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Communication

Reversible Control of DNA Binding with Cucurbit[8]uril-Induced Supramolecular 4,4′-Bipyridinium−Peptide Dimers

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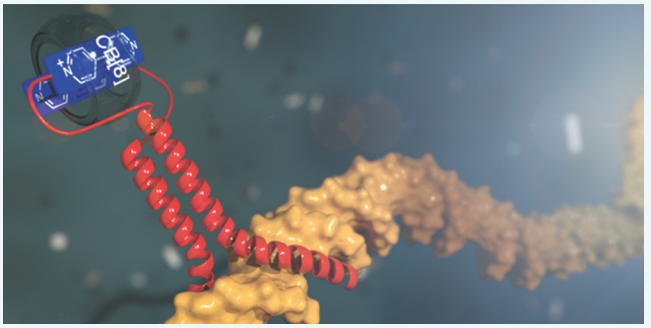
ABSTRACT: Many cellular processes in living organisms are regulated by complex regulatory networks, built from noncovalent interactions between relatively few proteins that perform their functions by switching between homo- and heterooligomeric assemblies or mono- and bivalent states. Herein, we demonstrate that the conjugation of a 4,4′-bipyridinium scaffold to the basic region of the GCN4 bZip transcription factor can be exploited to control the dimerization of the conjugate by formation of a supramolecular complex with cucurbit[8]uril. Importantly, this supramolecular complex is able to specifically recognize its target dsDNA, and this binding can be reversibly switched by the application of external stimuli.



Proteins regulate many cellular processes in living organisms, ranging from cell cycle progression to cell motility or gene expression. Interestingly, in eukaryotic cells, such regulation is achieved by employing relatively few proteins.[1,2] Consequently, intricate regulatory networks are created through the combinatorial formation of diverse noncovalent protein-based complexes, frequently by switching between homo- and heterooligomeric assemblies or mono- and bivalent states. Inspired by these natural complexes, chemists have reported a variety of supramolecular systems that have demonstrated a considerable potential for the regulation of protein assemblies, modulating their function by means of host−guest interactions.[3−7]

In the context of peptide dimerization, the macrocyclic host cucurbit[8]uril (CB[8]) has been reported to interact with phenylalanine-glycine-glycine (FGG) sequences,[8] and exploited to promote the association of functional peptide fragments.[5,9,10] However, the use of this short peptide tag is limited to the dimerization of peptides through their *N*-terminus, due to the structural requirements of the CB[8]:$(FGG)_2$ complex.[8] CB[8] has also been described to interact with middle-chain Phe and Trp residues to mediate peptide and protein dimerization.[11,12] Nevertheless, to the best of our knowledge, the use of these homoternary complexes to drive the dimerization and control the biological activity of functional peptides has not been explored.

Prompted by these facts, the unique ability of CB[8] to form 1:2 hetero- or homodimeric inclusion complexes with a variety of guests,[13−19] and our interest in the development of stimuli responsive host−guest and peptide-based systems,[19−22] we envisaged that *C*-terminal oligomerization of peptides could be effectively mediated by CB[8] through the introduction of a *N*-monoalkyl-4,4′-bipyridinium recognition motif ($_pV^+$), which interacts with the macrocyclic host yielding CB[8]:$(_pV^+)_2$ antiparallel homodimeric aggregates.[23] Furthermore, the well-known host−guest chemistry of the CB[8]-bipyridinium pair allows for further implementation of dynamic behavior on the designed artificial peptides, in particular, on the prospective endorsement of redox or acid−base stimulation into those.[19]

We focused our attention on the Basic Leucine Zipper (bZip) transcription factor GCN4, which binds to its target dsDNA (ATF/CREB) as a leucine zipper-mediated homodimer of continuous α-helices.[24] The group of Kim demonstrated that a simplified peptide, in which the leucine zipper region at the *C*-terminus of the GCN4 DNA binding domain is replaced by a cysteine residue, is able to bind its target DNA sequence with high affinity after dimerization through a disulfide bond.[25] Since then, other authors have exploited this strategy to incorporate new functionalities to the design of artificial GCN4 derivatives,[3,26−30] exemplifying both its utility as simplified model for the study of protein homodimerization and of protein−DNA interactions. Herein, we demonstrate that the incorporation of a 4,4′-bipyridinium moiety at the *C*-terminus of the GCN4 basic region (GCN4br) affords a peptide derivative able to recognize ATF/CREB, upon formation of the supramolecular homodimer with CB[8].

