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Biological Catalysis Regulated by Cucurbit[7]uril Molecular Containers

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Abstract: We report the synthesis of two-faced inhibitors **1**−**5** that contain both enzyme inhibitor and cucurbit[n]uril binding domains. The enzyme binding domains of **1**−**5** bind to the active sites of bovine carbonic anhydrase (BCA) or acetylcholinesterase (AChE) and inhibit their catalytic activities. Addition of CB[7] to BCA•**1** and BCA•**2** results in the transient formation of the BCA•**1**•CB[7] and BCA•**2**•CB[7] ternary complexes that undergo rapid dissociation to form free catalytically active BCA along with CB[7]•**1** and CB[7]•**2**. The on−off cycle can be performed repetitively by the sequential addition of competitive guest **8** and CB[7]. The detailed origins of this on−off switching of the catalytic activity of BCA is delineated by the combined inference of UV/vis catalytic assays, fluorescence displacement assays, ^{1}H NMR, along with measurement of the fundamental values of K_a, k_{on}, and k_{off} for the various complexes involved. In contrast, addition of CB[7] to AChE•$\mathbf{4}_4$ and AChE•$\mathbf{5}_4$ results in the formation of thermodynamically stable ternary complexes AChE•$\mathbf{4}_4$•CB[7]$_4$ and AChE•$\mathbf{5}_4$•CB[7]$_4$ that are catalytically inactive. We highlight some of the advantages and disadvantages of the strategy, based on the direct competition between two receptors (e.g., enzyme and CB[7]) for a common inhibitor, used in this paper to control enzyme catalytic activity compared to the strategy employed by Nature involving the binding of an allosteric small molecule remote from the enzyme active site.

Introduction

Over the past decade the cucurbit[n]uril (CB[n]) family of macrocycles has emerged as a premiere platform for basic and applied studies of molecular recognition and self-assembly processes in aqueous solution.[1,2] For example, the CB[n] family of macrocycles has been used in the construction of a variety of stimuli responsive (pH, photochemical, electrochemical, chemical) molecular machines, in chemical sensing applications, and as the critical stationary phase component for chromatographic and remediation applications.[2,3] Concurrent with these advances in technological applications, members of the CB[n] family have begun to be studied as molecular containers for a variety of biologically active pharmaceuticals[4] and first steps are now being taken to apply CB[n] compounds in drug delivery applications.[5] For many years, our group has been involved in the elucidation of the mechanism of CB[n] formation[6,7] that lead us to the isolation of new CB[n] (*i*-CB[n] and CB[10]),[8] *nor-seco*-CB[n] (bis-*ns*-CB[10], (±)-bis-*ns*-CB[6], *ns*-CB[6]),[9] and acyclic glycoluril oligomers[7] that exhibit a variety of interesting biomimetic phenomena including controlling folding of non-natural oligomers, mimics of metalloenzymes, homo-

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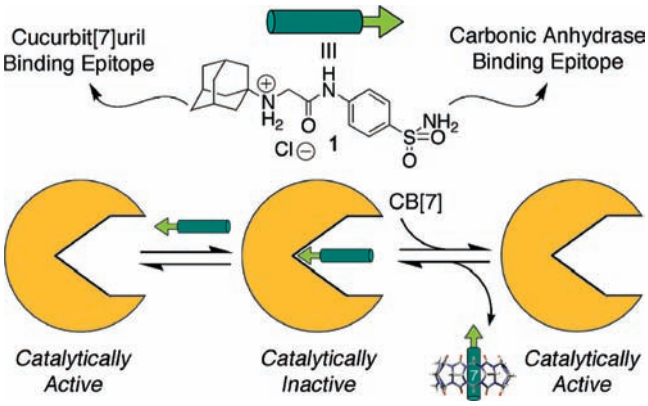
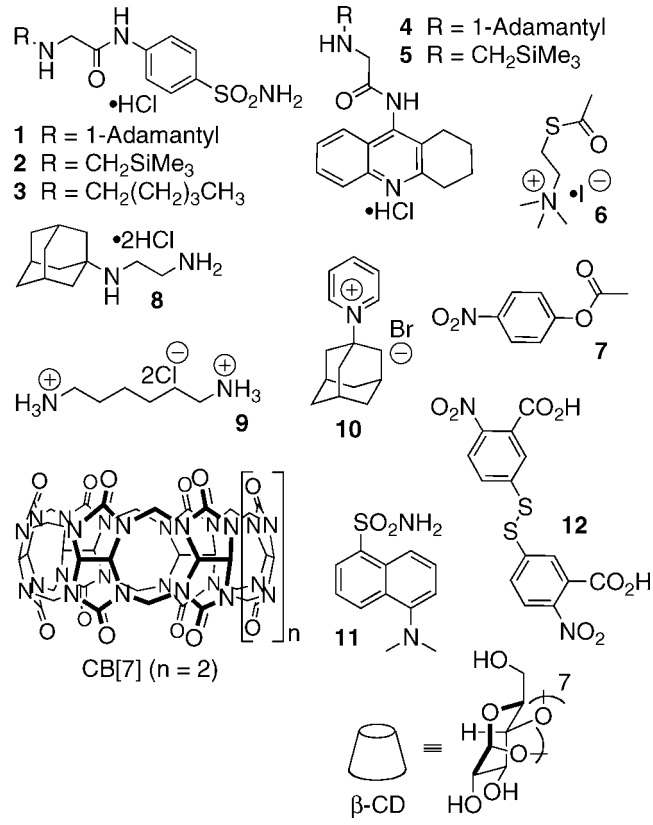
Scheme 1. Thermodynamic Shuttling of **1** between BCA and CB[7]



tropic allostery, chiral recognition, and metal-ion triggered folding and assembly reminiscent of natural RNA. The ability of CB[n] molecular containers to participate in high-fidelity biomimetic applications can be traced to the extremely high affinities (K_a up to 10^{15} M^{-1}) and selectivities (K_{rel} up to 10^7) that CB[n] display toward cationic species in water.[10−12] Such values of affinity and selectivity meet and exceed those typically observed in the interaction of proteins and antibodies toward their cognate ligands.[13] Accordingly, we wondered whether it would be possible to introduce CB[n] compounds into biological systems and thereby influence the behavior of biological receptors toward their guests and modulate their biological activity.

We have previously shown that the components of a series of well-defined aggregates from the literature are capable of reforming those aggregates even when present within a more complex multicomponent mixture through a self-sorting process.[14] Because of their high values of K_a and high selectivities CB[n] molecular containers are prime components for the construction of self-sorting systems under thermodynamic control.[11] As a strategy to develop systems that were also capable of self-sorting under kinetic control, which are immediately more relevant to biological systems that operate far from equilibrium, we have explored the behavior of compounds that contain two different binding epitopes that we refer to as two-faced guests.[15] Such hybrid guests are well precedented in medicinal chemistry where two drugs are often covalently linked in hopes of combining the desirable properties of both.[16] For example, a hybrid drug combining chloroquine with imipramine overcomes resistance problems by inhibiting the drug efflux transmembrane channel.[17] In this paper we continue this line

Chart 1. Compounds Used in this Study



of inquiry by investigating the behavior of two-faced guests that combine an enzyme inhibitor binding epitope with a CB[n] binding epitope.[18] We were confident that such two-faced guests would turn off the catalytic activity of their cognate enzyme by binding of the enzyme inhibitor binding epitope to the enzyme active site. We anticipated that CB[7] would be capable of turning on the enzyme activity by sequestering the two-faced inhibitor by binding to the CB[n] binding epitope. This strategy is illustrated in Scheme 1 based on two-faced guest **1** that contains adamantaneammonium ($K_a \approx 10^{12}$ M^{-1} toward CB[7]) and benzenesulfonamide binding epitopes. This strategy differs from the one typically used by Nature to regulate enzyme activity, allosteric regulation, in that direct competition between two receptors (enzyme and CB[7]) is involved. In this paper we describe the successful implementation of this strategy for the regulation of the catalytic activity of bovine carbonic anhydrase (BCA),[19] discuss some of the limitations of this approach illustrated by attempted regulation of acetylcholine esterase (AChE), and finally use these results to speculate on why Nature has evolved allosteric systems to regulate enzyme catalysis.

