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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
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Spin Exchange Monitoring of the Strong Positive Homotropic Allosteric Binding of a Tetraradical by a Synthetic Receptor in Water

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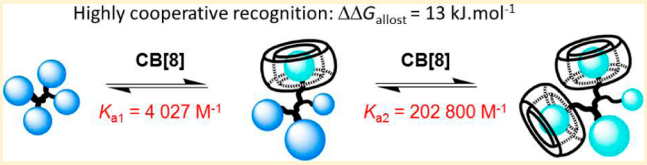
Supporting Information

ABSTRACT: The flexible tetranitroxide **4T** has been prepared and was shown to exhibit a nine line EPR spectrum in water, characteristic of significant through space spin exchange (J_{ij}) between four electron spins interacting with four nitrogen nuclei ($J_{ij} \gg a_N$). Addition of CB[8] to **4T** decreases dramatically all the J_{ij} couplings, and the nine line spectrum is replaced by the characteristic three line spectrum of a mononitroxide. The supramolecular association between **4T** and CB[8] involves a highly cooperative asymmetric complexation by two CB[8] (K_1 = 4027 M^{-1}; K_2 = 202 800 M^{-1}; α = 201) leading to a rigid complex with remote nitroxide moieties. The remarkable enhancement for the affinity of the second CB[8] corresponds to an allosteric interaction energy of ≈13 kJ mol^{-1}, which is comparable to that of the binding of oxygen by hemoglobin. These results are confirmed by competition and reduction experiments, DFT and molecular dynamics calculations, mass spectrometry, and liquid state NMR of the corresponding reduced complex bearing hydroxylamine moieties. This study shows that suitably designed molecules can generate allosteric complexation with CB[8]. The molecule must (i) carry several recognizable groups for CB[8] and (ii) be folded so that the first binding event *reorganizes* the molecule (unfold) for a better subsequent recognition. The presence of accessible protonable amines and H-bond donors to fit with the second point are also further stabilizing groups of CB[8] complexation. In these conditions, the spin exchange coupling between four radicals has been efficiently and finely tuned and the resulting allosteric complexation induced a dramatic stabilization enhancement of the included paramagnetic moieties in highly reducing conditions through the formation of the supramolecular **4T**@CB[8]$_2$ complex.



INTRODUCTION

Allostery is a collective property of some unusual chemical or biological systems occurring when this ensemble behaves differently with respect to expected interactions based on isolated, individual molecular components.[1] This property is usually closely related to positive cooperativity found when, for successive supramolecular events, the binding of a first component enhances the following binding events.[2] Allostery has been shown to play crucial roles in biology where subtle interaction-induced conformational changes are pivotal for new biological functions to occur.[3] This concept has then been relayed to chemists[4] that used it to prepare efficient catalysts[5] and advanced molecular architectures[6] even though the design of efficient allosteric systems is still challenging. Among allosteric systems, organic assemblies working in water remain rare,[7] but several interesting studies based on cucurbiturils have been reported.[8] However, quantification of the allosteric binding involving cucurbiturils has only been realized in two instances and for heterotropic systems.[8b,c] Nitroxides are routinely used as paramagnetic probes,[9] and in recent years, various reports described the preparation of supramolecular assemblies containing nitroxide free radicals.[10] One of the motivations for their use is related to the property of nitroxides to report subtle changes in their local environment and dynamic features induced by complexation or self-assembly phenomena. In the macrocyclic host/guest chemistry, nitroxides have been shown to be very useful to study the binding properties of cyclodextrins[11] and cucurbiturils[12] by electron paramagnetic resonance (EPR) spectroscopy. For instance, in mononitroxides, the nitrogen hyperfine coupling constant (a_N) changes as a function of its direct surrounding and the EPR line shape is a good reporter of the dynamics of the system.[13] For polynitroxides, additional magnetic interactions, i.e., electron−electron dipolar and electron spin exchange, are present in the molecule.

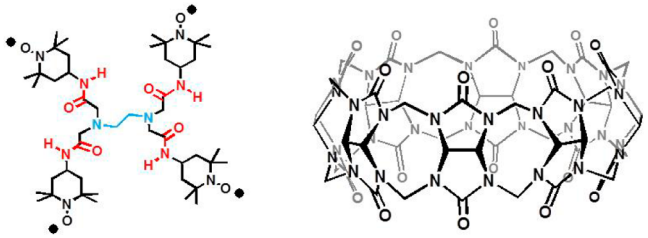
Because the spin exchange interaction is distance dependent, variations of the conformation of the molecule can be visualized

