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The Binding of 2-Acetylnaphthalene to Beta-Cyclodextrin Polymers Studied by Fluorescence Quenching

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THE BINDING OF 2-ACETYLNAPHTHALENE TO BETA-CYCLODEXTRIN POLYMERS STUDIED BY FLUORESCENCE QUENCHING

by

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Abstract

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Cyclodextrin (CD) molecules can form inclusion complexes with many different types of organics due to the hydrophobic environment of the CD internal cavity. The fluorophore 2-acetylnaphthalene (2-AN) has a high fluorescence intensity in polar solvents such as water. Therefore, its fluorescence is quenched when it forms a complex with a CD molecule and is contained in the non-polar cavity. By measuring the change in fluorescence intensity in the presence of CD, we can calculate the binding constant for CD:2-AN complex formation. The binding constants are temperature dependent as well. This allows us to calculate the thermodynamic properties, ΔH^0 and ΔS^0, for complex formation. This had previously been done for the CD molecules, α-, ß-, and γ-CD. The focus of this work is to present the findings for the ß-CD polymers and to compare those findings with the work already done on the monomer ß-CD molecule.

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I would like to thank my family for their love and support throughout my years at Union. I would especially like to thank my mother for her support, both financially and academically, over these four years. I hope someday to be able to repay her somehow.

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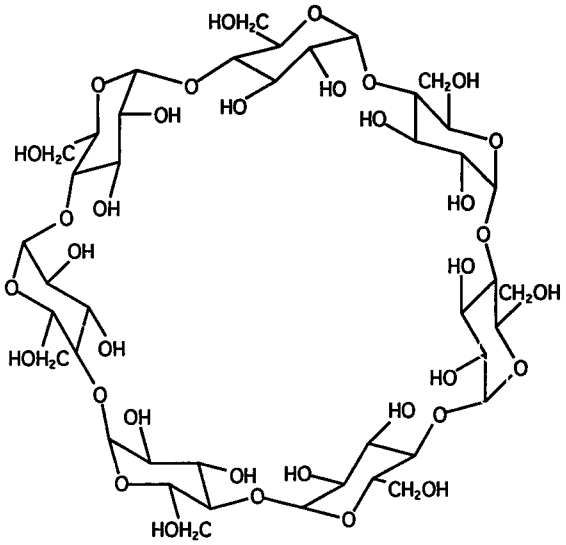
Introduction

Cyclodextrin (CD) molecules are rings composed of D(+)-glucopyranose units joined by α-(1,4)-linkages.[1] This gives them a torroidal shape with a height of 0.78 nm and varying internal diameters, depending on the type of CD molecule. The three common molecules are α-CD, ß-CD, and γ-CD, consisting of six, seven, and eight glucopyranose units, respectively (see Figure 1). These molecules have internal diameters of 0.57 nm, 0.78 nm, and 0.95 nm, respectively.[2]

Cyclodextrins are able to form host/guest type complexes with a large range and number of guest molecules by allowing them to bind in the internal CD cavities. The cavity of a CD molecule is relatively hydrophobic. This property makes it a good environment for many organics where size matches that of a given CD cavity. Cyclodextrins are often much more water soluble than the organics that may bind in them.

These properties give CDs a wide range of applications and makes them useful in many different types of systems. Applications such as solubility enhancement of organics in water,[3] improvement of analytical separations using CD mobile and bonded phases in HPLC,[4,5] controlling dye aggregation equilibria,[6] modeling protein complexes,[7] drug delivery and detection procedures,[8,9] and modifying photochemical behavior[10] have all been done with CDs.

Figure 1:
Structure of ß-CD Molecule



Another widely used application for CDs is in fluorescence work, since cyclodextrin molecules almost always enhance the fluorescence quantum yield of bound organic fluorophores.[11-16] This enhancement by complex formation is due to a number of factors. First, the fluorophore is exposed to a less polar environment as well as being protected from external quenchers like oxygen. The fluorophore is also held firmly in place and does not exhibit the "free rotor" effect, which often leads to quenching, once it is bound.

