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A Comparison of the Host–Guest Inclusion Complexes of 1,8-ANS and 2,6-ANS in Parent and Modified Cyclodextrins

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Abstract. The fluorescent molecules 1-anilinonaphthalene-8-sulfonate (1,8-ANS) and 2-anilinonaphthalene-6-sulfonate (2,6-ANS) are extremely sensitive to the polarity of their local environment, making them excellent probes for the study of heterogeneous systems, including cyclodextrin (CD) solutions. Both are only weakly fluorescent in a highly polar medium, such as water, but are extremely fluorescent in a relatively nonpolar medium, such as within a CD cavity. These two probes are isomers, with major structural differences: 1,8-ANS is much bulkier and more spherical, whereas 2,6-ANS is much more streamlined and rod-shaped. Thus, they show major differences in their formation of CD inclusion complexes. This is reflected both in the magnitude of the observed fluorescence enhancement upon CD inclusion, as well as in the value of the association constant for complex formation. The creation of a scale for each probe for their fluorescence in CDs relative to that in ethanol allows for direct comparisons to be made between the two probes. These results are obtained and compared for the host-guest inclusion complexes of 1,8-ANS and 2,6-ANS with six CDs: α, β, γ, and their hydroxypropylated analogs.

Key words: host–guest inclusion, cyclodextrins, fluorescence enhancement, association constants

1. Introduction

Anilinonaphthalene sulfonates (ANS) are very useful fluorescent probes because of the extreme sensitivity of their emission properties to the polarity of their local environment [1–8]. In general, they are highly fluorescent in a nonpolar environment, but only weakly fluorescent in a polar environment, such as in water. For this reason, ANS has found widespread application as a polarity probe in a variety of heterogeneous systems, including proteins [9–11], micelles [12–14], polystyrene microspheres [15], and cyclodextrin host–guest inclusion complexes [16–34].

Cyclodextrins (CDs) are cyclic amylose oligomers consisting of 6 (α), 7 (β), or 8 (γ) sugar units, with an overall truncated cone shape [35]. The CD molecules have an internal cavity, accessible to other molecules by openings of 5.7, 7.8, and 9.5 Å for α, β, and γ, respectively [35]. This internal cavity is relatively non-polar,

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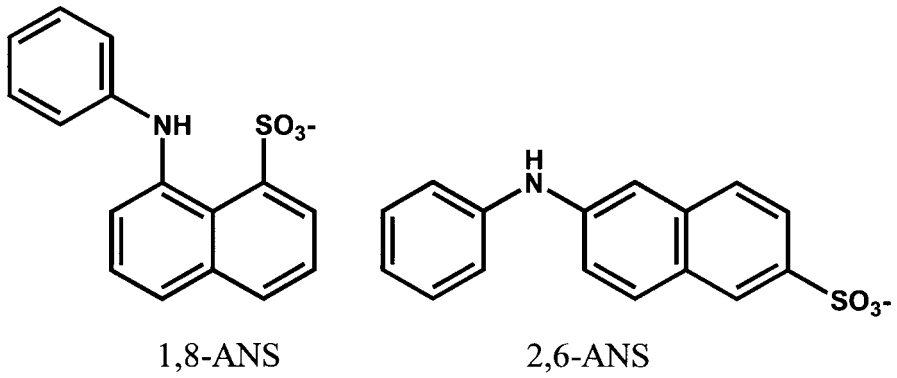

Figure 1. Structures of 1,8-ANS and 2,6-ANS.

therefore in aqueous solutions hydrophobic molecules tend to become included in the CD cavity, forming supramolecular host–guest complexes. In addition to the three naturally-occurring, or parent, CDs described above, there are a wide range of chemically-modified CDs, commercially or synthetically available [36]. These have been shown to have improved properties over the unmodified parents, including increased solubility, increased binding capacity, and increased fluorescence enhancement [28] of guest molecules.

If the guest molecule is a polarity-sensitive fluorescent probe, the complexation can be monitored by measuring the changes in the probe fluorescence upon addition of the CD host. Upon complexation of ANS from aqueous solution into the relatively less polar cavity of the CD, a significant increase in fluorescence is observed. This fluorescence enhancement, if measured as a function of CD concentration, can be used to determine the association constant for the host–guest complexation process.

