Ancestral AlaX Editing Enzymes for Control of Genetic Code Fidelity Are Not tRNA-specific*

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Background: Both AlaRS editing domains and their free-standing editing forms, AlaX, are thought to exclusively deaclylate mischarged tRNAAla.

Results: Contrary to this belief, we find that AlaX-S deacylates Ser-tRNAAla and Ser-tRNAThr.

Conclusion: AlaX-S is likely to be the ancestor of AlaX, AlaRS editing, and bacterial-like ThrRS editing domains.

Significance: The biochemical activities of AlaX-S link, for the first time, the activities of the AlaRS and ThrRS editing domains.

Accurate protein synthesis requires the hydrolytic editing of tRNAs incorrectly aminoacylated by aminoacyl-tRNA synthetases (ARSs). Recognition of cognate tRNAs by ARS is less error-prone than amino acid recognition, and, consequently, editing domains are generally believed to act only on the tRNAs cognate to their related ARSs. For example, the AlaX family of editing domains, including the editing domain of alanyl-tRNA synthetase and the related free-standing trans-editing AlaX enzymes, are thought to specifically act on tRNAAla, whereas the editing domains of threonyl-tRNA synthetases are specific for tRNAThr. Here we show that, contrary to this belief, AlaX-S, the smallest of the extant AlaX enzymes, deacylates Ser-tRNAAla in addition to Ser-tRNAAla and that a single residue is important to determine this behavior. Our data indicate that promiscuous forms of AlaX are ancestral to tRNA-specific AlaXs. We propose that former AlaX domains were used to maintain translational fidelity in earlier stages of genetic code evolution when mis-serylation of several tRNAs was possible.

Mistranslation is a biological phenomenon caused by the misacylation of tRNAs by aminoacyl-tRNA synthetases (ARSs). 3 Bona fide translation errors are often deleterious, but mistranslation can also be an adaptive strategy to correct other kinds of insults (1–3). The reported cases of toxic mistranslation are invariably due to errors in amino acid recognition. In contrast, adaptive mistranslation is mostly due to tRNAs that are charged promiscuously or by ARSs that acylate different tRNAs (4–8).

Toxic mistranslation is prevented by cells through proof-reading mechanisms (9). Error-prone ARSs are toxic to both bacterial and mammalian cells, and, to avoid mischarging, they have evolved editing domains to recognize mischarged tRNAs (10–15). In some instances, editing domains display tRNA selectivity, and, in other cases, the domains per se are not tRNA-specific and rely on the specificity of other domains for their activity (16–19). For example, YbaK is not tRNA-specific in vitro and will deacylate any tRNA charged with Cys (20, 21). In vivo, YbaK tRNA specificity requires interactions with ProRS (20). In contrast, AlaXs and ProXP-ala (a YbaK homolog) are tRNA-specific and recognize the same conserved tRNA acceptor stem identity elements (16, 22, 23).

Single domain AlaX editing proteins are widely distributed phylogenetically, and clear tRNAAla mischarged with Ser or Gly by AlaRS (Fig. 1) (24). They are homologous to the editing domains of alanyl- and threonyl-tRNA synthetases (AlaRS and ThrRS) (10, 22, 25–28). There are three types of freestanding AlaXs: AlaX-S, AlaX-M, and AlaX-L, which stand for small, medium, and large, respectively (Fig. 2A). AlaX-S is homologous to the C-terminal portion of the AlaRS editing domain (29, 30). AlaX-M contains an additional Gly-rich motif in its N-terminal region (31, 32). Finally, AlaX-L closely resembles the entire editing domain of AlaRS and contains an additional C-terminal domain (C-Ala) (27, 33).

The deacylation activities of the AlaXs vary. Although AlaX-M, AlaX-L, and AlaRS hydrolyze both Ser-tRNAAla and Gly-tRNAAla (22, 25, 27, 32), AlaX-S only hydrolyzes mischarged Ser-tRNAAla (29), possibly because of the lack of the Gly-rich domain (Fig. 2A), which results in an enlarged amino acid binding pocket and a much lower binding affinity toward Gly (29). In contrast, bacterial ThrRS editing domains hydrolyze Ser-tRNAThr (26, 34). AlaX freestanding domains are generally thought to be specific for tRNAAla, and their specificity has been linked to a highly conserved arginine residue (22) that
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Aminoacylation: Ala + tRNA_{Ala} \rightarrow \text{Ala-tRNA}_{Ala}

Misacylation: Ser + tRNA_{Ala} \rightarrow \text{Ser-tRNA}_{Ala}

Editing: Ser + tRNA_{Ala} \rightarrow \text{Ser-tRNA}_{Ala}

is believed to interact with the unique G3:U70 pair in the tRNA_{Ala} acceptor stem. A single R693K mutation in the editing domain of *Escherichia coli* AlaRS allows this enzyme to hydrolyze Ser-tRNA^{Thr} (22).

