Complex Formation of the Elongation Factor Tu from *Pseudomonas aeruginosa* with Nucleoside Diphosphate Kinase Modulates Ribosomal GTP Synthesis and Peptide Chain Elongation*

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The elongation factor Tu (EF-Tu) from *Pseudomonas aeruginosa* was purified as a 45-kDa polypeptide that forms a complex with both the 12- and 16-kDa forms of nucleoside-diphosphate kinase (Ndk) and predominantly synthesizes GTP. 70 S ribosomes of *P. aeruginosa* predominantly synthesize GTP, which is inhibited in presence of anti-Ndk antibodies. Anti-EF-Tu antibodies change the specificity of ribosomal GTP synthesis to all nucleoside triphosphate synthesis. Ndk has been shown to be a part of 30 S ribosomes, whereas EF-Tu is found to be associated with the 50 S ribosomal subunit. These data indicate that GTP synthesis in the ribosome is modulated both by Ndk and by EF-Tu. Peptide chain elongation as measured by polymerization of Phe-tRNA on a poly(U) template in presence of GDP can be inhibited by anti-Ndk antibodies and restored by the addition of GTP. Anti-EF-Tu antibodies similarly inhibit peptide chain elongation by *P. aeruginosa* ribosomes in the *in vitro* translation assay; however, this inhibition cannot be overcome by adding back GTP. Because the purified EF-Tu-16-kDa Ndk complex predominantly synthesizes GTP, it seems likely that this complex is a significant source of GTP for translational elongation in protein biosynthesis.

GTP binding proteins regulate a wide spectrum of intracellular processes. The protein elongation factor Tu (EF-Tu) is a well-documented abundant GTP binding protein in prokaryotes and plays a pivotal role in translational elongation. Elongation factor Tu binds aminoacyl-tRNA and GTP in a ternary complex that binds to the A site of the ribosome. When the tRNA anticondon matches the codon triplet of the programmed ribosome, GTP is hydrolyzed and EF-Tu-GDP complex dissociates, leaving the aminoacyl-tRNA in the A site, ready for peptidyl transferase. To participate in another round of elongation, the GDP in EF-Tu must be exchanged for GTP. This is normally catalyzed by EF-Ts, which upon binding to EF-Tu causes the release of GDP, which can then be converted to GTP for binding with EF-Tu. Without EF-Ts, EF-Tu has a high affinity for GDP and thus would remain in this inactive state. Whether or how GDP is subsequently converted to GTP in the ribosome is not clear.

Nucleoside-diphosphate kinase (Ndk) is an enzyme that catalyzes the transfer of terminal phosphate from ATP to any nucleoside (or deoxynucleoside) diphosphates to generate the corresponding NTPs and is thus important in maintaining the intracellular NTP pool. We have previously shown that in *Pseudomonas aeruginosa* 8830, the 16-kDa Ndk is cleaved by a protease to a 12-kDa form that is membrane-associated (4). The truncated 12-kDa form complexes with the enzyme pyruvate kinase (PK) and predominantly synthesizes GTP (5). GTP is a crucial molecule in signal transduction and is required for the process of translational elongation in protein biosynthesis (6). Under conditions of amino acid starvation, GTP is acted upon by the ribosome-associated enzyme RelA, along with SpoT, to generate the signal transducing molecule guanosine tetrathosphate, ppGpp (7). Though the role of GTP binding proteins has been well documented in eukaryotic cells, the significance of these proteins in prokaryotes remains to be deciphered. Because the enzyme Ndk has been implicated in generating GTP through complex formation with PK (5) and has also been reported to form complexes with a *P. aeruginosa* Ras-like GTP-binding protein Pfa (8), it was of interest to determine if other GTP-binding proteins that require GTP for their cellular function may also form complexes with Ndk. In this work we demonstrate that EF-Tu forms a complex with both the intact 16-kDa form as well as the truncated 12-kDa form of Ndk to synthesize GTP. We additionally demonstrate that 70 S ribosomes from *P. aeruginosa* predominantly synthesize GTP and that this synthesis is accomplished by 30 S ribosome-associated Ndk and 50 S ribosome-associated EF-Tu. Peptide chain elongation, normally inhibited by anti-EF-Tu antibodies, is also inhibited by anti-Ndk antibodies. This latter inhibition can be restored by the addition of GTP. The significance of our observations to the overall function of Ndk in providing GTP to EF-Tu through complex formation and thereby regulating translational elongation is discussed.

