Interaction between Virginiamycin S and Ribosomes Is Partly Provided by a Salt Bridge with a Mg^{2+} Ion†

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ABSTRACT: Type B streptogramins, such as virginiamycin S (VS), are cyclic hexadepsipeptides, inhibiting protein synthesis in prokaryotes. L-Thr connects a 3-hydroxypicolinyl residue (3-OH-Pic) to the peptide lactone ring. The fluorescence intensity of 3-OH-Pic is strongly increased by chelation to alkaline earth cations or binding to ribosomes. Similar behavior of the ribosome–VS complex and the VS–Mg chelate provides strong evidence for the presence of a VS–Mg chelate within the ribosomal binding site. Different models involving the ribosome binding of either members of the VS–Mg^{2+} chelate or both have been tested by fluorescence lifetime measurements, equilibrium titrations, and stopped-flow spectrofluorometry. Our data strongly suggest that (a) the interaction between VS and the ribosome is partly provided by a salt bridge between suitable acceptor atoms of the ribosome and the 3-OH-Pic residue, (b) Mg^{2+} can be exchanged by Mn^{2+} without dissociation of the ribosome–VS complex, (c) Mg^{2+} coordinates to the negative form of the 3-OH-Pic residue, probably via an interaction with the phenolate oxygen and the amide carboxyl group, and (d) the picolinyl residue is essential for the biological activity, as indicated by the lack of activity when the latter is replaced by a serine derivative.

Antibiotics of the streptogramin family (virginiamycin, pristinamycin, and mikamycin) contain two groups of chemically unrelated components: type A (such as virginiamycin M and pristinamycin II) and type B (such as virginiamycin S and pristinamycin I). Both types bind to 70S ribosomes and inhibit protein synthesis by acting on the peptidyltransferase domain of the 50S particle (Vãzquez, 1975; Cocito, 1979, 1985; Di Giambattista et al., 1989). The following considerations are restricted to type B components, which are cyclic hexapeptides containing unusual amino acid residues. Common to all known members is a L-Thr residue, carrying a 3-hydroxypicolinyl residue responsible for fluorescence (Di Giambattista et al., 1984). This property has largely contributed to elucidate various aspects of the interaction between different peptidyltransferase inhibitors and ribosomes (Moureau et al., 1984; Di Giambattista et al., 1984, 1986, 1987).

The observation that streptogramins inhibit respiration in rat liver and yeast mitochondria (Dixon et al., 1971; Groot Obbink et al., 1979) suggests the possible involvement of these molecules in the transport of protons and cations across membranes. Indeed, virginiamycin S (VS)[1] and mikamycin B were reported to behave as general cation carriers (Grell et al., 1977; Oberbäumer et al., 1983). Spectrophotometric titrations, at different pHs and solvent polarity, have revealed the presence of different absorption spectra, which were attributed to the different protolytic forms of the 3-hydroxypicolinyl residue (Grell et al., 1977). A cavity formed by the peptide lactone ring has been suggested as the binding site in the case of monovalent cations, whereas the transport of protons and divalent cations can be exclusively attributed to the physicochemical properties of the 3-hydroxypicolinyl residue, owing to the fact that model compounds, such as 3-hydroxypicolinamide, behave as VS. These results also indicate that alkaline earth cations probably coordinate to the twice-deprotonated form of VS (Oberbäumer et al., 1983).

As already mentioned, type B streptogramins are weakly fluorescent in aqueous solutions. However, the fluorescence increases strongly in the presence of alkaline earth ions such as Mg^{2+} or Ca^{2+}, and furthermore upon binding to ribosomal particles (Parfait et al., 1978). To learn more about the VS protolytic form coordinating to alkaline earth cations, the influence of pH, solvent polarity, and cation concentration on the fluorescence decay of VS has been analyzed by multifrequency phase fluorometry. In the preceding paper (Clays et al., 1991), different fluorescence lifetimes associated with the different protolytic forms of VS were reported: addition of alkaline earth cations resulted in the lengthening of the fluorescence lifetimes. This phenomenon was attributed to a reduction of the nonradiative decay rate due to a reduction of the number of degrees of freedom for nonradiative relaxation. A complexation of alkaline earth cations by the phenolate oxygen is likely to take place with the participation of the amide carboxyl group; a model in agreement with that already suggested (Grell et al., 1977; Oberbäumer et al., 1983). The following considerations led us to explore the possible role of magnesium chelation in the interaction of type B streptogramins with ribosomes: (i) the 3-OH-picolinyl residue is common to all known members of type B streptogramins; (ii) strong fluorescence increase occurs when VS is chelated to Mg^{2+} or bound to ribosomes; and (iii) there is a loss of biological activity when the picolinyl residue is replaced by serine derivative (Kessler et al., 1983).

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[1] Abbreviations: VS, virginiamycin S; 3-OH-Pic, 3-hydroxypicolinic residue.

