Natural Homolog of tRNA Synthetase Editing Domain Rescues Conditional Lethality Caused by Mistranslation*

Received for publication, August 1, 2008, and in revised form, August 22, 2008 Published, JBC Papers in Press, August 22, 2008, DOI 10.1074/jbc.M805943200

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AlaXp is a widely distributed (from bacteria to humans) genome-encoded homolog of the editing domain of alanyl-tRNA synthetases. Editing repairs the confusion of serine and glycine for alanine through clearance of mischarged (with Ser or Gly) tRNA<sub>Ala</sub>. Because genome-encoded fragments of editing glycine for alanine through clearance of mischarged (with Ser or Gly) tRNA synthetases, the unique widespread distribution of AlaXp arises from the singular difficulties, for translation, poised by alanine.

The editing activities of tRNA synthetases provide a major safeguard against mistranslation, the insertion of the wrong amino acid at a specific codon (1–4). This insertion arises from small amounts of tRNA mischarging, a phenomenon that is intrinsic to the active sites of many of the synthetases. This mischarging results from the inherent inability of the enzyme binding pockets to discriminate rigorously between closely similar amino acid side chains, especially to achieve the level of discrimination needed for the high accuracy of the genetic code. Examples include the synthesis of Val-tRNA<sub>leu</sub> by isoleucyl-tRNA synthetase (3, 5), Thr-tRNA<sub>val</sub> by valyl-tRNA synthetase (6, 7), Val-tRNA<sub>leu</sub> by leucyl-tRNA synthetase (8–10), Ala-tRNA<sub>pro</sub> by prolyl-tRNA synthetase (11, 12), Ser-tRNA<sub>thr</sub> by threonyl-tRNA synthetase (13), Ile-tRNA<sub>phe</sub> by phenylalanyl-tRNA synthetase (4), and Ser- or Gly-tRNA<sub>Ala</sub> by alanyl-tRNA synthetase (AlaRS)<sup>2</sup> (14, 15). Normally, the mischarged tRNAs are cleared by a distinct editing activity, specific to each synthetase. This activity is able to distinguish the correct from the incorrect amino acid fused to its cognate tRNA. In addition to the editing activity that is part of the synthetase and encoded as a separate domain (16–18), there are a few instances of free-standing editing domain homologs encoded by various genomes (12, 19–22). The most widespread (through all three kingdoms of life) of these is AlaXp, a small protein that is a homolog of the editing domain of AlaRS and has been shown to edit Ser-tRNA<sub>Ala</sub> and Gly-tRNA<sub>Ala</sub> <em>in vitro</em> (21–24). Not clear, however, is whether AlaXp plays a role <em>in vivo</em> in guarding against mistranslation.

The importance of editing for the (conditional) survival of bacteria is well established (7, 25, 26). The connection of editing defects to disease in mammalian systems has also been demonstrated from investigations <em>in vivo</em> of the editing activities of valyl-tRNA synthetase (ValRS) and AlaRS. For example, in the case of ValRS, inducing the expression of a transgene encoding an editing-defective ValRS in mammalian cells creates a transdominant, pathological phenotype (27). In the mouse, a mild editing defect in AlaRS leads to neurodegeneration characterized by ataxia and disintegration of Purkinje cells in the cerebellum (28). The editing-defective murine AlaRS synthesizes small amounts of Ser-tRNA<sub>Ala</sub> and the tiny amount of incorporation of Ser at codons for Ala eventually triggers the unfolded protein response and cellular markers for apoptosis.

Thus, the need for editing to guard against mistranslation is now recognized, but the contribution of components like AlaXp remains unanswered because activity <em>in vivo</em> has never been demonstrated. Because <em>Escherichia coli</em> is an exception to the usual case where AlaXp is present, it offers an opportunity to investigate an AlaXp <em>in vivo</em> in circumstances where the bacterium has an editing-defective AlaRS. Previous work showed that serine was toxic to <em>E. coli</em> when it harbored an editing-defective AlaRS (14), thus giving an example, in bacteria, of the significance of mistranslation at codons for alanine. Given these circumstances, the question we posed was whether a transgene encoding an AlaXp could rescue an editing-defective strain of <em>E. coli</em> and, if so, whether that rescue depended on determinants in AlaXp that were essential for its editing function.