We observed that, in archaeal AlaX-S, this arginine is substituted by glutamic acid (Fig. 2B and 2C). Intrigued by the lack of conservation of such a critical residue, we investigated the tRNA specificity of AlaX-S. We found that, although AlaX-S retains specificity for serylated tRNAs, it is not tRNA-specific and deacylates Ser mischarged onto tRNA_{Ala}, tRNA_{Thr}, and tRNA_{Pro}. These features set AlaX-S apart from the rest of the AlaX family of enzymes. A phylogenetic analysis of AlaX sequences shows that AlaX-S constitutes an ancient clade that separates from other AlaXs and that is more closely related to bacterial ThrRS editing domains than any other AlaX member. Therefore, on the basis of our biochemical and phylogenetic analyses, we propose a novel evolutionary model for the AlaX family in which the ancestral form of the AlaX family of editing enzymes likely was a tRNA-promiscuous enzyme whose function was to correct widespread mis-serylation of tRNAs by ancient primitive ARSs. This ancestral form would afterward have evolved to deacylate either tRNA_{Ala} (AlaX-M, AlaX-L, AlaRS editing domain) or tRNA^{Thr} (ThrRS editing domain), fulfilling the need for higher levels of discrimination before being incorporated as the editing domains now characteristic of contemporary synthetases.

**EXPERIMENTAL PROCEDURES**

Phylogenetic Analysis of AlaX Domains—Hidden Markov Model profiles were built for each protein containing an AlaX domain (AlaX-S, AlaX-M, AlaX-L, and AlaRS editing domain and ThrRS editing domain) using the HMMER package (35), which were used as query to find homologues in the Uniprot database. The resulting sequences were aligned using T-Coffee (36) with the structure-guided alignment mode, and phylogeny was built using the maximum likelihood method with bootstrapping using PHYLIP 3.68 (37). The final tree was visualized with TreeView (38).

Protein Expression and Purification—*Pyrococcus horikoshii* AlaX-S and AlaRS were a gift from Prof. Sokabe (University of California, Davis). The *Pyrococcus abyssi* ThrRS N-terminal editing domain (PaThrRS-NTD) was a gift from Prof. Sankaranarayanan (Centre for Cellular and Molecular Biology, India). Expression was induced in *E. coli* Rosetta™ BL21 (Novagen) at $A_{600} = 0.6$ with 1 mM isopropyl β-D-thiogalactoside for 6 h at 37 °C. Enzymes were prepared as described previously (29, 40).

In Vitro tRNA Transcription and Purification—Sequences for *P. horikoshii* tRNA_{Ala} and tRNA^{Thr} were taken from tRNAdb (41) (Fig. 3). In vitro-transcribed *P. horikoshii* tRNA_{Ala} and tRNA^{Thr} were prepared as described previously (42). *E. coli* tRNA^{Trp} was prepared following protocols published previously (43). Briefly, each tRNA was transcribed from an NspI-digested pUC19 plasmid template using T7 RNA polymerase. The sample was then loaded into a denaturating 10% polyacrylamide gel, and the resulting tRNA band was cut out, electroeluted, and ethanol-precipitated.
Preparation of Aminoacyl-tRNA Substrates—In vitro-transcribed E. coli tRNAPro, P. horikoshii tRNAAla and tRNAThr were 32P-labeled using E. coli tRNA nucleotidyltransferase and [α-32P]ATP (PerkinElmer Life Sciences) as described previously (44). Aminoacylation of tRNAs was carried out using biotinylated dFx (Thermo Scientific) using published conditions with minor modifications (45). Briefly, tRNA and dFx (42 μM each, 18-μl volume) and trace amounts of 32P-labeled tRNA were heated to 95 °C for 1 min, followed by addition of 25 mM MgCl2 and 5 mM of serine-3,5-dinitrobenzyl ester. dFx-catalyzed aminoacylation was carried out for 6 h on ice. Reactions were quenched with 120 μl of 0.3 M NaOAc (pH 5). Biotinylated dFx was removed by incubating the reaction mixture with 150 μl of streptavidin-agarose resin (Novagen) for ~15 min at room temperature, followed by centrifugation in a table-top centrifuge at 4 °C for 2 min at 2000 × g. The supernatant containing the aminoacylated tRNA was removed, phenol-chloroform-extracted, and ethanol-precipitated. The pellet was dissolved in diethylpyrocarbonate-treated water and stored at ~80 °C.

