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Articles

Predicted Structures of cAMP Binding Domains of Type I and II Regulatory Subunits of cAMP-Dependent Protein Kinase†

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ABSTRACT: The mammalian cAMP-dependent protein kinases have regulatory (R) subunits that show substantial homology in amino acid sequence with the catabolite gene activator protein (CAP), a cAMP-dependent gene regulatory protein from *Escherichia coli*. Each R subunit has two in-tandem cAMP binding domains, and the structure of each of these domains has been modeled by analogy with the crystal structure of CAP. Both the type I and II regulatory subunits have been considered, so that four cAMP binding domains have been modeled. The binding of cAMP in general is analogous in all the structures and has been correlated with previous results based on photolabeling and binding of cAMP analogues. The model predicts that the first cAMP binding domain correlates with the previously defined fast dissociation site, which preferentially binds N^6-substituted analogues of cAMP. The second domain corresponds to the slow dissociation site, which has a preference for C8-substituted analogues. The model also is consistent with cAMP binding in the syn conformation in both sites. Finally, this model has targeted specific regions that are likely to be involved in interdomain contacts. This includes contacts between the two cAMP binding domains as well as contacts with the amino-terminal region of the R subunit and with the catalytic subunit.

Cyclic AMP plays an important regulatory role in both prokaryotic and eukaryotic cells. The *Escherichia coli* catabolite gene activator protein (CAP) senses the level of cAMP and regulates transcription from several operons in the presence of cAMP (Zubay et al., 1970; Anderson et al., 1972). CAP binds to specific DNA sequences in the presence of cAMP and regulates transcription of several operons including lactose, galactose, and ara C [for review, see deCrombrugghe et al. (1984) and deCrombrugghe and Pastan (1978)]. The crystal structure of the CAP dimer with two bound molecules of cAMP has been determined (McKay & Steitz, 1981; McKay et al., 1982), and the amino acid sequence has been deduced from the DNA sequence of the gene (Aiba et al., 1982; Cossart & Gicquel-Sanzey, 1982). Each subunit of CAP folds into two domains; the larger amino-terminal domain binds cAMP between a long α-helix and a β-roll structure while the smaller carboxy-terminal domain forms the DNA binding site (Weber & Steitz, 1984; Steitz & Weber, 1985). The two subunits in the dimer have identical amino acid sequences but different orientations of the DNA binding domain with respect to the cAMP binding domain.

The major receptor for cAMP in eukaryotic cells is the regulatory subunit of cAMP-dependent protein kinase (EC 2.7.1.37) (Walsh et al., 1968). In the absence of cAMP, the kinase exists as an inactive tetramer containing two regulatory (R) and two catalytic (C) subunits. cAMP binds with high affinity to the regulatory subunit that promotes dissociation

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of the complex into a dimer of regulatory subunits and two monomeric catalytic subunits. The dissociated catalytic subunit is active as an ATP-protein phosphotransferase that phosphorylates a large number of target proteins on either serine or threonine residues. The regulatory effects of these phosphorylations have been well documented in many systems [for reviews, see Krebs and Beavo (1979) and Flockhart and Corbin (1982)].

There are two classes of cAMP-dependent protein kinases that by convention are designated type I and II (Corbin et al., 1975). These holoenzymes are distinguished primarily on the basis of their regulatory subunits (Hofmann et al., 1975; Rosen & Erlichman, 1975; Zoller et al., 1979), which also are classified as type I and II. The amino acid sequences have been determined for RII from bovine heart (Takio et al., 1984a) and for RI from bovine muscle (Titani et al., 1984). From the initial sequence of the type II regulatory subunit, it was clear that (1) the molecule contained two in-tandem gene duplicated sequences in the carboxy-terminal region, which was consistent with two cAMP binding sites, and (2) the two homologous regions of RII were also homologous to the sequence of the cAMP binding domain of CAP (Weber et al., 1982). When the crystal structure of CAP was examined, it was seen that the amino acids that formed the cAMP binding site in CAP were highly conserved in the RII sequence. One method of predicting the structure of a protein is to use the known structure of a homologous and functionally related protein as a model; some examples are spore coat protein (Wistow et al., 1985) and renin (Blundell et al., 1983). By use of the crystal structure of CAP as a framework, the amino acid sequences of RI and RII have been built into the three-dimensional structure of CAP in order to model the two cAMP binding domains. The validity of this model is confirmed by photoaffinity labeling studies, by correlation with the binding of cAMP analogues, and by correlation with fluorescence properties.

