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Slow Sequential Conformational Changes in *Escherichia coli* Ribosomes Induced by Lincomycin: Kinetic Evidence

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ABSTRACT

In a cell-free system derived from *Escherichia coli,* lincomycin produces biphasic logarithmic time plots for inhibition of peptide-bond formation when puromycin is used as an acceptor substrate and AcPhe-tRNA as a donor substrate. In a previous study, initial slope analysis of the logarithmic time plots revealed that the encounter complex CI between the initiator ribosomal complex (C) and lincomycin (I) undergoes a slow isomerization to C*I. During this change, the bound AcPhe-tRNA and lincomycin are rearranged to also accommodate puromycin, and this may account for the mixed noncompetitive inhibition ($K_i^* = 70\ \mu M$) established at higher concentrations of the drug. The above-mentioned effect was further investigated by analyzing the late phase of the logarithmic time plots. It was found that C*I complex reacts with a second molecule of I, giving C^*I_2 complex. However, the logarithmic time plots remain biphasic even at high concentrations of lincomycin, making possible the identification of another inhibition constant $K_i^{*\prime}$, which is equal to 18 μM. The simplest explanation of this finding is to assume the existence of a second isomerization step $C^*I_2 \rightleftharpoons C^*I_2'$, slowly equilibrated. The determination of $K_i^{*\prime}$ enables us to calculate the isomerization constant ($K_{isom} = 2.9$) with the formula $K_i^{*\prime} = K_i^*/(1 + K_{isom})$. Our results suggest that whenever a fast and reversible interaction of lincomycin with the elongating ribosomal complex C occurs, the latter undergoes a slow isomerization, which may be the result of conformational changes induced by the drug.

The use of antibiotics is a valuable strategy to analyze the detailed mechanisms of peptide bond formation and elongation. Conversely, knowledge of the structure and function of ribosomes is absolutely necessary to understand the mechanism of antibiotic action on protein synthesis and to develop new drugs.

Lincomycin belongs to the macrolide/lincosamide/streptogramin group of antibiotics and inhibits protein synthesis in prokaryotic cells by binding to ribosomes bearing short peptidyl-tRNAs (Gale et al.,1981). Early studies (Fernandez-Muñoz et al., 1971; Contreras and Vázquez, 1977) suggested that a single molecule of lincomycin is reversibly bound per 70S ribosome with a dissociation constant of ~5 μM. These experiments, on their own, cannot be characterized as fully informative because they have been performed in the presence of 33% ethanol and have been analyzed on the assumption that the equilibria that involve the inhibitor, are attained instantaneously. However, we have recently shown (Kallia-Raftopoulos et al., 1992, 1994) that lincomycin behaves as a slow-binding inhibitor of peptidyltransferase (EC 2.3.2.12) activity. Moreover, it has been reported that lincomycin protects from chemical modification more than one position in the V loop of 23S rRNA (Douthwaite, 1992).

Considerable interest has been generated in elucidating the mechanism of lincomycin action on ribosomes. Thus, it has been reported that lincomycin inhibits translation termination (Caskey and Beaudet, 1971; Lin et al., 1997) and stimulates peptidyl-tRNA dissociation from ribosomes (Menninger and Coleman, 1993). One suggestion in agreement with these effects is that lincomycin primarily affects the entrance of the tunnel that channels the nascent peptides away from the peptidyl transferase center (Kirillov et al., 1997). These results also have been supported by footprinting and mutagenesis studies (Douthwaite et al., 1995). This explains why lincomycin does not inhibit peptide bond formation on isolated native polyribosomes (Pestka, 1972) or in intact cells (Burns and Cundliffe, 1973). Nevertheless, the drug effect on peptidyltransferase activity has been detected in model systems that use isolated 70S ribosomes and simple substrates. For instance, lincomycin inhibits the formation of fMet-puromycin in the fragment reaction (Gale et al., 1981) or the AcPhe-puromycin synthesis in ribosomal complexes bearing the donor AcPhe-tRNA already bound (Kallia-Raftopoulos et al., 1992, 1994). In addition, it significantly reduces A-site binding of the e-type with AcPhe-tRNA as the A-site ligand (Hausner et al., 1988) and inhibits CACCA-AcLeu binding to 50S ribosomal subunits (Celma et al., 1970), thus pointing to an interference with both A- and P-sites of the

Dedicated to the memory of Professor C. Coutsogeorgopoulos, who established research on protein synthesis in our laboratory.

