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Polymerization of an *Escherichia coli* Elongation Factor Tu

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Elongation factor Tu (EF-Tu) from *Escherichia coli* is shown here to polymerize under conditions of low ionic strength and slightly acidic pH. Several factors, such as decreasing pH, decreasing ionic strength, increasing temperature, and increasing protein concentration (up to 2.5 μM) enhance the rate of polymerization. Both EF-Tu·GTP and EF-Tu·GDP polymerized equally well under the conditions studied. A lag time was observed between the lowering of the pH and the onset of measurable polymerization, which was not overcome by addition of preformed polymer "seeds." Finally, addition of unpolymerized EF-Tu·GDP to a solution of polymerized EF-Tu·GDP appears to lead to formation of new polymers instead of addition to preexisting ones, which may suggest a size limit for polymers of EF-Tu·GDP.

***Key Words:* elongation factor Tu (EF-Tu); polymerization; self-association.**

Elongation factor Tu (EF-Tu)[3] from *Escherichia coli* is a 43.2-kDa protein consisting of 393 amino acids. EF-Tu binds 1:1 with guanine nucleotides (GDP and GTP) and can hydrolyze GTP to GDP and P_i. The GTPase activity of EF-Tu is important for its role in prokaryotic protein biosynthesis as the GTP-bound form is the "on" form, and the GDP-bound form is the "off" form. That is, EF-Tu·GTP binds aminoacyl-tRNA's and carries them to the acceptor site of a mRNA-programmed or elongating ribosome. Binding to the ribosome enhances the GTPase activity of EF-Tu·GTP. After GTP hydrolysis, EF-Tu·GDP dissociates from the ribosome. Finally, EF-Tu·GTP is regenerated through interaction of EF-Tu·GDP with the exchange factor, a protein known as EF-Ts, which promotes exchange of GDP for GTP on EF-Tu. The function of EF-Tu has been reviewed recently (1).

Although the early evidence suggested that one molecule of GTP is hydrolyzed by EF-Tu per amino acid incorporated into a peptide, more recent evidence has suggested that two molecules of GTP are hydrolyzed per amino acid incorporated, which may indicate that a dimer of EF-Tu-GTP is the functional carrier of aminoacyl-tRNA's (2–5). The possibility of dimers or other aggregates present in solution along with monomers of EF-Tu has been suggested by techniques such as small angle neutron scattering, quasi-elastic light scattering, and fluorescence spectroscopy (6–8).

Besides a possible function for small aggregates of EF-Tu, the high concentration of EF-Tu *in vivo* (100–200 μM, making it 5–10% of the total cell protein) and its apparent membrane association have led to the speculation that large aggregates of EF-Tu may play a structural role (1, 9, 10). EF-Tu has been shown to polymerize under certain conditions, with the early evidence attributing polymerization to the presence of calcium or magnesium ions (10–12). A later study demonstrated that polymerization of EF-Tu could be induced by slightly acidic pH (6.0) and low ionic strength, forming polymers up to 0.7 μm in diameter and 200 μm in length (13). By way of comparison, the monomer of EF-Tu·GDP in solution has been shown by small angle X-ray scattering to be 4.08 × 4.08 × 1.18 nm, whereas the crystal structure of this monomer is 7.5 × 5.0 × 3.0 nm (14–17).

The conditions affecting the state of aggregation of EF-Tu have been examined here. Polymerization is shown to be induced by pH below 7.0. The rate of polymerization is increased by decreasing pH, increasing protein concentration (up to a point), increasing temperature, and decreasing ionic strength. Interestingly, both EF-Tu·GDP and EF-Tu·GTP polymerize equally well. The lag time observed early in polymerization

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[3] Abbreviations used: EF-Tu, elongation factor Tu; BSA, bovine serum albumin; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; Mes, *N*-morpholinoethanesulfonic acid.

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could not be abolished by addition of preformed polymers, or "seeds," suggesting the lack of an unfavorable nucleation step in polymerization. Finally, addition of unpolymerized EF-Tu·GDP to a solution of EF-Tu·GDP polymers apparently leads to formation of new polymers rather than addition onto preexisting polymers, which may suggest a size limit for polymers of EF-Tu·GDP.