Experimental Procedures

The sequences of RI and RII were aligned with the CAP sequence as given in Weber et al. (1982). The starting coordinates were taken from the CAP dimer structure that had been refined to 2.5-Å resolution (I. T. Weber and T. A. Steitz, unpublished results). The amino acids of the regulatory subunits were substituted for those of CAP in the positions where the sequences differed. The positions of the amino acid side chains were examined with the PS300 computer graphics system using the program FRODO (Jones, 1978). Occasionally an adjustment in the position of the amino acid side chain was required in order to avoid collisions with neighboring atoms. The amino acids of RI and RII in the region corresponding to the "C" helix of CAP were fitted to an α-helix, and it was possible to model the insertions and deletions by adjusting the positions of the two adjacent residues. Both syn and anti conformations of cAMP were examined. The covalent connections and photolabeling results were used to approximately position the A and B domains of the regulatory subunits with respect to each other.

Results

The overall homology between CAP and the four R domains for the sequences aligned with CAP from α-helix A through helix B is 37.0% for RIA, 36.1% for RIB, 40.6% for RIIA, and 37.1% for RIIB when both identical and similar residues are counted. Figure 1 gives the alignment of the sequences of the four R domains with CAP. The alignment is identical with the one previously reported (Weber et al., 1982) with the exception of the insertions and deletions between CAP β-strands 4 and 5. The modeled R domains are expected to differ from the CAP structure by at least as much as was observed for the two cAMP binding domains in the CAP dimer that have a root mean square deviation of 0.76 Å for equivalent α-carbon atoms. Here we describe the alignment in sections starting at the amino terminus. The CAP residue numbering is used for simplicity; however, the residue number of the corresponding R sequences can be obtained readily from Figure 1. Figure 2 illustrates the CAP structure with the shaded area indicating the regions of highest homology with the R sequences. In addition to the overall homology of 36–41% there are several regions where the sequences are invariant. In other regions all five sequences differ. In some cases, sequences are conserved due to similar secondary or tertiary folding of the proteins whereas in other cases conservation is due to the constraints of binding cAMP.

The amino-terminal region, from residues 10 to 28 in CAP, shows little sequence homology. Even the R domains show few conserved residues for the stretch from amino acids 18 to 25. This may be because β1 is mostly exposed to the solvent. The sequences of the R domains were placed into the CAP structure for helix A and β1.

The next segment corresponds to residues 29–45 in CAP and is the first highly conserved region. Four residues, Glu-32, Gly-33, Ile-42, and Gly-45, are invariant in all five domains. The hydrophobic nature of the last four residues of β-strand 3 is also conserved in all five domains. However, although this region is highly conserved, only one amino acid, Ile-30, forms part of the cAMP binding pocket. Gln-32 may be invariant since its side chain forms a hydrogen bond to the carbonyl oxygen of Ala-83, which lies close to cAMP.

Glycines-33, -45, and -71 are invariant in all five sequences, and Gly-67 is conserved in four of the domains. These residues are notable in the CAP structure for having positive ϕ torsion angles—conformations that are not energetically favorable for amino acids other than glycine. Each of these glycines occurs at the end of a β-strand or in a bend between two β-strands. Gly-33 lies between β2 and -3, Gly-45 between β3 and -4, Gly-67 between β5 and -6, and Gly-71 at the end of β6. This suggests that these residues are conserved because of the structural similarities among the domains and that the glycines are required for correct formation of the β-roll. These glycine residues also are conserved in another protein that is homologous to CAP, the fnr gene product (Shaw et al., 1983). The conserved glycines separate six out of the eight β-strands forming the β-roll structure in CAP, which suggests that they may play an important role in forming an antiparallel β-roll structure that is conserved in CAP, the R domains, and the fnr protein.

