Trans-editing of Cys-tRNA$_{Pro}^\text{Pro}$ by *Haemophilus influenzae* YbaK Protein*

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Prolyl-tRNA synthetases (ProRSs) from all three domains of life have been shown to misactivate cysteine and to mischarge cysteine onto tRNA$_{Pro}^\text{Pro}$. Although most bacterial ProRSs possess an amino acid editing domain that deacylates mischarged Ala-tRNA$_{Pro}^\text{Pro}$, editing of Cys-tRNA$_{Pro}^\text{Pro}$ has not been demonstrated and a double-sieve mechanism of editing does not appear to be sufficient to eliminate all misacylated tRNA$_{Pro}^\text{Pro}$ species from the cell. It was recently shown that a ProRS paralog, the YbaK protein from *Haemophilus influenzae*, which is homologous to the ProRS editing domain, is capable of weakly deacylating Ala-tRNA$_{Pro}^\text{Pro}$. This function appears to be redundant with that of its corresponding ProRS, which contains a canonical bacterial editing domain. In the present study, we test the specificity of editing by *H. influenzae* YbaK and show that it efficiently edits Cys-tRNA$_{Pro}^\text{Pro}$ and that a conserved Lys residue is essential for this activity. These findings represent the first example of an editing domain paralog possessing altered specificity and suggest that similar autonomous editing domains could act upon different mischarged tRNAs thus providing cells with enhanced proofreading potential. This work also suggests a novel mechanism of editing wherein a third sieve is used to clear Cys-tRNA$_{Pro}^\text{Pro}$ in at least some organisms.

Aminoacyl-tRNA synthetases (aaRSs) activate specific amino acids and synthesize aminoacyl-tRNAs, which are essential intermediates in protein synthesis. The high fidelity of this process must be ensured for cells to survive (1–3). In some cases, the amino acid binding pocket of an aaRS is unable to effectively discriminate among chemically and/or structurally related amino acid substrates. In these cases, the high likelihood of misactivation and misacylation of a noncognate amino acid requires an error correction mechanism (4, 5). Therefore, to ensure the exquisite specificity required for protein synthesis, aaRSs have evolved editing or proofreading functions. Specific hydrolysis of a noncognate aminoacyl-adenylate intermediate occurs via pretransfer editing, whereas a misacylated tRNA is hydrolyzed by a postransfer editing activity (5).

Both classes of synthetases have been shown to possess editing functions. Class I isoleucyl-tRNA synthetase, leucyl-tRNA synthetase, and valyl-tRNA synthetase use the highly conserved connective polypeptide 1 (CP1) domain to edit a variety of noncognate amino acids (6–13). Among class II synthetases, alanyl-tRNA synthetase (AlaRS) (14, 15), prolyl-tRNA synthetase (ProRS) (16–19), and threonyl-tRNA synthetase (ThrRS) (20, 21) possess editing domains that have been shown to hydrolyze Gly- and Ser-tRNA$_{Ala}^\text{Ala}$, Ala-tRNA$_{Pro}^\text{Pro}$, and Ser-tRNA$_{Thr}^\text{Thr}$, respectively. The internal editing domain of AlaRS and the N-terminal editing domain of ThrRS share sequence similarity (20, 22), while the editing domain of ProRS (INS) appears to be unique, sharing no sequence homology to any of the other known synthetase editing domains (17, 23).

Whereas the CP1 editing domain of class I synthetases is highly conserved through evolution, phylogenetic analyses have revealed that the class II-specific editing domain found in all AlaRSs and bacterial and eukaryotic ThrRSs is missing in archaeabacterial ThrRSs. However, a distinct domain for editing has recently been identified in the latter (22, 24). In the case of class II ProRS, the INS domain is only found in bacteria and is missing in most eukaryotes, archaea, and some bacteria. A region of weak homology to the INS has been identified at the N terminus of lower eukaryotic ProRSs (23). How species that lack a ProRS editing domain achieve the level of amino acid specificity required for the cell to survive is not known. Recently, a homolog of the ProRS INS domain (23, 25), present in all three kingdoms of life, was shown to possess hydrolytic editing activity (18, 26). In particular, it was demonstrated that the *Haemophilus influenzae* YbaK protein specifically hydrolyzes Ala-tRNA$_{Pro}^\text{Pro}$ but not Pro-tRNA$_{Pro}^\text{Pro}$ in vitro (18). *H. influenzae* is closely related to *Escherichia coli* (27) and therefore possesses a bacterial type ProRS containing a canonical editing domain with high sequence identity (73%) to the *E. coli* INS domain. In addition, the *H. influenzae* tRNA$_{Pro}^\text{Pro}$ sequence is 96% identical to *E. coli* tRNA$_{Pro}^\text{Pro}$ with only three differences at nonconserved positions in the TVC arm.

