Unraveling New Features of Clindamycin Interaction with Functional Ribosomes and Dependence of the Drug Potency on Polyamines

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The effect of spermine on the inhibition of peptide-bond formation by clindamycin, an antibiotic of the Macrolide-Lincosamide-Streptogramin family, was investigated in a cell-free system derived from Escherichia coli. In this system peptide bond is formed by puromycin, a pseudo-substrate of the A-site, and acetylphenylalanyl-tRNA, bound at the P-site of poly(U)-programmed 70 S ribosomes. Biphasic kinetics revealed that one molecule of clindamycin, after a transient interference with the A-site of ribosomes, is slowly accommodated near the P-site and perturbs the 70 S/acetylphenylalanyl-tRNA complex so that a peptide bond is still formed but with a lower velocity compared with that observed in the absence of the drug. The above mechanism requires a high temperature (25 °C as opposed to 5 °C). If this is not met, the inhibition is simple competitive. It was found that at 25 °C spermine favors the clindamycin binding to ribosomes; the affinity of clindamycin for the A-site becomes 5 times higher, whereas the overall inhibition constant undergoes a 3-fold decrease. Similar results were obtained when ribosomes labeled with N\(^1\)-azidobenzamidino-spermine, a photo-reactive analogue of spermine, were used or when a mixture of spermine and spermidine was added in the reaction mixture instead of spermine alone. Polyamines cannot compensate for the effect of the biphasic kinetics at 5 °C nor can they stimulate the clindamycin binding to ribosomes. Our kinetic results correlate well with photoaffinity labeling data, suggesting that at 25 °C polyamines bound at the vicinity of the drug binding pocket affect the tertiary structure of ribosomes and influence their interaction with clindamycin.

Lincomycin and clindamycin (Fig. 1) are lincosamides, still used as therapeutic agents in human diseases and some animal infections. Clindamycin, a semisynthetic derivative of lincomycin (7(S)-chloro-7-deoxylincomycin), is usually more active than the parent compound in the treatment of bacterial infections, in particular those caused by anaerobic species (1).

Lincosamides act on the large ribosomal subunit and share an overlapping binding site with macrolides, streptogramins B, chloramphenicol, and other antibiotics affecting the PTase\(^2\) center, as deduced from competition experiments and cross-resistance data (for review, see Ref. 2) as well as from two-dimensional transferred nuclear Overhauser effect spectroscopy analysis (3). In agreement with most of these observations, chemical footprinting analysis (4, 5) and crystallographic studies (6, 7) have revealed that lincosamides interact with both the A-site and the P-site on the 50 S ribosomal subunit and would be expected to hamper the positioning of aminoacyl moieties of tRNAs at both sites. This last view is consistent with earlier binding studies investigating the competition for binding to ribosomes between lincomycin and 3'-terminus pentanucleotide fragments from N-AcLeu-tRNA\(^{Leu}\), Leu-tRNA\(^{Leu}\), and Phe-tRNA\(^{Phe}\) (8, 9) as well as with the finding that lincosamides stimulate oligopeptidyl-tRNA dissociation from ribosomes (10). Tenson et al. (11) suggested that lincosamides bind to ribosomes allowing a space of ~4.6 Å for nascent peptide progression toward the exit tunnel. Further peptidyl-transfer is inhibited by steric hindrance, and this inhibition eventually leads to peptidyl-tRNA dissociation. Therefore, an increased probability of ribosome drop-off can be caused by enhanced peptidyl-tRNA dissociation, decreased PTase activity, or a combination of both. In consequence, termination is also affected by both lincomycin and clindamycin (12). In addition to the inhibitory effect on protein synthesis, each lincosamide exhibits a specific inhibitory effect on the 50 S subunit formation, an activity shared with 16-membered macrolide and streptogramin B antibiotics (13).

Molecular modeling approaches have been used in at least three previous attempts to establish structural relationships between lincosamides and residues in the 3'-terminus of aminoacyl-tRNAs or hypothetic transition states and intermediates in protein biosynthesis (14–16). These hypotheses were later either contradicted by crystallographic evidence or never verified.