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Materials and Methods

Buffers. The following buffers were used: TA, 20 mM Tris-HCl, pH 7.8, 100 mM NH_4Cl, and $MgCl_2$ and $MnCl_2$ as specified; KP, 100 mM KH_2PO_4/NaOH, pH 7.4; 7% EtOH was used in both buffers.

Antibiotics. Virginiamycin components M and S were kindly provided by Dr. J. I. H. Phillip (SKF-RIT Laboratories, Rixensart, Belgium); erythromycin was from Sigma.

Preparation of Ribosomes. Ribosomes from *Escherichia coli* A19 were prepared as previously described (Di Giambattista et al., 1984) and reactivated 10 min at 42 °C before use.

Absorption Measurements. Optical density measurements were performed on a single-beam DU-65 spectrophotometer (Beckman) using 1-cm path quartz cuvettes.

Exclusion Chromatography on Sepharose 4B Columns. The VS–ribosome complexes in TA buffer (1 mM $MgCl_2$) were separated from free VS by exclusion chromatography on 15-mL Sepharose 4B columns at 4 °C. TA buffer containing either 1 mM $MgCl_2$ or 8 mM $MnCl_2$ was used. Absorbance at 260 nm and fluorescence (excitation, 330 nm; emission, 420 nm) were recorded in each fraction (0.5 mL).

Equilibrium Fluorometric Titrations. Fluorescence was measured at 25 °C on a Aminco-Bowman SPR-Ratio II spectrofluorometer. The excitation and emission wavelengths were 330 and 420 nm, respectively, a 10-nm slit being used in both cases. The binding constant for VS–Mn^{2+} complexes was estimated from the plot of log ([VS–Mn^{2+}]/[VS]) versus log [Mn^{2+}]. Ratios of complexed to uncomplexed VS were calculated by using the equations:

$$[VS\text{–}Mn^{2+}]/[VS_0] = \Delta F/\Delta F_{max}$$

and

$$[VS]/[VS_0] = 1 - \Delta F/\Delta F_{max}$$

where [VS–Mn^{2+}] represents the concentration of complexed VS, [VS_0] is the total VS concentration, ΔF is the observed fluorescence change ($F_0 - F$) after addition of Mn^{2+} ions, and F_0 and F are the fluorescence values in the absence and presence of Mn^{2+}, respectively.

Stopped-Flow Fluorometry. Kinetic experiments were performed with a temperature-controlled stopped-flow apparatus, as previously described (Lambeir & Engelborghs, 1981). Kinetic data were stored in a digital storage oscilloscope (Gould Advance) and transferred to a PDP11/34 computer via a single interface (Hennau & Ceuterick, 1981). The excitation monochromator was set at the 365-nm mercury line with 10-nm slit width. For emission, a Kodak Wratten filter 2B (cutoff at 365 nm) was used. Fluorescence was collected over a wide angle (around 90°), and the transmitted beam was used as reference. The fluorescence data were analyzed with a general nonlinear least-squares program. Mg^{2+} displacement by Mn^{2+} has been analyzed by the equation (Gutfreund, 1974):

$$k_{obs} = \frac{k_{-Mg^{2+}}}{1 + k_{+Mg^{2+}}[Mg^{2+}]/k_{+Mn^{2+}}[Mn^{2+}]} + \frac{k_{-Mn^{2+}}}{1 + k_{+Mn^{2+}}[Mn^{2+}]/k_{+Mg^{2+}}[Mg^{2+}]}$$

This relation shows that at high concentration excess of Mn^{2+} over Mg^{2+}, k_{obs} is equal to $k_{-Mg^{2+}}$.

Fluorescence Lifetime Measurements. Fluorescence lifetime measurements were performed on a laboratory-built multifrequency phase fluorometer as described previously (Clays et al., 1989). The dyes used were Rhodamine 6G and DCM [4-(dicyanomethylene)-2-methyl-6-[*p*-(dimethylamino)-styryl]-4*H*-pyran]. After frequency doubling, excitation was produced at 330 nm. Laser pulses of 20 ps at a frequency of 400 kHz were provided. Phase measurements were made at 700-Hz frequency and 50 harmonics of the repetition frequency. Phase shifts and standard deviations were analyzed for the fluorescence decay parameter by the nonlinear least-squares Marquardt algorithm on a microVax 2000 minicomputer. All available statistical parameters were used to identify the exponentials involved in fluorescence decay. Details of phase measurements and analysis of the experimental data are described in the preceding paper (Clays et al., 1991).

