Effects of the Antibiotic Pulvomycin on the Elongation Factor Tu-Dependent Reactions. Comparison with Other Antibiotics

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ABSTRACT: The antibiotic pulvomycin is an inhibitor of protein synthesis that prevents the formation of the ternary complex between elongation factor (EF-) Tu·GTP and aminoacyl-tRNA. In this report, novel aspects of its action on EF-Tu are described. Pulvomycin markedly affects the equilibrium and kinetics of the EF-Tu−nucleotide interaction, particularly of the EF-Tu·GTP complex. The binding affinity of EF-Tu for GTP is increased 1000 times, mainly as the consequence of a dramatic decrease in the dissociation rate of this complex. In contrast, the affinity for GDP is decreased 10-fold due to a marked increase in the dissociation rate of EF-Tu·GDP (25-fold) that mimics the action of EF-Ts, the GDP/GTP exchange factor of EF-Tu. The effects of pulvomycin and EF-Ts can coexist and are simply additive, supporting the conclusion that these two ligands interact with different sites of EF-Tu. This is further confirmed on native PAGE by the ability of EF-Tu to bind the EF-Ts and the antibiotic simultaneously. Pulvomycin enhances the intrinsic EF-Tu GTPase activity, like kirromycin, though to a much more modest extent. As with kirromycin, this stimulation depends on the concentration and nature of the monovalent cations, Li^+ being the most effective one, followed by Na^+, K^+, and NH_4^+. In the presence of pulvomycin (in contrast to kirromycin), aa-tRNA and/or ribosomes do not enhance the GTPase activity of EF-Tu. The property of pulvomycin to modify selectively the conformation(s) of EF-Tu is also supported by its effect on heat- and urea-dependent denaturation, and tryptic digestion of the protein. Specific differences and similarities between the action of pulvomycin and the other EF-Tu-specific antibiotics are described and discussed.

The bacterial elongation factor (EF-)[1] Tu is a most useful model for the study of function−structure relationships and the mechanism of action of antibiotics (for refs, see *1* and *2*). EF-Tu is a GTPase, a family of proteins regulating signaling pathways in the cell or transporting biological components. The activity of these proteins depends on whether GTP ("on-state") or GDP ("off-state") is bound. In protein biosynthesis, EF-Tu·GTP is the carrier of aa-tRNA to the A-site of the mRNA-programmed ribosome. The codon−anticodon interaction is associated with a very fast hydrolysis of the bound GTP, inducing the release of EF-Tu·GDP due to its low affinity for aa-tRNA and the ribosome. This event is followed by peptide bond formation and the incorporation of aa-tRNA into the polypeptide chain bound to the ribosomal P-site. EF-Ts, the GDP/GTP exchange factor of EF-Tu, favors the regeneration of EF-Tu·GTP by enhancing the exchange rate of the EF-Tu-bound GDP with free GTP.

Four structurally unrelated families of antibiotics, which with analogues include ca. 30 components, have been identified that inhibit protein synthesis by acting specifically on EF-Tu: kirromycin, enacyloxin IIa, pulvomycin, and GE2270 A (for refs, see *1* and *2*). These drugs, which show specific spectra of antibacterial activity, played an important role in defining the functions of EF-Tu, its encoding genes, and ligands. They affect the allosteric regulation of EF-Tu and/or directly inhibit its interaction with the ligands. Interestingly, the action of kirromycin and enacyloxin IIa share similar properties (*3*, *4*), as does that of pulvomycin and GE2270 A (*5*, *6*). Whereas the former two antibiotics hinder the release of EF-Tu·GDP from the ribosome, preventing peptide bond formation and the recycling of the factor, pulvomycin and GE2270 A inhibit the interaction between EF-Tu·GTP and aa-tRNA, preventing the formation of the ternary complex EF-Tu·GTP·aa-tRNA. Despite the common target the actions of kirromycin and enacyloxin IIa and those of pulvomycin and GE2270 A display specific differences; for instance, enacyloxin IIa and kirromycin show a different effect on the intrinsic GTPase activity of EF-Tu (*3*; Parmeggiani, unpublished), and pulvomycin and GE2270 A on the dissociation of the EF-Tu·GDP complex (*5*, *6*).

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[1] Abbreviations: EF-, elongation factor; EF-Tu = EF-1A; EF-Ts = EF-1B; D1, D2, and D3, domains 1, 2, and 3 of EF-Tu, respectively; GST-EF-Tu(D1,D2), glutathione-*S*-transferase-fused EF-Tu truncated of domain 3; aa-, aminoacyl-; DTT, dithiothreitol, 3D, three dimensional; K', apparent dissociation constant; k'_{-1}, apparent dissociation rate constant; k'_{+1} apparent association rate constant.

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In the past years, the three-dimensional models of EF-Tu·GDP·GE2270 A (*7*) and EF-Tu·GDP·methylkirromycin (*8*) were resolved at high resolution, enlighting the structural background of the action of these two drugs.

