Asymmetric Configurations in a Reengineered Homodimer Reveal Multiple Subunit Communication Pathways in Protein Allostery

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ABSTRACT

Many allosteric proteins form homo-oligomeric complexes to regulate a biological function. In homo-oligomers, subunits establish communication pathways that are modulated by external stimuli like ligand binding. A challenge for dissecting the communication mechanisms in homo-oligomers is identifying intermediate liganded states, which are typically transiently populated. However, their identities provide the most mechanistic information on how ligand-induced signals propagate from bound to empty subunits. Here, we dissected the directionality and magnitude of subunit communication in a reengineered, single-chain version of the homodimeric transcription factor cAMP Receptor Protein (CRPSC). By combining wild-type and mutant subunits in various asymmetric configurations, we revealed a linear relationship between the magnitude of cooperative effects and the number of mutant subunits. We found that a single mutation is sufficient to change the global allosteric behavior of the dimer even when one subunit was wild-type. Dimers harboring two mutations with opposite cooperative effects had different allosteric properties depending on the arrangement of the mutations. When the two mutations were placed in the same subunit, the resulting cooperativity was neutral. In contrast, when placed in different subunits, the observed cooperativity was dominated by the mutation with strongest effects over cAMP affinity relative to wild-type. These results highlight the distinct roles of intrasubunit interactions and intersubunit communication in allostery. Finally, dimers bound to either one or two cAMP molecules had similar DNA affinities, indicating that both asymmetric and symmetric liganded states activate DNA interactions. These studies have revealed the multiple communication pathways that homo-oligomers employ to transduce signals.

INTRODUCTION

Allosteric proteins are the basic building blocks in the transmission of biological signals, allowing communication between and within cells, and from the extracellular environment to the cytosol (1). Because many allosteric proteins form homo-oligomeric complexes to modulate a ligand-induced biological response (2, 3), intersubunit communication must play a crucial role in the transduction of an allosteric signal (4, 5). Identifying the molecular mechanisms of intersubunit communication has important implications, from understanding how biological systems detect and transduce signals (6, 7), to reengineering of signaling proteins (8–10), to developing allosteric therapeutic modulators with enhanced affinities and specificities (11, 12). Despite its importance, dissecting the mechanisms of transduction of allosteric signals from one protein subunit to another has proven difficult because it requires monitoring intermediate
liganded states that are poorly populated, especially if the protein displays positive ligand binding cooperativity. Therefore, one of the remaining unresolved issues in allostery is dissecting the directionality of pathways of signal transmissions across protein subunits.

A widely-used strategy to perturb and examine the mechanisms of intersubunit communication in homo-oligomeric systems is to evaluate a mutation or combinations of mutations on binding or catalytic activities. However, in homo-oligomers mutations are present in all subunits, making it difficult to quantitatively dissect mutational effects from one subunit to another. To overcome this obstacle, in this study we reengineered the homodimeric transcription factor cAMP Receptor Protein (CRP) into a single-chain by covalently linking the two identical CRP subunits through an unstructured polypeptide linker (Figure 1A). The CRP single-chain dimer (CRPSC) allowed us to construct asymmetric CRP dimers harboring either a wild-type and a mutant subunit, or subunits with different mutation types. Specifically, the mutations S62F and D53H have been shown to reduce and enhance cAMP binding cooperativity, respectively. By placing these two mutations in various asymmetric configurations, we dissected the directionality and magnitude of mutational perturbations from one subunit to another, thereby providing a unique opportunity to examine communication pathways within and across CRP subunits.

Each CRP subunit has two functionally and structurally distinct domains: a cAMP-binding domain in the N-terminus that is also responsible for dimer formation, and a C-terminal DNA-binding domain (Figure 1B). CRP binds two cAMP molecules and undergoes a conformational change in the DNA binding domains that enables the protein to interact with high affinity and specificity with DNA promoter sequences (13–17). The molecular architecture of CRP is ideal for quantitative studies on the mechanisms of transduction of allosteric signals because: 1) cAMP binding reports on intersubunit communication and cooperative interactions and, 2) DNA binding reports on intrasubunit interactions and cAMP-induced conformational changes of the protein (13, 18).