ABBREVIATIONS: C, AcPhe-tRNA·poly(U)·ribosome; I, lincomycin; CI, encounter complex.

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ribosomes. Finally, molecular modeling approaches have been used to establish a structural relationship between lincomycin and residues of A- and P-substrates (Harris and Symons, 1973; Cheney, 1974).

With a cell-free system from *Escherichia coli* in which the ribosome participates in the form of the AcPhe-tRNA·poly(U)·ribosome (C), lincomycin (I) at 100 mM NH_4^+ produces biphasic logarithmic time plots for inhibition of peptide bond formation when puromycin is used as an acceptor substrate (Kallia-Raftopoulos et al., 1992). It has been postulated that the binding of lincomycin to the ribosome occurs in a two-step process; an initial, fast binding of the drug producing the encounter complex CI, followed by a slow isomerization to C*I responsible for the late phase of the biphasic logarithmic time plots. At higher ionic strength (150 mM NH_4^+) and with increasing concentrations of lincomycin, the inhibition pattern changes from competitive to mixed noncompetitive (Kallia-Raftopoulos et al., 1994). However, lincomycin still behaves as a slow-binding inhibitor and the time plots remain biphasic. In an attempt to further investigate the above-mentioned effect and to rationalize the behavior of lincomycin during the entire course of the reaction, we have extended our previous analysis to the late phase of the time plots. Our results support that the dissociation constant (K_i) of the encounter complex CI cannot be taken as a measure of the potency of lincomycin because it evaluates only the initial events of ribosome-drug interaction. Our observations are discussed on the basis of recent models for the formation of tRNA binding sites, especially those relevant to substrate movement through the peptidyltranferase center (Burkhardt et al., 1998).

Experimental Procedures

Materials. Puromycin dihydrochloride, GTP (disodium salt), ATP (disodium salt), phenylalanine, poly(U), and heterogeneous tRNA from *E. coli* strain W were obtained from Sigma Chemical Co. (St. Louis, MO). L-Phenyl-[2,3-^{3}H]alanine was purchased from Amersham Corp. (Arlington Heights, IL). Lincomycin was a gift from the Upjohn Company (Kalamazoo, MI). Cellulose nitrate filters (type HA, 24-mm diameter, 0.45-μm pore size) were purchased from Millipore Corp. (Bedford, MA).

Biochemical Preparations. Ribosomes from *E. coli* B cells, crude Ac[^{3}H]Phe-tRNA charged with 14.9 pmol of [^{3}H]Phe (86,000 cpm total) per A_{260} unit and complex C, i.e., the Ac[^{3}H]Phe-tRNA·poly(U)·ribosome complex that bears Ac[^{3}H]Phe-tRNA bound to the ribosomal P-site, were prepared as described previously (Kalpaxis et al., 1986). The formed complex C was adsorbed on cellulose nitrate filters and washed with three 4-ml portions of cold buffer [100 mM Tris-HCl, pH 7.2, 150 mM NH_4Cl, pH 7.2, 10 mM Mg^{2+} (acetate), 6 mM β-mercaptoethanol]. The adsorbed radioactivity was measured in a liquid scintillation spectrometer. Controls without poly(U) were included in each experiment, and the values obtained were subtracted.

