Hydrolytic editing by a class II aminoacyl-tRNA synthetase

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Editing reactions catalyzed by aminoacyl-tRNA synthetases are critical for accurate translation of the genetic code. To date, this activity, whereby misactivated amino acids are hydrolyzed either before or after transfer to noncognate tRNAs, has been characterized extensively only in the case of class I synthetases. Class II synthetases have an active-site architecture that is completely distinct from that of class I. Thus, findings on editing by class I synthetases may not be applicable generally to class II enzymes. Class II *Escherichia coli* proline-tRNA synthetase is shown here to misactivate alanine and to hydrolyze the noncognate amino acid before transfer to $tRNA^{Pro}$. This enzyme also is capable of rapidly deacylating a mischarged Ala-$tRNA^{Pro}$ variant. A single cysteine residue (C443) that is located within the class II-specific motif 3 consensus sequence was shown previously to be dispensable for proline-tRNA synthetase aminoacylation activity. We show here that C443 is critical for the hydrolytic editing of Ala-$tRNA^{Pro}$ by this class II synthetase.

The faithful translation of the genetic code is crucial for the survival of all organisms. The aminoacyl-tRNA synthetases play a central role in ensuring the fidelity of this process through selection and activation of the correct amino acid and by specific tRNA recognition (1–3). Aminoacylation of tRNAs is carried out in a two-step process. In the first step, the amino acid is activated with ATP to form the aminoacyl adenylate; in the second step, the activated amino acid is transferred to the tRNA. Misactivation of amino acids can be corrected through a number of different pathways (1). In the so-called "pretransfer" editing pathway, the noncognate aminoacyl adenylate is hydrolyzed by the synthetase in either a tRNA-dependent or tRNA-independent manner. In the "posttransfer" pathway, a tRNA aminoacylated with a noncognate amino acid is deacylated rapidly by the synthetase. A third pathway that may be used to expel a noncognate amino acid from the synthetase active site uses the chemical characteristics of the amino acid. For example, *Escherichia coli* methionine-tRNA synthetase misactivates homocysteine, which has been shown to cyclize via nucleophilic attack of the sulfur atom to form homocysteine thiolactone, with loss of AMP (4).

Amino acid editing was discovered first in class I isoleucine-tRNA synthetase (5, 6). Isoleucine-tRNA synthetase misactivates valine (7), hydrolyzes Val-AMP in a $tRNA^{Ile}$-dependent manner (5), and deacylates Val-$tRNA^{Ile}$ (6). It was determined that the D arm sequence of $tRNA^{Ile}$ is a critical determinant for triggering pretransfer editing (8) and appears to be required for efficient translocation of misactivated Val-AMP or Val-$tRNA^{Ile}$ from the synthetic to the editing active site (9). A large insertion in the active site of IleRS, designated connective polypeptide 1, has been shown to be responsible for the editing activity (10–13).

The editing reactions of class II synthetases have been much less studied than those of class I. Class II phenylalanine-tRNA synthetase specifically deacylates Ile-$tRNA^{Phe}$ (14), and alanine-tRNA synthetase has been shown to hydrolyze misactivated serine and glycine (15). *E. coli* lysine-tRNA synthetase hydrolyzes misactivated homocysteine, homoserine, cysteine, threonine, and alanine, whereas aspartic acid-tRNA synthetase and serine-tRNA synthetase do not (16). Additionally, lysine-tRNA synthetase apparently does not possess posttransfer editing (17). Although *E. coli* threonine-tRNA synthetase does not appear to edit via the pretransfer route, it misactivates serine, and recent experiments indicate that this class II enzyme can deacylate Ser-$tRNA^{Thr}$ (18, 19). An N-terminal domain has been proposed to function as the editing domain in this system (18, 19). *E. coli* proline-tRNA synthetase (ProRS) contains a large insertion domain of unknown function within its amino acid activation site (20). We hypothesized that ProRS might misactivate noncognate amino acids smaller than proline, such as alanine, and, therefore, would require editing activity. With this in mind, in this study, we examine amino acid misactivation and both pre- and posttransfer editing activities of this class II synthetase.

