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# Detection of Energy Transfer between Tryptophan Residues in the Tubulin Molecule and Bound Bis(8-anilinonaphthalene-1-sulfonate), an Inhibitor of Microtubule Assembly, That Binds to a Flexible Region on Tubulin†

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ABSTRACT: The fluorescent apolar probe bis(8-anilinonaphthalene-1-sulfonate) (Bis-ANS) is a potent inhibitor of microtubule assembly that binds to tubulin at a hitherto uncharacterized site distinct from those of the antimitotic drugs. We have found that energy transfer between tryptophan residues and bound Bis-ANS leads to quenching of the intrinsic tubulin fluorescence. The quenching is biphasic, implying two types of Bis-ANS binding sites. The estimated $K_d$ values are 2.7 and 22.2 $\mu$M, consistent with reported values for the primary and secondary Bis-ANS binding sites. Preincubation of tubulin at 37 °C results in increased quenching of tryptophan fluorescence without any effect on the $K_d$ values, suggesting localized structural change in the protein around the Bis-ANS binding sites. Concentration-dependent depolarization of Bis-ANS fluorescence was observed, suggesting energy transfer among bound Bis-ANS molecules. Such a concentration-dependent decrease in fluorescence polarization was not observed with 8-anilinonaphthalene-1-sulfonate (1,8-ANS), the monomeric form of Bis-ANS. Perrin-Weber plots were obtained for bound Bis-ANS and 1,8-ANS by varying the viscosity with sucrose. The rotational relaxation times calculated for Bis-ANS and 1,8-ANS are 18 and 96 ns, respectively. Comparison with the theoretical value (125 ns) suggests that Bis-ANS binds to a flexible region of tubulin. This, coupled with the fact that Bis-ANS, but not 1,8-ANS, inhibits microtubule assembly, suggests that the region in the tubulin molecule responsible for microtubule assembly is relatively flexible.

The fluorescent apolar molecule bis(8-anilinonaphthalene-1-sulfonate) (Bis-ANS)[1] binds tightly to tubulin ($K_d$ = 2 $\mu$M) and potently inhibits microtubule assembly (Horowitz et al., 1984). Because Bis-ANS is highly fluorescent, we have used it as a probe to learn about the region to which it binds, a portion of the tubulin molecule which is likely to be directly involved in microtubule assembly. The binding site of Bis-ANS on the tubulin molecule is unknown, but our previous work suggests that it does not overlap with the binding sites of colchicine, podophyllotoxin, vinblastine, or maytansine (Prasad et al., 1986). We report here the discovery of energy transfer between the tryptophan residues of tubulin and bound Bis-ANS and that the region to which Bis-ANS binds appears to be flexible.

## Materials and Methods

### *Materials*

Bis-ANS and 1,8-ANS were obtained from Molecular Probes Inc. (Junction City, OR). All other reagents were of analytical grade.

### *Methods*

*Tubulin Preparation.* Bovine brain tubulin was purified by phosphocellulose chromatography as described earlier (Prasad et al., 1986).

Protein measurements were made according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

*Fluorescence Measurements.* Fluorescence measurements were made with a Perkin-Elmer MPF-44A spectrofluorometer used in the ratio mode and equipped with temperature-controlled cell holder as described earlier (Prasad et al., 1986). The observed fluorescence intensities were corrected for the absorption of Bis-ANS using the relation (Lakowicz, 1983)

$$F_{cor} = F_{obsd} \text{ antilog } (OD_{ex} + OD_{em}/2)$$

where $OD_{ex}$ and $OD_{em}$ are the optical densities at the excitation and emission wavelengths, respectively.

*Polarization Measurements.* Fluorescence polarization measurements were made on an SLM Model 4800 spectrophotometer (SLM Instruments, Urbana, IL). The nature of

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[1] Abbreviations: Bis-ANS, bis(8-anilinonaphthalene-1-sulfonate); 1,8-ANS, 8-anilinonaphthalene-1-sulfonate; MAPs, microtubule-associated proteins.