Table I: Fluorescence Lifetimes and Fractional Contributions of VS, VS–Mg^{2+} Chelate, and Ribosome-Bound VS

	τ_1 (ns)	f_1	τ_2 (ns)	f_2
VS[a]	1.1 (0.1)[d]	0.75 (0.05)	1.7 (0.4)	0.25 (0.05)
VS–Mg^{2+} [b]	1.25 (0.02)	0.15 (0.02)	7.82 (0.02)	0.85 (0.02)
R–VS complex				
0.5[c]	0.57 (0.02)	0.10 (0.02)	8.73 (0.03)	0.90 (0.03)
1[c]	0.32 (0.01)	0.10 (0.01)	9.18 (0.02)	0.90 (0.01)
2[c]	0.28 (0.01)	0.10 (0.01)	9.22 (0.02)	0.90 (0.01)

[a] Fluorescence lifetimes were determined by phase fluorometry as described by Clays et al. (1991) in KP buffer, pH 7.4. [b] Fluorescence lifetimes were determined by phase fluorometry as described by Clays et al. (1991) in TM buffer, pH 7.8, and 10 mM Mg^{2+}. [c] Ribosome to VS ratio. [d] Values in parentheses are estimated uncertainties.

Results

Fluorescence Lifetimes of VS. An increase of VS fluorescence, after chelation of Mg^{2+} (Di Giambattista & Cocito, 1983) or binding to ribosomes (Parfait et al., 1978), has been previously noted. In the pH range of 7.4–7.8, measurements on the phase fluorometer revealed a biexponential fluorescence decay for VS in TA buffer (Table I). Lifetimes were 1.1 and 1.7 ns, the fractional contributions (f) being 0.75 and 0.25, respectively.

In TA buffer (pH 7.8), containing increasing amounts of Mg^{2+}, a biexponential fluorescence decay was also found, with fractional contributions dependent upon the cation concentration. At 10 mM Mg^{2+} (Table I), lifetimes were found to be 1.25 and 7.82 ns (0.15 and 0.85 being the corresponding f values). Addition of ribosomes (0.5, 1, and 2 VS equiv) resulted in an increase of the long lifetime and a decrease of the short lifetime. A saturation effect was observed when ribosome concentration was higher than equimolar (see Table I), the fractional contribution for the different lifetimes remaining unchanged. Although, a mixture of free VS, VS–Mg^{2+} chelate, and ribosome–VS complex (R–VS) was present (4 lifetimes would thus be expected), only two components were resolved, the two short and two long lifetimes being very close. Note that these lifetime values are in good agreement with those previously found by a simpler type of phase fluorometer (Di Giambattista et al., 1984; Ide et al., 1983). The finding that the long fluorescence lifetimes of VS–Mg^{2+} chelates and R–VS complexes were approximately the same suggests the presence, within the ribosomal complex, of a Mg^{2+} ion associated with VS. Three alternative models (Figure 1), all compatible with this observation, have been tested. In model 1, Mg^{2+} is assumed to be linked to the 3-hydroxypicolinyl residue and not interacting with ribosomal components. In model 2, the VS–ribosome interaction is provided by a single salt bridge (the macrocycle is not involved). In model 3, salt bridge and amino acid residues both contribute to the binding.

To identify the actual model, the dissociation rate constants of VS and Mg^{2+} have been measured in the presence and in

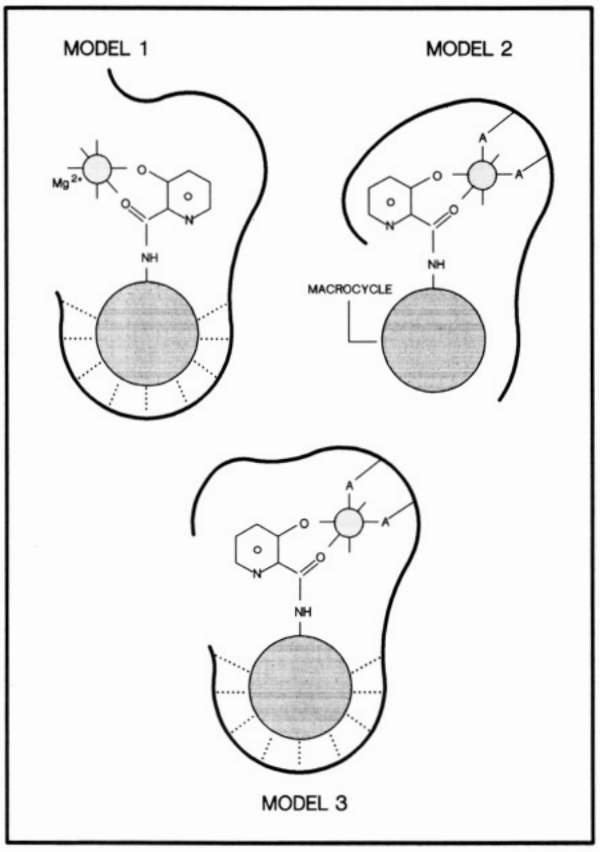

FIGURE 1: Schematic illustration of the VS binding site. The binding site of VS is represented as an open well on the ribosome surface. The 3-OH-picolinyl moiety is shown in detail, whereas the macrocycle has been reduced to a circle. The dotted lines represent potential interactions occurring between the macrocycle and ribosomal components. The hypothetical interactions of Mg^{2+} with ribosomal components have been represented by the coordination with suitable acceptor atoms (A). Concerning the binding model, it has to be noted that the Mg^{2+} ion is normally hexahydrated in solution and may be expected to bind to ribosomal components through hydrogen bonds between water molecules and suitable acceptor atoms. An alternative mode of binding would involve loss of one or more water molecules from the hydration shell and the formation of a direct bond with acceptor atoms. Model 1: Mg^{2+} is linked to VS through the coordination to the hydroxyl oxygen and the oxygen of the amide bond. Model 2: the interaction between VS and the ribosome is only provided by a single salt bridge. Model 3: the salt bridge (either with Mn^{2+} or Mg^{2+}) and amino acid residues of the macrocyle contribute to the interaction of VS with ribosomal components.