Deacylation Assays—Deacylation assays were performed as described previously (23). Briefly, reactions containing 50 mM HEPES (pH 7.5), 0.1 mg/ml BSA, 10 mM MgCl2, 5 mM 2-mercaptoethanol, 20 mM KCl, and ~1.5 μM aminoacyl-tRNA were initiated by addition of enzyme (2 μM P. horikoshii AlaX-S, 2 μM P. horikoshii AlaRS, or 0.5 μM P. abyssi ThrRS-NTD). At the indicated time points, the reactions were quenched into 6 μl of a solution containing 0.4 unit/μl P1 nuclease in 200 mM NaOAc (pH 5). The quenched solution (1 μl) was spotted onto PEI-cellulose thin-layer chromatography plates (prewashed in water) to separate aminoacyl-[32P]AMP and [32P]AMP. The data were analyzed as described previously (44), and the graphs were prepared using SigmaPlot (Systat Software, San Jose, CA), with error bars representing the standard deviation of triplicate data.

FIGURE 2. Structural comparisons of the AlaX-S, AlaX-M, AlaX-L, AlaRS, and ThrRS editing domains. A, domain organization of AlaRS, AlaXs, and ThrRS. The residue numbers shown correspond to P. horikoshii proteins, except for ThrRS, which corresponds to the E. coli enzyme. The core editing domain is common in all five proteins (red), AlaX-M, AlaX-L, and AlaRS contain the Gly-rich motif (orange) appended to the editing domain. The deacylation activities described to date are summarized in the right panel: AlaX-S (29), AlaX-M (25, 32), AlaX-L (25, 27), AlaRS (22, 25), and ThrRS (22). B, structure-guided multiple sequence alignment of AlaX-S, AlaX-M, AlaX-L, and AlaRS. The conserved Arg residue that confers specificity for tRNAAla is marked with an asterisk. C, superimposition of P. horikoshii AlaX-S, P. horikoshii AlaX-M, and E. coli AlaRS editing domain crystal structures. Residue Arg-693 of E. coli AlaRS corresponds to Glu-145 in P. horikoshii AlaX-S.

Preparation of Aminoacyl-tRNA Substrates—In vitro-transcribed E. coli tRNAPro, P. horikoshii tRNAAla and tRNAThr were 32P-labeled using E. coli tRNA nucleotidyltransferase and [α-32P]ATP (PerkinElmer Life Sciences) as described previously (44). Aminoacylation of tRNAs was carried out using biotinylated dFx (Thermo Scientific) using published conditions with minor modifications (45). Briefly, tRNA and dFx (42 μM each, 18-μl volume) and trace amounts of 32P-labeled tRNA were heated to 95 °C for 1 min, followed by addition of 25 mM MgCl2 and 5 mM of serine-3,5-dinitrobenzyl ester. dFx-catalyzed aminoacylation was carried out for 6 h on ice. Reactions were quenched with 120 μl of 0.3 M NaOAc (pH 5). Biotinylated dFx was removed by incubating the reaction mixture with 150 μl of streptavidin-agarose resin (Novagen) for ~15 min at room temperature, followed by centrifugation in a table-top centrifuge at 4 °C for 2 min at 2000 × g. The supernatant containing the aminoacylated tRNA was removed, phenol-chloroform-extracted, and ethanol-precipitated. The pellet was dissolved in diethylpyrocarbonate-treated water and stored at ~80 °C.

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APRIL 17, 2015 • VOLUME 290 • NUMBER 16 • JOURNAL OF BIOLOGICAL CHEMISTRY

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RESULTS

Sequence and Structural Analysis of the AlaX Domain Supersfamily—Previous work showed that the G3:U70 base pair is critical for aminoacylation of tRNA$^{\text{Ala}}$ by AlaRS (46, 47). This base pair interacts with a conserved Arg in the AlaRS editing domain (Arg-693 in *E. coli*), and the substitution of this residue with Lys results in loss of tRNA specificity (22). Although this residue was initially predicted to be universally conserved, our analysis here shows that AlaX-S enzymes contain a Glu residue (Glu-145 in *P. horikoshii* AlaX-S) (Fig. 2B). Structural superimposition of *E. coli* AlaRS, *P. horikoshii* AlaX-M, and *P. horikoshii* AlaX-S crystal structures demonstrates that Glu-145 is in a similar orientation as Arg-693 and Arg-210 of *E. coli* AlaRS and *P. horikoshii* AlaX-M, respectively (Fig. 2C). On the basis of these observations, we hypothesized that the presence of Glu-145 in AlaX-S alters its substrate specificity, allowing hydrolysis of Ser from different tRNAs.