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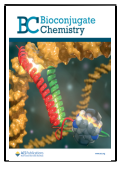

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Furthermore, we show how the application of external stimuli can be effectively used to reversibly control the homodimer formation and its DNA binding.

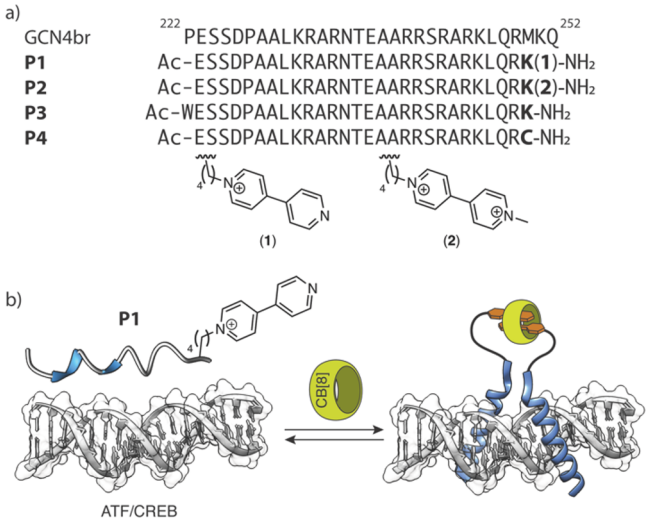

Figure 1. (a) GCN4br sequence, **P1** with the 4,4′-bipyripidinium unit (**1**) conjugated to the *C*-terminal Lys side chain, **P2** with the methylated bipyridinium unit (**2**), **P3** with the free Lys, and **P4** in which the Lys has been replaced by Cys to obtain the disulfide dimer $\mathbf{P4_2}$. (b) Formation and binding of the homoternary complex $\mathbf{P1_2}$:CB[8] with its target dsDNA.

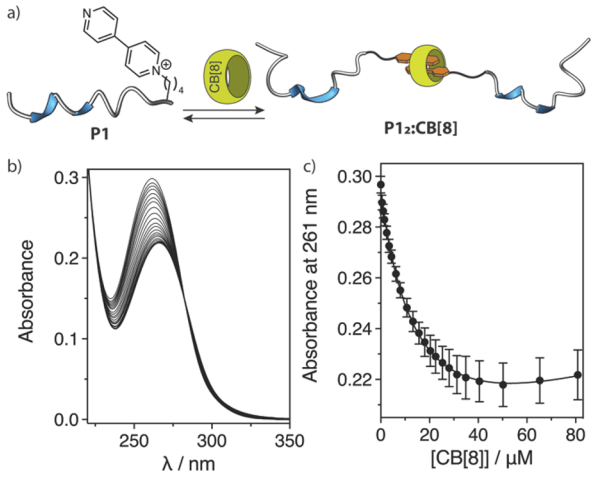

Figure 2. (a) Formation of the homoternary complex $\mathbf{P1_2}$:CB[8]. (b) UV titration of a 15 μM **P1** solution in 10 mM phosphate buffer (PB), pH 7.0, in the presence of increasing concentrations of CB[8]. (c) Mean absorbance at 261 nm and best fit to a sequential 2:1 binding model ($\mathbf{P1_2}$:CB[8]).[8]

As the starting point for our design, and based on the crystal structure of the GCN4-ATF/CREB complex,[31] we used the GCN4br fragment that comprises residues Glu^{223} to Arg^{249}. This sequence includes all the positive amino acids (Lys and Arg) that are important for the DNA recognition, to which we added an extra Lys residue at the *C*-terminus to attach the 4,4′-bipyridinium scaffold (peptide **P1**, Figure 1). Taking in consideration previous reports,[25,32] we expected that **P1** should not bind to ATF/CREB as monomer. However, the formation of a supramolecular homodimer with CB[8] ($\mathbf{P1_2}$:CB[8]) should trigger the recognition of ATF/CREB.