the absence of ribosomes. The rationale of our approach was as follows: (a) if Mg^{2+} interacts with both VS and suitable acceptor atoms of ribosome, the dissociation rate constant of R-bound VS–Mg^{2+}, is expected to be smaller than that of VS–Mg^{2+} chelate; (b) the role played by the VS macrocycle in the R–VS interaction can be estimated by comparing the dissociation constant of the VS–Mg chelate with that of R–VS.

Displacement of Mg^{2+} Bound to VS by Mn^{2+} and Dissociation by EDTA. In contrast to earth alkali ions, which increase the fluorescence quantum yield of VS, heavy metals, such as Mn^{2+}, are effective quenchers of VS fluorescence. In fact, paramagnetic ions are known to quench fluorescence by increasing the rate of singlet–triplet intersystem crossing. This property was exploited to measure the dissociation rate constant of the VS–Mg chelate. Kinetics of Mg^{2+} displacement were evaluated by the stopped-flow technique, whereby a fixed amount of VS–Mg^{2+} chelate was incubated with increasing concentrations of Mn^{2+}. For the whole range of concentrations used, fluorescence data fitted the relationship corresponding to a single relaxation process. Figure 2 represents the time

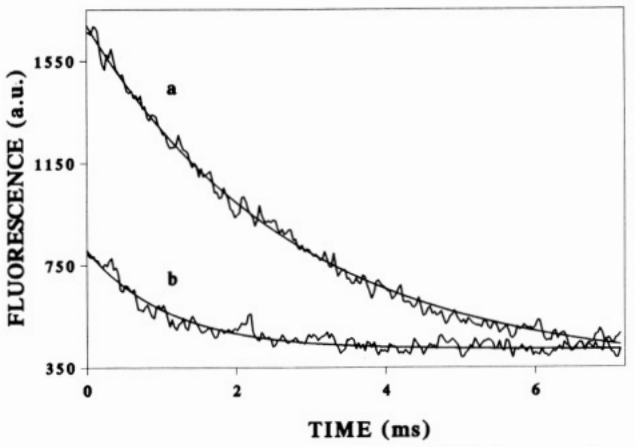

FIGURE 2: Fluorescence decrease caused by Mg^{2+} displacement from VS–Mg^{2+} chelates by Mn^{2+}. To a mixture of virginiamycin S (50 μM) and $MgCl_2$ (20 mM) was added 100 mM $MnCl_2$. The reaction was carried out at 6 °C (a, upper curve) and at 25 °C (b, lower curve). The continous lines are simulated curves according to a single exponential.

Table II: Binding Parameters of Various Complexes at 25 °C[a]

	k_- (s^{-1})	k_+ (M^{-1} s^{-1})	K_d (M)
VS–Mg^{2+}	690[b]	6.5×10^4[c]	10^{-2}
	610[d]		
VS–Mn^{2+}	270	4.8×10^5[c]	5.6×10^{-4}
VS–Mg^{2+} (+ribosome)	≈500[e]		
	≈65[e]		
ribosome–VS	0.043[f]	2.4×10^5[g]	1.8×10^{-7}

[a] In TA buffer, pH 7.8. The error in these constants is estimated to be 10–15%. [b] From displacement reaction with Mn^{2+}. [c] From k_- and K_d measured at equilibrium. [d] Dissociation with EDTA. [e] Mg^{2+} displacement reaction in the presence of ribosome. [f] Dissociation of R–VS complex by erythromycin. [g] From Moureau et al. (1984).

course of the fluorescence decrease occurring when the VS–Mg^{2+} chelate was mixed with Mn^{2+} at 6 °C (curve a) and 25 °C (curve b), respectively. At a high excess of Mn^{2+} over Mg^{2+}, the limiting k_{obs} value corresponding to $k_{-Mg^{2+}}$ was equal to 690 (±60) s^{-1}. Similar results were obtained when VS–Mg^{2+} chelates were mixed with an excess of EDTA (Table II). Since the dissociation reactions of VS–Mg^{2+} chelates at 25 °C had half-times in the millisecond range, experiments have been performed to check the possible occurrence of a faster process. Analysis of displacement reactions at different temperatures showed a linear variation of the amplitude (logarithmic scale) with the observed rate constant (the dead time was found to be 2.3 ms). Moreover, the amplitude value extrapolated at zero Mn^{2+} concentration fitted well the experimental point (data not shown). It is clear, therefore, that no fast event was overlooked.

