

Crystal structures of leucyl/phenylalanyl-tRNA-protein transferase and its complex with an aminoacyl-tRNA analog

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Eubacterial leucyl/phenylalanyl-tRNA protein transferase (L/F-transferase), encoded by the *aat* gene, conjugates leucine or phenylalanine to the N-terminal Arg or Lys residue of proteins, using Leu-tRNA^{Leu} or Phe-tRNA^{Phe} as a substrate. The resulting N-terminal Leu or Phe acts as a degradation signal for the ClpS-ClpAP-mediated N-end rule protein degradation pathway. Here, we present the crystal structures of *Escherichia coli* L/F-transferase and its complex with an aminoacyl-tRNA analog, puromycin. The C-terminal domain of L/F-transferase consists of the GCN5-related N-acetyltransferase fold, commonly observed in the acetyltransferase superfamily. The *p*-methoxybenzyl group of puromycin, corresponding to the side chain of Leu or Phe of Leu-tRNA^{Leu} or Phe-tRNA^{Phe}, is accommodated in a highly hydrophobic pocket, with a shape and size suitable for hydrophobic amino-acid residues lacking a branched β -carbon, such as leucine and phenylalanine. Structure-based mutagenesis of L/F-transferase revealed its substrate specificity. Furthermore, we present a model of the L/F-transferase complex with tRNA and substrate proteins bearing an N-terminal Arg or Lys.

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Introduction

Regulated degradation of intracellular proteins is essential for protein quality control in both eubacteria and eukaryotes. The N-end rule pathway, one of the most prevalent proteolytic pathways, functions in the *in vivo* half-life control of

selected proteins, by destroying them according to the N-terminal residue (Varshavsky, 1996, 2003). In eukaryotes, the N-end rule pathway is part of the ubiquitin (Ub) system (Hu *et al*, 2005; Nandi *et al*, 2006), which controls peptide import (Turner *et al*, 2000; Du *et al*, 2002), chromosomal segregation fidelity (Rao *et al*, 2001), apoptosis regulation (Ditzel *et al*, 2003; Varshavsky, 2003), and nitric oxide detection (Hu *et al*, 2005). In eukaryotes, the destabilizing N-terminal residues are hierarchical. The primary destabilizing residues are categorized into two types: the type-1 destabilizing residues are basic (Arg, Lys, or His), whereas the type-2 residues are bulky and hydrophobic (Phe, Leu, Trp, Tyr, or Ile). Multiple E3 Ub ligases (N-recognins) recognize these N-terminal residues, and conjugate a poly-Ub chain to the target protein. The resulting ubiquitylated proteins are thereafter progressively degraded by the 26S proteasome in an ATP-dependent manner. The secondary destabilizing N-terminal residues, Asp and Glu, are recognized by Arg-tRNA-protein transferase (R-transferase), which conjugates an arginine, the primary destabilizing residue, to them. The N-terminal Asn and Gln are tertiary destabilizing residues, in that they are deaminated to yield the secondary destabilizing N-termini of Asp and Glu, respectively. Recently, N-terminal Cys and oxidized Cys residues have also been reported to behave as tertiary and secondary destabilizing residues, respectively, in mammals (Kwon *et al*, 2002; Hu *et al*, 2005). Thus, the functions of the N-end rule pathway have been well elucidated in eukaryotes (Hu *et al*, 2005), whereas they still remain unknown in eubacteria.

Eubacteria have an analogous, but Ub-independent, N-end rule pathway (Tobias *et al*, 1991; Shrader *et al*, 1993). As in eukaryotes, the destabilizing residues in bacteria are also hierarchical. In *Escherichia coli*, the primary destabilizing N-terminal residues are Leu, Phe, Trp, and Tyr. Proteins with these N-terminal residues are degraded by ClpAP, a proteasome-like protease consisting of the AAA + chaperone ClpA and ClpP peptidase (ClpAP) (Tobias *et al*, 1991). Recently, the ClpAP-specific adaptor, ClpS, was identified as an N-recognin, an essential component of the pathway (Erbse *et al*, 2006). ClpS shares secondary structural features with UBR1, the E3 Ub ligase of the eukaryotic N-end rule pathway (Lupas and Koretke, 2003), and binds to a primary destabilizing residue of a substrate to deliver it to the ClpAP protease for degradation (Erbse *et al*, 2006). In *E. coli*, the secondary destabilizing N-terminal residues are Arg and Lys. These N-terminal residues are recognized by leucyl/phenylalanyl-tRNA-protein transferase (L/F-transferase), which conjugates leucine or phenylalanine to the N-terminal Arg or Lys, followed by ClpS recognition. However, the physiological functions of the eubacterial N-end rule pathway remain to be elucidated.

There are several similarities between the eukaryotic and eubacterial N-end rule pathways, such as the hierarchic structure of the destabilizing N-terminal residues, the require-

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ment of the specific N-recognin (E3 Ub ligase in eukaryotes and ClpS in eubacteria), and the involvement of ATP-dependent proteases (the 26S proteasome complex in eukaryotes and the proteasome-like ClpAP in eubacteria). Furthermore, in both systems, an aminoacyl-tRNA-protein transferase (R-transferase in eukaryotes and L/F-transferase in eubacteria) conjugates a primary destabilizing residue to the substrate's secondary destabilizing N-terminal residue. These similarities between the N-end rule pathways in the two kingdoms imply that these pathways and their components share a common origin. However, in contrast to the sequence similarity between the eukaryotic E3 Ub ligase and the eubacterial ClpS (Lupas and Koretke, 2003), no significant sequence similarity exists between the eukaryotic R-transferase and the eubacterial L/F-transferase.

E. coli L/F-transferase was identified about four decades ago (Kaji *et al*, 1965a,b). Biochemical studies revealed that *E. coli* L/F-transferase catalyzes the transfer of Leu and Phe, and Met and Trp less efficiently, using the cognate aminoacyl-tRNAs (Kaji *et al*, 1965a,b; Horinishi *et al*, 1975; Abramochkin and Shrader, 1996) to the N-terminal Arg and Lys of acceptor proteins (Soffer, 1973). It was also reported that the anticodon of the aminoacyl-tRNA is not a determinant for the enzyme recognition, but that the single-stranded acceptor region of the aminoacyl-tRNA is required for the L/F-transferase activity (Abramochkin and Shrader, 1996). However, the detailed molecular basis for the recognition of the aminoacyl moiety of an aminoacyl-tRNA by L/F-transferase has remained obscure.