Much work has been done with complexes formed between naphthalene and the derivatives of naphthalene because of this fluorescence enhancement effect. Many groups have used fluorescence enhancement to measure the binding constants of these complexes of ß-CD with naphthalene and some of its derivatives.[11, 13-14]

One of the problems with the use of CD molecules is that the most useful CD molecule of all, ß-CD, also happens to be the least water soluble. Researchers have attempted a number of different ways to increase the water solubility of CD molecules, including adding urea to CD solutions,[17] alkylation of CD units,[18] and polymerization of CD units using epichlorohydrin.[19] This latter reagent produces water soluble CD polymers.

Cyclodextrin polymers (CDPs) are a polydisperse mixture of CDs linked by repeating glyceryl units. They are formed by reacting the

proper amounts of CD monomer units and epichlorohydrin at 50°C for about three hours. These polymer units have the general formula

$$[CD\text{-}(CH_2\text{-}CHOH\text{-}CH_2\text{-}O)_n X]_p$$

where CD is α-, ß-, or γ-CD; X is H or CD; $1 < p < 6\text{-}8$; and $1 < n < 18$ (α-CD), < 21 (ß-CD), < 24 (γ-CD). The average value of n for the polymer chains is 12-15. The reported %CD is 54 (α-CDP), 55 (ß-CDP) and 57 (γ-CDP). The molecular weight distribution of these polymers ranges from <2000 to between 9-10,000. The polymer chains below 2000 contain a single CD unit per chain, while those chains between 9-10,000 are likely to contain 4-5 CD units per chain.[20] All of the CDPs are highly water soluble (at least 50% w/v).

Cyclodextrin polymers have potentially the same uses as monomer CD units. Their increased solubility makes certain applications even more appealing, such as using the polymers to enhance the solubilities of organics. Another use for CDPs is as a flavor-altering agent in citrus products.[21]

There have been a number of articles written comparing the host/guest binding abilities of ß-CD and ß-CDP.[19-20, 22-24] This is an area of potential interest because of the possible differences in the binding sites and in the complexes that may be formed. The glyceryl linker units may make a difference in binding. First of all, they may deepen the binding site, thereby increasing the hydrophobicity of the cavity.

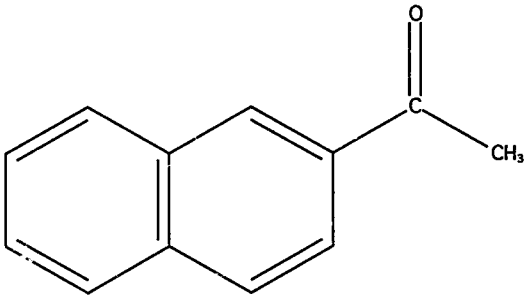
They might wrap around the molecule that is bound in the site, thereby increasing the strength of the complex formed. There is also the possibility that the linkers may compete with the guest for the binding site or sterically block the guest from entering the binding site. Evidence for the former has been shown in the use of aliphatic compounds as guests in forming host/guest complexes with CDs.[25] Moreover the polymers might form complexes with 2 CD:1 guest stoichiometry more easily than the monomer CDs because the monomer units can be on the same polymer chain. Complexes with 2 CD:1 guest stoichiometry have been reported for both CDs and CDPs.[19]

Our comparison of CD and CDP guest binding is done with the use of fluorescence. Fraiji et. al.[26] have already used static fluorescence quenching to determine the binding constant for the 1:1 complex that is formed between the fluorophore, 2-acetylnaphthalene (2-AN, see Figure 2), and α-, ß-, and γ-CD. Static quenching takes place when the 2-AN:CD complex is formed in the ground state as shown below.

$$\text{2-AN} + \text{CD} \longrightarrow \text{2-AN:CD}$$

The formation of the complex in the ground state prevents the fluorescence by bound 2-AN, and the fluorescence is said to be quenched statically. If the complex is indeed 1:1 and the fluorescence

Figure 2:
Structure of 2-Acetylnaphthalene



quenching is static then a modified Stern-Volmer equation, like Equation (1) below, can be used to determine the binding constant.

