Re-evaluation of Cyclodextrin as a Model of Chymotrypsin: Acceleration and Inhibition of Tertiary Anilide Hydrolysis

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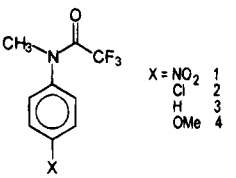
The hydrolysis of *p*-nitro-*N*-methyltrifluoroacetanilide (**1**), *p*-chloro-*N*-methyltrifluoroacetanilide (**2**), *N*-methyltrifluoroacetanilide (**3**), and *p*-methoxy-*N*-methyltrifluoroacetanilide (**4**) in the presence and absence of α- and β-cyclodextrin has been studied at 7.5 < pH < 10.6. For **1**–**3**, cyclodextrin (CD) exhibits simple Michaelis-Menten saturation kinetics, with no evidence for reaction via other than 1:1 CD–substrate complexes. The behavior of CD with **4** is more complex. Moreover, CD catalyzes the hydrolysis of **1** but inhibits the hydrolysis of **2**–**4** across the pH range studied. The nature of the buffer catalysis in the absence of CD, exhibited in the hydrolysis of **1**, also shows marked differences with that exhibited by **2**–**4**. The data are most simply interpreted by a mechanism in which CD accelerates formation of a tetrahedral intermediate **5**; in the case of **1**, the rate of breakdown of this intermediate is greater than the rate of buffer-catalyzed breakdown of the hydrolysis intermediate. The CD cavity may provide an environment complementary to the transition state for expulsion of the anilide leaving group. These results are compared with the previously reported effects of CDs on trifluoroacetanilide and phenyl ester hydrolysis and proposals of CD as a model of chymotrypsin.

Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides made up of α(1→4)-linked D-glucose residues, α- and β-CD having six and seven glucose residues, respectively. The hydrophobic interior of these torus-shaped molecules allow CDs to complex organic molecules of appropriate size in aqueous solution.[1] The ability of CDs to accelerate the hydrolysis of certain esters has led to the proposal that CDs "mimic" the mechanism of chymotrypsin. Hydrolysis occurs *via* binding of the substrate by CD followed by nucleophilic attack by a CD hydroxyl group, leading to acylation of the "enzyme model".[1,2] However, if the substrate is an ester, deacylation of CD is rate determining and the system is not truly catalytic.[3] Despite the frequent *simile* drawn with chymotrypsin, the primary function of which is to hydrolyze amides, surprisingly little of the study of CDs as enzyme models has examined amides as substrates, presumably due to the decreased reactivity of amides relative to esters. A recent critique draws attention to this discrepancy, among others, in the modeling of chymotrypsin.[4]

To examine amide hydrolysis under mild conditions, activated amides such as anilides,[5] trifluoroacetanilides,[5,6] acylimidazoles,[7] and strained lactams[8] have been studied. Komiyama and Bender originally studied the effects of CDs on the hydrolysis of acylimidazoles[7b] and trifluoroacetanilides.[3] More recently, CD-mediated trifluoroacetanilide hydrolysis has been studied by Granados and de Rossi.[9] This recent study contains results and conclusions which conflict with those of Bender, in particular the suggestion of a role for a 2:1 CD/substrate complex. In both cases, a small family of *NH* ionizable anilides were utilized as substrates, requiring kinetic analysis complicated by the necessity of accounting for substrate ionization.



The present study was undertaken using a series of four *N*-methyltrifluoroacetanilides **1**–**4**, which do not deprotonate under the experimental conditions. Full kinetic parameters have been obtained for hydrolysis, including buffer catalysis, providing a basis for comparison with CD-mediated hydrolysis. A simplified kinetic analysis is presented in which the effects of CDs on trifluoroacetanilide substrates may be determined unambiguously, resolving previous inconsistencies. Furthermore, the use of a family of amides as substrates represents an important reevaluation of the ability of CDs to provide a model for chymotrypsin and serine proteases.

Results

The kinetics of CD-mediated hydrolysis of esters or amides may be analyzed in terms of 1:1 complex formation[2,3] as shown in Scheme 1. The parameters K_d and k_{cat} are obtained from an Eadie–Hoftsee analysis, plotting $k_{obs} - k_u$ vs $(k_{obs} - k_u)/[CD]$ (eq 1). Both α- and β-CD

