

Essential Role of Histidine 84 in Elongation Factor Tu for the Chemical Step of GTP Hydrolysis on the Ribosome

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Elongation factor Tu (EF-Tu) is a GTP-binding protein that delivers aminoacyl-tRNA to the A site of the ribosome during protein synthesis. The mechanism of GTP hydrolysis in EF-Tu on the ribosome is poorly understood. It is known that mutations of a conserved histidine residue in the switch II region of the factor, His84 in *Escherichia coli* EF-Tu, impair GTP hydrolysis. However, the partial reaction which is directly affected by mutations of His84 was not identified and the effect on GTP hydrolysis was not quantified. Here, we show that the replacement of His84 with Ala reduces the rate constant of GTP hydrolysis more than 10^6 -fold, whereas the preceding steps of ternary complex binding to the ribosome, codon recognition and, most importantly, the GTPase activation step are affected only slightly. These results show that His84 plays a key role in the chemical step of GTP hydrolysis. Rate constants of GTP hydrolysis by wild-type EF-Tu, measured using the slowly hydrolyzable GTP analog, GTP γ S, showed no dependence on pH, indicating that His84 does not act as a general base. We propose that the catalytic role of His84 is to stabilize the transition state of GTP hydrolysis by hydrogen bonding to the attacking water molecule or, possibly, the γ -phosphate group of GTP.

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Introduction

Elongation factor Tu (EF-Tu) catalyzes the binding of aminoacyl-tRNA (aa-tRNA) to the A site of the ribosome. EF-Tu consists of three domains, a Ras-like domain 1 (the G domain) that binds GTP or GDP, and two β -barrel domains, 2 and 3, which together with domain 1 provide a binding surface for the acceptor domain of aa-tRNA. In its active GTP form, EF-Tu forms a high-affinity complex with aa-tRNA ("ternary complex") and delivers it to the A site of the ribosome. Upon codon-anticodon recognition, GTP is hydrolyzed, resulting in a major conformational change of EF-Tu. This triggers the release of the 3' end of aa-tRNA from EF-Tu and the subsequent dissociation of EF-Tu-GDP from the ribosome. The recycling of the inactive

GDP-bound form of EF-Tu to EF-Tu-GTP is catalyzed by elongation factor Ts (EF-Ts).

GTP hydrolysis is central to the function of EF-Tu, but its mechanism is not understood. By analogy to other GTP-binding proteins, the reaction is likely to proceed by a direct in-line attack of a water molecule on the γ -phosphorus atom of GTP.^{1,2} The catalytic residues involved in the reaction in EF-Tu and their role in catalysis have not been identified. Early work suggested that the conserved Gln residue present in the switch II region of all Ras-like and heterotrimeric G proteins could act as a general base by abstracting a proton from the water.^{1,2} However, both theoretical^{3,4} and experimental^{5,6} analysis showed that the pK_a of the Gln in question is too low for a general-base function. Rather, the γ -phosphate group itself appears to act as a base (substrate-assisted catalysis⁵). Crystal structures of several Ras-like and heterotrimeric G proteins indicated that the Gln residue in the switch II region is crucial for the stabilization of the GTPase transition state by correct positioning of both the water molecule and the γ -phosphate,

Abbreviations used: EF-Tu, elongation factor Tu; GTP γ S, guanosine 5'-[γ -thio]triphosphate; mant-GTP, 3'(2')-O-(*N*-methylanthraniloyl)-guanosine triphosphate.

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providing a likely explanation for the strong inhibitory effects of replacing the conserved Gln with other amino acid residues.⁷

In all members of the translation factors subfamily of GTPases,⁸ the position of the conserved Gln in the switch II region is occupied by His, His84 in EF-Tu from *Escherichia coli*. His84 could act in a similar way as Gln in stabilizing the GTPase transition state. However, in the structures of EF-Tu in the GTP form, His84 points away from the catalytic site and its access to the γ -phosphate group and the hydrolytic water molecule is prohibited by the side-chains of Val20 and Ile60 that form a hydrophobic gate.² It was suggested that, in order to accelerate GTP hydrolysis, one or both of the wings of the hydrophobic gate could open, providing access of His84 to the γ -phosphate.⁹ The switch II region that comprises His84 undergoes a major reorientation during the transition from the GTP to the GDP form, or upon interaction of EF-Tu with EF-Ts. Thus, a reorientation of His84 during GTPase activation is structurally possible. In fact, in the structure of EF-Tu·GDP in the complex with aureodox, an antibiotic which stimulates the GTPase activity of EF-Tu, the position of Ile60 is disordered and His84 is oriented towards the γ -phosphate position within the nucleotide-binding site.¹⁰ However, because the stimulation of GTP hydrolysis by aureodox is only tenfold (albeit measured under conditions of multiple turnover),¹¹ compared to the 10⁵-fold activation on the ribosome,¹² and the activation by the antibiotic and the ribosome may proceed by different mechanisms,¹³ the functional significance of these structural data remained unclear.¹⁰

In the isolated G domain of EF-Tu, the exchange of His84 for Gly led to a moderate (20-fold) reduction of the intrinsic GTPase activity measured in the absence of the ribosomes, much less than expected for the removal of a residue directly involved in catalysis.¹⁴ Substitution of His84 in full-size EF-Tu from *E. coli* reduced (H84Q) or abolished (H84A) the GTPase activity of EF-Tu alone or bound to non-programmed ribosomes.¹⁵ Similar results were obtained for EF-Tu from *Thermus thermophilus* when the H85Q and H85L substitutions at the position homologous to H84 in *E. coli* EF-Tu were studied, whereas the H85G mutation caused complete protein degradation *in vivo*.¹⁶ Upon ternary complex binding to poly(U)-programmed ribosomes, the rate of GTP hydrolysis was moderately reduced by the His substitution to Gln in EF-Tu from *E. coli* (no quantitative estimation of inhibition) or *T. thermophilus* (12-fold inhibition). These results suggest that Gln may take over part, but not all of the functions of the presumed catalytic His in the switch II region. The H85L substitution in *T. thermophilus* EF-Tu abolished the GTPase activity on poly(U)-programmed ribosomes.¹⁶ The activity of the H84A mutant of *E. coli* EF-Tu on mRNA-programmed ribosomes was not analyzed.¹⁵