Peptide Bond Formation Assay and First-Order Analysis. The reaction between complex C and excess puromycin (S) was carried out at 10 mM Mg^{2+} and 150 mM NH_4^+, as described elsewhere (Kallia-Raftopoulos et al., 1994). For comparison, some control experiments at 6 mM Mg^{2+} and 50 μM spermine also were performed. In the absence of lincomycin, the puromycin reaction

$$C + S \underset{}{\overset{K_S}{\rightleftarrows}} CS \xrightarrow{k_3} C' + P \qquad (1)$$

displayed pseudo-first-order kinetics and was analyzed as previously described (Synetos and Coutsogeorgopoulos, 1987; Kallia-Raftopoulos et al., 1994). The relationships

$$\ln \frac{100}{100 - x'} = k_{obs}t \qquad (2)$$

and

$$k_{obs} = \frac{k_3[S]}{K_S + [S]} \qquad (3)$$

hold, and the values of k_3 and K_S were determined from the double reciprocal plot of eq. 3 by linear regression.

In the presence of lincomycin, biphasic logarithmic time plots were obtained. The slope of the straight line through the origin was called initial slope and was taken as the value of the apparent rate constant (k) at the early phase of the puromycin reaction. Similarly, the slope of the second straight line was taken as the value of the apparent rate constant (k') at the late phase of the puromycin reaction.

Results

Progress Curve Analysis at Low Concentrations of Lincomycin ([I] < 60 μM). The progress curve of reaction 1, carried out at 10 mM Mg^{2+} and 150 mM NH_4^+, is a straight line at 200 μM puromycin (Fig. 1), corresponding to a k_{obs} value equal to 0.890 ± 0.08 min^{-1}. This value is similar to that obtained (k_{obs} =0.865 ± 0.07 min^{-1}) under conditions nearer to the in vivo ionic concentrations, i.e., at 6 mM Mg^{2+} and 50 μM spermine (Rheinberger and Nierhaus, 1987). However, when reaction 1 is carried out in the presence of lincomycin an initial as well as a late phase can be clearly seen in progress curves for all inhibitor concentrations tested (Fig. 1, lower lines and inset). Analysis of the early slopes (k) by double-reciprocal plots (not shown) and slope replot (Fig. 2A) confirmed the results of a previous study (Kallia-Raftopoulos et al., 1994), according to which lincomycin behaves as a competitive inhibitor over a narrow range of inhibitor concentrations ([I] <10 μM). Increase in the concentration of lincomycin alters the type of inhibition. This alteration becomes more pronounced when the late phase of puromycin reaction is analyzed; the corresponding slope replot (Fig. 2B) deviates from linearity and is characterized by a clear parabolic shape, indicating that there is more than one inhibitor

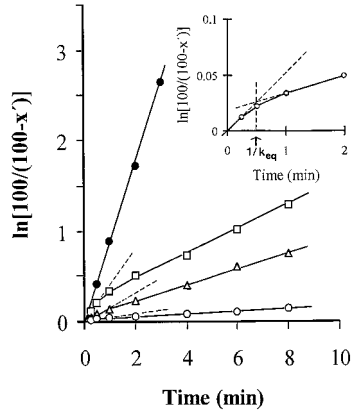

Fig. 1. First-order time plots for AcPhe-puromycin synthesis in the presence or absence of lincomycin. Complex C adsorbed on a cellulose nitrate filter, reacted in the presence of 10 mM Mg^{2+} and 150 mM NH_4^+, at 25°C, with (●) 200 μM puromycin or with a solution containing 200 μM puromycin and lincomycin at (□) 10 μM, (△) 30 μM, or (○) 200 μM. Inset, detail for the early stages of the reaction at 200 μM lincomycin.

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binding site (Segel, 1993). To evaluate the molecular order of lincomycin participation in the puromycin reaction, a modified formula of Hill equation (Kalpaxis and Drainas, 1993) was used, according to which the interaction coefficient (n) is given by the slope of the plot of $\log[k'/(k_{obs} - k')]$ versus log[I]. Such a plot obtained at 200 μM puromycin and at high concentrations of [I] (Fig. 3) is curved with limiting slope, which is two times greater than that obtained at low drug concentrations. Therefore, we can assume that two molecules of lincomycin are involved in the overall kinetic scheme of inhibition.