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(1) Bender, M. L.; Komiyama, M. *Cyclodextrin Chemistry*; Springer-Verlag: New York, 1978.
(2) (a) Van Etten, R. L.; Sebastian, J. F.; Clowes, G. A.; Bender, M. L. *J. Am. Chem. Soc.* **1967**, *89*, 3242; (b) *Ibid.* 3253.
(3) Komiyama, M.; Bender, M. L. *J. Am. Chem. Soc.* **1977**, *99*, 8021.
(4) Menger, F. M.; Ladika, M. *J. Am. Chem. Soc.* **1987**, *109*, 3145.
(5) Fife, T. H.; Bembi, R. *J. Am. Chem. Soc.* **1993**, *115*, 11358.
(6) (a) Biechler , S. S.; Taft, R. W., Jr. *J. Am. Chem. Soc.* **1957**, *79*, 4927. (b) Stauffer, C. E. *J. Am. Chem. Soc.* **1972**, *94*, 7887. (c) Pollack, R. M.; Dumsha, T. C. *J. Am. Chem. Soc.* **1973**, *95*, 4463. (d) Mader, P. M. *J. Am. Chem. Soc.* **1965**, *87*, 3191. (e) Komiyama, M.; Bender, M. L. *Bioorg. Chem.* **1978**, *8*, 133.
(7) (a) Fife, T. H. *Acc. Chem. Res.* **1993**, *26*, 325. (b) Komiyama, M.; Bender, M. L. *Bioorg. Chem.* **1977**, *6*, 323.
(8) (a) Bowden, K.; Bromley, K. *J. Chem. Soc., Perkin Trans. 2* **1990**, 2103. (b) Skorey, K. I.; Somayaji, V.; Brown, R. S. *J. Am. Chem. Soc.* **1989**, *111*, 1445.
(9) Granados, A.; de Rossi, R. H. *J. Org. Chem.* **1993**, *58*, 1771.

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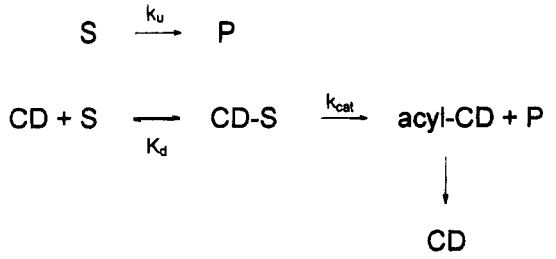
Scheme 1[a]



[a] S = substrate, P = products, CD–S = CD/substrate complex, K_d is the dissociation constant of the complex, k_{cat} is the pseudo-first-order rate constant for the conversion of complex to products, and k_u is the rate constant in the absence of CD. This scheme may be analyzed using an adapted Michaelis-Menten analysis.

$$k_{obs} - k_u = (k_{cat} - k_u)[CD]/(K_d + [CD]) \quad (1)$$

accelerate the hydrolysis of **1** at pH 8.5 (Figure 1), as might be expected from previous experiments using *p*-nitrotrifluoroacetanilide as substrate. The effects are not large, but do conform to Michaelis-Menten analysis, giving not only a good Lineweaver–Burke plot, but an excellent Eadie–Hoftsee correlation (Figure 2, Table 1). A pH–rate profile of the effects of 10 mM CD from pH 7.5 to 10.6 is linear and first order in hydroxide (Figure 3).

CD-mediated hydrolyses of **2** and of **3** also conform to the Michaelis-Menten analysis, showing saturation. However, as shown in Figure 4, β-CD does not accelerate, but rather retards hydrolysis. Such behavior is not unprecedented but has not been observed previously for such subtle changes in substrate structure. Eadie–Hoftsee plots may be used to analyze this retardation data. The parameters so obtained reflect a decrease in rate due to added CD ($k_{obs} - k_u < 0$), since $k_{cat} < k_u$ (Table 1). Addition of cyclohexanol had no effect on the CD-mediated hydrolysis. This is in agreement with the data of Tee on hydrolysis of *para*-substituted phenyl esters,[10] which is interpreted to indicate that the substrate is not bound within the CD cavity during reaction, but rather is to be found on the face of the CD.

As can be seen from Figures 1 and 4, the effects of β-CD on hydrolysis of **4** differ from those on hydrolysis of **1**, **2**, or **3** and do not conform to Michaelis-Menten analysis over the whole [CD] range. However, Eadie–Hoftsee analysis may be performed on the first portion of the data (Table 1). This result is consistent with the observation of a competitive process involving higher-order complexation which results in an even greater retardation of hydrolysis of **4**. β-CD produced no shift in the absorption spectrum of **4**; thus, higher-order complex formation could not be demonstrated in the manner of Granados and de Rossi.[9] These workers reported spectrophotometric evidence that β-CD and *p*-nitrotrifluoroacetanilide form a 2:1 CD–substrate complex at [CD] > 4×10^{-3} M and therefore incorporated termolecular binding terms in their kinetic analysis.[9] However, in the present study no spectrophotometric or kinetic evidence for complex formation, of a ratio greater than 1:1, between β-CD and **1** was observed.