Received: September 23, 2014
Published: November 22, 2014

dx.doi.org/10.1021/ja509586k | J. Am. Chem. Soc. 2014, 136, 17570−17577

by EPR.[14] Recently, several authors have reported the modulation of this interaction in dinitroxides using different strategies. For example, Feringa used light as the stimulus to control the cis/trans isomerization of an alkene bearing two TEMPO nitroxides.[15] Using host/guest complexation or self-assembly, Chechik,[16] Kaifer,[17] Lucarini,[18] and Ramamurthy and Ottaviani[19] controlled the distance separating the unpaired electrons by adding CB[*n*], CDs, octaacid, and resorcinarene capsules to the system, or by guanosine quadruplex formation. In our investigation at the interface between free radicals and macrocyclic hosts,[20] we recently reported the occurrence of a supratriradical based on 4-methoxy-TEMPO (4M) and cucurbit[8]uril (CB[8])[21] of composition $\{4M@CB[8]\}_3$ giving an EPR signature corresponding to a triangular assembly (seven line pattern).[22,23] Herein, we report the dramatic and reversible modulation of the spin exchange interaction in a tetranitroxide controlled by the allosteric complexation of CB[8] (Scheme 1). The EDTA core structure of the tetraradical offers a promising scaffold: (i) carrying four TEMPO units to monitor the allosteric complexation by subtle changes of the spin exchange interaction and (ii) presenting two protonable amine groups amenable for the attraction and complexation of CB[8] macrocycles and four additional amide groups as hydrogen bond donors. We also discuss structural requisites that are responsible for this allosteric behavior.

Scheme 1. Structures of Tetranitroxide 4T and of CB[8]



RESULTS AND DISCUSSION

Synthesis of Tetranitroxide EDTA4T. The tetranitroxide **EDTA4T** (hereafter referred to as **4T**) based on the EDTA skeleton was synthesized in two steps starting from 4-aminoTEMPO (Supporting Information Figure S1). First, the amine function was acetylated using 2-bromoacetyl bromide under argon at room temperature for 24 h. The purified product was then reacted with ethylenediamine in acetonitrile at 50 °C during 5 days, and **4T** was obtained with a global yield of 56% after purification.

EPR Spectroscopy. At 363 K, the aqueous solution EPR spectrum of **4T** (Figure 1) is composed of nine lines, the line spacing corresponding to about 1/4 of the nitrogen hyperfine splitting (a_N) for TEMPO in water.

These EPR features are characteristic of strong exchange couplings ($J_{ij} \gg a_N$) between the unpaired electrons, each electron interacting equally with the four equivalent nitrogen nuclei.[24] Neglecting the nonsecular terms of the equivalent nitrogen hyperfine couplings, the spin Hamiltonian for a polynitroxide bearing equivalent nitroxide moieties is written as

$$\sum_i g\beta_e B\hat{S}_{iz} + a_N \sum_i \hat{S}_{iz}\hat{I}_{iz} + \sum_{ij} J_{ij}\hat{S}_i\hat{S}_j$$

The first component is the Zeeman coupling between the unpaired electron spins and the magnetic field, the second component is the hyperfine coupling between the unpaired electron spin and the nitrogen nuclear spin of a nitroxide moiety, and the third component is the spin exchange between the unpaired electron spins. For a flexible molecule like **4T**, the exchange couplings have time dependence ($J_{ij}(t)$), and the shape of the EPR spectrum depends dramatically on the ratio between the magnitudes of the average exchange coupling ($\overline{J}$) and a_N.[24−26] When $\overline{J}$ is small compared to a_N ($\overline{J} \ll a_N$), each nitroxide moiety separately contributes to the EPR signal, which is therefore constituted of a 1:1:1 triplet. On the other hand, when $\overline{J} \gg a_N$, each unpaired electron interacts equally with the nitrogen nuclei and the number of lines depends on the number of nitrogen nuclei. With four nitrogen nuclei, a spectrum composed of nine lines separated by $a_N/4$ and with relative intensities of 1:4:10:16:19:16:10:4:1 is expected.[24−26] In water solution at 363 K the EPR spectrum of **4T** is indeed composed of nine lines (Figure 1a), with a line spacing (4.2 G) corresponding to ∼1/4 of the a_N value observed for TEMPO in water solution. However, at 363 K, the limit of very rapid exchange is not yet achieved, and the spectral lines do not exhibit the expected intensity ratios. In water at 298 K, a dramatic change of the EPR spectrum was observed (Figure 1b).

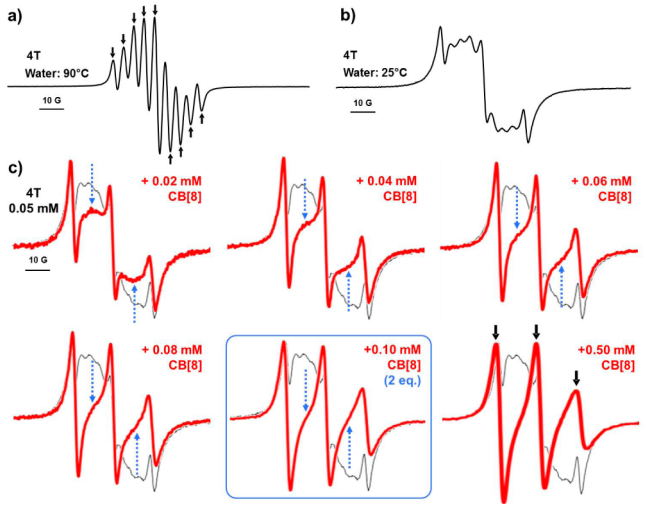


Figure 1. EPR spectra in water solution of (a) **4T** at 363 K, (b) **4T** at 298 K, and (c) **4T** in the presence of increasing amounts of CB[8] highlighting the absence of noticeable changes above 2 equiv of CB[8].

