Antibiotics MDL 62,879 and Kirromycin Bind to Distinct and Independent Sites on Elongation Factor Tu (EF-Tu)

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ABSTRACT: Antibiotic MDL 62,879 inhibits bacterial protein synthesis by acting on elongation factor Tu (EF-Tu). In this study we show that the inhibition of protein synthesis by MDL 62,879 in an *Escherichia coli* cell-free system was fully reversed by addition of stoichiometric amounts of EF-Tu but not by large excesses of EF-Ts, ribosomes, or aa-tRNA. MDL 62,879 bound tightly to EF-Tu and formed a stable 1:1 MDL 62,879:EF-Tu (M:EF-Tu) complex. We show that binding of MDL 62,879 to EF-Tu strongly affects the interaction of EF-Tu with aa-tRNA and causes rapid dissociation of preformed EF-Tu·aa-tRNA complex, suggesting that inhibition of aa-tRNA binding is due to a conformational change in EF-Tu rather than competition for the aa-tRNA binding site. Indication of a conformational change in EF-Tu induced by MDL 62,879 is further confirmed by proteolytic cleavage experiments: MDL 62,879 binding strongly protects EF-Tu against trypsin cleavage. The observed effects of MDL 62,879 appear to be different from those of the kirromycin class of antibiotics, which also inhibit protein synthesis by binding to EF-Tu, suggesting two distinct binding sites. Indeed, the M:EF-Tu complex was able to bind stoichiometric amounts of kirromycin to form a 1:1:1 M:EF-Tu:kirromycin (M:EF-Tu:K) complex, providing direct evidence that the two antibiotics bind to independent and distinct sites on the EF-Tu molecule. The interaction of the M:EF-Tu:K complex with aa-tRNA and other co-factors suggest that the contemporary binding of the two antibiotics locks EF-Tu into an intermediate conformation in which neither antibiotic exhibits complete dominance.

MDL 62,879 (formerly GE 2270 A) is an antibiotic active against Gram-positive bacteria, and is known to strongly inhibit cell-free protein synhtesis in *Escherichia coli* (Selva et al., 1991). It belongs structurally to the thioazolyl peptide family of antibiotics, whereas other members of this class, for example, thiostrepton, micrococcin and nosiheptide, are known to inhibit bacterial ribosome function [see Vazquez (1977) and Wiborg et al. (1994)], MDL 62,879 has been demonstrated to specifically inhibit elongation factor Tu (EF-Tu)[1] (Anborgh & Parmeggiani, 1991, 1993). EF-Tu is an abundant protein in *E. coli* and is essential for bacterial protein biosynthesis, being responsible for correctly positioning the aa-tRNA into the A site of the ribosome (Miller & Weissbach, 1977; Kaziro, 1978; Bosch et al., 1983). During protein synthesis, EF-Tu undergoes several conformational changes and interacts with different cofactors such as ribosomes, EF-Ts, and aa-tRNA (Weijland et al., 1992). EF-Tu can also bind both GTP and GDP, the EF-Tu·GDP complex being the more stable (Swart et al., 1982; Stouten et al., 1993). In addition, to its role in protein synthesis, EF-Tu is also known to associate with the cytoplasmic membrane (Jacobson & Rosenbusch, 1976), participate in RNA replicase activity of bacteriophage Qβ (Blumenthal et al., 1972), and may act as a positive regulator in RNA synthesis (Travers, 1973).

The essential nature of this protein in the bacterial cell is emphasized by the fact that EF-Tu is the target of several antibiotics, including the kirromycins, pulvomycin, and MDL 62,879. These antibiotics differ both in their chemical structures (see Figure 1), spectra of antimicrobial activity (Selva et al., 1991; Landini et al., 1993), and mechanism of EF-Tu inhibition. Kirromycins stimulate the intrinsic GTPase activity of EF-Tu which, under physiological conditions, is triggered by ribosomes and aa-tRNA, thereby blocking EF-Tu on the ribosome in a non-dissociable complex (Wolf et al., 1974). By contrast, pulvomycin inhibits aminoacyl-tRNA binding to EF-Tu (Pingoud et al., 1982), and this step has also been suggested as the point of inhibition for MDL 62,879 (Anborgh & Parmeggiani, 1993). However, there appear to be significant differences between these two antibiotics concerning their specific interactions with EF-Tu since pulvomycin has been shown to stimulate both association and dissociation of GDP, whereas MDL 62,879 has no effect on EF-Tu in its GDP-bound form (Anborgh & Parmeggiani, 1991, 1993).

In this report we have investigated the effects of MDL 62,879 on the interactions of EF-Tu with the other components of the bacterial protein sysnthesis machinery and in particular with aa-tRNA. We propose that inhibition of aa-tRNA:EF-Tu interaction by MDL 62,879 is not due to direct competition between the antibiotic and aa-tRNA for the same binding site but to a conformational change induced by MDL 62,879. Moreover, kirromycin can interact with MDL 62,879-complexed EF-Tu, suggesting two distinct and

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[1] Abbreviations: EF-Ts, elongation factor Ts; EF-T, elongation factor EF-Tu/EF-Ts; poly(U), polyuridylic acid; poly(phe), polyphenylalanine; aa-tRNA, aminoacyl-tRNA; phe-tRNA, phenylalanyl-tRNA; GDP (GTP), guanosine diphosphate (triphosphate); TCA, trichloroacetic acid; M:EF-Tu, MDL 62,879:EF-Tu complex; K:EF-Tu, kirromycin:EF-Tu complex; M:EF-Tu:K, MDL 62,879:EF-Tu:kirromycin co-complex.