The substituent parameter σ^- is required to describe the conversion of anilides to anilines.[11] This parameter has been used before when comparing trifluoroacetanilide hydrolysis in solution to its chymotrypsin-catalyzed reaction.[12] In an attempt to use this reactivity scale to explain the anomalous behavior of **1**, a correlation of log k_{cat} with σ^- has been drawn[13] (Figure 5a) which is surprisingly good, considering previous application of Hammett plots in CD-mediated reactions.[2] This would seem to indicate that the mechanism of the CD-mediated

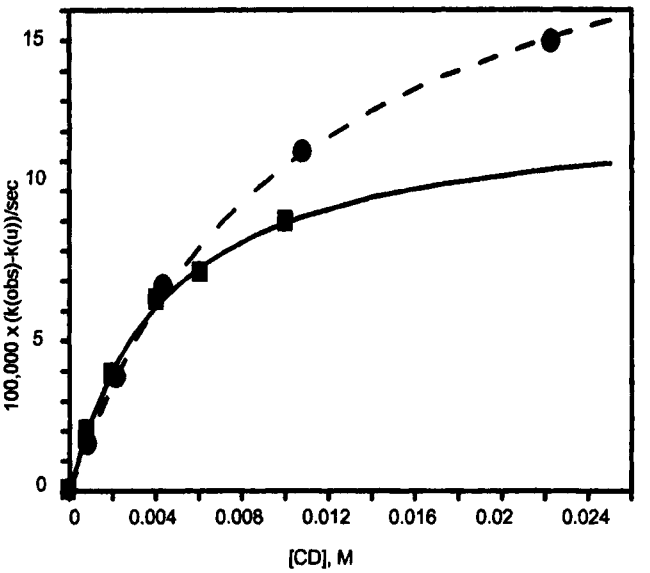


Figure 1. Effects of α-CD (dashed line, ●) and β-CD (solid line, ■) on the hydrolysis of **1** at pH 8.5. Lines are generated using equation 1 (see Table 1).

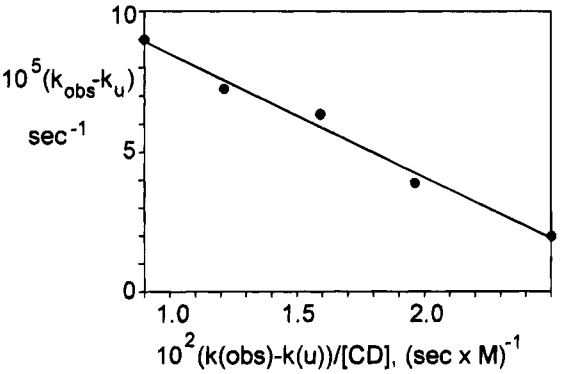


Figure 2. Eadie-Hoftsee plot of the effects of β-CD on hydrolysis of **1** at pH 8.5. The slope of the best-fit line is K_d and the intercept is k_{cat}-k_u.

Table 1. Kinetic Parameters for CD-Mediated Hydrolysis of Trifluoroacetanilides Derived from Eadie–Hoftsee Analysis[a]

substrate	CD	k_{cat}, 10^4 s^{-1}	k_{cat}/k_u	K_d, 10^3 M	k_{cat}/K_d, 10^2 s^{-1} M^{-1}	K_{TS}, 10^3 M
1	α	5.50 ± 0.09	1.68 ± 0.03	10.4 ± 0.7	5.3 ± 0.4	6.2 ± 0.4
1	β	5.10 ± 0.05	1.34 ± 0.02	4.4[b] ± 0.3	11.7 ± 0.9	3.3 ± 0.2
2	β	0.32 ± 0.02	0.64 ± 0.04	3.2 ± 0.6	1.0 ± 0.2	4.9 ± 0.9
3	β	0.13 ± 0.02	0.42 ± 0.07	2.3 ± 0.3	0.57 ± 0.18	5.5 ± 0.9
4[c]	β	0.10 ± 0.06	0.8 ± 0.5	3 ± 1	0.35 ± 0.31	3.9 ± 1.3

[a] See eq 1. Error limits in measured rate constants are <5%. Error in calculated constants is that given by the program used to fit the data. [b] Spectrophotometric determination at pH 6.9 gave a value of $(3.6 \pm 0.6) \times 10^{-3}$ M. [c] The parameters for hydrolysis of **4** are based on analysis of the data when [CD] < 6×10^{-3} M.

(10) Tee, O. S.; Hoeven, J. J. *J. Am. Chem. Soc.* **1989**, *111*, 8318.
(11) Jaffé, H. H. *Chem. Rev.* **1953**, *53*, 191.
(12) Komiyama, M.; Bender, M. L. *J. Am. Chem. Soc.* **1978**, *100*, 5977.
(13) Values for ρ were obtained from: (a) Swain, C. G.; Lupton, E. C., Jr. *J. Am. Chem. Soc.* **1968**, *90*, 4328. (b) Isaacs, N. S. *Experiments in Physical Organic Chemistry*; Collier-MacMillan: London, 1969.

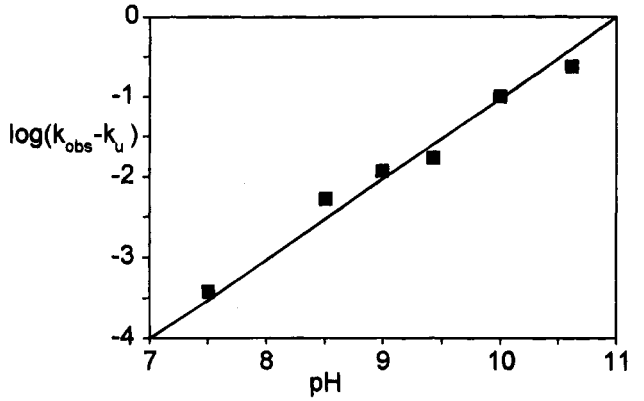


Figure 3. pH dependence of the hydrolysis of **1** in the presence of 1×10^{-2} M β-CD.