MATERIALS AND METHODS

Protein purification. EF-Tu·GDP was purified from frozen paste of *E. coli* MRE 600 (Center for Applied Microbiology and Research, Porton Down, UK) according to the method of Leberman *et al.* (18). An ammonium sulfate back extraction was performed as a last step. That is, the purified protein precipitate was dissolved in decreasing amounts of ammonium sulfate, with the highest specific activity of EF-Tu found in 30% ammonium sulfate. Purity was assessed by the appearance of a single band on sodium dodecyl sulfate–polyacrylamide gels stained with Coomassie blue. Protein concentration was determined with the Coomassie Plus assay (Pierce) using BSA as a standard. For long term storage, EF-Tu was quickly frozen in liquid nitrogen and kept at −80°C, otherwise it was kept on ice prior to use.

Buffer solutions. EF-Tu·GDP was purified and stored in buffer containing 25 mM Tris (pH 7.6 at 5°C), 10 mM $MgCl_2$, 1 mM NaN_3, 0.5 mM dithiothreitol (DTT), 0.01 mM phenylmethylsulfonyl fluoride (PMSF), and 5 μM GDP. For polymerization experiments, EF-Tu·GDP in the storage buffer was diluted at least 1:10 into either 2.5 mM Tris or 2.5 mM *N*-morpholinoethanesulfonic acid (Mes) (pH 7.5 at room temperature). The pH of the buffer could then be lowered by addition of one-tenth volume or less of either 50 mM KH_2PO_4 or 50 mM KHPhthalate (pH 5.0 at room temperature) to the 2.5 mM Tris buffer. Alternatively, the pH of the 2.5 mM Mes buffer could be lowered by the addition of one-tenth volume or less of 50 mM Mes buffer (pH 4.0 at room temperature).

The ionic strength of Mes buffer solutions was calculated using the Henderson–Hasselbalch equation to determine [conjugate base] and then calculating the ionic strength (μ) using the equation

$$\mu = \sum_{i=1}^{n} M_i \cdot z_i^2,$$

where M_i is the concentration of an ion i and z_i is the charge on that ion.

Addition of preformed polymers of EF-Tu. Polymers of EF-Tu·GDP, initially at 2.3 μM, were grown at pH 5.5 until a plateau in the absorbance at 340 nm was reached. A 10-μl aliquot of the polymer solution was then withdrawn and added to a solution of unpolymerized EF-Tu·GDP (2.3 μM) under polymerizing conditions (pH 5.5 for 1 min). As a control, the same quantity (2.3 μM) of unpolymerized EF-Tu·GDP was added.

Turbidity and scattering measurements. Turbidity was followed using either a Perkin–Elmer Lambda 5 or a Cary 4 uv/visible spectrophotometer. Optical density was monitored at 340 nm to avoid absorbance by the single tryptophan residue in EF-Tu and to maximize the scattering intensity, which exhibits an inverse dependence on the wavelength (initially a fourth power inverse function which holds for particles smaller than the wavelength of the exciting light) (19).

Scattering intensity at a right angle to the exciting light was followed using an SLM 8000C spectrofluorometer. Emission and excitation monochromators were both set to 340 nm to observe scattering. For both turbidity and scattering measurements, samples were placed in quartz cuvettes.

Quasi-elastic light scattering. Scattered intensity fluctuations were followed over time using a Brookhaven Instruments model 2030 quasi-elastic light scattering instrument equipped with a 72 channel autocorrelator. Exciting light was 514.5 nm from an argon ion laser (Lexel). The photomultiplier tube was set at a right angle to the exciting light. All solutions were filtered through 0.2-μm nylon filters (Gelman) to remove dust. As the diffusion coefficient grew smaller, i.e., as the polymers grew larger, the sample time was increased from 30 μs at the beginning of polymerization to 400 μs at the end of polymerization. Measurements were collected for a duration of 30 s. A single exponential nonlinear least squares analysis was used to recover the decay constant (Γ) from a fit of the autocorrelation function. The decay constant (Γ) could then be related to the translational diffusion coefficient (D) as follows:

$$\Gamma = K^2 D,$$

where K is an angular-dependent constant given by

$$K = \frac{4\pi n \sin\left(\frac{\theta}{2}\right)}{\lambda},$$

where n is the refractive index of the solution, θ is the angle of observation, and λ is the wavelength of the exciting light. The translational diffusion coefficient (D) is related to the radius of an equivalent sphere (R) by the Stokes–Einstein relation:

$$D = \frac{kT}{6\pi\eta R},$$

where k is Boltzmann's constant, T is the temperature in Kelvins, and η is the solvent viscosity.

