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Calorimetric Studies of *p*-Nitrophenol Binding to α- and β-Cyclodextrin

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The thermodynamics of the binding of *p*-nitrophenol to both α- and β-cyclodextrin has been investigated as a function of pH and solvent composition using the technique of flow microcalorimetry. A preferential binding of the anionic form of the ligand is found for both cyclodextrins. It is postulated that the additional affinity for *p*-nitrophenolate is due to a dispersion interaction between the cyclodextrin cavity and the delocalized charge of the ligand. Studies of the binding of *p*-nitrophenol analogs and of the binding of *p*-nitrophenol as a function of ionic strength and DMSO cosolvent composition are consistent with this theory.

INTRODUCTION

Cyclodextrins (CDs, also known as cycloamyloses) have been extensively studied in recent years as models for enzyme-catalyzed reactions and for understanding the thermodynamics of ligand–macromolecule interactions (*1*, *2*). In addition CDs have been found to interact with a number of drugs (*3*), making these molecules potentially interesting from a pharmacalogical point of view. The driving force for the formation of inclusion complexes between CDs and various guest molecules has been postulated to involve the hydrophobic effect (*4*), van der Waals interactions (*5*), the release of high-energy molecules from the CD cavity (*2*, *6*), and/or the release of strain energy in the CD ring structure upon ligand binding (*7*).

The binding of *p*-nitrophenol to α-cyclodextrin (αCD, cyclohexaamylose) has been studied by Cramer *et al.* (*8*) and Bergeron and co-workers (*5*, *9*, *10*). It has been found that the anionic form of this ligand, *p*-nitrophenolate, binds approximately 10-fold more strongly than the neutral ligand. Also both forms of the ligand are found to fit into the αCD cavity nitro end first. Bergeron and co-workers have suggested that the preferential binding of the anion results from improved London dispersion interactions with this form of the ligand.

Below we will report studies of the thermodynamics of the binding of *p*-nitrophenol to αCD and β-cyclodextrin (βCD, cycloheptaamylose) as a function of pH and solvent composition. These studies were performed in an attempt to characterize the nature of the binding forces involved, particularly those forces responsible for the enhanced affinity of the anionic form of the ligand. The

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enthalpy change, $\Delta H°$, and free energy change, $\Delta G°$, for the ligand–CD interaction were measured by using flow microcalorimetry (*11*).

MATERIALS AND METHODS

α- and β-cyclodextrin were obtained from Sigma Chemical Company and used without further purification. *p*-Nitrophenol was recrystallized from benzene. All other compounds were obtained commercially and used without purification.

A LKB flow microcalorimeter was used to determine the ligand–CD association constant, K, and $\Delta H°$ of interaction. A thorough description of this methodology has been given by Biltonen and Langerman (*11*). The heat effect, q, observed upon mixing the reactant solutions together is given by the equation (corrections for the heat of dilution of reactants being made when necessary)

$$q = \Delta H°' f[\mathrm{C}], \qquad [1]$$

where f is the total flow rate through the calorimetric cell, [C] is the concentration of ligand–CD complex formed in the calorimetric cell, and $\Delta H°'$ is the apparent enthalpy change for the reaction. For an association reaction between two reactants, A and B, the concentration of the complex, C, formed is given by the expression (when the concentration of reactant B is held at a constant level, lower than that of A; $[\mathrm{B}]_0$ in this expression is the total concentration of B)

$$[\mathrm{C}] = \frac{K[\mathrm{A}][\mathrm{B}]_0}{1 + K[\mathrm{A}]}. \qquad [2]$$

On combining Eqs. [1] and [2], one obtains

$$q = \frac{K[\mathrm{A}][\mathrm{B}]_0 f \Delta H°'}{1 + K[\mathrm{A}]}. \qquad [3]$$

