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High Fidelity Kinetic Self-Sorting in Multi-Component Systems Based on Guests with Multiple Binding Epitopes

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Abstract: The molecular recognition platforms of natural systems often possess multiple binding epitopes, each of which has programmed functional consequences. We report the dynamic behavior of a system comprising CB[6], CB[7], and guests cyclohexanediammonium (**1**) and adamantanealkylammonium (**2**) that we refer to as a two-faced guest because it contains two distinct binding epitopes. We find that the presence of the two-faced guest—just as is observed for protein targeting in vivo—dictates the kinetic pathway that the system follows toward equilibrium. The influence of two-faced guest structure, cation concentration, cation identity, and individual rate and equilibrium constants on the behavior of the system was explored by a combination of experiment and simulation. Deconstruction of this system led to the discovery of an anomalous host−guest complex (CB[6]·**1**) whose dissociation rate constant ($k_{out} = 8.5 \times 10^{-10}$ s^{-1}) is ≈100-fold slower than the widely used avidin·biotin affinity pair. This result, in combination with the analysis of previous systems which uncovered extraordinarily tight binding events ($K_a \geq 10^{12}$ M^{-1}), highlights the inherent potential of pursuing a systems approach toward supramolecular chemistry.

Introduction

The outcomes of the majority of designed self-assembly processes have been subject to thermodynamic rather than kinetic control.[1] Accordingly, supramolecular chemists have better intuition in estimating ground state − ground state rather than ground state − transition state energy differences. Notable examples of kinetic control in assembly processes include molecular chaperones, catenane and rotaxane threading, helicate formation, regioselective isotopomer exchange, capsule formation, cyclodextrin complexes, and even the folding of prion proteins.[2] In contrast, systems biologists—and biological systems—have numerous modules at their disposal for the top-down engineering of catalytic processes, compartmentation and segregation of incompatible species in time and space, and regulatory strategies that all depend on kinetically controlled in addition to thermodynamically controlled pathways.[3] As an approach toward systems chemistry through a bottom-up approach we,[4−6] and others,[7] have begun to study complex self-sorting systems that are under thermodynamic control. For the preparation of chemical systems that display functional aspects typically reserved for inherently nonequilibrium natural systems, it is necessary to incorporate kinetic and spatial control into our self-sorting systems. Herein we explore the hypothesis that the use of guests containing multiple binding epitopes may allow efficient control over both the kinetic and thermodynamic outcomes of multicomponent self-sorting systems. We report a four-component system whose deconstruction led to the discovery of an anomalous host−guest complex (CB[6]·**1**) whose

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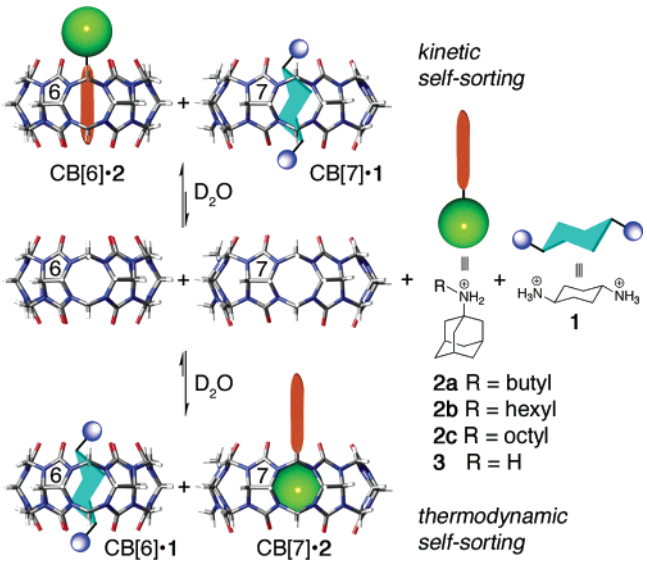
dissociation rate constant (k_{off} = 8.5 × 10^{-10} s^{-1}) is approximately 2 orders of magnitude slower than that of avidin·biotin.

Origin of the Hypothesis that Multiple Binding Epitopes Would Deliver Kinetic Control in Multicomponent Systems. Biological systems are inherently nonequilibrium in both time and space. A natural starting point for our investigations of kinetic control in self-sorting systems, therefore, was a literature review of the strategies that Nature uses to maintain its control over temporal (kinetic) and spatial distribution of important biological species such as proteins, nucleic acids, and ions. Although issues of kinetic control pervade nearly all aspects of biochemistry (e.g. control of transmembrane ionic gradients, spatial segregation of incompatible metabolic pathways, and chaperone-mediated protein folding), we concentrate in this section on three examples of protein targeting[8] that greatly influenced our design of the system described in this paper. Protein targeting—also referred to as a sorting process—is initiated during ribosomal peptide synthesis in the cytosol by the covalent attachment of a signal peptide sequence. The signal sequence directs the ribosome to the membrane of the endoplasmic reticulum (ER) where protein synthesis continues, the peptide is then translocated across the membrane where a signal peptidase removes the tagging peptide, and then the folded protein is released into the lumen of the ER. Subsequent modifications (e.g., glycosylation) are used to determine the ultimate destination of the protein (e.g., lysosomes, sectretory vesicles, or the plasma membrane). A second example is the co-translational modification of cytosolic proteins with myristoyl, palmitoyl, farnesyl, or geranylgeranyl groups that send them to the cytosolic side of plasma or compartment membranes. All four of these groups amount to long hydrocarbon tails that anchor the protein to a membrane. A final example involves the trafficking and adhesion of blood-borne cells to specific tissues which are critical components of immune response. As a specific example, the transition of leukocytes from unbound to rolling to adherent has been shown to depend on a number of factors including the presence of a specific binding epitope on the walls of the microvasculature and its valency (e.g., ligand density).[9] In all of these cases, spatio/temporal control is achieved by the appendage of a signaling group in the form of an additional binding epitope. These specific examples lead us to hypothesize that the study of guests containing multiple binding epitopes in complex multicomponent systems might result in interesting dynamic behavior.

Results

Design Aspects of the Chemical Components Used in this Study. On the basis of the observations described above for natural systems, we decided to explore the behavior of guests bearing multiple binding epitopes[10] in multicomponent systems. We hypothesized that the presence of multiple binding epitopes on a single guest might control the kinetic partitioning of species within a complex system (e.g. fast and weak versus slow and tight) resulting in a well-defined, kinetic, self-sorting state. For this study, we selected two members of the cucurbit[*n*]uril family[11,12] (CB[*n*]; *n* = 6, 7) as hosts because their complexes exhibit tight binding, high selectivity, and slow kinetics of exchange which allow the use of ^{1}H NMR as our primary analytical tool.[5,13−16] The two guests used in this paper were settled upon by an iterative process[17] that involved the preparation and screening of four-component mixtures with no prior knowledge of the precise equilibrium or rate constants of formation of their CB[6] or CB[7] complexes. This iterative process resulted in the selection of cyclohexanediamine (**1**) as one of the guests for detailed study. Two considerations that guided the iterative process toward **1** were (1) guests containing cyclohexane rings were given priority since Nau and co-workers have shown they display slow kinetics of association[15] that enables ^{1}H NMR monitoring of the kinetics, and (2) guests should form tight 1:1 complexes with both CB[6] and CB[7]. For our guests containing two-binding epitopes, we iteratively settled upon guests **2a**−**2c** which we refer to as *two-faced* guests because they contain two distinct (e.g., adamantylammonium and alkylammonium) binding epitopes (Scheme 1). Guests **2a**−**2c** can be used to study the influence of the length of the alkylammonium tail on the behavior of the system. For a control compound we selected adamantaneamine (**3**) which lacks a second binding epitope. Although we exclusively used the CB[*n*] hosts in this study because their dynamic processes are