**EXPERIMENTAL PROCEDURES**

*Preparation of Cell Lysate—* *P. aeruginosa* 8830 is a stable alginate producing strain. The organism was routinely grown in L broth in 1-liter batches at 37 °C to an *A*<sub>600</sub> value of 1.0. The cells from 1 liter of culture were harvested by centrifugation at 15,000 × *g* for 15 min at 4 °C. The pellet was suspended in 5 ml of buffer A (50 mM Tris-HCl, pH 7.6, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol) and lysed by French Press at 12,000 psi through two consecutive cycles. The cell lysate was centrifuged at 10,000 × *g* for 15 min to obtain the clear supernatant, and the pellet was discarded. This supernatant was used as the starting material for purification of the protein EF-Tu from *P. aeruginosa*. Protein was estimated by Bradford reagent (Bio-Rad).

*Immobilization of Ndk to a Sepharose 4B Matrix and Subsequent Analysis of Proteins Retained on the Matrix—* 3 g of activated Sepharose was suspended in 15 ml of chilled 0.2 mM NaHCO<sub>3</sub>, pH 8.5, and stirred in

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cold for 15 min. 1 ml of 5 mg/ml solution of the protein Ndk was dialyzed against 0.2 M NaHCO3, and added to the Sepharose suspension. Stirring was continued gently overnight in the cold. Powdered glycine was added to the suspension to a final concentration of 1 M and incubation continued for an additional 1 h at room temperature. The matrix was then washed sequentially with 0.5 M NaHCO3, 0.5 M sodium acetate, and 2.0 M urea. The matrix was finally washed with buffer A and stored in the cold until further use. 1 ml of the matrix was saturated with 0.5 mM GTP and added to 40 mg of supernatant protein obtained from the P. aeruginosa cell lysate. After the proteins were allowed to interact with the matrix-bound Ndk for 30 min on ice, the matrix was washed with buffer A alone followed by batches of buffer A containing increasing NaCl solution (0–200 mM) and 0.5 mM GTP. Approximately 10 μg of protein from each of the salt wash fractions were run on 15% SDS-polyacrylamide gel electrophoresis for detection of proteins that bound to the Ndk-Sepharose matrix. The N-terminal sequence of the proteins bound to the Ndk-Sepharose matrices and eluted at specific NaCl concentrations were carried out by Dr. K.-L. Ngai at the Noyes Genetic Engineering Facility of the University of Illinois at Urbana.

Glycerol Density Gradient Centrifugation—The glycerol density gradient centrifugation was carried out as described previously (5). A batch gradient of glycerol was prepared by successive layering of 60, 50, 40, 30, 20, 10, and 5% glycerol solutions, each of 1-ml volume, in Beckman 100 Ti ultracentrifuge tubes. The purified protein samples to be analyzed were carefully layered on top of the 5% band. The discontinuous gradient tubes were centrifuged at 40,000 × g for 1 h at 4 °C. Fractions were carefully collected by withdrawing 1-ml aliquots from the top of each gradient to microfuge tubes. All the fractions were analyzed by Western dot blot using anti-Ndk, anti-Pk, and anti-EF-Tu antibodies.

Effect of Purified EF-Tu on NTP Synthesizing Activity of Ndk—NTP synthesizing activity was measured in 20-μl reaction volumes containing 250 μM each of GDP, GTP, and UDP as described previously (4, 5). The reaction was initiated by adding different amounts of purified protein and 10.0 μCi of [γ-32P]ATP (3000 Ci/mmol) along with 0.125 mM radioactive ATP. The reaction mixture was incubated for 1 min, followed by addition of 2 μl of 10 × SDS buffer. 1 μl of the reaction mixture was spotted on to a polyethyleneimine TLC plate and the reaction products were separated in 0.75 M KH2PO4 buffer, pH 3.75, as described previously (4). The TLC plates were air dried, covered with plastic wrap, and autoradiographed.

Effect of Anti-Ndk and Anti-EF-Tu Antibodies on NTP Synthesizing Activity of 70 S Ribosomes—70 S ribosomes from P. aeruginosa were isolated according to the method used for isolation of ribosomes from Escherichia coli (9). Nucleoside triphosphate synthesizing activity of ribosomes was analyzed in a 20-μl reaction volume containing 250 μM each of GDP, GTP, and UDP. 10 μCi of [γ-32P]ATP along with 125 μM ATP was added to the reaction mixture, and the reaction was started by the addition of 10 μg of 70 S ribosomes. The reaction mixture was incubated for 1 min at room temperature, followed by addition of 2 μl of 10 × SDS buffer. Anti-Ndk and anti-EF-Tu antibodies were added at a dilution of 1:1000 separately to see their effect on NTP synthesizing activity by the ribosomes. In a parallel experiment, purified proteins EF-Tu and Ndk were added back in increasing amounts to determine the restoration of NTP synthesizing activity of the ribosomes.