Kinetics of the Fluorescence Decrease of the R–VS Complex with Increasing Concentrations of Mn^{2+}. In the previous section, the dissociation rate constant of the VS–Mg^{2+} chelate has been determined. Similar kinetic measurements were used to evaluate the dissociation rate constant of VS–Mg^{2+} in the presence of ribosomes. To ribosomes previously incubated with a fixed amount of VS were added increasing concentrations of $MnCl_2$, and the kinetics of the fluorescence decrease were monitored by the stopped-flow method. In contrast to the experiments with free VS, Mg^{2+} displacement from the R–VS complex showed biphasic kinetics. The two rate constants depended in a nonlinear way on the Mn^{2+} concentration (Figure 3, panels A and B). Such a concentration dependence, which is normally described by the simple displacement equation (Gutfreund, 1974), did not apply to our case. The

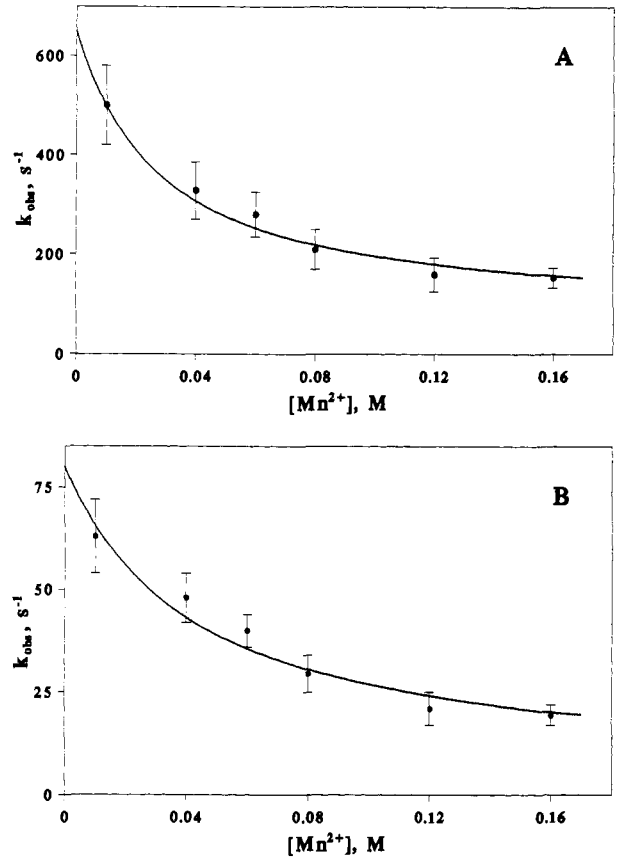

FIGURE 3: Kinetics of fluorescence decrease of ribosome-bound VS by mixing with increasing concentrations of Mn^{2+}. A suspension of ribosomes (0.5 μM) and VS (5 μM) in TA buffer (0.5 mM $MgCl_2$) was mixed with increasing concentrations (from 10 to 160 mM) of $MnCl_2$. The kinetics of fluorescence decrease were monitored at 25 °C by the stopped-flow method. For the whole range of concentrations used, two decay processes were discernible. The observed rate constants for the fast (panel A) and the slow (panel B) processes were plotted as functions of Mn^{2+} concentration. Continous lines are simulated curves according to the classical displacement equation (Gutfreund, 1974).

following inconsistencies were observed: (i) the recorded k_{obs} did not attain a positive plateau value; (ii) the equilibrium dissociation constant of Mn^{2+} was larger than that of Mg^{2+} (i.e., $k_{-Mn^{2+}} \gg k_{-Mg^{2+}}$; $k_{+Mg^{2+}} \gg k_{+Mn^{2+}}$); and (iii) the full amplitude of the fluorescence decrease occurred at the lowest Mn^{2+} concentrations, in contrast with the normal concentration dependence according to a simple displacement. Note that a K_d of VS–Mn^{2+} > K_d of VS–Mg^{2+} is incompatible with (a) the value of $k_{-Mn^{2+}}$ = 270 (±28) s^{-1} (estimated by the displacement of Mn^{2+} from the VS–Mn^{2+} chelate by Mg^{2+} in Figure 4), as compared to that of 690 s^{-1} for VS–Mg^{2+}, and (b) the isotherm of Mn^{2+} binding to VS (Table II), from which an equilibrium dissociation constant of 5.6×10^{-4} M was determined, as compared with the 10^{-2} M value previously determined for VS–Mg^{2+} (Di Giambattista & Cocito, 1983).

Assuming a concentration dependence due to an effect of Mn^{2+} on ribosomes (decrease of the dissociation rate constant of Mg^{2+}), the $k_{-Mg^{2+}}$ of R–VS complexes was taken as the k_{obs} at the lowest Mn^{2+} concentration.

