Kinetics of Binding of Macrolides, Lincosamides, and Synergimycins to Ribosomes*

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The synergistic effect of type A (virginiamycin M (VM)) and type B (virginiamycin S (VS)) synergimycins and their antagonistic effect against erythromycin (a 14-membered macrolide) for binding to the large ribosomal subunit (50 S) have been related. This investigation has now been extended to 16-membered macrolides (leucomycin A₈ and spiramycin) and to lincosamides (lincomycin). A dissociation of VS-ribosome complexes was induced as well by 16-membered macrolides as by lincosamides. The observed dissociation rate constant of VS-ribosome complexes was identified with the k₋ᵥₛ in the case of 16-membered macrolides, but linearly related to lincomycin concentration, suggesting a direct binding of the latter antibiotic to VS-ribosome complexes and the triggering of a conformational change of particles entailing VS release. Two different mechanisms were also involved in the VM-promoted reassociation to ribosomes of VS previously displaced by either macrolides or lincosamides. By binding to lincosamide-ribosome complexes, VM induced a conformational change of ribosomes resulting in higher affinity for VS and lower affinity for lincosamides. On the contrary, an incompatibility for a simultaneous binding of VM and 16-membered macrolides to ribosomes was observed. These results have been interpreted by postulating specific (nonoverlapping) and aspecific (overlapping) antibiotic binding sites at the peptidyltransferase domain. All the kinetic constants of five antibiotic families (type A and B synergimycins, 14- and 16-membered macrolides, and lincosamides) and a topological model of peptidyltransferase are presently available.

Antibiotics of the virginiamycin family (streptogramins or synergimycins) contain two groups of chemically unrelated components: type A (such as virginiamycin M or VM) and type B (virginiamycin S or VS). Single components have a bacteriostatic effect, whereas the mixture of A and B is bactericidal; the latter effect is due to a permanent block of protein synthesis (3–6). Type A components were recently shown to inactivate the acceptor and donor substrate binding sites of peptidyltransferase (7, 8). This effect has been correlated with a conformational change of the 50 S ribosomal subunit (9, 10). The VM-induced conformational change produces also an increase of ribosome affinity for type B components, which mimics in vitro the synergistic effect observed in vivo with mixtures of streptogramin components (11, 12). Such an increased affinity of ribosomes for type B components was proved to be due to a decrease of the dissociation rate constant (1).

Macrolides contain a large lactone ring substituted with one or more sugar residues, some being amino sugars (2). Three classes of macrolides including: 12-membered (methyloxymycin), 14-membered (erythromycin, oleandomycin), and 16-membered (leucomycin, tylosin, spiramycin) compounds have been recognized. Although macrolides are powerful inhibitors of protein synthesis in bacteria, their mode of action is largely unknown, because of controversial observations with different in vitro model systems. As a matter of fact, 14- and 16-membered macrolides were found to exert different inhibitory actions in cell-free systems for protein synthesis. The latter subgroup proved able to inhibit peptidyl transfer reactions in several assay systems (2, 13–18), whereas members of the former subgroup were inactive in this respect. There also have been claims for interference of 16-membered macrolides such as niddamycins and spiramycins with aminoseryl-tRNA binding to ribosomes, and of erythromycin, a 14-membered macroline, with translocation (19–21). Lincosamides (lincomycin, clindamycin, and others) were reported to inhibit CACCA-acetylleucine binding to 50 S subunits (16, 17) and the so called "fragment reaction" with puromycin (15), thus pointing to an interference with the function of both A and P sites of ribosomes.

An "undissociated" type of resistance can be induced by incubation of bacteria with sublethal amounts of erythromycin. Such an acquired phenotype, which is associated with a specific alteration of 23 S rRNA, entails a resistance toward all MLS antibiotics (22–24). Ribosomes from resistant cells were proved to bind erythromycin and lincomycin with decreased affinity (22, 25).

The purpose of the present investigation was to explore the competition mechanisms between virginiamycin S(a type B streptogramin) on the one hand, and, on the other hand, leucomycin, spiramycin (two 16-membered macrolides), and lincomycin (a lincosamide) for binding to ribosomes. The members of the macrolide, lincosamide, and streptogramin B families (MLS group of antibiotics) inhibit protein synthesis in bacteria by binding to 50 ribosomal subunits with a 1:1 stoichiometry. These antibiotics are known to interfere in unknown ways with the peptidyltransferase domain of the ribosome (2).