Scheme 1. Kinetic versus Thermodynamic Self-Sorting



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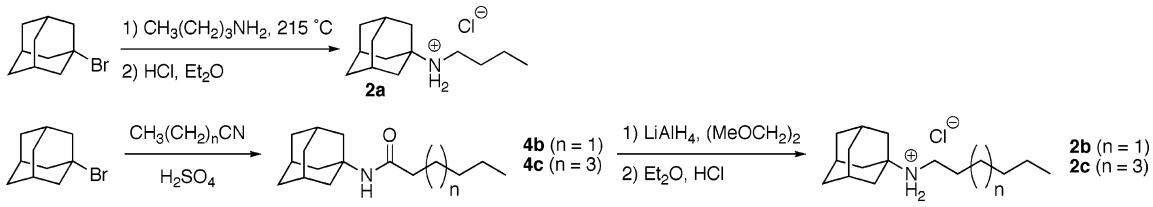
(17) The iterative process involved three steps: (1) preparation of four-component mixtures and observation of the outcome by ^{1}H NMR (e.g. fidelity of kinetic and thermodynamic sorting), (2) use of GEPASI to rationalize the outcome with guessed (estimated) kinetic and thermodynamic values as inputs and thereby inform chemical intuition, and (3) the use of these insights to guide the next round of iteration. We investigated approximately 20 pairs of guests—mainly derivatives of adamantylamines and cyclohexylamines—before discovering the system described in this paper. The discovery process took approximately 3 months including time for synthesis of the requisite two-faced guests.

Scheme 2. Synthesis of **2a**−**2c**



followed easily by ^{1}H NMR, we expect that the lessons learned with the CB[*n*] hosts will be transferable to other host systems (e.g., cyclodextrins and calixarenes), provided an analytical tool with sufficient time and structural resolution is available (e.g. stopped flow coupled with fluorescence or UV/vis).

Synthesis of Guests 2a−2c. The synthesis of two-faced guests **2a**−**2c** is shown in Scheme 2. For example, the reaction of 1-bromoadamantane with butylamine yielded the corresponding amine that was isolated and characterized as its hydrochloride salt **2a** in 64% yield. Separate treatment of 1-bromoadamantane with hexanenitrile and octanenitrile under strongly acidic conditions yielded amides **4b** (74%) and **4c** (70%) in good yield via a Ritter-type reaction. Reduction of amides **4b** and **4c** with $LiAlH_4$ in dimethoxyethane proceeded smoothly to give the corresponding amines which were isolated as the corresponding hydrochloride salts **2b** and **2c** in 84% and 87% yields, respectively.

A Single System Displays Both High-Fidelity Kinetic and Thermodynamic Self-Sorting. Our first observation that two-faced guests lead to well-defined dynamic behavior occurred when we mixed equimolar solutions of hosts CB[6] and CB[7] with guests **1** and **2a** (Scheme 1). Under kinetic control (6 min after mixing), two-faced guest **2a** used its slim and weakly binding butylammonium epitope to associate with CB[6] (e.g., CB[6]·**2a**), whereas **1** formed the CB[7]·**1** complex. Comparison of the ^{1}H NMR spectra recorded shortly after mixing (Figure 1e) with those of the individual complexes (Figure 1a,b) demonstrates the high-fidelity (95:5) of this kinetic self-sorting process. After 56 days we observed a dramatically different spectrum (Figure 1f) which is nearly the superposition of those recorded for CB[6]·**1** and CB[7]·**2a** (Figure 1c,d). Chemical shift analysis shows that two-faced guest **2a** has now used its bulkier, but much tighter binding, adamantylammonium face in the formation of the thermodynamically self-sorted state comprising CB[6]·**1** and CB[7]·**2a**.

Influence of Key Experimental Variables on the Fidelity of Kinetic and Thermodynamic Self-Sorting. Although the observation that two-faced guests such as **2a** control both the kinetic and thermodynamic outcomes of this system was intuitively appealing based on the precedent from natural systems, we wanted to explore the fundamental factors governing the efficiency of the process. For this purpose, we investigated the influence of two-faced guest structure (**2a**−**2c** and **3**), salt concentration, salt identity, and values of binding and rate constants by a combination of experiment and simulation.

Influence of the Length of the Alkylammonium Binding Epitope on the Fidelity of Kinetic Self-Sorting. We studied the influence of the presence and length of the alkylammonium chain of the two-faced guests (**2b**, **2c**, and **3**) on the fidelity of kinetic self-sorting for four-component mixtures with CB[6], CB[7], and **1**. When compound **3** was used, which lacks a CB[6] binding epitope, we observed the rapid formation of CB[7]·**3** along with free CB[6] and **1**. CB[6] and **1** then associate remarkably slowly (k_{in} = 0.0012 M^{-1} s^{-1}, Supporting Information) at room temperature to yield the thermodynamic self-sorted state. When guests **2b** and **2c** with their longer alkyl tails were used, we observed a decrease in the fidelity of the kinetic self-sorting (CB[6]·**2**:CB[7]·**2**; **2b** = 81:19; **2c** = 36:64; Supporting Information). The fidelity of the thermodynamic self-sorting state remains high when guests **2b**, **2c**, and **3** are used.

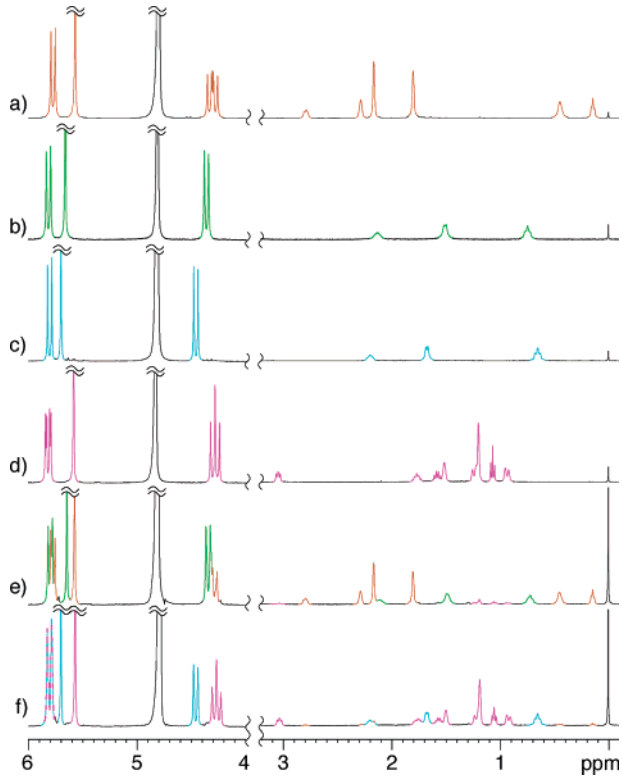


Figure 1. ^{1}H NMR spectra (400 MHz, 298 K, 5mM Na_2SO_4 in D_2O, pH 7.0) for: (a) CB[6]·**2a**, (b) CB[7]·**1**, (c) CB[6]·**1**, (d) CB[7]·**2a**, (e) mixture of CB[6]·**2a** and CB[7]·**1** recorded 6 min after mixing the components, (f) mixture of CB[6]·**1** and CB[7]·**2a** recorded 56 days after mixing the components. Color code: CB[6]·**2a**, orange; CB[7]·**1**, green; CB[6]·**1**, aqua; CB[7]·**2a**, pink.

