Delayed Release of Inorganic Phosphate from Elongation Factor Tu Following GTP Hydrolysis on the Ribosome†

Ute Kothe and Marina V. Rodnina*

Institute of Physical Biochemistry, University of Witten/Herdecke, 58448 Witten, Germany

Received June 15, 2006; Revised Manuscript Received August 22, 2006

ABSTRACT: The dissociation of inorganic phosphate (P_i) following GTP hydrolysis is a key step determining the functional state of many GTPases. Here, the timing of P_i release from elongation factor Tu (EF-Tu) and its implications for the function of EF-Tu on the ribosome were studied by rapid kinetic techniques. It was found that P_i release from EF-Tu is >20-fold slower than GTP cleavage and limits the rate of the conformational switch of EF-Tu from the GTP- to the GDP-bound form. The point mutation Gly94Ala in the switch 2 region of EF-Tu abolished the delay in P_i release, suggesting that P_i release is controlled by the mobility of the switch 2 region with Gly94 acting as a pivot. The rate of P_i release or the conformational switch of EF-Tu does not affect the selection of aminoacyl-tRNA on the ribosome. Rather, the slow P_i release may be a consequence of the tight interaction of the switch regions of EF-Tu with the γ-phosphate and the ribosome in the GTPase activated state of the factor.

Elongation factor Tu (EF-Tu[1]) is a GTP-binding protein that promotes the binding of aminoacyl-tRNAs (aa-tRNA) to the A site of the ribosome. EF-Tu consists of three domains, domain 1, or GTP binding domain, that is homologous among all GTP binding proteins, and domains 2 and 3 that are necessary to bind aa-tRNA (*1*). Binding of the ternary complex EF-Tu·GTP·aa-tRNA to the ribosome is a multistep process. It starts with the rapid and reversible initial binding of EF-Tu·GTP·aa-tRNA to the ribosome, which is most likely mediated by contacts between EF-Tu and the ribosomal protein L7/12 and allows for rapid sampling of different ternary complexes (*2*). On the ribosome, aa-tRNAs are selected according to the match between their anticodons and the mRNA codons in the A site (*3*). Correct codon recognition of cognate aa-tRNAs stabilizes the tRNA on the ribosome and accelerates the GTPase activation of EF-Tu by an induced-fit mechanism (*4*). GTP cleavage itself is rapid and limited by the preceding conformational changes leading to GTPase activation. Until now, it was assumed that inorganic phosphate (P_i) is expelled from EF-Tu immediately with GTP hydrolysis due to the strong charge repulsion between the reaction products, GDP and P_i. Following GTP hydrolysis, EF-Tu undergoes a large conformational change from the GTP- to the GDP-bound form; the rearrangement involves changes in the switch 1 and 2 regions of EF-Tu as well as large changes in the relative orientation of the domains (*5*, *6*). The rearrangements are inhibited by the antibiotic kirromycin (*7*) which stalls the ternary complex EF-Tu·GDP·aa-tRNA on the ribosome in a GTP-like conformation and prevents the subsequent steps (*8*). The GDP-bound form of EF-Tu binds aa-tRNA very weakly, resulting in the release of aa-tRNA and the dissociation of EF-Tu from the ribosome. After release from EF-Tu, the acceptor end of aa-tRNA is free to accommodate in the A site and to take part in peptide bond formation. In analogy to GTP hydrolysis, which is limited by the GTPase activation rearrangement, the rate of peptide bond formation is limited by the accommodation of aa-tRNA in the A site. The properties of EF-Tu as a GTP binding protein are crucial for the speed and fidelity of aa-tRNA delivery to the A site of the ribosome. The GTP to GDP switch causes the release of aa-tRNA and allows for EF-Tu dissociation from the ribosome. In addition, the GTPase rate is tightly controlled and dependent on correct codon recognition by aa-tRNA, and activated by contacts with the ribosome. Thereby, the properties of EF-Tu as GTP binding protein contribute to the selection of the correct aa-tRNA.

Most GTPases interact with their respective effectors in the active GTP-bound form. The intrinsically low GTPase activity is stimulated by interactions with GTPase activating proteins (GAPs). GTP hydrolysis and the subsequent switch from the GTP- to the GDP-bound form change the structure of GTPases, resulting in the loss of interactions with the effectors (and the GAPs) and the formation of the inactive GDP-bound form of the protein. P_i release is crucial for switching between the GTP- and GDP-bound forms; however, its importance for the regulation of signaling remains unclear. In small GTP binding proteins P_i release may take place rapidly after GTP hydrolysis or after a significant delay (*9*), while little data exist on P_i release from heterotrimeric G_α proteins, except for two studies with transducin which led to contradictory results (*10*, *11*).

† This work was supported by the Deutsche Forschungsgemeinschaft, the European Union, the Alfried Krupp von Bohlen und Halbach-Stiftung, and the Fonds der Chemischen Industrie. U.K. was supported by a fellowship of the Studienstiftung des deutschen Volkes.

* Corresponding author. Institute of Physical Biochemistry, University of Witten/Herdecke, Stockumer Str. 10, 58448 Witten, Germany. Tel.: 49-2302-926-205. Fax: 49-2302-926-117. E-mail: rodnina@uni-wh.de.

[1] P_i, inorganic phosphate; EF-Tu, elongation factor Tu; aa-tRNA, aminoacyl-tRNA; GAP, GTPase activating protein; EF-G, elongation factor G; eIF2, eukaryotic initiation factor 2; MDCC-PBP, MDCC-labeled phosphate-binding protein.

10.1021/bi061192z CCC: $33.50
Published on Web 10/03/2006

In contrast to Ras-like and heterotrimeric G_α proteins, some GTPases do not behave as classical switches. For example, GTP hydrolysis by elongation factor G (EF-G) induces structural changes within the ribosome that are required for the subsequent tRNA movement and precede P_i release (*12*). P_i dissociation from EF-G is controlled by the ribosomal protein L7/12 and is important for coupling of GTPase-induced conformational changes of EF-G, rearrangements of the ribosome, and movement of the tRNA, as well as for determining the timing of EF-G dissociation from the ribosome (*13*). P_i release from eukaryotic initiation factor 2 (eIF2) was shown to contribute to the function of the factor by ensuring that release of Met-tRNA^i from eIF2 occurs only upon correct AUG start site selection (*14*). In this respect, EF-G and eIF2 seem to resemble the motor proteins, such as myosin where P_i release is significantly delayed relative to ATP hydrolysis and triggers the power stroke (*15*). In contrast, other ATPases release P_i rapidly, such as motor protein kinesin, and the role of P_i release in the overall mechanism is not clear (*16*).

