Enacyloxin IIa Pinpoints a Binding Pocket of Elongation Factor Tu for Development of Novel Antibiotics*3

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Elongation factor (EF-) Tu-GTP is the carrier of aminoacyl-tRNA to the programmed ribosome. Enacyloxin IIa inhibits bacterial protein synthesis by hindering the release of EF-Tu-GDP from the ribosome. The crystal structure of the Escherichia coli EF-Tu- guanylylimidodiphosphate (GDPNP)enacyloxin IIa complex at 2.3 Å resolution presented here reveals the location of the antibiotic at the interface of domains 1 and 3. The binding site overlaps that of kirromycin, an antibiotic with a structure that is unrelated to enacyloxin IIa but that also inhibits EF-Tu-GDP release. As one of the major differences, the enacyloxin IIa tail borders a hydrophobic pocket that is occupied by the longer tail of kirromycin, explaining the higher binding affinity of the latter. EF-Tu-GDPNP-enacyloxin IIa shows a disordered effector region that in the P-site bound enacyloxin IIa complex (Thermus aquaticus)–GDPNP-cyloxin IIa complex, solved at 3.1 Å resolution, is stabilized by the interaction with tRNA. This work clarifies the structural background of the action of enacyloxin IIa and compares its properties with those of kirromycin, opening new perspectives for structure-guided design of novel antibiotics.

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5 The abbreviations used are: EF, elongation factor; aa-tRNA, aminoacyl-tRNA; D1, D2, and D3, EF-Tu domains 1, 2, and 3, respectively; GDPNP, guanylylimidodiphosphate; Ec, Escherichia coli; Tt, Thermus thermophilus; Ta, Thermus aquaticus; DTT, dithiothreitol; r.m.s., root mean square; MES, 4-morpholineethanesulfonic acid.

MATERIALS AND METHODS

Crystallization and Data Collection—EF-Tu-GDPNP was formed by calf intestinal alkaline phosphatase (molecular biological degree, Roche Applied Science; 2 units/mg EF-Tu) cleavage of GDP from purified EF-Tu-GDP in 20 mM Tris-HCl (pH 8.0), 200 mM (NH₄)₂SO₄, 2 mM dithiothreitol (DTT), 0.5 mM NaN₃, and a 2.5-fold molar excess of GDPNP. Stock solutions (10 mM) of enacyloxin IIa in absolute ethanol at −81 °C were stable for at least 1 year. ε 365 nm M⁻¹ cm⁻¹ = 91,000 in ethanol was used. For crystallization 60–180-μl solutions contained 4.0–4.5 mg/ml EF-Tu in 20 mM Tris-HCl buffer, pH 7.6, 20 mM NaCl, a 2-fold molar excess of GDPNP, 5–10 mM MgCl₂, 2 mM DTT, 1% glycerol, 0.5 mM NaN₃, and the antibiotic, added as the last component in a 1.2 molar ratio to EF-Tu. Higher molar excess of enacyloxin IIa over aa-tRNA into the ribosomal A-site, and peptide bond formation with the P-site bound peptidyl-tRNA. EF-Tu folds in three distinct domains: the nucleotide-binding domain 1 (residues 1–199 in Escherichia coli (Ec) EF-Tu-GDP) shows the classical GTPase mixed α/β Rossmann fold, whereas domains 2 and 3 (residues 209–299 and 300–393, respectively) are β-barrels. The association of the domains in EF-Tu-GDP with reduced interfaces and a central hole becomes more compact in EF-Tu-GTP with extensive interfaces and no hole (3–5). EF-Tu is the target of four structurally unrelated families of antibiotic inhibitors of protein synthesis (for review see Ref. 2), of which kirromycin and enacyloxin IIa hinder the release of EF-Tu-GDP from the ribosome after GTP hydrolysis, thus inhibiting its recycling and peptide bond formation. The high-resolution crystal structure of Thermus thermophilus (Tt) EF-Tu-GDP in complex with methylkirromycin (also called aurodox) showed that the antibiotic binds in the interface of domains 1 and 3, inducing a unique, compact EF-Tu-GTP-like conformation, which is the reason for its inhibition of the EF-Tu-GDP release (6). These conclusions have been further confirmed in studies of the Phe-tRNAHis EF-Tu-GDPNP-kirromycin. Specific differences between the enacyloxin IIa and kirromycin complexes with EF-Tu concern various aspects such as the electrostatic migration on native gel, binding affinity, GTase activity, different effects of mutations, etc. (7, 8).

