Self-Association of Rifamycin B: Possible Effects on Molecular Recognition

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The macrocyclic antibiotic rifamycin B was found to be highly surface-active and to aggregate in aqueous solution. The aggregational behavior was studied using small-angle neutron scattering (SANS). Aqueous solutions of rifamycin B showed pronounced scattering at both small-length scales ($Q \geq 0.1$ Å^{-1}) and at large-length scales ($Q \leq 0.1$ Å^{-1}). The larger association colloids appear to be rather open low-density aggregates. The addition of 10% 2-propanol greatly reduces the number and size of the aggregates. Somewhat higher amounts of alcohol appear to completely suppress or eliminate aggregation. The suppression of aggregation coincides with the appearance and enhancement of enantioselective association between rifamycin B and a variety of chiral amino alcohols. It appears that the self-aggregation of rifamycin B may be a factor that controls its ability to differentiate between enantiomers in aqueous and hydro-organic solutions.

Introduction

The rifamycin family of antibiotics are a subgroup of the ansamycins that includes streptovaricins, halomycins, mycotrienins, actomycin, and several other structurally related antibiotics.[1] The general name ansamycin refers to a specific macrocyclic structure that consists of a chromophoric moiety (generally aromatic) spanned by an aliphatic chain or bridge.[1,2] An example of the ansamycin structure is shown in Figure 1 for rifamycin B. Note that the aliphatic bridge must link to nonadjacent positions of the chromophore. This particular molecule has nine stereogenic centers and exists as a dibasic acid with pK_a's of approximately 2.8 and 6.7.[3] It has a molecular weight of 755.8 and is slightly soluble in water (more so when ionized) as well as in the smaller alcohols and acetone.[3,4]

The rifamycins are particularly important antituberculosis compounds[5,6] and are promising agents for the treatment of leprosy[7] rheumatoid arthritis and other chronic arthritic disorders.[8,9] Rifamycins also have been shown to be unusual but effective chiral selectors for high-efficiency separations of enantiomeric compounds (particularly those that contain amine functionalities).[4] Rifamycin B was the initial macrocycle in its family to be isolated and characterized. It is produced by fermentation as are other ansamycins (although they can be synthetically modified as well). Originally, it was thought that rifamycin B was produced by *Streptomyces mediterranei*. Later, on the basis of its cell wall chemistry, the organism was reassigned to the genus *Amycolctopsis*.[1]

Previously, in the course of evaluating enantioselective interactions between rifamycin B and a number of aminoalcohol drugs, two interesting observations were made (one of which was unique).[4] First, aqueous solutions of rifamycins readily produced foam when agitated or stirred. This indicated that rifamycin B may be surface-active. A review of the rifamycin literature revealed only a few corroborating observations of this type. These observations were in the purview of biotechnology/engineering where it was found that large-scale reactors for the biotransformation of rifamycins sometimes had a foaming problem and that the addition of small amounts of an antifoaming agent was beneficial.[10,11] The second observation was more puzzling. No enantioselective interactions between rifamycin B and the chiral aminoalcohols were observed in strictly aqueous or buffered solutions. However, when ~10% or more of a miscible organic solvent was added to the solution (e.g., methanol, ethanol, 1-propranol, 2-propranol, or acetonitrile), enantioselective binding was observed.[4] In many previous capillary electrophoresis (CE) studies (with other chiral selectors) it was found that addition of small amounts of miscible organic solvents could change the degree of enantioselective binding (most often decreasing it), but enantioselectivity was always observed in the optimized aqueous systems.[12,13]

If, indeed, rifmaycin B is surface-active and concentrates at the air−water interface, it is reasonable to propose that it may self-associate in aqueous solution. Just as micellization of ionic surfactants in water is impeded by increasing concentrations of miscible alcohols, rifamycin B self-assembly could be impeded as well. If so, then it appears that molecular chiral recognition by rifamycin B requires (at least in part) a significant fraction of monomers in solution. In this work, experiments are done and data generated to test the hypothesis outlined above. To our knowledge, there has never been a report on the self-association of rifamycin B or possible deleterious effects of self-

