THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 30, pp. 21139–21146, July 28, 2006

The Importance of P-loop and Domain Movements in EF-Tu for Guanine Nucleotide Exchange*

Received for publication, March 6, 2006, and in revised form, May 17, 2006 Published, JBC Papers in Press, May 22, 2006, DOI 10.1074/jbc.M602068200

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Elongation factor Ts (EF-Ts) is the guanine nucleotide exchange factor for elongation factor Tu (EF-Tu). An important feature of the nucleotide exchange is the structural rearrangement of EF-Tu in the EF-Tu·EF-Ts complex caused by insertion of Phe-81 of EF-Ts between His-84 and His-118 of EF-Tu. In this study, the contribution of His-118 to nucleotide release was studied by pre-steady state kinetic analysis of nucleotide exchange in EF-Tu mutants in which His-118 was replaced by Ala or Glu. Intrinsic as well as EF-Ts-catalyzed release of GDP/GTP was affected by the mutations, resulting in an ~10-fold faster spontaneous nucleotide release and a 10–50-fold slower EF-Ts-catalyzed nucleotide release. The effects are attributed to the interference of the mutations with the EF-Ts-induced movements of the P-loop of EF-Tu and changes at the domain 1/3 interface, leading to the release of the β-phosphate group of GTP/GDP. The K_d for GTP is increased by more than 40 times when His-118 is replaced with Glu, which may explain the inhibition by His-118 mutations of aminoacyl-tRNA binding to EF-Tu. The mutations had no effect on EF-Tu-dependent delivery of aminoacyl-tRNA to the ribosome.

The bacterial EF-Tu[3] is a GTP-binding protein that consists of three domains, of which domain 1 (the G domain) is responsible for the binding of guanine nucleotides (1). Like all GTP-binding proteins, EF-Tu alternates between an inactive, GDP-bound form and an active, GTP-bound conformation required for the binding of aminoacyl-tRNA (aa-tRNA) and its subsequent delivery to the A-site of the ribosome. EF-Tu has higher affinity for GDP than for GTP (K_d(GDP) ~ 10^{-9} M when compared with K_d(GTP) ~ 10^{-8} M), and the dissociation of GDP from EF-Tu is too slow to be physiologically relevant (0.002 s^{-1}) (2, 3). Rapid nucleotide exchange in EF-Tu requires the action of a guanine nucleotide exchange factor, EF-Ts. The exchange reaction is initiated by the binding of EF-Ts to EF-Tu·GDP to form the EF-Tu·GDP·EF-Ts complex, which is unstable and rapidly dissociates into GDP and EF-Tu·EF-Ts. Next, GTP binds to the EF-Tu·EF-Ts complex to form the intermediary EF-Tu·GTP·EF-Ts complex, which finally dissociates into EF-Ts and EF-Tu·GTP, thereby completing the exchange reaction (see Fig. 1*A*). All steps are reversible, and *in vivo*, the reaction is driven toward formation of active EF-Tu for two reasons: (i) the formation of GTP-bound EF-Tu is favored by the higher intracellular concentration of GTP over GDP (0.9 *versus* 0.1 mM, respectively (4)) and (ii) the high concentration of aa-tRNA shifts the equilibrium toward the GTP-bound state due to the formation of EF-Tu·GTP·aa-tRNA, with no effect on the kinetics of nucleotide exchange (2).

In the EF-Tu·EF-Ts complex, EF-Ts interacts with domains 1 and 3 of EF-Tu (5–7). Domain 1 of EF-Tu contacts the N-terminal domain, subdomain N, and the C-terminal module of EF-Ts, whereas domain 3 of EF-Tu interacts with subdomain C of EF-Ts. Structural and kinetic studies indicated that three factors contribute to the nucleotide exchange: (i) disruption of the Mg^{2+} binding site in domain 1 of EF-Tu, (ii) a structural change in the phosphate binding loop (P-loop) in domain 1 of EF-Tu that causes an altered binding of the phosphate moieties of GDP/GTP, and (iii) a relaxation of the interaction with the ribose and/or guanine base (2, 5–9). One of the most conspicuous interactions in the crystal structure of the *Escherichia coli* EF-Tu·EF-Ts complex is the intrusion of EF-Ts Phe-81 between two histidine residues of domain 1 of EF-Tu, His-84 of helix B and His-118 of helix C (see Fig. 1*B*). This intrusion causes a displacement of the two histidines, which was proposed to be propagated to cause the release of the bound nucleotide. The displacement of His-84 results in disruption of the Mg^{2+} binding site, whereas the displacement of His-118 disrupts the binding of the β-phosphate due to a successive displacement of residues Gln-114, His-19, and Val-20, of which the latter two are situated in the P-loop (the loop that connects strand a and helix A in EF-Tu and contains the consensus element G*XXXX*GK(S/T) involved in the coordination of the phosphates of GDP/GTP). The displacement of Val-20 causes a flip in the backbone of the P-loop that breaks the hydrogen bond to the β-phosphate (6). The flip of the P-loop was found in a number of other G-proteins in complexes with their respective guanine nucleotide exchange factors, suggesting that displacement of the P-loop is a universally important step in guanine nucleotide release (10). However, the contribution of EF-Ts-induced rearrangements in the P-loop of EF-Tu to the acceleration of nucleotide exchange remained unclear despite several

* This work was supported by the Danish Research Council of Natural Sciences, the Deutsche Forschungsgemeinschaft, the Alfried Krupp von Bohlen und Halbach-Stiftung, and the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[3] The abbreviations used are: EF-Tu, elongation factor Tu; EF-Ts, elongation factor Ts; aa-tRNA, aminoacyl-tRNA; mant-GDP/GTP, (2′/3′-*O*-(*N*-methylanthraniloyl-GDP/GTP); DTT, dithiothreitol; FRET, fluorescence resonance energy transfer.

mutagenesis studies (9, 11–13). In the present work, we examine the contribution of P-loop movement to nucleotide exchange by replacing one of the critical His residues, His-118, with Ala or Glu. These mutations are expected to hamper the successive displacement of residues in helix C of EF-Tu induced by EF-Ts binding and thereby abrogate the P-loop flip. The effects of these mutations on the elemental steps of the nucleotide exchange reaction were studied by using a pre-steady state kinetic approach (2, 8).

