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The elongation factor Tu.kirromycin complex has two binding sites for tRNA molecules

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The interaction of the polypeptide chain elongation factor Tu (EF-Tu) with the antibiotic kirromycin and tRNA has been studied by measuring the extent of protein modification with N-tosyl-L-phenylalanine chloromethylketone (TPCK) and N-ethylmaleimide (NEM). Kirromycin protects both EF-Tu.GDP and EF-Tu.GTP against modification with TPCK. Binding of aminoacyl-tRNA added at increasing concentrations to a solution of 40 μM EF-Tu.GDP.kirromycin complex re-exposes the TPCK target site on the protein. However, when the aminoacyl-tRNA concentration is raised beyond 20 μM, TPCK labeling drops again and is blocked completely at ~300 μM aminoacyl-tRNA. By contrast, addition of uncharged tRNA or N-acetylaminoacyl-tRNA enhances TPCK labeling of the protein over the entire tRNA concentration range studied. These data strongly suggest that kirromycin induces in EF-Tu.GDP an additional tRNA binding site that can bind uncharged tRNA, aminoacyl-tRNA, and N-acetylaminoacyl-tRNA. Support for this assumption is provided by measuring the modification of EF-Tu.GDP with the sulfhydryl reagent NEM. Moreover, NEM modification also indicates an additional tRNA binding site on EF-Tu.GTP.kirromycin, which could not be detected with TPCK. Mapping of the tryptic peptides of EF-Tu.GDP labeled with [^{14}C]TPCK revealed only one target site for this agent, i.e., cysteine-81. Modification occurred at the same site in the presence and in the absence of kirromycin and uncharged tRNA. The ability of EF-Tu to bind two tRNA molecules simultaneously is discussed in relation to the mode of action of kirromycin and to previous findings that EF-Tu.GTP-dependent binding of aminoacyl-tRNA to the A-site can only occur when the P-site is occupied either by uncharged tRNA or by peptidyl-tRNA.

Key words: elongation factor Tu/kirromycin/protein biosynthesis/tRNA-EF-Tu interaction

Introduction

The polypeptide chain elongation factor Tu (EF-Tu) is a multifunctional and remarkably abundant bacterial protein. Apart from its key role in the elongation process during protein biosynthesis (reviewed by Miller and Weissbach, 1977; Kaziro, 1978), EF-Tu fulfils several other functions (Blumenthal and Carmichael, 1979; Travers, 1973; Hamel and Cashel, 1974; Beck *et al.*, 1978; Jacobson *et al.*, 1976). The protein has been shown to interact with numerous ligands and thereby undergoes distinct conformational transitions (Miller and Weissbach, 1977; Kaziro, 1978).

The antibiotic kirromycin blocks polypeptide chain elongation by a 1:1 complex formation with EF-Tu (Wolf *et al.*, 1974), which results in the immobilization of the ribosome.EF-Tu.kirromycin complex on the messenger, as EF-Tu has now become unable to dissociate from the ribosomal A-site. Furthermore, kirromycin induces in EF-Tu a GTPase activity uncoupled from the presence of aminoacyl-tRNA and ribosomes (Wolf *et al.*, 1974) and facilitates the exchange between GDP and GTP (Blumenthal *et al.*, 1977). In this sense, it mimics the effect of complex formation with the ribosome and with EF-Ts, respectively. Wolf *et al.* (1977) showed that in the presence of the antibiotic EF-Tu.GDP has also acquired an aminoacyl-tRNA binding capacity. It has been suggested that kirromycin renders the conformation of EF-Tu.GDP more EF-Tu.GTP-like and *vice versa* (Douglass and Blumenthal, 1979).

N-Tosyl-L-phenylalanine chloromethylketone (TPCK) is known to react either with histidine (Schoelmann and Shaw, 1963) or cysteine (Whitaker and Perez-Villaseñor, 1980) residues. Jonák *et al.* (1973) demonstrated that the TPCK reaction with EF-Tu results in modification of one unique site in the polypeptide chain, presumably Cys-81 (Arai *et al.*, 1980). Similar findings have been reported by Arai *et al.* (1974). This has made TPCK a useful reagent to assay the interactions of EF-Tu with tRNA and its analogues (Sedláček *et al.*, 1971; Jonák *et al.*, 1976, 1979, 1980). The same site of modification has been shown to be exposed to the reagent N-ethylmaleimide (NEM) on EF-Tu in the presence of Mg^{2+}-ions and a guanine nucleotide (Arai *et al.*, 1974, 1980). Both the reaction with TPCK and that with NEM destroy the ability of EF-Tu.GTP to bind aminoacyl-tRNA (Jonák *et al.*, 1973; Arai *et al.*, 1974; Richman and Bodley, 1972).

We have studied the effect of kirromycin on the interactions of EF-Tu.GDP and EF-Tu.GTP with uncharged tRNA, aminoacyl-tRNA, and N-acetylaminoacyl-tRNA by measuring the extent of modification of the protein with TPCK and NEM.

Results

Modification of EF-Tu.GDP and EF-Tu.GTP by TPCK and NEM; effects of the antibiotic kirromycin

Under standard conditions (cf. Materials and methods) EF-Tu.GDP reacts with TPCK to an extent that 15% of the protein molecules are modified. With NEM, modification occurs up to 20%. For EF-Tu.GTP these figures are 14% and 24%, respectively. These data are in good agreement with those reported previously (Jonák *et al.*, 1980; Arai *et al.*, 1974).