Our results show that CRPSC is indistinguishable from the wild-type CRP homodimer based on solution structure, thermodynamic stability, and cAMP binding affinities and cooperativity. As seen in the wild-type CRP (13), the DNA binding activity of CRPSC is allosterically controlled by cAMP. We find that combinations of wild-type and mutant subunits in CRPSC result in cAMP binding affinities and cooperativities that are different from those of the parental proteins (i.e. wild-type CRP or variants with the same mutation in both subunits). Furthermore, we show that mutations with opposite effects on cAMP binding affinity have dramatically different consequences on cooperative interactions whether the mutations are in the same subunit or in different ones. Finally, we find that asymmetric mutants bound to one cAMP molecule have indistinguishable DNA binding affinity constants compared to the doubly liganded CRPSC, which suggests that a single cAMP molecule bound to CRP is sufficient to allosterically drive the conformational changes required for robust interactions with DNA, and underscores the role of asymmetric liganded states in the regulation of gene expression (19).

RESULTS


We engineered a CRP single-chain dimer (CRPSC) that connects the C-terminus of the first CRP subunit to the N-terminus of the second one through a flexible polypeptide linker (SGGGG)_7 (Figure 1A). Based on the CRP structures in the unliganded and cAMP-bound states (14–16), the distance between the C-terminus of one subunit and the N-terminus of the other is ~75 Å. The designed linker spans a larger distance (~130 Å) to accommodate the protein’s conformational changes induced by cAMP binding. CRPSC migrated as a 48 kDa protein in a SDS denaturing gel, twice the molecular weight of one CRP subunit (Figure 2A). During the purification process, gel filtration chromatograms of CRPSC had the same elution volume as of wild-type CRP corroborating that in native conditions CRPSC eluted as a pseudodimer (Figure 2B). During consecutive size exclusion runs, no peaks were observed before CRPSC elution (not shown), which otherwise would have indicated the formation of dimer of dimers, or other high-order oligomeric states. Moreover, CRPSC and wild-type CRP had
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identical CD spectra (Figure 2C), indicating that the global fold and secondary structure content of the two proteins are the same. We evaluated the effect of the polypeptide linker on the thermodynamic stability of CRPSC by monitoring changes in tryptophan fluorescence (Figure 2D) and circular dichroism (not shown) as a function of GdnHCl concentration. At the experimental conditions, CRPSC and wild-type CRP displayed a single unfolding transition and had similar fitted thermodynamic parameters ($\Delta G^\circ$ and $m$-values) that are in agreement with previous published data (Supplementary Table S1) (20).

Having established that the solution structure and the thermodynamic stability between CRPSC and wild-type CRP are very similar, we investigated whether the polypeptide linker affected the basic function of CRP, namely, the allosteric activation of DNA binding by cAMP. We used electrophoretic mobility shift assay (EMSA) to monitor the interaction of CRPSC and wild-type CRP to three different lengths of the lac promoter made by 26, 32 and 40 base pairs (bp) (Figure 2E). The different promoter lengths were used to test if the polypeptide linker in the CRPSC construct contributed to non-specific binding to DNA flanking sequences. In the presence of saturating amounts of cAMP, we observed robust DNA binding for both CRPSC and wild-type CRP. Moreover, for all promoter lengths we observed a single band, indicating that the polypeptide linker does not contribute to non-specific DNA binding. In the absence of cAMP, no DNA binding was observed for both proteins. These results indicate that the polypeptide linker in CRPSC does not affect the cAMP-dependent allosteric activation of the protein.

Quantification of cAMP Binding and DNA Interactions in CRPSC. To quantitatively determine whether CRPSC had cAMP-binding association constants similar to those reported for wild-type CRP, we used a published method based on changes in 8-anilino-1-naphthalenesulfonic acid (ANS) fluorescence to measure cAMP binding (13, 21). Figure 3A shows that the cAMP titration curves for CRPSC and wild-type CRP almost completely overlapped, resulting in indistinguishable microscopic association constants using a two-binding site model (Supplementary Table S1). We further validated the fluorescence data using isothermal titration calorimetry (ITC). The ITC data revealed similar microscopic cAMP binding association constants between CRPSC and wild-type CRP (Supplementary Figure S1 and Supplementary Table S2). As with wild-type CRP, the sequential binding of two cAMP molecules to CRPSC showed an initial exothermic phase followed by an endothermic phase. This biphasic behavior agrees with previous ITC studies (13, 22), and corroborates that the polypeptide linker does not affect the affinities nor the thermodynamic signatures associated with cAMP binding.

