Pulvomycin, an inhibitor of protein biosynthesis preventing ternary complex formation between elongation factor Tu, GTP, and aminoacyl-tRNA

(EF-Tu affinity for guanine nucleotides/EF-Tu GTPase reaction/ enzymatic binding of aminoacyl-tRNA/structural relationship/specificity of action)

HEINZ WOLF, DAGMAR ASSMANN, AND ECKHARD FISCHER

Institut für Biologie II, Lehrbereich Mikrobiologie, Universität Tübingen, D-7400 Tübingen, Federal Republic of Germany

Communicated by Bernard D. Davis, August 14, 1978

ABSTRACT Pulvomycin and the synonymous antibiotics labilomycin and 1063-Z are shown to inhibit prokaryotic protein synthesis by acting on elongation factor Tu (EF-Tu) in the presence of the antibiotic, the affinity of EF-Tu for guanine nucleotides is altered, the EF-Tu-GDP/GTP exchange is catalyzed, and the formation of the EF-Tu-GTP complex is stimulated. Hydrolysis of GTP by EF-Tu, induced by aminoacyl-tRNA, ribosomes, and mRNA or by kirromycin, is inhibited by pulvomycin. As shown by Millipore filtration, chromatographic analysis, and hydrolysis protection experiments, pulvomycin prevents interaction between aminoacyl-tRNA and EF-Tu-GTP to yield the ternary complex aminoacyl-tRNA-EF-Tu-GTP. Thus, enzymatic binding of aminoacyl-tRNA to ribosomes is blocked.

Virtually all antibiotics reported to interfere with peptide elongation inhibit reactions of the elongation cycle that depend on ribosomes. This is true even of antibiotics whose target is not the ribosome but rather the elongation factors. For the latter type, two examples are known: (i) the interaction of fusidic acid with elongation factor G (EF-G) leads to a breakdown of peptide translocation (1); (ii) kirromycin inhibits protein synthesis by virtue of its interaction with elongation factor Tu (EF-Tu) (2–4).

An example of an antibiotic acting upon an elongation factor without interfering with ribosome-dependent reactions is described in this publication. While working with 1063-Z (unpublished data), an antibiotic that is identical to the well-known substance pulvomycin (5) and synonymous with labilomycin (6, 7), we found pulvomycin to be a novel inhibitor of prokaryotic protein biosynthesis. It is shown here that, like kirromycin, pulvomycin interferes with EF-Tu partial reactions. In its presence, the affinity of EF-Tu for GTP is enhanced and the GTPase reaction is affected. Unlike kirromycin, pulvomycin blocks peptide elongation prior to the ribosome-requiring reactions by impairing ternary complex formation. In this way, enzymatic binding of aminoacyl-tRNA to ribosomes is inhibited (for review on EF-Tu, see ref. 8).

MATERIALS AND METHODS

EF-Tu, elongation factor T (EF-T), EF-G, and NH4Cl-washed ribosomes were isolated from Escherichia coli D-22 (9, 10). Kirromycin-resistant EF-Tu for experiments shown in Fig. 1 was isolated from E. coli D-2216 as described (9, 10). The elongation factor-containing fractions were >90% pure as judged by sodium dodecyl sulfate gel electrophoresis. One microgram of EF-Tu, EF-T, or EF-G was taken to correspond to 24, 15, or 12 pmol, respectively; and 1 A260 unit of ribosome was equated to 25 pmol (2). ATP, GDP, GTP, phosphoenolpyruvate, pyruvate kinase, poly(U), puromycin, and tRNA^Phe (E. coli MRE 600) were purchased from Boehringer Mannheim; [3H]GTP, [γ-32P]GTP, and [14C]phenylalanine were from Amersham Buchler (Braunschweig).