Materials and Methods

RNA Preparation. Wild-type *E. coli* and human $tRNA^{Pro}$ and the *E. coli* $tRNA^{Pro}$ G1:C72/U70 triple mutant were prepared as described (20, 21). RNA was prepared by *in vitro* transcription with T7 RNA polymerase (22). To determine the required time to reach plateau levels of aminoacylation, assays were performed at room temperature according to published conditions (23). Reactions to isolate mischarged tRNA contained 1 μM *E. coli* alanine-tRNA synthetase, 2 units/ml inorganic pyrophosphatase, and 10 μM *E. coli* G1:C72/U70-$tRNA^{Pro}$. All of the amino acids (at least 100 pmol) present in the reaction were tritiated. At the desired time, acetic acid was added to 1% final concentration to quench the reaction. The [^{3}H]Ala-tRNA was purified by repeated phenol extractions, followed by ethanol precipitation. Phenol was equilibrated against diethyl pyrocarbonate-treated water. Charged tRNA was quantified by scintillation counting.

Enzyme Preparation. Wild-type and C443G *E. coli* ProRS were prepared as described (24). *E. coli* alanine-tRNA synthetase was prepared according to the published protocol (25). Enzyme concentrations were determined by active-site titration (26).

Enzyme Assays. ATP-PP_i exchange assays were performed according to published conditions (27). 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 three determinations.

ATP hydrolysis assays were carried out essentially as described (7), with the following modifications: reactions contained 4 units/ml inorganic pyrophosphatase and were quenched with 25 vol of 7% $HClO_4$, 10 mM $NaPP_i$, and 3% charcoal. ProRS concentrations ranged from 1 to 4 μM. The charcoal-bound ATP/AMP was separated from the [^{32}P]P_i in solution by cen-

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Abbreviation: ProRS, proline-tRNA synthetase.

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Table 1. Kinetic parameters for activation of proline and alanine by *E. coli* ProRS

Amino acid	k_{cat}, sec^{-1}	K_M, mM	k_{cat}/K_M, $sec^{-1}\cdot mM^{-1}$	k_{cat}/K_M (relative)
Proline	70 ± 25	0.25 ± 0.035	280	1
Alanine	1.7 ± 0.56	140 ± 65	0.012	4.3×10^{-5}

trifugation. A 50-μl aliquot of the supernatant injected into 5 ml of scintillation fluor was quantified by liquid scintillation counting. For determination of kinetic parameters, the alanine concentration range used was 10–580 mM.

Aminoacylation assays to detect mischarged tRNA were carried out under standard assay conditions (23) with 2–5 μM ProRS, 8–10 μM $tRNA^{Pro}$, and either 214 μM [^{14}C]alanine or 22.5 μM [^{3}H]alanine. Proline aminoacylation assays were carried out under standard conditions of 0.1 μM ProRS, 2 μM $tRNA^{Pro}$, and 22.5 μM [^{3}H]proline (28).

Deacylation assays were performed as described (7), except that each reaction contained 150 mM KPO_4 (pH 7.0), 10 mM $MgCl_2$, 2 units/ml inorganic pyrophosphatase, and 0.1 mg/ml BSA.

Results

Noncognate Amino Acid Activation by *E. coli* ProRS. To test the hypothesis that *E. coli* ProRS has editing activity, we first measured kinetic parameters for alanine activation by ProRS. Indeed, we found that this enzyme misactivates alanine, albeit with a significantly reduced k_{cat} and elevated K_M relative to cognate proline (Table 1). Based on these data, the *in vitro* "discrimination factor" [1/(relative k_{cat}/K_M)] for activation of alanine compared with proline is 23,000. However, in *E. coli*, alanine is present at a much higher concentration than proline (148 μM vs. 9 μM, respectively) (29). Thus, the "effective discrimination factor" (30–32), which takes into account the higher frequency with which ProRS will encounter alanine *in vivo*, is only ≈1,200. Amino acid editing is predicted to be necessary when the discrimination factor is less than ≈3,300, the observed overall error rate for protein synthesis (33). Therefore, *E. coli* ProRS is likely to require editing to maintain fidelity in translation.