The antibiotic kirromycin protects EF-Tu.GDP and EF-Tu.GTP against modification by TPCK. Figure 1 shows the effect of increasing concentrations of kirromycin (open symbols). Complete inhibition of EF-Tu.GTP labeling with TPCK occurs at 90 μM of the antibiotic. A concentration of 180μM kirromycin is required for a complete block of EF-Tu.GDP labeling.

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No inhibition, but even stimulation, by the antibiotic is observed when the reaction of EF-Tu.GDP with the modifying agent NEM is studied (Figure 1, closed circles). The reaction with EF-Tu.GTP is unaffected (closed triangles). The differential response of EF-Tu.GDP and EF-Tu.GTP to kirromycin probably reflects a difference in conformation between the two EF-Tu.kirromycin complexes. Apparently, this difference is revealed more clearly by modification with NEM than with TPCK. The opposite effects of kirromycin upon EF-Tu.GDP labeling by TPCK and NEM as well as the differential effects of the antibiotic upon EF-Tu.GTP labeling may be ascribed to structural differences between the two modifying agents. Both NEM and TPCK react with one and the same site of the polypeptide chain, i.e., Cys-81, as will be shown below.

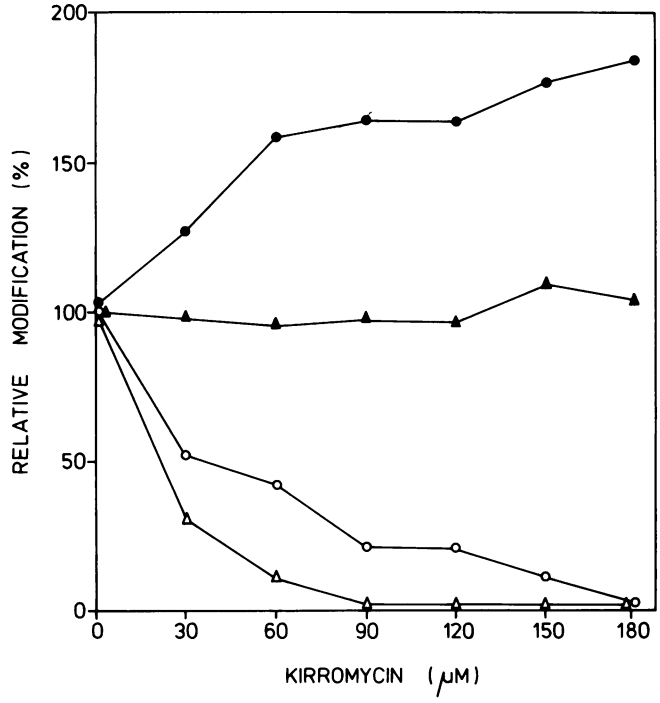

Fig. 1. The effect of kirromycin upon the modification of EF-Tu complexes by [^{14}C]TPCK (open symbols) or [^{3}H]NEM (closed symbols). Modifications were performed either with EF-Tu.GDP (circles) or with EF-Tu.GTP (triangles). For further details see Materials and methods.

Modification with TPCK reveals two tRNA binding sites on the EF-Tu.GDP.kirromycin complex

Kirromycin opens up an aminoacyl-tRNA binding site on EF-Tu.GDP as was reported previously (Wolf *et al.*, 1977). Figure 2A shows that the binding of aminoacyl-tRNA to this site can be monitored by modification of the EF-Tu protein with TPCK (closed triangles). In the absence of tRNA, the EF-Tu.GDP.kirromycin complex does not react with TPCK, in accordance with the results presented in Figure 1. Upon addition of increasing amounts of aminoacyl-tRNA, labeling by TPCK rises until the aminoacyl-tRNA reaches a concentration of ~20 μM. Surprisingly, the labeling drops again when the aminoacyl-tRNA level is raised further and is completely inhibited at a concentration of ~300 μM. This shielding of the EF-Tu.GDP complex against TPCK labeling is not observed with uncharged tRNA (closed circles) or N-acetyl-aminoacyl-tRNA (closed squares). Binding of the latter two species of tRNA to the EF-Tu.GDP.kirromycin complex enhances the labeling with TPCK over the entire concentration range of tRNA studied. These data strongly suggest that the antibiotic opens up not one but at least two binding sites for aminoacyl-tRNA. Binding to one site of the EF-Tu.GDP.kirromycin complex results in the exposure of the TPCK target, whereas binding to the second site blocks the reaction with the modifying agent. All three species of tRNA, uncharged, aminoacylated, and N-acetylaminoacylated-

