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ORIGINAL ARTICLE

Spectroscopic characterization of the binding and isomerization cycle of Brooker's merocyanine with α-, β-, and γ-cyclodextrins

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Abstract Complexes of Brooker's merocyanine dye with α-, β- and γ-cyclodextrin (CD) have been characterized to determine the relative strength and thermodynamics of binding, as well as the effect of binding on the protolytic-photochemical isomerization cycle of the dye. It was found that the dye binds most tightly to β-CD, with a binding equilibrium constant of 430 M^{-1}, in agreement with previous results (Hamasaki et al. J. Incl. Phenom. Mol. Rec. Chem. **13**, 349–359 (1992)), while α-CD and γ-CD complexes have a binding constant of approximately 110 M^{-1} and 70 M^{-1}, respectively, determined using absorbance and fluorescence spectroscopy. The isomerization cycle for the dye in α- and γ-CD complexes was found to be the same as for the free dye. Complexation with β-CD, however, resulted in depressed trans-to-cis photoisomerization in acidic conditions followed by spontaneous cis-to-trans isomerization (with the addition of base). Thermodynamic results also indicated differences between α-CD ($\Delta S° = -48$ J K^{-1}) and β-CD ($\Delta S° = +12$ J K^{-1}) complexes. There was no temperature dependence observed for the γ-CD complexes. These results can be justified in terms of the location of the dye molecule within the cyclodextrin cavity for each of the complexes.

Keywords α-Cyclodextrin · β-Cyclodextrin · Binding constant · Brooker's merocyanine · Dye · γ-Cyclodextrin · Isomerization · Thermodynamics

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Introduction

Cyclodextrin chemistry has become a prominent area of host-guest research because of the protection afforded guest species within the host cavity. This added stability leads to novel materials with a wide-range of applications, including drug encapsulation and delivery, waste treatment and odor elimination. Cyclodextrins are torus-shaped molecules with large hydrophobic interior cavities into which a guest molecule can be inserted. Common cyclodextrins include α-, β-, and γ-cyclodextrin (CD) which are made of six, seven or eight dextrin units, respectively. Binding between the guest molecule and the cyclodextrin occurs through weak van der Waals forces. Small molecules can be bound completely within the cavity, which is approximately 7.9 Å in length [1]. Larger molecules may be partially inserted, with one or both ends of the guest molecule extending beyond the cavity, allowing the external groups to be solvated. α-CD (inner diameter = 5.7 Å) [2] is known to encapsulate linear aliphatic molecules [3], while β-CD, with a diameter of 7.8 Å [2], is the appropriate size to accommodate simple aromatic molecules, such as stilbene [4] (similar to the guest molecule in this work). γ-CD, with a cavity size of 9.5 Å [2], can incorporate large molecules, as well as dimers of dye molecules [5] and PAHs such as pyrene [6]. The complexation reaction is given by:

$$x\ \text{Guest molecules} + y\ \text{Cyclodextrin} \rightleftarrows \text{Guest}_x\text{:Cyclodextrin}_y \tag{1a}$$

$$K = \frac{[\text{Guest} : \text{Cyclodextrin}]}{[\text{Guest}]^x[\text{Cyclodextrin}]^y} \tag{1b}$$

where K is the equilibrium binding constant, also known as the association constant.

The current study focuses on a particular aromatic dye molecule, Brooker's merocyanine ((4-[(1-methyl-4(1H)-pyridinylidene) ethylidene]-2,5-cyclohexadien-1-one), also known as stilbazolium betaine, to determine the complexation behavior as a function of the size of cyclodextrin cavity. Brooker's merocyanine was first synthesized in 1951 by Brooker et al. [7, 8] and is of interest in the field of spectroscopy due to the large solvatochromic response to the microenvironment surrounding the molecule [8–10]. The most stable form of Brooker's merocyanine is the deprotonated, planar [11] trans-isomer with primarily double bond character at the central bond (zwitterion form, shown in Fig. 1), even in low dielectric constant solvents [12]. Comprehensive studies have only recently been completed on Brooker's merocyanine in solution in order to understand the details of the UV/visible absorption spectrum [12, 13]. Several bands and shoulders have been observed in various solvents, which have been attributed to both planar and nonplanar conformers, and the formation of dimers and possibly tetramers, although the exact nature of these bands could not be determined by Morley et al. [12] There is no evidence for direct trans-cis isomerization of Brooker's merocyanine, although the cis isomer can be prepared by photoisomerization of the protonated molecule (pKa = 8.54) [14] followed by deprotonation, [15–17] as shown in Fig. 1. The protolytic-photochemical isomerization cycle of Brooker's merocyanine has been suggested as a model system for naturally-occurring photocyclic systems, including the behavior of retinal in rhodopsin or in the photosynthesis of *Halobacterium halobium* [18]. This type of cyclic process in which one step proceeds in only one direction has also been proposed as a model system for information storage [19].