Despite considerable interest for elucidation of the lincosamide binding site in ribosomes, kinetic studies concerning the inhibitory effect of these drugs on PTase activity are scant and exclusively focused on lincomycin. This drug cannot affect peptidyl- puromycin synthesis on native polyribosomes but inhibits transpeptidation with washed ribosomes and diverse synthetic peptidyl donors (17). Besides competitive kinetics (18, 19), there is also evidence for a mixed-noncompetitive mode of
action (20) depending on the inhibitor concentration and the buffer system used. From kinetic and binding studies, it has been demonstrated that both monovalent and Mg\textsuperscript{2+} ions are essential components for the PTase activity and the binding of lincomycin to ribosomes (20–22). This is in agreement with the finding that lincomycin forms metal complexes in solution (23). In fact, recent crystallographic studies (6, 7) do not detect any metal ion involved in the binding of clindamycin; however, one Mg\textsuperscript{2+} ion is displaced upon clindamycin binding. In addition to monovalent and divalent ions, polyamines are also essential for establishing an optimum ionic environment for ribosomal activity (24). Interestingly, polycations sensitize enteric bacteria to clindamycin (25). Consequently, a prerequisite when performing in vitro experiments is to take into account the physiological conditions and to try as closely as possible to imitate them. Unfortunately, most of the kinetic studies concerning the binding of lincosamides to ribosomes and the inhibition of PTase by these drugs have been performed exclusively in the presence of conventional buffers. Moreover, they have been analyzed on the assumption that the equilibria involved in the interaction of the drug with the ribosome are attained instantaneously. In fact, there is experimental evidence that the lincosamide-ribosome interactions display biphasic kinetics (3, 19).

Contradicting observations have been published concerning the inhibitory power of clindamycin. NMR studies revealed that this antibiotic compared with lincomycin exhibits higher affinity for the ribosome (3), in agreement with in vivo studies carried out in common pathogenic bacteria including Escherichia coli (1). In another study, however, the in vitro affinity of lincomycin to E. coli ribosomes was reported to be higher than that of clindamycin (4).

Kinetic studies of clindamycin interaction with functional ribosomal complexes have never been performed in the past nor has the effect of polyamines on this interaction been investigated. In the present study we examine the interaction of clindamycin with a post-translocation complex of poly(U)-programmed ribosomes isolated from E. coli cells. We employ an experimental approach by utilizing a series of polyamine buffers and by analyzing the peptide bond formation in the presence of clindamycin as a pseudo-first-order reaction. This approach allows us to have a picture of the entire course of the reaction. The use of polyamine buffers not only better resembles the physiological ionic environment but also gives us the opportunity to kinetically reveal the contribution of polyamines in the interaction of clindamycin with ribosomes.

### EXPERIMENTAL PROCEDURES

#### Materials
Puromycin dihydrochloride (disodium salt), clindamycin, spermine tetrahydrochloride, and heterogeneous tRNA from E. coli were supplied by Sigma. 1-[2,3,4,5,6-\textsuperscript{3}H]Phenylalanine and [\textgamma-\textsuperscript{32}P\textsuperscript{5}ATP were purchased from Amersham Biosciences. Avian myeloblastosis virus reverse transcriptase was from Roche Diagnostics. dNTPs and dideoxy-NTPs were from Roche Applied Science. ABA-spermine was synthesized and purified according to Clark et al. (26). Cellulose nitrate filters (type HA; 24-mm diameter, 0.45-\mu m pore size) were from Millipore Corp. (Bedford, MA).

#### Biochemical Preparations
Salt-washed (0.5 M NH\textsubscript{4}Cl), polyamine-depleted 70 S ribosomes and partially purified translation factors were prepared from E. coli K12 cells as reported previously (27). Before their use, ribosomes were activated in buffer containing 20 mM magnesium acetate and 150 mM NH\textsubscript{4}Cl by incubation for 20 min at 42 °C. Samples were then cooled to 0 °C, and the Mg\textsuperscript{2+} concentration was normalized to 4.5 mM. Ac-[\textsuperscript{3}H]Phe-tRNA\textsuperscript{Phe} was prepared from heterogeneous E. coli tRNA, as described by Xaplanteri et al. (28). It was charged to 77%, 100% being 28 pmol of Phe per A\textsubscript{260} unit (Sigma). Post-translocation complex of poly(U)-programmed ribosomes, complex C, bearing tRNA\textsuperscript{Phe} at the E-site and AcPhe\textsuperscript{[\textsuperscript{3}H]}tRNA\textsuperscript{Phe} at the P-site was prepared in buffer A (100 mM Tris/\textit{HCl}, pH 7.2, 4.5 mM magnesium acetate, 150 mM NH\textsubscript{4}Cl, and 6 mM 2-mercaptoethanol) and purified as shown in the same study. Whenever desired, 100 mM spermine or 50 mM spermidine and 2 mM spermidine were also included in buffer A. In the presence of polyamines, more than 50% of the used ribosomes was converted to complex C. This fraction was almost fully reactive toward puromycin.

