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Localization of Virginiamycin S Binding Site on Bacterial Ribosome by Fluorescence Energy Transfer

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ABSTRACT: Virginiamycin S, a type B synergimycin inhibiting protein synthesis in bacteria, competes with erythromycin for binding to the 50S ribosomal subunits; the mechanism of action of the two antibiotics is unclear. Energy-transfer experiments between virginiamycin S (which is endowed with inherent fluorescence due to its hydroxypicolinyl moiety) and fluorescent coumarinyl derivatives of ribosomal proteins L7 and L10 have been carried out to locate the binding site of this antibiotic on the ribosome. Previous studies have indicated that two L7/L12 dimers can attach respectively to a strong binding site located on the central protuberance and to a weak binding site located on the stalk of the 50S subunits and that protein L10 is located at the base of the stalk. The distance between ribosome-bound virginiamycin S and a fluorophore located on the strong binding site of proteins L7/L12 (Lys-51 of L7) was found to be 56 (±15) Å. Virginiamycin S, on the other hand, was located at a distance exceeding 67 Å from the weak binding site of L7/L12 dimers. A fluorophore positioned on the unique cysteine (Cys-70) of protein L10 and ribosome-bound virginiamycin S proved to be more than 60 Å apart. From data available on the location of proteins L7/L12 and L10, a model is proposed, whereby the virginiamycin S binding site is placed at the base of the central protuberance of the 50S subunits, in proximity of the presumptive peptidyl transferase center. The binding sites of macrolides and lincosamides (related antibiotics of the MLS group) are expected to be very close to that of virginiamycin S.

Virginiamycin-like antibiotics (synergimycins) contain two types of components (type A or virginiamycin M like and type B or virginiamycin S like), which inhibit synergistically protein synthesis in growing bacteria (each component increases 100-fold the inhibitory power of its partner) [for reviews, see Vázquez (1967, 1975), Tanaka (1975), Cocito (1979, 1983), and Cocito & Chinali (1985)]. Both components bind to the 50S ribosomal subunit: the association constant of the reaction for virginiamycin S–ribosome complex formation undergoes a 10-fold increase in the presence of virginiamycin M (Parfait & Cocito, 1980; Parfait et al., 1978; Cocito & Di Giambattista, 1978). Moreover, erythromycin ($K_a^{ery} = 7.2 \times 10^7$ M^{-1}), which displaces virginiamycin S ($K_a^{VS} = 2.5 \times 10^6$ M^{-1}) from its ribosome complex, is unable to exert this competition effect in the presence of virginiamycin M (Parfait et al., 1981). The latter produces a conformational change of the 50S subunit resulting in a permanent inactivation of the substrate acceptor and donor sites of peptidyltransferase (Cocito & Kaji, 1971; Chinali et al., 1981, 1984).

Identification of an antibiotic binding site yields information on both the mechanism of action of a given inhibitor and the structure and function of its target. Location of inhibitors on the ribosome surface has been obtained by photoaffinity labeling (Sonenberg et al., 1977; Nicholson et al., 1982a,b; Tejedor & Ballesta, 1985) and immune electron microscopy (Mc Kuskie Olson et al., 1982). The intrinsic fluorescence of type B synergimycins has suggested the possibility of localization of their ribosome binding site by fluorescence energy transfer. The latter approach, which is based on the distance dependence of long-range nonradiative transfer of excitation energy between chromophores, has been chosen for this study. According to previous studies (Di Giambattista et al., 1984) on quenching of virginiamycin S fluorescence,

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the corresponding binding site on the ribosome surface has the shape of an open well: the access to this well of the 3-hydroxypicolinyl residue of the antibiotic (which is responsible for its fluorescence) is partly controlled by electrostatic forces.

It is known that the large ribosomal subunit contains a unique acidic protein occurring, unlike the other L proteins, in multiple copies (Subramanian, 1975). Recent studies have shown that, although one dimer of L7/L12 is required for full activity in EF-G-dependent GTP hydrolysis, two L7/L12 dimers are required for polyphenylalanine synthesis (Möller et al., 1983). Two L7/L12 dimers (the two proteins have identical amino acid sequence, the terminal NH_2 group of serine being acetylated in L7) (Terhorst et al., 1972) have been recognized: they have separate binding sites, one being placed on the "stalk" and the other on the "central protuberance" of the 50S subunit (Zantema et al., 1982b; Thielen et al., 1984). Works from several laboratories suggest the location of L10 in proximity of the two L7/L12 dimers (Clegg & Hayes, 1974; Expert-Bezançon et al., 1976). Indeed, a complex of L7, L12, and L10 has been obtained by salt washing of ribosomes (Pettersson et al., 1976).

The strategy adopted in the experiments herewith described was to measure the energy transfer between virginiamycin S and fluorescence-labeled derivatives of L7 and L10. The L7 dimers were positioned respectively at the strong and weak binding sites. Preliminary to these determinations, the partial overlapping of the VS emission spectrum with the absorption spectra of the coumarinyl derivatives of L7 and L10 was assessed. This study has allowed location of the virginiamycin S binding site in a region of 50S subunits, which is close to the presumptive peptidyltransferase catalytic center.

Materials and Methods

Buffers. Buffer A_1 contained 20 mM Tris-HCl,[1] pH 7.6, 60 mM NH_4Cl, 10 mM $MgCl_2$, and 6 mM β-mercaptoethanol; buffer A_2 was as buffer A_1 but contained 1 M NH_4Cl; buffer A_3 was as buffer A_1 but contained 1 mM $MgCl_2$. Buffer B contained 20 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 200 mM NH_4Cl, and 6 mM β-mercaptoethanol.