A commercial tRNA^Phe preparation (1145 pmol of tRNA^Phe per A260 unit) was 76% charged (11). Purifying a phenylalanyl-tRNA synthetase partially purified from the 105,000 × g supernatant of the cell extract (E. coli A-19) by Sephadex G-200 gel filtration, [14C]Phe-tRNA^Phe was acetylated by the method of Haenni and Chapeville (12). Pulvomycin (molecular weight, 438) was isolated from Streptoverticillium moharaense Tü 1063; kirromycin (molecular weight, 796) was prepared from Streptomyces collinus Tü 365 as described (13). On storage in methanolic solution at ~20°C in the dark, both antibiotics were stable for at least several months. The solubility of pulvomycin at 30°C was found to be 0.12 mM in standard buffer (60 mM Tris-HCl, pH 7.6/30 mM KCl/30 mM NH4Cl/10 mM MgCl2/2 mM dithioerythritol) containing 2% methanol. Protein was estimated by the method of Lowry et al. (14) with bovine serum albumin as a standard. Analyses of EF-T by gel electrophoresis and GDP/GTP exchange experiments were performed as described (9).

RESULTS

Detection of the Pulvomycin Target. Pulvomycin is an inhibitor of prokaryotic protein biosynthesis and interferes with peptide chain elongation. Fig. 1 shows that the poly(U)-directed poly(Phe) synthesis in a cell-free system of E. coli is sensitive to the antibiotic; 50% inhibition of the polypeptide synthesis is obtained at 1 μM pulvomycin.

To locate the site of pulvomycin action, the EF-Tu-catalyzed binding of aminoacyl-tRNA to ribosomes was identified as the reaction of the elongation cycle susceptible to pulvomycin. The antibiotic did not affect nonenzymatic binding of Phe-tRNA^Phe to the ribosomal-poly(U) complex, whereas efficient EF-Tu-mediated binding was suppressed to the level of nonenzymatic binding (Fig. 2).

If pulvomycin were to inhibit the EF-Tu-catalyzed binding by blocking the aminoacyl site on the ribosome, Phe-tRNA^Phe binding ought to be prevented entirely. Because nonenzymatic binding of Phe-tRNA^Phe to the ribosomal aminoacyl site and to the peptidyl site can occur despite the presence of excess amounts of EF-Tu and GTP in the reaction mixture, these data suggest that pulvomycin inhibits the formation of the ternary complex between EF-Tu, GTP, and Phe-tRNA^Phe by its interaction with EF-Tu.

Abbreviations: EF-Tu, elongation factor Tu; EF-Ts, elongation factor Ts; EF-T, elongation factor T; the complex formed by EF-Tu and EF-Ts (EF-Tu-EF-Ts); EF-G, elongation factor G.
The effect of pulvomycin on other partial reactions of the elongation cycle was examined. At concentrations up to 0.12 mM, pulvomycin did not interfere with the formation of AcPhe-puromycin generated through the peptidyl transferase. Similarly, two parameters for translocation activity were found to be unaffected by the antibiotic: augmentation of the AcPhe-puromycin synthesis in the presence of EF-G plus GTP, and the EF-G-dependent GTPase activity.

Change of EF-Tu Affinity for Guanine Nucleotides. The effect of the antibiotic on the interactions of EF-Tu with its various ligands was examined.

The replacement of GDP by GTP in the EF-Tu-GDP complex is catalyzed by elongation factor Ts (EF-Ts) and may be considered to be the starting reaction of any new round in peptide elongation. Upon exposure of EF-T to pulvomycin, stimulation of the formation of the EF-Tu-GTP complex was achieved. At 3 μM pulvomycin, more than half of the EF-T present was converted to EF-Tu-GTP (Fig. 3), even at low GTP concentration (molar ratio of GTP to EF-T, 3:1). Under this condition little conversion occurred in the absence of the antibiotic.

The effect of pulvomycin on the affinity of EF-Tu for GTP is more pronounced than with kirromycin and does not depend on the presence of EF-Ts. The stimulation of GTP binding to EF-Tu can also be shown with crystalline EF-Tu-GDP. At 18 μM pulvomycin promoted a rapid exchange of free [γ-33P]GTP with GDP prebound to EF-Tu, even at 0°C (data not shown).