Nine lines are still observable, but the spectrum, while remaining symmetric, looks like the superimposition of a sharp triplet and six broad lines. This change is a consequence of the major contribution of the *J* modulation to the transverse electron relaxation time in polynitroxides.[27] The lines of the EPR spectrum arising from polyradicals in which all ^{14}N nuclei have the same spin state (i.e., $m_I = 1$, $m_I = -1$, or $m_I = 0$ for all *I*, where m_I is the ^{14}N spin quantum number for the *i*th nitroxide) do not have hyperfine fluctuations and are not broadened by the *J* modulation.[27] Thus, for **4T**, as shown in Figure 1b, the two extreme spectral lines ($M_I = \pm 4$) and one component of the central line ($M_I = 0$) remain relatively sharp, while the other lines are broadened to varying degrees. The influence of temperature on the **4T** EPR spectrum shape brings to light the high flexibility of the molecule and the dramatic influence of the *J* modulation. We thus aimed to study the effect on the spin exchange, of CB[8] that has (i) a suitable size to complex TEMPO moieties with respect to CB[7] and (ii)

dx.doi.org/10.1021/ja509586k | J. Am. Chem. Soc. 2014, 136, 17570−17577

carbonyl fringed portals which can stabilize the protonated forms of the two amine sites of **4T**. It rapidly appeared that very low concentrations of CB[8] dramatically changed the shape of the EPR signal of **4T** (Figure 1c). To determine the binding model and the association constants between **4T** and CB[8], we performed EPR titrations which were analyzed by the 2D_EPR program we have developed[28] (Table 1 and Supporting Information Figures S2−S4). Here, the crucial point of this approach is to precisely determine the variation, with respect to the CB[8] concentration, of the relative concentration of species contributing to the EPR signal. We considered four contributing species: the free **4T** and three **4T**@CB[8]$_n$ complexes with n = 1, 2, and 3. A curve fitting nicely the EPR signal of the free **4T** was obtained through the superimposition of a broad signal (BS, obtained with a hyperfine pattern using effective nitrogen splitting constants, Supporting Information Figure S4) to a 1.1.1 triplet identical to the TEMPO triplet (g = 2.0058, a_N = 16.99 G). Following the concentration variation in a broad range, we determined association constants for the three **4T**@CB[8]$_n$ complexes.

Table 1. EPR Parameters and Stepwise Binding Constants of All Species Considered in the Titration of Tetranitroxide 4T by CB[8] in Water

species	g	a_N/mT	K/M^{-1}
4T (**BS**[a] + **T**[b])			
4T-CB[8]	2.0060	1.638	4 027
4T-(CB[8])$_2$	2.0057	1.641	202 800
4T-(CB[8])$_3$	2.0058	1.597	157

[a]Fictive nitrogen coupling constant, see Supporting Information. [b]g = 2.0058, a_N = 16.99 G.

The spectra of the four components used to fit the calculated spectra are shown in the Supporting Information (Figure S4). The use of an artificial spectrum for the free **4T** does not affect the reliability of association constants, but the hfs couplings have no significance. After decomposing all the spectra into the five species, their EPR parameters (g, a_N, α, β, γ) and the association constants K_n were determined (29 parameters altogether) (Table 1, Figure 2, and the Supporting Information). The increase of relaxation parameters (Supporting Information Table S1) and the decrease of a_N are clear signs of binding events. However, it should be noted that the apparent a_N value is an average of the four hyperfine couplings (between included and free TEMPO moieties). A striking result is the propensity of CB[8] to rapidly form a 1:2 complex (Figure 2). A moderate binding constant was found for the first binding event ($K_1 \approx 4027$ M^{-1}), however, the association for the second event is exceedingly high ($K_2 \approx 202\ 800$ M^{-1}), indicating a strong cooperativity. To the best of our knowledge, this is one of the highest cooperative systems reported for synthetic complexes so far.[7,8] This 50-fold enhancement in CB[8] binding after the first complexation corresponds to an allosteric interaction energy of ≈13 kJ mol^{-1}, which is similar to the difference between the first and the last binding steps of oxygen by hemoglobin ($\Delta\Delta G_{allost} \approx 15$ kJ mol^{-1}).[6b,29] The cooperativity factor ($\alpha = 4K_2/K_1 \approx 201$)[2b,30] is large and reflects that the association of a second cucurbit[8]uril is promoted if already one CB[8] is associated. In essence, it means that the first binding event greatly enhances (by a factor of ≈200) the binding of a second CB[8]. The possible driving forces for this process are most likely a preorganization of the tetranitroxide in the 1:1 complex, where a suitable second TEMPO unit is favorably included in an additional CB[8] but also a large set of available C−H and N−H hydrogen bonds between the tetranitroxide skeleton toward the incoming oxygen rich cucurbituril rim of the second CB[8] molecule. As will be seen later (NMR, DFT), additional hydrogen bonds between the two cucurbiturils are also very likely to strengthen the assembly in a manner reminding of what is seen in crystal structures of cucurbiturils (partial self-closing of the cavities)[31] and in the CB[8] triangles.[21−23] Although this phenomenon is quite common in biology with systems displaying high α values,[32] it remains relatively rare for organic systems. If a third CB[8] was to be considered for binding, its association was found to be much less promoted than the second ($K_3 \approx 157$ M^{-1}) reflecting some hindrance or energy penalty for the binding event leading to the 1:3 complex (negative cooperativity). Figure 2 shows the species distribution over a small range of CB[8] concentrations. It shows that, at 0.3 mM, around 85% of the tetraradical is in the form of the 1:2 complex and, after 0.4 mM, there hardly remains free tetranitroxide in solution. Finally, electrospray mass spectrometry also supported these results[33,34] with the observation of all three complexes with guest:host stoichiometries from 1:1 to 1:3 (Supporting Information). The entire set of properties of this system (spin probes, EPR monitoring, high affinity cucurbituril hosts, tendency of CBs aggregation, protonable guest) is at the origin of the observed results.