#### Photoaffinity Labeling

#### Mapping of ABA-Spermine Cross-linking Sites in 23 S rRNA, and Chemical Modification
Complex C was photolabeled with 100 \mu M ABA-spermine in HEPES buffer containing 4.5 mM Mg\textsuperscript{2+} and 150 mM NH\textsubscript{4}Cl, and the photolabeled product was purified as described elsewhere (28). The sites in 23 S rRNA to which ABA-spermine was cross-linked were identified by primer extension analysis, as described by Stern et al. (29).

#### Binding of Ac\textsuperscript{[\textsuperscript{3}H]}Phe-tRNA to the P and A sites of Poly(U)- programmed Ribosomes
—Puromycin dihydrochloride —Disodium salt, clindamycin, spermine tetrahydrochloride, and heterogeneous tRNA from E. coli were supplied by Sigma. 1-[2,3,4,5,6-\textsuperscript{3}H]Phenylalanine and [\textgamma-\textsuperscript{32}P\textsuperscript{5}ATP were purchased from Amersham Biosciences. Avian myeloblastosis virus reverse transcriptase was from Roche Diagnostics. dNTPs and dideoxy-NTPs were from Roche Applied Science. ABA-spermine was synthesized and purified according to Clark et al. (26). Cellulose nitrate filters (type HA; 24-mm diameter, 0.45-\mu m pore size) were from Millipore Corp. (Bedford, MA).

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Photoaffinity Labeling. Mapping of ABA-Spermine Cross-linking Sites in 23 S rRNA, and Chemical Modification—Complex C was photolabeled with 100 \mu M ABA-spermine in HEPES buffer containing 4.5 mM Mg\textsuperscript{2+} and 150 mM NH\textsubscript{4}Cl, and the photolabeled product was purified as described elsewhere (28). The sites in 23 S rRNA to which ABA-spermine was cross-linked were identified by primer extension analysis, as described by Stern et al. (29).

Binding of Ac\textsuperscript{[\textsuperscript{3}H]}Phe-tRNA to the P and A sites of Poly(U)-programmed Ribosomes—70 S ribosomes programmed with poly(U) were incubated for 30 min at 25 °C in buffer A containing 0.4 mM GTP and uncharged tRNAPhe at a molar ratio to ribosomes 1.5:1 to pre-fill the P-site. Subsequently, Ac\textsuperscript{[\textsuperscript{3}H]}Phe-tRNA was added and incubated for up to 30 min at 25 °C to allow A-site binding. Whenever required, 30 \mu M clindamycin was included in the binding buffer. The level of the A-site bound Ac\textsuperscript{[\textsuperscript{3}H]}Phe-tRNA was measured by nitrocellulose filtration. Bound at this site, Ac\textsuperscript{[\textsuperscript{3}H]}Phe-tRNA was almost no reactive toward puromycin. Total binding was measured by using poly(U)-programmed 70 S ribosomes with no prefilled P-sites. The P-site bound Ac\textsuperscript{[\textsuperscript{3}H]}Phe-tRNA was estimated from the total binding by titration with puromycin (2 mM, 2 min at 25 °C).
Kinetics of the Puromycin Reaction—The reaction between untreated or photolabeled complex C and puromycin (S) (Scheme 1) was performed at 5 or 25 °C in buffer A containing 5 mM ribosomes in the form of complex C and puromycin at concentrations varying from 0.1 to 2 mM (30). Whenever required, 100 μM spermine or 50 μM spermine and 2 mM spermidine were also included in the reaction mixture. The product (P), Ac[3H]Phe-puromycin, was extracted in ethyl acetate, and its radioactivity was measured in a liquid scintillation spectrometer. Background controls (minus puromycin) were subtracted. The product was expressed as percentage (x) of the radioactivity contained in complex C. The values of x, appropriately corrected (30), were fitted into Equation 1,

