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Efficient Separation of *Thermus aquaticus* EF-Tu Functional Complexes

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A new method for fast separation of the main functional complexes of the elongation factor Tu from *Thermus aquaticus* has been developed. Binary complexes EF-Tu ∗ GDP and EF-Tu ∗ GDPNP as well as the ternary complex EF-Tu ∗ GDPNP ∗ Leu ∼ tRNA were separated from each other by means of HPLC on a hydrophobic sorbent TSK-Gel Phenyl 5PW in a reverse gradient of ammonium sulfate. This technique is suitable for monitoring EF-Tu activity, characterisation of the ratio between different EF-Tu forms in cell extracts, and isolation of individual EF–Tu complexes for structural and functional investigations. In order to illustrate the potentials of the method, we used HPLC on a TSK-Gel Phenyl 5PW matrix to determine the ratio between affinities of GDP and GDPNP for EF-Tu. We found that K_a(GDP) is about 27 times higher than K_a(GDPNP) at 37°C, the value being close to the one reported for *Thermus thermophilus* EF-Tu.

Key Words: **elongation factor Tu; *Thermus aquaticus;* binary complexes with GDP and GDPNP; ternary complex; chromatographic separation.**

Bacterial elongation factor Tu is one of the main participants of the ribosomal peptide elongation process. It promotes the binding of aminoacyl-tRNAs to the mRNA-programmed ribosome. During the elongation cycle EF-Tu consecutively forms a few different functional complexes depending on the type of cofactor (GTP or GDP) bound in the nucleotide binding site. Hydrolysis of bound GTP to GDP plays a regulatory role: It results in drastic decrease of EF-Tu affinity towards aminoacylated tRNA. Spontaneous GDP exchange for GTP is slow even if GTP is in significant excess over GDP. However, another elongation factor, EF-Ts, accelerates the exchange reaction (1).

Since the discovery of EF-Tu several techniques have been developed to purify this protein. EF-Tu is one of the most abundant bacterial proteins, therefore considerable amount of it can be isolated from bacterial cells by conventional methods. Such a "wild-type" protein can be heterogeneous because in many bacteria EF-Tu is encoded by two unlinked genes (1, 2); another source of heterogeneity is posttranscriptional modification (phosphorylation (3, 4) and methylation (5, 6)). Cloning and overproduction of EF-Tu in a convenient host is a way to overcome this problem, however a complicating factor during purification of the recombinant protein is the presence of large amounts of host-encoded EF-Tu. Therefore, cloned EF-Tu should possess some special properties in order to separate it from host EF-Tu. For example, *Thermus thermophilus* and *Thermus aquaticus* EF-Tu's overproduced in *E. coli* cells are both stable at 65°C when *E. coli* EF-Tu is completely denatured (7, 8). Overproduced *E. coli* EF-Tu with a C-terminal His-tag can be separated from wild-type *E. coli* EF-Tu on a Ni-nitrilotriacetic acid-agarose column (9). A more sophisticated technique is based on different affinities of cloned and host EF-Tu's to the antibiotic kirromycin and on subsequent EF-Ts complex formation with kirromycin-free EF-Tu (10). Finally, EF-Tu can be synthesized as a fusion product with a protein-carrier (11–14); in this case, the recombinant EF-Tu should be released by cleaving the chimeric protein with an appropriate protease.

All existing methods of EF-Tu purification are oriented generally towards the isolation of the EF-Tu ∗ GDP complex because EF-Tu ∗ GTP is intrinsically unstable due to spontaneous GTP-to-GDP hydrolysis (15); at the same time, nucleotide-free EF-Tu may loose its activity (as shown for the elongation factor from *E. coli* (16)). Purified EF-Tu ∗ GDP can be converted to EF-Tu ∗ GTP in presence of pyruvate kinase/phospho*enol*pyruvate. An alternative method to obtain EF-Tu in the GTP-conformation is to treat EF-Tu ∗ GDP with alkaline phosphatase in presence of GDPNP, a non-hydrolysable analogue of GTP. Then a ternary complex can easily be formed simply by addition of aminoacylated tRNA to EF-Tu ∗ GTP (or EF-Tu ∗ GDPNP).

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In order to separate the ternary complex from the initial components of the reaction mixtures, one can use gel-filtration or preparative native gel-electrophoresis. Separation of EF-Tu in the GTP-form from EF-Tu ∗ GDP is much more complicated and in fact, there is no satisfactory technique to resolve these two complexes. Here we describe a simple and efficient method to separate all three complexes from each other and from such components of the reaction mixtures as unbound aminoacyl-tRNA, GTP (GDPNP), GDP etc. The method can also be used as a final step of EF-Tu purification yielding homogeneous and fully active protein.