The role and timing of P_i release from EF-Tu is not known. Here, we employed rapid kinetic measurements to study the mechanism of P_i release by EF-Tu. Rate constants of P_i release were determined and compared to the rates of GTP hydrolysis and the EF-Tu conformational change reported previously (*17*, *18*). The importance of the mobility of the switch 2 region of EF-Tu for P_i release was studied using the mutant EF-Tu(G94A) that was defective in the conformational switch (*18*, *19*).

MATERIALS AND METHODS

Buffers and Reagents. Buffer A: 50 mM Tris-HCl, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 10 mM $MgCl_2$, and 2 mM dithiothreitol. GTP, phosphoenolpyruvate, and pyruvate kinase were from Roche Diagnostics. Radioactive compounds were from ICN. Kirromycin was a kind gift of A. Parmeggiani and P. Nissen (Aarhus, Denmark). All other chemicals were from Merck.

Ribosomes, tRNAs, and Factors. Ribosomes from *Escherichia coli* MRE 600 were prepared as described (*20*). AcPhe-tRNA^{Phe}, Phe-tRNA^{Phe}, wild-type EF-Tu, EF-Tu(G94A), and MDCC-labeled phosphate-binding protein (MDCC-PBP) were prepared and purified as described (*17*, *19*, *21*).

Preparation of Ribosome and Ternary Complexes. Ternary complexes containing Phe-tRNA^{Phe} from *E. coli* (0.4 μM prior to mixing with ribosome complexes) were prepared by incubating 20−50 μM EF-Tu (wild-type or G94A), 1 mM GTP, 3 mM phosphoenol pyruvate, 0.05 mg/mL pyruvate kinase, 0.05 μM EF-Ts in buffer A for 30 min at 37 °C followed by addition of 10−15 μM Phe-tRNA^{Phe} and purification by gel filtration on two Superdex 75 HR columns operated in tandem (Pharmacia) in the same buffer. Ternary complexes containing [γ-^{32}P]GTP were prepared in the same way, except that the concentration of [γ-^{32}P]GTP was 30 μM. In both cases, the excess of GTP was used to form the complex, and unbound GTP was removed by gel filtration. Ribosomes were programmed with 1 mg/mL poly(U) and a 1.1-fold excess of AcPhe-tRNA^{Phe} in buffer A for 15 min at 37 °C. When indicated, kirromycin was added to the ribosome complexes. Concentrations stated in the text are concentrations after mixing of the ribosome complex with ternary complex.

GTP Hydrolysis. GTP hydrolysis was measured using a KinTek quench-flow apparatus by rapidly mixing poly(U)-programmed ribosomes with AcPhe-tRNA^{Phe} in the P site (0.6 μM, after mixing) and ternary complex containing [γ-^{32}P]GTP (0.2 μM). Samples were quenched by 40% formic acid and analyzed by thin-layer chromatography on PEI-cellulose in 0.5 M potassium phosphate pH 3.5. The extent of GTP hydrolysis was quantified using a Bio-Rad phosphoimager. To obtain values for apparent rate constants, time courses were evaluated by fitting to a function with exponential terms (characterized by variable time constants, k_{app}, and respective amplitudes) and another variable for the final signal using TableCurve software (Jandel Scientific).

P_i Release. P_i release from EF-Tu after GTP hydrolysis was monitored by the fluorescence change of MDCC-PBP (*22*) in buffer A at 20 °C. Poly(U)-programmed ribosomes with AcPhe-tRNA^{Phe} in the P site (concentrations after mixing as indicated in the figure legends) and purified ternary complex (concentration after mixing, 0.2 μM) were rapidly mixed in a stopped-flow apparatus (Applied Photophysics). Both reaction mixtures contained MDCC-PBP (2.5 μM), purine nucleoside phosphorylase (0.1 U/mL), and 7-methylguanosine (200 μM) (the latter two components serving as "P_i mop" to take up trace amounts of contaminating P_i (*22*)). MDCC fluorescence was excited at 425 nm and measured after passing a cutoff filter (KV 450, Schott).

Determination of Rate Constants. The rate constant of P_i release (k_{Pi}) was calculated by global fitting of the respective time courses using Scientist for Windows software (Micromath Scientific software). Numerical integrations were based on the following kinetic scheme:

$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C \xrightarrow{k_2} D \xrightarrow{k_3} E \xrightarrow{k_{Pi}} F + P \underset{k_{-P}}{\overset{k_P}{\rightleftharpoons}} G$$

$$P \xrightarrow{k_x} H \qquad (1)$$

where the ternary complex EF-Tu·GTP·Phe-tRNA^{Phe} is A and the ribosomal complex is B. C represents the ribosomal complex after initial binding, D after codon recognition, E after GTP hydrolysis, and F after release of P_i (P). Liberated P_i binds to MDCC-PBP forming the P_i·MDCC-PBP complex (G); alternatively, P_i can be removed by the "P_i mop" resulting in ribose-1-phosphate (and guanine) (H). Rate constants k_1, k_{-1}, k_2, and k_3 for initial binding, codon recognition, and GTP hydrolysis were fixed to values determined previously for wild-type EF-Tu ($k_1 = 100\ \mu M^{-1}\ s^{-1}$, $k_{-1} = 25\ s^{-1}$, $k_2 = 100\ s^{-1}$, $k_3 = 500\ s^{-1}$) and for the mutant EF-Tu(G94A) ($k_1 = 120\ \mu M^{-1}\ s^{-1}$, $k_{-1} = 10\ s^{-1}$, $k_2 = 40\ s^{-1}$, $k_3 = 330\ s^{-1}$) (*17*, *18*). The dissociation rate of the codon recognition complex (k_{-2}) was neglected, as it is very low (0.2 s^{-1} for cognate tRNAs (*17*)). Rate constants for P_i binding to MDCC-PBP, k_P and k_{-P}, were determined in independent titrations of MDCC-PBP with phosphate and fixed in the calculations to $k_P = 100\ \mu M^{-1}\ s^{-1}$ and $k_{-P} = 60\ s^{-1}$, respectively. The rate constants for P_i release, k_{Pi}, and phosphate removal by the "P_i mob", k_x, as well as the amplitude factor for the fluorescence of MDCC-PBP·P_i (G) were determined by global fitting. Standard deviations of the amplitude factor (5%) and of elemental rate constants (4%) were calculated using Scientist for Windows software. The same global model (up to and including formation of

intermediate E) was previously used to describe the time courses of GTP hydrolysis (*17*).

