Isolation, crystallization and X-ray analysis of the quaternary complex of Phe-tRNA^{Phe}, EF-Tu, a GTP analog and kirromycin

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Abstract Kirromycin inhibits bacterial protein synthesis by acting on elongation factor Tu (EF-Tu). Complexes of the antibiotic, Phe-tRNA^{Phe}, the guanosine triphosphate analog GDPNP, and mesophilic (*Escherichia coli*), as well as thermophilic (*Thermus thermophilus*) EF-Tu were isolated. Crystallization was achieved at 4°C, pH 6.4, using ammonium sulphate as precipitant. Crystallographic data were recorded at cryogenic temperature on crystals exposed to synchrotron radiation. Crystals of the thermophilic complex are based on a rhombohedral lattice with cell dimensions of 137.3 Å, and angles of 54.0°. Although related, these cell parameters are different from those found in the crystals of the recently solved structure of the ternary complex of Phe-tRNA^{Phe}, GDPNP, and *Thermus aquaticus* EF-Tu (Nissen, P., Kjeldgaard, M., Thirup, S., Polekhina, G., Reshetnikova, L., Clark, B.F. and Nyborg, J. (1995) Science 270, 1464–1472 [1]), possibly indicating some allosteric effect caused by kirromycin. Crystals of the mesophilic complex belong to the cubic space P432, with cell axis of 196.26 Å. In both cases, the crystals contain one complex per asymmetric unit.

Key words: Protein synthesis; Elongation factor Tu; Kirromycin; Aminoacylated tRNA; Quaternary complex; Crystallization

1. Introduction

The ribosomal particle provides a large macromolecular structure, which serves as a scaffold for protein biosynthesis. The prokaryotic 70S ribosome is formed after assembly of the two constituting subunits, characterised as 50S and 30S in terms of sedimentation coefficients. For a given protein to be synthesised, genetic instructions are carried to the ribosome by means of messenger RNA (mRNA). Initially, the mRNA associates with the 30S subunit and an initiator transfer RNA (tRNA) molecule which binds to the appropriate start codon, in a catalytic process that is dependent on cytosolic protein factors. The 50S subunit joins the ensemble to form a functional particle, leaving the 70S ribosome in the state of an initiation complex. This step is the first of the three phases in protein synthesis: initiation, elongation, and termination. When a stop codon is exposed on the operating ribosome, release factors enter the scene and termination is effected.

Catalytically, the ribosome itself is central to the process of peptide chain elongation, but three additional protein factors are crucial to promote the reaction: elongation factor Tu (EF-Tu), nucleotide exchange factor Ts (EF-Ts), and elongation factor G (EF-G) [2–5]. A ternary complex of EF-Tu, aminoacyl-tRNA (aa-tRNA) and guanosine triphosphate (GTP) facilitate the recognition of an exposed codon on the mRNA, placing the aa-tRNA at the ribosomal A-site. The binary complex of guanosine diphosphate (GDP) and EF-Tu produced by ribosome induced hydrolysis, is then expelled from the A-site. Exchange of GDP for GTP to regenerate active EF-Tu:GTP, which can interact with another aa-tRNA, is mediated by EF-Ts. Peptide transfer from a peptidyl-tRNA in the ribosomal P-site to the aa-tRNA at the A-site is catalysed by a peptidyltransferase center on the ribosome. Translocation of deacylated P-site tRNA to the exit site (E-site) and A-site peptidyl-tRNA to the P-site, is catalysed by EF-G and cause the next codon on the mRNA to be exposed at the A-site for yet another round of processive protein synthesis [6].

Prokaryotic protein biosynthesis is the target for a wide range of antibiotics, and many are natural products [4,7]. Kirromycin is one in a family of antibiotics acting specifically on elongation factor Tu, binding in a stoichiometric one-to-one ratio. Nearly, all of the characteristics displayed by EF-Tu in peptide chain elongation are affected by the presence of kirromycin [8]. Briefly, formation of a EF-Tu:EF-Ts complex from EF-Tu:GDP and EF-Ts is prevented, EF-Tu:GDP/GTP equilibria are altered, EF-Tu mediated transfer of aa-tRNA to the A-site can take place in the absence of GTP, and the dependence on ribosomes and aa-tRNA for expression of EF-Tu GTPase activity is released [9].