Of the antibiotics acting on EF-Tu, pulvomycin is the one of which our knowledge suffers from the most gaps. It was therefore important to extend the characterization of its action. In this report we analyze a number of novel observations on the mode of action of pulvomycin, with particular attention to (i) its effect on the interaction of EF-Tu with GTP and GDP, (ii) the functional data related to structural aspects, and (iii) the differences from the actions of other antibiotics targeted on EF-Tu.

MATERIALS AND METHODS

Production and Purification of Pulvomycin. Pulvomycin was isolated from *Streptoverticillium netropsis* with a procedure in part similar to that described in ref *9*. The strain was fermented in a shaking flask on a medium composed of dextrose 20 g/L, yeast extract 2 g/L, soybean meal 8 g/L, NaCl 1 g/L and $CaCO_3$ 4 g/L in deionized water. After 5 days the broth was harvested and the mycelium cake was suspended in five volumes of acetone. After stirring of the sample, the mycelium was removed by filtration and the filtrate was concentrated under vacuum. The residue was extracted with an equal volume of ethyl acetate. The organic phase dissolved in methanol was fractionated on TLC chromatography using chloroform−methanol (10:1) as the solvent. The major portion of the antibiotic displayed an R_f of 0.4 and was at least 85% pure, as shown by HPLC on silica gel (GL. Science Com. Inertsil SIL. 150−5, 4.6 × 150 mm; solvent: chloroform−methanol 15:1 at a rate of 1 mL/min), containing some degradation products likely formed during the purification. Pulvomycin as powder is stable when kept at −80 °C under argon; as ethanol solution is at least stable for several months, when kept at −25 °C, and for years at −80 °C. Its concentration was determined using a molar absorbance coefficient (A_M) of 74 582 at 320 nm in methanol (*9*). To determine the precise amount of active pulvomycin, titration of the antibiotic with EF-Tu was performed by following the shift of pulvomycin-bound EF-Tu on native PAGE (*5*). This method yielded results close to those obtained with chromatography. GE2270 A was a gift of Dr. E. Selva, Gruppo Lepetit Research Center, Gerenzano, Italy, and kirromycin (mocimycin) was a gift of Dr. R. Beukers, Gist-Brocades, Netherlands. The concentrations of GE2270 A and kirromycin were obtained by using an A_M of 30 613 at 310 nm in methanol and 28 300 at 325 nm in 0.1 M KOH−ethanol (1:1, v/v), respectively.

Other Biological Materials and Assays. Pure, wild-type EF-Tu from *Escherichia coli* strain LBE1001 and other biological materials and reagents were as previously described (*6*, *10*). Trypsin (TPCK-treated) and benzamidine were obtained from Merck.

The standard assay buffer contained 50 mM Tris·HCl, pH 7.5 at 20 °C, 60 mM NH_4Cl, 10 mM $MgCl_2$, and 1 mM DTT. Association and dissociation rate constants and dissociation constants were determined by filtration on nitrocellulose filters as described (*5*, *6*, *11*). Hydrolysis of [γ-^{32}P]-GTP was measured as liberation of inorganic $^{32}P_i$, according to the isopropyl/molybdate or charcoal methods (*12*). The latter method was the only one used when the monovalent salt concentration exceeded 300 mM. Urea- or temperature-induced denaturation was determined according to ref *13*. Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA).

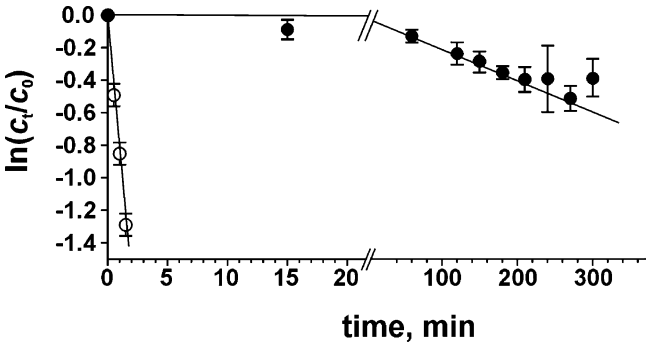

FIGURE 1: Dissociation of EF-Tu·GTP in the presence and the absence of pulvomycin. Nucleotide-free EF-Tu (20 pmol, prepared as in ref *19*) was incubated in 1 mL of standard buffer with 500 pmol of [γ-^{32}P]GTP (specific activity 10 000 cpm/pmol) in the absence (○) or presence of 30 μM pulvomycin (●). At the indicated times, a 100 μL aliquot was withdrawn and EF-Tu-bound radioactivity was measured by filtration through nitrocellulose membranes. The c_0 is the initial concentration of the EF-Tu-[γ-^{32}P]GTP complex and c_t is the concentration of EF-Tu-[γ-^{32}P]GTP at different times *t*. Data represent the mean ± SEM for five independent experiments.