Residues 46–66 in CAP form a region of lower homology with the regulatory subunits. Residues 49 and 50 are conserved in the five domains, and Val-49 forms part of the cAMP binding pocket in CAP. The pattern of hydrophobic residues in β4 is conserved. There is a tight turn between β-strands 4 and 5 (residues 54–55) that is the most variable region in this alignment since it contains both deletions and insertions in the other domains. This is most simply modeled by a variation in length of the loop between β4 and -5: RIA is four residues shorter than CAP, RIIA is the same length as in CAP, RIB is one amino acid longer, and RIIB is seven residues longer. The insertion in RIIB has been placed arbitrarily as a continuation of the β4–β5 loop, although it probably folds over another portion of the intact molecule. This loop lies on the surface of the domain so that these changes should not alter

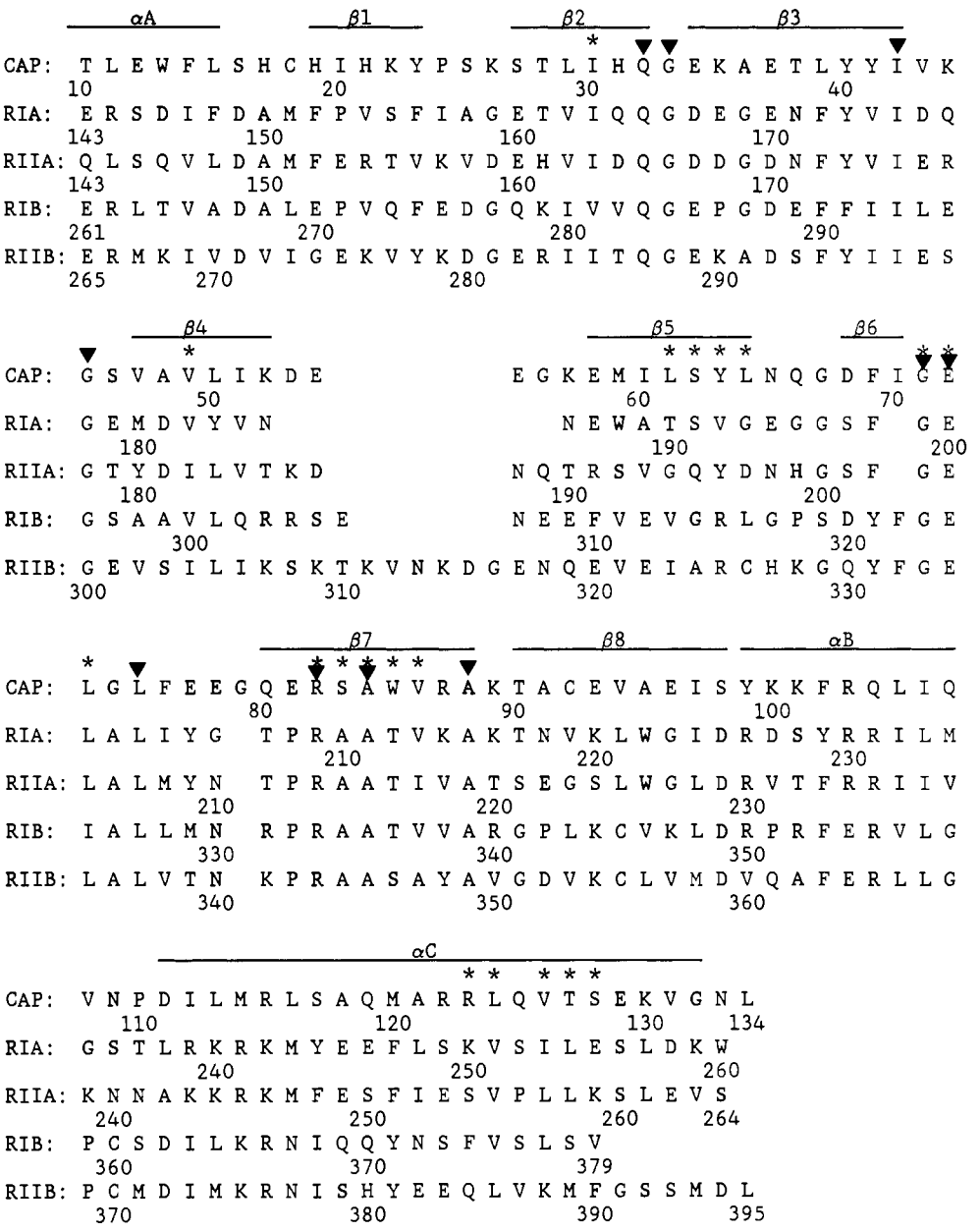

FIGURE 1: Amino acid sequence alignment of cAMP binding domains of CAP, RI, and RII. The A and B domains of RI and RII are continuous. The secondary structural elements of CAP are indicated by lines labeled α for α-helices and β for β-strands. The asterisks mark amino acids that in the CAP structure lie close to the cAMP molecule, and black triangles indicate residues that are identical in all five cAMP binding domains.

the internal folding. The residues corresponding to β5 show little homology among the five domains. There is not even internal homology between any pair of R domains. This is probably because β-5 is exposed to the solvent so that sequence conservation is not required.

The next segment, residues 67–88, shows high homology and contains several amino acids that directly participate in the binding of cAMP to CAP (Figure 3). The invariance of Gly-71 and its relation to secondary structure have already been addressed. The other invariant residues in this segment are Glu-72, Leu-73, Leu-75, Arg-82, Ala-84, and Ala-88. Five of these lie close to cAMP, and both Arg-82 and Glu-72 form direct interactions with cAMP in CAP. Despite the extent of homology in this region, there are two single