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New Aspects of the Kinetics of Inhibition by Lincomycin of Peptide Bond Formation

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SUMMARY

We have investigated the inhibition of peptide bond formation by the antibiotic lincomycin, at 150 mM NH_4Cl. We have used an *in vitro* system in which a ribosomal ternary complex, the acetyl[^{3}H] phenylalanine-tRNA-70 S ribosome-poly(U) complex (complex C), reacts with puromycin, forming peptide bonds. Complex C can be considered an analog of the elongating ribosomal complex and puromycin an analog of aminoacyl-tRNA. In a previous study we reported on the kinetics of inhibition by lincomycin at 100 mM NH_4Cl. In the present investigation, we find that an increase of the ammonium ion concentration to 150 mM causes profound changes in the kinetic behavior of the system, which can be summarized as follows. First, the association rate for complex C and lincomycin is increased. At a lincomycin concentration of 10 μM the apparent equilibration rate constant is 4.3 min^{-1} at 100 mM NH_4Cl, whereas it becomes 6.7 min^{-1} at 150 mM. Second, at 150 mM NH_4Cl, with increasing concentrations of lincomycin, there is a transition from competitive to mixed-noncompetitive inhibition. The prevailing notion is that lincomycin acts at the ribosomal A-site, a mechanism that agrees only with competitive kinetics (mutually exclusive binding between puromycin and lincomycin). At the molecular level, the change in the kinetics of inhibition that we observe may mean that the mutually exclusive binding between aminoacyl-tRNA and lincomycin is converted to simultaneous binding, as a result of conformational changes occurring in the elongating ribosomal complex.

Lincomycin belongs to the macrolide/lincosamide/streptogramin group of antibiotics and inhibits protein synthesis in sensitive cells; more specifically, it inhibits peptide bond formation and induces polysome breakdown (1). In cell-free systems, inhibition of peptide bond formation catalyzed by ribosomal peptidyltransferase (EC 2.3.2.12) can be demonstrated only if peptidyl-donors (peptidyl-tRNA) bear short peptide chains, i.e., if they are not like those found on polysomes (2).

One way of studying peptidyltransferase has been to consider this putative enzymic activity as an isolable part of the protein-synthesizing machinery. The development of the fragment reaction (3, 4) is the most representative example of this "reductionist" approach, in which the large ribosomal subunit alone is allowed to catalyze peptide bond formation between amino-protected aminoacyl oligonucleotides and puromycin; the addition of organic solvents is also important for the reaction to occur. The fragment reaction has been used to study the influence of the ammonium ion and several antibiotics on peptide bond formation (5), but kinetic analyses have not been carried out. Studies on the mechanism of action of lincosamides (lincomycin, clindamycin, celesticetin, etc.) have attracted attention because their sites of binding on the ribosome may be related to specific ribosomal functions (6–8).

We have employed an alternative approach, which can be described as "holistic," in which whole ribosomal complexes, bearing the donor AcPhe-tRNA already bound, react with puromycin, forming peptide bonds (the puromycin reaction). Using this system, we have investigated the puromycin reaction and its inhibition by lincomycin. In a previous paper (9) we showed that, at 100 mM NH_4Cl, lincomycin inhibits the puromycin reaction, exhibiting competitive kinetics, and behaves as a slow-binding inhibitor. In the present communication we show that, at 150 mM NH_4Cl and with increasing concentrations of lincomycin, there is a change in the type of inhibition from competitive to mixed-noncompetitive and there is an increase in the velocity of interaction between lincomycin and the ribosomal complex (complex C).

Experimental Procedures

Materials

Lincomycin was a gift from the Upjohn Co. Cellulose nitrate filters (type HA, 24-mm diameter, 0.45-μm pore size) were purchased from

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ABBREVIATION: AcPhe, acetylphenylalanine.

