The Effects of Buffers on the Thermodynamics and Kinetics of Binding between Positively-Charged Cyclodextrins and Phosphate Ester Guests

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Cyclodextrin derivatives in which the primary hydroxyl groups at the 6-positions of the sugar units have been replaced with 2-hydroxethylamino groups are known to bind phosphate esters with significant affinity. Association between the host and guest is mediated by a combination of hydrophobic and electrostatic interactions. Association constants for two cyclodextrin hosts with three phosphate ester guests are measured in a variety of buffer solutions, and it is found that the buffer has a large impact on the electrostatic component of the binding interaction. Negatively charged buffers such as phosphate and ADA (*N*-(2-acetamido)iminodiacetic acid) compete with the phosphate ester guests for binding to the positively charged cyclodextrins. This competition lowers the effective association constant between host and guest. Positively charged buffers such as Tris-HCl do not strongly compete and are thus more conducive to binding. The kinetics of dissociation of the host−guest complexes were measured in several buffers using ^{31}P EXSY experiments. These measurements demonstrate that negatively charged buffers decrease the equilibrium constant by lowering the rate of association of these complexes but in most cases do not effect the dissociation rates.

Introduction

In aqueous solution, cyclodextrins bind hydrophobic molecules in the interior of their cavities. The synthesis of modified cyclodextrins has expanded their recognition properties to include noncovalent binding motifs beyond simple hydrophobic interactions.[1] For example, the binding properties of a large number of modified cyclodextrins have been investigated that incorporate hydrogen bonding, electrostatic, and metal binding interactions.[2] Aminocyclodextrins (AminoCDs) are cyclodextrin derivatives that incorporate amino-containing functionality in place of the primary hydroxyl groups at the 6-positions of the sugar units. Such aminoCDs were first demonstrated to bind to phosphate esters such as benzyl phosphate through a combination of hydrophobic and electrostatic interactions by Knowles and Boger in 1979.[3] Since that time, a variety of aminoCDs have been developed that recognize phosphate esters,[4,5] including several with biological significance such as nucleotide mono- and triphosphates[6,7] and phosphotyrosine.[8] We have recently demonstrated that the binding interactions between phosphotyrosine and aminoCDs can be used to inhibit hydrolysis of the phosphate ester linkage in phosphotyrosine catalyzed by phosphatase enzymes.[9] Additionally, the investigations of Thatcher and co-workers have shown that aminoCDs bind to glycosaminoglycan sulfates and inhibit growth of neurites.[10] In vitro biological studies of this type typically require the use of solutions that contain buffers, and buffers are likely to mediate the strength of the electrostatic binding interactions between aminoCDs and negatively charged guest molecules. Therefore, the goal of the present study is to clarify the role that buffer structure and concentration plays in determining both the thermodynamics and kinetics of association between aminoCD hosts and phosphate ester guests. From a broader perspective, these studies provide a model system to study the effects of buffers on binding interactions, including small molecule−protein and protein−protein interactions, that occur in aqueous solution and that are mediated by electrostatic forces.

Results and Discussion

Cyclodextrin Hosts and Phosphate Ester Guests. Figure 1 shows the structures of compounds **1**β and **2**α, the two aminoCD hosts that are used in this study. The syntheses of these compounds has been reported by Darcy[7] and Thatcher,[11] respectively. Three aryl phosphates were used as guests; *p*-nitrophenyl phosphate (*p*-NPP), phosphotyrosine (Tyr(PO_3^{2-})), and *N*-acetyl-phosphotyrosine methyl ester (AcTyr(PO_3^{2-})OMe). The latter

(1) For a recent series of comprehensive reviews on all aspects of cyclodextrin chemistry, see: *Chem. Rev.* **1998**, *98*(5).

(2) For several recent examples of modified cyclodextrins binding to amino acids, see: (a) Bonomo, R. P.; Pedotti, S.; Vecchio, G.; Rizzarelli, E. *Inorg. Chem.* **1996**, *35*, 6873. (b) Liu, Y.; Zhang, Y.-M.; Qi, A.-D.; Chen, R.-T.; Yamamoto, K.; Wada, T.; Inoue, Y. *J. Org. Chem.* **1997**, *62*, 1826. (c) Liu, Y.; Zhang, Y.-M.; Qi, A.-D.; Chen, R.-T.; Yamamoto, K.; Wada, T.; Inoue, Y. *J. Org. Chem.* **1998**, *63*, 10085.

(3) (a) Boger, J.; Knowles, J. R. *J. Am. Chem. Soc.* **1979**, *101*, 7631. (b) Boger, J.; Brenner, D. G.; Knowles, J. R. *J. Am. Chem. Soc.* **1979**, *101*, 7630.

(4) Vizitiu, D.; Thatcher, G. R. J. *J. Org. Chem.* **1999**, *64*, 6235.

(5) Breslow, R.; Schmuck, C. *J. Am. Chem. Soc.* **1996**, *118*, 6601.

(6) (a) Eliseev, A. V.; Schneider, H.-J. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1331. (b) Eliseev, A. V.; Schneider, H.-J. *J. Am. Chem. Soc.* **1994**, *116*, 6081.

(7) (a) Schwinte, P.; Darcy, R.; O′Keeffe, F. *J. Chem. Soc., Perkin Trans. 2* **1998**, 805. (b) Ahern, C.; Darcy, R.; O′Keeffe, F.; Schwinte, P. *J. Incl. Phenom. Mol Recog. Chem.* **1996**, *25*, 43.