Pretransfer Editing by *E. coli* ProRS. We next wanted to establish whether *E. coli* ProRS carries out pretransfer editing *in vitro*. ATP hydrolysis activity is considered diagnostic of hydrolytic editing (1). The ATP hydrolysis activity of *E. coli* ProRS is stimulated in the presence of alanine but, as expected, not in the presence of 2–500 mM cognate proline (Fig. 1 and data not shown). Based on ATPase assays performed over a broad range of alanine concentrations, we determined a K_M^{Ala} of 216 mM and a pretransfer editing rate of 0.035 sec^{-1}. Using this assay, we determined that *E. coli* ProRS also edits the proline analogs *cis*- and *trans*-4-hydroxy-proline (Fig. 1). However, ProRS is unable to hydrolyze activated azetidine-4-carboxylic acid, a four-membered ring analog of proline that is toxic to *E. coli* cells (34, 35). Notably, the presence of an unmodified $tRNA^{Pro}$ transcript, or a mixture of native *E. coli* tRNAs, does not further stimulate ATP hydrolysis activity (data not shown).

Posttransfer Editing by *E. coli* ProRS. Posttransfer editing assays typically require the measurement of deacylation rates of preformed mischarged tRNAs (7). *E. coli* ProRS misaminoacylates $tRNA^{Pro}$ with alanine at an approximately 50,000-fold reduced efficiency relative to aminoacylation with proline. We also attempted to misaminoacylate $tRNA^{Pro}$ with alanine by using *Caenorhabditis elegans* mitochondrial and *E. coli* alanine-tRNA synthetases. These misaminoacylation assays were performed under conditions reported to increase mischarging, including the addition of up to 20% DMSO or methanol and inorganic pyrophosphatase (36). Using these methods, we were unable to produce sufficient amounts of mischarged $tRNA^{Pro}$ for use in deacylation assays.

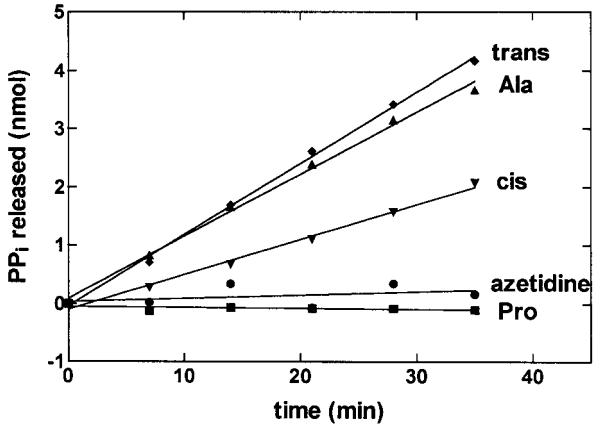

Fig. 1. Noncognate amino acids stimulate ATP hydrolysis by *E. coli* ProRS. Graph showing the ATP hydrolysis activity of ProRS (2 μM) in the presence of 250 mM *trans*-4-hydroxyproline (trans) (◆), 500 mM alanine (▲), 250 mM *cis*-4-hydroxyproline (cis) (▼), 250 mM azetidine-4-carboxylic acid (azetidine) (●), and 2 mM proline (■).

Because misacylated wild-type $tRNA^{Pro}$ was difficult to obtain in good yield, we decided to use an *E. coli* $tRNA^{Pro}$ variant containing three acceptor stem mutations, C1:G72 → G1:C72 and C70 → U70 (Fig. 2*A*) (21). This mutant is charged by *E. coli* ProRS, albeit at a reduced efficiency because of the absence of a major ProRS recognition element, G72 (28, 37). However, this variant is an excellent substrate for *E. coli* alanine-tRNA synthetase because it contains the G3:U70 base pair, a major determinant for aminoacylation with alanine (38, 39), and lacks G72, which is a known negative or blocking element (21). Using this triple mutant, it was possible to isolate mischarged G1:C72/U70-[^{3}H]Ala-$tRNA^{Pro}$ in high yield. We found that *E. coli* ProRS rapidly deacylates this Ala-$tRNA^{Pro}$ variant (Fig. 2*B*) but does not deacylate *E. coli* or human [^{3}H]Pro-$tRNA^{Pro}$ (Fig. 2 *C* and *D*). The rapid deacylation activity was specific to *E. coli* ProRS, because other enzymes, including human and *Methanococcus jannaschii* ProRSs and *E. coli* alanine-tRNA synthetase, did not exhibit this activity (Fig. 2*E*). To ensure that deacylation of G1:C72/U70-Ala-$tRNA^{Pro}$ does not result from the introduction of the three acceptor stem mutations, we also prepared G1:C72/U70-Lys-$tRNA^{Pro}$. This was accomplished by using human lysine-tRNA synthetase, which has nonspecific charging capabilities (T. Stello and K.M.-F., unpublished data). This mischarged variant was not deacylated by *E. coli* ProRS (Fig. 2*F*).