Progress Curve Analysis at High Concentrations of Lincomycin ([I] > 100 μM). Analysis of the initial slopes (k) by double-reciprocal plots, intercept replots, and slope replots confirmed previous results (Kallia-Raftopoulos et al., 1994) according to which lincomycin at high concentrations causes mixed noncompetitive inhibition on peptide bond formation. Given that in this range of drug concentrations two molecules of lincomycin bind the ribosome, a scheme that could adequately explain the above-mentioned kinetic results is presented in Fig. 4 (with the k_{10}/k_{11} step omitted). According to this model, lincomycin exhibits a transient phase of competitive inhibition followed by a mixed noncompetitive phase. Moreover, we assume that at high concentrations of I (>100 μM) the product comes mainly from C*I and not from C because the k_6/k_7 step is sufficiently slow (Kallia-Raftopoulos et al., 1994). However, the progress curves remain biphasic even at high concentrations of lincomycin (Fig. 1, inset). The deviation from linearity suggests a delay in the availability of complex C*I. Adopting the slow-onset inhibition theory (Morrison and Walsh, 1988) in our study, the apparent equilibration rate constant (k_{eq}) for the attainment of equilibrium between complex C and lincomycin can be determined from the intersection point of the two linear parts of the corresponding progress curve; at this point, $k_{eq} = 1/t$. For instance, the progress curve at 200 μM puromycin and 200 μM lincomycin gives a $k_{eq} = 2.09\ min^{-1}$ (Fig. 1, inset). Thus, the bimolecular rate constant ($k_{eq}/[I]$) associated with the binding of the second molecule of I equals $5 \times 10^2\ M^{-1}{\cdot}s^{-1}$. This value is much slower than $10^6\ M^{-1}{\cdot}s^{-1}$, which has been set as the upper limit for the characterization of slow-onset inhibition (Morrison and Walsh, 1988). Consequently, like the first molecule of lincomycin, the second one behaves as slow-binding inhibitor. The simplest explanation is to suggest, apart from the k_6/k_7 step, the existence of an additional slow isomerization step occurring after the attainment of the mixed noncompetitive equilibria.

Further analysis of the late phase of progress curves gives the double-reciprocal plots of Fig. 5 and the intercept replot of Fig. 6A, suggesting mixed noncompetitive type of inhibition (Segel, 1993). The straight line of Fig. 6A extrapolated meets the vertical axis at a point corresponding to the k_3* value, which is = $1.7\ min^{-1}$. Also, the [I]-axis intercept of the same plot gives a value for the equilibrium constant $\alpha K_i^* = 145$ μM. Both values are similar to those obtained by analyzing the initial phase of progress curves (Kallia-Raftopoulos et al., 1994). In contrast, the slope replots corresponding to the initial and late phases of the progress curves are given by different lines intercepting the vertical axis at a common point (Fig. 6B). Consequently, the second slow isomerization of the ribosomal complex is assigned to the k_{10}/k_{11} step (Fig. 4, part of the scheme in the box). The slope replot corresponding to the initial phase of logarithmic time plots, gives $K_i^* = 70$ μM. Similarly, the slope replot corresponding to the late phase of the logarithmic time plots gives $K_i^{*\prime} = 18$ μM. Once both K_i^* and $K_i^{*\prime}$ are calculated, the value of K_{isom} ($= k_{10}/k_{11}$) can be determined by the equation: $K_i^{*\prime} = K_i^*[k_{11}/(k_{10} + k_{11})]$, which is predicted by the slow-onset type of inhibition (Morrison and Walsh, 1988). This value equals 2.9.

