Ribosome-induced tuning of GTP hydrolysis by a translational GTPase

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GTP hydrolysis by elongation factor Tu (EF-Tu), a translational GTPase that delivers aminoacyl-tRNAs to the ribosome, plays a crucial role in decoding and translational fidelity. The basic reaction mechanism and the way the ribosome contributes to catalysis are a matter of debate. Here we use mutational analysis in combination with measurements of rate/pH profiles, kinetic solvent isotope effects, and ion dependence of GTP hydrolysis by EF-Tu off and on the ribosome to dissect the reaction mechanism. Our data suggest that—contrary to current models—the reaction in free EF-Tu follows a pathway that does not involve the critical residue H84 in the switch II region. Binding to the ribosome without a cognate codon in the A site has little effect on the GTPase mechanism. In contrast, upon cognate codon recognition, the ribosome induces a rearrangement of EF-Tu that renders GTP hydrolysis sensitive to mutations of Asp21 and His84 and insensitive to K^+ ions. We suggest that Asp21 and His84 provide a network of interactions that stabilize the positions of the γ-phosphate and the nucleophilic water, respectively, and thus play an indirect catalytic role in the GTPase mechanism on the ribosome.

GTPases are regulatory molecular switches that cycle between an active GTP-bound and an inactive GDP-bound state. Transitions between the two conformations are regulated by GTP hydrolysis, which is accelerated by GTPase-activating proteins (GAPs), and nucleotide exchange, often mediated by guanine nucleotide exchange factors (GEFs) (1–3). The α/β-fold structure of the GTP-binding domain (G domain) is essentially identical in all GTPases, despite limited sequence conservation. All GTPases contain highly conserved sequence motifs responsible for GTP/GDP recognition. The α- and β-phosphates of GTP/GDP bind the phosphate-loop (P-loop) motif, which is universally conserved not only among GTPases but also in some ATP-binding proteins (4). The presence of the γ-phosphate is sensed by two flexible regions in the G domain, termed switch I and switch II, which contact the γ-phosphate and the nucleotide-bound magnesium ion and undergo dramatic conformational changes upon GTP hydrolysis (3). The switch II region also contains a functionally important residue, typically glutamine or histidine, that is crucial for GTP hydrolysis and the conformational switching from the active to the inactive state (5–7). Despite decades of research the mechanism of GTP hydrolysis is still not fully understood, and there is significant controversy on both the reaction path and the origins of the catalytic power of GTPases (for reviews describing different mechanistic alternatives, see refs. 8–13). The proton emerging upon nucleophilic attack of a water molecule on the γ-phosphate of GTP can be transferred along the associative or dissociative paths in either a direct manner or with the assistance of a second water molecule. An enzyme could contribute to catalysis by extracting a proton from the nucleophilic water molecule. The general base function can be served either by a residue in the switch II region (14, 15) or by GTP itself; the latter mechanism is denoted as substrate-assisted catalysis (16). Recent theoretical work has suggested that in solution, GTP hydrolysis proceeds through the associative/concerted pathway (17) and that the electrostatic stabilization of the transition state is the key to activation of GTPases (8, 13, 18).

Translational GTPases (trGTPases), such as bacterial elongation factor Tu (EF-Tu), EF-G, initiation factor IF2, release factor RF3, SelB, and their homologs in Archaea and eukaryotes, form a subclass of the GTPase superfamily. Their GTPase activity is stimulated by the ribosome, suggesting that the ribosome acts as a GAP (2, 19, 20). Common sequence signatures of trGTPases are the P-loop consensus motif G/AHxDxGKT and a crucial His residue in switch II. One of the best-studied trGTPases is EF-Tu, a factor that in the elongation phase of protein synthesis delivers aminoacyl-tRNA (aa-tRNA) to the A site of the ribosome. Because EF-Tu releases GDP very slowly (21), the formation of the active GTP-bound form requires an interaction with its GEF, EF-Ts. EF-Tu can hydrolyze GTP in the absence of the ribosome; however, the reaction is very slow. On the ribosome, the GTPase activity of EF-Tu is stimulated by over six orders of magnitude upon cognate codon recognition by aminoacyl-tRNA on the small ribosomal subunit (30S subunit) (22). This entails conformational rearrangements of the EF-Tu–GTP–aa-tRNA complex bound to the large ribosomal subunit (50S subunit) more than 70 Å away (15, 22–26). As a consequence, the side chain of the crucial residue in switch II, His84 (H84) in *Escherichia coli* EF-Tu, is reoriented toward the γ-phosphate of GTP, at the same time contacting A2662 of the sarcin–ricin loop (SRL) of 23S rRNA in the 50S subunit and forming hydrogen bonds to the nucleophilic water molecule (Fig. 1*A*). Mutations of H84 strongly affect the GTPase activity of the factor (5, 7, 27–29). Several recent theoretical studies on the mechanism of GTP hydrolysis of EF-Tu (13, 27, 29–31) have