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1 The abbreviations used are: MLS, macrolide, lincosamide, and streptogramin families; VM, virginiamycin M; VS, virginiamycin S; ERY, erythromycin; LEU, leucomycin A₈; SPM, spiramycin; LIN, lincomycin; MA, macrolide.
mechanism by which VM induces rebinding of VS, in the presence of macrolides or lincosamides, has also been explored. This work aimed at unravelling the topology of the peptidyltransferase domain on 50 S and at determining the kinetic parameters for the binding reaction of each antibiotic family to its target.

MATERIALS AND METHODS

Buffer—The following buffer A, pH 7.8 (25 °C), was used for all kinetic measurements: 10 mM MgCl₂, 200 mM NH₄Cl, 20 mM Tris-HCl, and 7.5% ethanol.

Antibiotics—VM and VS were crystallized from fermentation products (SKF-RIT Laboratories, Rixensart, Belgium, courtesy of A. Biot). Erythromycin, lincomycin, spiramycin, and tylosin were obtained from Sigma. Leucomycin A₃ and 10(11,12,13-3H)tetrahydroleucomycin A₃, 1.37 Ci/ml/liter, were kindly provided by Prof. S. Omura (Tokyo). [14C]Erythromycin, 13 mCi/ml/mol, was a generous gift of Prof. H. Vanderhaeghe (Leuven).

Preparation of Ribosomes and 50 S Ribosomal Subunits—70 S ribosome particles and 50 S ribosomal subunits were prepared as previously described (7, 8).

Filtration of Ribosomal Complexes on Micropore Filter—Displacement reactions of bound radiolaabeled antibiotics were performed as follows. Reaction mixtures, in buffer A, contained 1 μM concentration of either 70 S ribosomes or 50 S ribosomal subunits, and either 0.08 μM [14C]erythromycin or 0.95 μM [1H]tetrahydroleucomycin A₃. After a 5-min incubation at 25 °C, a given competitor (100 or 200 μM) was added to the reaction mixture. At different times, aliquots of the reaction mixture were rapidly diluted with 5 ml of buffer A and filtered through 25-mm diameter membrane filters (0.45-μm pore size; HAWP, Millipore Corp.). The filters were then washed with two 5-ml portions of buffer A, dried, and counted in a scintillation spectrometer, using Omnifluor (New England Nuclear, at 4 g/liter in toluene) as a scintillation fluid.

Fluorescence Measurements—The kinetics of the interaction between the 50 S subunits of bacterial ribosomes and the antibiotics, virginiamycin S, virginiamycin M, and erythromycin have been described recently (1). Measurements, which were based on the enhancement of VS fluorescence upon its binding to ribosomes, were carried out at 25 °C with an SPR-Ratio spectrofluorimeter equipped with a thermostated cell housing. The excitation monochromator was set at 330 nm, and the fluorescence emission was recorded at 420 nm, a 10-nm slit being used in either case. Concentration of bound antibiotic (VSₕ) was determined from total VS in solution according to the equation

\[
(VS_{h}) = \frac{F - F_{r}}{F_{r} (\gamma - 1)} (VS_{c})
\]

where F and F_r were the fluorescence intensities, in the presence and absence of ribosomes, respectively, and γ was a constant representing the ratio of fluorescence per mole of bound VS to that of free VS. The γ value was determined by adding a large excess of ribosomes to VS solutions of known concentration.

Fluorescence Stopped Flow Measurements—Kinetic experiments were performed with a temperature-controlled (25 °C) stopped flow spectrofluorimeter, which was built in the laboratory of Chemical and Biological Dynamics (University of Leuven, Belgium) (26). The instrument was equipped with a 200-watt Hanovia Xe-Hg lamp. The dead time of the stopped flow, determined according to the method of Peterman (27), was 3 ms. The kinetic data were stored in a digital storage oscilloscope (Gould, Avance) and transferred to a PDP11/34 computer via a simple interface (28). The excitation monochromator was set at the 365 nm mercury line with a 10-nm slit width. For emission, a Kodak Wratten filter 2B (cut off at 395 nm) was used. Fluorescence was collected over a wide angle (around 90°), and the transmitted beam was used as reference.