RESULTS

Timing of P_i Release from EF-Tu. To investigate the kinetics of P_i release from EF-Tu, we used the indicator assay detecting free P_i upon its binding to phosphate-binding protein (PBP) which was fluorescently labeled with MDCC. The binding of P_i to MDCC-PBP results in a fluorescence increase which can be monitored in a time-resolved manner in a stopped-flow apparatus (*22*). Time courses of P_i release from EF-Tu were measured and compared to those of GTP hydrolysis measured by quench-flow using [γ-^{32}P]GTP. Both reactions were started by rapidly mixing the purified ternary complex EF-Tu·GTP·Phe-tRNA^{Phe} with poly(U)-programmed ribosomes containing AcPhe-tRNA^{Phe} in the P site. This model system was preferred over the system with natural mRNA (*23*, *24*), because all rate constants of A-site binding (except for P_i release) are available for the poly(U) system, including the kinetic parameters for wild-type EF-Tu in the presence of kirromycin as well as for mutant EF-Tu (*18*, *20*, *25*, *26*). Furthermore, the main mechanistic conclusions derived from previous experiments in the two systems were the same.

The time course of GTP hydrolysis at given concentrations of reactants was fitted to a single-exponential function with an apparent rate constant, $k_{app} = 14\ s^{-1}$ (Figure 1A), in agreement with previous studies (*17*). If P_i release would take place instantaneously following GTP cleavage, the time courses of the two reactions would be indistinguishable. However, the time course of P_i release deviated clearly from GTP hydrolysis and showed a lag phase of about 20 ms. Thus, the direct comparison of GTP hydrolysis and P_i release by EF-Tu indicates that the two steps have different kinetics.

The time course of P_i release could not be evaluated by single-exponential fitting, because at all ribosome concentrations, the time courses showed a clear lag phase (Figure 1B). The difference in the length of the lag phase reflects the concentration-dependence of the steps before P_i release at the respective concentration (*17*). A quantitative analysis of P_i release required global fitting by numerical integration of the time courses. For this purpose, all steps preceding and including GTP hydrolysis were taken into account with the rate constants reported previously for initial binding, codon recognition, and GTPase activation (*17*) (see Materials and Methods). Note that the k_{app} of GTP hydrolysis was only 14 s^{-1} (present data; refs *17*, *18*), compared to the true rate constant of GTP hydrolysis, $k_{GTP} > 500\ s^{-1}$ (Figure 1A). This is because in the experiment of Figure 1A subsaturating ribosome concentrations were used, and because the observed rate of GTP hydrolysis is largely limited by the preceding codon recognition step (100 s^{-1}; (*17*, *18*)). The unknown rate constant of P_i release following GTP hydrolysis, which was to be determined by global fitting, was added to the model. To fully account for the observed time courses it was also necessary to include steps for P_i binding to MDCC-PBP ($k_{on} = 100\ M^{-1}\ s^{-1}$, $k_{off} = 60\ s^{-1}$) and for the eventual removal of P_i by the "P_i mob" (k_x) (see Materials and Methods). The result of the global fitting closely matched the observed time courses (Figure 1B) indicating that the model was suitable to describe the data. Notably, it was not possible to fit the time courses of P_i release to a model without a distinct step for P_i release, i.e., assuming immediate P_i dissociation following GTP cleavage. The rate constant of P_i release obtained from the fit was $k_{Pi} = 23 \pm 1\ s^{-1}$. This value is much lower than the rate constant of GTP hydrolysis, which is $>500\ s^{-1}$ at the present conditions (*17*), indicating that P_i release is at least 20 times slower than GTP hydrolysis.

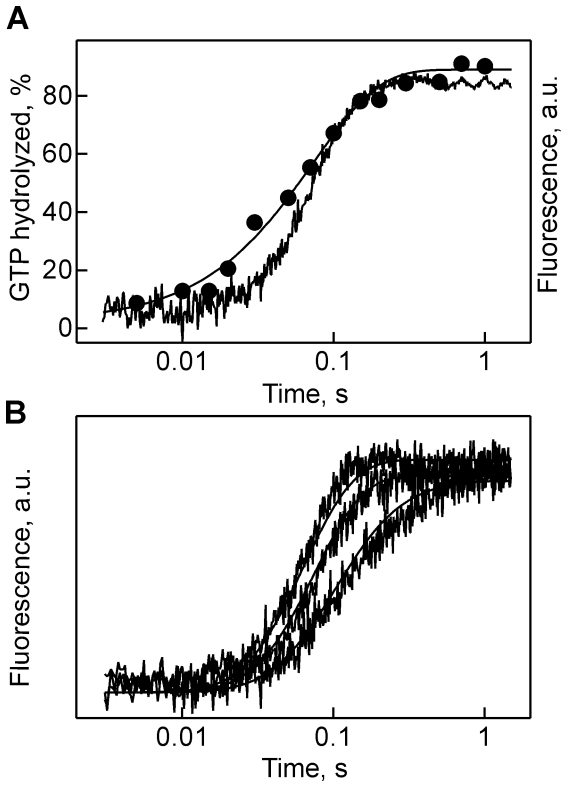

FIGURE 1: P_i release from EF-Tu during aa-tRNA binding to the ribosome. (A) Time courses of GTP hydrolysis (circles) and Pi release. Purified ternary complex EF-Tu·GTP·Phe-tRNA^{Phe} (final concentration 0.2 μM) was rapidly mixed with poly(U)-programmed ribosomes containing AcPhe-tRNA^{Phe} in the P site (final concentration 0.6 μM). The time course of GTP hydrolysis was measured by quench-flow using [γ-^{32}P]GTP; single-exponential fitting yielded $k_{app} = 14\ s^{-1}$. P_i release was monitored by the fluorescence change of MDCC-PBP in the stopped-flow apparatus. (B) P_i release measured at different concentrations of ribosome complexes (lower curve, 0.25 μM; middle curve, 0.6 μM; and upper curve, 1.0 μM). Smooth lines represent the results of least-squares fitting of the time courses by numerical integration using a model with a separate kinetic step for P_i release following GTP hydrolysis (Materials and Methods) with the rate constant for P_i release, $k_{Pi} = 23\ s^{-1}$.

