Functional Role of the Prokaryotic Proline-tRNA Synthetase Insertion Domain in Amino Acid Editing[†]

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ABSTRACT: Aminoacyl-tRNA synthetases catalyze the attachment of specific amino acids to cognate tRNAs in a two-step process that is critical for the faithful translation of genetic information. During the first chemical step of tRNA aminoacylation, noncognate amino acids that are smaller than or isosteric with the cognate substrate can be misactivated. Thus, to maintain high accuracy during protein translation, some synthetases have evolved an editing mechanism. Previously, we showed that class II *Escherichia coli* proline-tRNA synthetase (ProRS) is capable of (1) weakly misactivating Ala, (2) hydrolyzing the misactivated Ala-AMP in a reaction known as pretransfer editing, and (3) deacylating a mischarged Ala-tRNA^{Pro} variant via a post-transfer editing pathway. In contrast to most systems where an editing function has been established, pretransfer editing by *E. coli* ProRS occurs in a tRNA-independent fashion. However, neither the pre- nor the post-transfer editing active site(s) has been identified. Sequence analyses revealed that most prokaryotic ProRSs possess a large insertion domain (INS) between class II conserved motifs 2 and 3. The function of the ∼180-amino acid INS in *E. coli* ProRS is the subject of this investigation. Alignment-guided Ala scanning mutagenesis was carried out to test conserved amino acid residues present in the INS for their role in pre- and post-transfer editing. Our biochemical data and modeling studies suggest that the prokaryotic INS plays a critical role in editing and that this activity resides in a domain that is functionally and structurally distinct from the aminoacylation active site.

Aminoacylation of tRNAs with cognate amino acids is a two-step process catalyzed by aminoacyl-tRNA synthetases. In the first step, the amino acid is activated with ATP to form an aminoacyl−adenylate intermediate. In the second step, the amino acid is transferred to the 3′-end of the cognate tRNA (*1*, *2*). To ensure that aminoacylation occurs with high accuracy, synthetases must select the correct amino acid and discriminate against noncognate amino acids. Since synthetases do not recognize their substrates with absolute accuracy, amino acids that are smaller than or isosteric with the cognate substrate are sometimes misactivated at significant levels. If not corrected, these errors may result in mischarged tRNAs and, ultimately, incorporation of the incorrect amino acid into the growing polypeptide chain during protein synthesis. These mistakes in amino acid incorporation will accumulate over the life span of an organism and further compromise the accuracy of protein synthesis (*3*). Eventually, errors in protein synthesis will exceed the threshold required to maintain cell viability and lead to aging or cell death (*4*, *5*).

To correct errors of misactivation of amino acids and misaminoacylation of tRNAs, some synthetases have evolved a function termed amino acid editing or proofreading. Synthetases have been shown to carry out amino acid editing using two pathways. In pretransfer editing, the noncognate aminoacyl−adenylate intermediate is hydrolyzed, as measured by the level of amino acid-dependent hydrolysis of ATP (*6*). In the most common scenario, this pretransfer hydrolytic activity is stimulated by the presence of cognate tRNA. In post-transfer editing, mischarged tRNAs are deacylated by the synthetase (*6*).

Amino acid editing was first discovered in class I isoleucine-tRNA synthetase (IleRS)[1] (*7*, *8*). IleRS is capable of hydrolyzing misactivated Val-AMP in a tRNA-dependent pretransfer editing reaction (*7*). Additionally, mischarged Val-tRNA^{Ile} is readily deacylated via post-transfer editing (*8*, *9*). The active site of IleRS consists of a Rossmann nucleotide-binding fold (*10*, *11*) that is divided approximately in half by a large insertion domain termed connective polypeptide 1 (CP1). Previously, the isolated CP1 editing domain in class I IleRS, and the corresponding domain in class I valine-tRNA synthetase, were shown to deacylate the isolated mischarged

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[1] Abbreviations: CP1, connective polypeptide 1; IleRS, isoleucine-tRNA synthetase; INS, insertion domain; ProRS, proline-tRNA synthetase; ThrRS, threonine-tRNA synthetase.

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Val-tRNA^{Ile} and Thr-tRNA^{Val}, respectively (*12*). Amino acid residues that are critical in the overall editing activity have been identified in the CP1 regions of IleRS (*11*, *13*) and LeuRS (*14*). In *Escherichia coli* LeuRS, insertion of a 40-amino acid duplication within its CP1 domain allowed the enzyme to mischarge Ile and Met onto tRNA^{Leu} (*15*). Biochemical studies (*13*, *16*, *17*) and more recent structural data (*11*, *18*) strongly support the functional separation of the amino acid activation and editing sites in these class I systems.

Amino acid editing activity has also been observed in class II synthetases. For example, phenylalanine-tRNA synthetase has been reported to deacylate mischarged Ile-tRNA^{Phe} (*19*), while alanine-tRNA synthetase has been shown to hydrolyze misactivated Gly (*20*). More recently, threonine-tRNA synthetase (ThrRS) was shown to possess post-transfer editing against Ser. This class II enzyme misactivates Ser (*21*, *22*) and rapidly deacylates Ser-tRNA^{Thr} (*23*). A truncation mutant lacking the N-terminal domains (N1 and N2) of ThrRS was incapable of post-transfer editing (*23*). This mutant could attach Ser to tRNA^{Thr} at a significantly enhanced rate over that of wild-type ThrRS (*23*). This analysis together with site-directed mutagenesis of highly conserved residues resulted in the localization of the editing site to a cleft in the N2 domain (*23*, *24*). These mutational studies support the functional independence of editing and aminoacylation in this class II system. Interestingly, ThrRS does not appear to carry out pretransfer editing (*21*, *23*).