These studies suggested an important role of

His84 in the catalysis of GTP hydrolysis in EF-Tu on the ribosome. However, several important questions remained unresolved. First, GTP hydrolysis on the ribosome follows, and is rate-limited by, the preceding steps, including binding of the ternary complex to the ribosome, codon-anticodon interaction, and a conformational change of the factor leading to GTPase activation. Mutation of His84 could affect any of these preceding steps, rather than the chemistry step itself, resulting in an apparent effect on GTP hydrolysis. This issue has not been addressed in previous work. Second, the quantitative contribution of His84 to catalysis of GTP hydrolysis by ribosome-bound EF-Tu was not determined. Finally, unlike Gln, the side-chain of His has a pK_a at neutral pH and may act as a general base, in addition to the substrate-assisted catalysis, a possibility which has not been examined previously. Here, we analyze the role of His84 in catalysis of GTP hydrolysis in EF-Tu on poly(U)-programmed ribosomes. We have studied the H84A mutant of EF-Tu in order to identify the precise point within the functional cycle of EF-Tu where His84 may exert its function. Kinetic analysis was carried out to quantify the contribution of His to overall catalysis of GTP hydrolysis. Finally, in order to determine whether His84 may function in general acid-base catalysis, the pH dependence of GTP cleavage of the slowly hydrolyzable GTP analog, GTP γ S, was studied in the pH range between 6.5 and 8.5.

Results

Kinetics of GTP hydrolysis and peptide bond formation

The H84A mutant of EF-Tu was prepared and purified as described in Materials and Methods. Nucleotide and aa-tRNA binding were not affected by the mutation (data not shown), in agreement with previous reports for EF-Tu from *E. coli*¹⁵ and *T. thermophilus*.¹⁶ The ternary complex, EF-Tu(H84A)·GTP·Phe-tRNA^{Phe}, was stable enough to allow its purification from unbound ligands by gel-filtration as described.¹⁷

The rate of GTP hydrolysis was measured upon addition of the ternary complex, EF-Tu(H84A)·GTP·Phe-tRNA^{Phe}, to poly(U)-programmed ribosomes carrying an analog of peptidyl-tRNA, AcPhe-tRNA^{Phe}, in the P site (referred to as P site-blocked ribosomes) (Figure 1). GTP hydrolysis was very slow, with a rate constant $k_{\text{GTP}} = 3.0(\pm 0.3) \times 10^{-4} \text{ s}^{-1}$. Under the same conditions, GTP hydrolysis with wild-type EF-Tu is very rapid ($k_{\text{GTP}} > 500 \text{ s}^{-1}$ with both wild-type EF-Tu and EF-Tu with an oligohistidine tag as the H84A mutant studied here^{12,18}) and its actual rate is limited by the slowest of the preceding steps, i.e. codon recognition. On non-programmed ribosomes, GTP hydrolysis was extremely slow, $3 \times 10^{-5} \text{ s}^{-1}$, suggesting that GTP hydrolysis by

mutant EF-Tu is stimulated tenfold by a correct codon–anticodon interaction. In addition, peptide bond formation was very slow with EF-Tu(H84A), $k_{\text{pep}} = 2.6(\pm 0.2) \times 10^{-4} \text{ s}^{-1}$. With wild-type EF-Tu, the rate of peptide bond formation reached 8 s^{-1} and was limited by the accommodation of aa-tRNA in the A site.^{12,18} The fact that k_{GTP} and k_{pep} were the same with mutant EF-Tu indicates that peptide bond formation was limited by the rate of GTP hydrolysis.

Affinity of ribosome binding

The low rate of GTP hydrolysis may result from either inefficient catalysis or weak binding of the mutant ternary complex to the ribosome. To distinguish between these two possibilities, we tested the stability of ternary complex binding to the ribosome. The ribosome complex with EF-Tu(H84A)·[³H]GTP·[¹⁴C]Phe-tRNA^{Phe} was formed using a slight excess of P site-blocked ribosomes, and isolated from unbound ligands by gel-filtration on Sephacryl S300. The elution position of ternary complex was identified by immunoblotting using antibodies against the histidine tag of EF-Tu and by counting ³H and ¹⁴C radioactivity. Because of the large difference in the molecular mass between the ribosomes and the free ternary complex, a clear separation of the respective peaks was found, and in the presence of ribosomes practically the whole amount of ternary complex eluted in the ribosome peak (data not shown). The complex was stable and could be isolated and quantified by nitrocellulose filtration. Upon longer incubations (15–60 minutes), the amount of [³H]GTP bound to the complex decreased, due to GTP hydrolysis (Figure 1) and dissociation of EF-Tu·[³H]GDP from the ribosome (EF-Tu·[³H]GDP was poorly retained on the nitrocellulose filters under the filtration conditions used).

The reversibility of mutant ternary complex

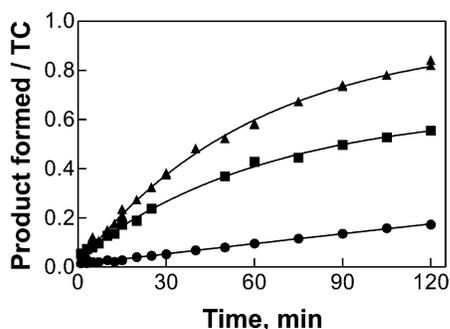


Figure 1. Functional activity of EF-Tu(H84A). Peptide bond formation (squares) and GTP hydrolysis were measured upon interaction of EF-Tu(H84A)·[³²P]GTP·[¹⁴C]Phe-tRNA^{Phe} (0.1 μM) with P site-blocked ribosomes (0.3 μM) (triangles) or non-programmed ribosomes (0.3 μM) (circles). Plotted is the extent of product formation relative to the amount of ternary complex (TC) added.