Although pulvomycin stimulates the formation of EF-Tu-GTP (with EF-Tu derived from either EF-T or EF-Tu-GDP), its activity differs from that of kirromycin (2, 9) with respect to the influence on EF-T if GTP is absent. As monitored by analytical polyacrylamide gel electrophoresis, pulvomycin did...
not induce dissociation of EF-T even when it was present together with GDP, both at a concentration of 50 μM in the reaction mixture and the upper chamber buffer (data not shown).

By comparison, kirromycin was found to cause dissociation of EF-T (9) and to prevent association of the EF-T complex (15).

Inhibition of Ternary Complex Formation. Aminoacyl-tRNA interacts with EF-Tu-GTP to form the ternary complex aminoacyl-tRNA-EF-Tu-GTP. This complex is not adsorbed to cellulose nitrate filters, whereas EF-Tu-GTP is adsorbed (16). Therefore this filtration technique has been used to assay the effect of pulvomycin on the formation of the ternary complex. As shown in Fig. 3, pulvomycin prevents the binding of Phe-tRNA^Phe\textsuperscript{a} to EF-Tu-GTP. Incubation with excess Phe-tRNA^Phe\textsuperscript{a} did not influence the amounts of EF-Tu-GTP retained on the cellulose nitrate filters; the values obtained in the presence of Phe-tRNA^Phe\textsuperscript{a} coincided with those found for EF-Tu alone. As a control, release of the resulting EF-Tu-GTP complex from the filters by Phe-tRNA^Phe\textsuperscript{a} due to the formation of the ternary complex was observed in the presence of kirromycin (2).

**Fig. 4.** Effect of pulvomycin on the interaction of Phe-tRNA^Phe with EF-Tu-GTP as measured by gel filtration. EF-Tu-GTP was formed by incubating 720 pmol of EF-Tu-GDP with 250 nmol of phosphoenolpyruvate and 20 μg of pyruvate kinase in 58 μl of elution buffer (60 mM Tris-HCl, pH 7.4; 30 mM KCl; 30 mM NH₄Cl/15 mM MgCl₂/2 mM dithioerythritol/1 mM GTP). After 20 min at 30°C, 1 μl of methanol containing, where indicated, 1 nmol of pulvomycin (C) or 1 nmol of kirromycin (D) and then 3 μl of [¹⁴C]Phe-tRNA^Phe solution (243 pmol) were added. The incubation was continued for 10 min at 0°C, after which 50 μl of the reaction mixture was filtered at 4°C on a Sephadex G-100 column (0.6 x 37 cm). Fractions (200 μl/20 min) were collected, and the radioactivity was determined on 100-μl aliquots dissolved in liquid scintillator KL 402 (Koch-Light). Controls were reaction mixtures with (A) and without (B) EF-Tu.

The same effect of pulvomycin on EF-Tu affinity for Phe-tRNA^Phe was also found when the formation of the ternary complex was measured by gel filtration (17). Chromatographic analysis of the reaction mixture containing 16 μM pulvomycin revealed no detectable formation of Phe-tRNA^Phe-EF-Tu-GTP eluting at a Ve/Vo of 1.4 (Fig. 4). In the presence of the antibiotic, uncomplexed Phe-tRNA^Phe (partially deacylated during the gel filtration process) eluted from the Sephadex G-100 column in the same way as in the absence of EF-Tu-GTP at a Ve/Vo of 1.7. When EF-Tu-GTP was treated with kirromycin, it was found that kirromycin caused a significant increase in the Phe-tRNA^Phe-EF-Tu-GTP peak. However, upon omission of GTP from the elution buffer, a decrease of the ternary complex was observed also with this antibiotic.

