Histidine 66 in *Escherichia coli* Elongation Factor Tu Selectively Stabilizes Aminoacyl-tRNAs*

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The universally conserved His-66 of elongation factor Tu (EF-Tu) stacks on the side chain of the esterified Phe of Phe-tRNA^Phe_. The affinities of eight aminoacyl-tRNAs were differentially destabilized by the introduction of the H66A mutation into *Escherichia coli* EF-Tu, whereas Ala-tRNA^Ala_ and Gly-tRNA^Gly_ were unaffected. The H66F and H66W proteins each show a different pattern of binding of 10 different aminoacyl-tRNAs, clearly showing that this position is critical in establishing the specificity of EF-Tu for different esterified amino acids. However, the H66A mutation does not greatly affect the ability of the ternary complex to bind ribosomes, hydrolyze GTP, or form dipeptide, suggesting that this residue does not directly participate in ribosomal decoding. Selective mutation of His-66 may improve the ability of certain unnatural amino acids to be incorporated by the ribosome.

Background: The cognate-esterified amino acid is critical for optimal delivery of aminoacyl-tRNAs to the ribosome by EF-Tu.

Results: Mutation of His-66 in EF-Tu alters the specific binding of many, but not all aminoacyl-tRNAs, but does not affect decoding.

Conclusion: His-66 is critical for the specificity of EF-Tu for the esterified amino acid.

Significance: Selective mutation of His-66 could improve the incorporation efficiency of unnatural amino acids into proteins.

The universally conserved His-66 of elongation factor Tu (EF-Tu) stacks on the side chain of the esterified Phe of Phe-tRNA^Phe_. The affinities of eight aminoacyl-tRNAs were differentially destabilized by the introduction of the H66A mutation into *Escherichia coli* EF-Tu, whereas Ala-tRNA^Ala_ and Gly-tRNA^Gly_ were unaffected. The H66F and H66W proteins each show a different pattern of binding of 10 different aminoacyl-tRNAs, clearly showing that this position is critical in establishing the specificity of EF-Tu for different esterified amino acids. However, the H66A mutation does not greatly affect the ability of the ternary complex to bind ribosomes, hydrolyze GTP, or form dipeptide, suggesting that this residue does not directly participate in ribosomal decoding. Selective mutation of His-66 may improve the ability of certain unnatural amino acids to be incorporated by the ribosome.

Bacterial elongation factor Tu (EF-Tu)\(^2\) in its activated, GTP-bound form binds all elongator aminoacyl transfer RNAs (aa-tRNAs) to form the ternary complexes that are substrates for the ribosome. The presence of the esterified amino acid is required for the aa-tRNAs to bind tightly to the protein (1–4), and experiments with misacylated tRNAs have established that the thermodynamic contribution of the esterified amino acid depends significantly on the identity of its side chain (5–8). A hierarchy of thermodynamic contributions for the different amino acids has been defined ranging up to as much as 2.8 kcal/mol between the “weak” amino acids such as glycine or aspartate and the “tight” amino acids such as tyrosine or glutamine (6, 8). Because tRNA sequences have evolved to thermodynamically compensate for the variable contributions of the esterified amino acids, correctly acylated aa-tRNAs bind to EF-Tu with similar affinities (8–10).

The differing thermodynamic contributions of the esterified amino acids can at least partially be explained by the interactions made between the side chain of the amino acids and a cleft or pocket formed between domains 1 and 2 that is large enough to fit all of the amino acids. In the cocrystal structure of the yeast Phe-tRNA^Phe_ and *Escherichia coli* EF-Tu-GMPPNP (Fig. 1), the side chain of the esterified phenylalanine stacks on His-66 at the top of the binding pocket, whereas its amino group forms hydrogen bonds with the carboxyl oxygen of Asn-273 and the backbone nitrogen from Phe-261 (11). In a structure of Cys-tRNA^Cys_ bound to the very similar *Thermus aquaticus* EF-Tu, the position of the cysteine side chain is quite similar to that of phenylalanine with the β carbons superimposing and the SH group in the plane of the phenylalanine ring (12). Two additional ribosome-bound ternary complex structures in pre- and post-GTP hydrolysis states are available that contain Trp-tRNA^Trp_ and Thr-tRNA^Thr_, respectively (13, 14). Although Trp-tRNA^Trp_ before GTP hydrolysis maintains a very similar environment around the esterified amino acid as the free ternary complexes, the post-GTP hydrolysis structure of Thr-tRNA^Thr_ shows subtle alterations of the pocket residues possibly induced by GTP hydrolysis. It is not known how other esterified amino acids fit into the amino acid binding pocket, although its asymmetric environment is expected to stabilize and/or sterically position each amino acid in a unique manner. In addition, two more distal acidic residues, Glu-215 and Asp-216, are primarily responsible for the overall negative charge of the pocket and help to account for the relatively weak binding of esterified aspartate and glutamate (6, 8, 15).

