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Abrahams, J.P.; Raaij, M.j. van; Ott, G.; Kraal, B.; Bosch, L.

Kirromycin Drastically Reduces the Affinity of *Escherichia coli* Elongation Factor Tu for Aminoacyl-tRNA†

Jan Pieter Abrahams,‡,§ Mark J. van Raaij,‡ Günther Ott,∥ Barend Kraal,*,‡ and Leendert Bosch‡

Department of Biochemistry, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands, and Laboratorium für Biochemie der Universität Bayreuth, P.O. Box 101251, d-8580 Bayreuth, FRG

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ABSTRACT: We have studied the interaction between EF-Tu·GDP or EF-Tu·GTP in complex with kirromycin or aurodox (N^1-methylkirromycin) and aminoacyl-tRNA, *N*-acetylaminoacyl-tRNA, or deacylated tRNA. Three independent methods were used: zone-interference gel electrophoresis, GTPase stimulation, and fluorescence. All three methods revealed that kirromycin induces a severe drop in the stability of the complex of EF-Tu·GTP and aminoacyl-tRNA of about 3 orders of magnitude. The affinities of EF-Tu·kirromycin·GTP and EF-Tu·kirromycin·GDP for aa-tRNA were found to be of about the same order of magnitude. We conclude that kirromycin and related compounds do not induce a so-called GTP-like conformation of EF-Tu with respect to tRNA binding. The findings shed new light on the mechanism of action of the antibiotic during the elongation cycle. In contrast to indirect evidence previously obtained in our laboratory [Van Noort et al. (1982) *EMBO J. 1*, 1199–1205; Van Noort et al. (1986) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4910–4914], we were unable to demonstrate complexes of EF-Tu·aurodox·GTP/GDP with *N*-acetylaminoacyl-tRNA or deacylated tRNA by direct detection using zone-interference gel electrophoresis. Modification with *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) decreases the affinity of EF-Tu·kirromycin·GTP for aminoacyl-tRNA, just like it does in the absence of the antibiotic.

Prokaryotic protein synthesis is known to be inhibited by kirromycin and related compounds, which form a complex with EF-Tu[1] in a 1:1 ratio. As a consequence of kirromycin binding, EF-Tu is not able to leave the ribosome, thereby effectively blocking further translation of the mRNA. Binding of kirromycin to EF-Tu also induces a conformational change of the protein in the absence of ribosomes. It causes a clear GTPase activity that can be enhanced by aminoacyl-tRNA (aa-tRNA). Furthermore, kirromycin levels the affinities of EF-Tu for GTP and GDP, which in the absence of the antibiotic differ by 2 orders in magnitude. For a review, see Parmeggiani and Swart (1985).

The current opinion is that kirromycin induces a GTP-like conformation of EF-Tu, irrespective of the nucleotide bound (Douglass & Blumenthal, 1979). The presence of tRNA at concentrations saturating both A and P sites of the ribosome·mRNA complex is necessary for the immobilization of EF-Tu·kirromycin·GDP (Wolf et al., 1977).

Earlier findings in our laboratory suggested the presence of a second tRNA binding site on EF-Tu, becoming manifest upon complex formation with kirromycin. The following data provided the operational characteristics of this site.

(1) The reactivity of Cys-81 of EF-Tu·kirromycin·GTP/GDP toward modifying agents such as *N*-ethylmaleimide (NEM) and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) is enhanced by aa-tRNA, Nacaa-tRNA, and HO-tRNA. At higher concentrations of aa-tRNA an inhibition of modification is found. In the absence of kirromycin a comparable inhibition is observed at much lower aa-tRNA concentrations, provided GTP is present. Such a shielding effect is not exhibited by Nacaa-tRNA or HO-tRNA. From these data it was concluded that all three tRNA species bind to a second, nonclassical site induced by kirromycin and that aa-tRNA has an additional affinity for the classical binding site as it exists in the absence of the antibiotic (Van Noort et al., 1982, 1986).

(2) The enhancement of GTPase activity of EF-Tu·kirromycin·GTP by aa-tRNA and Nacaa-tRNA was reported to be unaffected by modification of Cys-81 by TPCK (Van Noort et al., 1986), while in the absence of the antibiotic such a modification strongly inhibits binding of aa-tRNA (Jonâk et al., 1973). It was concluded that modification of EF-Tu with TPCK does not block the tRNA binding site induced by kirromycin and that the binding of tRNA to this site stimulates the GTPase of EF-Tu·kirromycin·GTP (Van Noort et al., 1986).

(3) High concentrations of Nacaa-tRNA were reported to inhibit the GTPase activity of EF-Tu·kirromycin·GTP, unless the protein was modified with TPCK. By consequence it was assumed that in the presence of kirromycin Nacaa-tRNA can also be bound by the classical tRNA binding site such as it exists in the absence of kirromycin and that this binding is anomalous since it does not induce a shielding of Cys-81 and that it inhibits GTPase (Van Noort et al., 1986).