amino acid deletions in the R domains relative to CAP. The two A domains have a deletion of amino acid 70 at the end of β6 in CAP, and all four R domains have a deletion of residue 79, which is at the start of β7. These deletions both occur at bends between strands of the β-roll structure and can easily be accommodated by small movements of adjacent residues.

The sequence alignment shows little homology for the region corresponding to β8 in CAP; however, the pattern of hydrophobic residues in β8 is conserved. Helix B in CAP shows residues that are conserved among all five domains from 102 to 106 (Figure 4). The pattern of hydrophobic and hydrophilic residues is also conserved, so that we expect the five structures to be homologous over this region of β8 and αB.

There is very little homology over the five domains for the residues 107 to the end of the C helix in CAP, even though the C helix has several direct interactions with cAMP in CAP. In this region RIA and RIIA show considerable homology as do RIB and RIIB. Residues 111–115 of CAP are similar to RIB and RIIB, which is some indication of a helix in the B domains of the regulatory subunits.

Discussion

A detailed comparison of the cAMP binding domain of CAP with the four cAMP binding domains of the type I and II

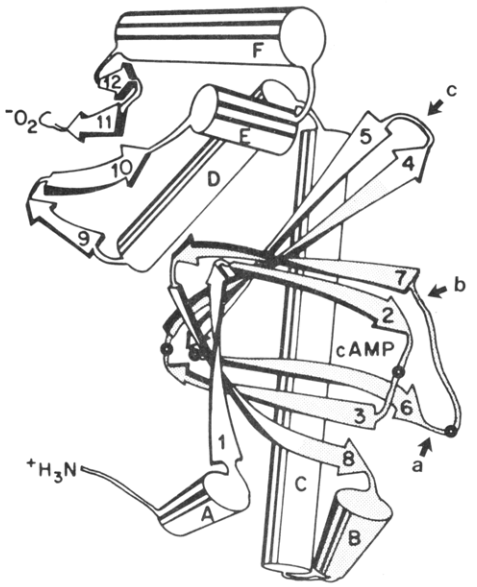

FIGURE 2: Schematic drawing of CAP subunit in which α-helices are indicated by cylinders and β-strands by numbered arrows. The larger amino-terminal domain binds cAMP and is connected to the DNA binding domain by a hinge region between helices C and D. The shaded region has a conserved amino acid sequence for CAP and the four R domains. The black dots mark the position of conserved glycines that have positive ϕ torsion angles in the CAP structure. The black arrows mark the position of insertions or deletions in R relative to CAP: (a) marks a single amino acid deletion of residue 79 in CAP that occurs in all four R subunits, (b) marks a single residue deletion of CAP residue 70 that occurs in the two A domains, and (c) marks the surface loop that varies in length from 4 residues shorter to 7 residues longer than in CAP.