Table 1: Apparent Dissociation Constants (K'_d), Half-life, Dissociation (k'_{-1}) and Association Rate Constants (k'_{+1}) of the GDP/GTP Complexes of EF-Tu in the Presence and the Absence of Pulvomycin[a]

systems	K'_d (nM)	half-life (min)	$10^4\,k'_{-1}$ (s^{-1})	k'_{+1} ($s\,M^{-1}$)
EF-Tu·GDP	0.9	52	2.3	28
EF-Tu·GDP + pulvomycin	9.8	2	59	60
EF-Tu·GTP	590	2	59	1
EF-Tu·GTP + pulvomycin	0.6	400	0.3	5.1

[a] At 0 °C. $K'_d = k'_{-1}/k'_{+1}$. For details of the method see refs *6* and *11*.

RESULTS

Selective Effects of Pulvomycin on the Interaction of EF-Tu with GTP or GDP. Previous reports indicated that pulvomycin destabilizes the EF-Tu·GDP complex favoring the formation of EF-Tu·GTP (*5*, *14*). We have quantified these effects by determining the dynamics and equilibrium of these two complexes in the presence of the antibiotic. While the equilibrium and kinetic constants of the EF-Tu·GDP complex display specific alterations, the most marked effect of pulvomycin concerns the dissociation rate of EF-Tu·GTP. As illustrated in Figure 1 and overviewed in Table 1 which reports the values of the various constants, the EF-Tu·GTP dissociation rate is dramatically decreased. At 0 °C in the presence of pulvomycin, this complex shows a half-life of about 400 min as compared to 2 min of the control. In contrast, its association rate is little affected showing a 5-fold stimulation. Also with EF-Tu·GDP, the dissociation rate is the kinetic constant most markedly affected, but, opposite to the EF-Tu·GTP complex, its rate is accelerated. Under the chosen conditions, a half-life of 2 min was calculated vs 52 min in the absence of the antibiotic, while the association rate only showed a 2-fold increase.

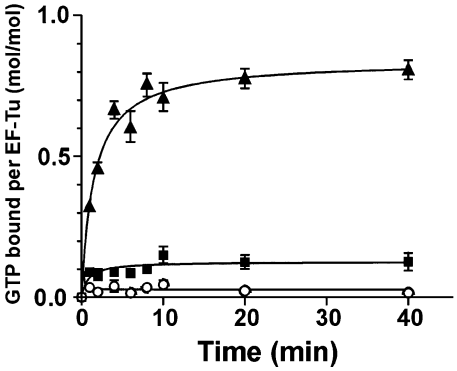

FIGURE 2: Exchange at 0 °C between EF-Tu·pulvomycin-bound GDP and free [γ-^{32}P]GTP. The reaction mixture contained in 1 mL of standard buffer 24 pmol of EF-Tu·GDP and 500 pmol of [γ-^{32}P]GTP (specific activity 3000 cpm/pmol) in the absence (○) or presence of 30 μM pulvomycin (▲) or GE2270 A (■). Data represent the mean ± SEM for three independent experiments.

These changes in the kinetic parameters regulating the levels of the two complexes cause a dramatic decrease by 3 orders of magnitude in the equilibrium constant of EF-Tu·GTP (from 590 to 0.6 nM) and a marked increase by 1 order of magnitude in the equilibrium constant of the EF-Tu·GDP complex (from 0.9 to 9.8 nM).

The consistent destabilization of the EF-Tu·GDP complex, due to the increased dissociation rate and the very strong stabilization of the EF-Tu·GTP complex, as a consequence of an extremely slowed dissociation rate, explains why the exchange rate of the EF-Tu-bound GDP with free [^{3}H]GDP and particularly with [γ-^{32}P]GTP is enhanced, as is shown for [γ-^{32}P]GTP in Figure 2.

EF-Tu-Dependent GTPase Activity. Pulvomycin was reported to be capable of stimulating the intrinsic GTPase activity of EF-Tu (*15*). To compare the effects of pulvomycin and GE2270 A with the well-known, marked stimulatory action of kirromycin, we have investigated the intrinsic GTPase activity of EF-Tu as a function of the concentration and nature of the monovalent cations, two conditions that greatly influence the kirromycin-induced GTPase activity (*12*). Also with pulvomycin the stimulation of the intrinsic GTPase activity of EF-Tu was found to be dependent on the nature and concentration of monovalent cations (Figure 3A), even though the extent of the effect was only approximately 10% that of kirromycin. Li^+ is the most active cation, followed by Na^+, K^+, and $[NH_4]^+$. At 1.75 M LiCl, the stimulation was more than 10-fold higher than that observed at 20−40 mM LiCl.

In marked contrast to pulvomycin, GE2270 A exerts little, if any, effect on the intrinsic GTPase activity. Only the presence of LiCl induces a significant stimulation of this activity (Figure 3B).

