

# Mechanism of Elongation Factor (EF)-Ts-catalyzed Nucleotide Exchange in EF-Tu

CONTRIBUTION OF CONTACTS AT THE GUANINE BASE\*

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**Nucleotide exchange in elongation factor Tu (EF-Tu) is catalyzed by elongation factor Ts (EF-Ts). Similarly to other GTP-binding proteins, the structural changes in the P loop and the Mg<sup>2+</sup> binding site are known to be important for nucleotide release from EF-Tu. In the present paper, we determine the contribution of the contacts between helix D of EF-Tu at the base side of the nucleotide and the N-terminal domain of EF-Ts to the catalysis. The rate constants of the multistep reaction between *Escherichia coli* EF-Tu, EF-Ts, and GDP were determined by stopped-flow kinetic analysis monitoring the fluorescence of either Trp-184 in EF-Tu or mant-GDP. Mutational analysis shows that contacts between helix D of EF-Tu and the N-terminal domain of EF-Ts are important for both complex formation and the acceleration of GDP dissociation. The kinetic results suggest that the initial contact of EF-Ts with helix D of EF-Tu weakens binding interactions around the guanine base, whereas contacts of EF-Ts with the phosphate binding side that promotes the release of the phosphate moiety of GDP appear to take place later. This “base-side-first” mechanism of guanine nucleotide release resembles that found for Ran-RCC1 and differs from mechanisms described for other GTPase-GEF complexes where interactions at the phosphate side of the nucleotide are released first.**

Bacterial elongation factor Tu (EF-Tu)<sup>1</sup> is a GTPase that catalyzes the binding of aminoacyl-tRNA to the ribosomal A site. EF-Tu cycles between the active GTP form, in which it interacts with aminoacyl-tRNA and the ribosome, and the inactive GDP form. Recycling of EF-Tu-GDP to EF-Tu-GTP is catalyzed by a guanine-nucleotide exchange factor, EF-Ts. The structures of EF-Tu-EF-Ts complexes from *Escherichia coli* (1) and *Thermus thermophilus* (2) are known. Although the architecture of the two complexes is distinctly different, as only one molecule of EF-Ts is found in the *E. coli* complex compared with an EF-Ts dimer in *T. thermophilus*, the interaction interface between EF-Tu and EF-Ts is remarkably similar in the two complexes. The G domain of EF-Tu contacts the subdomain N, the N-terminal domain, and the C-terminal module of EF-Ts

(helix 13), whereas domain 3 of EF-Tu is bound to subdomain C of the EF-Ts core (1, 2) (see Fig. 1). Mutational analysis demonstrated that the interactions of the subdomain N and the N-terminal domain of EF-Ts with the G domain of EF-Tu are essential for nucleotide dissociation, whereas the contacts that involve helix 13 and the subdomain C of EF-Ts are less important (3). The structures of EF-Tu-EF-Ts complexes suggest that the main factors that are important for the nucleotide exchange are (i) a conformational change of the P loop in the G domain of EF-Tu that leads to alterations in the binding site of the phosphate moieties of GDP/GTP, (ii) the loss of Mg<sup>2+</sup> coordination (1, 2, 4), and possibly (iii) the change in the relative orientation of the base and/or ribose binding sites (1). The relative contribution of these factors to the catalysis of nucleotide dissociation from EF-Tu is not known.

The contact between helix D of EF-Tu and the N-terminal domain of EF-Ts may be important for the formation of the EF-Tu-EF-Ts complex or nucleotide release. A movement of helix D in EF-Tu-EF-Ts complex may move residues Lys-136 and Asn-138 of EF-Tu, which are involved in interactions with the ribose and the guanine base, away from the nucleotide binding site, thereby relaxing the interactions with the base (1). Mutations of K9A/R12A and M19E/M20E in the N-terminal domain of EF-Ts abolished the factor-dependent nucleotide release from EF-Tu, whereas a more neutral replacement M19A/M20A decreased the stability of the EF-Tu-EF-Ts complex 8-fold (5). The contribution of the interactions of the N-terminal domain of EF-Ts to the mechanism of nucleotide exchange in EF-Tu has yet to be studied.