(8) Cotner, E. S.; Smith, P. J. *J. Org. Chem.* **1998**, *63*, 1737.

(9) Ghosh, M.; Sanders, T. C.; Zhang, R.; Seto, C. T. *Org. Lett.* **1999**, *1*, 1945.

(10) Borrajo, A. M. P.; Gorin, B. I.; Dostaler, S. M.; Riopelle, R. J.; Thatcher, G. R. J. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1185.

(11) Vizitiu, D.; Walkinshaw, C. S.; Borin, B. I.; Thatcher, G. R. J. *J. Org. Chem.* **1997**, *62*, 8760.

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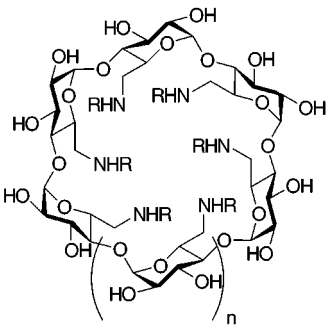
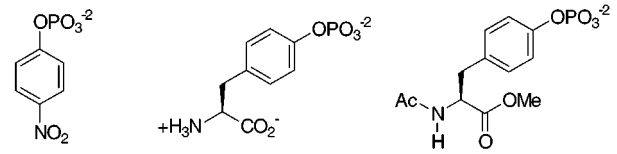


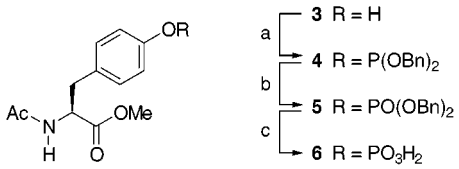
1β n = 2, R = $(CH_2)_2OH$
2α n = 1, R = $(CH_2)_2OH$



p-NPP Tyr(PO_3^{-2}) AcTyr(PO_3^{-2})OMe

Figure 1. Structures of positively charged cyclodextrin hosts and aryl phosphate guests.

Scheme 1[a]

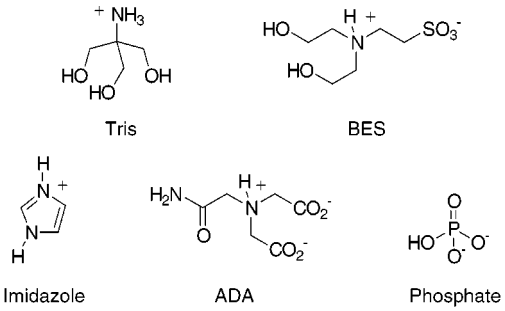


[a] Reagents and conditions: (a) $(BnO)_2PNEt_2$, tetrazole; (b) *m*-CPBA, 0 °C; (c) 5% Pd/C, H_2, MeOH.

compound was included in the study to determine if blocking the charged ammonium carboxylate portion of phosphotyrosine causes enhanced affinity to the cyclodextrin hosts. The synthesis of AcTyr(PO_3^{2-})OMe (**6**) is outlined in Scheme 1. *N*-Acetyltyrosine methyl ester was phosphorylated with dibenzyl-*N*,*N*-diethylphosphoramidite to give phosphite **4**. This compound was oxidized with *m*-CPBA to give the corresponding phosphate triester **5**. Finally, removal of the benzyl protecting groups with hydrogen and Pd/C gave the desired phosphate monoester **6**.

Measurement of Association Constants by NMR Spectroscopy. A variety of experimental methods have been used to measure association constants of cyclodextrins with guest molecules. These include UV and CD spectroscopies, calorimetry, potentiometric titration, and NMR spectroscopy. Potentiometric titration is one of the most common methods for investigating the binding interactions between positively charged cyclodextrins and phosphate esters.[6,7] This technique is useful because it provides information about both association constants and pK_a's of ionizable groups. However, potentiometric titration measurements must be performed in the absence of buffer, and buffers are typically required when investigating in vitro biological activity. Thus, association constants measured using this method may not accurately predict what will occur in biological systems.

Several reports show that nucleotide phosphates bind to positively charged cyclodextrins with significant affinity ($K_a \sim 10^4-10^5$ M^{-1}). These values were measured using potentiometric titration in nonbuffered aqueous NaCl solution.[6,7] Knowles has reported that benzyl phosphate binds to a positively charged cyclodextrin in triethanolamine buffer with similar affinity ($K_a \sim 3.2 \times 10^4$ M^{-1}).[3] In contrast, Breslow and Smith have shown that binding of aryl phosphates to positively charged cyclodextrins in *phosphate buffer* is somewhat weaker ($K_a \sim 10^2-10^3$ M^{-1}).[5,8] The 2- to 3-order of magnitude difference between these reported binding constants may simply reflect the differences in structures between the various cyclodextrin hosts and phosphate ester guests used in the studies. However, since electrostatic interactions are important in determining the strength of binding in all of these systems, it is also possible that the aqueous environment, specifically the buffer, plays a significant role in determining the association constants. Negatively charged buffers may have reasonable affinities for the positively charged cyclodextrins and effectively compete with a phosphate ester guest for binding with the cyclodextrin. This competition would have the effect of lowering the observed binding constant between host and guest.



Tris BES Imidazole ADA Phosphate

Figure 2. Structures of buffers.