In the case of kirromycin, aa-tRNA and ribosomes markedly enhance the intrinsic GTPase activity of EF-Tu at concentrations of monovalent cations lower than 200−250 mM (*12*). Table 2 shows the effect of these two physiological ligands on the pulvomycin-dependent stimulation of the intrinsic GTPase activity of EF-Tu. Not only Val-tRNA^{Val}, as expected from the property of pulvomycin to inhibit its interaction between EF-Tu and aa-tRNA, but also the ribosome alone or in combination with Val-tRNA^{Val} was unable to enhance the intrinsic GTPase of EF-Tu; the ribosome was even inducing some inhibition. This experiment was carried out using NH_4Cl, which, together with KCl, is the most used monovalent salt in measuring the EF-Tu-dependent activities.

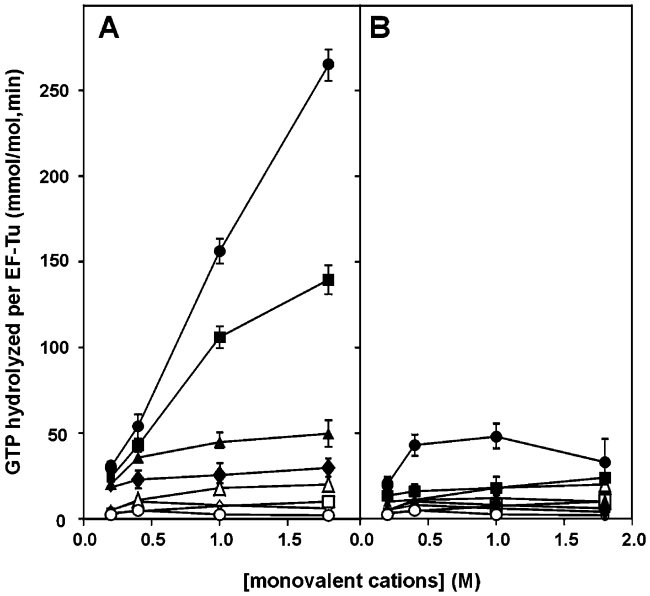

FIGURE 3: Dependence on the nature and concentration of monovalent cations of the intrinsic GTPase activity of EF-Tu·GTP·pulvomycin or EF-Tu·GTP·GE2270 A. EF-Tu·GDP (15 pmol) was converted into EF-Tu·GTP in 50 mM imidazole acetate, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 600 pmol of [γ-^{32}P]GTP (specific activity 3000 cpm/pmol), pyruvate kinase (40 μg/mL) and 2 mM phosphoenolpyruvate for 15 min at 30 °C. The hydrolysis reaction was started by adding LiCl (●, ○), NaCl (■, □), KCl (▲, Δ) or NH_4Cl (♦, ◊) to the indicated final concentrations and a shift of temperature to 37 °C. Incubations were in the presence (solid symbols) or absence (open symbols) of 50 μM pulvomycin (panel A) or GE2270 A (panel B). Samples (10 μL) were withdrawn after 4, 8, 12, and 16 min, during which time the reaction was linear, and the liberated $^{32}P_i$ was determined. Data represent the mean ± SEM for three independent experiments.

Table 2: Effect of Pulvomycin on the EF-Tu GTPase Activity in the Absence and in the Presence of aa-tRNA, Ribosomes, or Both[a]

	GTPase activity (mmol/mol EF-Tu min^{-1})	
components added	control	+ pulvomycin
EF-Tu	5.2	47.2
EF-Tu + Val-tRNA^{Val}	5.6	49.2
EF-Tu + ribosomes	35.2	40.8
EF-Tu + Val-tRNA^{Val} + ribosomes	334.8	37.6

[a] At 0 °C. The reaction mixture (50 μL) contained 50 mM imidazole acetate, pH 7.7, 40 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM DTT, 0.4 μM EF-Tu, 0.4 μM EF-Ts, 2 mM phospho*enol*pyruvate, 40 μg/mL pyruvate kinase, 5 μM γ-[^{32}P]GTP (specific activity 4000 cpm/pmol) and 50 μM pulvomycin, 0.9 μM ribosomes and 1.7 μM [^{3}H]Val-tRNA^{Val} (30% pure, specific activity 50 cpm/pmol) as indicated. Each mixture was preincubated for 10 min at 37 °C; thereafter, the reaction was started by the addition of [γ-^{32}P]GTP. Samples of 10 μL were withdrawn after 1, 2, 4, and 8 min and GTP hydrolysis was calculated from the slope of the kinetics.

The Binding of Pulvomycin and EF-Ts to EF-Tu·GDP Is Compatible. EF-Ts is known to markedly increase the association and dissociation rate of the EF-Tu·GDP complex. It was therefore important to determine whether its effect and that of pulvomycin on the dissociation rate of EF-Tu·GDP were additive or mutually exclusive. In Figure 4 we show that the effects of these two ligands on this kinetic