Role of Single Cysteine in *E. coli* ProRS Editing Activities. *E. coli* ProRS contains a single cysteine residue that is located in the class II-specific motif 3 sequence and aligns with residues that have been implicated in amino acid binding specificity in other class II systems (ref. 24 and references therein). Mutagenesis of C443 to alanine and glycine has a relatively small effect (4- and 7-fold, respectively) on the overall rate of the aminoacylation reaction (24). Our previous results suggested that the thiol located at position 443 is not essential for aminoacylation activity but is likely to form the prolyl-adenylate substrate-binding pocket (24). To establish the role of this single cysteine residue in amino acid editing activity, we tested the C443G mutant, which maintained the highest aminoacylation activity,

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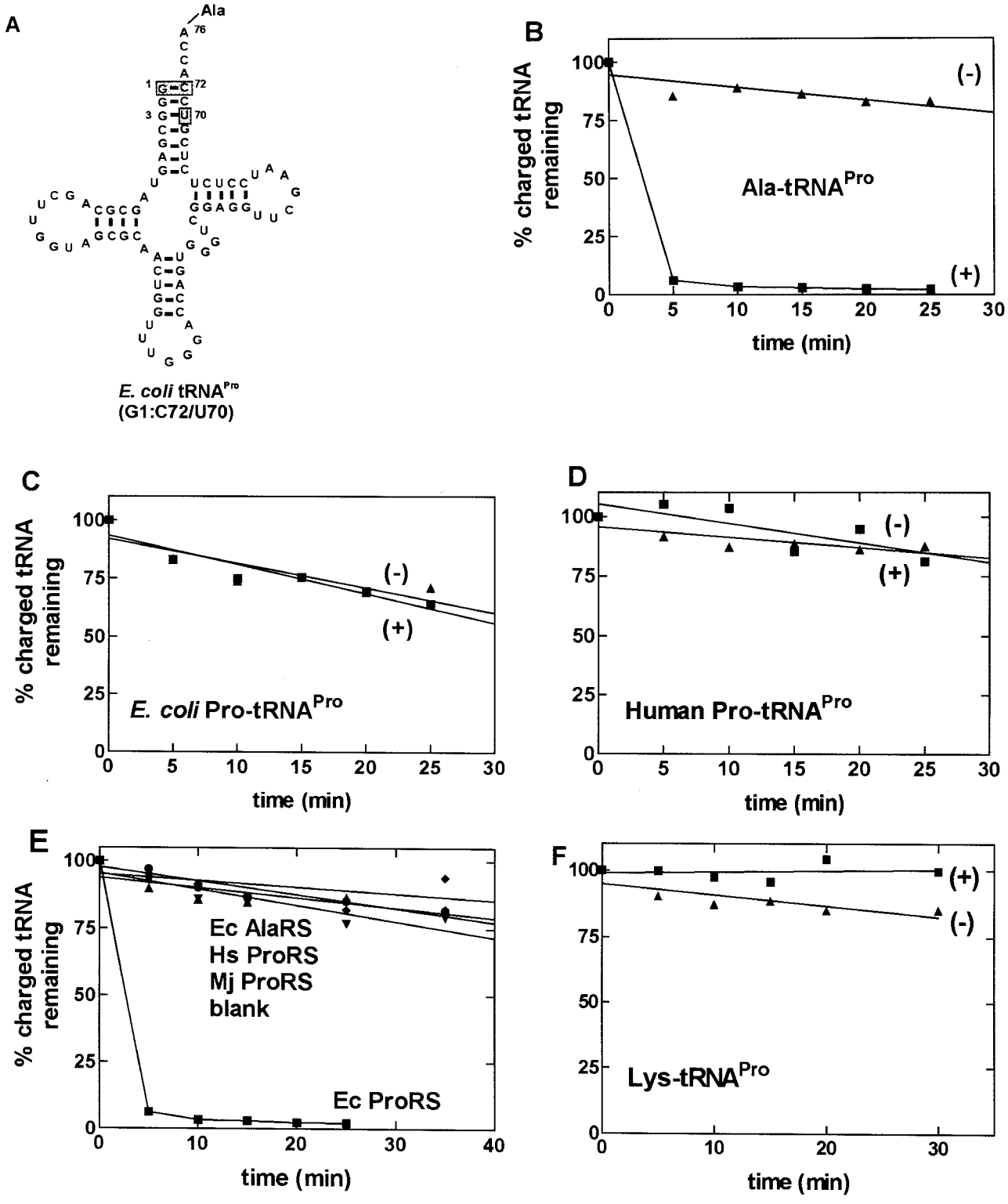