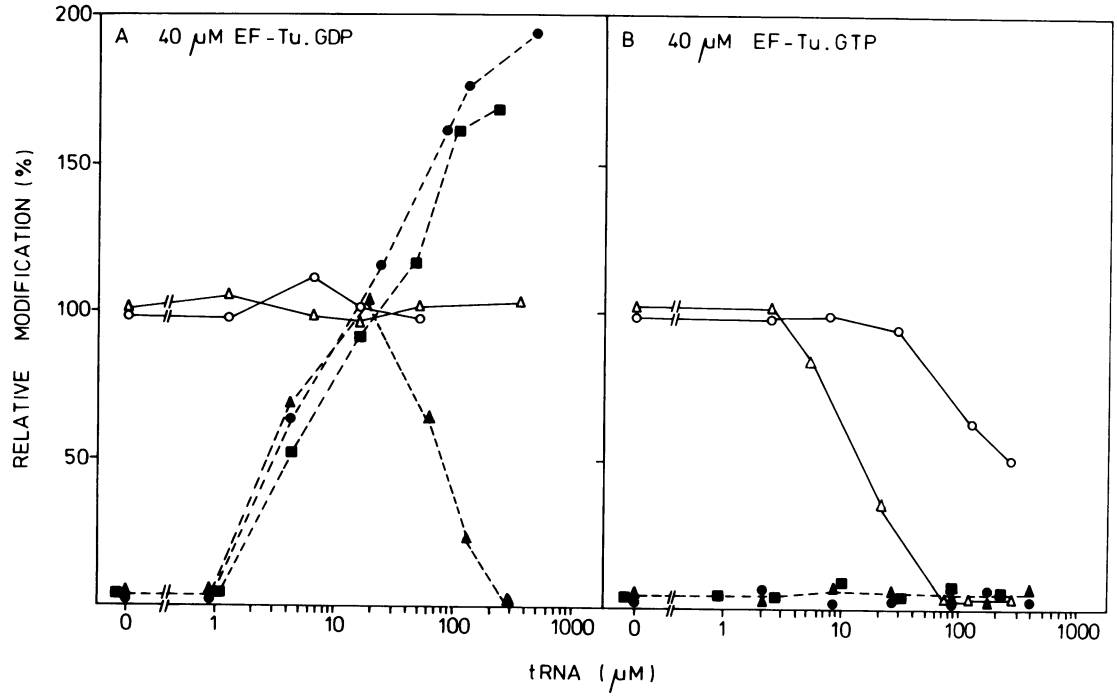

Fig. 2. The effects of uncharged tRNA (circles), aminoacyl-tRNA (triangles), or N-acetylaminoacyl-tRNA (squares) upon the modification of EF-Tu.GDP (**A**) or EF-Tu.GTP (**B**) by [^{14}C]TPCK. The modification was performed either in the absence (open symbols) or presence (closed symbols) of 180 μM kirromycin. For further details see Materials and methods.

tRNA, are able to fill the former site, whereas the data of Figure 2 reveal an interaction only for aminoacyl-tRNA with the latter. The binding of uncharged or N-acetylaminoacyl-tRNA to the latter site, though not detected by this assay, will be discussed below.

Control experiments performed in the absence of kirromycin show that neither uncharged (open circles) nor aminoacylated tRNA (open triangles) affect the labeling of EF-Tu.GDP with TPCK in the concentration range studied. Apparently, it is the antibiotic effector which opens up the two tRNA binding sites on EF-Tu.GDP. At tRNA concentrations up to 20 μM the site which can accept all three species of tRNA is preferentially filled. We propose to designate this site: site II. At aminoacyl-tRNA concentrations exceeding 20 μM the other site, designated site I, starts to be filled. Binding to site I results in inhibition of the TPCK modification. this is probably due to shielding of the TPCK target either by a conformational change of the protein or by a direct steric effect of aminoacyl-tRNA. Rejection of tRNA primarily bound to site II can be excluded since the modification curves for EF-Tu.GDP.kirromycin indicate a higher affinity of aminoacyl-tRNA for site II than for site I.

EF-Tu.GTP like EF-Tu.GDP undergoes a conformational change upon binding kirromycin (Blumenthal *et al.*, 1977; Fasano *et al.*, 1978). This is reflected in the shielding of the TPCK target (Figure 1). The EF-Tu.GTP.kirromycin complex, however, does not respond to the addition of either uncharged, aminoacylated, or N-acetylaminoacylated-tRNA with a re-exposure of this site (Figure 2B, closed circles, triangles, and squares, respectively). The occurrence of an additional binding site of EF-Tu.GTP.kirromycin thus cannot be established using the TPCK labeling technique. Modification with NEM, however, does reveal such an additional site as will be shown below.

The TPCK modification technique does not fail to reveal the classical binding site for aminoacyl-tRNA on EF-Tu.GTP in the absence of an antibiotic effector (see also Jonák *et al.*, 1980). This is illustrated in Figure 2B by the curve with the open triangles. Interaction of EF-Tu.GTP with uncharged tRNA, though less efficient than interaction with aminoacyl-tRNA, is also revealed by this technique.

Modification with NEM establishes the occurrence of two tRNA binding sites on complexes of kirromycin with both EF-Tu.GDP and EF-Tu.GTP

Labeling of EF-Tu.GDP with the modifying agent NEM is stimulated ~2-fold by addition of kirromycin (cf. Figure 1). Figure 3A shows that increasing amounts of uncharged, aminoacylated, and N-acetylaminoacylated-tRNA further enhance this type of labeling. The conclusion drawn from the studies with TPCK concerning two tRNA binding sites on the EF-Tu.GDP.kirromycin complex is confirmed by the NEM labeling: at concentrations up to ~20 μM aminoacyl-tRNA, modification of the NEM target on the protein is enhanced whereas shielding of this target occurs beyond this aminoacyl-tRNA concentration. The shielding dominates the exposure of the NEM target so that at concentrations of ~350 μM aminoacyl-tRNA the target site is completely blocked. It is possible that TPCK and NEM interact with a target which is located within or in close proximity to the tRNA binding site I. Alternatively, shielding of this target may be the result of a conformational change of the polypeptide chain.