Results and Discussion

This results and discussion section is organized as follows. First, we introduce the enzyme assays used to monitor the catalytic activity of BCA and AChE. Next, we demonstrate how

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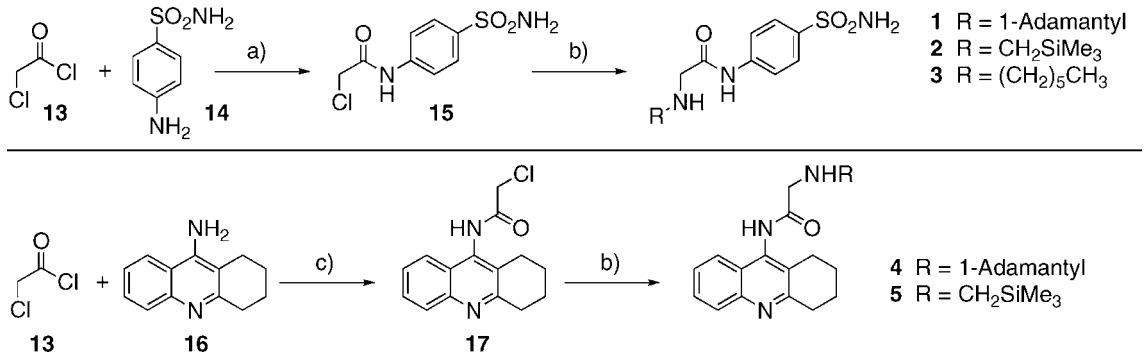
Scheme 2. Synthesis of **1**−**5**[a]



[a] Conditions: (a) K_2CO_3 or Et_3N, THF, reflux, (b) RNH_2, K_2CO_3, KI, anh. THF, reflux, (c) CH_3COCH_3, reflux.

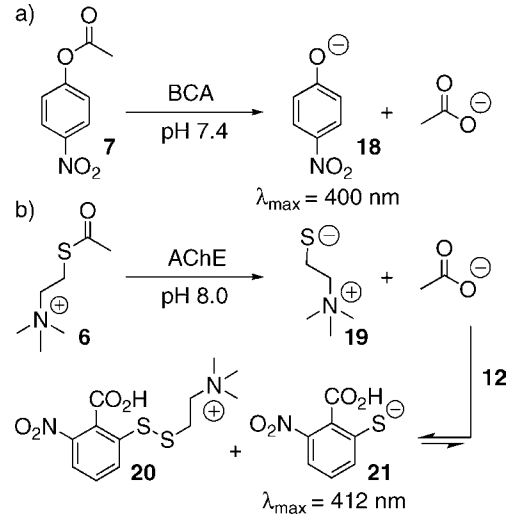
CB[7] can regenerate enzymatic activity of BCA by sequestering compound **1** and **2** from its active site. Subsequently, we discuss in detail the mechanism of the shuttling of **1** and **2** between CB[7] and BCA based on results from fluorophore displacement assays. In the final section of this paper we discuss the pitfalls encountered when we attempted to use two-faced guest molecules to control the enzymatic behavior of AChE.

Design Aspects of the Chemical Components Used in this Study. Chart 1 shows the chemical structures of molecular containers CB[7] and β-CD, two-faced guests **1**−**5**, colorimetric substrates for AChE (compounds **6** and **12**) and BCA (compound **7**), competitive binding guests for CB[7] and β-CD (**8**−**10**), and dye (**11**) used in fluorescence displacement assays with BCA that were used in this study. Compounds **1**−**3** and **4** and **5** each contain a single CB[7] binding epitope (adamantane ammonium ($K_a \approx 10^{12}$ M^{-1}), trimethylsilylmethyl ammonium ($K_a \approx 10^9$ M^{-1}), or hexylammonium ($K_a \approx 10^6$ M^{-1})) connected by a short linker to enzyme binding epitopes (benzenesulfonamide for BCA, ($K_a \approx 10^6$ M^{-1}), tacrine for AChE ($K_a \approx 10^9$ M^{-1})). We expected that by varying the strength of the CB[7] binding interaction relative to that of the enzyme•inhibitor binding strength that we would observe different behavior.

Synthesis of Guests 1−5. Scheme 2 shows the synthesis of two-faced guests **1**−**5**. For the synthesis of **1**−**3** we start by the reaction of aniline **14** with acid chloride **13** to deliver **15** in 83% yield according to the literature procedure.[20] Compound **15** was subsequently reacted with the appropriate amine under Finkelstein reaction conditions to deliver **1**−**3** in 68−75% yield. Compounds **4** and **5** were synthesized in two steps from **16** in a similar manner. Compound **17** was prepared by the acylation of **16** with **13**.[21] Subsequently, **17** was alkylated with adamantaneamine or $H_2NCH_2SiMe_3$ to give **4** and **5** in 60 and 65% yield, respectively.

Chemical Reactions Used to Monitor Enzymatic Activities. We used two straightforward colorimetric assays that allowed us to quantify the catalytic activity of BCA and AChE (Scheme 3). BCA is an esterase that catalyzes the hydrolysis of *p*-nitrophenylacetate (**7**) to form *p*-nitrophenolate (**18**), which is yellow colored and has an UV/vis absorption maximum at 400 nm. When the active site of BCA is bound to an inhibitor the catalytic hydrolysis is turned off and the formation of **18** is not observed. Consequently, the catalytic activity of BCA is reflected in the rate of hydrolysis of **7** and can be monitored by

Scheme 3. Chemical Reactions Used to Monitor Enzymatic Activities



measuring the absorbance at 400 nm by UV/vis spectroscopy (Scheme 3a).[22,23] Similarly, the enzymatic activity of AChE can be measured by the Ellman assay. The Ellman assay relies on the AChE catalyzed hydrolysis of acetylthiocholine (**6**) to **19** and acetate. Thiolate **19** reacts rapidly with disulfide **12** to generate thiophenolate **21**, which has a yellow color (λ_{max} = 412 nm). The rate of enzyme catalyzed hydrolysis can, therefore, be monitored by measuring absorbance at 412 nm using UV/vis spectroscopy (Scheme 3b).[24]

Regulation of Biological Catalysis of Bovine Carbonic Anhydrase. As a first test of our design strategy, we monitored the enzymatic hydrolysis of **7** by BCA alone (Figure 1, step 1) and after the addition of **1** (Figure 1, step 2). A shut down of the hydrolysis of **7** was observed, indicating that **1** inhibits the enzymatic activity of BCA as expected due to formation of BCA•**1**. Next (Figure 1, step 3) we added CB[7] to the solution of BCA•**1** and monitored the rate of hydrolysis by UV/vis spectroscopy. In this instance we observed only 45% regeneration of the initial enzymatic activity (Figure 1b). Given that the binding affinity of CB[7] toward **1** ($K_a = 4.1 \times 10^{12}$ M^{-1}, *vide infra*) was much higher than that of BCA toward **1** ((1.08 ± 0.10) × 10^8 M^{-1}), we found this result rather surprising.