Salt Washing of the Ribosome and Its Dissociation—70 S ribosomes from P. aeruginosa were isolated and extracted with 100 mM, 200 mM, 500 mM, and 1.0 M KCl solution in TMNM buffer (10 mM Tris, pH 7.5, 60 mM NaCl, 30 mM NH4Cl, and 4 mM 2-mercaptoethanol) sequentially, and at each step the pellet was resuspended while the supernatant was assayed for NTP synthesizing activity. In a separate experiment, 70 S ribosomes were separated into 30 and 50 S subunits by sucrose density gradient centrifugation as described for E. coli ribosomes (10). Both the subunits were assayed individually for NTP synthesizing activity.

Translation of Poly(U)—The poly(U) directed polyphenylalanine synthesis from [35S]Phe-tRNA was carried out in 0.1 ml of reaction mixture (9) consisting of 50 mM Tris-HCl, pH 7.4, 8 mM MgCl2, 60 mM NaCl, 6 mM β-mercaptoethanol, 0.2 mM GTP, 5 mM phosphoenol pyruvate, 1 μg of pyruvate kinase, 40 μg of poly(U), 50 pmol of [35S] Phe-tRNA, 25 pmol of ribosomes with EF-G, and 50 pmol of purified EF-Tu. The reaction was carried out at 37 °C for 10 min in presence or the absence of anti-Ndk or anti-EF-Tu antibodies. The reaction was stopped with 100 μl of 20% trichloroacetic acid, and kept on ice for 30 min. The precipitated samples were assayed for incorporation of [35S] Phe in trichloroacetic acid precipitated material using liquid scintillation spectrometry on a Pharmacia Biotech Inc. Rackbeta counter.

FIG. 1. Characterization of elongation factor Tu from P. aeruginosa 8830, A, EF-Tu was isolated from the Sepharose 4B-Ndk-GTP matrix by elution with 75 mM NaCl. About 5 μg of this protein was analyzed for size and electrophoretic homogeneity on a 15% SDS-polyacrylamide gel electrophoresis. Lane 1, molecular mass markers; lane 2, 5 μg of purified protein. B, sequence comparisons of amino acids located at the N terminus of various bacterial EF-Tu proteins and the isolated protein from P. aeruginosa.

RESULTS

Elution of P. aeruginosa EF-Tu from a Ndk-Sepharose 4B Column Saturated with GTP—We previously have reported the characterization of two proteins from P. aeruginosa that form complexes with Ndk and modulate its NTP synthesizing specificity to GTP. One is Pk, a 60-kDa protein (5), whereas the other is a 47-kDa Ras-like protein, Pra, with GTPase activity (8). There is a homolog of Pra in E. coli, known as Era, which is known to be an essential protein, but its exact function is unknown (11, 12). We have reported that the E. coli Era protein, having intrinsic GTPase activity, can also form complexes with P. aeruginosa 12-kDa Ndk to modulate its NTP synthesis to GTP (8). To see if other Ndk-binding proteins could also be present in P. aeruginosa, we cross-linked Ndk to Sepharose 4B and allowed the Sepharose-bound Ndk to interact with proteins present in the cell lysates of P. aeruginosa. Unbound or loosely bound proteins were removed by exhaustive washing of the Sepharose matrix with buffer A. A linear gradient of NaCl (0–200 mM) in buffer A was then used to elute proteins that may have been retained in the matrix through complex formation with Ndk. A protein of about 60 kDa, presumably Pk, was eluted at 150 mM NaCl, whereas a protein of about 47 kDa, presumably Pra, was eluted from the Sepharose-Ndk column at 200 mM NaCl concentration. Interestingly, a protein of about 45 kDa was eluted from the Sepharose-Ndk-GTP column at a NaCl concentration of 75 mM. Lower concentrations of NaCl did not allow its release, suggesting a specific association of this protein to the Ndk-Sepharose column. This protein appeared to be present in abundant amount and was isolated in a purified form. SDS-polyacrylamide gel electrophoresis demonstrated a single band with a molecular mass of about 45 kDa (Fig. 1A). N-terminal sequence determination of this protein revealed a high degree of sequence homology with EF-Tu from E. coli and Chlamydia (Fig. 1B). GTP-binding proteins such as IP-2, EF-Tu, and EF-G are known to mediate various steps in peptide chain initiation and elongation (13), and the isolation of EF-Tu
from an Ndk-Sepharose column appeared to suggest that Ndk may somehow form complexes with EF-Tu and modulate peptide chain elongation, perhaps through generation of GTP where it is needed.

