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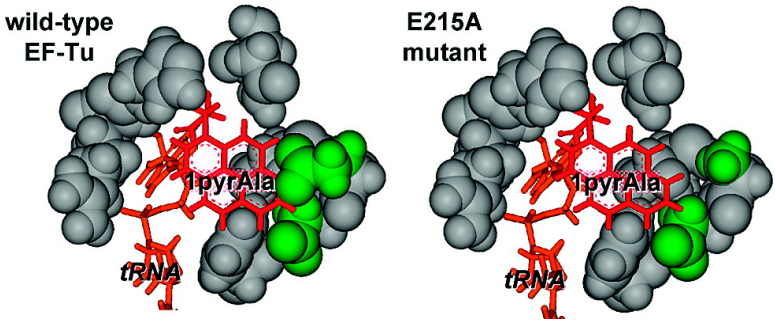

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Elongation Factor Tu Mutants Expand Amino Acid Tolerance of Protein Biosynthesis System

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Abstract: Nonnatural amino acids have been introduced into proteins using expanded protein biosynthesis systems. However, some nonnatural amino acids, especially those containing large aromatic groups, are not efficiently incorporated into proteins. Reduced binding efficiency of aminoacylated tRNAs to elongation factor Tu (EF-Tu) is likely to limit incorporation of large amino acids. Our previous studies suggested that tRNAs carrying large nonnatural amino acids are bound less tightly to EF-Tu than natural amino acids. To expand the availability of nonnatural mutagenesis, EF-Tu from the *E. coli* translation system was improved to accept such large amino acids. We synthesized EF-Tu mutants, in which the binding pocket of the aminoacyl moiety of aminoacyl-tRNA was enlarged. L-1-Pyrenylalanine, L-2-pyrenylalanine, and DL-2-anthraquinonylalanine, which are hardly or only slightly incorporated with the wild-type EF-Tu, were successfully incorporated into a protein using these EF-Tu mutants.

Introduction

In both in vitro and in vivo translation systems, nonnatural amino acids can be incorporated into proteins by delivery of an aminoacylated suppressor tRNA to a ribosome associated with mRNA containing an expanded codon/anticodon pair (reviewed in refs 1−4). This methodology provides a powerful tool for analyzing protein structure and function, and for producing proteins with new properties. However, nonnatural amino acids with large aromatic groups, such as fluorescent groups, are not always efficiently incorporated into proteins in the *Escherichia coli* in vitro translation system.[5] For example, incorporation efficiencies of 1-pyrenylalanine (1pyrAla) and 9-anthrylalanine (9antAla), which are fluorescent amino acids, into amino acid position 83 of streptavidin were 3% and less than 2%, respectively.[5] As incorporation of large amino acids is essential for various applications of nonnatural mutagenesis, it is necessary to improve this system to allow large amino acids. Factors that may determine incorporation efficiency include ribosome incompatibility of large amino acids and reduced binding efficiency of tRNAs carrying large amino acids to elongation factor Tu (EF-Tu)/GTP. As EF-Tu exhibits specificity for the aminoacyl moiety of aminoacyl-tRNA,[6−8] some nonnatural amino acids may be rejected from EF-Tu. Reduced binding efficiency of EF-Tu will not only suppress delivery of the aminoacyl-tRNA into ribosomes but also shorten the lifetime of the aminoacyl-tRNA due to spontaneous ester hydrolysis.

EF-Tu is a G protein responsible for delivering all elongator aminoacyl-tRNAs (aa-tRNAs) to ribosomal A site during translation. Although EF-Tu binds to natural elongator aa-tRNAs with similar affinities, it has considerable specificity against various amino acid side chains.[6−8] Our previous work suggested that tRNAs carrying large nonnatural amino acids bound less tightly to EF-Tu than tRNAs carrying natural amino acids.[9] To improve the binding ability of EF-Tu to large nonnatural amino acids, the amino acid binding pocket of EF-Tu needs to be enlarged. From the crystal structure of the ternary complex of EF-Tu/GTP/Phe-tRNA,[10] E215 and D216 of *E. coli* EF-Tu (E226 and D227 of *Thermus aquaticus* EF-Tu) were found to interfere with the side chains of large amino acids, such as 1pyrAla and 9antAla (Figure 1).