Here, we have determined the crystal structures of *E. coli* L/F-transferase and its complex with puromycin, an analog of the aminoacyl-tRNA. The structure of this complex, together with extensive biochemical mutational studies, revealed the mechanism by which L/F-transferase specifically recognizes the aminoacyl-moiety of aminoacyl-tRNAs. Based on the current structure, we also present a model of the L/F-transferase complex with tRNA and substrate proteins bearing an N-terminal Arg or Lys residue.

Results and discussion

Overall architecture of *E. coli* L/F-transferase and structural similarity with the FemABX enzyme family

The *E. coli* L/F-transferase was overexpressed in *E. coli* and crystallized. The *apo* structure was initially solved by multi-wavelength anomalous dispersion (MAD), using the selenomethionine-labeled protein, and the native structure was refined to an *R* factor of 27.5% (R_{free} of 22.2%) using reflections up to 2.4 Å resolution. Subsequently, the data sets were collected from crystals soaked in puromycin. The structure of the complex with puromycin was refined to an *R* factor of 27.5% (R_{free} of 22.5%) up to 2.8 Å resolution (Supplementary Table 1).

E. coli L/F-transferase forms a compact structure and consists of two domains: an NH₂-terminal domain (amino-acid residues 3–62) and a COOH-terminal domain (amino-acid residues 63–232) (Figure 1A). The NH₂-terminal domain is composed of four β-strands (β1–4) and one α-helix (α1),

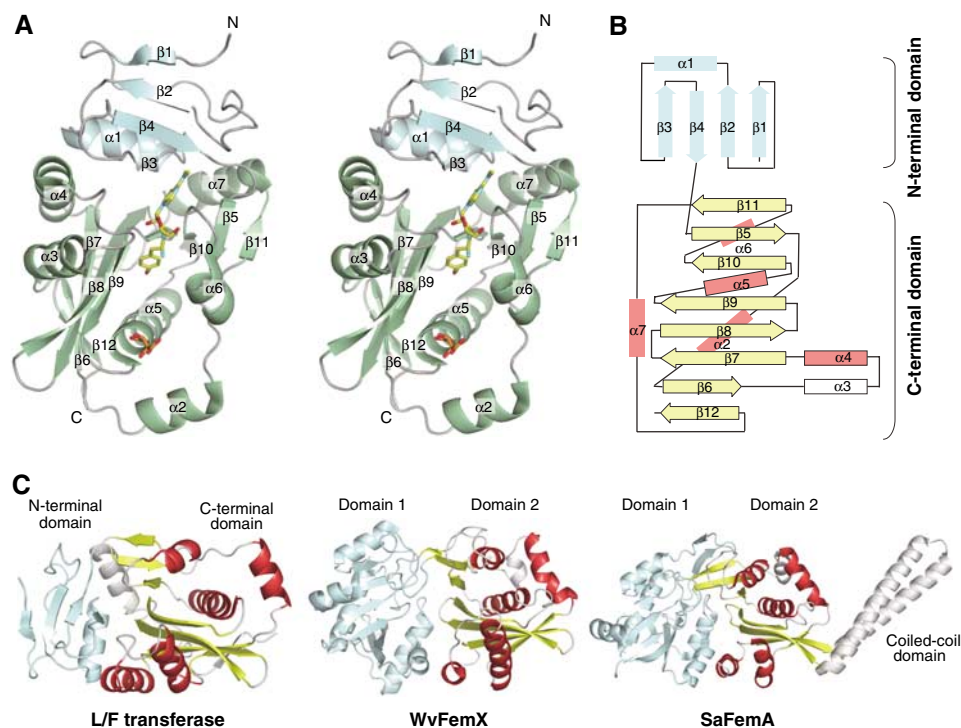


Figure 1 Overall architecture of *E. coli* L/F-transferase. (A) Stereo view of the *E. coli* L/F-transferase structure. The NH₂-terminal domain (residues 2–62) and the COOH-terminal domain (residues 63–232) are colored blue and green, respectively. The puromycin bound to the hydrophobic pocket is colored yellow. (B) Topology diagram of L/F-transferase. The rimmed elements in the COOH-terminal domain (α3–α5) and (β5–β12) are common to the GNAT superfamily fold. The α-helices and β-strands in the COOH-terminal domains are colored red and yellow, respectively. (C) Comparison of the structures of *E. coli* L/F-transferase (left), *W. viridescens* FemX (*wvFemX*; middle, PDB accession number 1P4N; Biarrotte-Sorin *et al*, 2004) and *S. aureus* FemA (*saFemA*; PDB accession number 1LRZ; Benson *et al*, 2002). The COOH-terminal domain of L/F-transferase is topologically similar to the domain 2's of *wvFemX* and *saFemA*. The conserved α-helices and β-strands in L/F-transferase, *wvFemX* and *saFemA*, are colored red and yellow, respectively.

whereas the COOH-terminal domain is composed of eight β -strands (β 5–12) surrounded by six α -helices (α 2–7) (Figure 1B). Puromycin, an aminoacyl-tRNA analog, binds to the cleft formed by β 9, α 5, and β 10 in the COOH-terminal domain and by β 3 in the NH₂-terminal domain (see below) (Figures 1A and 2).

The COOH-terminal domain of L/F-transferase is topologically similar to domain 2 of *Weissella viridescens* FemX (*wvFemX*; Biarrotte-Sorin *et al*, 2004) and that of *Staphylococcus aureus* FemA (*saFemA*; Benson *et al*, 2002) (Figure 1C, middle and right, respectively). Both *wvFemX* and *saFemA* belong to the FemABX family (Hegde and Shrader, 2001), containing a GCN5-related *N*-acetyltransferase (GNAT) fold (Sternier and Berger, 2000) (Figure 1B), and catalyze the transfer of an amino acid to a precursor of peptidoglycan, using an aminoacyl-tRNA as a substrate. *WvFemX* transfers an alanine to the ϵ -amino group of a Lys side chain using Ala-tRNA^{Ala} as a substrate, whereas *saFemA* transfers a glycine to the α -amino group of a Gly main chain, utilizing Gly-tRNA^{Gly}, to form an interpeptide bridge of the peptidoglycan. Therefore, the topological similarity between the COOH-terminal domain of L/F-transferase and the domain 2's of *wvFemX* and *saFemA* may reflect the similarity of their chemical reactions, where an amino acid is transferred from an aminoacyl-tRNA to the amino group of a protein (or peptide). It is interesting to note that, although the topological and structural similarity is readily apparent between L/F-transferase and the FemABX families (Figure 1C), there is no significant amino-acid similarity between these enzymes, suggesting that they might have arisen from a common ancestor, but have divergently evolved.