The function of the *H. influenzae* YbaK protein in the cell is not known. Its relatively weak Ala-tRNA$_{Pro}^\text{Pro}$ editing activity appears to be redundant with that of full-length ProRS. Interestingly, efficient editing of Ala-tRNA$_{Pro}^\text{Pro}$ was recently demonstrated by another INS domain homolog (designated PrdX) from *Clostridium sticklandii*, a bacterium that lacks a ProRS editing domain (26). This finding and the correlation with the occurrence of the prdX gene in organisms that lack an editing domain in the context of ProRS suggest that editing of Ala-tRNA$_{Pro}^\text{Pro}$ is performed by the autonomous editing domain in *vivo*.

ProRSs from all three domains of life have been shown to misactivate cysteine and to misacylate cysteine onto tRNA$_{Pro}^\text{Pro}$ (19, 28, 29, 30). Moreover, the x-ray crystal structures of ProRS from *Methanothermobacter thermautotrophicus* complexed with Cys- and Pro-sulfamoyl-adenylates show that ProRS accommodates both adenylates in a very similar manner (31). This finding provides structural support for the biochemical data showing that discrimination of Pro and Cys does not occur at the level of amino acid binding or adenylate synthesis. Surprisingly, Cys-tRNA$_{Pro}^\text{Pro}$ is resistant to editing by ProRS (28–
Materials—All amino acids and chemicals were purchased from Sigma unless otherwise noted. [3H]Alanine (54 Ci/mmol), [3H]lysine (79 Ci/mmol), [3H]serine (33 Ci/mmol), and [3H]glycine (23 Ci/mmol) were from Amersham Biosciences and [35S]cysteine (1705 Ci/mmol) was from PerkinElmer Life Sciences. All tRNAs used in this study were prepared by in vitro transcription as described before (16, 35).

Enzyme Preparation—Wild-type (WT) E. coli ProRS, E. coli INS, WT E. coli AlaRS, E. coli AlaRS-CQ, and WT human lysyl-tRNA synthetase (LysRS) were purified using Talon cobalt affinity resin (Clontech) as described previously (15, 36). H. influenzae YbaK protein was purified from E. coli B834 cells containing pCYB HI1434 using the IMPACT™ I system (New England Biolabs) as described before (18, 23). The YbaK K46A point mutant was created from plasmid pET15b HI1434 encoding the wild-type H. influenzae YbaK protein using the QuickChange site-directed mutagenesis kit (Stratagene). Expression of the mutant protein was induced with 1 mM isopropyl β-D-thiogalactopyranoside in LB21/DE53 E. coli cells (Novagen). The protein was purified using Ni2+-NTA resin (Qiagen) according to the manufacturer’s protocol, followed by removal of the His tag using the thrombin-cleavage capture kit (Novagen). The protein was concentrated using Centricon 10 concentrators (Amicon) and stored in 50 mM Tris-HCl (pH 7.5), 20 mM KCl, 20 mM 2-mercaptoethanol, 25 mM MgCl2, and 0.1 mg/ml bovine serum albumin. E. coli ProRS (0.5 μM) was used to aminoacylate [35S]Cys (59.9 μM) onto E. coli tRNA Pro (10 μM) in reaction buffer containing 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM MgCl2, 25 mM dithiothreitol, and 2 mM ATP as described previously (41). In some experiments, E. coli ProRS was premixed with the H. influenzae YbaK protein before initiating the charging reaction.

Aminoclylation Assays and Preparation of Aminoclyl-tRNAs—Aminoclylation assays with 3H-labeled amino acids were performed at room temperature as described previously using purified tRNA transcripts prepared in vitro (15, 19, 40). Briefly, E. coli AlaRS-CQ (3.4 μM) was used to aminoacylate the E. coli G1C72U70 tRNA Pro variant (5.0 μM) in the presence of both [3H]Ser (9.1 μM) and cold Ser (0.5 mM) in buffer containing 50 mM HEPES (pH 7.5), 4 mM ATP, 20 mM KCl, 20 mM 2-mercaptoethanol, 25 mM MgCl2, and 0.1 mg/ml bovine serum albumin. E. coli ProRS (0.5 μM) was used to aminoacylate [35S]Cys (59.9 μM) onto E. coli tRNA Pro (10 μM) in reaction buffer containing 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM MgCl2, 25 mM dithiothreitol, and 2 mM ATP as described previously (41). In some experiments, E. coli ProRS was premixed with the H. influenzae YbaK protein before initiating the charging reaction.