In this study, three *E. coli* EF-Tu mutants containing a single mutation (E215A or D216A) or a double mutation (E215A/D216A) were produced. We evaluated their binding abilities to tRNAs that carry aromatic nonnatural amino acids. We also investigated their effects on incorporation of 1pyrAla and 9antAla into proteins.

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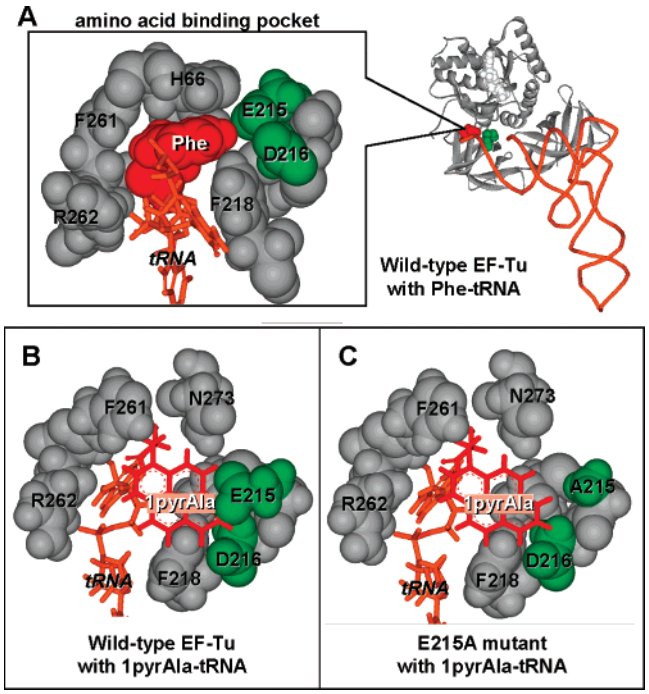

Figure 1. Structural model of amino acid binding pocket. (A) Crystal structure[10] of the ternary complex of *T. aquaticus* EF-Tu, GDPNP, and yeast Phe-tRNA^{Phe} (right), and amino acid binding pocket in the wild-type ternary complex (left). (B) Model of the amino acid binding pocket of wild-type EF-Tu with 1pyrAla-tRNA. (C) Model of the amino acid binding pocket of E215A mutant with 1pyrAla-tRNA. These models (B, C) were constructed simply by amino acid replacement of the crystal structure of the EF-Tu/GDPNP/Phe-tRNA ternary complex[10] using Discovery Studio 1.5 software (Accelrys Software Inc.). EF-Tu (excluding side chains 215 and 216) is shown in gray, and side chains 215 and 216 are highlighted in light green. Aminoacyl and tRNA moieties of aa-tRNA are shown in red and orange, respectively. GDPNP is shown only in the right panel of (A) in light gray as a space-filled model. EF-Tu residues are numbered according to amino acid numbering of *E. coli* EF-Tu.

Results

Binding of Wild-Type EF-Tu to aa-tRNAs That Carry Nonnatural Amino Acids. Binding of wild-type *E. coli* EF-Tu to aa-tRNAs that carry various aromatic nonnatural amino acids was analyzed using gel-shift assay. Nonnatural amino acids used were 1-naphtylalanine (1napAla), 2-naphtylalanine (2napAla), 1-pyrenylalanine (1pyrAla), and 9-anthrylalanine (9antAla) (Figure 2A). Wild-type *E. coli* EF-Tu bound to 1napAla-tRNA and 2napAla-tRNA as well as Phe-tRNA, but not to 1pyrAla-tRNA and 9antAla-tRNA (Figure 2A). These results agree with previous findings showing that 1napAla and 2napAla, but not 1pyrAla and 9antAla, were efficiently incorporated into proteins in an *E. coli* in vitro translation system.[5] The low binding ability of EF-Tu for 1pyrAla-tRNA and 9antAla-tRNA is likely the reason for the low incorporation efficiency of 1pyrAla and 9antAla.