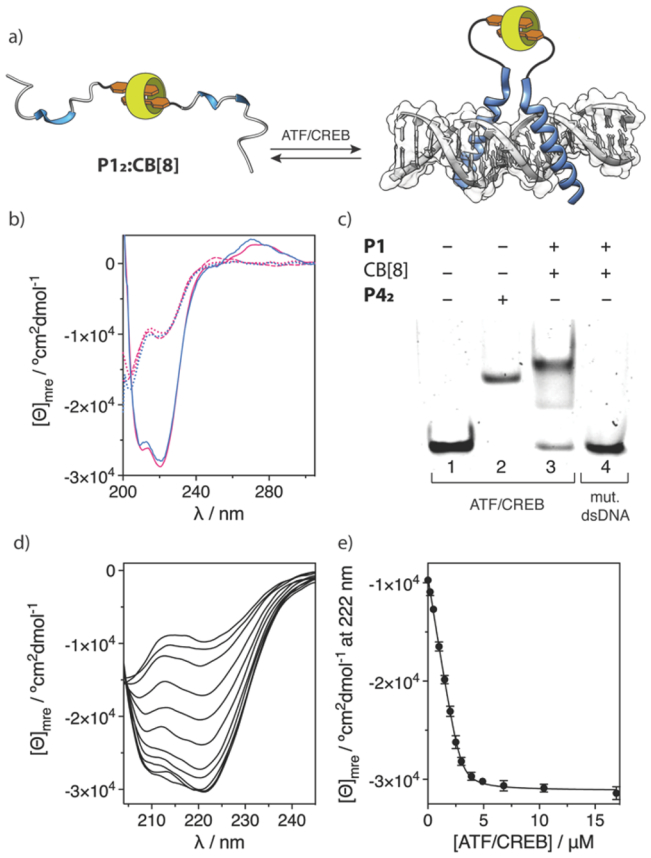

Figure 3. (a) Binding of the supramolecular dimer $\mathbf{P1_2}$:CB[8] with ATF/CREB. (b) CD spectra of a 10 μM **P1** and 18 μM CB[8] mixture (pink dotted line); the same mixture with 5 μM ATF/CREB (pink solid line) or 5 μM mutated dsDNA (pink dashed line); a 5 μM $\mathbf{P4_2}$ solution (blue dotted line) and the same solution with 5 μM ATF/CREB (blue solid line). All spectra were recorded at 23.2 °C in 10 mM PB, 100 mM NaCl, pH 7.5. (c) DNA binding analysis by electrophoretic mobility shift assay. Lanes 1−3:50 nM ATF/CREB with 500 nM $\mathbf{P4_2}$ (lane 2); 1 μM **P1**, 1.8 μM CB[8] (lane 3). Lane 4:50 nM mutated dsDNA, 1 μM **P1**, 1.8 μM CB[8]. (d) CD titration of a 10 μM **P1**, 18 μM CB[8] solution in 10 mM PB, 100 mM NaCl, pH 7.5 in the presence of increasing concentrations of ATF/CREB. (e) MRE at 222 nm and best fit to a 1:1 binding model ($\mathbf{P1_2}$:CB[8]:ATF/CREB, considering $\mathbf{P1_2}$:CB[8] as a single DNA binding element).

In this case, CB[8] should form a homoternary complex with the peptide derivative, in which the bipyridinium units are encapsulated in an antiparallel fashion in its hydrophobic cavity[23] (Figure 1b). Furthermore, $\mathbf{P1_2}$:CB[8] should be switched by acid−base stimuli,[33,34] allowing control of its binding to ATF/CREB.

Detailed synthetic protocols are described in the Supporting Information. In short, **P1** peptide was synthesized following standard Fmoc/*t*Bu solid-phase peptide synthesis protocols. For the coupling of the 4,4′-bipyridinium unit **1** to the orthogonally deprotected *C*-terminal Lys side chain, we followed our previously reported methodology based on a solid-phase Zincke reaction (Schemes S1 and S2).[35] In addition to **P1**, we also synthesized its methylated counterpart **P2** and **P3**, a peptide that lacks the 4,4′-bipyridinium moiety, as negative controls of the DNA binding, and the disulfide dimer of GCN4br, $\mathbf{P4_2}$,[25] as positive control (Figure 1a and Schemes S1, S2, S3, and S4).