$$\ln \frac{100}{100 - x} = k_{\text{obs}} \cdot t$$

(Eq. 1)

where \(t\) is the reaction time, and \(k_{\text{obs}}\) is the pseudo-first-order rate constant. As previously justified (31, 32), the \(k_{\text{obs}}\) value is related to the puromycin concentration by Equation 2,

$$k_{\text{obs}} = \frac{k_3[S]}{K_s + [S]}$$

(Eq. 2)

where \(k_3\) represents the catalytic rate constant of PTase, and \(K_s\) is the dissociation constant of the encounter complex between puromycin and complex C. The \(k_3\) and \(K_s\) values were calculated from DR plots (1/\(k_{\text{obs}}\) versus 1/[S]) derived from Equation 2.

Kinetics of the Puromycin Reaction Inhibition by Clindamycin—In this series of experiments, complex C in buffer A was added to a mixture containing puromycin and clindamycin. At 25 °C, biphasic time-plots (ln[100/(100 - x)] versus \(t\)) were obtained. The initial slope of each curve was taken as a measure of the apparent rate constant (\(k_{\text{obs}}o\)) at the early phase of the reaction. Similarly, the slope of the slow-onset straight line was taken as the value of the apparent rate constant (\(k_{\text{obs}}\)) at the late phase of the reaction. Alternatively, the (\(k_{\text{obs}}o\)) and (\(k_{\text{obs}}\)) values were estimated by fitting the data to a two-exponential function. Both methods of data processing resulted in the same pseudo-first-order values. At 5 °C, single-phase time plots were obtained, and one value for \(k_{\text{obs}}\) was calculated.

Statistics—One-way of variance was used to estimate the mean value and data variability. Statistically significant differences were determined using the unpaired Student’s \(t\) test.

RESULTS

Effect of Clindamycin on the Binding of Ac[3H]Phe-tRNA to the A and P sites of Poly(U)-programmed Ribosomes—Binding experiments were performed at 25 °C under several ionic conditions in the presence or in the absence of clindamycin. To estimate the maximum level of binding, we followed the entire course of the reaction. As shown in Fig. 2, clindamycin hardly affects the binding of AcPhe-tRNA to both sites of poly(U)-programmed ribosomes if the binding is performed in the absence of polyamines; although a small increase in binding is visible in the presence of clindamycin, the differences from the control values are not statistically significant (\(p < 0.05\)). In accordance with previous results (24, 33), spermine or a mixture of spermine and spermidine stimulates the AcPhe-tRNA binding and enhances the stability of the formed 70 S initiation complex. The addition of clindamycin into the polyamine buffer improves further the AcPhe-tRNA binding to both sites but impairs the stability of the initiation ribosomal complex formed.
Clindamycin Interaction with Functional Ribosomes

Inhibition of AcPhe-Puromycin Synthesis by Clindamycin—
The reaction shown in Scheme 1 represents the synthesis of AcPhe-puromycin from complex C and puromycin in excess. It proceeds as an irreversible pseudo-first-order reaction in which C is converted to C', a species of complex C stripped of Ac[3H]Phe-tRNA and, thus, unable to react with puromycin for a second cycle. The integrated kinetic law expressed by Equation 1 predicts that the progress curve of AcPhe-puromycin synthesis is expressed by a straight line at each concentration of puromycin. The predicted linearity is indeed observed experimentally. A representative plot obtained at 200 μM puromycin and 25 °C is shown in Fig. 3A, upper line. However, when complex C reacts with a mixture of puromycin and clindamycin at 25 °C, the reaction displays biphasic kinetics, characterized by an initial phase followed by a late phase (Fig. 3A, lower line). The deviation from linearity is of vital importance in our analysis because it suggests a delay in the onset of inhibition. Analysis of the initial slopes by DR plotting and slope reploting (Figs. 3, B and C) leads to the identification of an initial phase of simple competitive inhibition, with one molecule of clindamycin participating in the mechanism of inhibition. This type of inhibition survives over a narrow range of inhibitor concentrations ([I] < 10 μM). The K_i value of 5.5 μM found at 4.5 mM Mg^{2+} and 150 mM NH_4^+ agrees well with the value of 8 μM for clindamycin affinity measured by footprinting analysis (4). However, it does not markedly differ from the K_i value for lincomycin so as to justify the superiority of the in vivo clindamycin potency. It should be mentioned that this inhibition constant determines only the initial encounter of clindamycin with complex C, ignoring late events of the drug-ribosome interaction. On the other hand, comparisons between in vivo and in vitro processes should be made cautiously, given that several factors in vivo may render one drug more potent than another.