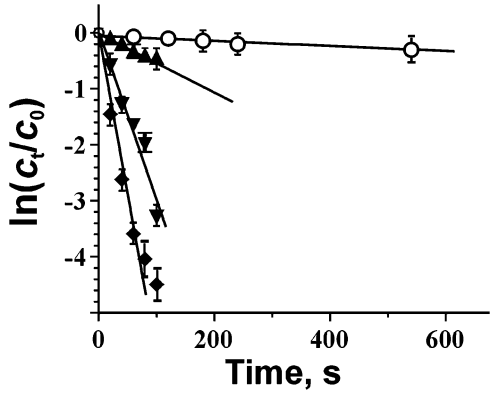

FIGURE 4: Dissociation of GDP: the effects of pulvomycin and EF-Ts are additive. The reaction mixtures kept at 0 °C contained in 1 mL of standard buffer 9 pmol of EF-Tu, 20 pmol of [^{3}H]GDP (specific activity: 6400 cpm/pmol), 1 mM DTT. Without additions (○); plus 330 pmol of pulvomycin (▼), 36 pmol of EF-Ts (▲), both pulvomycin and EF-Ts (◆). The dissociation reaction was started by adding a 1000-excess of unlabeled GDP. Aliquots (100 μL) were withdrawn at the indicated times and filtered through nitrocellulose filters (*6*, *11*). Data represent the mean ± SEM for three independent experiments.

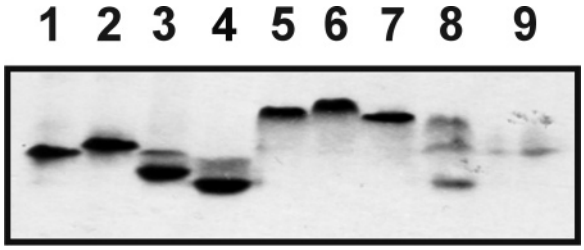

FIGURE 5: Pulvomycin does not induce the dissociation of the EF-Tu·EF-Ts complex. Native PAGE of the EF-Tu·EF-Ts complex was carried out in the presence or absence of pulvomycin, GE2270 A, or kirromycin. The reaction mixtures contained 46 pmol of EF-Tu·GDP (lanes 1−4); or 26 pmol of EF-Tu·EF-Ts (lanes (5−8) and were preincubated for 30 min at 0 °C in 10 μL of standard buffer in the absence of antibiotics (lanes 1, 5) or in the presence of 50 μM GE2270 A (lanes 2, 6), 50 μM pulvomycin (lanes 3, 7) or 50 μM kirromycin (lanes 4, 8). EF-Ts (16 pmol) was applied to lane 9 as control. Electrophoretic conditions were as reported (*5*). The buffer in the upper compartment contained 20 μM GDP to protect EF-Tu from denaturation phenomena.

parameter can coexist and are simply additive. This result supports the conclusion that EF-Ts and the antibiotic bind to different sites on EF-Tu. This possibility was further confirmed by the behavior of the EF-Tu·EF-Ts complex on native PAGE in the presence of pulvomycin. The binding of antibiotics to EF-Tu can be monitored by electrophoretic mobility (*5*, *16*), an effect indicative of modified electric charges of the molecular surface. By applying this method, we have observed that preincubation with pulvomycin does not promote any dissociation of the complex but merely causes a slight increase in the migration of EF-Tu·EF-Ts on PAGE. The direction of the shift is of the same nature as that of EF-Tu·GDP, though the effect is much less pronounced (Figure 5, cf lane 3 with lane 7). Also GE2270 A, which prevents the interaction between EF-Tu and aa-tRNA like pulvomycin, does not promote a dissociation of the EF-Tu·EF-Ts complex under the same conditions as tested for pulvomycin (Figure 5, lane 7). In this case however one can note a very slight decrease in the migration velocity slightly less accentuated than that of EF-Tu·GDP (Figure 5, cf lane 2 with lane 6). In agreement with the results of ref

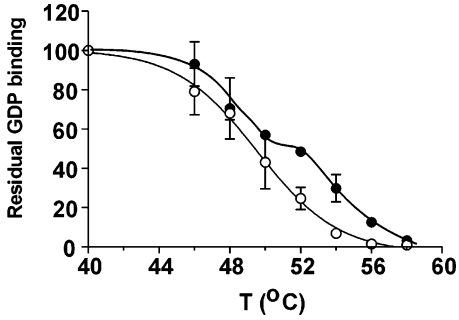

FIGURE 6: Pulvomycin and the temperature-induced inactivation of EF-Tu·GDP. EF-Tu·[^{3}H]GDP (550 pmol, specific activity: 270 cpm/pmol, in 480 μL of standard buffer) was incubated on ice for 15 min in the presence (●) or absence (○) of 40 μM pulvomycin. Subsequently, 30 μL portions were incubated for 8 min at the indicated temperatures and then cooled on ice. Aliquots of 25 μL were filtered on nitrocellulose membranes, which were washed twice with standard buffer. Data represent the mean ± SEM for three independent experiments.

17, kirromycin, used as control, promotes the dissociation of the EF-Tu·EF-Ts complex, indicating that EF-Ts and kirromycin compete for binding to EF-Tu (Figure 5, lane 8) (*18*).