We recently reported that *E. coli* ProRS is capable of misactivating and editing Ala via both the pre- and post-transfer editing pathways (*25*). This class II enzyme provides a unique system for further exploring editing activities of synthetases. Unlike most other synthetase systems, which require the presence of cognate tRNA for efficient pretransfer editing activity (e.g., class I IleRS), or which lack pretransfer editing activity altogether (e.g., class II ThrRS), class II ProRS is capable of tRNA-independent pretransfer editing (*25*). An open question in the field is whether pre- and post-transfer editing active sites overlap (*26*). Thus, in principle, the ProRS system offers a unique opportunity to address this question directly. As a first step, our aim here was to determine whether editing activity in ProRS resides in a domain that is functionally separate from the aminoacylation active site.

Extensive sequence alignments of ProRSs from all three domains of life resulted in their division into distinct "prokaryotic-like" and "eukaryotic-like" groups (*27*, *28*). This division is unusual in that the former group contains synthetases from bacteria and eukaryotic mitochondrial enzymes, whereas the latter group contains synthetases from eukarya, archaea, and bacteria (*27*, *29*). The division of ProRSs into two groups is not strictly correlated with the presence or absence of editing functions (*30*). In particular, whereas *Methanococcus jannaschii* ProRS edits via both pre- and post-transfer pathways, the human enzyme does not possess editing functions. Both prokaryotic-like and eukaryotic-like ProRSs contain class II-specific motifs 1, 2, and 3, as well as an anticodon binding domain. However, prokaryotic-like ProRSs have a large (185 amino acids in *E. coli*) insertion domain (INS) between motifs 2 and 3, which is not present in the eukaryotic-like group (Figure 1). Instead, a smaller amino acid extension is found at the C-terminus of eukaryotic-like ProRSs (*27*). While the functions of motif 1 (dimer formation), motifs 2 and 3 (amino acid activation), and the anticodon binding domain are well-characterized (*28*, *31*−*34*), the role of the prokaryotic ProRS INS is not known. The eukaryotic extension domain is known to form a separate motif that, at least in *Thermus thermophilus* ProRS, binds one atom of zinc and folds back such that its C-terminal residues interact with the aminoacylation active site (*34*). However, the functional significance of this interaction is presently unknown.

To date, the editing active site in class II ProRS has not been identified. Like the CP1 domain in class I synthetases, the novel INS in prokaryotic ProRS bisects the aminoacylation active site. In addition, the INS contains several highly conserved residues found in all prokaryotic ProRSs that contain an INS (Figure 1). These observations led us to hypothesize that the novel INS is the editing domain in *E. coli* ProRS (*30*). To test this prediction, we carried out Ala scanning mutagenesis of conserved amino acid residues in the *E. coli* ProRS INS. We characterized the mutant proteins for cognate and noncognate charging, as well as for pre- and post-transfer editing activities. Taken together, the results of this study provide strong support for the functional role of the prokaryotic INS in amino acid editing reactions, which take place in a site that is separate from the aminoacylation active site.

MATERIALS AND METHODS

RNA Preparation. Wild-type *E. coli* tRNA^{Pro} and a G1:C72/U70 tRNA^{Pro} triple mutant were prepared as described previously (*27*, *35*). All RNAs were transcribed from *Bst*NI-linearized plasmids using T7 RNA polymerase as described previously (*27*, *35*). Reaction mixtures for isolating mischarged tRNA for use in post-transfer deacylation assays contained 1 μM *E. coli* alanine-tRNA synthetase, 2 units/mL inorganic pyrophosphatase, and 10 μM *E. coli* G1:C72/U70-tRNA^{Pro}. Reactions were quenched by the addition of acetic acid to a final concentration of 1%. The mischarged Ala-tRNA^{Pro} variant was then isolated and purified as described previously (*25*). Formation of Pro-tRNA^{Pro} was carried out similarly using *E. coli* ProRS and tRNA^{Pro}. Purified charged tRNA was quantified by scintillation counting.

Enzyme Preparation. Mutant *E. coli* ProRS genes were created using the QuikChange site-directed mutagenesis kit (Stratagene). Expression of wild-type and mutant ProRSs was induced with 1 mM isopropyl β-D-thiogalactopyranoside in either SG13009 pREP4 or BL21(DE3) pLysE cells. Proteins were then purified and prepared as described previously (*36*). Enzyme concentrations were determined by active site titration (*37*).

Enzyme Assays. Aminoacylation assays were performed at room temperature using the published conditions (*38*, *39*). Cognate charging assays were generally carried out with 0.5 μM tRNA^{Pro} and 22.5 μM [^{3}H]Pro. In some reactions with mutant enzymes, a higher concentration of tRNA^{Pro} was used to achieve detectable levels of charging. Reactions were initiated by the addition of wild-type or mutant *E. coli* ProRS to a final concentration of 0.1 μM. Mischarging assays were carried out using the standard conditions with 5 μM tRNA^{Pro}, 22.5 μM [^{3}H]Ala, and 2 μM wild-type or mutant ProRS.