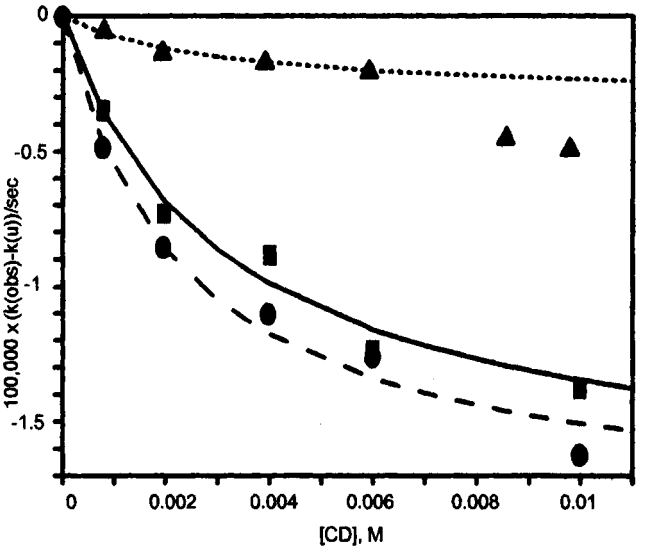


Figure 4. Effects of β-CD on the hydrolysis of **2** (solid line, ■), **3** (dashed line, ●), and **4** (dotted line, ▲) at pH 8.5. The lines are generated using equation 1 and the parameters of Table 1. The dotted line pertaining to **4** was generated using data at [CD] < 6×10^{-3} M.

reaction with all four substrates is identical; that is, rate-determining expulsion of substituted aniline from a tetrahedral intermediate occurs in the same manner for each substrate. Conversely, in the Hammett plot drawn for k_u, the point for **1** does not lie on the line drawn for the three less reactive substrates (Figure 5b). It was therefore appropriate to examine further the aqueous hydrolysis of the *N*-methyltrifluoroacetanilides.

Schowen has described the basic aqueous hydrolysis of *N*-methyltrifluoroacetanilides (Scheme 1) as "specific base catalysis superimposed on general base catalysis".[14] This can be expressed by eq 2, in which k_{Bt} and $[B]_t$ refer

$$k_{obs} = k_w + k_{OH^-}[OH^-] + k_{Bt}[B]_t[OH^-] \qquad (2)$$

to the total buffer concentration. Values of k_{OH^-} (assuming negligible k_w) and k_{Bt} were obtained from linear plots of $k_{obs}/[OH^-]$ vs $[B]_t$ in borate buffer (Tables 2 and 3). A Hammett plot of k_{OH^-} is linear for all substrates (not shown), but the plot of $\log(k_{Bt})[OH^-]$ versus σ^- again yields a straight line from which the point for **1** deviates considerably (Figure 5b). Further examination of buffer catalysis provides values for the general acid and base contributions for **1** and **3**, the latter representative of the three less reactive substrates (Table 3). Hydrolysis of **1** is dependent on the basic but not the acidic form of the buffer. In contrast, hydrolysis of **3** is dependent on both forms, with k_{BH} providing the larger contribution.

(14) (a) Schowen, R. L.; Zuorick, G. W. *J. Am. Chem. Soc.* **1966**, *88*, 1223. (b) Schowen, R. L.; Jayaraman, H.; Kershner, L. *Ibid.* **1966**, *88*, 3373. (c) Schowen, R. L.; Jayaraman, H.; Kershner, L.; Zuorick, G. W. *Ibid.* **1966**, *88*, 4008. (d) Kershner, L. D.; Schowen, R. L. *Ibid.* **1971**, *93*, 2014. (e) Drake, D.; Schowen, R. L.; Jayaraman, H. *Ibid.* **1973**, *95*, 454.