An important aspect of the NEM labeling is the demonstration that the antibiotic kirromycin also discloses an additional tRNA binding site on EF-Tu.GTP. As is illustrated in Figure 3B, both enhancement and inhibition of the NEM labeling of the EF-Tu.GTP.kirromycin complex is observed upon addition of aminoacyl-tRNA (closed triangles). Three stages in the labeling of this complex can be discerned depending on the concentration of aminoacyl-tRNA. At a concen-

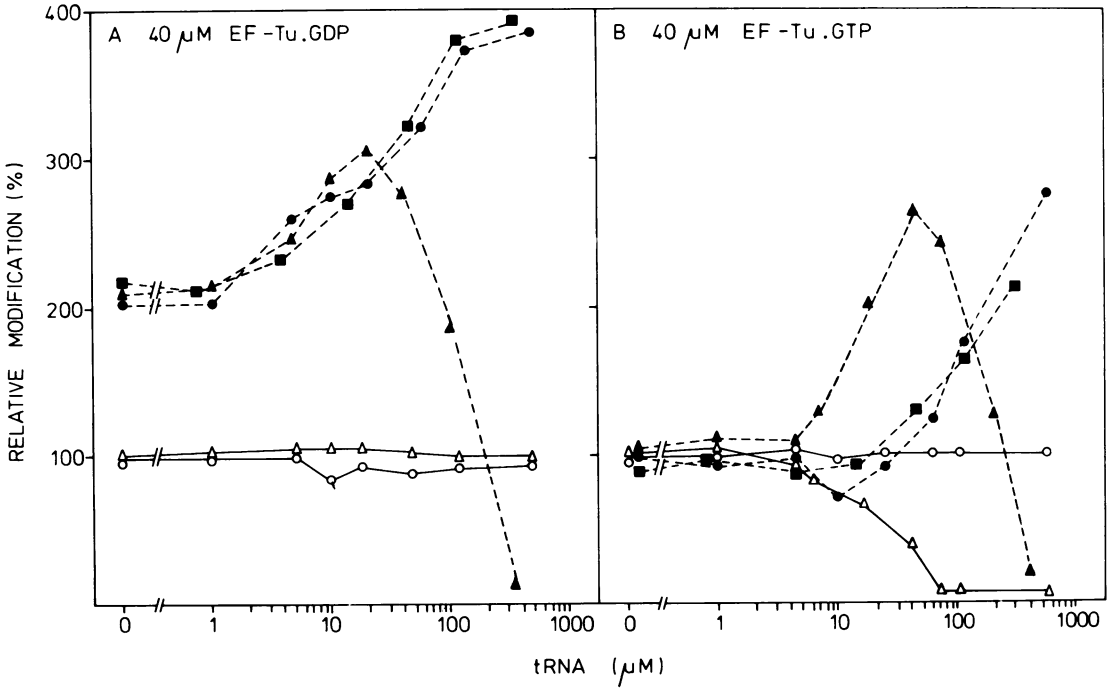

Fig. 3. The effects of uncharged tRNA (circles), aminoacyl-tRNA (triangles), or N-acetylaminoacyl-tRNA (squares) upon the modification of EF-Tu.GDP (**A**) or EF-Tu.GTP (**B**) by [^{3}H]NEM. The modification was performed either in the absence (open symbols) or presence (closed symbols) of 180 μM kirromycin. For further details see Materials and methods.

tration up to ~10 μM, addition of aminoacyl-tRNA does not detectably affect the labeling. Stimulation of the labeling occurs in the presence of 10–50 μM aminoacyl-tRNA, whereas an inhibition is observed with aminoacyl-tRNA concentrations >50 μM. Although different in the details of aminoacyl-tRNA dependence, NEM labeling of the EF-Tu.GTP.kirromycin complex is clearly reminiscent of the NEM labeling of the EF-Tu.GDP.kirromycin complex (Figure 3A) and strongly suggests the occurrence of two tRNA binding sites. The control experiments performed in the absence of kirromycin (open symbols) show that aminoacyl-tRNA addition only provides protection against NEM labeling but does not stimulate the labeling. This indicates that site II on EF-Tu.GTP is only accessible for tRNA binding when the protein has bound kirromycin. The control experiment with uncharged tRNA (open circles) shows that NEM labeling cannot be used to reveal interactions between EF-Tu.GTP and this type of tRNA.

Site II on the EF-Tu.GTP.kirromycin complex is more readily filled with aminoacyl-tRNA than with uncharged or N-acetylaminoacylated tRNA (cf. Figure 3B). Binding of the latter two tRNAs can only be monitored by NEM labeling at tRNA concentrations >50 μM (closed circles and squares), whereas binding of aminoacyl-tRNA to this site becomes apparent at concentrations exceeding ~10 μM. It can be concluded that the mode of tRNA charging affects the affinity of the tRNA for site II on the EF-Tu.GTP.kirromycin complex and furthermore, that this site differs from site II on the EF-Tu.GDP.kirromycin complex in its affinity for aminoacyl-tRNA (compare the curves with closed triangles of Figures 3A and 3B).

The data described so far have shown that site II on the EF-Tu.GDP.kirromycin complex does not discriminate between uncharged, aminoacylated, and N-acetylaminoacylated tRNA in contrast to site I which displays a strong preference for aminoacyl-tRNA. The question of the specificity of binding to site II therefore arises. Experiments with the synthetic polynucleotide poly(AGUC) did not reveal any effect upon TPCK or NEM modification of EF-Tu.GDP both in the presence and in the absence of 180 μM kirromycin (results not shown).