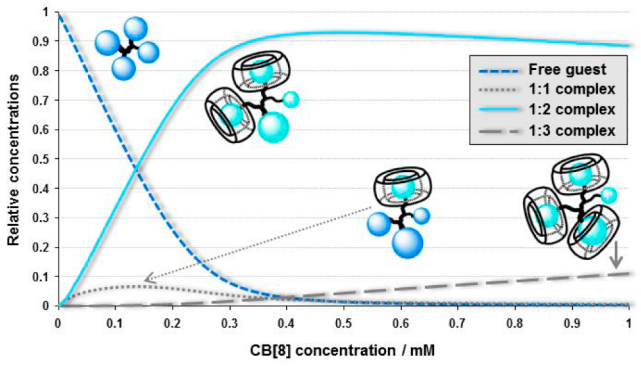


Figure 2. Product distribution for the binding of tetranitroxide **4T** (0.05 mM) with CB[8] in water.

NMR Spectroscopy. NMR study of the inclusion complexes was performed by reducing the paramagnetic nitroxides to diamagnetic hydroxylamines using ascorbic acid.[35] In these conditions, the ^{1}H NMR spectrum of **4T** exhibits a set of two well-resolved singlets occurring for the methyl groups around 1.5 ppm (Supporting Information Figure S6). However, when CB[8] is added to the solution, the two singlets become broader and less resolved and two new sets of broad signals are observed which are shifted upfield. These additional signals are in a slow exchange regime with regard to the NMR time scale. The values of the complexation induced chemical shift changes ($\Delta\delta$) are large (−0.60 and −0.65 ppm), in line with previous work for TEMPO diamagnetic analogues in the presence of CB[8] (≈0.70 ppm upfield shift).[23] The ratio between the integrals of the signals assigned to free and complexed species roughly correspond to a 1:2 complex. Interestingly, the NMR signals of CB[8] are modified upon mixing, displaying multiple unresolved peaks (Figure 3). For instance, the CB[8] equatorial methine protons display 7 peaks

dx.doi.org/10.1021/ja509586k | J. Am. Chem. Soc. 2014, 136, 17570−17577

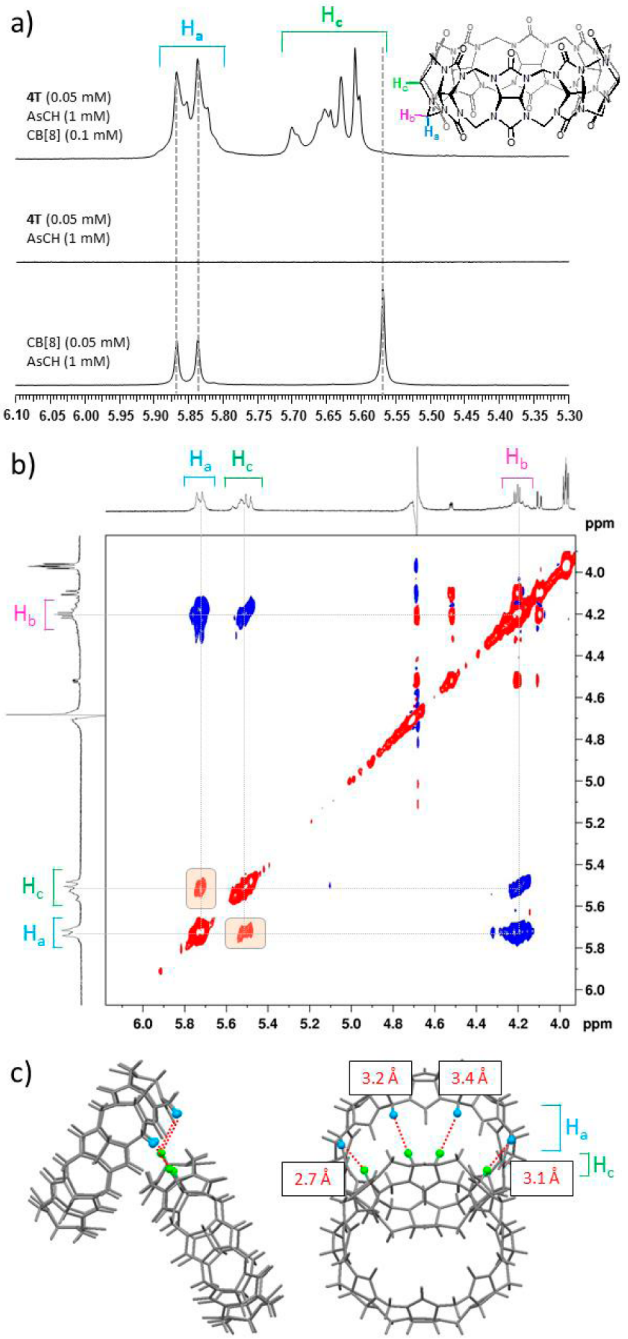
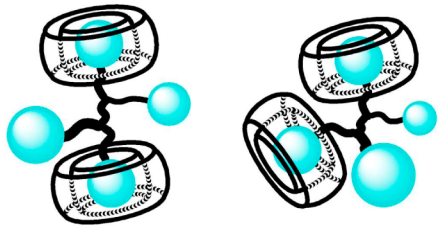


