Identification of the Residues Involved in the Unique Serine Specificity of *Caenorhabditis elegans* Mitochondrial EF-Tu2†

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Received March 17, 2006; Revised Manuscript Received June 9, 2006

ABSTRACT: In canonical translation systems, the single elongation factor Tu (EF-Tu) recognizes all elongator tRNAs. However, in *Caenorhabditis elegans* mitochondria, two distinct EF-Tu species, EF-Tu1 and EF-Tu2, recognize 20 species of T armless tRNA and two species of D armless tRNA^{Ser}, respectively. We previously reported that *C. elegans* mitochondrial EF-Tu2 specifically recognizes the serine moiety of serylated-tRNA. In this study, to identify the critical residues for the serine specificity in EF-Tu2, several residues in the amino acid binding pocket of bacterial EF-Tu were systematically replaced with corresponding EF-Tu2 residues, and the mutants were analyzed for their specificity for esterified amino acids attached to tRNAs. In this way, we obtained a bacterial EF-Tu mutant that acquired serine specificity after the introduction of 10 EF-Tu2 residues into its amino acid binding pocket. *C. elegans* EF-Tu2 mutants lacking serine specificity were also created by replacing seven or eight residues with bacterial residues. Further stressing the importance of these residues, we found that they are almost conserved in EF-Tu2 sequences of closely related nematodes. Thus, these three approaches reveal the critical residues essential for the unique serine specificity of *C. elegans* mitochondrial EF-Tu2.

Elongation factor Tu (EF-Tu)[1] forms a ternary complex with an aminoacyl-tRNA (aa-tRNA) and GTP and subsequently delivers the aa-tRNA to the ribosomal A site during the elongation step of protein synthesis in bacteria and eukaryotic organelles (*1*, *2*). Canonical EF-Tu is a RNA binding protein which recognizes all elongator tRNAs charged with one of 20 amino acids, except for the special tRNA for selenocysteine, which is specifically recognized by the EF-Tu homologue, SelB (*3*). Although EF-Tu had been generally considered a nonspecific binding protein, recent experiments have demonstrated that EF-Tu has significant specificity for both the esterified amino acid and the tRNA body (*4*, *5*) and that the specificity for the side chain of the esterified amino acid could be altered by mutations in the amino acid binding pocket of EF-Tu (*6*).

Previously, we found that the mitochondrial (mt) translation system of the nematode *Caenorhabditis elegans* has two distinct EF-Tu species, EF-Tu1 and EF-Tu2 (*7*, *8*). In *C. elegans* mitochondria, two distinct types of tRNAs with unusual structural topologies exist; both are extraordinarily truncated compared to the canonical tRNA structure (*9*). One type is a T armless tRNA (20 tRNA species), and the other is a D armless tRNA possessing a short T arm (two tRNA^{Ser} species) (*10*−*12*). EF-Tu1 specifically recognizes T armless aa-tRNAs (*7*). EF-Tu2 binds to D armless Ser-tRNAs by a unique tRNA recognition mechanism, which includes the interaction of EF-Tu2 with the phosphate groups in the T stem on the side opposite from that which canonical EF-Tu binds (*13*). EF-Tu2 has another unique characteristic feature in that it exclusively recognizes the serine moiety of serylated tRNA, while canonical EF-Tu binds to all 20 amino acids in aa-tRNAs (*8*). The mechanism by which *C. elegans* mt EF-Tu2 recognizes the serine moiety of the serylated tRNA is unknown. To understand the detailed molecular mechanism of serine specificity, it would be very useful to determine the crystal structure of the ternary complex containing EF-Tu2, GTP, and Ser-tRNA. However, the crystallization of EF-Tu2 is difficult because of its low solubility and low stability (*22*). An alternative way to understand the structural basis of serine specificity would be to identify the residues that are important for specificity by mutational analysis. However, systematic mutations of *C. elegans* EF-Tu2 are impractical because EF-Tu2 is unstable and most of its mutants would be insoluble. Thus, we designed systematic mutations in *Thermus thermophilus* EF-Tu by replacing its

† This work was supported by Grants-in-Aid from the Ministry of Culture, Sports, Science and Technology, Japan, to T.O., T.S., Y.W., and K.W. and by the Sumitomo Foundation (to T.O.).

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[1] Abbreviations: EF-Tu, elongation factor Tu; aa-tRNA, aminoacyl-tRNA; mt, mitochondrial; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; BSA, bovine serum albumin.

10.1021/bi060536i CCC: $33.50
Published on Web 08/19/2006

residues with those of EF-Tu2. An EF-Tu of *T. thermophilus* (thermophilic bacteria) is considered to be a canonical EF-Tu. If it were possible to obtain mutants of *T. thermophilus* EF-Tu with serine specificity, it would open up the possibility of determining their crystal structures, as they are more likely to be stable than EF-Tu2. The identification of the critical residues for serine specificity would also allow us to determine how serine-specific EF-Tu evolved from canonical EF-Tu.

In this study, we used three experimental approaches to identify the residues that are critical for serine specificity. First, we analyzed the amino acid sequences of EF-Tu2s from nematodes closely related to *C. elegans* (Nematoda, Chromadorea), *Caenorhabditis briggsae*, *Ascaris suum*, and *Strongyloides stercoralis*, to determine which residues were potentially important for nematode EF-Tu2 serine specificity. Second, we systematically replaced residues in the amino acid binding pocket (*14*, *15*) of *T. thermophilus* EF-Tu with those of EF-Tu2 and obtained a *T. thermophilus* EF-Tu mutant with serine specificity. We also prepared mutants of *C. elegans* EF-Tu2 in which several residues were replaced with those of bacterial EF-Tu. This resulted in mutants that lacked serine specificity. These complementary approaches allowed us to identify the critical residues involved in *C. elegans* EF-Tu2 serine specificity.