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* The work was supported, in whole or in part, by National Institutes of Health Grant GM 23562. This work was also supported by a fellowship from the National Foundation for Cancer Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: AlaRS, alanyl-tRNA synthetase; ValRS, valyl-tRNA synthetase; ThrRS, threonyl-tRNA synthetase.
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EXPERIMENTAL PROCEDURES

Preparation of tRNA—E. coli tRNA<sub>Ala</sub>(UGC) was produced in vitro as described previously (14), except the transcription was done using a MEGAshortscript kit (Ambion, Austin, TX). Mischarged [3H]Ser-tRNA<sub>Ala</sub> was produced in vitro using E. coli AlaRS C666A/584H as described previously (14).

Protein Expression and Purification—E. coli AlaRS and Methanosarcina mazei AlaXp proteins used in activity assays were prepared by expression from plasmid pET21b (Novagen, Madison, WI) as described previously (24).

Aminoacylation Assay—Aminoacylation was performed at room temperature in assay buffer (50 mM HEPES (pH 7.5), 20 mM KCl, 10 mM MgCl₂, 2 mM dithiothreitol) with ATP (4 mM) and tRNA<sub>Ala</sub> transcript (5 μM). Alanine charging was done in the presence of wild-type or mutant AlaRS (50 nM) and [3H]Ala (100 μM). Mischarging was done with wild-type or mutant AlaRS (5 μM) and [3H]Ser (100 μM). Aliquots were quenched and precipitated in 96-well Multiscreen filter plates (Millipore) as described previously (29). After washing and elution by NaOH, samples were counted in a MicroBeta plate reader (PerkinElmer Life Sciences).

Deacylation Assay—[3H]Ser-tRNA<sub>Ala</sub> (~5 μM) was incubated at room temperature with AlaRS (50 nM) or AlaXp (10 nM) in assay buffer before being quenched into 96-well Multiscreen filter plates as described previously (29). Liberated [3H]Ser was collected by centrifugation into 96-well flexible plastic plates and radioactive counts were measured on a MicroBeta plate reader.

Strain Construction—Unless otherwise noted, cells were grown in M9 minimal media (30) with 0.4% glyceral, 0.002% l-arabinose, 0.01 mg/ml thiamine, and some or all of the following antibiotics: kanamycin (50 μg/ml), ampicillin (100 μg/ml), chloramphenicol (30 μg/ml). The pBAD18/21 and pBAD33/21 plasmids consist of an insertion of a pET21b XbaI/StyI fragment into pBAD18 (Amp<sup>+</sup>, pBR origin) and pBAD33 (Cm<sup>+</sup>, pACYC origin) (31), respectively. E. coli strain W3110 (lac<sup>pl</sup> recAΔ1 Kan<sup>+</sup> lacS2 pM901[Tet<sup>+</sup>]) (32) was transformed with pBAD18/21-AlaRS(C666A/584H). The pMJ901 maintenance plasmid bears the <i>alaS</i> gene (encoding wild-type AlaRS) and a temperature-sensitive replicon, which fails to replicate at 42 °C. Transformants were selected on LB-Kan (25 μg/ml)/Amp (50 μg/ml)/Tet (50 μg/ml) at 30 °C. Colonies were grown on M9-Kan/Amp plates at 42 °C to clear the maintenance plasmid, leaving growth dependent on the editing-defective AlaRS. The resulting editing-defective AlaRS strain was made chemically competent by the rubidium chloride method and then transformed with pBAD33/21, pBAD33/21-AlaXp, or pBAD33/21-AlaXp (K251A/K253A).