Influence of Cation Concentration on the Fidelity of Kinetic Self-Sorting. It is well-known that cations (e.g., alkali metal ions and protons) bind to the ureidyl−carbonyl lined portals of CB[6] and CB[7] and thereby compete with guest binding which lowers the observed value of K_a for the host−guest complex.[11,12] We thought it would be interesting, therefore, to increase the complexity of the system even further by the addition of alkali metal cation and observe the influence on the fidelity of the kinetic and thermodynamic self-sorting states of the system. We found that as the concentration of Na_2SO_4 is increased (0−500 mM), the fidelity of the kinetic self-sorting state is compromised. Figure 2a shows a plot of the distribution

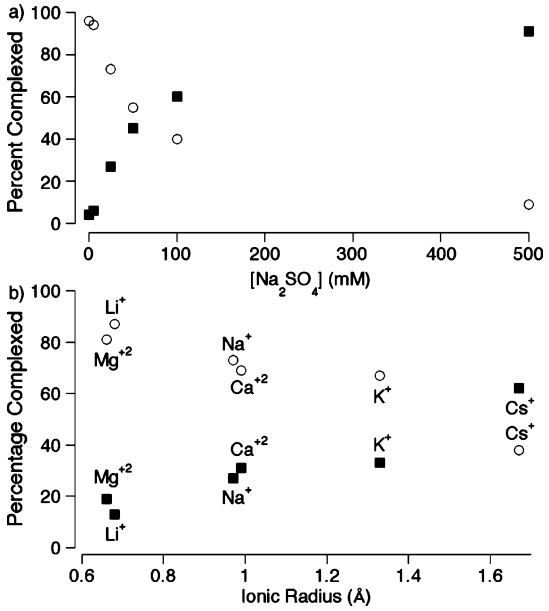


Figure 2. Fidelity of kinetic self-sorting. (a) Percentage of CB[6]·**2a** (○) and CB[7]·**2a** (■) formed after 6 min as a function of [Na_2SO_4]. (b) Percentage of CB[6]·**2a** (○) and CB[7]·**2a** (■) formed after 6 min as a function of ionic radius.

of **2a** into its CB[6]·**2a** and CB[7]·**2a** complexes as determined by ^{1}H NMR integration 6 min after mixing (Supporting Information) as a function of concentration of Na_2SO_4. Low concentrations of Na_2SO_4 (e.g., 0 or 5 mM) result in a high-fidelity kinetic self-sorting step, whereas at higher concentrations increasing amounts of the thermodynamically preferred CB[7]·**2a** complex is formed after only 300 s. The fidelity of the thermodynamic self-sorting state is high (≥95:5) over the full range of Na_2SO_4 concentrations.

Influence of Cation Identity on the Fidelity of Kinetic Self-Sorting. Given that the concentration of Na^+ controls the partitioning of the two-faced guest into CB[6]·**2a** and CB[7]·**2a** we wondered whether the identity of the cation would exhibit a similarly pronounced effect on the fidelity of kinetic self-sorting. For these experiments we used a common fixed concentration (25 mM) of metal sulfate salts. Figure 3 shows the ^{1}H NMR spectra recorded for the kinetically controlled partitioning in the presence of six different cations (Li^+, Na^+, K^+, Cs^+, Mg^{2+}, and Ca^{2+}). Integration of the resonances in Figure 3 allowed us to determine the ratio of CB[6]·**2a** to CB[7]·**2a**; these data were used to construct Figure 2b. Interestingly, the ionic radius of the cation shows a good correlation with the degree of fidelity of the kinetic self-sorting step.

X-ray Crystal Structure of CB[6]·1. Figure 4 shows the X-ray structure of one of the three molecules of complex CB[6]·**1** which appear in the unit cell of the crystal. The structure clearly demonstrates that the CB[6] cavity expands to accommodate the large cyclohexane ring (volume = 125 Å^3). Furthermore, the normally circular cavity of CB[6] undergoes a large ellipsoidal deformation (0.95−1.22 Å)[18] upon formation of CB[6]·**1** to accommodate the cyclohexane ring of guest **1**. Guest **1** fills the cavity of CB[6] so efficiently that the ground state of the complex CB[6]·**1** exhibits substantial distortions. The transition state to ingression of **1** through the *even smaller*

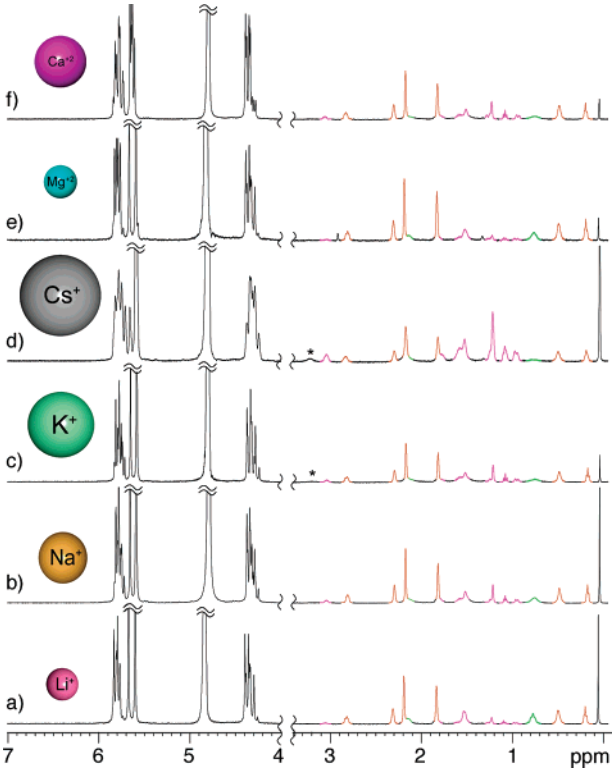


Figure 3. ^{1}H NMR spectra (400 MHz, D_2O, pD 7.4, 298 K, 2.5 mM components, 6 min after mixing) recorded for a mixture of CB[6], CB[7], **1**, and **2a** as a function of metal sulfate identity (25 mM): (A) Li_2SO_4, (B) Na_2SO_4, (C) K_2SO_4, (D) Cs_2SO_4, (E) $MgSO_4$, and (F) $CaSO_4$. Color code: CB[6]·**2a**, orange; CB[7]·**1**, green; CB[6]·**1**, aqua; CB[7]·**2a**, pink. The marked resonances (*) correspond to unbound **1**. The sphere sizes are scaled according to their ionic radii.

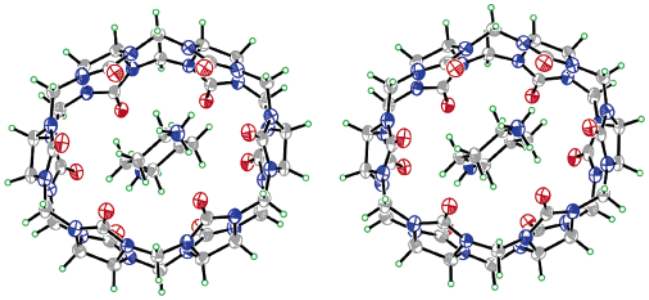


Figure 4. X-ray crystal structure of one of the three independent complexes of CB[6]·**1** in the unit cell. Solvating H_2O has been removed for clarity. Color coding: C, gray; H, white; N, blue, O, red.

ureidyl−carbonyl-lined portal of CB[6] should exhibit large structural distortions which provides a rationale for observed extremely slow association rate constant.[15]

Experimental Determination of Kinetic and Thermodynamic Parameters for Use in GEPASI Simulations. To assess the influence of the key thermodynamic and kinetic parameters in this system we first needed to determine or estimate values of k_{in}, k_{out}, and K_a for all four CB[*n*]·guest pairs to be used in simulations using GEPASI (vide infra).[19] GEPASI is a user-friendly program with a graphical user interface designed for biochemical simulations that uses symbolic reactions, component concentrations, and individual rate constants as input. GEPASI

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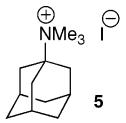
sets up the differential equations that govern the system and solves them to yield the concentrations of the components of the system as a function of time and at steady state as output.