Complex Formation of EF-Tu with Ndk—Because the protein EF-Tu was isolated from Ndk-Sepharose 4B affinity chromatography, we wanted to determine if this protein could form a complex with both the intact 16-kDa Ndk as well as the truncated 12-kDa form. We demonstrated previously that the other two Ndk-complexing proteins Pk and Pra could form complexes only with the 12-kDa form of Ndk and are associated with the membrane (5, 8). Because cytoplasmic EF-Tu also associates with ribosomes, it was of interest to see if it could be associated with the 16-kDa Ndk, which is predominantly cytoplasmic (4). As shown in Fig. 2A, the 12-kDa form of Ndk was found in 5% glycerol fraction and did not show migrational shift when incubated with bovine serum albumin; however, on incubation with the purified protein EF-Tu, which itself sediments in 10% glycerol fraction (Fig. 2B), there is a shift in mobility because both the proteins are observed in 40% glycerol (Fig. 2A, row EF-Tu-Ndk 12; Fig. 2B, row EF-Tu-Ndk 12). The 16-kDa Ndk, which itself sediments in 5% glycerol, also showed mobility shift (sediments at 60% glycerol) when incubated with EF-Tu but not with the control protein bovine serum albumin. Moreover, EF-Tu does not show complex formation with Pk (Fig. 2B, row EF-Tu-Pk). This lack of complex formation with Pk was also verified through use of anti-Pk antibodies that demonstrated a lack of mobility of Pk to a higher sedimenting stage (sediments at 60% glycerol) when incubated with EF-Tu but not with the control protein bovine serum albumin.

Effect of EF-Tu on NTP Synthesis by 12- and 16-kDa Forms of Ndk—The enzyme Ndk is capable of synthesizing all NTPs from nucleoside diphosphates in the cell without any apparent preference. Because both the intact 16-kDa form as well as 12-kDa form of Ndk form complexes with EF-Tu (Fig. 2), it was of interest to determine if this interaction can alter the specificity of NTP formation by Ndk. When either the 12-kDa or the 16-kDa Ndk was incubated with [γ-32P]ATP and nonradioactive CDP, UDP and GDP, the corresponding radioactive NTPs were formed. When EF-Tu was added to the 12-kDa or the 16-kDa form of Ndk, it resulted in predominant GTP synthesis (Fig. 3, lanes 4 and 10). However, upon addition of anti EF-Tu antibodies to the 12-kDa Ndk-EF-Tu complex or the 16-kDa Ndk-EF-Tu complex, the specificity of GTP synthesis was lost.
and the corresponding radioactive NTPs were formed (Fig. 3, lanes 6 and 12). Addition of anti-Ndk antibodies to the Ndk-EF-Tu complexes inhibited phosophotransfer as expected (Fig. 3, lanes 5 and 11), whereas EF-Tu itself had no phosphotransfer ability of its own (Fig. 3, lane 13).

Effect of Anti-Ndk and Anti-EF-Tu Antibodies on NTP Synthesis by 70 S Ribosomes—The process of translational elongation on ribosomes requires GTP. Because we found that the 16-kDa Ndk can form a complex with the purified protein EF-Tu and predominantly synthesizes GTP (Fig. 3), we decided to examine if 70 S ribosomes themselves can synthesize NTPs, particularly GTP, and if such NTP synthesis by 70 S ribosomes can be altered by anti-Ndk and anti-EF-Tu antibodies. 70 S ribosomes of P. aeruginosa predominantly synthesize GTP in the presence of $[^\gamma-^3P]ATP$ and nonradioactive CDP, GDP, and UDP (Fig. 4, lane 1), and this GTP synthesis can be inhibited by anti-Ndk antibodies (Fig. 4, lane 2). However, addition of increasing amounts of purified Ndk resulted in restoration of all NTP synthesis (Fig. 4, lanes 5 and 6), presumably independent of the complex. In contrast, anti-EF-Tu antibodies altered GTP synthesis by ribosomes to nonspecific NTP (CTP, UTP, and GTP) synthesis under identical conditions (Fig. 4, lane 3), suggesting that EF-Tu was responsible for the specificity to GTP synthesis in ribosomes. Indeed, addition of excess amounts of EF-Tu restored GTP synthesis by the 70 S ribosomes (Fig. 4, lane 9), suggesting that both Ndk and EF-Tu play an important role in ribosomal GTP synthesis.