Halo Assay—Media for the halo assay was made with 15 g/liter agar, and each plate contained 25 ml. 10 μl of an overnight culture was diluted into 1 ml of sterile water and spread on a M9-Kan/Amp/Cm plate. 100 μl of 1 M l-serine was added to a well cut into the center of the plate and allowed to diffuse into the agar, creating a radial gradient. Plates were incubated at 37 °C and imaged after 48 h.

Direct Growth Assay—To confirm the serine sensitivity of AlaRS C666A/584H, M9-Kan/Amp plates were poured with 0 or 5 mM l-serine. 10 μl of an overnight culture was streaked on the plates and incubated at 37 °C for 40 h. For testing AlaXp, M9-Kan/Amp/Cm plates were poured with 0, 5, 7.5, or 10 mM l-serine. Overnight cultures were diluted 1:100, and 5 μl was streaked on the plate. Plates were incubated at 37 °C and imaged after 72 and 96 h.

Immunoblotting—The primary antibody used was α-His (HIS.H8) from Santa Cruz Biotechnology (Santa Cruz, CA) at 1:10000 dilution.

Nitrocellulose Filter Binding Assay—In vitro transcribed tRNA<sub>Ala</sub> was end-labeled with [32P] using a KinaseMax kit (Ambion). The filter binding assays were carried out as described previously (33). Briefly, [32P]tRNA<sub>Ala</sub> (20 nM) was incubated with AlaXp (0–3.75 μM) in 60 mM KH<sub>2</sub>PO<sub>4</sub> (pH 5), 10 mM MgCl<sub>2</sub>, 0.1 mg/ml bovine serum albumin for 10 min at 25 °C. Reactions were spotted onto nitrocellulose filters (Millipore) soaked in 5% trichloroacetic acid and washed over a vacuum with assay buffer. Filters were dried, and captured [32P]tRNA<sub>Ala</sub> was measured by scintillation counting.

RESULTS

Overall Experimental Strategy—The challenge was to find a way to test in vivo for the activity of AlaXp in isolation from any contribution of editing activity from endogenous AlaRS. For this purpose, we needed a strain of bacteria that harbored an AlaRS with completely ablated editing activity. Because the sites for aminoacylation and editing are discrete, the activity for editing can in principle be abolished without disrupting the essential aminoacylation function. To abolish the editing function, two mutations were placed in the editing domain of AlaRS. The purpose of this mutant was to have total elimination of the editing function while maintaining aminoacylation function. The double mutant was introduced into a bacterial strain carrying a deletion of the endogenous AlaRS gene. With the endogenous editing activity from AlaRS completely abolished, we then set out to test AlaXp function by introducing an AlaXp-encoding transgene into the editing-defective strain.

Construction and Testing Editing-defective C666A/584H AlaRS—Cys-666 in the editing domain is conserved through evolution for all AlaRSs. It is important for editing, because substitution of Cys-666 with Ala severely diminishes the ability of AlaRS to edit mischarged Ser-tRNA<sub>Ala</sub> or Gly-tRNA<sub>Ala</sub> (14). As a consequence, C666A AlaRS misacylates tRNA<sub>Ala</sub> with Ser or Gly. (In work reported below, we focused on Ser-tRNA<sub>Ala</sub> to study editing activities of mutant and wild-type proteins.) Separately, the Q584H mutation was serendipitously discovered to further enhance mischarging by C666A AlaRS in vitro (14). To further investigate the properties of C666A/584H AlaRS, we first established that its aminoacylation activity with alanine was unchanged from that of wild-type AlaRS (Fig. 1A). However, in contrast to the wild-type enzyme, C666A/584H AlaRS mischarged Ser onto tRNA<sub>Ala</sub> (Fig. 1B). Consistent with this mischarging, deacylation of Ser-tRNA<sub>Ala</sub> by C666A/584H AlaRS was undetectable (at the level of background) (Fig. 1C).