We were interested in finding direct physical evidence for the existence of two binding sites for tRNA on EF-Tu·kirromycin·GTP/GDP. Since earlier undocumented attempts using Hümmel-Dreyer gel filtration and ultracentrifugation (A.

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* To whom correspondence should be addressed.

‡ Leiden University.

§ Present address: Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH, U.K.

∥ Laboratorium für Biochemie der Universität Bayreuth.

[1] Abbreviations: EF-G, elongation factor G; EF-Tu, elongation factor Tu; EF-TuTPCK, EF-Tu modified with TPCK; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); aa-tRNA, aminoacyl-tRNA; HO-tRNA, uncharged tRNA; Nacaa-tRNA, *N*-acetylaminoacyl-tRNA; Tyr-[AEDANS-s^2C]$tRNA^{Tyr}$, Tyr-$tRNA^{Tyr}$ modified at the penultimate cytidine residue with a thio group at position 2 of the pyrimidine ring, to which an *N*-(acetylaminoethyl)-5-naphthylamine-1-sulfonic acid fluorescence group is attached; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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Pingoud, personal communication) or gel electrophoresis (J. M. Van Noort, personal communication) failed to do so, we developed a new technique especially suited for studying weak protein–nucleic acid interactions. To our surprise, we could not detect properties of EF-Tu·kirromycin·GTP/GDP such as those predicted by the above-mentioned experiments. As a consequence, we repeated the qualitative GTPase experiments reported by Van Noort et al. (1986) in such a way that we could in the end quantitate their result. By doing so, we found in addition a few discrepancies with the original data that we cannot account for. What we could show with various independent techniques, however, is that the affinity of EF-Tu·kirromycin·GTP/GDP for aa-tRNA is much lower than is usually assumed, i.e., at least some 3 orders of magnitude lower than that of EF-Tu·GTP for aa-tRNA, see also our preliminary data (Abrahams et al., 1988; Kraal et al., 1989).

For the experiments described, we used kirromycin or N^1-methylkirromycin (also known as aurodox), alternatively. We could not detect differences in their behavior. All components except EF-Tu and tRNA were present in saturating amounts and when mentioning the K_d of a complex, we thus refer to the dissociation of the tRNA.

Materials and Methods

Guanine nucleotides, phosphoenolpyruvate, pyruvate kinase, and bulk tRNA were purchased from Boehringer Mannheim, FRG. Agarose was purchased from Bethesda Research Laboratories, Neu Isenburg, FRG. [^{14}C]Amino acids used to check the yield of aminoacylation of tRNAs were from Amersham, U.K. Kirromycin and aurodox were gifts from Gist Brocades, Delft, The Netherlands. Since the availability of pure kirromycin has become rather limited, most of the experiments were performed with aurodox.

EF-Tu·GDP from *Escherichia coli* was prepared according to Leberman et al. (1980). The aminoacylation of bulk tRNA and complex formation with EF-Tu was according to Van Noort et al. (1986). Zone-interference gel electrophoresis was performed as described by Abrahams et al. (1988). GTPase stimulation was studied according to Van Noort et al. (1986), although slightly different concentrations of EF-Tu were used (ranging from 1.4 to 2.7 μM instead of 2.5 μM).

The fluorescence assay is essentially described by Ott et al. (1989). To circumvent problems caused by the high specific extinction coefficient of kirromycin at 335 nm and the relatively high K_d of the complexes, the procedure was altered slightly. A 300-μL cuvette with an optical path length of 3 mm was used in order to minimize quenching of excitatory light by kirromycin. The tRNA and EF-Tu was dissolved in exactly the same buffer, so fluorescence only had to be corrected for dilution: there was no change in the concentration of kirromycin during the titration. After addition of EF-Tu to the tRNA and thorough mixing of the sample, this was left for 3 min in the dark before the fluorophore was excited at 335 nm. After 5 s, the fluorescence stabilized and its value was read, after which the excitation light beam was interrupted immediately. Separately we checked that a new equilibrium is reached well within 3 min after addition of EF-Tu·kirromycin. We also performed blank runs in which Tyr-[AEDANS-s^{2}C]tRNA^{Tyr} was substituted by H_2O in order to correct for the intrinsic fluorescence of EF-Tu. The fluorescence of the kirromycin-containing buffer in the absence of tRNA increases by a factor 3.07 upon addition of Tyr-[AEDANS-s^{2}C]tRNA^{Tyr}.