In an experiment in which [A] is varied and [B] is held at a low level, both K and $\Delta H°'$ can be determined from a plot of $1/q$ vs $1/[\mathrm{A}]$, where [A] is the concentration of free A ($[\mathrm{A}] = [\mathrm{A}]_0 - q/f\Delta H°'$; an initial estimate of $\Delta H°'$ being obtained from a plot of $1/q$ vs $1/[\mathrm{A}]_0$). Experiments were usually performed by varying the concentration of the quest molecule (from $\sim 1.5 \times 10^{-2}\ M$ to $\sim 4 \times 10^{-4}\ M$) and holding the CD concentration constant (at $\sim 5 \times 10^{-4}\ M$). Occasionally the procedure was reversed, with CD being varied. This was done in cases in which the solubility of the guest molecule is low. In test studies (see Fig. 1) we obtained the same $\Delta G°$ and $\Delta H°$ values (within 10%) by varying either reactant. The fact that the same $\Delta H°$ value is obtained in each manner also indicates that a one to one complex is formed.

If there is a release or uptake of protons from either reactant upon binding, the experimental heat of reaction, $\Delta H°'$, will contain a contribution from the heat of protonation of the buffer, ΔH_B, according to

$$\Delta H°' = \Delta H° + \Delta N\, \Delta H_B, \qquad [4]$$

where ΔN is the number of protons released per mole of complex formed and $\Delta H°$ is the molar enthalpy change for complex formation. ΔH_B will depend on the solution composition (*11*, *17*), as given by

$$\Delta H_B = \frac{\sum_i \beta_i \Delta H_i}{\sum_i \beta_i}, \qquad [5]$$

where ΔH_i is the heat of protonation of buffer component i and β_i is the function of the concentration of component i, C_i, and the ratio of the hydrogen ion concentration to the proton dissociation constant, K_i, of buffer component i:

$$\beta_i = \frac{C_i K_i/[H^+]}{(K_i/[H^+] + 1)^2}. \qquad [6]$$

Normally there will be one primary buffer component and the above equations will simplify greatly. In the present studies we have the complication that the ligand, *p*-nitrophenol, can serve as a buffering species. In experiments in which the concentration of *p*-nitrophenol is varied, this leads to a variation of ΔH_B (and hence $\Delta H°'$) with concentration. As a result of the concentration dependence of $\Delta H°'$, plots of $1/q$ vs $1/[L]$ will usually lead to an overestimate of $-\Delta H°'$ and an underestimate of K, if this effect is not realized. We have included this concentration dependence of $\Delta H°'$ in our analysis by use of the equation (where q is now the heat effect per mole of limiting reactant)

$$q = \frac{f K[L](\Delta H° + \Delta N \Delta H_B)}{1 + K[L]}, \qquad [7]$$

which upon rearrangement gives

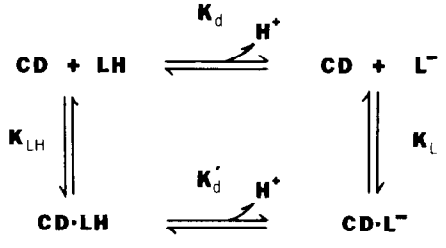
$$\left(q - \frac{f K[L]\Delta N \Delta H_B}{1 + K[L]}\right)^{-1} = \frac{1}{f K \Delta H°[L]} + \frac{1}{f \Delta H°}, \qquad [8]$$

where ΔH_B in the above equations is given by Eq. [5] and is a function of [L]. We have used Eq. [8] to obtain K and $\Delta H°$ in the following way: first an initial estimate of K and $\Delta H°'$ was obtained from a plot of $1/q$ vs $1/[L]_0$; second the free ligand concentration, [L], was calculated from $[L]_0$ using this value of $\Delta H°'$; third, ΔH_B was calculated as function of [L]; fourth, ΔN was calculated from the pK_a of *p*-nitrophenol and $\Delta\Delta G°$ (defined below); fifth, the above information was used to calculate the left-hand side of Eq. [8]. which was then plotted vs $1/[L]$ to obtain a second determination of K and $\Delta H°$; sixth, using the new K and $\Delta H°$ values, steps 2 through 5 can be repeated to further refine these values (we find repeating these steps to give no significant improvement).

The pK_a of *p*-nitrophenol and phenol at 25°C was determined by potentiometric titration using a London PHM 4 pH meter and a micrometer syringe. The heat of protonation of *p*-nitrophenol at 25°C, 0.1 *M* ionic strength, was determined calorimetrically.