Thermostability and Urea-Induced Inactivation. EF-Tu from *E. coli* is a thermo-unstable protein. In the absence of GTP or GDP, it becomes completely inactive within a few hours already at 0 °C (*19*). EF-Tu is stabilized up to 40−50 °C, depending on the experimental conditions, by GDP and GTP (*17*, *20*). Also kirromycin has a stabilizing effect, especially on EF-Tu·GDP (*19*). To determine the effect of pulvomycin on the heat-denaturation of EF-Tu, we have only tested EF-Tu·GDP, because previous results indicated that GDP is more efficient than GTP in stabilizing the factor against heat denaturation and, moreover, is much more stable than GTP against hydrolysis. As shown in Figure 6, in the absence of pulvomycin a 50% denaturation was observed at 49.5 °C; in its presence the denaturation curves seemed somewhat higher by ∼1 °C, but this difference was not significant. Interestingly, in the presence of the antibiotic, the inactivation appears to take place in two steps. In fact, at temperatures above 50 °C pulvomycin displays on EF-Tu a significant protective action against heat denaturation.

In contrast to heat denaturation, the urea-induced denaturation of EF-Tu·GDP is somewhat favored by pulvomycin, 50% inactivation taking place at 2.45 M urea in the absence of the drug vs. 1.9 M in its presence (Figure 7). A totally different picture becomes evident with EF-Tu·GTP, of which a 50% denaturation takes place at 2.2 M urea in the presence of the drug and at 0.95 M urea in its absence.

Tryptic Digestion. E. coli EF-Tu (M_r: 43 200) is readily cleaved by trypsin at residues Arg44 and Arg-58, yielding a 39 000 kDa product, via a 41 000 kDa intermediate. Kirromycin was reported to enhance the EF-Tu·GDP cleavage rate and to retard that of EF-Tu·GTP (*21*). Interestingly, the experiment illustrated in Figure 8 shows that pulvomycin displays an effect similar to that of kirromycin, for it enhances the EF-Tu·GDP cleavage rate and retards that of EF-Tu·GTP. GE2270 A does not influence the rate of cleavage of EF-Tu·GDP, while that of EF-Tu·GTP is inhibited, to about the same extent as is observed for EF-Tu·GTP·pulvomycin.

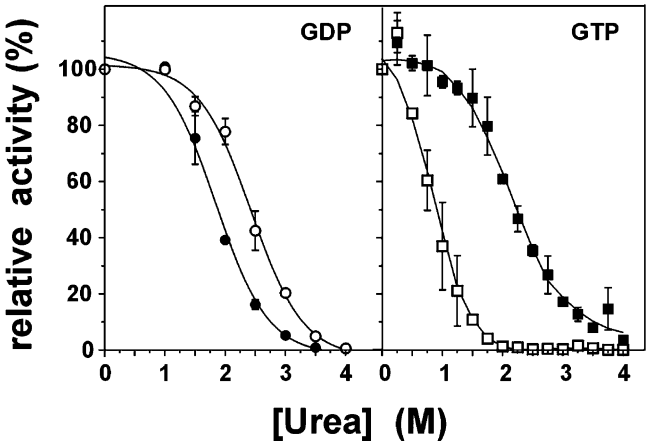

FIGURE 7: Effect of pulvomycin on the urea-induced denaturation of EF-Tu·GDP and EF-Tu·GTP. EF-Tu·[^{3}H]GDP (550 pmol) or EF-Tu·[γ-^{32}P]GTP (1500 pmol, prepared as described in the legend to Figure 3) were incubated in 640 μL of standard buffer. Subsequently, 6 μL of 2.5 mM pulvomycin (closed symbols) in dimethyl sulfoxide was added, while 6 μL of dimethyl sulfoxide was added to the control (open symbols). Subsequently, 30 μL portions were added to an equal volume of standard buffer containing urea to the indicated final concentrations. After 1 h incubation at 20 °C, aliquots of 50 μL were analyzed for GDP or GTP binding activity by the nitrocellulose filter method. (○, ●) EF-Tu.GDP; (□, ■) EF-Tu·GTP. Data represent the mean ± SEM for three independent experiments.