In contrast to the topological similarity between the COOH-terminal domain of L/F-transferase and the domain 2's of

wvFemX and *saFemA*, no significant topological similarity can be identified between the NH₂-terminal domain of L/F-transferase and the domain 1's of *wvFemX* and *saFemA* (Figure 1C).

Recognition of an aminoacyl-tRNA analog, puromycin, by L/F-transferase

The chemical structure of puromycin is similar to that of the 3'-terminus of an aminoacyl-tRNA; the carboxyl group of *p*-methoxyphenylalanine is linked to the 3'-amino group of 3'-amino-6-*N,N*-dimethyladenosine by an amide bond (Figure 3A). The *p*-methoxybenzyl group and the 6-*N,N*-dimethyladenosine correspond to the side chain of an amino acid and the adenosine at the CCA end of an aminoacyl-tRNA, respectively.

Puromycin reportedly inhibits the activity of L/F-transferase by preventing the aminoacyl-tRNA from binding to the enzyme, suggesting that puromycin binds to the same position in L/F-transferase as the 3'-end of an aminoacyl-tRNA (Horinishi *et al*, 1975; Abramochkin and Shrader, 1996). To elucidate the molecular basis of the recognition of the aminoacyl moiety of aminoacyl-tRNAs by L/F-transferase, *apo* L/F-transferase crystals were soaked in a solution containing puromycin, and the crystal structure was determined (Supplementary Table 1).

The electron density corresponding to the puromycin was clearly visible in the complex structure (Figure 3B), with no significant structural change observed between the *apo* and complex structures. The puromycin binds to a cleft formed by β 9, α 5, and β 10 in the COOH-terminal domain and β 3 in the NH₂-terminal domain (Figure 1A).

The *p*-methoxybenzyl group of puromycin is docked within a deep pocket at the bottom of the cleft (Figure 3C and D). The pocket is composed of the side chains of several hydro-

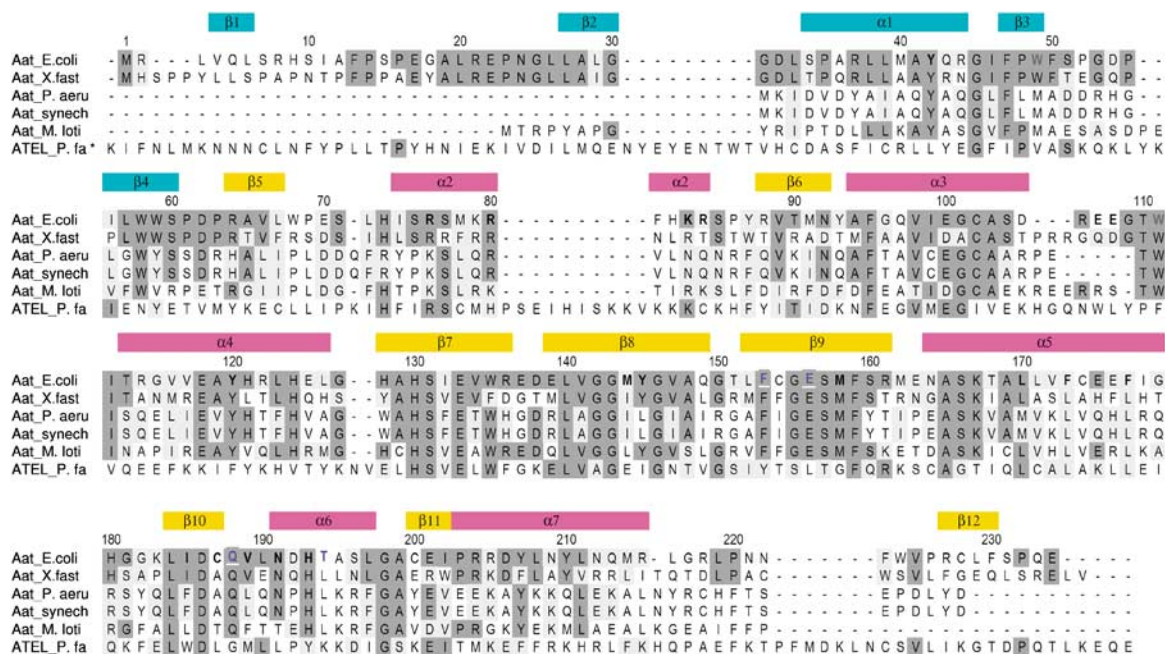


Figure 2 Sequence alignment of the L/F-transferases from *E. coli* (Aat_E.coli), *Xylella fastidiosa* (Aat_X-fast; accession number ZP_00683190.1), *Pseudomonas aeruginosa* (Aat_P.aeru; accession number ZP_00204849.1), *Synechocystis* (Aat_Synech; accession number NP_440931.1), and *Mesorhizobium loti* (Aat_M.lot; accession number NP_102051.1). The sequence of R-transferase from *Plasmodium falciparum* (ATEL_P_fa; accession number NP_473045.1). The secondary structure elements of *E. coli* L/F-transferase are indicated above the alignment. The α -helices and β -strands in the NH₂-terminal domain are colored blue, and those of the COOH-terminal domain are colored red and yellow, respectively, as shown in Figure 1B.

