Stepwise Binding of Tylosin and Erythromycin to Escherichia coli Ribosomes, Characterized by Kinetic and Footprinting Analysis*

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Erythromycin and tylosin are 14- and 16-membered lactone ring macrolides, respectively. The current work shows by means of kinetic and chemical footprinting analysis that both antibiotics bind to Escherichia coli ribosomes in a two-step process. The first step established rapidly, involves a low-affinity binding site placed at the entrance of the exit tunnel in the large ribosomal subunit, where macrolides bind primarily through their hydrophobic portions. Subsequently, slow conformational changes mediated by the antibiotic hydrophilic portion push the drugs deeper into the tunnel, in a high-affinity site. Compared with erythromycin, tylosin shifts to the high-affinity site more rapidly, due to the interaction of the mycosine sugar of the drug with the loop of H35 in domain II of 23S rRNA. Consistently, mutations of nucleosides U2609 and U754 implicated in the high-affinity site reduce the shift of tylosin to this site and destabilize, respectively, the final drug-ribosome complex. The weak interaction between tylosin and the ribosome is Mg2+ independent, unlike the tight binding. In contrast, both interactions between erythromycin and the ribosome are reduced by increasing concentrations of Mg2+ ions. Polyamines attenuate erythromycin affinity for the ribosome at both sequential steps of binding. In contrast, polyamines facilitate the initial binding of tylosin, but exert a detrimental, more pronounced, effect on the drug accommodation at its final position. Our results emphasize the role of the particular interactions that side chains of tylosin and erythromycin establish with 23S rRNA, which govern the exact binding process of each drug and its response to the ionic environment.

Erythromycin and tylosin (Fig. 1) are representatives of the 14- and 16-membered lactone ring macrolides, respectively, both of which have been proposed to inhibit protein synthesis by binding to the large ribosomal subunit in a ratio of one molecule per ribosome (1, 2). Erythromycin has only modest activity against Gram-negative enterobacteria (e.g. Escherichia coli), whereas tylosin is somewhat more potent against certain Gram-negatives, such as Pasteurella multocida and various species of Haemophilus (3). Nevertheless, erythromycin and tylosin show high specificity and affinity in vitro for the E. coli 50S ribosomal subunit, exhibiting dissociation constants of less than 10−7 and 10−8 M, respectively (1, 4–7).

Erythromycin and tylosin binding sites have been initially mapped by biochemical and genetic methods and they have been located in approximately the same region of the large ribosomal subunit, within a hydrophobic crevice of the peptide exit tunnel (8–13). This is consistent with the long standing view that both drugs act by hindering the progression of the nascent peptide through the ribosomal exit tunnel, which eventually leads to peptidyl-tRNA “drop off” (14). In fact, the footprinting pattern of erythromycin in 23S rRNA is somewhat different from that of tylosin (10–12), implying that, although both antibiotics exploit the same binding pocket, their specific interactions might vary in accordance with their chemical nature. Indeed, each one of the two antibiotics possesses distinctive side chains that make idiosyncratic interactions with 23S rRNA. These drug-specific contacts contribute dramatically to the drug affinity for the ribosome (6), molecular flexibility (15), activity against mutated or methylated ribosomes (15–17), induction of methyltransferases (3), tolerance against efflux pumps (3), and peculiar effects on translation (6, 12). Tylosin, for instance, can inhibit the puromycin reaction if the donor tRNA is acylated by a large amino acid, such as phenylalanine. In contrast, mild stimulation of PTase activity by erythromycin is observed in this model reaction. Consistently, biochemical and crystallographic studies have revealed that the mycamino/mycarose moiety of the 16-membered macrolides extends toward the PTase center and perturbs the relative positioning of the 3′-end of P-site bound tRNA and 23S rRNA in the ribosome (18, 19). Moreover, recent studies have indicated that erythromycin and tylosin interrupt the normal sequence of 50S subunit assembly, leading to a “stalled” intermediate precursor particle (20), and also affect the accuracy of translation by stimulating stop codon readthrough and frameshifting errors (21).

With the recent emergence of high-resolution crystal structures of the archaeal and bacterial large ribosomal subunits and their complexes with antibiotics, research on the molecular interactions of erythromycin and tylosin with the ribosome has made a significant progress (19, 22, 23). It should be mentioned that although the drug-ribosome structures observed with the

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1 The abbreviations used are: PTase, peptidyltransferase; DMS, dimethyl sulfoxide; CMCT, 1-cyclohexyl-3-(2-morpholinooethyl)carbodiimide; AcPhe, acetylphenylalan; TRNOE, transferred nuclear overhauser effect.
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archaeal and bacterial 50 S subunits show a close resemblance with respect to each other, they differ in some details (24). It has been postulated that among a few possible sources of these discrepancies, crystal environment differences resulting from the high-salt requirements for archaeal optimal growth and integrity may affect the conformation of the drug and/or mask several potential ribosomal entities that could interact with the drug (15). With respect to this postulation, noteworthy is the finding that the ionic environment can affect the interaction of antibiotics with the ribosome (25, 26), as well as the in vitro activity of selected antimicrobial agents against pathogens (27).

It is important to keep in mind that crystallographic structures provide only a static picture of the macrolide binding to the ribosome at the initial complex have been characterized by NMR measurements.

In the present study, we use a combination of chemical footprinting and kinetic analysis to characterize the steps involved in the binding of erythromycin and tylosin to poly(U)-programmed 70 S ribosomes bearing tRNA\(^{\text{Phe}}\) at the E-site and AcPhe-tRNA at the P-site, under various ionic conditions. Our results are discussed in view of NMR and crystallographic studies on the interactions of these drugs with the ribosome.