Significance

Translational elongation factor Tu (EF-Tu) utilizes GTP hydrolysis to control the speed and fidelity of decoding. We explored the reaction coordinate for GTP hydrolysis by EF-Tu and the origins of the million-fold activation effect of the ribosome. We show that amino acid residues in the P loop (Asp21) and switch II region (His84) are key to catalysis of GTP hydrolysis on the ribosome but are not essential for the intrinsic GTPase of EF-Tu. We conclude that the ribosome contributes to catalysis indirectly, by inducing a conformation that provides an electrostatic stabilization of the transition state. Translational GTPases provide yet another example of how the ribosome, an ancient RNA-based catalyst, solves problems posed by the limited catalytic power of RNA.

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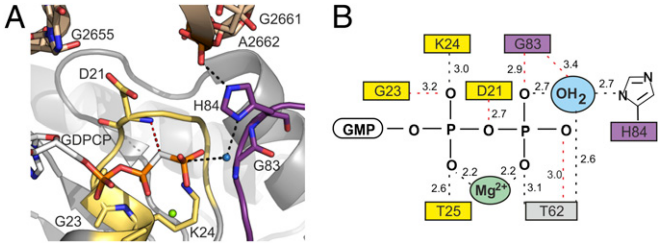

Fig. 1. GTP interactions in the active site of EF-Tu. (*A*) Nucleotide-binding pocket of EF-Tu in the ribosome-activated state [Protein Data Bank (PDB) ID codes 2XQD and 2XQE (15)]. Side chains of residues in the P loop (yellow) and switch II region (violet) important for nucleotide binding are shown as sticks. Universally conserved bases of the SRL are represented in salmon. (*B*) Schematic representation of the nucleotide-binding pocket (PDB ID code 2XQD) with GTP replacing GDPCP. Color coding is as in *A*. Black and red dashes indicate side-chain and main-chain interactions, respectively. Distances are given in Å.

provided a number of predictions on the role of H84; however, biochemical studies are needed to provide a coherent picture.

Here we reevaluated the GTPase mechanism of EF-Tu using mutational and biochemical approaches. We hypothesized that in addition to His84, Asp21 (D21) in the P loop is a striking candidate to be a catalyst. Theoretical work has suggested an important role of the P loop of Ras, a small GTPase of the Ras family, in electrostatic stabilization of the transition state (18, 32). Substitutions of the corresponding Gly13 in Ras completely abolished GTPase activation in vitro (33), and mutations of this residue were found in Ras proteins from several tumors (34–36). The P-loop Asp residue is universally conserved among trGTPases and occupies a critical position, with its main-chain amide nitrogen atom forming a hydrogen bond with the β–γ bridging oxygen of GTP (37) (Fig. 1*B*). In addition, a role for D21 as a general acid in EF-Tu was proposed (19), but this possibility has not been experimentally tested. Finally, a number of potassium-activated GTPases, such as MnmE and FeoB, contain a structurally related asparagine in the same position that seems to be crucial for coordination of an ion that contributes to acceleration of GTP hydrolysis (38, 39). Recently, a monovalent metal ion has been found coordinated to the homologous Asp residue in trGTPase eIF5B (40). We tested the effects of mutations of H84 and D21 on the intrinsic and ribosome-stimulated GTPase activity of EF-Tu. These results, combined with pH/rate profiles, kinetic solvent isotope effects, and the dependence on monovalent ions, provide insights into the path of GTP hydrolysis in free EF-Tu and the source of the catalytic effect on the ribosome.

Results

Intrinsic GTPase Activity of EF-Tu. Previous kinetic analysis suggested that the H84A substitution abolished GTP hydrolysis by EF-Tu but did not affect the steps preceding GTP hydrolysis, namely GTP binding and GTPase activation (5). H84 was thus suggested to position the water molecule for inline attack, a role that cannot be served by the short and hydrophobic side chain of Ala. Because Gln or Arg residues are found at that position in other GTPase families, we substituted H84 with Gln, Ala, or Arg. For D21, we chose Ala, Asn, and Glu substitutions to probe the contribution of charge and size of the side chain. We measured the intrinsic, slow GTPase reaction at multiple-turnover conditions, namely in the presence of an excess of [γ-^{32}P]GTP over EF-Tu. To ensure rapid exchange of the EF-Tu–bound GDP, EF-Ts was added to the reaction; at these conditions, GTP cleavage is the rate-limiting step of the GTPase cycle (21). Linear fitting of the time courses at initial velocity conditions yielded the rate of intrinsic GTP hydrolysis by EF-Tu off the ribosome. Contrary to what was expected for the substitution of a putative catalytic residue, mutations of H84 had no effect on the intrinsic GTPase rate (Fig. 2). This result is in contrast to a previous study (28), which reported an almost undetectable intrinsic GTPase activity of the H84A and H84Q mutants. However, this effect might, at least partially, be explained by the absence of EF-Ts in those experiments, which renders the reaction much more sensitive to the relative affinities of EF-Tu for GDP and GTP (21). In addition, mutations of D21 had only little effect on the intrinsic GTPase reaction, with the D21A mutant showing the largest reduction, about threefold (Fig. 2).