Localization of Ndk on the Ribosomal 30 S Subunit—The inhibition of ribosomal GTP synthesis by anti-Ndk antibodies implied that Ndk might be responsible for GTP synthesis by the ribosomes. To determine if Ndk is associated as a part of the ribosomes, the ribosomes were extracted with 100–1000 mM KCl solutions as described under “Experimental Procedures,” and the extracts were examined for NTP synthesizing activity in presence of $[^\gamma-^3P]ATP$ and 250 $\mu$M each of CDP, GDP, and UDP as described in the legend to Fig. 3. Lane 1, 70 S ribosomes; lane 2, + anti-Ndk antibody; lane 3, + anti-EF-Tu antibody; lane 4, 100 mM KCl wash; lane 5, + anti-Ndk antibody; lane 6, + anti-EF-Tu antibody; lane 7, 200 mM KCl wash; lane 8, + anti-Ndk antibody; lane 9, + anti-EF-Tu antibody; lane 10, 500 mM KCl wash; lane 11, + anti-Ndk antibody; lane 12, + anti-EF-Tu antibody; lane 13, 1000 mM KCl wash; lane 14, + anti-Ndk antibody; lane 15, + anti-EF-Tu antibody. B, NTP synthesizing activity of 70 S ribosomes and that of the dissociated 30 and 50 S subunits. The dissociation and isolation of individual ribosomal subunits have been described under “Experimental Procedures.” Lane 1, 70 S ribosomes; lane 2, + anti-Ndk antibody; lane 3, + anti-EF-Tu antibody; lane 4, 30 S ribosomal subunits; lane 5, + anti-Ndk antibody; lane 6, + anti-EF-Tu antibody; lane 7, 50 S ribosomal subunits; lane 8, + anti-Ndk antibody; lane 9, + anti-EF-Tu antibody; lane 10, 30 + 50 S ribosomal subunit mixture; lane 11, + anti-Ndk antibody; lane 12, + anti-EF-Tu antibody; lane 13, 30 S ribosomal subunit + purified EF-Tu; lane 14, + anti-Ndk antibody; lane 15, + anti-EF-Tu antibody.

![Figure 4](image4.png) **Fig. 4.** Effect of anti-Ndk and anti-EF-Tu antibodies on GTP synthesis by the P. aeruginosa 70 S ribosomal fraction. The 70 S ribosomal fraction was prepared as described under “Experimental Procedures” (9). Both the anti-Ndk as well as the anti-EF-Tu antibodies were used at a dilution of 1:1000 in the inhibition reaction. The NTP synthesizing reactions were as described in the legend to Fig. 3 except ribosomal fraction instead of purified Ndk with or without EF-Tu was used. Lane 1, 70 S ribosomal fraction; lane 2, + anti-Ndk antibodies; lane 3, + anti-EF-Tu antibodies; lane 4, same as lane 2 + 20 pmol of Ndk; lane 5, same as lane 2 + 30 pmol of Ndk; lane 6, same as lane 2 + 50 pmol of Ndk; lane 7, same as lane 3 + 20 pmol of EF-Tu; lane 8, same as lane 3 + 30 pmol of EF-Tu; lane 9, same as lane 3 + 50 pmol of EF-Tu.

