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Inhibition of Phosphatase Activity by Positively-Charged Cyclodextrins

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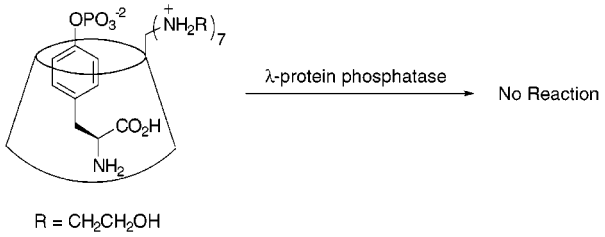
ABSTRACT



Aminocyclodextrins are known to bind phosphate esters such as phosphotyrosine and *p*-nitrophenyl phosphate. This paper describes the inhibition of phosphate ester hydrolysis, as catalyzed by λ-protein phosphatase and acid phosphatase, that is caused by such binding interactions. ROESY studies provide structural information about the cyclodextrin–aryl phosphate complexes. In addition, these experiments are used to generate approximations of the rates of dissociation of the noncovalent complexes.

Cyclodextrins are one of the most important classes of molecules in the field of molecular recognition. They have gained widespread use in such diverse areas as biomimetic catalysis and chromatography and as drug carriers.[1] Aminocyclodextrins are cyclodextrins in which one or several of the primary hydroxyl groups at the 6-position of the sugar units have been replaced by amines. In 1979 Boger and Knowles demonstrated that such molecules bind to benzyl phosphate through a combination of electrostatic and hydrophobic interactions.[2] Since then a number of investigators have examined the binding properties of aminocyclodextrins with a variety of phosphate esters including nucleotide mono- and triphosphates,[3,4] *p*-nitrophenyl phosphate and other 4-substituted phenyl phosphates,[4,5] phosphotyrosine,[6] and cyclic phosphate esters.[7] In this paper we demonstrate that aminocyclodextrins, because they are able to bind to phosphotyrosine and other aryl phosphate esters with significant affinity, can inhibit the hydrolysis of these phosphate ester guests catalyzed by λ-protein phosphatase and acid phosphatase.[8] The development of new phosphatase inhibitors is of current interest because of the important role that phosphatases play in intracellular signal transduction.[9] In addition, we use ^{1}H and ^{31}P NMR spectroscopy to examine intermolecular interactions between aminocyclodextrin hosts and phosphotyrosine guests and show that these experiments provide information about the kinetics of dissociation of the aminocyclodextrin−phosphotyrosine complexes.

The structures of the modified cyclodextrin hosts and the aryl phosphate guests used in this study are shown in Figure 1. Compounds **1**β and **2**α are derived from β- and α-cyclodextrin, respectively, and the syntheses of these molecules have been described previously by Darcy[4] and Thatcher.[10] The primary hydroxyl groups at the 6-positions of the sugar

(1) For a recent series of comprehensive reviews on all aspects of cyclodextrin chemistry, see: *Chem. Rev.* **1998**, *98*, No. 5.
(2) (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.
(3) (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.
(4) (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. Recognit. Chem.* **1996**, *25*, 43.
(5) Vizitiu, D.; Thatcher, G. R. J. *J. Org. Chem.* **1999**, *64*, 6235.
(6) Cotner, E. S.; Smith, P. J. *J. Org. Chem.* **1998**, *63*, 1737.
(7) Breslow, R.; Schmuck, C. *J. Am. Chem. Soc.* **1996**, *118*, 6601.
(8) For a demonstration that aminocyclodextrins can inhibit neurite growth by binding to glycosaminoglycan sulfates, see: Borrajo, A. M. P.; Gorin, B. I.; Dostaler, S. M.; Riopelle, R. J.; Thatcher, G. R. J. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1185.
(9) Burke, T. R.; Zhang, Z.-Y. *Biopolymers* **1998**, *47*, 225.
(10) Vizitiu, D.; Walkinshaw, C. S.; Borin, B. I.; Thatcher, G. R. J. *J. Org. Chem.* **1997**, *62*, 8760.

