Goodness of Fit in Complexes between Substrates and Ribonuclease Mimics: Effects on Binding, Catalytic Rate Constants, and Regiochemistry

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Received December 28, 1995[⊗]

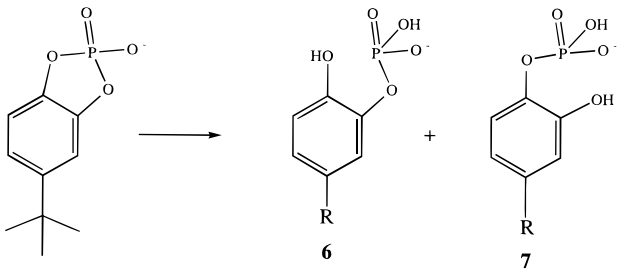
Abstract: The hydrolysis of 4-*tert*-butylcatechol cyclic phosphate and of 4-methylcatechol cyclic phosphate catalyzed by α-cyclodextrin-6A,6B-bisimidazolide, and by the corresponding derivatives of β-cyclodextrin (βCD) and γ-cyclodextrin (γCD), was examined. All three catalysts were able to hydrolyze the substrate derived from 4-methylcatechol, but only the βCD- and γCD-based catalysts could hydrolyze the substrate based on 4-*tert*-butylcatechol. Saturation kinetics were observed, from which k_{cat} and K_m values were derived. The k_{cat}'s showed a bell-shaped dependence on pH, indicating a bifunctional mechanism in which one imidazole acted as a base while the other, protonated, acted as an acid catalyst. The strongest binding was observed between the substrate derived from *tert*-butylcatechol and the βCD-based catalyst, and this combination also had the highest k_{cat}. An Arrhenius plot showed that the good fit of the *tert*-butyl group into the βCD cavity leads to an entropy advantage for catalysis within the complex, as well as an advantage in the binding. Furthermore, the combination with the best binding and catalytic constant also exhibited the highest regioselectivity in the substrate hydrolysis, being essentially completely selective for one mode of hydrolysis. All these effects reflect the result of a tight fit of the substrate into the catalyst binding cavity, leading to a well-defined reaction geometry.

Introduction

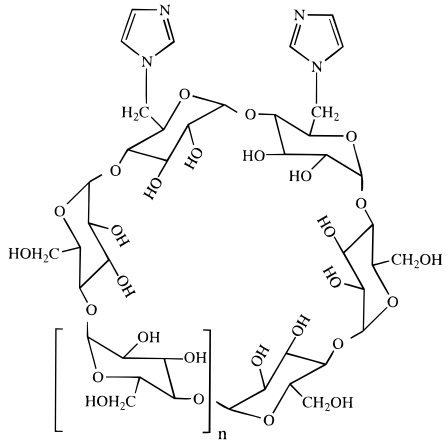
We have described the catalysis of several reactions by enzyme mimics with two imidazole rings attached to the primary carbons of β-cyclodextrin (βCD, cycloheptaamylose).[1−6] In our earliest study,[1] we examined the hydrolysis of a catechol cyclic phosphate carrying a 4-*tert*-butyl group (**1**) by three isomers of βCD bis-imidazole (cf. **2**). The substrate bound into the cavity of the catalyst, in water solution, and then was hydrolyzed by the combined action of one imidazole ring acting as a base and the other, protonated to its imidazolium state, acting as an acid.

Proton inventory studies showed that the acid−base catalysis was simultaneously bifunctional,[3] and the preferred geometry of the catalyst—the best isomer was that (**2**) with the two imidazoles attached on neighboring glucose units, the A,B isomer—showed that the mechanism of the process involved delivery of a water molecule by the base at the same time that the phosphate anionic oxygen was protonated by the imidazolium ion.[2] Thus a phosphorane monoanion was formed as an intermediate. We have also demonstrated a phosphorane monoanion intermediate for the catalysis of a dinucleotide by imidazole buffer[7] and have proposed a related mechanism for the enzyme ribonuclease A.[8]

In principle the bell-shaped pH vs rate profile that we observed only shows that the imidazolium ion plays a catalytic role, not that it acts as an acid. Thus one could propose that the delivery of water to the phosphate would lead to a phosphorane *dianion*, and that the imidazolium ion simply stabilized it electrostatically with a hydrogen bond. However, the basicity of a phosphorane dianion is such that it would capture the proton from an imidazolium ion, and the proton inventory studies showed that this occurs while the phosphorane



1 **6** **7**



2 n = 2
4 n = 1
5 n = 3

[⊗] Abstract published in *Advance ACS Abstracts,* July 1, 1996.