Mutations of P-loop residues may alter the affinity of EF-Tu for nucleotides (41, 42). We determined the affinity of the D21A mutant for both GDP and GTP, using Bodipy-FL (BOF)–labeled nucleotides as reporters (Fig. S1). D21A EF-Tu showed a markedly increased affinity for GDP and GTP (12- and 27-fold, respectively) compared with the WT protein. The effect can be explained by the thermodynamic advantage of the elimination of a negatively charged side chain in close proximity to the nucleotide phosphates, similar to the effect of substituting the conserved Asn in FeoB (38).

If H84 acts as a putative general base with a pK_a in the neutral range, changes in pH should affect the rate of GTP hydrolysis. In fact, early biochemical data suggested a moderate pH dependence of the intrinsic GTPase activity of EF-Tu (43). For quantitative analysis, we measured the intrinsic GTPase activity within the pH range of 6–8.5 (Fig. 3*A*); handling EF-Tu at lower or higher pH is not feasible. Because Gln can act as a proton acceptor, we also determined the pH profile of the reaction with EF-Tu(H84Q). Evaluation of the slope of the linear log/log plot in the pH range between 6.0 and 7.5—which indicates the number of ionizing groups potentially involved in catalysis—yielded 0.25 or 0.15 for WT and H84Q EF-Tu, respectively. Thus, with either protein the rate of GTP hydrolysis was essentially independent of pH. This finding argues against a role for the His/Gln residue as a general base in catalysis. Furthermore, because neither Arg nor Ala can act as a general base at neutral pH but both support the intrinsic GTPase activity, we conclude that general acid–base catalysis does not contribute significantly to the ribosome-independent GTPase activity of EF-Tu, in agreement with the conclusions of molecular dynamics simulations (13). For completeness, we also conducted the experiment with the D21A mutant, which showed no appreciable pH dependence.

Computer modeling of the transition state (TS) suggested that the intrinsic GTPase reaction may proceed through water molecules only, without participation of the catalytic H84 (31). In a substrate-assisted GTPase mechanism (16, 27, 29), where the GTP itself abstracts a proton from the nucleophilic water before or upon the hydroxide attack on the γ-phosphate, proton transfer may constitute the rate-limiting step. If this were the case, a kinetic solvent isotope effect (KSIE) of 2 or more is expected (44, 45). We thus measured the intrinsic GTPase rate of WT EF-Tu

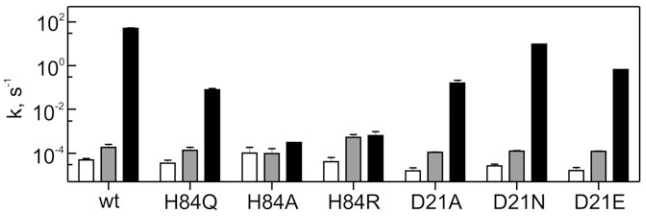

Fig. 2. Effect of H84 and D21 mutations on the GTPase activity of EF-Tu. Rates of GTP hydrolysis were measured using free EF-Tu (intrinsic GTPase, white bars), ternary complex EF-Tu–GTP–Phe-tRNA^{Phe} with nonprogrammed ribosomes (gray bars), and ternary complex with programmed ribosomes (0.6 μM) carrying an fMet-tRNA^{fMet} in the P site and a UUC codon in the A site (black bars). Error bars indicate the SE (SEM) of up to six independent measurements.