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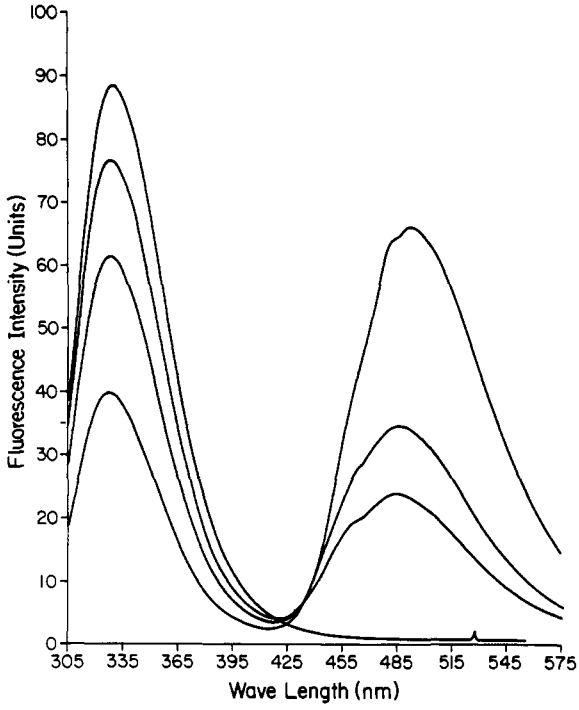


FIGURE 1: Energy transfer between tryptophan residues and bound Bis-ANS in tubulin molecule. Phosphocellulose-purified tubulin at 1 mg/mL in buffer was mixed with 0–20 $\mu$M Bis-ANS, and fluorescence emission spectrum was measured from 305 to 575 nm. Excitation was at 295 nm. Curves 1–4 were with 0, 2, 4, and 20 $\mu$M Bis-ANS, respectively.

the samples used for the polarization measurements are as in the figure legends.

The fluorescence polarization, $P$, was analyzed by the Perrin–Weber equation (Weber, 1952)

$$(1/P - \tfrac{1}{3}) = (1/P_0 - \tfrac{1}{3})(1 + 3\tau/\zeta_h)$$

where $P_0$ is the limiting polarization observed in the absence of fluorophore rotation or energy transfer, $\tau$ is the lifetime of the excited state, and $\zeta_h$ is the rotational relaxation time of the kinetic unit monitored by the probe. The reciprocal $\zeta_h$ is directly proportional to $T/\eta$, where $\eta$ is the viscosity of the solution at temperature $T$ in kelvin. A plot of $1/P$ vs. $T/\eta$ is predicted to give a straight line with an intercept equal to $1/P_0$. In the experiments described here the ratio $T/\eta$ was varied by changing the solution viscosity at a constant temperature of 25 °C by the addition of sucrose solution.

*Lifetime Measurements.* Lifetime measurements were made by using a PRA (Oak Ridge, TN) fluorescence lifetime instrument which uses a time-correlated single photon counting technique. Deconvolution of the observed decay curve was performed by using a least-squares fitting program kindly provided by Dr. W. Ware of the Chemistry Department at the University of Western Ontario. Tubulin (2 $\mu$M) was incubated with Bis-ANS (10 $\mu$M) for 2 h at 37 °C or with 1,8-ANS (25 $\mu$M) for 10 min at 37 °C, and then the lifetime was measured. Excitation was at 390 nm for Bis-ANS and at 370 nm for 1,8-ANS. Emission was at 490 nm.

## RESULTS

When excitated at 295 nm, tubulin shows a characteristic fluorescence emission spectrum with a maximum at 335 nm. Excitation at 295 nm was selected to minimize the emission due to tyrosine residues and is known to selectively excite tryptophan residues in proteins. When Bis-ANS was added to tubulin and excited at 295 nm, there was a decrease in emission at 335 nm and the appearance of a maximum at 490 nm characteristic of Bis-ANS. This suggested that there was energy transfer between tryptophan and the bound Bis-ANS. Analysis of a similar spectrum excited at 280 nm indicates that there is very little or no quenching of tyrosine fluorescence. Figure 1 shows the emission spectra of the Bis-ANS tubulin complex as a function of Bis-ANS concentration. Increasing concentration of Bis-ANS results in increased binding of the dye with a concomitant decrease in tryptophan fluorescence at 335 nm and an increase in Bis-ANS fluorescence at 490 nm. The quenching of the tryptophan fluorescence by Bis-ANS suggests that the energy absorbed by the tryptophan residues is being transferred to Bis-ANS molecules. The spectra show an isoemissive point at 432 nm. Bis-ANS–tubulin spectra do not change in emission maximum or bandwidth over the range of concentrations used. Controls were performed to eliminate the possibility of direct excitation of Bis-ANS at 295 nm. Thus, when 0–50 mM Bis-ANS in 50% ethanol was excited at 295 nm, there was very little fluorescence observed at 490 nm. In addition, Bis-ANS quenching of tubulin fluorescence was much more pronounced than that observed with *N*-acetyltryptophanamide.