To prepare aminoacyl-tRNAs, [3H]Ala and [3H]Lys were misaminoacylated onto the E. coli G1C72U70 tRNA Pro variant (8 μM) using WT E. coli AlaRS (2 μM) and WT human LysRS (4 μM), respectively, in the 7H-labeled amino acid buffer given above (16). [3H]Ser was mischarged onto E. coli tRNA Pro (8 μM) using E. coli ProRS (8 μM) in the [3H]Ser buffer described above. [3H]Gly-tRNA Pro and [3H]Gly-tRNA Pro were synthesized by mischarging E. coli tRNA Pro (8 μM) using E. coli AlaRS-CQ (5.7 μM) as described previously (15). The mischarged tRNAs were purified by repeated phenol extractions, followed by ethanol precipitation. The aminoacyl-tRNAs were quantified by scintillation counting and stored at −20 °C in 50 mM KPO4 (pH 7.0).

Decaylation Assays—Decaylation assays were carried out at room temperature according to published conditions (16, 29). Reactions contained ~0.8 μM [3H]Gly-tRNA Pro, 1 μM [3H]Ser-tRNA Pro, 0.5 μM [3H]Lys-tRNA Pro, 1 μM [3H]Gly-tRNA Pro, and 2 μM [3H]Cys-tRNA Pro and were initiated with the amount of protein indicated in the figure legends. In each case, a background reaction was carried out in which buffer (pH 7.0) was used to initiate the reaction. Each aminoacyl-tRNA had its own background reaction, although for clarity only one representative data set is shown in the figures.

RESULTS

To study the substrate specificity of the decaylation activity of the H. influenzae YbaK protein, we synthesized various aminoacyl-tRNAs, including Ala-, Lys-, and Cys-tRNA Pro and Ser- and Gly-tRNA Pro. The tRNA transcripts possessed ~70% acceptor activity on the basis of plateau-level aminoacylation assays with cognate amino acid. Our initial decaylation assays used high concentrations of purified YbaK protein (21 μM), similar to previous studies (18). As shown in Fig. 1, under these conditions we observe similar levels of decaylation of Ala-tRNA Pro and Ser- and Gly-tRNA Pro. In contrast, hydrolysis of Lys-tRNA Pro was not observed, which is in agreement with previous studies using E. coli ProRS (16). Strikingly, the YbaK protein was capable of rapidly hydrolyzing Cys-tRNA Pro under these conditions (Fig. 1).

We next tested the decaylation of the same set of aminoacyl-tRNAs using lower concentrations of YbaK (1.0 μM). Under these conditions, nearly complete decaylation (~90%) of Cys-tRNA Pro was observed after 20 min, but hydrolysis of the other aminoacyl-tRNAs, including Ala-tRNA Pro, was not detectable (Fig. 2A). In contrast, under similar reaction conditions, E. coli ProRS hydrolyzed Ala-tRNA Pro but not Cys-tRNA Pro (Fig. 2B). Thus, the YbaK protein appears to be specific for Cys-tRNA Pro, while ProRS is specific for Ala-tRNA Pro.

As shown in Fig. 1, even in the presence of high concentrations of YbaK, hydrolysis of Lys-tRNA Pro was not observed. Therefore, it is unlikely that an RNase contaminant is responsible for the Cys decaylation activity of the YbaK protein. To confirm that the editing reaction involves specific removal of the amino acid rather than cleavage by a nuclease, we showed that Cys-tRNA Pro, which had been hydrolyzed by YbaK and recovered via phenol-chloroform extraction and ethanol precipitation, could be re-aminoacylated with Pro by ProRS (data not shown). The extent of charging observed corresponded to the level expected based on the acceptor activity of the tRNA.

To determine whether the YbaK protein was functional in the presence of ProRS, aminoacylation assays were performed with E. coli ProRS in the absence and presence of varying concentrations of the YbaK protein. A concentration-dependent decrease in aminoacylation was observed in the presence of YbaK, with almost complete (~90%) elimination of charging achieved at a ProRS:YbaK ratio of 1:4 (Fig. 3). Thus, the YbaK protein performs its trans-editing reaction in the presence of a functional ProRS, thereby preventing Cys-tRNA Pro from being used in protein synthesis.