EXPERIMENTAL PROCEDURES

Reagents and Materials—Spermine tetrahydrochloride, spermidine trihydrochloride, puromycin dihydrochloride, erythromycin tartrate, tRNA\(^{\text{Phe}}\) from E. coli, DMS, and DMS stop solution were purchased from Sigma. \(l-[2,3,4,5,6-\text{H}]\) Phenylalanine and \([\alpha-\text{\text{32P}}]\) ATP were from Amersham Biosciences. Avian myeloblastosis virus reverse transcriptase, dNTPs, and ddNTPs were obtained from Roche Diagnostics and Roche Applied Science, respectively. Cellulose nitrate filters (type HA; 0.45-\(\mu\)m pore size) were from Millipore Corp. Kethoxal and CMCT were provided by MP Biomedicals and Fluka Biochemicals, respectively.

E. coli Strains and Biochemical Preparations—Salt washed (0.5 M NH\(_4\)Cl) 70 S ribosomes and partially purified translation factors were prepared from E. coli K12 cells as described elsewhere (29). E. coli TA531 cells that lack chromosomal rrr alleles but contain wild-type or mutant pKK3535 plasmids expressing wild-type or mutated 23 S rRNA (U2609C or U754A), respectively, were kindly provided by Prof. A. S. Mankin (University of Illinois) and used as a source of ribosomes in studying the effect of certain mutations on the mechanism of drug binding to E. coli ribosomes. Prior to their use, ribosomes were activated in buffer containing 20 mM Mg\((\text{CH}_3\text{COO})_2\) and 150 mM NH\(_4\)Cl by incubation for 20 min at 42°C. Samples were then cooled at 0°C and the Mg\(^{2+}\) concentration was normalized to 4.5 mM. Ac\([\text{\text{3H}}]\) Phe-tRNA, charged to 80%, was prepared from E. coli tRNA\(^{\text{Phe}}\) as described previously (30). Post-translocation complex of poly(U)-programmed ribosomes, complex C, carrying tRNA\(^{\text{Phe}}\) and Ac\([\text{\text{3H}}]\) Phe-tRNA at the E- and P-sites, respectively, was prepared in buffer A (100 mM Tris/HCl, pH 7.2, 4.5 mM Mg\((\text{CH}_3\text{COO})_2\), 150 mM NH\(_4\)Cl, and 6 mM 2-mercaptopethanol) and purified according to Dinos et al. (31).

Whenever required, 100 \(\mu\)M spermine or a mixture of 50 \(\mu\)M spermine and 2 \(\mu\)M spermidine was also included in buffer A. In the presence of polyanymes, the percentage of active ribosomes in AcPhe-tRNA binding was more than 65%. This fraction was almost fully reactive toward puromycin.

Inactivation of Complex C by Tylosin—Aliquots of complex C (5 pmol) in buffer A containing or not containing polyanymes (1 ml) were reacted with tylosin at various concentrations for the desired times of exposure. After removing excess tylosin, the percentage \((x)\) of the input complex C remaining active was determined by titration with puromycin (2 \(\mu\)M for 2 min, at 25°C). It should be mentioned that by removing the excess tylosin, the concentration of complex C in the reaction mixture does not change substantially within the reaction time of 2 min, because the \(k_{\text{off}}\) value of the ribosome-drug complex is very small. For each concentration of tylosin, Inx was plotted against time. The apparent rate constant of inactivation, \(k_{\text{in}}\), was estimated from the slope of these plots.

As indicated by a previous study (6), the kinetics of tylosin (T) binding to complex C can be described by two sequential reactions, the first one equilibrating much faster than the subsequent isomerization step (kinetic Scheme 1).

\[
\text{C} + \text{T} \xrightarrow{k_{\text{f}}} \text{CT} \xrightarrow{k_{\text{on}} T} \text{C*T}
\]

\[
\text{SCHEME 1}
\]

\(3\) Derivation of the kinetic equation used in this report are provided as supplemental data.


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\[
\begin{align*}
C + T & \xrightarrow[k_r]{k_r} CT \xrightarrow[k_{off,T}]{k_{on,T}} C^*T \\
+ & \\
E & \xrightarrow[k_{off,E}]{k_{off,E}} C^*E \\
K_F & \xrightarrow{k_{on,E}} CE \\
K_E & \xrightarrow{k_{off,E}} C^*E \\
\end{align*}
\]

**Scheme 2**

Therefore, \( k_{in} \) is given by Equation 1,

\[
k_{in} = k_{on,T} \times \frac{[T]}{K_T + [T]} + k_{off,T}
\]

(Eq. 1)

where \( K_T \) is the dissociation constant of the encounter complex CT. If the relationship \( k_{on,T} ([T]/(K_T + [T])) >> k_{off,T} \) holds, Equation 1 approximates to Equation 2.

\[
k_{in} = \frac{k_{on,T} [T]}{K_T + [T]}
\]

(Eq. 2)

Based on Equation 2, we determined the values of \( k_{on,T} \) and \( K_T \) by DR plotting.

The reversibility of the slow binding step was monitored by filtration of C*T complex through a cellulose nitrate filter, after which the filter was washed with buffer A in the presence or absence of polyamines and then immersed in 5 ml of the same buffer containing 2 mM puromycin for several time intervals. The rate constant of the enzymatic activity regain, \( k_{off,T} \), was estimated from the slope of the ln[100/(100 - x)] versus time plot. With \( k_{on,T} \) and \( k_{off,T} \) known, the equilibrium constant of the isomerization step (isomerization constant), was calculated after dividing \( k_{on,T} \) by \( k_{off,T} \).