Modification of EF-Tu with TPCK was performed as described by Van Noort et al. (1986), with a few changes in the procedure: 100 μL of EF-Tu (160 μM) was dialyzed against 50 mM Tris-HCl, pH 7.4/3.5 mM $MgCl_2$/75 mM KCl/10 μM PMSF/10 μM GDP. Thereafter, 10 μL of 25 mM TPCK dissolved in ethanol was added and the reaction mixture was incubated for 16 h at 0 °C and for 25 min at room temperature after which β-mercaptoethanol and glycerol were added to 10 mM and 10%, respectively. The total yield of protein after the procedure was 50% on the basis of GDP exchange activity.

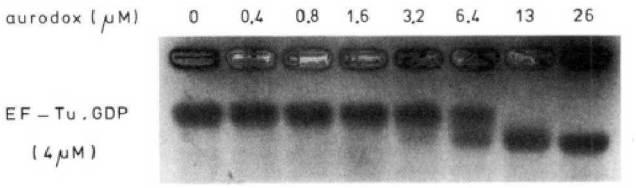

FIGURE 1: Horizontal 2% agarose gel electrophoresis of EF-Tu·GDP mixed with aurodox at the indicated concentrations. The electrophoresis buffer contained 20 mM Tris–acetate, pH 7.6, 3.5 mM magnesium acetate, and 10 μM GDP. The protein was stained with Coomassie Brilliant Blue.

Results

The Interaction between EF-Tu and Kirromycin. First we examined the influence of aurodox on the electrophoretic behavior of EF-Tu. Figure 1 shows the result of gel electrophoresis under nondenaturing conditions of EF-Tu·GDP preincubated with increasing amounts of aurodox. No aurodox was present in the gel or in the electrophoresis buffer. Note the drastic increase in electrophoretic mobility of the EF-Tu·aurodox·GDP complex. The difference in electrophoretic mobility between EF-Tu mutants differing one net charge (not shown) suggests that EF-Tu gains two net negative charges upon binding kirromycin. It seems unlikely that the extra charge of the complex is carried by kirromycin, in view of the chemical structure of the antibiotic. The increase is not due to a decreased Stokes' radius of the EF-Tu as judged by gel filtration on a TSK-125 HPLC column (not shown). Apparently, the charge of EF-Tu changes upon forming a complex with aurodox. Similar results were obtained with EF-Tu·GTP or when aurodox was substituted by kirromycin (not shown). From Figure 1 it is clear that all the EF-Tu in the preparation binds aurodox. Throughout all experiments we used saturating concentrations of aurodox as determined by electrophoresis.

The Interaction between EF-Tu·Kirromycin or EF-Tu·Aurodox and Aminoacyl-tRNA. We determined the dissociation constants of EF-Tu·aurodox·GTP·aa-tRNA and of EF-Tu·aurodox·GDP·aa-tRNA in three different ways.

Figure 2B shows the result of zone-interference gel electrophoresis of EF-Tu·aurodox·GDP with aa-tRNA. Using the graphical method described by Abrahams et al. (1988), we found a K_d with respect to tRNA binding of 11 μM for the EF-Tu·aurodox·GDP·aa-tRNA complex at 9 °C. In the case of EF-Tu·aurodox·GTP, two sets of bands show up in the gel (see Figure 2E), unless the slow-hydrolyzing GTP analogue [γ-S]GTPγS is used (not shown). In the latter case, only the lower set of bands remains. This indicates that the appearance of the upper set of bands is caused by the hydrolysis of complexed GTP during the preincubation. On the basis of Figure 2E, K_d values with respect to tRNA binding of 10 and 3 μM at 9 °C can be calculated for the complexes with GDP and GTP, respectively. In the absence of kirromycin, the electrophoretic mobility of the complex of EF-Tu·GTP·aa-tRNA relative to bromophenol blue is equal to that of the kirromycin-containing complex (Abrahams et al., 1991).

With a second and independent method, we determined the K_d for the EF-Tu·aurodox·GTP·aa-tRNA complex by making