In this work, we describe the structures of EF-Tu(Ec)GDPNP-enacyloxin IIa (Tt 2.3 Å and Phe-tRNAHis Thermus aquaticus (Ta) EF-Tu-GDPNP-enacyloxin IIa at 3.1 Å resolution. We have analyzed the antibiotic binding site and the induced structural and functional changes of EF-Tu in comparison with those of the GTP-like T. thermophilus EF-Tu-GDPNP-kirromycin complex. The similarities and differences in binding modes between the two antibiotics suggest new possibilities for structure-guided design of novel antibiotics with improved binding characteristics and possibly more selective action.
Binding of Enacyloxin IIa to EF-Tu

TABLE 1
Data collection and refinement statistics of EF-Tu-enacyloxin IIa (Enx) complexes

| Source | EF-Tu-GDPNP-Enx | Phe-tRNA-EF-Tu-GDPNP-Enx |
|--------|-----------------|--------------------------|
| Space group | E. coli EF-Tu | T. aquaticus EF-Tu |
| Cell dimensions (Å) | P4_2_2 | C2 |
| Wavelength (Å) | 0.9792 | a = 211.4, b = 99.8, c = 135.8 |
| Resolution (Å) | 30–2.3 (2.36–2.3) | β = 108.8° |
| R_res^a | 0.098 (0.51) | 0.9504 |
| R/α | 24.1 (2.8) | 30–3.1 (3.2–3.1) |
| Redundancy | 7.0 (6.6) | 0.064 (0.18) |
| Completeness (%) | 99.8 (100) | 14.1 (3.5) |
| B-value, Wilson plot (Å^2) | 42.1 | 2.4 (2.4) |
| Copies per asymmetric unit | 2 | 83.7 (52.0)^a |
| NCS model | Constrained, 3-fold | |
| Br/B_mean | RestraINED, 2-fold | |
| Ramachandran (%)^a | 89.4 / 0.6 | 62.7 / 0.6 |
| R.m.s. bond (Å) | 0.07 | 0.01 |
| R.m.s. angle (°) | 1.41 | 1.61 |
| Average B-value (Å^2) | 49.5 | 76.5 |

^a R_res = Σ(|Fo| - |Fc|)/Σ|Fo|, where |Fo| is the i-th measurement.

^a Strong anisotropy in the C2 form caused a low completeness in the higher resolution bins.

The figures were prepared using PyMol (33).

EF-Tu caused aggregation and precipitation of EF-Tu. After centrifugation, sitting drops of 2–5 μl of the clear supernatant mixed with a 0.4–0.5 volume equivalent from the reservoir (0.5 ml of 100 mM Tris-HCl, pH 7.4–7.6, 300–600 mM NaCl, 5–8% glycerol, and 20–23% polyethylene glycol 6000) were kept at 19°C. Tetragonal crystals (space group P4_2_2) with a faint yellowish color appeared within 24 h and grew as long rods to a maximum size of 1.5 × 0.5 × 0.3 mm within few days. The EF-Tu(Ta)-GDPNP-enacyloxin IIa-Phe-tRNA^Phe^ (Saccharomyces cerevisiae) complex, obtained as described for the quaternary complex with kirromycin (9), was precipitated with (NH_4)_2SO_4, resuspended to 10 mg/ml in a crystallization buffer containing 1.25 mM (NH_4)_2SO_4, 10 mM MgCl_2, 20 mM Tris-HCl, pH 7.2, 1 mM GDPNP, 0.5 mM DTT, and 50 μM enacyloxin IIa. Hanging drops (3–8 μl) were equilibrated at 4°C against reservoir solutions containing 1.8 mM (NH_4)_2SO_4, pH 6.4–7.0. Monoclinic crystals (space group C2) appeared within a few days and grew to maximum dimensions of 0.6 × 0.4 × 0.2 mm.