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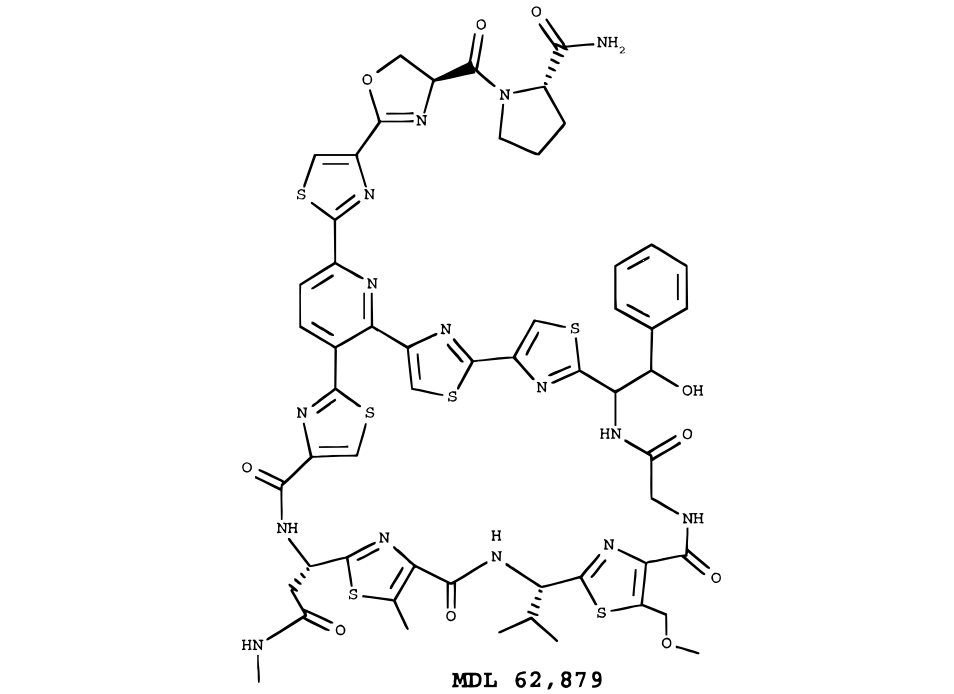

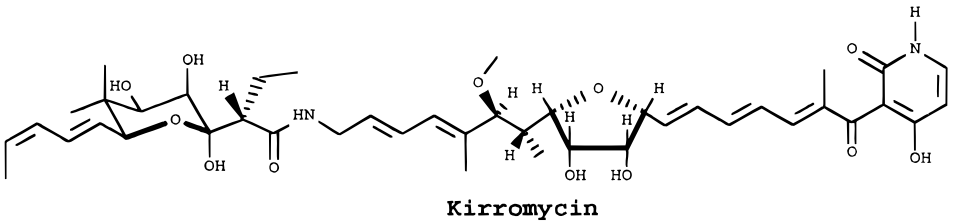

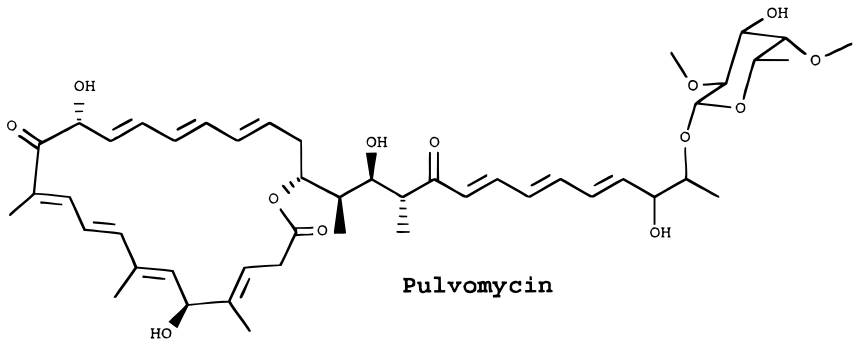

FIGURE 1: Chemical structures of antibiotics MDL 62,879, pulvomycin, and kirromycin.

independent sites for these antibiotics and co-dominant effects of the two antibitoics on EF-Tu.

MATERIALS AND METHODS

Chemicals. MDL 62,879 (molecular weight 1289) was prepared at the Lepetit Research Center, Gerenzano, Italy, and kirromycin (molecular weight 818) was a gift from Gist-Brocades laboratories. [^{3}H]Phenylalanine, [^{3}H]GDP and [^{32}P]GTP were obtained from Amersham, Bucks, U.K. Glass fiber filters (GF/C) were from Whatman, and nitrocellulose filters (HAWP 0045) were from Millipore. All other chemicals were from Sigma, St. Louis, MO.