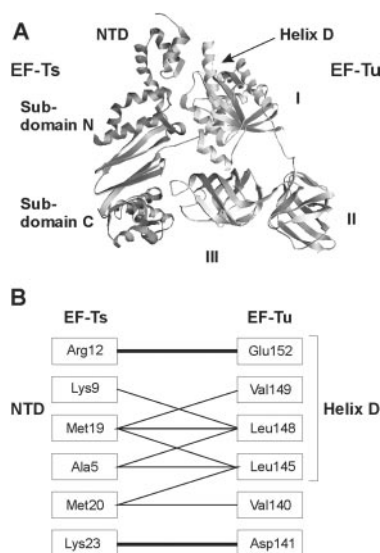
On the side of EF-Tu, the interaction between helix D (residues 144–161) and the N-terminal domain of EF-Ts involves Leu-145, Leu-148, Val-149, and Glu-152 (1) (Fig. 1). In the present work, we have performed alanine replacements at positions in helix D of *E. coli* EF-Tu that are located in the vicinity of EF-Ts in the EF-Tu-EF-Ts complex or are directly involved in the intermolecular contacts, Leu-145, Leu-148, Met-151, Glu-152, and Glu-155; a residue at the beginning of the helix, Glu-144, was replaced with glycine. The effects of the mutations on EF-Ts binding and GDP release were studied. The reaction mechanism, which is shown in Scheme 1, was analyzed using experimental approaches described recently for the wild-type EF-Tu (6).

Nucleotide binding and dissociation were studied using a derivative of GDP carrying the fluorescent methylantraniloyl group, mant-GDP/GTP. Fluorescence changes were monitored either upon direct excitation or upon indirect excitation via fluorescence resonance energy transfer (FRET) from the single tryptophan in EF-Tu, Trp-184, to the mant group. Trp-184 is located in the G domain of EF-Tu at a distance of about 20–25 Å from the mant group, allowing efficient energy transfer to mant-GDP bound to EF-Tu. To study the binding of EF-Tu to

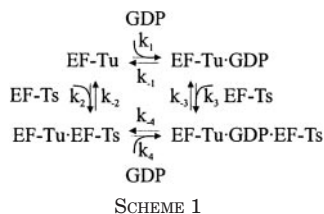
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<sup>1</sup> The abbreviations used are: EF-Tu, elongation factor Tu; EF-Ts, elongation factor Ts; mant, methylantraniloyl; mant-GDP, 2'(3')-O-(N-methylantraniloyl)guanosine 5'-diphosphate; GEF, guanine-nucleotide exchange factor; WT, wild type; FRET, fluorescence resonance energy transfer; NTD, N-terminal domain.



**FIG. 1. Interactions between EF-Tu and EF-Ts.** *A*, structure of EF-Tu-EF-Ts from *E. coli* (1). EF-Ts consists of four structural modules: the NTD, the core domain, which can be further divided into N-terminal and C-terminal subdomains, the dimerization insertion, and the C-terminal helix (the latter two elements are not indicated in the figure). *I-III*, the domains of EF-Tu; domain *I* is the G domain. *B*, schematic representation of the contacts between the NTD of EF-Ts and helix D of EF-Tu as revealed by the crystal structure.



EF-Ts, we monitored the fluorescence of Trp-184, which has an increased mobility and solvent accessibility in the EF-Tu-EF-Ts complex compared with EF-Tu-GDP (7). Our data reveal the functional role of the interactions between helix D of EF-Tu and the N-terminal domain of EF-Ts and show the relative contribution of the factors that are crucial for the nucleotide exchange.

#### EXPERIMENTAL PROCEDURES

**Buffer and Reagents**—Experiments were performed in buffer A (50 mM Tris-HCl, pH 7.5, 50 mM NH<sub>4</sub>Cl, 10 mM MgCl<sub>2</sub>) at 20 °C if not stated otherwise. Chemicals were purchased from Roche Molecular Biochemicals or Merck. Mant-GDP was purchased from Molecular Probes or JenaBioScience.

**Mutagenesis**—The plasmid pKECAHis (8) containing the full-length *E. coli tufA* gene was obtained from B. Kraal (Leiden, Netherlands). For mutagenesis, a BamHI/HindIII fragment containing the *tufA* gene was recloned into pET21(+) vector (Novagen) resulting in the plasmid pEECAHis. The Chameleon™ mutagenesis kit (Stratagene, Heidelberg, Germany) was used for site-directed mutagenesis using single-stranded DNA from the plasmid. Mutations were verified by sequencing.

**Protein Purification**—Wild-type and mutant EF-Tu containing a C-terminal oligohistidine tag was purified by Ni-nitrilotriacetic acid affinity chromatography under nondenaturing conditions. Proteins were overexpressed in *E. coli* BL21 DE3 cells. Cells were opened by sonication in buffer B (50 mM Tris-HCl at pH 8.0, 60 mM NH<sub>4</sub>Cl, 7 mM MgCl<sub>2</sub>, 7 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 50 μM GDP, 300 mM KCl, 10 mM imidazole, 15% glycerol). After centrifugation for 30 min at 10,000 rpm (A8.24 rotor, Kontron), the supernatant was mixed with Ni-nitrilotriacetic acid (Qiagen) equilibrated with buffer B and incubated for 30 min at 4 °C. The supernatant was removed, and the Ni-nitrilotriacetic acid was washed with buffer B and additionally with buffer B containing 20 mM imidazole. Proteins were eluted with 250 mM imidazole in buffer B and subsequently rebuffed to buffer A using Centricon 30 concentrators (Amicon).