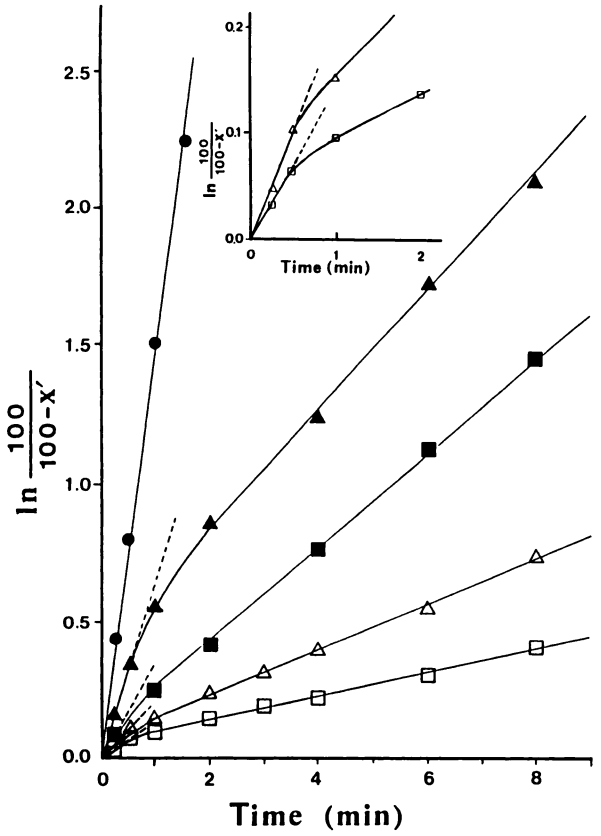

Fig. 1. First-order time plots for AcPhe-puromycin formation. Complex C reacted, in the presence of 150 mM NH_4Cl at 25°, with 0.4 mM puromycin (control) (●) or with a solution containing 0.4 mM puromycin and lincomycin at 10 μM (▲), 30 μM (■), 60 μM (△), or 200 μM (□). *Inset*, detail for the early stages of the reaction at the higher concentrations of lincomycin, 60 μM (△) and 200 μM (□).

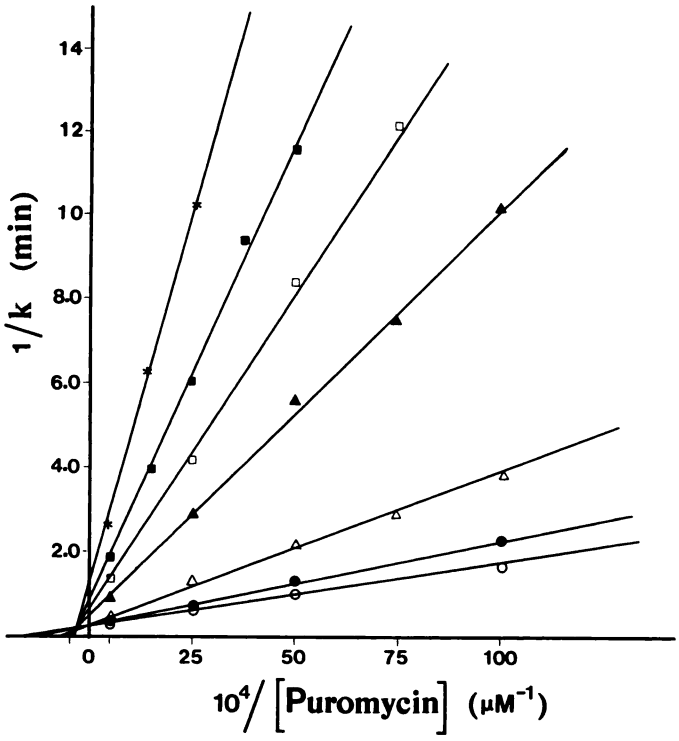

Fig. 2. Double-reciprocal plots (1/*k* versus 1/[puromycin]) according to the initial-slope analysis, in the presence of lincomycin. The data were obtained from the initial slopes of the logarithmic time plots of Fig. 1 and similar plots. The puromycin reaction was carried out at 150 mM NH_4Cl and 25°, with the indicated concentrations of puromycin in the absence of lincomycin (○) or with concentrations of lincomycin of 1 μM (●), 6 μM (△), 30 μM (▲), 60 μM (□), 100 μM (■), or 200 μM (*).