MATERIALS AND METHODS

Materials. *Thermus aquaticus* EF-Tu was overproduced in *E. coli* SCS1 carrying plasmid pTacTU2 with a *Thermus aquaticus* tufA gene. Homogeneous EF-Tu ∗ GDP was obtained generally according to (7, 8). The purification procedure included thermal denaturation of *E. coli* proteins, followed by chromatographies on DEAE-Sephadex A-50 and Sephacryl S100 HR. The final purification was done on the hydrophobic matrix TSK-Gel Phenyl 5PW (TosoHaas GmbH, Germany) in a reverse gradient of ammonium sulfate. The eluent contained 300 μM GDP. After the last purification step EF-Tu ∗ GDP was precipitated by addition of ammonium sulfate to 65% of saturation, the pellet was collected by centrifugation, redissolved in 25 mM Tris–HCl (pH 7.1), 0.2 M NH_4Cl, 0.5 mM GDPNP, 0.5 mM DTT and after flash-freezing kept at −80°C prior to use. *Escherichia coli* EF-Tu was overproduced as a fusion product with glutathione-*S*-transferase and purified according to (11, 29); native EF-Tu was obtained by cleavage of the chimeric protein with Factor Xa (Protein Engineering Technology ApS, Denmark). $tRNA^{Leu}$ was purified from total *Thermus thermophilus* HB8 RNA by chromatographies on Sepharose 4B and BND-Cellulose generally according to (17–19); the final acceptor activity was 600 pmol/A_{260}. *Thermus thermophilus* leucyl-tRNA synthetase was partially purified by successive chromatographies on Q-Sepharose and TSK-Gel Phenyl 5PW, was RNase-free and had specific activity 4.9 U/mg at 20°C (1 U corresponds to 1 nmol of L-leucine incorporated in TCA-insoluble fraction in 1 min). Alkaline phosphatase (bovine calf intestine, 6510 U/mg at 37°C) was from Sigma.

L-[U-^{14}C]Leucine (11.2 TBq/mol) and [8-^{3}H]Guanosine 5′-diphosphate (477 TBq/mol) were products of Amersham Life Science. 5′-Guanylylimidodiphosphate (GDPNP) was purchased from Sigma and additionally purified to homogeneity on Q-Sepharose. All other chemicals were from Sigma.

Preparation of EF-Tu ∗ GDPNP and EF-Tu ∗ GDPNP ∗ Leu∼tRNA. Unless otherwise mentioned, EF-Tu ∗ GDP was converted to EF-Tu ∗ GDPNP by incubation of 15 μM EF-Tu ∗ GDP in 20 mM Tris–HCl (pH 7.1), 0.2 M NH_4Cl, 1 mM GDPNP, 1 mM DTT for 3–5 h at 25–37°C. In some experiments alkaline phosphatase has been added (4–20 U/ml) in order to increase the yield of EF-Tu ∗ GDPNP. $tRNA^{Leu}$ was aminoacylated in a reaction mixture, containing 5 mM ATP, 50 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 50 μM L-leucine (or 10 μM L-[U-^{14}C]Leucine), 6.0 A_{260} units/ml tRNA and 0.5 U/ml leucyl-tRNA synthetase at 37°C for 5 h. EF-Tu ∗ GDPNP ∗ Leu∼tRNA was usually formed upon addition of the EF-Tu ∗ GDPNP-containing conversion mixture directly to the $tRNA^{Leu}$ aminoacylation mixture without prior isolation of EF-Tu ∗ GDPNP and Leu∼tRNA. After incubation for 30 min at 37°C ice-cold 3.8 M $(NH_4)_2SO_4$ was added to a final concentration of ammonium sulfate of 1.2–1.3 M and the reaction mixture was kept on ice prior to chromatographic analysis.

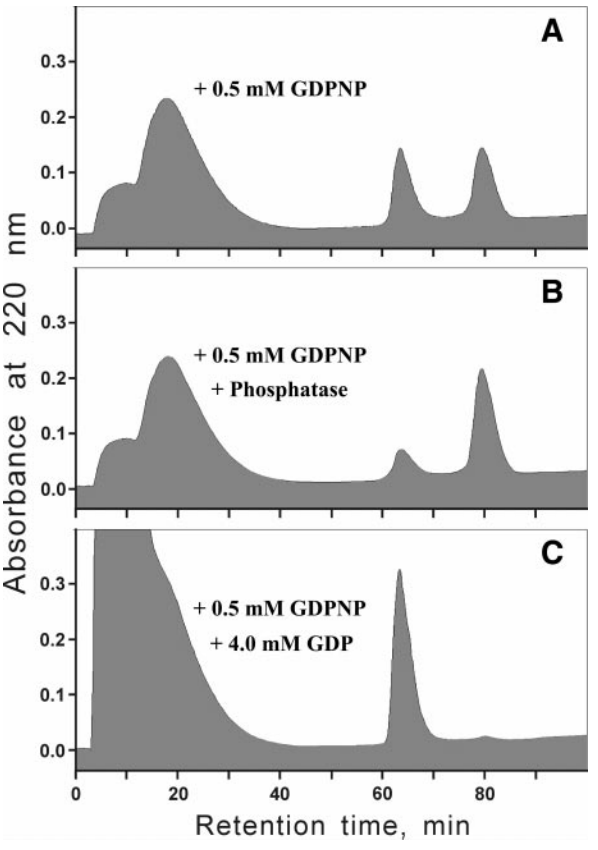

FIG. 1. Chromatographic separation of the components of nucleotide exchange reaction mixtures on a TSK-Gel Phenyl 5PW column. 6.5 μM EF-Tu was incubated for 2 h at 27°C in 20 mM Tris–HCl (pH 7.1), 0.2 M NH_4Cl, 1 mM DTT with 0.5 mM GDPNP (A), 0.5 mM GDPNP and 20 U/ml alkaline phosphatase (B) or 0.5 mM GDPNP and 4 mM GDP (C). Then cold 3.8 M $(NH_4)_2SO_4$ was added to a final concentration of 1.2 M and the reaction mixture was chromatographed as described under Materials and Methods.