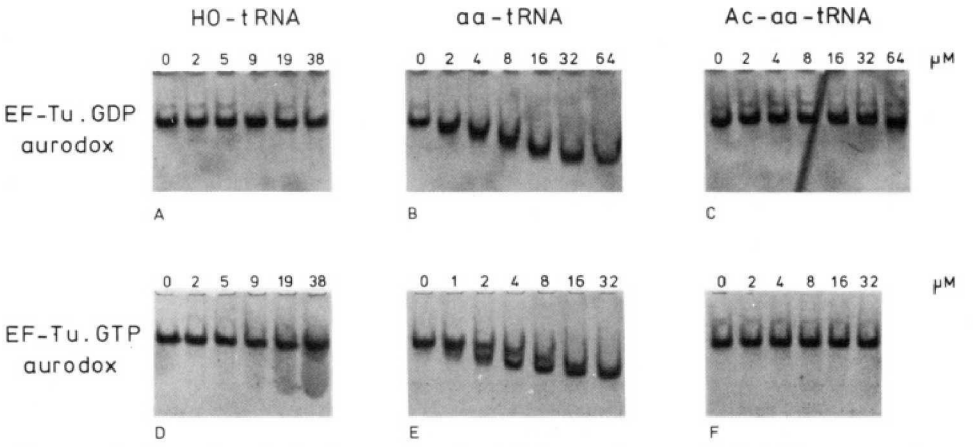

FIGURE 2: Zone-interference gel electrophoresis of mixtures of various EF-Tu complexes and bulk tRNA preparations at 9 °C. Samples of 4 μM EF-Tu·aurodox·GDP or 4 μM EF-Tu·aurodox·GTP were mixed with deacylated tRNA (A and D), aa-tRNA (B and E), or Nacaa-tRNA (C and F) in the presence of 20 μM aurodox, preincubated at 37 °C for 5 min, and subjected to electrophoresis in zones of the corresponding tRNA species. Protein bands were stained with Coomassie Brillant Blue. For further details, see Abrahams et al. (1988).

use of the fact that aa-tRNA stimulates the GTPase reaction of EF-Tu·kirromycin·GTP and EF-Tu·aurodox·GTP (Wolf et al., 1974). In a system with a large surplus of GTP and various concentrations of aa-tRNA, we measured the initial rates of GTP hydrolysis, which proved to be linear up to 10 min. We separately checked that the exchange of GDP with GTP was not rate-limiting (not shown), as was already reported (Fasano et al., 1977). On account of the latter finding, the high concentration and high molar excess of GTP over EF-Tu, and the measurements of initial rates, practically all the EF-Tu·aurodox is assumed to be in complex with GTP during the experiments. By consequence, eq 1 represents the contribution of EF-Tu·kirromycin·GTP on one hand and EF-Tu·kirromycin·GTP·aa-tRNA on the other to the overall GTPase activity.

$$k_{exp} = k_{EF\text{-}Tu}\frac{[EF\text{-}Tu]}{[EF\text{-}Tu_{tot}]} + k_{EF\text{-}Tu\cdot tRNA}\frac{[EF\text{-}Tu\cdot tRNA]}{[EF\text{-}Tu_{tot}]} \quad (1)$$

The parameters are defined as follows. k_{exp} is the experimentally determined apparent rate constant of GTP hydrolysis at a given aa-tRNA concentration. $k_{EF\text{-}Tu}$ is the experimentally determined rate constant of GTP hydrolysis of EF-Tu·aurodox·GTP in the absence of aa-tRNA. $k_{EF\text{-}Tu\cdot tRNA}$ is the rate constant of GTP hydrolysis of EF-Tu·aurodox·GTP·aa-tRNA. This value was determined by extrapolation of the titration data. [EF-Tu] is the concentration of EF-Tu·kirromycin·GTP. [EF-Tu·tRNA] is the concentration of EF-Tu·kirromycin·GTP·aa-tRNA. The following relations exist

$$[EF\text{-}Tu_{tot}] = [EF\text{-}Tu] + [EF\text{-}Tu\cdot tRNA] \quad (2)$$

$$[tRNA_{tot}] = [tRNA] + [EF\text{-}Tu\cdot tRNA] \quad (3)$$

Substitution of eq 1 with eqs 2 and 3 yields eqs 4, 5, and 6.

$$[EF\text{-}Tu\cdot tRNA] = [EF\text{-}Tu_{tot}]\frac{k_{exp} - k_{EF\text{-}Tu}}{k_{EF\text{-}Tu\cdot tRNA} - k_{EF\text{-}Tu}} \quad (4)$$

$$[EF\text{-}Tu] = [EF\text{-}Tu_{tot}]\frac{k_{EF\text{-}Tu\cdot tRNA} - k_{exp}}{k_{EF\text{-}Tu\cdot tRNA} - k_{EF\text{-}Tu}} \quad (5)$$

$$[tRNA] = [tRNA_{tot}] - [EF\text{-}Tu_{tot}]\frac{k_{exp} - k_{EF\text{-}Tu}}{k_{EF\text{-}Tu\cdot tRNA} - k_{EF\text{-}Tu}} \quad (6)$$

These equations, the right-hand sides of which now contain measurable quantities, can be substituted into the Scatchard equation (eq 7), where N is equal to the number of binding sites.