P_i Release and EF-Tu Rearrangement. The rate constant of P_i release from EF-Tu was the same as the rate constant of the conformational transition of EF-Tu from its GTP- to the GDP-form (k_4), $25 \pm 10\ s^{-1}$ (*18*). The latter rate constant was calculated from the lag phase of aa-tRNA accommodation in the A site in comparison to the rate of GTP hydrolysis and thus summarizes all steps that take place in between those two steps. As P_i release is a distinct step following GTP cleavage, the rate constant k_4 must comprise both the release of P_i and the conformational change of EF-Tu. This raises the question about the order of events in the active site of EF-Tu: either P_i release, at about 20 s^{-1}, precedes and limits the rate of the conformational change of EF-Tu, or the conformational change takes place first and limits the rate of subsequent P_i release which is intrinsically rapid. In

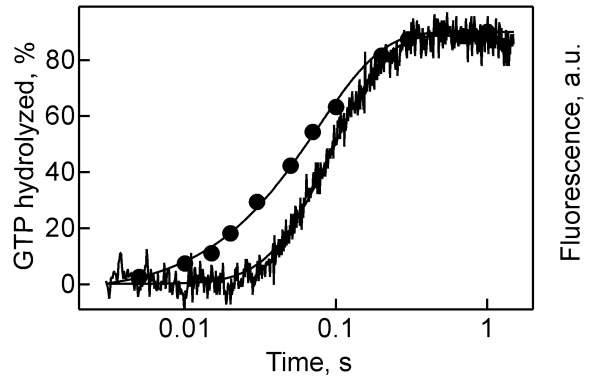

FIGURE 2: Influence of kirromycin on P_i release from EF-Tu. Time courses of GTP hydrolysis (circles) and P_i release were measured at the conditions of Figure 1A in the presence of 200 μM kirromycin. GTP hydrolysis takes place with an apparent rate constant $k_{app} = 14\ s^{-1}$ (single-exponential fit). P_i release in the presence of kirromycin was modeled using the same model and the same parameters as without antibiotic (smooth line), i.e. with a rate constant for P_i release, $k_{Pi} = 23\ s^{-1}$.

order to answer this question, we utilized the antibiotic kirromycin which inhibits the conformational change of EF-Tu and freezes EF-Tu·GDP in a GTP-like conformation (*7*). To assess the effect of kirromycin on the preceding steps, the rate of GTP hydrolysis by EF-Tu on the ribosome was measured in the presence of saturating amounts of kirromycin. The efficiency of blocking EF-Tu in the state prior to the conformational switch was verified by the inaccessibility of aa-tRNA for peptide bond formation with AcPhe-$tRNA^{Phe}$ in the P site. At the kirromycin concentrations used, <10% of Phe-$tRNA^{Phe}$ was found in dipeptides (data not shown), indicating that the majority of EF-Tu did not release aa-tRNA into the A site, i.e., was blocked in the GTP-like conformation.

The time courses of GTP hydrolysis in the presence and absence of kirromycin were identical and gave a $k_{app} = 14\ s^{-1}$, suggesting that kirromycin does not interfere with GTP hydrolysis (Figure 2). Also the time courses of P_i release in the presence or absence of kirromycin were very similar and showed a 20 ms lag phase relative to GTP hydrolysis (Figure 2). Indeed, P_i release in the presence of kirromycin could be fitted by the same model and same values of the rate constants as described above for the experiments without the antibiotic. Thus, kirromycin had no effect on P_i dissociation, whereas the conformational change of EF-Tu was completely inhibited (*8*, *27*). This indicates that P_i release takes place prior to the conformational switch of EF-Tu and excludes a model where P_i release follows and is rate-limited by the rearrangement of EF-Tu. An alternative would be a branched model in which P_i release and EF-Tu conformational change take place independently and both pathways were equally possible; the latter implies that the values of the intrinsic rate constants k_{Pi} and k_{conf} are similar. In such a model, the apparent rate constant of either P_i release or EF-Tu rearrangement equals the sum of the intrinsic rate constants of the two steps $k_{Pi} + k_{conf}$. In this case, kirromycin would block the pathway in which EF-Tu conformational change occurs first, whereas the pathway where P_i release takes place first could still be observed. This scenario would imply that the apparent rate constant of P_i release in the presence of kirromycin must be reduced by the value of the intrinsic rate constant of EF-Tu conformational change. However, this was not observed, and thus the branched model is unlikely. These data suggest that P_i release takes place prior to and is rate-limiting for the subsequent conformational change of EF-Tu from the GTP- to the GDP-form. The latter step must be rapid compared to P_i release, because the rate constant of P_i release is comparable to the overall rate of both steps (k_4) as determined previously.

Role of Gly94 in the Switch 2 Region of EF-Tu for P_i Release. Although P_i dissociates from EF-Tu prior to the gross conformational change of the protein from the GTP- to the GDP-bound form, local structural changes at the nucleotide binding site would be required for P_i release to occur. Most likely, residues in the switch 1 and 2 regions of EF-Tu restrict or facilitate P_i release. One interesting candidate residue is Gly94 at the end of the switch 2 region, which is believed to affect the conformational mobility of the preceding helix and the transition of EF-Tu from the GTP to the GDP form (*1*, *18*, *19*) (Figure 3A). Gly94 is not involved in direct contacts with the γ-phosphate of GTP but, together with Gly83, provides a pivot point for a rotation of the switch 2 helix (*19*). The mutation of Gly94 to Ala had no effect on the rate of GTP hydrolysis, but caused a more than 300-fold reduction in the rates of the reactions that lead to aa-tRNA accommodation in the A site and peptide bond formation, $0.075\ s^{-1}$ (*18*). The effect of the mutation on P_i release was not investigated previously, and thus it remained unclear whether P_i release, EF-Tu conformational change, or both steps are affected by the mutation. To assess the effect of the G94A mutation on P_i release, the rates of GTP hydrolysis and P_i release were measured (Figure 3B). Exponential fitting of the time courses of GTP cleavage resulted in $k_{app} = 14\ s^{-1}$, which is the same as for wild-type EF-Tu, in agreement with the notion that the rate constants of initial binding, codon recognition, and GTPase activation are not affected by the mutation (*18*). P_i release was rapid and completed after about 0.1 s under these conditions (Figure 3B). This suggests that the slow rearrangement reported earlier, $0.075\ s^{-1}$ (reaction transit time about 10 s), does not reflect P_i release but must reflect the slow conformational change of EF-Tu(G94A) to the GDP form, which is not limited by the preceding P_i dissociation. However, the time courses of P_i release from EF-Tu(G94A) and wild-type EF-Tu were markedly different, as the time courses of GTP hydrolysis and P_i release from EF-Tu(G94A) were essentially identical and no lag phase of P_i release was observed. Global fitting of the time course of P_i release from EF-Tu(G94A) using the model described above for wild-type EF-Tu suggested that the rate constant of P_i release was too high ($>1000\ s^{-1}$) to be distinguished from GTP hydrolysis (Figure 3C). Furthermore, the experimental data clearly differ from the fit obtained assuming the rate constant for P_i release of wild-type EF-Tu, $k_{Pi} = 23\ s^{-1}$ (Figure 3C, model 2). (The small decrease in the fluorescence of MDCC-PBP observed after 0.1 s, albeit reproducible, is probably due to an artifact of the coupled indicator assay appearing with EF-Tu(G94A) and was not considered in the modeling.) In summary, the results indicated that the mutation of Gly94 eliminated the delay between GTP hydrolysis and P_i release. Thus, conformational rearrangements with a pivot at Gly94 in the switch 2 region seem to be critically involved in the control of P_i release.