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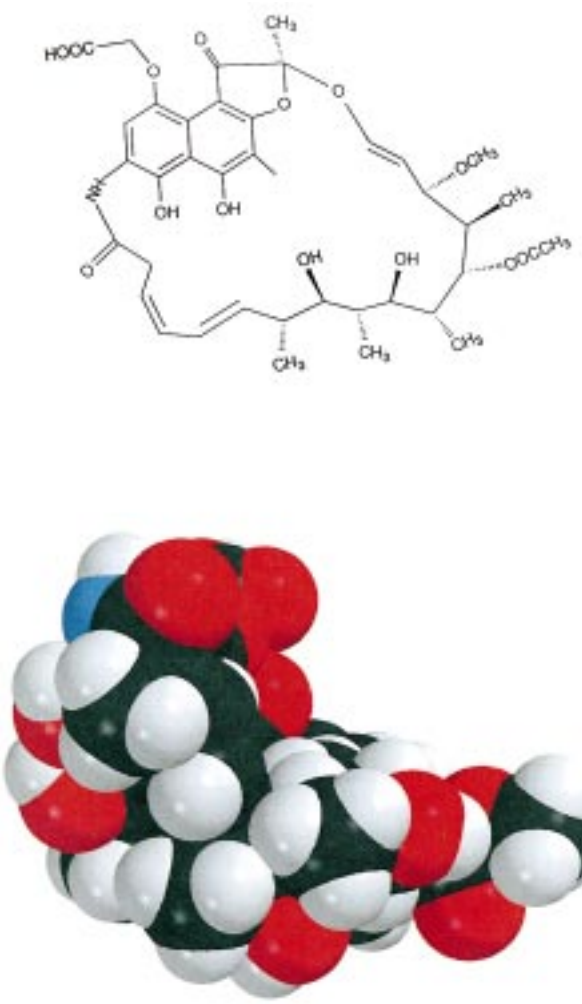

Figure 1. Structure of rifamycin B (top) and space-filling model (bottom) showing its configuration and 3-D shape. The atoms are color-coded as follows: carbon (black), oxygen (red), hydrogen (white), and nitrogen (blue).

aggregation on molecular recognition. The effects of rifamycin B concentration and ionic strength also were investigated.

Experimental Section

Preparation of Rifamycin B Samples for Small-Angle Neutron Scattering (SANS) Experiments. Deuterium oxide (99.9 atom % D), 2-propanol-d_8, sodium deuterioxide (40 wt % in D_2O), and sodium dihydrogen phosphate were purchased from Aldrich (Milwaukee, WI). Rifamycin B was purchased from Sigma (St. Louis, MO). Samples of 0.05 and 0.1 M phosphate buffers were prepared by dissolving appropriate amounts of NaH_2PO_4 in D_2O and adjusting to a pD of 7.00 using NaOD. Samples of 20, 15, and 10 mM rifamycin B samples were then prepared by dissolving appropriate amounts of rifamycin B in either a phosphate buffer or a phosphate buffer/2-propanol-d_8 mixture (v/v). The samples were then transferred to a quartz sample container for SANS analysis.

Small-Angle Neutron Scattering. The SANS measurements were performed on the NG-7 SANS spectrometer at the Cold Neutron Research Facility, National Institute of Standards and Technology, Gaithersburg, MD. The range of scattering, Q, used for the majority of the samples was $0.01-0.45$ Å^{-1}. Q is defined as $(4\pi/\lambda)\sin\theta$, where 2θ is the scattering angle and the incident neutron wavelength λ is 5 Å. For 20 mM rifamycin B in 0.05 M phosphate buffer scattering data were collected at lower Q ($Q_{min} - 0.002$ Å^{-1}, $\lambda = 10$ Å). The samples were contained in quartz spectrophotometric cells of 5 mm path length and mounted in a thermostated cell holder held at 25.0 ± 0.1 °C. Each two-dimensional set of raw scattering data was corrected for detector background and sensitivity and for scattering from the empty cell and then radially averaged.[15] The resulting $I(Q)$'s were converted to absolute intensities (in cm^{-1}) using precalibrated secondary standards provided by CNRF, NIST. The final step consisted of the Q-by-Q subtraction of the (flat) background scattering due to the aqueous buffer/alcohol solutions.

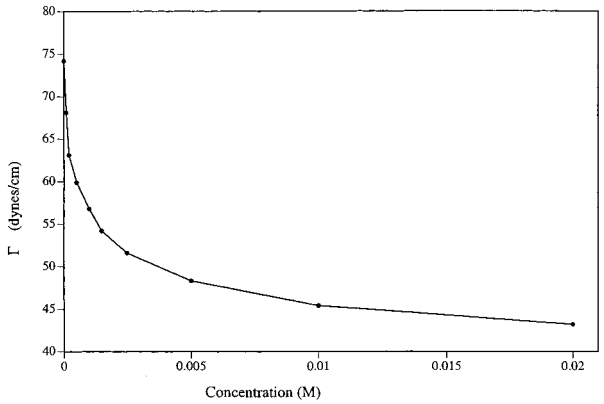

Figure 2. Plot of surface tension (distilled/deionized water) versus concentration of rifamycin B.