![Figure 5](image5.png) **Fig. 5.** A, NTP synthesizing activity of intact 70 S ribosomes and the various salt wash fractions. Ribosomes were suspended in TMNM buffer and extracted sequentially with 100, 200, 500, and 1000 mM KCl solutions, as described under “Experimental Procedures.” The extracts were tested for NTP synthesizing activity in presence of $[^\gamma-^3P]ATP$ and 250 $\mu$M each of CDP, GDP, and UDP as described in the legend to Fig. 3. Lane 1, 70 S ribosomes; lane 2, + anti-Ndk antibody; lane 3, + anti-EF-Tu antibody; lane 4, 100 mM KCl wash; lane 5, + anti-Ndk antibody; lane 6, + anti-EF-Tu antibody; lane 7, 200 mM KCl wash; lane 8, + anti-Ndk antibody; lane 9, + anti-EF-Tu antibody; lane 10, 500 mM KCl wash; lane 11, + anti-Ndk antibody; lane 12, + anti-EF-Tu antibody; lane 13, 1000 mM KCl wash; lane 14, + anti-Ndk antibody; lane 15, + anti-EF-Tu antibody. B, NTP synthesizing activity of 70 S ribosomes and that of the dissociated 30 and 50 S subunits. The dissociation and isolation of individual ribosomal subunits have been described under “Experimental Procedures.” Lane 1, 70 S ribosomes; lane 2, + anti-Ndk antibody; lane 3, + anti-EF-Tu antibody; lane 4, 30 S ribosomal subunits; lane 5, + anti-Ndk antibody; lane 6, + anti-EF-Tu antibody; lane 7, 50 S ribosomal subunits; lane 8, + anti-Ndk antibody; lane 9, + anti-EF-Tu antibody; lane 10, 30 + 50 S ribosomal subunit mixture; lane 11, + anti-Ndk antibody; lane 12, + anti-EF-Tu antibody; lane 13, 30 S ribosomal subunit + purified EF-Tu; lane 14, + anti-Ndk antibody; lane 15, + anti-EF-Tu antibody.
activity from 70 S ribosomes (Fig. 5A, lanes 4–12); however, this activity was released from the ribosomes by 1000 mM KCl (Fig. 5A, lane 13). The released activity was inhibited by anti-Ndk antibody (Fig. 5A, lane 14), but not by anti-EF-Tu antibodies, indicating that Ndk was released from the ribosomes free of EF-Tu. It is likely that EF-Tu might have been released by an earlier wash. To further delineate the localization of Ndk in the ribosomal subunits, the 70 S ribosomes were subjected to conditions that allowed their dissociation into 30 and 50 S components (10). Each component was isolated by sucrose density gradient centrifugation. As shown in Fig. 5B, the 30 S subunits harbored NTP synthesizing activity that was completely inhibited by anti-Ndk antibody (Fig. 5B, lanes 4 and 5); 50 S subunits had no Ndk-like activity (Fig. 5B, lanes 7–9). Interestingly, mixing the 30 S and the 50 S ribosomal subunits allowed GTP synthesis, which is inhibited by anti-Ndk antibody but whose specificity for GTP synthesis is eliminated by anti-EF-Tu antibody (Fig. 5B, lanes 10–12), reminiscent of intact 70 S ribosomes (Fig. 5B, lanes 1–3). This suggests that although the 30 S ribosomes harbor Ndk, the EF-Tu is associated with the 50 S ribosomes, so that an Ndk-EF-Tu complex is formed on reassociation of the two subunits. This is further confirmed by the fact that when the Ndk-containing 30 S ribosomal subunits are treated with purified EF-Tu, the preparation generates only GTP (Fig. 5B, lane 13) as contrasted with 30 S subunits alone, which generate all the three NTPs (Fig. 5B, lane 4). Although the association of Ndk with 30 S ribosomes has never been reported, EF-Tu is known to interact with ribosomal proteins present in the 50 S subunit (14).

The Role of Ndk in Ribosomal Peptide Chain Elongation—Whereas the role of EF-Tu on peptide chain elongation as well as the role of GTP bound to EF-Tu in promoting the binding of aminoacyl-tRNA to the A-site of mRNA-programmed ribosomes are well known events (6, 9, 13), much less is known about the source of the GTP or how the availability of GTP to EF-Tu is ensured for continued protein synthesis. Because both Ndk and EF-Tu are shown to exist in the form of a complex to direct the specificity of Ndk to GTP synthesis, an important question was if Ndk plays a critical role in peptide chain elongation as well. *P. aeruginosa* ribosomes are active in catalyzing the incorporation of 14C-Phe into trichloroacetic acid-precipitable material during an *in vitro* peptide elongation assay (Fig. 6, lane 1) where GTP is an essential component (Fig. 6, lane 2). However, GDP can replace GTP to a large extent in the presence of Ndk and an ATP-generating system (Fig. 6, lane 3), and this stimulation of peptide chain elongation by GDP is completely abolished in presence of anti-Ndk antibodies (Fig. 6, lane 4), suggesting that the conversion of GDP to GTP is mediated by the Ndk-EF-Tu complex. Indeed, addition of GTP to the reaction mixture, where GDP to GTP conversion is inhibited by anti-Ndk antibody, allows substantial restoration of 14C-Phe incorporation into the nascent polypeptide chain, emphasizing the role of Ndk in GTP synthesis for continued protein synthesis (Fig. 6, lane 5). As expected, anti-EF-Tu antibodies can effectively inhibit 14C-Phe incorporation in the nascent polypeptide chain in the presence of GDP (Fig. 6, lane 6), and such inhibition cannot be overcome by simple addition of GTP (Fig. 6, lane 7). Thus the role of Ndk appears to be to supply GTP to EF-Tu, and this role is abrogated in the presence of exogenously supplied GTP.