Determination or Estimation of the Values of K_a, k_{in}, and k_{out}. We estimated the values of K_a for CB[6]·**2a**, CB[7]·**2a**, and CB[7]·**1** using literature values recently determined by us for a similar solvent composition.[6,13] To determine the value of k_{in} for CB[6]·**1** ($k_{in} \approx 0.0012$ M^{-1} s^{-1}) we monitored its formation by ^1H NMR from a solution containing equimolar amounts of CB[6] and **1** (2.5 mM) and fitted the data to a simple bimolecular association model (Supporting Information). To determine the values of k_{out} for the CB[6]·**1**, CB[6]·**2**, CB[7]·**1**, and CB[7]·**2** complexes we performed competitive displacement assays[13,14] or used EXSY spectroscopy.[20] For example, we followed the dissociation of **2a**−**2c** from the CB[7]·**2** complex by NMR in the presence of 13 equiv of tighter binding guest **5**. Values of k_{out} for CB[7]·**2** (**2a**: 2.4×10^{-5} s^{-1}; **2b**: 2.3×10^{-5} s^{-1}; **2c**: 2.5×10^{-5} s^{-1}) at room temperature were determined (assuming a unimolecular irreversible dissociation model) and analyzed over greater than two half-lives using eq 1 in the standard way (Supporting Information). The comparable values of k_{out} for CB[7]·**2a**−CB[7]·**2c** is not surprising since the same binding epitope (e.g., adamantylammonium) is used for all three complexes. A similar experiment yielded a value of k_{out} for CB[6]·**2a** ($k_{out} = 2.2 \times 10^{-3}$ s^{-1}) by competition with 2 equiv of hexanediamine. We had to resort to EXSY spectroscopy to determine the value of k_{out} for the more quickly dissociating CB[7]·**1** ($k_{out} = 2.7$ s^{-1}) and CB[6]·**2c** ($k_{out} = 0.5$ s^{-1}) complexes.



5

$$\ln([\mathrm{CB[7]{\bullet}\mathbf{2}}]/[\mathrm{CB[7]{\bullet}\mathbf{2}}]_0) = -k_{out}t \quad (1)$$

Although the displacement of **1** from the CB[6]·**1** complex was impractically slow at room temperature, we were able to monitor its displacement by competition with 2 equiv of hexanediamine over the 80−96 °C temperature range by ^1H NMR. Figure 5a shows a plot of $\ln([\mathrm{CB[6]{\bullet}\mathbf{1}}]/[\mathrm{CB[6]{\bullet}\mathbf{1}}]_0)$ versus time used to determine the values of k_{out} for CB[6]·**1** at five temperatures; Figure 5b shows an Arrhenius plot of the data. From the Arrhenius plot we were able to extrapolate $k_{out,(CB[6]\cdot\mathbf{1})} = 8.5 \times 10^{-10}$ s^{-1} at room temperature along with an estimate of the activation energy for this dissociation process ($E_a = 30.0$ kcal mol^{-1}). The CB[6]·**1** pair has a half-life of 26 years at room temperature! Both values are remarkably anomalous for the dissociation of a monovalent host−guest pair. For example, avidin·biotin with its dissociation rate constant of a mere 10^{-7} s^{-1} is widely used in biological applications.[21] Despite its relatively low affinity ($K_a = 0.0012$ M^{-1} $s^{-1}/8.5 \times 10^{-10}$ $s^{-1} = 1.4 \times 10^6$ M^{-1}), the readily available CB[6]·**1** pair dissociates ∼100-fold slower than the natural pair which suggests its great potential in applications typically reserved for avidin·biotin (e.g., immobilization and affinity column purification applications).

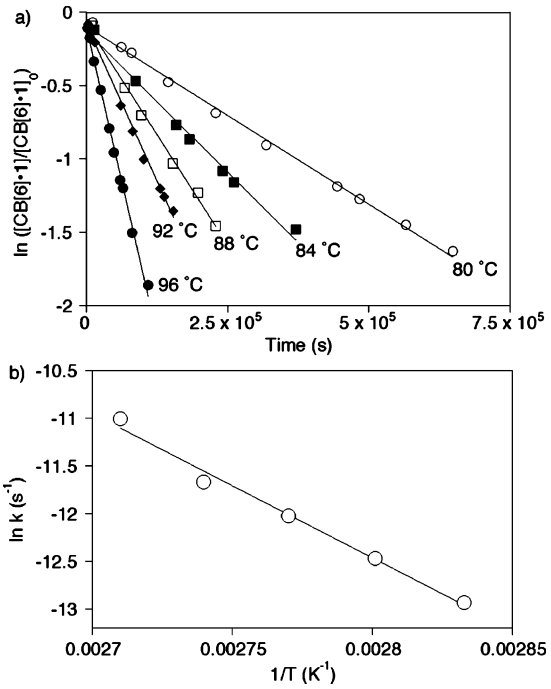


Figure 5. (a) Plot of the dissociation of CB[6]·**1** (2.5 mM, 298 K, 5 mM Na_2SO_4 in D_2O, pH 7.0) as a function of time at different temperatures. (b) Arrhenius plot of the data from part a.

Discussion

This discussion section has two goals. The first is to clarify the advantages of pursuing a systems chemistry approach toward the discovery of a multicomponent system with anomalous properties and collective function followed by its deconstruction relative to an approach based on an *a priori* study of all pairwise components of the system followed by its construction. Second, we use GEPASI simulations to rationalize the experimental results for the system comprising CB[6], CB[7], **1**, **2** (**3**), and metal ions which elucidate the key factors controlling the fidelity of the kinetic and the thermodynamic self-sorted states.

Reasons To Pursue a Systems Chemistry Approach toward Complexity. One of the common features of scientific studies—that has worked remarkably well for centuries—is the application of a reductionist approach. Reductionism holds that complex phenomena can be understood by analyzing the fundamental chemical, physical, or biological components operating in the system. The application of a reductionist approach in certain areas of 21st century science, however, seems less appropriate. Biologists, for example, appreciate the fact that the level of connectivity and complexity present in biological systems precludes a complete understanding of life processes by a reductionist approach and have responded by creating the field of systems biology[22] to tackle the issues that emerge from complex, highly interconnected systems. Computer scientists recognize that the form and connectivity of the worldwide web—which resembles biological systems in many ways—dictate that they must approach certain problems by an application of systems-wide approaches.[23] Despite the fact that all biological and life processes may be simply considered as

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(21) Green, N. M. *Methods Enzymol.* **1990**, *184*, 51−67.
(22) Aloy, P.; Russell, R. B. *Nat. Rev. Mol. Cell. Biol.* **2006**, *7*, 188−197.
(23) Milo, R.; Itzkovitz, S.; Kashtan, N.; Levitt, R.; Shen-Orr, S.; Ayzenshtat, I.; Sheffer, M.; Alon, U. *Science* **2004**, *303*, 1538−1542.