HPLC separation of EF-Tu functional complexes. Samples containing EF-Tu ∗ GDP, EF-Tu ∗ GDPNP, or EF-Tu ∗ GDPNP ∗ Leu∼tRNA were mixed with 3.8 M $(NH_4)_2SO_4$ to achieve a final concentration of ammonium sulfate of 1.2–1.3 M and loaded on a TSK-Gel Phenyl 5PW column (0.75 × 7.5 cm) equilibrated with 1.25 M $(NH_4)_2SO_4$, 50 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT. Elution was performed with a nonlinear gradient of $(NH_4)_2SO_4$ from 1.25 M to 0.01 M in 50 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and a flow rate 0.5 ml/min. The gradient was determined by breakpoints (($(NH_4)_2SO_4$ concentration/time): 1.25 M/0 min; 1.25 M/20 min; 0.88 M/35 min; 0.50 M/65 min; 0.01 M/85 min; 0.01 M/150 min. All chromatographies were done identically at room temperature on a LKB/Pharmacia HPLC-2150 system with a RSD-2140 diode array detector. UV-absorption has been recorded in the range 190–360 nm throughout the chromatographic run. Protein in collected fractions was analysed by SDS/PAGE according to (20). The [^{3}H]GDP-binding activity of the protein in fractions was determined generally according to (21). Protein concentration has been determined by Bradford's method with bovine serum albumin as a standard according to (22).

RESULTS

The purification procedure of *Thermus aquaticus* EF-Tu overproduced in *E. coli* included a thermal de-

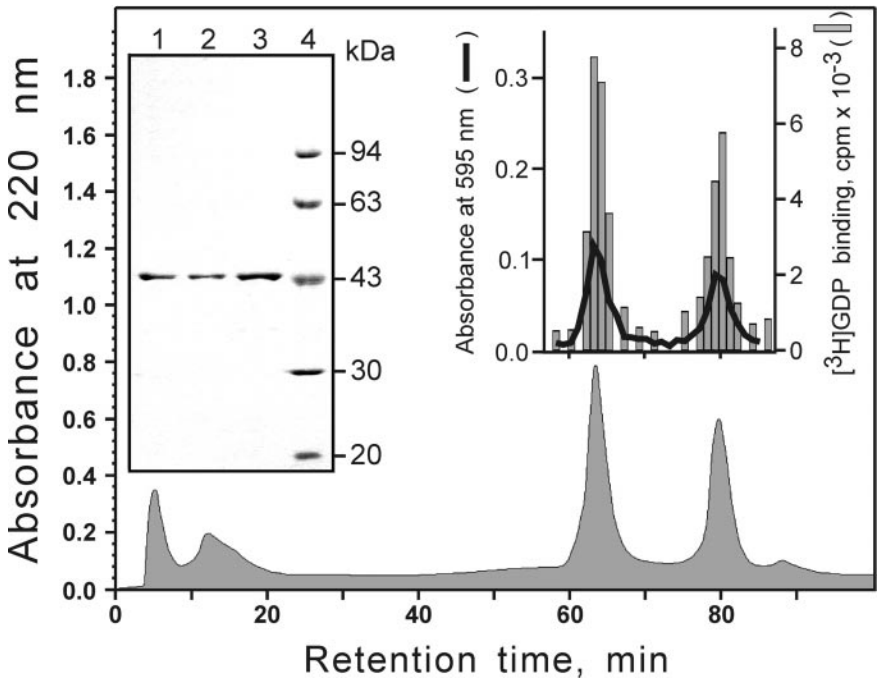

FIG. 2. Chromatography of EF-Tu after spontaneous GDP-to-GDPNP exchange and characterisation of material from separated protein peaks. 15 μM EF-Tu was incubated with 0.5 mM GDPNP for 4 h at 27°C in 20 mM Tris–HCl (pH 7.1), 0.2 M NH_4Cl, 5 mM $MgCl_2$, 1 mM DTT. Then cold 3.8 M $(NH_4)_2SO_4$ was added to a final concentration of 1.2 M and the mixture was separated on TSK-Gel Phenyl 5PW column. 20 μl aliquotes from collected fractions were mixed with 1 ml of Bradford's reagent and the absorbance was measured at 595 nm against a blank sample (bold line). 10 μl aliquotes from fractions were mixed with 90 μl of [^{3}H]GDP-containing binding mixture as in [21], incubated at 60°C for 30 min and filtered through a GN-6 acetylcellulose filter (Gelman Sciences). Then the filters were extensively washed with 10 mM Tris–HCl (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$ and the retained radioactivity was counted (bars). Insert: SDS–PAGE of samples taken at 63 min (line 1) and 80 min (line 2), control - EF-Tu prior to the exchange reaction (line 3), molecular weight markers (line 4). The gel was stained with Coomassie Brilliant Blue R-250.

naturation step, chromatography on anion-exchange resin, and gel-filtration. Protein obtained by this way is electrophoretically pure and migrates as a single peak on an appropriate gel-filtration column. At the same time hydrophobic interaction chromatography of this EF-Tu preparation on a TSK-Gel Phenyl 5PW column results in two peaks, the ratio between these two peaks varying from one purification to another. In order to estimate whether such a heterogeneity appertains to the ability of EF-Tu to fall into two different conformations, the GDP- or the GTP-induced state, we made the complex of EF-Tu with GDPNP (a stable analog of GTP) by different ways and compared the chromatographic behaviour of these preparations (Fig. 1). When EF-Tu ∗ GDP was incubated in presence of 0.5 mM GDPNP for 2 h at 27°C (the conditions of spontaneous exchange of GDP for GDPNP), two protein peaks of approximately equal size have been detected after separation of the mixture on a TSK-Gel Phenyl 5PW column, with a retention time of about 65 and 80 min (Fig. 1A). No protein has been found in the flow-through by Bradford's method. The group of peaks appearing right