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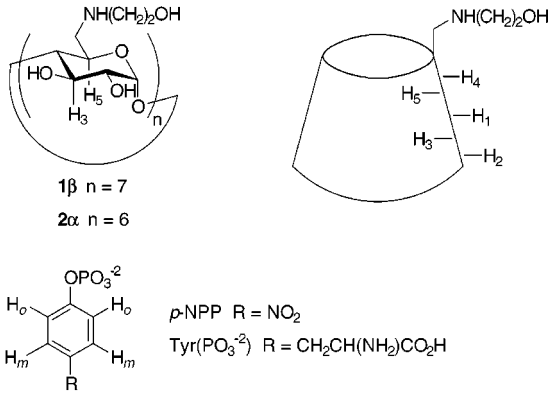

Figure 1. Structures of positively charged cyclodextrin hosts and aryl phosphate guests. The schematic diagram on the right shows the positions of the protons in the interior and exterior of the cyclodextrin cavity. The proton labels correspond to the annotated resonances in the ROESY spectra shown in Figure 3.

units have been replaced with 2-hydroxyethylamino groups. Several of these groups are protonated at pH 7, and they provide a positively charged binding site along one rim of the cyclodextrin cavity that forms electrostatic interactions with negatively charged guests. Two aryl phosphates were used as guests: *p*-nitrophenyl phosphate (*p*-NPP) and phosphotyrosine ($Tyr(PO_3^{-2})$).

The inhibition of λ-protein phosphatase and acid phosphatase by cyclodextrin **1**β was monitored using a UV assay to follow dephosphorylation of the aryl phosphate substrates. The data were analyzed using the reaction scheme shown in eq 1, where CD is cyclodextrin **1**β, S is the substrate, E is the enzyme, and P is the product. This reaction scheme is simply the Michaelis−Menten formalism that has been modified to incorporate a preliminary equilibrium between free substrate and the cyclodextrin−substrate complex (CD·S).

$$\mathrm{CD{\cdot}S} \overset{K_d}{\rightleftharpoons} \mathrm{CD} + \mathrm{S} + \mathrm{E} \overset{K_M}{\rightleftharpoons} \mathrm{E{\cdot}S} \xrightarrow{k_{cat}} \mathrm{E} + \mathrm{P} \qquad (1)$$

The rate of the enzyme-catalyzed reaction is given by eqs 2−4, where S_{app} is the concentration of free substrate, S_0 is the total substrate concentration, CD_0 is the total cyclodextrin concentration, and K_d is the dissociation constant of the CD·S complex.

$$\text{rate} = V_{max}S_{app}/(K_M + S_{app}) \qquad (2)$$

$$S_{app} = S_0 - \mathrm{CD{\cdot}S} \qquad (3)$$

$$\mathrm{CD{\cdot}S} = \frac{\mathrm{CD}_0}{2} + \frac{(S_0 + K_d)}{2}\left[1 - \left(\frac{\mathrm{CD}_0{}^2 - 2\mathrm{CD}_0(S_0 - K_d)}{(S_0 + K_d)^2} + 1\right)^{1/2}\right] \qquad (4)$$

Equation 4 can be derived from a simple 1:1 binding isotherm between CD and S.[11] The rate expression shown in eq 2 is based upon the assumption that the enzyme is able to hydrolyze only the free substrate and cannot react with substrate that is complexed with the cyclodextrin. This assumption is reasonable because in the complex the phosphate ester is completely surrounded by the cyclodextrin and binding to the enzyme is precluded by both steric and electrostatic repulsion. The initial rates of the enzyme-catalyzed reactions were determined using a single substrate concentration and a range of cyclodextrin concentrations.[12] The data were fitted to eqs 2−4 using a nonlinear curve fitting procedure, which gave estimates of K_d. An example of one set of data, along with the corresponding curve fit, is shown in Figure 2. K_M values for the substrates were determined separately using standard methods. Table 1 shows the association constants for the cyclodextrin−aryl phosphate complexes that were extracted from the kinetic data.

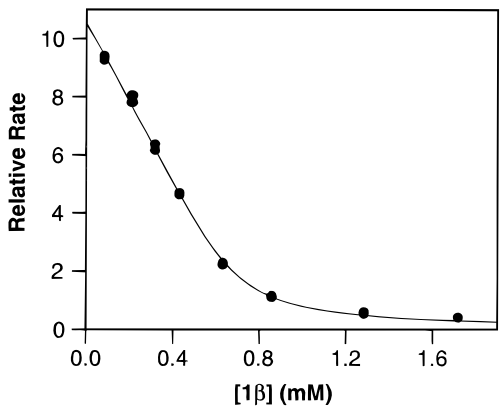

Figure 2. Inhibition of acid phosphatase by cyclodextrin **1**β in 100 mM Tris pH 7.0. The substrate, *p*-NPP, was present at a concentration of 0.64 mM. The curve corresponds to the best fit of the data to eq 2.