Biochemical and biophysical studies have indicated some degree of structural alteration caused by the presence of kirromycin in general, EF-Tu:GDP showing the most profound change adapting what has been described as an EF-Tu:GTP-like conformation [9–12]. EF-Tu, GTP, and aa-tRNA can still interact sequentially in the presence of kirromycin, thus forming a quaternary complex EF-Tu:GTP:aa-tRNA:kirromycin (QC). Although GTP hydrolysis occur readily at the ribosomal A-site, EF-Tu:GDP is no longer ejected from the ribosome, thus subsequent peptide-bond formation and further progress of protein biosynthesis is obstructed [8].

Macromolecules acting at the elongation step of translation have been the target for crystallographic investigations for decades. In 1968, the first report on diffracting crystals of *E. coli* initiator tRNA appeared [13], and EF-Tu:GDP was first crystallized in 1973 [14]. The structure of yeast tRNA^{Phe} was solved in 1974 [15,16], and crystallographic studies over nearly two decades have resulted in refined structures of EF-Tu:GDP from two species ([17], [18] and references therein). The structures of EF-Tu from *T. thermophilus* [19] and *T. aquaticus* [20] in their GTP conformation have been deter-

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mined as complexes with a GTP analog, EF-Tu:GDPNP, and recently the EF-Tu:EF-Ts complex from *E. coli* added to the list of solved structures [21]. Crystal structures have also been solved of nucleotide-free EF-G [22] and EF-G:GDP [23] from *T. thermophilus*. For years, an essential molecular component of protein biosynthesis was inaccessible to X-ray crystallographic analysis, despite numerous crystallization attempts [24,25], namely the ternary complex of yeast Phe-tRNA^{Phe}:*T. aquaticus* EF-Tu:GDPNP. The crystal structure of this complex was determined in 1995 [1], after successful crystallization [25]. Thus, the overall spatial structures of the macromolecular components of the quaternary complex Phe-tRNA^{Phe}:EF-Tu:GDPNP:kirromycin, reported on in this article, are known, thereby enabling a molecular replacement approach to be applied for structure determination.

Several kirromycin-resistant EF-Tu mutants have been isolated and found to involve a limited number of residues, located at the interface between domains I and III in the EF-Tu:GTP crystal structure [26]. Chemical cross-linking data implied that kirromycin is proximate to the conserved wild-type residue Lys^{357}, in the presence of aa-tRNA [27]. Truncated EF-Tu from *E. coli*, i.e., domains II and III only, does not bind the antibiotic [28]. The effect of kirromycin on EF-Tu has been most extensively studied in *E. coli*, but EF-Tu from *T. thermophilus*, as used in this study, is equally sensitive [26,29].

Elongation factor Tu is a monomeric protein of 393 amino acid residues in *E. coli*, while the *T. thermophilus* variant is only slightly larger and has 405 residues. Both yeast and *E. coli* tRNA^{Phe} has 76 nucleotides, and kirromycin has a molecular weight of 797 Da. Here we report the isolation and crystallization of quaternary complexes of Phe-tRNA^{Phe} from yeast and *E. coli* with EF-Tu:GDPNP:kirromycin from *T. thermophilus* and *E. coli*. The eventual structure determination by X-ray crystallography of the quaternary complex will provide a new basis to evaluate the action of kirromycin-like antibiotics in protein biosynthesis, and will undoubtedly be of value in the design of drugs against infectious diseases.