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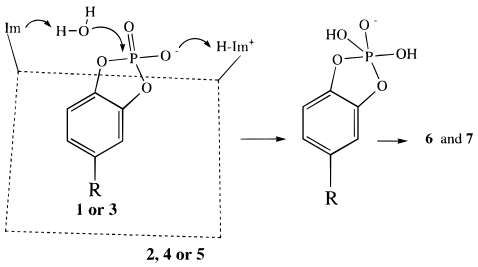

Figure 1. Schematic illustration of the hydrolysis of substrate **1** or **3** when bound in the cavity of catalyst **2**, **4**, or **5**. As shown one imidazole group of the catalyst acts as a general base to deliver a water to the phosphate, while the other imidazole, as an imidazolium ion, protonates the developing phosphorane anion intermediate. The direction of attack of the water that is shown will lead to a phosphorane that will decompose to product **7**, while product **6** arises from approach in an alternate direction. The best catalyst−substrate combination produces product **6** exclusively.

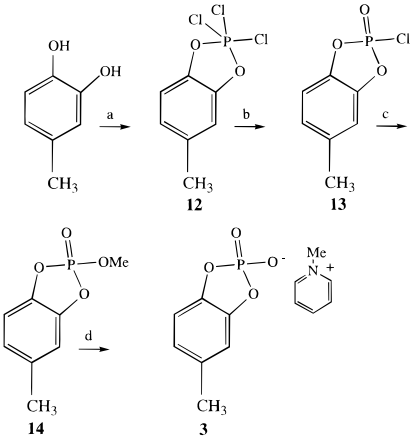

Figure 2. Synthesis of substrate **3**, following the one used previously[1] for the synthesis of substrate **1**: (a) PCl_5; (b) acetic anhydride, which becomes acetyl chloride; (c) methanol; (d) pyridine.

is being formed. There seems no way to avoid the conclusion that the mechanism involved is the one shown in Figure 1.

The *tert*-butyl group in **1**—and in a number of substrates we have used for other processes involving βCD-based catalysts—was present to promote strong hydrophobic binding into the cavity,[9] but it was not clear what other effect it had. βCD was used rather than αCD (cyclohexaamylose) or γCD (cyclooctaamylose) because of general availability, but again it was not clear how important this choice was. The smaller cavity of αCD—and the larger cavity of γCD—might change not only the affinity of the catalyst to the substrate but also the catalytic rate constants within the complex.

Since the catalysis by **2** is one of the most striking examples of bifunctional catalysis by an enzyme mimic that complexes the substrate, as well as the earliest, it was desirable to examine these questions. The results are of general interest with respect to catalysis within molecular complexes, including enzyme−substrate complexes.

Results and Discussion

Substrate **3** was prepared from 4-methylcatechol by a procedure (Figure 2) analogous to that used to prepare substrate **1**.[1] We compared the catalyzed (by **2**) hydrolysis of **1** with that of **3** , examining both the binding constants and the catalytic rate constants within the complexes. By varying the temperature we obtained the activation parameters of the two catalytic processes.

We also prepared two other catalysts: the A,B isomer of αCD-bis-imidazole **4** and the A,B isomer of γCD-bis-imidazole **5**. These were synthesized by a procedure (Figure 3) analogous to that used[2] in the preparation of **2**. The key step is bridging between the 6-OH groups of two adjacent glucose units using 1,3-dimethoxybenzene-4,6-disulfonyl chloride. The mass spectra and NMR spectra indicated that the new catalysts were pure, and with the assigned structures. In particular, the ^{1}H-NMR patterns in the imidazole region are characteristic for A,B substitution, in which the two imidazoles overlap (Figure 4). Quite different patterns are seen for the A,C and A,D isomers of **2** (supporting information).