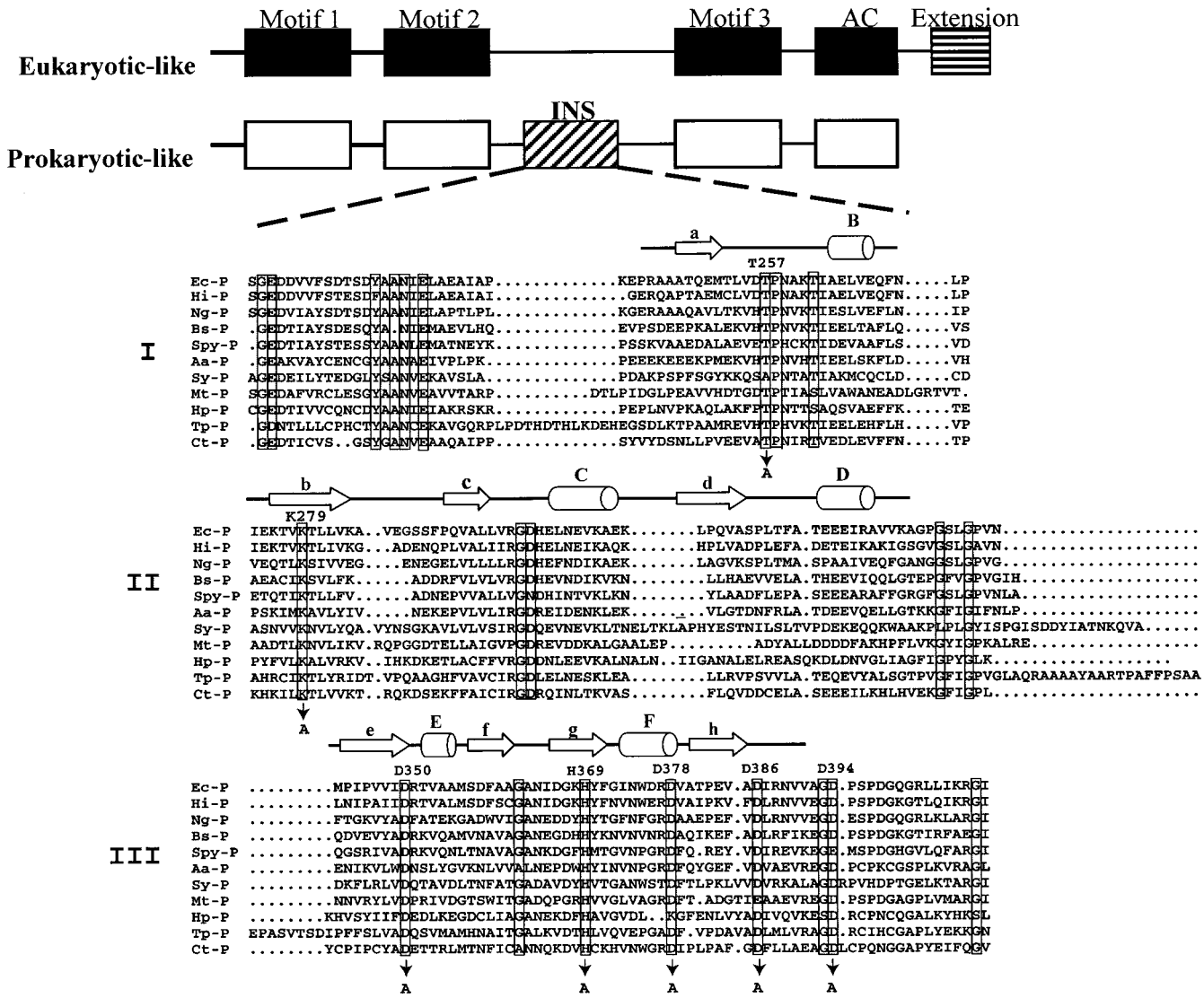

FIGURE 1: Schematic illustration of the domain structure of eukaryotic-like and prokaryotic-like ProRS and detailed alignment of the insertion domain (INS) sequence from 11 prokaryotic ProRSs. Highly conserved amino acids are boxed, and gaps in the sequence are denoted with dots. Predicted secondary structure elements are shown as arrows (β-strands) and cylinders (α-helices) and are based on the known crystal structure of the *H. influenzae* YbaK protein (*41*). β-Strands are labeled from a to h and α-helices from B to F. The numbers across the top refer to codon positions for *E. coli* ProRS. Abbreviations are as follows: Ec, *E. coli*; Hi, *H. influenzae*; Ng, *Neisseria gonorrheae*; Bs, *Bacillus subtilis*; Spy, *Streptococcus pyogenes*; Aa, *Aquifex aeolicus*; Sy, *Synechocystis* sp. PCC6803; Mt, *Mycobacterium tuberculosis*; Hp, *Helicobacter pylori*; Tp, *Treponema pallidum*; Ct, *Chlamydia trachomatis*.

ATP−PP_i exchange assays were performed using the published conditions (*40*). Amino acid concentrations were as follows: 0.05−2 mM proline and 25−500 mM alanine. The *E. coli* ProRS concentration was 1 nM in experiments with proline and 20 nM in experiments with alanine. Kinetic parameters were determined from Lineweaver−Burk plots and represent the average of at least two determinations.