TABLE 1

Comparison of the Retention Time of *Thermus aquaticus* and *Escherichia coli* EF-Tu Complexes with GDP and GDPNP

	GDP		GDPNP	
	Retention time, min	Number of independent runs	Retention time, min	Number of independent runs
Thermus aquaticus EF-Tu	64.2 ± 0.8	12	80.6 ± 0.6	11
Escherichia coli EF-Tu	78.7 ± 1.9	4	109.6 ± 0.2	2

Note. Prior to the chromatographic run EF-Tu ∗ GDP was preincubated in 20 mM Tris–HCl (pH 7.1), 0.2 M NH_4Cl, 1 mM GDPNP, 1 mM DTT for 2 h at 25°C. EF-Tu ∗ GDPNP was obtained by incubation of 15–30 μM EF-Tu ∗ GDP with alkaline phosphatase (30 U/ml) in 20 mM Tris–HCl (pH 7.1), 0.2 M NH_4Cl, 1 mM GDPNP, 1 mM DTT for 2 h at 25°C.

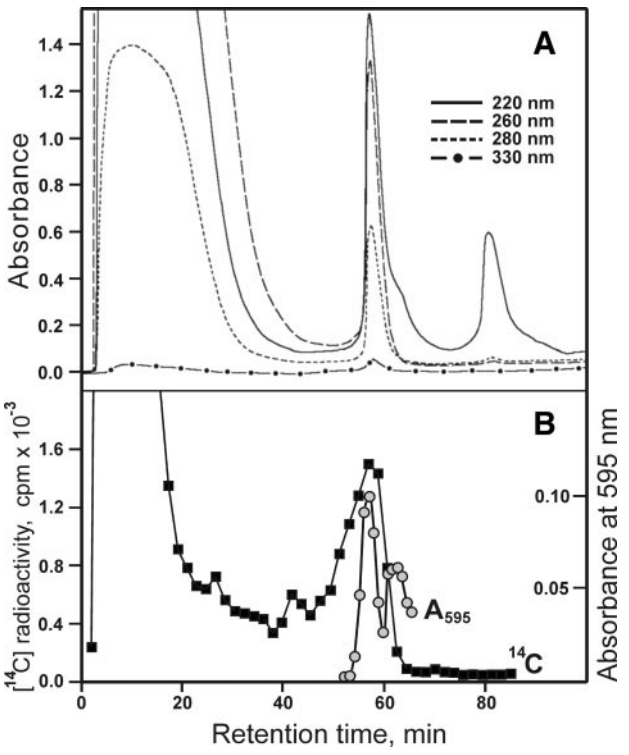

FIG. 3. Identification of the ternary complex EF-Tu ∗ GDPNP ∗ Leu-tRNA peak after chromatography of the reaction mixture on a TSK-Gel Phenyl 5PW column. (A) UV-absorbance profiles recorded at 220, 260, 280, and 330 nm. (B) Presence of protein and [^{14}C]Leu in selected fractions. 5 μl aliquotes were taken for [^{14}C] radioactivity counting (squares). 20 μl aliquotes were mixed with 1 ml of Bradford's reagent and the absorbance was measured at 595 nm against a blank sample (circles).

after the start of the gradient represents low molecular weight components of the exchange reaction mixture (data not shown). Longer incubation (up to 6 h) at the same conditions does not change the ratio between the peaks which means that the system reached equilibrium. Incubation of EF-Tu ∗ GDP in the mixture containing 0.5 mM GDPNP and alkaline phosphatase for 2 h (the conditions of selective GDP removal) results in pronounced domination of the last of these two protein peaks, with a retention time of 80 min (Fig. 1B). In a control experiment, after EF-Tu ∗ GDP incubation for 2 h with 4 mM GDP and 0.5 mM GDPNP, when the EF-Tu ∗ GDP complex should be predominant (1), only the first of the two peaks can be detected (Fig. 1C). Protein from both peaks migrates as a single band on SDS–PAGE, with a molecular weight of approx. 45 kDa, which corresponds to the molecular weight of native *Thermus aquaticus* EF-Tu. This means that the observed changes in the chromatographic mobility of the protein after incubation are not associated with polypeptide chain degradation. Material from both peaks was equally active in [^{3}H]GDP-binding tests (Fig. 2). Taking into account all these observations, we assumed that the first peak corresponds to EF-Tu ∗ GDP, and the second one is EF-Tu ∗ GDPNP.

A dramatic decrease of *Thermus aquaticus* EF-Tu chromatographic mobility after exchange of GDP for GDPNP may result from different hydrophobicity of the EF-Tu ∗ GDP and EF-Tu ∗ GDPNP complexes. Structural changes upon conversion of EF-Tu GDP-form to GTP-form have a profound functional significance: the corresponding conformational alteration leads to the formation of a high-affinity aminoacyl-tRNA binding site. This mechanism is considered to be universal among different organisms because of the same overall organisation of the translation machinery and high level of sequence homology between EF-Tu's. Therefore the observed difference in mobility of EF-Tu ∗ GDP and EF-Tu ∗ GDPNP on the hydrophobic matrix may be expected for EF-Tu's from other bacteria. We determined the retention time of *Escherichia coli* EF–Tu complexes with GDP and GDPNP under the same conditions as we used to study the *Thermus aquaticus* EF–Tu complexes. The *Escherichia coli* EF–Tu complex with GDP was eluted much earlier than that with GDPNP (Table 1). Both *Escherichia coli* EF–Tu complexes were retained stronger than the corresponding ones of *Thermus aquaticus* EF-Tu, revealing higher hydrophobicity of the *Escherichia coli* pro-