With increasing concentrations of clindamycin the competition at 25 °C becomes stronger (Fig. 3C). We suppose that at high concentrations of clindamycin a new relation of the drug to the puromycin binding site is established. This alteration becomes more pronounced when the late phase of puromycin reaction is analyzed. As shown in Fig. 4A, with increasing concentrations of clindamycin the DR plots meet the negative 1/[S] axis at the same point, whereas the slopes of the lines increase, approaching a plateau (Fig. 4B). Such a behavior characterizes inhibitors of the partial-noncompetitive type. Nevertheless, analysis of the Fig. 4A by Hill-plotting (not shown) reveals that again, only one molecule of clindamycin participates in the
mechanism of inhibition. Combined, these results suggest that clindamycin (I) reacts transiently with complex C to form the encounter complex CI, which is then isomerized slowly to a more stable complex, termed C*I. The partial-noncompetitive inhibition established at the late phase also implies that complex C*I, contrary to complex CI, is capable of accommodating the substrate and producing AcPhe-puromycin albeit with a lower catalytic rate constant than $k_3$. The two phases of inhibition obtained at 25 °C are compatible with a model shown in Scheme 2.

We assume that at the late phase of the puromycin reaction, the unimolecular change of CI to C*I is at equilibrium. Consequently, $k_7[C*I] = k_6[CI]$. This also implies that P is produced via both the $k_3$ and $k_3^*$ steps. The $K_s$, $k_3^*$, $k_6$, and $k_7$ values estimated as described previously (Refs. 30 and 34; see also supplemental data) are presented in Table 1. According to these values, the overall association constant at 4.5 mM Mg$^{2+}$ and 150 mM NH$_4^+$, $k_{assoc}$, concerning both steps of the clindamycin interaction with complex C, is equal to 3.81 μM$^{-1}$ min$^{-1}$. As a consequence, the overall dissociation constant, $K_s/k_{assoc}$, becomes 13-fold lower than $K_s$ a fact suggesting that the affinity of complex C for the drug is much higher than that expressed just by the $K_s$ alone.

In contrast to the results seen at 25 °C, single-phase time plots were obtained at 5 °C. A representative plot obtained at 200 μM puromycin and 1 μM clindamycin is shown in Fig. 3A (midline). As revealed by detailed kinetic analysis, increasing the concentration of the drug does not alter the type of inhibition, which remains simple-competitive ($K_s = 5.6$ μM) throughout the time course of the reaction.

**Polymines Enhance the Inhibitory Effect of Clindamycin—** To reveal the effect of spermine on the clindamycin potency, we re-analyzed the mechanism of inhibition using complex C, which was prepared in buffer A containing 100 μM spermine and then interacted at 25 °C with puromycin in the same buffer. In agreement with previous results obtained at 6 mM Mg$^{2+}$ (30, 33), the addition of spermine at this concentration increases the $K_s$ value of PTase without affecting the $K_s$ dissociation constant (Table 1). Consequently, the ratio $k_6/K_s$ expressing the activity status of PTase is enhanced by 43%. It should be noticed here that the $K_s$ values estimated in the present study agree with previous literature values (31, 36, 37), but they are lower than those measured by fast kinetics (32). This discrepancy seems to be due to the different experimental conditions and substrates used and, more probably, to a different accommodation of puromycin into the catalytic center. Nevertheless, the thermodynamic behavior of both systems is similar (38).

Despite the stimulatory effect on PTase, spermine does not change the type of inhibition by clindamycin. Thus, clindamycin again inhibits peptide-bond formation at 25 °C by binding initially to complex C in competition with puromycin. Subsequently, a slow isomerization occurs, resulting in a tighter complex C*I that accepts puromycin but produces AcPhe-puromycin with a much lower catalytic rate constant. Nevertheless, the values of certain kinetic parameters differ from those obtained in the absence of spermine (Table 1). Namely, the $K_s$ value becomes 5-fold smaller. In addition, the $K_s$ value is reduced by 52%, whereas the $k_7$ value remains essentially constant. As a consequence, the overall association rate constant, $(k_6 + k_7)/K_s$, becomes three times higher, a fact that favors the formation of complex C*I. Similar changes in the kinetic parameters, but slightly less pronounced, are recorded if the polyamine buffer contains 50 μM spermine and 2 mM spermidine (Table 1).

