The cAMP binding domain: An ancient signaling module

Helen M. Berman*, Lynn F. Ten Eyck†‡§, David S. Goodsell¶, Nina M. Haste¶, Alexandr Kornev†, and Susan S. Taylor†‡§**

*Department of Chemistry and Chemical Biology, Rutgers, The State University of New Jersey, 610 Taylor Road, Piscataway, NJ 08854-8087; Departments of §Pharmacology and ¶Chemistry and Biochemistry, ‡San Diego Supercomputer Center, and **Howard Hughes Medical Institute, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093; and †Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037

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Cyclic AMP (cAMP) (Fig. 1a) is an ancient signaling molecule that serves as an indicator of stress from the most primitive bacteria to humans. The module that binds cAMP and translates the signal into a biological response, the cyclic nucleotide-binding (CNB) domain, is also ancient. Although the best-known bacterial CNB domain is associated with the catabolite activator protein (CAP), it is linked to a DNA binding domain, and mediates gene transcription in the absence of glucose, the elucidation of many bacterial genomes indicates that this module is much more diverse. There are literally hundreds of proteins that are linked to CNB domains, and these proteins mediate many types of stress responses (1).

The CNB domain is a small module, typically ~120 aa, comprised of both β strands and helical elements. The general organization of secondary structure of the essential CNB-binding site is an eight-stranded β barrel that functions as a basket. The cyclic nucleotide phosphate lies at the base of the β barrel where it is well shielded from solvent and protected from phosphodiesterases (2). The most conserved feature of the β barrel is the phosphate binding cassette (PBC) comprised of β strand 6, a short turn of helix, and β strand 7. A conserved feature of this PBC is a buried arginine that binds to the exocyclic phosphate of cAMP and a glutamate that binds the ribose 2'-OH. In addition to the β barrel, the CNB module has a helical subdomain. The A helix precedes the β barrel and is in an antiparallel manner with the B helix that immediately follows β strand 8. The most variable feature of the domain is the C helix (Fig. 1b and c).

The structure of the CNB domain was first revealed when the structure of CAP was determined (3). Based on sequence similarities, it was predicted that the fold would be conserved in the CNB domains associated with mammalian protein kinases (4), protein kinase A (PKA) and cGMP-dependent protein kinase (5). This prediction was confirmed when the structure of a deletion mutant of the RIo subunit of PKA was determined (6). RIo has two tandem CNB domains, each with a fold that resembles the CNB domain of CAP. The subsequent structure solution of a similar deletion mutant of RIβ (2) showed once more a similar fold but revealed that the network of interactions that link the two domains was surprisingly different. In addition to serving as regulators of kinase activity, mammalian cells have cyclic nucleotide gated channels. A recently solved structure of a channel CNB domain (7) also has a similar fold. More recently, a CNB domain was discovered fused to a guanine nucleotide exchange protein, EPAC (8). Until the structure of EPAC was determined, all of the known structures of CNB domains contained cAMP. The apo form of the CNB domains of RIo has recently been solved as well (9).

Although considerable attention has been paid to the recognition of nucleic acids by proteins, relatively little is known about the binding properties of this ancient signaling module, the CNB domain. Here, we have undertaken to compare the CNB domains from several different proteins to determine systematically the properties of this recognition motif and how it interacts with the cyclic nucleotide. The focus of this work is to discover structural features that are conserved. To do this, we have carried out a systematic computational analysis involving structure-based sequence comparison, surface matching, affinity grid analysis, and analyses of the ligand–protein interactions. These analyses show distinctive roles of the sugar phosphate and the adenine in the cAMP-binding module.

Methods

Data Set. The Protein Data Bank (PDB) (10) was searched for proteins containing cAMP. There are 14 structures containing CAP, four containing the R subunits of PKA, and two of the hyperpolarization-activated cyclic nucleotide-modulated (HCN) channels (Tables 1–3, which are published as supporting information on the PNAS web site). After reviewing the sequence, structural, and experimental characteristics of all of these structures, four were selected as representative structures (Fig. 2):

• RIIβ subunit of PKA [PDB ID 1CX4 (2)] (Fig. 2a). The A and B domains [RIIβ(A) and RIIβ(B)] each contain one cAMP.

