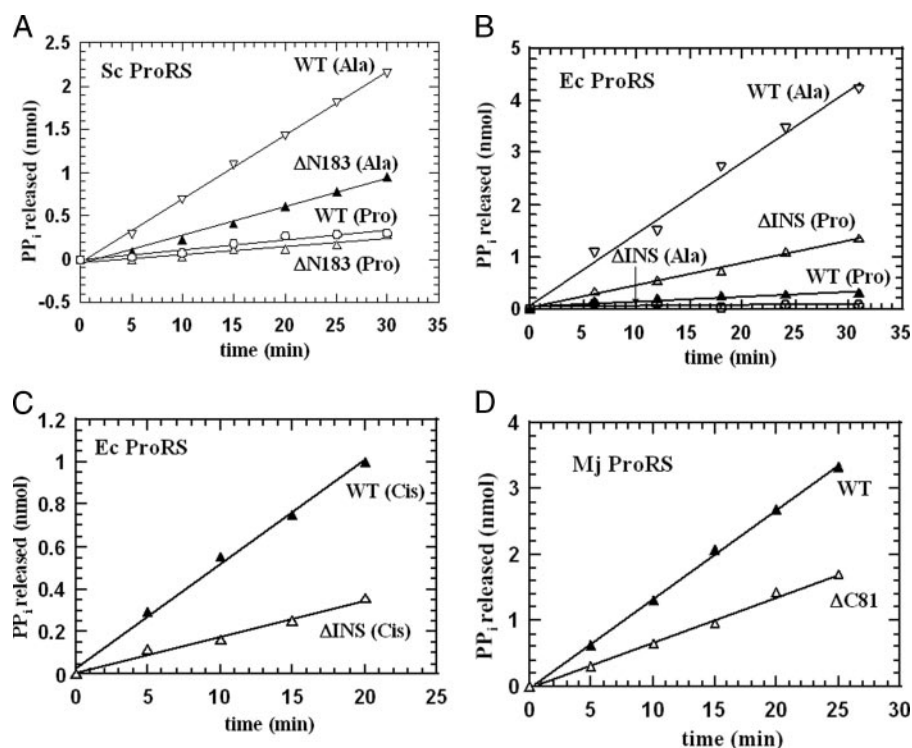


FIGURE 6. Pre-transfer editing activity of WT and truncated ProRSs. Plot showing stimulation of ATP hydrolysis by: WT Sc and Sc Δ N183 ProRS ($4 \mu\text{M}$) in the presence of 500 mM alanine (∇ , WT), (\blacktriangle , Δ N183)), and 10 mM proline (\circ (WT), (Δ , Δ N183)) (A); WT Ec ProRS and Ec Δ INS Gly₁₂Ser₄ ($4 \mu\text{M}$) in the presence of 100 mM proline (\blacktriangle (WT), (Δ , Δ INS)) and 500 mM alanine (∇ (WT), (\circ , Δ INS)) (B); WT Ec ProRS ($2 \mu\text{M}$) and Ec Δ INS Gly₁₂Ser₄ ($4 \mu\text{M}$) in the presence of 500 mM *cis*-4-hydroxyproline (Cis) (\blacktriangle (WT), (Δ , Δ INS)) (C); and WT Mj ProRS ($0.5 \mu\text{M}$) and Mj Δ C81 ($1.0 \mu\text{M}$) in the presence of 500 mM alanine (\blacktriangle (WT), (Δ , Δ C81)) (D). Assays were performed at 24°C with Ec and Mm ProRS, 30°C with Sc ProRS, and 60°C with Mj and Mt enzymes. Each assay was carried out in duplicate with the values differing by $<20\%$.