The complexation of Brooker's merocyanine with β-CD has previously been characterized by Hamasaki et al. [20]. This study will expand on those results in order to determine similarities and differences between the binding of Brooker's merocyanine to the three common cyclodextrins (α-, β-, and γ-CD). The thermodynamics of binding to each cyclodextrin will also be discussed. In addition, the effect of complexation on the rate of trans-to-cis conversion of the protonated molecule has been reported for β-CD complexes [20], but the deprotonated relaxation back to the trans isomer has not previously been discussed as a function of complexation. Brooker's merocyanine has been used to try to quantitatively characterize the microenvironment of the methyl-β-CD cavity, but a conclusive value could not be reached [21]. Therefore, we will use a qualitative description of the internal CD environment to understand the entire isomerization cycle of each of the Brooker's merocyanine-CD complexes.

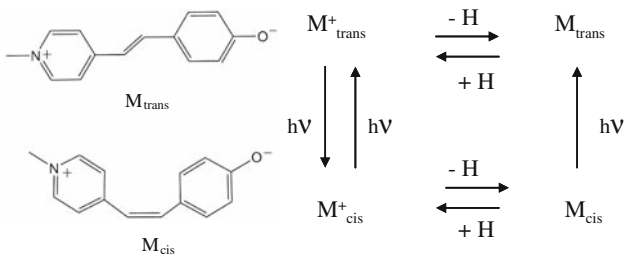

Fig. 1 The isomerization cycle and the molecular structure of the deprotonated, zwitterionic isomers of Brooker's merocyanine (M). Brooker's merocyanine can also be protonated (M^+) and can exist in the quinoidal form

Experimental

Brooker's merocyanine (Aldrich Chemicals), α-CD (Alfa Aeasar), and β-, and γ-CD (TCI International) were obtained and used without further purification. Concentrations were adjusted for the hydration of each cyclodextrin, reported as 10.5%, 2.7%, and 9.7%, for α-CD, β-CD and γ-CD, respectively. All solutions were prepared using deionized water with a standard carbonate buffer (pHydrion buffer capsules, Micro Essentials) at pH = 10.00 ± 0.02, unless otherwise stated. Carbonate buffer was chosen to ensure that competitive binding did not occur [22], which was verified by comparing the binding constant to results found using a weak solution of sodium hydroxide. Concentrations of cyclodextrin solutions were chosen to remain below the saturation limit for complexation.

Determination of the binding constant for deprotonated Brooker's merocyanine

A minimum of four different cyclodextrin concentrations, and as many as nine, were used to determine the binding constant for each cyclodextrin species. All data reported are the average of three experiments using fresh solutions. Absorption studies were performed using a Cary 300 Bio UV/Vis spectrometer at 20.0 ± 0.2 °C. Fluorescence emission spectra were recorded at room temperature using a Jobin-Yvon Fluoromax 3 spectrometer, with an excitation wavelength of 467 nm and a bandpass of 5.0 nm. Data were recorded at 500 nm (off-peak) for absorbance and 600 nm (peak) for fluorescence. Four samples of each cyclodextrin complex were prepared using a 2×10^{-5} M dye solution mixed with different cyclodextrin solutions, ranging from approximately 6×10^{-4} M to 1×10^{-3} M, in addition to the pure dye reference.

Thermodynamic studies

The Cary 300 Bio UV/Vis spectrometer was fit with a water bath temperature-regulated six-cell sample holder.

The solution concentrations used in the binding constant experiments were also used for the thermodynamic studies. Spectra were recorded from 20.0 to 50.0 °C in 10 ± 0.2 °C intervals.