His-118 has also been reported to play a role in aa-tRNA binding (12, 14, 15), which is, however, difficult to rationalize given that His-118 is located at least 16 Å from the aa-tRNA in the crystal structure of the ternary complex, EF-Tu·GDPNP·Phe-tRNA^{Phe} (16). On the ribosome, pairing between the mRNA codon and the anticodon of tRNA induces GTP hydrolysis in EF-Tu (17), and the transmission of the signal between the decoding and GTPase centers of the ribosome may be modulated by tRNA-EF-Tu interactions (18). Therefore, if His-118 is involved in aa-tRNA binding (12, 14, 15), this would imply a potential role of that contact in GTPase activation of EF-Tu on the ribosome. We studied this possibility by monitoring aa-tRNA delivery to the ribosome by pre-steady state fluorescence kinetics methods.

EXPERIMENTAL PROCEDURES

Mutagenesis—Site-directed mutagenesis was performed on a pGEX construct containing the *E. coli tuf A* gene (19) by using the QuikChange® II site-directed mutagenesis kit (Stratagene). The plasmid was constructed in such a way as to yield the native sequence of EF-Tu after cleavage of the protein with factor X_a. Mutations were confirmed by DNA sequencing.

EF-Tu—EF-Tu was expressed with an N-terminal glutathione *S*-transferase tag in *E. coli* JM109 as described previously (19). Purification was carried out at 4 °C. Cells were resuspended in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1% Triton X-100, 15 μM GDP, opened in a high pressure homogenizer (Avestin) and applied to a glutathione-agarose column (Sigma). The column was extensively washed first with 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 15 μM GDP and then with the same buffer containing 1 mM GDP to remove any traces of EF-Tu-bound EF-Ts. EF-Tu was eluted with 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM glutathione, 15 μM GDP, dialyzed against 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 15 μM GDP, and concentrated. Removal of the glutathione *S*-transferase tag was carried out by cleavage with 1 μg of factor X_a (Qiagen)/150 μg of fusion protein for 8 h at 4 °C. The cleaved sample was applied to the glutathione-agarose column, and EF-Tu was eluted with 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 15 μM GDP. Residual EF-Ts co-purified with the EF-TuH118A mutant was separated from free EF-TuH118A by gel filtration on Superdex 75 (Amersham Biosciences). Activity of all EF-Tu preparations in nucleotide binding was close to 90% as measured by nitrocellulose filtration techniques (20).

EF-Ts—EF-Ts was expressed in *E. coli* BL21 (DE3) cells as a fusion protein with the intein self-splicing element and a chitin binding domain (IMPACTI system, New England Biolabs) as described (21). The cells were resuspended in 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 10 mM $MgCl_2$, 1.0 g/liter Triton X-100, 50 μM GDP, 10 (v/v) % glycerol and lysed with 1 mg of lysozyme/g of cells in the presence of 2% deoxycholate and 40 μg of DNase/g of cells. After centrifugation, the supernatant was loaded on a chitin column equilibrated with pre-cleavage buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl). After fast equilibration with cleavage buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 60 mM DTT), the column was incubated for 20 h at 4 °C followed by 22 h of incubation at 21 °C, resulting in the cleavage of the tag from EF-Ts. EF-Ts was eluted with 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 1.0 g/liter Triton X-100 and dialyzed against storage buffer (20 mM Tris-HCl, pH 7.2, 40 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 20 (v/v) % glycerol).

Determination of Protein Concentration—Protein concentration was determined according to Bradford (22) and confirmed by comparing the intensity of a Coomassie Brilliant Blue R-stained band on an SDS-PAGE gel to that of a known standard by using an imaging densitometer (Bio-Rad GS-700). The concentration of nucleotide-free EF-Tu was determined according to Bradford and photometrically at 280 nm by using a molar extinction coefficient of 32,900 M^{-1} cm^{-1} (23).

Preparation of Nucleotide-free EF-Tu—To promote dissociation of GDP from EF-Tu, which forms a tight 1:1 complex during all purification steps, the protein was incubated in 50 mM Tris-HCl, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 1 mM DTT, 30 mM EDTA for 30 min at 37 °C. EF-Tu was separated from nucleotide by gel filtration on Superdex 75 and was immediately used in the stopped-flow experiments. Nucleotide-free EF-Tu was stable within the time of experiment and retained the characteristics of untreated EF-Tu with respect to the binding of EF-Ts and GDP/GTP (2).

Preparation of EF-Tu·mant-GTP, EF-Tu·mant-GDP, and EF-Tu·EF-Ts—Mant-GDP and mant-GTP (2′/3′-*O*-(*N*-methyl-anthraniloyl-GDP or -GTP) were purchased from Jena Bio-Science. EF-Tu was incubated with a 3-fold excess of mant-labeled nucleotide in 50 mM Tris-HCl, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$, and 1 mM DTT for 30 min at 37 °C. To remove GDP from reactions involving GTP, the experiments were performed in the presence of 3 mM phosphoenolpyruvate and 0.1 mg/ml pyruvate kinase. The EF-Tu·EF-Ts complex was prepared by incubation of EF-Tu and EF-Ts in equimolar amounts for 30 min at 37 °C in 50 mM Tris-HCl, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 1 mM DTT, 30 mM EDTA. The complex was purified by gel filtration on a Superdex 75 column.

Preparation of the Ternary Complex, EF-Tu·GTP·Phe-tRNA^{Phe}—Ribosome binding assays (24) showed that binding of the ternary complex to ribosomes saturated at a 2:1 ratio of EF-Tu·GTP·Phe-tRNA^{Phe} to ribosomes for the wild type or EF-TuH118A mutant and at a 4:1 ratio for the EF-TuH118E mutant. For ternary complex formation, 3.7 μM wild-type EF-Tu, 3.7 μM EF-TuH118A, or 7.4 μM EF-TuH118E mutant was incubated with 1.9 μM tRNA^{Phe}(proflavin 16/17), 3 mM ATP, 1 mM phenylalanine, 0.5% v/v yeast phenylalanyl-tRNA synthetase, 0.05 μM EF-Ts, 3 mM phosphoenolpyruvate, 1 mM GTP, 0.1 mg/ml pyruvate kinase in 50 mM Tris-HCl, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$, 1 mM DTT at 37 °C for 45 min. At least 65% of added tRNA was aminoacylated, yielding a concentration of ~1.2 μM ternary complex.

Ribosome Complexes—Ribosomes and tRNAs from *E. coli* MRE600 were prepared as described previously (25, 26). Ribo-

somes were programmed by incubating 0.4 μM purified ribosomes with 0.8 μM MFTI mRNA (26), 0.4 μM initiation factor 1, 0.4 μM initiation factor 2, 0.4 μM initiation factor 3, 0.8 μM [^{3}H]fMet-tRNA^{fMet}, 1 mM GTP in 50 mM Tris-HCl, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$, 1 mM DTT at 37° for 70 min. The efficiency of ribosome initiation was checked by nitrocellulose filtration and subsequent scintillation counting.