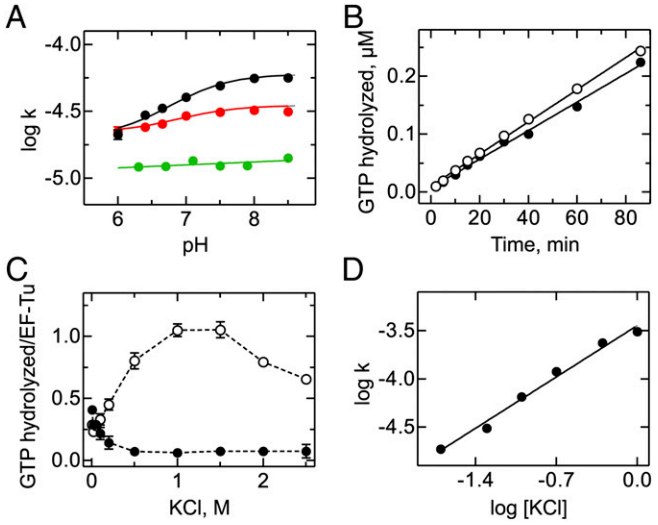

Fig. 3. Characterization of the intrinsic GTPase activity of EF-Tu. (*A*) Effect of pH on the intrinsic GTPase activity of WT (black), H84Q (red), and D21A (green) EF-Tu. (*B*) Time course of GTP hydrolysis by WT EF-Tu measured in buffer containing H_2O (open circles) or D_2O (closed circles). (*C*) Dependence on K^+ ions measured with WT (open circles) and D21A (closed circles) EF-Tu in buffer B. Error bars indicate SEM of three independent experiments. (*D*) Hill plot of the K^+ ion concentration dependence calculated from the data of *C*. Linear fitting yields a slope of 0.76 ± 0.04.

in the presence of D_2O-containing buffer and compared the rate to the reaction in H_2O buffer. The KSIE was very small, k_H/k_D = 1.16, where k_H and k_D are rates of reaction in water and D_2O, respectively (Fig. 3*B*), suggesting that proton transfer in the TS is not rate-limiting, in agreement with theoretical work (13).

The intrinsic GTPase reaction of EF-Tu is susceptible to stimulation by monovalent cations (46). In fact, for WT EF-Tu, the rate/KCl profile is bell-shaped, with the rate of GTP hydrolysis increasing almost linearly with the KCl concentration up to 1 M (Fig. 3*C*). Because in K^+-activated GTPases the residue corresponding to D21 of EF-Tu is crucial for ion coordination (38, 47, 48) and there is an Na^+ coordinated by an Asp residue in eIF5B (40), we tested whether D21 is responsible for monovalent ion binding and to what extent the GTPase activity depends on this ion. In contrast to WT EF-Tu, the intrinsic GTPase activity of the D21A mutant is not activated by K^+ ions, suggesting that the side chain of D21 is crucial for ion coordination. Assuming that the unspecific effects of K^+ ions are similar for WT and D21A EF-Tu, these results allowed us to deconvolute the bell-shaped curve obtained for the WT protein and estimate to what extent K^+ ions contribute to the GTPase activity (*SI Materials and Methods*). The log(k)/log[K^+] plot was linear with a slope close to 0.8 (Fig. 3*D*), suggesting that one K^+ ion is directly involved in catalysis and that this ion is coordinated by D21. Nevertheless, its contribution to the increase of the GTPase rate does not exceed a factor of 3 (Fig. 3 *C* and *D*).

In the Rab5a GTPase, mutation of Ala30, which occupies the place of EF-Tu D21, to proline abolished the GTPase activity, suggesting an important catalytic role for this P-loop backbone amide group (49). Therefore, one further experiment that could have strengthened our conclusions would be to test the importance of the hydrogen bond between the backbone nitrogen atom of D21 and the β–γ bridging oxygen of GTP by mutating D21 to Pro. Although Pro in this position did not alter the P-loop structure in Rab5a (50), the substitution in EF-Tu yielded an insoluble protein, presumably due to the inability to bind GDP.

GTPase Stimulation by Nonprogrammed Ribosomes. The extent to which ribosomes accelerate GTP hydrolysis by EF-Tu depends on the codon–anticodon base pairing (22, 51, 52). When there is no cognate codon–anticodon interaction, the ribosome only moderately stimulates the GTPase activity of EF-Tu. The ternary complex EF-Tu–GTP–Phe-$tRNA^{Phe}$ binds to nonprogrammed ribosomes with an affinity (0.5 μM) that is similar to that of near-cognate ternary complexes binding to programmed ribosomes (51). Because near-cognate ternary complexes bind to the ribosome in an orientation similar to that of cognate complexes (53), it is likely that the codon-independent contacts between the G domain of EF-Tu and the 50S subunit are similar regardless of the codon recognition, raising the question of whether the EF-Tu–50S subunit interactions alter the GTPase mechanism. To address this question, we measured the GTPase activity of EF-Tu in the presence of an excess of Phe-$tRNA^{Phe}$ and nonprogrammed 70S ribosomes (1.2 μM). The GTP hydrolysis was stimulated by about 2- to 10-fold. However, also in this case, replacements of H84 or D21 had very little effect (Fig. 2). In contrast to the intrinsic GTPase activity, stimulation by K^+ ions was not observed (Fig. S2).