Kinetics of isomerization

The isomerization of Brooker's merocyanine requires a change from acidic to basic conditions in order to observe the entire cycle. All acidic samples were kept in the dark during preparation. The pH was adjusted to 2.4 ± 0.2 using 1 M HCl, and samples were irradiated in 30 s intervals using a mercury lamp with a 254 nm filter (Mineralite Model UVG-11, Ultraviolet Products, Inc.). After the photostationary state was observed, 1 M NaOH was added to raise the pH to 11.6 ± 0.2. Absorbance spectra were obtained for each of the complexes, as well as the dye reference, at 25.0 ± 0.2 °C throughout the experiment.

Results

The absorbance spectrum of Brooker's merocyanine in water at pH = 10 exhibits a broad band at 444 nm, with a second weak band at 262 nm. The fluorescence emission peak is found at 574 nm. When Brooker's merocyanine is protonated, the absorbance bands undergo a hypsochromic shift to 374 and 248 nm respectively, while the emission peak shifts to 505 nm. The deprotonated Brooker's merocyanine is stable over long periods of time, but the protonated molecule will spontaneously isomerize from the trans to the cis isomer, leading to the presence of both isomers in a photostationary state. This state exhibits weaker absorbance than the pure trans isomer due to the smaller extinction coefficient for the cis form [15]. Determination of the binding constant of the protonated molecule is complicated by the existence of both isomers, so the binding studies reported here focus on the deprotonated dye molecule. A pH of 10 leads to the deprotonated form of Brooker's merocyanine (pKa = 8.54) [20], but is below the reported deprotonation of the cyclodextrin hydroxyl groups (pKa = 12.2 ± 0.2) [23, 24], allowing for favorable hydrogen bonding along the rim of the cyclodextrin cavity.

Binding to α-, β-, or γ-CDs can be measured by observing changes in the absorption spectra. As a result of complexation, the main absorption peak shifts to lower energy, and broadens slightly leading to a decrease in the peak intensity, as shown in Fig. 2a. β-CD complexes exhibit the largest effect, while α- and γ-CD complexes exhibit smaller, but measurable spectral shifts. The resulting intensity increase at 500 nm as a function of complexation can be fit using standard models for 1:1 complexes to determine the binding constant (K). Experimental conditions and absorbance values are reported in Table 1. Also reported is the complexation ratio ([complex]/$[dye]_o$), which is a measure of the degree of complexation. According to Hirose, the ratio of $[dye]_0$ to $[CD]_0$ values (approximately 70–350 [Cd]:[dye]) fall within appropriate ranges for accurate determination of the binding constant [25]. The first model is the linear modified Benesi-Hildebrand equation: [26]

$$\frac{1}{D - D_0} = \frac{1}{(D_\infty - D_0)\ K\ [CD]_0} + \frac{1}{(D_\infty - D_0)} \qquad (2)$$

where D is the absorbance of the sample, D_0 is the absorbance of the free dye, and D_∞ is the absorbance of the fully-complexed dye. $[CD]_0$ is the initial cyclodextrin concentration before complexation occurs. Data for each complex was fit to this equation using Microsoft Excel linear regression. The associated slope and intercept errors were also determined using Excel, and propagated to determine the accuracy of these results.

The second model involves a nonlinear regression analysis of the following equation for 1:1 complexes:

$$D = \frac{D_0 + D_\infty K[CD]_0}{1 + K\ [CD]_0} \qquad (3)$$

Nonlinear regression was performed using an iterative fitting procedure in SigmaPlot by fitting three parameters