DNA binding constants were obtained by monitoring anisotropy changes during the formation of the complex CRP-DNA using a 32-bp fluorescein-labeled lac promoter (Figure 3B). The data showed that both CRPSC and wild-type CRP have similar binding affinities for the lac promoter fragment in the presence of 200 µM cAMP, a concentration wherein both binding sites are occupied. Noteworthy, the maximal anisotropy levels were slightly higher with the CRPSC, suggesting a more rigid protein-DNA complex. Control experiments in the absence of cAMP showed negligible DNA binding (Supplementary Table S1). Altogether, the results from these quantitative studies demonstrate that the presence of the polypeptide linker that connects the two CRP subunits does not perturb cAMP binding or DNA binding activities.

Asymmetric CRP Dimers Composed of Wild-Type and Mutant Subunits. Two previously well-characterized mutations, S62F and D53H (13, 21, 23), were used in our studies to perturb cAMP binding affinity and cooperativity in the CRPSC construct. In wild-type CRP, the mutation S62F decreases the affinity to cAMP and generates negative cooperativity between the two cAMP binding domains. On the other hand, the mutation D53H has small effects on cAMP binding affinity to the first site, but significantly increases the affinity for the second one, thereby generating positive binding cooperativity.

We first placed these mutations in both subunits of the CRPSC (also referred as symmetric mutants) to corroborate the same mutational effects reported for wild-type CRP. The symmetric CRPSC mutant harboring S62F (CRPSC$^{S62F}$) had an association constant for the first cAMP binding site,
Asymmetric CRP Dimers Harboring Mutant Subunits with Opposite Cooperative Effects. The effects of asymmetric mutants on cAMP binding affinities resulted in intermediate values of cAMP binding cooperativity compared to symmetric CRP Sc mutants. The cooperativity for CRPSc WT/WT and CRPSc D/D were cS/W = 0.2 and cD/W = 4.1, whereas for CRPSc S/S and CRPSc D/D were cS = 0.12 and cD = 8.8, respectively. Interestingly, although the mutations S62F and D53H have opposite cooperative effects, the magnitude of their cooperative effect over the affinity of the second cAMP binding site was similar. For example, CRPSc S/W and CRPSc D/D reduced and increased the affinity for the second cAMP binding site 5-fold and 4-fold, respectively, while CRPSc S/S and CRPSc D/D reduced and increased the affinity 8-fold and 9-fold, respectively (Table 1).

To investigate the roles of intersubunit communication and intrasubunit interactions in cAMP binding cooperativity, we placed the S62F and D53H mutations in CRP Sc in two different configurations (referred as double asymmetric mutants). To study intersubunit communication, we constructed a CRP Sc harboring the S62F mutation in one subunit and the D53H in the neighboring one (CRPSc S/D). To study intrasubunit interactions, we constructed a CRP Sc harboring both mutations in the same subunit while the other one remained wild-type (CRPSc S/D/W). Given the similar magnitudes in their cooperative effects, the two CRP Sc mutant configurations allowed us to determine to what extent intersubunit communication or intrasubunit interactions neutralize the opposite cooperative effects of S62F and D53H.

The cAMP titrations of CRPSc S/D and CRPSc S/D/W revealed important differences in cAMP binding affinity and cooperativity (Figure 4C). CRPSc S/D—with the two mutations in opposing subunits—displayed negative cooperativity (cS = 0.25) as seen in CRPSc S/S and CRPSc S/D. However, the cooperativity in CRPSc S/D was not as low as in CRPSc S/S and CRPSc S/D, indicating that the positive cooperative effects exerted by the mutation D53H were transduced to the neighboring subunit harboring the mutation S62F. Surprisingly, CRPSc S/D/W—with the two mutations in the same subunit—showed neutral cooperativity (cS/D/W = 1.1), albeit k1 and k2 were 50% lower than those of the wild-type protein. Thus, despite the fact that the two mutations in CRPSc S/D/W significantly reduced the cAMP binding affinity in both binding sites, any cooperative effects transduced from the double-mutant subunit to the neighboring wild-type subunit were negligible. Thus, these results show that opposing cooperative effects are better counterbalanced through intrasubunit interactions than via intersubunit communication.
One could speculate that the functional differences between CRPSC\textsuperscript{S/D} and CRPSC\textsuperscript{S/D/WT} were due to destabilization of the secondary structures introduced by the mutations. However, chemical denaturation experiments monitored by CD and tryptophan fluorescence showed little differences in $\Delta G^\circ$ and $m$-values between these two proteins (Supplementary Figure S2 and Supplementary Table S3), which argues that the mutations do not alter the global fold of the protein, but instead their effects over cooperative interactions may arise from changes in the motions of the protein. Therefore, our results show that CRPSC is an ideal construct to examine how changes in protein motions in a single subunit modulate the behavior of the neighboring one