Chemicals. Analytical-grade glycerol was a product from Merck (Darmstadt, FRG). Quinine sulfate was purchased from Regis Chemical Co.

Antibiotics. Virginiamycin components M and S were crystallized from fermentation products (SKF-RIT Laboratories, Rixensart, Belgium, courtesy of Dr. J. I. H. Phillip). Erythromycin was from Sigma.

Preparation of Ribosomes and Subunits. Suspensions of *Escherichia coli* A19 in buffer A_1 were disrupted in a French pressure cell (Aminco), incubated with DNase I (EC 3.1.21.1, 1 mg/10 g) for 15 min at 20 °C, and centrifuged at 30000*g* for 10 min at 4 °C. Supernatants were centrifuged at 300000*g* for 2 h at 4 °C, and pellets of crude ribosomes were suspended overnight in buffer A_2. After centrifugation at 30000*g* for 10 min at 4 °C, supernatants were withdrawn and centrifuged for 4 h at 300000*g* (angular rotor 50.2 Ti from Spinco). Supernatants were discarded, and pellets of washed ribosomes were suspended in buffer A_3 and dialyzed for 14 h at 4 °C against the same buffer. Samples containing approximately 500 A_{260} units of ribosomal subunits were layered on a 7.5–40% (w/v) sucrose gradient in buffer A_3 and centrifuged for 17 h at 95000*g* at 4 °C in a SW-27 rotor from Spinco. Pellets of subunits, collected by centrifugation, were suspended in buffer A_1 and centrifuged at 10000*g* for 20 min at 4 °C; supernatants were dialyzed against buffer A_1 and stored in liquid nitrogen. Ribosomes were reactivated for 5 min at 40 °C before use.

Preparation of Cores and Split Proteins. The 50S ribosomal subunits lacking proteins L7/L12 (P_0 cores) were prepared by addition of cold ethanol (50% v/v final concentration) to a solution of 50S ribosomes in 1 M NH_4Cl at 0 °C (Hamel et al., 1972). P_{37} cores, i.e., 50S subunits lacking proteins L7/L12 and L10, were obtained by performing the same experiment at 37 °C. Ribosome concentrations were determined from the absorbance at 260 nm, assuming that 1 A_{260} unit corresponds to 39 pmol of 50S ribosome or equivalent core. Core proteins and split proteins were analyzed by bidimensional electrophoresis on polyacrylamide gels (Kaltschmidt & Wittmann, 1970).

Preparation of Fluorescence-Labeled Proteins L7 and L10. The preparations of specifically labeled protein L7 (COU-L7), carrying a coumarinyl moiety at Lys-51, and protein L10 (DACM-L10) modified at the single cysteine residue with *N*-[7-(dimethylamino)-4-methylcoumarinyl]maleimide were performed according to Zantema et al. (1982a). The degree of modification was based on an ϵ_{max} of 42000 M^{-1} cm^{-1} (Eastman catalog) for the L7 derivative and of 24000 M^{-1} cm^{-1} for DACM-L10 (Yamamoto et al., 1977). The amounts of coumarinyl derivatives incorporated were 0.86 mol/mol of L7 (75% at Lys-51) (the remaining 25% of fluorophore was in Lys-95 and Lys-100 of L7) and about 0.5 mol/mol of L10.

Preparation of Fluorescence-Labeled Ribosomal Subunits. Four different types of reconstituted 50S ribosome were used for energy-transfer measurements:

(1) P_0 cores lacking proteins L7 and L12 were incubated with 5 equiv of COU-L7 in buffer A_1 for 5 min at 25 °C. The resulting fluorescent 50S subunits were isolated either by centrifugation through a sucrose cushion (18% sucrose w/v, 6 h, 45000 rpm, SW 50.1 rotor of Spinco) or by chromatography on 5-mL Sepharose 6B columns. The number of isolated COU-L7 per 50S subunit was estimated from the extinction coefficient of the fluorophore (Table I). In this case, the two purification procedures yielded on the average 1.45 COU-L7 per 50S subunit. Although two COU-L7 dimers did bind to each subunit during the incubation step, the dimer positioned at the weak binding site was released from ribosomes during purification (Zantema et al., 1982b; Thielen et al., 1984).

(2) P_0 cores were first incubated with 5 equiv of L7/L12 prepared under denaturing conditions (6 M urea) followed by purification as in the previous section. Subunits carrying one unlabeled L7/L12 dimer positioned at the strong binding site were then titrated with COU-L7, in order to fill the weak binding site.

(3) Virginiamycin S-P_0 cores complexes were directly titrated with COU-L7 (0–8 equiv) without purification steps.

(4) P_{37} cores missing proteins L7, L12, and L10 were reconstituted by incubation with 2 equiv of DACM-L10 and 6 equiv of unlabeled L7/L12 and purified by chromatography on Sepharose 6B. An incorporation of 0.45 mol of DACM-L10 per 50S was estimated from the quantum yield of DACM-L10 presented in Table I.

Fluorescence Measurements. The association constants of complex formation of virginiamycin S with 50S subunits and their cores (P_0 and P_{37}) were determined by steady-state fluorescence measurements in buffer B according to Parfait

[1] Abbreviations: DACM, *N*-[7-(dimethylamino)-4-methylcoumarinyl]maleimide; DACM-L10, fluorescent derivative of protein L10 with a coumarinyl moiety on Cys-70; FPB-L7, 4-(4-formylphenoxy)butyrimidate derivative of protein L7; COU-L7, coumarin 314 hydrazide derivative of FPB-L7; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

et al. (1978). Increase of fluorescence intensity (ΔF_{416nm}) occurring upon addition of 50S particles to virginiamycin S solutions was previously shown to be proportional to the concentration of ribosome-bound antibiotic. For this purpose, reactivated 50S subunits (5 min incubated at 40 °C in buffer A_1) were incubated with increasing concentrations of virginiamycin S in buffer B.