Rapid Kinetic Measurements—The interaction of EF-Tu with guanine nucleotides and EF-Ts was studied essentially, as described previously (2, 3). Fluorescence stopped-flow measurements were performed on a SX-18MV spectrometer (Applied Photophysics) in buffer 50 mM Tris-HCl, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$, and 1 mM DTT at 20 °C. The interaction between EF-Tu and EF-Ts was observed directly by following the fluorescence change of EF-Tu Trp-184, which exhibits a fluorescence change upon EF-Ts binding (27). Tryptophan fluorescence was excited at 280 nm and measured after passing KV335 filters (Schott). The fluorescence of mant-GDP bound to EF-Tu was excited via fluorescence resonance energy transfer (FRET) from tryptophan excited at 280 nm and measured after passing KV408 filters (Schott). Proflavin fluorescence was excited at 470 nm and measured after passing KV 500 filters (Schott). Experiments were performed by rapidly mixing equal volumes (60 μl each) of the reactants and monitoring the time course of fluorescence change. Time courses depicted in the figures were obtained by averaging 5–10 individual transients. Data were evaluated by fitting to an exponential function with a characteristic time constant (k_{app}), amplitude (A), and another variable for the final signal ($F\infty$) according to the equation $F = F\infty + A \exp(-k_{app} \cdot t)$, where F is the fluorescence at time t. Calculations were performed by using TableCurve software (Jandel Scientific). Standard deviations for apparent rate constants, k_{app}, were calculated by using the same software.

RESULTS

Experimental Prerequisites to Measure Nucleotide Exchange—The kinetic scheme of nucleotide exchange in EF-Tu is shown in Fig. 1*A*. EF-Tu can bind GDP (association rate constant k_1), GTP (k_5), or EF-Ts (k_2) to form the respective binary complexes, which dissociate with the rate constants k_{-1}, k_{-5}, and k_{-2}, respectively. The interaction of EF-Tu·GDP or EF-Tu·GTP with EF-Ts can be described by two consecutive equilibria that represent the formation of the ternary complex and nucleotide release, respectively. EF-Ts interacts with EF-Tu·GDP to form the ternary complex EF-Tu·GDP·EF-Ts (association and dissociation rate constants k_3 and k_{-3}, respectively). GDP is released from the ternary complex, forming the binary complex EF-Tu·EF-Ts (k_{-4}); the reaction is reversible (k_4). By analogy, EF-Tu·GTP can bind EF-Ts (k_6), and the ternary complex can dissociate into either EF-Tu·GTP and EF-Ts (k_{-6}) or EF-Tu·EF-Ts and GTP (k_{-7}). The binding of GTP to the binary complex EF-Tu·EF-Ts is characterized by k_7. Note that the rate constants k_1, k_2, k_3, k_4, k_5, k_6, and k_7 are bimolecular association rate constants ($M^{-1}s^{-1}$), whereas the rate constants k_{-1}, k_{-2}, k_{-3}, k_{-4}, k_{-5}, k_{-6}, and k_{-7} are monomolecular dissociation rate constants (s^{-1}).

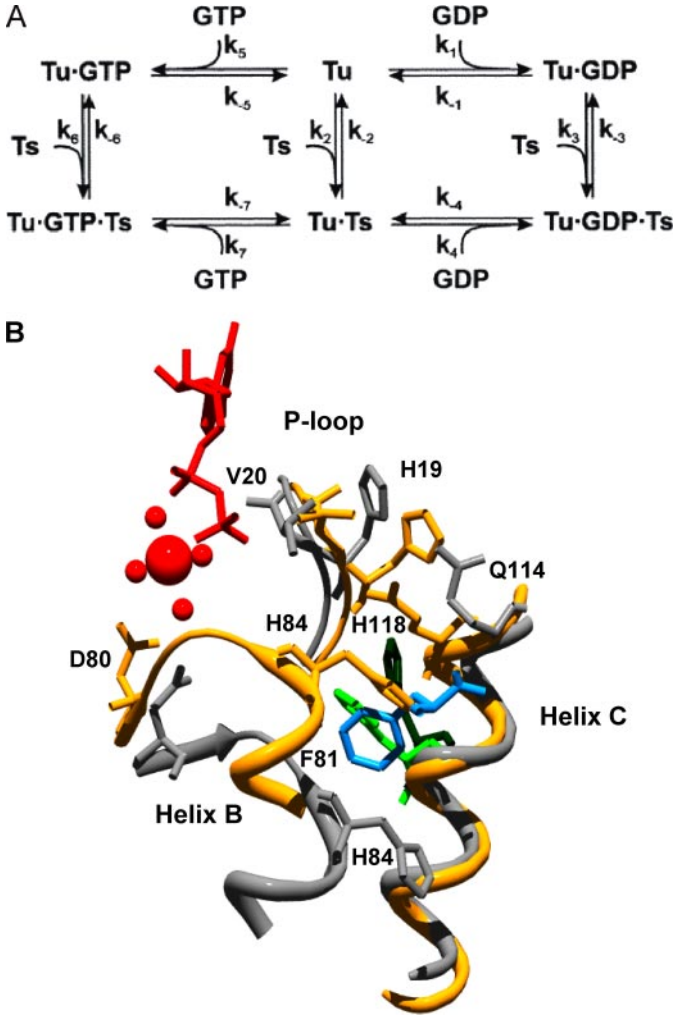

FIGURE 1. **Nucleotide exchange in EF-Tu.** *A*, kinetic scheme of EF-Tu interaction with EF-Ts and nucleotides (2). *B*, orientation of EF-Tu His-118 in the EF-Tu·GDP (*yellow*) or EF-Tu·EF-Ts (*gray*) complexes. EF-Tu His-118 is shown in *light* and *dark green* in the EF-Tu·GDP and EF-Tu·EF-Ts complexes, respectively. EF-Ts Phe-81 is shown in *blue*, and GDP with the water-coordinated Mg^{2+} is shown in *red*. The figure was prepared by using coordinates 1EFU and 1EFC in the Protein Data Bank (6, 33).