AlaX-S Is a Nonspecific Ser-tRNA Deacylase—We next examined the tRNA specificity of AlaX-S in vitro. We purified recombinant *P. horikoshii* AlaX-S, *P. horikoshii* AlaRS-N752 (PhAlaRS lacking the C-terminal C-Ala domain), and the isolated N-terminal editing domain of *P. abyssi* ThrRS (ThrRS-NTD). We also prepared *P. horikoshii* Ser-tRNA$^{\text{Ala}}$ and Ser-tRNA$^{\text{Thr}}$ and *E. coli* Ser-tRNA$^{\text{Pro}}$ using the Flexizyme (dFx) system (48). It should be noted that the *P. abyssi* ThrRS editing domain is not related to either the bacterial/eukaryotic ThrRS editing domains or the AlaX family (49–51).

As expected, deacylation assays confirmed robust editing activity of Ser-tRNA$^{\text{Ala}}$ by AlaX-S and PhAlaRS. In contrast, ThrRS-NTD does not possess significant deacylation activity for this substrate (Fig. 4A) but efficiently hydrolyzes Ser-tRNA$^{\text{Thr}}$ (50). PhAlaRS exhibited only weak deacylation activity for Ser-tRNA$^{\text{Thr}}$, whereas AlaX-S robustly edited this substrate, in support of our hypothesis (Fig. 4B). The lack of tRNA specificity of AlaX-S was further confirmed by showing that Ser-tRNA$^{\text{Pro}}$, which is not known to occur in nature, is also a substrate for the enzyme (Fig. 4C).

We then investigated whether the lack of tRNA specificity observed in AlaX-S is also accompanied by a lack of amino acid specificity. Gly-tRNA$^{\text{Ala}}$ had already been shown to not be a substrate of AlaX-S (29). We also tested the deacylation activity of AlaX-S upon Pro-tRNA$^{\text{Pro}}$, finding that AlaX-S is not capable of deacylating this substrate either (data not shown).

Taken together, these results support the idea that AlaX-S retains characteristics of an ancestral freestanding editing protein, able to deacylate multiple mischarged Ser-tRNAs. In contrast, the editing domain of archaeal *P. abyssi* ThrRS is capable of discriminating Ser-tRNAs and appears to be highly specific for Ser-tRNA$^{\text{Thr}}$.
Phylogenetic Analysis of the AlaX Superfamily—Several phylogenetic trees that include bacterial ThrRS, AlaRS, and free-standing AlaXs have been reported (10, 27). These studies did not distinguish AlaX-S from AlaX-M, which were previously classified together as AlaXp-I (27). For this reason, we performed a new phylogenetic analysis to try to resolve all three types of freestanding AlaX sequences (Fig. 5). We found that, although AlaX sequences are represented in the three domains of life, AlaX-S sequences are only found in Archaea. Previous studies have included several bacterial and eukaryal sequences within the set of AlaX-S sequences (27). However, we believe that these sequences may reflect misannotations because of the shorter sequence length of some bacterial and eukaryal AlaX-M proteins. To confirm the archaeal distribution of AlaX-S, we verified that shorter AlaX sequences from bacteria and eukarya had an Arg residue in the homologous position to Arg-693 of E. coli AlaRS and were evolutionary closer to AlaX-M sequences, indicating that AlaX-S members are only found in Archaea.

Our phylogenetic analysis shows that the editing domain of ThrRS is closest to AlaX-S, which positions AlaX-S as an intermediate evolutionary step between ThrRS and the group of AlaRS, AlaX-M, and AlaX-L, suggesting an early separation that split the original enzyme into two different specificities, one for tRNAThr and the other for tRNAAla. Therefore, our results suggest that all forms of AlaX evolved from a common ancestor and shows that both AlaX-M and AlaX-L are derived from AlaX-S.