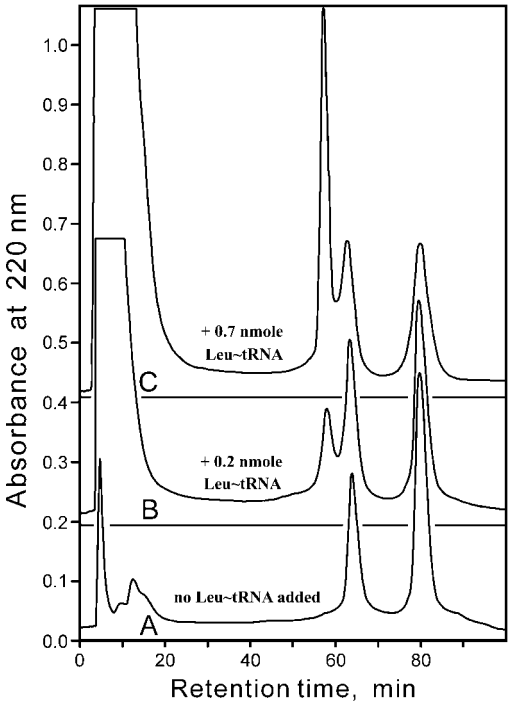

FIG. 4. Titration of a EF-Tu ∗ GDP and EF-Tu ∗ GDPNP mixture with Leu-tRNA. 4 nmoles of EF-Tu ∗ GDP were partially converted to EF-Tu ∗ GDPNP by incubation at 30°C for 4 h in 20 mM Tris–HCl (pH 7.1), 0.2 M NH_4Cl, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM GDPNP. Then none (A), 0.2 nmole (B) or 0.7 nmole (C) of Leu~tRNA was added and after additional 30 min incubation the mixture was separated on a TSK-Gel Phenyl 5PW column.

tein. Thus, the exchange of GDP for GDPNP in the case of *Escherichia coli* EF-Tu also results in drastic mobility decrease, as in the case of EF-Tu from *Thermus aquaticus*. This finding strongly favours our suggestion about universality of the observed phenomenon.

The mobility of the ternary complex between *Thermus aquaticus* EF-Tu, GDPNP and Leu ~ tRNA was determined under the same chromatographic conditions, as in the case of the binary complexes, EF-Tu * GDP and EF-Tu * GDPNP. The ternary complex was prepared by adding the EF-Tu * GDP conversion mixture to the $tRNA^{Leu}$ aminoacylation mixture without prior isolation of EF-Tu * GDPNP and Leu ~ tRNA, as described in Materials and Methods. The resulting solution was left for 30 min at 37°C, then cold 3.8 M $(NH_4)_2SO_4$ was added to a final concentration of 1.2 M and the mixture was applied onto a TSK-Gel Phenyl 5PW column. A EF-Tu * GDPNP * Leu ~ tRNA peak could be identified by its tRNA-like spectrum with a pronounced maximum at 260 nm, unlike the EF-Tu * GDP and EF-Tu * GDPNP peaks that have comparatively weak UV-absorbtion at 250–270 nm. We also used [^{14}C]Leu ~ tRNA in this experiment in order to introduce a radioactive label into the ternary complex. To exclude a possibility of misidentification, we performed control experiments with free tRNA (charged or uncharged) and [^{14}C]Leu and found that all these compounds are not retained by the sorbent at the given conditions (data not shown). Nucleotides (ATP, AMP, GDP, GDPNP) are also eluted from the column in the void volume. Thus, identification of the peak of EF-Tu * GDPNP * Leu ~ tRNA was quite straightforward (Fig. 3). The protein component of the peak was determined by reaction with the Bradford reagent, and the presence of Leu ~ tRNA was revealed from a UV-spectrum (Fig. 3A) or [^{14}C] radioactivity counts associated with the peak (Fig. 3B). The ternary complex was reproducibly eluted before EF-Tu * GDP, although quite close to it: the retention time for EF-Tu * GDPNP * Leu ~ tRNA was 57–58 min vs 64–65 min for EF-Tu * GDP. On the UV-absorbance profile EF-Tu * GDP appears only as a shoulder on the tail of the ternary complex peak (Fig. 3A), but the use of Bradford's method enables the resolution of the peaks of these two compounds (Fig. 3B).

Our identification of the EF-Tu * GDP, EF-Tu * GDPNP and EF-Tu * GDPNP * Leu ~ tRNA peaks has been validated by titration of an EF-Tu * GDP and EF-Tu * GDPNP mixture with aminoacyl-tRNA. EF-Tu * GDP (4 nmoles) was partially converted to EF-Tu * GDPNP, and none (Fig. 4A), 0.2 nmole (Fig. 4B) or 0.7 nmole (Fig. 4C) of Leu-tRNA was added to the mixture prior to chromatographic separation. As expected, upon titration the EF-Tu * GDPNP peak was decreasing in size, the EF-Tu * GDP peak was unchanged, and the EF-Tu * GDPNP * Leu ~ tRNA peak was rising.