RESULTS

Figure 1 illustrates the pH dependence of $\Delta G°$ and $\Delta H°$ for the binding of *p*-nitrophenol to both αCD and βCD. The inflection near pH 7 in each plot is due to the proton dissociation of *p*-nitrophenol. For both αCD and βCD preferential binding of the anionic form of the ligand is observed, with a more negative $\Delta\Delta G°$ ($\Delta G°$ for anion binding minus $\Delta G°$ for neutral ligand binding) seen for αCD ($\Delta\Delta G°$ = −1.35 and −0.9 kcal/mol for αCD and βCD, respectively). The $\Delta H°$ values are also found to vary with pH. The $\Delta H°$ for the binding of *p*-nitrophenol (low pH region) to both α- and βCD is found to be approximately −4.5 kcal/mol. For *p*-nitrophenolate, on the other hand (high pH region), the $\Delta H°$ for binding to αCD is −9.3 kcal/mol, while that for βCD remains at approximately −4.0 kcal/mol. The exact shape of the $\Delta H°$ vs pH curves, including the dip seen in the data for βCD, can be described by the following thermodynamic scheme, which includes the coupled proton dissociation of *p*-nitrophenol (in this scheme LH is the neutral *p*-nitrophenol molecule, L^- is *p*-nitrophenolate, K_{LH} and K_L are the respective association constants of these ligand forms, K_d is the acid dissociation constant of *p*-nitrophenol, and K'_d is the acid dissociation constant of the CD–*p*-nitrophenol complex).



According to this scheme the apparent $\Delta G°$ and $\Delta H°$ for *p*-nitrophenol binding will be given by

$$\Delta G° \text{ (app)} = \Delta G°_{LH} - RT \ln \left(\frac{1 + K'_d/[H^+]}{1 + K_d/[H^+]} \right), \quad [9]$$

$$\Delta H° \text{ (app)} = \Delta H°_{LH} + \frac{K(\Delta H°_L - \Delta H°_d - \Delta H°_{LH})}{[H^+] + K'_d} + \frac{K_d \Delta H°_d}{[H^+] + K_d}, \quad [10]$$

where K'_d is equal to $K_L K_d / K_{LH}$, $\Delta H°_{LH}$ and $\Delta H°_L$ are the enthalpy change for the binding of the neutral and anionic ligand forms, and $\Delta H°_d$ is the heat of protonation of *p*-nitrophenol. The solid lines in Fig. 1 are theoretical fits of the above equations to the data.

The fact that the experimental thermodynamic data can be described in a straightforward fashion in terms of linked binding processes is satisfying, but this does not explain why the anion binds preferentially to the CD cavity, or why the $\Delta\Delta G°$ and $\Delta\Delta H°$ are more negative for the binding to αCD than to βCD. In an attempt to characterize the nature of the additional binding force operating between αCD and *p*-nitrophenolate we studied the effect of ionic strength and