Surprisingly, a structural superimposition of all the homologous AlaX-type editing domains shows that an Arg residue is also found in bacterial ThrRS editing domains at the equivalent position to E. coli AlaRS Arg-693 (Fig. 6). This implies that the Arg residue by itself does not confer tRNAAla specificity, given that bacterial ThrRS has been shown to specifically deacylate Ser-tRNAThr (26). Additional sequence and structural elements are likely to contribute to specific G3:U70 recognition (52).
FIGURE 6. Multiple sequence alignment of the AlaX-S, AlaX-M, AlaX-L, AlaRS, and ThrRS editing domains. A, structure-guided multiple sequence alignment of AlaX-S, AlaX-M, AlaX-L, AlaRS, and ThrRS. The conserved Arg residue that confers specificity for tRNAAla is marked with an asterisk. B, superimposition of the P. horikoshii AlaX-S, P. horikoshii AlaX-M, E. coli AlaRS, and Staphylococcus aureus ThrRS editing domain crystal structures. Residue Arg-693 of E. coli AlaRS and its corresponding residues in P. horikoshii AlaX-S, P. horikoshii AlaX-M, and S. aureus ThrRS are displayed using stick representations.
DISCUSSION

Editing domains are universal features of some ARSs (10, 26, 53). Originally, however, their function might have been unrelated to protein synthesis quality control and linked to other roles in the RNA world (54). Structural data indicate that the incorporation of editing domains to the ARS ancient catalytic core happened first in class I enzymes (55). However, the existence of free forms of these domains raises the question of whether they originated as freestanding hydrolytic enzymes or as ARS-attached domains.

It is generally accepted that early genetic codes were prone to errors that were gradually minimized as organisms required more faithful genetic translation (56). Here we studied the tRNA specificity of a putative ancestral editing domain, AlaX-S. Our goal was to gain a better understanding of the original function of AlaX-type editing enzymes and the origin of tRNA specificity within AlaX-type editing enzymes. Our data show that AlaX-S is capable of deacylating Ser from multiple tRNA acceptors (Fig. 4), whereas AlaRS, other AlaXs, and the archaeal ThrRS editing domain (ThrRS-NTD) are tRNA-specific.

Our phylogenetic analysis shows that AlaX-S forms a distinct clade that separates AlaXs and AlaRS from ThrRS sequences (Fig. 5). One possibility is that an editing domain with relaxed tRNA specificity (AlaX-S) would have evolved in modern archaeal cells. However, this possibility would not explain the phylogenetic position of AlaX-S between ThrRS and other AlaX-type domains. The correct clustering of the different editing enzymes on the basis of their biochemical activities, and the robust bootstrap support, reinforce the possibility that AlaX-S is the closest relative of the ancestor of all AlaX-type editing domains. This ancestral nature of AlaX-S is further supported by the distribution of the Gly-rich motif responsible for the recognition of Gly in addition to Ser- (29), which is found in AlaX-M, AlaX-L, and AlaRS editing domain but not in the AlaX-S or ThrRS editing domains (Fig. 2A). Therefore, AlaX-S is likely an evolutionary intermediate between the AlaRS/AlaX-M/AlaX-L editing domains and bacterial ThrRS editing domains.

On the basis of both our biochemical and phylogenetic results, we propose that AlaX-S is a direct descendant of an ancestral deacylation enzyme that lacked tRNA specificity and is the functional ancestor of the AlaRS and ThrRS editing domains, including their freestanding forms (Fig. 7). This ancestral enzyme might have been in charge of deacylating mis-serylated tRNAs generated by synthetases with poor amino acid selectivity or by seryl-tRNA synthetases with poor tRNA specificity. This activity would be required in an earlier stage of genetic code development where mis-serylation of tRNAs was particularly frequent. Future work reconstructing ancestral sequences and testing their tRNA specificities will provide additional evidence on the evolution of this family.

The precise order by which amino acids entered the genetic code remains unknown (55, 57, 58), but it is generally accepted that similar amino acids were added through the reassignment of similar codons. The extant codons for Ala, Thr, Pro, and Ser occupy a common space in the genetic code and, possibly, initially coded for a single amino acid. Their diversification during the growth of the code might have generated a fidelity problem that was solved by AlaX-S, which can deacylate serylated tRNAAla, tRNAThr, and tRNAPro. By extension, it is plausible that other editing proteins or domains might have retained their ancestral tRNA specificities and, therefore, may provide additional information on the evolution and growth of the genetic code.

Acknowledgments—We thank Dr. Olivier Jaillon for discussions regarding the evolution of protein domains.
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APRIL 17, 2015 • VOLUME 290 • NUMBER 16 • JOURNAL OF BIOLOGICAL CHEMISTRY 10503