Millipore Corp. L-Phenyl[2,3-^{3}H]alanine was purchased from Amersham. Heterogeneous tRNA from *Escherichia coli* strain W, phenylalanine, poly(U), GTP (disodium salt), ATP (disodium salt), and puromycin dihydrochloride were from Sigma Chemical Co.

Methods

Preparation of Complex C. Washed ribosomes (0.5 M NH_4Cl), as well as the fraction of factors washable from ribosomes, were prepared from *E. coli* B cells as reported earlier (10). Ac[^{3}H]Phe-tRNA, charged with 14.9 pmol of [^{3}H]phenylalanine (82,000 cpm total/A_{260} unit), was prepared as described previously (10). Complex C, i.e., the Ac[^{3}H]Phe-tRNA-poly(U)-ribosome ternary complex that bears Ac[^{3}H]Phe-tRNA bound to the ribosomal P-site, was prepared in the presence of factors washable from ribosomes (11), adsorbed on cellulose nitrate filters, and washed twice with cold buffer (100 mM Tris·HCl, pH 7.2, 100 mM NH_4Cl, pH 7.2, 10 mM $MgCl_2$, 6 mM β-mercaptoethanol) and once with cold buffer containing 150 mM NH_4Cl. Complex C prepared under the conditions described above contained 37% of the input Ac[^{3}H]Phe-tRNA (3.2 A_{260} units/8.0 A_{260} units of ribosomes) and 8.5% of the ribosomes used.

Puromycin reaction. The puromycin reaction was carried out with complex C adsorbed on a cellulose nitrate filter, as reported previously (11). The reaction buffer contained 150 mM NH_4Cl and, whenever mentioned, lincomycin at the specified concentrations. If less than 100% of the bound Ac[^{3}H]Phe-tRNA (N_o) was converted to Ac[^{3}H]Phe-puromycin (P), the values of $x = (P/N_o) \cdot 100$ were corrected by division by the extent factor α, as if 100% of the bound Ac[^{3}H]Phe-tRNA were reactive toward puromycin. The value of the extent factor α used in the present experiments was 0.90 ± 0.03. Additional details on α are given in Ref. 11.

TABLE 1

Equilibrium and kinetic constants derived from primary and secondary kinetic plots

The K_s and k_3 values are calculated from the double-reciprocal plots in the absence of lincomycin (Fig. 2, *lower line*). The k_3* and αK_i* values are calculated from the intercept replot, on the assumption that, at high concentrations of I, product comes only from C*I. On the same assumption, the K_i* and K_s* values are calculated from the slope replot. The K_i value is calculated from the competitive phase of the reaction.

Parameter	Unit	Value for puromycin reaction at 25° with 150 mM NH_4Cl
k_3	min^{-1}	3.8
K_s	μM	400
k_3*	min^{-1}	1.7
K_s*	μM	1580
K_i	μM	3.0
K_i*	μM	70.0
αK_i*	μM	145.0

Initial-slope analysis of the puromycin reaction. The reaction between complex C and excess puromycin, in the presence of lincomycin, displays pseudo-first-order kinetics and was analyzed as described previously (12). The slope of the straight line through the origin is called the initial slope and is taken as the value of the apparent first-order rate constant (k).

Determination of the apparent equilibration rate constant k^I_{eq} (inactivation plots). To each one of a series of small beakers, 0.9