The presence of polyamines cannot compensate for the loss of biphasic kinetics observed at 5 °C. Moreover, at low temperature polyamines fail to sufficiently stimulate the clindamycin binding to ribosomes ($K_s = 5.1$ μM).

**ABA-Spermine Cross-linking in 23 S rRNA Interferes with Clindamycin Binding to Complex C**—ABA-spermine is a photoreactive analogue of spermine, which has an arylazido group attached to one of the terminal amino groups of the molecule (28). The photoreactive analogue retains a charge in the vicinity of the nearest amino group, closely resembling spermine. This may explain the fact that ABA-spermine in the dark displays similar biological activity to that determined for spermine when used as a component of the ionic environment of ribosomes (27). Photolabeling of ribosomes with ABA-spermine under mild irradiation conditions results in covalent and specific binding of spermine with ribosomal proteins (27) and various groups in rRNA (28). Previous studies of our group have demonstrated that complex C photolabeled by ABA-spermine at 6 mM Mg$^{2+}$ and 100 mM NH$_4^+$ exhibits at 25 °C kinetic behavior in peptide bond formation similar to that observed with native complex C reacting with spermine free in solution (28). Kinetic analysis of complex C modified by 100 μM ABA-spermine under the present ionic conditions is in agreement with the abovementioned observations (Table 1). It should be noted here that less than 3% of ribosomes carry spermine cross-linked after removing the excess of photoprobe. Therefore, the rest of ribosomes should behave as native ribosomes in polyamine-free solution. It is suggested that the remaining ribosomes, although in the majority, play a secondary role in the overall kinetic behavior of complex C.

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*Derivation of the kinetic equations used in this report and data processing for estimation of the $K_s$, $k_3^*$, $k_6$, and $k_7$ values are provided as supplemental data.*
Clindamycin Interaction with Functional Ribosomes

TABLE 1
Equilibrium and kinetic constants derived from analysis of the inhibition of AcPhe-puromycin synthesis by clindamycin

Data represent the mean ± S.E. values obtained from duplicate analysis of three independently performed experiments. Significant different values (p < 0.05) from the corresponding values obtained at 4.5 mM Mg²⁺ and 150 mM NH₄⁺ (first column) are shown with an asterisk.

| Constant (unit) | 4.5 mM Mg²⁺, 150 mM NH₄⁺ | 4.5 mM Mg²⁺, 150 mM NH₄⁺, 100 μM spermine | 4.5 mM Mg²⁺, 150 mM NH₄⁺, 50 μM spermine, 2 mM spermidine | 4.5 mM Mg²⁺, 150 mM NH₄⁺, complex C photolabeled by ABA-stermine |
|----------------|--------------------------|------------------------------------------|-------------------------------------------------|------------------------------------------------------|
|                 |                         |                                          |                                                 |                                                     |
| kₙ (min⁻¹)      | 2.00 ± 0.02             | 2.86 ± 0.05*                             | 2.50 ± 0.05*                                   | 2.63 ± 0.05*                                         |
| kₚ (μM⁻¹ min⁻¹) | 486 ± 28                | 510 ± 22                                 | 505 ± 25                                       | 496 ± 24                                             |
| kₛ (μM⁻¹)       | 5.50 ± 0.30             | 1.04 ± 0.06*                             | 1.29 ± 0.07*                                   | 1.50 ± 0.09*                                         |
| kₗ (min⁻¹)      | 19.40 ± 1.20            | 10.20 ± 0.70*                           | 10.26 ± 0.60*                                 | 8.70 ± 0.60*                                         |
| kᵦ (min⁻¹)      | 1.35 ± 0.12             | 1.46 ± 0.10                              | 1.34 ± 0.08                                   | 1.21 ± 0.09*                                         |
| kᵣ (min⁻¹)      | 0.049 ± 0.022           | 0.052 ± 0.028                           | 0.044 ± 0.027                                  | 0.049 ± 0.028                                         |
| kᵦ/kᵣ (μM⁻¹ min⁻¹) | 404 ± 90                | 445 ± 95                                | 403 ± 87                                     | 412 ± 90                                             |
| kᵦ/kᵣ (μM⁻¹)   | 3.81 ± 1.20             | 11.20 ± 0.90*                          | 9.00 ± 0.77*                                    | 6.60 ± 0.72*                                         |
| kᵦ/kᵣ (μM⁻¹)   | 0.44 ± 0.13             | 0.13 ± 0.01*                            | 0.15 ± 0.01*                                    | 0.18 ± 0.02*                                         |