In the different reconstituted particles, 1 μM ribosomes or equivalent cores and 0.1 μM virginiamycin S were used for the transfer measurements. The corresponding association constants were 5.0×10^6 M^{-1} for 50S–VS complex, 5.7×10^6 M^{-1} for P_0 core–VS complex, and 4.8×10^6 M^{-1} for P_{37} core–VS complex. In every case, more than 80% of total antibiotic was bound to the ribosomal particles.

Measurements were carried out with an Aminco Bowman SPR-Ratio II spectrofluorometer equipped with a thermostated cell housing (25 °C). The excitation monochromator was set at 330 nm, and the emission spectra were scanned in the range 350–550 nm; 10-nm slits were used in both cases. The excitation and emission wavelengths for virginiamycin S were 330 and 416 nm. Concentration of bound antibiotic [VS_B] was determined from total VS in solution [VS_T] according to the equation

$$[VS_B] = \frac{F - F_F}{F_F(\gamma - 1)}[VS_T] \quad (1)$$

where F and F_F are the fluorescence intensities in the presence and absence of ribosome particles, respectively, and γ a constant representing the ratio of the fluorescence per mole of bound VS to that of free virginiamycin S. The γ value was determined by adding a large excess of ribosome to a VS solution: under these conditions, virtually all virginiamycin S in solution was bound to the particles.

Fluorescence Polarization Measurements. Fluorescence polarization experiments were made by placing two Glan Prism polarizers with 10-nm slits in the paths of the excited and emitted beams. The emission anisotropy r was calculated from

$$r = (F_{VV} - TF_{VH})/(F_{VV} + 2TF_{VH}) \quad (2)$$

where F_{VV} and F_{VH} were the relative intensities of the vertical and horizontal fluorescent components with vertically polarized excitation. T was given by F_{HV}/F_{HH}, the ratio of vertical and horizontal fluorescent components with horizontally polarized light. When spectra were taken with vertically polarized excitation light and separate measurements of vertical (F_{VV}) and horizontal (F_{VH}) signals by the use of polarizers, total fluorescence intensity was then given by the equation:

$$F = F_{VV} + 2F_{VH} \quad (3)$$

Determination of Quantum Yield. Quantum yields of free and ribosome-bound virginiamycin S, Q_F and Q_B, were determined by reference to a standard of quinine sulfate. The Q value of quinine sulfate in 0.05 N H_2SO_4 was assumed to be 0.55 (Melhuish, 1965). Solutions of free and bound virginiamycin S were adjusted to give absorbancy values (<0.03) approximately equivalent to that of quinine sulfate, when measured at λ_{ex} 330 nm. Quantum yields were calculated with the relation

$$Q = (F_{VS}/F_S)(A_S/A_{VS}) \times 0.55 \quad (4)$$

where F_{VS} is the area under the emission spectrum of virginiamycin S, S is the area under the emission spectrum of quinine sulfate, and A_{VS} and A_S are the corresponding absorbancies at 330 nm.

Evaluation of Energy Transfer between Fluorophores. Energy transfer between ribosome-bound virginiamycin S (donor) and a coumarinyl derivative of ribosomal proteins L7 and L10 was measured by comparing the quantum yield or fluorescence intensity at 420 nm (λ_{ex} 330 nm) of virginiamycin S bound respectively to ribosomes (or to cores) and to reconstituted fluorescent particles. Conversely, the core–virginiamycin S complexes were titrated with the appropriate fluorescent proteins.

Energy-transfer measurements represent net (i.e., corrected) values. Indeed, for every donor–acceptor couple, the following fluorescence spectra were drawn at the excitation value (330 nm): (a) ribosomes or cores alone; (b) virginiamycin S–ribosome complexes; (c) ribosome-bound COU-L7 or DACM-L10; (d) couples of donor and acceptor fluorophores on ribosomes. A correction for donor contribution was, thus, feasible.

Efficiency of energy transfer, E, between a donor and an acceptor chromophore is related to their distance R by the relation (Förster, 1965):

$$R = (1/E - 1)^{1/6}R_0 \quad (5)$$

R_0, which is called the "Förster critical distance", corresponds to the distance allowing a transfer efficiency of 50% (Förster, 1965). R_0 is related to the spectral properties of both donor and acceptor fluorophores by the relation

$$R_0 = (9.765 \times 10^3)(K^2\bar{J}Q_D n^{-4})^{1/6} \quad (6)$$

where Q_D represents the donor quantum yields (virginiamycin S) in the absence of acceptor and n is the refractive index of the medium [for which a value of 1.4 is generally used, according to Beardsley & Cantor (1970)]. The overlap integral $\bar{J}$ represents the degree of resonance between donor and acceptor dipoles, and its value is obtained by integration of the overlapping area of donor emission spectrum F_D and acceptor absorption spectrum ϵ_A, according to the equation:

$$\bar{J} = \sum_{\Delta\lambda} F_D(\lambda)\epsilon_A(\lambda)\lambda^4\,\Delta\lambda / \sum_{\Delta\lambda} F_D(\lambda)\,\Delta\lambda \quad (7)$$

where λ = wavelength (nm) and ϵ = molar absorption coefficient. Limits of K^2, the orientation factor, and of the donor–acceptor distance were calculated according to Dale and Eisinger (1979).