Antibiotic EF-Tu Complex Formation. EF-Tu·GDP (2 mg/mL) was incubated with 100 μg of MDL 62,879/mL or 100 μg of kirromycin/mL or alternatively with 100 μg of MDL 62,879 and kirromycin/mL for 60 min at room temperature in 1 mL of Standard Buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, pH 7.7). At the end of the incubation period the protein was dialyzed overnight against three changes of 1 L volumes of Standard Buffer. A control solution of EF-Tu, in the absence of antibiotics, was dialyzed under the same conditions.

Determination of M:EF-Tu or K:EF-Tu Stoichiometry

(a) Antimicrobial Activity. After dialysis aliquots were removed, the protein content was determined, and the solutions were adjusted to 50% acetonitrile and kept on ice for 30 min. The precipitated protein was removed by centrifugation (12 000*g*, 15 min, 4 °C), and the supernatant was assayed for antimicrobial activity (Selva et al., 1991). The concentration of the dissociated antibiotic was determined by agar diffusion microbiological assay using *Staphylococcus aureus* and *Bacillus subtilis* as test microor-

ganisms. 5 μL aliquots were dispensed onto seeded plates, which were subsequently incubated at 37 °C for 20 h, and the diameter of the inhibition halo was measured. The minimal concentration which inhibited bacterial growth was determined, and the amount of antibiotic was calculated by comparison with a standard solution of the antibiotic.

(b) HPLC Analysis. MDL 62,879 or kirromycin complexed with EF-Tu was released from the protein by acetonitrile treatment as described in A above and chromatographed on a RP18 column (4 × 125; Merck) using a Varian model 5000 LC pump and Varian 2050 wavelength detector at 254 nm. The column was eluted with acetonitrile/ammonium formate buffer (3.15 g/L) 44:56 at a flow rate of 0.7 mL/min. The amount of MDL 62,879 or kirromycin was calculated by comparison of the integrated peak areas using a standard solution of the two antibiotics (standard error was 2.41% for MDL 62,879 and 5.48% for kirromycin).

The stoichiometry of the complex was calculated using molecular weights of 43 000, 1290, and 820 for EF-Tu, MDL 62,879, and kirromycin, respectively.

Fast Performance Liquid Chromatogarphy (FPLC). After dialysis the EF-Tu which had been pre-incubated with MDL 62,879 was applied to a Mono-Q ion-exchange column (P1.6/5) pre-equilibrated in the Standard Buffer. The column was washed with five volumes of the equilibration buffer and eluted with a linear salt gradient (0−0.5 M NaCl) at a flow rate of 100 μL/min, and the elution was monitored by absorbance at 280 nm. 100 μL fractions were collected and all fractions containing protein were analyzed by SDS−PAGE on 20% acrylamide gels (Pharmacia Phast System) and assayed for microbiological activity (see above).

Poly(U)-Directed Poly(phe) Synthesis. Cell-free poly(phe) synthesis was performed as described by Landini et al. (1992) using crude extracts from *E. coli* prepared as in Traub et al. (1971), using 3 pmol of ribosomes and 4 μg of crude supernatant factors. The amount of EF-Tu in these reactions was approximately 10 pmol as estimated by SDS−PAGE. [^{3}H]Phenylalanyl-tRNA (^{3}H-phe-tRNA) was charged and isolated as in Ravel and Shorey (1971). Assays were performed at 30 °C for 20 min in Standard Buffer containing 0.8 mM ATP, 1 mM GTP, and 80 mM NH_4Cl. Purified factors, EF-Tu, EF-Ts, and salt-washed ribosomes were used in inhibition reversion assays: MDL 62,879 was preincubated with either ribosomes or elongation factors for 5 min on ice, and the reaction was initiated by addition of crude elongation factors and ribosomes. The extent of radiolabeled incorporation was determined by filtration of hot TCA-precipitable material on glass fiber filters (Landini et al., 1992).

Hydrolysis Protection of aa-tRNA by EF-Tu. The binding of aa-tRNA to EF-Tu was measured by hydrolysis protection experiments of ^{3}H-phe-tRNA as described by Pingoud et al. (1982). Briefly, 12.5 pmol of ^{3}H-phe-tRNA in Standard Buffer containing 40 mM NH_4Cl and 0.5 mM GTP, was incubated at 28 °C in the presence or absence of EF-Tu (20 pmol) and GTP regenerating system (Pingoud et al., 1982), in a final volume of 500 μL. Aliquots of 80 μL removed at appropriate intervals were precipitated with cold 5% TCA and filtered onto glass fiber filters. This experiment was performed both with the M:EF-Tu complex, K:EF-Tu complex, M:EF-Tu:K complex, and with EF-Tu which had been preincubated with ^{3}H-phe-tRNA prior to the addition of MDL 62,879. In the latter case, 12.5 pmol of ^{3}H-phe-tRNA was incubated with 20 pmol of EF-Tu and 5 pmol of EF-Ts for 5 min at 28 °C followed immediately by addition of 50 pmol of MDL 62,879.