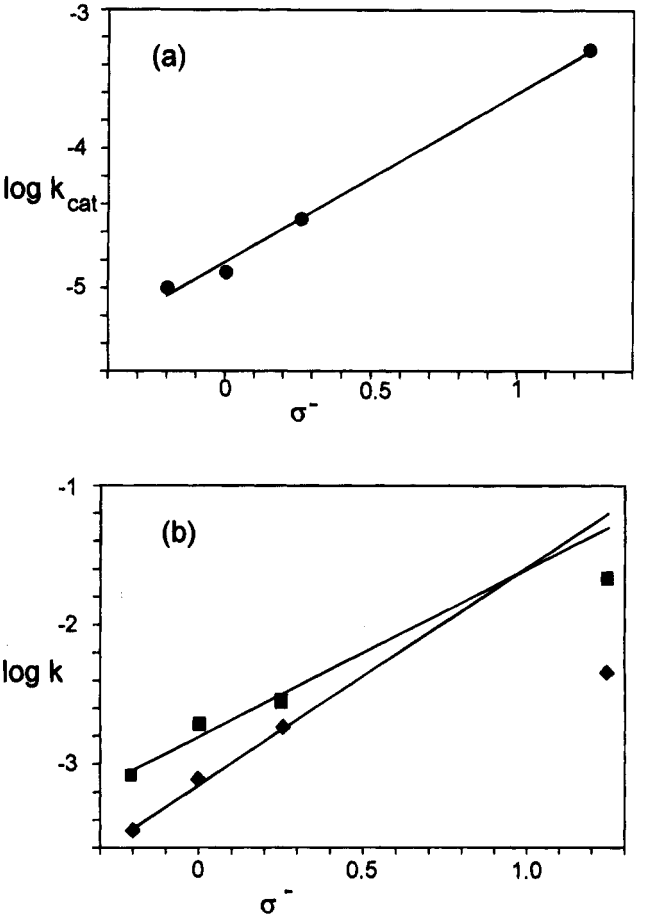


Figure 5. Hammett plots for (a) k_{cat} (●), and (b) k_u (■), and $k_{Bt}[OH^-]$ (◆) as defined in equations (1) and (2).

Scheme 2

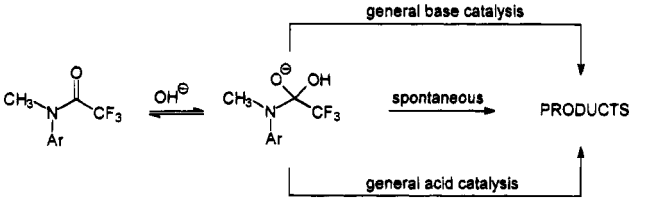


Table 2. Dependence of *N*-Methyltrifluoroacetanilide Hydrolysis on Borate Concentration at pH 9.54[a]

substrate	$10^4 \times$ slope = $k_{Bt}[OH^-]$, s^{-1} M^{-1}	$10^5 \times$ intercept = $k_w + k_{OH^-}[OH^-]$, s^{-1}
1	46 ± 1	168.7 ± 0.7
2	17 ± 1	11.9 ± 0.8
3	7.4 ± 0.4	7.2 ± 0.2
4	3.4 ± 0.3	3.5 ± 0.2

[a] See eq 2.

Discussion

Anilide Hydrolysis in the Absence of CD. Consideration of Hammett plots (Figure 5) and evaluation

Table 3. Evaluated Rate Constants for the Borate-Catalyzed Hydrolysis of *N*-Methyl-*p*-nitrotrifluoroacetanilide (1) and *N*-Methyltrifluoroacetanilide (3)[a]

substrate	$10^5 \times k_w$, s^{-1}	k_{OH^-}, $s^{-1}\,M^{-1}$	k_{BH}, $s^{-1}\,M^{-2}$	k_{B^-}, $s^{-1}\,M^{-2}$
1	34 ± 5	40 ± 1	0	213 ± 9
3	3.53 ± 0.03	1.090 ± 0.008	27.6 ± 0.2	18.6 ± 0.2

[a] See eq 2. BH and B^- refer to the acid and base forms of the buffer.

of buffer catalysis (Tables 2 and 3) clearly demonstrates that the aqueous hydrolysis pathways for 1 differ from those available to the less reactive anilides (**2**–**4**). It has been shown (*vide supra*) that hydrolysis of **1** shows no dependence on general acid in the pH region under study, but instead is dominated by a general base catalysis term. This hydrolysis reaction proceeds through a tetrahedral hydroxide adduct, which is deprotonated by general base to give the dianionic tetrahedral intermediate which breaks down to products, in a mechanism that has been termed general base with superimposed specific base catalysis.[14] The lower pK_a at nitrogen of the less reactive anilides, represented by **3**, permits and requires a general acid-catalyzed pathway which dominates over the general base mechanism seen for hydrolysis of **1**. This is consistent with Schowen's analysis of the basic aqueous hydrolysis in which it was suggested that for substrates with poorer leaving groups (such as **3** and **4**), breakdown of the monoanionic and dianionic tetrahedral intermedates formed by attack of hydroxide requires proton transfer to nitrogen from a general acid in the transition state or in the case of substrates with better leaving groups (such as **2**), solvent-assisted heavy atom bond breaking in the transition state, with subsequent proton transfer.[14d] Unfortunately, Schowen's work did not include the activated anilide **1** and thus does not provide a rationale for any difference in behaviour between **1** and the less reactive anilides **2**–**4**.