Fig. 2. Deacylation of wild-type $tRNA^{Pro}$ and mischarged $tRNA^{Pro}$ variants. (*A*) The sequence of alanine-accepting *E. coli* $tRNA^{Pro}$. The shaded boxes indicate nucleotides that are mutated relative to wild-type *E. coli* $tRNA^{Pro}$. (*B–D*) Graphs showing the deacylation of G1:C72/U70-[^{3}H]Ala-$tRNA^{Pro}$ (*B*), *E. coli* [^{3}H]Pro-$tRNA^{Pro}$ (*C*), and human [^{3}H]Pro-$tRNA^{Pro}$ (*D*) in the presence (+) and absence (−) of *E. coli* ProRS. (*E*) Graph showing that the efficient deacylation of the Ala-$tRNA^{Pro}$ variant is specific for *E. coli* (Ec) ProRS (0.5 μM). *E. coli* AlaRS (1 μM) (◆), human (Hs) ProRS (0.6 μM) (●), and *M. jannaschii* (Mj) ProRS (0.4 μM) (▼) were unable to deacylate *E. coli* G1:C72/U70-Ala-$tRNA^{Pro}$. (*F*) G1:C72/U70-[^{3}H]Lys-$tRNA^{Pro}$ is not deacylated by *E. coli* ProRS. In *B–F*, the data obtained in the presence of *E. coli* ProRS are represented by ■, and the control reaction carried out in the absence of enzyme is represented by ▲.

in both pre- and posttransfer editing assays. Fig. 3*A* shows that the C443G mutation abolishes the rapid deacylation of the Ala-$tRNA^{Pro}$ variant that is observed with wild-type ProRS. In this assay, the mutant enzyme concentration was the same as that of the wild-type enzyme (0.1 μM). When the concentration of C443G-ProRS is increased, weak deacylation activity is

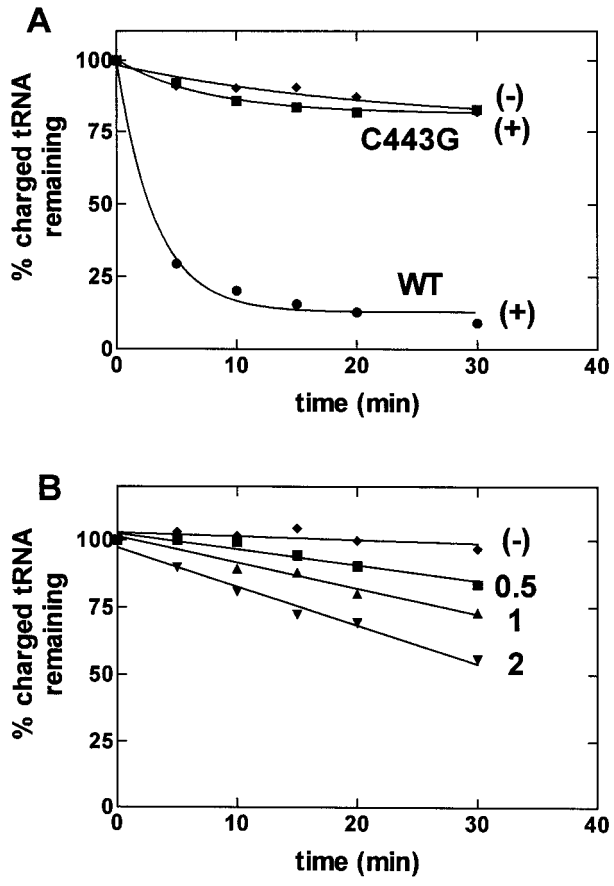

Fig. 3. Deacylation of G1:C72/U70-Ala-tRNA^{Pro} by wild-type (WT) and C443G ProRS. (*A*) Deacylation by 0.1 μM C443G-ProRS (■) and wild-type ProRS (●). (*B*) Deacylation by 0.5 μM (■), 1 μM (▲), and 2 μM (▼) C443G-ProRS. In both graphs, an assay performed in the absence of ProRS (◆) is shown.