Figure 3. (a) Excerpts of the 600 MHz ^{1}H NMR spectra of the reduced form of **4T** (hydroxylamines) in the absence and in the presence of CB[8] in water showing the splitting of the singlet of the methine protons of CB[8]. (b) Part of the 2D-ROESY spectrum of a mixture of **4T** (0.05 mM) and CB[8] (0.1 mM) with ascorbic acid (1 mM, 300 K, mixing time: 800 ms). (c) Part of the CB[8] supramolecular triangle (X-ray structure of ref 21) featuring two CB[8] interacting by multiple C−H···O interactions as a means to measure H···H contacts between $H_a(CB[8])_{TOP}$ and $H_c(CB[8])_{BOTTOM}$.

(Figure 3a) instead of a singlet for free CB[8]. This change reflects the break of the D_{8h} symmetry of CB[8] (H_c), which is not compatible with the occurrence of a 1:1 complex or with a symmetric 1:2 complex (Scheme 2 left).

Therefore, instead of a C_8 symmetry sandwich complex in which the two cucurbiturils would be maintained relatively far

Scheme 2. Two Possible Modes of Inclusion for the Complex 4T@CB[8]$_2$



from one another, it seems more plausible that the complex tends to be folded in a way in which the two CB[8] can be close to each other (Scheme 2 right). It is well-known that cucurbiturils tend to self-associate by means of multiple weak CH···O hydrogen bonds so that they self-close their cavities.[31] The inclusion of two TEMPO groups in two CB[8] may have brought two macrocycles close enough to interact as seen in crystals of CB[8]. Another explanation may be the occurrence of several conformers of the 1:2 complex exchanging slowly with respect to the NMR time scale. But the absence of noticeable changes in these NMR patterns with increasing temperature (283−343 K) does not favor this hypothesis.

In order to gain further insights on the complex structure, 2D NMR experiments were performed. The 2D-ROESY spectrum shows cross correlations due to H_b−H_a and H_b−H_c interactions that are most likely intramolecular (Figure 3b in blue). Indeed, these cross peaks have opposite signs with respect to diagonal peaks and suggest the occurrence of ROE contacts. However, the detection of a positive H_a−H_c cross peak (orange highlighted, Figure 3b) which is absent when **4T** is not present in the sample (i.e., for CB[8] alone at the same concentration) is in line with cucurbituril aggregates.[34,36] This observation is in good agreement with the splitting pattern observed for H_c in the 1D ^{1}H NMR spectrum highlighting asymmetric **4T** complexation by two CB[8] molecules (Figure 3a). The sign of the cross peak was intriguing, and after the recording of TOCSY, ROESY, and NOESY spectra at different mixing times, the building curves (volume integrals of the cross peak as a function of the mixing time) indicate that this correlation is most probably due to spin diffusion instead of direct dipolar coupling (NOE effect).[37]

This correlation indicates that there must be some protons acting as relays between H_a and H_c but also that the distance between these two kinds of protons must be rather short. All these data are in line with a tetranitroxide **4T** complexed by a V-shaped dimer of CB[8] (Figure 3c) reminding of the case of the supramolecular triangles for which one CB[8] is missing.[21]

These results are further supported by molecular dynamics simulations and DFT calculations of tetranitroxide **4T** (neutral, singly charged, and doubly charged), the 1:1 complexes (singly and doubly charged) and the 1:2 complex (doubly charged). The pK_a values of **4T** were measured by pH-metry in pure water. The titration curve allowed determination of two very close jumps corresponding to the two pK_a values 6.45 and 7.35 reflecting a very rapid transition from the unprotonated (**4T**) to the mono- (**4T**-H$^+$) and diprotonated (**4T**-2H^{2+}) forms.

Theoretical Calculations. Molecular dynamics (MD) simulations in water using Gromacs 4.6[38] package over a period of 5 ns allowed determination of the interspin distances between the four TEMPO units for 1:1 and 1:2 complexes (doubly charged form). Computational details are given in the