Experimental radii of gyration (R_g) were obtained from the corrected data over a range of Q from 0.12 to 0.35 Å^{-1} by making use of the Guinier approximation: $\ln I(Q) \propto -Q^2R_g^2/3$.

Surface Tension Measurements of Rifamycin B Solutions. Rifamycin B solutions were prepared by dissolving appropriate amounts of rifamycin B in 0.1 M NaH_2PO_4 (pH = 7.00). The samples were then transferred to a shallow watch glass, and the surface tension was measured using a surface tensiometer model 20 (Fisher Scientific Co.). Between each measurement, the platinum ring of the surface tensiometer was rinsed and flamed in a Mekker burner to ensure the removal of all residual materials.

Results and Discussion

Rifamycin B does not have a structure that is characteristic of traditional surfactant molecules (i.e., a hydrophilic "head" group and a hydrophobic "tail").[14] Nor is it completely similar to bile salt surfactants, i.e., steroids with a hydrophobic side and a hydrophilic edge.[14] However, the space-filling structure of rifamycin B shows a bent, disk-shaped molecule with distinct hydrophobic and hydrophilic regions (Figure 1). Furthermore, as shown in Figure 2, the addition of small amounts of rifamycin B to water lowers the surface tension drastically as would be expected of a more conventional surfactant. Although the simple lowering of surface tension of water and foaming behavior are no guarantee that a molecule will self-associate, these properties certainly warranted further investigation into the possibility.

Rifamycin B is anionic at pH 7. Its solutions are intensely colored. Consequently, it is not feasible to use light scattering as an experimental tool for the investigation of its aggregation behavior. Small-angle neutron scattering (SANS) is not affected by the light absorption of a sample. Furthermore, it is sensitive to the length scales that would occur in solutions containing monomeric and oligomeric rifamycin species.

SANS Studies. *Small-Length Scales.* All rifamycin B solutions studied show scattering at $Q \geq 0.1$ Å^{-1}. This is

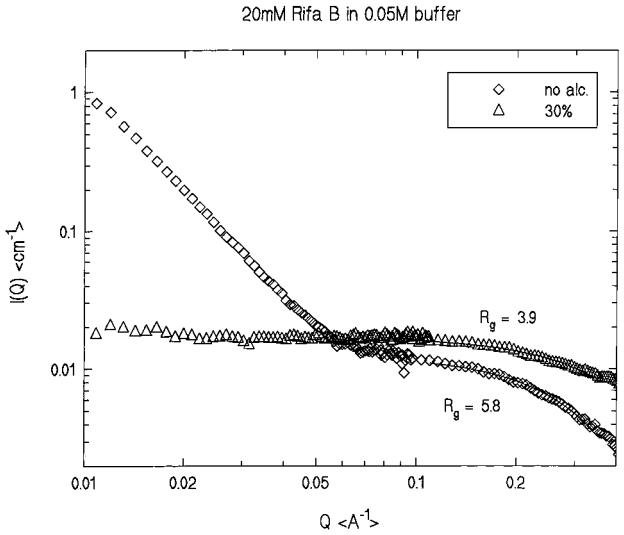

Figure 3. Small-length neutron scattering data for D_2O solutions containing 20 mM rifamycin B in 0.05 M phosphate buffer (pH 7.0). Note the flat profile for solutions containing 30% 2-propanol (△).