• RIo subunit of PKA [PDB ID 1N66 (11)] (Fig. 2b). The A and B domains [RIo(A) and RIo(B)] each contain one (5p)-cAMPs molecule.

• CAP (PDB ID 2CGP) (12) (Fig. 2c). Of the 14 CAP-containing structures, the highest-resolution CAP-DNA complex structure was selected. The biological unit of CAP is a dimer with two cAMPs per monomer unit. The CNB domain is at the N terminus. There is another cAMP in the C-terminal DNA binding domain. Some but not all CAP structures have this second cAMP.

• HCN (PDB ID 1Q43) (7) (Fig. 2d) is a tetramer with the CNB domain at the C terminus. The N-terminal domain forms the channel.

A schematic of the domain structure of the four representative structures is shown in Fig. 2e.

Abbreviations: CNB, cyclic nucleotide binding; CAP, catabolite activator protein; HCN, hyperpolarization-activated cyclic nucleotide-modulated channels; PBC, phosphate binding cassette; PKA, protein kinase A; PDB, Protein Data Bank; SAT, structure and transformation.

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Hydrogen-Bonding Analysis. The hydrogen-bonding environment of cAMP in each structure was examined by using LIGPLOT (13) and SWISSPDB VIEWER (14). Both the hydrogen-bonding and the hydrophobic interactions were examined.

Structure-Based Alignment. Structural alignments were done by using the structure and transformation (SAT) method (15). SAT is designed for close analysis of similarities and differences, rather than for the detection of remote relatives. The method is based on identification of pieces of the two molecules that have similar shapes, as determined by the local torsion and curvature of the peptide chain. Pieces that superpose within a specified tolerance are grouped according to the coordinate transformation that places the probe fragment on the target fragment. The analysis returns all of the sets of residues related by the same coordinate transformation and is thus very good at detecting conserved cores decorated with variable loops; it is also very good at detecting hinge motions. The algorithm was used as implemented on the web site www.npaci.edu/CCMS/wsatsat.

The sequences in the four representative structures were compared. 1CX4 was used as the standard against which all of the structures were compared. SWISSPDB VIEWER (14) was used to overlay the structures according to the alignments of SAT. Further visualization of the aligned and overlaid structures was done by using the graphics program CHIMERA (16).

Surface Matching. The surface-matching procedure was performed by the edge comparison and combination method (ECC) (A.K., S.S.T., and L.F.T.E., unpublished work) that provides detection of all similar patterns formed by amino acid side chains on two protein surfaces. ECC is designed to identify any similarity on any two protein surfaces, without regard to any knowledge of sequence or structural similarity. Details will be published elsewhere, but the essential features of the method are straightforward. First, all residues on each protein that are solvent-exposed [at least 2 Å² water-accessible surface, as calculated by the DSSP program (17)] were taken as neighbors. The surface description consists of a graph with Cα atoms as the nodes, where the edges connect neighbors. To determine similarity of surfaces each edge contains additional information based on the positions of the Cα atoms. The full description of an edge contains (i) the type of each amino acid connected; (ii) the Cα1-Cα2 distance; (iii) the Cα1-Cβ2 distance; (iv) the Cα2-Cβ1 distance; and (v) the torsion angle defined by the four atoms Cα1-Cα1-Cα2-Cβ2. The problem of finding “sim-

Fig. 1. Cyclic nucleotide-binding (CNB) domain. (a) The chemical structure of cAMP. (b) Tertiary structure of Rlα(A) showing the secondary structural units. The image was created by using PyMOL (Delano Scientific) (32). (c) Sequence of Rlα(A) showing the secondary structure color-coded as in a. The PBC is red.

