Structural basis for cAMP-mediated allosteric control of the catabolite activator protein

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The cAMP-mediated allosteric transition in the catabolite activator protein (CAP; also known as the cAMP receptor protein, CRP) is a textbook example of modulation of DNA-binding activity by small-molecule binding. Here we report the structure of CAP in the absence of cAMP, which, together with structures of CAP in the presence of cAMP, defines atomic details of the cAMP-mediated allosteric transition. The structural changes, and their relationship to CAP binding and DNA binding, are remarkably clear and simple. Binding of cAMP results in a coil-to-helix transition that extends the coiled-coil dimerization interface of CAP by 3 turns of helix and concomitantly causes rotation, by ~60°, and translation, by ~7 Å, of the DNA-binding domains (DBDs) of CAP, positioning the recognition helices in the DBDs in the correct orientation to interact with DNA. The allosteric transition is stabilized further by expulsion of an aromatic residue from the CAP-binding pocket upon cAMP binding. The results define the structural mechanisms that underlie allosteric control of this prototypic transcriptional regulatory factor and provide an illustrative example of how effector-mediated structural changes can control the activity of regulatory proteins.

allosteric regulation | cAMP binding | gene regulation | protein NMR | NMR structure

The Escherichia coli catabolite activator protein (CAP) (1) is a transcriptional activator known to regulate hundreds of transcription units by responding to fluctuations of the cellular concentration of cAMP (2). CAP is an ~50-kDa homodimer comprising 209 residues per subunit (supporting information (SI) Fig. S1). Each subunit is organized in 2 distinct domains: (i) an N-terminal cAMP-binding domain (CBD; residues 1–136), which contains the cyclic nucleotide-binding module and a long α-helix (C-helix) that mediates dimerization through formation of an intersubunit coiled coil, and (ii) a C-terminal DNA-binding domain (DBD; residues 139–209), which contains a helix-turn-helix motif for binding to DNA. The 2 domains are linked by a short hinge region (residues 137–138). CAP elicits an allosteric transition that switches CAP from the “off” state, which binds DNA weakly and nonspecifically, to the “on” state, which binds DNA strongly and specifically. In the CAP-bound state, CAP binds to DNA sites located in or adjacent to target promoters resulting in modulation of interactions of RNA polymerase with target promoters (1, 3).

The cAMP-mediated allosteric transition in CAP is a textbook example of modulation of DNA-binding activity by small-molecule binding and has been the subject of extensive structural, biophysical, biochemical, and genetic analysis (4). CAP was the first transcriptional regulatory protein to have its 3D structure determined (5). The 3D structures of CAP have been determined in its cAMP-bound state (6) and in its cAMP-bound state in complex with DNA (7, 8). Nevertheless, the structural basis of the cAMP-mediated allosteric transition in CAP has remained unclear, because the structure of CAP in the absence of cAMP was unknown.

Here, we report the solution structure of CAP in the absence of cAMP (apo-CAP). The structural changes, and their relationship to CAP binding and DNA binding, are remarkably clear and simple. The present results, together with previously reported structures of CAP in the presence of cAMP (6–8), complete the structural basis for understanding the allosteric control of CAP, a prototypic transcriptional regulatory factor.

Results

Structure Determination of apo-CAP. The structure of apo-CAP was determined in solution by NMR. Only 1 set of resonances is present for both subunits of CAP in the 2D 1H–15N HSQC spectra (Fig. S2A), suggesting that CAP exists as a symmetric dimer in solution. Because of the large size of CAP (~50 kDa), we applied multidimensional heteronuclear NMR experiments in combination with a series of strategically designed samples tailored to improve and simplify resonance assignment and structure determination and to yield the maximum number of structural restraints (Figs. 1A–C and S2). In addition, paramagnetic relaxation enhancement (PRE) experiments (9) provided a large number of very long distance restraints (up to ~28 Å) between the CBD and DBD, and residual dipolar couplings (RDCs) (10) were used to define the relative orientation of the CBD and DBD unambiguously (Fig. S3). In total, ~5,000 restraints (per subunit) were used to determine the structure of the dimeric ~50 kDa apo-CAP at high resolution (Table S1). The NMR solution structure of apo-CAP is represented by an ensemble of the 20 lowest-energy conformers (Fig. 1D).