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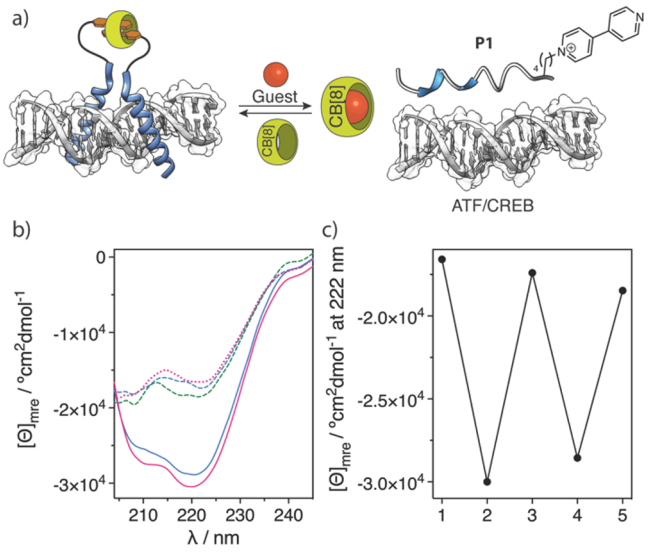

Figure 4. (a) Representation of the switching strategy. (b) ATF/CREB binding switching experiments by CD. A 10 μM **P1** and 5 μM ATF/CREB mixture in 10 mM PB, 100 mM NaCl, pH 7.5 (pink dotted line) was successively treated at room temperature with CB[8] and 1-adamantylamine, and the corresponding CD spectra were recorded after each addition. Starting mixture in the presence of 18 μM CB[8] (pink solid line); mixture after the addition of 1-adamantylamine (2 equiv with respect to CB[8], blue dashed line); mixture after the addition of a new aliquot of CB[8] (36 μM final concentration, blue solid line); mixture after the addition of 1-adamantylamine (other 2 equiv with respect to CB[8], green dashed line). (c) MRE at 222 nm for the successive spectra on the left.

With **P1** at hand, we studied its binding to CB[8] by UV spectroscopy. We observed that the absorption band at 261 nm, corresponding to the bipyridinium moiety, is red-shifted and decreases after the addition of increasing concentrations of CB[8] (Figure 2b). The UV data were fitted to a sequential 2:1 binding model using DynaFit 4.0 software.[36] We obtained good quality fittings for a wide range of stepwise dissociation constants that varied significantly. However, as previously observed by Urbach et al. for other CB[8] homoternary complexes,[8] the product of each pair of dissociation constants, i.e., the ternary dissociation constants K_D^{ter}, barely varied. Therefore, from the product of the stepwise dissociation constants obtained from the data fitting, we could estimate an apparent $K_D^{ter} \approx 680 \times 10^{-12}$ M^2 for the **P1**$_2$:CB[8] complex.

To study the DNA binding of the supramolecular dimer **P1**$_2$:CB[8], we first studied the interaction with ATF/CREB by circular dichroism (CD). Since it is well-known that bZip dimers are poorly structured in solution in the absence of their target DNA sequence (therefore, do not show the characteristic CD signature of a continuous α-helix), and that bZip monomers cannot bind to their target dsDNA,[25,26] we first recorded the CD spectra of a mixture of **P1** and CB[8] and a mixture of **P1** and ATF/CREB (Figures 3b and S1). As expected, none of these mixtures showed the characteristic CD signature of an α-helix. However, when we recorded the CD spectrum of a mixture containing **P1**, CB[8], and ATF/CREB we observed an intense negative CD band with a minimum at 222 nm, characteristic of an α-helical structure induced by the interaction of a bZip dimer and its target dsDNA, indicating that **P1**$_2$:CB[8] retains the DNA binding capabilities of GCN4. In addition, we also wanted to check that this interaction is due to the formation of the 2:1 homoternary complex between the 4,4′-bipyridinium unit and CB[8], as proposed in Figure 1b, and not to a 1:1 4,4′-bipyridinium:CB[8] binary complex or 2:1 ternary complexes between CB[8] and two amine groups from the peptide side chains instead the 4,4′-bipyridinium scaffold.[37] Interestingly, as shown in Figures S2, S4, S5, and S7, neither the **P1**:CB[7] binary complex, nor the control peptides **P2**, which includes the methylated 4,4′-bipyridinium unit that forms 1:1 inclusion complexes with CB[8],[19] and **P3**, that does not present the 4,4′-bipyridinium moiety, induced any increase in the negative CD bands at 222 nm, verifying that they do not interact with ATF/CREB in the presence of CB[8]. Furthermore, we also confirmed that the interaction between **P1**$_2$:CB[8] and ATF/CREB is sequence-specific by recording the CD spectrum of a mixture of **P1**, CB[8], and a mutated dsDNA, which did not induce any increase in the negative ellipticity at 222 nm (Figures 3b and S3). As positive control,[25] we recorded the CD spectrum of the disulfide dimer **P4**$_2$ in the presence of ATF/CREB, and it did not exhibit any significant difference with the CD spectrum of the **P1**$_2$:CB[8]:ATF/CREB complex (Figures 3b and S6). Moreover, we also confirmed the interaction between the supramolecular dimer **P1**$_2$:CB[8] and ATF/CREB by electrophoretic mobility shift assays. As expected, we observed a slower migration band for the **P1**$_2$:CB[8]:ATF/CREB complex that is not observed when **P1**$_2$:CB[8] is incubated with a mutated dsDNA (Figure 3c, lanes 3 and 4, respectively).