MATERIALS AND METHODS

Nematode EF-Tu2 cDNA Sequencing. The poly(A)$^+$ RNA from *A. suum* adult female body wall muscle was a gift from K. Kita (University of Tokyo). Reverse transcription was carried out with ReverTra Ace (Toyobo) and random hexamers. A partial cDNA fragment of putative EF-Tu2 was obtained by PCR using the degenerate primers P-748 [5′-AC(G/T)AT(A/T)GG(G/A/T/C)CA(T/C)(G/A)T(G/A/T/C)-GA(T/C)CA-3′] and P-750 [5′-TC(G/T)G(A/C)(G/A)TG(G/A/T/C)CC(G/A/T/C)GG(G/A)CA(G/A)TC-3′], or P-749 [5′-CA(T/C)(G/A)T(G/T)GA(T/C)CA(T/C)GG(G/A/T/C)AA(G/A)AC-3′] and P-750. The putative cDNA fragments were purified by agarose gel electrophoresis and then cloned using a TOPO TA cloning kit (Invitrogen). Positive clones were screened by colony PCR and sequenced. The full-length cDNA of EF-Tu2 (GenBank accession number AB212082) was reconstructed from RACE clones prepared with a GeneRacer kit (Invitrogen) and specific primers (5′-CGT-GTGCGCGTACCGTCGCTTATCGCTT-3′ and 5′-GCT-TATCGCTTTCATAGCCAACGTGAGC-3′ for 5′ RACE and 5′-TCAGCCAAGGGCAGGACAAAGTTTGTGA-3′ and 5′-TTGGCTATGAAAGCGATAAGCGACGGTA-3′ for 3′ RACE). *C. briggsae* AF16 (*16*) was obtained from The Caenorhabditis Genetic Center at the University of Minnesota (Minneapolis, MN) which is funded by the NIH National Center for Research Resources and grown with *Escherichia coli* OP50 as the food source, according to standard procedures (*17*). Worms of mixed stages were harvested, frozen, and crushed with an SK-200 device (Tokken), and then total RNA was extracted with TRIzol (Invitrogen). Poly-(A)$^+$ RNA was prepared with a PolyATtract system (Promega). The full-length cDNA (GenBank accession number AB234619) was reconstructed from RT-PCR clones prepared with a GeneRacer kit (Invitrogen) and specific primers (5′-GCCTGGACTCTTGTGATAGCCGAA-3′ and 5′-GCCCA-ATAGTGCCGACATTAACGT-3′ for 5′ RACE, 5′-ATGA-CATCTTCTGTGATATTTTCTTTCACA-3′ and 5′-CTA-GATTTTTTCAGCAGATTTTTTCAG-3′ for the internal portion, and 5′-CTACTGATGCCCGGTGAACACACT-3′ and 5′-CAGTAAGACTACCATTGCCAGAGGA-3′ for 3′ RACE). A tblastn search (*18*) against nematode ESTs in the public database using *C. elegans* EF-Tu2 as the query was carried out, and ESTs encoding putative EF-Tu sequences were identified. The EST clone possibly encoding a full-length cDNA of *S. stercoralis* (the GenBank accession number of the partial sequence is BI773024) was obtained from the Washington University Nematode EST project, and the entire cDNA (GenBank accession number AB212083) was sequenced. The alignment of the sequences was carried out with ClustalW, version 1.83 (*19*).

Preparation of EF-Tu Mutants. Expression vectors of all the EF-Tu mutants were prepared using the pET-15b-derived (Novagen) vector encoding an N-terminal His-tagged *T. thermophilus* EF-Tu or *C. elegans* mt EF-Tu2 (*8*). The mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene). The sequences of mutated plasmids were confirmed using an ABI prism 310 Genetic Analyzer. *E. coli* cells, BL21(DE3) for *T. thermophilus* EF-Tu mutants or Rosetta(DE3) for *C. elegans* mt EF-Tu2 mutants, were transformed by the plasmids encoding the His-tagged EF-Tu mutants. The expressed recombinant proteins were purified as described previously (*7*). The protein concentration was estimated using a Protein Assay Kit (Bio-Rad) with bovine serum albumin (BSA) as the standard.

Preparation of tRNA Transcripts. To generate DNA templates for transcription, primer extension was performed using two primers, which were designed to complement each other at each 3′ region (∼20 nucleotides). In the DNA templates, the promoter sequence for T7 RNA polymerase is inserted directly upstream of the tRNA sequence. The transcription reaction was performed at 37 °C for 4 h in a reaction mixture containing 40 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 1 mM spermine, 0.01% Triton X-100, 50 μg/mL BSA, 10 mM GMP, 2 mM ATP, 2 mM GTP, 2 mM CTP, 2 mM UTP, 20 μg/mL T7 RNA polymerase, and 50 nM template double-stranded DNA. The products were purified by 10% denaturing polyacrylamide gel electrophoresis (PAGE).