Table 1. Association Constants of Cyclodextrin 1β with Aryl Phosphates As Measured by Inhibition of Phosphatase Activity and by ^{1}H NMR Experiments

			K_{assoc} (M^{-1})	
Substrate	Enzyme[a]	Buffer[b]	Enzyme Kinetics	NMR Method
p-NPP	AP	100 mM Tris	37,000 ± 8,000	
p-NPP	λ-PP	100 mM Tris	13,000 ± 3,000	
p-NPP		100 mM Tris		60,000 ± 15,000[c]
p-NPP	AP	10 mM HPO_4^{-2}	1,400 ± 300	
p-NPP		10 mM HPO_4^{-2}		2,200 ± 400[c]
$Tyr(PO_3^{-2})$	AP	100 mM Tris	23,000 ± 5,000	
$Tyr(PO_3^{-2})$	λ-PP	100 mM Tris	19,000 ±4,000	
$Tyr(PO_3^{-2})$		100 mM Tris		130,000 ± 25,000[d]

[a] AP is acid phosphatase and λ-PP is λ-protein phosphatase. [b] Assays with AP and λ-PP were performed at pH 7.0 and 7.8, respectively. λ-PP was not stable at pH 7.0. [c] Measured by ^{1}H NMR titration. [d] Measured by ^{1}H NMR dilution.

We have also measured association constants by NMR spectroscopy for the sake of comparison (Table 1). The complex between **1**β and *p*-NPP was in fast exchange on the NMR time scale and showed only one set of resonances for the aryl phosphate protons. Thus, ^{1}H NMR titrations were used to determine association constants.[11] In contrast, the complex between **1**β and Tyr(PO_3^{-2}) was in slow exchange on the NMR time scale and displayed well-resolved signals for the free and bound aryl phosphate. In this case the relative integration of the aromatic protons of free and bound Tyr(PO_3^{-2}), along with the known concentrations of Tyr(PO_3^{-2}) and the cyclodextrin, were used to determine the association constant.

Comparison of the association constants derived from enzyme kinetics and from the NMR experiments shows that, for the **1**β−*p*-NPP complex with acid phosphatase in either Tris or phosphate buffers, there is reasonable agreement between values measured using the two different techniques. The association constants measured in 100 mM Tris buffer are significantly higher than those measured in phosphate buffer. In Tris-HCl buffer, the chloride anion does not strongly complex with the positively charged cyclodextrin and therefore does not compete significantly with *p*-NPP for binding to **1**β. In contrast, the phosphate buffer binds relatively tightly to the cyclodextrin and must be displaced before *p*-NPP can bind.[13] This competition by the buffer lowers the observed association constant.

For the **1**β−*p*-NPP complex with λ-protein phosphatase and the **1**β−Tyr(PO_3^{-2}) complex with either enzyme, the association constants measured using the enzyme kinetics method are significantly lower than the values measured using NMR spectroscopy. Two factors lower the sensitivity of the enzyme kinetics method and limit our ability to measure large association constants using this technique. First, the concentration of the substrate in these assays must be similar to the K_M value so that significant rates are achieved in the absence of inhibitor. Second, the difference in extinction coefficients between Tyr(PO_3^{-2}) and Tyr is relatively small. In addition, the assays with λ-protein phosphatase were performed at pH 7.8, while the assays with acid phosphatase and the NMR measurements were performed at pH 7.0. The higher pH decreases the protonation state of the cyclodextrin and lowers the electrostatic attraction between host and guest.[14] Despite the limitations of the kinetic method, the results presented here demonstrate that positively charged cyclodextrins, because they sequester phosphate ester guests, can effectively inhibit hydrolysis of the phosphate ester linkage as catalyzed by phosphatase enzymes.