Synthesis of *E. coli* EF-Tu Mutants. The crystal structure[10] of the ternary complex suggested that E215 and D216 in *E. coli* EF-Tu may prevent binding of large nonnatural amino acids, such as 1pyrAla and 9antAla (Figure 1). Thus, we synthesized three EF-Tu mutants, in which bulky side chains at amino acid positions 215 (glutamic acid) and/or 216 (aspartic acid) were replaced by alanine. These mutants (E215A, D216A, and E215A/D216A) were then purified from overexpressing *E. coli* cells. Growth rates for *E. coli* cells expressing EF-Tu mutants after IPTG induction were similar to cells expressing wild-type EF-Tu (data not shown), suggesting that these EF-Tu mutants are nontoxic for *E. coli* growth. As a result of the increased affinity to aa-tRNA, the two mutants (E215A and E215A/D216A) were obtained as complexes with some *E. coli* RNAs, most probably aa-tRNAs, even after purification on a Ni-NTA column. This was suggested by the similar mobility of purified EF-Tu samples on native gel to that of the ternary complex of EF-Tu/GTP/Phe-tRNA (Figure S1). To remove RNA contamination, EF-Tu mutants were treated with RNase A, and then purified again on a Ni-NTA column. This produced E215A and D216A mutants with marked reduction in RNA contamination, although the E215A/D216A mutant still contained a small amount of RNA contamination even after RNase treatment (Figure S1).

Binding of 1pyrAla-tRNA and 9antAla-tRNA to EF-Tu Mutants. Binding activities of EF-Tu mutants were analyzed by the gel-shift assay. E215A and D216A mutants bound to Phe-tRNA (Figure 2B) but not to uncharged tRNAs (Figure S2). Unexpectedly, E215A/D216A mutant bound to some uncharged *E. coli* tRNAs, although it did not bind to uncharged yeast tRNA$^{Phe}_{CGGG}$. Despite this unexpected function, translation in the *E. coli* S30 system was not inhibited by addition of 8 μM E215A/D216A mutant (data not shown).

Although 1pyrAla and 9antAla were not accepted by wild-type EF-Tu, all EF-Tu mutants bound tRNAs that carry those amino acids (Figure 2B). In particular, efficient binding was observed for E215A and E215A/D216A mutants. These observations suggest that the mutations successfully enlarged the amino acid binding site of EF-Tu and provided the chance for delivering large nonnatural amino acids to ribosomes.

Activity of EF-Tu Mutants in the in Vitro Protein Synthesizing System. Translation activity of EF-Tu mutants was analyzed using a PURE system that lacks EF-Tu (Figure 3A). Although we prepared the PURE (−EF-Tu) system in the present experiments, the same system is also available from Post Genome Institute Co. Ltd. (Japan). As expected, addition of wild-type streptavidin mRNA to the PURE (−EF-Tu) system did not produce streptavidin; however, it was successfully produced after addition of wild-type or mutant EF-Tu's. Synthesized protein was quantified by Western blotting (Figure 3A). The mRNA was efficiently translated with the E215A and D216A mutants over the concentration range between 0.25 and 16 μM. No translation product was detected with E215A/D216A mutant. Although translation efficiency with E215A mutant was lower than wild-type *E. coli* EF-Tu, translation efficiency with D216A mutant was similar to wild-type EF-Tu when the concentration was greater than 4 μM. Similar results were obtained when streptavidin mRNA containing a CGGG four-base codon at amino acid position 21 was translated in the presence of Phe-tRNA$_{CCCG}$ (Figure 3B). The range of effective concentrations for E215A and D216A mutants was 8−16 μM.