dx.doi.org/10.1021/ja509586k | J. Am. Chem. Soc. 2014, 136, 17570−17577

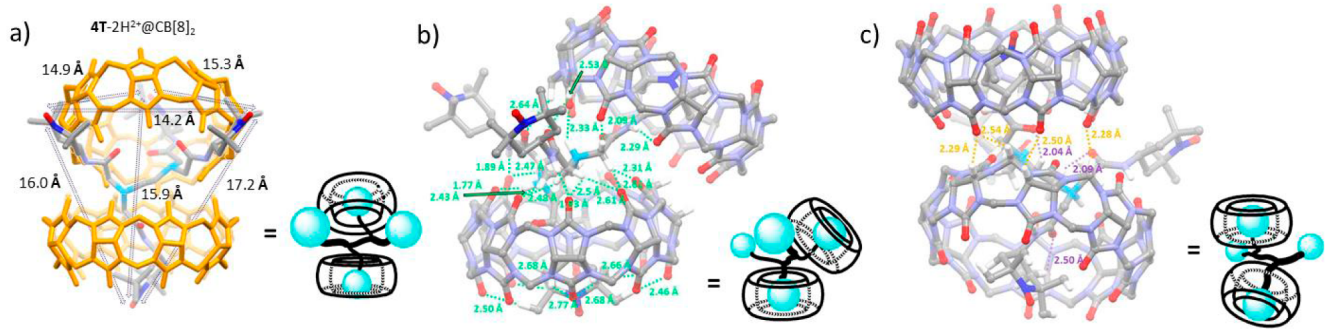


Figure 4. (a) Calculated structure (B3LYP/6-31G(d)) of the most stable conformer of the 1:2 complex of tetranitroxide **4T** in its doubly charged state with CB[8]. The large interspin distances (>14 Å) correlate well with experimental data (EPR). Host−guest (b) and host−host (c) hydrogen bonds and weak interactions found in the 1:2 complex of **4T** with CB[8] (see text and Supporting Information for details).

Supporting Information. For the 1:1 CB[8] inclusion complex, the tetranitroxide stayed included with one TEMPO unit in the CB[8] cavity. Average $O^{\bullet}-O^{\bullet}$ distances and complete range of $O^{\bullet}-O^{\bullet}$ distances are in a large window of ~9 Å (6−7 < $d_{O^{\bullet}-O^{\bullet}}$ < 15 Å, Supporting Information Figure S7). For the 1:1 complex, while four of the six interspin distances are above 11 Å, some conformations are found with distances $O^{\bullet}{}_1-O^{\bullet}{}_3$ and $O^{\bullet}{}_2-O^{\bullet}{}_3$ of 9 Å and 6−7 Å, respectively. These conformations correspond to a regime where the spin exchange interactions are large with regard to the hyperfine coupling, leading to an EPR spectrum exhibiting a number of lines >3 (not experimentally observed, see Figure 1c, 3 lines). Thus, geometrical considerations regarding the 1:1 complex are not sufficient to account for the observed EPR results. However, for the 1:2 complex, the two CB[8] molecules repelled the TEMPO units away from each other. The interspin distances are all above 11−12 Å, with averaged distances between 13 and 19 Å (Supporting Information Figure S8), which is in agreement with the observed three line EPR spectrum. Interestingly, the CB−CB distance (between the center of mass of the two closest oxygen crowns) is ~10 Å and the angle between the two planes defined by these crowns is 25 ± 14°, which compares favorably with the DFT minimized structure (33°, Figure 4a).

Quantum mechanics calculations have been performed to determine the geometries of the free and complexed (1:1 and 1:2) molecules.[39] DFT calculations were performed at the B3LYP/6-31G(d) level with a continuum of water (PCM method) using Gaussian 09 D.01.[40] For the tetranitroxide alone, at pH 6.8, at which the EPR experiments were performed, a mixture of **4T**-H^+ and **4T**-$2H^{2+}$ should be considered with a substantial population of **4T**. These three species were then considered for the DFT calculations. Because of the pK_a shift effect of cucurbiturils (supramolecular charge stabilization),[41] we only considered charged states for the CB[8] complexes. Thus, **4T**-H^+ and **4T**-$2H^{2+}$ were considered for the complexes (**4T**-H^+@CB[8], **4T**-$2H^{2+}$@CB[8], **4T**-$2H^{2+}$@$CB[8]_2$). The simulations for **4T** alone (neutral, singly charged, and doubly charged forms) indicate that for the vast majority of the 10 most stable conformations (Supporting Information Figure S9), the four TEMPO moieties tend to magnetically interact (folded conformations) with interspin distances in the range 8−13.8 Å, which is compatible with the EPR spectrum of **4T** in water (Figure 1b). In the four most stable conformers of the 1:1 complex (Supporting Information Figure S10), three uncomplexed TEMPO moieties are placed relatively close (8.3−13.5 Å) while the last TEMPO is pushed away from the others once included in the CB[8] cavity (d > 17 Å except for the monoammonium form). Thus, the first binding event reorganizes the molecule **4T** favoring the approach of a further CB[8]. Interestingly, the proximity of one of the two carbonyl crowns of the complexed CB[8] to the two amine functions of **4T** likely shifts their pK_a, and this may be an additional driving force for the binding of the second CB[8]. For the 1:2 complex, the two included TEMPO moieties are far from all others and the two remaining are also pushed away at distances >14.2 Å (Figure 4a). In this conformation, the spin exchange interactions are expected to be small, in agreement with the observed three line spectrum of Figure 1c.