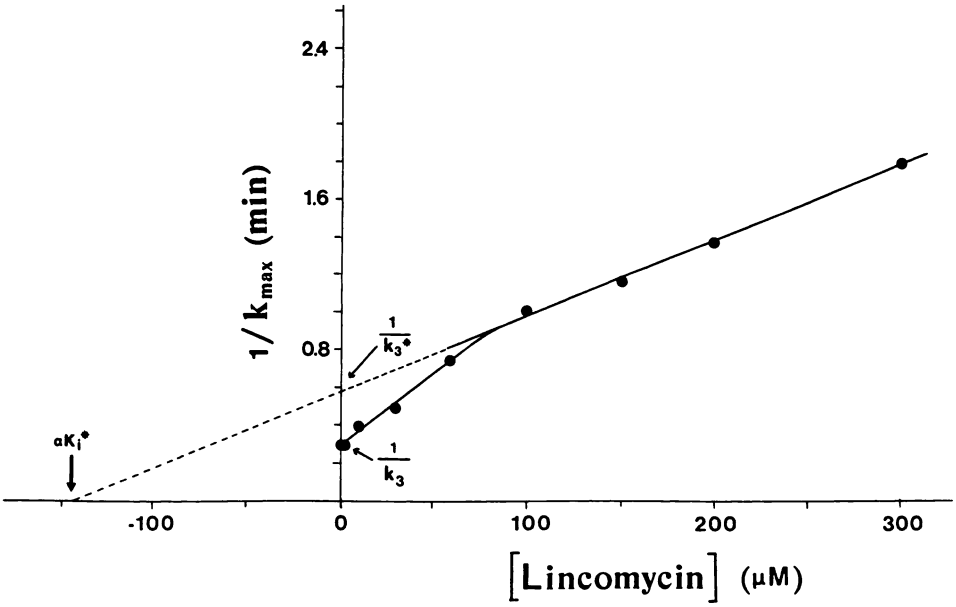

Fig. 3. Intercept replot ($1/k_{max}$ versus [lincomycin]) according to the initial-slope analysis. The data were taken from the double-reciprocal plots of Fig. 2 and similar plots.

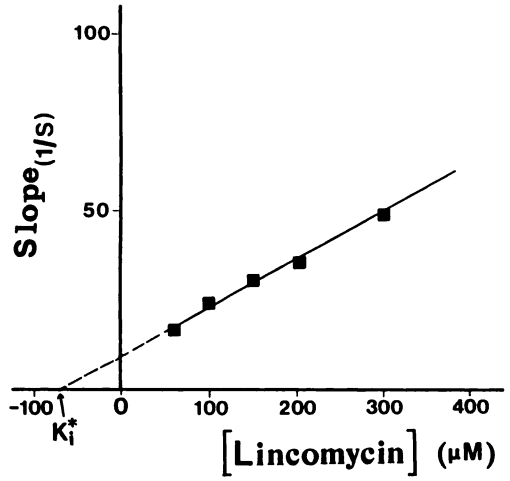

Fig. 4. Slope replot (slopes of double-reciprocal plots versus lincomycin concentration). The data were taken from the double-reciprocal plots of Fig. 2.

ml of reaction buffer (11), containing the appropriate amounts of NH_4Cl and lincomycin, was added so that the desired concentrations were achieved (final volume, 1 ml). The beakers were allowed to equilibrate at 25° for 5 min. At the time intervals indicated, the reaction between complex C and lincomycin was started by addition, to each beaker, of one half of a cellulose nitrate filter bearing complex C. After the desired reaction time had elapsed, 0.1 ml of 20 mM puromycin was added in the same buffer (containing the appropriate amount of lincomycin so that its final concentration remained constant) and allowed to react for 15 sec. The percentage $x/\alpha = x'$ for each time of exposure (t) and at each concentration of lincomycin was plotted against t, and a value of x' at equilibrium (x'_{eq}) was obtained. By assuming that the reaction between complex C and lincomycin proceeds toward equilibrium as a pseudo-first-order reaction, the values of k^I_{eq} were determined from the slope of the $\log(x' - x'_{eq})$ versus t plots (9).

Results and Discussion

Puromycin reaction in the absence of lincomycin. Equation 1 represents the reaction between complex C (C) and excess puromycin (S), which acts as a fraudulent substrate for peptidyltransferase. It constitutes the so-called "puromycin reaction" and it proceeds as an irreversible reaction in which C is converted to C′ (11).

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

In the absence of inhibitor this reaction follows pseudo-first-order kinetics and, at each concentration of S, the value of k_{obs} can be calculated from linear logarithmic time plots (Fig. 1, *upper line*). The relationship between k_{obs} and puromycin is given by $k_{obs} = k_3[S]/(K_s + [S])$.