apo-CAP DBD Adopts an Orientation That Is Incompatible with Specific DNA Binding. Superposition of the structure of apo-CAP on the structures of CAP-cAMP2-DNA (7, 8) reveals that the DBD adopts dramatically different positions relative to the CBD in apo-CAP vs. CAP-cAMP2-DNA (Fig. 2). In the structure of CAP-cAMP2-DNA, the recognition helices (F-helices) of the DBDs of the CAP dimer insert into successive DNA major grooves and make extensive contacts with DNA base pairs and the sugar-phosphate backbone (7, 8). In the structure of CAP-cAMP2 (6), the F-helices of the DBDs of the CAP dimer are positioned essentially as in the structure of CAP-cAMP2-DNA (i.e., positioned so as to be able to insert into successive DNA major grooves). In contrast, in the structure of apo-CAP, the F-helices are rotated by ~60° and translated by ~7 Å relative to their orientations and positions in the structure of CAP-cAMP2-DNA (Fig. 2C) and thus are not positioned to insert into successive DNA major grooves. In short, the cAMP-ligated state, the recognition

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Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 2WC2).

See Commentary on page 6887.

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helices are oriented correctly to interact with successive DNA major grooves, but in the cAMP-free state, the recognition helices are out of register with successive DNA major grooves (Fig. 2A and C).

The Coiled Coil Is Partially Unwound in apo-CAP. In the structures of CAP-cAMP2 and CAP-cAMP2-DNA the CBD C-helix, which forms an intersubunit C-helix/C’-helix (where ’ denotes the second CAP subunit) coiled coil, extends from residues Pro-110 to Phe-136. In contrast, in the structure of apo-CAP, the C-helix and the intersubunit C-helix/C’-helix coiled coil extend only to Gln-125 (11) (Fig. 2A and B and Fig. S1). This difference in the length of the C-helix, and of the C-helix/C’-helix coiled coil, represents 3 turns of helix. In apo-CAP, the 11-residue segment comprising Val-126 to Phe-136 segment and, correspondingly, nucleate and/or stabilize helix formation within the Val-126 to Phe-136 segment and, correspondingly, nucleate and/or stabilize intersubunit coiled-coil formation (Figs. 2B and 3A).

A third contributor to the coil-to-helix transition may be the cAMP-phosphate moiety and CAP (Fig. 3B). The cAMP sugar-phosphate moiety interacts with a conserved phosphate-binding cassette (PBC), which is the “signature” motif for all cAMP-binding proteins (residues Gly-71 to Ala-84 in CAP) (12). In apo-CAP, the guanidinium side-chain of Arg-82 forms a salt bridge with the carboxylate side-chain of Glu-129, potentially stabilizing the unstructured, non-helical conformation of the Val-126 to Phe-136 segment (Fig. 3B). Upon binding of cAMP, Arg-82 relocates, enters the CAMP-binding pocket, and forms a salt bridge with the cAMP phosphate (Fig. 3B); the relocation of Arg-82 disrupts the salt bridge with Glu-129 and thus potentially destabilizes the non-helical conformation of the Val-126 to Phe-136 segment (Fig. 3B). In apo-CAP, residues Glu-72 to Glu-77 of the PBC form a short α-helix that provides the scaffold for the formation of 2 favorable salt bridges involving Glu-77, Glu-78, and Arg-122’ (Fig. 3B). Binding of cAMP disrupts this short α-helix, thereby disrupting the salt bridges involving Glu-77, Glu-78, and Arg-122’ and causing Arg-122’ to relocate and form a salt bridge with Glu-129 that potentially stabilizes the helical conformational state of the Val-126 to Phe-136 segment (Fig. 3B).

A third contributor to the coil-to-helix transition may be the cAMP-forced expulsion of an aromatic residue from the CAMP-binding pocket. In the absence of cAMP, the CAMP-binding pocket, which is primarily hydrophobic, is occupied by the side chain of Trp-85 (Fig. 3C). Binding of cAMP displaces Trp-85, which is expelled from the hydrophobic core into the solvent (Fig. 3C). This
observation is in agreement with fluorescence data suggesting that binding of cAMP increases solvent exposure of Trp-85 (13). Displacement of Trp-85 drives the β-strand 4/β-strand 5 flap segment toward the C-helix (Fig. 3C), facilitating hydrophobic interactions between Ile-51, Lys-57, Met-59, and Leu-61 within the flap and Phe-136 (which resides at the tip of the extended C-helix) that potentially stabilize the helical conformational state of the Val-126 to Phe-136 segment (Fig. 2D).