When aminoacyl-tRNA is complexed with EF-Tu, the ester linkage between the amino acid and the -C-C-A3' terminus of tRNA in aminoacyl-tRNA is protected from nonenzymatic hydrolysis (18). Therefore, the effect of pulvomycin on the ternary complex formation was also examined by hydrolysis protection experiments (19). Addition of pulvomycin completely abolished the protective effect of EF-Tu-GTP on the nonenzymatic hydrolysis of Phe-tRNA^Phe (Fig. 5). By contrast, the effect of kirromycin is to render the ester bond in Phe-tRNA^Phe-EF-Tu-GTP more accessible to hydrolysis (4).

**Fig. 5.** Effect of pulvomycin on the interaction of Phe-tRNA^Phe with EF-Tu-GTP as measured by hydrolysis protection. EF-Tu-GDP was transformed into EF-Tu-GTP by incubating 600 pmol of EF-Tu-GDP with 250 nmol of phosphoenolpyruvate, 15 μg of pyruvate kinase, and 12.5 nmol of GTP in 100 μl of buffer (80 mM Tris-HCl, pH 7.4; 75 mM KCl/30 mM NH₄Cl/15 mM MgCl₂/2 mM dithioerythritol). After 20 min at 30°C, 2 μl of methanol containing, where indicated, 1.8 nmol of either pulvomycin (A) or kirromycin (B) was added. The reaction was started by adding 40 pmol of [¹⁴C]Phe-tRNA^Phe\textsuperscript{a} in 25 μl of buffer. Hydrolysis of [¹⁴C]Phe-tRNA^Phe\textsuperscript{a} during the incubation at 30°C was followed by measuring residual acid-insoluble radioactive material at different times in 25-μl aliquots. Controls were reaction mixtures with (●) and without (○) EF-Tu.
of EF-Tu-GDP from the ribosome (20). Induction of this EF-Tu-mediated GTPase reaction in vitro depends on the interaction of EF-Tu with the physiological effectors aminoacyl-tRNA, ribosomes, and mRNA, each of which considerably stimulates GTP hydrolysis (21). In the preceding section, pulvomycin was shown to disturb the association of aminoacyl-tRNA and EF-Tu-GTP. Consequently one might expect that this antibiotic would also interfere with the EF-Tu GTPase reaction. The results presented in Table 1 support this idea. The induction of EF-Tu GTPase activity by the addition of each effector was inhibited in the presence of pulvomycin.

Kirromycin affects the allosteric control of EF-Tu and induces the GTPase reaction with EF-Tu alone (2, 9). As shown in Table 1, pulvomycin acts in an analogous fashion, although this antibiotic is much weaker in triggering GTP hydrolysis than is kirromycin. Furthermore, pulvomycin activation of EF-Tu GTPase appears to be based on a different mechanism: upon addition of Phe-tRNA\(^{\text{Phe}}\), pulvomycin-induced activity was inhibited, whereas the kirromycin-induced reaction was stimulated (2, 9).

Pulvomycin not only interferes with the GTPase reaction induced by the physiological effectors but also, in the presence of both antibiotics, even the marked stimulation of EF-Tu GTPase by kirromycin (2, 9, 23) is affected (Table 1). Preliminary results indicate that pulvomycin competitively inhibits the kirromycin-induced activity.

Structural Relationship between Pulvomycin and Kirromycin. The mutual exclusion of pulvomycin and kirromycin in the GTPase reaction suggests a competition for a single binding site on EF-Tu due to their structural similarity. Indeed, pulvomycin resembles the \(5'\) substituent of the central tetrahydrofuran of kirromycin (Fig. 6).

Such structural homology and the availability of a kirromycin-resistant EF-Tu (10) prompted us to compare the pulvomycin sensitivity of EF-Tu from the parent strain D-22 and from the resistant mutant D-2216 in polypeptide synthesis. As shown in Fig. 1, no cross-resistance between pulvomycin and kirromycin could be observed. In contrast, the inhibition by pulvomycin appeared to be even more effective when poly-(Phe) synthesis was catalyzed by kirromycin-resistant EF-Tu than when catalyzed by wild-type EF-Tu. Poly(Phe) synthesis catalyzed by kirromycin-resistant EF-Tu was found to be about 1/90th as sensitive to kirromycin as catalysis by kirromycin-sensitive EF-Tu.