The occurrence of two phases is due either to two conformations of R–VS complexes in slow equilibrium or to a heterogeneity of the particle population. Since the measured amplitudes were independent of the Mn^{2+} concentration, the hypothesis of ribosome heterogeneity has been favored. One particle fraction showed for Mg^{2+} a dissociation constant similar to that for free VS (≈500 s^{-1}). The other fraction had

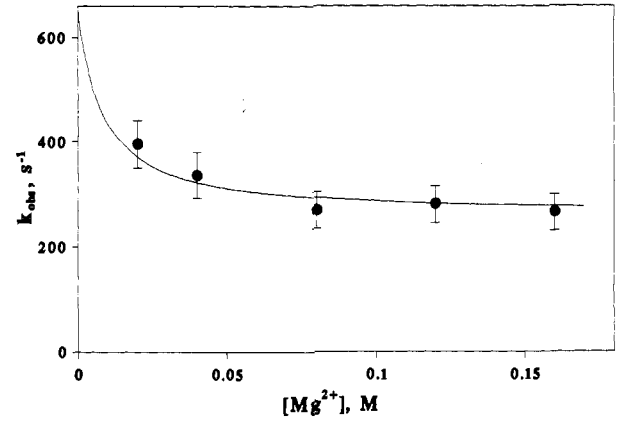

FIGURE 4: Dissociation of VS–Mn^{2+} chelates by Mg^{2+}. A fixed amount of VS [15 μM in TA buffer (1 mM Mn^{2+})] was mixed at 25 °C with increasing concentrations of Mg^{2+}. The kinetic curves (fluorescence increase) were fitted to a single exponential. The observed rate constants (k_{obs}) were plotted versus the final concentration of Mg^{2+}. The continuous line is the simulated curve according to the classical displacement equation (Gutfreund, 1974). The limiting value of k_{obs} is the dissociation rate constant of Mn^{2+} (270 s^{-1}).

a 10-fold lower value (≈65 s^{-1}). The possibility of a high dissociation rate constant independent of ribosome binding is in contradiction with the Mn^{2+} dependence of the observed k_{obs} (in this particular case, the k_{obs} is expected to be independent of Mn^{2+} concentration). Since Mn^{2+} had an effect on both types of population, ribosomes are likely to influence the k_- of the VS–Mg^{2+} chelate, thus ruling out model 1.

Exchange of Mg^{2+} by Mn^{2+} within the Ribosome–VS Complex. The puzzling action of Mn^{2+} on R–VS complexes is compatible with a dissociation of R–VS complexes when Mg^{2+} is replaced by Mn^{2+}. Complete fluorescence quenching of R–VS by Mn^{2+}, at equilibrium, can be explained in two ways: (i) addition of Mn^{2+} to the R–VS complex entails the dissociation of the latter, followed by fluorescence quenching of free VS; (ii) R–VS complexes are stable in the presence of Mn^{2+}, quenching being the result of Mg^{2+} exchange by Mn^{2+}.

To choose the correct model, a sample of ribosomes and VS in 1 mM Mg^{2+} TM was split into two parts, to one of which (sample B) $MnCl_2$ was added: fluorescence was measured. Free and ribosome-bound VS were separated on Sepharose 4B columns: fluorescence (tracing VS) and optical density (ribosomes) of the eluate were measured. As shown in Figure 5 (panel B, curve b), in the presence of 8 mM Mn^{2+}, fluorescence within the ribosome peak represented less than 5% of the control (Figure 5, panel A, curve b). When the Mg^{2+} concentration was increased to 50 mM in both samples, more than 65% of the fluorescence was recovered in sample B (Figure 5, panel B, curve c), the 100% value being that of control sample A (Figure 5, panel A, and curve c). Since in the sample containing 8 mM Mn^{2+} plus 50 mM Mg^{2+} VS was partly present as a nonfluorescent VS–Mn^{2+} complex, it can be inferred that a large fraction of VS was associated with ribosomes, in spite of the quenching of VS fluorescence by Mn^{2+}. In conclusion, R–VS complexes are stable in the presence of Mn^{2+} ions, and fluorescence quenching is due to Mg^{2+} replacement by Mn^{2+} rather to a dissociation of the R–VS complex followed by the quenching of free VS fluorescence.