Fig. 2. Four representative CNB domain structures. Helices are colored cyan; sheets are red. (a) Rlβ (PDB ID 1CX4). There are two domains: A and B. The A domain is at the top, and the B domain is at the bottom. Each domain has one cAMP. (b) Rlα (PDB ID 1NE6). There are two domains: A and B. The A domain is at the top, and the B domain is at the bottom. Each domain has one cAMP. (c) CAP (PDB ID 2CGP). There are two subunits related by 2-fold symmetry. For CAP in general each subunit can contain either one or two cAMP molecules. In this example there are two. The one in the N domain is the CNB domain. The other is in the domain that binds to DNA (shown in gray). (d) Cyclic nucleotide-gated channel (HCN) (PDB ID 1Q43). There are four subunits related by 4-fold symmetry. The C-terminal domain contains the cAMP. (e) A schematic of the four representative structures. Blue highlights the domains analyzed here. This image was created by using CHIMERA (16).
ilar” surfaces is now the problem of finding matching subgraphs of the two surfaces in which all of the edges are similar. For this work similar was defined as (i) a residue pair having a BLOSUM62 substitution value ≥2 compared with the candidate residue pair on the other surface; (ii) Cα-Cα distance difference <0.6 Å; (iii and iv) Ca-Cβ distances differing by <0.75 Å; and (v) Cβ1-Cα1-Cα2-Cβ2 torsion angles differing by <35°. The subgraph-matching problem was solved by considering only those edges that were similar in both surfaces, which greatly reduces the size of the problem. Graph layout was performed by using the BIOLAYOUT program (19).

The net effect of the edge comparison and combination method is that surfaces are considered similar if the side chains are of similar type and the relative position and orientation of the side-chain Ca-Cβ bonds are the same. This measure is not sensitive to the positional variability of disordered surface residues, but results in detection of corresponding regions of space that present similar spatial distributions of properties. This choice of effective resolution of surface descriptions appears to be very suitable for this application.

Affinity Grids. Affinity grids provide a visual method for analyzing the energetic properties of a protein-binding site. To calculate the affinity grid, the protein is embedded within a 3D grid and then a probe atom is placed successively at each grid point. The interaction energy between the probe and the protein is evaluated and recorded (20, 21). The result is a 3D volume that then a probe atom is placed successively at each grid point. The interaction energy between the probe and the protein is evaluated and recorded (20, 21). The result is a 3D volume that the energetic properties of a protein-binding site. To calculate the affinity grid, the protein is embedded within a 3D grid and then a probe atom is placed successively at each grid point. The interaction energy between the probe and the protein is evaluated and recorded (20, 21). The result is a 3D volume that. By contouring the edges that were similar in both surfaces, which greatly reduces the size of the problem. Graph layout was performed by using the BIOLAYOUT program (19).

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Affinity maps were calculated for three atom types: carbon, oxygen/nitrogen, and hydrogen. The protein structures included polar hydrogen atoms and united-atom charges (22), and grids were then calculated in AUTODOCK (23) by using a grid spacing of 0.375 Å. The interaction energy includes a 6/12 dispersion/repulsion term, a volume-based parameterization of carbon desolvation, a 10/12 directional hydrogen-bonding term, and a screened Coulombic electrostatic term. The oxygen/nitrogen probe was able to accept a hydrogen bond from the protein, and the hydrogen probe was able to donate a hydrogen bond. The maps were contoured at a consistent level of ~0.4 kcal/mol.

Results

Sequence Structure Analysis. As shown previously (1, 4, 12), sequences of CNB-containing domains found in many organisms are well aligned. Structural analyses confirmed that secondary structure assignments are also very similar to what is seen in Fig. 1b. Despite these similarities, however, the structure-based sequence alignments show important differences.

The sequences and structures of the A and B domains of the representative RIβ align well from residues 165 to 250 in the A domain and residues 287 to 379 in the B domain (Fig. 3a). The discontinuity in the structural alignment occurs in middle of the B helix. This causes the C helices of the two domains to not superimpose.

The alignments between RIβ(A) and RIα(A) result in two segments (Fig. 3b). The first segment involves residues 146–230 in RIα(A) and residues 163–251 in RIβ(B). The second segment includes residues 235–257 in RIα(A) and 256–278 in RIβ(B). There is a five-residue hinge region between the two segments that results in a large misalignment of the C helix region of the A domains in these two structures. In contrast, the structures of RIβ(B) and RIα(B) align well until the end of the C helices (Fig. 3c).