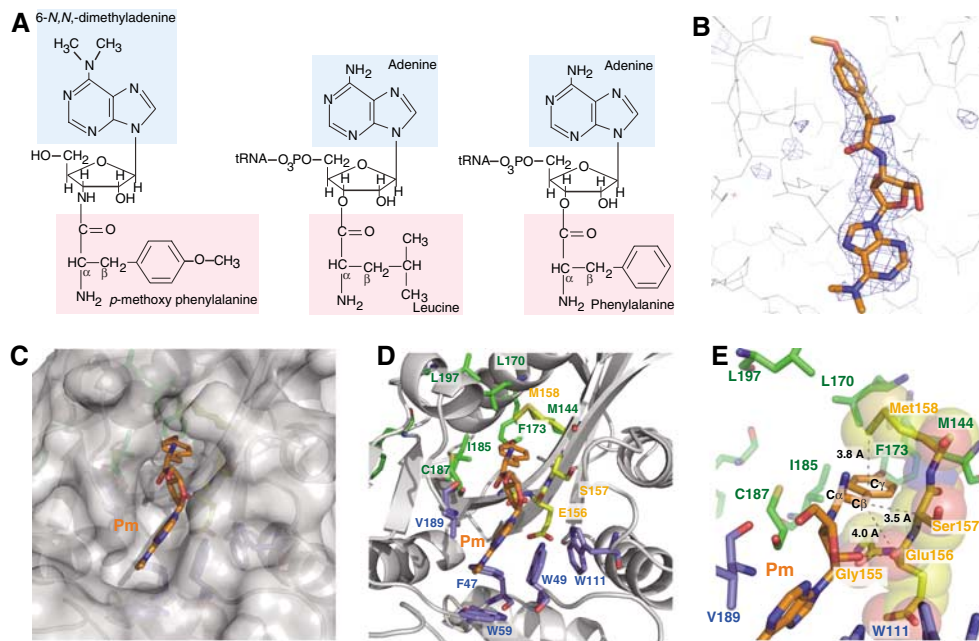


Figure 3 Recognition of the puromycin by *E. coli* L/F-transferase. (A) Chemical structure of puromycin (left) and that of the 3'-ends of Leu-tRNA^{Leu} and Phe-tRNA^{Phe} (middle and right, respectively). The amino-acid moiety and the base moiety are colored pink and blue, respectively. (B) [Fo-Fc] omit map of puromycin (contour level 3.0 σ). (C) Recognition of the *p*-methoxybenzyl group and the puromycin base by the hydrophobic pocket, as shown by a surface model. (D) Ribbon model of (C). The hydrophobic amino acid involved in the recognition of the *p*-methoxybenzyl group and the base moiety of puromycin are colored green and blue, respectively. (E) The C-shaped edge of the hydrophobic pocket is composed of continuous amino-acid residues (Gly155-Glu156-Ser157-Met158; colored yellow and highlighted). The α -, β - and γ -carbons of puromycin are also shown.

phobic residues (Met144, Phe153, Leu170, Phe173, Ile185), and thus the inner surface is quite hydrophobic. The recognition of the *p*-methoxybenzyl group of puromycin by L/F-transferase is achieved through a hydrophobic interaction with these amino-acid residues. The edge of the pocket is formed by the main chains of continuous residues (Gly155, Glu156, Ser157) and the side chain of Met158 (Figure 3E), adopting a C-shaped structure. The β -carbon of the *p*-methoxyphenylalanine group of puromycin is cramped by the C-shaped edge of the pocket: the side chain of Met158 and the peptide bond between Gly155 and Glu156 sandwich the β -carbon of puromycin (Figure 3E). The distance between the C γ of puromycin and the C ϵ of Met158 is 3.8 Å, and the distances between the C β of puromycin and the C α of Ser157 and the main chain N of Glu156 are 3.5 and 4.0 Å, respectively (Figure 3E).

The 6-*N,N*-dimethyladenine group of puromycin is stabilized mainly by a π - π stacking interaction with Trp49, which is further stabilized by a stacking interaction with Trp111 (Figure 3D). The hydrophobic amino-acid residues, Trp59, Phe47, and Val189, interact with the base moiety of puromycin through hydrophobic interactions, and these interactions are not specific to the 6-*N,N*-dimethyladenine of puromycin. The 2'-hydroxyl group of the ribose moiety of puromycin hydrogen bonds with the main-chain carbonyl group of Glu156 (Figure 3D).

Recognition mechanism of the amino-acid moiety of the aminoacyl-tRNA by L/F-transferase

The chemical structure of puromycin resembles that of the 3'-termini of aminoacyl-tRNAs, as described (Figure 3A). The reported inhibitory effect by puromycin on L/F-transferase

activity might reflect the similar hydrophobic properties of the *p*-methoxybenzyl group of puromycin to the side chains of phenylalanine and leucine (Figure 3A). Moreover, the 6-*N,N*-dimethyladenosine corresponds to the 3'-terminal adenosine at position 76 in the aminoacyl-tRNAs.

To explore the detailed molecular mechanism of the recognition of the 3'-end of Leu-tRNA^{Leu} or Phe-tRNA^{Phe} by L/F-transferase, a series of site-directed mutations were introduced into the L/F-transferase residues that are proximal to puromycin, based on the present complex structure, and the activities of leucyl and phenylalanyl transfer to α -casein bearing an NH₂-terminal Arg, from the respective aminoacyl-tRNAs, were analyzed (Figure 4A and B).

Mutations of hydrophobic amino-acid residues (Met144, Phe153, Leu170, Phe173, Ile185) that accommodate the *p*-methoxybenzyl group of puromycin (Figure 3C and D) considerably reduced the L/F-transferase activity (Figure 4A and B). Therefore, it is reasonable to assume that the hydrophobic leucyl and phenylalanyl moieties of Leu-tRNA^{Leu} and Phe-tRNA^{Phe}, respectively, are recognized by this highly hydrophobic pocket of L/F-transferase. Actually, superposition of the α and β carbons of Phe and Leu onto those of puromycin revealed that the side chains of Phe and Leu can enter the hydrophobic pocket without any steric clashes (Figure 5A and B). The corresponding amino-acid residues of L/F-transferases from other eubacteria are well conserved as hydrophobic amino-acid residues (Figure 2). These results explain why L/F-transferase recognizes aminoacyl-tRNAs attached to hydrophobic amino acids, and excludes those coupled to hydrophilic or charged amino acids.

The β -carbon of the aminoacyl moiety of puromycin is sandwiched between the side chain of Met158 and the

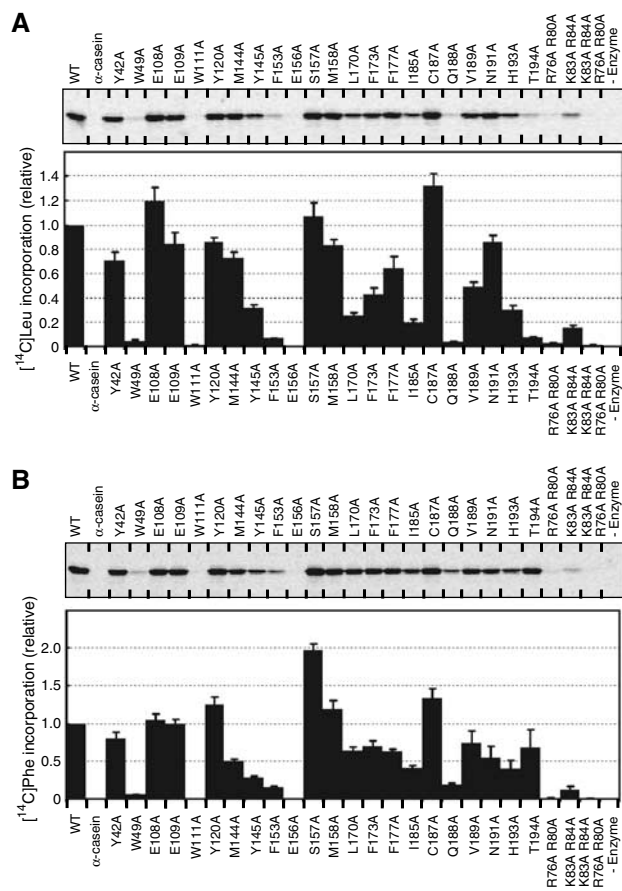


Figure 4 *In vitro* activity assays for various mutants of L/F-transferase. (A) The activity of [¹⁴C]Leu incorporation into α -casein from [¹⁴C]Leu-tRNA^{Leu} by mutant L/F-transferases (see Materials and methods). The upper gel shows the [¹⁴C]Leu-labeled α -casein. The lower graph shows the quantification of the relative intensity of the labeled product of the upper gel, where the incorporation of [¹⁴C]Leu into α -casein by wild-type L/F-transferase was taken as 1.0. The bars on the graph indicate the s.d. of more than three independent experiments. (B) The activity of [¹⁴C]Phe incorporation into α -casein from [¹⁴C]Phe-tRNA^{Phe} by mutant L/F-transferases, as in (A).