Displacement of Ribosome-Bound VS by Erythromycin. The ability of macrolides, such as erythromycin (ERY), to displace VS from R–VS complexes was previously reported (Moureau et al., 1984; Parfait et al., 1981). At high concentrations of erythromycin over VS, the k_{obs} was independent

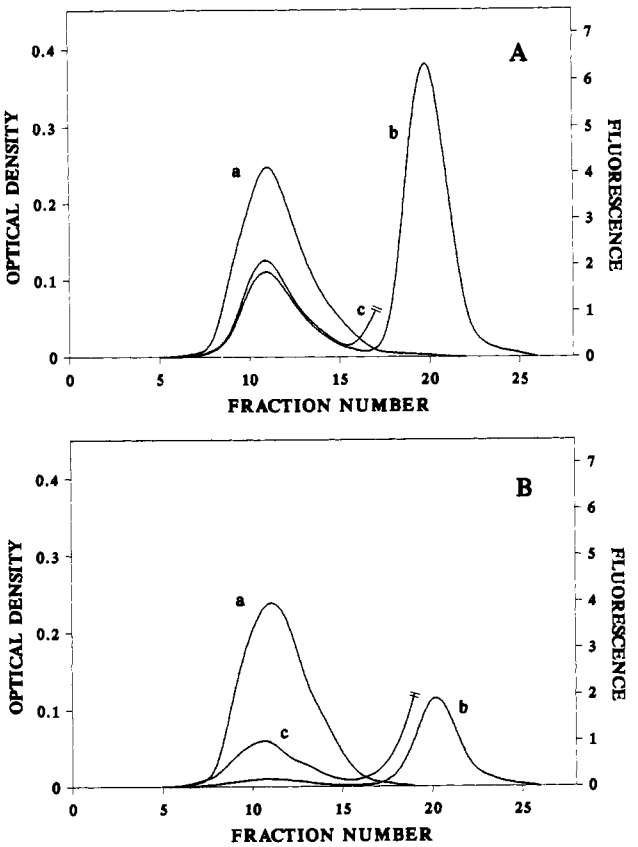

FIGURE 5: Exchange of Mg^{2+} by Mn^{2+} within the ribosome–VS complex. A sample (1 mL) containing 1 μM 70S ribosomes and 4 μM virginiamycin S in buffer TA (1 mM $MgCl_2$) was incubated 5 min at 4 °C and split into two parts: 4 μL of water or 4 μL of 1 M $MnCl_2$ was added to sample A and B, respectively. Samples were loaded on Sepharose 4B columns and eluted with TA buffer (1 mM $MgCl_2$) for sample A and with TA buffer (8 mM $MnCl_2$) for sample B. Fractions of 0.5 mL were collected, and the optical density at 260 nm (curve a) and the fluorescence (excitation 330 nm; emission 420 nm) (curve b) were recorded for each fractions. In the fractions containing ribosomal particles, the concentration of $MgCl_2$ was raised to 50 mM, and the fluorescence intensity was monitored again (curve c).

of erythromycin concentration and corresponded to the dissociation rate constant of VS (Moureau et al., 1984).

Kinetics of the fluorescence decrease in 1 mM $MgCl_2$ (monitoring the dissociation of VS) were measured in the presence of a 10-fold excess of erythromycin over VS (Figure 6). The observed rate constant was estimated to be 0.043 (±0.002) s^{-1}, in good agreement with the 0.042 s^{-1} value previously observed in 10 mM $MgCl_2$ (Moureau et al., 1984).

Since the dissociation rate constant of R–VS (0.043 s^{-1}) is much smaller than those of Mg^{2+} (±500 s^{-1} for the fast and ±65 s^{-1} for the slow process), the second model (interaction between VS and ribosome only provided by a salt bridge) can be excluded. Note that the presence of a single VS binding site on ribosome excludes model 2, since numerous Mg^{2+}, all potential receptors for VS, occurs at the particle surface.

Discussion

The essential role played by Mg^{2+} ions in translation complexes has been repeatedly stressed. Mg^{2+} is required for structural integrity of ribosomal particles and an efficient translational process (Gassen, 1980). Mg^{2+} stabilizes the association of the ribosomal subunits (Weiss et al., 1973; Zamir et al., 1971; Stahli et al., 1977; Wishnia & Boussert, 1977; Shimmel & Redfield, 1980), the tertiary structure of tRNA (Stein & Crothers, 1976; Jack et al., 1977), and the codon–

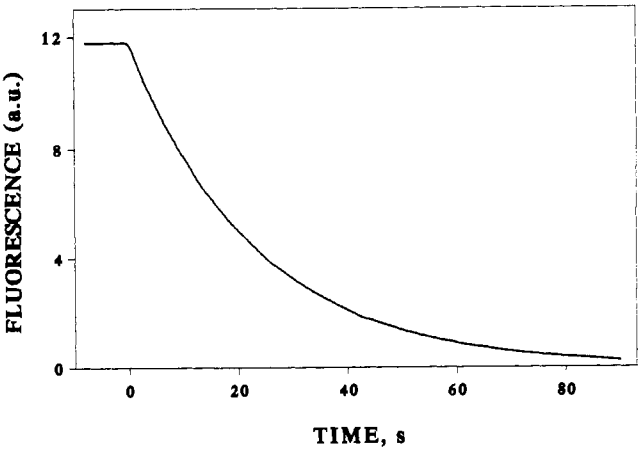

FIGURE 6: Displacement of VS bound to ribosomes by erythromycin. A suspension of 70S ribosomes (0.5 μM) and VS (2.5 μM) in buffer containing 1 mM $MgCl_2$ was mixed at 25 °C with a solution of erythromycin (20 μM). The kinetics of fluorescence decrease, monitoring the dissociation of VS, are presented. The observed rate constant, k_{obs} = 0.043 (±0.002) s^{-1}, has been calculated according to a simple exponential decay.