Incorporation of Large Nonnatural Amino Acids Using EF-Tu Mutants. As E215A and D216A mutants worked as effective EF-Tu's in the PURE system, the ability to accept large nonnatural amino acids by this system was evaluated. Incorporation of 1pyrAla and 9antAla into amino acid position 21 of streptavidin was attempted using mRNA-21CGGG. *Methanosarcina barkeri* tRNA$^{Pyl}_{CCCG}$ was employed as carrier for the nonnatural amino acids, as this tRNA can avoid recognition by *E. coli* endogenous aminoacyl-tRNA synthetases

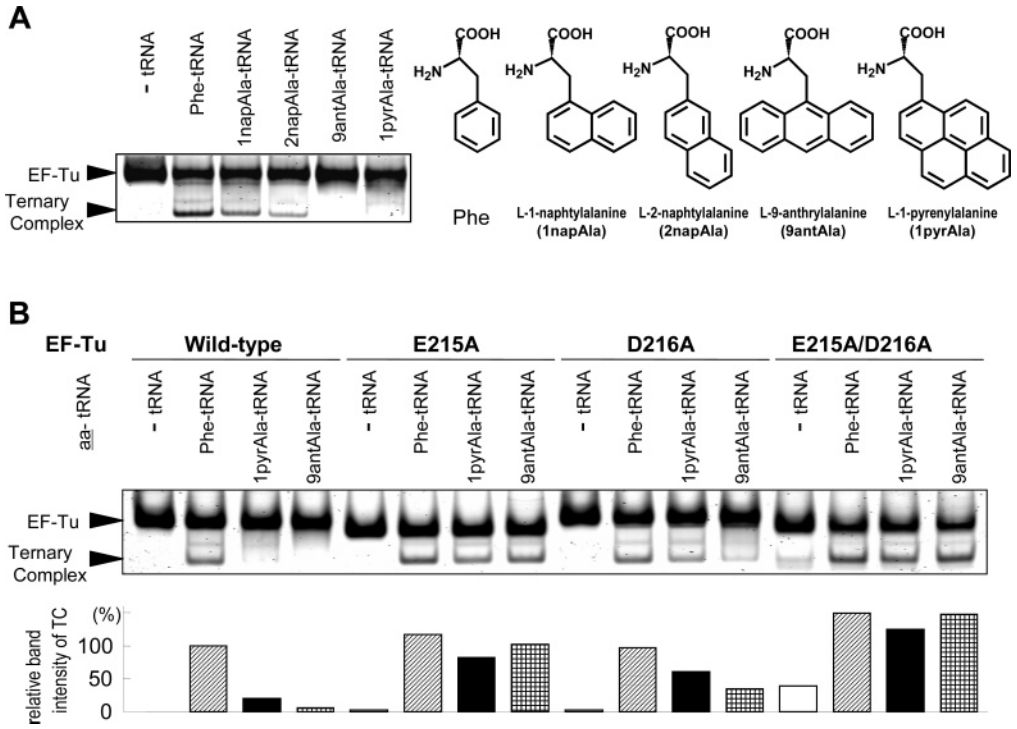

Figure 2. Binding of EF-Tu to aa-tRNAs, analyzed by gel-shift assay. (A) Binding of wild-type EF-Tu to various aa-tRNAs. The ternary complexes (EF-Tu/GTP/aa-tRNA) and EF-Tu/GTP were visualized using SYPRO Red protein gel stain (Takara). Chemical structures of the amino acids used here are shown at the right. (B) Binding of Phe-tRNA, 1-pyrAla-tRNA, and 9-antAla-tRNA to EF-Tu variants, analyzed by gel-shift assay (upper panel). Band intensities of ternary complexes on the gel are summarized in the lower panel. Band intensities were normalized relative to wild-type *E. coli* EF-Tu, GTP, and Phe-tRNA (100%).