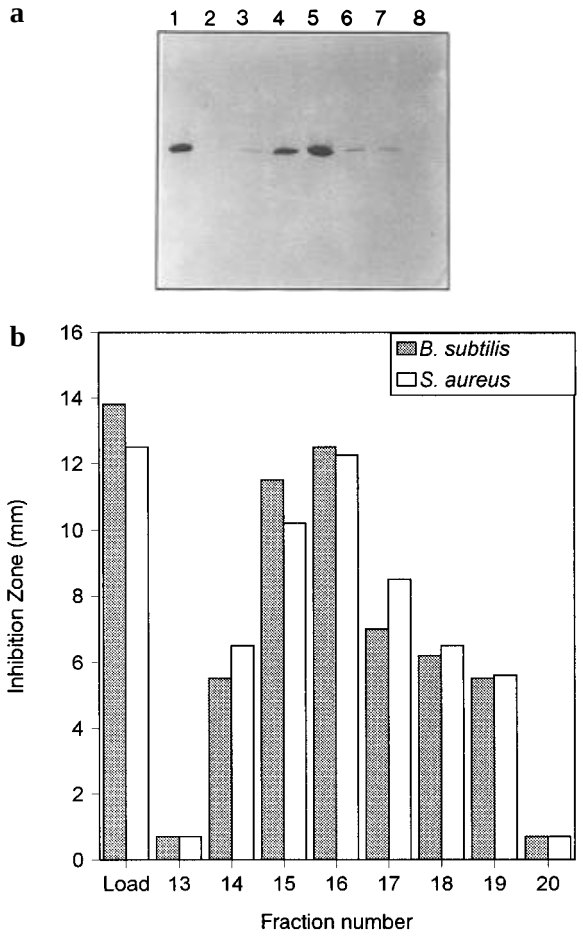

FIGURE 2: Purification of the M:EF-Tu complex by ion-exchange chromatography. EF-Tu was incubated with MDL 62,879 and dialyzed extensively (see Materials and Methods) prior to being loaded onto a Mono-Q resin. The column was pre-equilibrated in standard buffer (see Materials and Methods) and after washing was eluted with a linear NaCl gradient (0−0.5 M) in the pre-equilibration buffer. The fractions were analyzed by SDS−PAGE on a 10−15% gradient gel (a) and assayed for antimicrobial activity (b). Lanes 2−8 eluted fractions 13 to 20 in b, which shows the antibacterial activity of the starting material and the eluted fractions on *B. subtilis* and *S. aureus*.

Trypsin Cleavage Experiments. Proteolytic degradation of EF-Tu was perfomed essentially as described by Jacobson and Rosenbusch (1977).

RESULTS

M:EF-Tu Complex. The M:EF-Tu complex, when fractionated by FPLC, eluted as a sharp peak at 0.2 M NaCl. Protein containing fractions were analyzed by SDS−PAGE (Figure 2a) and assayed for microbiological activity (Figure 2b). Antimicrobial activity was present only in the EF-Tu containing fractions (cf. Figure 2a and 2b), and no antimicrobial activity was detected in the flow-through fraction (data not shown). Stoichiometry of MDL 62,879 and EF-Tu in the complex was determined to be 1:1, by HPLC and microbiological assays (see Materials and Methods).

Poly(U)-Directed Poly(phe) Synthesis. The addition of 10−20 pmol of MDL 62,879 to a crude protein synthesis cell-free system resulted in almost complete inhibition of poly(phe) synthesis. However, this inhibition of protein

Table 1: Poly(U)-Directed Poly(phe) Synthesis in an *E. coli* Cell-Free Extract: Reversion of Inhibition by MDL 62,879 by Addition of EF-Tu, Ribosomes, EF-Ts, and/or phe-tRNA

	cpm	% inhibition
crude extract		
control	80 130	–
MDL 62,879 0.25 (μg/mL)	8 874	88.6
+EF-Tu (20 pmol)		
control	106 739	–
MDL 62,879 0.25 (μg/mL)	91 896	4.9
+EF-Ts (250 pmol)		
control	120 779	–
MDL 62,879 0.25 (μg/mL)	19 169	84.1
+ribosomes (100 pmol)		
control	113 657	–
MDL 62,879 0.25 (μg/mL)	7 166	93.7
+phe-tRNA (250 pmol)		
control	58 185	–
MDL 62,879 0.25 (μg/mL)	5 441	91.6

synthesis by MDL 62,879 in a crude extract from *E. coli* was reversed by the addition of stoichiometric amounts, with respect to MDL 62,879, of purified EF-Tu. As shown in Table 1, 20 pmol of EF-Tu fully restored synthetic activity in a crude cell-free poly(U) system. By contrast, neither the addition of ribosomes (up to 100 pmol) nor EF-Ts or phe-tRNA (up to 250 pmol) was able to reverse the inhibition of protein synthesis by MDL 62,879.

aa-tRNA Hydrolysis Protection by EF-Tu. MDL 62,879 inhibits the binding of aa-tRNA to EF-Tu (Anborgh & Parmeggiani, 1991). However, it is unclear whether this inhibition of aa-tRNA binding is a consequence of competition for the same binding site or a conformational inactivation of EF-Tu. To distinguish between these two possibilities, we studied the effect of MDL 62,879 on preformed EF-Tu·GTP·phe-tRNA. The amount of phe-tRNA bound to EF-Tu was determined, using the method of Pingoud et al. (1982), as the amount of phe-tRNA protected by nonenzymatic hydrolysis. The stability of the ester bond between the amino acid and tRNA is increased by interaction with EF-Tu, so that the deacylation kinetics of free and bound phe-tRNA can be monitored by filtering samples onto glass fiber filters (phe-tRNA, but not the free amino acid, is retained by these filters). Figure 3a shows the results of a typical experiment using the M:EF-Tu complex wherein inhibition of ^{3}H-phe-tRNA binding appears to be complete. When MDL 62,879 was added to a preformed EF-Tu·^{3}H-phe-tRNA complex (Figure 3b), the deacylation kinetics were similar irrespective of whether the antibiotic was added before or after formation of the EF-Tu·^{3}H-phe-tRNA:EF-Tu complex. This strongly suggests that aa-tRNA does not affect MDL 62,879 binding to EF-Tu.