One goal of this paper is to evaluate the contribution of His-66 in *E. coli* EF-Tu to the specificity of the protein for binding different esterified amino acids. It is known that the H66A and H66L mutations of *E. coli* EF-Tu destabilize the binding to Phe-tRNA^Phe_ (16, 17), suggesting that the imidazole side chain can stabilize the esterified Phe. Here we test how the H66A mutation affects the binding of tRNAs esterified with other amino acids and how other mutations of His-66 can affect the specificity for aa-tRNAs.

A second goal of this paper is to use presteady state kinetics to evaluate the ability of the H66A mutant of EF-Tu to support tRNA decoding on *E. coli* ribosomes. Although the position of His-66 does not change substantially during initial binding, substantial structural rearrangements occur in nearby regions
EF-Tu Residue His-66 Selectively Stabilizes aa-tRNAs of both the tRNA and EF-Tu upon GTP hydrolysis (13, 14), suggesting that the H66A mutation may affect the kinetics of decoding. Although earlier experiments indicated that the H66A mutation had little effect on polyphenylalanine synthesis (16), this steady state assay could potentially obscure a kinetic effect in one of the sub-steps in decoding.

**EXPERIMENTAL PROCEDURES**

*tRNA Labeling and Aminoacylation Reactions*—All purified native tRNAs were purchased from Sigma, except tRNA\textsuperscript{Gly} and tRNA\textsuperscript{Ala}, which were purchased from Subriden. The tRNAs were 3'-\textsuperscript{32}P-labeled and aminoacylated for ribosome experiments as described in Ledoux and Uhlenbeck (18). All \textsuperscript{3}H]amino acids containing aminoacylation reactions for EF-Tu binding experiments were performed as described in Dale et al. (6).

EF-Tu Mutagenesis—The plasmid containing the *E. coli* tuf gene with a cleavable N-terminal His\textsubscript{6} linker was provided by Rachel Green (Johns Hopkins University School of Medicine). Mutations were introduced via the QuickChange XL site-directed mutagenesis kit (Stratagene). The sequence of each EF-Tu variant was confirmed by DNA sequencing.

Protein and Ribosome Purification—Tight-coupled 70 S ribosomes from *E. coli* MRE600 cells were purified as described in Powers and Noller (19). EF-Tu from *E. coli* was overexpressed and purified as described in Sanderson and Uhlenbeck (20) with the following exceptions; the EF-Tu plasmids were transformed into BLR(DE3) cells and grown to an A\textsubscript{600} of 0.5 followed by an incubation with 1 mM isopropyl 1-thio-\textbeta-d-galactopyranoside for another 2 h to an A\textsubscript{600} of 1.2. In buffer A, 4-(2-aminoethyl)benzenesulfonyl fluoride (ABSF) was replaced with a protease inhibitor mixture (Roche Applied Science complete mini). The cell lysate was cleared first by centrifugation at 10,000 \times g for 30 min then at 185,000 \times g for 90 min. Fractions containing EF-Tu were pooled and dialyzed against buffer A to remove imidazole before incubating with His\textsubscript{6}-tobacco etch virus protease for 3 h at 24 °C. The reaction was then run over a nickel-nitrioltriacetic acid column to remove uncleaved EF-Tu and tobacco etch virus protease, and the eluate was stored in buffer A with 50% glycerol.