EF-Ts-free EF-Tu has to be used to measure the kinetics of intrinsic nucleotide exchange. EF-Tu binds EF-Ts tightly (K_d = 3 nM (2)); therefore, EF-Ts often appears as a contaminant in EF-Tu preparations. However, the EF-Tu·EF-Ts complex is unstable in the presence of guanine nucleotides, and to avoid EF-Ts contamination, EF-Tu was purified in the presence of 1 mM GDP. The following experiment was carried out to test that no traces of EF-Ts were present in the resulting EF-Tu preparations. EF-Tu was preincubated with mant-GDP, and the complex was rapidly mixed with an excess of unlabeled GDP in the stopped-flow apparatus. The release of the bound mant-GDP from the EF-Tu·mant-GDP complex could be monitored by a decrease in mant-GDP fluorescence by using the FRET from Trp-184 in EF-Tu to mant-GDP or mant-GTP. In the absence of EF-Ts, the release was very low, 0.002 s^{-1} (2). If a given EF-Tu preparation does not contain EF-Ts, the addition of this EF-Tu together with GDP to the EF-Tu·mant-GDP complex should not affect the rate of mant-GDP release. If, in contrast, traces

of EF-Ts were present in the test sample, the rate of mant-GDP dissociation from the wild-type EF-Tu should increase. Testing different mutant EF-Tu preparations showed that the preparation of the EF-TuH118E mutant was free of EF-Ts (Fig. 2). Traces of EF-Ts that were present in the EF-TuH118A preparation were removed by gel filtration (Fig. 2).

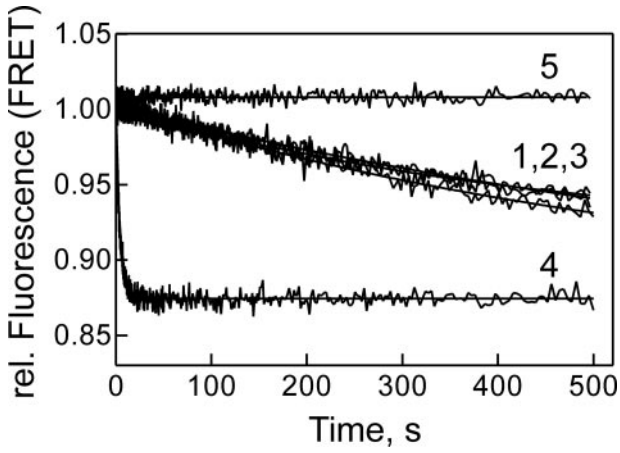

FIGURE 2. **Control of EF-Tu purity.** EF-Ts-free wild-type EF-Tu·mant-GDP complex (0.4 μM EF-Tu, 1.2 μM mant-GDP) was rapidly mixed with excess unlabeled GDP (120 μM) alone (*trace 1*) or together with EF-TuH118E (0.4 μM) (*trace 2*), EF-TuH118A (0.4 μM) after the additional purification by gel filtration (*trace 3*), or EF-Ts (0.04 μM) (*trace 4*). *Trace 5*, buffer control. *rel.*, relative.

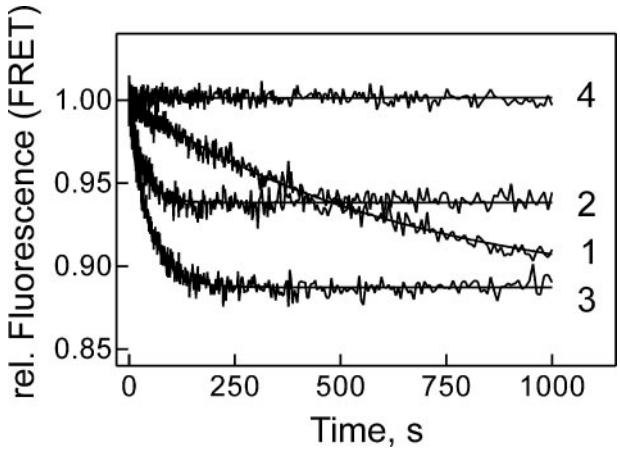

FIGURE 3. **Spontaneous dissociation of mant-GDP from EF-Tu (k_{-1}).** EF-Tu·mant-GDP complex (0.2–0.4 μM EF-Tu, 1.2 μM mant-GDP) was mixed with excess unlabeled GDP (120 μM) (*traces 1–3*) or buffer without GDP (*trace 4*). Dissociation of the binary complex was measured with wild-type EF-Tu (*traces 1* and *4*), EF-TuH118A; (*trace 2*); or EF-TuH118E (*trace 3*). *rel.*, relative.

Nucleotide Exchange—The interaction of EF-Tu with guanine nucleotides in the absence of EF-Ts was studied essentially as described (2, 3) by using FRET from Trp-184 in EF-Tu to mant-GDP or mant-GTP. FRET causes an increase of mant fluorescence upon binding of the nucleotide to the factor and a decrease of fluorescence upon dissociation of the complex. The rate constants of GDP release (k_{-1}) were 0.002 s^{-1} for wild type and 0.03 and 0.02 s^{-1} for the EF-TuH118A and EF-TuH118E mutants, respectively (Fig. 3). Thus, the rate constants for the two mutants were 10 times higher than for the wild-type EF-Tu. Similarly, the rate constants of GTP dissociation (k_{-5}) were 10 times higher for the EF-TuH118A and EF-TuH118E mutants, 0.2 and 0.5 s^{-1}, respectively, than that for wild-type EF-Tu, 0.02 s^{-1} (Table 1).

To measure the association rate constants k_1 and k_5, a constant amount of nucleotide-free EF-Tu was mixed with increasing amounts of mant-GDP or mant-GTP. Apparent rate constants, k_{app}, obtained by single-exponential fitting, were plotted against nucleotide concentration, yielding straight lines with slopes of k_1 (Fig. 4) or k_5 (data not shown). The association rate constants for both nucleotides were similar for the wild-type EF-Tu and the two mutants (Table 1). The K_d values for GDP and GTP calculated from the association and dissociation rate constants were 10–50 times lower for the mutants when compared with the wild type (Table 1).

To measure the rate constant of EF-Ts-catalyzed GDP release, k_{-4}, a constant amount of EF-Tu·mant-GDP was mixed with increasing amounts of EF-Ts. An excess of unlabeled GDP was included with the EF-Ts to prevent rebinding of mant-GDP (Fig. 5*A*). The apparent rate constants of mant-GDP release were plotted against increasing EF-Ts concentration (Fig. 5*B*). In the initial linear part of the hyperbolic curve (Fig. 5*C*), the association of EF-Ts to EF-Tu·mant-GDP was rate-limiting, and the association rate constant k_3 could be determined according to the equation: initial slope = $k_3/(1+k_{-3}/k_{-4})$ (28), provided that k_{-3} and k_{-4} are known (see below). At saturation with EF-Ts, the release reaction itself was rate-limiting. As the rate of mant-GDP rebinding was negligible in the presence of excess unlabeled GDP, the apparent rate constant at saturation

TABLE 1

Rate constants and equilibrium dissociation constants of interactions between EF-Tu, EF-Ts, and guanine nucleotides

Standard deviations were less than 10%, unless stated otherwise.