peptide-bond plane of Gly155–Glu156 (Figure 3E). Both the side chains of the leucyl and phenylalanyl moieties of the respective aminoacyl-tRNAs lack branched β -carbons (Figures 3A, 5A, and B). When the C α and C β of Ile and Val, possessing branched β -carbons, are superimposed onto those of puromycin (Figure 5C and D), the distances between the branched C γ 2 of Ile and the carbonyl carbon of Glu156, and between the branched C γ 2 of Val and the C α of Ser157 are 2.6 and 2.5 Å, respectively. These close distances between the branched methyl groups of Ile and Val and the C-shaped edge of the hydrophobic pocket would cause steric hindrance, thus precluding aminoacyl-tRNAs charged with β -branched amino acids, such as Ile and Val. These results explain the specificity of L/F-transferase for amino acids bearing an unbranched β -carbon (Abramochkin and Shrader, 1996).

For the other hydrophobic amino-acid residues, such as Ala, Pro, Trp, and Met, the size of the Ala and Pro side chains is not large enough to fit within the hydrophobic pocket (Supplementary Figure 1A and B). On the other hand, L/F-transferase reportedly transfers Met and Trp to proteins bearing an N-terminal Arg or Lys, although the activities are less efficient as compared with those of Leu and Phe (Kaji *et al*, 1965a; Abramochkin and Shrader, 1996). Our *in vitro* assays using Met-tRNA^{Met} and Trp-tRNA^{Trp} as substrates also showed that both Met and Trp could be transferred to the NH₂-terminal Arg of the α -casein fragment (Supplementary Figure 2D and E). However, in the presence of Phe-tRNA^{Phe}, Leu-tRNA^{Leu}, Met-tRNA^{Met}, and Trp-tRNA^{Trp}, Phe and Leu were predominantly transferred to the α -casein fragment (Supplementary Figure 2F).

The superposition of the C α and C β of Trp onto those of puromycin (Supplementary Figure 1C) revealed that the size of the Trp side chain (indole group) is too large to be properly accommodated in the hydrophobic pocket, and thus the indole group might become snagged on the C-shaped edge of the pocket. On the other hand, the size of the Met side chain is sufficiently large enough to be accommodated into the hydrophobic pocket properly (Supplementary Figure 1D). However, the contact area of the Met side chain with the

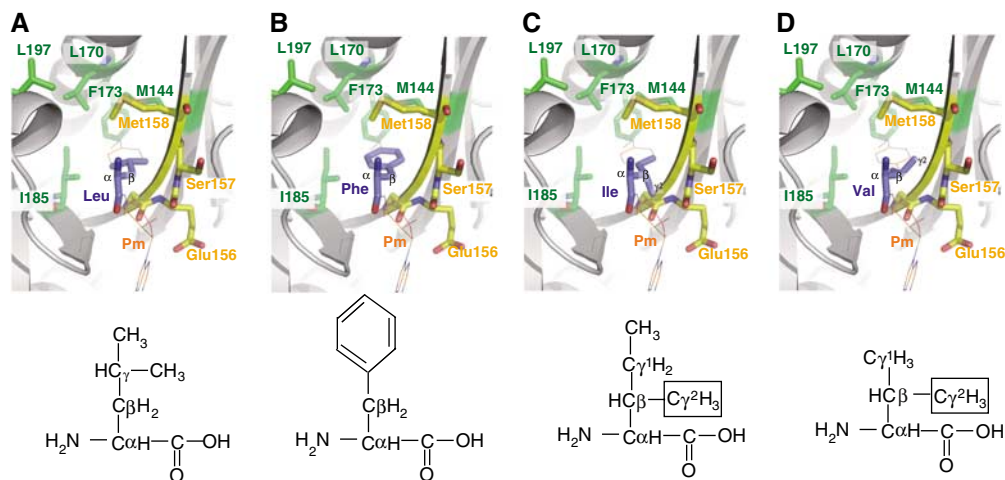


Figure 5 Recognition of the aminoacyl-moiety by L/F-transferase. Recognition of the side chains of Leu (A) and Phe (B) by L/F-transferase. The α - and β -carbons of Leu and Phe were superimposed onto those of puromycin (colored orange). Discrimination of Ile (C) and Val (D) by L/F-transferase. The α - and β -carbons of Ile and Val were superimposed as in (A) and (B). The steric hindrance between the branched methyl groups at the β -carbons of Ile and Val. The C-shaped edge of the hydrophobic pocket is colored yellow. The chemical structures of each amino acid are depicted in parentheses.

hydrophobic pocket is smaller than that of Leu and Phe. These findings explain the previous and present data showing that Trp and Met are incorporated into the protein by L/F-transferase less efficiently than Leu or Phe *in vitro*, and that Leu and Phe are incorporated predominantly *in vivo*.

Taken together, the hydrophobicity and size of the pocket and the confined C-shaped structure of the edge of the pocket could collaboratively discriminate Leu and Phe predominantly (and Trp and Met less efficiently), from other amino-acid residues to be transferred to acceptor proteins bearing Arg or Lys at the N-terminus. It is notable that the continuous amino-acid residues composing the C-shaped edge (Gly155–Met158) of the hydrophobic pocket are well conserved among the eubacterial L/F-transferases (Figure 2).

The 6-*N,N*-dimethyladenosine of puromycin corresponds to the 3'-terminal adenosine residue (A76) of tRNA, as described above (Figure 3A). The 6-*N,N*-dimethyladenosine of puromycin interacts with several amino-acid residues (Figure 3D). The mutations of Trp49 and Trp111 drastically reduced the L/F-transferase activity (Figure 4), suggesting that the stacking interaction between the base moiety of A76 and Trp49 and the further stabilization by Trp111 are crucial for recognition of the 3'-terminal nucleotide of the aminoacyl-tRNAs. This result is consistent with the previous finding that the activity of a recombinant L/F-transferase, lacking the NH₂-terminal 78 amino acids, is significantly reduced (Ichetovkin *et al*, 1997). Moreover, the mutation of Val189, which interacts with the base moiety through a hydrophobic interaction, reduced the activity, as expected (Figure 4).