RESULTS

Two sorts of experiments were performed. First, the ability of macrolides and lincosamides to cause the release of ribosome-bound VS was analyzed by competition and displacement experiments. In further experiments, induction by VM of VS rebinding in the presence of macrolides and lincosamides was explored.

Displacement of Virginiamycin S by Leucomycin A₃ and Spiramycin—The ability of erythromycin, a 14-membered macrolide, to displace VS from its complex with 50 S subunits has been previously reported (1). It was of interest to extend this study to 16-membered macrolides (e.g. leucomycin A₃ and spiramycin).

To ribosome suspensions previously incubated with a fixed concentration of VS, increasing concentrations of either leucomycin A₃ or spiramycin were added. The decrease in fluorescence intensity, which monitors VS release from ribosome, was recorded and described by a single exponential. As shown in Fig. 1, the observed rate constant was independent of the concentration of either leucomycin or spiramycin. This suggests the occurrence of a real displacement, with VS dissociation as the rate-limiting step. VS displacement by these macrolides (Ma) is governed by the following equation which is a straightforward and direct application of the formula derived by Gutierrez (29):

\[
k_{\text{obs}} = \frac{k_{\text{-VS}}}{1 + k_{\text{-VS}}[\text{VS}] + k_{\text{Ma}}[\text{Ma}]/(1 + k_{\text{VS}}[\text{VS}])}
\]

indicating that for macrolide concentrations largely exceeding that of VS, the rate constant of the observed reaction can be identified with \(k_{\text{-VS}}\). The experimental value \(k_{\text{-VS}} = 0.045 \pm 0.003 \text{ s}^{-1}\) is in good agreement with the value \(0.042 \pm 0.004 \text{ s}^{-1}\) previously determined with erythromycin (1).

FIG. 1. Displacement of ribosome-bound virginiamycin S by 16-membered macrolides. Ribosome suspensions (0.5 μM) in buffer A were equilibrated first at 25 °C with 5 μM VS and then increasing concentrations of either leucomycin A₃ (C) or spiramycin were added (D). The decrease in fluorescence emission (420 nm) monitoring the release of ribosome-bound VS, was recorded (Eₕ = 330 nm) and analyzed according to Equation 1. The limiting value of \(k_{\text{obs}}\) was found to identify with the dissociation rate constant of VS (\(k_{\text{-VS}} = 0.045 \text{ s}^{-1}\)).
constants were plotted against lincomycin concentrations. This equation is the direct application of the formula for binding reaction of leucomycin. The \( k_{oA} \) and the \( k_{o} \) of the binding reaction of leucomycin \( b_{A} \) and spiramycin to ribosomes were obtained from stopped flow fluorimetry determinations. From the amplitude decrease (A) and the increase of observed rate constants \( (k_{o} \text{obs}) \), the association rate constants were calculated according to Equation 2.

\[
\frac{k_{\text{obs}}}{k_{o}} = \frac{1}{k_{o} + [\text{lincomycin}]}
\]

The observed amplitude decrease matched the calculated observed rate constants that came from the observed rate constants, using the above equation.

Displacement of Virginiamycin S by Lincomycin—Data in the previous section have ruled out the possibility of a simultaneous binding of virginiamycin S and macrolides to ribosomes. Is there a similar incompatibility for VS and lincomycin? To answer this question, release of ribosome-bound VS was monitored according to simple first order kinetics. The calculated observed rate constants were plotted against lincomycin concentrations.

This equation is the direct application of the formula for parallel reactions (30), in which pseudo-first order binding kinetics are assumed for both VS and macrolide. The final displacement of VS by macrolide occurs on a much larger time scale and is therefore not taken into account.

As shown in Table I, the association rate constant for the binding reaction of leucomycin \( A_{1} \) and spiramycin to ribosomes was: 1.5 \( \pm 0.2 \) \( \times 10^{4} \) M\(^{-1}\) s\(^{-1}\) and 2.0 \( \pm 0.2 \) \( \times 10^{4} \) M\(^{-1}\) s\(^{-1}\), respectively. The observed amplitude decrease matched that calculated from the observed rate constants, using the above equation.