To prepare nucleotide-free EF-Tu, the protein was incubated in Mg<sup>2+</sup>-free buffer A with 10 mM EDTA for 20 min at 37 °C. Under these conditions, Mg<sup>2+</sup> bound to EF-Tu was removed, allowing the dissociation of GDP. The protein was purified by gel filtration on Superdex 75 in buffer A in the absence of Mg<sup>2+</sup>. The absence of nucleotide in EF-Tu preparations was verified by reversed phase high performance liquid chromatography on C<sub>18</sub>-MNP (Macherey-Nagel) in buffer C (65 mM potassium phosphate, pH 6.2, 2 mM tetrabutylammonium hydrogen sulfate, 15% acetonitrile).

Concentrations of EF-Tu were determined photometrically at 280 nm using a molar extinction coefficient of 32,900 M<sup>-1</sup> cm<sup>-1</sup> (9). EF-Ts was measured by absorbance at 210 nm (10) and 205 nm (11). Concentrations were verified by comparing the intensity of the Coomassie-stained protein band on a 12% SDS-polyacrylamide gel with that of a standard protein using an imaging densitometer (Bio-Rad GS-700) and the Molecular Analyst software.

A construct for EF-Ts expression, created as a fusion protein with an intein self-splicing element and chitin binding domain in the IMPACT I system (New England Biolabs), was provided by Charlotte Knudsen (Aarhus, Denmark). The protein was expressed in *E. coli* B834 DE3 cells for 1 h after induction with isopropyl-1-thio-β-D-galactopyranoside at 30 °C. Cells were opened by sonication in buffer B. After centrifugation at 15,000 × *g*, the supernatant was mixed with a suspension of chitin beads equilibrated with buffer B. After incubation for 30 min on ice, chitin beads were washed three times with buffer B and five times with buffer D (20 mM Tris-HCl at pH 8.0, 0.1 mM EDTA, 50 mM NaCl). The self-cleavage of the intein tag proceeded upon incubation in buffer E (20 mM Tris-HCl at pH 8.0, 0.1 mM EDTA, 50 mM NaCl, 60 mM dithiothreitol, and 0.4 KCl) for 25–35 h at 20 °C; EF-Ts was eluted with buffer E without dithiothreitol. The protein was further purified by gel filtration on Superdex 75 in buffer A, shock-frozen in liquid nitrogen, and stored at –80 °C.

**Rapid Kinetic Measurements**—Fluorescence stopped-flow measurements were performed on an SX-18MV spectrometer (Applied Photophysics) as described previously (6, 12). Tryptophan fluorescence was excited at 280 nm and measured after passing KV335 filters (Schott) (13). Fluorescence of mant-GDP was excited directly at 349 nm or via FRET at 280 nm and measured after passing KV408 filters (Schott). Experiments were performed in buffer A at 20 °C by rapidly mixing equal volumes (60 μl each) of reactants and monitoring the time courses of the fluorescence change. Data were evaluated by fitting to an exponential function with the characteristic apparent time constant ( $k_{app}$ ), the amplitude ( $A$ ), and the final signal ( $F_{\infty}$ ) according to the equation  $F = F_{\infty} + A \times \exp(-k_{app} \times t)$ , where  $F$  is the fluorescence at time  $t$ . Calculations were performed using TableCurve software (Jandel Scientific). Standard deviations of  $k_{app}$  values were calculated using the same software. All elemental rate constants of nucleotide exchange, except  $k_{-2}$ , were measured directly. The value of  $k_{-2}$  was estimated from all other rate constants according to the model depicted in Scheme 1. Standard deviations of the rate constants were estimated from the variation of values obtained in different experiments.

#### RESULTS

**Amino Acid Residues in Helix D of EF-Tu That Are Important for EF-Ts Function**—Mutagenesis was performed, and EF-Tu mutants were expressed and purified as described under “Experimental Procedures.” To prepare the EF-Tu-mant-GDP complex, EF-Tu was incubated with a 10-fold excess of mant-GDP for 15 min at 37 °C. The complex was purified from unbound nucleotide on a NAP10 column. The dissociation of mant-GDP in the presence of excess nonfluorescent GDP was measured upon addition of catalytic amounts of EF-Ts. Upon mixing EF-Tu-mant-GDP with EF-Ts and GDP, a fluorescence decrease was observed due to dissociation of mant-GDP from EF-Tu (Fig. 2). The mutations E144G, L145A, M151A, and E155A in EF-Tu had no effect. The mutation L148A reduced the rate of mant-GDP dissociation from EF-Tu, whereas the mutation E152A essentially abolished the reaction. Thus, amino acid residues 148 and 152 in helix D of EF-Tu are important for EF-Ts-dependent nucleotide exchange. These two mutants were studied in further detail.