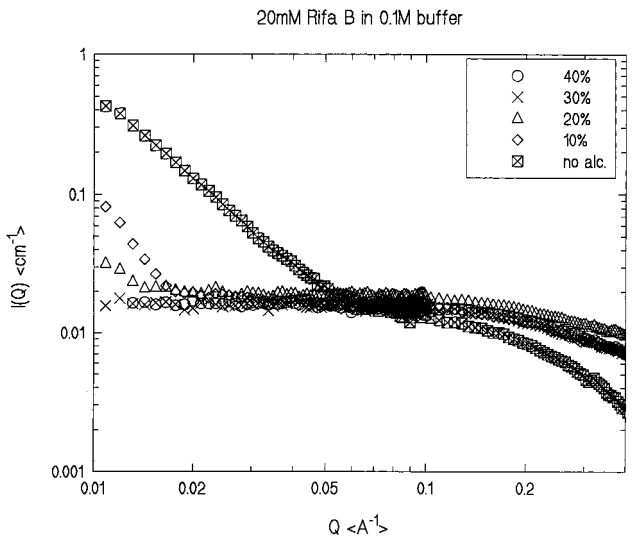

Figure 4. Plots showing the effect of increasing 2-propanol concentration on the small-length neutron scattering curves for D_2O solutions of 20 mM rifamycin B in 0.1 M phosphate buffer (pH 7.0).

TABLE 1: Radii of Gyration of Rifamycin B Aggregates[a]

[rifa B], mM	wt % 2-propanol	R_g, Å
in 0.05 M phosphate buffer		
20	0	5.82
	10	4.92
	20	3.93
	30	3.89
	40	4.08
in 0.1 M phosphate buffer		
10	0	4.80
15	0	5.08
20	0	5.92
	10	4.57
	20	4.07
	30	4.41
	40	4.57

[a] Error bars on the R_g values are ± 0.3 Å.

characteristic of species having a radius of gyration (R_g) in the 4−6 Å range. Figure 3 shows two typical scattering curves. For 0.05 and 0.1 M phosphate buffer containing 20 mM rifamcyin B, the R_g's are 5.8 and 5.9 Å, respectively (Table 1); at 10% 2-propanol, the R_g's are 4.9 and 4.6 Å, respectively. As Table 1 makes clear, in 0.05 M buffer further addition of alcohol (20−40%) causes the R_g to plateau for 20 mM rifamycin B at 4.0 ± 0.1 Å, while in 0.1 M buffer, there is somewhat greater dispersion, with fitted R_g's in a range from 4.1 to 4.6 Å. For 0.1 M buffer without 2-propanol, the scattering curves for 10 and 15 mM rifamycin B produce R_g's of 4.8 and 5.1 Å, respectively.

Crystal structures and molecular modeling of rifamycin B show an aromatic region with a protruding carboxylate-containing side chain and an aliphatic "belt" or side chain roughly perpendicular to the aromatic moiety (Figure 1). This molecule is somewhat Janus-faced, having a relatively more hydrophobic side and a hydrophilic one. For a discussion of the small R_g's reported in Table 1, it is sufficient to ignore the small hydrophobic cavity inside the belt and to approximate rifamycin B monomers as ellipsoids of revolution with three semiaxes, *a*, *b*, and *c*, giving

$$R_g^2 = (a^2 + b^2 + c^2)/5$$

From the modeling, we take dimensions for the full axes of 14.3 and 9.5 Å (length and width) and 6.2 Å (the "thickness", a compromise between the side of the molecule with the aromatic moiety plus side chain and the edge of the "belt"). These dimensions result in an R_g for the monomers of 4.1 Å. If the monomers were to dimerize via interaction between the more hydrophobic faces of their "belts", the 9.5 Å axis would be unaffected, while the thickness would approximately double and the long axis would increase by ca. 5 Å. This results in a dimeric object having an R_g of 5.6 Å. An alternative mode of dimerization, involving the faces of the aromatic moieties ("end-to-end" dimerization), would produce an object of dimensions 30 Å × 9 Å × 4.5 Å and an R_g of 6.9 Å, considerably larger than the values observed for rifamycin B in the absence of 2-propanol. The face-to-face belt dimers have the aromatic moieties at opposite ends of the dimers' longest axis, available for further aggregation in solution; end-to-end dimers have the hydrophobic faces of the belts available for further aggregation. Observations concerning scattering from longer-length scales are made below.

Face-to-face dimerization of the belts, as opposed to end-to-end dimerization, holds the potential to have more impact on the macrocycle's conformation and hence on molecular recognition. However, the differences between calculated R_g's for the two possible dimers and the experimental R_g's observed in the absence of 2-propanol are large enough to prevent a clear conclusion as to which dimer may predominate. Of course, other small aggregates such as trimers, etc. may contribute at $Q \geq 0.1$ Å^{-1} as well. The increase in R_g with increasing rifamycin B concentration in buffer alone is suggestive of indefinite association, with higher *n*-mers progressively becoming more important. In addition, the $I(Q)$'s observed for these three solutions at $Q \leq 0.1$ Å^{-1} increase much more rapidly with increasing concentration than the expected linear scaling that a shared distribution of aggregate sizes would suggest.