**Fig. 2.** Structural comparison of apo-CAP and CAP-cAMP-DNA. Dotted lines denote polar contacts (hydrogen bonds or salt bridges). (A) Superposition of apo-CAP (orange) and CAP-cAMP-DNA (blue) on the CBD (residues 10–125). F, recognition helix (see Fig. S1 for nomenclature). DNA is in light gray, and cAMP in dark gray. Proteins are shown as cartoons and DNA as sticks in semitransparent surface. (B) Close-up view of the intersubunit C-helix/C-helix coiled coil. In apo-CAP, the C-helix extends only to Gln-125; in contrast, in CAP-cAMP-DNA (and CAP-cAMP2) the C-helix extends to Phe-136. (C) View from above DNA showing the rigid-body rotation (indicated on the left subunit) and translation (indicated on the right subunit) that DBD undergoes upon cAMP binding. α-Helices are drawn as cylinders. DNA is in green. (D) View from above the C-helices highlighting crucial contacts made by residues located at the C-terminal of the C-helices. Phe-136 forms hydrophobic contacts with Ile-51, Lys-57, Met-59, and Leu-61 in the cAMP-bound state but not in the cAMP-free state. Lys-130 forms a salt bridge with Glu-54 in the cAMP-free state, but it forms a hydrogen bond with the carbonyl of Ile-60 in the cAMP-bound state. Residues involved in hydrophobic contacts are in green; nitrogen and oxygen atoms are in blue and red, respectively.

**Distinct Sets of Interactions Stabilize the Active and Inactive Conformations.** A network of newly formed contacts contributes to the stabilization of the C-helices and the intersubunit C-helix/C'-helix coiled coil upon binding of cAMP (Fig. 2D). For example, the side-chain amine of Lys-130 forms a hydrogen bond with the backbone of Ile-60 in CAP-cAMP2, whereas Lys-130 adopts a very different conformation in apo-CAP wherein it forms a salt bridge with Glu-54 located at the flap (Fig. 2D). Thus, disrup-

**Fig. 3.** Effect of cAMP binding on CAP. Color code is as follows: apo-CAP, orange; cAMP-DNA-CAP, blue; cAMP, gray. Structures are superimposed as in Fig. 1. Nitrogen and oxygen atoms are colored blue and red, respectively. (A) Close-up of the cAMP-binding site highlighting contacts between cAMP and the C-helix. (B) Close-up of the cAMP-binding site highlighting the rearrangement of the nearby electrostatic network. (C) Expulsion of Trp-85 from the nucleotide pocket to the solvent upon cAMP binding. cAMP is shown as thin lines.
tion of the salt bridge between Glu-54 and Lys-130 upon cAMP binding allows Lys-130 to relocate so as to interact with Ile-60, thereby stabilizing and properly orienting the C-helices, and also releases the strain from the flap region, which, coupled with the displacement of Trp-85 (Fig. 3C), is pushed closer to the C-helix. The new set of interactions established upon cAMP binding brings the D-helix much closer to the C-helix, leading to the formation of an extensive network of interactions between the 2 helices (Fig. 2D): the side chain of Arg-142 forms hydrogen bonds with the backbone carbonyl oxygens of Leu-134 and Leu-137 as well as with the carbonyl oxygen of Val-176 located near the F-helix; the side chain of Asp-138 hydrogen-bonds with the backbone amide of Gly-141; and Glu-129 forms a salt bridge with Lys-152 (6). These interactions result in the repositioning of the DBDs in cAMP_{2}-CAP relative to apo-CAP (Fig. 2A and C).