2. Materials and methods

EF-Tu:GDP was purified from *Escherichia coli* (MRE600) and *Thermus thermophilus* (DSM, No. 576, Germany). A full conversion of EF-Tu:GDP to EF-Tu:GDPNP was achieved by adding three units of a soluble alkaline phosphatase (Böhringer, Germany) per mg EF-Tu and a 5-fold excess of GDPNP to the protein solution [20,25], followed by incubation for 30 min at room temperature in the presence of a 5 times molar excess of kirromycin (Sigma). The mixture was subjected to ion exchange chromatography on a DEAE-Sepharose column as described in [30], allowing the alkaline phosphatase to be separated from EF-Tu:GDPNP:kirromycin before the ultimate complex formation with Phe-tRNA^{Phe}. The fractions containing EF-Tu bound kirromycin were concentrated in Centricon-30 tubes. Charging of tRNA^{Phe} from brewers yeast (Böhringer, Germany) and *E. coli* (Sigma) was mediated by incubation with [^{14}C]Phe, ATP and cognate Phe-tRNA synthethase [31]. The quaternary complex (QC) formed by combining equimolar amounts of Phe-tRNA^{Phe} and EF-Tu:GDPNP:kirromycin was added a further 2 times molar excess of the antibiotic and 30% ammonium sulphate (AS). Centrifugation of the sample before chromatography produced a slightly yellow pellet, indicating that the excess of kirromycin did not dissolve easily. The final chromatography of QC was carried out at pH 7.0 on a TSK Phenyl-5PW HPLC column (50 mM Tris-HCl, 10 mM $MgCl_2$, 0.5 mM DTT and AS gradient from 30 to 0.3%). The 0.5 ml QC fractions were collected in Eppendorf tubes and precipitated by direct addition of AS to 70%. Concentrations were estimated by a Bradford method using a BSA standard and by liquid scintillation counting of [^{14}C]Phe-tRNA^{Phe}. The presence of EF-Tu and Phe-tRNA^{Phe} in the fractions, as well as in the crystals, was verified by silver-stained SDS-PAGE.

3. Results and discussion

Many biochemical studies on interactions between EF-Tu and kirromycin have addressed the issue of affinity, i.e., providing binding and rate constants. The EF-Tu:GTP:kirromycin and EF-Tu:GDP:kirromycin complexes have been reported to be the more stable, with close to equal binding constants in the nanomolar range, while quaternary complexes of aa-tRNA and EF-Tu:GDP or EF-Tu:GTP with the antibiotic are of a more labile nature with reported equilibrium constants on a micromolar scale [32]. However, with the aim of crystallizing a macromolecular entity, it should be noted that the real questions are whether isolation is feasible and whether stability under conditions that can support crystal growth is obtainable.

Attempts in different laboratories have been made to get structural information on the kirromycin interactions, by soaking preformed crystals of both EF-Tu:GDP and EF-Tu:GDPNP in antibiotic containing solutions [9]. It seems that crystal packing have rendered these trials unsuccessful. In the present study, co-crystallization experiments were performed with *E. coli* EF-Tu:GDPNP, but all attempts resulted in fibrillar formations as in [33]. We tried to isolate complexes of EF-Tu:GDP:kirromycin with deacylated and phenylalanylated yeast tRNA, but were unable to observe the formation of such complexes under our experimental conditions.

Ammonium sulphate (AS) is known to increase the stability of ternary complexes [25,31,34,35], as well as being a useful precipitant for the crystallization of binary complexes of EF-Tu, thus making it reasonable to consider this salt for the purification and crystallization of the Phe-tRNA^{Phe}:EF-Tu:GDPNP:kirromycin complex (QC). The complex was separated by hydrophobic interaction chromatography on a phenyl substituted HPLC column (see Section 2 and Fig. 1). This procedure obviated the need for further purification by

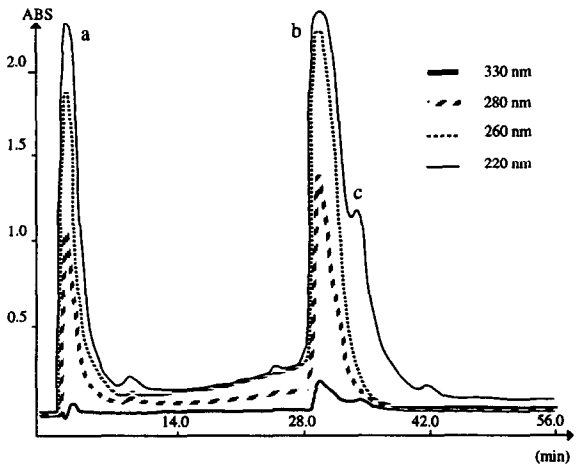

Fig. 1. The components of the quaternary complex were incubated and separated by hydrophobic interaction chromatography. a: Free nucleotides, tRNA^{Phe}, Phe-tRNA^{Phe} and kirromycin. b: Phe-tRNA^{Phe}:EF-Tu:GDPNP:kirromycin. c: EF-Tu:GDPNP:kirromycin.