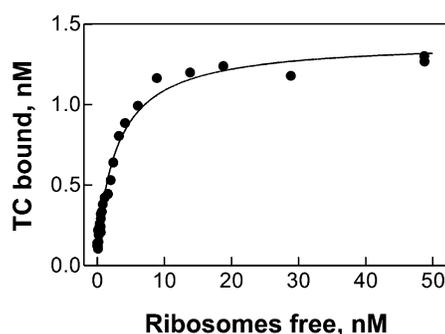


Figure 2. Affinity of EF-Tu(H84A)·GTP·[¹⁴C]Phe-tRNA^{Phe} to the ribosome complexes. Ternary complex (TC) binding to P site-blocked ribosomes was determined by nitrocellulose filtration one minute after mixing (Materials and Methods). For details, see the text.

binding to the ribosome was tested by chase experiments. The formation of the complex was complete within one minute after mixing. Upon short incubations (several minutes), when GTP was not hydrolyzed significantly, binding of EF-Tu(H84A)·GTP·[¹⁴C]Phe-tRNA^{Phe} to the ribosome was reversible, because the addition of excess unlabeled ternary complex resulted in rapid dissociation of the mutant ternary complex from the ribosome (data not shown). The equilibrium binding constant of mutant ternary complex, EF-Tu(H84A)·GTP·Phe-tRNA^{Phe}, to the ribosome was determined by titrating a constant amount of ternary complex with increasing amounts of ribosomes and determining complex formation by nitrocellulose filtration (Figure 2). The K_d of the complex was estimated to $3.1 \pm 0.3 \text{ nM}$, consistent with the fact that the complex could be isolated by gel-filtration, as described above. In comparison, the K_d of wild-type ternary complex binding to the ribosome was about 0.5 nM, as calculated from the rate constants of the steps preceding GTP hydrolysis.^{12,18} Thus, the affinity of ternary complex binding to the ribosome is not much affected by the H84A mutation.

Kinetic analysis of A-site binding

To identify the step in the mechanism of A-site binding which is impaired by the mutation and to quantify the effects on this and all other steps, a kinetic analysis was performed, as described previously.¹² The rate constants of initial binding of the ternary complex to the ribosome and subsequent codon recognition were determined using a fluorescent tRNA^{Phe} derivative, Phe-tRNA^{Phe}(Prf16/17).¹⁷ Initial binding of ternary complex to the ribosome was measured by monitoring the fluorescence change of EF-Tu(H84A)·GTP·Phe-tRNA^{Phe}(Prf16/17) upon mixing with non-programmed ribosomes, which is equivalent to initial complex formation with programmed ribosomes.^{12,19} Apparent rate constants

were determined from time-courses measured at different ribosome concentrations by exponential fitting and plotted *versus* the ribosome concentration (Figure 3(a)). From the slope of the linear plot, the bimolecular association rate constant was calculated, $k_1 = 120(\pm 20) \mu\text{M}^{-1} \text{s}^{-1}$. From the intercept with the Y-axis, the dissociation rate constant of the initial-binding complex, $k_{-1} = 75(\pm 25) \text{s}^{-1}$, was determined. The K_d of initial binding calculated from these values ($K_d = k_{-1}/k_1 = 0.63 \mu\text{M}$) was the same as that determined from the analysis of the concentration dependence of the fluorescence amplitudes (not shown), $0.6 \mu\text{M}$, and was similar to the value found for ternary complex containing wild-type EF-Tu.^{12,19}

When the ternary complex, EF-Tu(H84A)-GTP-Phe-tRNA^{Phe}(Prf16/17), was mixed with poly(U)-programmed ribosomes, an increase in fluorescence was observed within the first second (Figure 3(b)), followed by a slow decrease in fluorescence (not shown). Exponential fitting yielded apparent rate constants of about $10\text{--}40 \text{s}^{-1}$ and 6s^{-1} describing the increase in fluorescence. The decrease in fluorescence had an apparent rate of

about 0.5s^{-1} . The rate of the fastest step increased with ribosome concentration in a hyperbolic fashion (Figure 3(c)), consistent with a monomolecular rearrangement step following a bimolecular association reaction. The same experiments were performed monitoring the fluorescence of wybutine, a natural fluorophore at position 37 of tRNA^{Phe}, which specifically reports the formation of the codon-anticodon complex.^{20,21} This yielded the same rates as the fastest step seen with proflavin (Figure 3(c)), indicating that the step in question was codon recognition. Since the codon recognition complex with cognate aa-tRNA is rather stable, i.e. k_{-2} is small compared to k_2 ,^{12,22} the value of the rate constant of codon recognition can be estimated from the saturation of a hyperbolic fit, $k_2 = 50(\pm 10) \text{s}^{-1}$. The apparent K_M of the codon-recognition step calculated from the ribosome concentration at half-saturation was $0.7(\pm 0.2) \mu\text{M}$, in agreement with the K_M value calculated from the kinetic constants, $(k_{-1} + k_2)/k_1 = 1.0(\pm 0.4) \mu\text{M}$. The nature of the slow fluorescence changes is not known. The rates of these reactions were independent of the ribosome concentration, about 6s^{-1} and 0.5s^{-1} at saturation,