Displacement of Virginiamycin S by Lincomycin—Data in the previous section have ruled out the possibility of a simultaneous binding of virginiamycin S and macrolides to ribosomes. Is there a similar incompatibility for VS and lincomycin? To answer this question, release of ribosome-bound VS was monitored in the presence of increasing concentrations of lincomycin (Fig. 2). Fluorescence data were again analyzed according to simple first order kinetics. In this case, however, the observed rate constant depended linearly on the concentration of lincomycin indicating that direct binding of lincomycin was measured. This relation is described by the following equation:

\[
k_{oA} = k_{o\text{LIN}}[\text{LIN}] + k_{-\text{LIN}}
\]

From the slope of this straight line, the association rate constant \( k_{o\text{LIN}} \) was found to be 4.5 \( \pm 0.3 \) \( \times 10^{3} \) M\(^{-1}\) s\(^{-1}\). The intercept was, thus, interpreted as a \( k_{-\text{LIN}} \) value (0.25 \pm 0.01 s\(^{-1}\)), and the calculated equilibrium association constant was 1.80 \( \pm 0.05 \) \( \times 10^{4} \) M\(^{-1}\).

**Fig. 3. Displacement of ribosome-bound erythromycin by virginiamycin M.** Two ribosomes suspensions (1 \( \mu \)M) in buffer A were incubated at 25 °C for 5 min with 0.98 \( \mu \)M \([\text{C}]\text{erythromycin. At zero time, VM (10 \( \mu \)M) (●—●) was added to one sample, and erythromycin (100 \( \mu \)M) (○—○) to the second sample. At different intervals, aliquots of the reaction mixtures were filtered on Micropore filters, and retained radioactivity was measured. The stability of ribosome-erythromycin complexes in the absence of competitors (△—△) was likewise evaluated.

**Table I**

| Sample | VS (\( \mu \)M) | Leucomycin (\( \mu \)M) | Spiramycin (\( \mu \)M) | \( k_{oA} \) \( \times 10^{3} \) M\(^{-1}\) s\(^{-1}\) | A | \( k_{o} \) \( \times 10^{4} \) M\(^{-1}\) s\(^{-1}\) |
|--------|----------------|--------------------------|--------------------------|--------------------------------|---|--------------------------|
| 1      | 1              | 5                        | 0                        | 2.09 (± 0.17)                   | 164 (± 17) | 1.2 \( \times 10^{4}\) |
| 2      | 1              | 5                        | 40                       | 5.90 (± 2.00)                   | 110 (± 14) | 1.3 \( \times 10^{4}\) |
| 3      | 1              | 5                        | 80                       | 7.60 (± 2.10)                   | 94 (± 8)   | 1.5 \( \times 10^{4}\) |
| 4      | 1              | 5                        | 40                       | 3.88 (± 0.74)                   | 106 (± 6)  | 1.0 \( \times 10^{4}\) |
| 5      | 1              | 5                        | 80                       | 3.46 (± 0.63)                   | 75 (± 7)   | 2.0 \( \times 10^{4}\) |
| 6      | 1              | 5                        | 100                      | 9.73 (± 2.59)                   | 38 (± 8)   | 2.2 \( \times 10^{4}\) |

VF.

**FIG. 2. Displacement of ribosome-bound virginiamycin S by lincomycin.** To a suspension of ribosomes (1 \( \mu \)M) and VS (2.5 \( \mu \)M), increasing concentrations of lincomycin (5–240 \( \mu \)M) were added. The decrease in fluorescence intensity, monitoring the release of ribosome-bound VS, was stored in a digital oscilloscope and analyzed according to simple first order kinetics. The calculated observed rate constants were plotted against lincomycin concentrations.
addition. An increase in fluorescence intensity monitoring rebinding of VS to ribosomes was observed (Fig. 4). This effect, however, was much slower (time scale in hours) than that previously observed in the presence of erythromycin (time scale in seconds) (1).

Indeed, the observed rate constant under the experimental conditions described in Fig. 4 is about 3 \((\pm 0.25) \times 10^{-4}\) s\(^{-1}\), whereas that in similar experiments with erythromycin was about 0.2 \((\pm 0.01)\) s\(^{-1}\) (1). Results obtained with spiramycin were similar to those of leucomycin (not shown). In conclusion, while VM interaction with ribosomes was the limiting step for VS binding in the presence of erythromycin, this was not true for leucomycin or spiramycin.