**Inactivation of Complex C by Erythromycin**—Although erythromycin does not inhibit AcPhe-puromycin synthesis, it competes with tylosin for common binding sites (6). Assuming that erythromycin, like tylosin, binds to complex C via a two-step mechanism, the competition between erythromycin (E) and tylosin (T) for complex C can be described by kinetic Scheme 2.

This was exploited as follows: complex C was added into buffer A containing 4 \( \mu \)M tylosin and erythromycin at increasing concentrations. The apparent rate constant of inactivation, \( F \), estimated from the slope of inactivation plots, is given by Equation 3 (for the derivation of the kinetic equations, see supplemental data),

\[
F = F_o + (F_o - F_s) e^{-k' t}
\]

(Eq. 3)

where \( F_o \) and \( F_s \) are the inactivation constant at the steady state and zero time, respectively, and \( k' \) is the apparent equilibration rate constant for the attainment of equilibrium between complex C and the drugs. The \( k' \) value is related to the concentrations of erythromycin and tylosin by Equation 4,

\[
k' = \frac{k_{off,E}}{K_E} + \frac{k_{off,T}}{K_T}
\]

(Eq. 4)

\( F_o \) and \( F_s \) are related to the concentrations of the drugs by Equations 5 and 6, respectively,

\[
F_o = \frac{k_{on,T} [T]}{K_T \left(1 + \frac{[E]}{K_E} + [T]\right)}
\]

(Eq. 5)

\[
F_s = \frac{k_{on,T} [T]}{K_T \left(1 + \frac{[E]}{K_E} + [T]\right)}
\]

(Eq. 6)

where the above equations use the following.

\[
k^* = \frac{k_{off,E}}{k_{off,T} + k_{off,E}}
\]

(Eq. 7)

The values of \( k_{on,E} \), \( k_{off,E} \), and \( K_E \) were calculated by nonlinear regression fitting of the kinetic data to Equation 8,

\[
\ln x = 4.6 - t \times \frac{(F_o - F_s)}{k''} \left(1 - e^{-k'' t}\right)
\]

(Eq. 8)

which is derived from Equation 3 by integration.

**Binding of Antibiotics and Probing of Complexes**—Complex C at 100 nm was incubated alone or with antibiotics (I) at concentration equal to 50 \( \times \) \( K_i \) in 100 \( \mu \)l of buffer B (HEPES-KOH, \( \text{pH} \) 7.2, 4.5 mM Mg(CH\(_3\)COO)\(_2\), 150 mM NH\(_4\)Cl, 5 mM dithiothreitol) at 25 °C, either for 10 s or for 8 \( \times \) \( t_{1/2} \) min, dependent on whether the encounter complex CI or the final complex C*I was desired to be probed, respectively. The term \( t_{1/2} \), which represents the half-life for the attainment of equilibrium between complex C and the drug, was calculated through the relationship,

\[
t_{1/2} = \frac{0.693}{k''}
\]

(Eq. 9)

where \( k'' \) is the apparent equilibration rate constant, given by Equation 10.

\[
k'' = k_{off} + k_{on} \frac{[I]}{K_i + [I]}
\]

(Eq. 10)

Complexes CI or C*I were then probed at 37 °C for 10 min with DMS (3 \( \mu \)l of a 1:5 dilution in ethanol), kethoxal (5 \( \mu \)l of a 35 mg ml\(^{-1}\) solution in 20% ethanol), or CMCT (100 \( \mu \)l of a 42 mg ml\(^{-1}\) in buffer B). Reactions were stopped according to Stern et al. (32), and the rRNA was recovered from CI or C*I complexes by sequential extraction with phenol, phenol/chloroform, and chloroform, and by ethanol precipitation, before redissolving in Milli-Q water.

**Analysis of Chemical Footprinting by Primer Extension**—Identification of the modified sites in 23 S rRNA was achieved by primer-extension analysis according to Stern et al. (32), making use of the fact that reverse transcriptase reaction stops

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one position before a modified nucleoside by DMS (N1 position of adenine and the N3 of cytosine), kethoxal (N1 and N2 positions in guanine), or CMCT (N3 position of uracil and N1 of guanine). Helices H29–H35, H71–H76, and H86–H95 in 23S rRNA were screened using primers complementary to the sequences 888–906, 2099–2116, 2561–2578, and 2677–2694. Extension products were run on 6% polyacrylamide, 7 M urea gels. In parallel, dideoxy sequencing reactions were run on an unmodified rRNA template. Four replications were done for each experiment. Alterations in chemical modification of 23S rRNA residues were estimated by quantitative scanning of the gels (PhosphorImager, Fujifilm, FLA-3000, Berthold; ImageQuant software AIDA, Raytest). Background values in the “control” lanes were subtracted from the corresponding bands of interest. Each lane was searched for a reference nucleotide whose accessibility to a chemical probe is not affected by drug binding, and the intensity of the reference band was used to normalize the intensities of the bands of interest in this lane (vertical comparison). The relative intensity of this reference band between lanes is then used to correct for variability between lanes (horizontal comparison). Finally, the normalized values were compared with those corresponding to modified complex C in the absence of antibiotics.

Statistics—All data indicated in the kinetic diagrams denote mean values obtained from four independent experiments. Data variability and significant differences between mean values were determined by one-way analysis of variance.