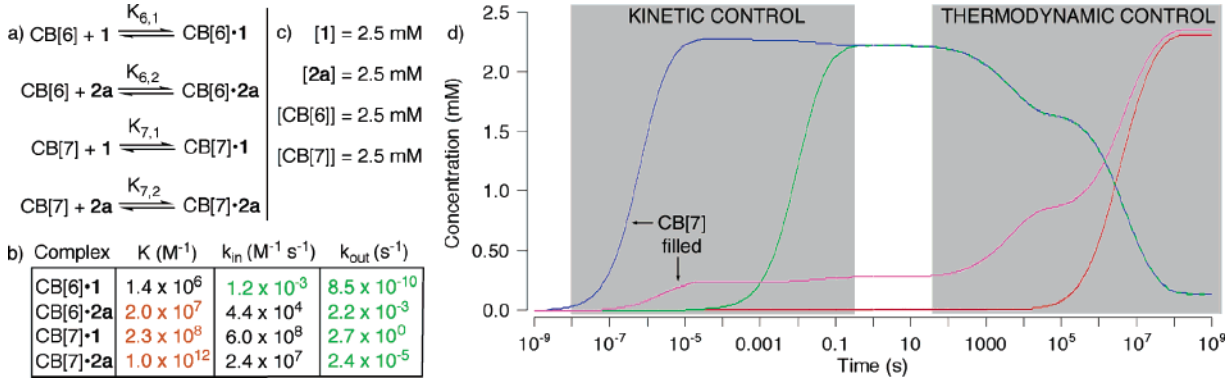

Figure 6. Simulation of the system comprising CB[6], CB[7], **1**, and **2a**: (a) equilibria considered, (b) values of K_a, k_{in}, and k_{out} (known values: green; literature estimates: orange; calculated from known or estimated values: black), and (c) concentrations used as input for the simulation, (d) plot of concentration versus time. Color code: CB[7]•**1**, blue; CB[7]•**2a**, pink; CB[6]•**2a**, green; CB[6]•**1**, red.

complex chemical systems, chemists have been slower to embrace the concept of a systems approach to chemistry.[24]

Within the molecular recognition community—whose general aim is to understand noncovalent interactions and use them to build up functional systems of our own design or to augment biological systems to respond to external stimuli—there are several significant reasons to pursue a systems chemistry approach toward the discovery of new function and fundamental insights. Some scientists assert that systems chemistry and self-sorting systems produce behaviors that are simply the sum of their parts and therefore could have been predicted beforehand with a detailed knowledge of all the values of K_a, k_{on}, and k_{off} for each of the possible pairwise interactions. Such assertions are, of course, correct in theory, but that approach would be inefficient and impractical to implement experimentally. For example, in a 10-component system there are 55 ($10^2/2 + 10/2$) pairwise interactions. Rarely, if ever, are such large collections of values of K_a, k_{on}, and k_{off} measured in common media (e.g., solvent, buffer, pH, ionic strength, temperature) for a series of homologous hosts and guests with sufficient accuracy to allow the *a priori* prediction of the behavior of a system of even modest complexity. Given that the measurement of 155 kinetic and thermodynamic constants would likely take more than 1 year and would allow the prediction of the behavior of *one* 10-component system which may have no interesting function, our view is that the systems chemistry approach which relies on the preparation and NMR observation of mixtures of modest complexity (e.g. a 10-component mixture requires at most 1 day) is far superior.

Second, as a community we need to devise strategies that allow us to uncover anomalies—extremely tight or weak (i.e., K_a) binding events, extremely fast or slow (i.e., k_{on} and k_{off}) binding events, or extremely high selectivity binding events—since those cases are most likely to require the development of new fundamental principles and lead to practical applications. The systems chemistry approach is quite efficient in discovering such anomalies. Most commonly, a single host−guest complex is studied in detail which yields—with large input of effort—information regarding K_a, k_{on}, and k_{off} for a single host−guest complex. In a systems chemistry approach multiple species (e.g., n components) are studied as a single entity whose overall behavior depends on the matrix of all pairwise interactions. Simple behavior in the form of self-sorting at modest concentrations signals the presence of a number of high affinity (K_a) and highly selective ($\Delta\Delta G$) binding events as components of the interaction matrix. As a specific example of this route to discovery, I note that our observation of 12-component self-sorting systems containing CB[n] hosts[11,12] leads us to measure values of K_a in excess of 10^{12} M^{-1} in water for complexes of CB[7] with cationic adamantane derivatives by competition experiments.[6] As such values exceed the upper limits of measurement by direct techniques, it is unlikely they would ever have been measured without the clues provided from the self-sorting experiments. Similarly, a systems chemistry approach that monitors concentrations as a function of time until equilibrium is achieved reflects the matrix of values of k_{on} and k_{off} and provides a route to discover systems and complexes with unusual dynamics. The discovery of the CB[6]•**1** pair which has a dissociation rate constant approximately 100-fold slower than avidin•biotin illustrates the great potential of this approach.[21]

Third, the systems chemistry approach is versatile in that it may be tailored to test specific hypotheses and goals (e.g. the influence of guests' multiple binding epitopes, vide infra) and also offers the opportunity for serendipitous discoveries that often result in significant scientific advancement. For example, given a large matrix of known values of K_a, k_{on}, and k_{off} it would be possible—using currently known principles—to predict the behavior of the complex self-sorting systems reported to date. Such an approach, however, would be unable to predict the behavior of a system whose underlying reductionist principles are not already known. As one of the goals of all science is to discover functional systems that do not obey current rules and to make them predictable on the basis of newly formulated principles, we believe that the systems chemistry approach—with its focus on collective output and function—offers significant advantages.

GEPASI Simulations of the System Comprising CB[6], CB[7], 1, and 2a. We used GEPASI to simulate the kinetic and thermodynamic outcomes of the four-component system comprising CB[6], CB[7], **1**, and **2a**. Figure 6a−c shows the equilibria considered, the kinetic and thermodynamic values, and the concentrations employed to generate the simulation shown in Figure 6d. The simulations reproduce the essential

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aspects of the experiments and validate the importance of the two-faced guests despite small differences in the degree of fidelity of the kinetic and thermodynamic self-sorting processes and the precise time course of the process.

The System Comprising CB[6], CB[7], 1, and 2a Undergoes High-Fidelity Thermodynamic Self-Sorting. The fidelity of this thermodynamic state depends on the initial concentrations of CB[6], CB[7], **1**, and **2a** and the values of K_a for CB[6]·**1**, CB[7]·**1**, CB[6]·**2a**, and CB[7]·**2a** but not on any of the individual rate constants. Interestingly, our estimates of K_a (Figure 6b) based on the literature suggest that guests **1** and **2** both prefer to bind to CB[7] (e.g. $K_{a\ (CB[7]\cdot\mathbf{1})} > K_{a\ (CB[6]\cdot\mathbf{1})}$ and $K_{a\ (CB[7]\cdot\mathbf{2a})} \gg K_{a\ (CB[6]\cdot\mathbf{2a})}$). Why then is high-fidelity thermodynamic self-sorting achieved? When there is a stoichiometric balance of hosts and guests (e.g. [CB[6]] = [CB[7]] = [**1**] = [**2a**]), the much larger free energy difference between the complexes of **2a** ($\Delta\Delta G = 6.4$ kcal mol^{-1}) can be used to drive **1** into its less thermodynamically stable CB[6]·**1** ($\Delta\Delta G = 3.0$ kcal mol^{-1}) complex. The behavior of the more complex four-component system is significantly different from those of the individual host−guest pairs because the overall free energy of the *entire system* is minimized.