Trypsin Cleavage. Partial proteolytic digestion of EF-Tu by trypsin resulted in three major fragments: 37, 24, and 14 kDa [Figure 4; see also Jacobson and Rosenbusch (1977)]. By contrast, digestion of the M:EF-Tu revealed the presence of only the 37 kDa fragment, suggesting protection against further cleavage. As shown in Figure 4, the 37 kDa fragment remains protected for up to 2 h, a time during which EF-Tu is almost completely cleaved to lower molecular weight (24 and 14 kDa) fragments. Even after 20 h (data not shown) no significant cleavage of the 37 kDa fragment of the M:EF-Tu was observed, whereas EF-Tu was completely digested.

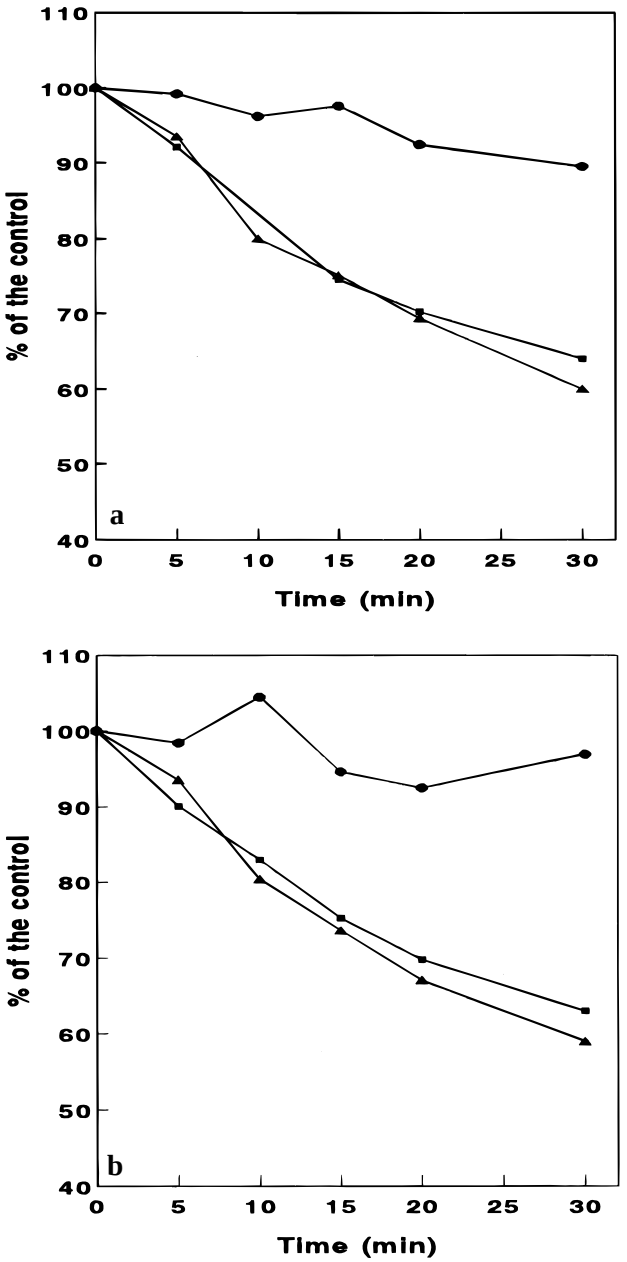

FIGURE 3: Protection of 3H-phe-tRNA against hydrolysis in the presence of either EF-Tu or M:EF-Tu. Experiments were performed as in Materials and Methods. Values are the average of duplicate samples from one experiment. (a) ^{3}H-phe-tRNA was incubated either with (●) or without (■) EF-Tu or with M:EF-Tu (▲) for 5 min at 26 °C. Aliquots of 80 μL were removed at the indicated times, the reaction was terminated with TCA, and the incorporation was determined after filtration. (b) ^{3}H-phe-tRNA was incubated at 26 °C with EF-Tu (●) in a final volume of 1 mL. After 5 min of incubation 50 pmol of MDL 62,879 (▲) was added to 500 μL of the reaction mix. At the indicated times samples (80 μL) were removed and hydrolysis of ^{3}H-phe-tRNA determined. ^{3}H-phe-tRNA hydrolysis in the absence of EF-Tu (■) was also determined.