A strictly conserved Lys residue (Lys279 in E. coli ProRS) present in all bacterial ProRS editing domains was previously shown to be critical for posttransfer editing of Ala-tRNA Pro by
E. coli ProRS (17). This amino acid is also found in the YbaK protein family (Lys 46 in H. influenzae YbaK). To determine whether this Lys residue is important for Cys editing, we mutated Lys46 to Ala in H. influenzae YbaK. Strikingly, this single point mutation abolished Cys-tRNA Pro deacylation activity even at high concentrations of the mutant protein (21 μM) (Fig. 4). In addition, K46A YbaK also failed to deacylate Ala-tRNAPro (data not shown). The Lys 46 residue lies within the putative substrate-binding pocket of the H. influenzae YbaK protein (23). Although its precise role in catalysis is unknown, the mutagenesis data suggest that this highly conserved Lys is a key catalytic residue of the protein family.

The separately cloned INS domain from E. coli ProRS was previously demonstrated to possess posttransfer editing activity (18). To establish whether the Ala specificity observed for editing by ProRS depends on protein domains outside of the editing domain or whether it is inherent to the INS domain, we tested the cloned INS domain for editing of Cys-tRNAPro. Whereas the cloned INS displayed a similar level of deacylation activity of Ala-tRNAPro as the YbaK protein, no hydrolysis of Cys-tRNAPro was observed, even in the presence of high concentrations of INS (10 μM) (data not shown). Thus, the substrate specificity is dictated by residues within the INS domain rather than by other regions of ProRS.

**DISCUSSION**

The findings reported here demonstrate at least one mechanism by which cells can survive despite the fact that they possess a ProRS that misactivates Pro and Cys with similar efficiencies. Previous studies had suggested that a double-sieve mechanism (32–34) for ProRS editing cleared Ala-tRNA Pro but was not capable of eliminating Cys-tRNAPro species. In particular, whereas all three amino acids (Pro, Ala, and Cys) pass through the course sieve (i.e. the aminoacylation active site) and are thus activated, only the smaller amino acid (Ala) enters into the second sieve and is edited, while Cys and Pro, which have similar molecular volumes (31), are rejected by this fine sieve. As shown in Fig. 2A, to eliminate Cys-tRNAPro, the mischarged tRNA appears to enter a third sieve and undergo a second round of proofreading by an autonomous trans-editing protein.

Intriguingly, previous studies suggest that not all INS domain homologs can hydrolyze Cys-tRNAPro as demonstrated here for the H. influenzae YbaK protein. In particular, a recent report demonstrated selective and efficient hydrolysis of Ala-tRNAPro by the INS domain homolog from the bacterium C. sticklandii (CSPrdX) (26). The ProRS expressed from C. sticklandii is classified as "eukaryotic like" and...
lacks the bacterial-specific editing domain. Unlike *H. influenzae*, which possesses both a ProRS with a canonical editing domain responsible for eliminating Ala-tRNA<sub>Pro</sub> and a YbaK protein with Cys-specific editing activity, the CSPrdX protein appears to be specific for Ala-tRNA<sub>Pro</sub>. Thus, the data obtained to date suggest the existence of at least two distinct classes of free-standing aa-tRNA<sub>Pro</sub> trans-editing factors, i.e. those with Ala specificity (ProX) and those with Cys specificity (YbaK).

The mechanism for Cys-tRNA<sub>Pro</sub> editing in *C. sticklandii* or in other species that appear to lack both a functional editing domain within ProRS and a YbaK protein (such as *Methanococcus jannaschii* and *Saccharomyces cerevisiae*) is still unknown. Some species may contain a ProRS that is more specific to start with and thus do not require editing (26). However, this is unlikely to be the explanation in the case of *M. jannaschii* ProRS, which very efficiently activates Cys and mischarges it to start with and thus do not require editing (26). However, this may be demonstrated for the *H. influenzae* YbaK protein.

In conclusion, the double-sieve mechanism for editing does not appear to be sufficient to eliminate all misacylated tRNA<sub>Pro</sub> species in some organisms. In particular, some bacteria, which contain a ProRS editing domain that clears Ala-tRNA<sub>Pro</sub>, additionally require an autonomous editing domain paralog that is specific for editing Cys-tRNA<sub>Pro</sub>. Although we have demonstrated that the highly conserved residue Lys<sup>46</sup> is critical for the editing activity, the detailed mechanism of trans-editing, as well as the molecular basis for the altered amino acid specificity of the free-standing editing domain, remain to be determined. A recent search using the Conserved Domain Architecture Retrieval Tool (CDART available at ncbi.nlm.nih.gov) identified 145 ProRSs containing a sequence homologous to the *H. influenzae* YbaK protein either as an internal domain or an N-terminal extension and an additional 213 homologous proteins expressed as independent domains. Additional studies of these intriguing ProRS paralogs from a variety of organisms will be required to gain a better understanding of how specific aminoacyl-tRNA synthesis is achieved in living cells.

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