The System Comprising CB[6], CB[7], 1, and 2a also Undergoes High-Fidelity Kinetic Self-Sorting. Which factors govern the fidelity of the kinetic self-sorting step? Initially, both CB[6] and CB[7] are in their free uncomplexed states. CB[7] with its wider carbonyl-lined portals exhibits faster association rate contants and is therefore filled first; the competition between **1** and **2a** greatly favors the formation of CB[7]·**1** kinetically because of the ≥20-fold difference in the values of k_{in}. The second critical variable governing the kinetic self-sorting step is the presence of the alkylammonium binding epitope on **2a** which allows it to be sequestered ($k_{in} = 4.4 \times 10^4$ M^{-1} s^{-1}) rapidly (e.g. complete in ∼0.1 s) as the CB[6]·**2a** complex. The final critical variable is that the dissociation rate constant for the CB[7]·**1** complex ($k_{out} = 2.7$ s^{-1}) is significantly slower than the amount of time needed to form the CB[6]·**2a** complex. If the CB[7]·**1** complex dissociates during the sequestration of **2a** as its CB[6]·**2a** complex then additional competitive opportunities occur between **1** and **2a** for the formation of CB[7]·**2a** and CB[7]·**1** which inevitably leads to increased amounts of the thermodynamically more stable CB[7]·**2a**. In summary, the fast sequestration of both **1** and **2a** into their respective kinetic complexes (CB[6]·**2a** and CB[7]·**1**) is critical because that keeps the concentrations of uncomplexed CB[6], CB[7], **1**, and **2a** low which reduces the rate of transition to the thermodynamic state by simple mass action considerations.

Interestingly, the simulations establish that the rate of approach to thermodynamic equilibrium after the kinetic self-sorting step is not governed by the individual rate constants, but rather by the value of K_a for CB[6]·**2a**. This value of K_a controls the amount of free CB[6] and **2a** after the initial kinetic self-sorting step. When K_a increases, the amount of free **2a** decreases, which in turn slows down the competition between **2a** and the even smaller amount of free **1** for CB[7]. Conversely, smaller values of K_a increase the rate of approach to equilibrium by increasing the concentration of uncomplexed **1**. Because the amount of uncomplexed **2a** is influenced by a number of variables—host:guest stoichiometry, total concentration, ionic strength, temperature, and the presence of competitors or chaperones—it should be possible to tune the behavior of the system by straightforward environmental changes.

The Fidelity of the Thermodynamic Self-Sorting State Is not Influenced by Guest Structure, Metal Ion Concentration, or Metal Ion Identity. Factors which decrease the difference in free energy between the various complexes (e.g. CB[7]·**2a** versus CB[6]·**2a**; CB[7]·**1** versus CB[6]·**1**) have the potential to decrease the fidelity of thermodynamic self-sorting. Increasing the length of the alkyl tail of the two-faced guest (**2b** or **2c**) or removing it completely (**3**) does not influence its affinity for CB[7] because a common adamantylammonium binding epitope is used to form this complex, whereas its affinity toward CB[6]—where the effect of chain length is well-known[13]—is decreased by the structural change. Therefore, the difference in free energy (e.g. CB[7]·**2a** versus CB[6]·**2a**) that drives the thermodynamic self-sorting process increases with the structural change. Accordingly, the fidelity of the thermodynamic self-sorting state remains high for all four guests (**2a**−**2c**, **3**).

Cations are well-known to bind to the carbonyl-lined portals of the CB[*n*] family.[11,12] For example, Buschmann has studied the interaction of CB[6] with a variety of metal cations (including Li^+, Na^+, K^+, Cs^+, and Ca^{2+}) and found that K_a does not vary significantly ($K_a \geq 10^3$ M^{-1}).[25] Kaifer demonstrated that increased concentrations of NaCl and $CaCl_2$ in the aqueous buffer decrease the observed binding constant of CB[7] toward methyl viologen.[26] Therefore, one might expect that the presence of high concentrations of Na^+ would also reduce the fidelity of the thermodynamic self-sorting state by competitive binding although that is not observed experimentally. We rationalize that the increase in [Na^+] concentration in this system reduces all four K_a (ΔG) values similarly and therefore does not significantly effect the overall values of $\Delta\Delta G$ (e.g. CB[7]·**2a** versus CB[6]·**2a**; CB[7]·**1** versus CB[6]·**1**) needed to drive thermodynamic self-sorting.

The Fidelity of Kinetic Self-Sorting Is Influenced by Guest Structure. Why is the fidelity of kinetic self-sorting decreased when the length of the alkylammonium binding epitope is increased (e.g., **2b** and **2c**) or when it is removed completely (**3**)? As described above for **2a**, the degree of kinetic self-sorting depends critically on the rate of sequestration of the two-faced guest as its CB[6]·**2a** complex. It is well-known in cucurbit[*n*]uril chemistry that butylammonium cation binds more tightly to CB[6] than longer alkylammonium ions (e.g., octyl- or hexylammonium) by approximately 10-fold per methylene group.[13] Using this information, we are able to extrapolate values of K_a for CB[6]·**2b** and CB[6]·**2c** ($K_a = 2.0 \times 10^5$ M^{-1} and 2.0×10^3 M^{-1}, respectively) in our solvent system. When combined with the experimentally determined value of k_{out} for CB[6]·**2c** (0.5 s^{-1})—which shows a decrease of 2.6-fold per methylene group relative to that for **2a**—and an interpolated value of k_{out} for CB[6]·**2b** (0.033 s^{-1}), it is apparent that the association rate constant for the formation of CB[6]·**2c** decreases as the length of the alkyl chain increases. In the extreme case of **3** which lacks an alkylammonium binding epitope we set K_a (CB[6]·**3**) = 0. Figure 7 shows kinetic simulations of these three systems based on the kinetic and thermodynamic parameters detailed above. In accord with the experimental findings (vide supra)

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(26) Ong, W.; Kaifer, A. E. *J. Org. Chem.* **2004**, *69*, 1383−1385.

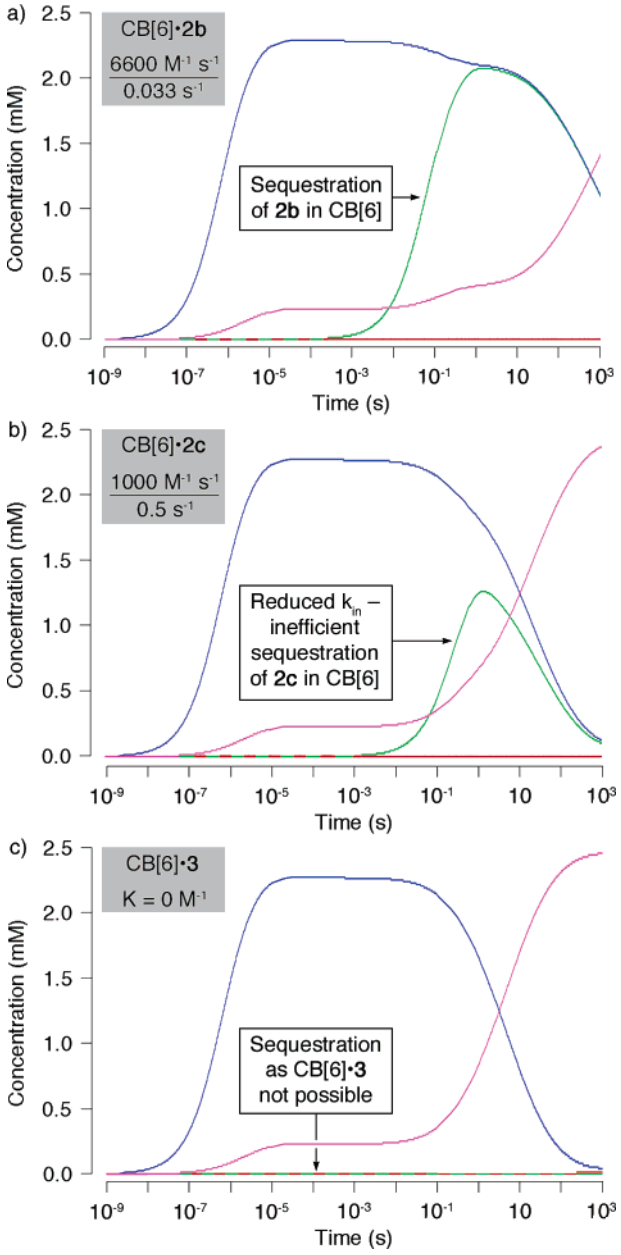

Figure 7. Simulation of the system comprising CB[6], CB[7], **1**, and (a) **2b**, (b) **2c**, and (c) **3**. The equilibria considered, the kinetic and thermodynamic values, and the intial concentrations of CB[6], CB[7], and **1** are the same as in Figure 6. Color code: CB[7]·**1**, blue; CB[7]·**2** (**3**), pink; CB[6]·**2** (**3**), green; CB[6]·**1**, red.