M:EF-Tu:K Complex. Kirromycin has been shown to inhibit prokaryotic protein biosynthesis by binding to EF-Tu and to form a stable 1:1 complex with EF-Tu (Parmegianni & Swart, 1985; Abrahams et al., 1991; Sottani et al., 1993). We investigated if MDL 62,879 could influence the ability of EF-Tu to bind kirromycin. Kirromycin was therefore incubated either with EF-Tu or with M:EF-Tu complex and, after extensive dialysis to remove unbound antibiotic (see Materials and Methods), the complexed

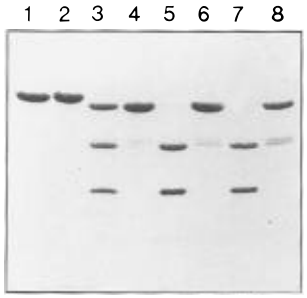

FIGURE 4: Time course of digestion of EF-Tu and the M:EF-Tu complex by trypsin. 150 μg of either EF-Tu or M:EF-Tu was incubated with trypsin (15:1 w/w) at 37 °C. Aliquots were removed at time 0 (lanes 1 and 2), 2 min (lanes 3 and 4), 30 min (lanes 5 and 6), and 120 min (lanes 7 and 8), and the reaction was terminated by boiling for 5 min in the gel sample buffer. The protein was analyzed by SDS−PAGE on 20% acrylamide Phast gels. Lanes 1, 3, 5, and 7, EF-Tu; lanes 2, 4, 6, and 8, M:EF-Tu.

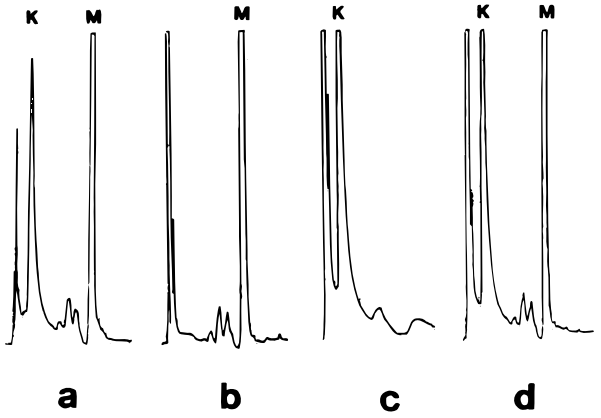

FIGURE 5: HPLC profiles of EF-Tu-complexed MDL 62,879 and kirromycin. HPLC analysis of the complexes was performed as described in Materials and Methods. The retention times for kirromycin and MDL 62,879 were 3.8 and 13.5 min, respectively. K and M indicate the respective positions of kirromycin and MDL 62,879 in the elution profiles. (a) A standard solution containing 50 μg of kirromycin/mL and 100 μg/mL of MDL 62,879, (b) M:EF-Tu, (c) K:EF-Tu, and (d) M:EF-Tu:K.

antibiotics were analyzed by HPLC. As shown in Figure 5, kirromycin bound both to the EF-Tu and to the M:EF-Tu complex (Figure 5, cf. spectra c and d). Calculation of the amount of complexed antibiotics, using the integrated peak areas and molecular mass, showed that both kirromycin and MDL 62,879 were present in a 1:1 stoichiometry with EF-Tu. The amount of kirromycin that bound to the M:EF-Tu was similar to that observed with EF-Tu alone, and the calculated stoichiometry of M:EF-Tu:K was 1:1:1 in the co-complex.

As kirromycin and MDL 62,879 have opposing effects as far as the aa-tRNA binding is concerned, the ability of the M:EF-Tu:K co-complex concerning this reaction was also examined. The protection of aa-tRNA binding was therefore examined by determining the amount of deacylation in the presence of EF-Tu alone or in the presence of K:EF-Tu, M:EF-Tu, or M:EF-Tu:K. The results of a typical experiment are shown in Figure 6a. In the absence of EF-Tu the aa-tRNA was deacylated but was protected against deacylation in the presence of EF-Tu. In the K:EF-Tu complex a slight stimulation of the aa-tRNA binding was observed, while the M:EF-Tu complex failed to bind aa-tRNA. Interestingly, the amount of aa-tRNA which bound to the M:EF-Tu:K co-complex was about 50% of control, suggesting a "metastate" between the properties of kirromycin to stimulate and those of MDL 62,879 to diminish the binding. Therefore both antibiotics appear to be able to promote their respective effects without either one being dominant.