RESULTS

Effect of pH upon Polymerization

Polymerization of EF-Tu·GDP was induced by lowering the pH of the buffer below 7.0. To a solution of EF-Tu·GDP in 2.5 mM Tris (pH 7.5 at 25°C), small aliquots of 50 mM KH_2PO_4 (pH 5.0 at 25°C) were added to lower the pH. For a solution of 4 μM EF-Tu·GDP, the effects of pH changes are demonstrated in Fig. 1A. The dramatic increase in absorbance at pH below 7.0 illustrates that slightly acidic pH induces polymerization of EF-Tu·GDP. In Fig. 1B, polymerization is also seen to occur using Mes as a buffer instead of Tris. The turbidity measurements of polymerizing EF-Tu·GDP are indistinguishable in the presence of either Tris or Mes. If the initial changes in absorbance (from data not shown) are plotted as a function of pH, the change in absorbance falls dramatically near pH 7.0, but increases rapidly below pH 7.0 as shown in Fig. 2. Also, brief exposure to pH below 7.0 caused polymerization that could be reversed by raising the pH to 7.5 or higher (data not shown).

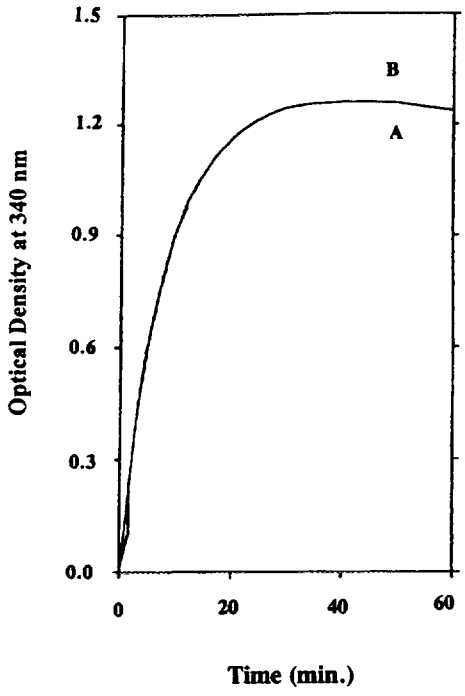

FIG. 1. Polymerization of EF-Tu · GDP in two different buffer systems: (A) 4 μM EF-Tu · GDP in 2.5 mM Tris (pH 7.5) with 50 mM KHPhthalate (pH 5.0) added to a final pH of 5.3 and (B) 4 μM EF-Tu in 2.5 mM Mes (pH 7.5) with 50 mM Mes (pH 4.0) added to a final pH of 5.75 at time 0. Optical density at 340 nm was followed as a function of time at 30°C.

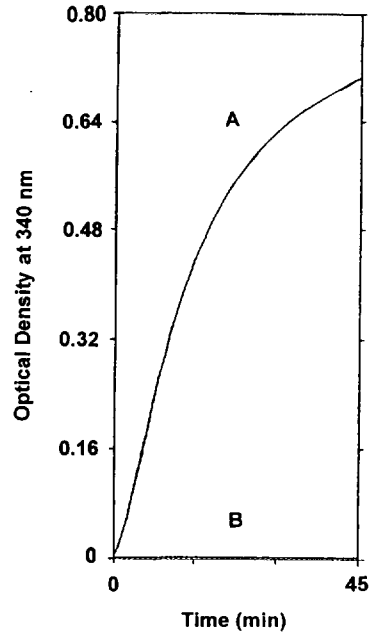

FIG. 3. Effect of an ionic strength jump on the state of aggregation of EF-Tu · GDP. A solution of 2 μM EF-Tu · GDP in 2.5 mM Mes at pH 7.6 with (A) 50 mM Mes added to a final pH of 5.75 added at time 0 or (B) NaCl added to 2.1 mM final concentration. The final ionic strengths were 2.1 mM for curve A and 4.5 mM for curve B. Optical density at 340 nm was followed as a function of time at 30°C.

Effect of Ionic Strength upon Polymerization

A solution of 2 μM EF-Tu · GDP in 2.5 mM Mes (pH 7.5 at 25°C) was adjusted to pH 5.75 by addition of 50 mM Mes (pH 4.0 at 25°C), which induced polymerization as shown in Fig. 3, curve A. Considering the change in pH, the concentration of Mes buffer and the pK_a of Mes, the ionic strength actually decreased after addition of the low pH buffer, going from 2.4 mM before