Editing assays were generally carried out at room temperature using the published conditions (*16*, *25*). ATPase assays for assessing pretransfer editing were performed in the presence of 500 mM Ala and initiated with ProRS to achieve a final concentration of 1 μM. Deacylation assays for assessing post-transfer editing were also performed using 1 μM enzyme (*16*, *25*).

Sequence Alignments. A multiple-sequence alignment was carried out using the PILEUP program provided by the Genetics Computing Group (Madison, WI). The alignment was further optimized manually. Sequences were obtained from public databases. For each subdomain, motif patterns were created from the alignments as follows: subdomain I, G-[DE]-x(6,12)-[FY]-[ASG]-A-N-x-E; subdomain ii, [TA]-[PG]-x-x-x-[TS]-x-x-x-[ILVM]-x(10,18)-[ILVM]-K-x-x-[ILVM]-x(13,18)-G-[ND]-x-x-[ILVM]; and subdomain III, D-x(11)-[GA]-x(6)-H-x(22,24)-[GS]-[DE]-x(8,9)-[LIY]-x(4)-[GS]. A search for proteins in the SWISS-PROT database that matched these motifs was performed using a search engine provided by GenomeNet (http://www.genome.ad.jp/).

Homology Model. A homology model of the ProRS INS was constructed on the basis of the known crystal structure of the homologous YbaK protein (*41*) from *Haemophilus influenzae* using the HOMOLOGY module in Insight (*42*). First, the two crystal structures of the *H. influenzae* YbaK protein, each from a different space group, were aligned. Next, the *E. coli* ProRS INS structure was approximated by aligning the sequence with the *H. influenzae* YbaK protein (*41*, *43*). Areas where there was no direct alignment were approximated by random loops generated with HOMOL-

OGY. The domain was visualized with Visual Molecular Dynamics (VMD) (*44*).

RESULTS

Sequence Alignments. Through alignment of sequences from 11 members of the prokaryotic-like group of ProRSs, highly conserved amino acid residues in the INS were identified (Figure 1). The INS could be divided into three subdomains. In the first subdomain (I), nine highly conserved amino acid residues were identified (Figure 1, boxed). Three of these conserved residues (T257, P258, and T262) are located in a Thr-rich cluster. A similar TP motif (T243 and P244 in *E. coli* IleRS) is also present within a Thr-rich region of the CP1 editing domain of class I IleRS. A cocrystal structure of *T. thermophilus* IleRS with bound Val showed that T230 (T243 in *E. coli* IleRS) is adjacent to and pointing toward the noncognate amino acid (*11*). In addition, a site-directed mutagenesis study showed that this residue is involved in the enzyme's post-transfer editing activity (*11*, *13*). Therefore, by analogy to class I IleRS, we chose to mutate T257 in our analysis of the ProRS INS.

As recently reported, the prokaryotic ProRS INS aligns well with a microbial protein superfamily known as the YbaK proteins (*41*, *43*). Although the functional role of the YbaK protein family is unknown, the crystal structure of the *H. influenzae* Ybak protein suggests that it may be involved in oligonucleotide binding (*41*). On the basis of a sequence alignment of the YbaK protein family and ProRS from different species, an invariant Lys residue (K279 in *E. coli* ProRS) was identified. This residue is in subdomain II of the INS (Figure 1). In the available crystal structure of the *H. influenzae* YbaK protein, the Lys residue is located at the opening of a putative small molecule-binding crevice. All of these observations indicated the potential role of this invariant residue in ProRS function, and the Lys residue was therefore targeted in the mutagenesis experiments described below.

Three motif patterns were created from subdomains present in the INS (see Materials and Methods), and a search for proteins having these patterns was conducted. The pattern from subdomain III aligns with several enzymes in the database, including a putative protease ydcP precursor. This subdomain contains a set of highly conserved acidic or basic residues (D350, H369, D378, D386, and D394) (Figure 1). The functional role of these five residues was also tested by Ala scanning mutagenesis.

tRNA^{Pro} Aminoacylation. As discussed above, seven highly conserved residues in the INS were targeted in the site-directed mutagenesis studies described here, including one in subdomain I (T257A), one in subdomain II (K279A), and five in subdomain III (D350A, H369A, D378A, D386A, and D394A). Mutant ProRSs were first tested in an aminoacylation assay to establish the effect on tRNA^{Pro} charging with cognate Pro. With the exception of H369A and D350A, all mutant ProRSs aminoacylated tRNA^{Pro} with an efficiency comparable (within ~2-fold) to that of wild-type *E. coli* ProRS (Figure 2). The H369A and D350A mutations resulted in 2.5- and 20-fold decreases in the level of cognate charging, respectively. The purity of the D350A variant (approximately 20−25%) was significantly lower than that of the other mutants examined in this study, which were all ≥90% pure