Next, we tested the ability of C666A/584H AlaRS to sustain cell growth. For this purpose, we used E. coli strain W3110...
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We postulated that the basic loop of M. mazei AlaXp contributed to tRNA binding affinity. However, when wild-type and mutant AlaXps were assayed for tRNA\(^{Ala}\) binding by the nitrocellulose filter binding method, the dissociation constants \(K_d\) values were 77 nM (wild-type), 71 nM (K253A), 48 nM (K251A), and 45 nM (K251A/K253A). Because the filter binding assays were done at pH 5.0, we wondered whether the lack of difference in the \(K_d\) values was a consequence of the low pH. For that reason, we did

3 Y. E. Chong, unpublished observation.
a kinetic analysis at pH 7.5 to determine relative values of $K_m$ for the wild-type and mutant proteins. The relative $K_m$ values were 1 (wild-type), 5.4 (K253A), 11.0 (K251A), and 11.4 (K251A/K253A). Thus, under these conditions, the wild-type protein associates (as defined by $K_m$) 5–10-fold more strongly. Still, most of the difference (over 500-fold) in catalytic activity ($k_{cat}/K_m$) of the wild-type versus the K251A/K253A mutant protein is in the $k_{cat}$ parameter. Thus, we conclude that the basic loop containing Lys-251 and Lys-253 may have an interaction with tRNA$^{Ala}$ that includes a component affecting $k_{cat}$, such as anchoring the acceptor stem to facilitate positioning of the CCA-3′ end in the catalytic site for editing.

### M. mazei AlaXp Rescues Serine Toxicity of Editing-defective Strain—

To investigate the biological function of AlaXp in vivo, we transformed a plasmid carrying M. mazei AlaXp into the editing-defective C666A/Q584H AlaRS-bearing strain. The flow of these experiments is shown in Fig. 3A. First, the editing-defective strain was established by introducing a maintenance plasmid encoding C666A/Q584H AlaRS into a ΔalaS strain. Next the plasmid encoding M. mazei AlaXp was introduced. Both plasmids were under the control of pBAD arabinose-inducible promoters (31), with distinct markers and origins of replication to allow for co-transformation.

To determine whether AlaXp expressed from the transgene was able to rescue the serine toxicity experienced by the editing-defective AlaRS strain, cells were subjected to high concentrations of serine. For this purpose, a radial gradient was generated by diffusion of serine from a central well cut into the agar media. Sensitivity to serine was seen as a “halo” of cell toxicity around the central well. The zone of growth inhibition in the strain containing wild-type AlaXp was markedly smaller in size compared with that of a strain carrying the empty vector (Fig. 3B). The reduction in halo size (Fig. 3C) showed that the presence of AlaXp allows editing-defective AlaRS to sustain growth at much higher concentrations of serine.

To confirm that the editing activity per se of AlaXp is responsible for this phenomenon, another strain was created with the K251A/K253A AlaXp double mutant that, as noted previously, is defective in its editing activity in vitro (Fig. 2C). Growth of this strain in the presence of the radial serine gradient was only marginally better than that of a strain carrying the empty vector (Fig. 3B). No toxicity was observed in any of the strains when water or alanine was added to the central well in place of serine. Thus, the editing activity of AlaXp is able to rescue serine toxicity in the editing-defective AlaRS strain by preventing mistranslation at alanine positions.
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To determine whether results seen in the halo assays were due to disparate levels of one or more of the proteins (C666A/Q584H AlaRS, AlaXp, K251A/K253A AlaXp) expressed from the introduced plasmids, cultures were inoculated from the plates at the conclusion of the halo assays. Cell lysates were prepared, and instead of a halo assay, colony growth was observed directly. The C666A/Q584H AlaRS strain, which failed to grow in the presence of 5 mM serine (Fig. 1D), was able to grow when transformed with wild-type AlaXp (Fig. 3E). Growth of the strain encoding wild-type AlaXp was also observed at 7.5 and 10 mM serine (although the growth rate was diminished at these higher concentrations). In contrast, strains carrying either the empty vector or K251A/K253A AlaXp failed to grow at the same serine concentrations (Fig. 3E). The data in Fig. 3 strongly support the conclusion that the free-standing editing domain homolog, AlaXp, is functional for clearance of mischarged tRNA$^{\text{Ala}}$ in vivo.