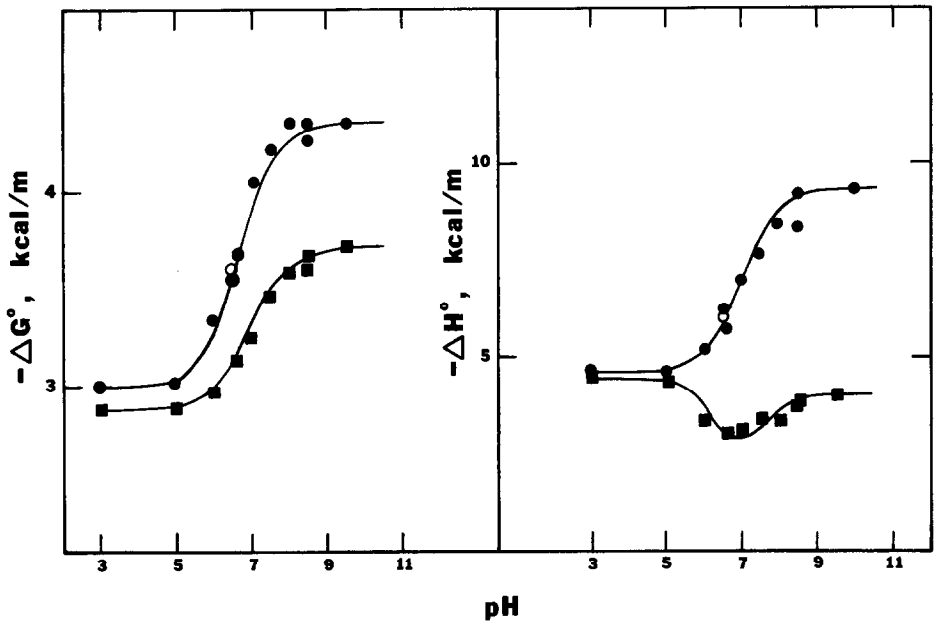


FIG. 1. The pH dependence of the free energy change and the enthalpy change for the binding of *p*-nitrophenol to α-cyclodextrin (●) and β-cyclodextrin (■). The $\Delta H°$ values are corrected for the heat of protonation of the buffer as described under Materials and Methods. The buffers used and their heats of protonation are as follows: Na^+ formate (pH 3–4), $\Delta H = 0$; Na^+ acetate (pH 5), $\Delta H = 0$; Na^+ cacodylate (pH 6–7), $\Delta H = -1.2$ kcal/mol; Tris (pH 7.5–8.5), $\Delta H = -11.3$ kcal/mol; glycine (pH 9.5), $\Delta H = -10.5$ kcal/mol. The solid lines are theoretical fits of Eqs. [9] and [10] to the data using the following fitting parameters: for αCD, $\Delta G°_{LH} = -3.0$ kcal/mol, $\Delta G°_{L} = -4.35$ kcal/mol, $\Delta H°_{LH} = -4.6$ kcal/mol, and $\Delta H°_{L} = -9.3$ kcal/mol; for βCD, $\Delta G°_{LH} -2.9$ kcal/mol, $\Delta G°_{L} = -3.7$ kcal/mol, $\Delta H°_{LH} = -4.5$ kcal/mol, and $\Delta H°_{L} = -4.0$ kcal/mol. Additional experimental (independently determined) data necessary for the fit are $K_d = 7.1 \times 10^{-8}$ *M*, and $\Delta H°_{d}$ (heat of protonation) $= -4.0$ kcal/mol.

additional organic cosolvent on the thermodynamics of binding. On increasing the ionic strength from 0.1 to 1.0 *M*, the $\Delta G°$ and $\Delta H°$ for binding were found to remain approximately the same (see Table 1), indicating that an electrostatic interaction between the CD and the anionic ligand is not important (such an interaction is not expected, of course, since the CD is uncharged). With dimethyl sulfoxide (DMSO) as cosolvent the $\Delta\Delta G°$ and $\Delta\Delta H°$ for *p*-nitrophenol binding to αCD are both found to become less negative, with $\Delta\Delta G°$ approaching zero at 50% DMSO, as shown in Fig. 2. Comments on the DMSO solvent effect will be given below.

The binding of several analogs of *p*-nitrophenol, both in their neutral and anionic form, have also been studied, as reported in Table 1.

DISCUSSION

The complexes formed between CDs and ligands such as *p*-nitrophenol have

TABLE 1

THERMODYNAMIC VALUES FOR THE BINDING OF SELECTED LIGANDS TO α-CYCLODEXTRIN[a]

Ligand	(kcal/mol)			
	$\Delta G°$	$\Delta\Delta G°$	$\Delta H°$	$\Delta\Delta H°$
p-Nitrophenol[b]	−3.0		−4.6	
		−1.35		−4.7
Na^+ *p*-nitrophenolate[b]	−4.35		−9.3	
p-nitrophenol, 1 *M* NaCl[c]	−3.45		−4.6	
		−1.0		−5.0
Na^+ *p*-nitrophenolate, 1 *M* NaCl[d]	−4.45		−9.6	
p-Cyanophenol[c]	−2.7		−4.6	
		−1.1		−1.4
Na^+ *p*-cyanophenolate[d]	−3.8		−6.0	
p-Nitrobenzoic acid[e]	−3.1		−9.2	
		0.9		4.4
Na^+ *p*-nitrobenzoate[f]	−2.2		−4.8	
p-Nitrophenyl acetic acid[e]	−2.3		−9.6	
		−0.2		4.6
Na^+ *p*-nitrophenyl acetate[g]	−2.5		−5.0	