EF-Tu-GDPNPEnacyloxin IIa crystals were mounted directly from mother liquor and flash-cooled in a 100 K cryostream (Oxford Cryosystems). X-ray crystallographic data extending to 2.3 Å resolution were collected from a single crystal at beam line I711 (MAX-Lab). Phe-tRNA^Phe^-EF-Tu-GDPNPEnacyloxin IIa crystals were flash-cooled in a cryoprotecting buffer consisting of 2.6 mM (NH_4)_2SO_4, 22% trehalose, 30 mM Tris-MES, pH 6.5, 10 mM MgCl_2, 0.5 mM DTT, and 50 mM enacyloxin IIa, and data were collected at 100 K at beam X13 (EMBL/ DESY). Diffraction data were integrated and scaled with the HKL package (10), and structure factor amplitudes were calculated using Truncate of the CCP4 package (11). Table 1 shows the data statistics. The tRNA-bound complex (Table 1) suffered from strong anisotropy in the diffraction pattern, compromising data quality and subsequent refinement procedures.

Structure Determination and Refinement—Structures were determined by molecular replacement using AMoRe (12) and search models derived from a kirromycin-bound complex (Protein Data Bank entry 1OB2). Two molecules of the EF-Tu-GDPNPEnacyloxin IIa complex related by a noncrystallographic 2-fold rotation axis (NCS) were located. Model phases were refined and extended by density modification using averaging, solvent flattening, and histogram matching in the program DM at 30–2.3 Å resolution (13). The resulting map was practically unbiased and showed clear density for enacyloxin IIa (Fig. 1B). Model building was performed in O (14), and an assigned C13-R, C14-S configuration for enacyloxin IIa provided the best fit. The model derived from the DM map was refined using crystallography NMR software (CNS) (15) initially at 5–2.3 Å resolution, enforcing strict NCS, and later at 30–2.3 Å resolution, using restrained NCS and bulk solvent correction. The r factor calculated for a subset of ~1200 reflections excluded from the refinement throughout.

RESULTS

Enacyloxin IIa Binding Site on EF-Tu—Enacyloxin IIa (for its structure, see Fig. 2A) is active against Gram-positive and Gram-negative bacteria, slightly active against fungi, but inactive against yeasts (17–19). It stabilizes the EF-Tu GDP to EF-Tu. The figures were prepared using PyMol (33). The antibiotic also reorients the side chain
of several interface residues, breaking the hydrogen bonds Gln\textsuperscript{124} N/H9280\textsuperscript{2-} Phe\textsuperscript{374} O and Tyr\textsuperscript{160} OH-Glu\textsuperscript{315} O/H9280\textsuperscript{2-}. Thus, it binds by an induced-fit mechanism. At \<3.8 \text{ Å} it contacts 16 amino acids (7 from domain 1 and 9 from domain 3) via 6 hydrogen bonds and extensive van der Waals and hydrophobic interactions (Fig. 2, A, C, E, and G). A salt bridge links the carboxyl group of the hexane ring with the side chain N\textsubscript{ε} of Lys\textsuperscript{313} (Fig. 2, A and C). It is noteworthy that all residues in the text are numbered according to EF-Tu\textsubscript{Ec}, even when referring to EF-Tu\textsubscript{Tt} or EF-Tu\textsubscript{Ta}, with the homologous residue number in italics between parentheses. For more details of the enacyloxin IIa binding site, see Table 2.