$$(1) \qquad F^o/F' = 1 + K\,[CD]$$

In equation (1), F^o/F' is the ratio of maximum fluorescence at 437 nm in the absence of quencher (F^o) to quenched fluorescence (F') at that wavelength, K is the binding constant of 1:1 complex formation, and [CD] is the concentration of cyclodextrin in the solution. If the plots of F^o/F' are linear and the intercept is near unity, then the fluorescence quenching is static in nature and the complex is 1:1. We can thus get the binding constant (K) from the slope of the line. Werner and Warner[27] have observed that this is indeed the case for 2-AN quenching by α-, ß-, and γ-CDPs at room temperature by getting high linearity in their Stern-Volmer plots. They also observe higher binding constant values for the polymer complexes compared to the published monomer complex binding constants.

The fact that Werner and Warner[27] obtained good linearity in their Stern-Volmer plots indicates two things about CDP binding with 2-AN. First of all, the binding sites of each CD unit for 2-AN must be nearly identical, and, second, the CD units must be acting independently of one another. Both of these conclusions are important to further work with these polymers.

The binding constant of the formation of the 2-AN:CD complex is useful information because we can use the temperature dependence of this value to calculate ΔH^o and ΔS^o for the binding. This is done by using the van't Hoff equation.

$$\ln K = - \Delta H^o/RT + \Delta S^o/R \qquad (2)$$

By plotting ln K versus the inverse of the temperature, we should get a line that will give us ΔH^o and ΔS^o by multiplying the slope and intercept of the line by the constant R (8.314 J/K·mol).

The focus of the work in this thesis is to explore the polymers further in a number of ways. First, we will focus on measuring the binding constant of 2-AN with ß-CDP as a function of temperature in order to get thermodynamic data about 2-AN:ß-CDP complex formation. We will then compare these data to the ß-CD monomer data obtained by Fraiji et.al. We would also like to see if evidence exists for multiple binding (i.e. 2:1 CD:2-AN complexes) from polymer chains containing more than one CD unit. This can be done by using 3500 MW cut off dialysis tubing to isolate the high and low molecular weight polymers. The low molecular weight polymer (CDPL) should have just a single CD unit per polymer chain. The high molecular weight polymer (CDPH) should contain 4-5 CD units per chain.[20] If the binding of these two polymers is similar and also consistent with that of the normal, whole polymer, we can rule out multiple binding.

We would also like to explore the environment of the binding site in a couple of ways. First, we would like to see if there is any competitive binding between the glyceryl linkages and the fluorophore. For this we have found a model for the linker units, polyethylene glycol (PEG). The polyethylene glycol can be checked for competition by putting it in solutions of 2-AN and ß-CDP and checking for an increase in fluorescence intensity as PEG displaces 2-AN from ß-CD binding site. If this method shows evidence of competitive binding, we can use these data as evidence for competition by the linker units.

We would also like to examine whether or not there are any conformational changes in the binding site at temperatures below room temperature. We can use the fluorophore 2-(N-methylanilino) naphthalene-6-sulfonic acid, sodium salt (2,6-MANS) to determine this by taking the corrected emission spectrum of the 2,6-MANS:CD complex over a range of temperatures and looking for a shift in the maximum.

and 350 nm for 2,6-MANS.

The fluorophore stock solutions were prepared by adding a small amount of solid fluorophore to either water (2-AN) or to 0.1 M phosphate buffer at pH 6.9 (2,6-MANS). These solutions were allowed to stir overnight. Five to ten milliliters of the stock solution was then passed through a 0.2 μ disposable filter obtained from Anotec. These solutions were then diluted with water (2-AN) or 0.1 M phosphate buffer, pH 6.9 (2,6-MANS) to give absorbances of 0.4-0.5 (1 cm cell) at their respective exciting wavelengths. CDP stock solutions were prepared by adding the required mass of CDP to an accurately known volume of water. Each set of 2-AN solutions to be measured were made up with a fixed concentration of 2-AN (0.04-0.05 absorbance at 340 nm) and increasing concentrations of CDP starting from zero.