Modification of EF-Tu.GDP with TPCK occurs at the same site as with NEM, cysteine-81

The large variation in TPCK labeling observed under various conditions (cf. Figures 1–3) raised the question whether binding of the modifying agent always occurred to one and the same target site on the EF-Tu polypeptide chain.

EF-Tu.GDP was therefore labeled under standard conditions with [^{14}C]TPCK in the presence of 180 μM kirromycin and 200 μM uncharged tRNA. In a control experiment the antibiotic and tRNA were omitted from the reaction mixture. After labeling, the reaction was prolonged with excess of non-radioactive TPCK. The protein was submitted to tryptic peptide mapping as described by Duisterwinkel *et al.* (1981) and the ^{14}C-labeled peptides were detected by autoradiography, eluted, hydrolyzed, and their amino acid compositions were analyzed. Figure 4 shows a tryptic peptide map of EF-Tu.GDP labeled in the presence of kirromycin and tRNA. It is clear that predominantly only one tryptic peptide has become labeled with [^{14}C]TPCK. Identical results were obtained in the absence of kirromycin and uncharged tRNA. Amino acid analysis of the labeled peptide revealed the following composition (residues/molecule; theoretical values between brackets): Asp 1.7 (2), Pro 1.1 (1), Ala 2.2 (2), Cys 0.6 (1), Val 2.1 (2), Tyr 1.9 (2), His 1.5 (3), Lys 0.7 (1). The yield of cysteine (determined as cystine) is always very low in amino acid analysis, even without TPCK modification. The low yield of histidine can be explained by the action of extensive fluorescamine staining on the N-terminal His-75. Although this result does not allow for an exact localization of the TPCK target site, it demonstrates that this site is positioned within the tryptic peptide with residue numbers 75–89.

In a subsequent experiment, EF-Tu.GDP was labeled with [^{14}C]TPCK in the absence of kirromycin and tRNA. One portion of the labeled protein was submitted to thermolytic peptide mapping; the ^{14}C-labeled peptide was identified as the one with residue numbers 79–86 (results not shown). The other portion of the labeled protein was combined with an amount of EF-Tu.GDP modified with [^{3}H]NEM, similarly digested with thermolysin and passed over a Sephadex G-25 column. In the eluate only one major radioactive peak with both isotopes was found as shown in Figure 5A. The fractions containing the radioactivity were lyophilized and their peptides submitted to the procedure of manual Edman degradation (Tomita *et al.*, 1978). The combined butylchloride extracts of the thiazolinone-derivative of the amino-terminal residues were assayed for their radioactivity after each successive step. The result, as presented in Figure 5B, clearly indicates that both [^{14}C]TPCK and [^{3}H]NEM modify the third residue of the thermolytic peptide, which is cysteine-81. The conclusion that it is the only site of modification is justified by the finding that the recoveries of ^{3}H and ^{14}C in the third step were identical, as is to be expected when the two

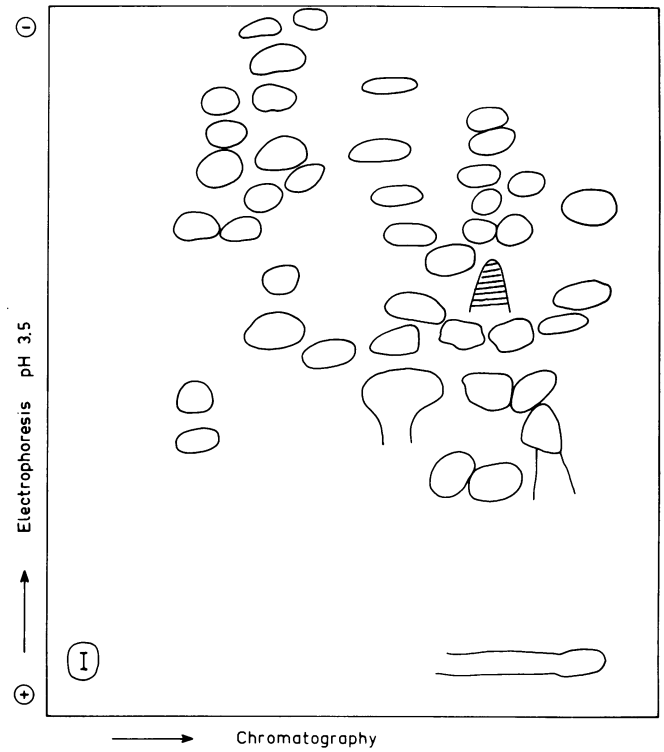

Fig. 4. Fingerprint analysis of a tryptic digest of EF-Tu.GDP modified by [^{14}C]TPCK in the presence of 180 μM kirromycin and 200 μM uncharged tRNA. The peptides were visualized by fluorescamine staining and subjected to autoradiography. The position of the ^{14}C-labeled peptide is indicated by the hatched area. See text for further details.

labels are attached to the same amino acid residue.

The degradation was performed up to the sixth step, which releases the thiazolinone-derivative of histidine-84. This residue might have also been the target of TPCK but the above-mentioned identical recoveries of ^{3}H and ^{14}C in the third step as well as the absence of any radioactivity in the butylchloride fraction of the sixth step demonstrate that histidine-84 is not modified by TPCK. That the latter residue is also not modified by TPCK in the presence of kirromycin and tRNA, can be concluded from the identical positions of the labeled peptide on the tryptic peptide map after labeling either in the presence or absence of kirromycin and tRNA. A modification of histidine would have resulted in the loss of one positive charge in contrast to that of cysteine and would have affected the electrophoretic mobility of the labeled peptide at pH 3.5.