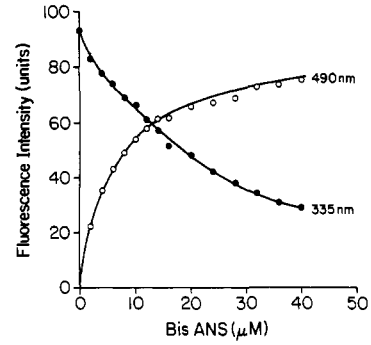


FIGURE 2: Concentration dependence of energy transfer between tryptophan and bound Bis-ANS molecules. Phosphocellulose-purified tubulin at 0.2 mg/mL in buffer was excited at 295 nm, and emission was measured at 335 and 490 nm. Bis-ANS at 1 mM was added in 5-$\mu$L aliquots, and emission was measured after 1 min. Measured fluorescence intensities were corrected for the absorption of Bis-ANS at 295, 335, and 490 nm, respectively.

Figure 2 shows the effect of adding increasing concentrations of Bis-ANS on tryptophan emission at 335 nm. Nearly 70% of the tryptophan emission could be quenched by a 20-fold excess of Bis-ANS over tubulin. This is accompanied by a proportional increase in Bis-ANS emission at 490 nm as a result of energy transfer between tryptophan and Bis-ANS molecules. When the results of these experiments were plotted in a manner similar to the modified Stern–Volmer plot (Eftink & Ghiron, 1981), Figure 3 was obtained. The plot is biphasic, suggesting the presence of two interaction sites, consistent with our earlier observation (Prasad et al., 1986). Analysis of the curve gave a $K_d$ of 2.7 $\mu$M for the primary site and 22.2 $\mu$M for the secondary site, and these values are comparable with the values of 2 and 19 $\mu$M obtained earlier by using a different method (Prasad et al., 1986).

Preincubation of tubulin alone or with Bis-ANS at 37 °C results in increased binding of Bis-ANS to tubulin (Prasad et al., 1986). Hence, it was of interest to know whether the extent of quenching of tryptophan fluorescence by Bis-ANS varied with time. The results of such an experiment are shown in Figure 4. For this, tubulin (2 $\mu$M) was mixed with Bis-ANS (20 $\mu$M) and incubated continuously at 37 °C. With time there was increased quenching of tryptophan fluorescence at 335 nm. Although it is difficult to quantitate exactly, it is interesting that the half-time for the quenching of tryptophan fluorescence (~45 min) is comparable to the half-time for the

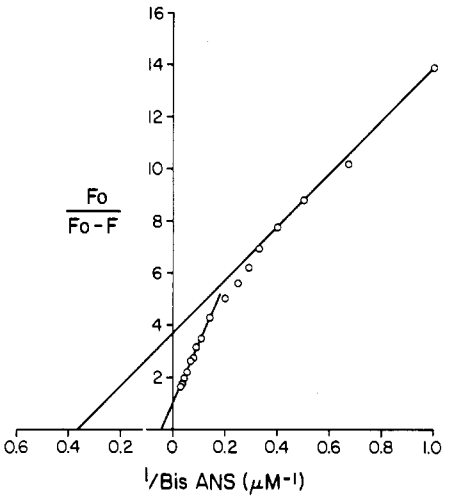


FIGURE 3: Modified Stern–Volmer plot for the quenching of tryptophan fluorescence by Bis-ANS. Phosphocellulose-purified tubulin at 0.2 mg/mL in buffer was titrated with increasing concentration of Bis-ANS as in Figure 2, and fluorescence intensity was measured at 335 nm. Excitation was at 295 nm. The corrected intensities were plotted vs. Bis-ANS concentration in a reciprocal manner. The intersection point on the axis gives the value of $K_d$ for the primary and secondary binding site.