logs. Although we could not observe any pre-transfer editing activity for Ec Δ INS Gly₁₂Ser₄ against alanine (Fig. 6B) or *trans*-4-hydroxyproline (data not shown), ATP hydrolysis was stimulated in the presence of *cis*-4-hydroxyproline (Fig. 6C). Addition of up to $10 \mu\text{M}$ cognate Ec tRNA^{Pro} transcript did not stimulate editing further (data not shown). Similarly, stimulation of ATP hydrolysis was not observed in the presence of $10 \mu\text{M}$ cloned INS domain (data not shown) (40). Because stimulation of ATP hydrolysis depends on the initial formation of adenylate, the lack of pre-transfer editing of alanine and *trans*-4-hydroxyproline by Ec Δ INS Gly₁₂Ser₄ is likely due to the weak adenylate synthesis activity observed in the presence of these non-cognate amino acids (Fig. 4). In addition, the reduced pre-transfer activity of the deletion mutant against *cis*-4-hydroxyproline relative to WT Ec ProRS (Fig. 6C) is consistent with their relative amino acid activation activities (Table 1). Interestingly, in contrast to WT Ec ProRS where very low levels of ATP hydrolysis are observed in the presence of proline, Ec Δ INS Gly₁₂Ser₄ exhibits significant pre-transfer editing of the cognate amino acid (Fig. 6B). The K_m^{Pro} measured for the Δ INS variant is elevated ~ 200 -fold relative to WT ProRS, suggesting that weak binding of the prolyl-adenylate may contribute to release and subsequent hydrolysis. The correlation between amino acid K_m and rate of pre-transfer editing is discussed further below.

The contributions of the extra domains of eukaryotic-like ProRS to pre-transfer editing were also examined. Only a minor

reduction (~ 2 -fold) in the pre-transfer editing activity of Mj ProRS was observed upon deletion of the C-terminal extension domain (Fig. 6D). Similarly, the pre-transfer editing activity of the N-terminal truncated Sc ProRS was reduced only ~ 3 -fold relative to the WT enzyme, suggesting that the N-terminal domain of Sc ProRS is not directly involved in the hydrolysis of non-cognate aminoacyl-adenylates (Fig. 6A). Taken together, these studies suggest that neither the INS domain of Ec ProRS nor the extension domains of eukaryotic-like ProRSs are the site of pre-transfer editing.

AMP Formation in the Presence of Proline and Alanine by Ec ProRS—As mentioned above, in the absence of tRNA, ProRS acts as an ATP hydrolase through the iterative formation and hydrolysis of non-cognate aminoacyl-adenylates, which results in a net conversion of ATP to AMP and PP_i. To characterize the pre-transfer editing activity of Ec ProRS in more detail, we utilized an assay in which we could simultaneously monitor AMP and aa-AMP formation in the presence of both

proline and alanine. Reaction products were separated by TLC (Fig. 7, A and B) and accumulation of [³²P]AMP and aa-[³²P]AMP in solution over multiple turnovers was quantified using PhosphorImager analysis. From the TLC images, it was apparent that the formation of AMP increased over time for both cognate and non-cognate substrates, but as expected, the alanine reaction exhibited a greater extent and rate of net ATP hydrolysis than the proline reaction. The rate of AMP formation in the presence of 500 mM alanine was 3-fold greater than the rate of AMP formation in the presence 1 mM proline (Table 3, Fig. 7, C and D). Similar results were obtained with 33.5 mM proline (data not shown), and ATP hydrolysis in the presence of alanine was not stimulated by the addition of up to $15 \mu\text{M}$ tRNA^{Pro} (data not shown), consistent with previously published data (21). Control reactions showed that there was very little formation of AMP detected in the absence of enzyme or amino acid. Therefore, the formation of the AMP could not have been due to spontaneous hydrolysis of ATP and must be the result of adenylate hydrolysis.

There exist two possibilities for the tRNA-independent hydrolysis of the aa-AMP observed in these reactions. AMP could be generated from an enzyme-catalyzed hydrolysis of the adenylate, or alternatively, the adenylate could be released from the enzyme and undergo non-catalytic hydrolysis in solution (Fig. 1, paths 1 and 2, respectively). Interestingly, for both alanine and proline reactions shown in Fig. 7, the amount of free adenylate present in solution exceeds the concentration of