We examined each of these as catalysts in the hydrolysis of the two substrates, **1** and **3**. The reactions were performed with 1 mM substrate and 50 mM phosphate buffer, and the concentrations of the (excess) catalyst, the pH, and the temperature were varied. To avoid inhibition by products, the reactions were carried to only a few percent hydrolysis and analyzed by initial rate methods. The rates for substrate hydrolysis in the absence of catalyst were also determined, and they were used in the calculation of k_{cat} values, assuming that only the unbound substrate undergoes background hydrolysis.[10] Kinetic runs were performed in triplicate, and the rate constants agreed within 8% or better. The kinetic data are listed in Table 1.

All three catalysts were able to hydrolyze the methyl substrate **3**, and the βCD catalyst **2** and the γCD catalyst **5** were able to hydrolyze the *tert*-butyl substrate **1**. In all these cases, a bell-shaped pH vs rate profile (cf. Figures 5 and 6) was seen as a function of pH when the catalyst was at 5 mM and T was 25.0 °C. The rate maximum in all these cases was at ca. pH 6.2, as we had seen with **1** and **2** previously.[2] Thus the bound substrates are undergoing bifunctionally catalyzed hydrolysis, with one basic imidazole and one acidic imidazolium catalytic group. Our subsequent studies were performed at this optimum pH.

By varying the concentrations of the catalysts over the range from 0.25 mM to 10.0 or 15.0 mM we were able to obtain saturation plots and extract the Michaelis constants K_m and the catalytic rate constants for the complexes k_{cat}.[10] The K_m is the dissociation constant for formation of the catalyst−substrate complex provided the rate of reaction within the complex is slow relative to the dissociation rate, which is true in our cases. These values are listed in Table 1.

As the data show, the methylated substrate **3** is bound most strongly by the βCD catalyst **2**, 3.2 times less strongly by the αCD catalyst **4**, and 4.5 times less strongly by the γCD catalyst **5**. Within the complexes, k_{cat} is least for **5**, 3.4 times greater with **2**, and 4.6 times greater with **5**. The contrasts are much greater with the *tert*-butyl substrate **1**. It is not hydrolyzed at all by **4**, the rate being simply that of the background with buffer alone. *tert*-Butylphenyl groups bind fairly strongly into αCD,[9] but apparently with a geometry that does not permit the hydrolysis of **1** by **4**. With the γCD catalyst **5**, **1** is bound 1.6 times as strongly as the methylated substrate **3** is, and it has a k_{cat} 11 times as large, but with the βCD catalyst **2** the binding of **1** is 4.1 times that of **3** and the k_{cat} is 43 times as large as for **3**.

These anionic substrates are binding not only to the cavity but also to the cationic imidazolium group. By titration calorimetry we have determined that 4-methylcatechol binds to simple αCD with a K_{diss} of 49 mM, and to simple βCD with

(9) Bender, M. L.; Komiyama, M. *Cyclodextrin Chemistry*; Springer-Verlag: Berlin, 1978.

(10) Since there is an appreciable rate of reaction with buffer alone, without the added catalysts, the equation used was $k_{obs} = k_{background} + \{(k_{cat} - k_{background})[catalyst]\}/\{[catalyst] + K_m\}$.