**DISCUSSION**

The genetic complementation assay here was able to provide a clear demonstration of the activity in vivo of AlaXp. When challenged with serine, cells harboring an editing-defective AlaRS could only survive by expression of AlaXp in trans. The results demonstrate the significance of “post-transfer” editing; that is, clearance of mischarged tRNA after the aminoacyl group has been transferred from the adenylate (as aminoacyl-AMP) to the 3′-end of the tRNA. (In pre-transfer editing, the adenylate is hydrolyzed before the amino acid is transferred to the tRNA.) Interestingly, AlaXp was able to capture and hydrolyze Ser-tRNA$^{\text{Ala}}$ before the amino acid is transferred to the tRNA.

To further confirm the ability of AlaXp to rescue the serine toxicity of the editing-defective strain, plates with various amounts of serine were prepared, and instead of a halo assay, colony growth was observed directly. The C666A/Q584H AlaRS strain, which failed to grow in the presence of 5 mM serine (Fig. 1D), was able to grow when transformed with wild-type AlaXp (Fig. 3E). Growth of the strain encoding wild-type AlaXp was also observed at 7.5 and 10 mM serine (although the growth rate was diminished at these higher concentrations). In contrast, strains carrying either the empty vector or K251A/K253A AlaXp failed to grow at the same serine concentrations (Fig. 3E). The data in Fig. 3 strongly support the conclusion that the free-standing editing domain homolog, AlaXp, is functional for clearance of mischarged tRNA$^{\text{Ala}}$ in vivo.
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ing editing domains. The work here provides a direct demonstration that, indeed, one example of these free-standing domains can provide the editing function in a way that ensures cell survival. That survival is dependent on AlaXp, in this particular experimental system, places further emphasis on the sensitivity of cells to mistranslation. In particular, the results stimulate further interest in the question of why a small, 2-fold defect in the editing activity of murine AlaRS should be sufficient to cause neurodegeneration in the sti mouse (28). In addition to neurodegeneration, these mice have other pathologies, such as substantially reduced body mass and altered fur. These pathologies are present even though the sti mouse encodes wild-type AlaXp. Thus, mistranslation appears to be near a “tipping point” presented by the singular difficulties of discriminating, in this instance, glycine and serine from alanine.

We can also raise the question of why AlaXp is the most widespread of these genome-encoded editing domains. No examples are known of separate, genome-encoded CP1 editing domains of IleRS, LeuRS, or ValRS, and only a limited evolutionary distribution of the ThrXp domain (that is closely related to the logos of IleRS, LeuRS, or ValRS, and only a limited evolutionary distribution of the ThrXp domain that is closely related to AlaXp) is seen (20, 21). ProXp (and the related Ybak) is also widely distributed and has been shown to clear Ala-tRNAPro (12, 21). Thus, the precise insertion of alanine into proteins (not confusing Gly or Ser for Ala, and not confusing Ala for Pro) seems to present a major problem for many species through evolution. The “alanine problem” may be more difficult to overcome than other examples of amino acid misrecognition, such as the confusion of valine for isoleucine, threonine for valine, or threonine for threonine. Whether the singular effects associated with mistranslation of alanine codons is due to the greater impact on protein folding of serine (or glycine) substitutions for alanine, or alanine substitutions for proline, is not clear but remains a formal possibility.