**GDP Binding to EF-Tu**—The rate constants of nucleotide binding to EF-Tu,  $k_1$  and  $k_{-1}$  (Scheme 1), were determined by FRET from Trp-184 in EF-Tu to mant-GDP, which results in

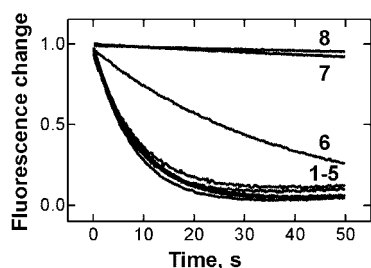


FIG. 2. Effect of mutations in helix D in EF-Tu on EF-Ts-induced dissociation of EF-Tu-mant-GDP. Dissociation of the EF-Tu-mant-GDP ( $0.15 \mu\text{M}$ ) was monitored upon addition of EF-Ts ( $0.01 \mu\text{M}$ ) and excess GDP ( $50 \mu\text{M}$ ). EF-Tu WT (trace 1) or mutants E144G (trace 2), L145A (trace 3), M151A (trace 4), E155A (trace 5), L148A (trace 6), E152A (trace 7), and WT in the absence of EF-Ts (trace 8).

an increase of mant fluorescence upon binding of the nucleotide to the factor. Time courses of binding were measured at a constant concentration of nucleotide-free EF-Tu and a varying concentration of mant-GDP (Fig. 3A). As only single-exponential time courses were observed, the data were treated on the basis of a one-step binding model,  $\text{EF-Tu} + \text{mant-GDP} \rightleftharpoons \text{EF-Tu-mant-GDP}$ . The bimolecular association rate constant,  $k_1$ , was determined from the slope of the linear concentration dependence of  $k_{\text{app}}$  (Fig. 3B) and was found to be similar for wild-type EF-Tu and the two mutants L148A and E152A: about  $2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  (Table I).

To measure  $k_{-1}$ , the EF-Tu-mant-GDP complex ( $0.3 \mu\text{M}$ ) was mixed with an excess of nonfluorescent GDP ( $25 \mu\text{M}$ ), and the decrease of mant-GDP fluorescence due to dissociation was monitored (Fig. 3C). Dissociation rate constants of  $0.003\text{--}0.004 \text{ s}^{-1}$  were obtained for both wild-type and mutant EF-Tu proteins (Table I). Thus, the nucleotide binding properties of EF-Tu were not affected by the mutations in helix D of EF-Tu.

**EF-Tu-EF-Ts Complex Formation**—The exchange of GDP may be inhibited either because EF-Ts does not bind to mutant EF-Tu or because a structural change of the EF-Tu-GDP-EF-Ts complex that is crucial for nucleotide release does not take place. According to both nondenaturing gel electrophoresis and gel filtration, mutations at positions 148 and 152 did not preclude EF-Tu-EF-Ts complex formation (data not shown). Although the data cannot be interpreted in terms of binding constants, they demonstrate that the effect of the mutations on nucleotide exchange cannot be attributed to an inhibition of complex formation between EF-Tu and EF-Ts. Thus, to assign the effect of the mutations on particular steps of the multistep reaction between EF-Tu and EF-Ts, a kinetic analysis was performed.

Rate constants of EF-Ts binding to nucleotide-free EF-Tu were measured using the change in Trp fluorescence in the presence of excess EF-Ts. Both mutations in helix D reduced the rate of binding (Fig. 4A). To determine the association constant, time courses were measured at increasing concentrations of EF-Ts, and values of  $k_2$  were determined from the slopes of the linear dependence of  $k_{\text{app}}$  on EF-Ts concentration (Fig. 4B). The values of the association rate constant were  $k_2 = (1.7 \pm 0.4) \times 10^7$ ,  $(4 \pm 1) \times 10^6$ , and  $(8 \pm 1) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  for the wild-type EF-Tu, L148A mutant, and E152A mutant, respectively. Thus, the mutations in helix D moderately (4-fold, L148A) or strongly (20-fold, E152A) slow down the association between EF-Tu and EF-Ts.