The CD-mediated reaction is an alcoholysis rather than a hydrolysis reaction, and thus the general base route (*vide supra*) is not available. Previous studies of the basic methanolysis of **2**–**4**[15] indicated a mechanism via specific base–general acid catalysis arising from acid-catalyzed breakdown of the methoxide adduct. Presumably, this is the same mechanism that is dominant in the buffer-catalyzed hydrolysis of **2**–**4**. Cyclodextrinolysis may operate by a similar mechanism or alternatively a simple specific base mechanism comparable to that described by k_{OH^-}. It is important to note that all of the pathways represented in eq 2 involve rate-determining breakdown of the tetrahedral intermediate. However, CD-accelerated ester hydrolysis has been argued to result from accelerated formation of a tetrahedral intermediate, despite the absence of "electrophilic assistance" to loss of the leaving group and breakdown of that intermediate.[4] Thus if, in the CD-mediated hydrolysis of anilides, the same factors influence the formation and breakdown of the tetrahedral intermediates as in CD-mediated hydrolysis of esters, one would predict CD to be incapable of catalyzing anilide hydrolysis over the background rate. This prediction is not borne out fully by experiment.

Conflicting Literature on CD-Mediated Anilide Hydrolysis. Bender reported that addition of α-CD increased the rate of hydrolysis of trifluoroacetanilide, as well as *p*- and *m*-nitrotrifluoroacetanilide at pH 9.[3] More recently, de Rossi observed[9] that β-CD caused a decrease in the rate of trifluoroacetanilide hydrolysis at pH 8 but also caused a small increase in the rate of *m*-nitrotrifluoroacetanilide hydrolysis at pH 10.[16] In studying the hydrolysis of *p*-nitrotrifluoroacetanilide, de Rossi observed the effects of CDs to vary with pH. These results indicate that the form of catalysis due to α- and β-CD's is dependent on the identity of CD and substrate and, furthermore, that the catalytic action of these CDs can change with pH. Such conclusions are somewhat surprising considering that hydrolysis effected by both α- and β-CD is thought to be operating by a similar mechanism, which rests on the ionization of a secondary hydroxyl group on CD, pK_a 12.1.[2b] Thus, it is reasonable to anticipate a linear increase in CD-mediated catalysis as solution pH increases toward 12.

An alternative explanation for the complex pH dependence reported for *p*-nitrotrifluoroacetanilide is not dependent on change in the action of CD, but rather on ionization of the substrate. Both Bender and de Rossi attempted to account for substrate deprotonation in the kinetic analysis by assuming that ionized substrate did not undergo hydrolysis. This treatment has been widely accepted, although it has been argued that the anions of trifluoroacetanilide[6d] and 2,4-dinitrotrifluoroacetanilide[5] do undergo hydrolysis. Further complexity was added by de Rossi in the analysis of the effect of β-CD on *p*-nitrotrifluoroacetanilde to account for reaction *via* 2:1 complexes. Absorption spectra were reported which showed two kinds of host–guest interactions; at [CD] > 4×10^{-3} M, a second set of spectra were observed, assigned to a 2:1 complex.[9] Our studies on β-CD with **1** or **4** showed no such effects up to [CD] = 10.3×10^{-3} M at pH 6.9. The linearity of the Eadie–Hoftsee plot (Figure 2) supports the conclusion that CD catalysis involves a 1:1 complex. This conclusion, along with the use of nonionizable substrates in the present study, allows for the simplified kinetic treatment described above.

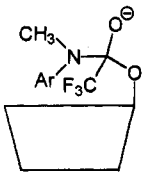
In this present study, at pH 8.5, both α- and β-CD are shown to have similar and consistent effects on all substrates investigated; those hydrolyses accelerated by β-CD were also accelerated by α-CD, and those which were retarded by β-CD were retarded by α-CD (Table 1). Hydrolyses of **1** and **2** in the presence of 10 mM β-CD were studied further at 7.5 < pH < 10.6. In both cases, the increase in rate due to CD was linear and dependent on $[OH^-]$.

Mechanism of CD-Mediated Hydrolysis. The effects of CD on the rate of hydrolysis of **1** appear to be strikingly different from those on the less reactive anilides (**2**–**4**): hydrolysis of **1** is accelerated by CD ($k_{cat} > k_u$) (Figure 1) but hydrolysis of **2**–**4** is retarded ($k_{cat} < k_u$) (Figure 4). Conversely, all substrates demonstrate saturation kinetics, show similar binding constants with CD, and in particular, conform to a linear Hammett correlation (Figure 5a). The simplest interpretation invokes CD mediating the hydrolysis of each anilide via a common mechanism, but only in the case of **1** is $k_{cat} > k_u$. Although CD accelerates the rate of formation of a tetrahedral intermediate, it is acceleration of the break-

(15) (a) Schowen, R. L.; Hopper, C. R.; Bazikian, C. M. *J. Am. Chem. Soc.* **1972**, *94*, 3095. (b) Hopper, C. R.; Schowen, R. L.; Venkatasubban, K. S.; Jayaraman, H.; *Ibid.* **1973**, *95*, 3280.

(16) see also: Palmer, D. R. J.; Buncel, E.; Thatcher, G. R. J. *Minutes of the 6th International Symposium on Cyclodextrins*, Editions de Santé **1992**, 86.

down of **5** to aniline and acylated-CD that is important for catalysis.