Discussion

The results of modification of EF-Tu with TPCK and NEM can be interpreted assuming a second tRNA binding site (site II) in addition to the classic one (site I) as depicted in the model of Figure 6. The conformational differences between EF-Tu.GDP and EF-Tu.GTP are symbolized in Figure 6, those induced by kirromycin are only indicated by the opening up of the tRNA binding sites I and II. EF-Tu.GDP has no detectable affinity for tRNA whereas EF-Tu.GTP can bind aminoacyl-tRNA and uncharged tRNA, though the latter 15-fold more weakly than the former, at site I.

The major result of the present investigation is the observation that interaction of the antibiotic with the protein induces an additional tRNA binding site II, both in EF-Tu.GDP and EF-Tu.GTP. In the former case, site II displays approximately equal affinities towards uncharged, N-acetylaminoacyl-, and aminoacyl-tRNA. Figures 2A and 3A show that site II on EF-Tu.GDP.kirromycin is filled preferentially at site I. It is not yet clear whether this is due to an intrinsically lower affinity of site I for aminoacyl-tRNA or to a cooperative effect induced by prior tRNA binding at site II. Site I on EF-Tu.GDP.kirromycin can bind aminoacyl-tRNA but binding of uncharged tRNA cannot be excluded as the labeling technique here employed might fail to detect such a binding. This would be the case if it is the aminoacyl moiety that is mainly responsible for the shielding of the TPCK and NEM target sites on EF-Tu. The possibility that acylation of aminoacyl-tRNA would again abolish the shielding by the latter seems rather remote. Most likely N-acetylaminoacyl-tRNA does not interact with site I.

In the case of EF-Tu.GTP.kirromycin, aminoacyl-tRNA was found to bind to site II at concentrations that are 10- to 15-fold lower than required for binding of uncharged and N-acetylaminoacyl-tRNA to this site. Again, site II is filled in preference to site I (see Figure 3B). A comparison of Figures 3A and 3B shows that occupation of site II on EF-Tu.GTP.kirromycin requires ~2-fold higher concentrations of aminoacyl-tRNA than occupation of site II on EF-Tu.GDP.kirromycin. Furthermore, the interaction of uncharged or N-acetylaminoacyl-tRNA with site II on EF-Tu.GTP.kirromycin occurs at 20- to 30-fold higher concentrations than with site II on EF-Tu.GDP.kirromycin.

Although the data do not permit a quantitative evaluation of dissociation constants, Figure 6 gives a qualitative impression of the various affinities between the interaction partners. Alternative models may also be considered. Here we will discuss and reject several possibilities.

Under appropriate conditions tRNA molecules with complementary anticodons can interact in solution. This interaction itself is not affected by aminoacylation of EF-Tu complexation (Yamane *et al.*, 1981). The possibility that such anticodon-anticodon interactions are responsible for the simultaneous binding of two tRNA molecules to an EF-Tu.kirromycin complex can therefore be excluded on account of the drastic differences between the effects of uncharged or