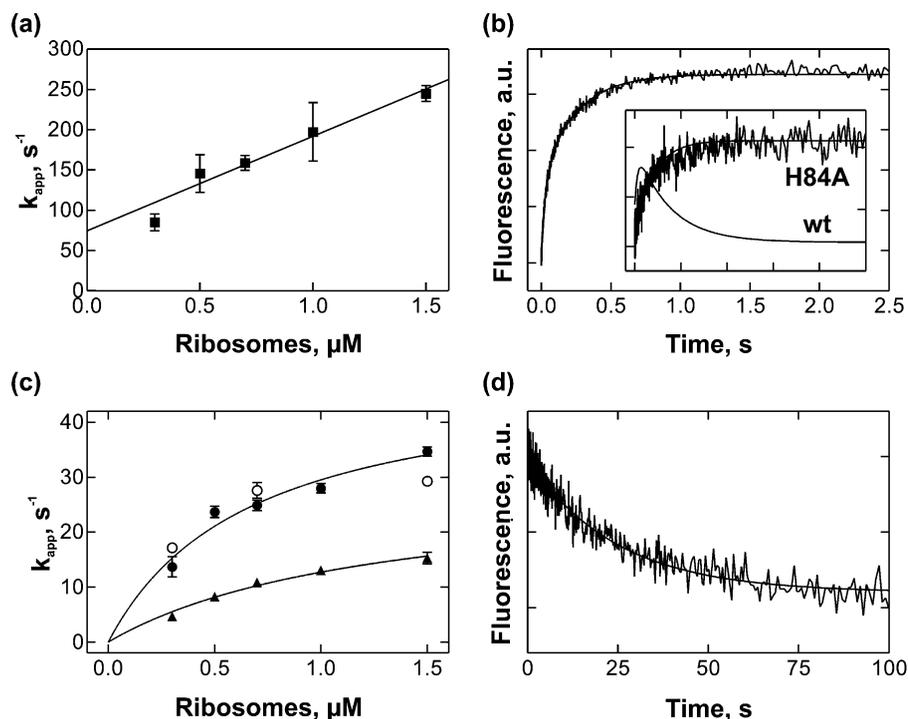


Figure 3. Kinetic analysis of A-site binding. (a) Initial binding. The apparent rate constants were determined by exponential fitting of the time-courses of the fluorescence changes measured after mixing of the purified ternary complexes EF-Tu(H84A)-GTP-Phe-tRNA^{Phe}(Prf16/17) ($0.1 \mu\text{M}$) with increasing amounts of non-programmed ribosomes. (b) Codon recognition and GTPase activation. Fluorescence changes of proflavin in tRNA^{Phe}(Prf16/17) were measured after mixing of ternary complex containing EF-Tu(H84A) ($0.1 \mu\text{M}$) with P site-blocked ribosomes ($0.3 \mu\text{M}$) in the stopped-flow apparatus. Inset, analogous experiment monitoring mant-GTP fluorescence; time-scale as in the main Figure. The fit of the analogous curve obtained the wt EF-Tu is shown for comparison. (c) Concentration dependence of $k_{\text{app}2}$ (codon recognition) and $k_{\text{app}3}$ (GTPase activation) monitored by the fluorescence of proflavin (filled circles), wybutine (open circles), or mant-GTP (triangles). (d) Dissociation from the ribosome (k_{-2}). The dissociation of EF-Tu(H84A)-GTP-Phe-tRNA^{Phe}(Prf16/17) from the ribosome was initiated by the addition of unlabeled ternary complex in tenfold excess and proflavin fluorescence was monitored.

and were much faster than GTP hydrolysis with mutant EF-Tu. Hence, the fluorescence changes may reflect rearrangements of the complex upon or after GTPase activation but before GTP hydrolysis. Because this step is not observable with wild-type EF-Tu due to rapid GTP hydrolysis and its structural and functional meaning is unclear, it was not included in the kinetic model.

The kinetic stability of the codon recognition complex, k_{-} , was determined by chase experiments (Figure 3(d)). The dissociation of EF-Tu(H84A)·GTP·Phe-tRNA^{Phe}(Prf16/17) from the ribosome was initiated by adding unlabeled ternary complex in large excess. Under these conditions, the dissociation rate represents k_{-2} . With cognate Phe-tRNA^{Phe}, $k_{-2} = 0.04(\pm 0.01) \text{ s}^{-1}$ was obtained by exponential fitting of the time-courses as in Figure 3(d). As expected, k_{-2} increased significantly, to about 2 s^{-1} , when near-cognate Leu-tRNA^{Leu2} (anticodon GAG) was used (not shown), because the stability of the codon–recognition complex strongly depends on the codon–anticodon match.^{22,23}

The crucial step for GTP hydrolysis is GTPase activation, i.e. the ribosome-induced rearrangement of EF-Tu that leads to the GTPase transition state and subsequent GTP hydrolysis. The GTPase activation rearrangement was monitored by the fluorescence of a derivative of GTP, mant-GTP.^{12,20,24} With wild-type EF-Tu, the interaction of the ternary complex with the ribosome led to a biphasic change in mant fluorescence, with an increase in fluorescence reflecting GTPase activation, and a decrease in fluorescence due to dissociation of EF-Tu·mant-GDP from the ribosome after GTP hydrolysis.^{12,18,20} Upon mixing of EF-Tu(H84A)·mant-GTP·Phe-tRNA^{Phe} with P site-blocked ribosomes, an increase in mant fluorescence was observed similar to that found with wild-type EF-Tu, while no decrease in fluorescence was observed within the time of the stopped-flow experiment (Figure 3(b), inset). This indicates that the mutation does not affect the GTPase-activation rearrangement, but rather inhibits subsequent steps. The analysis of the concentration dependence of the rates showed a hyperbolic behavior with saturation at $k_{\text{app}} = 30(\pm 5) \text{ s}^{-1}$ (Figure 3(c)). For the kinetic mechanism of A-site binding depicted in Figure 5, this value is equal to $k_2k_3/(k_2 + k_3)$.²⁵ Using the value of $k_2 = 50(\pm 10) \text{ s}^{-1}$, determined above, the rate constant of the GTPase activation was calculated to $k_3 = 70(\pm 20) \text{ s}^{-1}$. The summary of rate constants of A-site binding with EF-Tu(H84A) is shown in Figure 4.

The rate constant of the breakdown of the GTPase-activated state, k_{-3} , cannot be determined directly from kinetic data. Comparing the overall K_d , which may be calculated from the rate constant of Figure 4 (Materials and Methods), with that measured experimentally (Figure 2) suggests that the internal equilibrium $k_{-3}/k_3 = 6$, and thus $k_{-3} = 420 \text{ s}^{-1}$, which is too fast to be measured directly in chase experiments. However, given the

standard deviations of the values of the rate constants and the different experimental approaches used for the determination of equilibrium and rate constants, the value of k_{-3} should be considered as an estimate, and it is therefore not included in Figure 4.