Discussion

Kinetic studies provide a useful approach to gain insight into the mechanisms of antibiotic action. Lincomycin, like

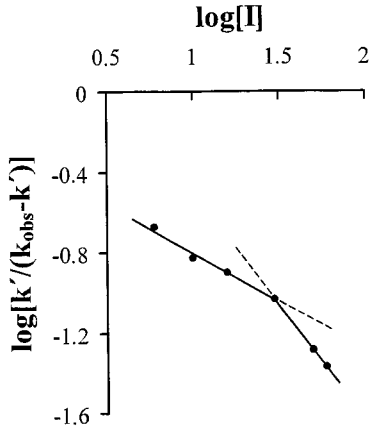

Fig. 3. Hill plot for the inhibition of puromycin reaction by lincomycin. Complex C adsorbed on a cellulose nitrate filter reacted with puromycin and lincomycin, as described in the legend of Fig. 1. The k' (in the presence of lincomycin) and k_{obs} (in the absence of lincomycin) values were calculated from the corresponding logarithmic time plots.

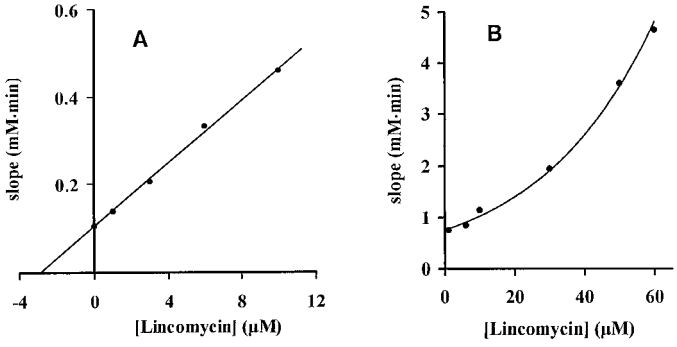

Fig. 2. Slope replots (slopes of double-reciprocal plots versus lincomycin concentration) for AcPhe-puromycin synthesis carried out at low concentrations of lincomycin. The data were obtained from double-reciprocal plot analysis of the early phase ($1/k$ versus 1/[puromycin]) (A) or of the late phase ($1/k'$ versus 1/[puromycin]) (B) of the logarithmic time plots of Fig. 1 and similar plots.

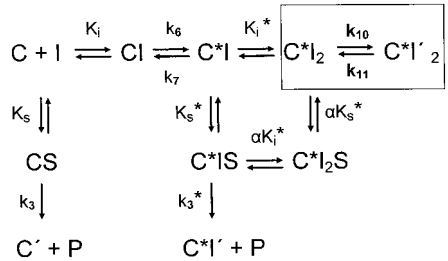

Fig. 4. Kinetic model for AcPhe-puromycin synthesis carried out in the presence of lincomycin, at 150 mM NH_4^+. Symbols: C, complex C; S, puromycin; I, lincomycin; C*I and C*I_2', modified complexes after their reaction with one or two molecules of lincomycin, respectively; P, AcPhe-puromycin; C′ or C*I′, complexes after their reaction with puromycin.

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other macrolide/lincosamide/streptogramin antibiotics, protects nucleotides within both the entrance to the peptide channel and the A- and P-substrate sites in the central loop of domain V of 23S rRNA (Douthwaite, 1992), where the peptidyltransferase center is thought to be located. Corroborative evidence is coming from studies on drug resistance conferred by relative mutations or base modifications in this region (Saarma and Remme, 1992; reviewed in Douthwaite et al., 1993, 1995; Spahn and Prescott, 1996). Because the effect of lincomycin on the ribosome is pleiotropic, the investigation of its biological action can be facilitated by dissecting the ribosome into functional subdomains and examining the drug effect on a certain subsite. We have, therefore, studied the inhibition of peptide bond formation by lincomycin with a system in which the ribosome participates in the form of AcPhe-tRNA·poly(U)·ribosome complex. We have succeeded to observe changes after the binding of the donor AcPhe-tRNA to the P-site. Furthermore, the analysis of puromycin reaction as a pseudo-first-order reaction provides us the means to trace the entire course of the reaction.