The plot of the reciprocal of k_{obs} versus the reciprocal of puromycin concentration (Fig. 2, *lower line*) is linear and gives a k_3/K_s value equal to 9.5 min^{-1} mM^{-1}. The k_3/K_s value represents the apparent second-order rate constant of the puromycin reaction and evaluates the activity status of peptidyltransferase. This value is about 2 times greater than the corresponding value at 100 mM NH_4Cl (9).

Puromycin reaction in the presence of lincomycin. In the presence of the inhibitor lincomycin (I) and at 150 mM NH_4Cl, eq. 2 expresses the initial stages of the reaction between complex C and lincomycin (eq. 2 is extended as shown in Fig. 6; see below).

$$C + I \overset{K_i}{\rightleftarrows} CI \rightleftarrows C^*I \qquad (2)$$

When reactions 1 and 2 take place simultaneously, pseudo-first-order analysis of product (P) formation gives logarithmic progress curves that are biphasic for all inhibitor concentrations tested (Fig. 1, *lower lines* and *inset*). This is in contrast to the linear time plots observed in the absence of inhibitor (Fig. 1, *upper line*, and similar lines at various concentrations of puromycin that are not shown). The initial-slope analysis gives the double-reciprocal plots of Fig. 2 and leads to the identification of an initial phase of competitive inhibition ($K_i = 3.0$ μM) (Table 1), which is observed only over a narrow range of inhibitor concentrations ([I] < 6 μM). Increase of the

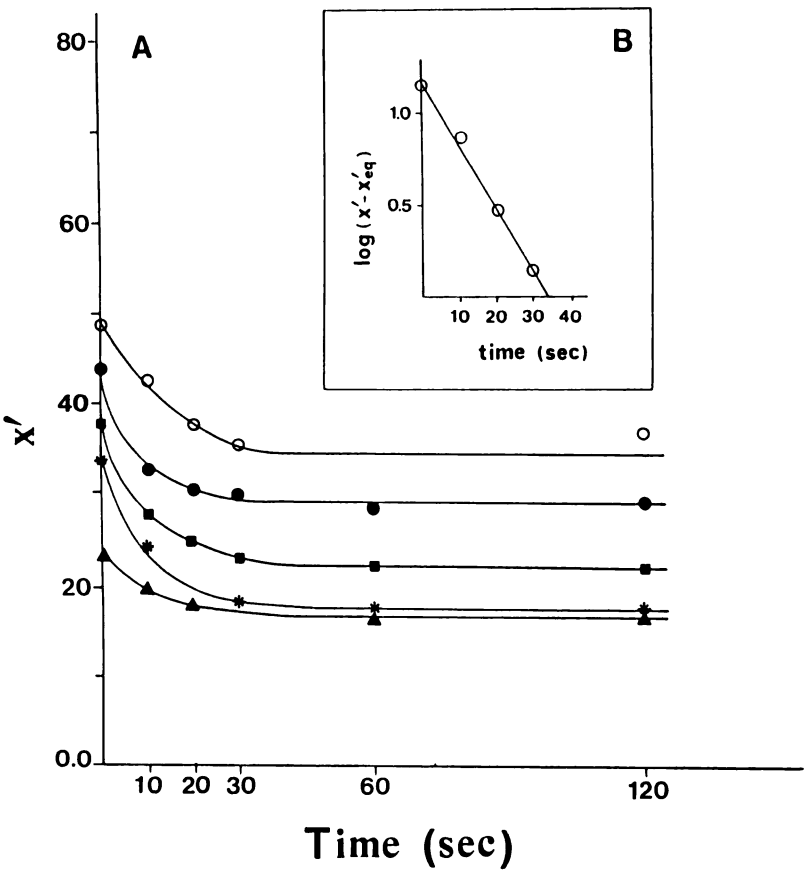

Fig. 5. Time plots for the attainment of equilibrium between complex C and lincomycin at 150 mM NH_4Cl. The disc-adsorbed complex C reacted with lincomycin at 3 μM (○), 10 μM (●), 20 μM (■), 25 μM (*), or 30 μM (▲) for the indicated time intervals. Complex C that had not reacted with lincomycin was then allowed to react with puromycin for 15 sec. *Inset*, the data from the *upper line* are replotted in the form of $\log(x' - x'_{eq})$ versus time.