the simulations show greatly reduced levels of kinetic self-sorting after 6 min as the length of the alkylammonium binding epitope is lengthened or removed. The reduced levels of kinetic self-sorting for **2b**, **2c**, and **3** relative to that for **2a** can be attributed to two factors: (1) the less efficient kinetic sequestration (e.g. reduced association rate constant k_{in}) as their CB[6] complexes, and (2) the increased amounts of free two-faced guest as the valued of K_a for the alkylammonium binding epitope decreases which enhances competition with **1** for binding to CB[7].

The Fidelity of Kinetic Self-Sorting Is Influenced by Sodium Concentration. Alkali metal cations are well-known to bind to the members of the CB[*n*] family with a relatively constant value of K_a ($\approx 10^3$ M^{-1}).[11,25] As such, one might expect that increasing the concentration of sodium cation would simply

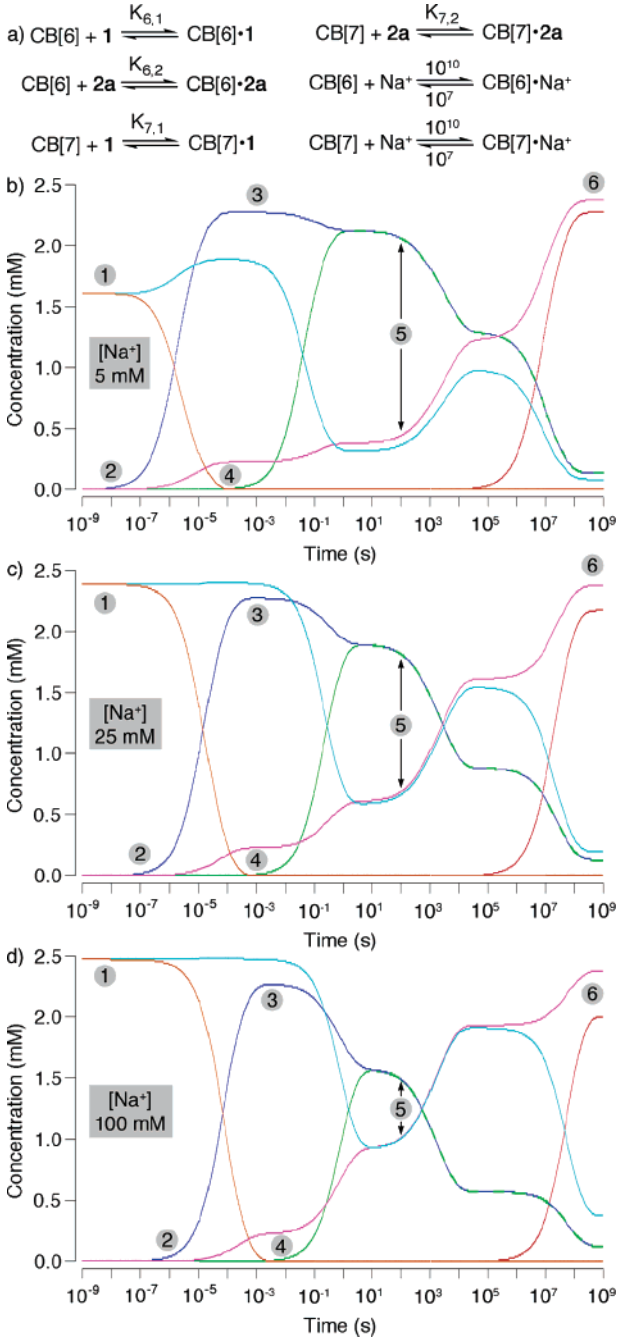

Figure 8. Simulation of the system comprising CB[6], CB[7], **1**, **2a** (all 2.5 mM), and Na_2SO_4. (a) Equilibria considered, (b) $[Na^+] = 5$ mM, (c) $[Na^+] = 25$ mM, (d) $[Na^+] = 100$ mM. The kinetic and thermodynamic constants for complexes of CB[6], CB[7], **1**, and **2a** are the same as in Figure 6. Color code: CB[7]·**1**, blue; CB[7]·**2a**, pink; CB[6]·**2a**, green; CB[6]·**1**, red; CB[6]·Na^+, aqua; CB[7]·Na^+, orange.

reduce the concentration of free CB[6] and CB[7] by formation of CB[6]·Na^+ and CB[7]·Na^+ and thereby slow the kinetics of the system, but that the degree of kinetic and thermodynamic self-sorting would not change. Why then does the fidelity of kinetic self-sorting decrease as the concentration of sodium cation increases? Figure 8 shows a simulation of the system as a function of Na^+ concentration that serves as a basis for discussion. First, note that at the beginning of the simulation (Figure 8, point 1), the higher the Na^+ concentration, the higher the concentration of CB[6]·Na^+ and CB[7]·Na^+ and the lower the concentration of free CB[6] and CB[7] available to form

host−guest complexes with **1** and **2a**. The competition of Na^+ with **1** for binding to CB[7] results in a delay in the formation of the CB[7]·**1** complex (Figure 8, point 2) from $\sim 10^{-8}$ to 10^{-6} s as Na^+ increases (5, 25, 100 mM). The plateau region for CB[7]·**1** concentration under kinetic control occurs at $\sim 10^{-3}$ s at a relatively constant value of 2.25 mM (Figure 8, point 3). Similarly, competition of Na^+ with **2a** for CB[6] delays the formation of CB[6]·**2a** from $\sim 10^{-4}$ to 10^{-2} s as sodium ion concentration increases (Figure 8, point 4). Sodium ion competition does, in fact, slow the formation of both CB[7]·**1** and CB[6]·**2a** similarly, although it does so at *different times*. The measured fidelity of kinetic self-sorting (Figure 8, point 5) decreases as $[Na^+]$ increases because the sequestration of **2a** as CB[6]·**2a** and dissociation of the initially formed CB[7]·**1** complex ($k_{out} = 2.7\ s^{-1}$, half-life = 0.26 s) begin to occur on the same time scale, allowing for increased competition between **1** and **2a** for CB[7]. The Na^+ ion and chain length effects are completely analogous since both variables act by slowing down the sequestration of the two-faced guest and thereby decrease the degree of kinetic self-sorting. The simulations reveal a small change in the fidelity of thermodynamic self-sorting (Figure 8, point 6) due to competition between Na^+ and **1** for CB[6] at the higher Na^+ concentrations. We do not observe this decrease experimentally; small changes in one or more K_a values could account for this discrepancy. As mentioned above, the simulations reproduce the overall behavior of the system although the precise fidelity of kinetic and thermodynamic self-sorting depends quite sensitively on the input kinetic and thermodynamic constants.