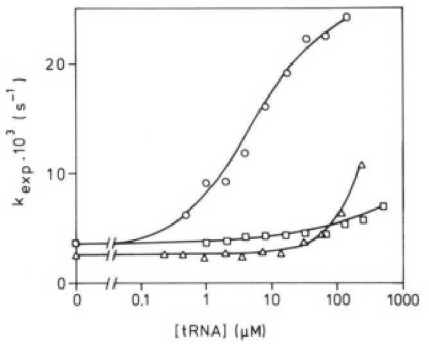

FIGURE 3: GTPase stimulation of EF-Tu·aurodox·GTP by aa-tRNA, HO-tRNA, or Nacaa-tRNA at 37 °C. For each plot, 1.35 μM EF-Tu·aurodox·GTP was mixed with aa-tRNA (○), HO-tRNA (□), or Nacaa-tRNA (△) at the indicated concentrations in a buffer containing 50 mM Tris-HCl, pH 7.4, 3.5 mM $MgCl_2$, 75 mM KCl, 0.12 mM aurodox, and 77 μM [^{3}H]GTP. Aliquots were taken after 10 min and analyzed for their GTP to GDP ratio on polyethyleneimine cellulose plates. The results were corrected for the appropriate backgrounds. Maximally 25% of the GTP was hydrolyzed. From the amount of hydrolyzed GTP, the apparent rate constants were calculated. In the case of aa-tRNA, the concentrations indicated in the graph were corrected for hydrolysis of the aminoacyl ester bond.

$$\frac{[EF\text{-}Tu\cdot tRNA]}{[tRNA][EF\text{-}Tu_{tot}]} = \frac{N - [EF\text{-}Tu\cdot tRNA]}{K_d[EF\text{-}Tu_{tot}]} \quad (7)$$

The total concentration of aa-tRNA present has to be also corrected for the inevitable hydrolysis of the aminoacyl ester bond occurring during the incubation. To this aim, we determined the half-life of the ester bond (which was 39 min), then calculated the definite integral of the degradation curve over the time of incubation and divided the resulting value by the time of incubation (10 min). This yields the average concentration of aa-tRNA during the incubation. From the data in Figure 3 we calculated a dissociation constant with respect to the binding of tRNA for the complex EF-Tu·aurodox·GTP·aa-tRNA at 37 °C of about 6 μM.

Finally, we determined the dissociation constant of the quaternary complex with the fluorescence titration method described by Ott et al. (1989). In contrast to most other experiments described in this paper, the complex contained kirromycin instead of aurodox, since the former has a lower extinction coefficient at 335 nm, the wavelength at which the

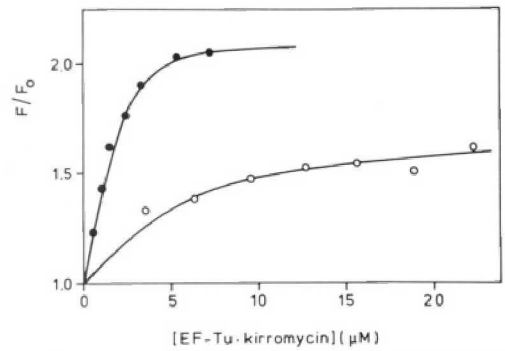

FIGURE 4: Fluorescence titration of Tyr-[AEDANS-s^{2}C]tRNA^{Tyr} with EF-Tu·kirromycin·GTP or EF-Tu·kirromycin·GDP. Tyr-[AEDANS-s^{2}C]tRNA^{Tyr} (100 pmol in a volume of initially 200 μL) was titrated with EF-Tu·kirromycin·GTP (●) or with EF-Tu·kirromycin·GDP (○) at 4 °C in a buffer containing 18 mM sodium borate, pH 7.0, 18 mM NH_4Cl, 3.5 mM $MgCl_2$, 2.3% ethanol, 110 μM kirromycin, 750 μM GDP or 5.5 mM GTP, 5.5 mM PEP, and 33 μg/mL PK. The fluorescence indicated in the graph was corrected for the intrinsic fluorescence of EF-Tu, the fluorescence of uncharged tRNA, and the changes in volume caused by the addition of EF-Tu.

Table I: K_d Values for the Complexes EF-Tu·Aurodox/Kirromycin·GTP/GDP·aa-tRNA As Obtained with Various Techniques

method	K_d of complex with GDP (μM)	K_d of complex with GTP (μM)
zone-interference gel electrophoresis	10 ± 2 (9 °C)	3 ± 1 (9 °C)
GTPase stimulation assay		6 ± 2 (37 °C)
fluorescence titration	7 ± 2 (4 °C)	2 ± 0.6 (4 °C)

fluorophore is excited. Tyr-[AEDANS-s^{2}C]tRNA^{Tyr} modified with the fluorescence label at C_{75} was titrated with increasing concentrations of EF-Tu, and the increase in fluorescence of the AEDANS group at 480 nm was measured after complex formation. The Tyr-[AEDANS-s^{2}C]tRNA^{Tyr} and the unmodified Tyr-tRNA^{Tyr} were shown to have practically the same association constant for EF-Tu·GTP (Ott et al., 1989). A practical problem we encountered was that kirromycin rapidly decays upon irradiation with UV light of 335 nm during the excitation of the fluorophore. At high concentrations of the antibiotic this became manifest by a slow increase in fluorescence at 480 nm upon prolonged irradiation. It was accompanied (and probably caused) by a minute opalescence in the cuvette. As a consequence, measurements were as short as possible and therefore error-prone. Nevertheless, the dissociation constants as calculated from the data in Figure 4, were in agreement with those obtained by zone-interference gel electrophoresis and the GTPase stimulation assay. Scatchard analysis yielded a K_d of 2 μM (±30%) in the case of EF-Tu·kirromycin·GTP·Tyr-[AEDANS-s^{2}C]tRNA^{Tyr} and a K_d of 7 μM (±30%) in the case of EF-Tu·kirromycin·GDP·Tyr-[AEDANS-s^{2}C]tRNA^{Tyr}, both at 4 °C and both with respect to tRNA binding. We interpret the difference in maximal fluorescence change as a difference in binding.