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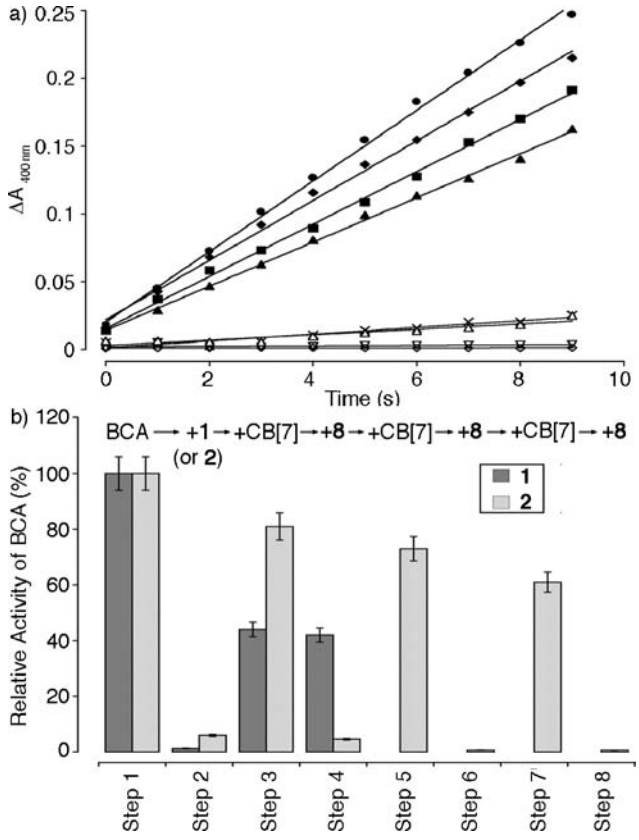

Figure 1. (a) Plot of change in absorbance versus time for the hydrolysis of **7** by BCA (1 μM) in response to the addition of **2** (4 μM) followed by sequential additions of CB[7] (8 μM) and **8** (8 μM). (b) Plot of the relative activity of BCA at each step determined from the initial slope data shown in part a. Step 1 (●), step 2 (×), step 3 (◆), step 4 (△), step 5 (■), step 6 (◇), step 7 (▲), and step 8 (▽). The kinetic assays were performed as described in the literature.[22]

Although we were disappointed with the 45% regeneration of enzymatic activity, we decided to take the experiment one step further and try to turn off the enzymatic activity of BCA by the addition of **8**. Compound **8** binds more tightly to CB[7] than **1** does, and therefore, we expected the formation of CB[7]•**8** along with the regeneration of **1** that would fully inhibit the enzyme by the formation of BCA•**1**. In the experiment we found that addition of **8** (Figure 1, step 4) did not result in a significant change in enzymatic activity. This result indicates that addition of **8** failed to release **1** from CB[7]•**1** over the time scale of the experiment (45 min incubation). It is known from the literature that dissociation of the CB[7]• adamantaneammonium ion complex is slow ($k_{off} = 2 \times 10^{-5}$ s^{-1}; $t_{1/2} = 9$ h). This slow dissociation allows us to rationalize the inability of **8** to function in Figure 1b, step 4. Although **1** appears at first glance to be ideally suited to control the enzymatic activity of BCA in concert with CB[7] because of its very high thermodynamic binding constant ($K_a = 4.1 \times 10^{12}$ M^{-1}) in reality the slow kinetic dissociation of CB[7]•**1** limits its utility to a single on−off cycle.

The partial regeneration of enzymatic activity observed using **1** was intuitively promising and inspired us to modify our design to improve the performance over multiple on−off cycles. We synthesized two-faced guest **2** bearing a (trimethylsilyl)methyl ammonium group. Trimethylsilylmethylammonium ion ($Me_3SiCH_2NH_3^+$) is known from the literature[11] to bind tightly ($K_a \approx 8.9 \times 10^8$ M^{-1}) to CB[7] with exchange kinetics that are fast on the laboratory time scale. Figure 1, panels a and b, show that addition of **2** to a solution of BCA (Figure 1, steps 1 and 2) nearly completely inhibits the enzymatic activity of BCA as monitored by UV/vis spectroscopy. When we added CB[7] to a solution of BCA•**2** (Figure 1, step 3) we observed 83% regeneration of the initial enzymatic activity. In step 4, the addition of **8** triggers the dissociation of CB[7]•**1** that results in the release of free **1** that then inhibits the enzymatic activity of BCA by formation of BCA•**1**.[25] We were able to turn the enzymatic activity of BCA off and on over several additional cycles (Figure 1, steps 5−8) by adding CB[7] and **8** in an alternating sequence (Figure 1, panels a and b).[26]

Strength of the CB[7]•Two-Faced Inhibitor Complex Is Critically Important. If the swapping of two-faced inhibitor **2** from the active site of BCA to CB[7] was driven by the fact that **2** has a higher affinity toward CB[7] than BCA, then the enzymatic activity should not be regenerated if a related two-faced inhibitor (e.g., **3**) was used whose CB[7]•two-faced inhibitor complex was weaker than the BCA•sulfonamide interaction. Based on the literature we expected that the hexylammonium ion tail of **3** would bind relatively weakly to CB[7] ($K_a \approx 10^6$ M^{-1}).[10,11] Experimentally, we did not observe any regeneration of enzymatic activity after addition of CB[7] to a solution containing BCA•**3**. As a second control experiment we attempted to use two-faced inhibitor **1** along with β-CD to control enzymatic activity. β-CD is known to have a modest affinity toward adamantane derivatives ($K_a \approx 10^4$ M^{-1}).[27] As expected, the affinity of β-CD toward the adamantaneammonium epitope of **1** was insufficient to cause the dissociation of BCA•**1** and enzymatic activity was not regenerated. These control experiments indicate that one important factor governing the observed on−off cycling of enzymatic activity is the thermodynamically controlled shuttling of the two-faced inhibitor between BCA and CB[7] based on differences in the value of K_a of the binding epitopes toward their cognate receptors.

Fluorescence Spectroscopy Provides Indirect Evidence for Exchange of the Two-Faced Inhibitor Between BCA and CB[7] under Thermodynamic Control. We used a competitive fluorescence assay to further confirm the binding location of the two-faced inhibitor inferred from the UV/vis studies. For this process we use dye **11** that has a low quantum yield in water that increases markedly (at 460 nm) when bound inside the active site of BCA.[28,29] Accordingly, addition of **1** or **2** to a solution containing BCA•**11** resulted in a decrease in fluorescence intensity at 460 nm that indicates the displacement of **11** from the active site of BCA by **1** or **2** (Scheme 4). In this manner, fluoroscent probe **11** signals whether or not the two-faced guest is bound to the enzyme. Interestingly, when CB[7]

(25) Mohanty previously used competitive binding of sodium to CB[7] to induce transfer of neutral red to bovine serum albumin. See Shaikh, M.; Mohanty, J.; Bhasikuttan, A. C.; Uzunova, V. D.; Nau, W. M.; Pal, H. *Chem. Commun.* **2008**, 3681–3683.

(26) We do not fully understand the steady decrease in activity over several cycles. We note that the entire assay shown in Figure 1 requires more than 12 h to complete and that substantial amounts of CB[7]•**8** accumulate over the various steps. From control experiments we know that some enzymatic activity (~15%) is lost over this time frame and suspect that the remaining 25% may be due to the presence of CB[7]•**8**.

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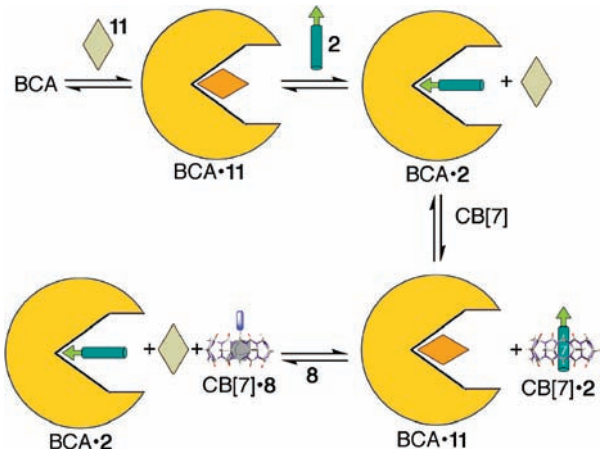
Scheme 4. Schematic Representation of the Fluorophore Displacement Assay Under Thermodynamic Control



was added to the solution of BCA•**2** and **11**, we observed an increase in fluorescence intensity at 460 nm. This observation is consistent with the formation of a mixture comprising BCA•**11** and CB[7]•**2**. Finally, addition of **8** to this mixture of BCA•**11** and CB[7]•**2** results in a decrease in fluorescence intensity at 460 nm signaling the formation of a mixture consisting of CB[7]•**8**, BCA•**2**, and free **11**. The alternate increase and decrease of fluorescence intensity at 460 nm supports our interpretation that the regulation of the biological catalysis of BCA by **2** and CB[7] is due to binding and dissociation of **2** from the active site of BCA triggered by the presence of CB[7].