There are a series of ANS isomers which have been used as sensitive fluorescent probes; these differ only in the positions of the anilino and sulfonate groups on the naphthalene fluorophore. Of these, the two which have found the most application are the 1,8-ANS and 2,6-ANS isomers, which are shown in Figure 1. Both show great sensitivity of their fluorescence and have been used in studies of CD inclusion complexes, but are quite different in their geometries. Figure 1 illustrates that in the 1,8 isomer, the sulfonate and anilino groups are substituted on the same side of the naphthalene moiety, in close proximity, whereas in the 2,6 isomer, the two groups are substituted at opposite ends of the naphthalene ring. Therefore, 1,8-ANS is a much bulkier molecule with a more spherical overall shape (especially when intramolecular mobility is taken into account [8]), whereas 2,6-ANS is more rod-like; significantly narrower and longer. As a result of these large structural differences, these two related probes are expected to exhibit significant differences in their formation of host–guest inclusion complexes with CDs.

In this paper, we compare the fluorescence enhancement and host–guest complexation association constants for these two probes with six CDs: α, β, and γ, and their hydroxypropylated derivatives. Although there have been previous reports in the literature on the association constants for CD complex formation for 1,8-ANS [18–20, 22, 27, 28, 34], and to a lesser degree 2,6-ANS [19, 31], these have been mainly with the parent unmodified CDs, have shown some rather wide variations, and have not been systematic.

2. Experimental

1-Anilinonaphthalene-8-sulfonic acid (1,8-ANS) and 2-anilinonaphthalene-6-sulfonic acid (2,6-ANS) were obtained from Molecular Probes; α, β, HP-α, HP-β, and HP-γ were obtained from Aldrich Chemical Co.; γ was obtained from Cerestar USA. All compounds were used as received. Tests of the water content of the CDs showed values ranging from 3.3 to 11.8% for all of the CDs used (based on mass loss after heating for 4 hours in a vacuum oven at 180 °C). The CDs were not dried before use, however the calculated CD concentrations were corrected using the determined water content values. Solutions of the ANS probes were prepared in aqueous phosphate buffer (pH = 6.80 $\pm$ 0.10).

Absorption spectra were measured on a Cary 50 Bio UV-Visible Spectrophotometer, to obtain the absorbance of the probe solutions at the excitation wavelengths of 335 nm (1,8-ANS) and 325 nm (2,6-ANS), and to ensure that no significant increase in absorbance occurred upon addition of the various CDs. Probe concentrations used were 3.0×10^{-5} M (1,8-ANS) and 2.0×10^{-5} M (2,6-ANS), giving absorbances of 0.31 and 0.27, respectively.

Fluorescence spectra were obtained on a Perkin-Elmer LS-5 luminescence spectrometer, with excitation and emission monochrometer bandpasses set at 5 nm and 3 nm, respectively, in 1 cm^2 quartz fluorescence cells. All spectra were obtained at 21 $\pm$ 2 °C. Solutions were not oxygen-purged, as preliminary investigations showed a negligible effect of purging on the observed fluorescence intensity ($<$ 5%). Fluorescence enhancements (F/F_o) were determined as the ratio of the integrated area under the corrected fluorescence spectrum (I_F *vs.* wavenumber) of the probe in the presence and absence of the CD of interest.

3. Results

3.1. FLUORESCENCE ENHANCEMENT

Large increases in the measured integrated fluorescence intensity were observed for both 1,8-ANS and 2,6-ANS upon addition of the various CDs. The relative fluorescence spectra of 2,6-ANS in the absence of CD and in the presence of various CDs at 10 mM concentration are shown in Figure 2. Although the fluorescence of both probes in the absence of CD is relatively low, it is significantly greater than the negligible solvent emission, and provides an accurate value for F_o, the