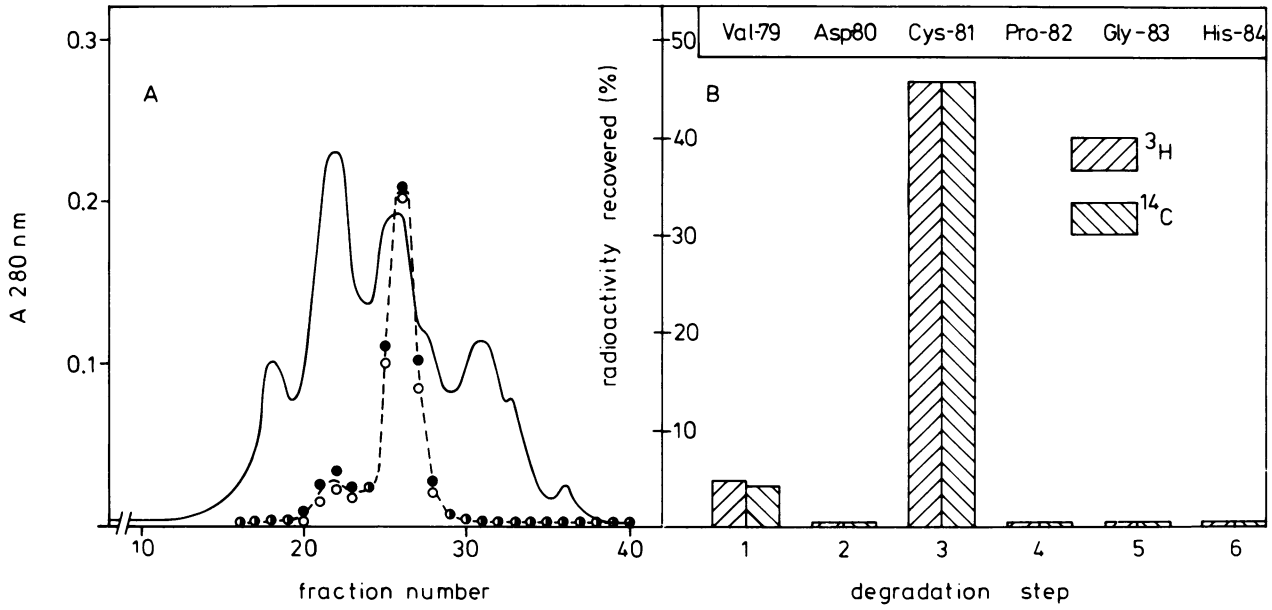

Fig. 5. (**A**) Separation on a Sephadex G-25 column (1 x 191 cm) of a thermolytic digest of a mixture of EF-Tu.GDP labeled by [^{14}C]TPCK and EF-Tu.GDP labeled by [^{3}H]NEM. Fractions of 6.0 ml were collected and their A_{280} (———) as well as their ^{14}C (○---○) and ^{3}H (●---●) contents were determined. The fractions 25–27 were pooled and submitted to manual Edman degradation. (**B**) A plot of the ^{14}C and ^{3}H contents of thiazolinone-containing butylchloride fractions obtained during the manual Edman degradation. The amino acid residues concerned are indicated for the corresponding fractions. From the yields of ^{14}C and ^{3}H in the third step an average repetitive yield of ~80% was estimated for the degradation procedure.

N-acetylaminoacyl-tRNA on one hand and aminoacyl-tRNA on the other.

It has been reported that kirromycin enhances the tendency of EF-Tu to form multimers (Gulewicz *et al.*, 1981). This might block their reactivity towards TPCK and one could think that increasing concentrations of tRNA would gradually dissociate these multimers, half of the tRNA-binding sites being protected against TPCK and the other half becoming exposed. This supposition does not explain, however, why aminoacyl-tRNA and uncharged tRNA in concentrations up to 20 μM affect TPCK modification of EF-Tu.GDP.kirromycin in an identical fashion whereas their effects become completely opposite at tRNA concentrations beyond 20 μM. EF-Tu.GDP behaves as a monomer in the absence of kirromycin. Therefore, it would not be expected that the extent of labeling in the presence of kirromycin and tRNA would exceed that of the monomer. The latter observation also excludes the possibility that tRNA binding displaces kirromycin and relieves its inhibition of TPCK and NEM labeling.

It is also possible that tRNA possesses two sites of interaction with EF-Tu.kirromycin, one of which is induced only upon interaction of the protein with the other. Interaction of the protein with the latter site would stimulate modification of cysteine-81 whereas that with the former would shield this residue. Such a model is inconsistent, however, with the dependence upon tRNA concentration of the biphasic labeling of EF-Tu.kirromycin.

The interpretation of our results as presented in Figure 6 may well have a bearing on the mode of action of kirromycin. The kirromycin-induced ability of EF-Tu.GTP to bind two tRNA molecules simultaneously, one of which bears an aminoacyl and the other a peptidyl moiety, can account for the formation of a stable complex of EF-Tu with tRNAs at the ribosomal A- and P-sites. The induction of GTP hydrolysis will even enhance the stability of this complex, because site II on EF-Tu.GDP.kirromycin has a higher affinity for peptidyl-tRNA than site II on EF-Tu.GTP.kirromycin. Such an anchoring of EF-Tu to aminoacyl- and peptidyl-tRNA may explain the tenacity with which EF-Tu.GDP.kirromycin binds to the ribosome, thus immobilizing the latter on the messenger during protein biosynthesis.

In the Introduction it has been pointed out that kirromycin in a way mimics the ribosome in its action on EF-Tu.GTP. Both the antibiotic and the ribosome can trigger the intrinsic GTPase activity of EF-Tu. It is possible, therefore, that association of the ternary complex EF-Tu.GTP.aminoacyl-tRNA with the ribosome-mRNA complex opens up site II, enabling EF-Tu to interact with aminoacyl-tRNA in the A-site and peptidyl-tRNA in the P-site. This would account for previous observations (Lürhmann *et al.*, 1979; De Groot *et al.*, 1971) that the EF-Tu.GTP-dependent binding of aminoacyl-tRNA to the A-site can only occur when the P-site is occupied either by uncharged tRNA or by peptidyl-tRNA. It could also explain the finding that GTP hydrolysis on EF-