**DNA Interactions with Asymmetric CRP Configurations.** It is well-established that CRP bound to two cAMP molecules interacts with high affinity and specificity to DNA promoter sequences (13). Less understood, however, is the role of the singly cAMP-bound conformation in transcription regulation and DNA interactions. Because both CRPSC\textsuperscript{WT/WT} and the wild-type CRP displayed positive cooperativity between the two cAMP binding domains (Table 1 and Supplementary Table 1), the singly cAMP-bound intermediate state is poorly populated and thus difficult to isolate and characterize. However, the asymmetric mutants CRPSC\textsuperscript{S/D}, CRPSC\textsuperscript{S/WT} and CRPSC\textsuperscript{S/S} displayed negative cAMP binding cooperativity, enabling us to populate the singly cAMP-bound conformation and examine its interaction and binding affinity for the lac promoter.

Based on the cAMP binding data for CRPSC\textsuperscript{S/D} and to CRPSC\textsuperscript{S/WT}, the highest ratio of singly to doubly cAMP-bound populations was obtained when [cAMP] = 30 µM. At this cAMP concentration, the distribution of populations of unliganded, singly cAMP-bound and doubly-bound states is approximately 34 %, 60 % and 6 %, respectively (Supplementary Figure S3). Because of the negligible interaction between the unliganded CRP and the lac promoter, the total change in anisotropy at 30 µM cAMP, which reflects the formation of the CRP-DNA complex, corresponds to $\sim 91$ % to the singly cAMP-bound conformation and 9 % to the doubly-bound state. Figure 5A shows the DNA binding data of CRPSC\textsuperscript{S/D} using [cAMP] = 0, 30 and 1000 µM. Surprisingly, the titrations at 30 and 1000 µM revealed that both the singly cAMP-bound and doubly cAMP-bound conformations had very similar DNA binding affinities constants: $k_{DNA-cAMP} = 8.0 \cdot 10^7$ M$^{-1}$ and $k_{DNA-cAMP-2} = 8.9 \cdot 10^7$ M$^{-1}$, respectively. We estimated the DNA binding affinity of the unliganded conformation, $k_{DNA(empty)}$, assuming a similar total change in anisotropy as seen for the singly cAMP or doubly-bound conformations. This assumption provided an upper limit on the affinity for the singly cAMP-bound and doubly cAMP-bound conformations (Figure 5B). Similar results were obtained for CRPSC\textsuperscript{S/WT}, namely, the singly cAMP-bound and doubly cAMP-bound conformations interacted with the lac promoter with high affinity, $8.0 \cdot 10^7$ M$^{-1}$ and $2.2 \cdot 10^8$ M$^{-1}$, respectively, whereas the affinity of the unliganded conformation was more than twenty times lower, $3.0 \cdot 10^6$ M$^{-1}$ (Figure 5B).

For CRPSC\textsuperscript{S/S}, the highest ratio between singly and doubly cAMP-bound populations was obtained with [cAMP] = 200 µM (Supplementary Figure S3). At this concentration, the population of doubly cAMP-bound is $\sim 2$ %, whereas the unliganded and singly cAMP-bound populations are $\sim 40$ % and $\sim 58$ %, respectively. In agreement with the results from CRPSC\textsuperscript{S/D} and to CRPSC\textsuperscript{S/WT}, the singly and doubly cAMP-bound conformations for CRPSC\textsuperscript{S/S} also have comparable affinities, $6.9 \cdot 10^7$ M$^{-1}$ and $6.3 \cdot 10^7$ M$^{-1}$, respectively (Figure 5B and Supplementary Methods).

Altogether, our data suggests that binding of a single cAMP molecule to CRP is sufficient to allosterically drive the conformational changes required for robust interactions with DNA promoter sequences. Importantly, all the CRPSC mutants studied here displayed significantly higher DNA binding affinity constants in saturating cAMP concentrations than in the absence of cAMP. Thus, the basic allosteric activation mechanism in all CRPSC constructs remained unperturbed (Figure 5C).