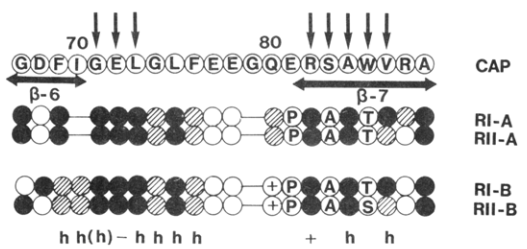

FIGURE 3: Homologies around the region from CAP residue 67 to CAP residue 88 where there are many interactions with cAMP. Identical residues are indicated by solid circles and conservative changes by hatched circles. Hydrophobic (h), basic (+), and acidic (−) residues also are indicated. The arrows mark residues that lie in the cAMP binding site of CAP.

regulatory subunits of cAMP-dependent protein kinase strongly indicates that the general features of both secondary and tertiary structure are conserved in all five domains (Figures 5 and 6). This conclusion is reinforced by correlating the highly conserved regions with functional sites and structural constraints imposed in CAP. The absolute conservation of certain glycine residues, for example, is striking in that each glycine is located at a restricted bend between β-strands. In addition, the few deletions and insertions all occur at bends and can be accommodated easily without significant alteration of the structure. On the basis of this overall homology, selected regions can be considered in more detail and correlated with other experimental evidence.

General structural features and functional sites associated with the R subunits and CAP are summarized in Figure 7. Each monomeric subunit consists of three regions. The amino-terminal third, which includes the dimer interaction site (Zick & Taylor, 1982; Potter & Taylor, 1979; Reimann, 1986), is followed by two in-tandem cAMP binding sites

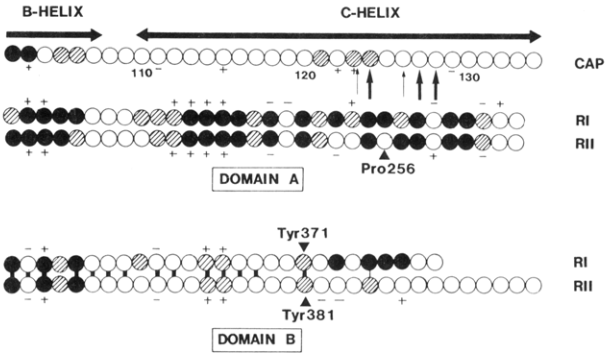

FIGURE 4: Homologies within the B and C helices relative to the A domains of the R subunits. Identical and conserved residues are designated by solid and cross-hatched circles, respectively. The triangles indicate the tyrosine residues that are modified by 8-azido-cAMP and the proline (Pro-256) that is expected to terminate the α-helix in domain A. Basic (+) and acidic (−) residues are also indicated. Residues that are identical in domain B of the two R subunits are linked by a solid bar. The thicker arrows indicate residues that interact with cAMP in CAP, whereas the thin arrows indicate residues that are in close proximity to cAMP in CAP.

(Takio et al., 1982). Limited proteolysis at a hinge region (Potter & Taylor, 1979; Corbin et al., 1978; Takio et al., 1980) cleaves the R subunit into a monomeric cAMP binding segment that in some cases retains the ability to form a complex with the C subunit (Weldon & Taylor, 1985). Mapping the cAMP binding sites with analogues of cAMP has established that (a) there is a slow and a fast dissociation site (Rannels & Corbin, 1981; Ogreid & Doskeland, 1980), (b) the two sites differ in their ability to bind various cAMP analogues with the slow dissociation site preferring C8-substituted analogues and the fast dissociation site having a general preference for N^6-substituted analogues (Corbin et al., 1982; Doskeland et al., 1983), and (c) there is a synergistic relationship between the two sites (Corbin et al., 1982; Robinson-Steiner & Corbin, 1983). Specific sites of interaction with cAMP have been identified by photolabeling with 8-N_3cAMP and are indicated in Figure 6. This information can now be considered more specifically in relation to the CAP-based structures proposed here for each cAMP binding domain.