The combined results of the various measurements are summarized in Table I.

The Interaction between EF-Tu·Aurodox·GTP/GDP and HO-tRNA or Nacaa-tRNA. Interactions between EF-Tu·aurodox·GTP/GDP and HO-tRNA or Nacaa-tRNA were suggested by protection assays with modifying agents such as NEM and TPCK (Van Noort et al., 1982, 1986). With regard to the influence of Nacaa-tRNA on the stimulation of the reactivity of Cys-81 of EF-Tu·kirromycin·GTP, the above-mentioned papers are contradictory: according to the former one, an effect can already be seen at 2 μM of Nacaa-tRNA,

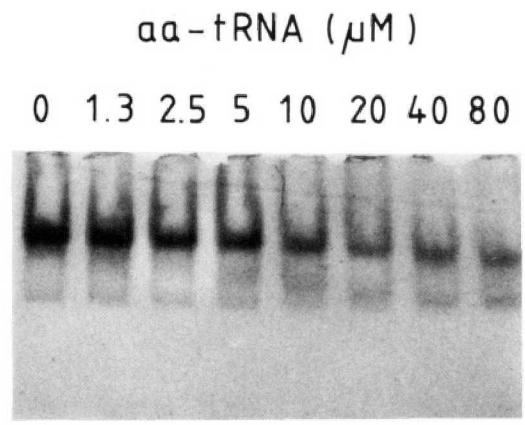

FIGURE 5: Zone-interference gel electrophoresis of TPCK-modified EF-Tu·aurodox·GTPγS with aa-tRNA at the concentrations indicated. The procedure was essentially the same as in Figure 2. Graphical calculation as described by Abrahams et al. (1988) yielded a K_d of 5 μM for the faint band of unmodified EF-Tu present in the gel, and a K_d of 50 μM for the darker band migrating more slowly.

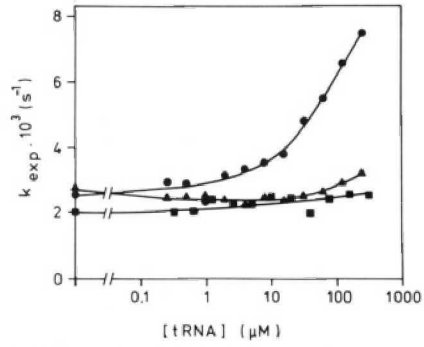

FIGURE 6: GTPase stimulation of TPCK-modified EF-Tu·aurodox·kirromycin by HO-tRNA, aa-tRNA, or Nacaa-tRNA at 37 °C. The procedure was essentially the same as in Figure 3.

while the latter suggests that at least 50 μM is needed. Zone-interference gel electrophoresis did not reveal an interaction between either EF-Tu·aurodox·GTP or EF-Tu·aurodox·GDP on the one hand and either Nacaa-tRNA or HO-tRNA on the other at tRNA concentrations up to 40 μM (Figure 2). Figure 3 illustrates that, with respect to Nacaa-tRNA, we could not reproduce the results of the GTPase experiments reported by Van Noort et al. (1986). We have no simple explanation for the discrepancy. Reexamination of the particular EF-Tu preparation used by Van Noort et al. (1986) revealed a contamination of about 10% with a protein of the size of EF-G.

The Interaction of EF-TuTPCK·Aurodox·GTP with HO-tRNA, Nacaa-tRNA, and aa-tRNA. EF-Tu was modified with TPCK at cysteine-81 as described in Materials and Methods. Exchange studies confirmed earlier reports that the protein remained active in binding the nucleotide (Jonák et al., 1973), while the mobility shift during native gel electrophoresis (cf. Figure 1) indicated that all the protein was capable of binding aurodox.

Complexes of EF-TuTPCK·kirromycin·GTPγS were incubated with various amounts of aa-tRNA and subjected to zone-interference gel electrophoresis. As can be seen in Figure 5, a trace of the protein shows an increase in electrophoretic mobility comparable to that of unmodified EF-Tu·kirromycin·GTP (compare Figure 2). Graphical determination of the dissociation constants according to Abrahams et al. (1988) shows that the bulk of the protein has a K_d of about 50 μM, while the small trace of protein has a K_d of 5 μM with respect to tRNA binding.