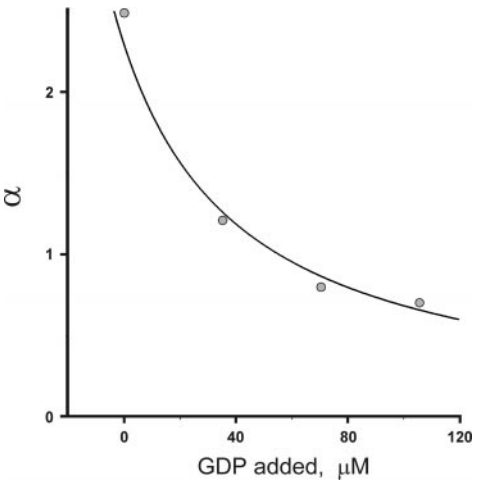

FIG. 5. Dependence of the ratio [EF-Tu * GDPNP]/[EF-Tu * GDP] (parameter α) on the amount of GDP added. 10.7 μM EF-Tu was incubated at 37°C for 2 and 4 h in a mixture containing 50 mM Tris–HCl (pH 7.6), 0.2 M NH_4Cl, 5 mM $MgCl_2$, 1 mM DTT, 2.56 mM GDPNP and the appropriate amount of GDP. Then the mixtures were analysed on a TSK-Gel Phenyl 5PW column at standard conditions. The ratio [EF-Tu * GDPNP]/[EF-Tu * GDP] was determined from the areas of the corresponding peaks on the chromatograms. The curve represents the best fit of equation (5) to the experimental data.

In order to evaluate potential applications of the method, we used analytic chromatography on a TSK-Gel Phenyl 5PW matrix to compare EF-Tu affinities to GDP and GDPNP. Equilibrium between EF-Tu * GDP and EF-Tu * GDPNP in the presence of both guanine nucleotide cofactors in an exchange reaction mixture can be described by the equation:

$$\text{EF-Tu} * \text{GDP} + \text{GDPNP} \leftrightharpoons \text{EF-Tu} * \text{GDPNP} + \text{GDP}$$

$$K = \frac{[\text{EF-Tu} * \text{GDPNP}][\text{GDP}]}{[\text{EF-Tu} * \text{GDP}][\text{GDPNP}]} \qquad [1]$$

Taking into account that the partial reactions of the complex formation between the elongation factor and GDP or GDPNP can be expressed as:

$$\text{EF-Tu} + \text{GDP} \leftrightharpoons \text{EF-Tu} * \text{GDP}$$

$$K_a(\text{GDP}) = \frac{[\text{EF-Tu} * \text{GDP}]}{[\text{EF-Tu}][\text{GDP}]} \qquad [2]$$

$$\text{EF-Tu} + \text{GDPNP} \leftrightharpoons \text{EF-Tu} * \text{GDPNP}$$

$$K_a(\text{GDPNP}) = \frac{[\text{EF-Tu} * \text{GDPNP}]}{[\text{EF-Tu}][\text{GDPNP}]} \qquad [3]$$

the equilibrium constant K for the nucleotide exchange reaction (1) may serve as a measure for the comparison

TABLE 2

Comparison of the Guanine Nucleotide-Binding Affinities of EF-Tu's

Source (reference)	K_a(GDPNP)/ K_a(GDP)	K_a(GTP)/ K_a(GDP)	Temperature, °C	Buffer	Method
Thermus aquaticus (this study)	0.0371	—	37	50 mM Tris–HCl (pH 7.6), 200 mM NH_4Cl, 5 mM $MgCl_2$, 1 mM DTT	Chromatographic separation of equilibrium mixture of EF-Tu complexes
Thermus thermophilus ([24])	0.054	0.092	5	50 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 0.5 mM DTE, 1 mM NaN_3	Rapid kinetics of nucleotide cofactor binding, monitored by change of intrinsic protein fluorescence
	—	0.064	15		
E. coli ([25])	—	0.0015	0	50 mM Tris–HCl (pH 7.8), 60 mM NH_4Cl, 7 mM $MgCl_2$, 1 mM DTT	Kinetics of radiolabeled nucleotide cofactor binding to EF-Tu, monitored by nitrocellulose filter assay
	—	0.0094	30		
E. coli ([26])	—	0.0023	0	20 mM Tris–HCl (pH 7.5), 10 mM Mg acetate, 10 mM 2-mercaptoethanol	Kinetics of radiolabeled nucleotide cofactor binding to EF-Tu, monitored by nitrocellulose filter assay
	—	0.0019	12		
	—	0.0018	21		
	—	0.0018	30		
E. coli ([27])	—	0.00181	0	50 mM Tris–HCl (pH 7.6), 60 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM DTT	Kinetics of radiolabeled nucleotide cofactor binding to EF-Tu, monitored by nitrocellulose filter assay
E. coli ([28])	—	0.00179	0	50 mM Tris–HCl (pH 7.6), 60 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM DTT	Kinetics of radiolabeled nucleotide cofactor binding to EF-Tu, monitored by nitrocellulose filter assay
E. coli ([29])	—	0.00350	0	50 mM Tris–HCl (pH 7.6), 60 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM DTT	Kinetics of radiolabeled nucleotide cofactor binding to EF-Tu, monitored by nitrocellulose filter assay

of the affinities of these two nucleotide cofactors to EF-Tu:

$$K = K_a(GDPNP)/K_a(GDP) \quad [4]$$

Quantification of the chromatographic profiles thus allows the calculation of the ratio between EF-Tu ∗ GDP and EF-Tu ∗ GDPNP in a sample loaded onto the column. We assumed that the concentrations of both EF–Tu complexes do not change during preparation of the sample for chromatography (normally includes addition of cold 3.8 M $(NH_4)_2SO_4$ to the final concentration 1.2–1.3 M and brief centrifugation) and during separation, and that the ratio between EF–Tu complexes in the nucleotide exchange mixture is the same as the ratio between areas of the corresponding peaks on the chromatographic profiles. Now we can rewrite equation (1) in such a way that K will depend only on parameters, which can be determined experimentally:

$$K = \alpha \cdot \frac{C_t(GDP) - C_t(EF\text{-}Tu)/(1 + \alpha)}{C_t(GDPNP) - C_t(EF\text{-}Tu)/(1 + 1/\alpha)} \quad [5]$$

where α is the ratio of equilibrium concentrations of EF–Tu complexes [EF-Tu ∗ GDPNP]/[EF-Tu ∗ GDP], and C_t(EF-Tu), C_t(GDP), C_t(GDPNP) are total concentrations of EF-Tu, GDP and GDPNP in the exchange mixture, respectively. The ratio [EF-Tu ∗ GDPNP]/[EF-Tu ∗ GDP] was measured after 2 and 4 h of incubation of the reaction mixtures and found to be the same, which indicates that the system was in equilibrium. Concentrations of GDPNP and EF-Tu were fixed, and concentration of added GDP varied from 0 to 105.7 μM. The results of the measurements are presented in Fig. 5. Fitting equation (5) to the data has been performed at C_t(GDPNP) = 2.56 mM, C_t(EF-Tu) = 10.7 μM, and C_t(GDP) = C_{added}(GDP) + C_0(GDP), where C_{added}(GDP) is the concentration of added GDP, and C_0(GDP) is the concentration of GDP, which arose from the EF-Tu ∗ GDP stock solution used to prepare the exchange mixtures. C_0(GDP) is the same in all the exchange mixtures, it is unknown but constant. Thus, the only parameters to be determined in the equation are K and C_0(GDP). The value of K has been found to be 0.0371 ± 0.0005, and C_0(GDP) was 44.6 ± 1.2 μM.

DISCUSSION

Separation of the *Thermus aquaticus* EF-Tu functional complexes on a TSK-Gel Phenyl 5PW matrix in a reverse gradient of ammonium sulfate is a typical example of the hydrophobic interaction chromatogra-

phy (HIC). Protein molecules stick to a nonpolar surface of the sorbent at high salt concentration, then selective desorbtion occurs upon elution with a decreasing salt gradient. The observed significant difference in EF-Tu ∗ GDP and EF-Tu ∗ GDPNP retention times correlates with the pronounced structural difference between these two EF–Tu complexes (23) and reveals higher EF-Tu ∗ GDPNP affinity to the hydrophobic stationary phase (or lower solubility in the ammonium sulfate-containing mobile phase) compared with that of EF-Tu ∗ GDP. The phenomenon is not limited to the case of *Thermus aquaticus* EF-Tu: A similar difference of chromatographic mobilities has been observed for *Escherichia coli* EF–Tu complexes with GDP and GDPNP. Taking into account the high level of sequence homology among bacterial elongation factors, we expect the same behaviour of EF-Tu's from other sources on a hydrophobic interaction matrix.

A possibility to separate simultaneously EF-Tu ∗ GDP, EF-Tu ∗ GDPNP and EF-Tu ∗ GDPNP ∗ aminoacyl-tRNA by a single chromatographic run makes this method suitable for fast characterisation of the ratio between different EF–Tu complexes in reaction mixtures or cell extracts. Chromatography on a TSK-Gel Phenyl 5PW sorbent can also be used to purify certain EF–Tu complexes for structural or functional investigations.

In order to demonstrate the possible application of the method, we used chromatography on TSK-Gel Phenyl 5PW to determine the ratio between association constants for EF–Tu ∗ GDP and EF–Tu ∗ GDPNP complexes. We found that the *Thermus aquaticus* elongation factor Tu has 27 times higher affinity for GDP than for GDPNP at 37°C. The value is close to that determined by a stop-flow technique for *Thermus thermophilus* EF–Tu ∗ GDP and EF–Tu ∗ GTP (or EF–Tu ∗ GDPNP) complexes (24). The preference for GDP over GTP (or GDPNP) in the case of both thermophilic elongation factors is smaller than in the case of *E. coli* EF-Tu (Table 2). The difference between *Thermus* and *E. coli* EF-Tu's in guanine nucleotide cofactor binding may reflect a peculiarity of the protein biosynthesis at elevated temperature and reveals a functional adaptation of the translation machinery of thermophilic bacteria to the extreme environments.