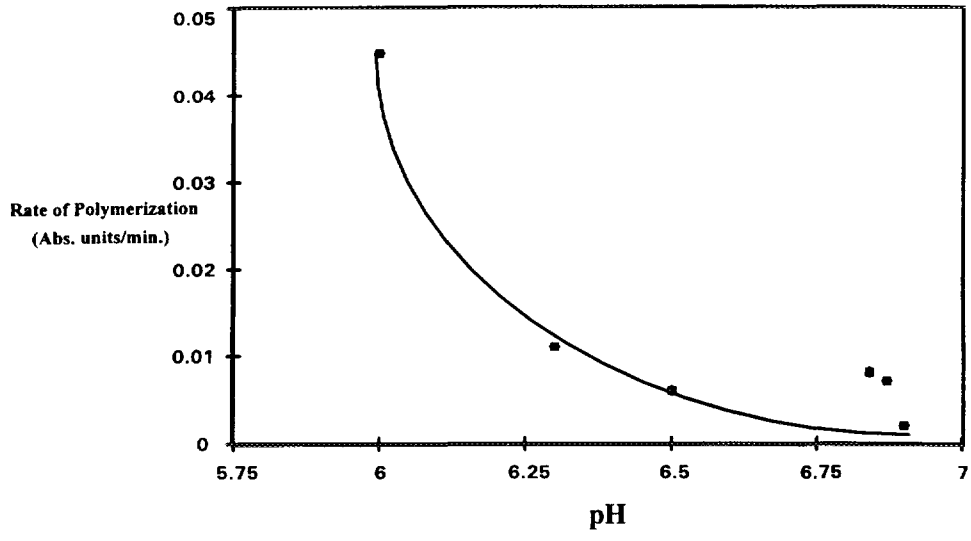

FIG. 2. Initial rate of polymerization as a function of pH. The initial rates were obtained from data not shown here.

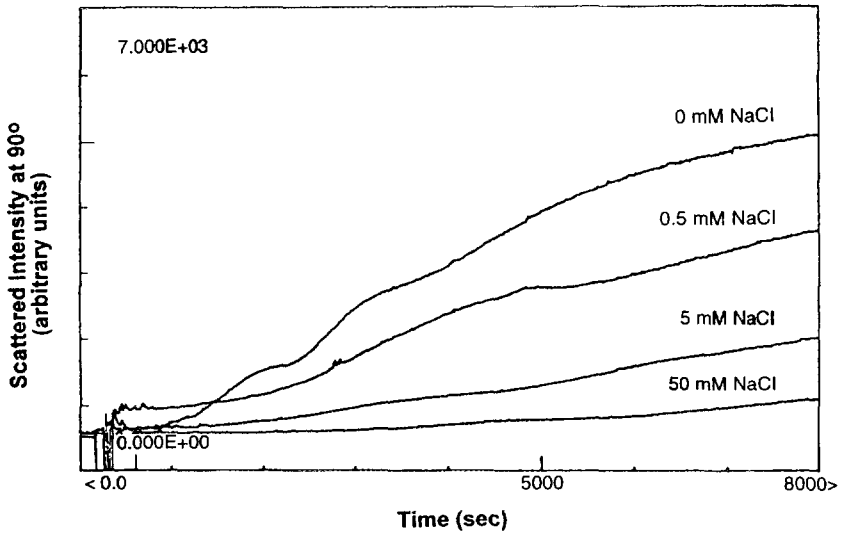

FIG. 4. Scattering intensity at a right angle to the exciting light (340 nm) for solutions containing 1.2 μM EF-Tu · GDP, 2.5 mM Tris (pH 7.5), and varying amounts of NaCl. The pH was lowered to 5.8 by addition of small amounts of 50 mM KH_2PO_4 (pH 5.0). The temperature was 25°C.

addition to 2.1 mM after addition. For comparison, NaCl was added to raise the ionic strength of 2 μM EF-Tu · GDP in 2.5 mM Mes (pH 7.5 at 25°C), thus increasing the ionic strength from 2.1 mM before addition to 4.5 mM after addition. The lack of an absorbance increase is demonstrated in Fig. 3, curve B.

EF-Tu · GDP was also polymerized in solutions of increasing ionic strength, as shown in Fig. 4. The rate of change of scattered intensity at a right angle was found to decrease with increasing concentrations of NaCl.

Effect of Temperature upon Polymerization

EF-Tu · GDP was polymerized at different temperatures, as shown in Fig. 5. The rate of increase in absorbance was found to decrease with decreasing temperatures over the range of 15–25°C.

Effect of Protein Concentration upon Polymerization

Several concentrations of EF-Tu · GDP over the range of 0.25–9.8 μM were polymerized at pH 5.8 to ascertain the effect of protein concentration on the rate of polymerization. The change in absorbance at 340 nm increases with increasing concentration of EF-Tu · GDP up to 2.5 μM, as shown in Fig. 6A. However, concentrations of EF-Tu · GDP up to 9.8 μM show a surprising lengthening of the lag time as concentration increases (Fig. 6B).