The problem of K^2 evaluation in relationship 6 has been thoroughly analyzed by Haas et al. (1978) and by Dale et al. (1979). According to the latter authors, steady-state emission anisotropy of bound fluorophores yields information on both their orientational freedom and their relative orientation. The K^2 value can be obtained either by anisotropy determination or by polarization measurement: the former measurement was chosen in the present work. According to Dale et al. (1979), the observed depolarization factors $\langle d_D \rangle$ and $\langle d_A \rangle$ are the ratio of limiting anisotropies (i.e., the anisotropy values extrapolated to infinite viscosity, which reflect the orientations of bound fluorophores following excitation) and of fundamental anisotropy ($r_f = 0.4$). The observed axial depolarization factors $\langle d_A^x \rangle$ and $\langle d_D^x \rangle$ are the numerical square roots of the depolarization factor themselves. When depolarization factors of both donor and acceptor fluorophores are determined, the contour plots provided by the above authors can be used to obtain the maximum and minimum values of K^2. Actually, two K^2 values are reported in Table II: one was obtained from the anisotropy measurement and the other by assuming a $^2/_3$ value of approximation. Minor differences between the two kinds of measurement were recorded.

It can be objected that R_0 values were calculated by assuming the occurrence of a single coumarinyl per L7 dimer. In case of two fluorescent monomers, correctness of the R_0

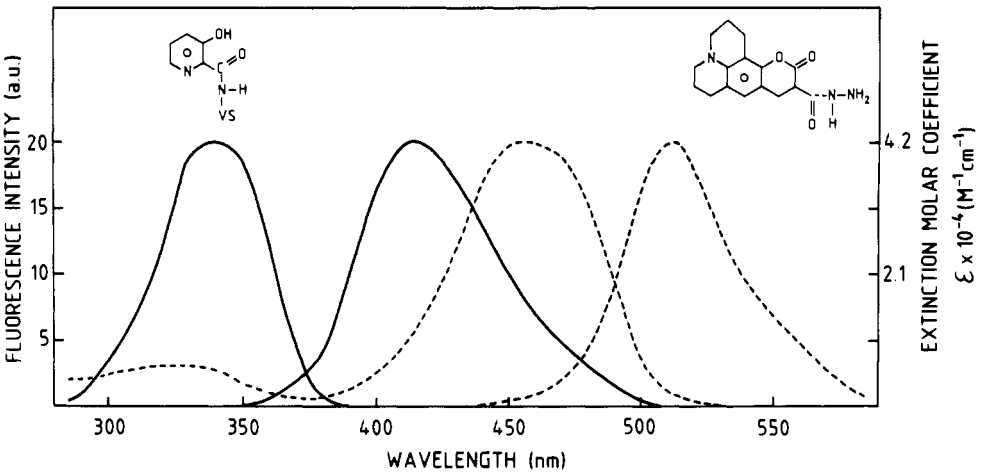

FIGURE 1: Absorption and emission spectra of virginiamycin S (donor) (continuous lines) and of a coumarinyl derivative of ribosomal protein L7 (acceptor) (dotted lines). The spectra of 0.5 μM VS and 5 μM COU-L7 (carrying a coumarinyl group at lysine-51 of protein L7) in buffer A were normalized. The fluorescence spectrum of VS was recorded at an excitation wavelength of 330 nm. (J, the overlap integral, was calculated for the pair VS–COU-L7 to be 7.4×10^{-14} cm^6/mol.)

Table I: Spectroscopic Properties of Virginiamycin S and Fluorescence Derivatives of L7 and L10

	λ_{abs} (nm)[a]	ϵ ($\times 10^3$ M^{-1} cm^{-1})[a]	λ_{emi} (nm) for bound	Q[a] free	Q[a] bound	r[b]	r_0
virginiamycin S	340	7.7	416	0.30	0.51	0.30[b]	0.31[d]
COU-L7	453	42	503	0.23	0.10	0.31[c]	0.31[c]
DACM-L10	402	24	472	0.31	0.25	0.32[c]	0.32[c]

[a] Q = quantum yield, as defined by eq 4. [b] r = emission anisotropy, as defined in eq 2. [c] According to Zantema et al. (1982). [d] See Figure 3.

values in Table II would rely on the closeness of the acceptor coumarinyl residue to donor virginiamycin S. Under those circumstances, the actual R_0 value would be $1.12(2^{1/6})$ of that reported in Table II (Gennis & Cantor, 1972). This corresponds to an upper deviation value of 12%, which lies within the uncertainty limits of K^2 estimations.

In the case of the coumarinyl derivative of protein L7 (COU-L7), whereby two acceptors were present, the simplified equation of Gennis and Cantor (1972) was used:

$$E_{(N)} = \sum_{i=1}^{N}(R_0/R_i)^6/[1 + \sum_{i=1}^{N}(R_0/R_i)^6] \quad (8)$$

In this relation, the two acceptors were considered as equivalent to a single acceptor with an extinction coefficient twice as high. The transfer efficiency E was determined by measuring the quenching of donor fluorescence emission at a wavelength (420 nm) where no acceptor emission was recorded. For this purpose, the following relation was used (Epe et al., 1982):

$$E = (1 - Q_{DA}/Q_D)(1/\chi_A) \quad (9)$$

where Q_{DA} and Q_D are the relative quantum yields of the donor in the presence and in the absence of acceptor and χ_A is the degree of labeling of ribosomal protein with the acceptor fluorophore.

Conversely, transfer efficiency E was determined by measuring the increase in fluorescence intensity of the acceptor fluorophore. For this purpose, the relationship

$$E = (H - 1)(\epsilon_A/\epsilon_D)/\chi_d \quad (10)$$

was used, in which H represents the ratio of fluorescence intensities of the acceptor in the presence and in the absence of the donor, ϵ_D and ϵ_A are the molar extinction coefficients of donor and acceptor at the excitation wavelength (330 nm), and χ_d is the degree of ribosome labeling by the acceptor fluorophore.