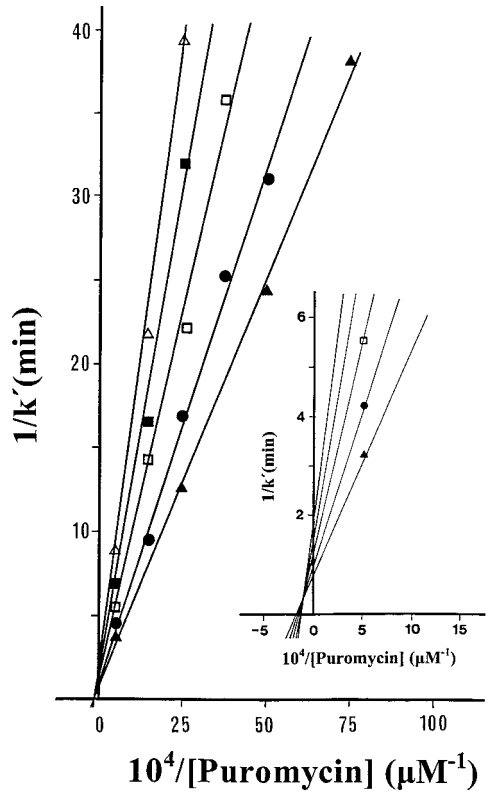

Fig. 5. Double-reciprocal plots ($1/k'$ versus 1/[puromycin]) for AcPhe-puromycin synthesis carried out in the presence of lincomycin. The data were obtained from the late slopes of the corresponding logarithmic time plots. The puromycin reaction was carried out at 150 mM NH_4^+ and 25°C, in the presence of lincomycin at (▲) 60 μM, (●) 100 μM, (□) 150 μM, (■) 200 μM, or (△) 300 μM. Inset, detail for the intercepts on the $1/k'$ axis.

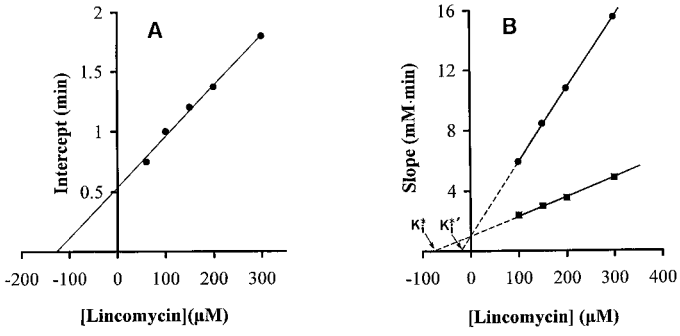

Fig. 6. Intercept replot (A) and slope replots (B) for AcPhe-puromycin synthesis carried out at high concentrations of lincomycin. The data were taken from the double-reciprocal plots of Fig. 5. For comparison, the corresponding slope replot (■) to the analysis of the initial phase of progress curves also is given.

In a previous work (Kallia-Raftopoulos et al., 1994), with the conventional analysis of the initial apparent rate constants, we demonstrated that lincomycin at high concentrations of NH_4^+ (150 mM) exhibits a biphasic inhibition pattern, i.e., a transient competitive phase followed by a mixed noncompetitive phase. The present investigation pertains to the analysis of the late apparent rate constants and leads to some interesting conclusions: The parabolic slope replot (Fig. 2B) corresponding to the transient phase of the inhibition pattern supports the assumption that two molecules of lincomycin are involved in the kinetic scheme of inhibition. Corroborative evidence is coming from the finding that exhaustive washing of the modified complex C*I shifts the equilibrium between complex C and I to the left and to almost complete recovery of complex C activity. This fact eliminates an alternative model for the interaction between lincomycin and complex C of the type