To test the possibility that the dissociation rate constant of leucomycin might be the limiting step of the VM-induced VS rebinding, the dissociation of ribosome-bound \[^{3}H\]leucomycin in the presence of VM was measured and compared to the displacement reaction occurring in the presence of an excess of unlabeled leucomycin. As shown in Fig. 5, overlapping curves were obtained. Consequently, the observed rate constant of VS binding in the presence of both leucomycin and virginiamycin M can be identified with the \(k_{\text{LEU}} = 3.2 \pm 0.2 \times 10^{-4}\) s\(^{-1}\).

VM-induced Rebinding of VS in the Presence of Lincomycin—The kinetics of VS rebinding in the presence of both lincomycin and VM are depicted in Fig. 6. Ribosome suspensions containing a fixed concentration of both VS and lincomycin were mixed with increasing concentrations of VM, and fluorescence intensity was measured. The experiments were carried out at two levels of lincomycin. Under these circumstances, the rate-limiting step for appearance of the fluorescence change (VS rebinding to ribosomes) was expected to be the action of VM. Although the observed curves were slightly sigmoidal, if the first 15% of the amplitude was neglected, the rest of the curve could be analyzed by simple first order kinetics. With this assumption, the observed rate constant depended linearly on the concentration of VM, at least in the lower region. At higher concentrations, a downward curvature became apparent: this was more pronounced in the presence of higher concentrations of lincomycin. The slope of the lower linear region corresponded to the \(k_{0.2}\) value of VM \((1.4 \times 10^{4}\) M\(^{-1}\) s\(^{-1}\)).

**DISCUSSION**

Ability of 16-membered macrolides, such as leucomycin and spiramycin, to induce the dissociation of virginiamycin S-ribosome complexes has been supported by data in Fig. 1. With these antibiotics, the observed rate constant of the dissociation reaction was independent on macrolide concentration and identified with the \(k_{\text{VS}}\). The same conclusion was previously reached for erythromycin (1). This points to a competitive effect occurring between VS (a streptogramin B) and 14-membered macrolides such as erythromycin or 16-membered macrolides such as leucomycin and spiramycin.

Two alternative models can account for these observations: (i) overlapping of the binding sites for type B streptogramins and macrolides; (ii) occurrence of different sites for type B streptogramins and macrolides, and of two ribosome conformations in equilibrium, each conformation being specific for one antibiotic family only. The second model accounts for the complete unrelatedness of the chemical structures of the two...
antibiotic families. However, the overlapping sites model cannot be excluded, owing to the size of the antibiotic molecules and the flexibility of ribosomal particles. The possibility that macrolides trigger a conformational ribosome change, resulting in the displacement of VS, is excluded by data in Fig. 1. Such a conformational change would alter the dissociation rate constant of VS, which is clearly not the case. The competition between VS and 16-membered macrolides (leucomycin and spiramycin) for binding to ribosome has been exploited to determine the association rate constant of macrolides. Direct competition experiments in Table I have yielded the values $k_{b,LEU} = 1.5 \pm (0.2) \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ and $k_{b,SPM} = 2 \pm (0.2) \times 10^4 \text{ M}^{-1} \text{s}^{-1}$. Such values differ from that previously obtained for erythromycin ($k_{b,ERY} = 3.2 \pm (0.15) \times 10^6 \text{ M}^{-1} \text{s}^{-1}$) by 1 order of magnitude (1).

It is well established that incubation of sensitive bacteria with low levels of erythromycin induces resistance to all the members of three antibiotic families: macrolides, lincosamides, and type B streptogramins (MLS). This inducible "undissociated" type of resistance was shown to be due to the methylation of 23 S rRNA by a specific methylase coded for by inducible plasmid pEI94 (31). Indeed, 50 S subunits possessing $N^6,N^6$-dimethyladenine in their large rRNA component proved unable to bind macrolides and lincosamides (22-24). Binding of MLS antibiotics to closely related or overlapping ribosomal sites would account for this multiresistance effect. Accordingly, similar behavior of macrolides and lincosamides toward streptogramins B would reasonably be expected: experimental data in Fig. 2, however, did not fulfill this expectation. In fact, the observed rate constant of the virginiamycin S displacement by lincomycin varied linearly with the concentration of the latter. This is just what can be expected when direct binding of lincomycin is measured. The simplest interpretation is that lincomycin is able to bind directly to a ribosome-VS complex and triggers a fast conformational change resulting in the immediate dissociation of VS. This model implies that the binding site of lincosamides is different from that of type B streptogramins. The association and dissociation rate constants for lincomycin binding was estimated to be $4.5 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ and $0.25 \text{ s}^{-1}$, respectively. From these data, the dissociation constant was calculated to be $5.6 \pm (0.5) \times 10^{-5} \text{ M}$, a value which agrees with that of $3.4 \pm (1) \times 10^{-8} \text{ M}$ reported by others (32).