Preparation of Aminoacyl-tRNAs. Aminoacylation reactions with the yeast $tRNA^{Phe}$ derivative were performed at 37 °C for 15 or 30 min in a reaction mixture containing 100 mM Hepes-KOH (pH 7.8), 10 mM $MgCl_2$, 20 mM KCl, 2 mM ATP, 1 mM DTT, 20−55 μM ^{14}C-labeled amino acid (5−17 GBq/mmol), 6 μM tRNA, aminoacyl-tRNA synthetase, and, in some cases, 15% DMSO. To aminoacylate the tRNA with serine, alanine, and phenylalanine, we used 230 μg/mL bovine mt seryl-tRNA synthetase (SerRS), 170 μg/mL *E. coli* alanyl-tRNA synthetase (AlaRS), and *E. coli* extract (as phenylalanyl-tRNA synthetase), respectively. The *A. suum* mt $tRNA^{Ser}_{UCU}$ derivative with an alanine identity was aminoacylated as described previously (*8*). The aminoacylated tRNA was purified as described previously (*7*) and finally dissolved with 6 mM KOAc (pH 5) at a concentration of 2 or 4 μM. The concentration of aa-tRNA was estimated from labeled amino acids incorporated into the tRNA.

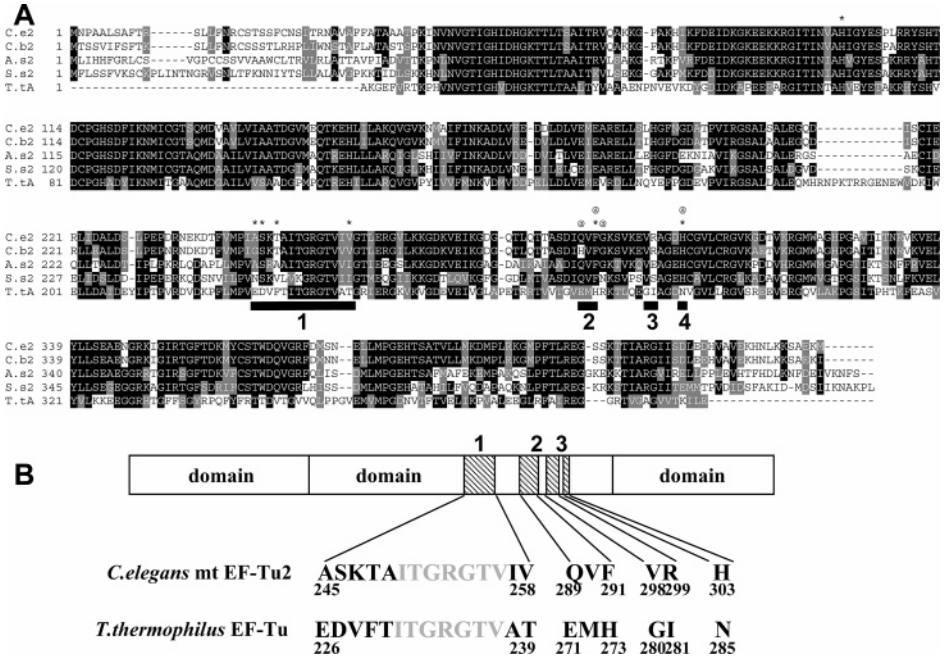

FIGURE 1: (A) Alignment of mitochondrial EF-Tu2s of *C. elegans* (C.e2, GenBank accession number BAA31345), *C. briggsae* (C.b2, this study), *A. suum* (A.s2, this study), and *S. stercoralis* (S.s2, this study) together with *T. thermophilus* EF-Tu (T.tA, GanBank accession number Q5SHN6). The alignment was modified using BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). Asterisks denote the residues lining the side chain pocket of the aminoacyl group, and the commercial at symbols denote the positions of residues in contact with the aminoacyl group (*14*, *15*). Identical residues have a black background. Conservatively substituted residues have a gray background, and nonconserved residues have a white background. (B) Schematic diagrams of the EF-Tu mutants. The lengths of the domains are not to scale. The amino acid sequences of the mutated positions are shown. Each number (1−4) corresponds to the positions indicated in panel A.

Hydrolysis Protection Assay. The assay was performed by following previously described methods (*7*, *20*). The deacylation reaction mixture contained 75 mM Tris-HCl (pH 7.5), 75 mM NH_4Cl, 15 mM $MgCl_2$, 7.5 mM DTT, 60 μg/mL BSA, 0.1 mM GTP, 2.25 mM phosphoenolpyruvate, 2.5 units/mL pyruvate kinase, 1.2 μM EF-Tu, and 0.2 or 0.4 μM aa-tRNA. The reaction mixture without aa-tRNA was preincubated for 10 min, and then the aa-tRNA was added. The preincubation and the deacylation reaction were performed at 37 °C using *T. thermophilus* EF-Tu mutants or at 30 °C using *C. elegans* EF-Tu2 mutants. Each assay was performed at least three times.

Structural Modeling of the Serine-Specific EF-Tu Mutant. The structure of the Tt134 mutant complexed with Ser-tRNA was predicted by amino acid replacement of the crystal structure of the EF-Tu−GTP−Phe-tRNA ternary complex (*14*) followed by energy minimization calculations (300 iterations) using the Insight II/Discover package (BIOSYM/Molecular Simulations). With the exception of the mutated residues, EF-Tu and tRNA structures were fixed during the calculation.