anticodon interaction (Labuda & Pörschke, 1982). Ribosome-bound Mg^{2+} mainly interacts with rRNA (0.4 Mg^{2+} per phosphate) (Goldberg, 1966; Wishnia & Boussert, 1977). To explain the Mg^{2+} dependence of subunit association, a specific-sites model (Zitomer & Flasks, 1972; Klotz, 1976) and an electrostatic model (Wishnia et al., 1975) have been proposed. The effects of alkaline earth and transition-metal ions on subunit association are compatible with a charge-neutralization binding of metal ions (Wishnia & Boussert, 1977). In contrast, very little is known on the possible role of mono- and divalent ions on antibiotic–ribosome interaction (Gale et al., 1980). A work along these lines is that of White and Cantor (1971), on the Mg^{2+}-dependent binding of tetracycline to *E. coli* ribosomes.

VS and 3-OH-picolinic methylamide had similar spectrophotometric behavior with respect to alkaline earth cations (shifts in absorption spectra and linear dependence of log K_a versus cationic radius, which represents a predominant electrostatic contribution to the overall binding interaction) (Grell et al., 1977, 1985; Oberbäumer et al., 1983). Accordingly, a possible coordination of the Mg^{2+} with the lactone ring has been excluded, and that of alkaline earth cations with the negatively charged form of the 3-hydroxypicolinyl residue has been inferred. The addition of Mg^{2+} to VS, at pH higher than 9, whereby the negatively charged form predominates, results in an increase of the fluorescence lifetimes [cf. the preceding paper (Clays et al., 1991)]. This effect can be attributed to a reduced nonradiative decay rate, caused by Mg^{2+} complexation. The latter involves the phenolate oxygen and the carboxyl amide group, as previously suggested (Grell et al., 1977).

The number of degrees of freedom for nonradiative relaxation is expected to decrease, when the VS–Mg^{2+} chelate interacts with the ribosome, and to cause an additional lengthening of the fluorescence lifetimes. These effects were indeed experimentally verified (Table I). They agree with previous studies of fluorescence quenching with ribosome–VS complexes, showing that the 3-hydroxypicolinyl residue is buried within the corresponding ribosome binding site and partially shielded from solvent access (Di Giambattista et al., 1984).

The ability of VS to chelate bivalent ions (alkaline earth and transition metals) might play a role in the stabilization of VS within ribosome–VS complexes: part of the binding

energy would then be due to a salt bridge between Mg^{2+} and ribosomes. To select among the three models of interaction of VS–Mg^{2+} with ribosomes (Figure 1), the dissociation rate constants of VS–Mg^{2+} chelate, in the absence and in the presence of ribosomes, and of the VS–ribosome complex have been measured. The k_- of the VS–Mg^{2+} chelate was evaluated from the Mg^{2+} displacement by Mn^{2+}. In the case of the VS–ribosome complex, Mn^{2+} and ERY were used as competitors. Our results indicate that Mg^{2+} at the VS binding site interacts with both the antibiotic and the ribosome: this is clearly shown by the 10-fold difference between the k_- of the VS–Mg^{2+} chelate in the absence (650 s^{-1}) and in the presence (65 s^{-1}) of ribosomes. Such a difference was not observed, however, with a fraction of the ribosome population (500 s^{-1}). One could argue that Mg^{2+} ions do not interact with these particular kinds of particles, but such a hypothesis is contradicted by the variation of the Mg^{2+} k_- (from the R–VS complex) as a function of Mn^{2+} concentration (Figure 3). These observations indicate that ribosomes modify the k_{obs} of VS–Mg^{2+} complexes and that Mg^{2+} replacement by Mn^{2+}, being incompatible with a charge neutralization model, produces a conformational change of the VS binding site which is responsible for a decrease of the Mg^{2+} k_- value.

The 3-hydroxypicolinyl residue of VS is essential for its antibiotic activity, as indicated by the lack of antimicrobial power of a virginiamycin S analogue in which the picolinyl moiety was replaced by a serine derivative (Kessler et al., 1983). This lack of activity could be due either to impaired penetration or to lowering of the association constant of the synthetic derivative to ribosomes. Competition experiments between VS and the serine analogue for in vitro binding to ribosomes have demonstrated the lack of affinity of the serine analogue (not shown). In conclusion, our data indicate that the 3-OH-picolinyl moiety of VS plays an essential role in VS interaction with ribosomes and that the stability of the linkage of this antibiotic with its target relies on a salt bridge with Mg^{2+}.

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Registry No. VS, 9040-14-6; 3-OH-Pic, 874-24-8; Mg^{2+}, 7439-95-4.

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