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Conformational alteration of protein synthesis elongation factor EF-Tu by EF-Ts and by kirromycin

(trypsin cleavage/Qβ replicase/protein renaturation/antibiotic)

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ABSTRACT **Alterations of the structure of EF-Tu have been investigated by using the rate of EF-Tu cleavage by trypsin as a conformational probe. The presence of EF-Ts bound to EF-Tu results in a 10-fold increase in the cleavage rate. The antibiotic kirromycin, which inhibits protein synthesis by virtue of its interaction with EF-Tu, mimics this effect of EF-Ts. Both kirromycin and EF-Ts also facilitate the exchange of free GDP with GDP bound to EF-Tu. The results suggest that EF-Ts and kirromycin induce a similar conformational change in EF-Tu, thereby "opening" the guanine nucleotide binding site. The trypsin-cleaved EF-Tu still can bind GDP and EF-Ts and can function in Qβ replicase, but it no longer spontaneously renatures following denaturation in urea.**

Protein synthesis elongation factor EF-Tu is responsible for catalyzing the binding of aminoacyl-tRNA to ribosomes by means of the following sequence of events (see ref. 1 for review):

$$\text{EF-Tu} \cdot \text{GTP} + \text{aminoacyl-tRNA} \rightarrow \text{EF-Tu} \cdot \text{GTP}\cdot\text{aminoacyl-tRNA} \quad [1]$$

$$\text{EF-Tu} \cdot \text{GTP} \cdot \text{aminoacyl-tRNA} + \text{ribosome} \rightarrow \text{ribosome} \cdot \text{aminoacyl-tRNA} + \text{EF-Tu} \cdot \text{GDP} + \text{Pi} \quad [2]$$

$$\text{EF-Tu} \cdot \text{GDP} + \text{EF-Ts} \rightarrow \text{EF-Tu} \cdot \text{Ts} + \text{GDP} \quad [3]$$

$$\text{EF-Tu} \cdot \text{Ts} + \text{GTP} \rightarrow \text{EF-Tu} \cdot \text{GTP} + \text{EF-Ts} \quad [4]$$

Recent evidence suggests that these elongation factors perform additional functions. The EF-Tu·Ts complex performs an unknown function as part of the Qβ RNA replicase complex (2–5). Furthermore, EF-Tu may be associated with the cell membrane (6) and may be related to eukaryotic actin (7, *).

It has recently been shown that treatment of EF-Tu with trypsin at 0° cleaves approximately 65 residues from the NH_2-terminal end, quantitatively converting the 45,000-dalton polypeptide to a fragment of 39,000 daltons (8–10). This fragment retains the ability to bind guanine nucleotides (8–10). Arai *et al.* (8) reported that the aminoacyl-tRNA binding capacity is lost after trypsin cleavage, whereas Jacobson and Rosenbusch (10) reported that this capacity is retained. The reason for this discrepancy is not known.

EF-Tu has been shown to undergo conformational transitions upon conversion of the bound nucleotide GDP to GTP (11–15) and upon displacement of GDP by EF-Ts (15). It has also been shown that the rate at which trypsin cleaves EF-Tu is decreased when aminoacyl-tRNA is bound, suggesting the possibility of a conformational change (8). In the experiments described in this paper, we have used sensitivity to trypsin cleavage to investigate the effects of EF-Ts and the antibiotic kirromycin on the conformation of EF-Tu.

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Kirromycin inhibits protein synthesis in *Escherichia coli*. It specifically affects a number of EF-Tu-dependent *in vitro* assays, including inhibition of protein synthesis, stimulation of GDP and GTP binding to EF-Tu, and, most notably, release of the EF-Tu GTPase reaction from dependence on ribosomes (16–18). In addition, kirromycin prevents renaturation of denatured Qβ replicase by preventing formation of the complex between EF-Tu and EF-Ts (4). In this paper, we show that when kirromycin is bound to EF-Tu it mimics the action of EF-Ts in two assays: it increases the rate of trypsin cleavage of EF-Tu, and it facilitates the rate of exchange of free and bound GDP. We suggest that kirromycin interacts with EF-Tu, possibly at the EF-Ts binding site, thereby inducing a conformational transition similar to that induced by EF-Ts. We also show that the trypsin-cleaved EF-Tu functions in Qβ replicase but does not spontaneously renature from urea-containing solutions.