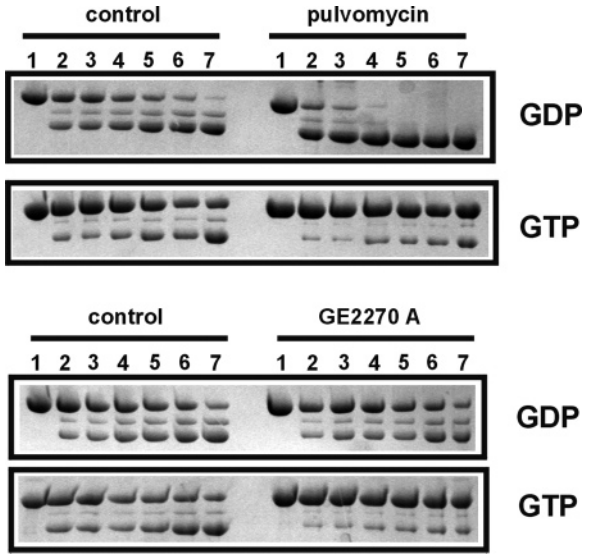

FIGURE 8: Influence of pulvomycin and GE2270 A on the tryptic digestion of EF-Tu·GDP and EF-Tu·GTP. The reaction mixture, kept on ice, contained 780 pmol of EF-Tu·GTP (prepared as described in the legend of Figure 3) or EF-Tu·GDP in 82 μL of standard buffer supplemented with 1 mM $CaCl_2$. It was divided in two portions of 39 μL each to one of which was added 0.5 μL of a 5 mM solution of the antibiotic (pulvomycin in methanol or GE2270 A in dimethylformamide) while to the other the organic solvent without antibiotic was added. Prior to the addition of trypsin (2 μL of a 0.05 μg/mL freshly prepared TPCK-treated trypsin solution in 0.5 mM HCl), a sample of 5 μL from each portion was taken as 0 time (lane 1). Thereafter, 5 μL aliquots were withdrawn after 30 s, 1, 2, 4, 8, and 16 min lanes 2−7) and added to 1.8 μL of a 100 mM benzamidine solution to stop the tryptic digestion. They were then analyzed on 12% polyacrylamide SDS−PAGE.

Pulvomycin and GE2270 A Display a Different Binding Affinity for Truncated EF-Tu. In a previous report (*18*), pulvomycin and GE2270 A were shown to be able to retard to a similar extent the dissociation rate of the D3-truncated GST-EF-Tu(D1,D2)·GDP, whereas they were unable to affect the dissociation rate of GST-EF-Tu·GDP deleted of domain 2. We have carried out a similar experiment by determining the migration velocity in native gel. As shown in Figure 9 using EF-Tu(D1,D2), GE2270 A is able to enhance the migration velocity of EF-Tu, whereas pulvomycin does not, suggesting that domain 3 of EF-Tu is involved in the binding of the antibiotic. However, one should mention that in this experiment part of the GST-EF-Tu(D1,D2)·GDP complex was unable to enter the gel due to aggregation phenomena. In the case of GST-EF-Tu·GDP deleted of domain 2 the aggregation was so pronounced that the entire construct was excluded from entering the gel (not shown).

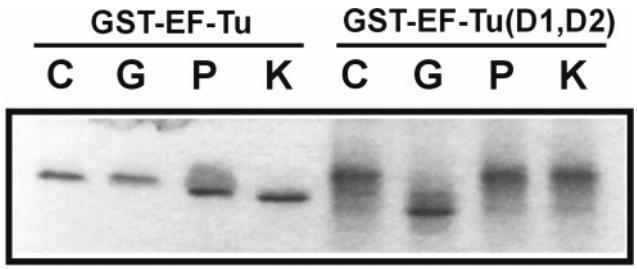

FIGURE 9: Pulvomycin and GE2270 A display a different binding affinity for EF-Tu truncated of domain 3. The reaction mixtures containing 50 pmol of GST-Tu·GDP or of partially purified GST-Tu(D1,D2)·GDP were incubated in 10 μL of standard buffer in the presence of 0.5 mM GTP, 25 μM antibiotic, pyruvate kinase (40 μg/mL), and 1 mM phospho*enol*pyruvate for 15 min at 30 °C. Lanes: (C) control (no additions); (G) plus 25 μM GE2270 A; (P) plus 25 μM pulvomycin; (K) plus 25 μM kirromycin. Native PAGE was carried out as reported in ref *5*.

DISCUSSION

In this report, several aspects of the action of the antibiotic pulvomycin on the EF-Tu-dependent reactions are investigated; similarities to and differences from the actions of other EF-Tu-specific antibiotics are discussed. Although pulvomycin affects both the EF-Tu·GTP and the EF-Tu·GDP complexes, the former appears to be the privileged target, as has been found for all other antibiotics acting specifically on EF-Tu. The stability of the GTP complex is increased a thousand times, due to a dramatic decrease in the dissociation rate of the same order as that reported for GE2270 A (*5*) and 1 order of magnitude higher than in the case of kirromycin (*11*) and enacyloxin IIa (*3*). Pulvomycin markedly increases the dissociation rate of EF-Tu·GDP, like EF-Ts, kirromycin, or enacyloxin IIa (*11*, *3*), whereas GE2270 A does not affect at all this parameter (*5*). Differently from EF-Ts, kirromycin, and enacyloxin IIa, the effect of pulvomycin is targeted on the dissociation rate of EF-Tu·GDP, and has little effect on the association rate. Moreover, unlike kirromycin and enacyloxin IIa, the effect of pulvomycin can coexist with that of EF-Ts, emphasizing the lack of interference between the actions of EF-Ts and this antibiotic. This difference is particularly evident on native PAGE, where kirromycin promotes the dissociation of EF-Tu and EF-Ts (*18*), whereas pulvomycin (or GE2270 A) only induces a shift of the migration of the EF-Tu·EF-Ts complex of the same kind as that observed with EF-Tu·GDP in the absence of EF-Ts. The observation that pulvomycin (or GE2270 A) and EF-Ts can be bound simultaneously to EF-Tu demon-

strates that this antibiotic (or GE2270 A) and EF-Ts neither compete for the same binding site on EF-Tu nor interfere via long-range effects.