The sequence and structure comparisons of the RIβ(B) and the CNB domains of HCN and CAP show good alignment until the middle of the B helix. The C helices do not superimpose at
Domains of both RI
the hydrogen map have no features in the vicinity of the adenine
phosphate (Fig. 5). The second set of residues occurs between the
amino acids that form hydrogen bonds to the cAMP sugar phos-
resides in the region of the PBC of each structure and includes
residues in the molecules studied. One set of conserved residues
RI
conservation of the corresponding C
are shown as the graph vertices. The lines between them illustrate
present as a pair of graphs; the residues that form similar patterns
consences between the residues. RII(B) was compared with RII(A),
Rlα(A), Rlα(B), CAP, and HCN. There are 11 highly conserved
residues in the molecules studied. One set of conserved residues
resides in the region of the PBC of each structure and includes
amino acids that form hydrogen bonds to the cAMP sugar phos-
phate (Fig. 5). The second set of residues occurs between the
β2 and β3 regions. A detailed description of the role of these conserved
residues is given in a later section (cAMP Interactions).

Affinity Grids. In the sugar and cyclic phosphate portion of cAMP,
the affinity grids generated with an oxygen probe show consistent
features that cover the phosphate oxygen atoms and the
sugar O2' atom (Fig. 6 and Fig. 8, which is published as
supporting information on the PNAS web site). These features
indicate the presence of hydrogen-bond donors that are appro-
priately placed to interact with the ligand. The carbon contour
tightly encloses the entire sugar and phosphate, showing that the
active site surrounds this portion of the molecule.

The contours associated with the adenine ring are far more variable in these affinity grids, indicating that there is not a consistent mode of recognition. The oxygen/nitrogen map and the hydrogen map have no features in the vicinity of the adenine ring, indicating that hydrogen bonding is not used in binding or recognition. The carbon map is different for the A and B domains of both Rlα and RIIβ. In RIIβ(A) and Rlα(A) there are contours around the five member rings of the adenines indicating hydrophobic interactions around that region. In Rlα(B) and RIIβ(B), there is a strong, form-fitting contour indicating that the adenine ring is sandwiched between hydrophobic residues. The contours in CAP are similar to what is seen in the B domains of the R-subunit structures. The carbon density in HCN is intermediate, with the adenine ring contacted by hydrophobic residues but not surrounded.

cAMP Interactions. In all of the systems studied, the sugar phosphate moiety of cAMP is involved in very similar hydrogen bonding and hydrophobic interactions with residues in the conserved set found by using the surface matching method (Fig. 5).

As an example in RIIβ(A), O2' is hydrogen-bonded to
Glu-221 and the O1P is hydrogen-bonded to Arg-230. There are also nonspecific hydrogen bonds to Gly-220. These hydrogen bonds are conserved in all six domains of the structures studied here. Two nonsurface-matched residues, Ala-223 and Ala-231, also form nonspecific hydrogen bonds with cAMP. In addition to these hydrogen bonds, surface-matched conserved residues Leu-222, Ala-232, Gly-183, Ile-180, and Val-179 form hydrophobic interactions. The carbons of Gln-182 are involved in hydrophobic interactions with Ala-232 and the polar atoms face solvent. Tyr-190 interacts with the conserved Ile-180. As a group, these hydrophobic residues serve to shield the specific hydrogen bonds by forming a protective hydrophobic cap.

In some of the structures, the adenine forms hydrogen bonds with protein residues and in others it does not; there is no consistent pattern. The affinity grid method showed that the adenine ring is always involved in hydrophobic interactions as has been observed previously in analyses of structures containing adenine cofactors or ligands (24). Analysis of the structures allowed us to identify the nature of the interactions.

The adenine ring of cAMP in RIIβ(A) forms a hydrophobic interaction with the carbon chain of Arg-381, which is in the B helix in the B domain. Ile-199 in the A domain is on the other side of the adenine forming a sandwich. The adenine of cAMP in RIIβ(B) forms a similar hydrophobic sandwich with Tyr-397 in the C helix and Ile-321, which are both in the B domain. The adenine of cAMP in Rlα(A) interacts with Trp-260, which is at the interface between Rlα(B) and Rlα(A), and Val-182 in Rlα(A). In Rlα(B), the adenine interacts with Tyr-371 in the C

![Fig. 4. Examples of surface match graphs RIIβ(B) and CAP. The PBC is shown in red.](image)