**DISCUSSION**

**Covalent Linkage of an Allosteric Protein Complex.** In this study we used several quantitative approaches to demonstrate that the wild-type CRP can be successfully reengineered
and expressed as a single-chain (CRP\textsubscript{SC}) without compromising the fold, stability, and function of the protein. The strategy of linking individual subunits in a homo-oligomeric protein offers important advantages to study hybrid functional states (i.e., combinations of mutant and wild-type subunits) (24). First, it eliminates the statistical degeneracy that occurs when mixing unlinked chains of mutant and wild-type subunits (25, 26), which can be further complicated if the mutant and wild-type subunit have different oligomerization association constants. Second, because of the high local concentration of protein subunits in the linked oligomer, subunit exchange throughout the course of an experiment is largely minimized. Finally, hybrid functional states allow for a detailed quantitative examination of the mechanism of intersubunit communication and coordination in homo-oligomeric proteins (24). Here, we compared symmetric and asymmetric CRP\textsubscript{SC} mutant configurations to investigate the mechanisms of communication within and across CRP subunits, and determined how different communication pathways play unique roles in cooperative interactions.

**Intersubunit Communication Independent of Ligand Binding.** An important question in the field of allostery is whether coupling interactions between protein subunits emerge exclusively from ligand binding or, instead, the native state ensemble already manifests coupling interactions whose magnitude is amplified by the presence of the ligand (5, 27, 28). A comparison of the cAMP binding data between symmetric and asymmetric CRP\textsubscript{SC} mutant interactions or intersubunit communication are at work. In fact, previous studies have shown a correlation between protein motions and cAMP binding cooperativity in CRP (23, 29). The dimer interface, i.e., intersubunit communication mediated by quaternary interactions.

**Arrangement of Asymmetric Mutations Plays a Crucial Role in cAMP Binding Cooperativity.** Interestingly our results show that a single mutation in one CRP subunit was sufficient to drive the same cooperative behavior seen in the parental proteins but with an intermediate magnitude (Table 1), indicating that the effects of mutations over cooperative interactions between the two cAMP binding domains scale linearly proportional with the number of mutant subunits.

Furthermore, our results show that the arrangement of the mutations has dramatic consequences depending on whether intrasubunit interactions or intersubunit communication are at play. In fact, by just placing the two mutations S62F and D53H in different configurations, we engineered a family of CRP\textsubscript{SC} variants with fine-tuned binding energies covering a broad range of positive-to-negative cooperativities (Figure 6). The results obtained with the CRP\textsubscript{SC} construct therefore illustrate how the different protein’s communication pathways can be exploited to modulate binding and enzymatic activities without directly altering active binding pockets or interaction surfaces.
Role of Asymmetric Conformations in the Mechanism of CRP Activation and Interaction with DNA. The main structural transition of CRP that accompany cAMP binding involves a ~60-degree rotation of the DNA-binding domains relative to the cAMP-binding domains (14–16). Such structural transition enables tight interactions with the major groove of the DNA, and provides a structural basis for the activation and affinity enhancement of CRP for DNA promoter sequences. While high-resolution structures offer detailed information about the unliganded and doubly-cAMP bound conformations, the allosteric activation pathway of CRP must involve, in addition, a singly-cAMP bound intermediate. This intermediate is not only important for a mechanistic understanding of the activation pathway of CRP, but it may also represent an additional conformer in the regulation of gene expression as proposed by others (19). In this study, we took advantage of the negative cAMP binding cooperativity of CRPScS/D and CRPScS/WT to interrogate the DNA binding properties of CRP bound to one cAMP molecule.

We found that in the singly-cAMP bound state, CRPScS/D, CRPScS/WT, CRPScS/S have similarly high affinity for the 32-bp lac promoter compared to the doubly cAMP-bound conformation (Figure 5B). These results suggest that a single cAMP binding event triggers a conformational change in CRP that allows tight interactions with the DNA. NMR studies with the CRP homodimer showed that cAMP binding to either wild-type or D53H subunits elicits an active conformation of the DNA-binding domains (30). Thus, one possible model to explain our results is that after the first cAMP binding event, the cAMP-bound subunit elicits a reorientation of the DNA-binding domain of the neighboring, unliganded subunit that is compatible with DNA binding (Figure 7).

An alternative model is that DNA interactions occur with a CRP dimer in a hybrid conformation, namely, the cAMP-bound subunit is in the active conformation, whereas the unliganded one remains in the inactive state. A DNA-CRP complex formed with only one active DNA-binding domain would establish approximately half of the interactions and thus result in half of the binding energy compared to a CRP homodimer with both DNA-binding domains active. The observed DNA binding energies ($\Delta G^\circ = -RT \cdot \ln[k_{DNA}]$) for the singly and the doubly-cAMP bound states were essentially the same, around $-10.7$ kcal/mol. Therefore, we favor a model in which binding of cAMP to one CRP subunit elicits a conformational change in the neighboring one. Such conformational change in the unliganded subunit does not need to be the exact same as in the cAMP-bound subunit, but needs to be compatible with strong DNA interactions. This model also provides a plausible explanation for the transduction of positive cooperative effects from one subunit to another seen in asymmetric CRPSc mutants, especially for CRPScS/D where the D53H subunit bound to cAMP increases the affinity of the S62F subunit by 2-fold.