^{1}H NMR Provides Direct Evidence for the Guest Exchange Process. The UV/vis and fluorescence assays described above serve as indirect evidence of the sequential exchange of **2** between BCA and CB[7]. Direct evidence of guest shuttling can be obtained from ^{1}H NMR spectroscopy by monitoring the chemical shift of the resonance for the $(CH_3)_3SiCH_2NH_2$ group of **2**. Figure 2a shows the ^{1}H NMR spectrum of BCA in the region from +0.5 to −1 ppm. The appearance of a new peak at −0.27 ppm upon addition of **2** to BCA is indicative of the formation of the BCA•**2** complex (Figure 2b). Figure 2e shows the resonances for free **2** and the CB[7]•**2** complex. Figure 2c shows the ^{1}H NMR spectrum of a mixture containing **2**, BCA•**2**, and BCA•**2**•CB[7]. After addition of excess CB[7] the resonance for BCA•**2** disappeared completely and only the resonance for BCA•**2**•CB[7] was observed (Figure 2d). These ^{1}H NMR experiments further support the swapping of **2** between BCA and CB[7] as depicted in Scheme 1 in accord with the UV/vis results.[30]

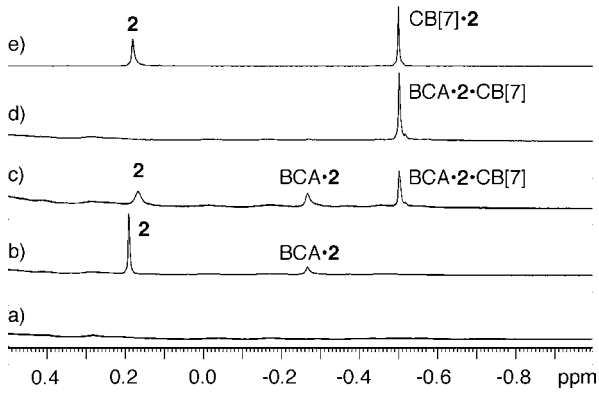


Figure 2. ^{1}H NMR spectra recorded for (400 MHz, 30 mM $Na_2D_2PO_4$ buffer, pD 7.3, 298 K): (a) BCA (0.75 mM, 1 equiv); (b) BCA (0.75 mM, 1 equiv) and **2** (1.32 mM, 1.76 equiv); (c) BCA (0.75 mM, 1 equiv), **2** (1.32 mM, 1.76 equiv), and CB[7] (0.38 mM, 0.5 equiv); (d) BCA (0.75 mM, 1 equiv), **2** (1.32 mM, 1.76 equiv), and CB[7] (1.50 mM, 2.0 equiv); and (e) **2** (2.0 mM, 2 equiv) and CB[7] (1.0 mM, 1 equiv).

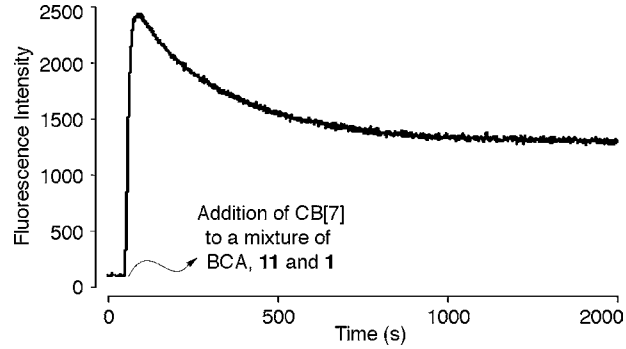


Figure 3. Plot of fluorescence intensity (at 460 nm) versus time after addition of CB[7] (50 μM) to a mixture of **11** (2 μM), **1** (20 μM), and BCA (80 nM). All measurements were done in 20 mM NaH_2PO_4 buffer, pH 7.3, 298 K.

Kinetic Profile Observed During the CB[7] Assisted Shutting of the Two-Faced Inhibitor from the BCA•Two-Faced Inhibitor Complex to the CB[7]•Two-Faced Inhibitor Complex is Unusual. While we were carrying out the fluorophore displacement assay described above, we observed unexpected dynamic (kinetic) behavior during the removal of the two-faced inhibitor from the active site of BCA upon addition of CB[7]. For example, when CB[7] was added to a solution of BCA•**1** and **11** (also to a solution of BCA•**2** and **11**, Supporting Information, Figure S20), we observed an initial rapid increase, reflecting the formation of BCA•**11**, followed by a slower exponential decrease in fluorescence intensity (Figure 3).[31] To begin to understand this complex behavior, we added CB[7]•**1** to a solution of BCA•**11** and observed only the slower exponential decrease in fluorescence intensity. This result strongly suggested to us that CB[7]•**1** (and CB[7]•**2**) are also capable of acting as inhibitors of BCA (*vide infra*). Given the unusual kinetic profile displayed in Figure 3, we decided to determine the fundamental kinetic and thermodynamic parameters controlling this process.

Determination of the Values of K_a, k_{on}, and k_{off}. As described above, the successful alternate turn on and off sequence of the biological catalysis of BCA using **2**, the results obtained from ^{1}H NMR, and control experiments suggested the shuttling of guests operates under thermodynamic control. Conversely, the unusual kinetic profile of the approach to equilibrium (Figure 3) suggested that a knowledge of the kinetics was important. To fully understand the complex behavior of this system we decided to determine the values of K_a, k_{on}, and k_{off} for the various receptor•guest pairs.

Measurement of the K_a Values of the Two-Faced Inhibitors to CB[7]. The association constants for **1** and **2** toward CB[7] were measured by competition with appropriate guests of known values of K_a by using ^{1}H NMR competition experi-

(30) Initially, we were a little surprised that the $(CH_3)_3Si$ resonance appears at the same chemical shift for both CB[7]•**2** and BCA•**2**•CB[7]. On further reflection we realized that in both the CB[7]•**2** and BCA•**2**•CB[7] complex the $(CH_3)_3Si$ group is bound within CB[7] so its chemical shift is not influenced by the protein BCA.

(31) Initially we found this kinetic profile to be counter-intuitive since the concentration of BCA•**11** (fluorescence intensity) overshoots the equilibrium point. This intuition, of course, was based on standard two component systems where the approach to equilibrium occurs asymptotically and overshooting does not occur.

Table 1. Thermodynamic and Kinetic Parameters for BCA•Inhibitor Interactions

	bovine carbonic anhydrase				
	K_a (M^{-1})	ΔG (kcal mol^{-1})	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	$t_{1/2}$ (s)
1	$(1.08 \pm 0.10) \times 10^8$	−10.92	5.1×10^5	$(4.30 \pm 0.01) \times 10^{-3}$	161
2	$(2.73 \pm 0.24) \times 10^7$	− 10.11	2.8×10^5	$(1.40 \pm 0.01) \times 10^{-2}$	49.5
11	$(3.87 \pm 0.34) \times 10^6$	− 8.96	4.7×10^5	$(1.20 \pm 0.10) \times 10^{-1}$	5.8
CB[7]•**1**	$(9.14 \pm 0.77) \times 10^5$	−8.10	3.1×10^4	$(4.81 \pm 0.58) \times 10^{-2}$	14.3
CB[7]•**2**	$(2.56 \pm 0.21) \times 10^5$	− 7.36	6.5×10^4	$(2.70 \pm 0.13) \times 10^{-1}$	2.6

ments (Supporting Information, Figure S1) as described previously.[11] All binding experiments were performed in 50 mM NaO_2CCD_3-buffered D_2O (pD 4.75) at 25 °C. The association constants of **1** ($K_a = 4.1 \times 10^{12}$ M^{-1}) and **2** ($K_a = 2.5 \times 10^8$ M^{-1}) to CB[7] were measured by competition with **10** and **9**, respectively, for a limiting quantity of CB[7].[32] On the basis of our previous studies of binding constants for CB[6]−CB[8] and those of Mock and co-workers,[10,11] we estimate the binding constant for CB[7]•**3** as $\sim 10^6$ M^{-1}.

