DNA Recognition

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Controlling Self-Assembly by Linking Protein Folding, DNA Binding, and the Redox Chemistry of Heme**

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Biological molecules are being used extensively as self-assembling materials for the "bottom-up" creation of useful nanoscale structures with non-natural functions.[1–3] New proteins have been created that form linear, branched, and meshed "wires",[4–6] recognize inorganic surfaces,[2,7] assemble and solubilize carbon nanotubes,[8,9] create molecular wires by the organization of conducting materials,[10,11] and generate organized protein networks.[6] As a result of these functions, the ability to control self-assembly processes through an external signal would be very useful. Allowing added ligand molecules to direct assembly is one such method and is used extensively in nature to switch conformational states which then regulates the associated activity. Artificial systems have also been designed in which a small molecule acts as the link between two separate protein components. The problem with such a system is that when the small molecule simply bridges the protein components, the signaling event is only dependent on the presence or absence of that small molecule. A system in which control is exerted by an external electron transfer or a photochemical event would be more useful for nanotechnology applications. Extending the principle of electron-transfer-triggered folding reactions[17] and learning from natural heme-based sensors,[18,19] we sought to create a macromolecular assembly system that can be controlled by the electronic state of the small molecule — a step towards true electronic control of macromolecular assembly and function.

Previously, we constructed a novel protein that incorporates both cytochrome b_{562} (cyt b_{562}) and the DNA binding basic helix region (BHR) of the leucine zipper transcription factor (bZIP) GCN4.[20] This design created a protein capable of being assembled on a designated template (DNA) that could potentially both carry and control current flow. The novel DNA-binding cytochrome (DBC) exhibits spectral characteristics and heme affinity that is comparable to those of the parent cyt b_{562} and also has the ability to bind DNA sequences based around those recognized by GCN4. This new protein provides the starting point for the creation of a controllable protein-based assembly system in which the parent protein is split into two separate fragments. Each fragment contains one half of the heme binding site and one of the two DNA binding elements that is required for DNA recognition (Figure 1). The assembly of the two protein components is dependent on heme and/or DNA. The oxidation-state-dependent affinity of heme for protein can then be exploited to control the assembly, and conditions can be identified under which DNA binding could be controlled by electron-transfer reactions.

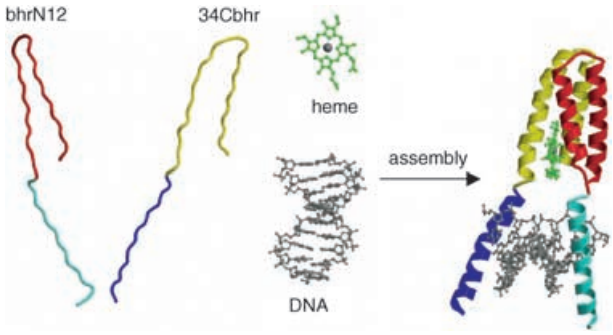

Figure 1. Schematic representation of each system component. The original DNA-binding cytochrome was fragmented to create bhrN12 (residues Met1 to Thr70) and 34Cbhr (residues Met84 to Arg156). DsNC1 is the optimum DNA binding sequence and corresponds to the DNA sequence 5′-caacgATGAcgATGAcggtt-3′ (capital letters designate the potential recognition site).

The original design of the single-molecule scaffold[20] has the BHR of GCN4, attached to the termini of the four-helix bundle of cyt b_{562}, thus creating a DBC. The design specifically arranges the conducting material (heme) on a template (DNA), and events that occur at the heme center can be transmitted to the DNA-binding region and vice versa, which

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would allow the attenuation of current flow by DNA binding. The DBC binds to specific DNA sequences with a K_D value in the low nM range, but little change in DNA-binding affinity was observed in either the presence or absence of heme, and the affinity was seen to be independent of the oxidation state of heme. We have deconstructed the single protein to create two mutually compatible fragments, bhrN12 and 34Cbhr. Each of these contains one half of the heme-binding site together with one BHR that defines half of the DNA-recognition motif. The two residues that provide axial ligands to the heme iron center, Met7 and His102 (wild-type cyt b_{562} numbering), are separated into the two different molecules in this arrangement. The assembly of these two fragments can be driven by the binding of either heme or DNA and then regenerates an analogue of the original intact complex (Figure 1). The three classes of interaction that become thermodynamically linked in the assembly process are protein–protein, protein—DNA, and protein–heme interactions. As the latter is dependent on the oxidation state of heme, the oxidation state is therefore coupled to the DNA-binding process. The fragments should not self-assemble in the absence of heme or DNA, and the individual halves should not bind heme on their own but can have low affinity for DNA. (In the context of wild-type GCN4, a single BHR can bind its target DNA but with a much lower affinity than the dimeric forms.)[21,22]