The proposed model in Figure 7 provides a framework to further dissect the thermodynamic cycle describing the linkage between cAMP and DNA interactions. Specifically, we interrogated the effect of the CRP-DNA interaction over cAMP binding cooperativity, which in Figure 7 corresponds to the binding affinity constant for the second cAMP molecule when CRP is bound to DNA, $k_2(DNA)$, and can be calculated from the relationship $k_2(DNA) = k_2^0 k_{DNA(cAMP)}$/$k_{DNA(cAMP)}$. Using the results from this study, we obtained values of $k_2(DNA)$ that are slightly larger than $k_2$, indicating that DNA interactions with a CRP molecule in intermediate liganded states also modulate the protein’s response to cAMP concentration. This result sheds lights into the bidirectional interplay between DNA interactions and cAMP binding cooperativity during transcription regulation. Future studies using asymmetric CRPSc mutants that simultaneously perturb cAMP binding and DNA interactions will be used to further dissect the communication pathways between the cAMP- and DNA-binding domains within and across protein subunits.

MATERIALS AND METHODS

Cloning, Expression and Purification of CRPSc. The DNA sequence of wild-type CRP from Escherichia coli was used to synthesize (GenScript) the CRP single-chain dimer (CRPSc) with a sequence encoding (SGGGG)7 as linker connecting the two CRP subunits. The protein purification protocol is described in detail in Supplementary Methods and Supplementary Figure S4. The wild-type CRP was kindly provided by Dr. James C. Lee from the University of Texas Medical Branch.
Circular Dichroism (CD). Measurements were performed in an Aviv Model 202-01 spectrometer with 10 µM of protein in PBS buffer pH 7.4 over the range of 195–260 nm. For each sample, two repetitive scans were performed, averaged and baseline corrected.

Chemical Denaturation with Guanidine Hydrochloride (GdnHCl). Protein unfolding was monitored by changes in tryptophan fluorescence (λ<sub>ex</sub> = 295 nm and λ<sub>em</sub> = 340 nm) and circular dichroism absorption (at 222 nm) using 10 µM of protein in 20 mM Tris, 50 mM NaCl, 1 mM EDTA at pH 7.8. At least two independent titrations were performed for each protein, and corrected for buffer absorption. Data were fitted to a two-state unfolding model according to the linear extrapolation method (31).

cAMP Binding Monitored by Isothermal Calorimetry (ITC). Experiments were performed in 20 mM Tris, 50 mM NaCl, 1 mM EDTA, 0.2 mM TCEP, pH 7.8 at 25°C in a VP-ITC MicroCalorimeter (1.4 mL chamber volume). All solutions were filtered and degassed prior use. The protein and cAMP concentrations were 16 µM and 0.57 mM, respectively. The experiment consisted of a first 5 µL injection, followed by 17 injections of 18 µL each. A reference titration of cAMP into buffer was subtracted from the cAMP titration to the protein. The data were analyzed using a sequential two-site binding model (MicroCal ITC-Origin).

Electrophoretic Mobility Shift Assay (EMSA). Reaction mixtures contained 40 nM of DNA, 1.25 µM wild-type CRP or CRP<sub>SC</sub>, 10 mM DTT and 200 µM cAMP in 20 mM Tris, 50 mM NaCl, 1 mM EDTA, pH 7.8. After a 45-min equilibration at room temperature, samples were loaded onto an 8.5 % polyacrylamide gel in 0.5X TBE buffer. Gels were run at 10V/cm for 55 min in 0.5X TBE buffer with 1mM DTT and 200 µM cAMP.

cAMP Binding Monitored by 8-anilino-1-naphthalenesulfonic acid (ANS) Fluorescence. Measurements were collected with a PTI spectrometer. All experiments were conducted in 20 mM Tris, 50 mM NaCl, 1 mM EDTA, pH 7.8 at 25°C. The reaction mixture contained 47.7 µM ANS and 3.6 µM of protein. cAMP binding to wild-type CRP or CRP<sub>SC</sub> was measured by the quenching of the fluorescent signal from the CRP-ANS complex (λ<sub>ex</sub> = 350 nm and λ<sub>em</sub> = 480 nm). Intensity count as a function of cAMP concentration was fitted to a two-site binding model as follows:

\[
F_{480nm} = \frac{F_0 + F_1 x + F_2 k_1 x + F_2 k_2 x^2}{1 + k_1 x + k_2 x^2}
\]  
(Equation 1)

where \(F_{480nm}\) is the observed signal; \(F_0, F_1\) and \(F_2\) correspond to the fluorescent signal of the free, singly and doubly cAMP-bound states; \(k_1\) and \(k_2\) correspond to the microscopic cAMP affinity constant for the first and second binding events, and \(x\) to the concentration of cAMP.