[a] All values at 25°C, 0.1 *M* ionic strength, unless otherwise stated.
[b] See Fig. 1.
[c] Formate buffer, pH 4.0.
[d] Glycine buffer, pH 10.0.
[e] Glycine buffer, pH 2.0, αCD varied.
[f] Acetate buffer, pH 5.5.
[g] Phosphate buffer, pH 7.0.

been widely studied as models for protein–ligand interactions, and the nature of the driving force for the formation of such CD inclusion complexes has been a matter of much discussion (*2, 5–7, 12, 13*). The release of configurational strain in the CD ring has been proposed to be a major driving force for complex formation (*7*). However, conformational energy calculations by Tabushi *et al.* (*12*) and experimental studies of Bergeron and Meeley (*14*) do not support this contention. Also the soundness of this proposal is questionable. An induced change in the CD conformation upon ligand binding must be at an expense in free energy and cannot be a source of driving force (*15*). The CD ring in solution will exist in its most stable state (be it distorted or not). If the ligand binds preferentially to some altered state of the CD ring, then the (positive) free energy change for this induced transition (and the associated ΔH and ΔS for the transition) will contribute to the apparent thermodynamic parameters for ligand binding. The real driving force, however, will be the intrinsic interaction between the ligand and the altered state of the CD.

These intrinsic binding forces may include the release of high-enthalpy cavity

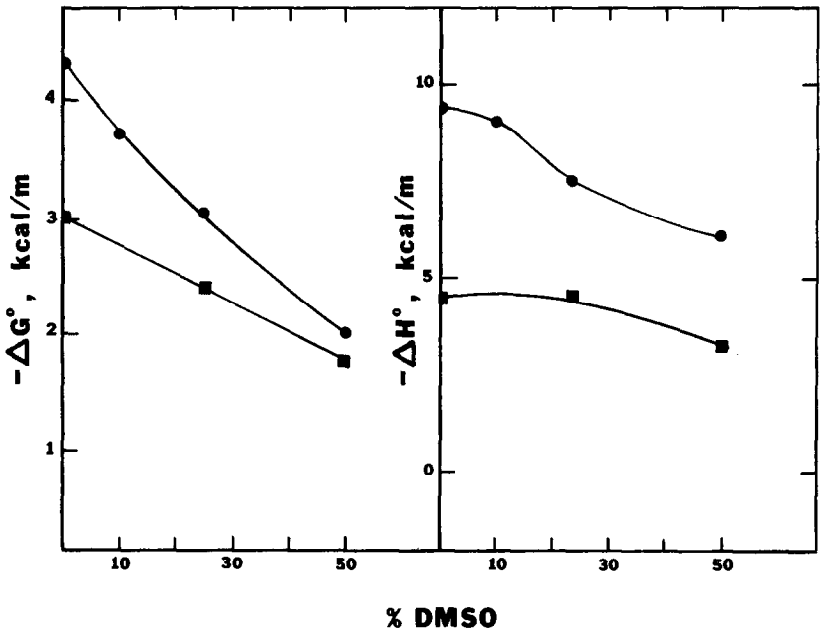


FIG. 2. The dependence of the free energy change and enthalpy change for the αCD–*p*-nitrophenol interaction on the weight percentage of the organic cosolvent dimethyl sulfoxide. The binding of *p*-nitrophenolate (●) was studied at pH 9.5, 0.05 *M* glycine buffer. At this pH the ligand is at least 95% in the anionic form at all solvent compositions. The binding of *p*-nitrophenol (■) was studied at pH 4.0, 0.1 *M* formate buffer.

water (*2, 6*), the hydrophobic effect (*2, 4*), and van der Waals/London dispersion interactions (*5, 12*). Water molecules within the CD cavity are thought to be unstable (high in enthalpy) due to their inability to satisfactorily hydrogen bond with other water molecules or with groups lining the cavity. The displacement of cavity water by a ligand could thus result in a favorable drop in enthalpy. Such a driving force would be expected to be relatively nonspecific, operating for all ligands able to enter the cavity and displace the water molecules.