GTP Hydrolysis upon Cognate Decoding. To determine the extent of GTPase activation provided by the ribosome after the recognition of the codon by the cognate aa-tRNA, we measured GTP hydrolysis upon interaction of the ternary complex EF-Tu–[γ-^{32}P]GTP–Phe-$tRNA^{Phe}$ with an excess of UUC-programmed ribosomes, namely at single-round conditions (Figs. 2, 4*A*, and 5*A*). The H84 mutants showed a drastically reduced GTPase rate (Fig. 2). Notably, although measured at single-round conditions, the rates determined with the H84R and H84A mutants practically coincided with those of the intrinsic GTPase reaction measured without ribosomes, indicating that the ribosome- and codon-specific acceleration requires H84.

Because the lack of a stimulatory effect can arise from either the effects on the chemistry step or inhibition of any preceding step of decoding, we tested whether the H84Q mutation affected binding of the ternary complex to the ribosome or the GTPase activation by following the respective conformational rearrangements using established fluorescence reporter assays (22, 23, 51). The fluorescence changes of a proflavin (Prf) attached to the D loop of $tRNA^{Phe}$ were used to monitor ternary complex binding to the ribosome and codon recognition, followed by the release of $tRNA^{Phe}$(Prf) from EF-Tu and accommodation in the A site (Fig. 4*B*). To increase the sensitivity of the assay, the concentration of programmed ribosomes used in this experiment, 0.4 μM, was chosen to be in the linear range of the concentration dependence. The rate of the fluorescence increase, which reports on initial binding and codon recognition, was similar for ternary complexes with WT and H84Q EF-Tu, 18 and 13 s^{-1}, respectively, indicating that codon recognition was not affected by the mutation. However, the rate of tRNA accommodation observed with H84Q EF-Tu, 0.06 s^{-1}, was much slower than with WT EF-Tu, 4 s^{-1}, and coincided with the GTPase rate, 0.07 s^{-1} (Fig. 2).

To monitor the GTPase activation, we measured the change in mant-GTP fluorescence upon addition of increasing concentrations of programmed 70S complexes to the EF-Tu–mant-GTP–Phe-$tRNA^{Phe}$ complex (Fig. 4 *C* and *D*). The fluorescence of the mant group changed in a biphasic fashion, which reports on the conformational rearrangement in the nucleotide-binding pocket of EF-Tu leading to GTP hydrolysis (fluorescence increase) and the dissociation of EF-Tu–GDP from the ribosome (fluorescence decrease) (22). With H84Q, the fluorescence change was somewhat smaller than with WT EF-Tu (Fig. 4*C*), which may reflect some structural differences in the GTP binding site. However, the rate of GTPase activation was very similar for WT and H84Q EF-Tu, suggesting that this step is not affected by the mutation (Fig. 4*D*). Whereas GTP hydrolysis was very slow with mutant EF-Tu, for the WT factor the rates of GTPase activation and hydrolysis coincided, saturating at the same value