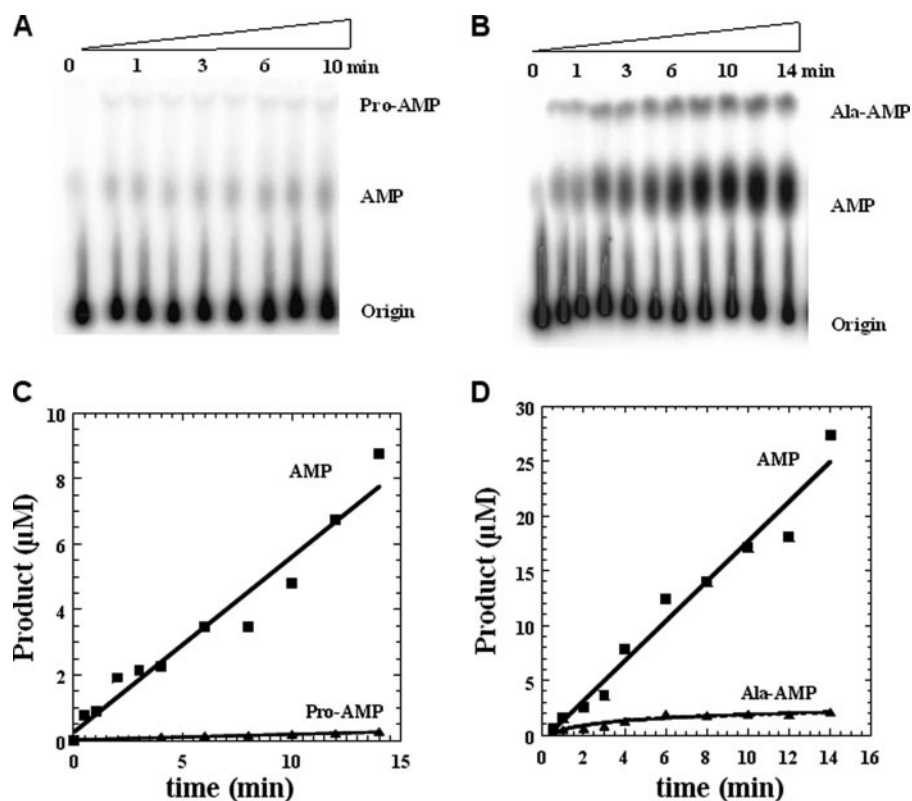


FIGURE 7. TLC-based assay for pre-transfer editing. *A*, reaction time course showing chromatographic separation of Ala- ^{32}P AMP and ^{32}P AMP. *B*, reaction time course showing chromatographic separation of Pro- ^{32}P AMP and ^{32}P AMP. *C*, graphical representation of TLC data shown in panel *A*. *D*, graphical representation of TLC data shown in panel *B*.

TABLE 3
Observed rate constants for overall AMP formation and non-enzymatic adenylate hydrolysis in the presence of Ec ProRS

| | Proline | Alanine |
|---------------------------------------|------------------------------------|---------------------------------|
| | $k_{\text{obs}} (\text{min}^{-1})$ | |
| AMP formation ^a | $(1.95 \pm 0.2) \times 10^{-2}$ | $(5.44 \pm 1.5) \times 10^{-2}$ |
| Non-enzymatic hydrolysis ^b | $(17.8 \pm 2.4) \times 10^{-2}$ | $(11.3 \pm 1.7) \times 10^{-2}$ |

^a Rates were determined using the adenylate synthesis TLC assay described under "Experimental Procedures."

^b Rates were determined by monitoring the rate of adenylate decay using the L-PSA chase assay. All rates represent the average of 3 trials with the standard deviations indicated.

enzyme used. This is consistent with the selective release mechanism of pre-transfer editing. To gain further evidence for this pathway, the non-enzymatic rates of Pro-AMP and Ala-AMP hydrolysis were measured. This was accomplished by carrying out standard reactions with labeled ATP in the presence of alanine or proline for an initial period of time (~10 min) followed by a chase with high concentrations of L-PSA. This non-hydrolyzable adenylate analog binds to ProRS with low nanomolar affinity (49) and is expected to result in irreversible release of bound substrates. The hydrolysis in solution of released aa-AMP was monitored by TLC. The rate constant for non-enzymatic hydrolysis of Pro-AMP was $17.8 \times 10^{-2} \text{ min}^{-1}$ (Fig. 8, *A* and *C*, Table 2). This rate was ~9-fold greater than k_{obs} determined in the ATP turnover assay, which measures the total rate of ATP hydrolysis (both catalytic and non-catalytic) observed in the presence of Ec ProRS. The k_{obs} for the non-enzymatic hydrolysis of Ala-AMP was $11.3 \times 10^{-2} \text{ min}^{-1}$ (Fig. 8*B*, Table

2), which was 2-fold greater than the total hydrolysis rate determined in the ATP hydrolysis reaction. These data demonstrate that the rate of adenylate hydrolysis following the release of the adenylate into solution is greater than the rate of total hydrolysis, measured in the presence of ProRS. Thus, adenylate hydrolysis is not the rate-limiting step in the alanine-stimulated ATP hydrolysis reaction.