RESULTS

Kirromycin Increases the Rate of Trypsin Cleavage of EF-Tu. When EF-Tu·GDP is treated with trypsin at 0°, it is quantitatively converted to a polypeptide of approximately 39,000 daltons (8–10). We find that when low trypsin concentrations (5 μg/ml) are used and the reaction is stopped by the addition of trypsin inhibitor within 5 min after initiating the reaction, the approximate rate of the reaction can be measured. Fig. 1 shows a sodium dodecyl sulfate/polyacryamide gel of such an experiment with homogeneous EF-Tu·GDP. It can be seen that approximately 30% of the EF-Tu has been converted to a 39,000-dalton fragment after a 10 min incubation at 0°. The other band seen (approximately 41,000 daltons) is an intermediate cleavage product. [It predominates when Qβ replicase is treated with trypsin at high salt concentrations (T. Blumenthal, unpublished observations).] When 50 μM kirromycin is incubated with the EF-Tu·GDP before the addition of trypsin, the rate of cleavage is dramatically increased. Approximately 30% of the EF-Tu has been cleaved by 1 min. We estimate from a similar experiment in which the gels were scanned densitometrically and subsequently quantitated that the initial rate is increased approximately 10-fold by the antibiotic. Fig. 2 shows that the rate of cleavage of EF-Tu·GDP is dependent on the kirromycin concentration and that the maximal rate is achieved at about 10 μM.

EF-Ts Increases the Rate of Trypsin Cleavage of EF-Tu. The experiment shown in Fig. 3 is identical to that shown in Fig. 1 except that a partially purified preparation of EF-Tu·Ts complex was treated in place of EF-Tu·GDP. Comparison of the two gels shows that the EF-Tu had been cleaved much more rapidly in the EF-Tu·Ts complex than in the EF-Tu·GDP complex. In fact, the rate of cleavage of the former was indis-

* B. D. Beck, X. Arscott, and G. R. Jacobson, personal communication.

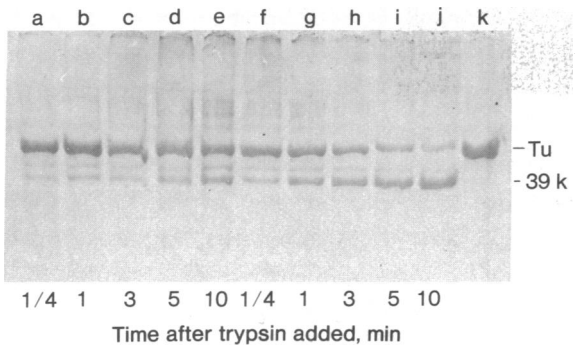

FIG. 1. Trypsinization of EF-Tu in the presence (f–j) and absence (a–e) of kirromycin. EF-Tu was prepared by the method of Arai *et al.* (21). Fifty microliters of elongation factor (0.3 mg/ml) in standard buffer [50 mM Tris·HCl, pH 7.5/5 mM $Mg(OAc)_2$/1 mM EDTA/20% (wt/vol) glycerol], with or without 50 μM kirromycin (a gift of H. Wolf, Tübingen, Germany), was mixed with trypsin (bovine pancreas, A grade, Calbiochem) (5 μg/ml) in the presence of 2.5 mM $CaCl_2$ in an ice bath. Samples (10 μl) were removed to tubes containing 2.5 μg of trypsin inhibitor (soybean, B grade, Calbiochem) at the times shown. All samples (plus 3 μg of untreated EF-Tu in the right-hand well) were mixed with sample buffer, heated, and subjected to electrophoresis on a sodium dodecyl sulfate/10% polyacrylamide slab gel according to Weber and Osborn (22). 39 k, 39,000-dalton fragment.

tinguishable from the rate of cleavage of EF-Tu·GDP in the presence of kirromycin (Fig. 1). When the antibiotic was added to the EF-Tu·Ts complex, it caused little further increase in the rate of EF-Tu cleavage by trypsin (Fig. 3). These experiments indicate that either saturating kirromycin concentration or saturating EF-Ts concentration alters the conformation of EF-Tu such that the accessible cleavage site becomes more exposed. In additional experiments (not shown), we have found that whereas the rates are directly dependent on the trypsin concentration, they are independent of the EF-Tu·GDP or EF-Tu·Ts concentration. Thus, the difference between the rates of cleavage of the two complexes cannot be a function of different protein concentrations. We also found that the presence of GDP (which separates EF-Tu from EF-Ts) decreases the rate of cleavage of EF-Tu in the EF-Tu·Ts complex to that found with EF-Tu·GDP alone but that the presence of either kirromycin or both GDP and kirromycin does not affect the rate (not shown).