Only two solutions were necessary for 2,6-MANS measurements, one with no CDP and one with CDP, with a fixed amount of 2,6-MANS. The amount of 2,6-MANS used was determined by its absorbance at 347 nm. The desired absorbance for the solutions to be measured was similar to that of 2-AN (between 0.04-0.05).

For the competition experiment, the solutions were made up with fixed amounts of 2-AN and CDP, and the concentrations of PEG were varied. One solution had a PEG concentration equal to the concentration of ß-CDP, another had 10 times that amount, and the last had 25 times the initial amount.

All solutions, except 2,6-MANS solutions, were allowed to sit overnight to ensure that they were equilibrated. The 2,6-MANS solutions sat for about eight hours, which should have been sufficient time for equilibration.

Quenching runs using the 2-AN solutions were initially run at temperatures varying from 10°C to about 50°C at roughly 10° intervals. Later 5, 15, and 25°C readings were added. Temperature control for these experiments was provided by a circulating water bath through a thermostated cell block using a Neslab Endocal Refrigerated Circular Bath.

Temperature control was critical for the 2-AN experiments due to the sensitivity of 2-AN fluorescence to temperature. The solution to be measured had to be placed in the cell block and then left alone for several minutes with the excitation shutter closed to avoid photodecomposition until it reached the desired temperature. The temperature was checked by a digital thermometer to verify that the desired setting had been reached. To ensure complete thermal equilibration, it was then necessary to monitor the intensity reading until it stopped changing. This whole process takes about 20 minutes at each temperature. Four solutions were allowed to sit in the cell block at one time in order to speed up the process.

The high and low molecular weight ß-CDP (ß-CDPH and ß-CDPL) were obtained by dissolving 4-5 grams of the polymer in about 10-15

milliliters of water. The resulting solution was then placed in 3500 MWCO dialysis tubing obtained from Spectrum. The tubing was suspended in one liter of water and was stirred continuously for about a week. The liter of water, which should now contain ß-CDPL, was evaporated down to about 15-20 ml using a Rotovap. This solution was then placed in a freeze-drying apparatus which removed the remaining water and left us with about 1.6 g of ß-CDPL. The solution left in the tubing was allowed to sit in the water stirring for about two more weeks with a change of water every two or three days. That solution was frozen for recovery of the ß-CDPH. The ß-CDPH used in these experiments had been dialyzed and recovered previously in a similar fashion.

Results

Table 1 shows a sample of the fluorescence intensity data that were collected for each experiment. F represents the fluorescence intensity measured by the instrument. F' is the corrected fluorescence intensity which is calculated by using Equation (3).

(3) $F' = F - ([\text{ß-CDP solution}]/[\text{ß-CDP stock}]) \times F_{\text{stock}}$

F^o/F' is the same as in Equation (1).

The data in Table 1 were obtained at 10°C. This set of solutions was run at higher temperatures as well. Each solution set was run through the temperature range at least once, and twice if time permitted.

Once we have obtained F^o/F', we can plot F^o/F' versus the concentration of ß-CDP for each temperature. Figure 3 shows a Stern-Volmer plot for the temperature run from which the data from Table 1 were taken.

The slope of each of the lines in the plot is the binding constant for 2-AN complex formation with ß-CDP at the given temperature (see Equation (1)). Notice that the lines appear to be linear and that the intercepts of the lines are all about 1. Also, note that the lines at 10 and 20°C are almost on top of one another, signifying a similar binding constant at both temperatures. This will be discussed later.