Determination of K_i for L-ASA—The non-enzymatic pathway of pre-transfer editing requires that non-cognate adenylates are more readily released from the enzyme active site than the cognate substrate (42). To gain insights into the relative binding affinities of Pro-AMP and Ala-AMP, inhibition studies were carried out using the adenylate analogs L-PSA and L-ASA. Enzyme binding to these non-hydrolyzable analogs should closely approximate binding of the corresponding adenylates and dissociation constants for the analogs can reasonably be used as proxies for those of the adenylate intermediates. L-PSA was previously

shown to be a potent inhibitor of the ATP-PP_i exchange reaction ($K_i = 4.3 \text{ nM}$) (49). Here, the ATP-PP_i exchange assay was used to determine the inhibition constant for L-ASA in the presence of varying proline concentrations. Fig. 9 shows a plot of the slopes obtained from the Lineweaver-Burk plots against the L-ASA concentration. From the x intercept of this plot, the K_i for L-ASA was found to be $88.0 \pm 5.6 \text{ nM}$. The K_i for L-ASA was much higher than the previously reported K_i for L-PSA (49). Hence, Ec ProRS binds L-PSA with a 20-fold greater affinity relative to L-ASA.

To further establish whether there is a correlation between binding affinity and pre-transfer editing, we measured the rate of pre-transfer editing by Ec ProRS in the presence of proline, alanine, *cis*-4-hydroxyproline, and *trans*-4-hydroxyproline. The K_m of these amino acids in adenylate formation was determined by ATP-PP_i exchange assays as mentioned previously. Fig. 10 shows that there is a positive correlation between the rate of pre-transfer editing by Ec ProRS and the K_m of the amino acid analogs examined. Taken together, these results suggest that noncognate amino acids and the corresponding adenylates, which are less tightly bound in the synthetic active site of the enzyme relative to the cognate adenylate, are selectively released and undergo solution hydrolysis.

DISCUSSION

Aminoacyl-tRNA synthetases are highly modular and editing functions tend to reside in appended domains of these enzymes (37, 56). The latter may be insertion domains within

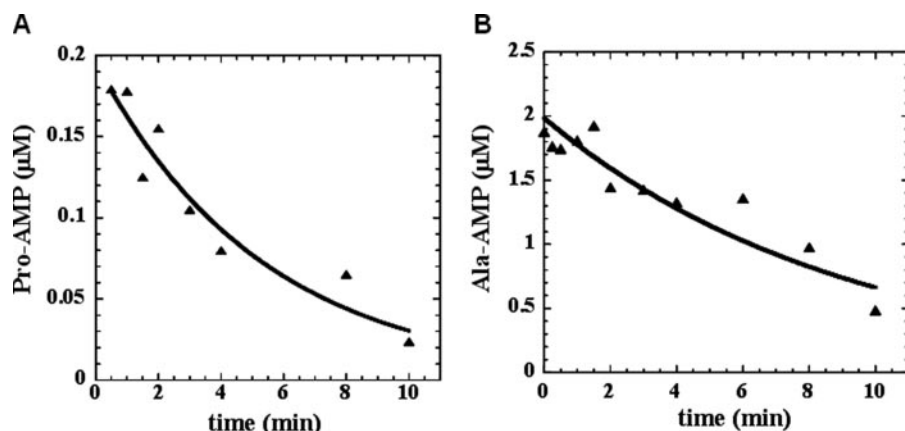


FIGURE 8. **TLC-based chase assay.** Chase assay showing non-enzymatic hydrolysis of Pro-AMP (A) and Ala-AMP (B) measured in solution after addition of the nonhydrolyzable Pro-AMP analog, L-PSA. The data are fit to the equation $y = Ae^{-kt}$.