**DISCUSSION**

In this report, we describe the isolation of *P. aeruginosa* EF-Tu on the basis of its association with Ndk by Sepharose 4B affinity chromatography, implying that it may exist, at least in part, in association with Ndk on the ribosomes. Western blotting of the proteins isolated from the *P. aeruginosa* ribosomes, using anti-Ndk antibodies, has shown the presence of Ndk in ribosome fractions (data not shown), and *in vitro* complex formation between Ndk and EF-Tu has been demonstrated in this report (Fig. 2). The implication of this complex formation in the ribosome, where Ndk has been localized in the 30 S subunit and EF-Tu as part of the 50 S subunit (Fig. 5B), is apparent from the fact that either anti-Ndk or anti-EF-Tu antibodies will inhibit ribosomal GTP synthesis (or change the specificity of GTP synthesis to all NTPs) as well as inhibit peptide chain elongation (Figs. 4, 5, and 6), strongly implying this complex in GTP-mediated peptide chain elongation. The role of EF-Tu in protein synthesis is well established and involves delivery of aminoacyl-tRNA to the A site on the ribosome as a ternary complex with GTP (13). Subsequent hydrolysis of GTP to GDP results in release of an EF-Tu-GDP complex, which must be recycled to EF-Tu-GTP in order for EF-Tu to participate in another round of elongation. EF-Tu is a typical G protein in that it has a higher affinity for GDP than for GTP and thus requires a GDP-GTP exchange factor such as EF-Ts to be recycled to the active GTP-bound form. EF-Ts enhances phosphorylation of EF-Tu by a ribosome-associated kinase, and the phosphorylation of EF-Tu abolishes its ability to bind aminoacyl-tRNA (15). EF-Ts then catalyzes GDP-GTP exchange on EF-Tu such that in presence of EF-Ts, GDP is released from EF-Tu-GDP complex and a EF-Tu-EF-Ts complex is formed. In presence of GTP, EF-Ts is released and EF-Tu-GTP complex is formed for binding with aminoclyl-tRNA for continued peptide chain elongation. It is not known, however, from what source the GTP is acquired for complex formation with EF-Tu.

It is generally accepted that GTP cannot be generated directly from a complex of EF-Tu-GDP. The results of our study show clearly that EF-Tu forms a complex with Ndk in *in vitro* and changes its substrate specificity predominantly to GDP (Figs. 2 and 3). Ndk is associated with the 30 S, whereas EF-Tu is associated with the 50 S ribosomal subunits so that it is likely that Ndk and EF-Tu form an association in the 70 S ribosomes under certain conditions. Preliminary results from our laboratory show that when *E. coli* EF-Tu is treated with GDP, the excess GDP is removed by biospin column chromatography, and the EF-Tu-GDP complex is treated with Ndk and
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[γ-32P]ATP, radioactive GTP is formed. Inclusion of EF-Ts in this reaction results in only marginal enhancement of GDP release. Although the [32P]GTP synthesis is blocked by anti-Ndk antibody, anti-EF-Tu antibody has no effect, suggesting that [32P]GTP is formed due to release of GDP from EF-Tu by Ndk. Other GDP-binding or nonbinding proteins such as Pra (8) or bovine serum albumin did not allow GTP synthesis under such conditions, suggesting that Ndk releases GDP specifically from EF-Tu-GDP complex. It is tempting to speculate that part of the EF-Tu-GDP complex, released after the aminoacyl tRNA is transferred to the A site in the ribosome, is acted upon by the ribosome-associated Ndk, resulting in the release of GDP. This GDP is then converted to GTP by Ndk and made available for translational elongation. Some EF-Tu-GDP may additionally be acted upon by EF-Ts outside the ribosome, resulting in EF-Tu-EF-Ts complex formation and GDP release. This GDP can then be accessible to cytoplasmic Ndk. Preliminary experiments on the determination of Ndk levels in the cytoplasm and isolated ribosomes demonstrated variability depending upon the growth stage of the bacteria, suggesting that Ndk is firmly associated with the ribosomes during the log phase of growth but is largely dissociated at the stationary phase, presumably because of the proteolytic cleavage to a 12-kDa form that associates with the membranes (4). We are in the process of purifying the EF-Ts from *P. aeruginosa* to study the relative rate of GDP exchange from EF-Tu-GDP complex by either Ndk or EF-Ts.