Measurement of the K_a Values of the Two-Faced Inhibitors to BCA. We directly measured the value of K_a for BCA•**11** by monitoring the increase in fluorescence intensity at 460 nm of BCA with the concomitant increase of the concentration of **11** and fitting the data by nonlinear least-squares analysis to a 1:1 binding model (Supporting Information, Figures S5 and S6). The binding constant values for the interaction of BCA with two-faced inhibitors **1** and **2** were subsequently measured by a competitive fluorophore displacement assay where we monitored the decrease in fluorescence intensity at 460 nm of BCA•**11** as the concentration of competitors **1** and **2** were increased (Figure 4). The values of K_a for BCA•**1** and BCA•**2** were calculated by fitting the decrease in fluorescence intensity to eq 1 (Table 1 and Figure S7).[33]

$$\frac{F - F_{Min}}{F_{Max} - F_{Min}} = \frac{1}{1 + \left(\frac{K^d_{DNSA}}{[DNSA]}\right)\left(1 + \frac{[Inh]}{K^d_{Inh}}\right)} \quad (1)$$

Measurement of the K_a Values of CB[7]•1 and CB[7]•2 to BCA. Based on the kinetic profiles observed in the fluorescence assays described above we surmised that the CB[7] complexes CB[7]•**1** and CB[7]•**2** might be capable of acting as inhibitors of BCA. As described above, we observed the change in fluorescence of a solution of BCA•**11** at thermodynamic equilibrium as a function of the concentration of added CB[7]•**1** or CB[7]•**2** as competitor (Table 1, Figures S8 and S9). As expected the CB[7]•**1** and CB[7]•**2** bind substantially weaker (118- and 107-fold, respectively) to BCA than do the free two-faced inhibitors **1** and **2**, presumably due to steric interactions between the CB[7] moiety and the walls of the active site of BCA.

Measurement of the Values of k_{off} for the BCA•11 and BCA•Two-Faced Inhibitor Complexes. To measure the value of k_{off} for the BCA•**11** complex, we treated a solution of BCA•**11** with **1** and observed the decrease in fluorescence intensity as **11** was released into free solution. The decrease in fluorescence intensity could be fitted to a single exponential decay in the standard manner to yield an observed value of k_{off} (Table 1). The overall process can be described according to the equilibria given in eq 2 controlled by the fundamental dissociation (k_{-1} and k_{-2}) and association (k_1 and k_2) rate constants. To ensure that the observed value of k_{off} is equal to the fundamental dissociation rate constant k_{-1}, we measured k_{off} as a function of [**1**]. As the concentration of **1** is increased, we initially observed an increase in the observed value of k_{off} that plateaus at a limiting value at high concentrations of **1** (Supporting Information, Figure S15). At low concentrations of **1**, addition of **1** to BCA is the rate-determining step; hence, the observed rate constant is a function of both concentration of **1** and **11**. At high values of [**1**], the dissociation of the BCA•**11** complex is the rate determining step ensuring that the observed value of k_{off} is equal to k_{-1} as given in Table 1. In a similar manner, we calculated the value of dissociation rate constant of **1** and **2** from BCA by adding excess **11** to BCA•**1** or BCA•**2** to ensure that dissociation of **1** and **2** is the rate-determining step (Table 1 and Figures S13−S16).

$$\text{BCA•}\mathbf{11} + \mathbf{1} \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} \text{BCA} + \mathbf{11} + \mathbf{1} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{BCA•}\mathbf{1} + \mathbf{11} \quad (2)$$

Addition of CB[7] Enhances the Rate of Dissociation of 1 and 2 from the Active Site of BCA. Figure 5a shows the change in fluorescence intensity at 460 nm upon addition of **11** to a solution containing BCA•**1**. This change in fluorescence intensity is directly related to the concentration of the fluorescent BCA•**11** complex. Therefore, the observed slow change in fluorescence reflects the rate determining dissociation of BCA•**1** followed by the fast formation of BCA•**11**. Somewhat surprisingly, when we added CB[7] to a solution containing BCA•**1** and **11**, we observed a significantly faster (Table 1, 11-fold) kinetic profile (Figure 5b and Supporting Information Figure S17). In the same

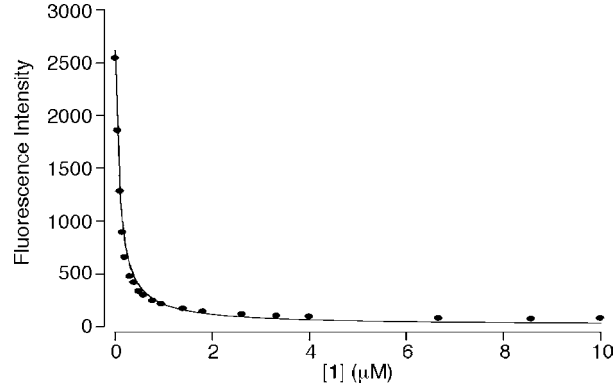


Figure 4. Plot of fluorescence intensity versus [**1**] in the titration of BCA•**11** with **1**. Concentration of BCA (67.5 nM), **11** (2 μM), and **1** (50 nM to 10 μM). All measurements were performed in 20 mM NaH_2PO_4 buffer, pH 7.3, 298 K.

(32) In this paper we use different conditions for the catalytic processes (30 mM Na_2DPO_4 buffer, (pD 7.3) at 25 °C). Because the values of pK_a of the ammonium groups of **1** and **2** should be at least two pK_a units higher than the working pH, we believe the binding constants obtained by ^{1}H NMR competition experiments at pD 4.75 will be comparable at pD 7.3.

(33) Nair, S. K.; Krebs, J. F.; Christianson, D. W.; Fierke, C. A. *Biochemistry* **1995**, *34*, 3981–3989.

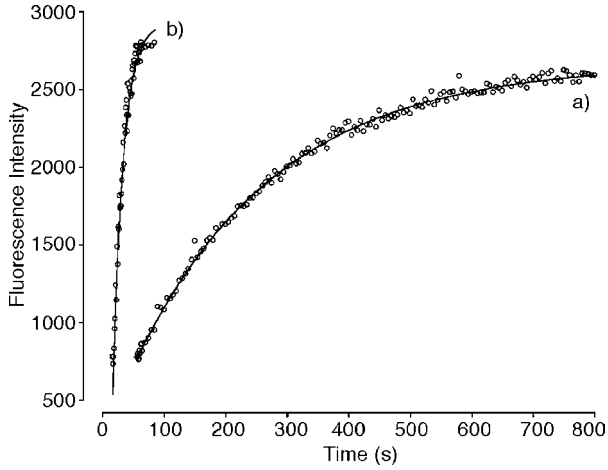
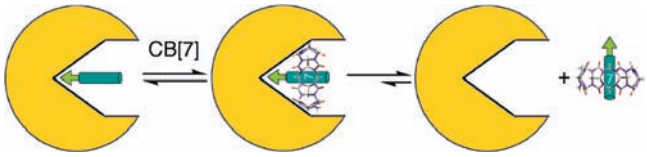


Figure 5. Plot of fluorescence intensity versus time: (a) Addition of **11** to a solution of BCA and **1**. Final concentration of BCA, **11**, and **1** are 76 nM, 98 μM, and 2 μM. (b) Addition of CB[7] to a solution containing BCA, **11**, and **1**. Final concentrations of BCA, **11**, **1**, and CB[7] are 75 nM, 25 μM, 18 μM, and 50 μM, respectively. All experiments were done in 20 mM NaH_2PO_4 buffer, pH 7.3, 298 K.