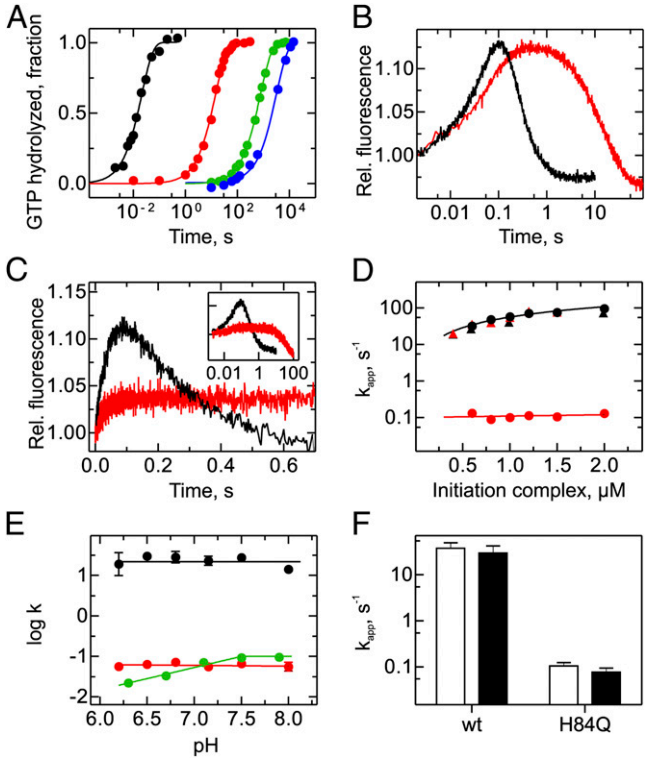

Fig. 4. Effects of H84 and D21 substitutions. (*A*) Time courses of the GTP hydrolysis on programmed ribosomes measured under single-round conditions with WT (black), H84Q (red), H84R (green), and H84A (blue) EF-Tu. Data points are normalized to the extent of the reaction. (*B*) Time courses of codon recognition and tRNA accommodation measured by stopped-flow experiments. Ternary complexes of Phe-tRNA^{Phe}(Prf) with WT (black) or H84Q (red) EF-Tu–GTP (0.2 μM) were rapidly mixed with initiated ribosomes (0.4 μM). (*C*) Time courses of GTPase activation. Ternary complexes containing WT (black) or H84Q (red) EF-Tu and mant-GTP (0.2 μM) were mixed with programmed ribosomes (1 μM). (*Inset*) Longer time window. (*D*) Concentration dependence of the GTPase activation rates (triangles) compared with GTP hydrolysis (circles) measured with WT (black) and H84Q (red) ternary complexes and increasing concentrations of programmed ribosomes. (*E*) pH dependence measured as in *A* by mixing WT (black), H84Q (red), and D21A (green) ternary complexes (0.2 μM) with programmed ribosomes (1 μM). Error bars indicate the SEM of two independent measurements. (*F*) KSIEs. GTPase rates were measured as in *A* using ternary complexes containing WT or H84Q EF-Tu (0.12 μM) and programmed ribosomes (0.4 μM) in buffer made up with H_2O (white bars) or D_2O (black bars). Error bars indicate the SEM of at least three measurements.

of about 300 s^{-1} (Fig. 4*D*) (51). Thus, the H84Q mutation specifically impairs the catalytic step of GTP hydrolysis, confirming previous observations with the H84A substitution (5). The rate of the fluorescence decrease was much slower with H84Q EF-Tu than with WT EF-Tu, 0.02 and 2.7 s^{-1}, respectively (Fig. 4*C*, *Inset*); the former value was similar to the rate of GTP hydrolysis with H84Q, 0.07 s^{-1}, measured over the whole range of ribosome concentrations (Fig. 4*D*), suggesting that slow GTP hydrolysis limits the rate of all of the following steps, such as aa-tRNA release from EF-Tu, accommodation in the A site, and dissociation of EF-Tu–GDP from the ribosome.

Previous experiments with GTPγS showed that hydrolysis was not pH-dependent (5), suggesting no general acid–base catalysis. However, it was pointed out that the lack of pH dependence may be due to the use of the GTP analog and may not pertain to the natural substrate (31, 54), although there is no indication that GTPγS is not an authentic GTP analog. To clarify this point, we determined the pH dependence with unmodified GTP. As expected, with the WT EF-Tu, the rate of GTP hydrolysis was independent of pH (Fig. 4*E*). This suggests that the chemistry step is pH-independent or may reflect the fact that GTP activation is rate-limiting for GTP hydrolysis, apparently over the whole pH range tested. Because Gln can in principle act as a general base, and in H84Q EF-Tu the hydrolysis rate is not limited by the activation step, we also measured the pH dependence of GTP hydrolysis with this mutant. Also in this case, the rate of GTP hydrolysis was independent of pH (Fig. 4*E*).

Finally, we wanted to determine the KSIE for the ribosome-activated GTPase activity. Because in WT EF-Tu the reaction is limited by the activation step, we measured the solvent effect with both WT and H84Q EF-Tu. The KSIE was rather small, in the range of 1.3 in both cases (Fig. 4*F*), suggesting that proton transfer from the hydrolytic water is not rate-limiting for GTP hydrolysis on the ribosome, consistent with theoretical predictions (13). For comparison, a KSIE of about 4 with one proton in flight in the TS was observed for the hydrolysis of peptidyl-tRNA in the catalytic center on the 50S subunit (55).

Because D21 turned out to have an unexpected role in stabilizing a K^+ ion that had a moderate catalytic effect on the intrinsic GTPase, we also examined the effect of D21 mutations on the reaction on programmed ribosomes. In this case, mutations of D21 strongly inhibited GTP hydrolysis, in contrast to the lack of effect in free EF-Tu and on nonprogrammed ribosomes. With the exception of D21N EF-Tu, which retained substantial activity compared with the WT factor, Glu and Ala substitutions resulted in about 70- and 640-fold reduction in the GTPase rate, respectively (Figs. 2 and 5*A*). A similar effect has been observed for mutations of the corresponding residue in Ras, Gly13 (33). This phenotype might be explained by the loss of K^+ ion coordination, especially because the mutation to Asn is less severe than the others. However, GTP hydrolysis on the ribosome stimulated by codon recognition was largely insensitive to the K^+ ion concentration, at least in the concentration range compatible with unimpaired ribosome activity (Fig. S2). GTPase activation—monitored by mant-GTP fluorescence, as described above—proceeded at virtually the same rate as with WT EF-Tu (Fig. 5*B*). With D21A EF-Tu, the fluorescence increase due to GTPase activation was followed by a very slow fluorescence decrease, 0.04 s^{-1}, which coincided with the GTP hydrolysis rate measured at the same conditions (Fig. 2). Because also in this case the hydrolysis rate was independent of the ribosome concentration (Fig. S3), we conclude that D21 mutations specifically limited the catalytic proficiency of EF-Tu but did not interfere with the conformational rearrangements triggered by codon recognition at the A site. Curiously, the D21A mutant exhibited a moderate pH dependence (fivefold rate increase in the range between 6.3 and 8.0) with a slope of about 0.5 and an apparent pK_a of 7.5; the origin of this effect is not clear at present.