EF-Tu	GDP cycle								
	k_1	k_{-1}	K_{d1}	k_3	k_{-3}	K_{d3}	k_4	k_{-4}	K_{d4}
	$10^6\ s^{-1}\ M^{-1}$	s^{-1}	nM	$10^7\ s^{-1}\ M^{-1}$	s^{-1}	μM	$10^7\ s^{-1}\ M^{-1}$	s^{-1}	μM
Wild-type	2.0	0.002	1	3[a]	240	10[a]	0.7[a]	150	20[a]
H118A	3.5	0.03	9	7	280	4	0.5	2.3	0.5
H118E	1.2	0.02	17	1[b]	160[a]	20[b]	0.1[b]	2.8	3[b]
EF-Tu	**GTP cycle**								
	k_5	k_{-5}	K_{d5}	k_6	k_{-6}	K_{d6}	k_7	k_{-7}	K_{d7}
	$10^5\ s^{-1}\ M^{-1}$	s^{-1}	nM	$10^7\ s^{-1}\ M^{-1}$	s^{-1}	μM	$10^6\ s^{-1}\ M^{-1}$	s^{-1}	μM
Wild-type	2.7	0.02	70[b]	3[a]	80	3[a]	1.6[b]	100	60[b]
H118A	2.5	0.2	800	9	120	1.4	0.4[b]	4.4	10[b]
H118E	1.6	0.5	3100	1.7	76	4.5	0.1[a]	9	90[a]

[a] Standard deviations are between 10 and 20%.
[b] Standard deviations are between 20 and 30%.

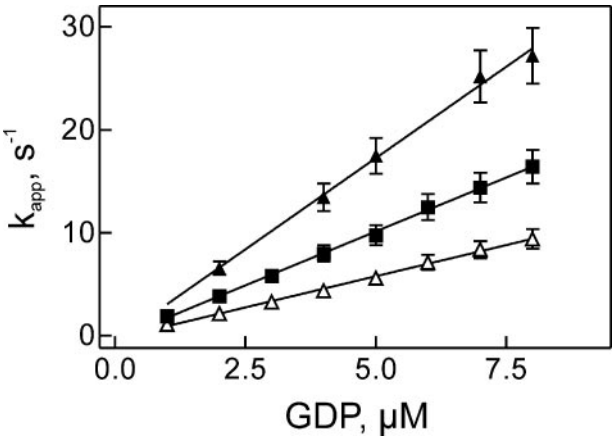

FIGURE 4. **Association of mant-GDP with EF-Tu (k_1).** Nucleotide-free EF-Tu (1 μM) was mixed with increasing amounts of mant-GDP. Symbols are as follows: *squares*, wild-type EF-Tu; *closed triangles*, EF-TuH118A; *open triangles*, EF-TuH118E. k_{app} values were obtained by single-exponential fitting of the average of 5–10 time courses. *Error bars* represent the standard deviations of the fits.

was equal to the rate constant k_{-4}. The following values of k_{-4} for mant-GDP were obtained from a fit to the hyperbolic function (Fig. 5*B*): 150 s^{-1} for wild-type EF-Tu, 2.3 s^{-1} for EF-TuH118A, and 2.8 s^{-1} for EF-TuH118E (Table 1). Similar experiments were performed with mant-GTP, yielding values of k_{-7} of 100 s^{-1} for the wild-type EF-Tu, 4.4 s^{-1} for EF-TuH118A, and 9 s^{-1} for EF-TuH118E (Table 1). Thus, the rate constants of EF-Ts-catalyzed GDP and GTP release from EF-Tu were much lower for the mutants when compared with wild-type EF-Tu.

Finally, the rate constants of GDP binding to EF-Tu·EF-Ts (k_4) and the subsequent release of EF-Ts (k_{-3}) were measured (Fig. 6) by mixing a constant amount of EF-Tu·EF-Ts with increasing amounts of unlabeled nucleotide. A single exponential increase in intrinsic fluorescence of Trp-184 of EF-Tu was observed, reflecting the release of EF-Ts from EF-Tu (Fig. 6*A*). Rates of EF-Ts release were plotted against the increasing concentration of nucleotide, yielding a hyperbolic curve (Fig. 6*B*). The rate constant of EF-Ts release (k_{-3}) was estimated at nucleotide saturation, in which the reverse reaction, *i.e.* rebinding of EF-Ts to EF-Tu, was negligible. The initial slope of the curve (Fig. 6*C*) yielded the value of the association rate constant of GDP to EF-Tu·EF-Ts (k_4) according to the equation: initial slope = $k_4/(1+ k_{-4}/k_{-3})$ (28) (Table 1). The rate constants, k_{-6} and k_7, were determined from the titration of EF-Tu·EF-Ts with GTP (data not shown). Knowledge of k_{-3} and k_{-6} allowed for the calculation of the remaining rate constants, k_3 and k_6, from the initial slopes of the hyperbolic concentration dependences (shown for k_3 in Fig. 5*C*) (Table 1). The results show that only the rate constants of nucleotide release from the EF-Tu·GTP/GDP·EF-Ts complexes (k_{-4} and k_{-7}) were affected by the His-118 mutations, resulting in a 10–50 times slower nucleotide release from EF-TuH118A and EF-TuH118E.

Delivery of aa-tRNA to the Ribosome—The effects of His-118 mutations on aa-tRNA delivery to the ribosome were investigated by stopped-flow experiments monitoring the fluorescence changes of proflavin-labeled aa-tRNA (29). The ternary complex, EF-Tu·GTP·Phe-$tRNA^{Phe}$(proflavin 16/17), was rapidly mixed with mRNA-programmed ribosome complexes with fMet-