TABLE 2

Apparent equilibration rate constants for the attainment of equilibrium between complex C and lincomycin at different concentrations of lincomycin, with 150 mM NH_4Cl

The k'_{eq} values are determined from the slope of the $\log(x' - x'_{eq})$ versus time (t) plots (e.g., Fig. 5, *Inset*).

Lincomycin concentration	k'_{eq}
μM	min^{-1}
3.0	3.7
6.0	5.2
10.0	6.7
15.0	7.2
20.0	7.7
25.0	8.3
30.0	8.6

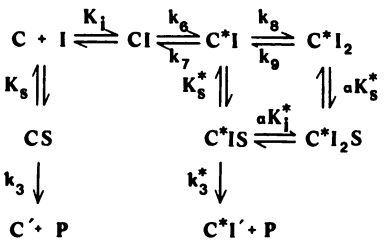

Fig. 6. Kinetic scheme proposed for the puromycin reaction in the presence of lincomycin, at 150 mM NH_4Cl. *C*, Complex C; *I*, lincomycin; *C*I*, modified complex C; *S*, puromycin; *P*, AcPhe-puromycin; *C'* or *C*I'*, complexes after their reaction with puromycin.

concentration of lincomycin alters the type of inhibition to mixed-noncompetitive (Fig. 2). The change in the type of inhibition is also evident from the intercept replot, which is not linear. Linearity develops above 60 μM ($\gg 20 \times K_i$) (Fig. 3). Because the linear part of the intercept replot does not extrapolate (for [I] = 0) to the intercept of the control ($1/k_3$, according to eq. 1), we surmise that, above 60 μM, the species that interacts with S and I to give mixed-noncompetitive kinetics is the conformationally altered species C*I (see Fig. 6). The slope replot that corresponds to the mixed-noncompetitive phase is given in Fig. 4 and affords the inhibition constant K_i^* of 70 μM (Table 1). It should be noted that the encounter complex CI consists of the donor AcPhe-tRNA, the ribosome, the message [poly(U)], and lincomycin. The fact that the type of inhibition changes from competitive to mixed-noncompetitive suggests that there is a change in the kinetic behavior so that the bound AcPhe-tRNA and lincomycin are rearranged to accomodate also puromycin.

Interaction between complex C and lincomycin in the absence of puromycin. In the absence of substrate (puromycin), inactivation plots at various concentrations of lincomycin were obtained. These plots, not yet expressed in a logarithmic form, are shown in Fig. 5 for lincomycin concentrations up to 30 μM. Under our experimental conditions it becomes possible to observe the delayed equilibration. The x'_{eq} value obtained from each equilibration plot can be used to replot the data in a logarithmic form, as shown in Fig. 5, *inset*. The slope of this replot gives the value of k^I_{eq} (see also Ref. 9).

The values of k^I_{eq} at different concentrations of lincomycin are given in Table 2. In the range of lincomycin concentrations between 5 and 10 × K_i, the overall equilibration, which is assumed to proceed as a pseudo-first-order reaction, is faster than that observed with 100 mM NH_4Cl. For example, at a lincomycin concentration of 10 μM and with 100 mM NH_4Cl, the k^I_{eq} value equals 4.3 min^{-1} (9); at the same concentration of lincomycin but with 150 mM NH_4Cl, k^I_{eq} equals 6.7 min^{-1} (Table 2). The k^I_{eq} values pertain to slow binding behavior, as evidenced by the following calculations, which were carried out according to the method of Morrison and Walsh (13). At [I] = 10 μM, k^I_{eq} equals 6.7 min^{-1} and the bimolecular rate constant associated with the interaction of C and I ($k^I_{eq}/[I_o]$) equals 1.1 × 10^4 M^{-1} sec^{-1}. This value is below 10^6 M^{-1} sec^{-1}, which is set (13) as the upper limit for the characterization of slow-onset inhibition ($[I_o] \simeq [I]$). Thus, despite the increase in the association rate between C and I at 150 mM NH_4Cl, lincomycin remains a slow-binding inhibitor.