DNA Binding Monitored by Anisotropy. Measurements were collected with a PTI spectrometer using a 32-bp lac promoter (5'-GCAATTAATGTGAGTTAGCTCACTCATTAGGC-3') covalently linked to a fluorescein molecule (λ<sub>ex</sub> = 480 nm and λ<sub>em</sub> = 518 nm). The reaction mixture contained 5-10 nM of fluorescein-labeled DNA and various concentrations of cAMP (see main text). Data were analyzed as described previously by Heyduk and Lee (32).

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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest with the contents of this article.

AUTHOR CONTRIBUTION
M.F.L. design, conducted, and analyzed research, and wrote the manuscript. F.G. conducted and analyzed research, and wrote the manuscript. A.J.E. conducted research. R.A.M. design and analyze research, and wrote the manuscript.
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FOOTNOTES
The abbreviations used are: CRP, cAMP receptor protein; CRPsc, CRP single-chain dimer; EMSA, electrophoretic mobility shift assay; ITC, isothermal titration calorimetry; GdnHCl, guanidine hydrochloride; ANS, 8-Anilinonaphthalene-1-sulfonic acid; “c,” cooperativity.
### Table 1. cAMP and DNA binding affinity constants to CRP<sub>SC</sub>

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<th>cAMP binding affinity and cooperativity (c)</th>
<th>DNA binding affinity</th>
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<tr>
<td></td>
<td>$k_1$</td>
<td>$k_2$</td>
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<tr>
<td>CRP&lt;sub&gt;SC&lt;/sub&gt;</td>
<td></td>
<td></td>
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<tr>
<td>WT/WT</td>
<td>3.4 ± 0.6</td>
<td>6.1 ± 2.3</td>
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<tr>
<td>S/S</td>
<td>0.34 ± 0.06</td>
<td>0.04 ± 0.03</td>
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<tr>
<td>D/D</td>
<td>4.4 ± 1.9</td>
<td>38.9 ± 8.9</td>
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<tr>
<td>S/WT</td>
<td>2.3 ± 0.3</td>
<td>0.47 ± 0.06</td>
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<tr>
<td>D/WT</td>
<td>4.2 ± 0.8</td>
<td>17.1 ± 3.4</td>
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<tr>
<td>S/D</td>
<td>2.8 ± 0.7</td>
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<tr>
<td>S+D/WT</td>
<td>1.5 ± 0.2</td>
<td>1.7 ± 0.2</td>
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Error corresponds to standard deviation from fitted parameters using a two-site binding model as described in Equation 1 in materials and methods. The units of $k_1$ and $k_2$ are $10^4$ M$^{-1}$ and $c = k_2/k_1$. The units of $k_{DNA$(empty)}$ and $k_{DNA(cAMP-2)}$ are $10^7$ M$^{-1}$. 
FIGURE 1

Figure 1. Design and construction of a CRP single-chain dimer (CRP_{SC}). (A) Model of a CRP_{SC} that connects the two CRP subunits (cyan and orange) through a flexible polypeptide linker (red). The model was rendered in PyMOL based on the CRP structure bound to cyclic nucleotide (PDB 1G6N). (B) Structure of CRP in the unliganded (left, PDB 2WC2) and cAMP bound conformations (right, PDB 1G6N). cAMP is shown in magenta.
Figure 2. Biophysical and functional characterization of CRP<sub>SC</sub>. (A) SDS-PAGE showing the molecular weight of CRP<sub>SC</sub> vs. wild-type CRP (labeled CRP in all figure panels). (B) Size-exclusion chromatogram of CRP<sub>SC</sub> and wild-type CRP. (C) CD spectra of CRP<sub>SC</sub> and wild-type CRP. (D) Chemical denaturation of CRP<sub>SC</sub> and wild-type CRP monitored by changes in tryptophan fluorescence. The line corresponds to the fit of a two-state unfolding model as described in materials and methods. (E) CRP-DNA interactions monitored by electrophoretic mobility shift assay using increasing lengths of the lac promoter in the absence and presence of 200 µM cAMP for CRP<sub>SC</sub> and wild-type CRP.
Figure 3. Quantification of the functional behavior of CRPsc. (A) cAMP binding to CRPsc and wild-type CRP (labeled CRP in all figure panels) monitored by changes in ANS fluorescence. The solid lines represent the fit using a two-site binding model as described in materials and methods. (B) Binding of CRPsc or wild-type CRP to a 32-bp fluorescein-labeled lac promoter monitored by changes in fluorescence anisotropy. The solid lines represent the fit as described in reference 32. Residuals of the fit for both experiments are shown below the titrations.
Role of Asymmetric Conformations in Protein Allostery