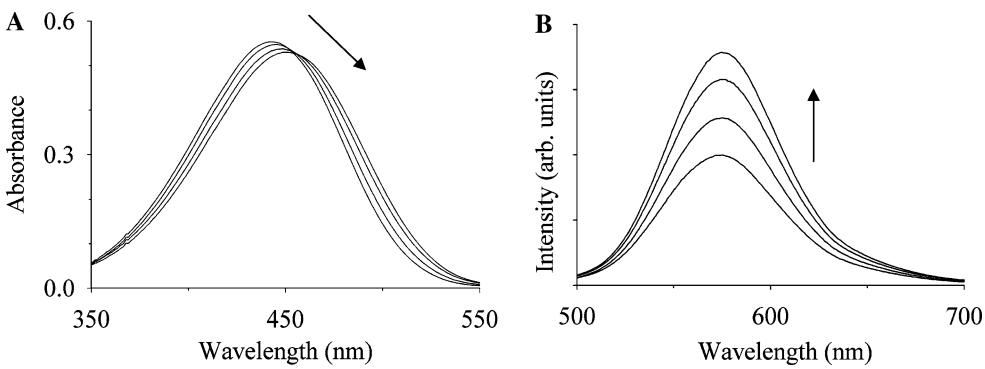

Fig. 2 The effect of α-CD complexation on the absorbance (**a**) and fluorescence emission (**b**) spectrum of Brooker's merocyanine at room temperature. The arrow indicates the direction of change as a result of increased complexation. β-CD and γ-CD complexes exhibit similar behavior. Note that intermediate spectra were removed for clarity

Table 1 Experimental conditions used to determine the binding constant using absorbance data

	$[CD]_o$	Absorbance	Complexation ratio
α-CD complexes	0	0.163	–
	$1.30 * 10^{-03}$	0.197	11.5
	$2.27 * 10^{-03}$	0.221	19.8
	$3.89 * 10^{-03}$	0.247	28.7
	$6.49 * 10^{-03}$	0.282	40.5
β-CD complexes	0	0.167	–
	$1.41 * 10^{-03}$	0.212	36.2
	$2.47 * 10^{-03}$	0.231	51.6
	$4.23 * 10^{-03}$	0.247	64.5
	$7.05 * 10^{-03}$	0.259	73.9
γ-CD complexes	0	0.162	–
	$1.31 * 10^{-03}$	0.167	9.4
	$2.29 * 10^{-03}$	0.170	16.3
	$3.93 * 10^{-03}$	0.174	23.2
	$6.55 * 10^{-03}$	0.180	34.8

All dye solutions were $2.06 * 10^{-5}$ M

Table 2 Determination of the binding constant (K) using absorbance and fluorescence data fit with linear and nonlinear models (Eqs. 2 and 3, respectively)

	Absorbance binding constant		Fluorescence binding constant	
	Linear fit	Nonlinear fit	Linear fit	Nonlinear fit
α-CD	100 ± 10	110 ± 10	140 ± 10	150 ± 10
β-CD	400 ± 20	430 ± 20	500 ± 20	470 ± 20
γ-CD	100 ± 20	70 ± 20	130 ± 20	120 ± 20

All units are in M^{-1}

(D_0, D_∞, and K). The associated errors with each value were determined as part of the fitting procedure. Due to the inverse nature of Eq. 2, the linear model tends to overestimate the importance of low cyclodextrin concentrations, which are also prone to small changes upon complexation and therefore more error. However, by using a data set weighted to more concentrated solutions, this can be overcome, and the results of both linear and nonlinear methods are found to be in agreement, as shown in Table 2.

The change in absorbance for each of the Brooker's merocyanine-CD complexes is fit well by both equations, as shown in Fig. 3, indicating a 1:1 complex is formed. In addition, there is a unique isosbestic point at 456 nm, indicating a single equilibrium between free and complexed dye molecules. None of the absorbance spectra exhibit any additional peaks or shoulders, indicating that Brooker's merocyanine dimers are not formed within the cavity.

Binding to α-, β-, and γ-CD can also be measured by observing changes in the emission spectra. However, in comparison with the absorbance spectra, there is no observable shift in the fluorescence emission peak as a result of increased complexation. Instead, the complexation leads to fluorescence enhancement, as shown in Fig. 2b. Enhancement may occur for a variety of reasons, including protection of the dye molecule against collisional quenching, changes in the polarity of the microenvironment, or an increase in the rigidity of the guest as a result of complexation [27]. The peak intensity can be fit using similar linear (Eq. 2) and nonlinear (Eq. 3) models, where D represents the fluorescence emission intensity [28–31]. Binding constants determined by absorbance and fluorescence data are in agreement, as shown in Table 2, although the fluorescence binding constants tend to be slightly higher than the absorbance results.