We have provided data (1) suggesting that the limiting step for the VM-promoted VS rebinding in the presence of erythromycin was not the dissociation rate constant of ribosome-erythromycin complexes. The present work furnishes a direct proof for this inference, by showing that the $k_{d,VM}$ of erythromycin, in the presence of VM, is far higher than the $k_{b,ERY}$ (Fig. 3). Indeed, the two observed rate constants differed by a factor of 10. Our present and previous data (1) thus confirm the following reactions sequence:

$$R + ERY \rightleftharpoons R . ERY$$
$$R . ERY + VM \rightarrow VM . R^* . ERY$$
$$VM . R^* . ERY \rightleftharpoons VM . R^* \rightleftharpoons ERY$$
$$VM . R^* + VS \rightleftharpoons VM . R^* . VS$$

where VM binds to ribosome-erythromycin complexes and triggers a conformational change ($R^*$), which results in the dissociation of erythromycin and in the rebinding of VS. Such a sequence postulates the occurrence of different binding sites for type A streptogramins, such as VM, and 14-membered macrolides, such as erythromycin.

The basic differences between 16-membered and 14-membered macrolides are: the number of atoms of the lactone ring and the nature and relative arrangement of their sugar moieties. Emphasis upon the sugar residues stems from the observation that macrolides devoid of one or more of their sugar components show diminished activity (33). In view of the similarity of the two kinds of chemical structures, similar behavior of 16- and 14-membered macrolides would reasonably be expected. Data in Fig. 4, which actually infringe this expectation, were rather a surprise. The observed rate constant of the VS rebinding to ribosome, in the presence of leucomycin, was indeed about 700-fold smaller than that of erythromycin under similar experimental conditions. The dissociation rate constant of leucomycin might be the rate-limiting step for the VM-promoted VS rebinding to ribosome. Such a hypothesis was checked by comparing the rate constants of the dissociation reactions of [H]tetrahydroleucomycin-ribosome complexes in the presence of a large excess of either VM or unlabeled leucomycin, respectively. As shown in Fig. 5, a perfect overlapping between the two curves was obtained, suggesting the occurrence of a true displacement event, with leucomycin dissociation rate constant as the rate-limiting step. Three alternative models are compatible with these results: (i) VM and leucomycin bind to overlapping sites on the ribosome surface; (ii) the binding sites for the two antibiotic families are different, and there are two ribosomal conformations, one specific for 16-membered macrolides, and one for type A streptogramins; (iii) VM binds directly to the ribosome-leucomycin complexes but the induction of the conformational change $R \rightarrow R^*$ takes place only after detachment of leucomycin (without modification of $k_{b,ERY}$).