RESULTS

Sequencing of Nematode EF-Tu2. To investigate which residues are possibly important in the function of nematode mt EF-Tu2, we determined full-length cDNA sequences from three nematodes belonging to the class Chromadorea (Figure 1A). All chromadorean mt DNAs published so far encode 20 T armless tRNAs and two D armless serine tRNAs, as found in *C. elegans* mt DNA (reviewed in ref *21*), suggesting that mt EF-Tu2s in chromadorean nematodes share a characteristic unusual specificity for both the tRNA body and the esterified amino acid (*8*, *13*). The *C. briggsae* EF-Tu2 cDNA we obtained allowed us to correct the predicted peptide sequence already in the public database (GenBank accession number CAE66814). The predicted splicing of the putative third intron does not occur, but the 5′ part of the putative third intron does exist in the cDNA sequence as part of the third exon followed by a 3′ poly(A) tail. All of the nematode EF-Tu2 proteins have a C-terminal extension which includes several lysine residues. The C-terminal extension is essential for tRNA binding by *C. elegans* mt EF-Tu2 (*13*). All of the residues in the amino acid binding pocket (*14*, *15*) (asterisks and @ symbols in Figure 1A) in *T. thermophilus* EF-Tu differ completely from those of nematode EF-Tu2s, but the corresponding residues are highly conserved among nematode EF-Tu2s. Thus, these residues in nematode EF-Tu2 seem to have a role for the unique serine specificity.

Design of EF-Tu Mutations. Our previous work showed that *C. elegans* mt EF-Tu2 exclusively recognizes the seryl moiety of Ser-tRNA (*8*), while canonical EF-Tu can bind to all 20 amino acids in their respective aa-tRNAs. To identify residues important for the serine specificity of *C. elegans* mt EF-Tu2, we designed and prepared mutants of *T. themophilus* EF-Tu (canonical EF-Tu), in which the residues involved in binding to the aminoacyl moiety of an aa-tRNA (*14*, *15*) were replaced with those of *C. elegans* mt EF-Tu2 (Figure 1B). If serine specificity could be conferred on *T. thermophilus* EF-Tu through mutation, then the mutated amino acids that give rise to the serine specificity could be considered to be those that are critical for the serine specificity. In our first mutant, residues from position 226 to 239 and from position 269 to 286 of *T. thermophilus* EF-

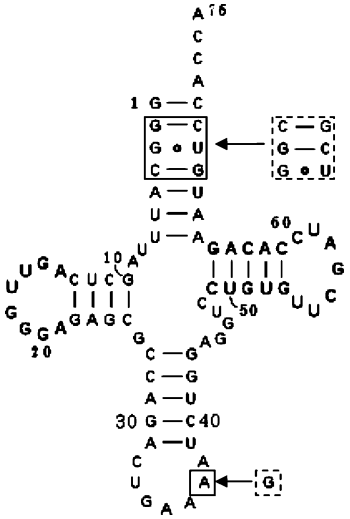

FIGURE 2: Secondary structure of the yeast $tRNA^{Phe}$ derivative used in the binding assays presented in Figure 3. This $tRNA^{Phe}$ derivative can be charged with serine, alanine, or phenylalanine. The residues in the dashed boxes represent sequences in the native $tRNA^{Phe}$ that were changed into the sequences enclosed by the solid boxes.

Tu, which include all of the aminoacyl binding (aa-binding) residues, were replaced with those from the corresponding regions of *C. elegans* mt EF-Tu2. However, binding to any aa-tRNA was almost completely eliminated by this simple approach (not shown). This may be due to differences in the molecular environment around the aa-binding residues in bacterial EF-Tu versus EF-Tu2. Therefore, we divided the aa-binding residues into four regions (Figure 1B) and prepared bacterial EF-Tu mutants bearing combinations of these four regions of EF-Tu2. The residues interacting with the amino acid side chain of the aminoacyl-tRNA (*14*, *15*) were primarily localized in region 1, but single residues in regions 2 and 4 also interacted with the side chain (Figure 1). Region 3 does not interact directly with the amino acid side chain of the aminoacyl-tRNA. However, since region 3 is juxtaposed with regions 2 and 4, from the perspective of the amino acid side chain, mutations in region 3, especially the bulky G280V mutation, seem to affect the amino acid specificity (Figure 6C). Thus, mutations in regions 1−4 were studied.

T. thermophilus EF-Tu Mutant Tt134 Has Serine Specificity. We prepared a series of *T. thermophilus* EF-Tu mutants, and all of these mutants were obtained as soluble proteins. The mutated regions are numbered 1−4, as indicated in Figure 1B, and were used to indicate the constitution of the mutants. For example, the *T. thermophilus* EF-Tu mutant,