In the wild-type cyt b_{562}, the dissociation constant for reduced heme is in the pM range, but rises to 10 nM when the heme is oxidized.[23] In the context of our fragments, the difference in free energy between the two states results in a redox-dependent complex assembly that can be used to influence DNA binding. Titration of bhrN12 and 34Cbhr with ferric heme (2 μM) revealed a low affinity for the cofactor (Figure 2a); $K_D > 27000$ nM ± 3000 is at least 1000-fold higher than that observed for the intact DBC.[20] Under reducing conditions, the bhrN12 and 34Cbhr fragments bind heme with an affinity ($K_D = 435$ nM ± 85 nM) much lower than that of the oxidized form (Figure 2b). In both oxidation states, the spectrum was identical to that of wild-type cyt b_{562}, and the DBC was observed on the addition of 5 equivalents of bhrN12 and 34Cbhr (relative to heme), indicating that the helical bundle had assembled correctly (Supporting Information).

Circular dichroism (CD) spectra of bhrN12 and 34Cbhr suggest that there is little helical structure present when the domains are separated (Supporting Information). Upon mixing of the two components, the CD spectra suggest that no additional structure is induced (Figure 3). The addition of either an equimolar (data not shown) or excess amount of oxidized heme to the mixture of the two proteins, however, results in significant changes in the CD spectrum. The shift in the wavelength of the minimum at 205 nm to 208 nm and the increase in ellipticity at 222 nm are consistent with large increases in the helical content as a result of complex formation. It was not possible to perform the same experiments with the reduced state of heme owing to the absorbance of the reductant required to keep the heme in the reduced state.

To determine if DNA can influence complex assembly, the optimum double-stranded DNA sequence for the chimera[20]

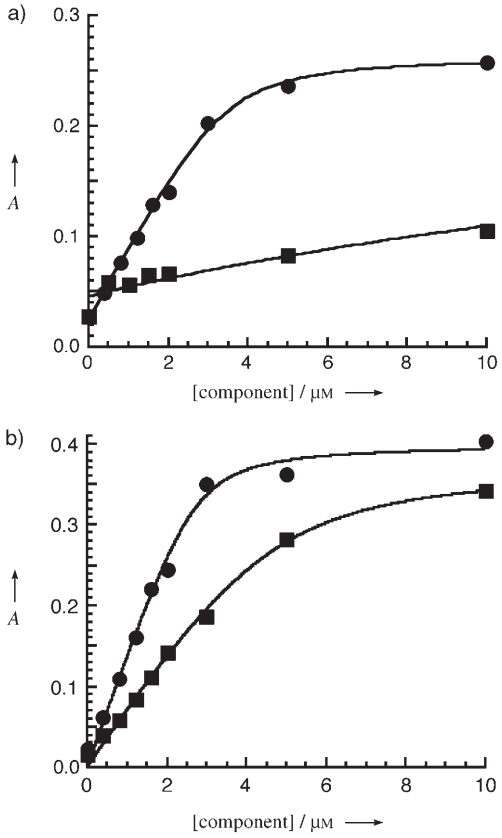

Figure 2. Determination of heme affinity for bhrN12 and 34Cbhr. Data were extracted at a) 417 nm (oxidizing conditions) and b) 427 nm (reducing conditions) in the absence (■) and presence (●) of dsNC1 DNA (Supporting Information). In all samples, heme concentration remains constant at 2 μM in tris-HCl (20 mM), pH 7.5. BhrN12 and 34Cbhr were titrated in equimolar amounts of 0.4, 0.8, 1.2, 1.6, 2.0, 3.0, 5.0, and 10 μM. When present, dsNC1 DNA was also at the same concentration as bhrN12 and 34Cbhr. Curve fitting was performed as described in the Supporting Information.