The value of  $k_{-2}$  could not be determined with precision because in the linear plot the intercept with the y axis was close to 0. Therefore  $k_{-2}$  was calculated from all other rate constants (see below) on the basis of the law of mass action. From the rate constants of Scheme 1,  $k_{-2} = k_{-1} \times k_2 \times k_{-3} \times k_4 / (k_1 \times k_3 \times k_{-4})$ , values of  $k_2$  of 0.03, 0.03, and  $0.04 \text{ s}^{-1}$  were calculated for

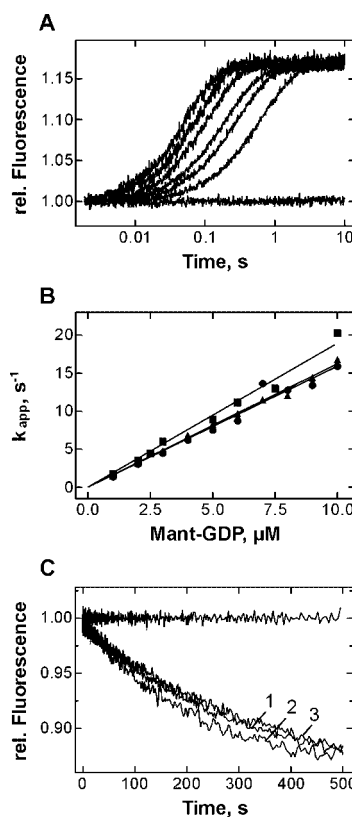


FIG. 3. Interaction of mant-GDP with EF-Tu. A, time courses of mant-GDP binding to nucleotide-free EF-Tu L148A ( $0.5 \mu\text{M}$ ) measured by FRET. From right to left: 1, 2, 3, 5, 7, and  $10 \mu\text{M}$  mant-GDP; baseline, control without nucleotide. B, concentration dependence of  $k_{\text{app}}$ . Values of  $k_{\text{app}}$  were determined as in Fig. 4A. Circles, WT EF-Tu; triangles, L148A; squares, E152A. From the slopes of the plots, the values of the bimolecular association rate constant ( $k_1$ ) were calculated. C, determination of the rate constant of GDP dissociation ( $k_{-1}$ ) from WT EF-Tu (trace 1), L148A (trace 2), and E152A (trace 3). rel., relative.

TABLE I  
Effects of mutations in helix D of EF-Tu on kinetics and thermodynamics of EF-Tu interactions with EF-Ts and GDP

Constants <sup>a</sup>	EF-Tu		
	WT	L148A	E152A
$k_1 \text{ (M}^{-1} \text{ s}^{-1})^b$	$1.6 \times 10^6$	$1.6 \times 10^6$	$2.0 \times 10^6$
$k_{-1} \text{ (s}^{-1})^c$	$3.4 \times 10^{-3}$	$3.8 \times 10^{-3}$	$3.0 \times 10^{-3}$
$K_1 \text{ (M)}^d$	$2.1 \times 10^{-9}$	$2.4 \times 10^{-9}$	$1.6 \times 10^{-9}$
$k_2 \text{ (M}^{-1} \text{ s}^{-1})^e$	$1.7 \times 10^7$	$4.0 \times 10^6$	$8.0 \times 10^5$
$k_{-2} \text{ (s}^{-1})^f$	$2.6 \times 10^{-2}$	$2.9 \times 10^{-2}$	$3.7 \times 10^{-2}$
$K_2 \text{ (M)}^d$	$1.5 \times 10^{-9}$	$7.4 \times 10^{-9}$	$4.6 \times 10^{-8}$
$k_3 \text{ (M}^{-1} \text{ s}^{-1})^g$	$4.0 \times 10^7$	$1.7 \times 10^7$	$4.6 \times 10^6$
$k_{-3} \text{ (s}^{-1})^g$	350	240	170
$K_3 \text{ (M)}^d$	$7.5 \times 10^{-6}$	$1.4 \times 10^{-5}$	$3.7 \times 10^{-5}$
$k_4 \text{ (M}^{-1} \text{ s}^{-1})^g$	$1.1 \times 10^7$	$1.4 \times 10^7$	$7.6 \times 10^6$
$k_{-4} \text{ (s}^{-1})^g$	120	50	9
$K_4 \text{ (M)}^d$	$9.1 \times 10^{-6}$	$3.6 \times 10^{-6}$	$1.2 \times 10^{-6}$

<sup>a</sup> According to the mechanism of Scheme 1.

<sup>b</sup> From Fig. 3B.

<sup>c</sup> From Fig. 3C.

<sup>d</sup> Calculated from the respective rate constants.

<sup>e</sup> From Fig. 4B.

<sup>f</sup> Calculated according to the mechanism shown in Scheme 1 from all other rate constants.

<sup>g</sup> From Fig. 5.

wild-type EF-Tu, the L148A mutant, and the E152A mutant, respectively (Table I), suggesting that the mutations in helix D of EF-Tu have no effect on the dissociation of the EF-Tu-EF-Ts complex.