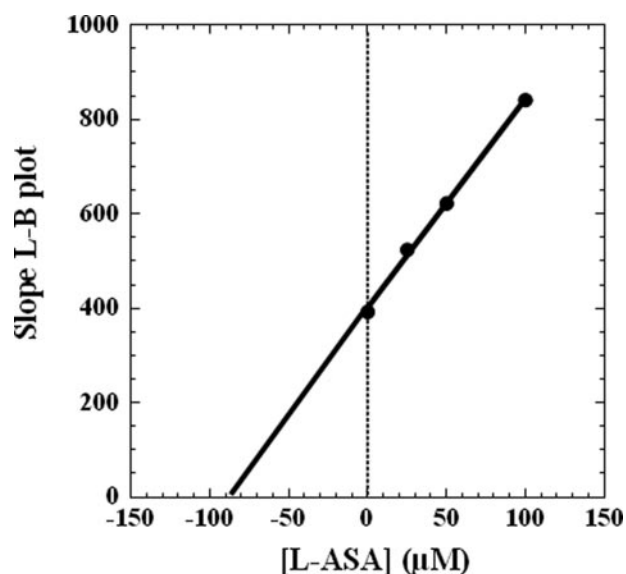


FIGURE 9. **Determination of K_i for L-ASA.** Data were obtained using the ATP-PP_i exchange assay as described under "Experimental Procedures." The intercept of the x axis corresponds to $-K_i$.

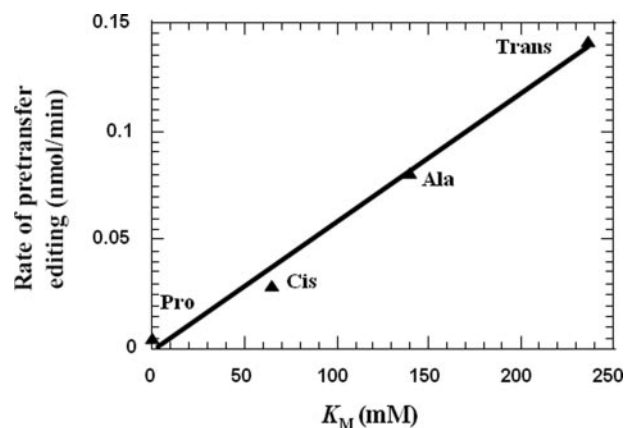


FIGURE 10. **Correlation between the rate of net ATP hydrolysis by Ec ProRS and the K_m of various amino acids.** The x axis shows the K_m of the amino acid determined using the standard ATP-PP_i exchange assay, and the y axis corresponds to the ATP hydrolysis activity of ProRS (2 μM) measured at 24 °C in the presence of 10 mM proline (Pro), 250 mM *cis*-4-hydroxyproline (Cis), 500 mM alanine (Ala), and 500 mM *trans*-4-hydroxyproline (Trans).

the aminoacylation active site, such as the CP1 domain of class I synthetases (9, 10, 27). Alternatively, they may exist as additional domains at the termini, such as the N-terminal domain of Ec threonyl-tRNA synthetase (20). A divergence in editing functions is observed between prokaryotic-like and eukaryotic-like ProRSs. Whereas most prokaryotic-like ProRSs contain an INS domain and possess both pre-transfer and post-transfer editing activities, most eukaryotic-like ProRSs lack an INS domain and possess only pre-transfer editing activity (21, 22, 41).

An earlier mutagenesis study exploring the role of the INS domain in editing by Ec ProRS established that this is the site of post-transfer editing of Ala-tRNA^{Pro}, and suggested that this domain may also play a role in pre-transfer editing (31). To further investigate the role of INS in pre-transfer editing, we previously reported the preparation of a Δ INS construct containing a deletion from residues 249 to 418, which resulted in removal of 86% of the editing domain and 11 flanking residues (40). The end points for this internal deletion were chosen to encompass the majority of the editing domain and also reflected convenient sites for cloning without disrupting sequences within the class II consensus motifs 2 and 3. Although this variant could still activate alanine and was defective in both pre- and post-transfer editing, it was unable to activate proline or to aminoacylate tRNA^{Pro}. In the present work, the deletion construct was re-designed in an attempt to ensure that all critical residues for proline activation were maintained. We also incorporated a Gly_xSer_y flexible linker between motifs 2 and 3 of the aminoacylation catalytic domain (Fig. 2). The optimal Ec Δ INS Gly₁₂Ser₄ construct displayed ~1200-fold reduced proline activation efficiency but aminoacylated tRNA^{Pro} with an overall k_{cat}/K_m that was comparable with WT ProRS.