Similarities to and Divergences from the Kirromycin Binding Site—In this work kirromycin is often used as synonym for methylkirromycin (Fig. 2B), because it is the prototype and most studied member of this family and displays the same effect on EF-Tu (20). From the three-dimensional model of the GTP-like EF-Tu(Tt)-GDP-kirromycin com-
FIGURE 2. Comparisons between enacyloxin IIa (left panels, yellow) and (1-methyl)-kirromycin (right panels, purple) binding sites showing their chemical structures (A and B), hydrogen bonds with EF-Tu (A–D), relationships with Gln-124/125 (E and F), and location in the interface gap between domains 2 and 3 with the hydrophobic pocket occupied by the tail of kirromycin (G and H). A and B, the structures are drawn schematically to facilitate comparison with C and D. The heads are on the left, and the “tails” on the right. Hydrogen bonds to EF-Tu structural elements are indicated by dotted lines and the salt bridge in A by larger dots. C and D, close-up of the antibiotics bound to EF-Tu in a “back” view compared with Fig. 1A. The antibiotics are shown as stick figures surrounded by transparent molecular surfaces. Hydrogen bonds are shown as green dotted lines and the salt-bridge in C as a red dotted line. Selected side chains and stretches of the EF-Tu backbone that make hydrogen bonds are shown as sticks on the schematic representation. Oxygen atoms are red, nitrogens are blue, and the chlorines in enacyloxin IIa are bright green. Note that the heads of the two antibiotics point in opposite directions; that of enacyloxin points toward the viewer, whereas that of kirromycin points away (into the EF-Tu). Also note that the tail of kirromycin points away into the interior of EF-Tu. This latter point is illustrated...
TABLE 2
Connections between enacyloxin IIa and its EF-Tu binding site and hydrogen bonds interconnecting residues at <3.8 Å from the antibiotic.

| Contacts at <3.8 Å | Enacyloxin IIa (res: 16) | Leu<sup>373</sup> - Arg<sup>373</sup> - Lys<sup>319</sup> - Val<sup>312</sup> - Gln<sup>159</sup> - Tyr<sup>144</sup> - Leu<sup>111</sup> - Lys<sup>115</sup> - Asp<sup>114</sup> - Glu<sup>131</sup> - Tyr<sup>123</sup> - Arg<sup>123</sup> - Arg<sup>123</sup> - Phe<sup>124</sup> - Ala<sup>126</sup> |
|-------------------|--------------------------|---------------------------------------------------------------------------------|
| Hydrogen bonds | Al<sup>90</sup> - O-ENX NH<sub>4</sub> 43; Arg<sup>23</sup> - O-ENX OH<sub>33</sub>; Lys<sup>144</sup> - O-ENX OH<sub>33</sub>; Tyr<sup>123</sup> - OH-ENX OH<sub>41</sub>; Arg<sup>123</sup> - N<sub>2</sub>-ENX O45; Arg<sup>123</sup> - N<sub>2</sub>- ENX OH<sub>45</sub> |
| Methylkiorromycin (res: 8) | Leu<sup>121</sup> - O-Arg<sup>121</sup> - N<sub>6</sub> - Leu<sup>98</sup> - O-Gln<sup>98</sup> - N<sub>6</sub> - Gln<sup>129</sup> - N<sub>2</sub>- Gln<sup>110</sup> - O; Ala<sup>126</sup> - O-Tyr<sup>121</sup> N |
| Methylkiorromycin (res: 8) | Leu<sup>121</sup> - O-Arg<sup>121</sup> - N<sub>6</sub> - Leu<sup>98</sup> - O-Gln<sup>98</sup> - N<sub>6</sub> - Gln<sup>129</sup> - N<sub>2</sub>-Gln<sup>110</sup> - O; Ala<sup>126</sup> - O-Tyr<sup>121</sup> |