Recently, Heffron and Jurnak (*7*) have obtained from X-ray diffraction studies a three dimensional (3D) model at high resolution (2.35 Å) of EF-Tu·GDP in complex with GE2270 A. In this model, the binding site for the antibiotic is embedded in the domain 2. Since its binding site partially overlaps the position that in EF-Tu·GTP is occupied by the aminoacylated acceptor stem of tRNA, it was concluded that GE2270 A through steric hindrance prevents the interaction between EF-Tu·GTP and aa-tRNA. From the model of the EF-Tu·GDP·GE2270 A complex, the antibiotic does not interact with the structures involved in the binding of EF-Ts that include several elements of the domain 1 and the tip of domain 3 of EF-Tu (*22*, *23*). Noteworthy, the substitution of Arg230, one of the amino acids of the EF-Tu binding pocket for GE2270 A contacting the antibiotic, can induce pulvomycin resistance (*24*, *25*). Moreover, competition phenomena between the two antibiotics characterize their action. In fact, bound GE2270A prevents pulvomycin from enhancing the GDP-dissociation rate (*5*). Taken together, these results strongly suggest that also pulvomycin interacts with EF-Tu domain 2 and that its binding site overlaps, at least partially, that of GE2270 A.

On the other hand, a number of differences between the actions of the two antibiotics point to the existence of selective structures in their binding site. Besides specifically affecting the dissociation rate of EF-Tu·GDP, pulvomycin significantly increases the intrinsic GTPase activity of EF-Tu, while GE2270 A exerts little, if any, influence on these two reactions. Whereas the amino acid residues, whose substitution induces resistance toward GE2270 A have so far only been found in domain 2 (V226, G257 and G275, refs *26*−*28*), in the case of pulvomycin they are located in both domains 2 (R230 and R230/233, refs *24* and *25*) and 3 (R333 and T334, ref *25*). Concerning methylkirromycin and GE2270A, the binding sites on EF-Tu as predicted from residues inducing resistance (*18*) turned out to agree with the location identified by the crystallographic analysis. If these conclusions can be extended to pulvomycin, its binding site in addition to domain 2 should involve the three domains junction and domain 3. In line with this is also the observation that GE2270 A can affect the electrophoretic mobility of GST-EF-Tu truncated of the C-terminal domain, whereas pulvomycin does not.

Interestingly, the effect of pulvomycin on the tryptic digestion of EF-Tu displays similarity to that of kirromycin, since it enhances the EF-Tu·GDP cleavage rate and retards that of EF-Tu·GTP. GE2270 A has no influence on the rate of cleavage of EF-Tu·GDP in agreement with its lack of effect on the dynamics of the EF-Tu·GDP complex, whereas it inhibits the cleavage of EF-Tu·GTP to an extent similar to that of EF-Tu·GTP·pulvomycin or EF-Tu·GTP·kirromycin. Another similarity to kirromycin is the property of pulvomycin to activate the catalytic center of the intrinsic GTPase activity of EF-Tu (*15*; see also below). Moreover, the extent of the intrinsic GTPase activity of EF-Tu stimulated by pulvomycin is dependent on the concentration and nature of the monovalent cation, and is directly proportional to their electron density, like the kirromycin-induced GTPase activity of EF-Tu. This suggests that despite the different target, pulvomycin and kirromycin share some common mechanisms on EF-Tu.

On the basis of competition phenomena between pulvomycin and kirromycin, it was concluded (*15*) that more than one molecule of pulvomycin may bind to EF-Tu. Although interesting, this possibility remains speculative. An involvement of the three interfaces junction in the binding site of EF-Tu for pulvomycin could affect the organization of the three domains in a stepwise manner, causing a functional multistep response such as the biphasic course of the heat denaturation of EF-Tu·GDP. The mechanism of action of EF-Ts and of kirromycin has emphasized the possibility and importance of long-range effects in the interactions between EF-Tu and its ligands (*1*, *2*, *22*, *23*, *29*).

Pulvomycin can moderately enhance the urea-dependent inactivation of the EF-Tu·GDP complex, whereas it exerts a marked protection on EF-Tu·GTP. This further shows that this antibiotic influences more strongly the conformation of EF-Tu induced by GTP than that induced by GDP, in agreement with its effect on the interactions with GTP and GDP. GE2270 A little affects the urea-dependent denaturation of EF-Tu·GDP or EF-Tu·GTP, except that EF-Tu·GTP displays a biphasic course for concentrations of urea exceeding 1.3 M (*6*).

In conclusion, pulvomycin and kirromycin share a number of similar properties, even if they have a different overall mode of action, whereas some selective differences distinguish pulvomycin from GE2270 A, despite the same main target. It can be expected that the elucidation of the 3D structure of the EF-Tu·pulvomycin complex will play an important role in shedding light on the various similarities and differences in the actions of the EF-Tu-specific antibiotics.

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