Table 1:
Fluorescence Intensity Data Sample

Solution No.	[ß-CDP] (M)	F	F'	F^0/F'
0	0	500	500	1.00
1	2.4×10^{-4}	406	404	1.24
2	4.8×10^{-4}	341	337	1.48
3	7.3×10^{-4}	289	283	1.77
4	9.7×10^{-4}	257	249	2.01
5	1.2×10^{-3}	229	219	2.28
ß-CDP stock	2.4×10^{-3}	19		

Figure 3:
Stern-Volmer Plot

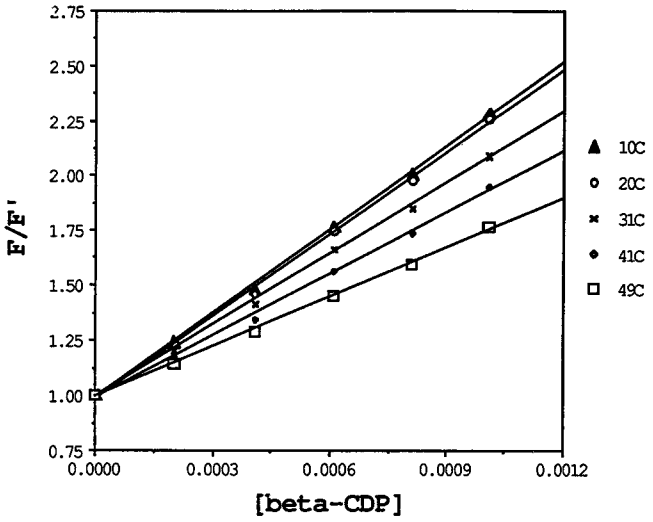


Table 2 is a summary of all binding constant data for the 2-AN temperature experiments with all three ß-CDPs (ß-CDP, ß-CDPH, ß-CDPL). The range, mean, and standard deviation are all given. Notice the temperature dependence of the binding constants. Again, the data show that the binding constants for 10 and 20°C agree in the three cases to plus or minus one standard deviation.

We can use the temperature dependence of the binding constants to construct a van't Hoff plot for our data. To do this, we simply plot ln K versus the inverse of the temperature. Figures 4 and 5 are van't Hoff plots for the ß-CDP data and the ß-CDPH data using data obtained at 20°C and above. A plot is not included for ß-CDPL because there are only data for three temperatures. The fact that the binding constant data is so similar between the ß-CDPL and the other two polymers allows us to conclude that the thermodynamic data for the ß-CDPL will be similar as well.

We can now get the values for ΔH° and ΔS° for the formation of the complex (eq. (2)). Table 3 shows these calculated values and their related error. Notice the agreement in values for ß-CDP and ß-CDPH.

The thermodynamic values that we obtained were calculated using only the data from temperatures above and including 20°C. This is because the binding constants at the temperatures lower than that do not change as expected with decreasing temperature. The binding constants level off and seem to go back down as the temperature is

Table 2:
Binding Constants for All Runs

T(°C)	N(1)	ß-CDP Range	Mean (+/- SD)
10	4	1240 - 1350	1290 (+/-51)
20	4	1220 - 1270	1250 (+/-21)
30	4	1010 - 1120	1080 (+/-47)
40	4	890 - 950	930 (+/-25)
49	3	760 - 800	780 (+/-21)

T(°C)	N(1)	ß-CDPH Range	Mean (+/- SD)
10	6	1230 - 1330	1270 (+/-40)
20	6	1200 - 1360	1250 (+/-61)
30	4	1040 - 1160	1100 (+/-61)
40	4	880 - 940	900 (+/-26)
49	5	740 - 820	780 (+/-40)

T(°C)	N(1)	ß-CDPL Range	Mean (+/- SD)
10	4	1300 - 1390	1350 (+/-38)
20	4	1260 - 1310	1290 (+/-23)
30	4	1110 - 1170	1140 (+/-24)

(1) Number of runs at each temperature.