Kirromycin Increases GDP Exchange at 0°. At 0°, exchange between GDP bound to EF-Tu and unbound GDP is

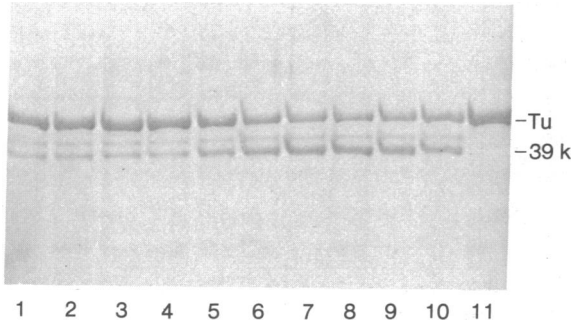

FIG. 2. Effect of kirromycin concentration on trypsin cleavage of EF-Tu. EF-Tu was treated with trypsin as follows: 20 μl of EF-Tu (0.3 mg/ml) was mixed with 2.5 μl of kirromycin diluted in standard buffer and treated with trypsin (final concentration, 5 μg/ml) for 3 min as described in the legend to Fig. 1. Wells: (*1*) no kirromycin; (*2*) 0.1 μM kirromycin (final concentration); (*3*) 0.5 μM; (*4*) 1 μM; (*5*) 5 μM; (*6*) 10 μM; (*7*) 50 μM; (*8*) 0.1 mM; (*9*) 0.5 mM; (*10*) 1 mM. Samples (10 μl) of each fraction were electrophoresed as described in the legend to Fig. 1. Well (*11*) contained 3 μg of untreated EF-Tu. 39 k, 39,000-dalton fragment.

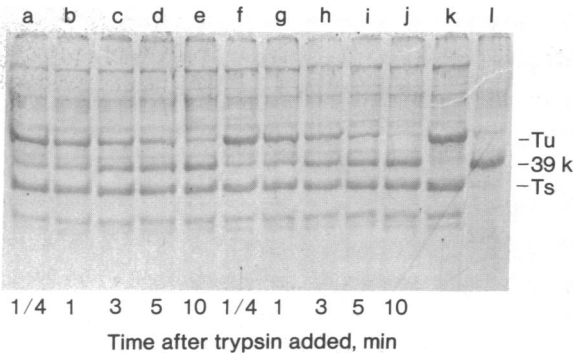

FIG. 3. Trypsinization of EF-Tu·Ts in the presence (f–j) and absence (a–e) of kirromycin. EF-Tu·Ts was prepared by the method of Arai *et al.* (21). Fifty microliters of elongation factor (0.3 mg/ml), with or without 50 μM kirromycin, was treated with trypsin as described in the legend to Fig. 1. Well (k) (right-hand side) contained 3 μg of untreated EF-Tu·Ts. Unlabeled bands are contaminants of our EF-Tu·Ts preparation. Well (l) contained 1 μg of EF-Tu treated with trypsin [50 μl of EF-Tu (3 mg/ml) plus 5 μg of trypsin] for 10 min in an ice bath, followed by addition of 10 μg of trypsin inhibitor. 39 k, 39,000-dalton fragment.

slow. The presence of EF-Ts (in catalytic amounts) stimulates the rate of exchange. This observation serves as the basis for the standard *in vitro* assay for EF-Ts (19). We have measured the amount of exchange in the standard assay (5 min at 0°) with various concentrations of unbound [^{3}H]GDP in the presence and absence of 20 μM kirromycin. The data presented in Fig. 4 *lower* show that the antibiotic increases the rate of exchange at all GDP concentrations at 0°. A reciprocal plot of this data (not shown) indicates that kirromycin apparently does not alter the K_m for GDP but dramatically increases the maximal exchange at 5 min at 0° (V_{max}). Because exchange at 30° between EF-Tu·GDP and free GDP is rapid in the absence of EF-Ts, kirromycin has only a small stimulatory effect at this temperature (Fig. 4 *upper*). We tested GDP exchange at 0° as a