Elongation factor Tu is now known to be the third protein, besides Pk and Pra, that allows complex formation with Ndk to modulate its NTP synthesizing activity to GTP. Unlike Pk and Pra that are membrane-associated proteins, EF-Tu is primarily cytoplasmic and can therefore form complexes with the 16-kDa Ndk, which is known to be soluble (4). It is important to emphasize that Pk and Pra can form complexes with only 12-kDa Ndk, which is known to be membrane-associated, and not with the cytoplasmic 16-kDa Ndk (5, 8). Thus the cytoplasmic 16-kDa Ndk not only generates NTPs for cellular metabolic activities, but also provides GTP to specific GTP-requiring proteins such as EF-Tu. It is interesting to note that proteins homologous to EF-Tu and Pk, capable of complexing with Ndk to modulate its NTP synthesizing specificity to GTP, have also been isolated from other microorganisms such as *Mycobacterium smegmatis* (16). Nucleoside-diphosphate kinase has been shown to be a part of the ribosomes in *Dictyostelium* and has been inferred to interact with ribosomal proteins or translational elongation factors to modulate protein synthesis (17), although no specific mode of action was proposed. It would be interesting to see if other ribosomal GTP-requiring proteins or G proteins in general might demonstrate complex formation with Ndk to modulate their GTP synthesis as a way to ensure the availability of GTP, as previously suggested (18, 19).

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REFERENCES

1. Zhang, Y.-X., Shi, Y., Zhou, M., and Petsko, G. A. (1994) J. Bacteriol. 176, 1184–1187
2. Sprinzl, M. (1994) Trends Biochem. Sci. 19, 245–250
3. Ehrenberg, M., Rojas, A. M., Diaz, I., Bilgin, N., Weiser, J., Classen, F., and Kurland, C. G. (1990) in *The Ribosome: Structure, Function, and Evolution* (Hill, W. E., Dahlberg, A., Garrett, R. A., Moore, P. B., Schlessinger, D., and Warner, J. A., eds) pp. 373–379, American Society for Microbiology Press, Washington, D. C.
4. Shankar, S., Kamath, S., and Chakrabartty, A. M. (1996) J. Bacteriol. 178, 1777–1781
5. Sundin, G. W., Shankar, S., Chugani, S. A., Chepade, B. A., Kavanaugh-Black, A., and Chakrabartty, A. M. (1996) *Mol. Microbiol.* 20, 965–979
6. Kaziro, Y., Itoh, H., Kosasa, T., Nakafufu, M., and Satoh, T. (1991) *Annu. Rev. Biochem.* 60, 349–400
7. Casthel, M. (1994) Methods Mol. Genet. 3, 341–356
8. Chepade, B., Shankar, S., Sundin, G. W., Mukhopadhyay, S., and Chakrabartty, A. M. (1997) *J. Bacteriol.* 179, 2181–2188
9. Garrilova, L. P., and Spirin, A. S. (1974) Methods Enzymol. 30, 452–462
10. Rheinberger, H.-J., Geigenmüller, U., Wedde, M., and Nierhaus, K. H. (1986) Methods Enzymol. 164, 658–662
11. March, P. E. (1992) *Mol. Microbiol.* 6, 1253–1257
12. Pühltul, R. C., Shaler, J. D., Gutati, P. S., Wu, E., Yamashita, Y., Lerner, C. G., Inouye, M., and March, P. E. (1995) *J. Bacteriol.* 177, 2194–2196
13. Moore, P. B. (1996) *Science* 270, 1453–1454
14. Spirin, A. S., and Vasiliev, V. D. (1989) *Bio. Cell.* 66, 215–223
15. Alexander, C., Bilgin, N., Landschau, C., Mesters, J. R., Kraal, B., Hilgenfeld, R., Erdmann, V. A., and Lippmann, C. (1995) *J. Biol. Chem.* 270, 14541–14547
16. Shankar, S., Herscherger, C. D., and Chakrabartty, A. M. (1997) *Mol. Microbiol.* 24, 477–487
17. Sonnenmann, J., and Mutzel, R. (1995) *Biochem. Biophys. Res. Commun.* 209, 490–496
18. Rominaar, A. A., Molijn, A. C., Pestel, M., Veron, M., and Haastert, P. J. M. V. (1990) *EMBO J.* 12, 2275–2279
19. Klinker, J. F., and Seifert, R. (1995) *Biochem. Biophys. Res. Commun.* 209, 575–581

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2 S. Mukhopadhyay, unpublished observations.