To investigate this issue, we have measured the association constants of cyclodextrins **1**β and **2**α with three aryl phosphates in five different buffer systems in order to clarify the role that buffer structure and concentration plays in determining the magnitude of the binding interactions. Association constants were measured using NMR spectroscopy because this method is compatible with a variety of aqueous solutions. The structures of the buffers used in this study are shown in Figure 2. These buffers encompass a range of functional groups, sizes, and charge states that vary from −2 for phosphate at pH 7 to +1 for imidazole and Tris.

Three NMR methods were used to measure the association constants: ^{1}H NMR titrations and ^{1}H and ^{31}P NMR dilution experiments. We chose a particular experiment for a given complex based upon two considerations; signal dispersion of the aromatic protons of the guest and the rate of exchange between the free and bound guest. In general, complexes with *p*-NPP as the guest were in fast exchange on the NMR time scale, while complexes with Tyr(PO_3^{2-}) and AcTyr(PO_3^{2-})OMe were in the slow exchange regime. For complexes that displayed good signal dispersion and that were in fast exchange on the NMR time scale, the ^{1}H NMR titration method was used. In these experiments, the chemical shift of the aryl phosphate protons were monitored as a function of added cyclodextrin. Data were analyzed using a nonlinear curve fitting procedure to an equation, reported by Wilcox, that is based upon a simple 1:1 binding isotherm.[12] An

Table 1. Association Constants for Cyclodextrins 1β and 2α with Aryl Phosphates in a Variety of Buffers[a]

		K_{assoc} (M^{-1})						
CD	guest	Tris	imidazole	D_2O, NaCl	BES	ADA	10 mM HPO_4^{-2}	100 mM HPO_4^{-2}
1β	*p*-NPP	60 000[b] ± 15 000	210 000[b] ± 60 000	65 000[b] ± 13 000	34 000[b] ± 8000	2700[b] ± 600	2200[b] ±400	410[b] ± 80
1β	Tyr(PO_3^{2-})	130 000[c] ± 25 000			16 000[c] ±3000	1500[d] ± 300		710[d] ± 140
1β	AcTyr(PO_3^{2-})OMe	610 000[c] ± 120 000		430 000[c] ± 90 000				
2α	*p*-NPP	30 000[b] ± 6000		70 000[b] ± 10 000				370[d] ± 80
2α	Tyr(PO_3^{2-})	42 000[c] ± 8000						
2α	AcTyr(PO_3^{2-})OMe	150 000[c] ± 30 000		33 000[c] ± 6000				

[a] Buffer concentrations are 100 mM unless noted otherwise. The pH of all solutions was 7.0. [b] Measured by ^{1}H NMR titration experiments. [c] Measured ^{1}H NMR dilution experiments. [d] Measured by ^{31}P NMR dilution experiments.

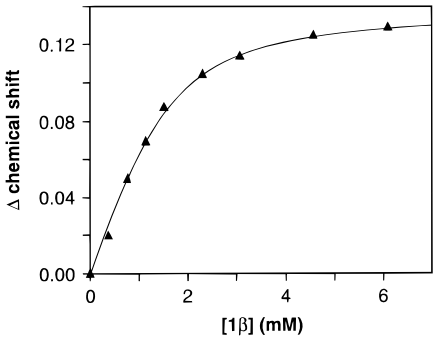


Figure 3. Titration of *p*-NPP (1.5 mM) with cyclodextrin **1**β in 100 mM ADA buffer as monitored by ^{1}H NMR spectroscopy. The curve corresponds to the best fit of the data to a simple 1:1 binding isotherm.

example of one data set, along with the curve fit, is shown in Figure 3.

Complexes that were in slow exchange on the NMR time scale showed well-resolved signals for the free and bound guest. Integration of these resonances gave a measure of the relative concentrations of free and bound aryl phosphate. In these cases, an NMR sample containing aryl phosphate and a slight excess of cyclodextrin was prepared. The solution was diluted with buffer in several steps, and the ^{1}H NMR spectrum was monitored until it showed approximately a 1:1 mixture of free and bound aryl phosphate. At this point, the signals were integrated and the binding constant calculated on the basis of the known concentrations of aryl phosphate and cyclodextrin.

For several of the complexes in this study, the ^{1}H NMR spectra were not useful for measuring association constants because either exchange was intermediate on the NMR time scale and resonances were broad or the protons of the aryl phosphate were overlapping. In these cases, the ^{31}P NMR spectra showed well-resolved resonances for free and bound aryl phosphate. Thus, ^{31}P NMR dilution experiments were used to measure association constants. In all cases, there was a significant chemical shift difference between free and bound aryl phosphate in the ^{31}P spectrum, reflecting the large difference in environment experienced by the phosphate group in the free and bound forms. Relaxation rates (T_1) were measured for several of the free and complexed aryl phosphates to ensure that the relaxation delays used in the ^{1}H and ^{31}P NMR spectra were long enough to allow for accurate integration of resonances.

Association constants for the two cyclodextrin hosts with the three aryl phosphate guests in a variety of buffers are shown in Table 1. From these data, it is apparent that buffers have a dramatic impact on the value of the measured association constants. Differences of 3−4 orders of magnitude are observed on the basis of the structure and concentration of the buffer. The weakest binding constants were measured in 100 mM phosphate. Phosphate can associate with the ammonium groups of the cyclodextrin[13] and must be displaced before the aryl phosphate guest can bind. Part of the free energy that is gained from binding the aryl phosphate to the cyclodextrin is offset by the loss of binding interactions between the buffer and the cyclodextrin. This has the effect of lowering the observed binding constant. Lowering the phosphate concentration from 100 to 10 mM increases the association constant of *p*-NPP with **1**β by a factor of 5 because there is less competition from the buffer. Measurements in 100 mM ADA buffer, which has one negative charge compared to phosphate with two negative charges, gives higher association constants. This result indicates that ADA is bound less tightly by the cyclodextrin than is phosphate.