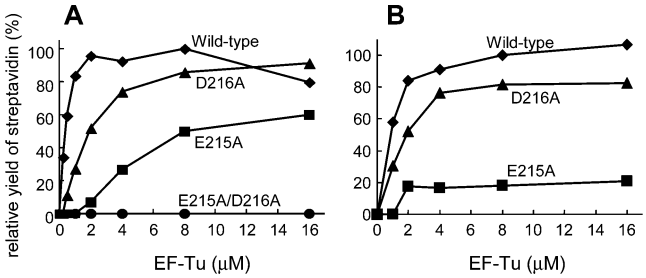

Figure 3. Protein synthesis efficiency with only natural amino acids for EF-Tu mutants. (A) Translation of wild-type streptavidin mRNA. (B) Translation of streptavidin mRNA-21CGGG with 20 μM Phe-tRNA$_{CCCG}$. Translation was performed with various amounts of wild-type *E. coli* EF-Tu (diamonds), E215A (squares), D216A (triangles), or E215A/D216A (circles). Yields were normalized relative to streptavidin obtained from reaction of 8 μM wild-type EF-Tu (100%).

and can decode the CGGG codon efficiently.[11] Neither wild-type EF-Tu nor EF-Tu mutants could incorporate 9antAla into the protein; however, 1pyrAla was successfully incorporated into amino acid position 21 of streptavidin by adding EF-Tu mutants to the system (Figure 4A). Relative yields of streptavidin containing 1pyrAla in the presence of 8 μM wild-type EF-Tu, E215A mutant, and D216A mutant were 1.9%, 7.6%, and 11.2%, respectively. Yield relative to full-length streptavidin containing Phe-21 in the presence of wild-type EF-Tu is expressed as a percentage (Figure 4B). Both EF-Tu mutants activated 1pyrAla incorporation more efficiently than wild-type *E. coli* EF-Tu. To examine the generality of the effect of these EF-Tu mutants, we further examined incorporation of L-2-pyrenylalanine (2pyrAla) and DL-2-anthraquinonylalanine (anqAla) into position 21 of streptavidin. Both of the E215A and D216A mutants assisted incorporation of 2pyrAla and anqAla much more efficiently than wild-type *E. coli* EF-Tu (Figure 5). We also confirmed that incorporation of 1pyrAla and anqAla into protein was enhanced by the addition of D216A mutant to the standard PURE system (PURESYSTEM classic II, Post Genome Institute Co. Ltd.) containing wild-type EF-Tu (data not shown).

Discussion

The binding ability of wild-type *E. coli* EF-Tu against tRNAs charged with aromatic nonnatural amino acids was evaluated. *E. coli* EF-Tu bound 1napAla-tRNA and 2napAla-tRNA, but not 1pyrAla-tRNA and 9antAla-tRNA (Figure 2A). These findings are consistent with incorporation efficiencies of these nonnatural amino acids into proteins.[5] Thus, we postulated that the very low incorporation efficiencies for 1pyrAla and 9antAla in an *E. coli* in vitro translation system may be due to binding to EF-Tu being too weak. Previous studies had shown that mutations of the amino acid binding pocket of EF-Tu altered its amino acid specificity.[7,12] Therefore, the amino acid binding pocket of EF-Tu was expanded by mutations E215A and/or D216A in an attempt to improve binding ability to large nonnatural amino acids (Figure 1A).

E215A and D216A mutations improved binding activity of EF-Tu to 1pyrAla and 9antAla (Figure 2B). Effect of the E215A mutation was more marked than that of D216A, particularly for 9antAla. Modeling studies are consistent with these findings. E215 overlaps both side chains of 1pyrAla and 9antAla when their aromatic moieties are located on the same plane as the