This latter model seems less likely because we have previously shown (1) that the binding of VM to ribosome is followed by a conformational change of the particles. A possible relationship between the ribosome binding sites of VM and lincomycin has been explored by measuring the VM-induced rebinding of VS to ribosomes in the presence of lincomycin. In these experiments, ribosomes were premixed with VS and lincomycin, leading to an equilibrium mixture of ribosome-leucomycin complex and free VS. When VM was added in the stopped flow equipment, the reappearance of the fluorescence signal due to bound VS was observed. The curves thus obtained were sigmoidal; the rate constant of the last part was plotted as a function of VM concentration in Fig. 6. In the case of a common binding site shared by VM and lincomycin, attachment of the former would require a release of the latter from its ribosome complex. A simple calculation, using Equation 1, shows that the observed rate constant does not correspond to a direct displacement of bound lincomycin by VM. Pure displacement reactions would not give sigmoidal curves either. Therefore, we conclude that lincomycin and VM bind to different sites, and that, at low VM concentrations, binding of VM is rate-limiting for the rebinding of VS. From the initial slope of the curve in Fig. 6, a value of $k^* = 1.4 \pm (0.2) \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ has been calculated, which is very close to that obtained in a similar experiment with erythromycin (1). The nonlinear relationship between the observed rate constants and the concentration of both VM and lincomycin remain to be explained. The possibility that the dissociation of lincomycin is the rate-limiting step for VS rebinding at high VM concentrations seems unlikely, since the $k_{d,VM}$ for lincomycin, after VM attachment to ribosomes, would be much larger than the observed rate constants. To explain our results, we propose the occurrence of a low affinity binding of lincomycin to VM: $R^*$ (the complex with altered conformation triggered by VM attachment), only the lincomycin-free form being capable of binding VS directly. In this case, the rebinding of VS is slowed down, due to a continuous re-equilibration
of VM·R* with lincomycin. Since experiments in Fig. 2 show that VS and lincomycin bind to different sites, the competition effect between the two antibiotics is likely to rely on ribosome conformation. Accordingly, the following sequence of reactions are proposed, neglecting the off rates (or dissociation rates) for VM and VS:

\[ R\text{-LIN} + VM \rightarrow VM\cdot R^*\text{-LIN} \]
\[ VM\cdot R^*\text{-LIN} \rightleftharpoons VM\cdot R^* + LIN \]
\[ VM\cdot R^* + VS = VM\cdot R^*\cdot VS \]

The first step of this scheme can be described by the following equation:

\[
\frac{d[VM\cdot R^*\cdot LIN]}{dt} = +k_{VM}\cdot [VM]\cdot [R\cdot LIN]
\]

By assuming a fast equilibration of VM·R* with lincomycin, the following equilibrium constant is proposed:

\[ K_{LIN} = \frac{[VM\cdot R^*\cdot LIN]}{[VM\cdot R^*]\cdot [LIN]} \]

Further developments of this equation are:

\[ [VM\cdot R^*] = \frac{1}{(1 + K_{LIN}[LIN])} [VM\cdot R^* + VM\cdot R^*\cdot LIN] \]

and

\[ [VM\cdot R^*] = \phi [VM\cdot R^* + VM\cdot R^*\cdot LIN] \]

where \( (1 - \phi) \) represents the fractional saturation of the available sites with lincomycin.

From the mass balance, it is clear that:

\[ [VM\cdot R^*] + [VM\cdot R^*\cdot LIN] = [VM\cdot R^*] - [VM\cdot R^*\cdot VS] \]

where VM·R* is the formal concentration of ribosomes in the R* conformation. Consequently

\[ [VM\cdot R^*] = \phi [VM\cdot R^*] - [VM\cdot R^*\cdot VS] \]

The appearance of the ribosome-VS complex can therefore be written as

\[
\frac{d[VM\cdot R^*\cdot VS]}{dt} = k_{VS}\cdot [VS]\cdot [VM\cdot R^*] - k_{VS}\cdot [VS]\cdot [VM\cdot R^*\cdot VS]
\]

Assuming pseudo-first order conditions, and neglecting the off-rate constants, the formula of two irreversible consecutive reactions can be applied (30). With \( k_{VM}/[VM] \) as the rate constant of the first step and \( k_{VS}/[VS] \) for the second step, the following integrated rate equation is obtained by direct substitution (30):

\[
\Delta F(t) = \Delta F(0)
\]

\[
+ \left( \frac{k_{VS}/[VS]}{k_{VS}/[VS] - k_{VM}/[VM]} \right) \exp \left( -\frac{k_{VM}/[VM]}{[VS]} - k_{VS}/[VS] \cdot t \right)
\]

where \( \Delta F(0) \) is the fluorescence change that occurs upon binding of VS to R*. This equation leads to predict sigmoidal progress curves of the type observed experimentally. In reality, more than 85% of the total amplitude followed a simple first order approach to equilibrium, the rate constants of which are shown in Fig. 6. At the experimental concentrations of 20 and 50 \( \mu M \) lincomycin, good fits were obtained for \( \phi \) values of 0.55 and 0.12. These values being incompatible with a single binding constant, it seems obvious that our assumption of fast equilibrium is oversimplified, and that the dissociation kinetics of lincomycin possibly influences the process of VS rebinding to ribosomes.