A zwitterionic, but overall neutral, buffer such as BES does not compete as strongly for the cyclodextrin host. The sulfonate anion of BES is significantly stabilized by the intramolecular ammonium ion and gains only a small amount of additional stabilization by complexing with the cyclodextrin. Therefore, association constants measured in this buffer are significantly higher than those measured in ADA and phosphate buffers. In D_2O/NaCl solution that has been pH adjusted to 7.0, the association constants for *p*-NPP and AcTyr(PO_3^{2-})OMe with **1**β are in the range of 10^4−10^5 M^{-1}. These values are similar to those reported for binding of nucleotide phosphates to positively charged cyclodextrins as measured by potentiometric titration under similar conditions.[6,7] Association constants measured in buffers that bear a positive charge such as Tris and imidazole are of similar orders of magnitude. These solutions allow optimal interactions between the aryl phosphates and positively charged cyclodextrins, with the chloride counterion of the buffers providing little competition to the binding event.

Figure 4 shows a plot of the free energy of association for the various cyclodextrin−aryl phosphate combinations as a function of the buffer. From this pictorial represen-

(12) Wilcox, C. S.; Cowart, M. D. *Tetrahedron Lett.* **1986**, *27*, 5563.

(13) Schneider has reported that the binding constant between an aminoCD and inorganic phosphate is 3.7×10^3 M^{-1}. See ref 6.

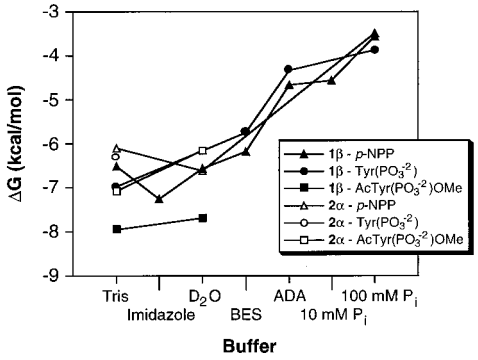


Figure 4. Free energy for the binding interaction between the cyclodextrin hosts and the aryl phosphate guests as a function of buffer structure.

tation of the binding data it becomes apparent that the two cyclodextrin hosts bind the three aryl phosphate guests with similar affinity in any particular buffer system. The only exception is the complex between **1**β and AcTyr(PO_3^{2-})OMe, which has an association constant that is significantly higher than the other complexes. The difference in affinity between the complexes of **1**β with Tyr(PO_3^{2-}) and AcTyr(PO_3^{2-})OMe suggests that the later compound makes better hydrophobic contacts with the cyclodextrin host. In addition, molecular models suggest that there is a good steric compatibility between AcTyr(PO_3^{2-})OMe and cyclodextrin **1**β, while the cavity of **2**α is too narrow for an optimal fit.

The binding data presented here demonstrate that negatively charged buffers weaken the association between aryl phosphate guests and positively charged cyclodextrin hosts by competing with the guest for the available binding sites. If these cyclodextrin derivatives are used to influence the activity of biological macromolecules in vitro, positively charged buffers should be used in order to maximize binding interactions between negatively charged guests and the aminoCD hosts.

Measurement of Dissociation Rates by Exchange Spectroscopy. The equilibrium constants discussed in the previous section provide insights into how buffers affect the thermodynamics of aryl phosphates binding to cyclodextrins. We are also interested in how buffers influence the kinetics of such binding events. In previous work we have used ROESY experiments to make rough estimates of the dissociation rate constants of several of the complexes.[9] To determine these rate constants more precisely, we have measured them using ^{31}P 2D exchange spectroscopy (EXSY). These experiments were performed on the complex between Tyr(PO_3^{-2}) and cyclodextrin **1**β in a variety of buffers in order to examine the relationship between dissociation rate and buffer structure.

The EXSY experiment uses the same pulse sequence as a NOESY experiment, and cross-peaks of opposite sign are observed for NOEs and exchange. For the complex between Tyr(PO_3^{2-}) and **1**β, we observe separate resonances for free and bound Tyr(PO_3^{2-}) in the ^{31}P NMR spectrum, and no cross-peaks can occur for NOE interactions. If the mixing time used in the experiment is short compared to the rate of exchange between free and bound Tyr(PO_3^{2-}), then no cross-peak is observed between these species. However, if the mixing time is of the same order of magnitude or longer than the exchange rate, then a cross-peak will be observed. The volume of the cross-peak is proportional to the amount of exchange that has occurred during the mixing time and, thus, can be used to calculate the exchange rate. One EXSY spectrum is shown in Figure 5 as an example, and a summary of the exchange data is given in Table 2.