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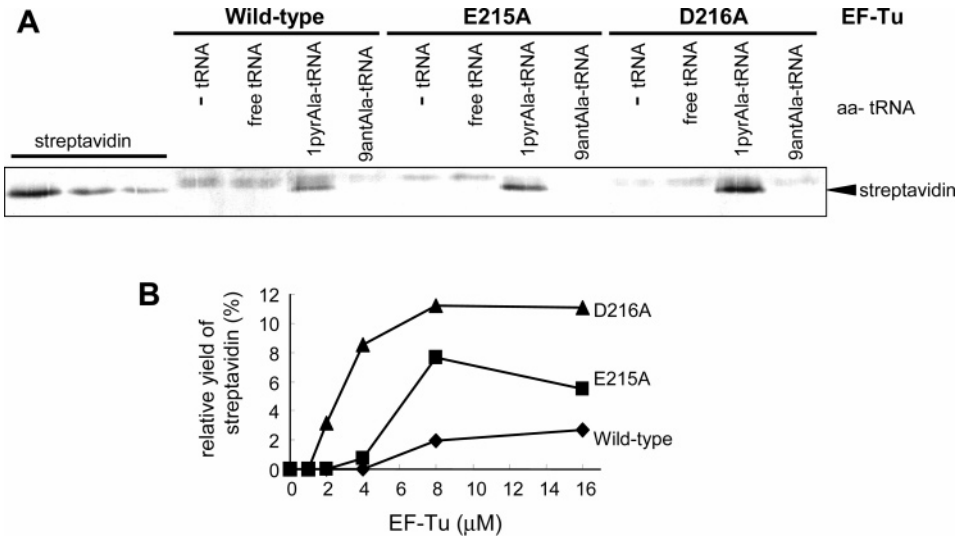

Figure 4. Incorporation of nonnatural amino acids into position 21 of streptavidin. (A) Products obtained by translating streptavidin mRNA-21CGGG with 8 μM EF-Tu (wild type or mutant) and 20 μM aa-tRNA$_{CCCG}$ were analyzed by Western blot. (B) Protein synthesis efficiency of EF-Tu mutants using 1pyrAla. Translation of streptavidin mRNA-21CGGG with 20 μM 1pyrAla-tRNA$_{CCCG}$ was evaluated with various amounts of wild-type *E. coli* EF-Tu (diamonds), E215A (squares), or D216A (triangles). Yields were normalized relative to the translation product of mRNA-21CGGG with 8 μM wild-type EF-Tu and 20 μM Phe-tRNA$_{CCCG}$ (100%).

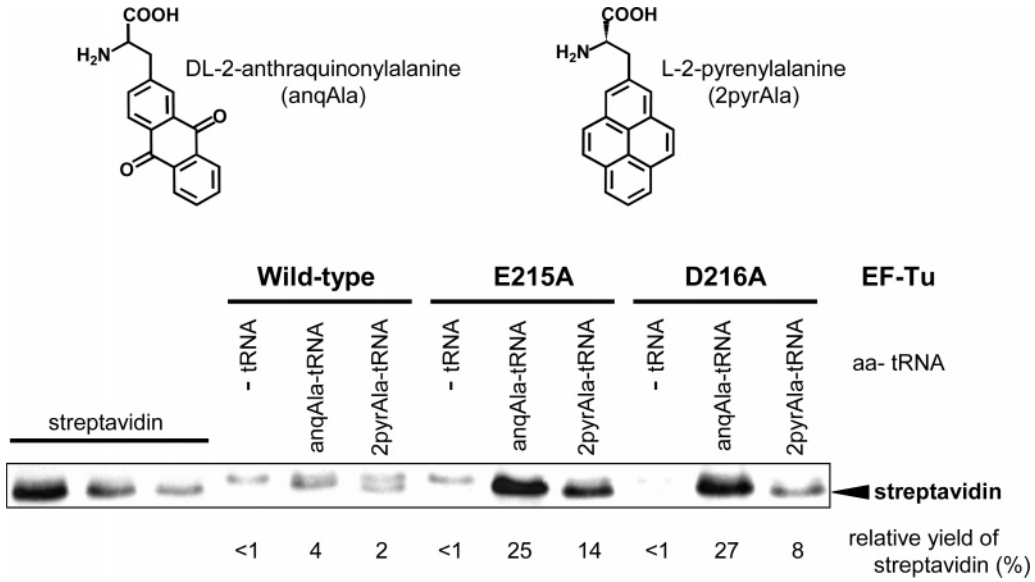

Figure 5. Incorporation of anqAla or 2pyrAla into position 21 of streptavidin. Products obtained by translating streptavidin mRNA-21CGGG with 8 μM EF-Tu (wild type or mutants) and 20 μM aa-tRNA$_{CCCG}$ were analyzed by Western blot. Relative yield to the translation product of mRNA-21CGGG with 8 μM wild-type EF-Tu and 20 μM Phe-tRNA$_{CCCG}$ is indicated below each lane.