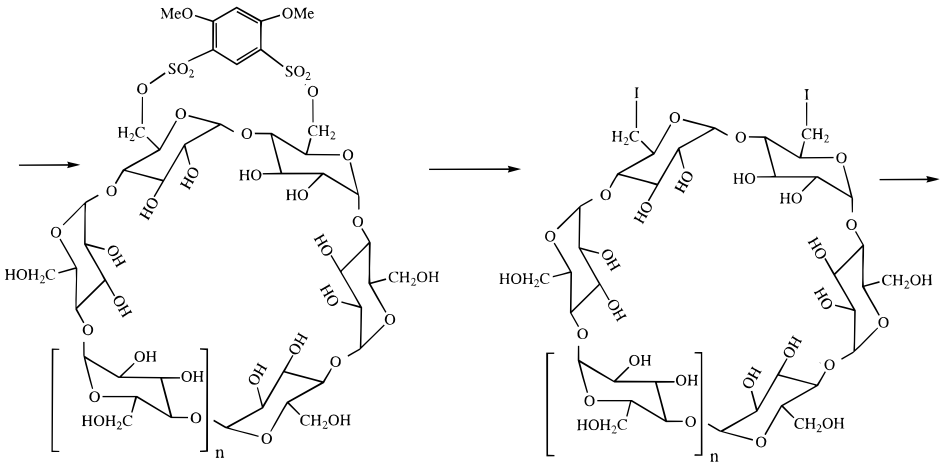

Figure 3. Conversion of cyclohexaamylose and cyclooctaamylose to catalysts **4** and **5**. In the first step a bridged disulfonate is formed using 1,3-dimethoxy-4,6-benzenedisulfonyl chloride, and in the second step this is converted to the 6A,6B diiodide with KI. In the last step, reaction of the diiodide with excess imidazole produces the two catalysts. The path is analogous to that used previously[2] for the synthesis of catalyst **2**.

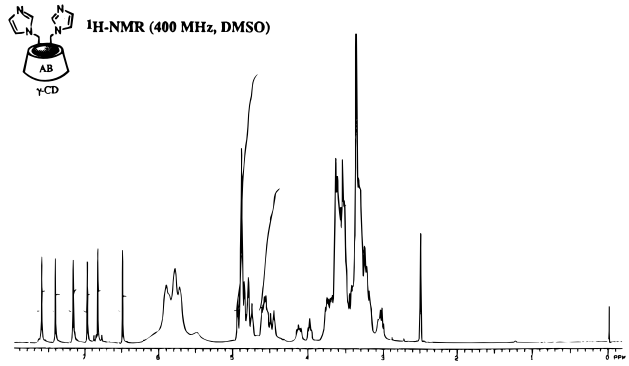

Figure 4. 400 MHz ^{1}H-NMR spectrum of catalyst **5**, in DMSO. As the spectra in the supporting information show, the A,B isomers have a characteristic pattern in the imidazole region resulting from the spatial overlap of the two imidazole rings.

Table 1. Catalytic Rate Constants, Binding Constants, and Product Ratios for Reactions at 25.0 °C in Water with 50 mM Phosphate Buffer at pH 6.2

catalyst	constant	*t*-Bu substrate **1**	methyl substrate **3**
α **4**	k_{cat}[a]		3.8×10^{-5} s^{-1}
	K_m[a]		5.5 mM
	6:**7**[b]		77:23
β **2**	k_{cat}[a]	120×10^{-5} s^{-1}	2.8×10^{-5} s^{-1}
	K_m[a]	0.41 mM	1.7 mM
	6:**7**[b]	>99:1	82:18
γ **5**	k_{cat}[a]	9.0×10^{-5} s^{-1}	0.83×10^{-5} s^{-1}
	K_m[a]	4.8 mM	7.6 mM
	6:**7**[b]	78:22	50:50
none	$k_{background}$	1×10^{-5} s^{-1}	1.3×10^{-5} s^{-1}

[a] From the average of three values for each kinetic point, which agreed within 8% or better. [b] The ratio of the products carrying either a methyl or a *tert*-butyl group. Cleavage of either substrate by NaOH solution affords an approximately 50:50 mixture of the two products. The ratios listed are corrected for products from uncatalyzed reactions of unbound substrates.

a K_{diss} of 18 mM. The binding of **3** to catalyst **4** is 9 times stronger when the anionic substrate group and cationic catalyst group are present than is the binding of 4-methylcatechol to αCD. Its binding is 10 times stronger to catalyst **2** than is the binding of 4-methylcatechol to simple βCD. Data in the literature confirm the stronger binding of **1** to **2**[2] than of a simple *tert*-butylphenyl derivative to βCD,[9] by a similar factor of 10-fold or so, This stronger binding, which must reflect ion pairing