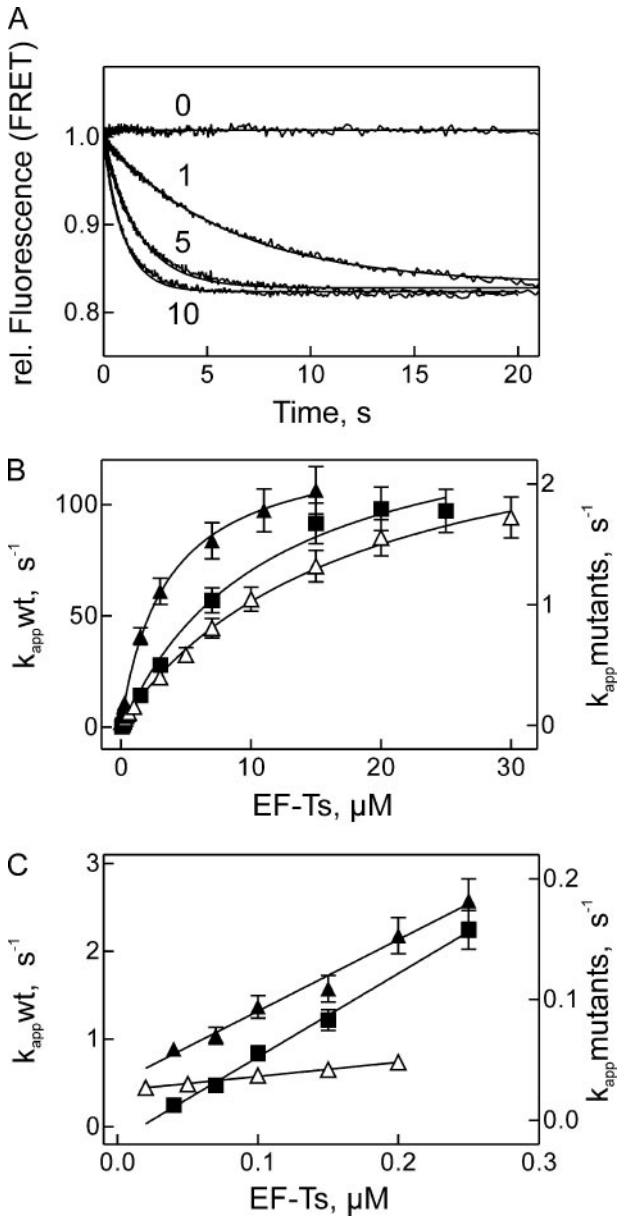

FIGURE 5. **Dissociation of mant-GDP from EF-Tu in the presence of EF-Ts.** *A*, time courses of mant-GDP release from EF-TuH118E·mant-GDP (1 μM) at 1 (*trace 1*), 5 (*trace 5*), and 10 μM (*trace 10*) EF-Ts and a control without EF-Ts (*trace 0*). Time courses shown represent averages of 5–10 individual traces. *rel.*, relative. *B*, nucleotide concentration dependence of k_{app}. The values of k_{app} were calculated by single-exponential fitting from the time courses shown in *A*. Note the different ordinates for the wild-type EF-Tu and the mutants. *C*, initial slopes of the hyperbolic curves in *B* used to determine $k_3/(1+k_{-3}/k_{-4})$. Symbols in *B* and *C* are as follows: *squares*, wild-type EF-Tu; *closed triangles*, EF-TuH118A; *open triangles*, EF-TuH118E. *Error bars* represent the standard deviations of the fits.

$tRNA^{fMet}$ in the P site and a UUC codon exposed in the A site (Fig. 7). Upon ternary complex binding to the ribosome, biphasic fluorescence changes were observed. The increase in fluorescence reports the initial binding of the ternary complex to the ribosome and codon recognition, whereas the decrease in fluorescence reports the accommodation of the aa-tRNA in the A site (29). The rate of binding and codon recognition, 26 s^{-1}, was essentially unchanged by the His-118 mutations, as was the apparent rate of A-site accommodation (6 s^{-1}). With increasing concentrations of reactants, the apparent rate constant of codon recognition

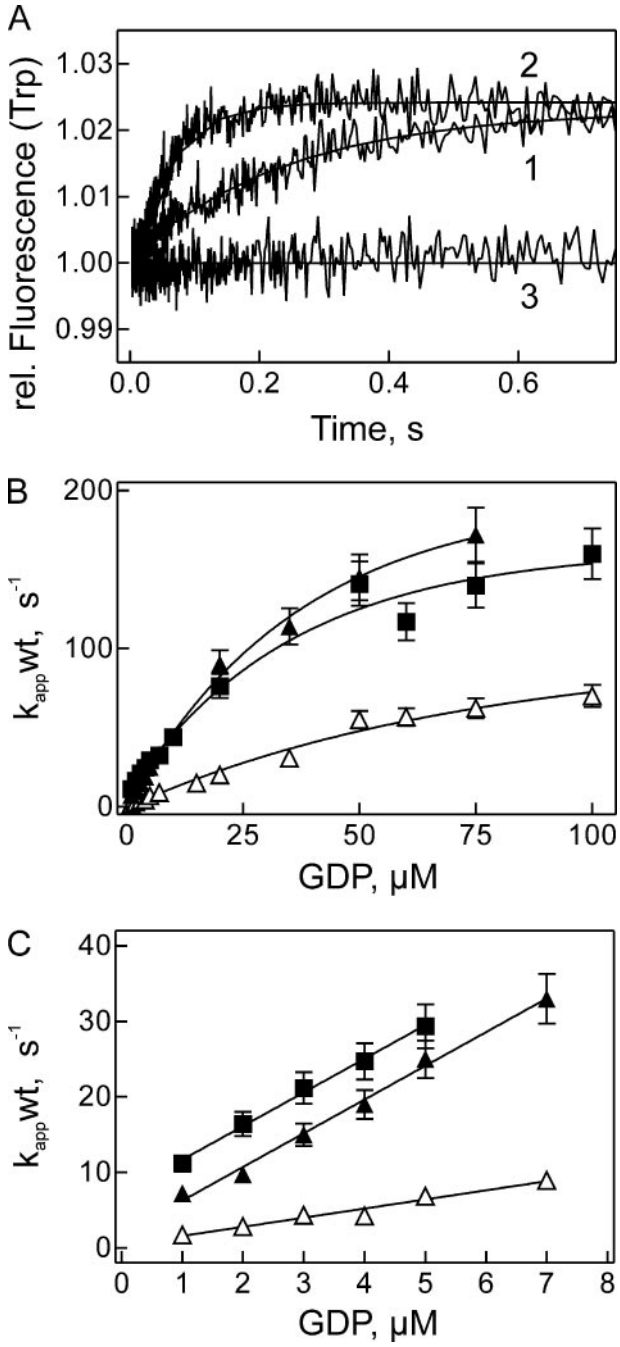

FIGURE 6. **Dissociation of EF-Ts from the binary EF-Tu·EF-Ts complex induced by addition of GDP.** Fluorescence of EF-Tu Trp-184 was measured. *A*, time courses of EF-Ts release from EF-TuH118E·EF-Ts (2 μM) at 3 (*trace 1*) or 20 μM (*trace 2*) GDP or without GDP (*trace 3*). Time courses shown represent averages of 5–10 curves. *rel.*, relative. *B*, nucleotide concentration dependence of k_{app}. k_{app} values were determined by single exponential fitting of the time courses shown in *A*. *C*, initial slopes of the hyperbolic curve in *B* used to calculate $k_4/(1+k_{-4}/k_{-3})$. Symbols in *B* and *C* are as follows: *squares*, wild-type EF-Tu; *closed triangles*, EF-TuH118A; *open triangles*, EF-TuH118E. *Error bars* represent the standard deviations of the fits.

increased and reached saturation at 100 s^{-1}, whereas the rate of the accommodation was almost independent of concentration, 7–8 s^{-1} (30, 31). Because the GTPase activation step follows codon recognition but precedes the A site accommodation step, the results indicate that His-118 is not involved in the GTPase activation step, as well as in any step preceding A site accommodation, which would otherwise have been delayed.