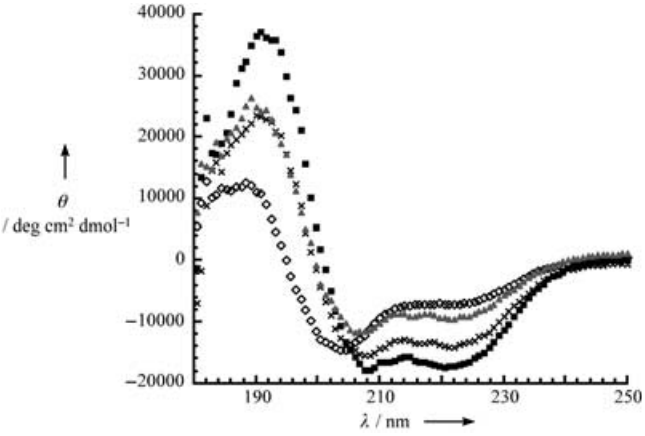

Figure 3. Effect of heme on the structure of the protein components. CD spectra of 5 μM each of bhrN12 and 34Cbhr in the absence of any ligand (◇) or in the presence of either 30 μM heme (▲), 5 μM dsNC1 DNA (×), or both 5 μM dsNC1 DNA and 5 μM heme (■). CD spectroscopy methods are outlined in the Supporting Information.

dsNC1 was employed to maximize potential binding. It has been observed that the presence of only one BHR region attached to either the N or C terminus of the intact cyt b_{562} does not promote tight, specific DNA binding.[20] However, the increase in helical content of the fragments in the presence of DNA (Supporting Information) suggests that both the bhrN12 and 34Cbhr fragments bind to DNA alone. This crucial difference should allow DNA to direct the assembly and so influence heme binding.

This is indeed the case, as in the presence of dsNC1 DNA, the affinity of bhrN12 and 34Cbhr for oxidized heme is 100-fold higher (K_D decreases to 207 nM ± 27) than in the absence of DNA (Figure 2). Under reducing conditions, DNA again increased the affinity of bhrN12 and 34Cbhr for heme, to give $K_D < 3$ nM, which is more than a 100-fold increase in affinity relative to that in the absence of DNA. It is therefore clear that DNA preassembles the bhrN12 and 34Cbhr fragments to generate a heme-binding site. In the presence of different DNA sequences (comparable length to dsNC1, but with no recognition site), tight binding of heme to the fragments was not observed, regardless of the oxidation state (data not shown). These observations suggest that specific DNA binding is required for optimum bundle-assembly enhancement and is reinforced by the changes observed in the CD spectra upon the addition of DNA to the protein fragments, which indicate an increase in helicity (Supporting Information). The increase in the helical signal is consistent with a coil-to-helix transition known to occur upon BHR binding DNA.[24] The CD spectrum in the presence of both DNA and heme together with bhrN12 and 34Cbhr indicates that further structural events occur when the complete quaternary complex is formed (Figure 3). The final spectrum in either oxidation state closely resembles that of the wild-type cyt b_{562}, which suggests that the core of the four-helix-bundle structures are very similar. The specific protein–protein interactions allow precise assembly of the complex thus deterring "off pathway" assembly events such as bhrN12 or 34Cbhr binding heme, despite the presence of the appropriate ligands (Supporting Information). The reduction potential of the DNA-bound, monomeric NCb_{562} protein is close to that of the wild-type cyt b_{562} (180 mV vs. normal hydrogen electrode at pH 7[25]). In general, the potential of cyt b_{562} is very sensitive to the electrostatic environment, and hence the heme bound species in the thermodynamic scheme (Figure 4) may have different reduction potentials. The electrochemical properties of these assemblies are complicated by multiple equilibria and are being studied by direct electrochemical methods.

CD spectroscopy was also used to observe the binding properties of bhrN12 and 34Cbhr with DNA. The traditional gel-shift assay, as was employed for the intact DBC,[20] is not useful when complexes can dissociate during electrophoresis.[22] The changes in the CD spectrum as DNA was titrated against a solution of equimolar concentrations of bhrN12 and 34Cbhr are included in the Supporting Information. Maximum intensity was almost reached at 1 equivalent of DNA. The dissociation constant for DNA binding ($K_D = 186$ nM) is about twofold higher than that observed for the intact apo-, chimeric DNA-binding cytochrome, but is still lower than that