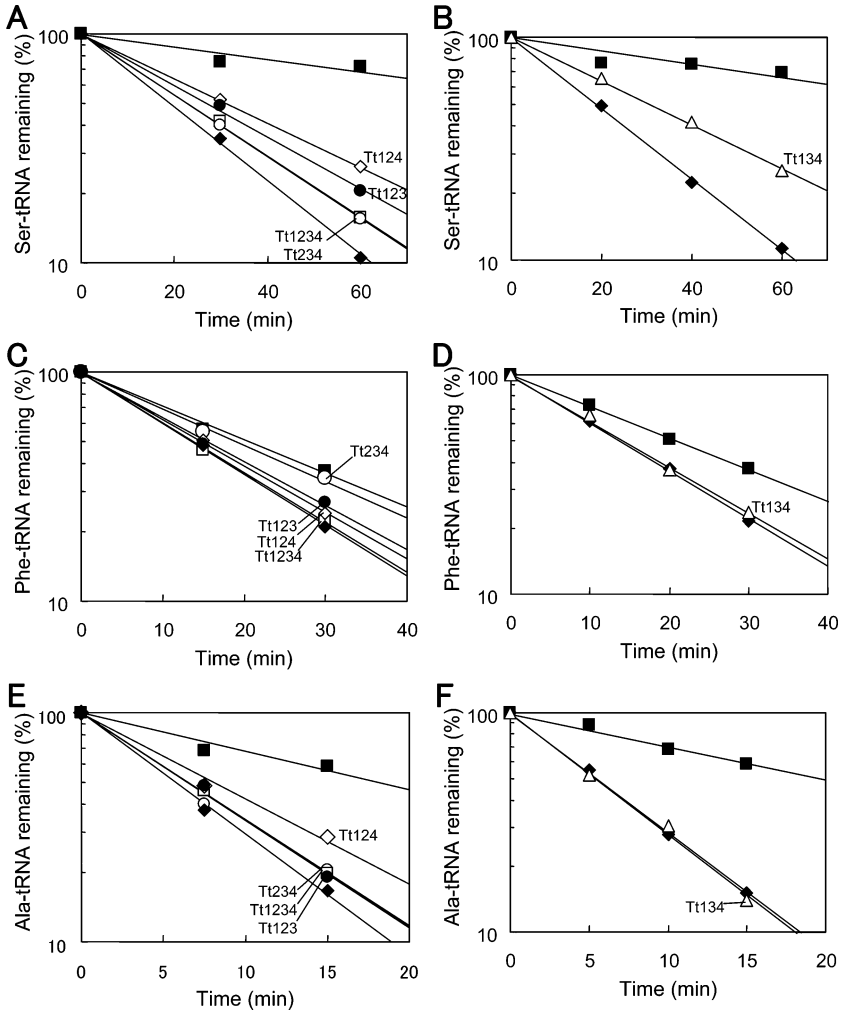

FIGURE 3: Binding activity of *T. thermophilus* EF-Tu mutants toward Ser-tRNA (A and B), Phe-tRNA (C and D), and Ala-tRNA (E and F). (A, C, and E) Deacylation protection assays were performed in the presence of Tt1234 (□), Tt123 (●), Tt124 (◇), Tt234 (○), or *T. thermophilus* EF-Tu (■) or in the absence of EF-Tu (◆). (B, D, and F) Deacylation protection assays were performed in the presence of Tt134 (△) or *T. thermophilus* EF-Tu (■),or in the absence of EF-Tu (◆).

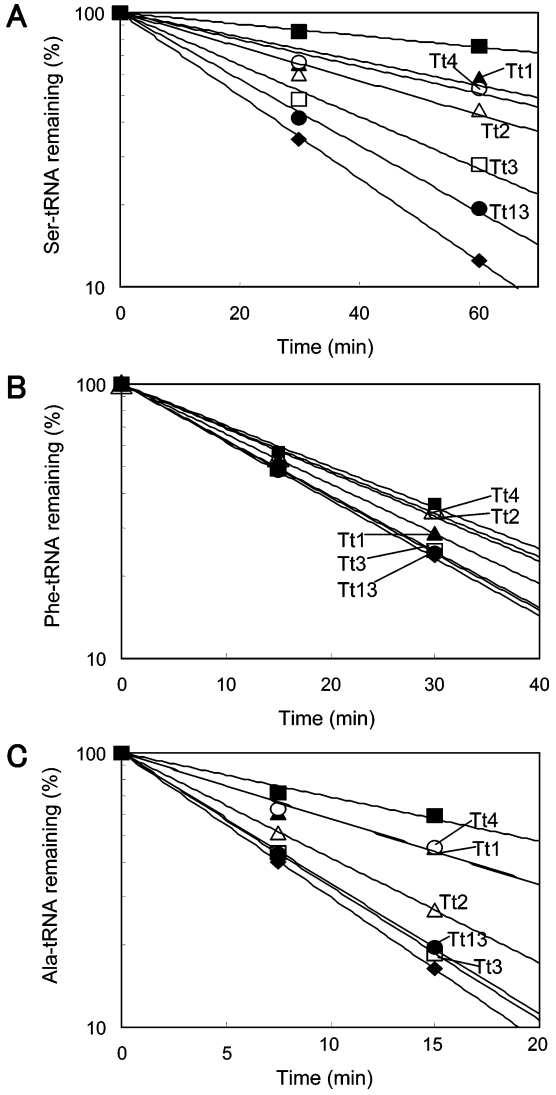

FIGURE 4: Binding activity of *T. thermophilus* EF-Tu mutants toward Ser-tRNA (A), Phe-tRNA (B), and Ala-tRNA (C). Deacylation protection assays were performed in the presence of Tt1 (▲), Tt2 (△), Tt3 (□), Tt4 (○), Tt13 (●), or *T. thermophilus* EF-Tu (■) or in the absence of EF-Tu (◆). In panel C, the deacylation curves for Tt1 and Tt4 overlap.

in which regions 1−3 are replaced with those of *C. elegans* EF-Tu2, was named Tt123. To examine the amino acid specificity of *T. thermophilus* EF-Tu mutants, hydrolysis protection assays using the yeast $tRNA^{Phe}$ derivative were performed. The yeast $tRNA^{Phe}$ derivative contains three altered base pairs in the acceptor stem and an altered base at position 37 (Figure 2). The tRNA derivative could then be charged with serine, alanine, or phenylalanine by bovine mt SerRS, *E. coli* AlaRS, or *E. coli* PheRS, respectively. The serylation, alanylation, and phenylalanylation efficiencies of this tRNA were approximately 10, 30, and 70%, respectively. Thus, we were able to compare the binding activities of each EF-Tu mutant toward Ser-tRNA, Phe-tRNA, and Ala-tRNA without changing the tRNA sequence. Figure 3 (A, C, and E) demonstrates that Tt1234, which includes all of the EF-Tu2 residues shown in Figure 1B, shows almost no aa-tRNA binding activity, even for Ser-tRNA. We then prepared four mutants (Tt123, Tt124, Tt134, and Tt234) containing three of the four EF-Tu2 regions indicated in Figure 1B. Tt123 seems to have a serine-specific tendency,