RESULTS

Inactivation of Complex C by Tylosin—In agreement with previous results obtained at 10 mM Mg\(^{2+}\) and 100 mM NH\(_4\)\(^+\) (6), tylosin inhibits AcPhe-puromycin synthesis at 4.5 mM Mg\(^{2+}\) and 150 mM NH\(_4\)\(^+\), producing nonlinear progress curves whose initial slope and plateau vary as a function of the drug concentration (data not shown). The mechanism of tylosin binding to complex C was studied by exposing the ribosomal complex to various tylosin concentrations for several time intervals and titrating the remaining PTase activity by puromycin. Representative inactivation plots are shown in Fig. 2A. Each of them is characterized by a descending straight line suggesting that the inactivation of complex C follows pseudo-first order kinetics. The \(k_\text{in}\) values, estimated from the slope of each inactivation line, if plotted against the concentration of tylosin, give a rectangular hyperbolic curve suggesting that a two-step mechanism of binding may exist (kinetic Scheme 1). Consistently, the DR-plot is given by a straight line intersecting the vertical axis at a point above zero (Fig. 2B). The values of \(K_T\) and \(k_\text{on, T}\) determined from this plot are given in Table 1. The linearity of the plots shown in Fig. 2B suggests irreversible inhibition (\(k_\text{off, T} = 0\)). Nevertheless, a very low value of \(k_\text{off, T}\) may be escaping somewhat. To distinguish irreversible from slowly reversible inhibition, complex C*T was diluted into a large volume of buffer containing near-saturating puromycin. A very slow recovery of activity is actually observed at the steady state (Fig. 2C). The value of \(k_\text{off, T}\) measured in this manner is also shown in Table 1.

In a previous study we observed that spermine, the most effective of the naturally occurring polyamines in stabilizing the
RNA folding, inhibits the binding of spiramycin, another 16-membered lactone ring macrolide (33). This prompted us to re-examine the interaction of tylosin with complex C in the presence of spermine. We found that the affinity of tylosin during the first step of binding is enhanced by 50% at 100 μM spermine, whereas the isomerization constant, $k_{\text{on}}/k_{\text{off,D}}$, is reduced by 72%. By using 50 μM spermine and 2 mM spermidine instead of spermine alone, the isomerization constant was further decreased (Table 1).

Nucleosides U2609 and U754 of 23 S rRNA are both located in regions implicated in tylosin binding and placed on the side of the exit tunnel that is opposite to that forming the A2058–A2059 hydrophobic crevice (10, 12, 15, 19). Mutations U754A reduce the dissociation constant of the encounter complex CT, although both have a detrimental effect on the formation or stability of complex C*T (97 and 95% reduction of the isomerization constant, respectively; Table 1).

Binding of Erythromycin to Complex C—Previous studies have indicated that erythromycin does not inhibit the AcpPhemuporycin synthesis under conventional ionic conditions (10 mM Mg$^{2+}$, 100 mM NH$_4$Cl) (6, 12). In agreement with these studies, we observed that erythromycin at 4.5 mM Mg$^{2+}$ and 150 mM NH$_4$Cl also fails to inhibit this model reaction. In fact, erythromycin at concentrations ranging from 0.5 to 10 μM stimulates PTase activity by 20% (data not shown). This renders the study of erythromycin interaction with complex C impossible, if we try to follow the experimental approach applied for tylosin. Nevertheless, erythromycin competes with tylosin for binding to complex C (6, 34). This allows investigation of the binding process of erythromycin by competition kinetic experiments. As shown in Fig. 3, when complex C is incubated in a solution containing tylosin at 4 μM and erythromycin at increasing concentrations, it produces nonlinear inactivation curves whose initial slopes vary as a function of erythromycin concentration. High concentrations of erythromycin (>10 μM) provide complex C with full protection against tylosin. Both observations support the hypothesis that erythromycin competes with tylosin for binding to complex C, following kinetic Scheme 2. The values of $k_{\text{on,E}}$, $k_{\text{off,E}}$, and $K_E$ estimated by nonlinear regression fitting of kinetic data obtained at 4.5 mM Mg$^{2+}$ and 150 mM NH$_4$Cl for Equation 8, are shown in Table 1. By repeating the experiments in the presence of 100 μM spermine, we observed that the $K_E$ value undergoes a 6-fold enhancement, whereas the isomerization constant is decreased by 52%. Similar alterations were recorded with complex C reacting in a mixture of 50 μM spermine and 2 mM spermidine (Table 1).

![FIGURE 3. Effect of erythromycin on the inactivation of complex C by tylosin.](image)

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### Table 1

| Constant (unit) | 4.5 mM Mg$^{2+}$, 150 mM NH$_4$Cl | Ionic conditions |
|-----------------|----------------------------------|------------------|
|                 | Wild type | U2609C | U754A | 4.5 mM Mg$^{2+}$, 150 mM NH$_4$Cl, 100 μM spermine, wild-type | 4.5 mM Mg$^{2+}$, 150 mM NH$_4$Cl, 50 μM spermine, wild-type | 10 mM Mg$^{2+}$, 100 mM NH$_4$Cl, wild-type |
| Tylosin $K_E$ (μM) | 2260 ± 260 | 1900 ± 153 | 1730 ± 108 | 1180 ± 87 | 1038 ± 61 | 3000 ± 30 |
| $k_{\text{on}}$ (min$^{-1}$) | 1.23 ± 0.18 | 0.38 ± 0.03 | 1.17 ± 0.07 | 0.53 ± 0.04 | 0.39 ± 0.02 | 1.50 ± 0.04 |
| $k_{\text{off,T}}$ (min$^{-1}$) | 0.0015 ± 0.0001 | 0.016 ± 0.001 | 0.028 ± 0.002 | 0.0023 ± 0.0002 | 0.0033 ± 0.0001 | 0.0025 ± 0.0002 |
| $k_{\text{on,E}}/k_{\text{off,E}}$ | 820 ± 132 | 241 ± 7.5 | 41.8 ± 3.5 | 230.4 ± 24.2 | 118.2 ± 6.8 | 600 ± 150 |
| $K_E^{s}$ (μM) | 2.75 ± 0.54 | 75.81 ± 9.87 | 40.43 ± 4.29 | 4.99 ± 0.65 | 8.84 ± 0.71 | 4.99 ± 0.42 |