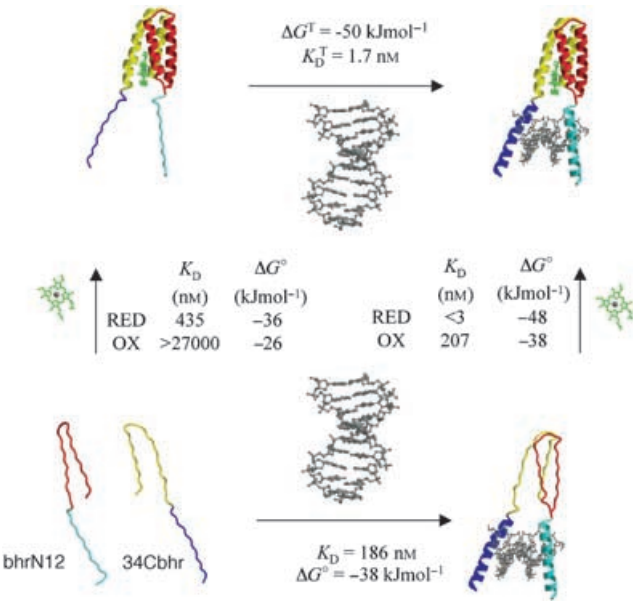

Figure 4. Thermodynamic square based on the calculated K_D values. The K_D values were converted into free energies by using the classical equation $\Delta G^\circ = -RT\ln K_D$. The terms ΔG^T and K_D^T are theoretical values for the bhrN12-heme-34Cbhr complex binding to DNA, calculated by using the determined values of reduced heme for the other three binding equilibria.

observed for the intact DBC binding to a non-optimal DNA-binding sequence.[20]

Measurement of the dissociation constant of DNA from the heme-bound complex has not yet been possible as measurements cannot be made at concentrations well above the K_D value required for the saturated ternary complex between ferric heme and the two fragments. Below the K_D value, binding experiments result in convolution of multiple equilibria, and there is no measurable signal that reports the contribution of each binding event. We are, however, pursuing the use of fluorescently labeled DNA to access this equilibrium constant. The K_D value can be estimated for bhrN12 ≈ heme ≈ 34Cbhr for DNA through the construction of a classical thermodynamic square (Figure 4). The sum of the free energies of the formation of the quaternary complex (DNA-bound bhrN12-heme-34Cbhr complex; top right corner of Figure 4) from bhrN12 and 34Cbhr should be equal, irrespective of the pathway taken. The calculated standard free energy for the formation of the bhrN12-heme-34Cbhr complex with DNA is -50 kJ mol^{-1}, which translates into $K_D = 1.7$ nM and is independent of the oxidation state of the heme. This value is very close to that of the intact DBC binding to dsNC1 in the absence of nonspecific DNA ($K_D \approx 10$ nM).[20] The magnitude of the changes in the DNA-binding affinity of our proteins in the absence and presence of heme is similar to the changes observed when naturally occurring GCN4 peptides dimerize upon DNA binding.[22] Therefore, we relate heme binding in our system to leucine zipper dimerization in GCN4. The magnitude of the energy of the coupling between DNA and heme binding (two orders of magnitude in K_D) compares favorably with the coupling of small-molecule binding (by maltose-binding protein) with β-

lactamase activity, as was recently reported for a different chimeric protein.[26,27]

To assess the influence of DNA on protein assembly, equimolar amounts of bhrN12 and 34Cbhr were incubated with varying concentrations of DNA prior to the addition of heme. As the DNA concentration increases to the equivalence point, so does absorbance at 417 nm, therefore indicating complex formation (Figure 5). After the equivalence point, the absorbance begins to decrease, suggesting a decrease in complex assembly. Increasing the DNA concentration beyond that of the protein concentration results in the binding of the protein fragments to different DNA molecules, inhibiting complex assembly. Together with the observation that the binding of DNA alone induces helical structure in bhrN12 and 34Cbhr (Supporting Information), these data suggest the binding of a monomeric BHR peptide to DNA. This is in contrast to our previous result that the complete cytochrome with only one BHR unit attached to either terminus cannot bind DNA.[20] Although we currently have no explanation for this, it is interesting that the loop that links helices 2 and 3 of the wild-type cyt b_{562} is present in the intact DBC, but absent in our current work (having been deleted in making the fragments described herein). Modeling studies suggest that this loop may interact in a negative fashion with the DNA, and our initial study revealed a complex relationship between loop length and DNA affinity.[20] Complete removal of the constraints imposed by this loop (as is the case in our current work) may promote the binding of the fragments to DNA.