Figure 4:
van't Hoff Plot for Beta-CDP

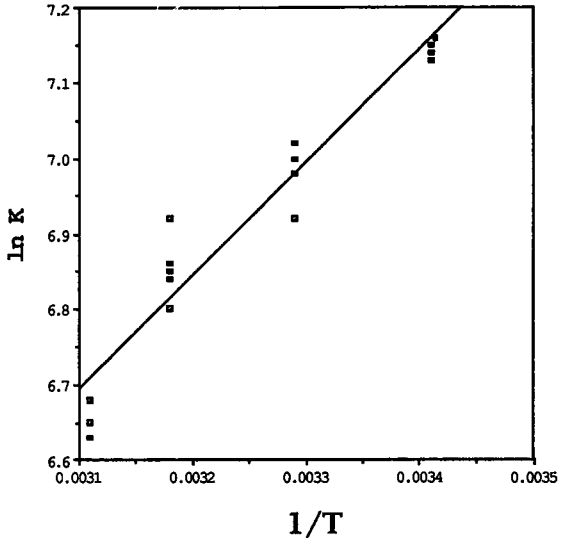


Figure 5:
van't Hoff Plot for Beta-CDPH

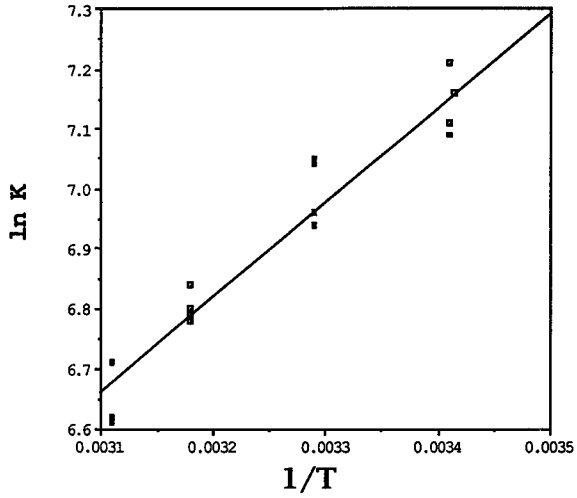


Table 3:
Thermodynamic Data for Complex Formation

Polymer	ΔH^0 (kJ/mol)	ΔS^0 (J/mol deg)
ß-CDP	-12.5 +/- 0.9	17 +/- 3
ß-CDPH	-13.1 +/- 0.8	15 +/- 6
ß-CD[1]	-11.9 +/- 0.5	13 +/- 2

(1) From reference 26.

lowered. Figure 6 shows a plot of the binding constants versus temperature for ß-CDP and ß-CDPH over a broader temperature range with additional points at 5 and 15°C.

Table 4 shows the results for the PEG competition experiment. The fluorescence intensity does not change due to the increased concentration of PEG in the measured solutions.

Finally, we ran the 2,6-MANS experiments to see if there is some sort of conformational change in the cavity of CD at low temperature. Figures 7 and 8 show the results of those experiments. We were looking for a shift in the fluorescence maxima of 2,6-MANS to tell us if there was some change happening. Figure 7 shows the corrected emission spectra at 10 and 40°C for 2,6-MANS alone in solution. Figure 8 shows the spectra after complex formation with ß-CDP.

Figure 6:
Binding Constants Versus Temperature

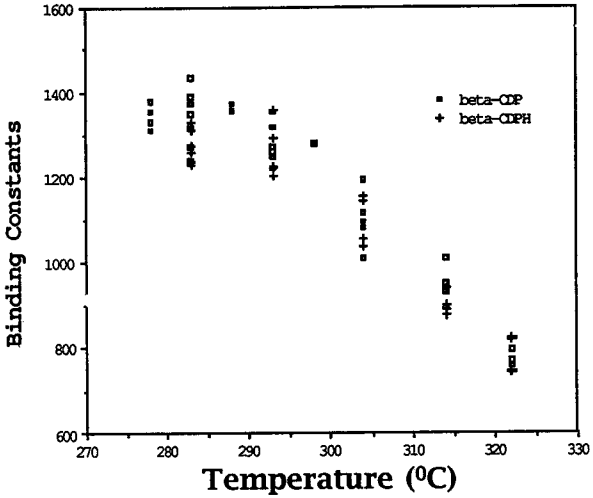


Table 4:
Fluorescence Intensity Data for PEG Experiment

Solution No.	[PEG] (M)	[ß-CDP] (M)	F
0	0	0	500
1	0	1.2×10^{-3}	208
2	1.2×10^{-3}	0	501
3	1.2×10^{-3}	1.2×10^{-3}	206
4	1.2×10^{-2}	1.2×10^{-3}	206
5	3.0×10^{-2}	1.2×10^{-3}	205