In summary, the H84A mutation reduces the rate of GTP cleavage by more than six orders of magnitude, while the rate constants of other elemental steps of A-site binding, including GTPase activation, are little affected. The small effects on the rate constants k_{-1} , k_2 , k_{-2} , and k_3 may be explained by a slightly different conformation of the ternary complex containing mutant EF-Tu, compared to the wild-type factor.

pH dependence of GTP hydrolysis

As His84 in EF-Tu plays a crucial role in the catalysis of GTP hydrolysis, we next asked the question whether it could act as a general base in abstracting a proton from the water molecule during the approach to the transition state of hydrolysis. If a general base were involved in catalysis, the rate of the reaction would be expected to depend on pH according to the $\text{p}K_a$ of the catalytic base. For histidine, the $\text{p}K_a$ is expected to be near 7. Thus, the experiment to perform would be to determine the rate constants of GTP hydrolysis by wild-type EF-Tu on the ribosome at different pH values. However, this experiment is not feasible, because GTP hydrolysis by wild-type EF-Tu is rapid intrinsically and its actual rate is limited by the preceding steps; thus, k_{GTP} cannot be determined in such an experiment. Therefore, we have used a slowly hydrolyzable derivative of GTP, GTP γ S, which is known to be a suitable analog to study the function of EF-Tu on the ribosome²⁶ following the same mechanism with respect to substrate-assisted catalysis as GTP, at least in Ras.⁵ Furthermore, in order to control whether GTPase activation is also affected by pH, we used a fluorescent derivative of GTP γ S, mant-GTP γ S. Upon interaction of EF-Tu·mant-GTP γ S·Phe-tRNA^{Phe} with P site-blocked ribosomes at pH 7.5, an increase of mant fluorescence was observed, followed by a very slow decrease (Figure 5(a)). As determined with mant-GTP, the increase in fluorescence reports the GTPase activation rearrangement of EF-Tu.²⁰ The rate of this step as reported by mant-GTP γ S, $7.2 \pm 0.8 \text{ s}^{-1}$, is somewhat slower than that obtained with mant-GTP at the same ribosome concentration, 18 s^{-1} ,^{12,18} but is still within the same kinetic domain, indicating that the modification of the γ -phosphate of mant-GTP γ S had little effect on the steps of A-site binding up to and including the GTPase activation. However, the rate of the slow step is decreased about 400-fold, from about 3 s^{-1} to 0.008 s^{-1} , upon replacement of mant-GTP by mant-GTP γ S, consistent with very slow hydrolysis of GTP γ S.²⁶ This suggests that the slow decrease in mant fluorescence is determined by the rate of the preceding

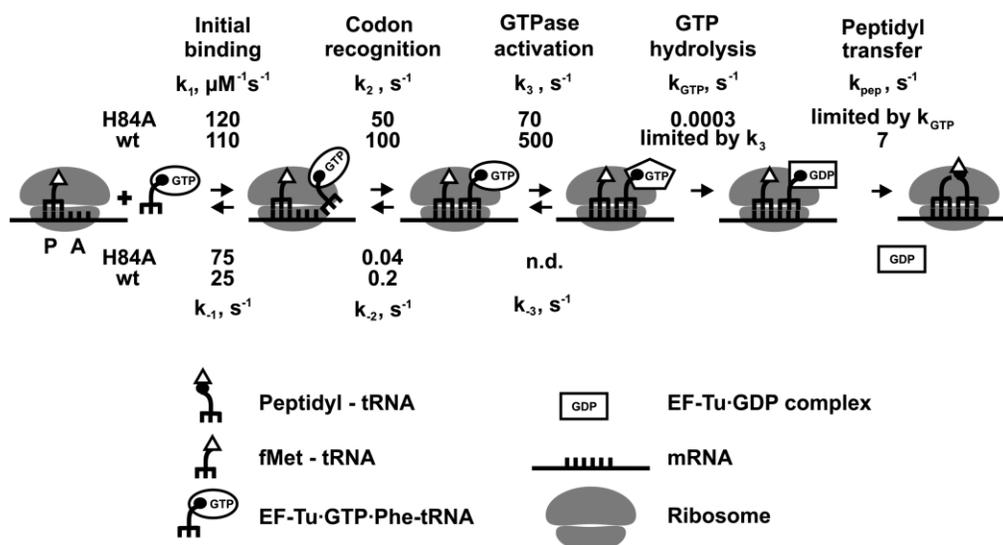


Figure 4. Kinetic scheme of A-site binding with EF-Tu(H84A). The scheme summarizes the rate constants determined by kinetic analysis, as described in the text. The rate constants for wild-type EF-Tu¹² are shown for comparison.

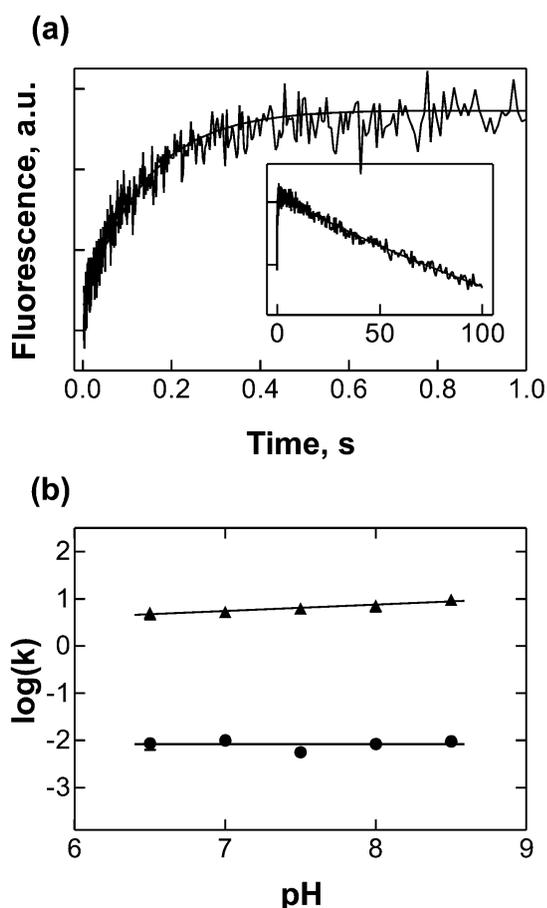


Figure 5. pH dependence of GTPase activation and GTP hydrolysis. (a) Time-course of GTPase activation (rapid fluorescence increase) and GTP hydrolysis (slow decrease in fluorescence, inset) measured with wild-type EF-Tu with a slowly hydrolyzable fluorescent GTP analog, mant-GTP γ S, at pH 7.5. Main Figure, short time-window; inset, long-time window of the same experiment. (b) pH dependence of the rates of GTPase activation (triangles) and GTP hydrolysis (circles).

hydrolysis of mant-GTP γ S. Thus, the rate of the slow step provides a measure for $k_{\text{GTP}\gamma\text{S}}$ and can be monitored to determine the pH dependence of the reaction.