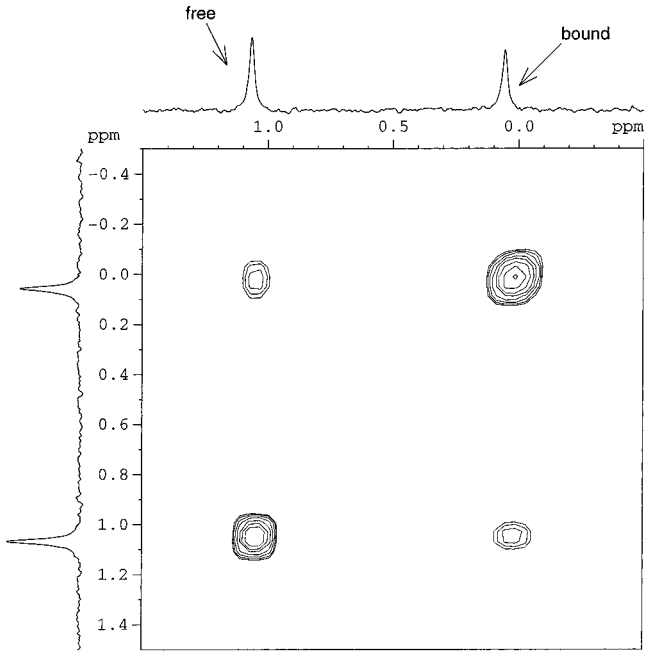


Figure 5. ^{31}P EXSY spectrum of the complex between cyclodextrin **1**β and Tyr(PO_3^{-2}) in 100 mM ADA buffer at pH 7.0. The mixing time in this experiment was 50 ms.

Table 2. Rate Constants, k_{off}, for Dissociation of Complexes between Positively-Charged Cyclodextrins and Tyr(PO_3^{2-}) Determined by ^{31}P EXSY Experiments (T = 25 °C)

CD	buffer	k_{off} (s^{-1})	k_{on}[a] ($M^{-1}s^{-1}$)
1β	100 mM phosphate	45 ± 5	32 000
1β	100 mM ADA	7 ± 1	10 500
1β	100 mM BES	7 ± 1	110 000
1β	100 mM Tris	7 ± 1	910 000
2α	100 mM Tris	$2 \times 10^{-3} \pm 0.4 \times 10^{-3}$ [b]	84

[a] k_{on} values are inferred based upon the measured k_{off} and K_{assoc} values shown this table and Table 1. [b] This value is extrapolated from measurements that were performed at higher temperatures (see text).

When ADA, BES, and Tris are used as buffers, the dissociation rate constants are 7 s^{-1}, despite the fact that the binding constants in these buffers range from 1500 M^{-1} for ADA to 130 000 M^{-1} for Tris. The fact that these off-rates are idential shows that the buffer does not participate during the rate-limiting step for dissociation. Thus, the dissociation rate constant of 7 s^{-1} simply represents the rate at which phosphotyrosine dissociates from the cyclodextrin in aqueous solution. This slow step is then followed by a second fast step that involves binding of the buffer to the empty cyclodextrin. In contrast, the buffer is critical for determining the rate that Tyr(PO_3^{2-}) associates with the cyclodextrin. Association rate constants (k_{on}) are provided in Table 2 and have been calculated on the basis of the measured K_{assoc} and k_{off} values. These data are consistent with an interpretation in which the cyclodextrin, in the absence of Tyr(PO_3^{2-}), binds to buffers that incorporate a negatively charged functional group. In order for Tyr(PO_3^{2-}) to bind, the buffer must first dissociate, and it is this dissociation reaction that controls the overall rate at which Tyr(PO_3^{2-})

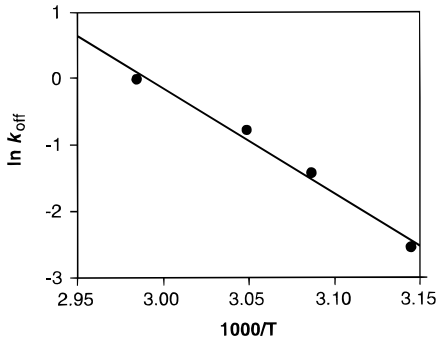


Figure 6. Arrenhius plot for dissociation of the complex between cyclodextrin **2**α and Tyr(PO_3^{-2}) in 100 mM Tris buffer at pH 7.0. The *y*-intercept gives the preexponential factor ($A = 1.4 \times 10^{22}$ s^{-1}) and the slope gives E_a/R. The activation energy for this dissociation reaction is 34 kcal/mol. The rate constants for dissociation at the four temperatures are $k =$ 0.064 s^{-1} (45 °C), 0.22 s^{-1} (51 °C), 0.42 s^{-1} (55 °C), and 0.98 s^{-1} (62 °C).

binds to the cyclodextrin. After the buffer dissociates from the cyclodextrin, the Tyr(PO_3^{2-}) can bind to the empty cavity in a faster second step. Tris, which is positively charged and has chloride as the counterion, is not likely to complex strongly with the cyclodextrin and provides the fastest association rate between Tyr(PO_3^{2-}) and the cyclodextrin. BES, which is neutral overall but possesses a negatively charged sulfonate group, slows the association rate by almost 1 order of magnitude. The association rate is slowest in ADA buffer, which has an overall negative charge and is likely to bind well to the cyclodextrin.

In phosphate buffer, the dissociation rate constant is six to seven times faster than what is observed in ADA, BES, and Tris buffers. The higher off-rate suggests that phosphate, unlike the other buffers, is playing an active role in facilitating dissociation of Tyr(PO_3^{2-}) from **1**β. We suggest two possible interpretations of this result. First, phosphate may actively catalyze displacement of the Tyr(PO_3^{2-}) from the cyclodextrin by stabilizing the transition state for dissociation through formation of electrostatic interactions with the ammonium groups of the cyclodextrin. A second possibility is that the ground state of the complex actually involves a ternary aggregate between Tyr(PO_3^{2-}), **1**β, and inorganic phosphate. Electrostatic interactions between inorganic phosphate and the cyclodextrin would weaken the interactions with Tyr(PO_3^{2-}) and thus decrease the amount of energy necessary for dissociation.