more clearly in H. In E–H, the EF-Tu is shown as a molecular surface with selected elements identified by labels. The color scheme is again red for domain 1 and blue for domain 3. In E and F, the complex relationships between kiorromycin and Gln<sup>124</sup> (green area) with several contacts is compared with that of enacyloxin IIa. For a close comparison, E and F also outline the overlap with kiorromycin and enacyloxin IIa, respectively. The green area in G and H is the hydrophobic surface of atoms within 4 Å of the kiorromycin tail (or the equivalent atoms in G). Clearly the tail of enacyloxin IIa borders this pocket, whereas that of kiorromycin occupies it. Note that in C and D, domains 1 are in the exact same position, whereas domains 3 are pushed down and to the left as a result of the greater bulk of kiorromycin. This larger gap between the domains can be recognized in H on the left side (cf. G).
helix C (residues 89–93) is slightly pushed up, whereas helix B is pulled roughly along its axis toward the tail of the antibiotic. As a consequence the effector region, residues 41–65, that in EF-Tu-GDPNP contacts this helix is destabilized and changes conformation. Its N-terminal part (residues 41–50) shows no electron density, although in the residual part (Switch 1 region) the weak electron density reveals an unwound α-helix A* collapsed into domain 2 (Fig. 1A). Arg58 and Thr61, two conserved residues important for GTPase activity and interaction with the ribosome (21, 22), are moved away by ~15 Å toward the vacant binding pocket for the aminoacyl residue of tRNA. Fig. 1D illustrates the superimposition of EF-Tu-GDPNP-enacyloxin IIa and EF-Tu-GDP-kirromycin. Also the kirromycin complex shows a disordered effector region (6). For the changes in the Switch 1 region of the two antibiotic complexes, see also Fig. 6. Most important, on binding of Phe-tRNA<sub>III</sub>Phe to EF-Tu-GDPNP-enacyloxin IIa the disordered effector region is restructured by the interaction of the α-helix A* with tRNA (Fig. 4). The acceptor stem and part of the CCA-end of tRNA are tugged by the effector region to follow the displacement of domain 1 induced by the antibiotic binding, whereas the remaining tRNA regions follow domains...
2 and 3. As a result, the acceptor stem twists relative to the T-stem and displays a unique conformation intermediary between free tRNA and tRNA in the ternary complex (Fig. 4). The phenylalanyl group has a reduced electronic density, indicating a defective accommodation (Fig. 5) that is reflected in the decreased protection by EF-Tu-GTP against the spontaneous deacylation of Phe-tRNA^Phe, which is more pronounced than in the case of kirromycin (see supplemental Fig. 1). The Switch 2 region of the enacyloxin IIa complex shows no marked changes. The side chain of His^{85(61)}, a residue important for the GTPase activity dependent on EF-Tu (23–25), points away from the γ-phosphate of the nucleotide similarly to the native conformation (Fig. 6). In contrast, in the kirromycin complex it is turned toward the phosphate groups of GDP. Enacyloxin IIa enhances the intrinsic GTPase activity of EF-Tu but much more weakly than kirromycin (Fig. 7; note the difference in scale between panels A and B). Interestingly, with enacyloxin IIa the different ions show similar concentration dependence, the “soft” NH₃⁺ being however the strongest stimulator, whereas with kirromycin EF-Tu displays a GTPase increase as a function not only of the increasing concentration of “hard” monovalent ions but also of their charge density.

**DISCUSSION**

This work describes for the first time the structure of the EF-Tu-enacyloxin IIa complex, defining the antibiotic binding site on EF-Tu, which in the domains 1–3 interface overlaps that of kirromycin. There is strong evidence that despite a number of functional differences both compounds inhibit protein synthesis by inducing a unique GTP-like conformation of EF-Tu-GDP (8). Accordingly, the overall structures of EF-Tu-GDP and kirromycin, and the binding sites of the two antibiotics share marked similarities. Nonetheless there are important, specific differences, such as the less complex contact of enacyloxin IIa with Glu^{124} and the behavior of the short tail of enacyloxin IIa, which borders an empty hydrophobic pocket, whereas the longer tail of kirromycin fits into this pocket. This, we believe, represents the main reason for the lower binding affinity of enacyloxin IIa to EF-Tu. Related to this aspect is the differential effect caused by substituting the bordering Ala^{375(87)} with the bulkier Val that fills this pocket. EF-Tu becomes 3-fold more sensitive to enacyloxin IIa (8) but ~300 times more resistant to kirromycin (26). The insertion of the Val^{375} side chain stabilizes the short tail of enacyloxin IIa by extending the contacting hydrophobic surface (Fig. 8A), whereas it inhibits the fitting of the longer tail of kirromycin by steric hindrance (Fig. 8B).