**DISCUSSION**

Pulvomycin is unique among the known antibiotic inhibitors (27) of EF-Tu with respect to its size and function. Comparison of the structures of pulvomycin and kirromycin reveals similarities between pulvomycin and the \(5'\) substituent of the central tetrahydrofuran of kirromycin. Both antibiotics have a similar skeleton of a pyranose and an aliphatic moiety coupled to the C-1 position of the sugar. The two fragments of kirromycin, derived by hydrolysis of the peptide bond, are inactive as inhibitors of protein synthesis (23). This would suggest that the affinity of either drug for EF-Tu depends upon their common skeleton, so that pulvomycin might therefore be considered a "minimal-size" EF-Tu antibiotic inhibitor.

These data do not exclude binding of the antibiotics to EF-Tu due to features other than their common skeleton. The influence of different chemical substitutions is not yet understood. Differences in chemical substitution on the two antibiotics likely modulate the action of their common binding property to EF-Tu. In the presence of either antibiotic, the affinity of
EF-Tu for GTP is enhanced and the EF-Tu-GDP/GTP exchange is catalyzed. Despite this underlying similarity, differences are found in the influence of the drugs on other partial reactions of EF-Tu. The GTPase reaction of EF-Tu is stimulated by aminoacyl-tRNA, ribosomes, and mRNA; pulvomycin acts to prevent the reaction. Even the artificial stimulation of EF-Tu GTPase activity by kirromycin (2, 9) is decreased by pulvomycin. In contrast, kirromycin is a potent stimulus of EF-Tu GTPase reactions, both with EF-Tu alone and through induction by its physiological effectors (9, 23).

The most striking contrast between the mode of action of the two antibiotics is their different influences on ternary complex formation between EF-Tu-GTP and aminoacyl-tRNA. Although both antibiotics stimulate the formation of EF-Tu-GTP, formation of the ternary complex is influenced in opposite ways. In the presence of kirromycin, EF-Tu-GTP is active in aminoacyl-tRNA binding, and ternary complex formation is stimulated by excess GTP. Pulvomycin inhibits interaction of EF-Tu-GTP and aminoacyl-tRNA. Because cooperative binding of aminoacyl-tRNA is important for the induction of the EF-Tu GTPase reaction, a direct block of the aminoacyl-tRNA binding site of pulvomycin could explain both inhibition of GTPase reaction and inhibition of the ternary complex formation.

Alternatively, pulvomycin and kirromycin may induce different configurations of the EF-Tu-GTP complexes. Kirromycin could invoke a conformational change in EF-Tu similar to that of EF-Tu-GTP and regardless of GDP or GTP being bound to the elongation factor. This would explain splitting of EF-Tu-EF-Ts (9), formation of a nonfunctional ternary complex aminoacyl-tRNA-EF-Tu-GDP (9), and failure of EF-Tu-GDP to dissociate from the ribosome in the presence of kirromycin (2, 23). Prevention of EF-Tu-GTP binding to aminoacyl-tRNA, stabilization of EF-Tu-EF-Ts, and inhibition of the GTPase activity leads to the idea that pulvomycin, on the other hand, may induce the binary complex EF-Tu-GDP configuration. This notion suggests that pulvomycin and kirromycin bind to a common site on EF-Tu; such a site may be important for allosteric control of EF-Tu as a function of phosphorylation of the bound guanine nucleotide.

In conclusion, our data show that pulvomycin prevents binding of aminoacyl-tRNA to EF-Tu-GTP, and tentatively suggest that protein synthesis might be inhibited by substrate limitation.

We thank Drs. J. W. Coulton and F. Lippmann (Tübingen) for critical reading of the manuscript. This investigation was supported by Grant SFB-76 of the Deutsche Forschungsgemeinschaft.