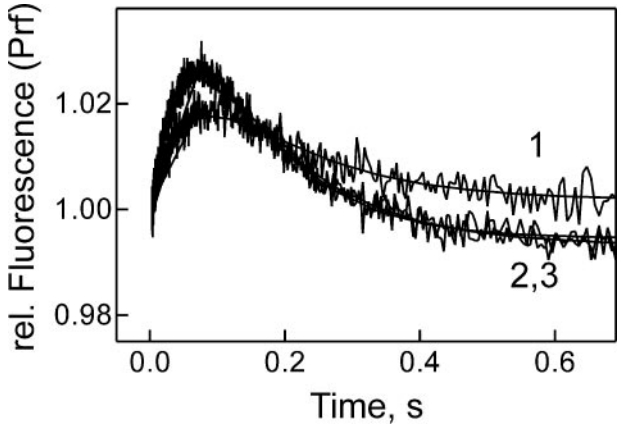

FIGURE 7. **Kinetics of EF-Tu·GTP·Phe-tRNA^{Phe} binding to the ribosome.** EF-Tu·GTP·Phe-tRNA^{Phe}(proflavin (*Prf*) 16/17) (1.2 μM) was mixed with ribosomes (0.4 μM) programmed with MF-mRNA (coding sequence AUGUUC) and containing fMet-tRNA^{fMet} in the P site. The fluorescence of proflavin in the tRNA was monitored. Apparent rate constants of fluorescence increase were 26(±4), 24(±4), and 26(±4) s^{-1} for wild-type EF-Tu, EF-TuH118A, and EF-TuH118E, respectively. Apparent rate constants of fluorescence decrease were 6.0 (±0.6), 6.5 (±0.9), and 6.0 (±0.6) s^{-1}, respectively. Traces represent wild type (*trace 1*), EF-TuH118A (*trace 2*), and EF-Tu-H118E (*trace 3*). *rel.*, relative.

DISCUSSION

Role of His-118 in Intrinsic and EF-Ts-catalyzed Nucleotide Release—Mutations of His-118 in EF-Tu affect several steps of nucleotide exchange. The largest effects are found on the rate constants of EF-Ts-stimulated release of nucleotides, k_{-4} and k_{-7}. The rate of GDP dissociation from the EF-Tu·EF-Ts complex is decreased particularly strongly, by more than 50-fold. The intrusion of Phe-81 of EF-Ts between His-84 and His-118 of EF-Tu is predicted to cause a flip of the P-loop as well as the disruption of the Mg^{2+} binding site via the displacement of helix B in the switch 2 region of EF-Tu. However, the EF-Ts mutants D80A and F81A were only 2–3-fold less active than the wild type in promoting GDP exchange on EF-Tu, whereas a double mutant combining the two single-point mutations was 10-fold less active (9, 11). Unless other residues of the conserved motif 79-TDFV-82 in EF-Ts act to displace helix B, this suggests that movement of this helix by EF-Ts Phe-81 is an important, but not an essential, feature in guanine nucleotide exchange. Therefore, the large inhibitory effect of the His-118 mutations on dissociation of nucleotides is unexpected and suggests that other EF-Tu·EF-Ts contacts, apart from the Phe-81 intrusion, contribute to the nucleotide exchange.

The mutants EF-TuH118A and EF-TuH118E have lower affinities for nucleotide binding than the wild-type EF-Tu, due to 10-fold higher intrinsic dissociation rate constants (k_{-1} and k_{-5}). The effects of the mutations are similar for GDP and GTP, indicating that the phenomenon is not limited to one form of EF-Tu. The explanation for the altered behavior is not straightforward as His-118 is not part of the nucleotide binding pocket in the crystal structure. However, His-118 forms a hydrogen bond to the P-loop residue Gly-18 in both GDP-bound and GTP-bound forms of EF-Tu, which may serve to rigidify the P-loop. A mutation that causes the loss of that hydrogen bond may lead to higher mobility of the P-loop, thereby weakening nucleotide binding. Other local structural perturbations caused

by the mutations may contribute to the weaker binding as well, *e.g.* effects of His-118 mutations on the structure of the domain 1/3 interface or on the position of the switch 2 region, both of which may affect guanine-nucleotide binding (32–34).

The His-118 mutants of EF-Tu show slightly (<3-fold) altered association rate constants for the binding of EF-Ts to the EF-Tu·GDP and EF-Tu·GTP complexes (k_3 and k_6, respectively). The lack of a bulky side chain in the EF-TuHis-118A mutant may slightly facilitate binding, whereas the charged side chain of glutamate in EF-TuH118E may hamper the intrusion of the hydrophobic side chain of EF-Ts Phe-81. However, as the effects are small, and it appears that a number of residues are required for EF-Tu·EF-Ts binding, His-118 seems not to be crucial for the complex formation. This may explain why EF-Ts-catalyzed GDP release from another His-118 mutant, EF-TuH118G, appeared to be only two times slower when compared with the wild-type EF-Tu (13). Those experiments were carried out at very low concentrations of the factors (15 nM), in which the association of EF-Ts to EF-Tu is rate-limiting, thus causing a potential underestimation of the effect on the rate of GDP release.