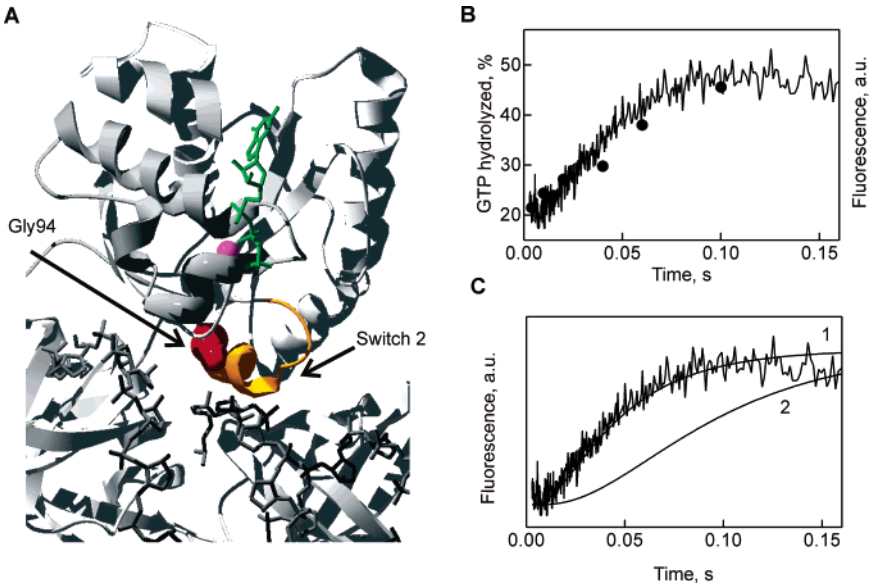

FIGURE 3: Role of Gly94 for P_i release. (A) Structural context of Gly94 in the ternary complex EF-Tu·GTP·Phe-tRNA^{Phe} (1TTT (*1*)). Gly94 (red) is located at the end of the switch 2 region (yellow) of EF-Tu; switch 2 region contacts the γ-phosphate of GTP (green) prior to GTP hydrolysis and presumably P_i after GTP cleavage. The hydrolysis of GTP induces transitions starting from Gly83 at the N terminus of the switch 2 region by breaking the hydrogen bond between the main chain amide nitrogen and the γ-phosphate, causing a rotation of the switch 2 region by 42°. The position of the switch helix is shifted from residues 87−97 in the GTP form to residues 83−93 in the GDP form. Mutation G94A severely inhibits the overall conformational change of EF-Tu to the GDP form (*18*). (B) Time courses of GTP hydrolysis (circles) and P_i release measured with purified ternary complex EF-Tu(G94A)·GTP·Phe-tRNA^{Phe} (0.2 μM) and poly(U)-programmed ribosome complexes (0.6 μM). The apparent rate constant of GTP hydrolysis was 14 s^{-1}. (C) Comparison of time course of P_i release from EF-Tu(G94A) with kinetic models assuming instantaneous P_i release after GTP hydrolysis (>1000 s^{-1}; curve 1) or a slow separate step of P_i release (23 s^{-1}; curve 2) as calculated for wild-type EF-Tu. Both models take into account the rate constants for initial binding, codon recognition, and GTP hydrolysis with EF-Tu(G94A) reported previously (*18*).

DISCUSSION

Kinetic Mechanism of A-Site Binding. The present kinetic measurement of P_i release from EF-Tu indicates that the reaction is significantly slower than the preceding GTP hydrolysis (k_{GTP} > 500 s^{-1}) which generates P_i. Thus, the kinetic scheme of A-site binding has to be expanded to include an additional step for P_i release, which proceeds at a rate constant $k_{Pi} = 23\ s^{-1}$ (Figure 4). P_i release takes place prior to, and limits the rate of, the conformational switch of EF-Tu from the GTP- to the GDP-bound form. The intrinsic rate constant of the conformational switch appears to be high compared to P_i release, resulting in an essentially instantaneous EF-Tu rearrangement following P_i dissociation. Thus, EF-Tu seems to behave like a "loaded spring" (*28*) which is held together by interactions with the γ-phosphate even after GTP hydrolysis. Only upon P_i release, the tension within EF-Tu is relieved and instantaneous relaxation into the GDP conformation takes place.

Structural Requirements for Delayed P_i Release. Charge repulsion between the products of GTP hydrolysis, P_i and GDP, would be expected to cause rapid P_i release. The delayed release of the highly negatively charged P_i indicates that certain structural elements block its immediate dissociation. Most likely, the contacts between the switch regions and the γ-phosphate of GTP might persist in the P_i·GDP state as long as the overall structure of EF-Tu does not change. In the free ternary complex, contacts with switch 1 and 2 regions to do not close the P_i pocket completely (*1*); hence P_i release from free EF-Tu may be rapid and spontaneous, consistent with the notion that the rates of GTP hydrolysis by EF-Tu and P_i release from EF-Tu·GDP·P_i are similar (*29*). However, during the interaction of the ternary complex with the ribosome the GTP-binding pocket of EF-Tu may be closed by ribosomal elements, such as the sarcin-ricin loop of 23S rRNA (*8*). Furthermore, during GTPase activation by the ribosome, the catalytic His84 in the switch 2 region of EF-Tu presumably moves toward the γ-phosphate, thereby additionally shielding the phosphate pocket (*30*). Therefore, the interaction of EF-Tu with the ribosome may contribute to the P_i retention in EF-Tu, by shielding the binding pocket and/or stabilizing a closed conformation of EF-Tu.