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REFERENCES

1. Krab, I. M., and Parmeggiani, A. (1998) EF-Tu, a GTPase odyssey. *Biochim. Biophys. Acta* **1443,** 1–22.
2. Bosch, L., Kraal, B., Van der Meide, P. H., Duisterwinkel, F. J., and Van Noort, J. M. (1983) The elongation factor EF-Tu and its two encoding genes. *Prog. Nucleic Acid Res. Mol. Biol.* **30,** 91–126.
3. Lippmann, C., Lindschau, C., Vijgenboom, E., Schroder, W., Bosch, L., and Erdmann, V. A. (1993) Prokaryotic elongation factor Tu is phosphorylated *in vivo*. *J. Biol. Chem.* **268,** 601–607.
4. Alexander, C., Bilgin, N., Lindschau, C., Mesters, J. R., Kraal, B., Hilgenfeld, R., Erdmann, V. A., and Lippmann, C. (1995) Phosphorylation of elongation factor Tu prevents ternary complex formation. *J. Biol. Chem.* **270,** 14541–14547.
5. Ames, G. F., and Niakido, K. (1979) *In vivo* methylation of prokaryotic elongation factor Tu. *J. Biol. Chem.* **254,** 9947–9950.
6. Young, C. C., and Bernlohr, R. W. (1991) Elongation factor Tu is methylated in response to nutrient deprivation in *Escherichia coli*. *J. Bacteriol.* **173,** 3096–3100.
7. Voss, R. H., Hartmann, R. K., Lippmann, C., Alexander, C., Jahn, O., and Erdmann, V. A. (1992) Sequence of the tufA gene encoding elongation factor EF-Tu from *Thermus aquaticus* and overproduction of the protein in *Escherichia coli*. *Eur. J. Biochem.* **207,** 839–846.
8. Ahmadian, M. R., Kreutzer, R., and Sprinzl, M. (1991) Overproduction of the *Thermus thermophilus* elongation factor Tu in *Escherichia coli*. *Biochimie.* **73,** 1037–1043.
9. Boon, K., Vijgenboom, E., Madsen, L. V., Talens, A., Kraal, B., and Bosch, L. (1992) Isolation and functional analysis of histidine-tagged elongation factor Tu. *Eur. J. Biochem.* **210,** 177–183.
10. Anborgh, P. H., Swart, G. W., and Parmeggiani, A. (1991) Kirromycin-induced modifications facilitate the separation of EF-Tu species and reveal intermolecular interactions. *FEBS Lett.* **292,** 232–236.
11. Knudsen, C. R., Clark, B. F., Degn, B., and Wiborg, O. (1992) One-step purification of *E. coli* elongation factor Tu. *Biochem. Int.* **28,** 353–362.
12. Soslo, M., Amati, G., Cappellano, C., Sarubbi, E., Monti, F., and Donadio, S. (1996) An elongation factor Tu (EF-Tu) resistant to the EF-Tu inhibitor GE2270 in the producing organism *Planobispora rosea*. *Mol. Microbiol.* **22,** 43–51.
13. Cappellano, C., Monti, F., Sosio, M., Donadio, S., and Sarubbi, E. (1997) Natural kirromycin resistance of elongation factor Tu from the kirrothricin producer *Streptomyces cinnamoneus*. *Microbiology* **143**(Pt 2), 617–624.
14. Kim, S. I., Kim, H. Y., Kwak, J. H., Kwon, S. H., and Lee, S. Y. (2000) Cloning, overexpression, and purification of *Bacillus subtilis* elongation factor Tu in *Escherichia coli*. *Mol. Cells* **10,** 102–107.
15. Parmeggiani, A., and Sander, G. (1981) Properties and regulation of the GTPase activities of elongation factors Tu and G, and of initiation factor 2. *Mol. Cell. Biochem.* **35,** 129–158.
16. Fasano, O., Crechet, J. B., and Parmeggiani, A. (1982) Preparation of nucleotide-free elongation factor Tu and its stabilization by the antibiotic kirromycin. *Anal. Biochem.* **124,** 53–58.
17. Watanabe, K., Oshima, T., Iijima, K., Yamaizumi, Z., and Nishimura, S. (1980) Purification and thermal stability of several amino acid-specific tRNAs from an extreme thermophile, *Thermus thermophilus* HB8. *J. Biochem. (Tokyo)* **87,** 1–13.
18. Hatfield, G. W. (1979) Sepharose chromatography of transfer ribonucleic acids using reverse salt gradients. *Methods Enzymol.* **59,** 215–218.
19. Keith, G., Pixa, G., Fix, C., and Dirheimer, G. (1983) Primary structure of three tRNAs from brewer's yeast: $tRNA^{Pro}_2$, $tRNA^{His}_1$, and $tRNA^{His}_2$. *Biochimie* **65,** 661–672.
20. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

21. Arai, K. I., Kawakita, M., and Kaziro, Y. (1972) Studies on polypeptide elongation factors from *Escherichia coli*. II. Purification of factors Tu-guanosine diphosphate, Ts, and Tu-Ts, and crystallization of Tu-guanosine diphosphate and Tu-Ts. *J. Biol. Chem*. **247,** 7029–7037.
22. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **72,** 248–254.
23. Andersen, G. R., Stepanov, V. G., Kjeldgaard, M., Thirup, S. S., and Nyborg, J. (2000) Ternary complex of EF-Tu and its action on the ribosome. *in* The Ribosome: Structure, Function, Antibiotics, and Cellular Interactions (Garret, R. A., Douthwaite, S. R., Liljas, A., Matheson, A. T., Moore, P. B., and Noller, H. F., Eds.), pp. 337–345, ASM Press, Washington, DC.
24. Wagner, A., Simon, I., Sprinzl, M., and Goody, R. S. (1995) Interaction of guanosine nucleotides and their analogs with elongation factor Tu from *Thermus thermophilus*. *Biochemistry* **34,** 12535–12542.
25. Fasano, O., Bruns, W., Crechet, J. B., Sander, G., and Parmeggiani, A. (1978) Modification of elongation-factor-Tu-guanine-nucleotide interaction by kirromycin. A comparison with the effect of aminoacyl-tRNA and elongation factor Ts. *Eur. J. Biochem*. **89,** 557–565.
26. Arai, K., Kawakita, M., and Kaziro, Y. (1974) Studies on the polypeptide elongation factors from *E. coli*. V. Properties of various complexes containing EF-Tu and EF-Ts. *J. Biochem. (Tokyo)* **76,** 293–306.
27. 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.
28. Rattenborg, T., Nautrup Pedersen, G., Clark, B. F., and Knudsen, C. R. (1997) Contribution of Arg288 of *Escherichia coli* elongation factor Tu to translational functionality. *Eur. J. Biochem*. **249,** 408–414.
29. Mansilla, F., Knudsen, C. R., Laurberg, M., and Clark, B. F. (1997) Mutational analysis of *Escherichia coli* elongation factor Tu in search of a role for the N-terminal region. *Protein Eng*. **10,** 927–934.