The present structural and biochemical studies of L/F-transferase (Figures 3 and 4) strongly suggest that the 3'-terminus of the aminoacyl-tRNA is recognized by the combination of the hydrophobic aminoacyl moiety recognizing pocket and the 3'-nucleotide recognition site, although the 3'-nucleotide binding site is not specific for the adenosine.

Docking model of L/F-transferase and aminoacyl-tRNA

The electrostatic potential surface of L/F-transferase reveals the highly biased distribution of charged residues (Figure 6A,

lower panel). Especially, the $\alpha 2$ helix in the COOH-terminal domain contains a cluster of positively charged residues (Arg76, Arg80, Lys83, and Arg84). These amino-acid residues protrude toward the solvent and are conserved among the eubacterial L/F-transferases (Figure 2). Moreover, next to the charged region, a cleft suitable for the accommodation of the 3'-region of a tRNA-acceptor helix is formed by helices $\alpha 5$ and $\alpha 6$, extending toward the puromycin-binding pocket (Figure 6A, lower and right panels). The amino-acid residues in the NH₂-terminal half of $\alpha 5$ (Asn164, Ser166, and Lys167) and Asn191 and His193 in $\alpha 6$ are well conserved among the eubacterial L/F-transferases (Figure 2). Mutations of the positively charged residues in $\alpha 2$ (Arg76, Arg80, Lys83, and Arg84) and $\alpha 6$ (His193) remarkably reduced the L/F-transferase activity (Figure 4). A comparison of the surface electrostatic potential of L/F-transferase with that of *wvFemX* and *saFemA* revealed similar distributions of positively charged amino-acid residues, in the regions corresponding to the $\alpha 2$ helix of L/F-transferase, and the distances between the positively charged region and the substrate-binding pocket were almost the same as those observed in L/F-transferase (Figure 6B).

These observations and present biochemical studies allowed us to build a tRNA docking model (Figure 6A, upper panel). In this model, the positively charged cluster in $\alpha 2$ interacts with the phosphate backbone at the bottom of the tRNA D stem, whereas the phosphate backbone of the 3'-acceptor region interacts with the positively charged cleft, consisting of helices $\alpha 5$ and $\alpha 6$. The present tRNA docking model is consistent with the previous biochemical studies showing that L/F-transferase recognizes tRNA molecules in a sequence-independent manner, but in a manner depending on the aminoacyl moiety attached to the 3'-end of the tRNAs (Abramochkin and Shrader, 1996). When C-C-Pm (cytidine-cytidine-puromycin) was soaked into the L/F-transferase *apo* crystals, the electron density of puromycin was clearly visible, whereas that of the C-C was not. This observation also suggests that the aminoacyl-tRNA specificity of L/F-transferase is determined by the aminoacyl moiety

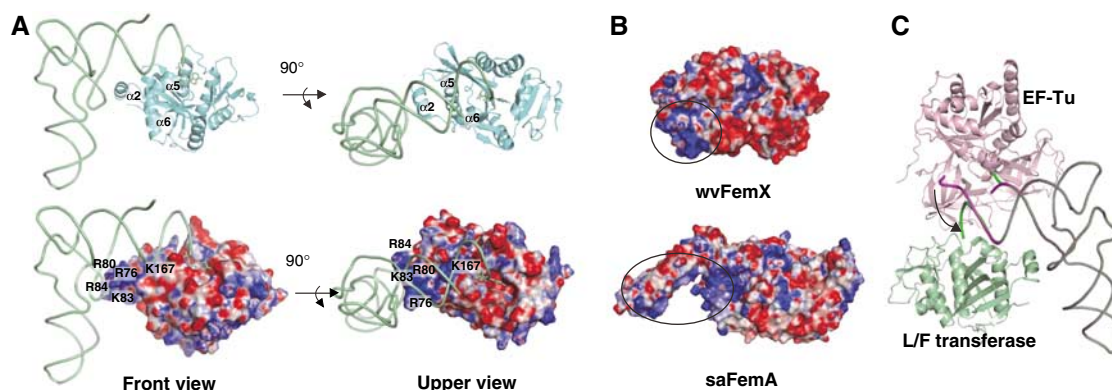


Figure 6 Model of aminoacyl-tRNA binding to L/F-transferase. (A) Two views of a ribbon diagram of the docking model of L/F-transferase and tRNA. The tRNA backbone is shown as a green line (upper two panels). Two views of the L/F-transferase-tRNA complex model, showing the surface colored according to its calculated electrostatic potential (lower panel; blue, positively charged +8KT; red, negatively charged -8KT). The electrostatic surface model was calculated by the program APBS (Baker *et al*, 2001). The upper view displays the top side of the complex of front views. (B) Electrostatic potentials of *wvFemX* (upper) and *saFemA* (lower). The marked regions on the diagrams show the positively charged regions corresponding to $\alpha 2$ of L/F-transferase and predicted as RNA binding regions. (C) The simultaneous tRNA binding to L/F-transferase (colored green) and EF-Tu (colored pink). The tRNA phosphate backbones are shown as a green line and a pink line for the L/F-transferase-tRNA complex and the EF-Tu-tRNA complex, respectively. The EF-Tu and Cys-tRNA^{Cys} complex structure was from PDB 1B23 (Nissen *et al*, 1999). The 3'-terminus of Cys-tRNA^{Cys} was manually modeled to accommodate the puromycin binding pocket of L/F-transferase.

attached to the 3'-terminus of the tRNA, rather than by the tRNA itself.