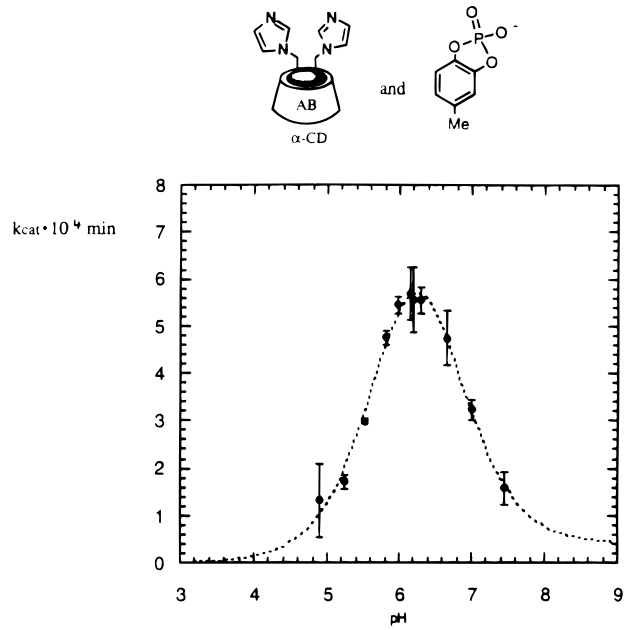

Figure 5. pH vs k_{cat} profile for the hydrolysis of substrate **3** (1 mM) catalyzed by the αCD compound **4** (5 mM) at 25 °C with 50 mM phosphate buffer at various ratios, but 50 mM acetate buffer used for the pH 4.9 and 5.2 points.

between the substrate and a catalyst group, indicates that the K_m involves *productive* binding to the cavity. That is, substrate binds so as to put the phosphate group on the primary side of the cyclodextrin, where the catalysts are located. This may not be true in the case of the *inactive* complex of **1** with **4**, and indeed binding in the reverse sense may explain its inactivity.

The precise fit of the *tert*-butyl group into the βCD cavity causes not only an increase in the binding constant but also an even larger increase in the catalytic rate constant. The advantage is smaller with the γCD catalyst, with its larger cavity in which both substrates fit loosely.

We have done an Arrhenius plot (supporting information) for the ($k_{observed} - k_{background}$) of hydrolysis of substrates **1** and **3** by catalyst **2**. The background rate is negligible for the strong complex between **1** and **2**, but with substrate **3** a correction had to be made for the background reaction. This correction means that the values may not be exactly correct for **3**. The data with 5 mM catalyst and 1 mM substrate show that the E_a's for the

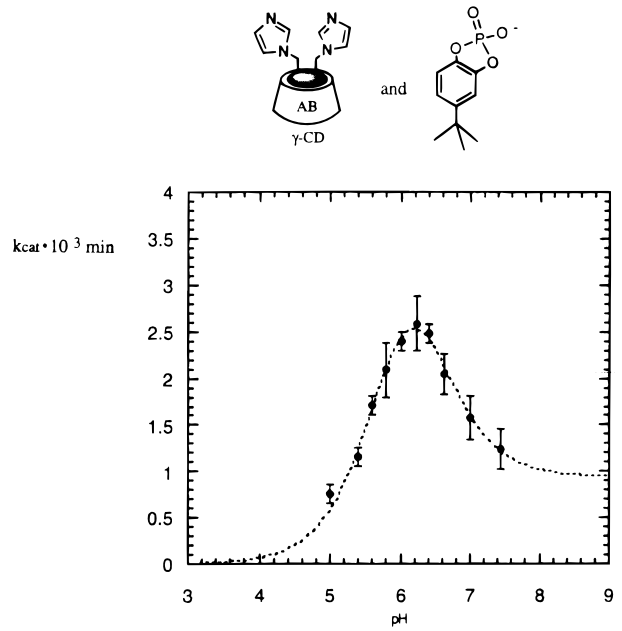

Figure 6. pH vs k_{cat} profile for the hydrolysis of substrate **1** catalyzed by the γCD compound **5**. Conditions as in Figure 5.