We have acquired ROESY spectra of several of the cyclodextrin−aryl phosphate complexes (Figure 3). These

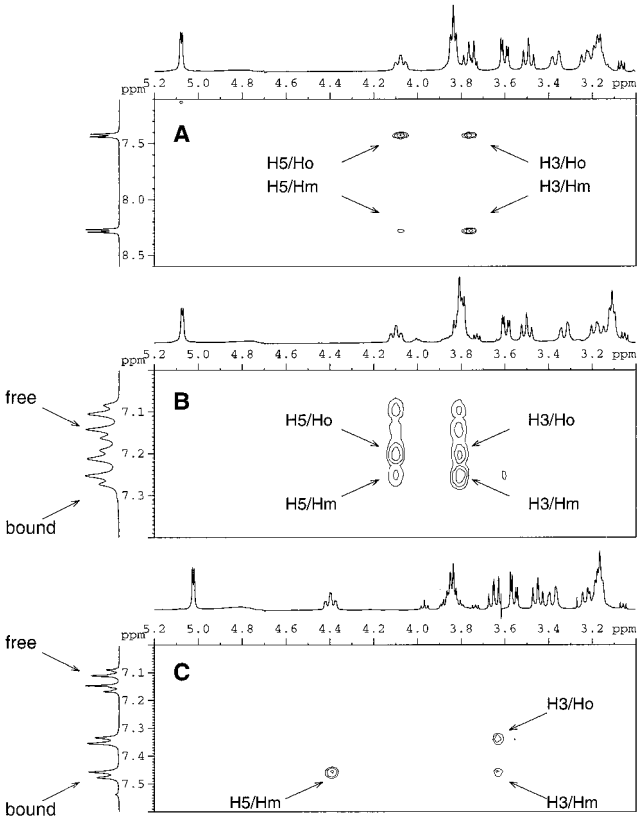

Figure 3. ROESY spectrum showing intermolecular NOEs between protons on the interior of the cyclodextrin cavity and the *ortho* and *meta* protons of *p*-NPP and Tyr(PO_3^{-2}). Spectrum A: **1**β + *p*-NPP. Spectrum B: **1**β + Tyr(PO_3^{-2}). Spectrum C: **2**α + Tyr(PO_3^{-2}).

spectra not only give information about the structure and conformation of the noncovalent complexes[15] but they also provide insights into the kinetics of their dissociation. Spectrum A (Figure 3) shows the complex between cyclodextrin **1**β and *p*-NPP. Exchange between free and bound *p*-NPP is fast on the NMR time scale, and thus only one set of resonances is observed for the aromatic protons of *p*-NPP. The ROESY spectrum shows strong intermolecular NOE cross-peaks between the *ortho* protons of *p*-NPP and both the H3 and H5 protons on the interior wall of the cyclodextrin cavity (see Figure 1 for the positions of the protons). In addition, there is a strong cross-peak between the *meta* protons of *p*-NPP and H3 and a weaker cross-peak between the *meta* protons and H5 of the cyclodextrin.[16] These NOEs are consistent with a binding conformation in which the phosphate interacts with the ammonium groups of the cyclodextrin and the aromatic ring is positioned inside the cyclodextrin cavity. The *ortho* protons are relatively deep in the cavity and are proximal to both H3 and H5, while the

(11) Wilcox, C. S.; Cowart, M. D. *Tetrahedron Lett.* **1986**, *27*, 5563.
(12) Addition of unmodified α- or β-cyclodextrin to the enzyme-catalyzed reactions has no effect on the reaction rate.
(13) A related positively charged cyclodextrin has been shown to bind inorganic phosphate with an association constant of 3.7×10^3 M^{-1}. See ref 3b.
(14) The association constant for the complex between cyclodextrin **1**β and Tyr(PO_3^{-2}) measured by ^{1}H NMR spectroscopy in 100 mM Tris buffer at pH 7.8 is 10 000 ± 2000 M^{-1}.
(15) Schneider, H.-J.; Hacket, F.; Rudiger, V.; Ikeda, H. *Chem. Rev.* **1998**, *98*, 1755.
(16) The assignments of the resonances in the NMR spectra were performed using COSY spectra of the aryl phosphate−cyclodextrin complexes.

meta protons occupy a more shallow position and interact strongly only with H3. From the limiting chemical shift values of the aromatic protons in the free and bound forms, along with the observation that this acquisition, which was recorded at 25 °C, is well above the coalescence point, we estimate that the dissociation rate constant for this complex is much greater than 150 s^{-1}.[17]