cGMP Fails to Stimulate DNA Binding by CAP Because it Fails to Elicit the Coil-to-Helix Transition. To understand why cGMP, a non-effector molecule, binds to CAP but does not stimulate DNA binding by CAP (14, 15), we used NMR to characterize the CAP-cGMP_{2} complex. The NMR data show that, in contrast to cAMP (Fig. 4A), cGMP does not allosterically alter the conformation of the DBD (Fig. 4B), which remains in the inactive conformation incompatible with DNA-binding. NOE, chemical shift, and relaxation data reveal that the Val-126 to Phe-136 segment remains unstructured in CAP-cGMP_{2}. Thus, in contrast to cAMP, cGMP fails to elicit the coil-to-helix structural transition to the C-helix. To determine the conformation of cGMP in complex with CAP, we used transferred 2D NOE experiments. The NOE data (Fig. S5) reveal that, in contrast to cAMP that binds to CAP in the anti conformation (6), cGMP binds to CAP in the syn conformation (Fig. 4C). Structural modeling based on NMR chemical shift and sparse NOE data (Fig. S5) suggest that cGMP fails to elicit the active conformational state because the cGMP guanine base cannot make key contacts, involving residues Thr-127 and Ser-128, that elicit the coil-to-helix transition in the C-helix (Fig. 4C).

Substitutions Resulting in Constitutively Active Phenotype Stimulate DNA Binding by CAP by Eliciting the Active Conformation. Mutants of CAP have been isolated that are able to bind to DNA and to activate CAP-dependent promoters in the absence of cAMP (CAP* mutants). Most CAP* mutants involved substitutions located in or close to the hinge region (16, 17), which undergoes major conformational changes during the cAMP-mediated allosteric transition (Fig. 2), and substitutions of Thr-127 and Ser-128 (18), 2 residues that are located within the intersubunit C-helix/C-helix coiled coil and seem to serve as initiation points for the cAMP-mediated coil-to-helix transition. Here we have characterized 3 of these CAP derivatives, CAP*-G141S, CAP*-R142H/A144T, and CAP*-T127L/S128I, by NMR.

Our NMR data reveal that the G141S substitution has dramatic effects both on the structure and on the dynamics of CAP (Fig. 5). (Results for the R142H/A144T substitution are very similar to those for G141S.) The chemical shifts of all DBD residues change significantly and move toward the chemical shift positions in the CAP-cAMP_{2} complex (Fig. 5B). This observation indicates that the G141S substitution shifts the conformational ensemble toward...
the active conformation (Fig. 5B). The significant resonance broadening observed for almost all the DBD resonances indicates that the 2 states, active and inactive, interconvert on the micro-to-millisecond (µs-ms) time scale (Fig. 5). Addition of cAMP shifts the population further toward the active conformation with the spectra of CAP-cAMP, and CAP-G141S-cAMP being almost identical (Fig. 5B). Interestingly, whereas cGMP binding to WT CAP has no effect on DBD (Figs. 4B and 5B), cGMP binding to CAP*-G141S seems to activate the CAP* protein further by shifting the equilibrium toward the active state (Fig. 5B), in agreement with biochemical data (19, 20). Moreover, the pattern and direction of chemical shift changes in the residues located at the unstructured segment of the coiled coil in the CAP* mutant, together with the observed severe exchange broadening, suggest that this region undergoes a coil-to-helix transition in apo-CAP*-G141S. This observation is important, because it indicates that the coil-to-helix transition is a prerequisite for inducing and stabilizing the active conformation of the DBD, even in the absence of cAMP. It is likely that polar substitutions at CAP* sites near the hinge region involve the formation of hydrogen bonds or electrostatic interactions that cause translation of the D-helix toward the C-helix and rotation of the DBD toward the active conformation. A similar mechanism was observed for a constitutively active mutant of PrfA (21), a structural homologue of CAP.

NMR characterization of CAP*-T127L/S128I indicates that the double substitution elicits the active conformational state in the absence of cAMP, albeit with less efficiency than in the presence of cAMP (Fig. 6). The structural basis for the induction of the active conformation by this double mutant seems to be the stabilization of the metastable C-helix/C'-helix coiled coil. The intrinsic instability of the C-terminal portion of the intersubunit C-helix/C'-helix coiled coil probably is attributable to the atypical coiled-coil heptad-repeat sequence (Fig. S6A). The atypical presence of polar residues at heptad-repeat positions a and d (i.e., Ser-117 and Thr-127 at positions a and d, respectively), together with the atypical lack of intra- and inter-helical electrostatic interactions between residues at heptad-repeat positions e and g, are likely to reduce coiled-coil stability (22). The single substitution of Thr-127 by Leu, a hydrophobic residue that is particularly favored at position d of the heptad sequence, is not sufficient by itself to stabilize the coiled coil, as judged by NMR analysis of the CAP*-T127L mutant (Fig. S6B). NMR chemical shift analysis of CAP*-T127L/S128I suggests that Ile-128 engages in hydrophobic interactions with Ile-51, Met-59, Leu-61, and Leu-73, all located in the cyclic nucleotide-binding module, thereby contributing to the stabilization of the C-helix (Fig. 6A). Therefore, in the absence of inter-helical contacts between residues at positions e and g, interactions between the C-helix and the effector domain seem to be essential for the stabilization of the C-helix/C'-helix coiled coil.