EF-Tu Assays—The dissociation rates (k\textsubscript{off}) of \textsuperscript{3}H]aa-tRNAs from EF-Tu-GTP were determined as described in Sanderson and Uhlenbeck (20) at 0 °C in buffer A containing 50 mM Hepes (pH 7.0), 20 mM MgCl\textsubscript{2}, 50 mM NH\textsubscript{4}Cl, 5 mM DTT, 20 \mu M GTP, 3 mM phosphoenolpyruvate, and 50 \mu g/ml pyruvate kinase. The association rate constants for aa-tRNAs to EF-Tu were determined as described in Schrader et al. (21) at 0 °C in buffer A. The equilibrium dissociation constant (K\textsubscript{D}) was measured in buffer A as described in Sanderson and Uhlenbeck (20). The apparent rate of GTP hydrolysis was performed in buffer B (50 mM Hepes (pH 7.0), 30 mM KCl, 70 mM NH\textsubscript{4}Cl, 10 mM MgCl\textsubscript{2}, and 1 mM DTT) with 1 \mu M ribosomes as described in Schrader et al. (22). The rate of dipeptide formation (k\textsubscript{p2}) was performed in buffer B as described in Schrader et al. (22). The ternary complex binding assay was performed in buffer B as described in Ledoux and Uhlenbeck (23).

**RESULTS**

The H66A Mutation Only Destabilizes Binding to Certain aa-tRNAs—As shown in Fig. 1, the imidazole ring of His-66 is stacked between the esterified phenylalanine and the side chain of Val-79 and is positioned 4 – 6 Å from three potential hydrogen bond partners Gln-97, Asn-273, and Glu-215. As a result, the H66A mutation would be expected to enlarge the amino acid binding pocket and thereby reduce the affinity for esterified amino acids with aromatic or polar side chains without affecting the affinity for smaller esterified amino acids which are unlikely to be stabilized by the imidazole. To test this prediction, a ribonuclease protection assay was used to determine the dissociation rates of \textsuperscript{14}C]aa-tRNAs from wild-type *E. coli* EF-Tu and the H66A protein (Table 1). As had been reported previously for both *Thermus thermophilus* and *E. coli* EF-Tu (8, 9), the dissociation rates of different aa-tRNAs from wild-type *E. coli* EF-Tu are quite similar, with k\textsubscript{off} values ranging from 0.08 ± 0.02 min\textsuperscript{-1} for Tyr-tRNATyr to 0.21 ± 0.06 min\textsuperscript{-1} for Lys-tRNALys\textsuperscript{\textsuperscript{\textgamma}}. In contrast, the H66A protein shows a broader range of k\textsubscript{off} values, ranging from 0.16 ± 0.02 min\textsuperscript{-1} for Gly-tRNAGly\textsuperscript{\textsuperscript{\textgamma}} to 1.5 ± 0.38 min for Trp-tRNATrp. The values of k\textsubscript{off} for Gly-tRNAGly and Ala-tRNA\textsuperscript{Ala} are quite similar for the wild-type and mutant proteins, indicating that the removal of the imidazole ring does not globally distort the amino acid binding pocket. However, most of the other aa-tRNAs, especially those esterified with aromatic amino acids, show significantly faster k\textsubscript{off} values from the H66A protein, indicating that His-66 par-
Gly-tRNAGly and Ala-tRNAAla. This clearly establishes that EF-Tu is fairly uniform, the H66A mutant protein destabilizes Gly-tRNAGly and Ala-tRNAAla. The destabilization observed for Phe-tRNAPhe is similar to the identity of the esterified amino acid nor the H66A mutation affected the rate of association of EF-Tu with aa-tRNA, k_on values for Gly-tRNAGly with H66A EF-Tu. Because this assay does not easily yield an accurate k_on value for the weak complex of Tyr-tRNA^Tyr with H66A EF-Tu, k_on values were verified indirectly by measuring the fraction of ternary complex formed at equilibrium as a function of EF-Tu concentration to give K_D and then by calculating k_on = k_off/K_D. Although these calculated k_on values were all about 2-fold lower, all four complexes gave similar k_on values despite a more than 55-fold variation in k_off (Table 2). Taken together, it appears that neither the identity of the esterified amino acid nor the H66A mutation affects k_on.