**Erythromycin $K_E$ (μM) | 83.0 ± 7.9 | 75.0 ± 4.9 | 89.0 ± 6.5 | 512.0 ± 42.9 | 425.0 ± 26.7 | 390.0 ± 16.0 |
| $k_{\text{on}}$ (min$^{-1}$) | 0.39 ± 0.03 | 0.40 ± 0.02 | 0.36 ± 0.03 | 0.35 ± 0.03 | 0.29 ± 0.02 | 0.60 ± 0.07 |
| $k_{\text{off,T}}$ (min$^{-1}$) | 0.054 ± 0.004 | 0.050 ± 0.003 | 0.065 ± 0.005 | 0.100 ± 0.008 | 0.098 ± 0.006 | 0.060 ± 0.005 |
| $k_{\text{on,E}}/k_{\text{off,E}}$ | 7.26 ± 0.87 | 8.00 ± 0.73 | 5.57 ± 0.25 | 3.49 ± 0.39 | 3.00 ± 0.25 | 10.00 ± 1.43 |
| $K_E^{s}$ (μM) | 10.10 ± 1.42 | 8.33 ± 0.87 | 13.54 ± 1.51 | 114.0 ± 13.8 | 106.0 ± 9.5 | 36.0 ± 4.83 |
shown in Table 2, tylosin at the CT binding state and in the probing data obtained should be considered as minimal. As modifications or stable structures of the wild-type 23 S rRNA, during the treatment of ribosomes or from post-transcriptional were observed in control samples arising from cuts incurred in Table 2. Because autonomous pauses of reverse transcriptase 5, and the modified nucleosides by the probes are summarized obtained by primer-extension analysis, are shown in Figs. 4 and 10.

Representative autoradiograms lanes 3

lanes 4

lanes 5

lanes 6

lanes 7–12

lanes 1

lanes 2

lanes 8

lanes 9

lanes 10

lanes 11

lanes 12

absence of polyamines strongly protects A2058 and A2062 from DMS, U2609 from CMCT, and G2505 from kethoxal modification, while enhancing the accessibility of DMS to A792. Weak protection effects on A2059, A2572, U2506, and surprisingly on C2611 are also seen, despite the fact that the latter position is not normally accessible to DMS, because of base-pairing with G2057. After longer exposure of complex C to tylosin, protection effects appear on A752, A788, U2609, and U2506 protections become stronger, whereas the accessibility of A792, A2058, and A2062 to DMS is slightly enhanced. In the presence of polyamines, the protections of A2058, A2059, and U2609 in complex CT soften, whereas the protection of A2062 is abolished. In contrast, the protections of U2506 and A2572 become stronger. In complex C*E, polyamines cause in general a stimulatory effect on the accessibility of all nucleotides implicated, except for A792 and A2572. Interestingly, in the presence of polyamines, DMS fails to modify C2611 in CT or C*T complex, nor can it modify complex C. Therefore, it is easy to envisage that at low Mg2+ concentrations, polyamines correct the local ribosomal conformation by re-establishing the C2611/G2057 base pair.

Erythromycin in the CE binding state and in the absence of polyamines strongly protects A2058 and G2505, whereas slightly decreasing the accessibility of A2059 and U2609. A tentative protection effect on A788 is often observed but in general, this footprint is very weak. Whereas the protection of A2058, A2059, and G2505 in complex C*E is reduced, a fact reminiscent of that observed in C*T, alterations in the reactivity of other bases are less evident than those seen in the C*T complex and, in some cases (A752, A2062), are in the opposite direction. In the presence of polyamines, the protection of A2058 in complex CE is reduced. Similar reduction is also observed in the protection of A2059 and U2609 in complex C*E, whereas enhancement in the accessibility of A752 is suppressed.

DISCUSSION

In recent years, the crystal structures of several macrolide antibiotics complexed with the large ribosomal subunit were
published, providing important insights into the molecular basis of antibiotic action and explaining how alterations to ribosomal components confer resistance (reviewed in Ref. 3). Nevertheless, the ribosome is a dynamic machine and, therefore, crystal structures should not necessarily be assumed to offer a complete picture of the drug binding process. In the present work, we use a combination of chemical footprinting and pseudo-first order kinetic analysis to investigate the entire course of tylosin and erythromycin binding to E. coli ribosomes. At the same time, we seize the chance to monitor the effects of the ionic environment on the binding process and to discuss them on the basis of the flexibility of the ribosome.