**EF-Tu Interactions with EF-Ts and GDP**—Time courses of nucleotide dissociation from EF-Tu-mant-GDP were measured

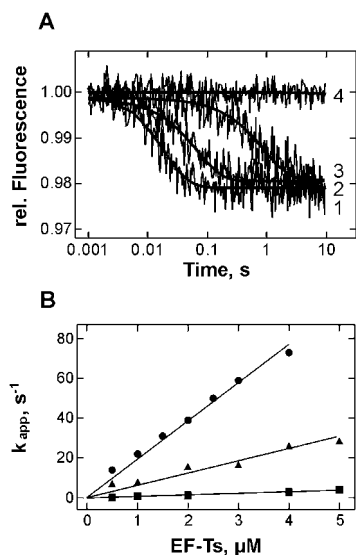


FIG. 4. **EF-Tu-EF-Ts interactions.** *A*, time course of EF-Ts ( $2 \mu\text{M}$ ) binding to nucleotide-free EF-Tu ( $0.5 \mu\text{M}$ ): WT (trace 1), L148A (trace 2), E152A (trace 3), control WT without EF-Ts (trace 4). The fluorescence of Trp-184 in EF-Tu was measured. *B*, concentration dependence of  $k_{\text{app}}$ . From the slope of the plot, the values of  $k_2$  were calculated. Circles, WT EF-Tu; triangles, L148A; squares, E152A. *rel.*, relative.

at different concentrations of EF-Ts in the presence of excess nonfluorescent GDP to suppress the rebinding of mant-GDP to the EF-Tu-EF-Ts complex. At a fixed EF-Ts concentration, the dissociation of mant-GDP from the EF-Tu mutants was slower than from the wild-type factor (Fig. 5A). The rate of dissociation increased with EF-Ts and saturated at  $k_{\text{app}} = k_4$  (6) (Fig. 5B). The mutations in EF-Tu reduced the dissociation rate 2-fold (L148A) or 13-fold (E152A) (Table I). The initial slopes of the titration curves, which equal  $k_3/(1 + k_{-3}/k_{-4})$  (14), were also affected by the mutations in helix D of EF-Tu (Fig. 5C).

To measure  $k_3$  and calculate the remaining constants  $k_3$  and  $k_4$ , the dissociation of the EF-Tu-EF-Ts complex was induced by the addition of GDP and monitored by the small change of tryptophan fluorescence. Release of EF-Ts from EF-Tu-GDP results in fluorescence increase as shown for wild-type EF-Tu (Fig. 5D). Similar time courses were obtained with both EF-Tu mutants (not shown), although the rate of dissociation was somewhat reduced. Mutations in helix D had little effect on the values of  $k_{-3}$ , which were determined at saturation of  $k_{\text{app}}$  (Fig. 5E). Also the initial slopes, which give  $k_4/(1 + k_{-4}/k_{-3})$ , were similar (Fig. 5F). From the initial slopes of Fig. 5, C and F, and the values of  $k_{-3}$  and  $k_{-4}$  determined at saturation, the remaining rate constants were calculated (Table I). The rate constant of EF-Ts binding to the EF-Tu-GDP complex was reduced 2-fold (L148A) or 10-fold (E152A), whereas the rate of GDP binding to EF-Tu-EF-Ts was essentially unaffected.

#### DISCUSSION

**Role of Helix D of EF-Tu in EF-Ts Binding and Nucleotide Release**—Interactions between helix D of EF-Tu and the N-terminal domain of EF-Ts include hydrophobic contacts between Leu-145, Leu-148, and Leu-149 of EF-Tu with Met-19 and Met-20 of EF-Ts and a salt bridge between Glu-152 of EF-Tu and Arg-12 of EF-Ts (Fig. 1; Val-140 and Asp-141, which also interact with the N-terminal domain (NTD) of EF-Ts, do not belong to helix D and will not be discussed further). Our data show that the replacements of Leu-148 and Glu-152 reduced the rates of both EF-Ts binding to EF-Tu and nucleotide release, whereas the replacements of other amino acids in the hydrophobic patch had little effect in agreement with previous results (3). Comparison of the equilibrium dissociation con-