observed (Fig. 3*B*). The mutant enzyme does not deacylate cognate *E. coli* Pro-tRNA^{Pro} under these high enzyme conditions (data not shown). Thus, the activity is specific for the mischarged tRNA and is not a result of an increase in the nonspecific background rate of deacylation that some synthetases have been shown to exhibit (6, 40). Based on the weak deacylation activity observed at high mutant ProRS concentrations, we determined that the C443G mutation results in a 150-fold (±44) decrease in the efficiency of posttransfer editing. In contrast, C443G-ProRS exhibited wild-type levels of pretransfer editing of alanine, as measured by the ATP hydrolysis assay (data not shown).

Discussion

We show here that class II *E. coli* ProRS misactivates the noncognate amino acid alanine and possesses both pre- and posttransfer editing activity. Additionally, we find that some nonproteinaceous amino acids appear to be misactivated and subsequently hydrolyzed by *E. coli* ProRS. 4-Hydroxy-proline is incorporated into collagen via a posttranslational modification (41). To avoid its random incorporation into proteins, its adenylate, when formed, must be prevented from being transferred to tRNA^{Pro}. Indeed, *cis*-4-hydroxy-proline and its isomer, *trans*-4-hydroxy-proline, both stimulate ATP hydrolysis, suggesting a pretransfer editing pathway. In contrast to misactivated 4-hydroxy-proline, the adenylate of the four-membered ring analog of proline, azetidine-4-carboxylic acid, is not hydrolyzed by *E. coli* ProRS. This is consistent with the finding that this compound is toxic to *E. coli* and can be incorporated into proteins in place of proline (34, 35, 42).

We also show that the rapid, alanine-specific posttransfer deacylation activity observed in the presence of *E. coli* ProRS depends on a single cysteine residue located within the amino acid activation active site. This residue is located at position 443 within motif 3, and sequence alignments indicate that there is a conserved cysteine, serine, or threonine at this position in all ProRSs sequenced to date (20). The C443 residue previously was shown to be dispensable for aminoacylation (24) and, as shown here, does not appear to be important for pretransfer editing. In contrast, the active-site cysteine plays a critical role in the observed posttransfer deacylation activity. Although C443G-ProRS is severely defective in posttransfer hydrolysis, it is not completely inactive. However, a much higher concentration of enzyme is required to observe deacylation in the absence of C443. From the data presented here, we cannot distinguish between a direct or an indirect involvement of C443 in the catalytic reaction. Based on the ''double-sieve'' mechanism of amino acid editing, a second active site that is distinct from the activation active site contains key catalytic residues responsible for editing (32). This mechanism indeed has been observed in the case of several class I enzymes (10–13, 43). A recent report on class II threonine-tRNA synthetase suggests that a separate editing active site also may be present in this enzyme (18, 19). By analogy, the large insertion domain located between motifs 2 and 3 of prokaryotic-like ProRSs is a good candidate for the second sieve (20, 44). Therefore, an indirect role for C443 in posttransfer editing is more likely than a direct catalytic role. Although C443 is dispensable for aminoacylation, it is involved in the formation of the proline-binding pocket (24). Thus, this residue may be required for optimal binding of the end of a mischarged tRNA before its transfer to the editing active site. The efficiency of transfer of alanine to tRNA^{Pro} by C443G-ProRS is not increased by the mutation. However, this is not too surprising given the observation that the mutant enzyme maintains full pretransfer editing capabilities.

Taken together, our results strongly suggest that in addition to C443G, other residues are likely to be involved in both editing reactions. By analogy to class I synthetases, these catalytic residues may be located in a domain that is distinct from the activation active site.

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