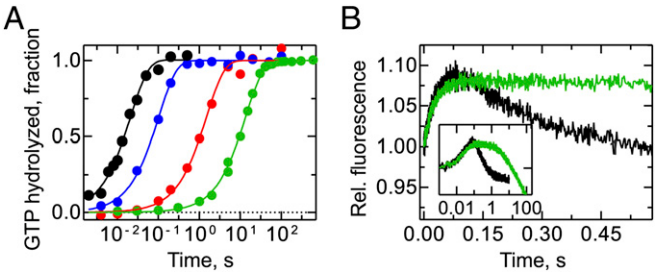

Fig. 5. Anticatalytic effect of D21 substitutions. (*A*) Time courses of the GTP hydrolysis. Ternary complexes containing WT (black), D21N (blue), D21E (red), and D21A (green) EF-Tu (0.2 μM) were rapidly mixed with programmed ribosomes (0.6 μM). (*B*) Time courses of GTPase activation measured upon interaction of WT (black) and D21A (green) ternary complexes (0.2 μM) containing mant-GTP with programmed ribosomes (1 μM). (*Inset*) Longer time window.

Discussion

The results of the present mutational and kinetic analysis have implications for understanding the mechanism of both intrinsic and ribosome-stimulated GTP hydrolysis by EF-Tu. In the absence of the ribosome, GTP hydrolysis proceeds through a mechanism that does not involve the side chains of D21 and H84 in the P loop and the switch II region, respectively. A monovalent K^+ ion coordinated by D21 has a small stimulatory effect. In the simplest model, the lack of effects of replacement of the side chains of H84 and D21 on the intrinsic GTPase activity, the insignificant pH dependence, and the small KSIE may indicate that the reaction follows a dissociative pathway, as suggested for the nonenzymatic and enzyme-catalyzed reactions (9, 56). However, as pointed out by the theoretical work, these findings are equally consistent with the associative-concerted mechanism both in solution and in the context of enzymes (8); further calculations, which will take into account the present findings, would be necessary to accurately describe the path of intrinsic GTP hydrolysis in EF-Tu. Notably, in all known structures of free EF-Tu–GTP (19, 57) or the ternary complex (58), H84 is oriented away from the γ-phosphate and shielded from the nucleophilic water molecule by the hydrophobic side chains of Val20 and Ile60 (the hydrophobic gate) (2). It is likely that in free EF-Tu a reorientation of H84 toward the γ-phosphate is not required for the reaction to occur and H84 is not involved in catalysis. Also, the suggestion that the main chain of D21 may act as a general acid (19) is not supported by our analysis, because the reaction is independent of pH and the effect of D21 mutations is small. Stabilization of the TS may be mediated mainly by interactions with residues surrounding the β-phosphate [D21 (main chain), Gly23, Lys24, and Thr25 in the P loop, and Thr62) and the magnesium ion (29) (Fig. 1), which are also crucial for nucleotide binding. Notably, these residues are conserved among all GTPases, which raises the possibility that this mechanism is universally conserved. Furthermore, the small KSIE on the intrinsic GTPase reaction suggests that proton transfer from the activated water molecule is not rate-limiting, in agreement with theoretical predictions (13).

The findings that the side chain of D21 coordinates a K^+ ion and that mutation of this residue to Ala abolishes the stimulation by K^+ ions of the intrinsic GTPase reaction (Fig. 3*C*) indicate that this monovalent ion plays a role as structural cofactor in the free protein. Furthermore, a D21-coordinated metal ion was suggested to catalyze the GTPase activity (40) in a way similar to the arginine finger in the GAPs of small GTPases (3). However, for EF-Tu, the contribution to catalysis of this putative mechanism is small, as the K^+-independent intrinsic activity is reduced only by threefold (Fig. 2). Apart from K^+ ion coordination, the D21 side chain does not seem to contribute to the GTPase reaction off the ribosome.