![Fig. 5. Molecular graphics showing the cAMP interactions. Conserved residues from surface matching are shown by using chemical colors. The domain in which the cAMP resides is cyan. The other domain is white. The PBC and the residue forming the hydrophobic interaction is in red. Conserved specific hydrogen bonds are in yellow. (a) RIIβ(A). The residue numbering for the conserved residues is shown. The B domain is white. The A domain is cyan. (b) RIIβ(B). (c) Rlα(A). The B domain is white. (d) Rlα(B). (e) CAP. (f) HCN. This image was created by using CHIMERA (16).](image)
helix and Val-300, both in the B domain. In CAP, the hydrophobic sandwich is formed by Arg-123 and Val-49; in HCN, it is formed by Val-564 and Arg-632.

The hydrophobic sandwiches occur in the R subunits of PKA, the N-terminal domain of CAP, and the C-terminal domain of HCN. However, there is a subtle difference in the nature of the interactions of the cAMPs that reside in the A domain of the R subunits and those that reside in the B domain. In the two examples here, the adenines of the cAMPs bound to the B domains interact with hydrophobic residues found in the C helices of the same domain. On the other hand, the adenines that are part of the cAMPs that reside in the A domains interact with hydrophobic residues that are part of the B domain. Furthermore, the residues that are involved in these interactions are in different parts of the B domain.

Discussion

We analyze here a ubiquitous signaling module that senses cAMP and then translates that message into a biological response. Although the module has been recognized for some time, its architecture has not been analyzed in depth nor has there been a systematic consideration of precisely how cAMP binding is communicated to other proteins or domains. The elements of this module, shown in Fig. 7a, consist of the PBC, a second layer of hydrophobic residues, and a hydrophobic lid to the adenine binding pocket.

In all cases, the sugar phosphate of the cAMP is bound to a highly conserved PBC between β strands 6 and 7 (Fig. 1b and c) where glutamic acid and arginine residues form specific hydrogen bonds with the O2’ of the sugar and the O1P of the phosphate (1, 12). These hydrogen bonds anchor the cAMP in a hydrophobic environment.

Our study has identified two further sets of conserved residues. One is a region containing six conserved hydrophobic residues between β2 and β4 that shield the hydrogen bonds from solvent and the cyclic phosphate group from phosphodiesterases. One conserved residue in this region closely contacts the adenine. This protective layer was noted by Diller et al. (2) in RIA and RIIB. Our surface matching analysis finds it in all other cAMP-binding sites presently in the PDB. The other conserved feature consists of a single hydrophobic residue that contacts on the other side of the adenine. This particular residue cannot be predicted by sequence alignment (Fig. 7b), but is spatially conserved. This residue seals a hydrophobic pocket in which the adenine ring sits. The affinity grids demonstrate that this pocket is a satisfactory position for an aromatic amine in the nonionized state. This feature has been observed in other proteins bound to adenine-containing ligands (24). Although the adenine does form hydrogen bonds in some of the structures studied here, there is no consistent or conserved pattern.

In the B domains of each of the PKA R-subunit structures, as well as the CNB domains of CAP and HCN, the adenine of cAMP stacks in exactly the same way with a hydrophobic residue in the C helix, anchoring this helix. The interaction of the adenine in the A domain of each of the PKA R subunits is different in that these adenosines stack with a hydrophobic residue in the B domain. Trp-260 [R1α(A) A helix] and the hydrocarbon portion of Arg-381 [RIIB(A) B helix], respectively, provide the hydrophobic lids.

All of the proteins in this study regulate physiological functions by changing protein–protein interactions in response to cAMP. A change in binding affinity in response to binding a second ligand is classical allostery (25–27). The putative allosteric sensing module is shown in Fig. 7c as it exists in the R1α(A) CNB domain. The common feature of this module is the presence of the hydrophobic residue positioned to interact with...
the adenine. Removal of cAMP eliminates interaction between the hydrophobic residue and the adenine. Releasing the helix can thus initiate a cascade of structural changes modifying interactions among domains and proteins. This model is consistent with the following previous experimental observations and proposals.

In the case of CAP, Passner and colleagues (12, 28) noted that cAMP helps orient the C helix. The absence of cAMP would change the relationship of the C helices in the dimer interface, which would then change the orientation of the helices involved in the DNA interaction. Our studies pinpoint the precise residues required for the adenine–C helix interaction.