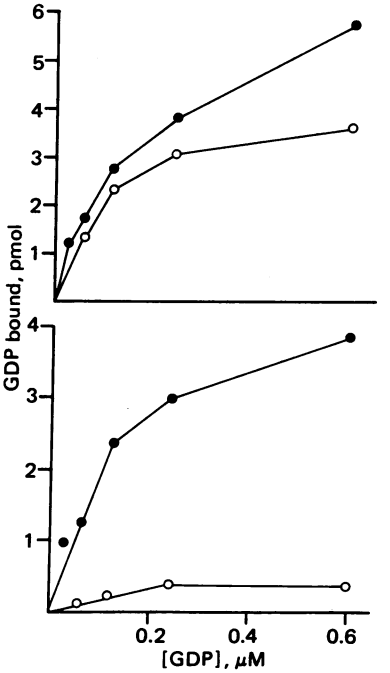

FIG. 4. Kirromycin effect on EF-Tu·GDP binding. EF-Tu·GDP (11 pmol per tube) was mixed with [^{3}H]GDP, at the concentrations shown, in standard buffer and incubated for 5 min at 0° (*Lower*) or 30° (*Upper*). Open circles, no kirromycin; solid circles, 20 μM kirromycin. Samples were filtered on 6-mm nitrocellulose filters (Schleicher and Schuell, B6) as described (20).

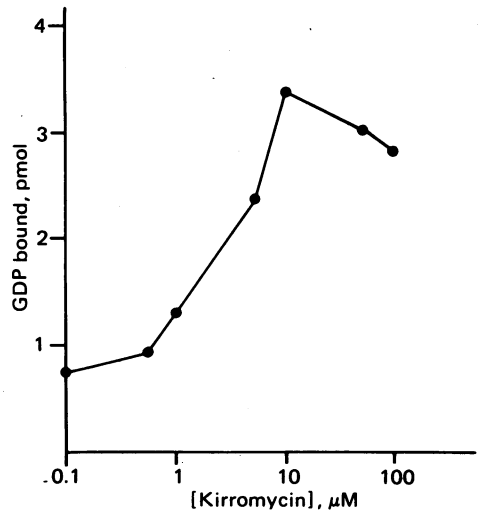

FIG. 5. Effect of kirromycin concentration on EF-Tu GDP-binding at 0°. Assays were performed as described in the legend to Fig. 4 at a [^{3}H]GDP concentration of 0.6 μM in the presence of kirromycin at the concentrations shown.

function of kirromycin concentration (Fig. 5). The effective antibiotic concentration is approximately the same as in the trypsin-cleavage experiment (Fig. 2).

In other experiments (not shown), we found that addition of saturating levels of EF-Ts to EF-Tu·GDP resulted in a much faster exchange rate than that seen with saturating levels of kirromycin. When saturating levels of both EF-Ts and kirromycin were added, the rate seen was equivalent to that with kirromycin alone, presumably because the antibiotic prevents EF-Tu interaction with EF-Ts (4). When a similar experiment was performed with the preformed EF-Tu·Ts complex, the highest exchange rate was observed, but in this case the kirromycin did not lower the rate, presumably because kirromycin will not attack an EF-Tu·Ts complex (4). Thus, in these functional assays, kirromycin and EF-Ts are seen to give qualitatively similar results (both increase the GDP exchange rate), but EF-Ts has a quantitatively greater effect.

Trypsin-Cleaved EF-Tu Function in Qβ Replicase. Using a previously developed denaturation–renaturation system, we can replace the endogenous EF-Tu in Qβreplicase with exogenously added EF-Tu (20). When denatured Qβ replicase is renatured at 0°, it renatures rapidly in the presence of native EF-Tu but fails to renature in its absence. The renatured enzyme contains only the exogenous EF-Tu under these conditions. Fig. 6 shows the recovery of poly(C)-dependent poly(G) polymerase activity of Qβ replicase after renaturation of the enzyme in the presence of either native EF-Tu or EF-Tu that had been cleaved with trypsin (Fig. 3, well *12*). The two preparations were nearly equally effective in permitting reformation of active Qβ replicase. The enzyme reconstituted from the cleaved EF-Tu was also found to recover normal activity when assayed for *in vitro* Qβ RNA-dependent RNA synthesis (not shown).

When native Qβ replicase was treated with trypsin for up to 60 min at 0°, the enzyme was found to retain full activity in the poly(C)-dependent poly(G) polymerase assay in spite of the fact that the endogenous EF-Tu was wholly converted to the 39,000-dalton fragment (unpublished observations).