For relatively apolar ligands the release of clustered water molecules from around the ligand upon complex formation, the so-called hydrophobic effect, is expected to be a favorable binding force, with an associated positive entropy change (and near zero enthalpy change). Again this driving force is expected to be relatively nonspecific and could be important for many ligands. CD–ligand complex formation is, however, typically found to be enthalpy driven rather than entropy driven. This suggests that while the hydrophobic effect may be important, the $\Delta H°$ and $\Delta S°$ for complex formation are dominated by contributions from other intrinsic binding forces.

In addition to the above-mentioned release of cavity water, other binding forces that can give rise to a negative $\Delta H°$ are van der Waals/London dispersion forces. These forces are also most likely to provide a basis for specificity in ligand

binding, with complementary (snug fitting) CD–ligand complexes being the most stabilized.

In the present work we have made a comparative study of the thermodynamics of the interaction of the neutral and anionic forms of the ligand *p*-nitrophenol to both α- and βCD. Making such a comparative study allows us to focus on the difference in the way the two forms of the ligand bind to the host, as well as the effect of changing the size of the cavity on the thermodynamics of binding. As the data presented above indicate, there is a preferential binding of the anionic form of the ligand to both CDs, with the selectivity being greater for αCD. For both the neutral and anionic forms of the ligand one would expect contributions to the binding energy from the release of cavity water, the hydrophobic effect, and van der Waals forces. The fact that the $\Delta G°$ for the binding of *p*-nitrophenol is approximately the same for both α- and βCD indicates that—at least for the neutral form of the ligand—the size of the cavity has little bearing on the sum of the above binding forces. The similarity of the $\Delta H°$ values for the binding to both CDs also suggests that the elementary binding forces are the same for the two hosts. It should, however, be realized that the similarity of the $\Delta H°$ values may arise due to a compensation between the van der Waals interactions (weaker for more loosely fitting βCD cavity) and the displacement of enthalpy rich water molecules (more such molecules to be displaced from the larger cavity of βCD).

The preferential binding of *p*-nitrophenolate to both CDs must be the result of the existence of some binding force in addition to those mentioned above. In agreement with Bergeron and co-workers (*5, 10*), we postulate that the improved binding of *p*-nitrophenolate is due to the added contribution of London dispersion interactions between the CD cavity and the delocalized charge of the anionic ligand. This explanation is similar to the argument presented by Grunwald and Price (*16*) in interpreting the effect of solvent on the pK_a of picric acid. The formal negative charge on the oxyanion of *p*-nitrophenolate will be delocalized about the aromatic ring, with charge density building up in particular on the *para* nitro group. Interaction between the delocalized charge of the anion and groups lining the CD cavity (i.e., monopole–dipole and monopole-induced dipole interactions) will lead to an additional binding force not found for the neutral ligand. Such a dispersion interaction, although expected to give rise to a negative contribution to the $\Delta H°$ of binding, would be expected to be a significant driving force for binding only if the interaction with the CD cavity is stronger than that with the aqueous solvent. It is reasonable for this to be the case since the CD cavity is lined with polar hydroxyl groups and is more densely packed than liquid water. On increasing the size of the CD cavity one would expect such a dispersion force to be weakened, consistent with our observation that *p*-nitrophenolate has less affinity for βCD than for αCD.

To test the above hypothesis we have studied the binding of several analogs of *p*-nitrophenol, both in their neutral and anionic forms, to αCD. One of these ligands was *p*-nitrophenyl acetic acid. Like *p*-nitrophenol, the anionic form of this ligand has a negative charge *para* to the nitro group; however, the charge is not delocalized about the aromatic ring. As seen in Table 1, the $\Delta G°$ for the binding of the anionic form of *p*-nitrophenyl acetic acid to αCD is not significantly different

from that for the neutral ligand.[1] Another ligand studied that can experience charge delocalization when ionized is *p*-cyanophenol. The anionic form of this ligand was also found to bind preferentially to αCD, with a $\Delta\Delta H°$ being negative as well. Studies of the binding of *p*-cresol, in its neutral and anionic form, were also attempted. However, due to small values for the association constant of both forms of this ligand ($K < 50\ M^{-1}$ at both pH 4 and 10.5) we can only conclude that there appears to be no significant binding preference for the anionic form of this ligand. This is as expected since the *para* methyl group of *p*-cresol is electron donating and would tend to oppose the delocalization of charge around the aromatic ring. These studies with analogs of *p*-nitrophenol are thus consistent with the above postulate that dispersion interactions between the CD cavity and the anionic form of the ligand are responsible for the preferential binding of the latter.