Local conformational changes are necessary to open the binding pocket on EF-Tu and to release P_i. The mutation of Gly94 in the switch 2 region, a residue that does not have a direct contact with the γ-phosphate of GTP, eliminates the delay of P_i release and results in P_i dissociation concomitantly with GTP cleavage. It seems likely that the mutation causes a slightly different orientation of the switch 2 region. The structural alteration does not impair GTP hydrolysis, but appears to affect the contacts at the P_i binding pocket.

Mutations in the P loop and in the beginning of the switch 2 region of $G_{i\alpha1}$ allowed the crystallization of a $G_{i\alpha1}$·GDP·P_i complex in a conformation that was distinct from both GDP- and GTP-bound forms, and formed a stable P_i binding pocket (*31*, *32*). This suggests that two structural rearrangements in the switch 2 region may be required: the first one that stabilizes the P_i binding pocket, and the second that allows for P_i dissociation (*32*). The mutation of Gly94 in EF-Tu seems to facilitate the second rearrangement, in contrast to the mutations in $G_{i\alpha1}$ which seem to inhibit it. Recently, the crystal structure of a small GTP binding protein, Rab11, in the complex with GDP and P_i has been solved. In the complex, the P_i moiety retains the classical

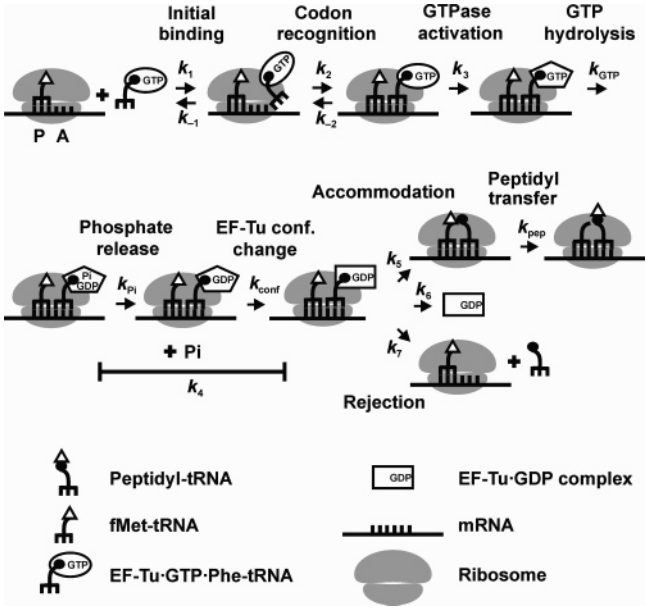

FIGURE 4: Kinetic mechanism of EF-Tu-dependent aa-tRNA binding to the A site of the ribosome. P_i release from EF-Tu (k_{Pi}) is a separate step following rapid GTP hydrolysis. It takes place with a rate constant of 23 s^{-1} and limits the subsequent conformational change of EF-Tu to the GDP conformation (k_{conf}); the latter step is intrinsically fast. In the previous kinetic analysis, P_i release and EF-Tu conformational change were not distinguished as independent steps and were grouped as k_4. All other steps are defined as described before (*42*). EF-Tu is depicted in different shapes to indicate different conformational states: ellipse, GTP bound form; pentagon, GTPase-activated state and posthydrolysis state with bound GDP and P_i; rectangle, GDP bound form.

contacts of the γ-phosphate, suggesting that the structure represents an early intermediate following GTP hydrolysis (*33*). Compared to the structure of Rab11 in the GTP-bound form, the only significant change is a slight displacement at the switch 2 region that accommodates P_i. This underlines the general importance of the switch 2 region for the formation of the P_i binding pocket and for subsequent P_i release.

In addition to the sarcin-ricin loop of 23S rRNA, EF-Tu contacts other elements of the ribosome, e.g., the ribosomal protein L7/12. L7/12 is involved in the stimulation of rapid GTP hydrolysis by both EF-Tu and EF-G (*2*, *34*). Several mutations in the C-terminal domain of L7/12 were shown to reduce the rate of P_i dissociation from EF-G, while GTP hydrolysis was unaffected, indicating that the ribosomal protein is involved in control of the P_i release. However, the same mutations did not affect P_i release from EF-Tu, suggesting that either the contact between EF-Tu and L7/12 is transient and does not persist beyond the initial binding step (*35*), or the interactions of L7/12 with EF-Tu and EF-G are different in details.

In addition to EF-Tu, EF-G, and eIF2, slow P_i release has been reported for several other GTPases. The small GTP-binding protein Ras in the complex with one of its GAPs, p120GAP, releases P_i rapidly, but the release is delayed when Ras is bound to another GAP, neurofibromin (*9*, *36*, *37*). This indicates that the rate of P_i release is a property not only of the G protein alone but also of its activating interaction partner. In the case of Ras, the delayed P_i release correlates with a strong interaction between the GTP-binding protein and the GAP (K_d < 0.3 μM for Ras·GTP·neurofibromin (*38*)) whereas rapid P_i release correlates with moderate affinity (K_d > 10 μM for Ras·GTP·p120GAP (*39*)). On the other hand, the affinity of RapGAP to Rap·GTP is moderate with a K_d of about 14 μM (*9*) and yet P_i release is 5- to 8-fold slower than GTP hydrolysis stimulated by RapGAP (*9*, *40*). Thus, the data on P_i release and GAP interaction for different GTP-binding proteins are limited and do not establish a clear correlation between these two parameters.

Functional Significance of P_i Release from EF-Tu. Slow P_i release compared to GTP hydrolysis has been observed in EF-G and eIF2 (*14*, *21*). In both cases, the delay in P_i dissociation is important to control the coupling between GTP hydrolysis and the function of the factors in tRNA translocation and initiator Met-$tRNA^i$ release from the factor, respectively (*12*, *14*). The question arises whether the delay in P_i release from EF-Tu may also fulfill a control function in aa-tRNA delivery to the ribosome. GTP hydrolysis in EF-Tu is an important determinant for the speed and fidelity of aa-tRNA selection on the ribosome (*41*, *42*). If the rates of P_i release from cognate and near-cognate ternary complexes were grossly different, i.e., fast with cognate and slow with near- or non-cognate aa-tRNA, this could further contribute to the discrimination of incorrect aa-tRNAs. However, the rate constant previously assigned to the EF-Tu conformational change (k_4) was the same for cognate and near-cognate aa-tRNAs (*4*). As the rate of the EF-Tu rearrangement is limited by P_i release, the observed rate represents the rate of P_i dissociation which thus does not depend on correct codon−anticodon pairing, suggesting that P_i release does not contribute to the accuracy of aa-tRNA selection.