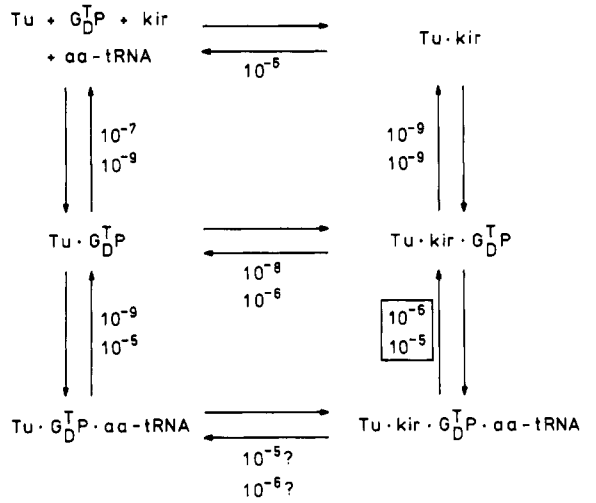

FIGURE 7: Diagram of the equilibria of complexes between EF-Tu, GTP or GDP, aa-tRNA and kirromycin/aurodox with the order of magnitude of the corresponding K_d values (M) indicated [see also Parmeggiani and Swart (1985) and Kraal et al. (1989)]. Of each pair of K_d values, the upper one refers to the EF-Tu complex with GTP and the lower one to the complex with GDP. More recent proposals for the K_d of EF-Tu·GTP·aa-tRNA (Abrahamson et al., 1985; Louie & Jurnak, 1985; Johnson et al., 1986; Ott et al., 1989) point to lower values (down to the subnanomolar range) than reports before 1985 do. The boxed values originate from the experiments described above. The question mark refers to calculated values without experimental confirmation.

GTPase experiments similar to the experiments described above yielded the results shown in Figure 6. They indicated a loss in affinity of EF-TuTPCK·aurodox·GTP for aa-tRNA, since higher concentrations of aa-tRNA were needed to stimulate the GTPase activity of EF-Tu (compare Figure 3). If EF-Tu had become partially inactivated, a smaller increase at lower aa-tRNA concentrations would have been expected. They also indicate a loss in activity of the catalytic center; EF-TuTPCK seems to have an approximately 2-fold decrease in k_{cat}. A more drastic loss in GTPase activity was already reported by Van Noort et al. (1986).

Discussion and Conclusions

It has been established that the dissociation constant of EF-Tu·GTP·aa-tRNA is in the (sub)nanomolar range (Abrahamsom et al., 1985; Louie & Jurnak, 1985; Johnson et al., 1986; Ott et al., 1989). With three independent methods, we have shown that the dissociation constant with respect to tRNA binding for the complex EF-Tu·kirromycin/aurodox·GTP·aa-tRNA is at least 3 orders of magnitude higher. This is a surprising outcome in view of the current opinion that kirromycin would only cause a 2-fold weakening of the binding of aa-tRNA to EF-Tu as earlier results, obtained with the technique of aminoacyl ester bond protection, suggested (Pingoud et al., 1978). Nevertheless, productive interactions between EF-Tu·kirromycin·GTP/GDP and aa-tRNA are still possible in view of the high concentrations of tRNA in the cell. A K_d value in the micromolar range could in retrospect already be deduced from results presented by Van Noort el al. (1986) and Parlato et al. (1981), which concern the effect of aa-tRNA on the GTPase of EF-Tu·kirromycin·GTP. When GTP in the complex is replaced by GDP, the affinity for aa-tRNA drops only by a factor of 3, while in the absence of the antibiotic, the affinity of EF-Tu·GDP for aa-tRNA is at least 3 orders of magnitude lower than that of EF-Tu·GTP (Johnson et al., 1986). Kirromycin seems to level the affinity of EF-Tu·GTP and EF-Tu·GDP for aa-tRNA to that of EF-Tu·GDP. The equilibrium diagram of Figure 7 gives a summary of these results. In a recent publication (Dell et al., 1990) the drop in affinity of EF-Tu·GTP for aa-tRNA induced by aurodox was claimed to be only about 6-fold. The reason for this discrepancy is unknown to us, but since in the experiments described in the latter paper only the behavioral change of the tRNA was monitored, it cannot be completely excluded that this change was brought about by traces of EF-Tu·GTP not in complex with aurodox, since the excess of EF-Tu over tRNA was large during the titrations reported. The dissociation constant reported by Dell et al. (1990) for EF-Tu·kirromycin·GDP in complex with Phe-tRNA^{Phe} (about 1.5 μM) is in good agreement with the value we report.