Discussion

We provide atomic details of the cAMP-mediated allosteric transition in CAP, a classic model system for understanding how small molecules effect gene regulation (Fig. 7 and Movie S1). The basic feature of the mechanism is a bistable coiled coil, in which a small-molecule effector determines the balance between the 2 alternative coiled-coil states and in which the 2 alternative coiled-coil states are linked, essentially directly and mechanically, to 2 alternative orientations of a DNA-binding domain. The bistability

Fig. 6. Effect of the CAP* T127L/S128I substitution on CAP. (A) Residues that experience significant chemical shift change as a result of the T127L/S128I double substitution are mapped (pink) on the structure of CAP-cAMP2. Effector domain residues that are involved in hydrophobic interactions with Ile-128, as assessed on the basis of chemical shift changes, are shown as green sticks. (B) Overlaid 1H-15N HSQC spectra of characteristic DBD residues of apo-CAP (blue), CAP-cAMP2 (green), and apo-CAP-T127L/S128I (red).

Fig. 7. Mechanism of allosteric control of CAP. Schematic models of CAP in the 3 structurally characterized states: apo-CAP (this work), CAP-cAMP2 (6), and CAP-cAMP2-DNA (7). The proposed primary mechanism of allosteric control is clear and simple: cAMP binds to the CBD of CAP and makes direct contacts with Thr-127 and Ser-128. These contacts induce a coil-to-helix transition that extends the C-helix, and the intersubunit C-helix/C'-helix coiled coil, by 3 turns of helix. This coil-to-helix transition results in rotation of the DBDs of the CAP dimer by ~60° and translation of the DBDs of the CAP dimer by ~7 Å (distance of intersubunit F-helices is 41 Å in apo-CAP and 34 Å, matching the distance between successive DNA major grooves in CAP-cAMP2). This rotation and translation places the F-helices (“recognition helices”) of the DBDs of the CAP dimer in the correct orientation and correct position to interact with successive DNA major grooves. (See also Movie S1).
of the coiled-coil and disorder-order transitions coupled to effector binding permit efficient, precise allosteric control. The results highlight the versatility of the coiled-coil motif both in mediating oligomerization and in functioning as an allosteric switch.

The allosteric mechanism proposed herein is consistent with, and accounts for, a large array of biophysical, biochemical, and genetic data. The present results provide a framework for understanding and discussing allosteric control of the more than 400 members of the CAP regulator of fumarate and nitrate reduction (FNR) protein superfamily of transcriptional regulatory factors (23), which share the same overall structural organization as CAP but respond to a broad, chemically diverse, range of allosteric effectors. In carbon monoxide oxidation activator (CooA; effector = CO) and o-chlorophenol reductive dehalogenase transcription regulator (CprK) (effector = ortho-chlorophenolacetic acid), 2 members of the CAP/FNR superfamily for which structural data are available (24–26), the C-helix and the intersubunit C-helix/C-helix coiled coil seem to be fully structured in the absence of effector. Like CAP, CooA and CprK suffer from irregularities in coiled-coil heptad-repeat sequences. However, in contrast to the situation with CAP, in CooA and CprK, a multitude of interactions between the C-helices and the remainder of the effector domain seem to provide the critical interactions needed for the coiled-coil stability (24–26). Apparently, the structural mechanisms that allow effector-mediated activation of CooA and CprK are distinct and do not involve the coil-to-helix transition seen in CAP. In N-oxide sensing and denitrification transcription factor (DNR; effector = NO), a member of the CAP/FNR superfamily for which a structure of the isolated effector-binding domain in the absence of effector is available (27), the C-helix and the intersubunit C-helix/C-helix coiled coil seem to be partly unstructured in the absence of effector and to extend only to the residue corresponding to Gln-125 in CAP. The striking similarity to CAP in the length of the C-helix and the intersubunit C-helix/C-helix and the C-helix and the intersubunit C-helix/C-helix coiled coil in the absence of effector suggests that DNR employs the same mechanism of allosteric control mechanism as CAP. We anticipate that other members of the CAP/FNR superfamily also use this mechanism. The present results also may provide a framework for understanding and discussing allosteric control of the numerous, functionally diverse, proteins that use cyclic nucleotides as allosteric effectors and conserved cyclic-nucleotide–binding domains as allosteric control units (28). This group includes, among many others, protein kinase A (29), guanine nucleotide exchange factors (30), and ion channels that regulate metal ion gating (31).