Prepared under these conditions. This may be explained by the fact that complex C formed in the absence of polyamines at 4.5 mM Mg²⁺ and 150 mM NH₄⁺ is very unstable (Fig. 2). In addition, the affinity of P-site for AcPhe-tRNA is much higher in ribosomes cross-linked to ABA-stermine compared with native ribosomes. Our results also show that photolabeled complex C behaves against clindamycin similarly to complex C interacting reversibly with polyamines (Table 1). Localization of the binding sites for spermine in 23 S rRNA by primer-extension analysis revealed no differences in the cross-linking pattern of ABA-stermine between the present work (Table 2) and a previous study performed at 6 mM Mg²⁺ and 100 mM NH₄⁺ (30). Interestingly, some of the ABA-stermine cross-linking sites coincide or are adjacent to nucleosides implicated in clindamycin binding to the large ribosomal subunit.

DISCUSSION

The results of this study demonstrate that at 25 °C clindamycin derivates its potency through interactions with both A and P sites. The initial accommodation of clindamycin in the ribosome interferes transiently with the binding of puromycin to the A-site (competitive kinetics). Soon after this initial interaction, the antibiotic is slowly accommodated in its final position (C*I complex), so that puromycin is accepted and peptide bond formation may be associated with the cross-linking of CI to C*I by clindamycin.

The apparent association rate constant of clindamycin binding at 25 °C, (kₚ + kᵦ)/kₛ, is equal to 6.33 × 10⁹ M⁻¹ s⁻¹, a value considerably lower than the upper limit of 10⁸ M⁻¹ s⁻¹ set for the characterization of a drug as a slow binding inhibitor (35). Moreover, the kᵦ value is less than that determined for the forward rate constant kᵦᵦ, rendering the dissociation of C*I the rate-limiting step of complex C recycling. The slow reversal of the C*I complex is consistent with studies on the post-antibiotic effect of the drug (retention of antibiotic activity even after the circulating levels of clindamycin have dropped below the minimal inhibitory concentration) (41) and, therefore, is of high significance in pharmaceutical applications.

The interference of clindamycin with both the acceptor and donor sites of the ribosomes is consistent with early biochemical studies (8, 9) and agrees with recent footprinting and crystallographic data that emphasize the A- and P-site character of clindamycin positioning within the catalytic cavity (4, 6, 7, 11). Nevertheless, the results of the present work, in agreement with some previous observations (42), suggest that clindamycin stimulates rather than inhibi-
its aminoacyl-tRNA binding to ribosomes. A hypothesis explaining the above controversial effects is that clindamycin directly or indirectly may block the access of the 3′-end of the aminoacyl-tRNA to the catalytic center without disrupting the interaction of the remaining tRNA backbone with the ribosome. The above interpretation is consistent with cross-linking studies (43), which indicate that the parent compound lincomycin does perturb the relative positioning of the 3′-terminal adenosine of P/P′-site-bound tRNA and the PTase loop region of 23 S rRNA. It is nevertheless possible that the interaction of the 3′ terminal sequence as well as other contacts of the tRNA body with the ribosome may stabilize an active conformation of the PTase center with different susceptibility against clindamycin. In conclusion, although the values of the kinetic parameters determined here might differ somewhat from the in vivo situation, the use of puromycin is appropriate to investigate the mechanism of clindamycin interaction with ribosomes.

Our results cannot support functional correlation of clindamycin with transition-state substrates or hypothetical intermediates in the peptide elongation cycle, as proposed by other studies (14–16). Both the dissociation constant (K_d) for the encounter complex CI and the apparent dissociation constant (k_d/k_assoc) for the final complex C*I are much higher than the K_d predicted for the transition state of the PTase-catalyzed puromycin reaction (38). This implies that the conformation of clindamycin either in complex CI or in complex C*I does not imitate transition-state structures. Nevertheless, to better quantify the extent to which clindamycin resembles the transition-state structure, one should correlate the dissociation constant of clindamycin with those for natural intermediates.