Experiments analogous to those described above at pH 7.5 were carried out at pH 6.5 to 8.5. A broader range of pH values could not be tested, because of EF-Tu aggregation at pH values below 6.5²⁷ (T.D., H.-J.W. & M.V.R., unpublished results) and significant hydrolysis of Phe-tRNA^{Phe} above pH 8.5, both rendering the ternary complex inactive. The rate constants of GTPase activation and GTP γ S hydrolysis showed no dependence on pH (Figure 5(b)). This is at variance with the expected behavior if a general base with a pK_a of about 7 were involved in catalysis. These results strongly argue against the involvement of a general base ionizing at neutral pH in the catalytic mechanism of GTP hydrolysis by EF-Tu on the ribosome.

Discussion

His84 in the switch II region of EF-Tu plays a key role in the mechanism of GTP hydrolysis on the ribosome. The present kinetic results show that the side-chain of His84 is directly involved in the chemistry step of the reaction, while all the steps preceding GTP cleavage are practically not affected by the H84A mutation. Notably, the GTPase activation rearrangement of domain 1 of EF-Tu, which can be envisaged as a conformational change leading to opening of the hydrophobic gate,^{2,9} is not inhibited by the mutation. The replacement of His84 by Ala decreased the rate of GTP hydrolysis by more than six orders of magnitude, which translates into an increase in the activation energy barrier by 35 kJ/mol. EF-Tu with an H84Q substitution hydrolyzed GTP with rates that were somewhat lower, but comparable to those of wild-type

EF-Tu.^{15,16} Furthermore, the present data on the pH dependence of the rate of GTP hydrolysis disfavors a general-base function of His84 in the reaction. Thus, the likely catalytic role of His84 is to form hydrogen bond(s) to the substrates of the reaction, the attacking water molecule and/or the γ -phosphate, to precisely align groups directly involved in the reaction, in a similar way as Gln stabilizes the transition state of GTP hydrolysis in Ras-like or heterotrimeric G proteins⁷ (Figure 6).

Spontaneous GTP hydrolysis in EF-Tu is very slow, about $5 \times 10^{-5} \text{ s}^{-1}$.²⁸ In the structure of EF-Tu-GTP, His84 points away from the γ -phosphate, and its rotation towards the active site is hindered by Ile60 and Val20 (Figure 6). The conformation of

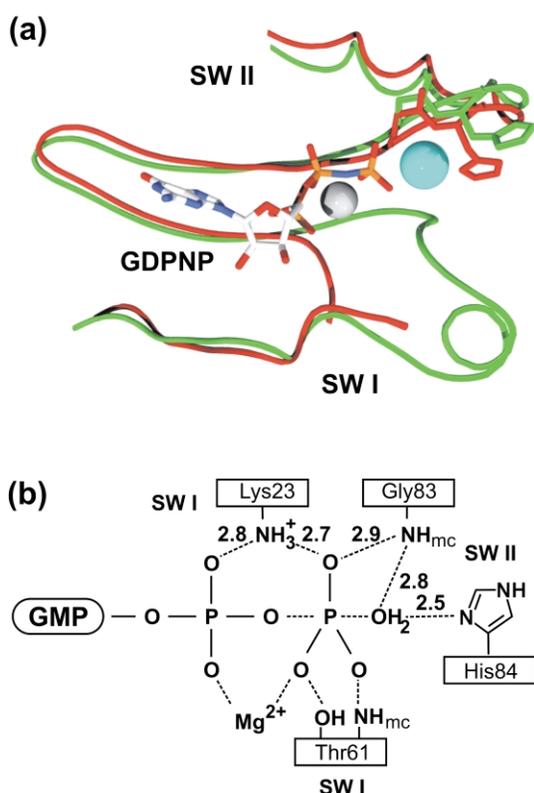


Figure 6. Orientation of His84 in the active site of EF-Tu. (a) Positions of His84 in the crystal structures of EF-Tu-GDPNP (green, PDB 1EXM) and EF-Tu-GDPaurodox (red, 1HA3), are shown relative to the hydrolytic water molecule (cyan), Mg^{2+} (gray) and GDPNP (1EXM). Structures were aligned on Lys23 of EF-Tu which occupies the same position in the different structures. SWI and SWII, switch I and II regions. Part of the switch I region (amino acid residues 53–61) is not resolved in the EF-Tu-GDPaurodox structure. (b) Model of the transition state of GTP hydrolysis. Interactions between residues important for catalysis and GTP are inferred from the superposition of the EF-Tu-GDPNP and the EF-Tu-GDPaurodox structures. The positions of Lys23, Gly83 and His84 are taken from the EF-Tu-GDPaurodox complex (1HA3), those of water, Mg^{2+} , GDPNP, and Thr61 from EF-Tu-GDPNP (1EXM). The distance between N of His84 and O of γ -phosphate is between 3 Å and 5 Å depending on the His84 rotamer used.

the switch II region, and the orientation of His84 must change in order to position the His84 side-chain in the catalytic site to stabilize the GTPase transition state. Intrinsic GTP hydrolysis in EF-Tu is slow, probably because the spontaneous structural rearrangement of His84 takes place rarely or not at all. In the latter case, the mechanism of GTP hydrolysis would take a pathway different from that supported by the ribosome, relying on different catalytic residues in the active center. The antibiotic aurodox induces a structure of EF-Tu with His84 oriented towards the GTP-binding pocket in a conformation that is unique in comparison to all other EF-Tu structures solved so far.¹⁰ In view of the crucial role of His84 for GTP hydrolysis, it appears likely that the structure of the active site in the EF-Tu-antibiotic complex is similar to that in the GTPase transition state induced on the ribosome. The fact that aurodox (or kirromycin) stimulated GTP hydrolysis only moderately (tenfold), measured under conditions of multiple turnover, compared to the extensive stimulation by the ribosome, can be explained by slow turnover. Thus, the antibiotic may induce the GTPase-activated conformation of EF-Tu, while it blocks subsequent structural rearrangements that determine the rate of turnover.