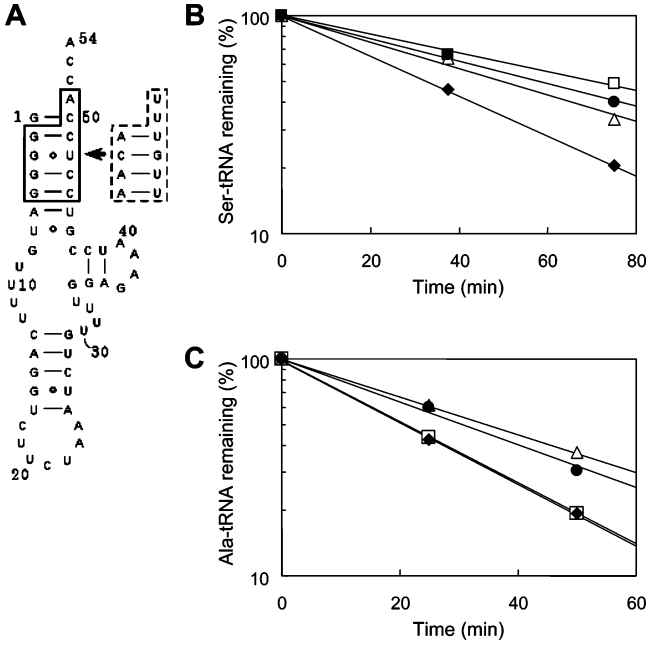

FIGURE 5: (A) Secondary structure of the *A. suum* mt $tRNA^{Ser}_{UCU}$ derivative, which can be charged with alanine as well as serine. The residues in the dashed box represent the sequence of wild-type $tRNA^{Ser}_{UCU}$. (B and C) Binding activities of *C. elegans* mt EF-Tu2 mutants toward nematode mt Ser-$tRNA^{Ser}_{UCU}$ (B) and Ala-$tRNA^{Ser}_{UCU}$ (C). Deacylation protection assays were performed in the presence of EF-Tu2 (□), Ce1 (△), or Ce14 (●) or in the absence of EF-Tu (◆).

in that it protected Ser-tRNA, but it also slightly protected Phe-tRNA and Ala-tRNA. Tt124 did not bind Phe-tRNA but did bind both Ser-tRNA and Ala-tRNA. Tt234 bound weakly to Ser-tRNA and Ala-tRNA, but its binding activity to Phe-tRNA was almost the same as that of wild-type *T. thermophilus* EF-Tu. Figure 3 (B, D, and F) demonstrates that Tt134 bound to Ser-tRNA but not to Phe-tRNA and Ala-tRNA, indicating that Tt134 is the most specific for Ser-tRNA of all the *T. thermophilus* EF-Tu mutants.

C. elegans mt EF-Tu2 Mutants Lost Serine Specificity. With regard to the method used to identify the critical residues, systematic mutations of bacterial EF-Tu seem to be more suitable than systematic mutations of EF-Tu2, because EF-Tu2 is unstable (*22*) and most of its mutants would be insoluble as shown below. Thus, we attempted to create EF-Tu2 mutants, but only for the purpose of confirming the results of the mutation study of bacterial EF-Tu. To conduct experiments which were the converse of Figure 3, we tried to prepare seven kinds of *C. elegans* mt EF-Tu2 mutants associated with regions 1, 3, and 4. Three mutants (Ce3, Ce4, and Ce134) could not be obtained as soluble proteins. Four soluble mutant proteins (Ce1, Ce13, Ce14, and Ce34) were obtained, but Ce13 and Ce34 did not bind to any of the aa-tRNAs used in the experiment shown in Figure 5. We examined the binding activity of the two remaining *C. elegans* mt EF-Tu2 mutants (Ce1 and Ce14) toward the *A. suum* mt $tRNA^{Ser}_{UCU}$ derivative using the hydrolysis protection assay. The *A. suum* mt $tRNA^{Ser}_{UCU}$ derivative (Figure 5A) could be charged with serine and alanine by *A. suum* mt SerRS and *E. coli* AlaRS, respectively (*8*). Figure 5 (B and C) shows that Ce1 and Ce14 bound to both Ser-$tRNA^{Ser}_{UCU}$ and Ala-$tRNA^{Ser}_{UCU}$, whereas wild-type *C. elegans* mt EF-Tu2 bound to Ser-$tRNA^{Ser}_{UCU}$ but not to