Large-Length Scales. In the absence of 2-propanol, there is significant scattered intensity at $Q \leq 0.1$ Å^{-1}; Figure 4 illustrates this for 20 mM rifamycin B in 0.1 M phosphate buffer. The low-Q scattering is greatly reduced already at 10% alcohol and disappears completely by 30% alcohol (Figure 4) at both buffer concentrations. Attempts to fit these data assuming either cylindrical or disklike morphologies failed.

The sample showing the most intense low-Q scattering, 20 mM rifamycin B in 0.05 M phosphate buffer, was investigated

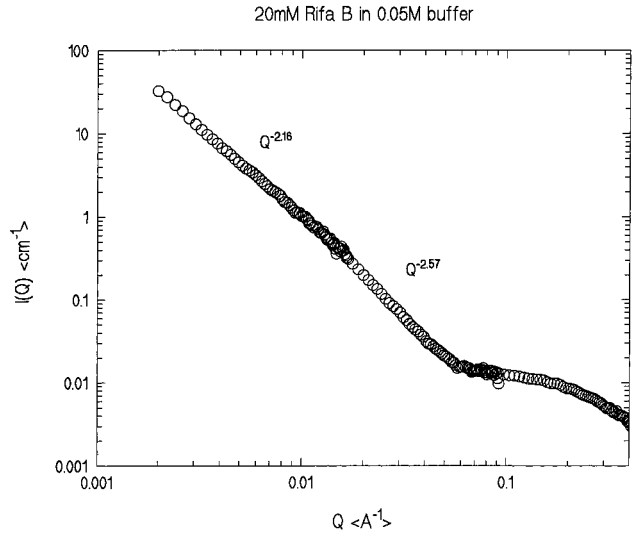

Figure 5. Long-length neutron scattering data for a D_2O solution of 20 mM rifamycin B with 0.05 M phosphate (pH 7).

down to 0.002 $Å^{-1}$. Figure 5 shows that there is a wide range of Q over which $I(Q)$ displays a power-law behavior; the appearance of the scattering curve is reminiscent of data for polymeric fractal materials such as polymer foams or silica aerosols. These materials have multiple length scales, from micrometers to angstrons, and scattering curves having both power-law and Guinier regimes. The Guinier regimes result from R_g's of the overall structure and from various structural subunits; in each case the power-law decay comes after the exponential regime.[17−19] Unfortunately, the Q range for our rifamycin B samples cannot be further extended by either light scattering (intensely colored solutions) or by ultralow SANS or X-ray scattering (insufficient scattered intensity). As a result, we are unable to obtain an R_g for the overall structure but apparently see only its power-law regime. The observed low-Q dependence, roughly $I(Q) \propto Q^{-2}$, suggests a mass fractal dimension of 2. This relatively low mass fractal dimension implies that the aggregate has a somewhat open structure of low density.

Conclusions

Despite having a molecular structure unlike those of other traditional surfactants, rifamycin B both concentrates at the air−water interface, depressing surface tension, and shows self-aggregation in aqueous solutions. Neutron scattering studies have identified both small aggregates, probably concentration-dependent indefinite association type, monomer, dimer, trimer, etc., and much larger aggregates with a relatively open structure of low density. Small amounts of 2-propanol (∼10%) greatly reduces aggregation. At higher concentrations of 2-propanol (∼30−40%) there is little evidence of aggregation. The disappearance of aggregation with increasing concentrations of alcohol seems to correspond to the appearance of molecular chiral recognition between rifamycin B and a variety of chiral aminoalcohol drugs.[4] There are at least two possible scenarios that can be proposed on the basis of this information. One is that the self-aggregation of rifamcyin B may, in some way, impair its ability to differentiate enantiomers of aminoalcohols in aqueous solution. An alternative scenario is that the added alcohol plays some other beneficial (but unknown) role in molecular recognition and that its concurrent inhibition of aggregation is coincidental. Note that the self-aggregation of rifamycin B does not need to completely disrupt the binding of a chiral guest molecule to the rifamycin B.[4] Simply altering one of the simultaneous interactions necessary for stereoselective association is sufficient to alter chiral molecular recognition. Hence, one possible interpretation of the results of this study is that they provide some evidence for aggregational mediated chiral molecular recognition. To our knowledge, this is the first report on the aggregational behavior of rifamycin B.

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