The Fidelity of Kinetic Self-Sorting Is Influenced by the Identity of the Cation. Figure 2b shows that the fidelity of kinetic self-sorting decreases as the ionic radius of the cation increases. As mentioned above, the affinities of most cations for CB[6] are similar ($\approx 10^3\ M^{-1}$) although the ionic radius of the larger cations should match better to the van der Waals radius of the CB[6] portal (1.95 Å).[27] Our first hypothesis, therefore, was that the rate constants for CB[*n*]·M^+ might be the source of the observed cation identity dependence. Simulations (not shown) establish that, while rate constants for CB[*n*]·M^+ can influence the observed fidelity of kinetic self-sorting, they only do so when k_{on}/k_{off} are unreasonably small (e.g. ,$10^3\ M^{-1}\ s^{-1}/1\ s^{-1}$) given the structure of CB[6]. A second hypothesis is based on the potential direct conversion of CB[*n*]·M^+ and guest into CB[*n*]·guest and M^+. In none of the simulations reported above (Figures 6−8) did we allow such a process. One can imagine, however, that the smaller cations (e.g. Li^+, ionic radius = 0.68 Å) which block the portal of CB[6] less completely than the larger cations (e.g. Cs^+, ionic radius = 1.67 Å) might participate more readily in such a direct conversion process. When such direct conversion is allowed, the larger cations would be predicted to result in lower-fidelity kinetic self-sorting processes as is observed experimentally. A detailed assessment of the importance of this direct conversion process is not possible with our current experimental data.[28]

Conclusions

This paper advocates the use of systems chemistry—as opposed to a more traditional approach based on single host−guest complexes—as a route to discover aggregates with anomalous properties and systems with collective function. This paper explored the hypothesis that compounds that contain multiple binding epitopes might result in systems with unusual dynamic behavior. We employed an iterative approach involving the preparation of four-component mixtures and observation of their dynamic properties by ^1H NMR spectroscopy which ultimately lead to the system comprising CB[6], CB[7], **1**, and **2** (**3**) which was deconstructed in detail in this paper. Just like their natural counterparts, complex systems containing two-faced guests such as **2** are found to display unusual dynamic phenomena including kinetic self-sorting. For example, under kinetic control two-faced guest **2a** uses its slim alkylammonium binding epitope to form CB[6]·**2a**, whereas **1** engages in the CB[7]·**1** complex; at thermodynamic equilibrium the roles are completely reversed. We have explored several of the factors governing the fidelity of the kinetic self-sorting step including the concentration and identity of metal cations present in solution, the length of the alkylammonium binding face of **2**, and the influence of individual rate and equilibrium constants. We find that factors which retard the rate of sequestration of the two-faced guest within CB[6] reduce the fidelity of the kinetic self-sorting step.

More fundamentally, we believe that systems chemistry—with its focus on collective output and function—has great potential to enhance the rate of discovery of compounds with anomalous recognition properties and functional supramolecular systems. For example, the study of a thermodynamic self-sorting system previously led us to the discovery of compounds with affinities exceeding $10^{12}\ M^{-1}$ in water.[6] In this paper, we discovered a four-component system whose deconstruction lead to the measurement of a host−guest system with truly anomalous properties—namely CB[6]·**1** with a dissociation rate constant of $8.5 \times 10^{-10}\ s^{-1}$ which is approximately 2 orders of magnitude slower than avidin·biotin. The CB[6]·**1** complex has a half-life of 26 years at room temperature! The remarkably slow dissociation rate constant for CB[6]·**1** suggests that this binding pair may find use in immobilization and affinity column applications typically reserved for avidin·biotin. These results highlight how the kinetic and thermodynamic behavior of complex *n*-component systems—which depend on the matrix of possible pairwise interactions—efficiently directs the researcher toward recognition anomalies that stimulate a deeper understanding of recognition phenomena.

Finally, the systems chemistry approach is quite versatile and allows the testing of hypotheses derived from observations of more complex natural systems that have always served as stimulation for chemists. In this paper we explored the hypothesis that the presence of multiple binding epitopes plays

(27) The observed correlation with the ionic radius of the desolvated cations may seem strange given that these cations are hydrated in water. Upon complexation to CB[*n*], some of the hydrating H_2O molecules are displaced by coordination with O-atoms of the carbonyl-lined portals of CB[*n*].

(28) One approach to address this question would involve measurement of the association rate constant for CB[6] and **2a** as a function of metal ion concentration. Unfortunately, the analytical tools at our disposal do not allow the measurement of such rapid processes (e.g., $10^4\ M^{-1}\ s^{-1}$); Tarmyshov, K. B.; Muller-Plathe, F. *J. Phys. Chem. B* **2006**, *110*, 14463−14468.

a key role in controlling the dynamic behavior within complex systems. By using an iterative approach based on these broad design principles—in concert with simulation to inform chemical intuition along the way—it is possible to program both the thermodynamic outcome of self-sorting systems and the kinetic pathways by which they evolve toward equilibrium. When the strategy for temporal control introduced in this paper is combined with future modules that enable catalytic processes, feedback loops, compartmentation, and metastable states, we anticipate that the toolbox available to systems chemists will be sufficiently diverse to allow the engineering of functional systems currently only accessible to systems biologists.

Experimental Section.

Sample Preparation. The four-component kinetically self-sorted mixture comprising CB[6], CB[7], **1**, and **2** was prepared as follows: (1) the calculated amounts of each component were weighed out separately. The hosts CB[6] and CB[7] and the guests **1** and **2** were transferred, respectively, into two separate 5-mL screw-capped vials; (2) CB[6] and CB[7] were dissolved in 1 mL of D_2O containing the desired amounts of Na_2SO_4; (3) **1** and **2** were dissolved in 1 mL of D_2O containing the desired amounts of Na_2SO_4. The solutions containing the components were maintained at 5 mM; (4) the pD of each solution was adjusted to 7.4 with concentrated NaOD or DCl solution; (5) 0.3 mL of solution from each of the vials was transferred using a Hamilton syringe to an NMR tube for analysis; (6) the NMR spectra were recorded at 298 K within 10 min after mixing. Similar procedures were followed for preparing the other four-component mixtures. Samples for the variable Na ion concentration experiments were prepared using 0−500 mM of Na_2SO_4 in step 2. Samples for the variable metal ions experiments were prepared using 25 mM solutions of the corresponding salt ($Li^+/K^+/Cs^+/Mg^{2+}/Ca^{2+}$) in step 2. For variable pD experiments samples were prepared by adjusting the pH from 2.8 to 12.8 in step 3. The same procedure was followed for all the other steps.

Determination of Values of k_{out}. The values of k_{out} at 298 K for CB[7]·**2a**, CB[7]·**2b**, CB[7]·**2c** were determined using 13 equiv of **5** as displacing ligand as described in the text and detailed in the Supporting Information. The value of k_{out} for the CB[6]·**2a** complex was determined in a similar manner at 298 K using 1,6-hexanediamine as the displacing ligand. The value of k_{ex} for CB[7]·**1** was determined by standard 2D-EXSY experiments using a solution containing CB[7]·**1** and free **1**. The spectra were recorded at 298 K with mixing time of 200 ms. EXSY measurements were also used to determine k_{out} CB[6]·**2c** = 0.50 s^{-1}. Compound **2b** in the complex CB[6]·**2b** did not show cross-peaks in the 2D-EXSY spectrum over a broad range of mixing times (50−900 ms); accordingly we could not measure a value of k_{ex} for this pair by this method.

Simulations. Simulations were performed using Gepasi 3.30 running on a Windows XP workstation. The Gepasi model files used in these simulations are deposited in the Supporting Information.

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Supporting Information Available: Synthetic procedures and characterization data, selected ^1H NMR spectra for CB[*n*]·guest complexes, determination of k_{out} and k_{in} values, ^1H and ^{13}C NMR spectra for all new compounds; Gepasi model files; and details of the X-ray structure of CB[6]·**1** in CIF format. This material is available free of charge via the Internet at http://pubs.acs.org.

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