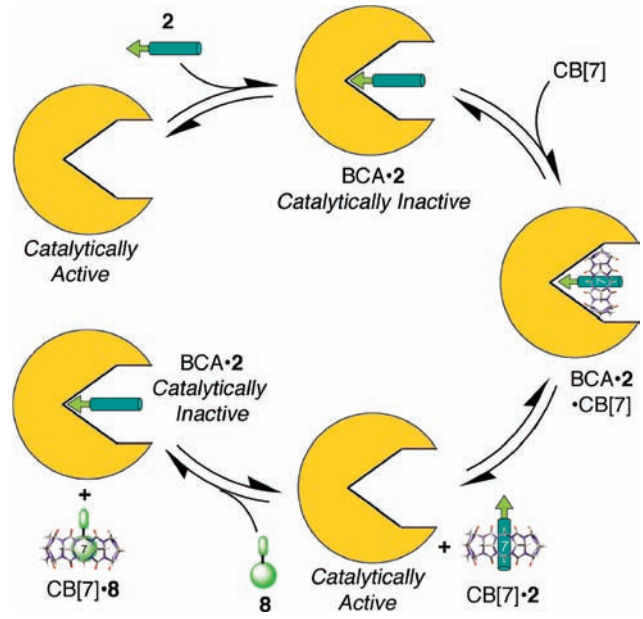
Scheme 5. Proposed Mechanism of CB[7] Assisted Dissociation of **1** and **2** from BCA



manner, addition of CB[7] to a mixture of BCA•**2** and **11** displayed a 19-fold enhancement of the rate of dissociation of **2** from BCA (Figure S18). Scheme 5 shows our interpretation of the mechanism of this process. Initially, CB[7] binds to the adamantaneammonium ion binding epitope present in BCA•**1** to form the ternary complex BCA•**1**•CB[7]. The ternary complex BCA•**1**•CB[7] then undergoes dissociation to free catalytically active BCA and CB[7]•**1** as a consequence of the fact that CB[7]•**1** binds 118-fold weaker to BCA than **1** (Table 1). In this manner the binding free energy of the CB[7]•**1** interaction ($K_a = 4.1 \times 10^{12}$ M^{-1}) pays the thermodynamic price to transform **1** from a small and tight binding BCA inhibitor into a sterically hindered weaker binding BCA inhibitor (CB[7]•**1**). In this manner CB[7] enhances the dissociation of **1** (and **2**) from the active site of BCA.

Overall Understanding of the Regulation of BCA Catalysis Using CB[7] and Two-Faced Inhibitors 1 and 2. Some issues remain that require explanation. What is the cause of the biphasic kinetics observed in Figure 3? Binding of CB[7] to BCA•**1** results in ternary complex BCA•**1**•CB[7] that dissociates under the experimental conditions to form free BCA and CB[7]•**1**. An initial fast increase in fluorescence occurs when free **11** interacts with BCA to form BCA•**11**. The system overshoots the equilibrium point because the association rate constant for the formation of BCA•**11** (4.7×10^5 M^{-1} s^{-1}, Table 1) is substantially larger than that for the formation of BCA•**1**•CB[7] from BCA and CB[7]•**1** (3.1×10^4 M^{-1} s^{-1}, Table 1). The slower phase reflects the transformation from the kinetically preferred state comprising mainly BCA•**11** and CB[7]•**1** to the thermodynamic state comprising containing a substantial fraction of BCA•**1**•CB[7] (Figure 1, step 3, loss of 55% of initial catalytic activity). This reduction in activity is as expected based on the

Scheme 6. Representation of the Control of Enzymatic Activity of BCA usnig **2** and CB[7]



value of K_a for the formation of BCA•**1**•CB[7] from BCA and CB[7]•**1** ($K_a = 9.14 \pm 0.77 \times 10^5$ M^{-1}) and the working concentrations of BCA (80 nM) and **1** (20 μM). Why does **2** perform better in the off-on regulation of enzymatic activity of BCA? In addition to the slow dissociation kinetics of CB[7]•**1** that effectively limits the on−off behavior to a single cycle, the thermodynamics of the system are also important. The catalytic activity of BCA is better regenerated using two-faced inhibitor **2** than **1** because CB[7]•**2** is a weaker inhibitor of BCA than CB[7]•**1** ($K_a = (2.56 \pm 0.21) \times 10^5$ M^{-1} for BCA•**2**•CB[7] versus $K_a = (9.14 \pm 0.77) \times 10^5$ M^{-1} for BCA•**1**•CB[7]; Table 1). Scheme 6 summarizes the overall reaction scheme that uses two-faced inhibitors **1** and **2** in concert with CB[7] to regulate the activity of BCA. The most notable feature is the ability of CB[7] to accelerate the dissociation of BCA•**1** and BCA•**2** complexes by transient formation of the corresponding ternary complexes.

Can Two-Faced Inhibitors Be Used to Regulate the Activity of Enzymes in General? To address the question of whether other enzymes could be regulated in a manner similar to that described above for BCA, we decided to investigate acetylcholine esterase. Based on an identical design strategy, we synthesized compounds **4** and **5** featuring a cationic amine group linked to the well-known AChE inhibitor tacrine.[34] On the basis of our experience with BCA, we anticipated a similar behavior under thermodynamic control where the addition of CB[7] would regenerate activity of AChE by sequestering **4** (or **5**) from AChE•$\mathbf{4}_4$ (or AChE•$\mathbf{5}_4$).

Regulation of the Biological Catalysis of Acetylcholinesterase. The rate of hydrolysis of **6** catalyzed by AChE was measured using Ellman's assay before and after the addition of two-faced inhibitors **4** or **5** (Figure 6).[24] As expected, we observed a decrease in rate of hydrolysis of **6** as the concentration of two-faced inhibitor **4** or **5** was increased. These results demonstrate

(34) Marco, J. L.; Carreiras, M. C. *Mini-Rev. Med. Chem.* **2003**, *3*, 518–524. Musial, A.; Bajda, M.; Malawska, B. *Curr. Med. Chem.* **2007**, *14*, 2654–2679. Villarroya, M.; Garcia, A. G.; Marco, J. L. *Curr. Pharm. Des.* **2004**, *10*, 3177–3184.

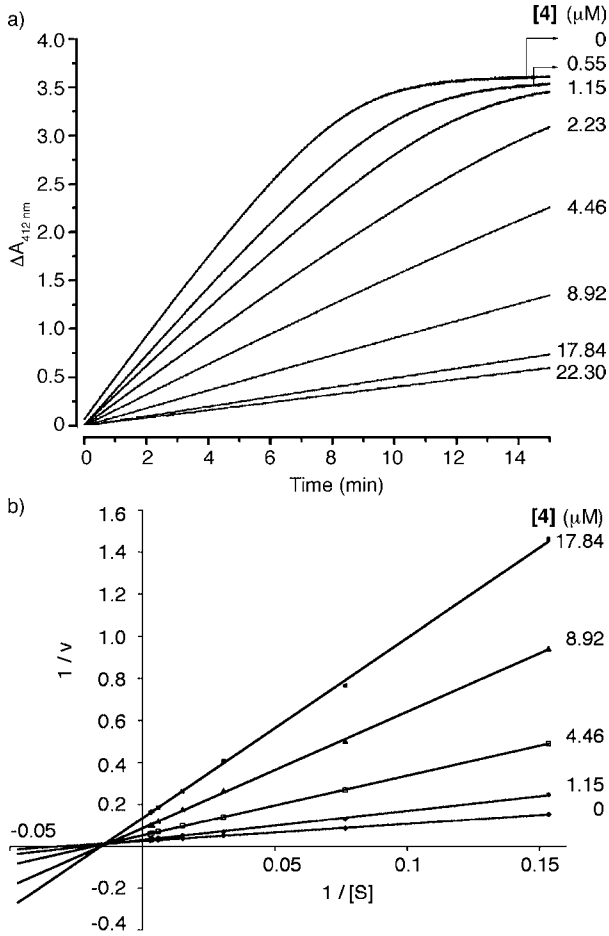


Figure 6. (a) Plot of absorbance versus time due to the AChE (0.084 units) catalyzed hydrolysis of **6** as the concentration of **4** is increased. (b) Lineweaver−Burk plot of the activity of AChE (0.84 units) in the presence of increasing concentrations of **4**. Conditions: 20 mM potassium phosphate buffer, pH 8.0, [**6**] = 6.5−325 μM, [**12**] = 353 μM. By definition, one unit of AChE hydrolyzes 1.0 μmol of acetylcholine to choline and acetate per minute at pH 8.0 and 37 °C.