In the present model, the acceptor end of the tRNA might be disrupted or bent for the aminoacyl moiety to be accommodated into the pocket. In the previous complex structures of glutamyl-tRNA synthetase and tRNA (Rould *et al*, 1989) and of methionyl-tRNA_f^{Met} transformylase and formylmethionyl-tRNA_f^{Met} (Schmitt *et al*, 1998), the 3'-CCA ends of the tRNAs adopt similar bent hairpin conformation, with the base pair between 1 and 72 disrupted. *E. coli* tRNA^{Gln} and tRNA_f^{Met} possess U1-A72 and C1-A72 base pairs, respectively. The weak base pair at the top of the acceptor helix enables glutamyl-tRNA synthetase and methionyl-tRNA_f^{Met} transformylase to disrupt the base pair, allowing the tRNA 3'-end to adopt the bent conformation. Both *E. coli* tRNA^{Leu} and tRNA^{Phe} have G-C pairs at the top of the acceptor helices. It should be noted that the L/F-transferase activity is reportedly increased when a tRNA^{Leu} isoacceptor with weaker base pairs in the acceptor helix is used as the substrate, and when the acceptor helix of Leu-tRNA^{Leu} is disrupted by a complementary oligonucleotide (Abramochkin and Shrader, 1996). These observations suggest that the disruption or bending of the 3'-acceptor region of aminoacyl-tRNAs might be required for efficient accommodation of the aminoacyl moiety into the hydrophobic pocket of L/F-transferase. The crystal structure of the archaeal leucyl-tRNA synthetase and tRNA^{Leu} complex revealed that the tRNA CCA end adopts a bent conformation (Fukunaga and Yokoyama, 2005), where the G-C pair at the top of the acceptor helix of tRNA^{Leu} is not disrupted. Therefore, it is also possible that the 3'-CCA of tRNA could adopt a bent conformation by L/F-transferase, and the aminoacyl moiety of the aminoacyl-tRNA could be accommodated in the hydrophobic pocket, without the disruption of tRNA acceptor helix. It is interesting to note that the elongation factor (EF-Tu) binds to all aminoacyl-tRNAs uniformly. The uniform binding of EF-Tu to all aminoacyl-tRNAs is suggested to be ensured by combinational recognition of amino acid and tRNA moieties of aminoacyl-tRNAs (Ibba, 2001; LaRiviere *et al*, 2001). At present, it is not clear whether similar principle can be applied to the binding of aminoacyl-tRNA to L/F-transferase. The further experimental studies are required to clarify this point.

As shown in Figure 6C, our tRNA-docking model allows the simultaneous binding of EF-Tu to the tRNA. An *in vitro* UV crosslinking experiment showed that the ternary complex of L/F-transferase, EF-Tu, and aminoacyl-tRNA is not formed, and that EF-Tu competes with L/F-transferase for binding with the aminoacyl-tRNA (Supplementary Figure 3A and B). The initial rate of L/F-transferase activity is reduced in the presence of an excess amount of EF-Tu, suggesting that the recognition of aminoacyl-tRNAs by L/F-transferase and EF-Tu is competitive (Supplementary Figure 3C and D). Therefore, L/F-transferase might overcome the competition with EF-Tu in the uptake of the aminoacyl-tRNA by using a different binding site in the tRNA, which does not overlap with the EF-Tu binding site. The transfer of aminoacyl-tRNAs between L/F-transferase and EF-Tu does not require full dissociation of the tRNA molecule, and thus only the structural change of the acceptor end of the tRNA might be required, as shown in Figure 6C.

Recognition of an acceptor protein bearing Lys or Arg at the N-terminus

L/F-transferase catalyzes the transfer of the aminoacyl moiety of Leu-tRNA^{Leu} or Phe-tRNA^{Phe} to the amino group of an acceptor protein bearing Lys or Arg at its NH₂-terminus. This enzymatic activity is similar to peptide-bond formation by ribosomes. Our extensive efforts to locate the acceptor peptides, such as the α -casein fragment peptide (RYLGYL) and other peptides (RGDS and RFDS), by soaking the L/F-transferase crystals into solutions containing both puromycin and these acceptor peptides, were unsuccessful, although the α -casein fragment peptide can accept Leu from Leu-tRNA^{Leu} and Phe from Phe-tRNA^{Phe} by L/F-transferase *in vitro* (Supplementary Figure 2); only the electron density corresponding to puromycin was visible (data not shown).

In the complex structure of L/F-transferase with puromycin, the loop region between $\alpha 3$ and $\alpha 4$ occupies the space where the acceptor peptide would enter to approach the puromycin. This might explain why the acceptor peptides were not visible in our structure. The binding of aminoacyl-tRNAs to L/F-transferase probably induces a global conformational change accompanied by the movement of the loop region between $\alpha 3$ and $\alpha 4$. As a result, the NH₂-terminus of acceptor peptides can approach the aminoacyl moiety of the aminoacyl-tRNAs (Supplementary Figure 4A).

Our functional studies showed that the mutation of Glu156 abolished and that of Gln188 significantly impaired the L/F-transferase activity (Figure 4). As the side chains of these residues do not interact with the puromycin directly, they might be involved in the interaction with the acceptor protein. We can speculate here that the terminal carboxyl groups of Glu156 and Gln188 interact with the positively charged side chain of the NH₂-terminal Arg or Lys of the acceptor protein, to fix the amino group of the N-terminal residue in the vicinity of the aminoacyl bond of the aminoacyl-tRNA, thus facilitating peptide-bond formation. The replacement of Glu156 with Lys or Arg abolished the L/F-transferase activity, whereas that of Glu156 with Asp did not impair the activity completely (Supplementary Figure 4B). Therefore, it is possible that an electrostatic interaction between the positively charged Arg or Lys at the NH₂-terminus of the substrate proteins and the negatively charged side chain of Glu156 of L/F-transferase is essential for substrate protein recognition.

To elucidate the exact molecular mechanism of peptide-bond formation by L/F-transferase, further structural analyses using more authentic substrates, such as an aminoacyl-tRNA and a transition state analog of the substrates, are now underway.

Concluding remarks

Recent phylogenetic and biochemical analyses have revealed the presence of new types of aminoacyl-tRNA protein transferases. In the human pathogen *Vibrio vulnificus*, a eubacterial protein transferase, named Bpt, was identified (Graciet *et al*, 2006). Bpt is a homolog of the eukaryotic R-transferase, but it conjugates leucine to the N-terminal Asp or Glu. Moreover, in the eukaryotic pathogen *Plasmodium falciparum*, a eukaryotic protein transferase, named ATEL1, was also identified (Graciet *et al*, 2006). ATEL1 is a homolog of the eubacterial L/F-transferase (Figure 2), but its activity is the same as that of eukaryotic R-transferases. Therefore, the specificities for the donor and acceptor amino acids,

as well as the hierarchic structure of the N-end rule, of aminoacyl-tRNA-protein transferases are diverse among living organisms. It is noteworthy that the amino-acid residues comprising the C-shaped edge of the hydrophobic pocket in L/F-transferase (Figure 3E) are not conserved in ATEL1 (Figure 2). In contrast, the cluster of positively charged residues, which might interact with the D stem region of the aminoacyl-tRNA, are conserved in ATEL1 (Figure 2), suggesting that the tRNA-binding mode of ATEL1 might be similar to that of L/F-transferase (Figure 6). Further structural analyses of various aminoacyl-tRNA protein transferases will provide the structural basis for the evolution and the physiological meaning of the N-end rule protein degradation pathway in living organisms.