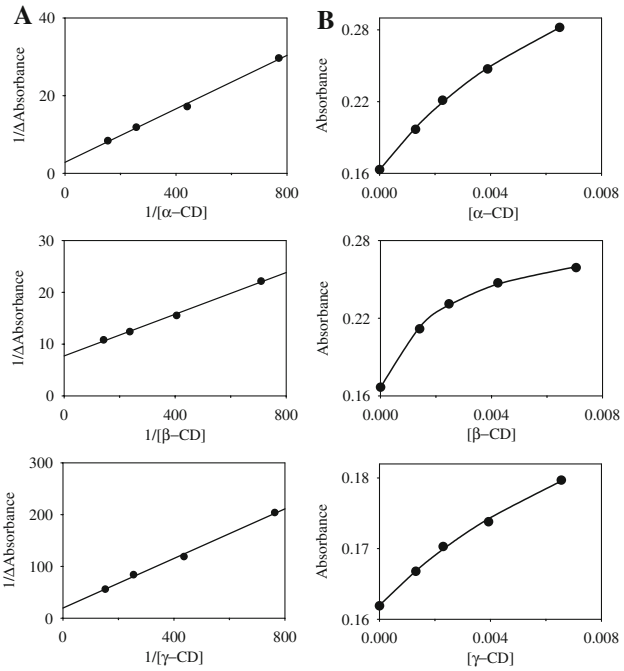

Fig. 3 Absorbance data for each of the CD-Brooker's merocyanine complexes (data points) with a trendline representing the linear modified Benesi-Hildebrand (**A**) and nonlinear (**B**) equation results

Thermodynamic studies

In addition to characterizing the dye-cyclodextrin equilibrium for each cyclodextrin, the thermodynamics of binding was also investigated. Equilibrium constants for each complex were measured as a function of temperature between 20 and 50 °C. The van't Hoff equation was used to determine ΔG° from the equilibrium constants using the nonlinear mathematical treatment of the absorbance data at 20 °C. ΔH° and ΔS° were determined from the slope and intercept of the following equation:

$$R \ln K = \frac{-\Delta H^{o}}{T} + \Delta S^{o} \quad (4)$$

The results for ΔG°, ΔH°, and ΔS° are reported in Table 3. All thermodynamic results are within the normal range for these types of complexes, and are similar in sign and magnitude to a variety of related guest molecules, as summarized by Rekharsky and Inoue [3]. It is interesting to note that the size of the cavity leads to a change in the sign of ΔS°. This has been reported for similar molecules, such as the acidic form of methyl orange, a conjugated, aromatic dye with an N-N central bond. Tawarah reported that the ΔS° for the methyl orange-α-CD complex is -20.4 J mol^{-1} K^{-1} and $+10.9$ J mol^{-1} K^{-1} for β-CD complexes [32], which agree in sign and magnitude with the current results. ΔG° found using the van't Hoff equation at 20 °C was compared with ΔG° found from ΔH° and ΔS° over the entire temperature range, and were within 1%. It should be noted that no appreciable temperature dependence was found for the γ-CD complexes, as the equilibrium constant was within experimental error across the temperature range investigated.

Table 3 Determination of the thermodynamic parameters from temperature-dependent binding equilibrium constants

	ΔH° (kJ mol^{-1})	ΔS° (J mol^{-1} K^{-1})	ΔG° at 20 °C (kJ mol^{-1})
α-CD	−26 ± 1	−48 ± 3	−11.5 ± 0.2
β-CD	−11.2 ± 0.3	12 ± 1	−14.8 ± 0.2
γ-CD	−10.5 ± 0.5	–	−10.5 ± 0.5

The γ-CD-dye complex binding equilibrium constant does not exhibit a measurable temperature dependence

Kinetics of isomerization

The characterization of Brooker's merocyanine isomerization cycle is shown in Fig. 4. The difference between the initial (low pH) and final (high pH) absorbance values indicates that there was enough thermal energy at room temperature to initiate the spontaneous trans-to-cis isomerization of the protonated molecule before spectra were recorded. Acidic samples were irradiated with UV light to increase the rate of isomerization. It was reported by Hamasaki et al. [20] that the final ratio of cis and trans isomers was not affected by the presence of cyclodextrin at low pH. However, we found this not to be the case. Although the effect was small, the β-CD complex (filled squares) showed depressed trans-to-cis isomerization (~5%), when compared to the dye solution (open circles). This differs from the results obtained for the α- and γ-CD complexes, which exhibited no appreciable difference from the free dye in solution.