Spectrophotometric Measurements. Absorption spectra were obtained with a Beckman double-beam spectrophotometer in 1-cm path quartz cuvettes. The reconstitution buffer A_1 was used in all cases. The λ_{abs}(max) and ϵ values that were determined for the free fluorophores were also used for bound fluorophores.

Results

Analysis of Spectroscopic Properties of Virginiamycin S and of Fluorescence-Labeled Derivatives of L7/L12 Ribosomal Proteins. Preliminary to experiments of energy transfer between chromophores, it was necessary to explore the spectroscopic properties of the reacting components of our system. In fact, transfer of excitation energy requires an overlapping of the emission spectrum of the donor and the absorption spectrum of the acceptor.

In Figure 1 the emission spectrum of virginiamycin S and the absorption spectrum of the coumarinyl derivative at lysine-51 of protein L7 (COU-L7) are reported. There is evidence for a partial overlapping of the two spectra. Similar conclusions can be drawn for the coumarinyl derivative at the cysteine of protein L10 (DACM-L10) according to Figure 2. The structure of the picolinyl moiety of virginiamycin S (the part of the antibiotic molecule involved in the fluorescence effect) and of the coumarinyl derivatives used for labeling proteins L7 and L10 is displayed in Figures 1 and 2.

The spectroscopic properties of virginiamycin S and of the coumarinyl derivatives of L7 and L10 are summarized in Table I. The latter relates the quantum yields of the three reagents, in the free form and after complexing with ribosomes. It is known that measurements of the steady-state emission anisotropy yield information on both the degree of freedom and the orientation of bound fluorophores. A plot of the reciprocal anisotropy of virginiamycin S against the temperature/viscosity ratio is displayed in Figure 3: a straight line of the Perrin–Weber plot is evident. The reciprocal anisotropy $1/r$ value for $T/\eta = 0$ (the ordonate intercept by the curve in Figure 3) yields the limiting anisotropy value that is required for K^2 estimation, as indicated under Evaluation of Energy Transfer

Table II: Parameters of Energy Transfer between Virginiamycin S and Fluorescence-Labeled L7 and L10

fluorophore acceptor	J (cm^6/mol)	K^{2a}	$R_{0,2/3}$ (Å)^b	R_0 (Å)	E^c	R (Å)	$R_{2/3}$ (Å)
COU-L7 (strong binding site)	7.4×10^{-14}	0.10–3.3	42	31–55	0.18 ± 0.014	56 ± 15	54
COU-L7 (weak binding site)	7.4×10^{-14}	0.10–3.3	42	31–55	<0.01^d	>67	>70
DACM-L10	2.9×10^{-15}	0.10–3.6	25	18–33	<0.005^d	>60	>60

^{a} Minimum and maximum values were determined according to Dale and Eisinger (1975). $^b R_0$ was calculated from eq 6 assuming $K^2 = {}^2/_3$. $^c E$ was measured by two independent procedures, i.e., quenching of donor fluorescence (eq 9 under Materials and Methods) and enhancement of acceptor fluorescence (eq 10). The variation range between the two sets of measurements, each represented by the mean value of three independent determinations was of the order of 5–7%. ^{d} The E values were too small to be accurately measured.

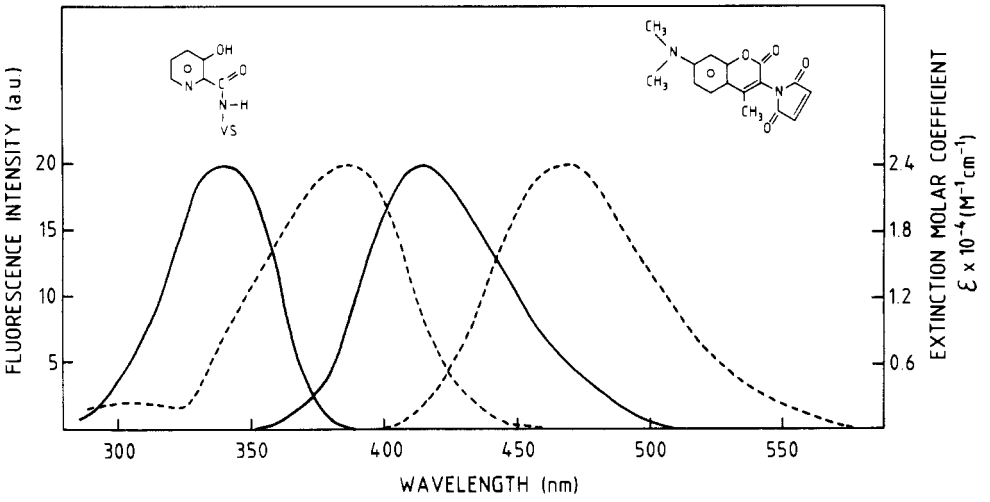

FIGURE 2: Absorption and emission spectra of virginiamycin S (donor) (continuous lines) and of a coumarinyl derivative of ribosomal protein L10 (acceptor) (dotted lines). The conditions were similar to those in Figure 1, except that 8 μM DACM-L10 carrying an *N*-[7-(dimethylamino)-4-methylcoumarinyl] moiety at the cysteine residue of L10 was used. (J, the overlap integral, was calculated for the pair VS–DACM-L10 to be 2.9×10^{-15} cm^6/mol.)