The ribosome stimulates the intrinsic GTP hydrolysis by EF-Tu in the ternary complex by five orders of magnitude.²⁸ This can be achieved either by promoting the conformational rearrangements that correctly position the own catalytic groups of EF-Tu, i.e. His84 or other residues at the active site, or by donating additional catalytic groups *in trans*. A residue next to His84, Gly83, plays an important role in both the rearrangement of the switch II region upon GTPase activation, due to the great flexibility inherent to Gly residues, and in GTP hydrolysis itself, probably by helping to position the catalytic water by hydrogen-bonding with the main-chain oxygen atom of Gly83 (Figure 6).¹⁸ The intrinsic arginine residue (Arg58), that is located in the switch I region of EF-Tu in a position homologous to that of the catalytic arginine in G_{α} proteins, is not essential for GTPase activity,²⁹ and an extensive mutagenesis search did not identify any other side-chains important for GTP hydrolysis in *E. coli* EF-Tu.^{29,30–32} Thus, two residues in EF-Tu, Gly83 and His84, seem to provide the most important contributions to GTP hydrolysis.

The cryo-EM reconstruction of the ternary complex stalled on the ribosome after GTP hydrolysis, but prior to the conformational rearrangement into the GDP form, shows extensive interactions of the switch regions of the G domain with the sarcin-ricin loop (SRL) of 23 S rRNA,^{33,34} indicating that the SRL stabilizes the transition state conformation of the switch regions of the factor. Another contact is formed between the elbow region of aa-tRNA in the ternary complex and ribosomal protein L11³³ or the L11-binding region of 23 S rRNA,³⁴ and there is a transient contact of ribosomal protein L7/12 to helix D of EF-Tu³⁵ (unpublished results). On the basis of biochemical and

genetic analyses, the contacts with L7/12, L11, and the L11-binding region of 23 S rRNA are involved in stimulating GTP hydrolysis by EF-Tu on the ribosome.^{36,37} However, all these contacts take place far from the nucleotide-binding pocket and therefore must act indirectly by inducing conformational transitions of EF-Tu, rather than donate catalytic groups for GTP hydrolysis, as in the case of Ras.⁷

Finally, another key player in determining the rate of GTP hydrolysis appears to be aa-tRNA, which in the ternary complex contacts both switches I and II regions of the G domain of EF-Tu. The correct geometry of the codon-anticodon interaction on the 30 S subunit²² and an intact tRNA molecule in the ternary complex³⁸ are both required to induce rapid GTP hydrolysis. In the ternary complex stalled on the ribosome in the presence of kirromycin, the interactions between aa-tRNA and EF-Tu are distorted^{33,34} such that the contacts of the acceptor domain of tRNA with EF-Tu are likely to be altered and the switch regions of the factor may undergo a tRNA/ribosome-driven conformational change resulting in repositioning of His84. Thus, although the molecular details of GTPase activation and GTP hydrolysis remain to be determined directly, the combined influence of the ribosome and the tRNA appear to promote rearrangements of the G domain of EF-Tu that position the catalytic residue(s), in particular the essential His84, into the active site such as to stabilize the reactive arrangement of the attacking water molecule or, possibly, the γ -phosphate of GTP.

Materials and Methods

Buffer and reagents

All experiments were performed in buffer A (50 mM Tris-HCl (pH 7.5), 50 mM NH₄Cl, 10 mM MgCl₂, 1 mM DTT). The experiments were performed at 20 °C, if not stated otherwise. Chemicals were purchased from Roche or Merck, radioactive compounds from ICN. mant-GTP and mant-GTP γ S were from JenaBioScience.

Ribosome complex

Ribosomes from *E. coli*, AcPhe-tRNA^{Phe} from *E. coli*, [¹⁴C]Phe-tRNA^{Phe}, and tRNA^{Phe}(Prf16/17) from yeast were prepared as described.^{17,39} To prepare P site-blocked ribosomes, 70 S ribosomes were incubated in buffer A for 15 minutes at 37 °C with a 1.3-fold excess of AcPhe-tRNA^{Phe} or tRNA^{Phe} from *E. coli* and 0.5 mg/ml of poly(U).

EF-Tu mutant

Single-stranded DNA from the plasmid pEECAHis⁴⁰ containing the *E. coli* *tufA* gene was used for site-directed mutagenesis with the ChameleonTM mutagenesis kit (Stratagene, Heidelberg), using the primer 5'CATAGTCGGCGCCCCCGGGCAGTCTACG3'. The mutation

resulting in the H84A replacement was verified by DNA sequencing. Mutant EF-Tu(H84A) containing a C-terminal histidine tag was expressed in *E. coli* BL21 DE3 and purified by Ni-NTA-affinity chromatography. Cells were opened by sonication in buffer B (50 mM Tris-HCl (pH 8.0), 60 mM NH₄Cl, 7 mM MgCl₂, 7 mM 2-mercaptoethanol, 1 mM PMSEF, 50 μ M GDP, 300 mM KCl, 10 mM imidazole, 15% glycerol). After centrifugation for 30 minutes at 10,000 rpm (A8.24 rotor, Kontron), the supernatant was mixed with Ni-NTA Sepharose (Qiagen) equilibrated with buffer B and incubated for 30 minutes at 4 °C. The supernatant was removed, and the Ni-NTA Sepharose was washed three times with 20 volumes of buffer B and an additional three times with 20 volumes of buffer B containing 20 mM imidazole. Protein was eluted with 250 mM imidazole in buffer B and subsequently rebuffed to buffer A using Centricon 30 concentrators (Amicon).

The concentration of EF-Tu was determined photometrically at 280 nm⁴¹ using a molar extinction coefficient of 32,900 M⁻¹ cm⁻¹ and verified by comparing the intensity of the Coomassie-stained protein band on an SDS-12% (w/v) polyacrylamide gel with that of a standard protein using an imaging densitometer (Biorad GS-700).