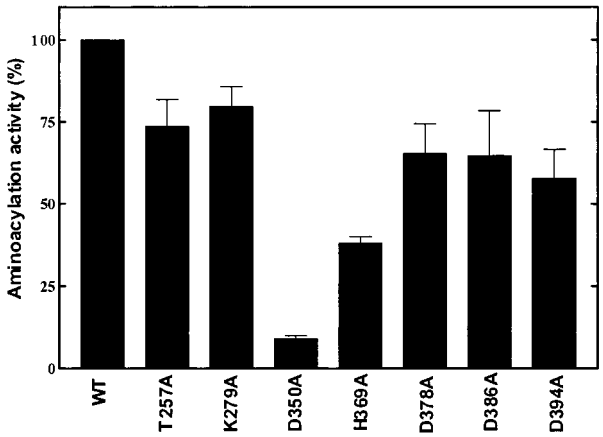

FIGURE 2: Bar graph comparing the aminoacylation activity of *E. coli* ProRS mutants. Assays were carried out as described in Materials and Methods using 0.5 μM tRNA^{Pro} and 0.1 μM ProRS, except for the D350A mutant. Due to the low activity of the latter variant, assays were carried out using 4 μM tRNA^{Pro} and 0.1 μM enzyme. In all cases, enzyme concentrations are based on active site titration as described in Materials and Methods. Results are based on the initial rate of aminoacylation, which under these conditions is proportional to k_{cat}/K_M, and reported as percent activity with wild-type ProRS activity set at 100%. Assays were carried out in triplicate.

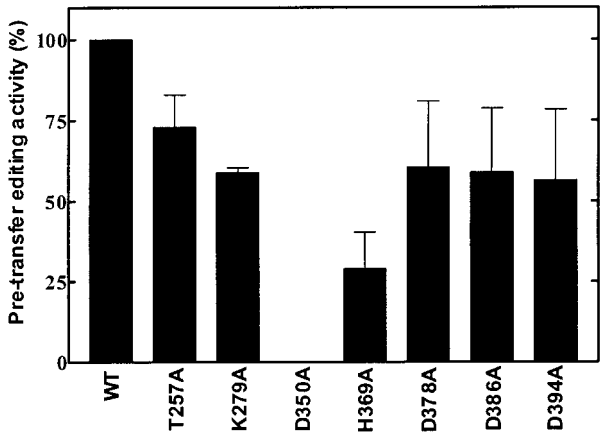

FIGURE 3: Bar graph comparing the pretransfer editing of Ala by *E. coli* ProRS mutants. Results are based on the initial rate of ATP hydrolysis and reported as percent activity with wild-type ProRS activity set at 100%. None of the mutations resulted in cognate Pro hydrolysis, the level of which was consistently below the background level (data not shown).

based on denaturing polyacrylamide gel electrophoresis. Despite repeated attempts, the D350A ProRS variant was resistant to further purification and appeared to be unstable. Although these results are consistent with D350 playing a functionally important role, we cannot rule out the possibility that this residue is primarily involved in protein folding and stability rather than playing a direct role in catalysis.

Pretransfer Editing. Previously, we showed that wild-type *E. coli* ProRS is capable of pretransfer editing. This activity can be monitored by an ATPase assay because the cycle of adenylate hydrolysis and reactivation consumes ATP. *E. coli* ProRS stimulates ATPase activity in the presence of non-cognate Ala as well as hydroxy-Pro analogues, while cognate Pro-AMP is not hydrolyzed (*25*). Figure 3 summarizes the results of ATPase assays conducted with each of the INS domain mutants. Five of the mutations (T257A, K279A, D378A, D386A, and D394A) resulted in a less than 2-fold

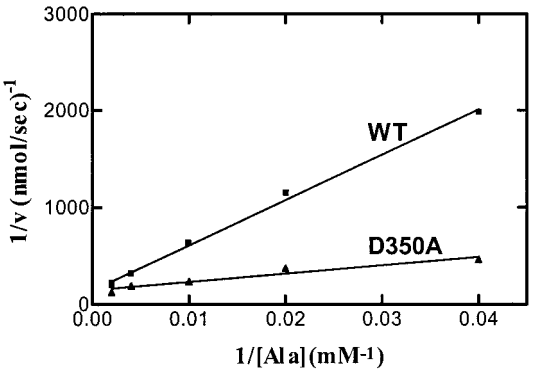

FIGURE 4: Lineweaver−Burk plot comparing the Ala activation activity of wild-type ProRS vs the D350A ProRS variant determined using the ATP−PP_i exchange assay. The data points represent the average of two determinations.

reduction in pretransfer editing activity. Pro and Ala activation were also unaffected by these mutations (data not shown). The H369A mutation resulted in an approximately 2.5-fold decrease in pretransfer editing activity relative to that of wild-type ProRS. This effect is similar to the decreases observed in the level of aminoacylation (Figure 2) and both Pro and Ala activation (data not shown). Thus, we cannot conclude that H369 plays a direct role in pretransfer editing. However, a single Asp residue (D350) appears to be involved in pretransfer editing, as the D350A mutation abolished the enzyme's ability to hydrolyze Ala-AMP, despite the fact that Ala is activated more efficiently by this variant than by wild-type ProRS. Figure 4 shows the results of Ala activation as measured by the ATP−PP_i exchange assay for the wild-type enzyme and the D350A ProRS variant. The k_{cat}/K_M of the latter (0.058 s^{-1} mM^{-1}) is approximately 5-fold enhanced relative to that of wild-type ProRS (0.011 s^{-1} M^{-1}). As discussed above, the purity and stability of this mutant protein are low. Nevertheless, the complete elimination of pretransfer editing activity is striking given the lack of a negative effect on Ala activation. Although Ala activation is stimulated approximately 5-fold by the D350A variant relative to the wild-type enzyme, the level of Pro activation is approximately 10-fold reduced (data not shown), similar to the effect of mutating this residue on cognate charging (Figure 2).