Trypsin-Cleaved EF-Tu Does Not Spontaneously Renature from Urea. We have previously demonstrated that when EF-Tu is denatured in an 8 M urea-containing buffer, the EF-Tu-dependent GDP-binding activity can be recovered within 4 hr after dilution in a high-salt glycerol-containing

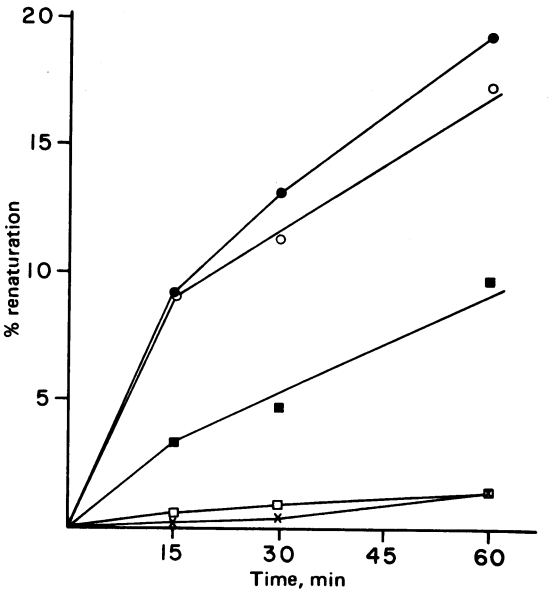

FIG. 6. Renaturation of Qβ replicase stimulated by trypsin-treated EF-Tu. Qβ replicase was denatured in 8 M urea and renatured at 2° by a 1:10 dilution in renaturation buffer as described (20). Qβ replicase concentration after dilution was 0.5 μM. Renaturation buffer contained: x, no added EF-Tu; ●, 0.8 μM EF-Tu treated with a 1:5 mixture (wt/wt) of trypsin/trypsin inhibitor (this mixture does not cleave EF-Tu); ○, 0.8 μM EF-Tu treated with trypsin (0.2 mg/ml) for 5 min at 0° before the addition of trypsin inhibitor to 1 mg/ml; ■, 2.3 μM EF-Tu treated with the 1:5 mixture of trypsin/trypsin inhibitor, denatured, and renatured for 30 min at 30° as described (20); □, 2.3 μM EF-Tu treated with trypsin as above, then denatured, and renatured for 30 min at 30°. Samples (5 μl) were assayed in the poly(C)-dependent poly(G) polymerase assay (20) at the times shown. Percent renaturation was calculated from a control reaction in which an equivalent amount of nondenatured Qβ replicase was assayed.

buffer (20). For the experiment shown in Fig. 7, we used trypsin-cleaved EF-Tu purified by Sephadex G-100 column chromatography and denatured the sample (as well as control untreated EF-Tu) with 8 M urea. It can be seen that, whereas we regained >90% of the input EF-Tu GDP-binding activity by 3 hr following denaturation and renaturation, we could recover only 6% of denatured cleaved EF-Tu binding activity.

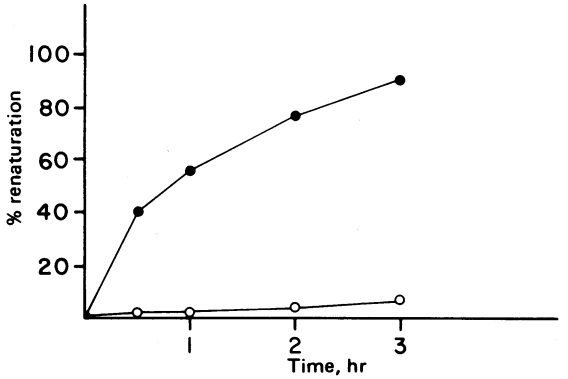

FIG. 7. Renaturation of EF-Tu (●) and trypsin-cleaved EF-Tu (○). A 250-μl sample of EF-Tu (5 mg/ml) was treated with trypsin (0.1 mg/ml) in the presence of 2.5 mM $CaCl_2$ at 0° for 15 min before the addition of inhibitor (0.2 mg/ml). The trypsin-cleaved EF-Tu was purified by Sephadex G-100 column chromatography and then denatured by the addition of crystalline urea to 8 M. An equivalent concentration (1 mg/ml) of untreated EF-Tu was denatured similarly, and both protein solutions were diluted in renaturation buffer and assayed for GDP-binding as described (20). Percent renaturation was calculated as before.