Whereas free tetranitroxide **4T** is shown to have at least three intramolecular hydrogen bonds (number independent of protonation states, Supporting Information Figure S9), the 1:1 complex shows one TEMPO moiety included in CB[8] with the tetraradical skeleton multiply hydrogen bonded for the three conformers of the bisammonium form (Supporting Information Figure S10). Besides the hydrophobic effect (TEMPO inclusion), a strong charge stabilization is likely to direct the structure of the 1:1 complex as reflected by the large number (12 for **4T**-$1H^{1+}$@CB[8] and 17 for **4T**-$2H^{2+}$@CB[8]) of intramolecular and intermolecular CH···O and NH···O interactions featuring activated (amide, α-amine, or α-ammonium) or nonactivated (TEMPO) hydrogen atoms. The three strongest H bonds (1 $N^+-H\cdots O{=}C$ and 2 $N-H\cdots O{=}C$) appear to anchor the tetraradical near the entrance of one rim of CB[8]. In addition to these stabilizing interactions, there are a number of CH···O close contacts, ranging from close (2.1 Å, 159.8°) to some near the limit of the sum of van der Waals radii (~2.8 Å, 165.0°). The $N-H\cdots O{=}C$ hydrogen bonds occur to orientate two of the three remaining TEMPO units at opposite directions in the plane perpendicular to the C_8 axis of CB[8]. Thus, one TEMPO moiety is isolated from the three other moieties with distances ranging from 9.5 to 13.5 Å contrary to 7.0−12.4 Å for the free tetraradical. The inclusion process likely benefits from (i) the hydrophobic effect due to the encapsulation of one TEMPO unit and (ii) additional stabilizing interactions, mainly host−guest hydrogen bonds plus one charge-assisted hydrogen bond due to the presence of the ammonium cation.

However, the addition of a second CB[8] to produce the 1:2 complex resulted in TEMPO moieties being further separated from one another. As a consequence, the interspin distance is now between 14.2 and 17.2 Å, precluding the occurrence of any strong spin exchange interaction (Figure 4a).[42] In this

dx.doi.org/10.1021/ja509586k | J. Am. Chem. Soc. 2014, 136, 17570−17577

geometry, the two cationic charges are stabilized by ion−dipole interactions and there are numerous host−guest hydrogen bonds (21) plus 3 intramolecular hydrogen bonds and 4 host−host C−H···O═C hydrogen bonds further stabilizing the architecture and reflecting the very strong binding between the guest and the two hosts (Figures 4b and 4c). The observed host−host multiple weak interactions (Figure 4c) also support the asymmetric binding model deduced from NMR experiments (Scheme 2 right and Figure 3c).

The first inclusion promotes the isolation of one TEMPO unit from the other spin carrying units, but the carbonyl fringe complexing the ammonium cation is still highly hydrogen bond demanding and thus entails two other TEMPO moieties to position perpendicular to the CB[8] D_{8h} symmetry axis by means of amide hydrogen bonds. In our opinion, the first inclusion followed by the positioning of the three other TEMPOs causes the second CB[8] to bind to the 1:1 complex tightly. The presence of the first carbonyl crown is also believed to shift the pK_a of the two amines (protonated form) to further promote the second binding event. This new structure thus offers an ideal means for a second CB[8] to complex a pendant TEMPO moiety benefiting extra energy stabilization by a preorganized skeleton which is multiply hydrogen bond donating. Therefore, theoretical calculations showed how the first binding event reorganized the molecule to propose a more favorable issue for the second binding in accord with the Monod−Wyman−Changeux model.[43] Finally, the second CB[8] can be anchored after the first binding event with the aid of the first CB[8] (host−host interactions, Figure 4c) further strengthening the **4T**@CB[8]$_2$ assembly. Taken altogether, these considerations are likely to explain the experimentally observed very high cooperativity.

Competition and Reduction Experiments. The initial nine line pattern of the EPR spectrum is restored after addition of a competitor (benzylamine or adamantylamine) to the solution of **4T** and CB[8] (Figure 5a and Supporting Information Figure S11).

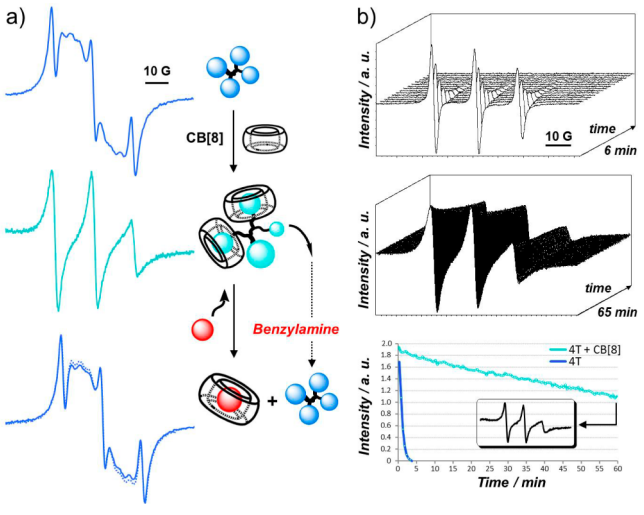


Figure 5. (a) EPR spectra of tetranitroxide **4T** in water (0.1 mM) and the observed three line spectrum after addition of CB[8] (1 mM) before coming back to the nine line pattern after the addition of a competitor (dashed line: initial spectrum). (b) Kinetics of the signal decay before (top) and after (middle) addition of CB[8] (1 mM) with ascorbic acid (5 mM) to a solution of tetraradical **4T** (0.1 mM) in water.