When the pH of the photostationary state is increased, leading to the deprotonation of the dye molecule, the cis isomer is known to return to the more stable trans isomer with additional thermal or photochemical energy [15]. With no irradiation, the free dye molecule was observed to follow zeroth-order kinetics for the cis-to-trans isomerization, indicating that a small percentage of molecules randomly underwent thermal isomerization. This process is shown in Fig. 4b. α- and γ-CD complexes exhibited the same kinetic behavior. When these samples were irradiated, the process changed dramatically to a first-order kinetic process, returning to the pure trans isomer, as shown in Fig. 4c. It is interesting to note that the behavior of β-CD complexes was very different. When the pH was increased, the dye molecules complexed with β-CD

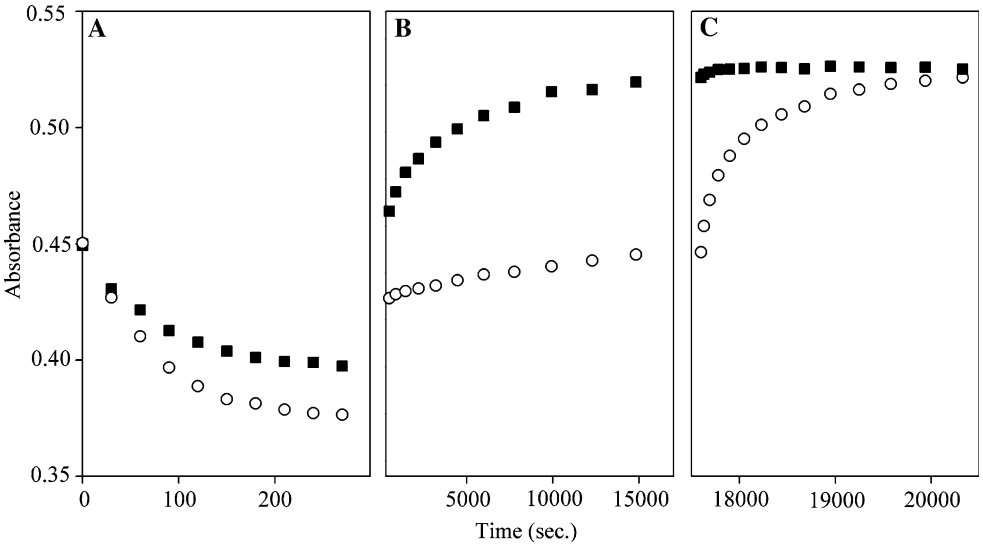

Fig. 4 Isomerization of Brooker's merocyanine in solution (*open circles*) and complexed to β-CD (*filled squares*). Region A, B and C represent a continuous experiment with a different time scale for each isomerization step. Region A–Samples are irradiated with UV light at low pH (pH = 2.4) to generate the cis/trans photostationary state. Region B–the pH is increased to 11.6, while both samples are kept in the dark. Region C–samples are then irradiated with UV light at pH = 11.6

spontaneously underwent thermal isomerization following first-order kinetics, and completely returned to the pure trans isomer with no additional photochemical energy needed.

Discussion

It is expected that β-CD will bind most favorably to Brooker's merocyanine because of the size of the interior cavity. The binding constant for β-cyclodextrin, found to be approximately 450 M^{-1}, is in agreement with that reported by Hamasaki et al. [20]. The β-CD complex also exhibits the largest ΔG° value of −14.8 kJ mol^{-1} and therefore the most favorable complexation of all three cyclodextrins investigated. The positive ΔS° value for the β-CD complex is typically ascribed to the dye molecule being inserted deeply into the cyclodextrin cavity causing displacement of water molecules found within the cavity [33]. The isomerization studies also indicate that the central C–C bond is fully encapsulated within the hydrophobic cavity.