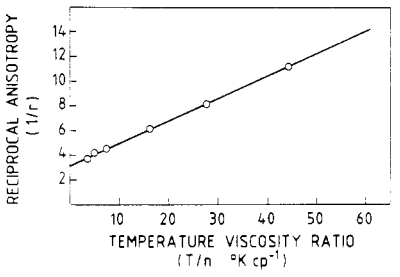

FIGURE 3: Perrin–Weber plot of the fluorescence emission anisotropy of virginiamycin S. To a solution of virginiamycin S (5 μM in buffer A), an increasing amount of glycerol was added to increase the viscosity (a constant temperature, 25 °C, was maintained throughout the experiment), and fluorescence intensity was measured in two perpendicular directions (F_{VV} and F_{VH}). The excitation and emission wavelengths were 330 and 416 nm, respectively. Emission anisotropy was calculated from eq 2 and plotted against the temperature viscosity ratio.

between Fluorophores. The corresponding r and r_0 values are reported in Table I.

In conclusion, data in Figures 1–3 and Table I indicate that virginiamycin S and the two coumarinyl derivatives of L7 are suitable reagents for energy-transfer experiments and furnish all the parameters necessary for a measurement of the transfer and for an interpretation of the results.

Energy Transfer between Ribosome-Bound Virginiamycin S and Fluorescence-Labeled L7/L12 Dimers. It has been previously shown that two different binding sites for L7/L12 dimers can be recognized on the surface of 50S subunits: L7/L12 can be removed from the weak binding site by density gradient centrifugation, a treatment that does not affect dimers located at the strong binding site. Isolation of L7/L12 under denaturing conditions facilitates its subsequent removal from the weak binding site. These observations would allow a positioning of L7/L12 dimers carrying a fluorescent probe at each of the two binding sites, in view of performing energy-transfer experiments with ribosome-bound virginiamycin S.

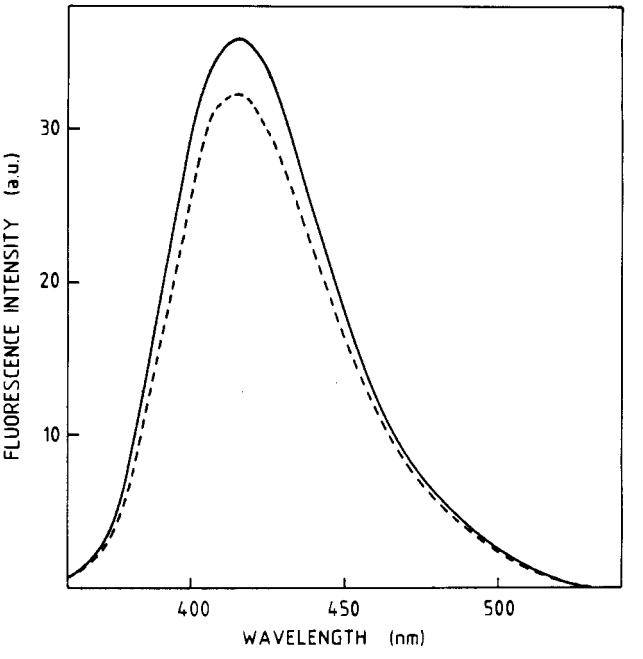

FIGURE 4: Typical fluorescence energy transfer between virginiamycin S (donor) and the coumarinyl derivative of protein L7 (acceptor). P_0 cores–virginiamycin S complexes were titrated with 8 equiv of L7 carrying a coumarinyl group at lysine-51 (cf. Materials and Methods). (—) Virginiamycin S (donor) in the absence of COU-L7 (acceptor); (– –) virginiamycin S (donor) in the presence of COU-L7 (acceptor).

In a first series of experiments, P_0 cores of L7/L12-depleted 50S subunits were incubated with an excess of COU-L7. The reaction mixture was submitted either to density gradient centrifugation or to exclusion chromatography, to selectively

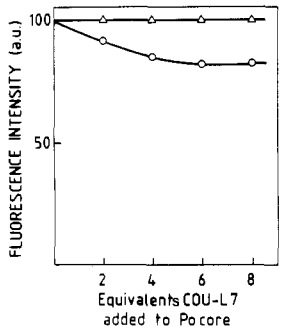

FIGURE 5: Quenching of the fluorescence of ribosome-bound virginiamycin S by fluorescent L7 protein (○). To a suspension of virginiamycin S–P_0 core complexes, increasing amounts of L7 carrying a coumarinyl group at lysine-51 were added, and the fluorescence intensity of the samples was measured. (△) Fluorescence intensity measurements of the same sample, after addition of 5 μM erythromycin. Similar titration with COU-L7 of VS–P_0 cores in which the strong binding site was filled with nonfluorescent L7 yielded a curve overlapping that of the erythromycin sample (cf. the column of E values in Table II).

remove the L7/L12 dimer from the weak binding site. After binding of VS, the energy transfer between the latter donor chromophore and coumarinyl-L7 as a recipient chromophore positioned at the L7/L12 strong binding site was measured (Figure 4). As shown in Table II (first line), data calculated according to two different estimations of K^2 led to a distance value of 56 (±15) Å between the two chromophores. In reality, energy-transfer experiments were carried out by two methods: quenching of donor fluorescence and enhancement of acceptor fluorescence.

A second set of experiments was carried out by incubating P_0 cores with unlabeled L7/L12 prepared under denaturing conditions. After chromatographic or ultracentrifugal fractionation, leading to removal of the L7/L12 dimer from the "weak" binding site, the latter was filled with COU-L7. Virginiamycin S was then added, and energy transfer between the latter donor chromophore and the COU-L7 recipient positioned at the weak binding site was measured. Data summarized in Table II (second line) suggest that the distance between the virginiamycin S binding site and the chromophore on the L7/L12 weak binding site exceeds 70 Å.