Figure 7:
2,6-MANS Fluorescence

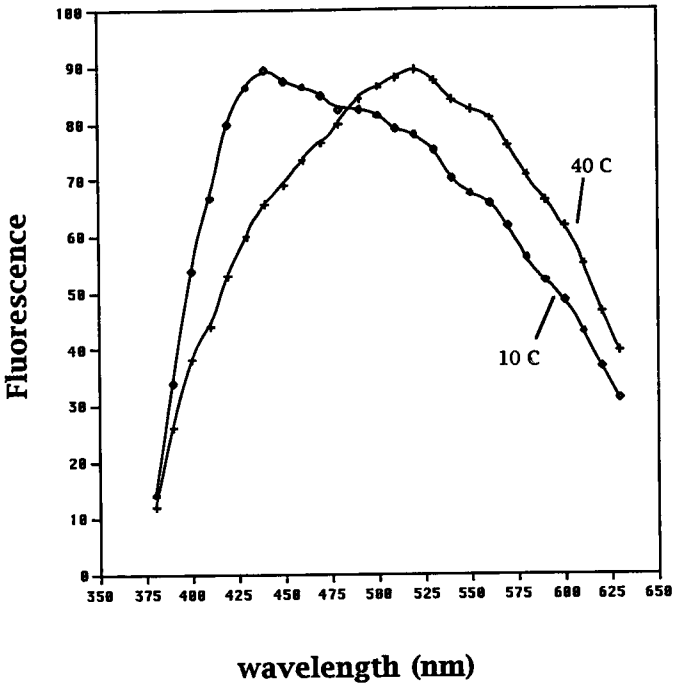
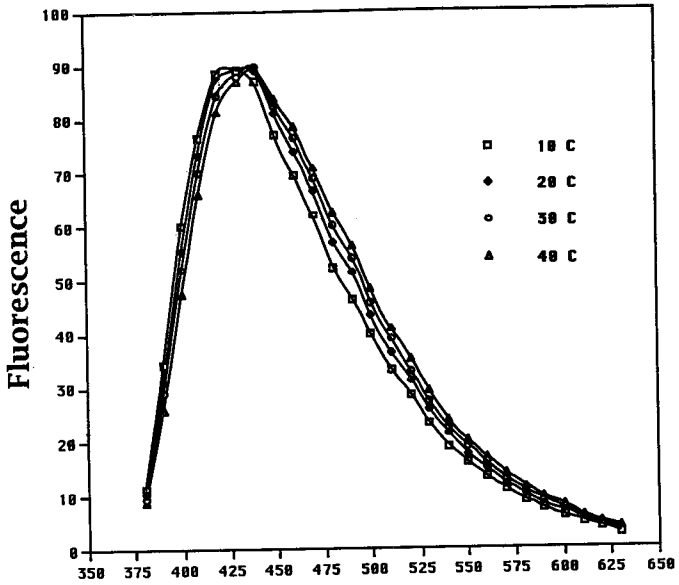


Figure 8:
2,6-MANS Complexed With beta-CDP



Discussion

All of the Stern-Volmer plots obtained during this work are similar to the one shown in Figure 3. The plots show the same high linearity as the lines in Figure 3 and have an intercept around 1. This supports our original assumption that the 2-AN:ß-CDP complex is indeed a 1:1 complex. It also means that the quenching is static in nature.

The average binding constants in Table 3 are in good agreement for the polymers at each temperature. The low molecular weight polymer has only one CD unit per chain, which makes it impossible for there to be any intrachain cooperative binding. Because the average binding constants are so similar for all three molecular weight polymers, we can conclude that there is no intrachain cooperative binding for the higher molecular weight polymers as well. This means that each CD unit on each chain is behaving similarly and independently.

The similarities in the binding constants, as expected, give us thermodynamic data for ß-CDP and ß-CDPH which are also in agreement, as shown in Table 3. We would also expect the same thermodynamic data for ß-CDPL, due to the similarity of its binding constants to those of the other two polymers.