While the functional significance of the delayed P_i release from EF-Tu appears unclear, it seems to be a consequence of the interactions of EF-Tu with its ligands. First, the EF-Tu complex with GDP is thermodynamically much more stable than that with GTP. The stabilization of the GTP-bound structure must be provided by interactions with the γ-phosphate of GTP. It may be inevitable that these interactions persist for some time after GTP cleavage, until the local conformational rearrangement of the switch regions has taken place. Second, the interaction of EF-Tu with the ribosome is very tight, with a K_d of the codon recognition complex in the range of 1 nM (*30*), which may be important to keep the GTPase activity of EF-Tu tightly controlled (*4*). Binding to the ribosome might close the P_i binding pocket of EF-Tu, resulting in slow P_i release. Only in special cases, such as EF-G and eIF2, delayed P_i release might have evolved into an additional control step.

In summary, we have shown that phosphate release from EF-Tu is significantly slower than GTP hydrolysis and limits the subsequent global conformational change of EF-Tu from the GTP-bound to the GDP-bound form. P_i release requires a local conformational change which involves movements of the switch 2 region. In EF-Tu, delayed P_i release does not seem to have a role for aa-tRNA selection, but appears to be a consequence of the tight interaction of the switch regions of EF-Tu with the γ-phosphate and the ribosome.

ACKNOWLEDGMENT

We thank Charlotte Knudsen for plasmid coding for EF-Tu(G94A); Andrea Parmeggiani and Poul Nissen for kirro-

mycin; Yuri Semenkov and Vladimir Katunin (Petersburg Nuclear Physics Institute, Russia) for purified tRNA; and Petra Striebeck, Carmen Schillings, Astrid Böhm, and Simone Möbitz for expert technical assistance.