In agreement with previous studies carried out at 10 mM Mg$^{2+}$ and 100 mM NH$_4$$_2$$O}$, the binding of tylosin to the ribosome at 4.5 mM Mg$^{2+}$ and 150 mM NH$_4$$_2$$O}$ is accomplished via a two-step mechanism. This is supported by two experimental findings: first, the initial slopes of the semilogarithmic time plots of the puromycin reaction vary as a function of the inhibitor concentration (data not shown) and, second, the $k_{on}$ values if plotted against tylosin concentration give a rectangular hyperbolic curve. On the other hand, the apparent association rate constant of binding, $(k_{on,T} + k_{off,T})/K_d$ equals 8.86 × 10$^3$ M$^{-1}$ s$^{-1}$, a value well below the upper limit of 10$^6$ M$^{-1}$ s$^{-1}$, set for the characterization of a drug as a slow binding slowly reversible inhibitor (36). In addition, the values of the $k_{on,T}/k_{off,T}$ ratio is much higher than one (Table 1). Collectively, these findings suggest that an initial recognition step rapidly equilibrates is followed by a slower drug accommodation step, which falls within the minute time scale. Regarding the $K_T$ value, we conclude that at 4.5 mM Mg$^{2+}$ the affinity of tylosin for complex C during the initial step of binding becomes higher than that previously found at 10 mM Mg$^{2+}$ (6). Moreover, the isomerization constant $k_{on,T}/k_{off,T}$ undergoes a 37% increase. Instead, polyamines exert a beneficial effect on the initial step of binding by lowering the $K_T$ value, but exhibit a negative, more pronounced effect on the isomerization step. The optimized polyamine buffer (50 μM spermine and 2 mM spermidine) was found to exhibit the strongest effect; a 48% reduction of $K_T$ value and a 6.8-fold decrease of the $k_{on,T}/k_{off,T}$ value.

The erythromycin binding mechanism resembles that exhibited by tylosin. The relative low value of the apparent association constant, $(k_{on} + k_{off})/K_E = 8.95 × 10^3$ M$^{-1}$ s$^{-1}$, and the fact that the reverse rate constant, $k_{off,E}$ is less than the forward rate constant, $k_{on,E}$ allow us to classify erythromycin as a slow binding, slowly reversible drug. This finding confirms our previous results obtained at 10 mM Mg$^{2+}$ (6) and is in agreement with TRNOE measurements (28). Evidence supporting the two-step mechanism is derived from Fig. 3; the initial slopes of the inactivation plots vary as a function of erythromycin concentration. It should be mentioned here that the initial slope of each inactivation plot is equal to $F_o$ which is related to the erythromycin concentration by Equation 5. If erythromycin binding could proceed via a one-step mechanism, then $K_E = \infty$, and $F_o$ should be independent of erythromycin concentration. In a recent publication, Lovmar et al. (14) failed to detect a two-step mechanism for erythromycin binding to pre-initiated ribosomes, but their kinetic data still allow for comparisons to be made. It should be mentioned here that $K_E^*$ represents the overall dissociation constant, concerning both steps of erythromycin binding to complex C, and its value approximates numerically the classical $K_E$ value. We measure a $K_E^*$ value of 10.1 nM compared with a $K_q$ value of 10.8 nM obtained by these investigators. However, we observe somewhat slower kinetics for binding and dissociation. These differences may be explained as arising from different experimental conditions, in particular, from different temperature and ionic conditions upon which our and their experiments were performed. Also, another inconsistency, regarding the Mg$^{2+}$ effect, exists with the data obtained by Bertho et al. (28). They found by means of TRNOE analysis that the weak interaction between erythromycin and the ribosome is Mg$^{2+}$ independent, unlike the tight binding. In contrast, we found that both steps of erythromycin binding are affected by Mg$^{2+}$. Namely, a decrease of Mg$^{2+}$ concentration from 10 to 4.5 mM causes a 5-fold reduction in $K_E$ value and a smaller, but measurable reduction in the isomerization constant (Table 1). Such a strengthening of erythromycin binding upon decrease in Mg$^{2+}$ concentration has also been observed previously (26). Binding of erythromycin is influenced by polyamine at both steps; $K_E$ value becomes over 5-fold

| 23 S rRNA residue | Ionic conditions | Ionic conditions | Ionic conditions |
|-------------------|-----------------|-----------------|-----------------|
|                   | C   | CT  | C'T | CE | C'E | C   | CT  | C'T | CE | C'E | C   | CT  | C'T | CE | C'E |
| A752              | ++  | ++  | (+) | ++ | ++  | ++  | ++  | (+) | ++ | ++  | ++  | ++  | ++  | ++  | ++  | ++  |
| A788              | ++  | ++  | +   | ++ | ++  | ++  | ++  | +   | ++ | ++  | ++  | ++  | ++  | ++  | ++  | ++  |
| A792              | ++  | ++  | ++   | ++ | ++  | ++  | ++  | ++   | ++ | ++  | ++  | ++  | ++  | ++  | ++  | ++  |
| A2058             | ++  | ++  | ++   | ++ | ++  | ++  | ++  | ++   | ++ | ++  | ++  | ++  | ++  | ++  | ++  | ++  |
| A2059             | ++  | ++  | ++   | ++ | ++  | ++  | ++  | ++   | ++ | ++  | ++  | ++  | ++  | ++  | ++  | ++  |
| A2062             | ++  | +   | ++   | ++ | ++  | ++  | ++  | ++   | ++ | ++  | ++  | ++  | ++  | ++  | ++  | ++  |
| A2439             | (+) | +   | (+)  | (+) | (+) | 0   | 0    | 0   | 0  | 0   | 0    | 0    | 0    | 0    | 0    | 0    |
| G2505             | +   | 0   | (+)  | 0   | (+) | +   | 0    | (+) | (+) | (+) | (+)  | (+)  | (+)  | (+)  | (+)  | (+)  |
| U2506             | ++  | ++  | (+)  | ++ | ++  | ++  | ++  | +   | ++ | ++  | ++  | ++  | ++  | ++  | ++  | ++  |
| A2572             | ++  | +   | +    | ++ | ++  | ++  | ++  | +   | ++ | ++  | ++  | ++  | ++  | ++  | ++  | ++  |
| U2609             | ++  | +   | 0    | ++ | ++  | ++  | ++  | 0   | ++ | ++  | ++  | ++  | ++  | ++  | ++  | ++  |
| C2611             | +   | (+) | (+)  | (+) | (+) | 0   | 0    | 0   | 0  | 0   | 0    | 0    | 0    | 0    | 0    | 0    |