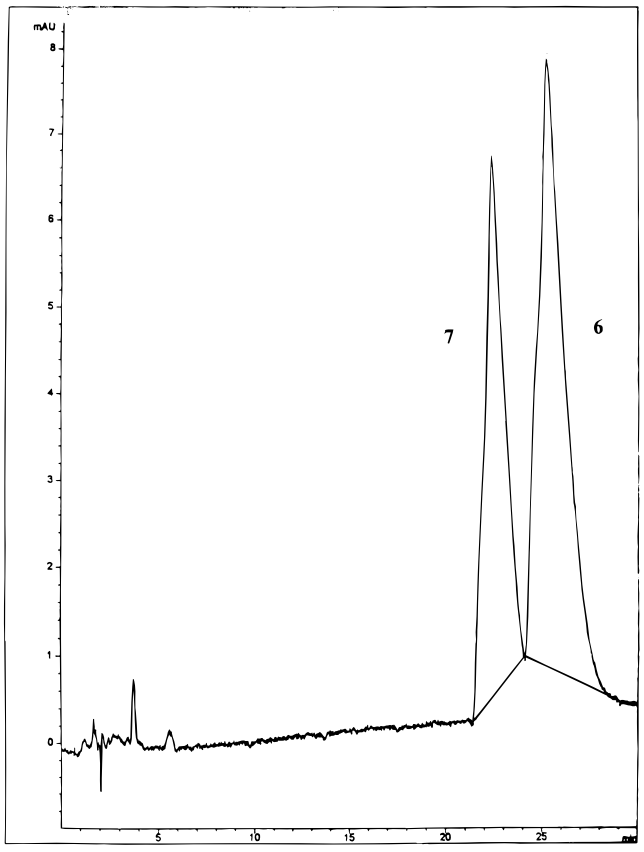

Figure 7. HPLC of products **6** and **7** from the hydrolysis of **1** with simple NaOH.

two processes are almost identical—7.46 kcal/mol for **1** and 7.91 kcal/mol for **3**—and that the pre-exponential *A* factors differ by a ratio of 14. Thus most of the 43-fold rate advantage for **1** over **3** with catalyst **2** reflects an entropy advantage for reaction within the complex.

We have also examined the selectivities of these processes, since substrates **1** and **3** can hydrolyze either to product **6** or to product **7**. The results are listed in Table 1 and illustrated in Figures 7 and 8. The hydrolysis of the substrates by NaOH is

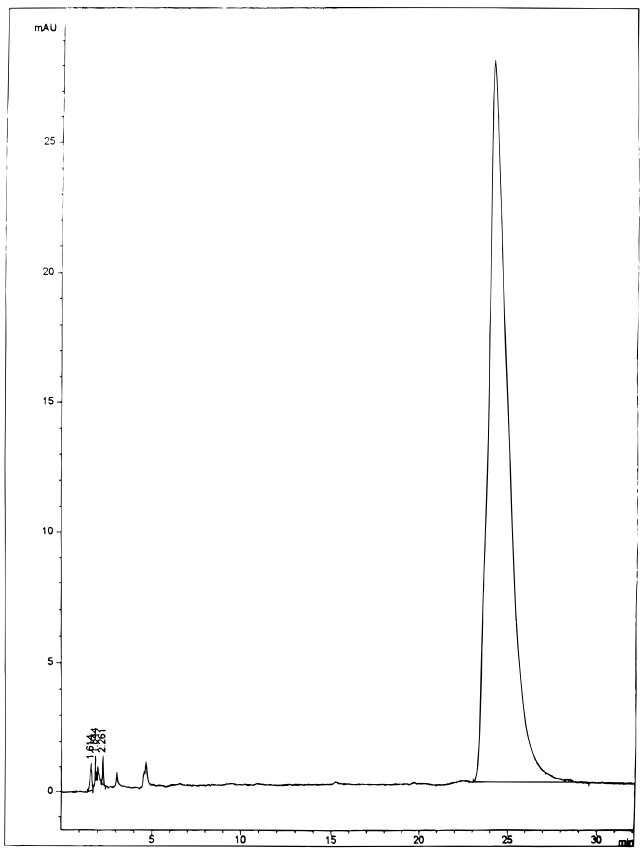

Figure 8. HPLC of the products from the hydrolysis of **1** with catalyst **2**. The reaction is specific for product **6**, and less than 1% of product **7** is formed.

essentially random, but it is completely selective in the hydrolysis of **1** by **2**. The increased flexibility of the catalyst−substrate complex in the other cases leads to a decrease in selectivity compared with the **2**/**1** combination, down to an almost random result in the very flexible complex of **3** with the γCD catalyst **5**.