REFERENCES

1. Nissen, P., Kjeldgaard, M., Thirup, S., Polekhina, G., Reshetnikova, L., Clark, B. F., and Nyborg, J. (1995) Crystal structure of the ternary complex of Phe-tRNA^{Phe}, EF-Tu, and a GTP analog, *Science 270*, 1464−1472.
2. Diaconu, M., Kothe, U., Schlunzen, F., Fischer, N., Harms, J. M., Tonevitsky, A. G., Stark, H., Rodnina, M. V., and Wahl, M. C. (2005) Structural basis for the function of the ribosomal L7/12 stalk in factor binding and GTPase activation, *Cell 121*, 991−1004.
3. Rodnina, M. V., Gromadski, K. B., Kothe, U., and Wieden, H. J. (2005) Recognition and selection of tRNA in translation, *FEBS Lett. 579*, 938−942.
4. Pape, T., Wintermeyer, W., and Rodnina, M. (1999) Induced fit in initial selection and proofreading of aminoacyl-tRNA on the ribosome, *EMBO J. 18*, 3800−3807.
5. Abel, K., Yoder, M. D., Hilgenfeld, R., and Jurnak, F. (1996) An α to β conformational switch in EF-Tu, *Structure 4*, 1153−1159.
6. Polekhina, G., Thirup, S., Kjeldgaard, M., Nissen, P., Lippmann, C., and Nyborg, J. (1996) Helix unwinding in the effector region of elongation factor EF- Tu-GDP, *Structure 4*, 1141−1151.
7. Vogeley, L., Palm, G. J., Mesters, J. R., and Hilgenfeld, R. (2001) Conformational change of elongation factor Tu (EF-Tu) induced by antibiotic binding. Crystal structure of the complex between EF-Tu.GDP and aurodox, *J. Biol. Chem. 276*, 17149−17155.
8. Stark, H., Rodnina, M. V., Wieden, H. J., Zemlin, F., Wintermeyer, W., and van Heel, M. (2002) Ribosome interactions of aminoacyl-tRNA and elongation factor Tu in the codon-recognition complex, *Nat. Struct. Biol. 9*, 849−854.
9. Kraemer, A., Brinkmann, T., Plettner, I., Goody, R., and Wittinghofer, A. (2002) Fluorescently labelled guanine nucleotide binding proteins to analyse elementary steps of GAP-catalysed reactions, *J. Mol. Biol. 324*, 763−774.
10. Ting, T. D., and Ho, Y. K. (1991) Molecular mechanism of GTP hydrolysis by bovine transducin: pre-steady-state kinetic analyses, *Biochemistry 30*, 8996−9007.
11. Guy, P. M., Koland, J. G., and Cerione, R. A. (1990) Rhodopsin-stimulated activation-deactivation cycle of transducin: kinetics of the intrinsic fluorescence response of the alpha subunit, *Biochemistry 29*, 6954−6964.
12. Savelsbergh, A., Katunin, V. I., Mohr, D., Peske, F., Rodnina, M. V., and Wintermeyer, W. (2003) An elongation factor G-induced ribosome rearrangement precedes tRNA-mRNA translocation, *Mol. Cell 11*, 1517−1523.
13. Savelsbergh, A., Mohr, D., Kothe, U., Wintermeyer, W., and Rodnina, M. V. (2005) Control of phosphate release from elongation factor G by ribosomal protein L7/12, *EMBO J. 24*, 4316−4323.
14. Algire, M. A., Maag, D., and Lorsch, J. R. (2005) Pi release from eIF2, not GTP hydrolysis, is the step controlled by start-site selection during eukaryotic translation initiation, *Mol. Cell 20*, 251−262.
15. Takagi, Y., Shuman, H., and Goldman, Y. E. (2004) Coupling between phosphate release and force generation in muscle actomyosin, *Philos. Trans. R. Soc. London, Ser. B 359*, 1913−1920.
16. Cross, R. A. (2004) The kinetic mechanism of kinesin, *Trends Biochem. Sci. 29*, 301−309.
17. Pape, T., Wintermeyer, W., and Rodnina, M. V. (1998) Complete kinetic mechanism of elongation factor Tu-dependent binding of aminoacyl-tRNA to the A site of the E. coli ribosome, *EMBO J. 17*, 7490−7497.
18. Knudsen, C., Wieden, H. J., and Rodnina, M. V. (2001) The importance of structural transitions of the switch II region for the functions of elongation factor Tu on the ribosome, *J. Biol. Chem. 276*, 22183−22190.
19. Knudsen, C. R., Kjaersgard, I. V., Wiborg, O., and Clark, B. F. (1995) Mutation of the conserved Gly94 and Gly126 in elongation factor Tu from Escherichia coli. Elucidation of their structural and functional roles, *Eur. J. Biochem. 228*, 176−183.
20. Rodnina, M. V., and Wintermeyer, W. (1995) GTP consumption of elongation factor Tu during translation of heteropolymeric mRNAs, *Proc. Natl. Acad. Sci. U.S.A. 92*, 1945−1949.
21. Rodnina, M. V., Savelsbergh, A., Matassova, N. B., Katunin, V. I., Semenkov, Y. P., and Wintermeyer, W. (1999) Thiostrepton inhibits the turnover but not the GTPase of elongation factor G on the ribosome, *Proc. Natl. Acad. Sci. U.S.A. 96*, 9586−9590.
22. Brune, M., Hunter, J. L., Corrie, J. E., and Webb, M. R. (1994) Direct, real-time measurement of rapid inorganic phosphate release using a novel fluorescent probe and its application to actomyosin subfragment 1 ATPase, *Biochemistry 33*, 8262−8271.
23. Gromadski, K. B., and Rodnina, M. V. (2004) Kinetic determinants of high-fidelity tRNA discrimination on the ribosome, *Mol. Cell 13*, 191−200.
24. Gromadski, K. B., Daviter, T., and Rodnina, M. V. (2006) A uniform response to mismatches in codon-anticodon complexes ensures ribosomal fidelity, *Mol. Cell 21*, 369−77.
25. Pape, T., Wintermeyer, W., and Rodnina, M. V. (2000) Conformational switch in the decoding region of 16S rRNA during aminoacyl-tRNA selection on the ribosome, *Nat. Struct. Biol. 7*, 104−7.
26. Rodnina, M. V., Fricke, R., and Wintermeyer, W. (1994) Transient conformational states of aminoacyl-tRNA during ribosome binding catalyzed by elongation factor Tu, *Biochemistry 33*, 12267−12275.
27. Valle, M., Zavialov, A., Li, W., Stagg, S. M., Sengupta, J., Nielsen, R. C., Nissen, P., Harvey, S. C., Ehrenberg, M., and Frank, J. (2003) Incorporation of aminoacyl-tRNA into the ribosome as seen by cryo-electron microscopy, *Nat. Struct. Biol. 10*, 899−906.
28. Vetter, I. R., and Wittinghofer, A. (2001) The guanine nucleotide-binding switch in three dimensions, *Science 294*, 1299−1304.
29. Kalbitzer, H. R., Goody, R. S., and Wittinghofer, A. (1984) Electron-paramagnetic-resonance studies of manganese(II) complexes with elongation factor Tu from *Bacillus stearothermophilus*. Observation of a GTP hydrolysis intermediate state complex, *Eur. J. Biochem. 141*, 591−597.
30. Daviter, T., Wieden, H. J., and Rodnina, M. V. (2003) Essential role of histidine 84 in elongation factor Tu for the chemical step of GTP hydrolysis on the ribosome, *J. Mol. Biol. 332*, 689−699.
31. Raw, A. S., Coleman, D. E., Gilman, A. G., and Sprang, S. R. (1997) Structural and biochemical characterization of the GTPγS-, GDP.Pi-, and GDP-bound forms of a GTPase-deficient Gly^{42} → Val mutant of $G_{i\alpha1}$, *Biochemistry 36*, 15660−15669.
32. Berghuis, A. M., Lee, E., Raw, A. S., Gilman, A. G., and Sprang, S. R. (1996) Structure of the GDP-Pi complex of Gly203→Ala $G_{i\alpha1}$: a mimic of the ternary product complex of Gα-catalyzed GTP hydrolysis, *Structure 4*, 1277−1290.
33. Pasqualato, S., and Cherfils, J. (2005) Crystallographic evidence for substrate-assisted GTP hydrolysis by a small GTP binding protein, *Structure 13*, 533−540.
34. Mohr, D., Wintermeyer, W., and Rodnina, M. V. (2002) GTPase activation of elongation factors Tu and G on the ribosome, *Biochemistry 41*, 12520−12528.
35. Kothe, U., Wieden, H. J., Mohr, D., and Rodnina, M. V. (2004) Interaction of helix D of elongation factor Tu with helices 4 and 5 of protein L7/12 on the ribosome, *J. Mol. Biol. 336*, 1011−1021.
36. Phillips, R. A., Hunter, J. L., Eccleston, J. F., and Webb, M. R. (2003) The mechanism of Ras GTPase activation by neurofibromin, *Biochemistry 42*, 3956−3965.
37. Allin, C., Ahmadian, M. R., Wittinghofer, A., and Gerwert, K. (2001) Monitoring the GAP catalyzed H-Ras GTPase reaction at atomic resolution in real time, *Proc. Natl. Acad. Sci. U.S.A. 98*, 7754−7759.
38. Ahmadian, M. R., Hoffmann, U., Goody, R. S., and Wittinghofer, A. (1997) Individual rate constants for the interaction of Ras proteins with GTPase-activating proteins determined by fluorescence spectroscopy, *Biochemistry 36*, 4535−4541.
39. Brownbridge, G. G., Lowe, P. N., Moore, K. J., Skinner, R. H., and Webb, M. R. (1993) Interaction of GTPase activating proteins (GAPs) with p21ras measured by a novel fluorescence anisotropy method. Essential role of Arg-903 of GAP in activation of GTP hydrolysis on p21ras, *J. Biol. Chem. 268*, 10914−10919.
40. Chakrabarti, P. P., Suveyzdis, Y., Wittinghofer, A., and Gerwert, K. (2004) Fourier transform infrared spectroscopy on the

Rap.RapGAP reaction, GTPase activation without an arginine finger, *J. Biol. Chem. 279*, 46226−46233.
41. Daviter, T., Gromadski, K. B., and Rodnina, M. V. (2006) The ribosome's response to codon-anticodon mismatches, *Biochimie 88*, 1001−1011.
42. Rodnina, M. V., and Wintermeyer, W. (2001) Fidelity of aminoacyl-tRNA selection on the ribosome: kinetic and structural mechanisms, *Annu. Rev. Biochem. 70*, 415−435.

BI061192Z