At 25 °C, the affinity of complex C for clindamycin is enhanced by the addition of polyamines or by prelabeling complex C with 100 μM ABA-spermine (Table 1). Instead, the K_d value is reduced by ~50%. Despite the negative effect on the isomerization of CI toward C*I, the contribution of polyamines to the formation of CI is beneficial and more pronounced. Consequently, the overall effect of polyamines on clindamycin potency is positive. Positive effects of the ionic environment on the interaction of lincosamides with ribosomes have been previously reported (20, 21). Thus, it was found that K^+ or NH_4^+ exerts a profound positive influence on lincomycin binding to ribosomes, whereas Mg^{2+} has no effect at concentrations between 0.5 and 50 mM. The present work complements these studies and supports the notion that polyamines cannot be simply replaced by Mg^{2+} in accordance with other functional studies (24) but in contrast to the opinion of some researchers in the field (32). Nevertheless, polyamines cannot stimulate the clindamycin binding to ribosomes at 5 °C.

To formulate a hypothesis explaining the polyamine effect at 25 °C, the detailed knowledge of polyamine binding sites on ribosome is a prerequisite. Progress in localizing binding sites for spermine in 50 S ribosomal subunits from E. coli has recently been made by our group (28, 30) using ABA-spermine as a photoprobe reagent. Although E. coli cells contain primarily spermidine and putrescine, we chose to use a photoreactive analogue of spermine because almost all the cellular functions can be fulfilled by this polyamine (44). In addition, its use offers many advantages to our analysis, as discussed previously (28). When adapting the photolabeling techniques to the present ionic conditions and comparing the obtained data with those already published (20, 30), we found no differences in the cross-linking of ABA-spermine to 23 S rRNA (Table 2). Among the cross-links identified within the central loop of domain V of 23 S rRNA, there are two regions of ABA-spermine cross-linking of high importance, a long stretch of strong cross-links around C2452 and a second weaker site at U2506 (Fig. 5). Various experimental approaches, including footprinting (4, 5), mutational analysis (40), and x-ray crystallography (6, 7), have indicated that both regions are located in the heart of PTase cavity and are implicated in the binding of lincosamides. Combined, these observations reveal that polyamines bind at the vicinity of the clindamycin binding pocket. Therefore, an interference of polyamines in the clindamycin binding could be expected. We suppose that attachment of polyamines to the ribosome may induce conformational changes to it, which reduce the entropic cost of clindamycin binding. It is worth noting that alterations in the tertiary structure of the central loop of domain V of 23 S rRNA, induced by ABA-spermine photoincorporation, have been identified recently by dimethyl sulfate-protection experiments (28). Nevertheless, the possibil-

**FIGURE 5. The binding site of clindamycin on the 50 S ribosomal subunit.** Clindamycin (white) interacts with 23 S rRNA from Deinococcus radiodurans through an extensive hydrogen bond network (dashed lines, PDB 1JZX, see Schlu¨ nzen et al. (6)). Nucleoside residues implicated in clindamycin binding (4–6) are shown with blue or orange, whereas nucleosides susceptible to ABA-spermine cross-linking are indicated with pink or orange. Nucleosides are numbered according to E. coli.
ity of a direct involvement of polyamines in the binding of clindamycin to ribosomes cannot easily be precluded.

In conclusion, our results demonstrate that clindamycin behaves as a slow binding inhibitor, affecting both the A and P sites of ribosomes. Polyamines bound at the vicinity of the clindamycin binding pocket favor the interaction of the drug with the ribosome. Both effects are temperature-dependent. In addition, the present work suggests that whenever a drug interaction with ribosomes is studied, the experimental system must be able to search possible late events of the interaction. If such events indeed occur, then the in vitro affinity of the drug for the ribosome is not expressed just by the $K_i$ alone but requires additional constants for full characterization. Naturally, many additional factors, such as membrane permeability, efflux systems, and enzymes modifying the target molecule or the antibiotic may influence the in vivo effectiveness of a drug, and this may be beyond the reach of most in vitro techniques.

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