Materials and Methods

Protein Preparation. Isotopically labeled samples for NMR studies were prepared as detailed in SI Text.

NMR Spectroscopy. NMR experiments were performed on Varian 900-, 800-, and 600-MHz and Bruker 700-MHz spectrometers. Backbone and side-chain assignment of apo-CAP was achieved using triple-resonance pulse sequences optimized for large proteins (32). NOEs were measured using 2D, 3D, and 4D NOESY using mixing times of 70 ms for protonated samples and 150–300 ms for deuterated samples. The structure of cGMP bound to CAP was determined using 2D transferred NOE spectroscopy with a mixing time of 100 ms. Concentration of cGMP was 1 mM, whereas concentration of CAP was 50 μM. 1H-15N NOE data were obtained as described previously (33). All NMR samples were prepared in 50 mM KPi (pH 6.0), 500 mM potassium phosphate, 1 mM DTT, and 1 g l1 Na3. Concentration was 0.6–0.8 mM. His-tagged and non-His-tagged CAP samples provided essentially identical spectra. Spectra were recorded at 32 °C. PRE (9, 34) and RDC data were collected and analyzed as detailed in SI Text.

Structure Calculation. Structure calculations were performed with CNS software (35) as detailed in SI Text.

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Supporting Information

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SI Text

Protein Preparation. The plasmids encoding for WT-CAP and His
tagged CAP were transformed in BL21(DE3) cells and grown at 37 °C in the presence of ampicillin. Protein synthesis was induced by addition of 1.0 mM of isopropyl-ß-d-
thiogalactopyranoside (IPTG) at OD600 = 0.4. Cells were harvested at OD600 ~0.8 and resuspended in the lysis buffer containing 20 mM Tris-HCl (pH 7.9), 500 mM NaCl, 5 mM imidazole, and 1 mM ß-mercaptoethanol. Isotopically labeled samples for NMR studies were prepared by growing the cells in minimal (M9) medium. For the backbone assignment a U-13C/15N-labeled sample was prepared by supplementing the growing medium with 1 g l⁻¹ of 15NH₄Cl and 2 g l⁻¹ of 2H₂O-glucose in 99.9% 2H₂O (CIL). For the production of methyl-protonated samples the following were used (1, 2): 50 mg ml⁻¹ of alpha-ketobutyric acid (for Ile-51 [13C₃] labeling); 100 mg ml⁻¹ of alpha-ketoisovaleric acid (for Val, Leu-[13C₅,13C₇] labeling); 250 mg ml⁻¹ of [13C₆]-methionine (methyl-[13C₅]); and 100 mg [13C₆,13C₇]-alanine, prepared as described previously (3). For Phe, Tyr, and Trp labeling, U-[13C₃]-labeled amino acids were added. All precursors and amino acids were added to the culture 1 h before addition of IPTG, except Ala, which was added 30 min before induction. Cells were sonicated and centrifuged at 50,000 x g. Two purification steps were used for all protein samples. The first step involved a nickel-chelated Sepharose fast-flow resin (Amersham), and the second step involved a Superdex-200 size exclusion column (Amersham). Protein concentration was determined spectrophotometrically at 278 nm using an extinction coefficient of 19,940 M⁻¹ cm⁻¹. The second step involved a nickel-chelated Sepharose fast-flow resin (Amersham), and the second step involved a Superdex-200 size exclusion column (Amersham). Protein concentration was determined spectrophotometrically at 278 nm using an extinction coefficient of 19,940 M⁻¹ cm⁻¹. The second step involved a nickel-chelated Sepharose fast-flow resin (Amersham), and the second step involved a Superdex-200 size exclusion column (Amersham). Protein concentration was determined spectrophotometrically at 278 nm using an extinction coefficient of 19,940 M⁻¹ cm⁻¹.