Post-Transfer Editing. We next examined the ability of the mutant ProRSs to deacylate a mischarged Ala-$tRNA^{Pro}$ variant. Because misacylated $tRNA^{Pro}$ is difficult to prepare in good yield, we carried out these post-transfer editing assays using a $tRNA^{Pro}$ variant containing three acceptor stem mutations that facilitate aminoacylation with Ala. Previously, we showed that wild-type *E. coli* ProRS rapidly deacylates the Ala-$tRNA^{Pro}$ variant but does not deacylate cognate Pro-$tRNA^{Pro}$ (*25*). Figure 5 summarizes the effect of each of these INS point mutations on post-transfer editing relative to the activity of wild-type *E. coli* ProRS. Mutation of the Lys residue (K279) within subdomain II of the INS severely affected the ability of ProRS to deacylate mischarged Ala-$tRNA^{Pro}$. The K279A mutant retains only ~5% of the post-transfer editing activity of wild-type ProRS. This 20-fold effect was the most severe defect observed in the post-transfer editing assay. Several other ProRS mutants (T257A, D350A, and H369A) also displayed significantly reduced post-transfer editing activities (4−5-fold reductions), whereas the D386A and D394A mutations resulted in more modest

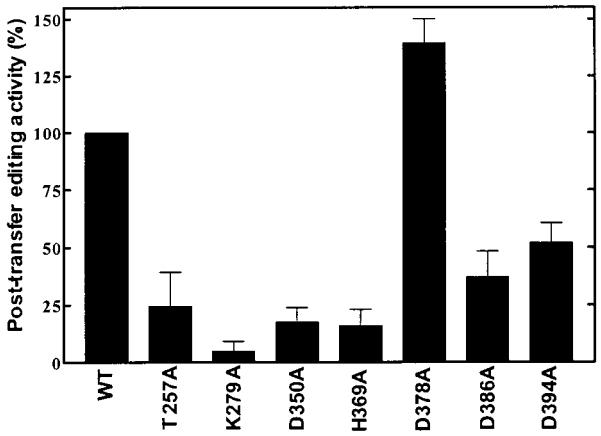

FIGURE 5: Bar graph comparing the post-transfer editing activity of an Ala-$tRNA^{Pro}$ variant by an *E. coli* ProRS mutant. Results are based on the initial rate of deacylation with wild-type ProRS activity set at 100%.

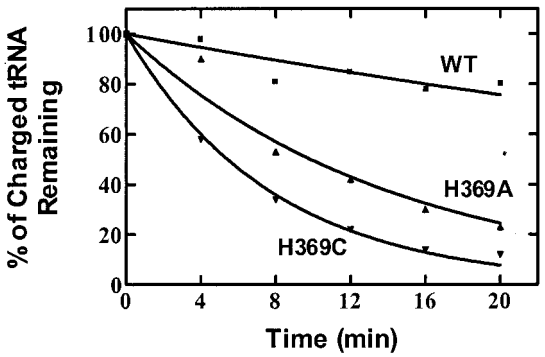

FIGURE 6: Deacylation of cognate Pro-$tRNA^{Pro}$ by the wild type and H369A and H369C ProRS variants. Reactions were initiated by the addition of enzyme to a final concentration of 1 μM (based on active site titration).

decreases (approximately 2-fold). Interestingly, one mutant (D378A) has ~1.4-fold increased activity relative to that of wild-type ProRS.

Deacylation of Cognate Pro-$tRNA^{Pro}$ by Mutant ProRS. As mentioned earlier, wild-type *E. coli* ProRS readily deacylates noncognate Ala-$tRNA^{Pro}$, while cognate Pro-$tRNA^{Pro}$ is not deacylated. Like wild-type ProRS, the majority of the INS mutants do not deacylate cognate Pro-$tRNA^{Pro}$ (data not shown). However, mutagenesis of H369 to Ala results in a loss of specificity in deacylation. In particular, the H369A mutant ProRS, which displayed 5-fold reduced activity against Ala-$tRNA^{Pro}$ (Figure 5), can deacylate cognate Pro-$tRNA^{Pro}$ (Figure 6). The loss of deacylation specificity is consistent with the significantly reduced aminoacylation activity of the H369A ProRS variant (−60%) (Figure 2). To confirm this effect, we constructed a H369C variant. This change results in a mutant ProRS that displays slightly more efficient deacylation of cognate Pro-$tRNA^{Pro}$ than the H369A variant (Figure 6), and as expected, the aminoacylation activity is reduced even further (−75%) relative to that of wild-type ProRS (data not shown).

Misaminoacylation of $tRNA^{Pro}$ by Mutant ProRS. Using wild-type ProRS, we are unable to misacylate $tRNA^{Pro}$ with Ala, presumably due to the effective pre- and post-transfer editing functions. To establish whether any of the INS mutants with defective editing functions are capable of misacylating $tRNA^{Pro}$ with a noncognate amino acid, we next

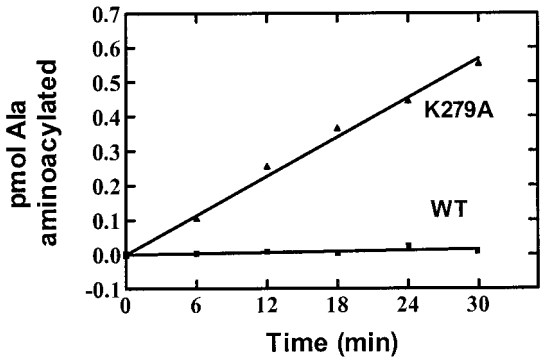

FIGURE 7: Mischarging of Ala onto $tRNA^{Pro}$ by mutant ProRS. Assays were carried out as described in Materials and Methods using 2 μM wild-type or mutant ProRS and 10 μM $tRNA^{Pro}$.

tested the mutant ProRSs in misacylation assays with Ala. One mutant is indeed capable of generating significant levels of Ala-$tRNA^{Pro}$ (Figure 7). Interestingly, this mutant K279A ProRS displayed the most severe defect ($\geq$95%) in post-transfer editing. None of the other mutant ProRS enzymes were capable of mischarging $tRNA^{Pro}$ to a detectable extent in vitro.