Additional experiments were performed with P_0 cores of L7/L12-depleted 50S ribosomal subunits. To these cores virginiamycin S was bound, and a titration was done with COU-L7 (Figure 5). These experiments indicate that for saturating concentrations of L7/L12 dimers leading to occupation of the two L7/L12 sites maximum energy transfer is about 18%. This value is very close to that obtained when only the strong L7/L12 site is filled (Figure 4). The finding that erythromycin displaces VS from its binding site on ribosome has allowed a control experiment showing that quenching of donor VS fluorescence in Figure 5 is due to a specific transfer of energy. Indeed, addition of erythromycin to a VS–ribosome–COU-L7 system completely abolished energy transfer (legend to Figure 5).

In conclusion, our data show that the virginiamycin S binding site is relatively close to the fluorescence marker in the "strong" L7/L12 binding site and far from that of the "weak" binding site.

Energy Transfer between Ribosome-Bound Virginiamycin S and Fluorescence-Labeled Protein L10. The synthesis of fluorescence-labeled preparations of L10 by inserting a coumarinyl group on the single cysteine (Cys-70) of this protein has prompted further energy-transfer experiments. As a matter of fact, the reported location of L10 in the proximity of the two L7/L12 dimers would further help the location of the virginiamycin S binding site.

Accordingly, P_{37} cores missing proteins L7, L12, and L10 were prepared and incubated with 2 equiv of the DACM-L10 and 6 equiv of L7/L12 dimers. After purification on chromatography columns, the 50S complexes were incubated with virginiamycin S, and the energy transfer between this antibiotic and the recipient L10 chromophore was measured. As shown in Table II (third line), the distance between the two chromophores largely exceeded 60 Å.

Discussion

Experimental Approach. Transfer measurements between fluorophores were used in this work to locate the virginiamycin S binding site on the ribosome surface. Similar approaches were already used to solve other problems of ribosome topology. This technique was used, for instance, to measure the distance between the 3′ ends of the three rRNAs within ribosomes, which were reconstituted with fluorescence-labeled 5S, 16S, and 23S rRNA (Odom et al., 1980). Likewise, energy-transfer measurements between fluorescent derivatives of tRNA, which were positioned at the A and P sites of ribosomes, were made, and changes of the degree of freedom of ribosome-bound aminoacyl-tRNA, which were triggered by translocational events, were recorded (Robertson & Wintermeyer, 1981; Paulsen et al., 1983). Distances between the 3′ end of 16S rRNA within the 30S subunit and fluorescent probes in P site bound tRNA have been measured (Robbins et al., 1981). By this method, it was possible to obtain a triangulation of L6, L11, and L10, specifically labeled at their single thiol group (Steinhaüser et al., 1983).

Energy Transfer between Fluorophores. In this paper, the inherent fluorescence of virginiamycin S was used as a donor fluorophore, while the coumarinyl derivatives of ribosomal proteins L7 and L10 did function as recipient fluorophores. Natural fluorescence of type B synergimycins is due to their 3-hydroxypicolinyl moieties, as shown by previous experiments in which the behavior of this component and of the whole molecule was compared in the presence of dynamic quenchers and ribosomes (Di Giambattista et al., 1984). Properties of the coumarinyl derivatives of proteins L7 and L10 have been described previously (Zantema et al., 1984a,b).

As shown in Table II, the distance between ribosome-bound virginiamycin S and L7 located on the strong binding site was determined with sufficient precision. Instead, approximate distance values (due to low efficiencies of transfer) between the antibiotic and Cys-70 of protein L10, or Lys-51 of L7 in the weak binding site, were determined. Yet, these minimal values have enabled the location of the binding site of virginiamycin S (cf. Topological Location of Virginiamycin Binding Site under Discussion).

Relationship among Antibiotics of MLS Group. The occurrence of an inducible undissociated type of resistance against the members of the MLS group of antibiotics (which includes macrolides, lincosamides and type B synergimycins) has been exhaustively investigated in *Staphylococcus*. Incubation of these bacteria with low concentrations of erythromycin (a macrolide) entails the acquirement of a multiple resistance against the members of the related families, lincosamides and type B synergimycins (Weisblum & Demohn, 1969). Such a resistance was proven to be due to a dimethylation of 23S rRNA by an RNA methylase, which is coded for by the inducible plasmid pE 194 (Weisblum et al., 1979). Indeed, 50S ribosomal subunits harboring N^6,N^6-dimethyladenine in their 23S rRNA do not bind antibiotics of the MLS group (Lai et al., 1973). The 23S rRNA segment

bearing the N^6,N^6-dimethyladenine presumably lies within the overlapping portions of the binding sites of MLS antibiotics.

Competition between erythromycin and virginiamycin S for binding to the 50S subunits is well documented (de Bêthune & Nierhaus, 1978, Parfait et al., 1978; Parfait & Cocito, 1980). In fact, erythromycin ($K_A = 7.2 \times 10^7$ M^{-1}) prevents fixation of virginiamycin S ($K_A = 2.5 \times 10^6$ M^{-1}) to ribosomes and displaces the latter from its binding site on the particles. Stopped-flow kinetics experiments clearly indicate that the two antibiotics are mutually exclusive, for only one of them can bind at any time to a ribosome (Moureau et al., 1983). The simplest interpretation of data in Figure 5, whereby erythromycin prevented the energy transfer between virginiamycin S and fluorescence labeled L7 on the ribosomes, is that the former inhibitor removed the latter (donor fluorophore) from the ribosome.

Topology of Ribosomal Proteins. According to our approach, the virginiamycin S binding site is mapped on the ribosome surface, with respect to the topographical location of proteins L7, L12, and L10. Although the application of immune electron microscopy techniques pointed to the presence of L7/L12 proteins on the "stalk" (Lake, 1976; Kastner et al., 1981), it was subsequently shown that the two L7/L12 dimers have different locations on the ribosomes. Indeed, the occurrence of a strong binding site at the base of the central protuberance, and of a weak binding site on the stalk, was recognized by three different approaches based respectively on immune electron microscopy (Maassen et al., 1984), fluorescence energy transfer (Thielen et al., 1984), and radioimmunological techniques (Möller et al., 1983). Evidence for a location of the strong L7/L12 binding site on the central protuberance is provided by immunoelectromicroscopy work with stalkless ribosomes (Stöffler et al., 1980) as well as by experiments in which cross-linking was obtained between L7/L12 and several L proteins known to be located on the central protuberance (Traut, 1983). Our data give further support to this conclusion.