The ΔS^0 values for both the monomer and polymer complexes are in relatively good agreement with one another within plus or minus one

standard deviation (see Table 3). The ΔH^p values, however, are slightly more favorable for the polymer complexes than for the monomer complex. This implies that the difference in binding is due to stronger binding interactions in the site. The linkers may affect the binding site of the CD molecule. For example, they may deepen the binding site by wrapping around the sides or the end of the fluorophore, thereby providing a more hydrophobic site for the fluorophore to bind.

Figure 6 shows a plot of the binding constants for the 2-AN:polymer complex versus temperature. Some points have been added at 5 and 15°C. We would expect the K value to show a continuous increase with decreasing temperature. This is not the case, however. Below 20°C the binding constants level off and even seem to go down instead of continuing to increase. The plots are still quite linear, however, which implies that a single binding constant value still characterizes binding.

One explanation for this occurrence may involve a competition between the linkers and 2-AN, which become significant at lower temperatures. The reactions would proceed as follows.

(4) A + P <----> AP

(5) L + P <----> LP

where A represents 2-AN, P represents CDP and L represents the linkers. Equation (4) shows the binding of the fluorophore with the polymer, and equation (5) shows the binding of the linkers with the

polymer. The binding constant expression for equation (4) is

$$(6) \qquad K = [AP]/[A][P]$$

This would be the true binding constant if there were no competition. To take into account competition by L at lower temperatures, we must introduce α_P which is defined in equation (7).

$$(7) \qquad \alpha_P = [P] / ([P] + [LP]) = [P] / C'_P$$

Equation (7) shows that α_P is a ratio of the concentration of free polymer to the sum of the concentrations of free and linker bound polymer (C'_p). We can substitute these new terms in for K.

$$(8) \qquad K = [AP] / \alpha_P C'_P [A]$$

What we have now is a new binding constant shown in equation (9).

$$(9) \qquad K' = \alpha_P K = [AP] / C'_P [A]$$

The new binding constant, K', will be less than K because α_P is less than one, whenever LP formation is significant. Hence, if the linkers are competing with the 2-AN for the binding site, we would see a lower binding constant than we would expect. We would still, however, see a single valued binding constant which is consistent with the high linearity of the Stern-Volmer plots at 10°C.

The PEG experiment was designed to determine if competition occurs. Table 4 shows the results of the PEG experiment. The important things to note are the fluorescence intensities of solutions 1, and 3 through 5. Solution 1 contains the given concentration of ß-CDP and has an intensity of 208. Solutions 3, 4, and 5 contain PEG in the

same concentration as ß-CDP, 10 times that amount, and 25 times that amount respectively. If there were any competition between PEG and 2-AN for the ß-CDP site, we should see an increase in intensity for each solution as more and more 2-AN is kicked out of the cavity and into the water. We do not see this, since fluorescence intensity remains the same for all four solutions. This does not necessarily rule out competition, however, it may simply mean that PEG is not a suitable model for the linker units on the polymer chains. Other models for the linkers may be explored in the future, such as polymerized epichlorohydrin. Epichlorohydrin may be a better linker model because it contains the repeating hydroxide unit that PEG does not.

Figures 7 and 8 show the results of the 2,6-MANS experiments. If there was some sort of conformational change in the binding site at lower temperatures, we would expect to see a shift in the fluorescence emission spectrum of the 2,6-MANS:ß-CDP complex as temperatures are lowered. Figure 7 shows that there is some shift in the fluorescence emission maximum for 2,6-MANS alone going from 10 to 40°C. The fluorescence maximum shifts from around 430 nm to around 525nm, almost a 100 nm shift. The fluorescence intensity of 2,6-MANS is relatively weak when it is free in solution. Figure 8 shows the spectra of 2,6-MANS in the presence of ß-CDP. There is very little shift in the fluorescence emission maxima over the same range of temperatures as Figure 7. There is only about a 20 nm shift in the wavelength over this

same range. This suggests that there is no significant conformational change in the binding site at lower temperatures.

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