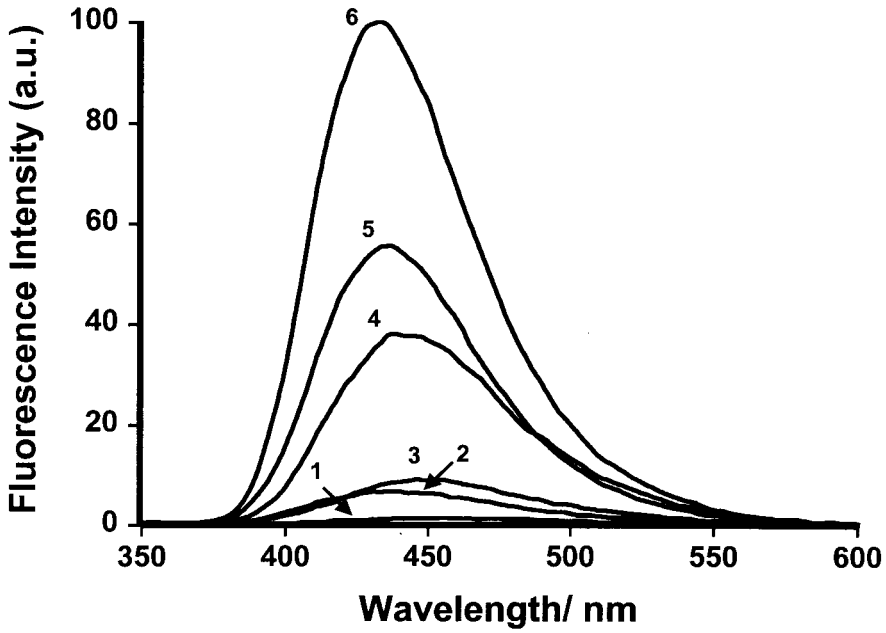

Figure 2. Relative fluorescence spectra of 2,6-ANS in various cyclodextrin solutions (10 ± 1 mM in phosphate buffer): **1** no CD; **2** α; **3** γ; **4** β; **5** HP-α; **6** HP-β.

reference value for the enhancement calculation. The enhancements at 10 mM CD concentration, F/F_o (10 mM), for the two probes in the six CDs are listed in Table I.

Table I. Fluorescence enhancements, F/F_o, for 1,8-ANS and 2,6-ANS in six cyclodextrins, expressed in two ways: F/F_o (10 mM) (fixed CD concentration of 10 ± 1 mM) and F_∞/F_o (100% guest complexation, obtained from fit to Equation (3)). F_∞/F_o values for cases in which the data did not fit well to Equation (3) were estimated from the experimental data; these are given in { }

Cyclodextrin	1,8-ANS		2,6-ANS	
	F/F_o (10 mM)	F_∞/F_o	F/F_o (10 mM)	F_∞/F_o
α	1.6	{5}	4.7	26
β	7.6	16	31	32
γ	10	{17}	7.5	{20}
HP-α	6.6	35	36	70
HP-β	104	125	82	81
HP-γ	17	25	25	35

Table II. Fluorescence enhancements, F/F_o (10 mM) and F_∞/F_o, for 1,8-ANS and 2,6-ANS in six cyclodextrins scaled to F(ethanol)/F(water) = 100 (see text)

Cyclodextrin	1,8-ANS		2,6-ANS	
	F/F_o (10 mM)	F_∞/F_o	F/F_o (10 mM)	F_∞/F_o
α	1.3	{3}	4.1	22
β	4.4	8.6	26	27
γ	5.6	{9}	6.5	{17}
HP-α	3.9	18	30	58
HP-β	53	64	69	68
HP-γ	9.1	13	21	29

Since the 12 different ANS:CD complexes have different association constants, a comparison of enhancement values for equal CD concentrations is of limited use, as the fraction of guest which is actually complexed by each CD will be quite different. In order to eliminate this dependence on the fraction of guest complexed, a second measure of fluorescence enhancement will be used, namely the extrapolated value at 100% guest complexation (i.e., at infinite host concentration); this will be referred to as F_∞/F_o. These values can best be obtained by fitting the enhancement data to Equation 3 (see below); the resulting values of F_∞/F_o are listed in Table I. In the case of three complexes, the data could not be fit well to this equation, so F_∞/F_o values were estimated by graphical extrapolation; these are indicated by { } in Table I.