Although the new Δ INS construct failed to exhibit pre-transfer editing activity against alanine or *trans*-4-hydroxyproline, this was not unexpected due to the lack of adenylate formation for these substrates under normal reaction conditions. In contrast, the Ec Δ INS Gly₁₂Ser₄ variant did exhibit pre-transfer editing against *cis*-4-hydroxyproline. Although the efficiency of *cis*-4-hydroxyproline activation was reduced ~350-fold relative to the WT enzyme, the pre-transfer editing activity was only reduced 6-fold (Table 1, Fig. 6C). In the case of *cis*-4-hydroxyproline, the rate of adenylate formation is significantly higher than that of the *trans*-isomer, and the K_m for the *cis*-isomer is the same as that of WT Ec ProRS (~50 mM) (Table 1). Surprisingly, pre-transfer editing of the cognate proline by Ec Δ INS Gly₁₂Ser₄ is also observed, and the measured K_m^{Pro} is the same as the K_m for *cis*-4-hydroxyproline. Taken together, these results suggest that the INS domain of Ec ProRS is not essential for pre-transfer editing *per se*, but appears to play a role in proper formation or stabilization of active site residues that facilitate aa-AMP formation.

The C-terminal extension domain of the archaeal ProRS species is also dispensable for pre-transfer editing, as Mj Δ C81 displayed only ~2-fold reduced activity relative to WT Mj ProRS (Fig. 5D). Although the precise role of the C-terminal domain is not known, structural studies show that it folds into an independent structural domain, and that the strictly conserved terminal tyrosine residue interacts with and stabilizes the aminoacylation active site (55, 57). The N-terminal extension domain of Sc ProRS resembles the Ec INS domain (~13% sequence identity), but lacks post-transfer editing activity (41).⁴ This domain is also dispensable for pre-transfer editing. The small decrease (~3-fold) in pre-transfer editing activity of Sc Δ N183 is consistent with the decreased rate of adenylate formation displayed by this variant,⁴ and further supports the conclusion that the appended domains play a role in proper assembly of the synthetic active site pocket, but do not play a crucial role in pre-transfer editing activity. Structural changes upon substrate binding have been observed for other class II synthetases. For example, in the case of eukaryotic-like *Thermus thermophilus* ProRS, residues located distantly from the synthetic active site contribute to proper substrate selection and binding (55). It is possible that some of the residues in the INS or extension domains of ProRS are involved in similar induced-fit rearrangements required for optimal substrate binding and catalysis.

The TLC assay used to investigate editing by Ec ProRS in more detail allowed direct observation of adenylate release from the enzyme. The steady-state levels of adenylate are significantly higher for Ala-AMP than for Pro-AMP (Fig. 7, A and B) and in both cases exceed the amount of ProRS present. As expected, net ATP hydrolysis by Ec ProRS with Ala-AMP as an intermediate occurs 3 times faster than ATP hydrolysis when cognate Pro-AMP is formed. In contrast, the rate of non-enzymatic hydrolysis of Pro-AMP is ~2-fold greater than that of Ala-AMP, consistent with earlier reports (58, 59). In both cases, the rate of adenylate hydrolysis in solution exceeds the rate of total ATP hydrolysis in the presence of ProRS. These findings together with measured inhibition constants for substrate analogs L-ASA and L-PSA support a selective release model for pre-transfer editing by Ec ProRS. The direct correlation between the K_m of various proline analogs in adenylate synthesis and the rate of pre-transfer editing as measured by net ATP hydrolysis (Fig. 10), is also in accord with this conclusion. The total amount of ATP consumption in the presence of alanine and tRNA^{Pro} should reflect the summed contributions of selective release and post-transfer editing. The lack of additional stimulation of ATP hydrolysis in the presence of tRNA suggests that pre-transfer editing may be the dominant pathway of alanine proofreading by ProRS.

In summary, the insertion/extension domains of prokaryotic and eukaryotic ProRSs are not involved in pre-transfer editing function. However, their presence enhances adenylate synthesis, most likely by facilitating proper formation of the synthetic active site. This result is in contrast to the pre-transfer editing pathways proposed for class I isoleucyl-tRNA synthetase (32, 60, 61) and leucyl-tRNA synthetase (9), which involve enzy-

matic hydrolysis in a distinct editing domain (30), but is in good agreement with a recent report showing an enzyme-catalyzed pre-transfer editing-like reaction in the aminoacylation active site of class I glutamyl-tRNA synthetase (39). The data presented here suggest that the major pathway for pre-transfer editing by class II Ec ProRS is not enzyme-catalyzed, but involves selective release of the noncognate amino acid from the active site of the enzyme followed by hydrolysis in solution. To definitely establish the existence of kinetic proofreading, it will be necessary to determine the elementary rates of amino acid transfer to the tRNA *versus* the rate of release/hydrolysis, and these studies are currently underway.

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