There are two possible explanations for the differences in isomerization behavior between the free and β-CD-complexed dye molecules. The simple explanation is steric effects. The observed depressed trans-to-cis isomerization in acidic solution indicates that rotation around the central C–C bond may be sterically hindered within the cavity and there may be less favorable intermolecular interactions between the cyclodextrin and the cis isomer. These steric effects would be eased when base is added through the spontaneous cis-to-trans thermal isomerization, as the trans form with the phenyl groups anti to one another should more easily reside within the cavity. Another explanation is "solvatokinetic" isomerization [17]. It is well known that as the polarity of the solvent increases, the zwitterionic character of this dye becomes favored [7]. Therefore, if the dye enters the hydrophobic cyclodextrin cavity, the quinoidal form will be enhanced. As a result, rotation around the central bond will be more facile, and the more stable trans isomer will be favored. This spontaneous behavior is in direct contrast to the behavior of free dye, which undergoes only limited isomerization because it is in a much more polar environment, favoring the zwitterionic form. When the dye is complexed to other hosts, such as the hydrophobic heme pocket in metmyoglobin, enhanced dark cis-trans conversion is observed [34], in agreement with these results. This observation can be contrasted with the behavior of azobenzene dyes, with a central N–N bond that undergoes depressed thermal cis-trans isomerization in the presence of β-CD [35]. However, the isomerization mechanism for this molecule is quite different. Azobenzene is believed to undergo isomerization via inversion of one of the nitrogen atoms [36] instead of rotation around the central bond, which may explain the differences between azobenzene and Brooker's merocyanine complexes.

Aromatic molecules are generally considered to be too large to completely enter the cavity of α-cyclodextrin. Instead, one end group of the guest molecule is incorporated into the cavity, while the remainder of the dye is left to be solvated outside of the cavity [3]. The lack of isomerization dependence on binding indicates that the dye behaves as if it is free in solution because most of the dye, including the central C–C bond, is not hindered by the presence of the α-cyclodextrin. The negative ΔS° term is in agreement with this observation, as the α-CD complex does not displace the internal water molecules to the same extent as the dye in the β-CD complex. The ΔH° term, which is larger for the α-CD complex than for the β-CD complex, becomes the dominant factor in the determination of ΔG° and complexation spontaneity. Although the ΔH° and ΔS° terms are very different, the overall ΔG° is only slightly smaller in magnitude than the ΔG° of the β-CD complex. The smaller binding constant, although on the same order of magnitude as the β-CD binding constant, also indicates a slightly weaker complex is formed by Brooker's merocyanine with α-CD than with β-CD.

Binding within γ-CD appears to be similar to α-CD, although in this case, the dye molecule likely enters the cavity, but is not tightly bound. This can be concluded from the smaller binding equilibrium constant when compared to the snug-fitting dye within β-CD. ΔG° of −10.5 kJ mol^{-1} also indicates weaker binding when compared to the other complexes. Finally, the lack of temperature dependence of the binding constant indicates that the dye will move in and out of the cavity with no additional energy required to overcome the intermolecular binding forces. Although γ-CD has been reported to be large enough to contain dimers [5] and form excimers, as reported for pyrene [6] and thioflavin T complexes, [37] no additional spectral peaks were observed for Brooker's merocyanine with γ-CD. This indicates only 1:1 complexes are formed, and is confirmed by the linearity of the Benesi-Hildebrand plots. The interior cavity of γ-CD appears to be large enough to incorporate either the cis or trans isomer, since there is no effect on the isomerization cycle when the dye is bound to γ-CD.

Conclusions

Spectroscopic characterization of the complexation of Brooker's merocyanine to α-, β- and γ-cyclodextrin has been completed in order to fully characterize the binding and isomerization processes of the dye molecule as a function of cyclodextrin cavity size. β-CD complexes result in strong association and deep penetration into the

cavity. The binding of the cis isomer is not favored within β-CD, likely due to steric hindrance or "solvatokinetic" effects within the cavity, causing depressed trans-to-cis photoisomerization in acidic conditions and spontaneous cis-to-trans thermal isomerization in basic conditions, when compared to the free dye. In contrast, α-CD and γ-CD form weak complexes, and allow for free rotation around the C–C central bond through the entire isomerization cycle. For the α-CD complex, this is likely due to partial insertion of the dye into the cavity, in agreement with the negative entropy value. For the γ-CD complex, the dye appears to be free to enter and leave the cavity, exhibiting weak binding and no temperature dependence of the binding constant.

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