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p-Nitrotrifluoroacetanilide has been shown to hydrolyze with rate-determining formation of the tetrahedral intermediate at pH < 9[6c,9] or 9.6.[6d] An in-depth examination by Fife[5] argues against this being the case for the hydrolysis of **1** under conditions similar to ours. The linearity of the Hammett plot for k_{cat} indicates that the rate-determining step does not change for the CD-mediated reactions: breakdown is rate-determining for all four substrates.

How does the CD cavity stabilize the transition state for breakdown via C—N bond cleavage? The secondary face hydroxyls may provide a general acid or "electrophilic catalysis" mechanism. However, one would then expect **2**–**4** to show catalysis based upon the importance of the general-acid, buffer-catalyzed breakdown pathway for these compounds in the absence of CD (Table 3). That **1** shows the greater catalysis suggests instead that the CD cavity is complementary to the transition state for C—N bond cleavage. Indeed, molecular models demonstrate that for both the substrate and the CD-adduct, steric constraints prevent favorable interactions of both of the hydrophobic groups, trifluoromethyl and aryl, with the hydrophobic cavity. Cleavage of the C—N bond allows such stabilizing interactions.

In considering the possibility that CD stabilizes the tetrahedral intermediate, it is interesting to note that in only four cases have tetrahedral intermediates of acyl transfer reactions to or from nitrogen been detected,[17] consistent with calculations by Guthrie which indicate that such intermediates should be less stable than those of acyl transfer between two oxygen atoms.[18] All four are intermediates of an intramolecular acyl transfer (making the carbon atom part of a ring structure), and all contain a group capable of conjugating with nitrogen (such as phenyl) which reduces the propensity to expel alkoxide. A common feature of detectable tetrahedral intermediates is a trifluomethyl group. Many of these features are incorporated into the CD-adduct.

Menger has coined the term "*p*-nitrophenyl ester syndrome" to refer to enzyme modeling studies, particularly using CDs, on highly activated substrates.[4,19] It was shown that tetrahedral CD-intermediates tend to partition toward products only when the leaving group pK_a is less than 10. In the specific example discussed, rate enhancement at pH 10 dropped by 5 orders of magnitude when the leaving group was changed from *p*-nitrophenyl to *p*-nitrobenzyl.[4] Our results are in agreement with these findings, since a very small rate enhancement is observed for the hydrolysis of **1**, which has leaving group pK_a of about 18.5.[20] The phenomenon of CD-induced retardation of a hydrolysis reaction has been observed previously. Bender found that in the α-CD mediated hydrolysis of *p*-carboxyphenyl esters, changing the ester function from acetate to 3,3-dimethylbutyrate caused k_{cat}/k_u to drop from 5.3 to 0.19.[2a] However, the rationale in this system rests on a different binding mode for the the two esters with CD. Tee has studied such reactions and rationalized Bender's results in terms of differences in the apparent binding of the transition state (K_{TS}) and the ground state to CD. K_{TS} is defined as the apparent dissociation of the transition state of the catalyzed (CD-bound) reaction into the transition state of the uncatalyzed reaction and CD.[21] Since stabilization of the transition state relative to the ground state is the origin of catalysis, one can rationalize effects of catalysts by comparing trends in K_{TS} relative to K_d.[22] Tee states that Bender's data show that the change from rate acceleration to retardation by α-CD occurs because there is more of an increase in the strength of substrate binding than of apparent binding of the transition state as the ester moiety becomes more hydrophobic. The values of K_{TS} for the hydrolyses of **1**–**3** show the same trend noticed in Bender's results (Table 1). The decrease in k_{cat}/k_u is accompanied by an increase in substrate binding (K_d decreases) but a deterioration of apparent transition state binding (K_{TS} increases). However, it is doubtful in this present case that the small change in aromatic substituent causes a change in the mode of substrate binding *in simile* with Bender's system. The changes in K_{TS} and K_d are small, apparently confirming that binding is similar for all substrates (**1**–**3**).

Cyclodextrin and Chymotrypsin. Testimony as to the importance of CDs as enzyme models can be found in contemporary textbooks. Bender has pointed out that the rate-determining step in chymotrypsin-catalyzed peptide hydrolysis is acylation of the enzyme and that acylation is also the rate-determining step for CD-catalyzed trifluoroacetanilide hydrolysis.[3] On this basis, Komiyama and Bender called CDs "excellent models of hydrolytic enzymes". This conclusion should apply equally well to *N*-methyltrifluoroacetanilides, but in most cases the effect of CD on tertiary anilide hydrolysis (i.e., inhibition of hydrolysis) is opposite to that of chymotrypsin on peptide hydrolysis. Because the *N*-methylated substrates in this present study do not ionize under mild conditions, they may be considered closer analogues of peptides than the anilides previously studied. It is clear that CD is poorly suited to assist in breakdown of the tetrahedral intermediate, whereas such assistance is essential to the mechanism of chymotrypsin, which is proposed to employ a protonated histidine residue to facilitate this process.[12,23] Furthermore, in contrast to chymotrypsin,[12] CD-mediated hydrolytic reactions, including those of **1**–**4**, show positive ρ values.