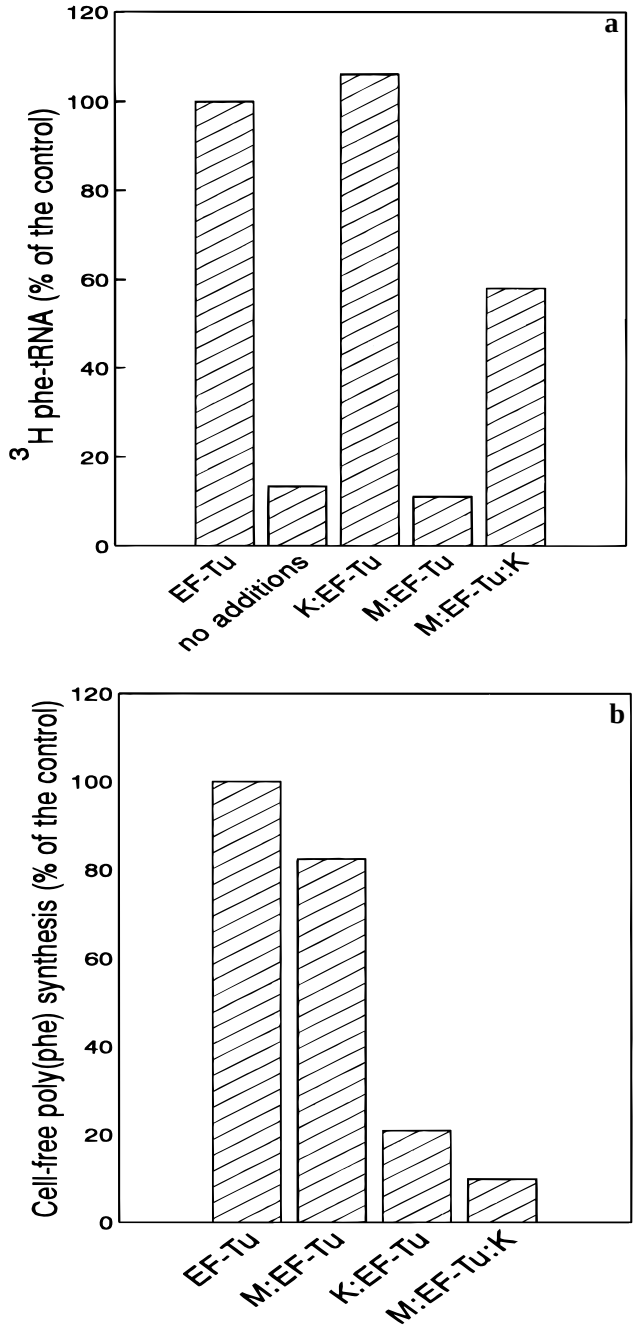

FIGURE 6: Effects of M:EF-Tu, K:EF-Tu, and M:EF-Tu:K complexes on (a) protection against ^{3}H-phe-tRNA hydrolysis and (b) cell-free poly(phe) synthesis. (a) ^{3}H-phe-tRNA was incubated either in the absence of EF-Tu or in the presence of either EF-Tu or the indicated complexes. After 120 min of incubation at 26 °C, the reaction was terminated by addition of TCA and the incorporation determined after filtration. The data are expressed as a percentage of the counts incorporated (52 248 cpm) in the presence of EF-Tu. (b) Effect of addition of either EF-Tu or the complexes to a crude cell-free poly(phe) synthesis system. The reaction was terminated after 30 min, and the TCA precipitated counts were determined and expressed as a percentage of those observed (50 350 cpm) in the presence of EF-Tu.

We therefore also examined the effect of the co-complex on protein synthesis to determine if the dominant property of kirromycin to inhibit the release of EF-Tu from the

ribosome after GTP hydrolysis, thereby blocking the elongation cycle, was altered in the co-complex. The effect of addition of either EF-Tu, M:EF-Tu complex, K:EF-Tu, or M:EF-Tu:K co-complex on the poly(phe)-synthesizing cell-free system was examined. In contrast to EF-Tu and M:EF-Tu complex, which typically had little or no effect on poly(phe) synthesis, K:EF-Tu and M:EF-Tu:K complex inhibited the poly(phe) synthesis (Figure 6b), suggesting that the ability of kirromycin to form an extremely stable K:EF-Tu:ribosome ternary complex, remains unaltered in the M:EF-Tu:K co-complex.

DISCUSSION

During the elongation cycle of bacterial protein synthesis, EF-Tu undergoes several conformational changes as a result of its interaction with different cofactors such as guanine nucleotides, EF-Ts, and aa-tRNA [see Weijland et al. (1992), Stouten et al. (1993), and Wiborg et al. (1994)]. The novel antibiotic MDL 62,879 inhibits bacterial protein synthesis by binding to EF-Tu and locking the protein in a GTP-like conformation (Anborgh & Parmeggiani, 1991, 1993; Landini et al., 1992). Although MDL 62,879 does not affect EF-Tu·GDP-mediated reactions, it retains the ability to bind to the EF-Tu·GDP form (Anborgh & Parmeggiani, 1993). The strong interaction of MDL 62,879 with the EF-Tu·GDP allows an M:EF-Tu complex to be recovered by ion-exchange chromatography [Figure 2; see also Anborgh and Parmeggiani (1993)]. HPLC analysis and antimicrobial activity indicate that the stoichiometry of MDL 62,879 to EF-Tu in the complex is 1:1.

The strong interaction between MDL 62,879 and EF-Tu allowed us to perform enzymatic assays with the M:EF-Tu complex in the absence of free MDL 62,879 in the reaction medium. A major effect of MDL 62,879 on the interaction of aa-tRNA binding to EF-Tu is observed. This effect may be due to direct competition between MDL 62,879 and aa-tRNA for the aa-tRNA binding site on EF-Tu. Alternatively, binding of MDL 62,879 to EF-Tu may induce a conformational change which affects the aa-tRNA binding site. Several of our observations suggest that aa-tRNA and MDL 62,879 probably do not compete for the same binding site: (a) a stoichiometric excess of phe-tRNA did not reverse MDL 62,879 inhibition of poly(phe) synthesis (Table 1), (b) experiments using labeled ^{3}H-phe-tRNA preincubated with EF-Tu demonstrated that addition of MDL 62,879 promoted rapid dissociation of the complex (Figure 3b), and (c) phe-tRNA is known to protect the 6 kDa fragment of EF-Tu from tryptic cleavage (Jacobson & Rosenbusch, 1977) while MDL 62,879 afforded no protection of this fragment (Figure 4). These observations strongly suggest that inhibition of the EF-Tu·aatRNA interaction is not due to direct competition for the same binding site but is the result of a MDL 62,-879-induced conformational change in EF-Tu. Furthermore, MDL 62,879 has no affect on the binding of fMet·tRNA to initiation factor 2 (C. O. Gualerzi, personal communication). Interestingly, initiation factor 2 is also a GTP-binding protein, and the observation that MDL 62,879 has little or no affect on this protein would also be consistent with the previously proposed action of this antibiotic in stabilising the EF-Tu·-GTP form (Anborgh & Parmeggiani, 1993).