8C illustrates the overlap of enacyloxin IIa and kirromycin, underlining their overall structural divergences and similarities; it also indicates the active core of kirromycin containing the tail half-molecule as deduced from the functional analysis of fragments and chemical modifications of the antibiotic (27–29). Our observations hint at new approaches for a structure-guided antibiotic design on the basis of the hybridization of enacyloxin and kirromycin structural components, with the aim of obtaining novel EF-Tu binding compounds with specific properties. From our results, combination of the head moiety of enacyloxin IIa with the tail moiety of kirromycin should increase the binding affinity of the resulting compound. The binding efficiency of an antibiotic can affect the degree of the antibacterial effect and reduce the onset of resistance by microorganisms. One can extend this approach by creating hybrids between structural parts of the two antibiotics and other cyclic compounds (e.g. sugars, lactones, macrolides, etc.). By taking into account the common properties of the two antibiotics binding sites, one could combine any structural moieties making hydrogen bonds with amino acids around the head of the antibiotic, e.g. Tyr^{160(162)} with compounds bonding residues around the tail, such as Glu^{124} and Lys^{113(125)}.

Another interesting result of this work is the ability of aa-tRNA to revert the disorder of the effector loop caused by the binding of the antibiotic. This effect is associated with a unique distortion of the acceptor stem of tRNA. This, however, does not impair the EF-Tu-mediated binding of aa-tRNA to the programmed ribosome, which in the presence of enacyloxin IIa is even improved (7). A possible explanation for this effect can be derived from the observation that the similarly distorted tRNA bound to EF-Tu-GDPNP/kirromycin shares common features with the conformation of the ribosome-bound EF-Tu-GDPNP/kirromycin complex. Concerning the high mobility of the effector region, a plausible mechanism can be found in the distortion of helices B and C induced by the accommodation of the antibiotic in the domain 1–3 interface. The signal arising from the binding of the antibiotic can thus be transferred to other areas of EF-Tu.

It is not clear why EF-Tu-enacyloxin IIa shows a lower intrinsic GTPase activity than EF-Tu-kirromycin. Site-directed mutagenesis did not prove the involvement of His^{61} side chain in a nucleophilic attack on the γ-phosphate; however, it supported an indirect key role of this residue in both the intrinsic and the very fast GTP hydrolysis evoked by programmed ribosomes (23, 24). To date the most convincing hypothesis about the mechanism of the low intrinsic EF-Tu GTPase activity is “substrate-assisted” GTP hydrolysis (30). The level of intrinsic GTPase activity of EF-Tu likely depends on selective local pH effects influencing

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**FIGURE 8. Structural divergences between enacyloxin IIa and kirromycin and some specific effects.** A and B show the structural background of the effect of the A375V mutation on the sensitivity of EF-Tu toward enacyloxin II and kirromycin. The panels show close-up views in the same orientation of the enacyloxin IIa (A) and the kirromycin (B) complex of EF-Tu. Ala^{375} has been substituted in silico by Val (gray). The transparent van der Waals sphere indicates the space occupied by an Ala side chain. The open surface indicates the space occupied by Val. Clearly, the tail of enacyloxin IIa is not hindered, but rather it makes a more extensive hydrophobic contact with the Val^{375} side chain. In contrast, there is a steric clash for the kirromycin tail entering the hydrophobic pocket; the transparent van der Waals envelope around the tail intersects with the Val^{375} van der Waals envelope (glossed). C, selective differences in the tail moiety of the overlap between enacyloxin IIa (yellow) and kirromycin (pink), likely to affect the binding affinity and activity of the two antibiotics. The active core area of kirromycin is circled.
the stability of the γ-phosphate, which could be differently affected by the two antibiotics and which may also relate to the differential influence of cations on this activity.

In conclusion, the structural similarities and differences between enacyloxin IIa and kirromycin will not only explain central features of their action but also evoke selective approaches for obtaining novel inhibitors of protein synthesis, a proven target and a crucial aspect for society, taking into account the general increase in resistance toward antibiotics.

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