For HCN (7, 12), Arg-591 in the C helix interacts with the adenine of cAMP. It was proposed that binding cAMP changes the conformation of the C helix. This change propagates through the A and B helices to the C-linker region to change the interface with the other subunits. When this occurs, the channel opens. Our studies shed light on the role and interrelationships of the residues that are identified as being important for this transition.

Tyr-371 is the key hydrophobic residue in the C helix that serves as the lid for the adenine ring in the RII(B) CNB domain. This interaction is important for communication between the A and B domains. In our model, the absence of cAMP in the B domain would allow the C helix to flip out, altering interactions with the A domain. RII(B) would be similar. The recent crystal structure of RII(C) with a nucleotide bound in the B domain (9) shows that the C helix in the B domain is extended in the absence of cAMP exactly as our model suggests.

The effects of the absence of cAMP in the A domains of the PKA R subunits are more complex because the adenine lid residue comes from the B domain rather than the A domain. The positioning of these residues is directly determined by the relative position and orientation of the A-domain C helix. Further, the C helix of the A domains is kinked. Release of the A-domain cAMP allows the C helix of the A domain to straighten and possibly reposition, which would cause a dramatic rearrangement of the relative positions of the A and B domains.

Each cAMP binding domain studied has at least two binding partners, cAMP and another protein or domain. In the case of the PKA R subunits, the protein partners include the C subunit of PKA and the other domain of the R subunit. CAP binds another subunit of CAP and the dimer binds DNA. The HCN channel functions as a tetramer and the cyclic nucleotide determines the relative orientation of the four subunits, which in turn determines the open or closed state of the channel.

The conformational mechanism involves a two-state situation with different affinities for the ligands, as originally described by Monod, Wyman, and Changeux (25). In this model, the presence of C subunits would reduce the binding affinity for cAMP by stabilizing a conformation of the R subunits in which the adenine ligand is not closed; binding of cAMP would reduce the affinity of R for C by repositioning the C helix so that the lid is closed. Similar considerations apply to CAP and the HCN channel. This speculation is consistent with the observations in PKA that the binding of R and C subunits is linked to cAMP and substrate binding as expected (29, 30). The structure of cAMP-free RIIα shows that the C helix of the B domain straightens out (9). Finally, a truncated construct of RIIα, in which the entire B domain from Trp-260 onward is deleted, is capable of inhibiting the C subunit and the inhibition is released by addition of cAMP. This apparently contradictory result (because Trp-260 is the adenine lid for the A domain) is explained by a photoaffinity labeling experiment in which it was demonstrated that 8-azidoadenosine 3′,5′-monophosphate labels Trp-260 and Tyr-371 in full-length RIIα, but labels Tyr-244 in the truncated RIIα (29).

Examination of Fig. 5 shows that the truncated form should be able to assume a conformation very similar to that of the RII(B) domain, with Tyr-244 assuming the role of the adenine lid. This reorganization would convert the A domain into the more conventional form assumed by the B domains.

Structural studies on cAMP-free EPAC2 (8) and the RIIβ subunit of PKA (2) identified a “greasy hinge” at the point where the B helix contacts the PBC. Conserved hydrophobic residues in the B domain interact with conserved hydrophobic residues in the B helix. In the case of RIIα, this is the site where the C subunit docks (31). This region is also the last point at which structural alignments are strongly conserved as determined by the SAT structural alignment algorithm (15). Thus, it is likely that the hydrophobic hinge facilitates repositioning of the C helix.

We propose that the cAMP-binding regulatory proteins function by providing a system with at least two stable states in which the presence or absence of cAMP produces a substantial structural change through the loss of hydrophobic interactions with the adenine ring and consequent repositioning of the C helix. This allosteric pathway was suggested previously for communication between the A and B domains of the R subunit of PKA (9), but it appears from the other examples studied here that the mechanism applies to all known classes of cAMP-binding proteins. The modified positioning of the helix is recognized by a protein-binding event, completing the allostery. The story of these regulatory proteins is not fully told until the binding partners are all identified and the specificity determinants of the protein–protein interactions are found.

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