Some evidence already exists for a conformational change in EF-Tu induced upon binding MDL 62,879. Partial proteolytic degradation of EF-Tu by trypsin results in a time-dependent generation of multiple fragments (Jacobson & Rosenbusch, 1977) with an initial fragment of about 37 kDa, followed by fragments of 24 and 14 kDa. When M:EF-Tu was treated with trypsin, only the 37 kDa fragment was formed and was not further cleaved by the protease for up to 20 h (Figure 4). Anborgh and Parmeggiani (1991) have also reported that EF-Tu complexed with MDL 62,879 shows an altered mobility on native gels, indicative of altered charges.

Our findings also highlight important differences in the mode of action of this antibiotic compared with the kirromycins. Like MDL 62,879, kirromycin antibiotics also form a 1:1 complex with EF-Tu but the M:EF-Tu and K:EF-Tu complexes behave quite differently. The K:EF-Tu complex is unable to leave the ribosome, thereby blocking further translation of mRNA [see review by Parmeggiani and Swart (1985)]. Moreover, kirromycins have been demonstrated to induce the EF-Tu GTPase, to level the affinities of EF-Tu for GDP and GTP, and to compete with EF-Ts for binding to EF-Tu (Swart et al., 1982; Wolf et al., 1974, 1977; Hall et al., 1991), while MDL 62,879 leads to GTP stabilization and has no affect on the EF-Ts interaction. MDL 62,879 dramatically affects phe-tRNA binding to EF-Tu while kirromycins do not affect the phe-tRNA binding to EF-Tu and promote the interaction of aa-tRNA with EF-Tu in the absence of GTP. Furthermore, there is a marked difference in the protection afforded by MDL 62,879 and by kirromycin against proteolytic degradation of EF-Tu by trypsin. Although both antibiotics protect the 37 kDa fragment against cleavage [Jacobson and Rosenbuch (1977) and Figure 4), this effect is temporary for kirromycin; 24 and 14 kDa fragments are generated within 20−30 min (unpublished observation) when compared with MDL 62,879.

In view of the differences in the mode of action of the two antibiotics we reasoned that there was a possibility that kirromycins and MDL 62,879 may bind to different sites on the EF-Tu molecule, and this possibility was directly tested, as both antibiotics form stable complexes, by determining the ability of kirromycin to bind to the M:EF-Tu complex (Figures 5 and 6). The observation that kirromycin forms a 1:1 complex with the M:EF-Tu complex clearly demonstrates that the two antibiotics bind to distinct and independent sites. This is further confirmed by recent observations that resistance to kirromycins is conferred by mutations in the interface between EF-Tu domains I and III (Abdulkarim et al., 1994) while resistance to MDL 62,879 is conferred by mutations in domain II (Shiminaka et al., 1995; M. Sosio, F. Monti, V. Wright, F. Ripamonti, S. Lociuro, and K. Islam, unpublished observations). Indeed, the binding of MDL 62,879 does not affect the kirromycin binding site. In the M:EF-Tu:K co-complex the effect of MDL 62,879 on aa-tRNA binding is modified by the co-presence of kirromycin and is suggestive of a metastate in which either both antibiotics promote their individual effects, thereby counterbalancing each other or, alternatively, kirromycin promotes a conformation of EF-Tu which cannot be fully modified to the MDL 62,879 only conformation. This metastate would once more strongly argue that the influence of MDL 62,879 on aa-tRNA binding is mediated via conformational alterations in EF-Tu rather than direct competition between these two ligands. The dominance of kirromycin with respect to ribosomal release of EF-Tu after GTP hydrolysis is not

affected by MDL 62,879, suggesting that the latter antibiotic does not influence the kirromycin-induced conformation concerning this property.

The demonstration that MDL 62,879 can bind EF-Tu·GDP indicates that this antibiotic binds to several different conformations of EF-Tu in its reaction cycle. M:EF-Tu·GDP binds EF-Ts [Anborgh and Parmeggiani (1993) and unpublished observations] which catalyzes the GDP/GTP reaction to form a stable M:EF-Tu·GTP complex. This complex is no longer able to bind aa-tRNA to form an M:EF-Tu·GTP·aa-tRNA complex although EF-Tu·GTP·aa-tRNA can bind MDL 62,879, but with consequent dissociation of aatRNA from the complex. We are currently attempting to determine the precise binding site of this antibiotic along the primary sequence of EF-Tu. These studies will help in gaining insights into the structure−function aspects of this exciting protein.

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