Upon complex formation with kirromycin or aurodox, the charge of EF-Tu at neutral pH changes dramatically: its electrophoretic mobility increases about 30% (Figure 1), while its size remains the same. Conformational changes of EF-Tu upon complex formation with kirromycin are consistent with findings reported by Balestrieri et al. (1989) and Douglass and Blumenthal (1979). The change in charge induced by kirromycin was also observed by ion-exchange chromatography (A. Parmeggiani and co-workers, personal communication). The experiment in Figure 1 also shows that the dissociation rate of the complex is very low, which is in contradiction with a half-life of 10 min as calculated by Eccleston (1981). This calculation used a K_d value for the complex of EF-Tu·GDP with kirromycin as determined by circular dichroism measurements at 340 nm (Pingoud et al., 1978). As pointed out in Results, kirromycin is quite sensitive to light of this wavelength, and this property of the antibiotic could account for the discrepancy between the calculated dissociation rate and the observed one. A low dissociation rate is also reflected by the experimental difficulties in completely removing the antibiotic from the protein by dialysis (Swart et al., 1987).

Earlier reports from our laboratory gave evidence for the existence of a second tRNA binding site on EF-Tu, becoming manifest upon complex formation free in solution of the protein with kirromycin (Van Noort et al., 1982, 1984, 1986). We were not able to confirm this model with zone-interference gel electrophoresis within the range of tRNA concentrations used: the plots constructed from Figure 2 are linear and indicate a 1:1 stoichiometry. This means that if a second tRNA would bind, the dissociation constant of tRNA in such a complex has to be higher that 80 μM. We cannot exclude the possibility, however, that EF-Tu complexes with one and with two tRNA molecules comigrate, the effect of the higher negative charge of the latter being compensated by an increased Stokes' radius.

Our present results indicate that the affinity of EF-Tu·kirromycin·GDP for aa-tRNA is at least 1–2 orders of magnitude higher than that for either HO-tRNA or Nacaa-tRNA. This is not incompatible with the results presented by Van Noort et al. (1982), which show that even in a reaction mixture containing 40 μM EF-Tu·kirromycin·GDP and 200 μM Nacaa-tRNA or 500 μM HO-tRNA a plateau in stimulation of the modification of EF-Tu with NEM or TPCK is still not reached. This means that much higher concentrations are necessary for saturation and consequently that the complexes involved are quite weak in comparison to the one containing aa-tRNA.

We could not confirm data presented by Van Noort et al. (1986), which pointed to comparable affinities of Nacaa-tRNA and aa-tRNA for EF-Tu·kirromycin·GTP as measured by GTPase assays and modification tests. We have no easy explanation for this, although the EF-Tu preparation used by Van Noort et al. (1986) appeared to be less homogeneous upon reexamination. The same is true for our experiments on the

affinity of aa-tRNA for TPCK-modified EF-Tu in the presence of aurodox as measured by the GTPase assay. Data presented by Van Noort et al. (1986) suggest that TPCK modification does not impair the usual ternary complex formation (Jonák et al., 1973) if kirromycin is present. Figures 5 and 6 here show that the affinity of EF-TuTPCK·aurodox·GTP for aa-tRNA is at least 1 order of magnitude lower than that of unmodified EF-Tu·aurodox·GTP. Figure 6 furthermore shows that the GTPase activity of the present EF-TuTPCK·aurodox preparation can still be enhanced by aa-tRNA and not by Nacaa-tRNA. If we had only the measured the residual GTPase of the small fraction of unmodified EF-Tu, a small increase with a half-maximum effect at about 6 μM aa-tRNA would be expected.

The most plausible explanation for the data presented in this paper is that EF-Tu·kirromycin binds only one tRNA at a site overlapping with the once existing in EF-Tu·GTP, taking into account that the experiments have been performed in the absence of ribosomes. On the other hand, cross-linking experiments in our laboratory showed that in the absence of kirromycin EF-Tu·GTP can make contact with aa-tRNA at the ribosomal A site as well as with peptidyl-tRNA at the P site (Van Noort et al., 1985).

A question that remains to be answered is why kirromycin prevents EF-Tu from leaving the ribosome. It has been proposed that, upon binding kirromycin and after GTP hydrolysis on the ribosome, EF-Tu remains stuck because its affinity for the aa-tRNA at the ribosomal A site is still high. The results presented in this paper do not support this hypothesis, although they are compatible with the view expressed by Chinali et al. (1977). This view states that kirromycin brings about a hybrid conformation of EF-Tu, with characteristics of both the GTP and the GDP conformations. The residual affinity of EF-Tu for the tRNA at the ribosomal A site together with the affinity for another site on the ribosome (for example the site that induces EF-Tu to hydrolyze its nucleotide cofactor) could result in an overall low dissociation rate of the complex. In fact, it might be the transient conformation EF-Tu adopts on the ribosome during GTP-hydrolysis, which is induced and stabilized by kirromycin.

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Registry No. GTP, 86-01-1; GDP, 146-91-8; GTPase, 9059-32-9; kirromycin, 50935-71-2; aurodox, 12704-90-4.

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