NMR Restraints. In the first step, we used a U-13C/15N-labeled sample that contained specifically protonated methyl groups of Ala, Val, Leu, Met, and Ile (51) and protonated aromatic residues Phe, Tyr, and Trp in an otherwise deuterated background (Fig. S2B). The high sensitivity and resolution of the methyl region, coupled with the fact that the interior of CAP is highly enriched with these residues (~45% of CAP residues), provided a significant number of NOEIs (Fig. 1 B and C) sufficient to yield an initial well-defined structure. The initial structure was used to guide the subsequent collection of a much larger number of NOEs, using fully protonated (Fig. 1A) and fractionally deuterated samples that resulted in further refinement to precise and accurate coordinates. In addition, PRE experiments provided a large number of very long distance restraints (up to ~28 Å) between the CBD and DBD, and RDCs were used to define the relative orientation of the CBD and DBD unambiguously (Fig. S3).

Paramagnetic Relaxation Enhancement Measurements. Nitroxide spin labels (MTSL; Toronto Research Chemicals Inc.) were introduced via cysteine-specific modification of engineered CAP derivatives containing single-solvent-accessible cysteine residues at sites of interest (K26, K57, G66, D155, Q170, and S197) (4). Mutants and MTSL derivatives that did not perturb the CAP structure, as assessed by 1H-13N heteronuclear single quantum coherence (HSQC) spectra, were used for measuring PRE rates. After purification, proteins were exchanged into phosphate buffer (50 mM KPi, 500 mM KCl, pH 8.0) free of any reducing agent, and the mixture was concentrated (~8 μM). MTSL was added from a concentrated stock in acetonitrile at a 10-fold excess, and the reaction was allowed to proceed at 4 °C for ~12 h. The completion of the reaction was confirmed by mass spectrometry. Excess MTSL was removed by extensive dialysis using an Amicon stirred cell, and the pH was corrected to 6.0. PRE-derived distances were determined from 1H-15N-transeverse relaxation optimized spectroscopy (TROSY)-HSQC spectra of U-13C/15N-CAP by measuring peak intensities before (paramagnetic) and after (diamagnetic) reduction of the nitroxide spin label with ascorbic acid. PRE values then were converted to distances by using a modified Solomon-Bloembergen equation for transverse relaxation, as described previously (5). Ensemble simulated annealing refinement was used as described (6). Two sets of restraints were incorporated into subsequent structure calculations. Amide resonances strongly affected by the presence of the spin label in the peptide (Ipara/Ipara < 0.15) and whose resonances broaden beyond detection in the paramagnetic spectrum were restrained with only an upper-bound distance estimated from the noise of the spectrum plus 4 Å. Amides whose resonances appear in the paramagnetic spectrum (Ipara/Ipara < 0.85) were restrained as the calculated distance with ± 4 Å upper/lower bounds.

Residual Dipolar Coupling Measurements. Alignment of the protein for RDC measurements was achieved using poly(ethylene glycol)/alcohol mixtures (7). A 5% C12E5/hexanol (molar ratio = 0.96) mixture, which is stable in the temperature range 26–39 °C, was prepared. C12E5 was used to a final concentration of 5% (wt/wt) in a 90% H2O:10% D2O solution. The pH was adjusted using sodium hydroxide. The amount of hexanol was added by drops, during vigorous shaking, to a final molar C12E5:hexanol ratio of 0.96. Air bubbles were removed by centrifugation at 5000 × g for a few minutes. The HDO quadrupolar deuterium splitting was checked to confirm the presence of the crystalline phase (a splitting of ~27 Hz was observed). For the measurement of RDCs in the protein, 250 μl of the C12E5:hexanol stock solution was added to 50 μl of protein in buffer. 13N-HSQC (in-phase anti-phase)- and TROSY-HNCO–based experiments were used to measure 1-bond N-H and Ca-C RDCs (8). The alignment tensor was determined as described (9).