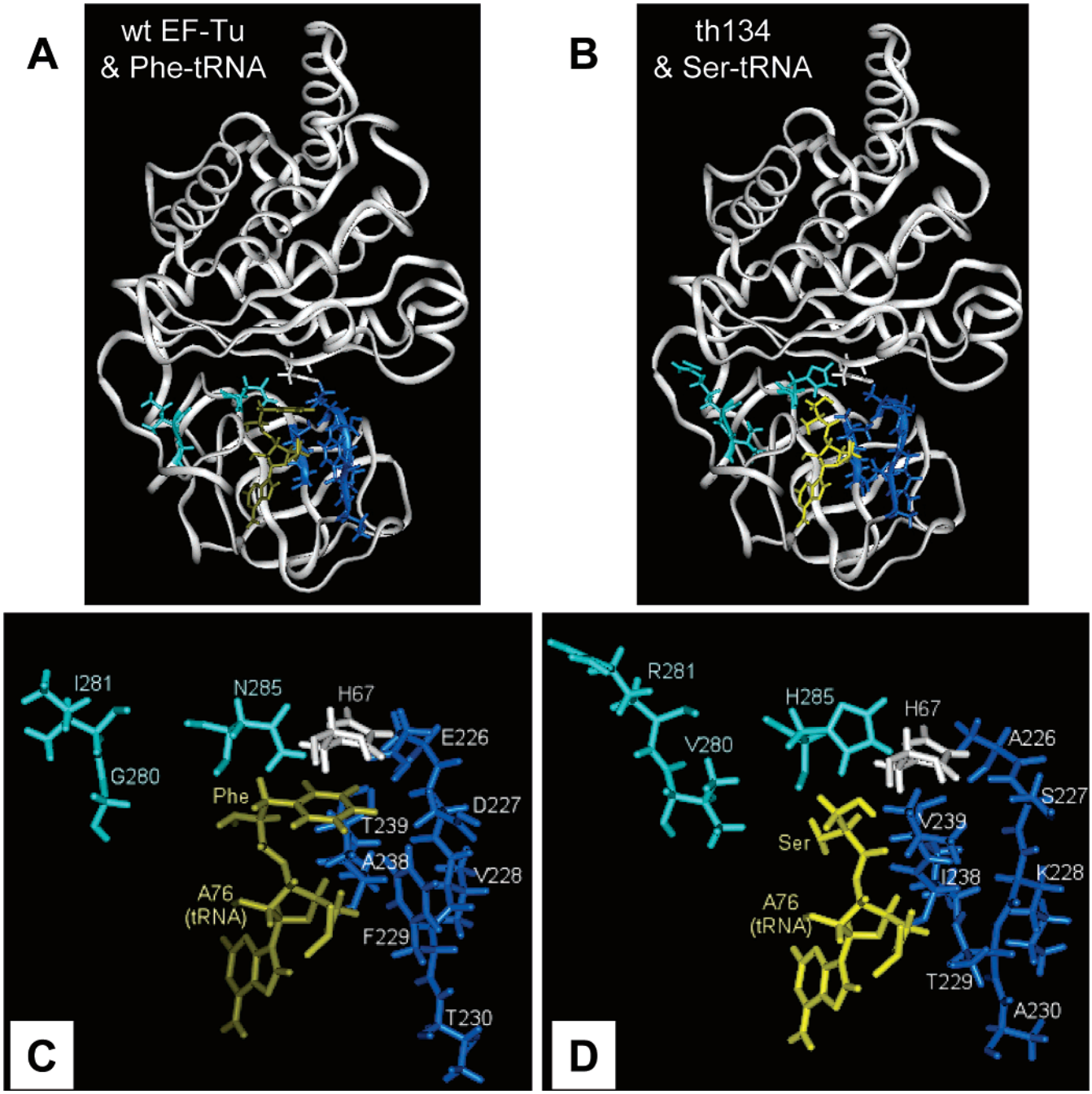

FIGURE 6: Prediction of the tertiary structure of the Tt134 mutant. (A and B) Domains 1 and 2 of *T. thermophilus* EF-Tu (*14*) (A) or the Tt134 mutant (B) are shown with the 3′ terminus of Phe-tRNA (A) or Ser-tRNA (B). (C and D) The residues surrounding the aminoacyl moiety in panels A and B are magnified: yellow for aminoacyl-tRNA, blue for residues of mutation region 1 (see Figure 1B), and cyan for residues of mutation regions 3 and 4 (see Figure 1B).

Ala-tRNA$^{Ser}_{UCU}$. This indicates that the *C. elegans* mt EF-Tu2 lost its serine specificity when region 1 was mutated.

DISCUSSION

In this study, we succeeded in transplanting the unique serine specificity of the *C. elegans* mt EF-Tu2 into a bacterial EF-Tu. The role of each region can be roughly characterized by the systematic analyses of these mutants, as described below.

Four mutants which included mutations of region 1 (Tt123, Tt124, Tt134, and Tt1234) showed almost no binding of Phe-tRNA, whereas binding of Tt234 to Phe-tRNA was similar to that of wild type EF-Tu (Figure 3C,D). This observation suggests that region 1 functions to block binding of EF-Tu2 to Phe-tRNA. This hypothesis is supported by the assays presented in Figure 4 which show that the *T. thermophilus* EF-Tu mutant containing the EF-Tu2 region 1 sequence (Tt1) bound to Ser-tRNA and Ala-tRNA but that binding to Phe-tRNA was minimal.

The four mutants containing a mutated region 3 of EF-Tu2 (Tt123, Tt234, Tt134, and Tt1234) showed a severely reduced level of binding to Ala-tRNA, whereas Tt124 did bind Ala-tRNA (Figure 3E,F). These data suggest that region 3 plays a role in excluding Ala-tRNA binding. This hypothesis is supported by Figure 1 of the Supporting Information showing that the Tt3 mutant bound Ser-tRNA

and slightly bound Phe-tRNA but did not bind Ala-tRNA. Thus, the serine specificity of Tt134 could be generated by region 1 and region 3 blocking the binding of Tt134 to Phe-tRNA and Ala-tRNA, respectively.