FIGURE 4

Figure 4. Effect of symmetric and asymmetric mutations on cAMP binding affinity. (A) cAMP titrations to CRP\textsubscript{SC}\textsuperscript{WT/WT} (green circles) and the symmetric mutants CRP\textsubscript{SC}\textsuperscript{D/D} (red circles) and CRP\textsubscript{SC}\textsuperscript{S/S} (dark purple circles). (B) cAMP titrations to the single asymmetric mutants CRP\textsubscript{SC}\textsuperscript{D/WT} (light pink squares) and CRP\textsubscript{SC}\textsuperscript{S/WT} (light purple squares). (C) cAMP titrations to the double asymmetric mutants CRP\textsubscript{SC}\textsuperscript{S+D/WT} (dark brown diamonds) and CRP\textsubscript{SC}\textsuperscript{S/D} (beige diamonds). For comparison the dashed lines corresponding to the fits of the parent symmetric proteins obtained from panel A were included in panels B and C. The solid lines in all three panels represent the fit using a two-site binding model as described in materials and methods. Error bars correspond to the standard deviation of at least three repeats.
FIGURE 5

Figure 5. DNA-CRP<sub>Sc</sub> interactions using saturating and non-saturating cAMP concentrations. (A) Interaction of CRP<sub>Sc</sub><sup>S/D</sup> with the 32-bp lac promoter using 0, 30 and 1000 µM cAMP, which correspond to unbound (□), singly (▲) and doubly (●) cAMP-bound states. The solid lines represent the fit as described in reference 32. (B) DNA binding affinity constants of CRP<sub>Sc</sub><sup>S/D</sup>, CRP<sub>Sc</sub><sup>S/WT</sup> and CRP<sub>Sc</sub><sup>S/S</sup> when the proteins are in the unbound (□), singly (▲) and doubly (●) cAMP-bound states. (C) DNA binding affinity constants obtained from fluorescence anisotropy experiments for symmetric and asymmetric CRP<sub>Sc</sub> in the absence (□) and presence of saturating cAMP concentrations (●). For CRP<sub>Sc</sub><sup>WT/WT</sup>, CRP<sub>Sc</sub><sup>D/D</sup> and CRP<sub>Sc</sub><sup>D/WT</sup>, [cAMP] = 200 µM. For CRP<sub>Sc</sub><sup>S/S</sup>, [cAMP] = 2000 µM. For CRP<sub>Sc</sub><sup>S/D</sup>, CRP<sub>Sc</sub><sup>S/WT</sup> and CRP<sub>Sc</sub><sup>S+D/WT</sup>, [cAMP] = 1000 µM.
FIGURE 6

Figure 6. cAMP binding energies and cooperativities of CRP<sub>sc</sub> variants. Light and dark squares correspond to the cAMP binding energy for the first (ΔG<sub>1</sub>) and second (ΔG<sub>2</sub>) sites, respectively. The order of CRP<sub>sc</sub> mutants is sorted from most positive cooperativity (ΔG<sub>2</sub> – ΔG<sub>1</sub> < 0) to most negative (ΔG<sub>2</sub> – ΔG<sub>1</sub> > 0) as indicated by the dashed lines.
Figure 7. Mechanism of CRP activation and interaction with DNA. In the absence of cAMP, CRP is in an inactive state. Binding of cAMP to one CRP subunit triggers a conformational change in the DNA binding domain of the bound subunit, generating an asymmetric conformation within the dimer. The cAMP-bound subunit induces a re-orientation in the unliganded, neighboring subunit that is compatible with strong DNA interactions. Binding of a second cAMP molecule can occur to either the singly cAMP-bound CRP or to the ternary complex DNA-CRP-cAMP.
Asymmetric Configurations in a Reengineered Homodimer Reveal Multiple Subunit Communication Pathways in Protein Allostery
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