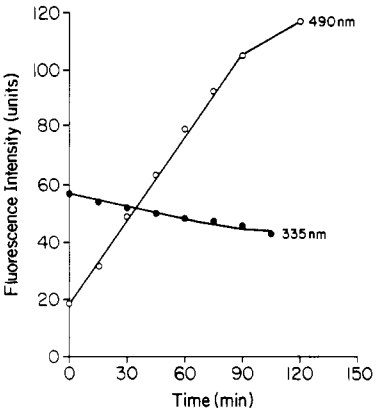


FIGURE 4: Time course for the quenching of tryptophan fluorescence and appearance of Bis-ANS fluorescence. Phosphocellulose-purified tubulin at 0.2 mg/mL in buffer was mixed with 20 μM Bis-ANS, and fluorescence intensity was measured every 15 min at 335 and 490 nm. Excitation was at 295 nm.

increase in Bis-ANS fluorescence (~55 min). When the experiment in Figure 2 was repeated with tubulin preincubated for various intervals of time, essentially the same pattern was obtained, but the extent of quenching at 335 nm and increase in fluorescence at 490 nm were greater. Figure 5 shows the results obtained. For this, 2 μM tubulin was incubated at 37 °C for 0, 1, 2, and 3 h, then an increasing amount of Bis-ANS was added, and fluorescence intensity was measured at 335 nm with excitation at 295 nm. The results are shown in the form of a modified Stern–Volmer plot. As shown in the figure, preincubation increased the effectiveness of Bis-ANS as a quencher of tryptophan fluorescence. Only the maximum amount of quenching obtained varied with time with no effect on the $K_d$ value for both the primary and the secondary sites. The enhanced quenching of the tryptophan fluorescence and energy transfer must be due to localized conformational changes in the tubulin molecule which results in increased binding of the probe to tubulin. The change that is taking place in the protein must be very subtle as it is not reflected in circular dichroism and ultraviolet absorption spectral studies of the protein (Prasad et al., 1986; Bhattacharyya & Wolff, 1975).

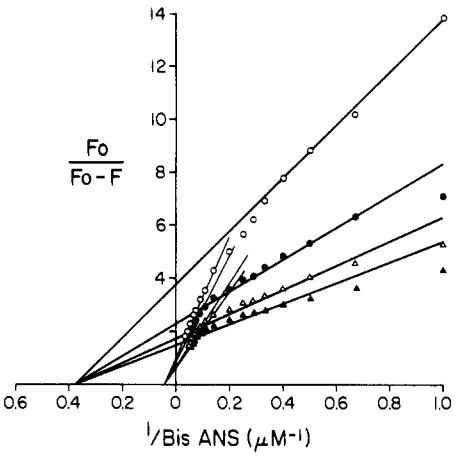


FIGURE 5: Effect of preincubation time on the binding of Bis-ANS to tubulin. Phosphocellulose-purified tubulin at 0.2 mg/mL was incubated at 37 °C for 0, 60, 120, and 180 min, respectively. Bis-ANS (1 mM) was added in aliquots to this tubulin, and fluorescence intensity was measured at 335 nm 1 min after the addition of Bis-ANS. Excitation was at 295 nm. Figure shows a plot similar to a modified Stern–Volmer plot. (○) 0, (●) 1, (△) 2, and (▲) 3 h.