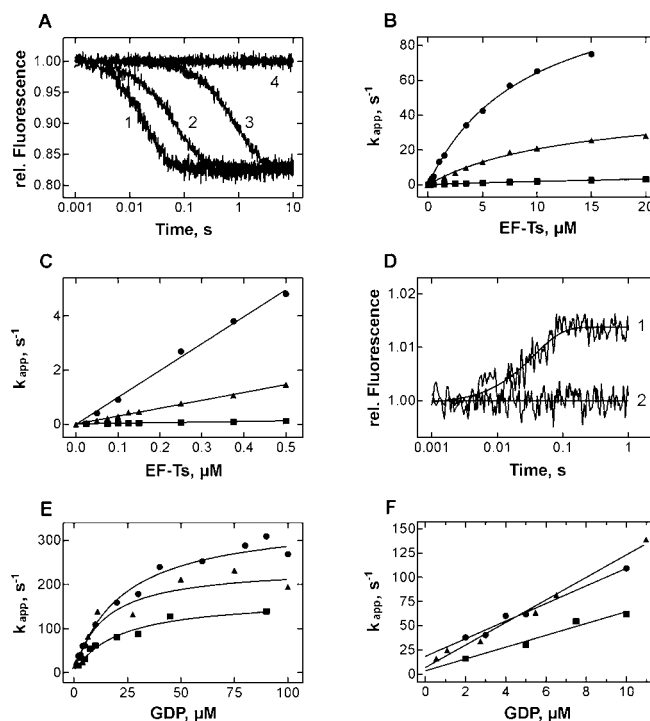


FIG. 5. **Interactions of EF-Tu with EF-Ts and GDP.** *A*, time course of dissociation of EF-Tu-mant-GDP ( $0.15 \mu\text{M}$ ) WT (trace 1), E148A (trace 2), L152A (trace 3) in the presence of EF-Ts ( $5 \mu\text{M}$ ), and excess unlabeled GDP ( $25 \mu\text{M}$ ) or WT in the absence of EF-Ts (trace 4). Fluorescence of mant was monitored. *B*, concentration dependence of  $k_{\text{app}}$  of EF-Tu-mant-GDP dissociation. Values of  $k_{\text{app}}$  were calculated by single-exponential fitting from time courses as shown in *A*. Circles, WT EF-Tu; triangles, L148A; squares, E152A. Fitting of the concentration dependence to a hyperbolic function gave values of  $k_{-4} = 120 \pm 20 \text{ s}^{-1}$  (WT),  $50 \pm 8 \text{ s}^{-1}$  (L148A), and  $9 \pm 2 \text{ s}^{-1}$  (E152A) (Table I). *C*, initial slopes from *B* given by  $k_3/(1 + k_{-3}/k_{-4}) = 9.8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  (WT),  $2.9 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  (L148A), and  $0.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  (E152A). *D*, time course of dissociation of EF-Tu-EF-Ts ( $0.5 \mu\text{M}$ ) in the presence of GDP ( $3.75 \mu\text{M}$ ) (trace 1) or in the absence of nucleotide (trace 2). Fluorescence of Trp-184 in EF-Tu was monitored. *E*, concentration dependence of  $k_{\text{app}}$  of EF-Tu-EF-Ts dissociation. Circles, WT EF-Tu; triangles, L148A; squares, E152A. Fitting the concentration dependence to a hyperbolic function gave the values of  $k_{-3} = 350 \pm 50 \text{ s}^{-1}$  (WT),  $240 \pm 40 \text{ s}^{-1}$  (L148A), and  $170 \pm 30 \text{ s}^{-1}$  (E152A) (Table I). *F*, initial slopes from *E* given by  $k_4/(1 + k_{-4}/k_{-3}) = 9.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  (WT),  $1.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$  (L148A), and  $6.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  (E152A). *rel.*, relative.

stants of the binary EF-Tu-EF-Ts complexes,  $K_2$  (Table I), shows that the mutations resulted in free energies of interaction that were less favorable by 3.9 kJ/mol (L148A) or 9.0 kJ/mol (E152A) in keeping with the loss of the respective hydrophobic interaction or the salt bridge between Glu-152 of EF-Tu and Arg-12 of EF-Ts (Fig. 1). In the ternary complexes, EF-Tu-GDP-EF-Ts, the energetic penalty for the mutations was smaller than in the binary complexes, 1.5 kJ/mol for L148A and 3.9 kJ/mol for E152A, suggesting that the loss of interactions between helix D of EF-Tu and the NTD of EF-Ts is partially compensated by newly formed contacts in the ternary complex.

The bimolecular rate constants of EF-Ts binding to EF-Tu and EF-Tu-GDP,  $k_2$  and  $k_3$ , are reduced by mutations in helix D (Table I), indicating that contacts between helix D of EF-Tu and the N-terminal domain of EF-Ts are important for complex formation. The crystal structures suggest that contacts to the P loop of EF-Tu may be involved as well (1, 2). However, the rate constants of EF-Ts binding to EF-Tu-GTP, EF-Tu-GDP, or EF-Tu are the same (6), indicating that the different structure of the phosphate binding pocket does not affect the rate of EF-Tu-EF-Ts complex formation. Taken together these data suggest that helix D of EF-Tu is an important element of the primary binding surface and that the formation of the contacts

between helix D and the NTD of EF-Ts is the rate-limiting step for binding; the other interactions between EF-Tu and EF-Ts, including those with the P loop, seem to be established later and to be rapid compared with the preceding bimolecular binding step.