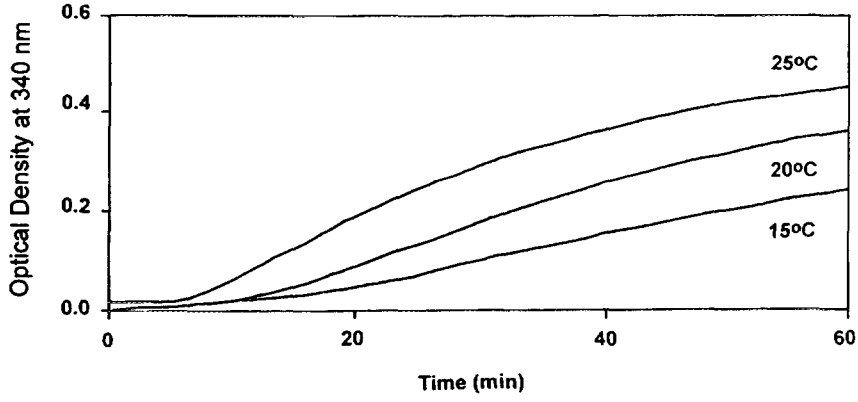

FIG. 5. Effect of temperature on the rate of polymerization of EF-Tu · GDP. Solutions of 4 μM EF-Tu · GDP in 2.5 mM Tris (pH 7.5) were adjusted to a final pH of 5.5 at time 0 by addition of 50 mM KHPhthalate (pH 5.0). Optical density at 340 nm was followed with time at the temperatures indicated on each curve.

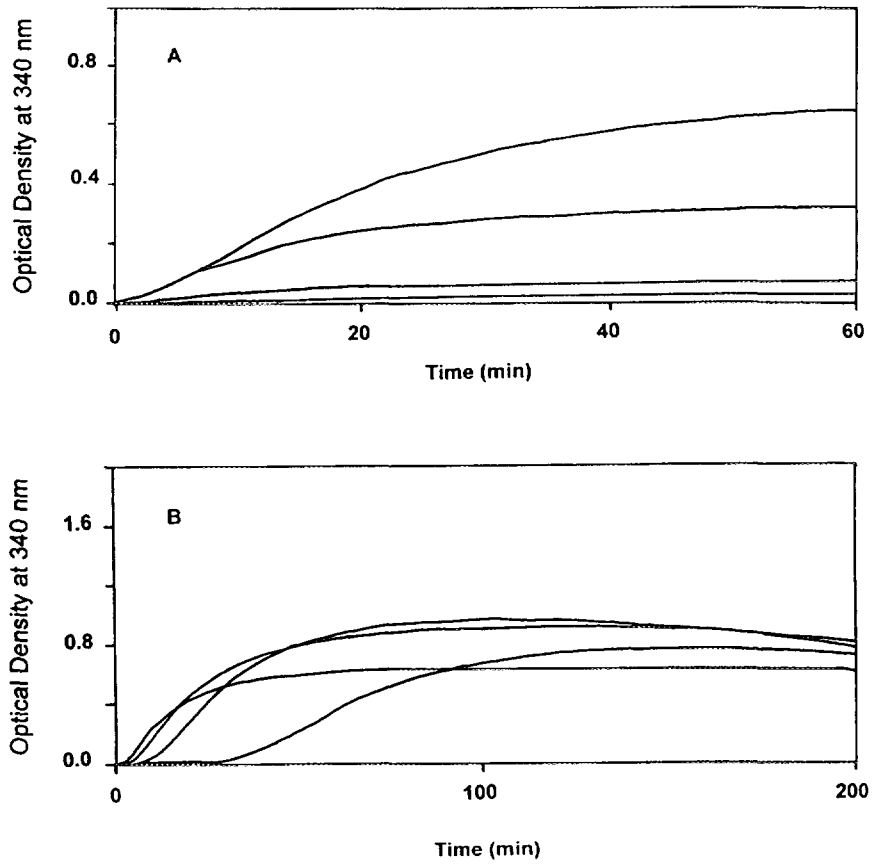

FIG. 6. Polymerization of EF-Tu·GDP at varying concentrations ranging from 0.25 to 9.8 μM. The solutions were all 2.5 mM Mes (pH 7.5) and the final pH was adjusted to 5.8 at time 0 by addition of 50 mM Mes (pH 4.0). Optical density at 340 nm was followed with time and the temperature was 25°C. (A) Protein concentrations from the bottom curve are 0.25, 0.5, 1.0, and 2.5 μM. (B) Protein concentrations from the left-most curve are 1.2, 2.5, 4.9, and 9.8 μM.