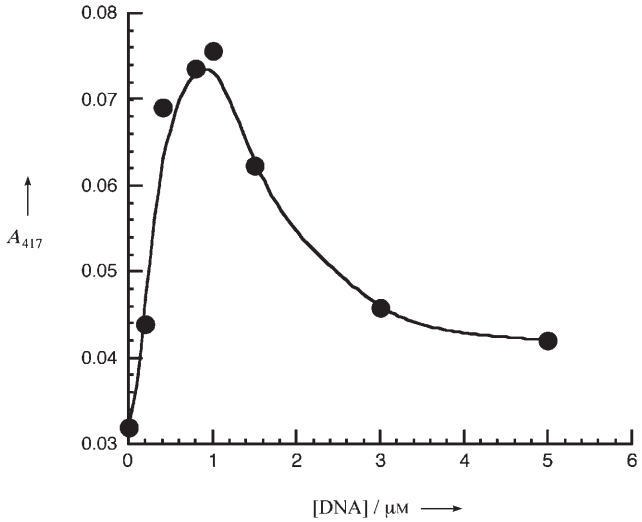

Figure 5. DNA as a competitive inhibitor to complex formation. The bhrN12 and 34Cbhr components were present at 1 μM, in the presence of 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 3.0, or 5.0 μM dsNC1 DNA. Oxidized heme (1 μM) was added as the last component of the mixture, and the system was allowed to equilibrate. Absorbance at 417 nm was used to determine complex assembly (Supporting Information).

The mechanism by which GCN4 binds DNA is dependent on how the whole complex assembles. It is known that GCN4 can bind DNA either as a monomer or as a dimer, but to generate a stable, high-affinity protein–DNA complex, the dimeric form is required.[22] GCN4 can form the dimer through the leucine zipper prior to binding, with the BHR disordered until it binds to DNA,[24,28] but the dimerization event is thought to be rate-limiting with respect to DNA binding.[22] It has also been reported that certain bZIP proteins bind sequentially as monomers to DNA and then assemble into dimers,[21] whereas other studies have shown that bZIP proteins can bind as monomers.[22,29] It is therefore not surprising that we have observed evidence of DNA binding of the individual fragments.

We have successfully taken the original design of the DNA-binding cytochrome (DBC) and converted it into a highly cooperative system in which heme and DNA binding influence the self-assembly of the complex. Splitting the complex into two fragments essentially mimics the original GCN4 mechanism, but in this case, a heme-binding domain replaces the leucine zipper. The result is that heme and DNA-binding processes are linked through protein conformation and assembly, which can be controlled electronically. This therefore adds an extra functional component to the toolbox of molecules that could be used in device construction.[3,30] To avoid heme dissociation during the switching, a covalent linkage between the protein and the cofactor can be introduced into the system,[31,32] though this will change the relative stabilities of the different assembled states. To compensate for this, heme-iron–ligand mutations[33] will be needed to exaggerate the oxidation-state dependence of assembly. Direct electron transfer between cyt b_{562} and solid-state electrodes is facile,[34] and we are currently investigating the DNA-promoted electrochemistry of these DBCs. Our system therefore has the properties required of a molecular transducer in an electronic device that can control an assembly process. The scaffold in its present forms represents the fundamental core of such a device and can be modified further to change or enhance its character.

Experimental Section

All the materials used are described in the Supporting Information. The bhrN12 and 34Cbhr genes were constructed by using the original DBC as the template. A more-detailed description of fragment construction and purification is in the Supporting Information. Heme-binding affinity was determined spectrophotometrically as outlined in both the figure legends and Supporting Information. Absorbance at 417 nm and 427 nm was used to monitor heme binding to bhrN12 and/or 34Cbhr under both oxidizing and reducing conditions (2 mM sodium ascorbate as the source of reducing equivalents). The heme-binding affinity for bhrN12 and 34Cbhr was determined by extracting the data at the above wavelengths and plotting against component concentration (bhrN12, 34Cbhr, and/or DNA). The data was then analyzed in a manner similar to that used by Bosshard and co-workers for dimeric derivatives of the GCN4 protein.[22] DNA inhibition of complex formation was determined as described in the legend to Figure 5 and in the Supporting Information. CD spectroscopy methods are described in the Supporting Information.

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