A constant value of k_on and the values of k_off determined in Table 1 permit calculation of the ΔG° of formation for each ternary complex. These data, depicted graphically in Fig. 2, clearly show that whereas the binding of aa-tRNAs to wild-type EF-Tu is fairly uniform, the H66A mutant protein destabilizes all of the aa-tRNAs by differing amounts with the exception of Gly-tRNA^Gly and Ala-tRNA^Ala. This clearly establishes that His-66 contributes substantially to the specificity of EF-Tu for different esterified amino acids.

His-66 Substitutions Alter Specificity for aa-tRNAs—In an attempt to obtain a mutation of EF-Tu, which bound certain aminoacyl-tRNAs tighter than wild-type, His-66 was mutated to Trp, Phe, Tyr, and Arg. Although these bulkier amino acids may not position themselves in the same orientation as histidine, the size of the pocket is large enough to accommodate them, and they may be better than histidine at stabilizing hydrophobic amino acids. Although all bacterial and most archaeal EF-Tus have a histidine at the orthologus position, it is interesting that about 15% of archaea have a Phe or, rarely, a Tyr in the corresponding site in their somewhat different amino acid binding pockets. When the dissociation rates of Gly-tRNA^Gly from each of the four His-66 mutations were determined, the H66W and H66F proteins had k_off values very similar to wild-type and the H66A protein (Tables 1 and 3). However, the values of k_off for H66Y and H66R were 3- and 6-fold faster than wild type (data not shown). Because the esterified glycine would be more than 5 Å away from the residue 66, Gly-tRNA^Gly would not be expected to be affected by His-66 mutations. Thus, the faster off rates for the H66Y and H66R proteins indicates that the structure of EF-Tu was compromised outside of the amino acid binding pocket. Because of this, the H66Y and H66R proteins were not studied in further detail.

k_off values for the 10 different aa-tRNAs from the H66W and H66F mutants are presented in Table 3, and the corresponding ΔG° values are shown in Fig. 2. It is clear the affinities of the H66W and H66F proteins for the different aa-tRNAs are different from each other as well as from the wild-type and the H66A proteins. For example, Phe-tRNA^Phe and Tyr-tRNA^Tyr bind much better to the H66W and H66F proteins than to the H66A protein, consistent with a productive stacking interaction between the aromatic esterified amino acid and the aromatic Trp-66 and Phe-66 residues. However, it is interesting that His-66, present in the wild-type protein, is even better than Trp-66 or Phe-66 at binding the esterified Phe and Tyr even though it would not be expected to stack any better. It is possible that the partial protonation of His-66 (25) may aid its positioning and thereby improve its ability to stabilize aromatic amino acids. Additional intriguing examples of ΔG° differences between the four proteins include the fact that the ΔG° of Glu-tRNA^Glu is reduced to a similar extent in all three mutant EF-Tus, whereas the ΔG° of Gln-tRNA^Gln is only reduced in the H66A mutation and even slightly stabilized in the H67F mutation. Ala-tRNA^Ala and Gly-tRNA^Gly bind to all four proteins with a similar ΔG°. In general, Fig. 2 makes it clear that the identity of residue 66 is an important feature in establishing the specificity of aa-tRNAs to EF-Tu.

Decoding Properties of H66A Mutant—Three assays were used to evaluate the role of His-66 in ribosomal decoding. The apparent binding affinity (K_D) of ternary complex to ribosomal entry site, the rate of GTP hydrolysis (k_\text{GTP}) and the rate of dipeptide bond formation (k_\text{p}) were measured for wild-type and for Gly-tRNAGly with H66A EF-Tu. Because of the restricted GTPase activity of EF-G, k_\text{GTP} was determined by a direct determination of k_off (21, 24). As summarized in Table 2, k_off values for Gly-tRNAGly and Tyr-tRNATyr binding to the wild-type and H66A proteins were determined, the H66W and H66F proteins had k_off values very similar to wild-type and the H66A protein (Tables 1 and 3). However, the values of k_off for H66Y and H66R were 3- and 6-fold faster than wild type (data not shown). Because the esterified glycine would be more than 5 Å away from the residue 66, Gly-tRNA^Gly would not be expected to be affected by His-66 mutations. Thus, the faster off rates for the H66Y and H66R proteins indicates that the structure of EF-Tu was compromised outside of the amino acid binding pocket. Because of this, the H66Y and H66R proteins were not studied in further detail.