Effect of Bound Nucleotide upon Polymerization

The dependence of polymerization of EF-Tu upon bound GDP or GTP was examined by comparison of the rate of polymerization of EF-Tu·GDP and EF-Tu·GTP. (EF-Tu·GTP was prepared as described above). When 9.2 μM EF-Tu·GDP or EF-Tu·GTP were induced to polymerize at pH 5.7, the change in absorbance at 340 nm was very similar for both samples (Fig. 7).

Effect of Adding Polymer "Seeds" to EF-Tu·GDP under Polymerizing Conditions

To determine if the lag time observed in the polymerization curves of EF-Tu·GDP is indicative of the unfavorable formation of a "critical nucleus," preformed polymers of EF-Tu·GDP grown at pH 5.5 were added to a solution of unpolymerized EF-Tu·GDP under polymerizing conditions. The addition of preformed polymers did not abolish the lag time in the absorbance change at 340 nm over time, as shown in Fig. 8. Also, addition of unpolymerized EF-Tu·GDP, in the same amount as with the preformed polymers, did not abolish the lag time (Fig. 8).

Effect of Adding Unpolymerized EF-Tu·GDP to Polymers of EF-Tu

To illustrate the growth of EF-Tu·GDP over time at low pH, the translational diffusion coefficient of EF-Tu·GDP was followed with time using a quasi-elastic light scattering instrument with the observation angle set at 90°. The scattered intensity was also followed over time at low pH. Figure 9 shows the increase in both the log of the diffusion coefficient and the scattered intensity of a solution of 5.6 μM EF-Tu·GDP at pH 5.6, which suggests an increase in the average size of the scattering particles, i.e., polymers of EF-Tu·GDP.

Unpolymerized EF-Tu was then added to the final concentrations of 1.1 and 3.3 μM as indicated by the

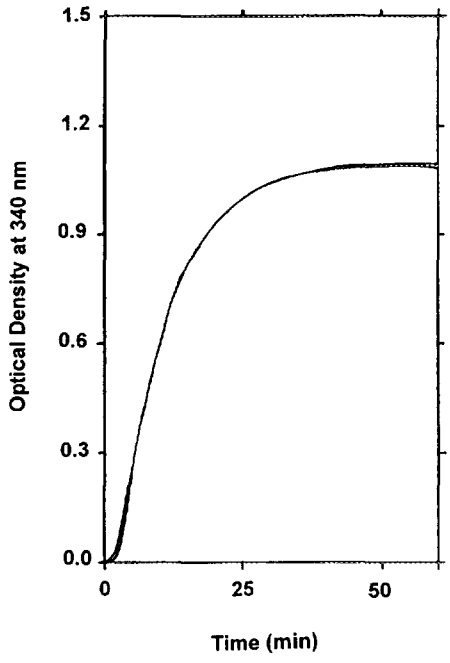

FIG. 7. Polymerization of 9.2 μM EF-Tu·GDP and EF-Tu·GTP. Both solutions contained 2.5 mM Tris (pH 7.5) and the pH was adjusted to a final value of 5.7 at time 0 by addition of 50 mM KH_2PO_4. The temperature was 30°C and optical density at 340 nm was followed with time.

arrows in Fig. 9. The scattered intensity was observed to increase after a lag time each time that unpolymerized EF-Tu·GDP was added to the solution of EF-Tu·GDP at low pH (5.6), as shown in Fig. 9A, which suggests that growth continues. On the other hand, the log of the translational diffusion coefficient did not change significantly upon addition of unpolymerized EF-Tu·GDP, suggesting that the size did not change significantly (Fig. 9B).

DISCUSSION

The conditions affecting the state of aggregation of EF-Tu·GDP have been examined here. Polymerization of EF-Tu·GDP is shown to be induced by pH below 7.0, with the change in turbidity over time evident after the pH is lowered (Fig. 1 and 2). The effect of pH upon the state of aggregation of EF-Tu·GDP is consistent with a previous report in the literature which suggested that EF-Tu·GDP could polymerize at pH 6.0 and low ionic strength (13).

The effect of ionic strength upon polymerization has also been examined here since it was reported in the literature that EF-Tu·GDP could be induced to polymerize in the presence of calcium or magnesium ions (12). As demonstrated in Fig. 3, a decrease in ionic strength and a concomitant decrease in pH induced polymerization. However, addition of NaCl did not induce polymerization. Instead, when EF-Tu·GDP was polymerized in the presence of increasing concentrations of NaCl, the rate of polymerization was decreased (Fig. 4). Salt ions may interfere with charge interactions between protein surfaces. In fact, other salts, such as NH_4Cl, KCl, $MgCl_2$, and $CaCl_2$ all had similar effects except that the salts with divalent cations were more effective at slowing polymerization than those with monovalent cations when both salts are at the same ionic strength (data not shown).