These enhancement results still do not allow for direct comparisons between 1,8-ANS and 2,6-ANS, as these two probes have different polarity sensitivity. In identical environments, they will exhibit different fluorescence intensities relative to that in water. Thus, in order to make direct comparisons of enhancements, the fluorescence in ethanol relative to water (using the same excitation wavelength and correcting for differing absorbance) was determined for each probe. These values of F(ethanol)/F(water) were determined to be 197 and 120 for 1,8-ANS and 2,6-ANS, respectively, indicating that 1,8-ANS is the more polarity-sensitive of the two. The observed enhancements in the six CDs were scaled for each probe using these ethanol results, by setting the enhancement in ethanol *versus* water equal to 100. These scaled enhancements, both F/F_o(10 mM) and F_∞/F_o, are listed in Table II.

3.2. ASSOCIATION CONSTANTS FOR THE ANS:CD COMPLEXES

For a 1 : 1 ANS:CD complex, the association constant K can be defined as follows:

$$\mathrm{CD} + \mathrm{ANS} \rightleftharpoons \mathrm{CD{:}ANS}, \tag{1}$$

Table III. Association constants, K, for 1,8-ANS and 2,6-ANS in six cyclodextrins, from the fit to Equation 3 (assumes the formation of a 1 : 1 complex only)

Cyclodextrin	K/M^{-1}	
	1,8-ANS	2,6-ANS
α	≤ 20	≤ 20
β	80 ± 25	1350 ± 450
HP-α	21 ± 18	110 ± 20
HP-β	480 ± 80	7200 ± 1500
HP-γ	240 ± 80	250 ± 80

$$K = \frac{[CD{:}ANS]}{[ANS][CD]}. \tag{2}$$

The numerical value of K can be obtained from observed fluorescence enhancement F/F_o as a function of added CD concentration ($[CD]_o$) [37, 38]:

$$F/F_o = 1 + (F_\infty/F_o - 1)\frac{[CD]_o K}{1 + [CD]_o K}, \tag{3}$$

where F is the integrated fluorescence intensity in the presence of CD, F_o is the integrated fluorescence intensity in the absence of CD, and F_∞ is the integrated fluorescence intensity when all of the ANS probe molecules have been complexed by CD molecules. This equation assumes that only a 1 : 1 complex is formed; this assumption can be readily tested using a reciprocal plot (also known as a Benesi–Hildebrand plot [39]) of F_o/F versus 1/[CD]. This plot will be linear if only a 1 : 1 complex is formed, but will show curvature if complexes of other stoichiometry are being formed [37, 38].

Of the twelve inclusion complexes studied, six exhibited excellent linear reciprocal plots: 1,8-ANS in HP-β and HP-γ, and 2,6-ANS in β, HP-α, HP-β, and HP-γ. These form only 1 : 1 inclusion complexes, and can be analyzed using Equation 3. For example, Figure 3 shows the plot of F/F_o *versus* [CD] for both 1,8-ANS and 2,6-ANS in HP-β, along with the resulting fits to Equation 3 (using a non-linear least squares fitting routine) of K = 480 and 7200 M^{-1}, respectively. The fit result values for K for all six of these 1 : 1 inclusion complexes are listed in Table III.

In the cases where non-linear reciprocal plots were obtained, higher-order complexes must be involved. Analysis equations for various types of higher-order host:guest complexes are available in the literature, including 2:1 [40], and 1:2 and 2:2 [41–43]; a nonlinear least-squares fitting program was written for the 2:1 case, but not for the 1:2 and 2:2 case, due to the complexity of the equations. In