DISCUSSION

Previously, we showed that *E. coli* ProRS hydrolyzes misactivated Ala-AMP via a pretransfer editing pathway, and deacylates mischarged Ala-$tRNA^{Pro}$ via post-transfer editing (*25*). Because it is capable of tRNA-independent pretransfer editing, *E. coli* ProRS provides an opportunity to study pre- and post-transfer editing independently, and to address the open question of whether these activities reside within the same active site. As a first step toward this goal, we wanted to determine whether editing activity resides in a domain that is separate from the aminoacylation active site. One distinguishing feature of prokaryotic-like ProRS is the presence of a unique insertion domain (INS) between motifs 2 and 3 (*27*). Although it is not significantly homologous to other synthetase editing active sites, it was previously suggested that this ~180-amino acid INS may be involved in *E. coli* ProRS editing activity (*30*, *41*). To test this hypothesis, we chose to target seven highly conserved residues in the INS for mutagenesis (Figure 1). Each mutant was tested for cognate $tRNA^{Pro}$ aminoacylation with Pro, cognate Pro and noncognate Ala activation, pretransfer editing of Pro and Ala, post-transfer editing of cognate Pro-$tRNA^{Pro}$ and mischarged Ala-$tRNA^{Pro}$, and misacylation of $tRNA^{Pro}$ with Ala.

Of the seven mutants tested via Ala scanning mutagenesis, a single Asp residue (D350) in subdomain III of the INS appeared to be involved in pretransfer editing activity. The D350A ProRS was unable to hydrolyze misactivated Ala-AMP (Figure 3) despite the fact that it retained full Ala activation capability (Figure 4). Interestingly, subdomain III of the INS aligns with a putative protease in the protein database (Figure 1). In the protease family of enzymes, Asp residues are commonly utilized as catalytic residues to facilitate the hydrolysis of peptide amide bonds (*45*). Although it is not clear whether destabilization of the much more labile mixed anhydride bond by enzyme active site residues is required, such a role may be played by D350 to facilitate hydrolysis of the misactivated Ala-AMP. Due to the low purity of the D350A variant, additional mutations at this position and nearby residues will be necessary before the role of D350 in pretransfer editing can be conclusively established. Furthermore, as discussed in more detail below, mutation of this residue is likely to significantly destabilize the putative ligand binding site in the ProRS INS.

In testing the ProRS mutants for their capability to deacylate a mischarged Ala-$tRNA^{Pro}$ variant, we identified four residues (T257, K279, D350, and H369) that upon mutagenesis to Ala displayed significantly reduced post-transfer editing activity. On the basis of these data, the role of D350 in post-transfer editing is unclear, since this variant has significantly reduced aminoacylation activity relative to that of wild-type ProRS. Among these four mutants, the K279A change has the most dramatic effect on post-transfer editing. This mutant retains merely 5% of its post-transfer editing activity, and hence, it is capable of mischarging Ala onto $tRNA^{Pro}$ to a significant extent (Figure 7). In the cocrystal structure of class I *T. thermophilus* ValRS complexed with $tRNA^{Val}$, two charged residues, K270 and R216, are observed near the binding site for A76 of $tRNA^{Val}$ (*18*). In a more closely related class IIa ThrRS system, an H73A/H77A double mutation in the N-terminal editing domain resulted in a loss of post-transfer editing activity, and this mutant enzyme is capable of mischarging Ser onto $tRNA^{Thr}$ (*23*). Although the precise role of positively charged basic residues in post-transfer editing is unknown, several functions can be envisioned. For example, ProRS may utilize K279 as a catalytic residue that is directly involved in deacylating Ala-$tRNA^{Pro}$. Alternatively, it may serve to facilitate the positioning of the 3′-end of this mischarged tRNA into the editing active site. Additional experiments are required to distinguish between these possibilities.

In the cocrystal structure of *T. thermophilus* IleRS complexed with Val, T230 (T243 in *E. coli* IleRS) is 5 Å away from the carboxyl group of the bound L-Val (*11*). In a later study, a T242P (or T242A/N250A) *E. coli* IleRS mutant was also observed to have reduced post-transfer editing activity (*13*). T242 was thought to assist in positioning the Val-$tRNA^{Ile}$ moiety into the editing site of IleRS. Thus, the observed reduction in post-transfer editing activity is presumably caused by the compromised binding of the substrate. In *E. coli* ProRS, we found that a T257A mutant displays 4-fold reduced post-transfer editing activity. As the T257A ProRS still maintains 25% of its activity, it is unlikely to function as a key catalytic residue, but is more likely to facilitate proper positioning of Ala-$tRNA^{Pro}$ into the enzyme's editing site.