benzene ring of Phe-tRNA (Figure 1B); however, D216 does not overlap with 9antAla, only with 1pyrAla. The D216A mutation raised the acceptability of even 9antAla, probably because the space made by truncating the D216 side chain is flexible. Enhanced binding by the E215A mutant to 1pyrAla and 9antAla can be explained in terms of the computer-predicted structure shown in Figure 1C. This model suggests that A215 does not hinder the binding of aromatic groups of these amino acids. Enhanced binding to 1pyrAla and 9antAla by E215A and D216A mutations may also be due to hydrophobic nonnatural amino acids preferring alanine to hydrophilic glutamic or aspartic acid. These mutations may also affect the binding of EF-Tu to the tRNA body of aa-tRNAs as shown in Figure S2. Removal of two negative side chains (E and D) from the amino acid binding pocket of EF-Tu might reduce repulsion of a positively charged tRNA.

E215A and D216A mutants worked in the translation system with natural amino acids (Figure 3A), but less effectively than wild-type EF-Tu. No protein synthesis was observed with the E215A/D216A mutant. It seems that this result contradicts the fact that the mutant tightly binds aa-tRNAs carrying natural and nonnatural amino acids (Figure 2B). However, it is possible that the E215A/D216A mutant may be sluggish in releasing the tightly bound aa-tRNAs in the ribosome A site, resulting in less efficient delivery of the aa-tRNAs. LaRiviere et al.[6] also suggested that a too tight binding of aa-tRNAs to EF-Tu may compromise the accommodation efficiency of aa-tRNAs into the ribosomal A site.

Incorporation efficiency of 1pyrAla into proteins was markedly increased by the E215A and D216A mutants compared to wild-type EF-Tu (Figure 4A). Relative yields of full-length streptavidin synthesized using mRNA-21CGGG/1pyrAla-tR-

NA_{CCCG} pair in the presence of 8 μM wild-type EF-Tu, E215A mutant, and D216A mutant were 1.9%, 7.6%, and 11.2%, respectively (Figure 4B). These values do not represent incorporation efficiency of 1pyrAla into the protein. As translation efficiencies using mRNA-21CGGG/Phe-$tRNA_{CCCG}$ pair in the presence of 8 μM E215A and D216A mutants relative to wild-type EF-Tu were 18% and 82%, respectively (Figure 3B), incorporation efficiencies of 1pyrAla in the presence of 8 μM wild-type EF-Tu and the E215A and D216A mutants were estimated to be 1.9%, 42%, and 14%, respectively, close to proportional to the EF-Tu binding ability of 1pyrAla.

Incorporation efficiencies of 2pyrAla and anqAla into proteins were also markedly increased by both EF-Tu mutants compared to wild-type EF-Tu (Figure 5). This result suggests that the expanded amino acid tolerance of E215A and D216A mutants is widely applicable to those aromatic amino acids.

In contrast, 9antAla was not incorporated even in the presence of the E215A and D216A mutants (Figure 4A), although the binding capability against 9antAla-tRNA was much improved by these mutations (Figure 2B). These findings suggest that 9antAla was rejected not only by wild-type EF-Tu but also by ribosome. For this type of amino acids to be successfully incorporated into proteins, concomitant use of ribosome mutant[13] and EF-Tu mutant may be helpful.