The DFT calculations indicate that even though one TEMPO nitroxide protrudes well from CB[8] (bisammonium 1:1 complex), this is not the case for the other 1:1 and the highly favored 1:2 complex. In these cases, the N−O oxygen atoms of the included TEMPOs stay near the carbonyl crown of the bulk exposed rim. In order to have a more detailed indication of the position of the nitroxide inside the cavity, EPR experiments were performed in the presence of ascorbate as reductant. The ascorbate anion is known to be a strong reductant for nitroxides,[35] and monitoring the reduction can be a good assay to probe the deepness of inclusion or accessibility of the nitroxides once complexed with CB[8]. Ascorbic acid (5 mM) was added to a solution of **4T** (0.1 mM) and CB[8] (0.4 mM; >92% 1:2 complex, see Figure 2). Whereas all the nitroxides are reduced within 2.5 min ($t_{1/2} \approx 20$ s) in the absence of CB[8] (Figure 5b and Supporting Information Figure S12), an EPR signal can be detected after 1 h if CB[8] was added to the mixture (Figure 5b). This result indicates that most of the nitroxides are protected against the reduction reaction. Without CB[8], the half-lifetime of the **4T** EPR signal is around 22 s. In the presence of CB[8], the decay curve is more flat and cannot be fitted according to a first order process. Two half-lifetime values were obtained assuming two sets of kinetics for the nitroxide reduction (Supporting Information Figure S13). The first component is approximately as fast as the reduction of **4T** without CB[8] ($t_{1/2} \approx 136$ s). The second component is longer, indicating a strong protection due to the inclusion of the two remaining TEMPO units in the two CB[8] cavities ($t_{1/2} \approx 31$ min) which represents an 85-fold decrease of the nitroxide reduction rate (Supporting Information Table S3). Moreover, after ≈200 s, half of the signal disappeared (two nitroxides over four have been reduced) and the remaining three line spectrum remains for more than 1 h. The monitoring of the nitrogen coupling constant (Supporting Information Figure S14) and the line width (Supporting Information Figure S15) shows significant variations during the first 200−250 s (a_N decrease and line width increase) before it reached plateau values corresponding to included radicals. This observation indicates that the remaining species hold the two nitroxides deeply immersed and immobilized in the CB[8] cavities. If the nitroxides would have protruded from the cavities or if a rapid exchange process had occurred (high k_{out}), a rapid loss of the EPR signal would have been observed. If a higher concentration of CB[8] was used (until 1 mM), a larger protection was obtained ($t_{1/2} \approx 75$ min for the second component) corresponding to a 210-fold decrease in nitroxide reduction. The competition and reduction experiments further confirm the stoichiometry and the strength of the complex formed when the tetranitroxide **4T** and CB[8] are mixed in water.

CONCLUSION

Overall, these results report the modulation of the through space spin exchange interaction in a tetranitroxide (**4T**) upon complexation by the synthetic host CB[8] in water. The affinity of CB[8] for the tetranitroxide is very high, as shown by the need of only three equivalents (Figure 1) to entirely change the conformation of **4T** and so the mean distance between each radical center. This property of communication of magnetic information between several spin centers was used to finely investigate the allosteric binding of two CB[8] molecules. The binding of a first CB[8] induces a profound conformational change of the molecule which highly favors binding of a second CB[8]. The well-known capacity of cucurbiturils to shift pK_a of

dx.doi.org/10.1021/ja509586k | J. Am. Chem. Soc. 2014, 136, 17570−17577

amines to higher means may also be a further driving force explaining this surprisingly effective positive cooperativity. The inclusion process resulted in a complete suppression of electron−electron spin exchange interaction between all four nitroxides, and this phenomenon could be restored by adding a suitable CB[8] competitor. The extent of allosteric interaction energy is surprisingly high ($\Delta\Delta G_{allos}$ **4T**/CB[8]$_2$ ≈ 13 kJ mol^{-1}) but can be rationalized by the conformational properties of *folded vs unfolded*, and information stored in *hydrogen bonds and protonable sites*, the tetraradical and the binding properties of CB[8]. Previously, peptides[44] and molecules carrying amide bonds[45] have been shown to display new and exciting properties in the context of supramolecular chemistry. The possibility to tune magnetic spin exchange interaction on demand in the present report is particularly attractive for molecular scale information processing and the production of memory devices. More generally, we believe that the assignment of the role of each subcomponent (amide functions, protonated amines, hydrophobic spin centers, size, and flexibility of the scaffold) in the reported strong cooperativity will contribute to the design and preparation of efficient allosteric systems based on cucurbiturils.

■ ASSOCIATED CONTENT

Supporting Information

Details of chemical syntheses and additional EPR and NMR spectra. Additional molecular dynamics and DFT results, especially absolute energies and the coordinates of the atoms for all the molecules whose geometries have been optimized. Competition and kinetic results of radical decay in reducing conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENTS

The CNRS and the Aix-Marseille Université are acknowledged for financial support. This work was supported by the computing facilities of the CRCMM, "Centre Régional de Compétences en Modélisation Moléculaire de Marseille".

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