Role of His-118 of EF-Tu in the Interaction with aa-tRNA and GTPase Activation—His-118 is involved in the protection of the aminoacyl bond in the ternary complex, EF-Tu·GTP·aa-tRNA (data not shown and (14, 15)). The structures of the ternary complexes containing yeast Phe-tRNA^{Phe} (16) or *E. coli* Cys-tRNA^{Cys} (35) do not provide an explanation for these observations since His-118 is buried at the interface between domains 1 and 3 of EF-Tu, at least 16 Å away from the tRNA. Previously, the involvement of His-118 in aa-tRNA binding was sought in the dynamic properties of EF-Tu, implying a temporary dissociation of domains 1 and 3 during the formation of the ternary complex (16). The importance of conformational changes at the interface between domains 1 and 3 was supported by early results that suggested protection against photooxidation of EF-Tu His-118 in the ternary complex but not in free EF-Tu (15). According to the present results, the reduced stabilities of ternary complexes containing mutant EF-Tu are explained by lowered GTP binding, which reduces the amount of EF-Tu·GTP complex capable of aa-tRNA binding. In support of this view, we find a correlation between the lower stability of the ternary complex with EF-TuH118E and its higher K_d for GTP, when compared with EF-TuH118A (data not shown).

Interaction between the codon of the mRNA and the anticodon of the aa-tRNA carried by EF-Tu to the ribosomal A site induces GTP hydrolysis by EF-Tu. The mechanism of GTPase activation is unknown but appears to require an intact conformation of tRNA (36, 37) as well as ribosomal elements (38–42). Mutation of His-118 may alter the contacts of EF-Tu with aa-tRNA and thereby influence the transmission of the GTPase activation signal. A delay in GTPase activation should cause a retardation in the accommodation of tRNA in the A site. However, our data show that A site accommodation takes place at the same rate for mutants and wild-type EF-Tu; thus, His-118 is not essential for the GTPase activation. Previous studies on an H118G mutant indicated that the mutation had a stimulatory effect on GTP hydrolysis (12). The effect was observed upon addition of aa-tRNA to EF-Tu in a multiple turnover assay on the ribosome. EF-Ts was not included, and thus, the regeneration of EF-Tu·GTP from EF-Tu·GDP was rate-limiting. Therefore, the apparent stimulatory effect of the H118G mutation (12) may be explained by accelerating the turnover reaction due to faster dissociation of GDP (k_{-4}) from the mutant EF-Tu.

Mechanism of Guanine Nucleotide Exchange—The mechanism of nucleotide exchange in EF-Tu entails several rearrangements of the factor. The insertion of EF-Ts Phe-81 causes the displacement of residues 80–92 of the switch 2 region and loss of Mg^{2+} coordination (5–7). The loss of the Mg^{2+} ion accounts for a 150–300-fold acceleration of nucleotide dissociation in the absence of EF-Ts (2). Changes in the binding site of the β-phosphate of the guanine nucleotide also contribute to the release (5–7, 9, 11). A flip in the peptide bond between Val-20 and Asp-21 disrupts the interaction between the P-loop and the β-phosphate; replacement of Val-20 by Gly results in an ~10-fold increased rate of both intrinsic and EF-Ts-catalyzed nucleotide exchange (43). Finally, the displacement of helix D moves Lys-136 and Asp-138 away from the nucleotide binding site and destabilizes the binding of the ribose and the guanine base of the nucleotide, respectively; mutations in helix D of EF-Tu reduce the rate of nucleotide exchange ~10-fold (8). The present data indicate an important contribution to nucleotide exchange of movements involving His-118. However, if the intrusion of EF-Ts Phe-81 between EF-Tu His-84 and His-118 was the primary mechanism governing guanine nucleotide exchange, the effects of mutating His-118 of EF-Tu and Phe-81 of EF-Ts were expected to be similar. Yet the mutations of His-118 of EF-Tu resulted in a much larger inhibition of EF-Ts-dependent nucleotide release than did the replacement of Phe-81 of EF-Ts (9). The additional effect caused by mutations of His-118 seems to be indirect and could be caused by interference with the EF-Tu interdomain dissociation induced by EF-Ts. Domain 1 is displaced by up to 18° relative to domains 2 and 3 in the structure of the EF-Tu·EF-Ts complex when compared with the structure of GDP-bound EF-Tu (6). It is likely that EF-Ts increases the rate of nucleotide dissociation from EF-Tu by facilitating the dissociation of domain 1 from domains 2 and 3 (6), which in the free EF-Tu constrain the movements of domain 1 and regulate nucleotide binding (44, 45). His-118 is positioned in the middle of helix C, which together with helix B forms the interface of domain 1 to domain 3. Although the side chain of His-118 points away from the domain 1/3 interface, a mutation at this position could lead to disturbances in the helix, which would alter the interface and the propensity of EF-Ts to induce the open conformation of EF-Tu. If the strong inhibition of the nucleotide dissociation caused by the His-118 mutations is due to changes at the domain interface, the relatively large effect of the mutations suggests that relative movements of the domains in the EF-Tu-EF-Ts complex provide an important contribution to the acceleration of the nucleotide exchange in addition to the direct disruption of the nucleotide binding site.

Among other GTP-binding proteins, EF-Tu has the largest similarity to the small GTP-binding protein Ran with respect to guanine nucleotide exchange, which in both proteins seems to follow the "base side-first," rather than the "phosphate side-

first," mechanism (46). In the structure of the complex between Ran and its guanine nucleotide exchange factor, RCC1, RCC1 interacts directly with a P-loop glycine. The importance of this interaction is supported by mutation of the P-loop glycine to valine, which strongly affects the ability of RCC1 to induce GDP release on Ran (47). Similarly to EF-Tu His-118, the corresponding residue in Ran, Trp-104, is flipped in the Ran-RCC1 complex, breaking a hydrogen bond to the P-loop. Furthermore, helix α3 of Ran (corresponding to EF-Tu helix C) forms a contact to RCC1 residue Asp-128, which has been shown to be important for the exchange reaction (48, 49). One might therefore speculate that movements in Ran helix α3 induced by interactions with RCC1 could affect the P-loop structure and β-phosphate binding in a manner similar to EF-Tu.

The picture emerging from the present and previous studies (*e.g.* Refs. 2, 8, 9, and 50) on the mechanism of EF-Ts-catalyzed guanine nucleotide exchange on EF-Tu is that no single residue or event accounts for the 60,000-fold stimulation of guanine nucleotide exchange obtained by the action of EF-Ts (2). Rather, the effect of EF-Ts comes from many smaller contributions that affect the binding of Mg^{2+}, phosphate, sugar, and base moieties of the nucleotide and the interactions between the domains of EF-Tu. Therefore, the mechanism of the EF-Ts-catalyzed nucleotide release is most likely a global event involving multiple interactions and conformational changes.

Acknowledgments—We thank Karen Margrethe Nielsen, Carmen Schillings, Astrid Böhm, Simone Möbitz, and Petra Striebeck for expert technical assistance.

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