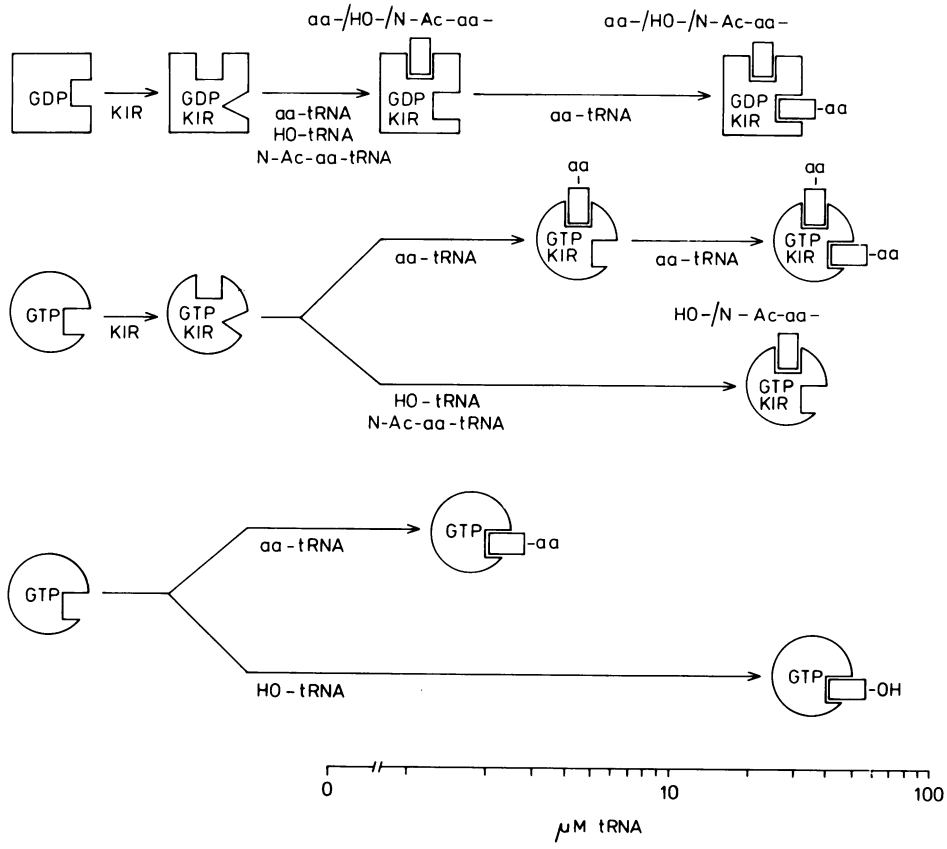

Fig. 6. A tentative model of complex formation between EF-Tu and tRNA in the presence or absence of kirromycin. The protein molecule is represented by a circle (EF-Tu.GDP) or a square (EF-Tu.GTP). The indentations in the outlines represent the tRNA binding sites on the elongation factor; the triangular indentations indicate that we do not know whether or not the affinity of aminoacyl-tRNA for site I of the protein.kirromycin complex increases upon occupation of site II by tRNA (see also text). The presence of a tRNA binding site on EF-Tu.GDP is suggested by previous findings (Jonák *et al.*, 1980). All tRNA molecules are represented by rectangles. At the bottom of the figure a logarithmic tRNA concentration scale is drawn. The positions of the complexes relative to this scale indicate the tRNA concentrations at which the interactions were detected in Figures 2 and 3 between aminoacyl-tRNA (aa-tRNA), uncharged tRNA (HO-tRNA) or N-acetylaminoacyl-tRNA (N-Ac-aa-tRNA), and 40 μM EF-Tu.

Tu.ribosome complexes in the presence of 2′(3′)-O-L-phenylalanyladenosine is stimulated by N-acetylphenylalanyl-tRNA prebound to the ribosome (Campuzano and Modolell, 1981).

Materials and methods

Materials

GDP, GTP, phosphoenol pyruvate, pyruvate kinase (EC 2.7.1.40), and tRNA from *Escherichia coli* MRE 600 were products of Boehringer Mannheim. [^{3}H]NEM (22 mCi/mMol) was purchased from New England Nuclear (Boston, MA). Kirromycin was a gift of Gist Brocades N.V. (Delft, The Netherlands) while [^{14}C]TPCK (4.2 mCi/mmol) was prepared as described previously (Jonák *et al.*, 1979).

Electrophoretically pure EF-Tu from *E. coli* MRE 600 was prepared as described by Leberman *et al.* (1980) and stored in 20 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 150 mM KCl, 10 mM 2-mercaptoethanol, 100 μM phenylmethylsulphonylfluoride (PMSF), 10 μM GDP, and 10% (v/v) glycerol at −80°C. The preparation had a GDP binding capacity of 19 nmol per mg of protein.

Aminoacylation of tRNA

A mixture of tRNAs was aminoacylated with a mixture of all amino acids, except methionine and cysteine (Verhoef *et al.*, 1968). The amount of covalently linked amino acids was determined on a Chromaspek amino acid analyzer (Rank Hilger) after hydrolysis of the aminoacyl ester bond at pH 9.0. A representative aminoacyl-tRNA preparation contained 63.75 OD_{260} units (~102 nmol tRNA) per 100 nmol of amino acids in the following amounts (nmol): Asp 7.3; Thr, Asn, and Gln together 10.8; Ser 5.5; Glu 4.5; Pro 2.3; Gly 8.1; Ala 4.8; Val 13.7; Ile 6.9; Leu 17.7; Tyr 3.6; Phe 3.7; His 2.8; Trp 0; Lys 3.4, and Arg 4.5.

Modification with TPCK or NEM

EF-Tu.GDP was dialyzed against 70 mM Tris-HCl pH 7.6, 12 mM $MgCl_2$, 260 mM KCl, 100 μM PMSF, and 10 μM GDP. Exchange of bound GDP by GTP was performed by incubation with 6 mM GTP, 6 mM phosphoenolpyruvate, and 50 μg/ml pyruvate kinase at 37°C for 15 min. The modification was performed in the presence of 65 μM TPCK or NEM in a final reaction mixture containing 40 μM EF-Tu, 20 mM Tris-HCl pH 7.6, 3.5 mM $MgCl_2$, and 75 mM KCl. The concentration of methanol, added as the solvent of NEM, TPCK, and kirromycin, was limited to a value of 10% (v/v). After 30 min at 0°C the reaction was terminated by the addition of 25 μl of 20 mM 2-mercaptoethanol, followed by 10 μl of a bovine serum albumin solution (10 mg/ml) and 3 ml of 7% (w/v) trichloroacetic acid. The mixtures were filtered and the precipitates counted in a liquid scintillation counter (for further details, see Jonák *et al.*, 1980). A control experiment showed that the modification reactions only took place with native EF-Tu: upon heat denaturation no radioactivity was incorporated under these conditions.

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