With the purpose of better understanding the interaction between **P1**$_2$:CB[8] and ATF/CREB, we performed CD titrations by adding increasing concentrations of ATF/CREB to a mixture of **P1** (10 μM) and CB[8] (18 μM). As shown in Figure 3d, the addition of ATF/CREB induced a continuous increase in the intensity of the negative CD band at 222 nm, characteristic of the α-helical structure induced by the DNA binding. The data could be fitted to a 1:1 binding model by considering **P1**$_2$:CB[8] as a single DNA binding element (Figure 3e, Scheme S6). As a result, we could calculate an apparent dissociation constant, $K_D \approx 75 \pm 16$ nM, for the formation of the **P1**$_2$:CB[8]:ATF/CREB complex, in line with the low nanomolar dissociation constants reported for other GCN4br dimers.[38,39]

Seeing that **P1** dimerization and its DNA binding depends on the formation of a supramolecular complex with CB[8], and taking in consideration that the 4,4′-bipyridinium scaffold in **P1** can be protonated yielding acid−base responsive switches,[34,40,41] **P1**$_2$:CB[8] has the potential to behave as a supramolecular switch. Therefore, we investigated by CD if **P1**$_2$:CB[8] could respond to pH changes and, thus, control its DNA binding. We registered the CD spectrum of a starting mixture containing **P1**, CB[8], and ATF/CREB in 10 mM PB, NaCl 100 mM, pH 7.5, and over it we added successive aliquots of HCl and NaOH. We observed that the intensity of the negative CD band at 222 nm decreased significantly after the addition of an aliquot of HCl (pH $\approx$ 4) and that the intensity of this negative CD band was considerably recovered after adjusting the solution back to pH $\approx$ 8 with NaOH, showing that the DNA binding can be reversibly controlled by a pH change (Figure S8). We also wanted to corroborate that it is possible to reversibly control the formation of **P1**$_2$:CB[8]:ATF/CREB by the addition of competitive guests, such as 1-adamantylamine and cobaltocenium. Hence, we registered the CD spectrum of a **P1**, CB[8], and ATF/CREB mixture, and again after the addition of successive aliquots of the competitive guests. As shown in Figures 4 and S9, the intensity

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of the negative CD band at 222 nm increased and decreased after each addition, demonstrating that the formation of $\mathbf{P1}_2$:CB[8] and its DNA binding capability can be reversibly controlled with external stimuli.

In summary, we have presented a new approach to the supramolecular control of peptide assemblies. We have shown that the incorporation of a 4,4′-bipyridinium scaffold at the *C*-terminus of a GCN4br monomer can be used to control its dimerization through the formation of a homoternary supramolecular complex with CB[8]. Moreover, this supramolecular dimer retains the DNA binding properties of the natural transcription factor GCN4. We further demonstrated that the CB[8]-induced dimerization, and thus the DNA binding, can be conveniently disrupted in a reversible fashion by disassembly of the host−guest complex upon the addition of a specific competing guest. Therefore, this strategy offers the possibility of designing new peptide conjugates that can be implemented into complex protein-based networks, and could be reversibly controlled in a straightforward fashion by using the robust and well-established host−guest chemistry of CB[8].

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.1c00063.

Peptide synthesis, experimental procedures, analytical data, CD experiments, and DNA binding assays (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CB[8], cucurbit[8]uril; bZip, basic leucine zipper; GCN4br, GCN4 basic region; Fmoc, fluorenylmethyloxycarbonyl; *t*Bu, *tert*-butyl; CD, circular dichroism; PB, phosphate buffer; K_D, dissociation constant; MRE, mean residue ellipticity

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