Another effect of the mutations in helix D is the decrease of the rate constant of guanine nucleotide dissociation from the EF-Tu-GDP-EF-Ts, whereas the rate of GDP dissociation from EF-Tu-GDP in the absence of EF-Ts is unchanged (Table I). The interaction between helix D of EF-Tu and the NTD of EF-Ts may result in a displacement of helix C, thereby affecting the interactions of EF-Tu with the ribose and guanine moieties of the nucleotide (1). If helix D is involved in primary contacts between EF-Tu and EF-Ts, then the distortion of the base binding site of EF-Tu may constitute one of the early structural changes among the rearrangements that have to take place during nucleotide release. The loss of contacts with the base would be followed by further changes in the P loop and in the Mg<sup>2+</sup> binding site that result in the release of the phosphate side of the nucleotide. According to the microscopic reversibility of the reaction pathway, one may expect that in the reverse reaction, that is during binding of the nucleotide to EF-Tu-EF-Ts, the phosphate part of the nucleotide would come in first, whereas the contacts with the base are established later.

*Contribution of Different Factors to the Interactions of EF-Tu with EF-Ts and GDP*—EF-Ts enhances the dissociation of GDP from EF-Tu by a factor of 60,000, whereas the loss of Mg<sup>2+</sup> accounts for a factor of 150–300, suggesting that the disruption of the Mg<sup>2+</sup> binding site alone does not explain the EF-Ts effect (6). The insertion of Phe-81 of EF-Ts, a residue of the highly conserved TDFV sequence motif, into a hydrophobic pocket formed by His-84, Leu-121, and His-118 of EF-Tu, was proposed to trigger a peptide flip in the P loop, thereby disrupting hydrogen bonds to the phosphates, and to reposition the carbonyl group of Val-20 such as to sterically and electrostatically eject GDP (2). However, mutations of Asp-80 and Phe-81 in EF-Ts reduced the rate of GDP dissociation only 2–10-fold, indicating that the conformational change in the P loop of EF-Tu is not the main factor to bring about the nucleotide exchange either (15). The present data show that the disruption of interactions between helix D of EF-Tu with the N-terminal domain of EF-Ts also decrease the rate of nucleotide release 2–13-fold. This indicates that none of the structural elements suggested by x-ray crystallography as important determinants of nucleotide exchange alone accounts for the rapid EF-Ts-induced nucleotide dissociation from EF-Tu. Rather the 60,000-fold acceleration of GDP release from the EF-Tu-GDP-EF-Ts complex is attributed to various structural rearrangements at both the phosphate and base sides of the nucleotide.

*Comparison to Other GTP-binding Proteins*—Structures of several GTPases complexed with their respective GEFs determined so far include Ras-Sos (16), Rac-Tiam (17), Arf1-Sec7 (18), and Ran-RCC1 (4) in addition to EF-Tu-EF-Ts (1, 2). While all GEFs have unrelated structures and their interactions with the respective GTPases are different in detail, there are common features in the mechanism of nucleotide exchange. The general strategy of GEFs is to release positive charges, *i.e.* the

Mg<sup>2+</sup> ion and the invariant P-loop lysine, from their interactions with the phosphates of the nucleotide. At the same time, other parts of the nucleotide binding pocket, such as the switch I region and the base binding site, remain flexible in the complex to allow rapid entry of the nucleotide (4).

The interactions of Ras with Sos, Rac with Tiam, and Arf1 with Sec7 are limited mainly to the switch I and II regions of the GTPase, whereas regions homologous to helix D in EF-Tu are not involved. However, in the Ran-RCC1 complex, helix  $\alpha 4$  in Ran, which corresponds to helix D in EF-Tu, is an important element of the interaction with the GEF. The pathway of nucleotide release and rebinding to Ran-RCC1 is different from that of other GTP-binding proteins. The structure of Ran-RCC1 suggests that the base is released prior to the phosphate, and during binding the nucleotide comes in with the phosphate moiety first in contrast to the mechanism proposed for Ras-Sos, where the base moiety was proposed to come in first (4). Thus, the order of events during nucleotide release and rebinding to Ran-RCC1 and to EF-Tu-EF-Ts (see above) are similar and different from other G proteins. The reason might be the contact of helix  $\alpha 4$  (Ran) or D (EF-Tu) with the respective GEF. Provided this contact is established early during binding of the GEF to the GTPase, residues binding the base could be displaced, and the interaction with the base might be released first, whereas the contacts of the GEF with the phosphate binding side that result in the release of the phosphate moiety of the nucleotide may take place later. Thus, the contact of helix D of EF-Tu and its homolog in Ran with their respective GEFs may be the structural reason why in these systems the “base-side-first” mechanism of nucleotide release is favored.

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(EF)-Ts-catalyzed Nucleotide Exchange in  
EF-Tu: CONTRIBUTION OF  
CONTACTS AT THE GUANINE BASE**

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