Ternary complex

Ternary complex, EF-Tu(H84A)-GTP-Phe-tRNA^{Phe}, was prepared and purified by gel-filtration on Superdex 75 in buffer A as described¹⁷ with the following modifications. To prepare EF-Tu(H84A)-GTP-Phe-tRNA^{Phe}(Prf16/17) complexes, 10 μ M tRNA^{Phe}(Prf16/17) were incubated with 30 μ M [¹⁴C]Phe, 5% (v/v) S100 cell extract as a source of phenylalanyl-tRNA synthetase, 30 μ M EF-Tu, 1 mM GTP, 3 mM ATP, 3 mM phosphoenol pyruvate, and 0.1 mg/l of pyruvate kinase in buffer A for 30 minutes, 37 °C. EF-Tu(H84A)-mant-GTP-Phe-tRNA^{Phe} complexes were prepared in the same way, except that 300 μ M mant-GTP was used instead of GTP. To prepare EF-Tu-mant-GTP γ S-Phe-tRNA^{Phe}, 17 μ M [¹⁴C]Phe-tRNA^{Phe} were incubated with 50 μ M EF-Tu and 200 μ M mant-GTP γ S in the presence of 0.02 μ M EF-Ts in buffer A for 30 minutes, 37 °C, and the ternary complex was purified by gel-filtration.

Ribosome-EF-Tu-tRNA complex

The *K_d* of ternary complex binding to ribosomes was determined using non-purified ternary complex prepared with a tenfold excess of EF-Tu(H84A) over [¹⁴C]Phe-tRNA^{Phe}. Ternary complex (2 nM) was incubated with 0.1–50 nM P site-blocked ribosomes for one minute at 20 °C and then passed through nitrocellulose filters (Sartorius 0.45 μ m). Filters were washed with 5 ml of buffer A, dissolved in scintillation fluid (QS361, Zinsser Analytic) and counted. For chase experiments, ternary complex and P site-blocked ribosomes were mixed to form a codon-recognition complex at a concentration of 0.6 μ M. An equal volume of 6 μ M ternary complex with unlabeled Phe-tRNA^{Phe} was added, samples were taken after different incubation times and analyzed by nitrocellulose filtration as described above.

Kinetic experiments

Fluorescence stopped-flow measurements were performed as described previously¹⁷ using a stopped-flow

apparatus from Applied Photophysics. Proflavin fluorescence was excited at 470 nm and measured after passing KV 500 filters (Schott). The fluorescence of mant-GTP was excited at 366 nm and measured after passing KV 408 filters (Schott). Experiments were performed by rapidly mixing equal volumes (60 μ l each) of ternary complex (0.2 μ M), purified by gel filtration, and P site-blocked ribosomes (0.6–3 μ M) at 20 °C.

For the chase experiments, equal amounts of P site-blocked ribosomes and ternary complex, EF-Tu(H84A)·GTP·Phe-tRNA^{Phe}(Prf16/17), were mixed to form 0.2 μ M codon-recognition complex. The dissociation of the complex was initiated in the stopped-flow apparatus by mixing with an equal volume of 2 μ M non-fluorescent ternary complex, EF-Tu(H84A)·GTP·Phe-tRNA^{Phe}.

Time-courses for GTP hydrolysis and dipeptide formation were measured manually under conditions identical with those employed in the stopped-flow experiments. Purified ternary complex, EF-Tu(H84A)·[γ -³²P]GTP[¹⁴C] Phe-tRNA^{Phe}, and P site-blocked ribosomes were mixed to obtain final concentrations of 0.1 μ M and 0.3 μ M, respectively, in a reaction volume of 800 μ l. To measure GTP hydrolysis rates, samples were quenched with 25% (v/v) formic acid and analyzed by thin-layer chromatography on polyethyleneimine–cellulose plates in 0.5 M potassium phosphate, pH 3.5, and quantified using a Biorad phosphorimager. To measure rates of dipeptide formation, samples were quenched with 0.6 M KOH, incubated for 30 minutes at 37 °C, neutralized, and dipeptide formed was analyzed by HPLC.

The pH dependence of GTP hydrolysis was measured using ternary complex containing wild-type EF-Tu and mant-GTP γ S in the fluorescence stopped-flow apparatus. Prior to the experiment, ternary and ribosomal complex were diluted 1:10 into buffer A additionally containing 20 mM Bis-Tris at pH 6.5–8.5. Equal volumes of ternary complexes and P site-blocked ribosomes, 0.1 μ M and 0.3 μ M, respectively, in the buffer of the respective pH were rapidly mixed and the mantGTP γ S fluorescence monitored.

To obtain values for apparent rate constants, time-courses were evaluated by fitting an expression which contained one or more exponential terms (characterized by variable time constants, k_{app} , and amplitudes) and another variable for the final signal using TableCurve software (Jandel Scientific) or GraphPad Prism™.

Calculation of rate constants

Values of k_1 and k_{-1} were determined from the slope and Y-axis intercept, respectively, of the concentration dependence of k_{app} of initial binding measured in a model system where only this step was observed.¹⁹ For the calculation of k_2 and k_3 , the concept of net rate constants was used.²⁵ The suitability of this approach for the analysis of data obtained under pre-steady-state conditions was shown earlier.⁴² Rate constants were calculated for the model depicted in Figure 5 from the concentration dependencies of the rates of codon recognition (step 2, k_{app2}) and GTPase activation (step 3, k_{app3}), values for k_{app} at saturation (k_{app2} and k_{app3} , respectively), and the concentration of ribosomes at half saturation, K_{M2} and K_{M3} , respectively, were determined by hyperbolic fitting. Derivation of the concentration dependencies of the apparent rates (k_{app})

of steps 2 and 3 yields:²⁵

$$k_{app2} = k_{sat2}[Rs]/(K_{M2} + [Rs]),$$

where $k_{sat2} = k_2$ and $K_{M2} = (k_{-1} + k_2)/k_1$,

$$k_{app3} = k_{sat3}[Rs]/(K_{M3} + [Rs]),$$

where $k_{sat3} = k_2k_3/(k_2 + k_3)$

and $K_{M3} = (k_{-1} + k_2)/k_1k_3/(k_2 + k_3)$

where [Rs] is the concentration of ribosomes. The value of K_d of steps 1–3 was calculated as $(k_{-1}k_{-2}k_{-3})/(k_1k_2k_3)$ from the rate constants of Figure 5 and compared with the value determined experimentally (Figure 3).

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