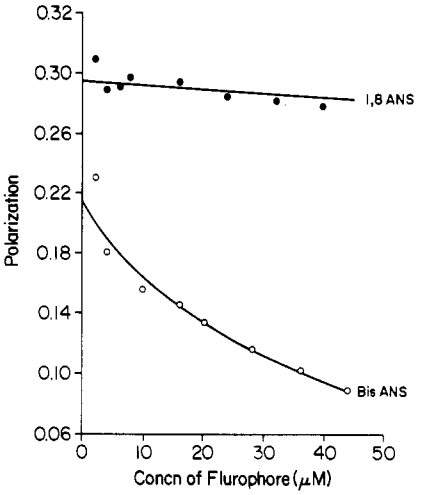


FIGURE 6: Polarization of Bis-ANS and 1,8-ANS as a function of fluorescent probe concentration. Phosphocellulose-purified tubulin at a concentration of 0.2 mg/mL was titrated with 1 mM Bis-ANS and 1,8-ANS, separately. Excitation was at 385 nm, and emission was at 490 nm. Polarization was calculated as described under Methods.

Fluorescence polarization measurements can reveal the presence of segments of proteins that are flexible that are not normally revealed by CD and UV absorption measurements. In addition they yield information about energy transfer among the ligands. Hence, polarization of Bis-ANS fluorescence was measured. As a comparison to Bis-ANS, the same experiments were done with 1,8-ANS, the monomeric form of Bis-ANS, which does not inhibit microtubule assembly. When polarization was measured as a function of Bis-ANS concentration, measured polarization decreased with time (data not shown) and reached a steady value in 2 h. This decrease was not due to any gross changes in the tubulin structure since there was no significant change in the UV spectrum or the CD spectrum of the protein (Prasad et al., 1986). The time-dependent decrease was more pronounced at higher Bis-ANS concentrations and may reflect changes in energy transfer among bound Bis-ANS molecules as time-dependent binding proceeds. A time-dependent increase in the flexibility of the Bis-ANS site may also contribute. Figure 6 shows the values obtained at the end of 2 h. An immediate observation that we can make

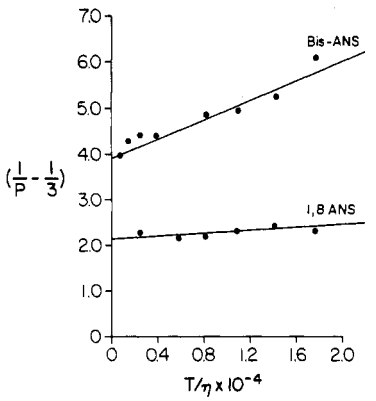


FIGURE 7: Perrin–Weber plot for Bis-ANS and 1,8-ANS. Fluorescence polarization was measured as a function of viscosity at 25 °C. Tubulin (2 μM) was preincubated for 2 h with 10 μM Bis-ANS or with 25 μM 1,8-ANS at 37 °C. Excitation was at 385 nm, and emission was at 490 nm.

Table I: Fluorescence Lifetime in Nanoseconds for Bis-ANS and 1,8-ANS in Propanol and as a Conjugate of Tubulin[a]

| condition | Bis-ANS | 1,8-ANS |
|---|---|---|
| propanol | 4.19 ± 0.008 | 9.21 ± 0.017 |
| tubulin in buffer | 5.42 ± 0.010 | 8.62 ± 0.016 |

[a] Tubulin (4 μM) was preincubated for 2 h with 10 μM Bis-ANS or with 25 μM 1,8-ANS for 10 min at 37 °C. Lifetimes were measured for the same concentrations of the two probes dissolved in 1-propanol. Under these conditions the primary Bis-ANS binding site is predominantly the one occupied (Prasad et al., 1986).

is that the value of polarization decreases with increasing concentration of Bis-ANS. Such concentration-dependent depolarization of fluorescence has been observed for the binding of 1,8-ANS to bovine serum albumin (Weber & Daniel, 1966). In the case of 1,8-ANS there was no decrease in the polarization observed as a function of the concentration of the probe. This is not surprising as 1,8-ANS binds to a single site on tubulin (Horowitz et al., 1984; Bhattacharyya & Wolff, 1975). In addition we have observed that the binding of 1,8-ANS to tubulin is not affected by time (data not shown).