CD is truly catalytic in hydrolysis of **1**. Michaelis-Menten saturation kinetics are observed. Catalysis of both formation and breakdown of the tetrahedral intermediate is indicated by the data. Catalysis is observed at neutral pH. All these indicators are comparable to features of chymotrypsin catalysis of amide hydrolysis.[24] However, the rate acceleration for CD-catalysis is only

(17) Capon, B.; Dosunmu, M. I.; Sanchez, M. N. M. *Adv. Phys. Org. Chem.* **1985**, *21*, 37.
(18) (a) Guthrie, J. P. *J. Am. Chem. Soc.* **1974**, *96*, 3608; (b) **1973**, *95*, 6999.
(19) Breslow, R.; Singh, S. *Bioorg. Chem.* **1988**, *17*, 408.
(20) Dolman, D. D.; Stewart, R. *Can. J. Chem.* **1967**, *45*, 911.
(21) Tee, O. S. *Carbohydr. Res.* **1989**, *192*, 181.
(22) (a) Kurz, J. L. *J. Am. Chem. Soc.* **1963**, *85*, 987. (b) Lienhard, G. E. *Science (Washington, D.C.)* **1973**, *180*, 149. (c) Pregel, M. J.; Dunn, E. J.; Buncel, E. *J. Am. Chem Soc.* **1991**, *113*, 3545.
(23) Walsh, C. *Enzymatic Reaction Mechanisms*; W. H. Freeman: New York, 1979; p 94–97.

1.4−1.7, and most importantly, CD cannot provide general acid catalysis of breakdown of the tetrahedral intermediate, the key function of enzymes designed to hydrolyze amide bonds.

Conclusions

Both α- and β-cyclodextrin catalyze the hydrolysis of *N*-methyl-*p*-nitrotrifluoroacetanilide (**1**) and inhibit hydrolysis of the *p*-chloroanilides, unsubstituted anilides, and *p*-methoxyanilides **2**−**4**. Comparison with data for hydrolysis in the absence of cyclodextrin yields a unified mechanism for cyclodextrin-mediated hydrolysis of all four anilides via 1:1 CD−substrate complexes. Catalysis of *N*-methyl-*p*-nitrotrifluoroacetanilide hydrolysis by β-cyclodextrin requires acceleration of C−N bond cleavage from the tetrahedral intermediate. Although this catalysis and acceleration bear comparison with chymotrypsin, the inhibition observed for the less reactive anilides and the absence of general acid catalysis of C−N bond cleavage suggest cyclodextrin to be a poor chymotrypsin model.

Experimental Section

N-Methylanilines and α- and β-CDs were purchased from the Aldrich Chemical Co. β-CD was recrystallized before use. *N*-Methyltrifluoroacetanilides were prepared as described in the literature.[14d] The aniline was stirred in dry THF under N_2 at 0 °C, and an excess of trifluoroacetic anhydride was added slowly. The solution was allowed to warm to room temperature, ice−water was added, and the precipitated *N*-methyltrifluoroacetanilide was filtered. In the case of **4**, the product was an oil which was extracted into $CHCl_3$ and concentrated *in vacuo*. The solid was recrystallized from MeOH (**1**), MeOH/H2O (**2**), or ether/petroleum ether (**3**, **4**). Melting points were determined: **1**, 126−128 °C (lit.[13d] mp 126.5−128 °C); **2**, 65-67 °C (lit.[13d] mp 66−68 °C); **3**, 26−27 °C (lit.[13d] mp 26−28 °C); **4**, 40.5−42 °C (lit.[13d] mp 42−43.5 °C).

All kinetic measurements were made at 20 °C using 0.1 M buffer, 0.1 M KCl, and 50 μM substrate. Buffers used were phosphate (pH < 8.0), borate (pH 8.0− 9.9), or carbonate (pH > 9.9). Reactions were initiated by injection of 20 μL of a stock solution of substrate in dry CH_3CN into 2.18 mL of buffer solution. Reactions were followed by monitoring increase in absorbance due to product aniline at the following wavelengths using a CARY 3 or HP452A UV−vis spectophotometer: **1**, 408 nm; **2**, 246 nm; **3**, 238 nm; **4**, 299 nm. Good pseudo-first-order kinetics were observed for >4 half-lives in all cases. Potential inhibition experiment was carried out using 10×10^{-3} M β-CD and 60×10^{-3} M cyclohexanol. Analysis of buffer dependence was performed using 0.008 M < [borate] < 0.100 M for all substrates at pH 9.54 and also for **1** and **3** at pH 9.0 and 9.84.

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(24) Direct comparison of the catalytic rates of CD and chymotrypsin for hydrolysis of aryl acetates has been employed to support CD's as serine protease mimics. Indeed, CD is a better catalyst of hydrolysis of **1** at neutral pH than chymotrypsin, but only because **1** is not a substrate for this enzyme.