Structure Calculation. Structure calculations were performed with CNS software (10). The 13Ca, 13CB, 13C, Hα, 13N, and NH chemical shifts served as input for the TALOS program (11) to extract dihedral (φ and ψ) angles. Distance restraints derived from the NOE SY spectra acquired using longer mixing times (150–300 ms) were categorized in 4 upper-bound bins (4.0, 5.0, 6.0, and 7.5 Å) based on the cross-peak intensities. An additional 0.5 Å was added for NOEs involving methyl groups. Additional distance restraints derived from NOESY measurements on protonated samples were categorized in 3 bins with upper-bound distances of 2.8, 3.5, and 5.0 Å. Only unambiguous long-range (ij > 3) NOE restraints involving methyl, aromatic, and amide protons were used in initial structure calculations. The initial structures were used as a reference for the assignment of additional NOE cross-peaks, particularly from side chains. Restraints from PRE and RDC data were included in the final stages of the calculation. For the structure calculations a simulated annealing protocol was used using both torsion angle dynamics and Cartesian dynamics.

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Fig. S1. Secondary structure of apo-CAP and CAP-cAMP$_2$. $\alpha$-Helices are shown as cylinders and $\beta$-strands as arrows. Labeling of secondary structure is shown on the structure of cAMP$_2$-CAP. CDB, DBD, and the hinge are colored light blue, green, and red, respectively. Regions that have different secondary structure in apo-CAP and cAMP$_2$-CAP are colored yellow. The flap region ($\beta$4-loop-$\beta$5) is indicated also.
Fig. S2. NMR structural characterization of apo-CAP by NMR. (A) $^1$H-$^{15}$N-TROSY-HSQC of U-$^2$H,$^{15}$N apo-CAP recorded at 32 °C. Only 1 set of peaks is present for both subunits, indicating that apo-CAP exists as a symmetric dimer in solution. (B) $^1$H-$^{13}$C HMQC of U-$^1$H,$^{12}$C, Ala-, Leu-, Met-, Val-, Ile-$^1$-$^{13}$CH$_3$ apo-CAP.

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Fig. S3. Experimental versus calculated RDC values ($\Delta_{\text{HN}}$) for apo-CAP. RDCs of fixed-bond vectors, such as the backbone N–H and C$^{\text{a}}$/H$_{11032}$–C$^{\text{a}}$/H$_{9251}$ bond vectors, are dependent on the orientation of the bond vectors relative to the alignment tensor and provide a very powerful tool to determine unambiguously the relative orientations of domains.
Fig. S4. NMR relaxation analysis of picosecond-to-nanosecond (ps-ns) time-scale motions of apo-CAP and CAP-cAMP$_2$. $^1$H-$^{15}$N NOE values of apo-CAP (red) and cAMP$_2$-CAP (green) plotted against residue number. The region (Val-126 to Phe-136) that undergoes the coil-to-helix transition is highlighted. Lower values indicate flexibility, whereas higher values indicate rigidity of motions on the ps-ns time scale.
Fig. S5. NMR analysis of cGMP binding to CAP. (B) Characteristic NOEs (blue dotted lines) observed by 2D trNOESY for cGMP in complex with CAP indicating that cGMP binds in the syn conformation. (A) Overlaid $^1$H-$^{15}$N HSQC spectra of apo- (blue), cAMP-bound (green), and cGMP-bound (pink) CAP highlighting chemical shift changes of residues located in the C-helix (V126-K130) and the PBC (G71, A82). These data show that although the sugar moiety of cGMP and cAMP binds to the PBC in a very similar manner, cGMP does not elicit the coil-to-helix transition in region V126-F136 of the C-helix.
Fig. S6. Effect of coiled-coil substitutions. (A) Helical-wheel representation of the intersubunit C-helix/C-helix coiled coil of CAP (P110-F136). Heptad-repeat sequence positions a, b, c, d, e, f, and g are shown as circles. Residues T127 and S128 are in green. (B) Overlaid $^1$H-$^1$N HSQC spectra of apo-CAP (blue), apo-CAP-T127L (yellow), and apo-CAP*-T127L/S128I (red).
**Movie S1.** Mechanism of cAMP-mediated allosteric transition in CAP.
Table S1. NMR and refinement statistics for apo-CAP structures

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<th>NMR Restraints (per subunit)</th>
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<td>NOEs</td>
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<td>Violations (mean and SD)</td>
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*Statistics applied in rigid regions (residues 10–209)