The dissociation rate constant for the complex between Tyr(PO_3^{2-}) and the α-cyclodextrin derivative **2**α is too small to measure at room temperature using the ^{31}P EXSY experiment. The phosphorus nuclei relax completely to the ground state before any exchange takes place, and therefore, no cross-peaks are observed in the EXSY spectrum no matter how long the mixing time that is used. To circumvent this problem, we have measured the dissociation rate constant at several different temperatures above 25 °C in a range in which the rate can be measured conveniently by EXSY spectroscopy. Extrapolation of the rate constant back to room temperature gives the value for this complex shown in Table 2. Figure 6 shows an Arrenhius plot of the exchange data at four different temperatures, and these data can be used to calculate thermodynamic parameters for the transition-state of the dissociation reaction. The calculated values are $\Delta G^{\ddagger} = 21$ kcal/mol, $\Delta H^{\ddagger} = 33$ kcal/mol, and $\Delta S^{\ddagger} = 41$ eu ($T\Delta S^{\ddagger} = 12$ kcal/mol at 25 °C). The reaction is entropically favored as is expected for a dissociation process, and the enthalpy change on going to the transition-state is surprisingly high for a reaction that involves only noncovalent interactions. There is likely to be significant error in the entropy calculation because the temperature range covered by these experiments is fairly small.

The dissociation rate constant for the Tyr(PO_3^{2-})−**2**α complex is 3 orders of magnitude smaller than the complex with **1**β, and the calculated rate constant for association is also small. The interior diameter of the cavity in α-cyclodextrin is significantly narrower than that of β-cyclodextrin, and presents a sterically constrained hydrophobic pocket through which the phosphate group of Tyr(PO_3^{2-}) must pass in order for association or dissociation to occur. In the case of **1**β, the cavity is large enough to allow the phosphate group, perhaps in the solvated form, to pass through it during these processes. This lowers the energy of the transition state for exchange. However, in the case of **2**α the tight confines of the cavity do not easily allow Tyr(PO_3^{2-}), in the free or solvated form, to pass through it during exchange. Therefore, there is a large energy barrier for both association and dissociation. This interpretation is supported by molecular models of the two complexes.[14]

Conclusions

The structure and concentration of buffers play a major role in controlling both the thermodynamics and kinetics of binding interactions of aminoCDs **1**β and **2**α with aryl phosphate guest molecules. Negatively charged buffers compete with the aryl phosphate guest for the positively charged binding site of the cyclodextrin, and this competition lowers the effective binding constant. Based upon these results, we conclude that positively charged buffers such as Tris and imidazole should be used when studying the influence of these cyclodextrins on biological systems. The ^{31}P EXSY experiments show that, with the exception of phosphate, buffers exert a major influence on the association rates of aryl phosphates with cyclodextrins, but have no effect on the dissociation rates.

One of the disadvantages of the aminoCDs in their present form is that they show low selectivity and will thus bind to many sterically compatible phosphate esters. To make the aminoCDs more useful for biological studies, we are modifying their structure to make them more specific for particular phosphotyrosine-containing sequences and structures within peptides and proteins. Such modifications include addition of a second CD unit that is designed to bind to the side chain of a nearby hydrophobic residue, and functionalization of the aminoCDs with Lewis acidic groups such as activated carbonyl compounds or boronic acids that are designed to interact with the side chains of proximal nucleophilic amino acids.

Experimental Section

General Methods. Reactions were conducted under an atmosphere of dry nitrogen in oven-dried glassware. Anhy-

(14) Thatcher and co-workers have recently reported that the coalescence temperature for free 4-isopropylphenyl phosphate and 4-isopropylphenyl phosphate that is bound to an aminoCD is approximately 100 °C. These authors also attribute the large barrier to exchange to the passage of the phosphate group through the hydrophobic cavity of the CD. See ref 4.

drous procedures were conducted using standard syringe and cannula transfer techniques. THF was distilled from sodium and benzophenone. Other solvents were of reagent grade and were stored over 4 Å molecular sieves. All other reagents were used as received. Organic solutions were dried over $MgSO_4$ unless otherwise noted. Solvent removal was performed by rotary evaporation at water aspirator pressure.