On the basis of present and previous (1) data, some general conclusions can be drawn, concerning the topology of the

### TABLE II

Kinetic parameters for the binding reactions of macrolides, lincosamides, and synergimycins to ribosome

| Antibiotics | Peptidyl Transferase Domains |
|-------------|-----------------------------|
| Type B streptogramins | 
| 14-membered macrolides | 
| 16-membered macrolides | 
| Type A streptogramins | 
| Lincosamides | 

\( k_a \) and \( k_d \) are the measured association and dissociation rate constants. \( K_a \) and \( K_d \) are the association and dissociation constants calculated from kinetic data. \( K^0_a \) and \( K^0_d \) are the association and dissociation constants measured at equilibrium.

| Antibiotics | \( k_a \) | \( k_d \) | \( K_a \) | \( K_d \) |
|-------------|----------|----------|--------|--------|
| **VS** | 2.8 \( \times \) 10^6 | 0.042 | 6.6 \( \times \) 10^6 | 5.5 \( \times \) 10^6 |
| **VS (+VM)** | 2.1 \( \times \) 10^6 | 0.009 | 2.6 \( \times \) 10^7 | 2.5 \( \times \) 10^6 |
| **ERY** | 3.2 \( \times \) 10^6 | 0.0039 | 8.2 \( \times \) 10^7 | 7.2 \( \times \) 10^8 |
| **LEU A3** | 1.5 \( \times \) 10^5 | 0.00015 | 4.7 \( \times \) 10^7 | 6.7 \( \times \) 10^7 |
| **SPM I** | 2.0 \( \times \) 10^5 | 0.00035 | 6.7 \( \times \) 10^7 | 7.8 \( \times \) 10^7 |
| **LIN** | 4.5 \( \times \) 10^4 | 0.25 | 1.8 \( \times \) 10^5 | 2.9 \( \times \) 10^4 |
| **VM** | 1.4 \( \times \) 10^4 | 0.25 | 1.8 \( \times \) 10^5 | 5.0 \( \times \) 10^4 |

\( a \) Ref. 1.
\( b \) Ref. 35.
\( c \) Ref. 36.
\( d \) The dissociation rate constant of the ribosome-spiramycin complex was \( \approx 3 \times 10^{-4} \) \( s^{-1} \).

\( \Delta F(t) = \Delta F(0) \)

\( \frac{d[VM\cdot R^*\cdot VS]}{dt} = k_{VS}\cdot [VS]\cdot [VM\cdot R^*] - k_{VS}\cdot [VS]\cdot [VM\cdot R^*\cdot VS] \)

Assuming pseudo-first order conditions, and neglecting the off-rate constants, the formula of two irreversible consecutive reactions can be applied (30). With \( k_{VM}/[VM] \) as the rate constant of the first step and \( k_{VS}/[VS] \) for the second step, the following integrated rate equation is obtained by direct substitution (30):
peptidyltransferase domain on the 50S ribosomal subunit, the common target of the MLS group of antibiotics. We wish to propose a model with the spatial distribution of the different binding sites for MLS antibiotics. An inherent restriction of this model is that it assumes the existence of a single binding site, whenever an antibiotic is displaced by another without change of the dissociation rate constant. Accordingly, three binding sites for: (a) type B streptogramins and macrolides, (b) lincosamides, and (c) type A streptogramins can be recognized in the peptidyltransferase domain of the 50S subunits (Fig. 7). The (partial) overlapping of different sites is shown in this figure. The postulated overlapping of lincosamide and macrolide binding sites was not verified experimentally. The proposed topological model for the peptidyltransferase domain may account for many conflicting results obtained by submission of different antibiotics of the MLS group to different functional tests in vitro. It agrees also with recent genetic works, whereby mutants endowed with "dissociated" types of resistance toward MLS antibiotics were characterized and referred to specific changes of the rRNA molecules (34).

In conclusion, the present work has furnished all the kinetic parameters for antibiotics of the MLS group (Table II) and proposed a topological model for the peptidyltransferase domain of prokaryotic ribosome (including the different antibiotic binding sites).

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