that the tacrine subunits in **4** and **5** retain their inhibition capabilities even after derivatization with the CB[7] binding epitopes. Unfortunately, addition of CB[7] to a solution of AChE•**4** or AChE•**5** did not result in an increase in the rate of hydrolysis of **6**. We conclude that CB[7] is incapable of driving the dissociation of **4** or **5** from AChE. We found this result somewhat puzzling since tacrine and most of its derivatives are known to bind to AChE with moderate affinities (10^6−10^9 M^{-1})[35] that are significantly weaker than the interaction of CB[7] with adamantaneammonium derivatives.[11]

Measurement of Association Constants. To assess the influence of the thermodynamic parameters on the inability of CB[7] to regenerate the activity of AChE we determined the K_a values for AChE•inhibitor and CB[7]•guest pairs (Table 2). We measured the association constant of the two-faced inhibitors **4** and **5** to AChE using Lineweaver−Burk analysis, which also demonstrates the mixed inhibition behavior of **4** and **5** (Figure 6). It is well-known that AChE has two binding sites: 1) a deeply buried active site, and 2) a peripheral site that binds certain inhibitors including tacrine. The fact that the Lineweaver−Burk plot indicates mixed inhibition means that inhibitors **4** and **5**

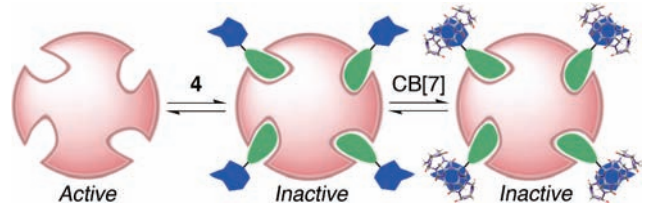
(35) Porcelli, F.; Delfini, M.; Del Giudice, M. R. *Bioorg. Chem.* **1999**, *27*, 197–205.

Table 2. Thermodynamic Parameters for the Interaction of **4** and **5** with BCA and CB[7]

	K_a (M^{-1})	
	AChE	CB[7]
4	4.0×10^{6a}	3.7×10^{12b}
5	1.5×10^{6a}	5.6×10^{8c}

[a] Measured by Lineweaver−Burk analysis. [b] Measured by competition with **10** for a limiting quantity of CB[7]. [c] Measured by competition with **9** for a limiting quantity of CB[7].

Scheme 7. Formation of Ternary Complex Upon Addition of CB[7] to AChE•**4**



are capable of binding to both AChE and the enzyme•substrate (e.g., AChE•**6**) complex. Both AChE•inhibitor complexes show reduced rates of catalytic hydrolysis of **6**. The association constant of **4** and **5** with CB[7] were measured by ^{1}H NMR competition experiments as described above (Table 2 and Supporting Information). Table 2 shows that both **4** and **5** have a strong thermodynamic preference to bind to CB[7]. So, the inability of CB[7] to regenerate enzymatic activity must be due to another factor.

On the basis of the values of K_a and results of the colorimetric assays, we considered two possibilities: either (1) CB[7] does not interact with AChE•$\mathbf{4}_4$ or AChE•$\mathbf{5}_4$ under the experimental conditions or (2) CB[7] binds to AChE•**4** or AChE•**5** to form a ternary complex AChE•$\mathbf{4}_4$•CB[7]$_4$ or AChE•$\mathbf{5}_4$•CB[7]$_4$.[36] In either situation, inhibitors **4** or **5** are bound to AChE in both the absence or presence of CB[7] and therefore the regeneration of the biological catalysis would not be observed. Additionally, when we added the separately prepared 1:1 complexes CB[7]•**4** or CB[7]•**5** to AChE, we also observed a decrease in the rate of hydrolysis of **6**. This result strongly suggests that both CB[7]•**4** and CB[7]•**5** are capable of binding to AChE and therefore inhibit the catalytic activity by formation of the ternary complexes as indicated in Scheme 7.

Secondary Effects of CB[7] on the Enzymatic Activity of AChE. As one of our control experiments we changed the concentration of CB[7] used in the attempted regeneration of AChE. In these experiments, we observed a decrease in the enzymatic activity of AChE as assessed by Ellman's assay as the concentration of CB[7] was increased (Figure 7). We considered two possibilities: (1) CB[7] acts as an inhibitor of AChE or (2) CB[7] reduces the observed enzymatic activity of AChE by binding to and thereby sequestering cationic substrate **6**. Accordingly, we prepared a solution of CB[7] and **6** and observed an upfield shift of the $(CH_3)_3N$ resonance in the ^{1}H NMR spectrum indicating that the quaternary ammonium group of **6** binds to CB[7] to form the CB[7]•**6** complex. CB[7] thereby reduces the concentration of free **6** that in turn reduces the observed rate of hydrolysis of **6** by AChE. Similar observations

(36) The Nau group has previously observed the formation of ternary complexes between bovine serum albumin, brilliant green, and CB[7]. See: Bhasikuttan, A. C. ; Mohanty, J. ; Nau, W. M. ; Pal, H. *Angew. Chem., Int. Ed.* **2007**, *46*, 4120−4122.

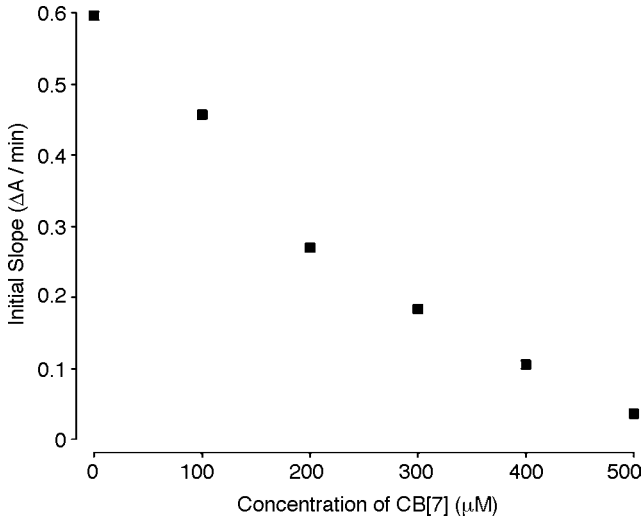


Figure 7. Plot of the initial slope of a plot of absorbance versus time for the hydrolysis of **6** (521 μM) by AChE (0.084 units) versus concentration of CB[7].

have been made by Nau and co-workers for the Trypsin catalyzed hydrolysis of peptides containing CB[7] binding sites.[37]