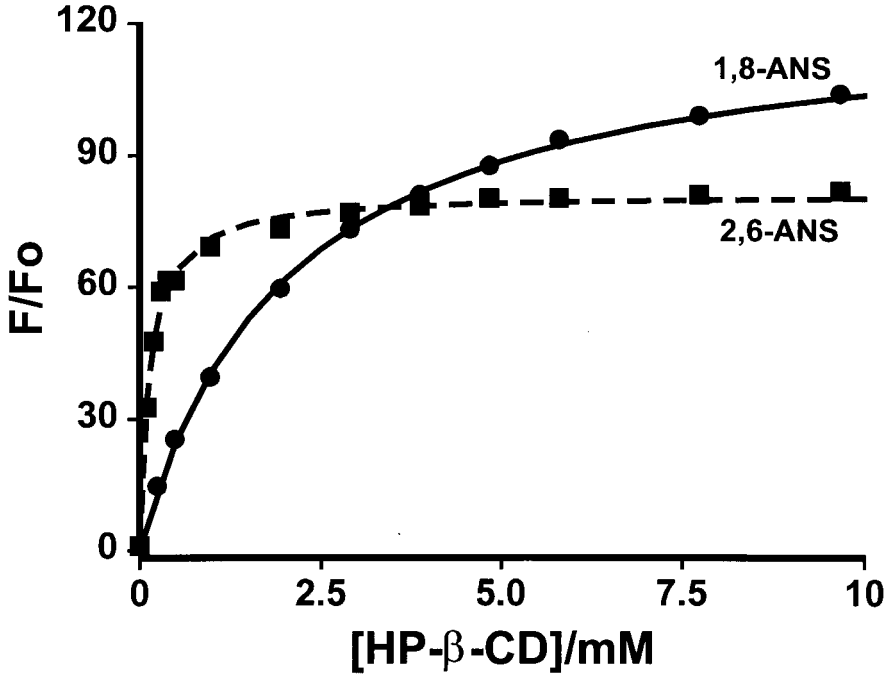

Figure 3. Fluorescence enhancement of 1,8-ANS (●) and 2,6-ANS (■) as a function of HP-β concentration. The lines show the best fits to Equation 3: —— 1,8-ANS, K = 480 M^{-1}; - - - - 2,6-ANS, K = 7200 M^{-1}.

the case of α, the curvature of the observed plots of F/F_o *versus* [CD] were too small over the concentration range studied to obtain a proper fit; an upper limit to the value of K can be estimated as $K < 20\ M^{-1}$. In the cases of 1,8-ANS in β and HP-α, although the reciprocal plots did deviate from linearity (R = 0.939 and 0.933, respectively), the data fit very well to a 1 : 1 complex model, but very poorly to the model involving 2:1 complexes. Thus, the values of K obtained for these two complexes, assuming the formation of a 1 : 1 complex only, are also included in Table III, with the possibility of a minor involvement by 1:2 and 2:2 complexes. Finally, the data for both probes in γ fit very poorly to both of the models tried. This agrees with previous reports that γ can include two naphthalene moieties, with both 1:2 and 2:2 stoichiometries [42–43]. Thus, no association constants are listed in Table III for γ.

Of the ten ANS:CD complexes listed in Table III, K values have been previously reported for only four (values in M^{-1}): α:1,8-ANS: (K from 0 to 26 [18, 22, 34]); β:1,8-ANS (K from 64 to 115 [18–20, 22, 27, 34]); β:2,6-ANS (K = 2080 [19]); and HP-β:1,8-ANS (K = 430) [28]. The values obtained in this work for 1,8-ANS in α, β and HP-β agree well with the literature values, while that for 2,6-ANS in β is significantly lower than that reported in the literature.

4. Discussion

Before discussing the enhancement and association constant results, it is useful to review what is known from the literature on the mode of inclusion, i.e., orientation, of ANS and other naphthalene derivatives into CD cavities. Harata and Uedaira [44] showed that in the β cavity, 2-substituted naphthalene derivatives exhibit axial inclusion of the naphthyl moiety, whereas 1-substituted derivatives may exhibit axial or equatorial inclusion. However, Muñoz de la Peña *et al.* [37] proposed axial-included naphthalene moieties for both 1- and 2-substituted naphthalene derivatives in β. Nishijo *et al.* [26] showed that in the case of 1,8-ANS in β, the preferred inclusion mode in fact involves the phenyl ring, with most of the naphthyl ring outside of the cavity. Similarly, Schneider *et al.* [22] found that α weakly binds the phenyl but not the naphthyl moiety of 1,8-ANS, while β and γ show equal or greater affinity for binding the phenyl as compared to the expected naphthalene moiety.

Although the polarity of the water-solvated cavities probably increase in the order of $\alpha < \beta < \gamma$ due to the number of water molecules included, if inclusion of a probe molecule results in removal of these included waters, then the polarities of the three CD cavities are relatively similar, due to the identical chemical structures. For example, Lichtenthaler and Immel [45] conclude that the three CDs have very similar polarities. Cox *et al.* [46] reported $E_T(30)$ values of 57 and 58 for α and β, respectively. Street and Acree [47] reported the dielectric constants of the β and γ cavities to be 48 and 55, again quite similar. Thus, it will be assumed that the polarity of the bare cavities of the three parent CDs is roughly the same, and that differences observed in fluorescence enhancement can be attributed to differences in the degree of encapsulation of the probe into the CD cavity, or in the number of waters co-included with the guest.