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maximizing any potential effect of the mutation. The rates of GTP hydrolysis were similar for Ala-tRNA\textsuperscript{Ala} with wild-type and H66A EF-Tu, suggesting that His-66 does not significantly affect the final step of initial selection, \( k_{\text{GTP}} \). Phe-tRNA\textsuperscript{Phe} did not form a stable enough complex with the H66A protein to allow purification of the ternary complex from excess \([32P]\)GTP, thereby prohibiting the measurement of \( k_{\text{GTP}} \).

To investigate whether His-66 alters the rate of peptide bond formation (\( k_{\text{pep}} \)), Ala-tRNA\textsuperscript{Ala} and Phe-tRNA\textsuperscript{Phe} were both assayed with wild-type and H66A EF-Tu on \( E. coli \) ribosomes (Table 4). As previously observed, both aa-tRNAs undergo \( k_{\text{pep}} \) with wild-type EF-Tu with equivalent rates (23). When assayed with H66A EF-Tu, no impact for Ala-tRNA\textsuperscript{Ala} was observed on \( k_{\text{pep}} \) as expected by the lack of interaction between the residues. This suggests that the loss of the stacking energy between the aminoacyl phenylalanine and His-66 does not dramatically affect the rates of accommodation or peptide bond formation on the ribosome. Interestingly, despite a reduced affinity of H66A EF-Tu for Phe-tRNA\textsuperscript{Phe} off the ribosome, only a slight impact was observed in \( k_{\text{pep}} \) similar to what had been previously observed in polyphenylalanine synthesis (16).

**DISCUSSION**

Because His-66 is the residue that contributes the most surface area to the amino acid binding pocket of EF-Tu, it is not surprising that His-66 is critical in determining the specificity of the protein for different esterified amino acids. The mutation of His-66 to alanine in \( E. coli \) EF-Tu causes larger amino acids to be destabilized by as much as 18-fold, whereas the smaller amino acids such as glycine or alanine are not affected at all. Although mutation of Glu-215 and Asp-216 in the back of the pocket have also been found to alter the specificity for certain esterified amino acids (6, 15), the effects are considerably smaller. Understanding the physical mechanism of stabilization by the His-66 residue is not straightforward. Although the structure of the ternary complex supports the presence of stabilizing stacking interactions between the imidazole and aromatic side chains, our data suggest several non-aromatic amino acids also appear stabilized by His-66. However, an understanding of the amino acid binding specificity also requires consideration of the structures of the unbound forms of EF-Tu-GTP and aa-tRNA. The x-ray structure of the free EF-Tu-GTP indicates that the position of His-66 with respect to the neighboring side chains is identical to the ternary complex. Although no high resolution structure of a tRNA with an ester-
modified EF-Tu must be used in the presence of the wild-type EF-Tu.

An initial partially successful example of such an “orthogonal” EF-Tu system was the discovery that the E215A or D216A mutations of *E. coli* EF-Tu improved incorporation of DL-2-anthrachinonylalanine, L-2-pyrenelyalanine, and L-1-pyrenylalanine into protein compared with the wild-type EF-Tu (30). However, the overall incorporation efficiency of these Uaas remained low, possibly because the activity of EF-Tu was compromised by the mutations.

A second example of an orthogonal EF-Tu system is an elegant selection of an *E. coli* EF-Tu variant that can function with phosphoserine, a Uaa that does not work well with the wild-type protein (31). In this case six residues in the amino acid binding pocket, including His-66, were changed to create an EF-Sec that promoted efficient incorporation of a single phosphoserine residue into several proteins. However, this system was not efficient at introducing multiple phosphoserines into proteins, again perhaps because the intrinsic activity of EF-Sec was not very high.

Our experience with various mutations of His-66 indicate that whereas it is possible to enlarge the amino acid binding pocket without compromising the function of EF-Tu, the identity of the introduced amino acid can be critical to the activity of the protein. One convenient way to test whether the function of a mutant EF-Tu is altered is to test it using Gly-tRNA<sup>Gly</sup> or Ala-tRNA<sup>Ala</sup>, which are minimally sensitive to mutations in the pocket. If these aa-tRNAs are fully active, the mutations do not disrupt the structure of the binding site.

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