***N*-Acetyl-L-tyrosine Dibenzyl Phosphate Methyl Ester 5.** 1*H*-Tetrazole (1.68 g, 23.5 mmol), *N*-acetyl-L-tyrosine methyl ester (2.0 g, 7.8 mmol), and dibenzyl *N*,*N*-diethylphosphoramidite (4.7 mL, 15.6 mmol) were combined in 8 mL of dry THF, and the solution was stirred at 25 °C for 1 h. The mixture was then cooled to −40 °C in a dry ice−acetone bath, and a solution of 77% *m*-CPBA (2.6 g, 11.6 mmol) dissolved in 20 mL of methylene chloride was added via syringe. After the addition was complete, the reaction was warmed to 25 °C and stirred for 30 min, then 10 mL of a 10% aqueous solution of $NaHSO_3$ was added and the reaction was stirred for an additional 10 min. The mixture was extracted with ether, and the organic layer was washed twice with 10% aqueous $NaHSO_3$, twice with 5% aqueous $NaHCO_3$, and once with brine and dried over $MgSO_4$. The solvent was removed by rotary evaporation, and the product was purified by flash chromatography (1:9 hexanes/ethyl acetate) to give 1.9 g (3.9 mmol, 50%) of *N*-acetyl-L-tyrosine dibenzyl phosphate methyl ester: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.94 (s, 3H), 3.02−3.08 (m, 2H), 3.67 (s, 3H), 4.82 (dd, J = 14.6, 6.3 Hz, 1H), 5.10 (d, J = 8.3 Hz, 4H), 6.43 (d, J = 7.7 Hz, 1H), 7.03 (d, J = 8.6 Hz, 2H), 7.07 (d, J = 8.6 Hz, 2H), 7.31 (s, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 22.7, 36.8, 52.1, 69.5 (d, J = 5.6 Hz), 119.8 (d, J = 4.7 Hz), 127.8, 128.3, 128.4, 130.3, 132.8, 135.1 (d, J = 7.1 Hz), 149.4 (d, J = 7.1 Hz), 169.7, 171.8; HRMS-FAB ($M + H^+$) calcd for $C_{26}H_{29}NO_7P$ 498.1680, found 498.1692.

***N*-Acetyl-L-tyrosinephosphate Methyl Ester 6.** To a solution of **5** (0.77 g, 1.4 mmol) in 2 mL of MeOH was added a catalytic amount of 5% Pd/C, and hydrogen gas was bubbled into the reaction mixture. The reaction was stirred for 3 h at 25 °C under an atmosphere of hydrogen. The catalyst was removed by filtration, and the solvent was removed by rotary evaporation to give 421 mg (1.27 mmol, 91%) of compound **6** as a clear oil: ^{1}H NMR (300 MHz, MeOH-d_4) δ 1.93 (s, 3H), 2.95 (dd, J = 13.6, 9.3 Hz, 2H), 3.14 (dd, J = 13.6, 5.7 Hz, 1H), 3.69 (s, 3H), 4.65 (dd, J = 9.3, 5.7 Hz, 1H), 7.15 (d, J = 8.6 Hz, 2H), 7.21 (d, J = 8.6 Hz, 2H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 21.2, 36.6, 51.6, 54.3, 120.3 (d, J = 4.7 Hz), 130.3, 133.6, 150.7 (d, J = 6.2 Hz), 172.2, 172.4; HRMS-FAB ($M + Na^+$) calcd for $C_{12}H_{16}NNaO_7P$ 340.0561, found 340.0562.

^{1}H NMR Titration Experiments. For the ^{1}H NMR titration experiments, the conditions that were used during each titration are provided in the following format: Complex: buffer, cyclodextrin concentration or range of concentrations used, aryl phosphate concentration or range of concentrations used, identity of the proton or protons that were monitored during the titration experiment. **1**β−*p*-NPP: 100 mM Tris, 0−0.25 mM, 0.05 mM, aromatic protons of the aryl phosphate. **1**β−*p*-NPP: 100 mM imidazole, 0.05 mM, 0−0.20 mM, H4 of the cyclodextrin. **1**β−*p*-NPP: D_2O/NaCl, 0−0.38 mM, 0.1 mM, aromatic protons of the aryl phosphate. **1**β−*p*-NPP: 100 mM BES, 0−1.0 mM, 0.21 mM, aromatic protons of the aryl phosphate. **1**β−*p*-NPP: 100 mM ADA, 0−6.1 mM, 1.5 mM, aromatic protons of the aryl phosphate. **1**β−*p*-NPP: 10 mM phosphate, 0−4.4 mM, 0.74 mM, aromatic protons of the aryl phosphate. **1**β−*p*-NPP: 100 mM phosphate, 0−10 mM, 1.0 mM, aromatic protons of the aryl phosphate. **2**α−*p*-NPP: 100 mM Tris, 0.05 mM, 0−0.25 mM, H4 of the cyclodextrin. **2**α−*p*-NPP: D_2O/NaCl, 0.1 mM, 0−0.63 mM, H4 of the cyclodextrin.

^{1}H NMR Dilution Experiments. For the ^{1}H NMR dilution experiments, the conditions that were used during each experiment are provided in the following format: Complex: buffer, final cyclodextrin concentration, final aryl phosphate concentration, identity of the proton or protons that were monitored during the dilution experiment. **1**β−AcTyr(PO_3^{-2})-OMe: 100 mM Tris, 0.027 mM, 0.025 mM, *N*-Ac protons of the aryl phosphate. **1**β−Tyr(PO_3^{-2}): 100 mM Tris, 0.019 mM, 0.018 mM, aromatic protons of the aryl phosphate. **1**β−Tyr-(PO_3^{-2}): 100 mM BES, 0.14 mM, 0.087 mM, aromatic protons of the aryl phosphate. **1**β−AcTyr(PO_3^{-2})OMe: D_2O/NaCl, 0.026 mM, 0.022 mM, *N*-Ac protons of the aryl phosphate. **2**α−Tyr-(PO_3^{-2}): 100 mM Tris, 0.093 mM, 0.067 mM, aromatic protons of the aryl phosphate. **2**α−AcTyr(PO_3^{-2})OMe: 100 mM Tris, 0.063 mM, 0.050 mM, *N*-Ac protons of the aryl phosphate. **2**α−AcTyr(PO_3^{-2})OMe: D_2O/NaCl, 0.055 mM, 0.050 mM, *N*-Ac protons of the aryl phosphate.