Similarly, the ability of the trypsin-cleaved EF-Tu to function in Qβ replicase was not recovered following dilution with renaturation buffer (Fig. 6). Attempts to renature the cleaved EF-Tu (not purified by Sephadex G-100 chromatography) by varying such conditions as temperature, glycerol or salt concentration, or presence of GDP or EF-Ts all met with failure. Removal of the urea by dialysis instead of by dilution also did not allow recovery of the GDP-binding activity. In a mixing experiment, the presence of the denatured cleaved EF-Tu did not affect the ability of denatured EF-Tu to renature (not shown).

DISCUSSION

EF-Tu is a multifunctional protein. In its role in the elongation of polypeptide chains, it interacts with GTP, GDP, aminoacyl-tRNA, EF-Ts, and ribosomes. It is now apparent that EF-Tu undergoes conformational transitions associated with many, if not all, of these interactions (8, 11–15). Like the EF-Tu·Ts complex, the elongation factor also plays a critical (but unknown) role in the replication of RNA-phage RNA as part of the replicase enzyme (2–5). In addition, EF-Tu has recently been shown to interact with itself to form filaments.* Indeed, there are apparently enough similarities between EF-Tu and eukaryotic actin to suggest a relatively close evolutionary relationship as well as a common function (8, *).

Recently, a number of laboratories have used the technique of trypsin cleavage to study EF-Tu structure and function (8–10). It is clear from this work that the polypeptide undergoes a rapid cleavage (or cleavages) of approximately 65 amino acids from its NH_2 terminus with the result that a fragment of 39,000 daltons is formed. (It is not known whether the small fragments remain bound to the large one.) The trypsin-cleaved EF-Tu retains its guanine nucleotide and EF-Ts binding sites, as well as its capacity to function in Qβ replicase (Fig. 6). There is disagreement about whether it retains its aminoacyl-tRNA binding (8,10) and its ability to refold spontaneously from urea-containing buffers (10) (Fig. 7). The reasons for these discrepancies are unknown. Our demonstration that the trypsin-cleaved EF-Tu cannot renature suggests that the NH_2-terminal region of EF-Tu is critical to the development of this polypeptide's tertiary structure.

The antibiotic kirromycin promises to provide considerable new information about the functions and modes of action of EF-Tu. It has been shown to increase guanine nucleotide binding by the elongation factor, to inhibit *in vitro* protein synthesis catalyzed by EF-Tu, and to allow EF-Tu to express GTPase activity in the absence of ribosomes (16–18). Kirromycin does not inhibit Qβ replicase activity, but it does inhibit renaturation of denatured Qβ replicase by preventing formation of the EF-Tu·Ts complex (4, 17). We show here that kirromycin is able to mimic qualitatively the action of EF-Ts in two assays. First, whereas aminoacyl-tRNA has been found to decrease the rate of cleavage of EF-Tu·GTP by trypsin (8), either EF-Ts or kirromycin increases the rate of EF-Tu·GDP cleavage 10-fold. No further increase in the cleavage rate is produced when both EF-Ts and the antibiotic are present together. Second, both EF-Ts and kirromycin increase the rate of exchange of free GDP with EF-Tu-bound GDP (Fig. 4) (17), although EF-Ts has a quantitatively greater effect. The same concentration of kirromycin is required for both effects (Figs. 2 and 5). These data, taken together, indicate that kirromycin binds to EF-Tu, possibly at the EF-Ts binding site, thereby preventing EF-Ts binding (4, 17) and inducing a similar conformational change to that induced by EF-Ts. When this occurs, the guanine nucleotide binding site is "opened," resulting in the rapid exchange of bound and free GDP.

Our results indicate that EF-Ts and kirromycin affect EF-Tu similarly and suggest that kirromycin may inhibit protein synthesis by preventing EF-Ts interaction with EF-Tu. Wolf *et al.* (18) have recently reported that kirromycin prevents release of EF-Tu from the ribosome. Thus, it is possible that EF-Ts may normally catalyze the release of EF-Tu from the ribosome, perhaps as part of the process by which is displaces bound GDP from EF-Tu.

Note Added in Proof. G. E. Wilson, Jr. and M. Cohn [J. Biol. Chem. (1977) **252**, 2004–2009] using nuclear magnetic resonance spectroscopy have shown recently that antibiotic X5108, a close relative of kirromycin, induces a conformational change in EF-Tu. G. R. Jacobson and J. P. Rosenbusch (Eur. J. Biochem.) have found that kirromycin decreases the rate of an additional trypsin cleavage of EF-Tu that normally yields two fragments of 24,000 and 14,000 daltons. We have confirmed this observation and have found, in addition, that this cleavage is also prevented by EF-Ts.

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