Figure 7 shows the Perrin–Weber plot obtained for Bis-ANS and 1,8-ANS binding to tubulin. The experiment was done by changing the viscosity of the solution by the addition of sucrose. The choice of sucrose was based on the fact that glycerol and temperature affect the polymerization of tubulin and hence the measured polarization. The plot is linear, and the plot with Bis-ANS shows a higher slope than the one with 1,8-ANS. Lifetimes of the excited state of Bis-ANS, of 1,8-ANS in propanol, and of the Bis-ANS–tubulin and the 1,8-ANS–tubulin complexes were measured as described under Methods. The results are shown in Table I. The lifetime obtained for Bis-ANS in propanol is close to the value measured by Rosen and Weber (1969). Using the values obtained for the lifetime and the $P$ and $P_0$ values from Figure 7, we have calculated the rotational relaxation times. The estimated values for Bis-ANS–tubulin and 1,8-ANS–tubulin complexes are 18 and 96 ns, respectively.

## Discussion

The results presented above demonstrate the presence of energy transfer from the tryptophan residues of tubulin to bound Bis-ANS and confirm the presence of more than one site for Bis-ANS on tubulin. Binding constants obtained from the quenching of the tryptophan fluorescence in the present study are in conformity with the values reported earlier (Horowitz et al., 1984; Prasad et al., 1986). Brain tubulin has four tryptophan residues per subunit (Krauhs et al., 1981; Ponstingl et al., 1981). Sequence studies have shown that three of these residues at positions 21, 346, and 407 are in the same position in both the $\alpha$ and the $\beta$ subunits. Tryptophan residues at positions 346 and 407 are likely to be closer to the subunit contact region (Ponstingl et al., 1983) and are close to cysteine residue 354, which may be critical for assembly (Litlle & Luduena, 1985). It would be of interest to know whether all of these residues are quenched by Bis-ANS or only a few of them, each one to a different extent.

The present study has also shown some differences in the binding characteristics of Bis-ANS and 1,8-ANS. There was no time-dependent increase in the binding of 1,8-ANS as in the case of Bis-ANS. We found that the fluorescence polarization decreased with time and concentration of the Bis-ANS. This was not so in the case of 1,8-ANS.

Rotational relaxation ($\zeta_0$) calculated by assuming tubulin to be a globular hydrated sphere of $M_r$ 100 000 is 125 ns (Cantor & Schimmel, 1980). The Perrin–Weber plot in Figure 7 gave a value of 18 ns for the Bis-ANS–tubulin conjugate ($\zeta_h/\zeta_0 = 0.14$) and 96 ns for the 1,8-ANS–tubulin conjugate ($\zeta_h/\zeta_0 = 0.78$). In general the ratio $\zeta_h/\zeta_0$ is expected to be $\geq 1$ unless there is flexibility that can influence the site of labeling. Hence, the observed low value for the $\zeta_h$ of the Bis-ANS conjugate suggests that Bis-ANS binds to a flexible segment of the tubulin molecule. 1,8-ANS seems to bind at a more rigid portion on the tubulin molecule. Since Bis-ANS is an inhibitor of microtubule assembly, it is tempting to speculate that the region responsible for microtubule assembly is a very flexible one. Ide and Engelborghs (1981) have also suggested very subtle conformational changes in the tubulin in the presence of various anions from their study of quenching of tryptophan fluorescence by iodide. The observation that the circular dichroism spectrum is independent of the presence of Bis-ANS appears to confirm that the flexibility observed is a localized change in the protein structure.

It is perhaps significant that a region on the tubulin molecule that plays a role in assembly is both flexible and apolar. An inherent flexibility in the tubulin molecule is a reasonable explanation for its well-known ability to decay, and its ability to polymerize is apparently the first property to be lost, well before the loss of colchicine binding (Wiche et al., 1977). This suggests that certain regions of the tubulin molecule may be more flexible than others, consistent with our finding that 1,8-ANS binds to a less flexible region than does Bis-ANS. It is tempting to speculate that microtubule assembly is accompanied by, and may require, a localized, limited, and controlled conformational change in a region of the tubulin molecule such that apolar surfaces become exposed and ready to interact with similar regions on other tubulin molecules. On the other hand, under conditions where assembly is not possible, the flexibility of this region may result in a less controlled conformational change leading to random aggregation and the decay of tubulin.

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**Registry No.** 1,8-ANS, 82-76-8; Bis-ANS, 25551-04-6.

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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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