Materials and methods

Purification and crystallization of L/F-transferase

The *E. coli aat* gene, encoding L/F-transferase, was PCR-amplified from the genomic DNA and cloned into the pET15b vector (Novagen) between the *NdeI* and *BamHI* sites. The recombinant L/F-transferase contains additional vector-encoded histidine residues (MGSSHHHHHHSSGLVPRGSH) at its N-terminus for affinity purification. *E. coli* BL21(DE3) Codon Plus (Stratagene) was transformed by the plasmid, inoculated in LB medium containing 50 µg/ml ampicillin and 20 µg/ml chloramphenicol, and grown at 37°C until the A_{600} reached 0.8. The expression of L/F-transferase was induced by the addition of IPTG (isopropyl-β-D thiogalactopyranoside) to a 0.1 mM concentration for 12 h at 25°C. The cells were harvested, sonicated in a buffer containing 50 mM Tris-HCl, pH 6.8, 500 mM KCl, 6 mM β-mercaptoethanol, 10 mM imidazole and 5% (w/w) glycerol (buffer A), and centrifuged at 10 000 g for 30 min at 4°C. The clear supernatant was applied to a 5 ml Ni²⁺ attached Hi-trap chelating column (GE Healthcare Bio-Sciences) equilibrated with buffer A, which was washed with 200 ml of buffer A. The protein was eluted with a 75 ml linear gradient of imidazole (10–500 mM) in buffer A. The fractions containing L/F-transferase were pooled and concentrated by NH₄(SO₄)₂ precipitation. The pellet was dissolved in a buffer containing 50 mM Tris-HCl, pH 8.0, and 10 mM β-mercaptoethanol, and was dialyzed against 2 l of buffer, containing 50 mM Tris-HCl, pH 8.0, 100 mM NH₄(SO₄)₂, and 10 mM β-mercaptoethanol (buffer B), at 4°C overnight. The solution was applied to a 5 ml Hi-Trap Q column (GE Healthcare Bio-Sciences) equilibrated with buffer B. The flow-through fractions were collected and concentrated to 5 mg/ml. The purity of L/F-transferase was >95%, as judged by SDS-PAGE, and about 3 mg of L/F-transferase was obtained per liter of LB. The selenomethionine L/F-transferase derivative was expressed in the methionine auxotrophic *E. coli* B834 strain and was purified as described above.

Data collection, structure determination, and structural refinement of L/F-transferase

For the crystallization of L/F-transferase, 1 µl of protein solution (5 mg/ml) was mixed with 1 µl of crystallization solution, containing 50 mM HEPES, pH 8.0, and 0.7 M trisodium tartrate (reservoir A), and the drop solution was equilibrated against reservoir A at 20°C by the hanging drop vapor diffusion method (condition A). L/F-transferase was also crystallized under different conditions, in which the protein solution was mixed with a solution containing 50 mM HEPES, pH 8.1, 0.2 M trisodium citrate, and 17% (v/v) polyethylene glycol 3550 (reservoir B), and the drop solution was equilibrated against reservoir B (condition B).

For structure determination, three data sets for the MAD method with the selenomethionine derivative were collected at the beamline BL41XU at SPring-8 (Harima, Japan), and the data set of the

native crystal were collected at BL-5A and MW12 at KEK (Tsukuba, Japan). The crystals were cryo-protected with 20% (v/v) ethylene glycol, and were flash-frozen in a 100 K nitrogen stream. All of the data were processed using the program HKL2000 (Otwinowski and Minor, 1997). The native crystals from conditions A and B belong to the space group *P*2₁2₁2, with *a* = 114.0, *b* = 129.6, *c* = 38.8 Å, and contain two molecules in the asymmetric unit cell. The selenomethionine crystal showed the same space group and unit cell parameters of *a* = 116.5, *b* = 129.8, *c* = 39.0 Å. The data set at the peak wavelength up to 2.80 Å resolution was used for locating the selenium atoms with the program SnB (Weeks and Miller, 1999), and 14 peaks were picked out of 16 atoms in the asymmetric unit. An initial phase set was calculated by the MAD method, using data to 2.80 Å resolution, with the program SHARP (de La Fortelle and Bricogne, 1997), and density modification with solvent flattening and NCS averaging was performed using the program DM (CCP4, 1994; Cowtan, 1994). A model with two L/F-transferase molecules in the asymmetric unit was built using the program XFIT (McRee, 1999), and was refined with the native data by the program CNS (Brunger *et al*, 1998). For the preparation of the complex with puromycin, a soaking solution containing all of the solutes of condition A and 5 mM puromycin was used. A native crystal crystallized under condition A was soaked in the soaking solution for 30 min, and after the diffraction data of the complex crystal were collected and processed, the model structure was refined as described above.

In vitro aminoacyl-transfer assay

Mutations were introduced into the L/F-transferase overexpression plasmid by site-directed mutagenesis, and the mutant enzymes were expressed and purified as described above. *E. coli* tRNA^{Phe} and tRNA^{Leu} were synthesized by T7 RNA polymerase *in vitro* using the linearized pT7trnaF and pT7trnaL plasmids, which encode the tRNA^{Phe} and tRNA^{Leu} genes downstream of the T7 promoter, respectively. The transcripts were fractionated by anion-exchange chromatography. The assays for leucine (or phenylalanine) transfer onto the amino group of the α-casein fragment (Sigma) were carried out in a buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM KCl, 10 mM Mg(OAc)₂, 1 mM DTT, 2 mM ATP, 0.5 µM tRNA^{Leu} (or 1.0 µM tRNA^{Phe}), 16.4 µM [¹⁴C]Leu (11.3 GBq/mmol) (or 11.0 µM [¹⁴C]Phe, 17 GBq/mmol), 10 µM α-casein, an excess amount of leucyl-tRNA synthetase (or phenylalanyl-tRNA synthetase), and 0.12 µM L/F-transferase (or 0.06 µM L/F-transferase). After a 7 min (or 4 min) incubation at 37°C, the reactions were stopped by adding SDS-PAGE loading buffer and were incubated at 100°C for 8 min, to deacylate the aminoacyl-tRNA. The mixtures were separated by 15% SDS-PAGE, dried, and visualized with a BAS-5000 bioimaging analyzer (Fuji Film). The intensities of the [¹⁴C]-labeled α-casein were quantified. Under these assay conditions, the reactions proceeded in the linear range; therefore, the assay conditions used here are sensitive enough to evaluate the effect of each mutation on the L/F-transferase activity.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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