The most effective catalyst−substrate combination in this series is the originally examined **1** with **2**, not just because the binding is best, but also because the rigidity of the complex promotes the best regioselectivity and best rate within the complex. Even better rates can be expected if the flexibility of the catalytic groups is also restricted.

Experimental Section

Syntheses. β-Cyclodextrin-6A,6B-bis-imidazole (**2**) and 4-*tert*-butylphenylene-1,2-phosphate (**1**) as its *N*-methylpyridinium salt were synthesized as reported previously.

Bis-6A,6B-(1,1′-imidazolyl)-6A,6B-dideoxycyclohexaamylose (4). Dried cyclohexaamylose (21.0 g, a gift from the Amerchol Corp.) in 700 mL of pyridine was treated with 7.0 g of 2,6-dimethoxybenzene-1,3-disulfonyl chloride, added in two portions as a solid, for 3 h at room temperature. The solvent was evaporated *in vacuo* at $T < 50$ °C, and the crude product was dissolved in 50 mL of water and added dropwise to 50 mL of water in 500 mL of acetonitrile. The white precipitate of β-cyclodextrin was discarded, and the residue, from evaporation of the solvent, was chromatographed on a 5 cm × 15 cm reverse phase column with increasing concentrations of MeOH in water. The combined product fractions yielded 0.6 g of a white product (2.3% yield), the 6A,6B bridged disulfonate **8**. The mass spectrum (M + 1 = 1235) and 400 MHz ^{1}H-NMR spectrum were consistent with the assigned structure.

This was converted to the corresponding 6A,6B diiodide **9** by adding 1.2 g of **8**, in 50 mL of dry DMF, to 1.6 g of carefully dried KI and then stirring at 80 °C for 3 h. The solvent was evaporated, and the

residue was chromatographed on a 5 cm × 15 cm reverse phase column with increasing concentrations of MeOH in water. The combined product fractions yielded 0.9 g (80% yield) of the white product **9**. The FAB mass spectrum had peaks at 1193 (M + 1) and 1215 (M + 23) units.

Then 0.9 g of the diiodide **9** and 0.9 g of dried imidazole were heated in 10 mL of dry DMF at 80 °C for 24 h. The solvent was evaporated, the residual imidazole was removed with 10 mL of acetone, and the residue was dissolved in 5 mL of water and chromatographed as above. The combined product fractions yielded 0.5 g (60% yield) of a white product, compound **4**. This has a peak at 1073 (M + 1) and a 400 MHZ ^{1}H-NMR spectrum (supporting information) that is characteristic of 6A,6B bis-imidazoles. The ^{13}C-NMR spectrum (supporting information) was also as expected. The mass spectrum showed no evidence for any mono-imidazole or tris-imidazole derivatives.

Bis-6A,6B-(1,1′-imidazolyl)-6A,6B-dideoxycyclooctaamylose (5). This was prepared in a fashion similar to that described above, starting with dried γ-cyclodextrin (a gift from the American Maize Products Company). However, no precipitation procedure was used, but the product **10** from sulfonation was directly chromatographed after pyridine evaporation. The corresponding diiodide **11** had a mass spectral signal at *m/e* 1517 (M + 1), and the bis-imidazole derivative **5** had a signal at *m/e* 1397 (M + 1). The mass spectrum showed no evidence for any mono-imidazole or tris-imidazole derivatives. The 400 MHZ ^{1}H-NMR spectrum (Figure 4) is characteristic of 6A,6B bis-imidazoles, and the ^{13}C-NMR spectrum (supporting information) was also as expected.

4-Methylphenylene-1,2-phosphate (3). 4-Methylcatechol (31.0 g) was added in four portions to a suspension of PCl_5 (57.4 g) in 400 mL of dry benzene. After the violent HCl evolution ceased, the clear solution was heated under reflux for 2 h. The solvent was evaporated, and the solid residue was distilled under vacuum. The excess PCl_5 sublimed first, and the product trichloride **12** was collected at 94−96 °C and 0.2 Torr as 30.0 g of a clear liquid (50% yield).

This trichloride was converted to the phosphorochloridate **13** by stirring with an excess of acetic anhydride, with cooling at the beginning, and **13** was collected by vacuum distillation as 15.0 g (64% yield) of a nearly colorless liquid at 78−80 °C and 0.2 Torr.