Spectrum B shows the complex between cyclodextrin **1**β and Tyr(PO_3^{-2}). Unlike the complex between **1**β and *p*-NPP, this complex is in slow exchange on the NMR time scale so that separate resonances are observed for free and bound Tyr(PO_3^{-2}). The ROESY spectrum shows cross-peaks between the aromatic protons of both free and bound Tyr(PO_3^{-2}) and H3 and H5 of the cyclodextrin. We expect only bound Tyr(PO_3^{-2}), and not free Tyr(PO_3^{-2}), to have NOE interactions with the cyclodextrin. However, the exchange rate between free and bound Tyr(PO_3^{-2}) is fast compared to the 350 ms spinlock time used in the ROESY experiments. During the spinlock period, bound Tyr(PO_3^{-2}) molecules that experience NOEs with the cyclodextrin can dissociate to the unbound form. Therefore, the spectrum shows cross-peaks with both the free and bound forms, even though the NOE interactions occur only between bound Tyr(PO_3^{-2}) and the cyclodextrin. Since this complex shows slow exchange on the NMR time scale, but ROESY cross-peaks for both the free and bound forms using a 350 ms spinlock time, we estimate that the dissociation rate constant for this complex must fall in the range between 3 and 75 s^{-1}.[18] ^{31}P 2D exchange spectroscopy (EXSY) can be used to more accurately determine exchange rates.[19] Using this method we have found that the dissociation rate constant for the complex between **1**β and Tyr(PO_3^{-2}) is 7 $\pm$ 1 s^{-1}, a value that is consistent with the estimate derived from the ROESY experiments.

For the complex between Tyr(PO_3^{-2}) and cyclodextrin **2**α (spectrum C), exchange between free and bound Tyr(PO_3^{-2}) is again slow on the NMR time scale. However, in this case the exchange rate is slow compared to the 350 ms spinlock time used in the ROESY experiments. As a result we observe NOE cross-peaks between the aromatic protons of only the bound form, and not the free form, of Tyr(PO_3^{-2}) and H3 and H5 of **2**α. These results demonstrate that only bound Tyr(PO_3^{-2}) molecules are in close proximity to the cyclodextrin as we expect and put an upper limit on the dissociation rate constant for this complex of 3 s^{-1}. Slow dissociation of Tyr(PO_3^{-2}) from **2**α when compared to **1**β is likely caused by the narrower, more restrictive cavity of the α-cyclodextrin derivative. The association constant of this complex in 100 mM Tris buffer is 30 000 $\pm$ 4500 M^{-1}.[20]

The pattern of NOE intensities that we observed in the **2**α−Tyr(PO_3^{-2}) complex is different than the pattern that appears in the complex with **1**β. With the α-cyclodextrin derivative, there are strong interactions between H3 of the cyclodextrin and both the *ortho* and *meta* protons of the aryl phosphate. However, there is a strong interaction between H5 and the *meta* protons, while the interaction between H5 and the *ortho* protons is absent. These observations are consistent with a binding conformation in which the Tyr(PO_3^{-2}) molecule is positioned "upside down" in the cyclodextrin cavity with the phosphate group oriented away from the ammonium groups. This conformation is somewhat surprising since it precludes a strong electrostatic interaction between the negatively charged phosphate group and the positively charged rim of the cyclodextrin. On the other hand, this conformation allows electrostatic interactions to form with the carboxylate group of Tyr(PO_3^{-2}).

The experiments presented in this paper demonstrate that the binding interactions between positively charged cyclodextrin hosts and aryl phosphate guests inhibit the hydrolysis of the guest molecules catalyzed by phosphatase enzymes. The ROESY studies show that the aryl phosphates bind in the interior of the cyclodextrin cavity and also provide limits on the dissociation rates of the aryl phosphate−cyclodextrin complexes. These limits can be confirmed using ^{31}P 2D exchange spectroscopy. We are currently investigating in greater depth the kinetics and thermodynamics of these types of binding interactions.

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(17) Sandstrom, J. *Dynamic NMR Spectroscopy*; Academic Press: New York, 1982.

(18) The upper limit for the exchange rate is derived from the difference in chemical shift values for free Tyr(PO_3^{-2}) and Tyr(PO_3^{-2}) that is fully bound to cyclodextrin **1**β, along with the fact that this acquisition which was recorded at room temperature is well below the coalescence point. See also ref 17.

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

(20) Reference 5 provides some related coalescence temperature data for similar aminocyclodextrin−aryl phosphate complexes.