REFERENCES

1. Jakubowski, H., and Goldman, E. (1992) Microbiol. Rev. 56, 412–429
2. Schreier, A. A., and Schimmel, P. R. (1972) Biochemistry 11, 1582–1589
3. Eldred, E. W., and Schimmel, P. R. (1972) J. Biol. Chem. 247, 2961–2964
4. Yarus, M. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 1915–1919
5. Fersht, A. R. (1977) Biochemistry 16, 1025–1030
6. Fersht, A. R., and Kaether, M. M. (1976) Biochemistry 15, 3342–3346
7. Doring, V., Mootz, H. D., Nangle, L. A., Hendrickson, T. L., de Crecy-Lagard, V., Schimmel, P., and Marliere, P. (2001) Science 292, 501–504
8. Englisch, S., Englisch, U., von der Har, F., and Cramer, F. (1986) Nucleic Acids Res. 14, 7529–7538
9. Chen, J. F., Guo, N. N., Li, T., Wang, E. D., and Wang, Y. L. (2000) Biochemistry 39, 6726–6731
10. Martinis, S. A., and Fox, G. E. (1997) Nucleic Acids Symp. Ser. 36, 125–128
11. Beuning, P. J., and Musier-Forsyth, K. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 8916–8920
12. Wong, F. C., Beuning, P. J., Silvers, C., and Musier-Forsyth, K. (2003) J. Biol. Chem. 278, 52857–52864
13. Dock-Bregeon, A.-C., Sankaranarayanan, R., Romby, P., Caillet, J., Springer, M., Rees, B., Francklyn, C. S., Ehresmann, C., and Moras, D. (2000) Cell 103, 877–884
14. Beebe, K., Ribas de Pouplana, L., and Schimmel, P. (2003) EMBO J. 22, 668–675
15. Tsui, W. C., and Fersht, A. R. (1981) Nucleic Acids Res. 9, 4627–4637
16. Schimmel, P., and Ribas de Pouplana, L. (2000) Trends Biochem. Sci. 25, 207–209
17. Larkin, D. C., Williams, A. M., Martinis, S. A., and Fox, G. E. (2002) Nucleic Acids Res. 30, 2103–2113
18. Jasin, M., Regan, L., and Schimmel, P. (1983) Nature 306, 441–447
19. An, S., and Musier-Forsyth, K. (2005) J. Biol. Chem. 280, 34465–34472
20. Korencic, D., Abel, I., Schelert, J., Sacher, M., Ruan, B., Stathopoulos, C., Blum, P., Ibba, M., and Soll, D. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 10260–10265
21. Abel, I., Korencic, D., Ibba, M., and Soll, D. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 15422–15427
22. Sokabe, M., Okada, A., Yao, M., Nakashima, T., and Tanaka, I. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 11669–11674
23. Fukunaga, R., and Yokoyama, S. (2007) Acta Crystallogr. Sect. D Biol. Crystallogr. 63, 390–400
24. Beebe, K., Mock, M., Merriman, E., and Schimmel, P. (2008) Nature 451, 90–93
25. Bacher, J. M., de Crecy-Lagard, V., and Schimmel, P. R. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 1697–1701
26. Nangle, L. A., de Crecy-Lagard, V., Doring, V., and Schimmel, P. (2002) J. Biol. Chem. 277, 45729–45733
27. Nangle, L. A., Motta, C. M., and Schimmel, P. (2006) Chem. Biol. 13, 1091–1100
28. Lee, J. W., Beebe, K., Nangle, L. A., Jang, J., Longo-Guess, C. M., Cook, S. A., Davisson, M. T., Sundberg, J. P., Schimmel, P., and Ackerman, S. L. (2006) Nature 443, 50–55
29. Beebe, K., Waas, W., Druza, Z., Guo, M., and Schimmel, P. (2007) Anal. Biochem. 368, 111–121
30. Miller, J. H. (1972) Experiments in Molecular Genetics, p. 431, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
31. Guzman, L. M., Belin, D., Carson, M. J., and Beckwith, J. (1995) J. Biol. Chem. 270, 4121–4130
32. Jasin, M., and Schimmel, P. (1984) J. Bacteriol. 159, 783–786
33. Regan, L., Bowie, J., and Schimmel, P. (1987) Science 235, 1651–1653
34. Hou, Y. M., and Schimmel, P. (1988) Nature 333, 140–145