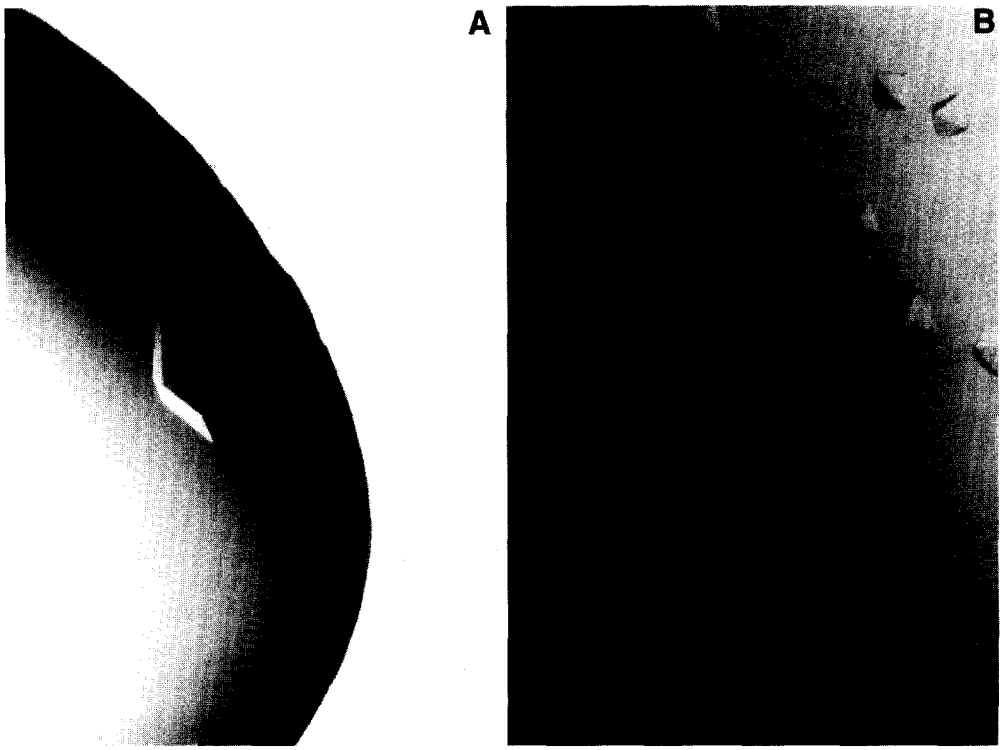

Fig. 2. Single crystals of quaternary complexes. A: Rhombohedral crystal of yeast Phe-tRNA^{Phe}:*T. thermophilus* EF-Tu:GDPNP:kirromycin. B: Cubic crystals of yeast Phe-tRNA^{Phe}: *E. coli* EF-Tu:GDPNP:kirromycin.

reverse fractional extraction [25]. The intensity of the characteristic kirromycin absorption at 335 nm is known to decrease upon binding to EF-Tu, while the maximum position is unaffected [8]. In an equivalent purification of the ternary complex, no detectable absorption in the neighbourhood of this red-shifted wavelength was observed. The crystallization procedure was the same for the different variant complexes in this study. Precipitated fractions from the central part of the QC-peak were combined and added a 2-fold molar excess of kirromycin. The precipitate was collected by centrifugation and redissolved in crystallization buffer at a concentration of 3–4 mg QC/ml (50 mM Tris-HCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM GDPNP, 30% AS). Equilibration at 4°C of 10 μl droplets on siliconized cover slips placed over the reservoirs (45–49% AS, 10 mM Mes, 3.9 mM Tris-HCl, pH 6.4), resulted in crystal growth within 3–7 days.