Although mutations of D21 and H84 do not affect the intrinsic GTPase activity or the GTP hydrolysis stimulated by nonprogrammed ribosomes, these mutations disrupt the acceleration of the GTPase activity in the cognate EF-Tu–GTP–aa-tRNA complex stimulated by programmed ribosomes. This change of the reaction mechanism must be caused by conformational rearrangements in EF-Tu in response to cognate codon–anticodon interaction. Notably, the replacements do not affect the conformational changes leading to GTPase activation; rather, the following GTP cleavage step itself is impaired. From the ratio of the GTPase rates at saturation, the penalty associated with the H84Q substitution amounts to an increase of the activation energy by 4.8 kcal/mol, which is much higher than previously estimated for *Thermus thermophilus* EF-Tu (7). The difference can be explained by the suboptimal temperature used in those experiments or the incomplete activity of the *T. thermophilus* in vitro translation components. However, computer simulations also predicted a smaller anticatalytic effect for mutations of H84 (27, 29); further structural information about the TS would be necessary to understand this discrepancy. The penalties for the H84A and H84R replacements are 8 and 7.2 kcal/mol, respectively, in line with the inability of these residues to coordinate the nucleophilic water molecule.

The pH independence of ribosome-stimulated GTP hydrolysis by EF-Tu further supports the notion that neither H84 nor D21 acts as a general base or acid catalyst at neutral pH. Moreover, because the KSIE is small, proton transfer in the TS appears not to be rate-limiting. In this scenario, the only way H84 can contribute to catalysis appears to be by positioning of the nucleophilic water molecule for attack, which is triggered by the interaction of EF-Tu with A2662 of the SRL upon codon recognition (13, 15). This suggestion is consistent with the proposed substrate-assisted catalysis model (16) and with work on the phosphoryl transfer mechanism suggesting that the orientation of the nucleophilic water molecule can improve catalysis in both dissociative and associative mechanisms (29).

The role of D21 is more difficult to explain. The penalty for the D21A substitution is 4.4 kcal/mol, which is comparable to the effect of the H84Q mutation. The effect depends on the nature of the amino acid side chain: The size seems to be more important than the charge, as the D21N mutant hydrolyzed GTP only sixfold slower than the WT, whereas the longer glutamate side chain in the same position had a much stronger effect. The moderate stimulatory effect of K^+ ions on catalysis observed in free EF-Tu is lost on ribosomes (Fig. S2). It is possible that the K^+ ion that is bound to the side chain of D21 in the free protein is exchanged by a water molecule (or a different cation) upon ribosome binding and/or codon-induced conformational change of EF-Tu. This hypothesis is corroborated by a recent ribosome-bound structure of EF-G (59) that suggests that upon SRL binding the side chain of the corresponding Asp22 rearranges into a conformation that would bring a second water molecule close to the γ-phosphate, possibly contributing to the stabilization of the γ-phosphate [we note that EF-G does not require an additional activation by codon–anticodon interaction and appears to adopt the activated form spontaneously (60)]. A water molecule has often been seen or modeled at hydrogen-bonding distance to the side chain of D21 or its corresponding aspartate residues in different trGTPases (13, 29, 59, 61). D21 together with its ligands may contribute to the acceleration of GTP hydrolysis by providing an optimal orientation of the γ-phosphate. Thus, codon recognition appears to change the structure of the EF-Tu active site to allow the universally conserved residues in the P loop and switch II region to reorient and form a network of interactions that stabilize the transition state. In this model, the ribosome would act in a similar way as those GAPs or regulators of G protein signaling that do not directly provide catalytic residues but rather accelerate the hydrolysis reaction by stabilizing the switch regions in a productive way (62). This would explain why crystal structures and extensive mutagenesis did not identify a specific catalytic Arg or other group crucial for the GTPase activation in trGTPases, except for the SRL, which is required for factor binding. If our hypothesis is correct, then the million-fold rate acceleration brought about by the ribosome is achieved solely by electrostatic stabilization and shielding effects, which were collectively denoted as "allosteric" based on computer simulations (13, 27). This would be similar to the catalysis at the peptidyl transferase center of the ribosome, which may provide yet another example of how an ancient RNA-based catalyst solves the problem posed by the limited catalytic power of RNA (63).

Materials and Methods

All experiments were carried out at 20 °C in buffer A (50 mM Tris·HCl, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$) except for the K^+ ion dependence experiment, which was carried out in buffer B (50 mM Tris·HCl, pH 7.5, 7 mM $MgCl_2$, and KCl added as indicated). To measure the intrinsic GTPase activity, EF-Tu (0.4 μM) was incubated for 1 h in buffer A containing phosphoenol pyruvate (3 mM), pyruvate kinase (0.1 μg/μL), [γ-^{32}P]GTP (10 μM), EF-Ts (0.02 μM), and, where indicated, Phe-tRNA^{Phe} (2 μM) and 70S ribosomes (1.2 μM). Quench-flow assays

were performed by rapidly mixing purified ternary complex (0.2 μM) and 70S initiation complex at the indicated concentrations. Reactions were quenched with formic acid (25% vol/vol) and the extent of GTP cleavage was determined by TLC (51). Stopped-flow experiments were performed by mixing a fixed concentration of purified ternary complexes (0.2 μM) and initiation complexes at the indicated concentrations.

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