In our mutagenesis study, a functionally important His residue (H369) was also identified. In addition to its 5-fold reduced post-transfer editing activity, the H369A ProRS mutant has altered its specificity, as cognate Pro-$tRNA^{Pro}$ is readily deacylated by this variant (Figure 6). In accordance with this new activity, the H369A enzyme is also significantly reduced in cognate tRNA aminoacylation (Figure 2). Recently, a residue with a similar function was identified in class I LeuRS. A T252A *E. coli* LeuRS mutant was observed to lose its substrate specificity in the deacylation reaction and to be totally defective in cognate aminoacylation (*14*). This T252A LeuRS variant does not discriminate between cognate Leu-$tRNA^{Leu}$ and noncognate Ile-$tRNA^{Leu}$ or Met-$tRNA^{Leu}$, and these tRNAs are all readily deacylated. Like

the role proposed for this conserved Thr in the LeuRS CP1 domain, the role of H369 in the ProRS INS appears to be to function as a gatekeeper to prevent the cognate Pro-tRNA^{Pro} from entering the editing active site.

In this study, we have identified amino acid residues that are important for either pre- or post-transfer editing function. One mutation (K279A) resulted in a 20-fold reduction in post-transfer editing activity, yet this change only modestly affected pretransfer editing activity (<2-fold). This is the only mutation that resulted in a ProRS variant that could stably mischarge Ala onto tRNA^{Pro}. Conversely, mutagenesis of D350A was found to completely abolish pretransfer editing, yet this mutant retained approximately 25% of its post-transfer editing activity. A third functionally critical residue (H369) acts as a gatekeeper to prevent cognate Pro-tRNA^{Pro} hydrolysis. All of these residues reside in the unique INS located between motifs 2 and 3, thus firmly establishing the role of this domain in amino acid editing by *E. coli* ProRS.

Although a crystal structure of a prokaryotic ProRS is presently unavailable, a high degree of similarity between the INS and the YbaK family of proteins has been noted (*41*, *43*). Moreover, the structure of one member of this protein family of unknown function has recently been elucidated (*41*). On the basis of this structure and the reported sequence alignment (*41*), we constructed a homology model of the ProRS INS (see the Supporting Information). The YbaK protein structure contains a mixed seven-stranded β-sheet motif surrounded by six short helices. The four longest β-sheets in the core domain of the structure form part of a putative oligonucleotide or oligosaccharide binding fold, and at least three of these strands are present in the homology model of the ProRS INS. A similar well-defined β-sheet motif is found in the aminoacylation active site domain of class II synthetases and serves to bind the cognate aminoacyl−adenylate intermediate as well as the 3′-end of the tRNA (*23*, *32*, *46*−*48*). Thus, we propose that in the ProRS INS, this β-sheet motif is likely to form the binding surface for the misacylated tRNA acceptor stem as well as for the noncognate aminoacyl−adenylate intermediate.

This proposal is supported by the following observations. As reported by Zhang et al. (*41*), the only invariant residue, K279, which is present in all of the aligned YbaK proteins as well as in all prokaryotic INS sequences resides on one of the central β-sheets in a crevice that has been proposed to bind a small ligand such as an oligonucleotide or oligosaccharide (*41*). As reported previously, the INS homology model maintains the putative active site crevice (*41*). The proximity of K279 to a putative ligand binding crevice is consistent with its important role in editing.

Residue H369, which is conserved in all INS domains, but not in the YbaK family, is also close to this binding site in the model, consistent with its role in deacylation specificity. In fact, in the homology model, the two other residues with the largest effects on editing function (D350 and H369) are positioned approximately 8 and 13 Å from the critical K279 residue, respectively. Like K279, D350 is also highly conserved in the YbaK family. Although residue D350 is present in 8 of 10 sequences, it is replaced with an Asn (N117) in the known crystal structure of the *H. influenzae* YbaK protein (*41*). This residue lies at the base of one of the β-sheets constituting the putative ligand binding site. Importantly, N117 is involved in an intricate network of hydrogen bonds with at least four different residues. This observation is consistent with our findings that the D350A substitution results in decreased protein stability. Residues T257, D386, and D378, which had only modest or negligible effects on editing, are farther removed from the putative ligand-binding site in the model, consistent with their less critical role in editing function. These residues are 15−21 Å away from K279.

In summary, although the detailed mechanism of pre- and post-transfer editing by ProRS remains to be elucidated, this study provides the first definitive evidence that the prokaryotic INS is the functional domain for post-transfer editing and is likely to play a role in pretransfer editing as well. Moreover, this domain is distinct from the aminoacylation active site and is not present in eukaryotic-like ProRSs. Whereas higher eukaryotic ProRSs do not appear to possess editing activity, two archaebacterial ProRSs, which also lack a domain homologous to the prokaryotic INS, have been shown to carry out both types of editing reactions (*30*) (M. Nagan, F.-C. Wong, and K. Musier-Forsyth, unpublished data). Ongoing studies are aimed at addressing the question of whether the shorter C-terminal extension in these ProRS systems serves a functional role in editing.

SUPPORTING INFORMATION AVAILABLE

Homology model of the prokaryotic ProRS INS constructed on the basis of the known crystal structure of the homologous YbaK protein from *H. influenzae* (*41*). This material is available free of charge via the Internet at http://pubs.acs.org.

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