Origin of the Difference in the Behavior of Two Enzymes

What factors result in the very different behavior observed for BCA and AChE in the presence of two-faced inhibitors **1**−**5** and CB[7] (Scheme 6 versus Scheme 7)? One obvious difference between BCA and AChE is the size and location of their active and peripheral binding sites. Bovine carbonic anhydrase has an unusually deep (∼15 Å) and narrow conical binding site.[38] Sulfonamide inhibitors bind to the Zn^{2+} cofactor that is located at the bottom of the cleft. This Zn^{2+}•inhibitor coordination interaction is highly directional and the majority of the strength of the BCA•inhibitor complex originates from this interaction.[29] On the other hand AChE is not a metalloprotease. An analysis of the crystal structure of AChE form Torpedo California shows that the active site of the enzyme is present in a ∼20 Å deep gorge that widens out near the base. At the rim of the gorge, (∼14 Å from the surface) AChE also has a peripheral binding site.[39] The binding of ligands to these sites are driven by nondirectional forces like hydrophobic effects, $\pi-\pi$ interactions, cation-π interactions, and electrostatic interactions.[40] For example, in the crystal structure of tacrine•torpedo acetylcholinesterase, the acridine is stacked against the indole of Trp-84. Compounds **4** and **5** show mixed inhibition behavior indicating that they bind to the peripheral site. In contrast to the active site of BCA that is well-structured and interacts with its inhibitors by directional interactions, the active and peripheral sites of AChE are flexible and can accommodate a variety of ligands (inhibitors) of different shapes and sizes. CB[7] is able to effectively regulate the catalysis of BCA in concert with two-faced inhibitor **2** because the CB[7]•**2** complex binds 106-fold weaker to BCA. This reduction in affinity can be attributed to the sterically bulky CB[7]•**2** complex being relatively poorly accommodated by the rigid active site of BCA. In contrast, the more open binding sites of AChE readily accommodate the more bulky CB[7]•**4** and CB[7]•**5** resulting in the AChE•$\mathbf{4}_4$•$CB[7]_4$ and AChE•$\mathbf{5}_4$•$CB[7]_4$ complexes that are catalytically inactive.

Summary and Conclusion

In summary, we have described the use of two-faced inhibitors (**1** − **5**), comprising enzyme inhibitor and cucurbit[n]uril binding domains, in combination with CB[7] to control enzymatic activity. We find that the two-faced inhibitors retain their ability to inhibit enzymatic activity by binding to the enzyme active site (BCA) or peripheral sites (AChE). In the case of BCA the addition of CB[7] to BCA•**1** or BCA•**2** results in the regeneration of enzymatic activity due to sequestration of the two faced inhibitor as the CB[7]•**1** or CB[7]•**2** complex. One of the key controlling factors in this process is the relative binding affinity of the two-faced inhibitors toward BCA versus CB[7]. The high binding free energy of CB[7] toward its best guests in water, which exceeds that of enzyme•inhibitor complexes, pays the thermodynamic price associated with the on−off cycling of catalytic activity. Perhaps the most intriguing aspect of the study is the ability of CB[7] to enhance the rate of dissociation of BCA•**1** or BCA•**2** by the transient formation of the BCA•**1**•CB[7] and BCA•**2**•CB[7] ternary complexes. In contrast, it is not possible to regulate the activity of AChE in this manner. The relatively open active and peripheral sites of AChE bind to both two-faced inhibitors **4** and **5** and their CB[7] complexes (e.g., CB[7]•**4** and CB[7]•**5**).

Beyond the system specific results described above, our results allow us to speculate on the reasons why Nature evolved allostery, rather than the strategy described in this paper involving direct competition between receptors for a common target, as a method to control enzymatic catalysis. In allosteric enzymes, a small molecule binds to a site remote from the enzyme active site resulting in decreased (allosteric inhibitor) or increased (allosteric activator) enzymatic activity. In contrast, the direct competition between receptors (e.g., BCA and CB[7]) for a two-faced inhibitor (e.g., **1** or **2**) is only capable of decreasing enzymatic activity relative to its natural rate because our strategy relies on occupation of the enzyme active site. Second, because allosteric activator or inhibitor concentration can be varied independently of substrate concentration in allosteric systems it is not necessary for the affinity of the allosteric activator (or inhibitor) toward the enzyme to exceed that of the enzyme•substrate interaction. As we have seen for the BCA case study presented above, the requirement that the K_a of the two-faced inhibitor toward the synthetic host exceeds that toward the enzyme may result in slow kinetics of on−off switching (e.g., CB[7]•**1** has a half-life of hours). Lastly, in contrast to BCA whose active site is highly structured and buried deeply, the active site of many enzymes are located close to their surface in sterically less demanding environments. Since the direct competition between receptors relies on the fact that the CB[7]•two-faced inhibitor complex is a poor enzyme inhibitor due to the increased steric bulk of the complex this strategy is limited to enzymes like BCA with deep active sites. In contrast, allosteric control operates efficiently regardless of the location of the active site. For living systems, which operate based on complex networks of interactions, it is clearly

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advantageous to employ allosteric small molecules to control catalytic processes because they are capable of participating at multiple points in the network resulting in feedback and regulation.

What then are the future prospects for the use of the strategy described here involving the direct competition between synthetic CB[n]-type and biological receptors for a common two-faced inhibitor described here? The largest advantage is selectivity. Because the binding sites of synthetic receptors (e.g., CB[n]) are built from synthetic (e.g., nonpeptidic) building blocks our expectation is that they will display affinity and selectivity profiles that are distinct from their natural counterparts. Therefore, building on the initial steps described in this paper, we expect that it will be possible to use CB[n]-type receptors in concert with two-faced guests to compete with and thereby control the activity of specific portions of the interaction networks of complex biological systems. A second major advantage is the growing technology available to synthetically modify CB[n]-type receptors.[2,41] For example, CB[n]-type receptors embedded inside a dendrimer core should be bulky enough to sequester two-faced inhibitors even from enzymes with shallow active sites thereby greatly expanding the applicability of the system described herein. Furthermore, with synthetic modification comes the ability to attach groups tailored for targeting and imaging applications. For all these reasons, we envision that CB[n]-type receptors will become valued tools that allow scientists to interface with and exert control over complex biological systems.

Experimental Section

Materials. Carbonic anhydrase (Sigma C3934); acetylcholinesterase (Sigma C3389); compounds **6**, **7**, and **9**−**12**; and β-CD were obtained from commercial sources. The synthesis and characterization of **1**−**5** are described in the Supporting Information.

^{1}H NMR Competition Experiments. ^{1}H NMR competition experiments were performed on a 400 MHz NMR spectrometer as described previously.[11] The temperature was maintained at 298 ± 0.5 K with a temperature control module that had been calibrated using the separation of the resonances of methanol. Samples for K_a determinations contained CB[7] and an excess of the two competitive guests. ^{1}H NMR spectra were acquired with a delay time of five times the longest T_1 of the guest to ensure that systematic errors due to differences in relaxation times were eliminated. All spectra were referenced relative to residual HOD at 4.79 ppm.

UV−vis Experiments. UV−vis spectra were recorded on a Cary 100-Bio UV−visible spectrophotometer using 1 cm path length cells. The temperature was held constant at 25.0 °C using a RTE bath/circulator containing a microprocessor controller. The kinetic assays were performed using the literature procedures.[22−24] The relative rates of reaction were determined from the initial slope of plots of absorbance versus time.

Fluorescence Spectroscopy. Solutions for fluorescence titrations were prepared in 20 mM NaH_2PO_4 buffer, pH 7.3. All spectra were measured on a Hitachi F-4500 fluorescence spectrophotometer with excitation and emission band passes set at 5 nm. The temperature was maintained at 25.0 °C using a Neslab RTE-111 bath/circulator. An excitation wavelength of 290 nm was used to excite BCA. The change of fluorescence at 460 nm was measured by integrating the area (from 400 to 500 nm) under each spectrum. The change in area under the emission spectra versus [**11**] was used to determine the K_a value of **11** by nonlinear least-squares analysis fitting to a 1:1 binding model using Associate 1.6.[42]

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Supporting Information Available: Synthetic procedures and characterization data; ^{1}H and ^{13}C NMR spectra for all new compounds; details of the kinetic assays; ^{1}H NMR spectra for competitive binding experiments; Scatchard plots for BCA•**1** and BCA•**2**; Measurement of dissociation rate constants of **1** − **3**, CB[7]•**1**, CB[7]•**2**, and **11**; Lineweaver−Burk plots for AChE•**4** and AChE•**5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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