The measured fluorescence enhancements F_∞/F_o provide a direct indication of the local polarity of the probe. A larger enhancement for a given probe in the presence of one CD as compared to another indicates that the probe is in a more nonpolar environment, either because the cavity itself is less polar (modified *vs.* parent), or because the fluorophore fits inside the cavity better. Comparisons of the fluorescence enhancements for the two probes reveal a number of interesting trends. The HP-substituted CDs provide substantially larger enhancements than do the corresponding parent CDs; this is true for both probes. For example, HP-β enhances 1,8-ANS by a factor of 125, whereas β itself results in an enhancement of only 16. Similar comparisons can also be made for both probes in the case of HP-α *vs.* α and the case of HP-γ *vs.* γ. These observations of larger enhancements by modified CDs are in agreement with previous reports [25, 28], and have been explained previously as resulting from the relatively less polar cavity provided by the replacement of hydroxyl groups in the parent CD by hydroxypropyl groups, and the extension of the non-polar cavity provided by the alkyl chains of the HP groups along the rims of the cavity [28]. This would be particularly important if it is the

phenyl moiety which is included in the cavity, with the naphthalene fluorophore mainly outside.

When the enhancement trends of the two probes are compared, significant differences are observed. In terms of the unmodified CD cavity size, there is little difference for 1,8-ANS between β and γ, as indicated by the very similar enhancement values of 16 and 17, respectively. However, in the case of the HP derivatized CDs, HP-β gives a much larger enhancement than does HP-γ: 125 *vs.* 25. This must be a result of the effect of the HP groups on the two cavities. In both cases, the substitution with HP groups results in a larger enhancement; however, this increase is much more pronounced in the case of the β cavity. The cavity size of α is too small to significantly encapsulate the 1,8-ANS naphthalene moiety, giving a very small enhancement of 5.0. The extension of the α cavity by HP groups does however result in a significant enhancement of 35 for HP-α. It is also interesting to note that the order of increasing enhancement of 1,8-ANS is different in the parent ($\gamma \approx \beta > \alpha$) and modified CDs (HP-$\beta$ > HP-α > HP-γ). This indicates that the addition of the hydroxypropyl groups is not a simple additive effect of decreasing the cavity polarity, but must involve specific interactions of the HP groups with the host–guest structure.

In the case of 2,6-ANS, the probe matches best with the β cavity, with larger enhancements than in α or γ, for both parent and unmodified CDs. The γ-cavity is apparently too large, so that the 2,6-ANS molecule is not held as tightly, and more water molecules can presumably be included in the cavity along with the probe. This results in the significantly smaller enhancement in γ as compared with β (20 *vs.* 32), as well as in HP-γ as compared with HP-β (35 *vs.* 81). In the case of HP-γ, the HP groups must be able to hold the 2,6-ANS more tightly, or prevent the presence of water molecules, resulting in the significant (but smaller than in HP-β) enhancement. Unlike the case of 1,8-ANS, the enhancement of 2,6-ANS in α-CD is very significant (26), and that in HP-α (70) is even larger. These differences in the two probes are clearly a consequence of the narrower shape of 2,6-ANS, which is a much better match to the size of the β-cavity. Furthermore, 2,6-ANS would be more likely to be included axially, whereas 1,8-ANS would have to be included equatorially, leaving more of the naphthalene exposed.

The enhancements scaled to ethanol allow for the direct comparison of the enhancements for the two probes in the same CD. These numbers in effect represent the degree of nonpolarity of the probe medium on a scale where water equals 1 and ethanol equals 100. 1,8-ANS and 2,6-ANS show a similar scaled enhancement (64 and 68) in HP-β, signifying that a similar environment is being experienced for both probes. The larger value of 125 *vs.* 81 for 1,8-ANS *vs.* 2,6-ANS in HP-β given in Table I is thus a result of the greater sensitivity of 1,8-ANS to polarity. However, in the parent β, there is a large difference in the enhancements: 8.6 for 1,8-ANS and 27 for 2,6-ANS. This is a reflection of the difference in shape of the two probes, with 2,6-ANS fitting better into the β cavity; this effect is diminished in the HP substituted CD.