The kinetic model. A scheme that can adequately explain the kinetics of inhibition by lincomycin at 150 mM NH_4Cl is shown in Fig. 6. According to this model lincomycin exhibits a transient phase of competitive inhibition, followed by a mixed-noncompetitive phase. We assume that at high concentrations of I (above 60 μM) the product comes mainly from C*I and not from C. This assumption implies that there is an isomerization of the encounter complex CI to C*I. The isomerized complex C*I gives product with an altered k_3 (k_3^*). The mixed-noncompetitive kinetics (Fig. 2) suggest that C*I binds S and I simultaneously. The simplest explanation is to tentatively accept the formation of a species such as C^*I_2S, containing two molecules of lincomycin. It might be relevant to note that, as recent evidence suggests, lincomycin binds to more than one position of the central loop of 23 S rRNA (14). Because with increasing concentrations of I the initial-slope analysis leads to mixed-noncompetitive inhibition, the experimentally determined inhibition constant K_i^* is assigned to the k_8/k_9 step and equals $[C^*I][I]/[C^*I_2]$. On the assumption that at high concentrations of I (above 60 μM) the product comes mainly from C*I and not from C, we can explain the fact that the linear part of the intercept replot does not extrapolate, for [I] = 0, to the intercept of the control (Fig. 3). This is predicted by the right side of the model depicted in Fig. 6. On this assumption, the $1/k_{max}$ intercept from Fig. 3 can give an estimate of the rate constant k_3^*, which equals 1.7 min^{-1}. This value is about 2 times smaller than k_3 (Table 1), suggesting a decrease in the reactivity toward puromycin of the AcPhe-tRNA bound to C*I. The term "reactivity toward puromycin" has been defined previously (15). The [I]-axis intercept of the intercept replot shown in Fig. 3 gives the value αK_i^*, which equals 145 μM (Table 1).

Concluding remarks. Ribosomal peptide bond formation is a field in which there is considerable debate about the mechanism of catalysis (16–20). The holistic approach of examining the kinetics of the reaction between puromycin and preformed ribosomal complexes bearing the donor already bound offers unique advantages in the effort to examine the mechanism of ribosomal peptide bond formation; especially beneficial is the absence of recycling of the donor (for more details, see Ref. 21). The present kinetic analysis shows that there is an increase in the ratio of k_3/K_s, which is a measure of the activity status of peptidyltransferase. This increase is compatible with the stimulation of protein synthesis observed in sea urchin eggs when the concentration of NH_4Cl is increased (22). Moreover, we find that the rate of association between complex C and lincomycin (I) is also increased. Such a kinetic analysis cannot be applied when, in the reductionist approach, one uses the fragment reaction; in that case there is recycling of the donor and the kinetics become rather complex (23, 24).

The data of this communication allow for some interpretations at the molecular level. We propose that at 150 mM NH_4Cl and at increased concentrations of lincomycin there is simultaneous binding of lincomycin and puromycin to the C*I complex (mixed-noncompetitive kinetics) and at lower concentrations of lincomycin the simultaneous binding of puromycin and lincomycin is not allowed (competitive kinetics). The prevailing notion is that lincomycin acts at the ribosomal A-site (1, 9), a mechanism that agrees only with the competitive kinetics. In that case, lincomycin competes with puromycin and, by inference, with aminoacyl-tRNA. The observed transition from competitive to mixed-noncompetitive kinetics may be related to a newly recognized facet of the mechanism of action of lincomycin in protein synthesis. Provided that the analogy between puromycin and aminoacyl-tRNA is valid, the change in the kinetics of inhibition may mean that the mutually exclusive binding between aminoacyl-tRNA and lincomycin is converted to simultaneous binding, as a result of conformational changes occurring in the elongating ribosomal complex.

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