The above-mentioned studies with DMSO–water mixtures are also consistent with this explanation. The $\Delta\Delta G°$ for *p*-nitrophenol binding to αCD approaches zero as the percentage of DMSO increases. This suggests that the additional dispersion force between the delocalized charge of *p*-nitrophenolate and the αCD cavity makes less of a contribution to the overall binding constant in the DMSO–water mixtures. For this to be the case, similar (canceling) dispersion interactions must occur between *p*-nitrophenolate and the mixed solvent. As discussed by Grunwald and Price (*16*), the strength of such solute–solvent dispersion interactions is expected to increase as the size of the solvent molecules increases. This is thought to be due to the fact that atoms of the solvent, when held together by covalent bonds, will form a more closely packed surface with which the solute can interact. For smaller solvent molecules there will be more space between nonbonded atoms, thus providing a less well packed solvent cage around the solute. Independent evidence for the existence of favorable dispersion interactions between *p*-nitrophenolate and the DMSO–water mixture comes from a study of the effect of the added DMSO on the pK_a of *p*-nitrophenol and phenol. In water the former is a 5.5-fold stronger acid than the latter but is a 40-fold stronger acid in

[1] The binding of *p*-nitrobenzoic acid was also studied. The neutral form of this ligand is found to bind five-fold better to αCD than the anionic form, a binding preference opposite of that for *p*-nitrophenol. The anionic form of *p*-nitrobenzoic acid may experience some charge delocalization; however, there is reason to believe that this ligand (particularly its neutral form) may not bind to αCD is the same manner as *p*-nitrophenol. Whereas *p*-nitrophenol is found to penetrate the αCD cavity nitro end first, Bergeron *et al.* (*18*) have presented evidence that benzoic acid (an analog of *p*-nitrobenzoic acid) penetrates the αCD cavity carboxyl group first (others, however, have suggested the opposite orientation for bound benzoic acid (*19*)). If the carboxyl group of *p*-nitrobenzoic acid does in fact bind first, this means than an inclusion complex having the nitro end penetrating the cavity is a less stable state. The possible difference in the mode of binding should be kept in mind when comparing the binding of *p*-nitrobenzoic acid and *p*-nitrophenol.

We would also like to add at this point that we find nitrobenzene to be able to bind to αCD with $\Delta G° = -3.1$ kcal/mol and $\Delta H° = -1.1$ kcal/mol at pH 4 (similar values found at pH 10 also). The binding constant for nitrobenzene is thus similar to that for the neutral *p*-nitrophenol species. This suggests that the nitro group is of primary importance in determining the binding of these ligands and that the direct formation of a hydrogen bond between the hydroxyl group of *p*-nitrophenol and the 2- or 3-hydroxyl groups of αCD does not contribute significantly to the binding.

50% DMSO–water. This result suggests that the DMSO mixture stabilizes the anionic form of *p*-nitrophenol (relative to its ability to stabilize phenolate) by a dispersion interaction. Thus the effect of DMSO on the *p*-nitrophenol–αCD interaction can be understood in terms of a balance between the ability of *p*-nitrophenolate to interact with the αCD cavity and with the solvent environment.

CONCLUSION

The preferential binding of the anionic form of *p*-nitrophenol to CDs has been attributed to the existence of a dispersion interaction between the CD cavity and the delocalized charge of the anion. Solvent dependence studies as well as studies of the binding of analogs of *p*-nitrophenol are consistent with this explanation. If such a dispersion interaction involving a ligand with a delocalized charge is of major importance in a model system such as this, one might expect such an interaction force to also possibly play a role in the binding of certain ligands to biological macromolecules, if the ligands have delocalized charges.

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