^{31}P NMR Dilution Experiments. For the ^{31}P NMR dilution experiments, the conditions that were used during each experiment are provided in the following format: Complex: buffer, final cyclodextrin concentration, final aryl phosphate concentration. In all of these experiments, the resonance of the phosphate group of the aryl phosphate was monitored. **1**β−Tyr(PO_3^{-2}): 100 mM ADA, 1.40 mM, 1.12 mM. **1**β−Tyr(PO_3^{-2}): 100 mM phosphate, 2.9 mM, 2.6 mM. **2**α−*p*-NPP: 100 mM phosphate, 8.0 mM, 4.5 mM.

^{31}P 2D Exchange Spectroscopy Experiments. The phase-sensitive 2D ^{31}P−^{31}P EXSY spectra were recorded at 27 °C at 161.9 MHz on a Bruker AMX 400 spectrometer equipped with an SGI computer, using a 5 mm QNP probe. The 2D EXSY maps were obtained from the basic NOESY pulse sequence that involves three 90° pulses with time-proportional phase increments and 16-order phase cycling. Sixteen scans were accumulated for each of the 128 t_1 increments, zero filled to 512 W in the F_1 dimension and using 512 W in the F_2 dimension with no zero filling, a 2 s relaxation delay and a spectral window of 3221 Hz. Preliminary experiments with longer relaxation delays did not reveal any significant change in the relative integrals of the resonances, indicating essentially full relaxation within the recycling delay. The free induction delay was multiplied by a sine bell function with $\phi = \pi/2$. The 2D spectra were phase and baseline corrected in both dimensions and diagonal and cross-peak volumes were determined using the standard Bruker Avance software. The rate constants were evaluated using the method of Harzell and co-workers.[15]

For cases in which the T_1 values of the free and bound aryl phosphate were much less than the exchange rate constant ($k_{ex} \equiv k_{off}$), the effects of longitudinal cross relaxation rates were ignored. For cases in which the T_1 values were of the same order of magnitude as the exchange rate constant, the k_{ex} value was corrected for the effects of longitudinal cross relaxation using the method described by Macura and Ernst.[16] The equation relating the values of k_{ex} to the ratios of the areas of bound diagonal to cross-peaks, I_{ii}/I_{ij}, for the case of equal concentration of free and bound species, is shown below and is taken from ref 16.

$$I_{ii}/I_{ij} = 1/2(R_c/k_{ex})[(1 + e^{-Rc\tau m})/(1 - e^{-Rc\tau m})] - {}^1/_2(\Delta R/k_{ex})$$

In this equation, $\Delta R = R_{(bound)} - R_{(free)} = 1/T_{1(bound)} - 1/T_{1(free)}$, and $R_c = [\Delta R^2 + 4k_{ex}^2]^{1/2}$. The value of k_{ex} was obtained using a computer program to solve in an iterative fashion for I_{ii}/I_{ij}, starting from a given value of k_{ex} and the observed values of T_1 for each temperature. Iterations were continued until the calculated I_{ii}/I_{ij} ratio agreed to better than 1% with the experimentally observed value. Data for the complex between cyclodextrin **2**α and Tyr(PO_3^{2-}) in 100 mM Tris buffer at four different temperatures are as follows, where $k_{ex,uncorr}$ corresponds to the experimentally observed values and $k_{ex,corr}$ are the values that have been corrected for the effects of longitudinal cross relaxation. 45 °C: $k_{ex,uncorr}$ = 0.078 s^{-1}, $k_{ex,corr}$ = 0.064 s^{-1}; 51 °C: $k_{ex,uncorr}$ = 0.239 s^{-1}, $k_{ex,corr}$ = 0.217 s^{-1}; 55 °C: $k_{ex,uncorr}$ = 0.457 s^{-1}, $k_{ex,corr}$ = 0.415 s^{-1}; 62 °C: $k_{ex,uncorr}$ = 1.09 s^{-1}, $k_{ex,corr}$ = 0.980 s^{-1}.

***T*$_1$ Measurements.** Phosphorus T_1 relaxation times were measured using a ^{31}P inversion recovery sequence with increasing delays between the 180° inversion pulse and the

(15) Hartzell, C. J.; Mente, S. R.; Eastman, N. I.; Beckett, J. L. *J. Phys. Chem.* **1993**, *97*, 4887.

(16) Macura, S.; Ernst, R. R. *Mol. Phys.* **1980**, *41*, 95, eq 23.

90° observe pulse. The intensities of the peaks were plotted against the delay times, and the T_1 values were calculated using standard Bruker software. To measure the T_1 value of the phosphate group in a cyclodextrin−aryl phosphate complex, a solution of the complex was prepared in which greater than 99% of the aryl phosphate was present in the complexed form. **1**β−Tyr(PO_3^{2-}): $T_1 = 1.01$ s (100 mM phosphate, 25 °C); Tyr(PO_3^{2-}): $T_1 = 6.29$ s (100 mM phosphate, 25 °C); **2**α−Tyr-(PO_3^{2-}): $T_1 = 1.89$ s (100 mM Tris, 62 °C); **2**α−Tyr(PO_3^{2-}): $T_1 = 1.33$ s (100 mM Tris, 45 °C); Tyr(PO_3^{2-}): $T_1 = 6.88$ s (100 mM Tris, 62 °C); Tyr(PO_3^{2-}): $T_1 = 5.23$ s (100 mM Tris, 45 °C).

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Supporting Information Available: Copies of ^{1}H and ^{13}C NMR spectra for compounds **5** and **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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