What then is the role of regions 2 and 4 in EF-Tu2? The role of region 2 is unclear at present. However, region 4 seems to have a role in restricting the binding of Ala-tRNA because the Tt13 mutant slightly protected the Ala-tRNA (Figure 4) whereas the Tt134 mutant did not (Figure 3D,F). Although the amino acid specificity of the Tt4 mutant was almost the same as that of wild-type *T. thermophilus* EF-Tu (Figure 4), EF-Tu2 region 4 might be able to influence the serine specificity only when regions 1 and 3 of EF-Tu2 exist near region 4.

We were able to analyze only two of the *C. elegans* mt EF-Tu2 mutants because most of the mutants were insoluble or inactive. *C. elegans* mt EF-Tu2 mutants Ce1 and Ce14 lost serine specificity. Since the mutation of region 1 alone impaired the serine specificity of EF-Tu2, creating EF-Tu2 mutants without serine specificity was easier than creating a serine-specific bacterial EF-Tu mutant. These results indicate that region 1 is necessary for the specific recognition of the seryl moiety of Ser-tRNA.

Panels B and D of Figure 6 present a model of the complex of the Tt134 mutant and Ser-tRNA. Figure 6D shows that mutations which are in region 1 (A238I and T239V) narrow the space available for the aminoacyl side chain. This observation may explain why region 1 of EF-Tu2 restricts binding to Phe-tRNA which has a larger aminoacyl group than Ser-tRNA. These positions (257 and 258 in *C. elegans* EF-Tu2) are semiconserved as bulky hydrophobic amino acids (Ile or Val) among nematode mt EF-Tu2s (Figure 1A), suggesting that they may be important for serine specificity. Though it is not known whether all nematode mt EF-Tu2s exhibit serine specificity, all published nematode mt DNAs of the class Chromadorea encode 20 T armless tRNAs and two D armless serine tRNAs as found in *C. elegans*, suggesting that all chromadorean nematodes are likely to have mt EF-Tu2 with serine specificity. The mutation of region 3 affected the amino acid specificity of EF-Tu, as described above, but positions 280 and 281 are slightly removed from the seryl moiety. Figure 6D shows that the G280V mutation would add a bulky side chain in the direction of the seryl group and it might indirectly narrow the aa-binding pocket to restrict binding of aa-tRNAs other than Ser-tRNA. Position 285 in *T. thermophilus* EF-Tu (position 303 in EF-Tu2) is located very close to the serine side chain. Thus, the imidazole group of H303 might interact with the serine side chain. Since V298 and H303 (positions 280 and 285, respectively, in *T. thermophilus* EF-Tu) are completely conserved in nematode EF-Tu2s (Figure 1A), these residues are likely to be important for the serine specificity.

The detailed mechanism of recognition of the seryl moiety by EF-Tu2 still needs to be clarified. To define the details of the molecular mechanism of serine-specific recognition by *C. elegans* mt EF-Tu2, the detailed structure of EF-Tu2 complexed with Ser-tRNA should be determined. However, EF-Tu2 is particularly unstable; for example, this protein is easily precipitated in low-salt solutions or at a concentration higher than 1 mg/mL (*22*), so crystallization of the protein may be difficult. In this study, we obtained a stable *T. thermophilus* EF-Tu mutant that has serine specificity. Thus, it should be possible to analyze the crystal structure of the serine-specific EF-Tu using the mutant obtained here.

Canonical EF-Tu can bind to all 20 amino acids in aa-tRNAs, while *C. elegans* mt EF-Tu2 exclusively recognizes the seryl moiety of Ser-tRNA. The evolutionary process that led to the development of a serine-specific EF-Tu2 from a canonical EF-Tu remains a mystery. Chromadorean nematode mitochondria have two distinct EF-Tu species; EF-Tu1 binds to T armless aminoacyl-tRNAs, and EF-Tu2 binds only to D armless Ser-tRNAs. Plants and fungi have only a single mt EF-Tu, which is closely related to bacterial EF-Tu, suggesting that the original mt EF-Tu was highly homologous to the canonical EF-Tu when mitochondria were generated from ancestral bacteria. After the emergence of metazoa, the mt EF-Tu gene was duplicated, at least in the common ancestor of chromadorean nematodes, and then the two EF-Tu genes co-evolved together with different types of truncated tRNAs into distinct forms (*7*, *8*). As all known eumetazoan mitochondria have at least one D armless $tRNA^{Ser}$ (*9*), an ancestor of EF-Tu2 might first have acquired specificity for the tRNA backbone of D armless tRNA (*13*). It is likely that the unique serine specificity of EF-Tu2 was generated after the role of EF-Tu2 was limited to the delivery of only Ser-tRNAs. An important conclusion of this study is that the specificity for an esterified amino acid can be significantly altered by mutating a relatively small number of amino acids. Thus, it is suggested that the evolution from a canonical EF-Tu to an EF-Tu specific for a particular esterified amino acid(s) can occur easily when two conditions are fulfilled: (i) when the EF-Tu is relieved of its obligation to bind all elongator aa-tRNAs and (ii) when it is obliged to bind to tRNAs specific for one amino acid.

ACKNOWLEDGMENT

We thank Prof. K. Kita (University of Tokyo) for the *A. suum* and Prof. G. Kawai (Chiba Institute of Technology, Chiba, Japan) for structural modeling of EF-Tu mutants.

SUPPORTING INFORMATION AVAILABLE

Binding activity of *T. thermophilus* EF-Tu mutants toward Ser-tRNA, Phe-tRNA, and Ala-tRNA. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI060536I