**TABLE 2**

Footprinting of tylosin and erythromycin in domain II and the central loop of domain V of 23 S rRNA, at the initial and final binding state

Reactivity against chemical probes is assigned as follows: ++ +++, very strong; ++ +, strong; ++, medium; +, weak; (+) very weak; 0, no modification. The quantification denotes the average results from four independent experiments. Abbreviations: C, complex C; CT, encounter complex between C and tylosin; C'T, final complex resulting from CT by isomerization; CE, encounter complex between C and erythromycin; C'E, final complex resulting from CE by isomerization.
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higher, whereas the isomerization constant undergoes a 50% reduction.

The slow binding interaction between complex C and the antibiotics under examination acquires biological significance. All macrolides block the entrance to the ribosomal tunnel, an event leading to arrest of protein synthesis during the early rounds of elongation. This eventually leads to drop off of peptidyl-tRNA from the ribosome (14). Complex CI is formed and dissociated very rapidly. If tylosin or erythromycin and a peptidyl-tRNA are simultaneously bound to a stalled ribosome, CI complex might dissociate before drop off of the peptidyl-tRNA. Subsequently, protein elongation is resumed rapidly, and the ribosome becomes refractory to the drug. However, in the case of C*I complex, the situation is different. The rate constant for drop off of a di- or a tripeptidyl-tRNA calculated by Lovmar et al. (14) is a hundred times higher than $k_{off,T}$ (Table 1), which implies that tylosin at saturation concentrations completely shuts down the synthesis of longer peptides. On the other hand, $k_{off,E}$ is ~40 times higher than $k_{off,T}$, a fact rendering erythromycin dissociation before drop off of the peptidyl-tRNA more probable. This can explain the higher activity of tylosin relative to erythromycin against permeable E. coli strains (17) and Gram-positive bacteria (37). Our results can also explain why a 16-membered lactam ring macrolide, such as tylosin, causes a longer suppression than erythromycin of optimal bacterial growth that persists after removal of the drug from the growth medium (post-antibiotic effect) (38), and why tylosin that dissociates much slower from the ribosome appears to be a poor inducer of erm genes (39). It is known that the inducible erm cassette consists of the ermC gene encoding a methyltransferase and a constitutively translated leader sequence that precedes ermC. According to a model suggested by Weisblum (40), the leader mRNA segment normally assumes a conformation in which the Shine-Dalgarno sequence and the initiation codon of the ermC gene are sequestered by secondary structure. In the presence of an inducer macrolide, binding of the drug to ribosomes engaged in synthesis of the leader peptide causes the translation to stall after a few rounds of elongation. In turn, stalling of ribosomes triggers a conformational change in the leader region resulting in the “opening” of the ermC gene. However, ribosomes translating the ermC gene should be free of the drug. Hence, induction of methyltransferase synthesis may significantly depend on macrolide concentration and drug dissociation from the ribosomes.

Insight into the structural characterization of complexes CI (encounter complex) and C*I (final complex) between each drug and ribosome was gained by footprinting analysis. As shown in Table 2, the footprinting pattern of complex CT does not significantly differ from that previously reported (10, 12), except for the absence of protection at A2572 and a slight enhancement of DMS reactivity at A792. Unexpectedly, C2611 in complex C exhibits a small, but measurable reactivity toward DMS that might be an indication that at 4.5 mM Mg$^{2+}$ and 150 mM NH$_4$ the local ribosomal conformation is changed. In complex CT, base C2611 is protected, a fact suggesting that this change is compensated upon binding of tylosin. Accommodation of tylosin at its final position (complex C*T) favors drug interaction with the groove formed by nucleosides A752, A788, and A792 in domain II of 23 S rRNA, but weakens the interactions with nucleosides A2058, A2062, and G2505 in domain V of 23 S rRNA (Table 2). The footprinting pattern of complex C*T bears better resemblance than the CT pattern to that published by other groups (10, 12) and generally correlates well with crystallographic data (19). This may be due to the fact that both chemical footprinting and crystallographic analyses have been performed on tylosin-ribosome complexes prepared under prolonged incubation with high concentrations of the drug. One could rationalize our findings by invoking a two-step mechanism of binding, where initially tylosin binds through its hydrophobic portion to residues of domain V and then the mycinose portion seeks out its binding pocket in domain II to initiate an insertion of the drug deeper into the tunnel. Corroborative evidence is coming from kinetic studies with ribosomes bearing mutations U2609C and U754A. Both mutants hinder the shift of tylosin to the high affinity site. As stated before, U2609 and U754 are placed on the same side of the exit tunnel, opposite to that forming the A2057–A2059 hydrophobic crevice. Nucleoside U754 base pairs with A743, and therefore it is easy to envisage that U754A mutation would disrupt H35 and thus indirectly affect the conformation of A752 that is believed to contact tylosin. In the presence of 100 μM spermine, the footprinting pattern of complex C is altered; the accessibility of A2572 and U2506 against DMS and CMCT, respectively, is reduced, whereas the reactivity of A2439 and C2611 against DMS is lost. This may be due either to the sensitivity of DMS and CMCT to compounds containing amino residues (41), or to a competition of spermine and chemical probes for binding to certain residues of 23 S rRNA. It is tempting to adopt the second hypothesis because first, a general depression of chemical probe reactivities is not observed and second, the alterations are restricted to nucleosides susceptible to spermine cross-linking (42). Beyond the effects of spermine on the modification of complex C by the chemical probes, alterations in the footprinting pattern of complex CT and C*T are also notable. Loss of protection at A2062 as well as a softening of A2058 and U2609 protections are evident in complex CT, whereas protections at U2506 and A2572 become stronger. In complex C*T, the effect of spermine on the accessibility of A2058 and A2062 remains the same, but A752 becomes protected. Consequently, it could be hypothesized that polyanines bound to the region of 23 S rRNA encompassing U2506 and A2572 facilitate the establishment by the ribosome of a complementary shape to the C5-disaccharide branch of tylosin, a fact explaining the positive effect of polyanines on the formation of the encounter complex CT. Spermine cross-linking to U2506 and A2572 has been already detected (42). On the other hand, the negative effect of polyanines on the overall affinity of complex C for tylosin may be related to the instability of the covalent bond formed between the ethylaldehyde substituent at the C6 position of the lactone ring and the N6 of A2062 (19). This bond is reversible and vulnerable to aminolysis by juxtaposed polyamines. Loss of the covalent bond to A2062 would, in turn, cause a shift in the A2058 position, weakening its interaction with mycinose.