$$C + I \rightleftarrows CI \rightleftarrows C^*I \rightleftarrows C^* + I$$

It should be mentioned that lincomycin protects more than one site of the central loop of 23S rRNA (Douthwaite, 1992), although some of them may result from rRNA perturbation. One-to-one complex of lincomycin with the 50S subunit of the bacterial ribosome was reported during the 1970s (Fernandez-Muñoz et al., 1971; Contreras and Vázquez, 1977); however, it is relevant to note that these equilibrium dialysis studies have been carried out under the prevalent notion that the interaction between the ribosomal subunit (R) and the inhibitor (I) is expressed by a simple equilibrium of the form $R + I \leftrightharpoons RI$. The present work postulates that this equilibrium represents only the initial encounter between the ribosomal complex and lincomycin, and that the use of constants additional to K_i is required to describe late events of the inhibition pattern. Moreover, according to the previously reported kinetic scheme (Kallia-Raftopoulos et al., 1994) all the reactions involved in the mixed noncompetitive phase have been assumed rapidly equilibrated. However, this assumption is not consistent with the finding that at high concentrations of lincomycin the first-order time plots continue to be biphasic. The present analysis overcomes this discrepancy revealing that a second slow step of isomerization occurs after the binding of the second molecule of lincomycin. By comparing the intercept and slope replots obtained after analysis of the initial and late phases of the progress curves, we suggest that the second slow isomerization of the ribosomal complex is assigned to the k_{10}/k_{11} step (Fig. 4). It should be mentioned that the bimolecular rate constant associated with the binding of the second molecule of lincomycin has a value much lower than 1.1×10^4 $M^{-1}\cdot s^{-1}$, which characterizes the binding of the first molecule of lincomycin (Kallia-Raftopoulos et al., 1994). Considering the overall kinetic scheme of Fig. 4, it seems that, whenever a fast and reversible interaction of lincomycin with the ribosomal complex C occurs, the latter

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undergoes a slow isomerization. Given that the inhibition pattern is sensitive to ionic strength (Kallia-Raftopoulos et al., 1992, 1994), the slow isomerization events may arise from drug-induced conformational changes of the ribosome, permitting the movement of the drug closer to or/and into the catalytic cavity thus accounting for the mixed noncompetitive kinetics exhibited at higher concentrations of lincomycin. It should be mentioned that our hypothesis about lincomycin-induced conformational changes of the ribosome needs to be confirmed by additional physicochemical methods. Thus, it should be regarded as one of a number of possible explanations for the observed results.

Recent models for the formation of tRNA binding sites during the elongation cycle (reviewed in Burkhardt et al., 1998) provide us with the opportunity to rationalize further the molecular basis of inhibition by lincomycin. Taking into account the hybrid site model (Moazed and Noller, 1989), lincomycin should affect primarily the P-substrate site. Paradoxically, footprinting analysis (Douthwaite, 1992), binding studies (Fernandez-Muñoz and Vázquez, 1973; Odom and Hardesty, 1992) and kinetic data (Gale et al., 1981; Kallia-Raftopoulos et al., 1992, 1994) suggest that this drug is also potent in blocking the entrance to the tunnel that channels away the nascent peptides as well as the A-site in the catalytic center. This conflicting evidence may be overcome by assuming that lincomycin after binding to the entrance of the peptide channel, either extending into the catalytic center or changing allosterically the conformation of the peptidyltransferase center, may interfere with transition states or short-lived intermediate states of A- and P-substrates. Other investigators also have characterized lincomycin as a transition state analog (Kirillov et al., 1997; Fitzhugh, 1998). More recently, a powerful new approach of the elongation cycle mechanism in protein synthesis has been postulated, i.e., the α-ϵ model (Nierhaus et al., 1995). In terms of this model, lincomycin inhibits competitively the binding of puromycin by blocking the α domain of the α-ϵ conveyor. Subsequently, the initial encounter between ribosomal complex C and lincomycin is slowly rearranged, so that puromycin and a second molecule of drug also can be accommodated. This mode of action precludes the binding of the second molecule of lincomycin to the puromycin binding site (mixed noncompetitive inhibition) and suggests that it may interfere with the shift of the α-ϵ conveyor from the P-E to the A-P positions. Corroborative evidence comes from a previous study (Hausner et al., 1988), which has demonstrated a strong inhibition by lincomycin of the A-site occupation of the e-type (E-site occupied), in contrast to a marginal interference with the A-site occupation of the i-type (E-site free), i.e., an effect similar to that observed with the translocation inhibitors thiostrepton and viomycin. However, complete understanding of lincomycin interaction with the ribosome will take time until the resolution of the ribosome structure at the atomic level is elucidated.

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