Several works yield converging evidence for a location of protein L10 in close contact with both L7/L12 dimers. First of all, extraction of ribosomes with a 1 M NH_4Cl–50% ethanol mixture at 37 °C yields a complex of proteins L7, L12, and L10 (Highland & Howards, 1975; Strycharz et al., 1978). In addition, the location of L7/L12 relative to protein L10 was obtained by energy-transfer studies involving a series of fluorescence probes inserted into different regions of L7, L12, and L10 molecules (Zantema et al., 1982a,b). Two of these fluorescence-labeled proteins were, in fact, used in this work. In turn, L10 was located on the ribosome surface with respect to other ribosomal proteins. Thus, a proximity of L6, L10, and L11 was demonstrated by energy transfer (Steinhäuser et al., 1983). Reconstitution experiments indicated also that L7/L12 binding is dependent on L10 and the binding of the latter on the presence of L11.

Topological Location of Virginiamycin Binding Site. In Figure 6, the two L7/L12 dimers and protein L10 have been mapped on the surface of the 50S subunit, according to the data cited in the previous section. By taking the strong L7/L12 site and the L10 site as centers, two spheres have been traced, the corresponding radii representing the minimal distances between virginiamycin S and the reference proteins. The hatched surface shows the proposed location of the antibiotic binding site, at the base of the central protuberance. This site would, then, be in proximity of the peptidyltransferase domain.

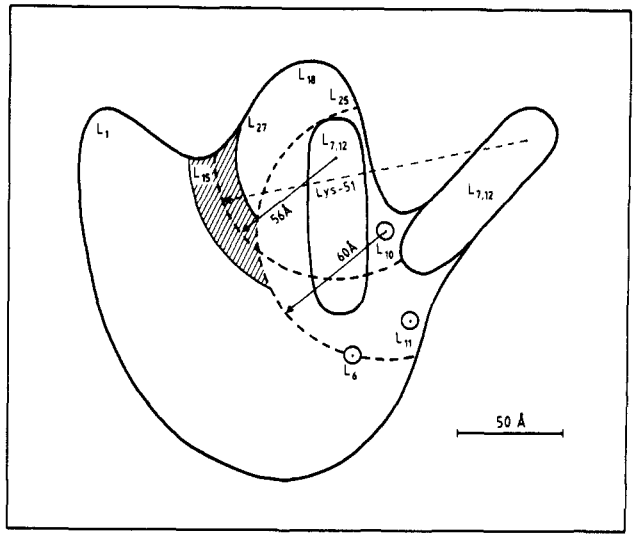

FIGURE 6: Hypothetical model for the location of the virginiamycin S binding site with respect to the two L7/L12 dimers and to protein L10 on the surface of the 50S ribosomal subunits. The distances reported have been drawn by assuming for the 50S particle and L7/L12 dimer sizes of 200 and 100 Å, respectively. The position of lysine-51 carrying the fluorescence probe was chosen according to the results of Maassen et al. (1984). Fluorescence-labeled cysteine residues of L10, L6, and L11 are positioned according to Steinhäuser et al. (1983).

The catalytic center of this enzyme overlaps the binding sites of a series of ribosomal proteins including L1, L2, L14, L15, L26, and L27. In fact, affinity labeling of the P site with aminoacyl-tRNA derivatives led to recognition of L2, L15, and L27 (L14, L26) (Pellegrini et al., 1972, 1974; Sapori et al., 1974; Küchler & Ofengand, 1979). Proteins localized by immune electron microscopy were L1, L15, and L27 (Lake, 1980; Stöffler et al., 1980). Also, the use of chloramphenicol (a reference inhibitor of peptidyltransferase reactions) and of puromycin (a reference reagent for peptidyltransfer reactions) in affinity labeling experiments led to identification of proteins L2 and L27 (Sonenberg et al., 1973) and of L15, L18/22, and L23 (Nicholson et al., 1982a–c), respectively. Consequently, available data tend to locate the peptidyltransferase domain in the hollow between the central protuberance and the L1 shoulder: this region houses the chloramphenicol and puromycin binding sites.

The overall conclusion of our studies is that the binding sites of virginiamycin S and erythromycin are located at the base of the central protuberance of 50S subunits, in close contact with the presumptive peptidyltransferase domain and the L15 binding site (Lotti et al., 1983). A portion of the 23S rRNA molecule including nucleotide 2058, which carries the dimethyladenine responsible for the resistance to MLS antibiotics (Weisblum & Demohn, 1969), presumably lies within this domain. In fact, the 23S rRNA region including nucleotides 2090–2200 has been localized on the L1 arm, in close contact with the peptidyltransferase domain at the base of the central protuberance. Moreover, the 23S rRNA portion protected by L27 includes nucleotides 2300–2350 [for a review article, see Giri et al. (1984)]. All these data further support the model displayed in Figure 6. The latter agrees also with previous energy-transfer measurements whereby a fluorescent derivative of erythromycin on the ribosome was found to be located at 23 Å from the 3′ end of $tRNA^{Phe}$ at the P site (Langlois et al., 1976) and at 70 Å from the terminal COOH of L7 (Wong & Paradies, 1974).

Registry No. Virginiamycin S, 9040-14-6; peptidyltransferase, 9059-29-4.

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