In complex CE, erythromycin binding protects strongly nucleosides A2058 and G2505, whereas it causes a weak protection at A2059 and U2609. It has been demonstrated by crys-
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U2609, which has been postulated to interact with A752 (24, 43). Supportive is the finding that enhancement of A752 reactivity coexists with strong protection at U2609 (Table 2). Partial loss of A2058 interaction with erythromycin destabilizes drug binding to the hydrophobic G2057–A2059 crevice, resulting in a shift of erythromycin toward the other side of the tunnel where U2609 is placed. Consistent is the finding that mutation U2609C does not significantly change the kinetics of erythromycin binding to complex C, because neither the contact of position 2609 with the lactone ring, nor the interaction with A752 are interrupted (44). Similarly, mutation U754A, which could affect the interaction chain A754–A752–U2609, causes only small changes in the kinetics of shifting and the overall drug affinity (Table 1). These results are in excellent agreement with resistance and antibiotic binding data produced by Garza-Ramos et al. (13). Polyamines attenuate the erythromycin affinity for the ribosome at both steps of binding, by reducing the interactions of the drug with A2058 and U2609 (Table 2). This implies that polyamines affect diversely the binding process of tylosin and erythromycin. The molecular basis of this diversity is related to the distinct structural features of each macrolide. The positions and orientations of the tylosin and erythromycin lactone ring within the ribosomal exit tunnel, are depicted in Fig. 6. It is conceivable that the binding state that is adopted at the initial and final position of the drug accommodation is difficult to extrapolate from one macrolide to another. It is also difficult to distinguish at this time if certain interactions with 23S rRNA, such as interactions with U2609 and A752, are actually prerequisite or induced by the drug binding.

In conclusion, our results demonstrate that erythromycin and tylosin behave as slow binding inhibitors, following a two-step mechanism. Despite this apparent similarity, the interaction of each drug with the ribosome shows specific characteristics at both the transient and the final binding site, due to distinctive side chains that each drug possesses. These differences may explain the diverse influence of mutations or the ionic environment on the tightness of each drug-ribosome complex and the efficiency by which each drug shifts from the

![Erythromycin and Tylosin Binding](image)

**FIGURE 6.** Schematic representations of erythromycin and tylosin binding at the initial and final position of their accommodation within the ribosomal exit tunnel. The dashed line represents the tunnel axis, whereas the position of the PTase center is indicated by an arrow. A and B, illustrations of erythromycin and tylosin structures, respectively; the orientation of each drug is represented by four (tylosin) or two (erythromycin) connected circles reflecting the planes of the lactone ring and its sugar substituents. The plane of the cladinose sugar in erythromycin molecule is omitted for clarity. C, D and E, F, positional and orientational clustering of erythromycin and tylosin, respectively, at the initial (C and E) and final (D and F) sites within the exit tunnel. Representations are based on the results of the present study and on crystallographic data taken from Refs. 19 and 22.

tallography that A2058, A2059, and G2505 interact with the mycaminose portion, whereas U2609 forms a hydrogen bond with the OH-group at the C11 position of the lactone ring of erythromycin bound to the 50S ribosomal subunit of Deinococcus radiodurans (22). Our results also correlate well with TRNOE spectroscopy data regarding the weak binding of erythromycin to E. coli ribosomes (28). Significant alterations in erythromycin footprinting patterns occur, when the drug is incubated with complex C for a prolonged time; the reactivity of A752, A2058, A2062, and G2505 is enhanced, whereas U2609 is strongly protected. Enhancement of A2062 reactivity caused by erythromycin binding has also been recorded by Hansen et al. (11), although this effect is less obvious in another study performed by Poulsen et al. (12). Enhanced accessibility of A752 could be due to interaction of the lactone ring with
transient to the final site. Along with recent crystallographic advances on the localization of these drugs in ribosomes, this study provides a new basis for drug design. Thus, securing an orientation of lactone ring plane perpendicular with the tunnel and an efficient blocking of exit tunnel further away from the PTase center may cause a stronger inhibition of protein synthesis. In this case, monitoring of constants additional to \( K_i \) may be a prerequisite for rational drug design.

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