The association constants determined for these CD:ANS complexes provide different information. Whereas the enhancements as described above indicate the polarity of the ANS environment in the complex, the association constants indicate how strong the complex is, i.e., how tightly bound the probe is in the CD cavity. These are related properties, but are not necessarily the same. In all of the measured CD:ANS complexes, there is a correspondence between these two properties: complexes with a large enhancement invariably have a large K value, indicating that the probes are bound more strongly in the less polar cavities. For example, for both probes, HP-β gives the largest value of both K and F/F_o. Thus, a larger difference in polarity between the bulk water solution and the CD cavity results in a larger enhancement (fluorescence increases as polarity decreases), as well as a greater affinity of the hydrophobic part of the probe for the cavity as opposed to solution, and hence a larger association constant. However, the correspondence is not direct. For example, in the comparison of the results for HP-β *vs.* β, in the case of 1,8-ANS, K increases by a factor of 6 (480 *vs.* 80), whereas F/F_o increases by a slightly larger factor of 7.8 (125 *vs.* 16). By contrast, in the case of 2,6-ANS, K increases by a factor of 5.3 (7200 *vs.* 1350), whereas F/F_o only increases by a factor of 2.5 (81 *vs.* 32). Thus, the relative increases in K and F/F_o depend on the specific host–guest pair involved.

Comparison of the K values for the two probes in a given CD dramatically indicates the differences in their ability to form CD host–guest complexes. In the case of α, both probes have very small K values, indicating that the α cavity is too small in both cases. For HP-α, K is still very small for 1,8-ANS (21 M^{-1}), but has increased significantly in the case of 2,6-ANS to a value of 110 M^{-1}; the addition of the HP groups has extended this small cavity enough to result in a significant binding of the less bulky probe. In the case of β, K = 80 M^{-1} for 1,8-ANS, but K = 1350 M^{-1} for 2,6-ANS, an increase of a factor of 17. This result again indicates that the size and shape of 2,6-ANS provide a much better match for the β cavity than in the case of the bulkier 1,8-ANS. This is seen again in the case of HP-β, in which K = 480 M^{-1} for 1,8-ANS, but K = 7200 M^{-1} for 2,6-ANS, a similar increase by a factor of 15. However, in the case of HP-γ, the cavity size is large enough that there is very little discrimination between 1,8-ANS and 2,6-ANS, giving K values that are identical within experimental error.

The results discussed above for both relative fluorescence enhancements and association constants for these two probes in the six CDs are in general agreement with the "size-fit" concept for host–guest complexation [48]. This simply states that the host/guest pair with the best match between the overall size and shape of the guest and the size and shape of the host cavity will form the strongest complex. This is a result of the distance dependence of the van der Waals forces, the main forces of attraction between the host and guest, and hence the driving force for the complexation processes. These are extremely short range forces, and are greatly increased by close contact between the host and guest, especially if the size match

allows contact at various positions on the guest. This is analogous to the well-known “lock and key” model for enzyme/substrate behavior.

5. Conclusions

The fluorescent probes 1,8-ANS and 2,6-ANS both form host–guest inclusion complexes with parent and modified CDs in aqueous solutions, with significant enhancement of their fluorescence upon complexation. In all cases, the modified CDs provide larger enhancements than do their corresponding unmodified parents, by providing an extended nonpolar cavity allowing for more of the fluorophore to be included. There is a significant effect of the relative size and shape of the guest and host molecules, with those pairs with the best match in these properties giving the strongest-bound complexes. The more streamlined 2,6-ANS probe was found to form much stronger complexes than the bulkier 1,8-ANS analog. However, the 1,8-ANS was found to be more sensitive, and gave the highest fluorescence enhancement. The establishment of a polarity scale for the two probes relative to ethanol provided a means for the direct comparison of results for the two probes in a given CD; this should prove to be a generally useful method for comparing the encapsulation of different guests (with different fluorescence sensitivities) in the same host CD. The use of the two probes in complementary fluorescence enhancement experiments is recommended for investigating inclusion complexes of host molecules, since the differences in results for the two probes will provide useful information on the size and shape of the cavity of the host of interest.

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