Temperature is also shown to affect the rate of polymerization of EF-Tu·GDP. As shown in Fig. 5, increasing temperature favors higher rates of polymerization, at least over the range of 15–25°C.

Varying the concentration of EF-Tu·GDP had an unexpected effect, which is shown in Fig. 6. Over the range of 0.25–2.5 μM EF-Tu, the rate of polymerization increased with increasing protein concentration, which has been shown to be the case for other polymerizing proteins such as tubulin and actin (19, 20). However, in the range of 2.5–9.8 μM, the lag time actually increased as protein concentration increased (Fig. 6B). There may be a higher aggregate of EF-Tu·GDP formed at higher concentrations which does not poly-

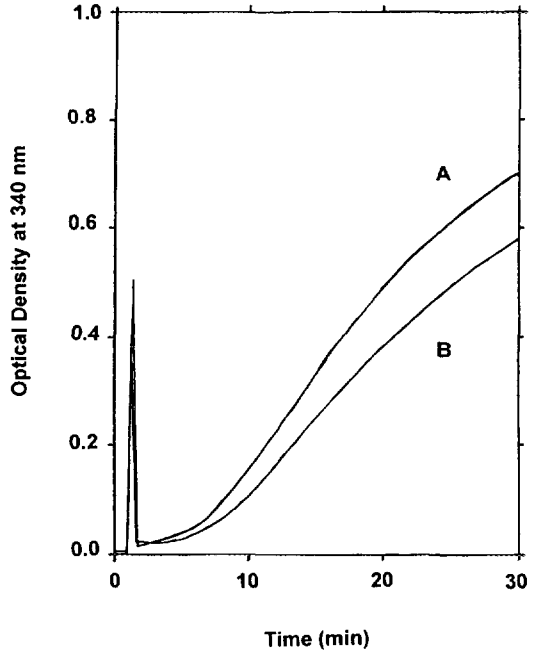

FIG. 8. Addition of polymers or monomers of EF-Tu·GDP to a polymerizing solution of EF-Tu·GDP. A solution of 2.3 μM EF-Tu·GDP in 2.5 mM Tris (pH 7.5) was adjusted to a final pH of 5.5 by addition of 50 mM KHPhthalate (pH 5.0) at time 0. After 1 min, either (A) 2.3×10^{-11} mol of EF-Tu·GDP monomers or (B) 2.3×10^{-11} mol of EF-Tu·GDP polymers was added, with both solutions at pH 5.5. The temperature was 30°C.