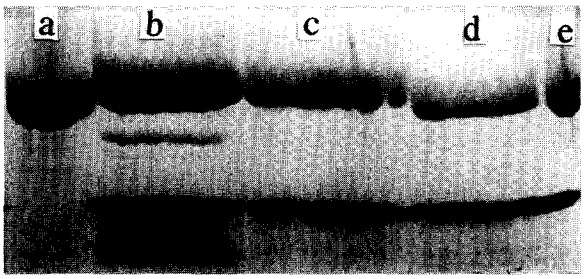

Fig. 3. Silver-stained gradient gel of washed and redissolved crystals (SDS-PAGE, 12.5–25%). Lanes a,e: *E. coli* EF-Tu molecular weight marker (43 kDa). Lane b: Outdated crystals of the ternary complex [25] used as a standard to indicate a 1:1 molar ratio of EF-Tu/tRNA^{Phe}. Lane c: Crystals of yeast Phe-tRNA^{Phe}:*T. thermophilus* EF-Tu:GDPNP:kirromycin. Lane d: Crystals of yeast Phe-tRNA^{Phe}:*E. coli* EF-Tu:GDPNP:kirromycin.

For the yeast Phe-tRNA^{Phe}:*E. coli* EF-Tu:GDPNP:kirromycin complex single crystals of maximum size $0.2\times0.2\times0.2$ mm^3 and for *E. coli* Phe-tRNA^{Phe}:*E. coli* EF-Tu:GDPNP:-kirromycin sizes of $0.03\times0.03\times0.03$ mm^3, were obtained within 2 weeks. Morphologically, these crystal are indistinguishable and so it is believed that the source of the tRNA has no significant structural implication. A similar time span was needed for rhombohedral crystals of yeast Phe-tRNA^{Phe}:*T. thermophilus* EF-Tu:GDPNP:kirromycin to grow to a size of $0.5\times0.5\times0.5$ mm^3 (Fig. 2). The presence of EF-Tu and Phe-tRNA^{Phe} in the crystals was verified by silver-stained SDS-PAGE of redissolved crystals after careful washing (Fig. 3).

Collection of diffraction data from the reported crystals is feasible only by the use of intense synchrotron radiation. A low-resolution data set to 3.5 Å was measured at SRS, Daresbury (station 9.6) on the yeast Phe-tRNA^{Phe}:*E. coli* EF-Tu:GDPNP:kirromycin complex using a sucrose concentration of 25% at cryogenic temperatures. The crystals belong to the cubic spacegroup P432, with cell dimensions of 196.26 Å. The diffraction limit extends beyond 3 Å with sufficient exposure provided. Judged from spot shape, background appearance and a mosaicity of 0.2° for the collected data, the crystals seem well ordered. It should be noted that unstable hexagonal plates were obtained in attempts to produce crystals of the ternary complex from *E. coli* EF-Tu:GDPNP and yeast Phe-tRNA^{Phe} [25]. Assuming a realistic solvent content, calculations give one quaternary complex per asymmetric unit.

The crystals of yeast Phe-tRNA^{Phe}:*T. thermophilus* EF-Tu:GDPNP:kirromycin were likewise tested at Daresbury. The diffraction potential was similar to that of the *E. coli* complex, possibly with slightly less demand to radiation in-

tensity. The crystal system is rhombohedral with cell dimensions of 137.3 Å and angles of 54.0°. The asymmetric unit is estimated to contain one quaternary complex.

The crystallization of the quaternary complex reported here provides the basis for an eventual structure determination by X-ray crystallography, that will be a valuable supplement to our understanding of kirromycin action. Elongation factor G is affected by the antibiotic fusidic acid and also by kirromycin but at very high concentrations [8,36]. Considering the revealed similarity between the ternary complex and EF-G [1], the results from a crystallographic analysis made possible through this study may be of more general interest. Furthermore, the structure of the quaternary complex is expected to reveal new details concerning the GTPase activity, and thus will provide further support for speculations on this issue.

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