Experimental Section

Preparation of tRNA Transcripts. tRNA transcripts were prepared as described.[11] Aminoacyl-tRNAs were prepared by ligating transcribed tRNA without the 3′-CA sequence and the aminoacyl dinucleotide (pdCpA) with T4 RNA ligase as described.[5,14,15]

Preparation of EF-Tu Mutants. Vectors expressing *E. coli* EF-Tu mutants were prepared according to supplementary experimental section in the Supporting Information. BL21 (DE3) *E. coli* cells were transformed with the plasmids and the expressed recombinant proteins were purified as described[16] with the following modifications. After first purification on Ni-NTA column, RNA contaminants which were included in purified EF-Tu mutants were digested by RNase A. The digestion was performed at 4 °C for 30 h in a reaction mixture that included 2 mg/mL EF-Tu mutants, 300 units/mL RNase A, 50 mM HEPES−KOH (pH 7.5), 100 mM $(NH_4)_2SO_4$, 150 mM imidazole, 20% glycerol, 2 μM GDP, and 5 mM β-mercaptoethanol. The reaction mixture was then purified again by Ni-NTA column chromatography. N-terminal His-tag of purified proteins was excised by thrombin during dialysis as described before,[16] except for using different dialysis buffer (50 mM HEPES−KOH [pH 7.6], 50 mM KCl, 1 mM DTT, 2 μM GDP, and 10% glycerol).

Gel-Shift Analysis. Binding of EF-Tu to tRNAs charged with various amino acids was investigated using a yeast $tRNA^{Phe}$ mutant, in which anticodon GAA was mutated to four-base anticodon CCCG. The ternary complex of EF-Tu, GTP, and aa-tRNA was prepared as follows: 10 μM EF-Tu was preincubated with 1 mM GTP at 37 °C for 15 min in 5 μL total volume containing 70 mM HEPES−KOH (pH 7.6), 52 mM NH_4OAc, 8 mM $Mg(OAc)_2$, 30 mM KCl, 0.8 mM DTT, 1.6 μM GDP, 6% glycerol, 10 mM phosphoenolpyruvate, and 0.08 U/μL pyruvate kinase. To the preincubated EF-Tu solution, 3 μL of 8.3 μM aa-tRNA (dissolved in 6 mM KOAc) and 2 μL of ternary complex buffer, containing 150 mM HEPES−KOH (pH 7.6), 195 mM NH_4OAc, and 30 mM $Mg(OAc)_2$, were added. The mixture, containing 50 pmol of wild-type EF-Tu and 25 pmol of aa-tRNA, was incubated at 37 °C for 10 min. Electrophoresis of samples was performed using 8% polyacrylamide gels at 4 °C in a buffer containing 50 mM Tris·HCl (pH 6.8), 65 mM NH_4OAc, and 10 mM $Mg(OAc)_2$. Gels were stained with SYPRO Red (Takara, Japan). Band intensity was evaluated using ImageQuant Tools v2.2 (Molecular Dynamics Inc.). All gel-shift assays were repeated twice or more, and the reproducibility of the results was confirmed.

In Vitro Translation Using the *E. coli* PURE System. Streptavidin mRNA with a CGGG codon at amino acid position 21 (streptavidin mRNA-21CGGG) was prepared as previously described.[5] A T7-tag sequence was encoded at the N-terminus of streptavidin mRNA so that its protein product could be detected with an anti-T7 antibody. To decode the CGGG codon, we used *M. barkeri* $tRNA^{Pyl}$ mutant, in which the anticodon is mutated to a CCCG four-base anticodon. Components used in the PURE system were purified as previously reported.[17] Ribosomes were prepared according to Ohashi et al.[18] Reaction conditions for the PURE system were as described in a previous report,[19] except that the concentrations of the 20 amino acids, *E. coli* EF-Tu (or EF-Tu mutants), and aa-tRNA were 0.1 mM, 0.25−18 μM, and 10 μM, respectively. Reactions were started by addition of 1.6 mg/mL mRNA and incubated for 1 h at 37 °C. Synthesized proteins were analyzed by Western blotting as described previously.[5] All translation experiments were repeated twice or more, and the reproducibility of the results was confirmed.

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Supporting Information Available: Supplementary experimental section and Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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