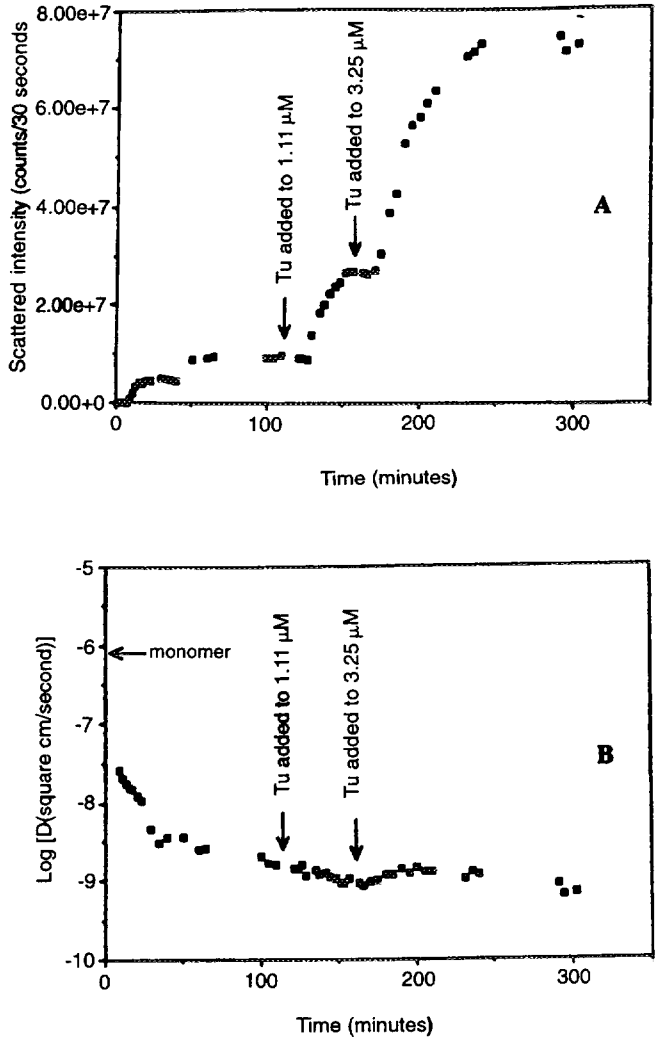

FIG. 9. (A) Scattering intensity at 90° versus time for a solution of 0.557 μM EF-Tu · GDP at pH 5.7 and 25°C. Arrows indicate addition of monomers of EF-Tu · GDP. (B) Log of the translational diffusion coefficient measured for the same solution of EF-Tu · GDP.

merize, but instead requires time to dissociate into a form which does polymerize.

Another interesting aspect of EF-Tu is that both EF-Tu · GDP and EF-Tu · GTP polymerize equally well at pH 5.7 (Fig. 7). Both actin and tubulin have been shown to require bound ATP or GTP, respectively, in order to polymerize (21–23). Apparently, the conformational difference between EF-Tu · GDP and EF-Tu · GTP is important for its function in protein biosynthesis, but not for polymerization.

The model used to explain polymerization of many other proteins consists of a nucleation step and an elongation step (24, 25). The nucleation step is thought to be unfavorable, thus requiring time and giving rise to the lag time observed in polymerization curves. Once a "critical nucleus" is formed, elongation proceeds rapidly. Thus, the presence of an unfavorable nucleation step may be demonstrated by addition of preformed polymers to a solution of unpolymerized protein. The preformed polymers provide nuclei or "seeds" for subunits to add onto, and elongation should then proceed rapidly. Seeds of actin polymers have been shown to abolish the lag time in actin monomer solutions under polymerizing conditions (26). On the other hand, when preformed polymers of EF-Tu · GDP are added to unpolymerized EF-Tu · GDP at pH below 7.0, the lag time was not overcome (Fig. 8), suggesting that an unfavorable nucleation step may not be involved. The mechanism of polymerization of EF-Tu · GDP and intermediates is not suggested here, as more experimental results are required.

Finally, the opposite experiment was done, whereby unpolymerized EF-Tu · GDP was added to a solution of polymerized EF-Tu · GDP. The size of the polymerized EF-Tu · GDP was followed in the form of a translational diffusion coefficient (D) using a quasi-elastic light scattering instrument. The scattered intensity was also followed with time. As polymerization proceeds, the scattered intensity is seen to increase and reach a plateau, as with the other curves presented above (Fig. 9A). When unpolymerized EF-Tu · GDP was added, a lag phase followed by growth and a plateau was observed (Fig. 9A), suggesting that either more scattering particles are formed or the particles get larger. In the meantime, the logarithm of the diffusion coefficient decreased over time, but did not change significantly upon addition of unpolymerized EF-Tu · GDP (Fig. 9B), suggesting that the size of the polymers did not change. Thus, formation of more scattering particles appears to be a likely explanation. That is, addition of unpolymerized EF-Tu · GDP leads to formation of new polymers rather than elongation of preexisting ones, which may suggest a size limit for polymers of EF-Tu · GDP.

In summary, polymerization of EF-Tu · GDP has been shown to be induced by pH below 7.0. The rate of polymerization has been shown to be enhanced by decreasing pH, increasing protein concentration (up to 2.5 μM), decreasing ionic strength, and increasing temperature. Both EF-Tu · GDP and EF-Tu · GTP appear to polymerize equally well. High concentrations of EF-Tu · GDP show an anomalously long lag time, which may suggest the presence of an unpolymerizable higher aggregate. Addition of preformed polymers did not function to "seed" polymerization, suggesting that an unfavorable nucleation step is not involved in polymerization of EF-Tu · GDP as it is in other polymerizing proteins. Finally, addition of unpolymerized EF-Tu · GDP to a solution of polymerized EF-Tu · GDP resulted in formation of new polymers rather than adding onto preexisting polymers, which may suggest a size

limit for EF-Tu · GDP polymers. The polymerizing ability of EF-Tu may be relevant to recent suggestions that two molecules of EF-Tu are involved in binding of one aminoacyl-tRNA to the ribosome (2–5). The high concentration of EF-Tu *in vivo* (100–200 μM (1)) suggests the existence of aggregates larger than monomers. In the cellular milieu (shown to be pH 7.5 on average (27)), there may be factors which enhance polymerization, and a more complete *in vivo* characterization of this process is in order.

ACKNOWLEDGMENTS

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