The phosphorochloridate **13** (6.2 g) was dissolved in 10 mL of dry dioxane under argon, and excess methanol was added dropwise. After 30 min of refluxing, the solvent was evaporated and the triester **14** was collected by high-vacuum distillation (5.4 g, 90% yield). This was dissolved in 10 mL of dry dioxane, and excess pyridine was added. After stirring overnight, the suspension was filtered and the white crystalline (hygroscopic) product was collected and washed with cold dioxane under argon to yield 1.7 g (23%) of **3**. The 400 MHz ^{1}H-NMR spectrum (supporting information) was as expected.

Kinetic Studies. Normal 0.2 M phosphate and acetate buffer solutions in deionized water were made up to ionic strength 0.6 M with NaCl. The catalysts were made up as solutions 10 mM in water, and the substrates were made up as solutions 100 mM in DMSO. Kinetic experiments to determine the pH vs rate profiles were started by mixing 300 μL of buffer solution, 600 μL of catalyst solution, and 300 μL of water. The pH was measured and adjusted to the desired value with NaOH or HCl if necessary. Then 12 μL of substrate solution was added, the mixture was transferred to a cuvette, and the reaction was monitored in a thermostated (25.0 °C) cell compartment of a Beckman DU-8 spectrophotometer. The absorbency increase due to hydrolysis was continuously monitored at 290 nm, and at the end of the run the pH was redetermined. The change was generally less than 0.05 unit. The phosphate buffer was always used, in various buffer ratios, except for two kinetic points near pH 5.

For the kinetic saturation studies,[10] the concentrations of the catalysts were varied over the range 0.25 mM to 10 or 15 mM while the substrates were kept at 1 mM and the buffer at 50 mM. The studies were done at pH = 6.20, the observed pH optimum for the catalyzed reactions.

For the determination of activation parameters, the rate of hydrolysis of the *tert*-butyl substrate **1** and the methyl substrate **3** were determined with 5 mM catalyst **2**, 1 mM substrate, and 50 mM pH 6.20 buffer at six temperatures over the ranges 30−70 °C for **1** and 30−80 °C for **3**. With the *tert*-butyl substrate **1** the background rate is negligible (<2%) under these conditions, but with the methyl substrate **3** the k_{cat} was obtained by correcting the total observed rate by the background rate for unbound substrate, using the K_m determined at 25 °C to calculate this.

In general the reactions were run to only a few percent hydrolysis of the substrate, to avoid problems with product inhibition. The data were treated by the method of initial rates to determine the rate constants.

Product Studies. The reactions were run under the same conditions as with the kinetics, except that the substrate was added as a solid and the reactions were run overnight to convert all the substrate. The products were analyzed by HPLC on a reverse phase 18 column. For the methyl substrate **3** the eluent was 500 mM phosphate buffer pH 7.00, while for the *tert*-butyl substrate **1** the eluent was 100 mM phosphate buffer pH 7.00 with 20% methanol. The peaks were detected at 290 nm.

Two of the resulting chromatograms are exhibited in Figures 8 and 9. Previous work[2] had shown that the dominant product in the hydrolysis of **1** catalyzed by **2** was the 2-phosphate **6**. It was assumed that this was also true with the methyl substrate **3**, whose chromatographic behavior after hydrolysis similarly caused the minor product to elute first. Coinjections confirmed the assignments, since the HPLC retention times varied a bit.

Binding Studies. A solution of 4-methylcatechol (100 mM) in 50 mM aqueous phosphate buffer (pH 6.20) was titrated into a solution of the cyclodextrin (10 mM) in the same buffer at 25 °C in a Microcal OMEGA titration calorimeter. The background was obtained, and corrected, by titrating the 4-methylcatechol solution into pure buffer. The data were fitted to a single binding site model using the software supplied with the calorimeter.

Acknowledgment. Support of this work by the NSF, NIH, and ONR and a Humboldt Fellowship to C.S. are gratefully acknowledged.

Supporting Information Available: Some NMR spectra and the Arrhenius plot (7 pages). See any current masthead page for ordering and Internet access instructions.

JA954307N