Interdomain Contacts. The major interdomain contacts in the R subunit are (1) between the two cAMP binding domains, (2) between the cAMP binding domains and the amino-terminal region, (3) between the R and C subunits in the holoenzyme, and (4) between the two protomers in the dimer. Both photolabeling and proteolysis can provide a chemical basis for predicting and confirming these domain contacts. The major intersubunit contacts in CAP are associated with the two C helices that lie parallel and side by side in the CAP dimer (Steitz & Weber, 1985). Since the intersubunit contacts in R are well segregated from the cAMP binding domains (Figure 7), they are distinct from CAP and cannot be considered in the present model. On the other hand, both C helices in the R subunits are likely to participate in other interdomain contacts. The contacts between domains A and B will be considered first. The C helix in domain A terminates in the covalent linkage joining the two domains. The lack of sequence homology in the C helix of CAP relative to R (Figure 4) made it difficult to predict the exact structures; however, a C helix in domain A probably would terminate at residue 124 in CAP, since RIIA-256 is proline, which tends to break the α-helical structure. Although there is no Pro to terminate the C helix in RIA, the high degree of sequence homology between RI and RII in this region suggests that it, too, will have a shortened C helix ending at Ser-252.

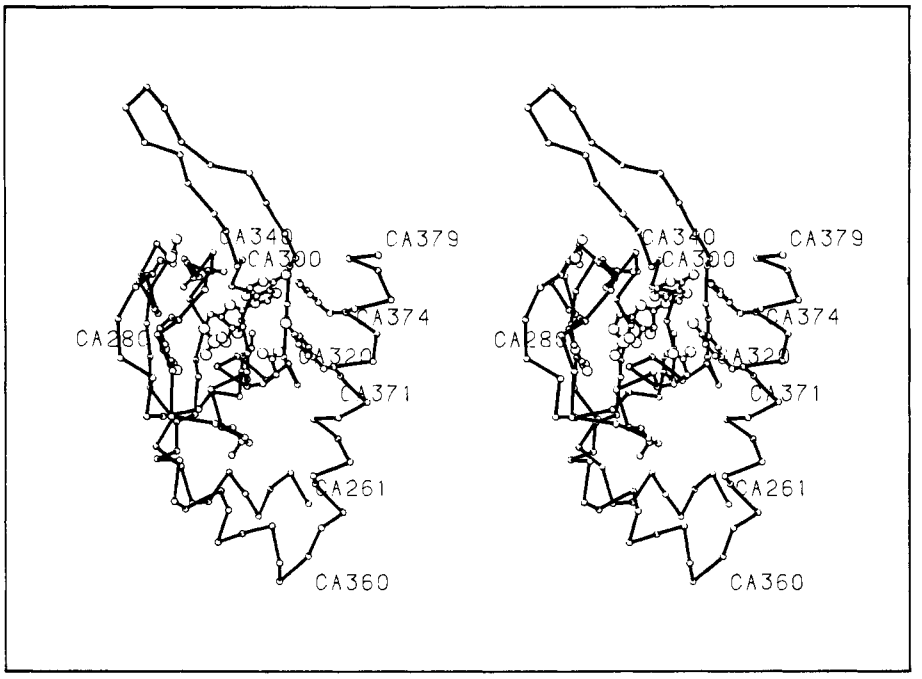

FIGURE 5: Stereo drawing of cAMP binding domain RIB with cAMP bound in the syn conformation. The α-carbon atoms of each residue are illustrated and the side chains of amino acids that are identical in CAP and the four R domains. The cAMP is shown with the nearby Tyr-371 and Phe-374 from the C helix.

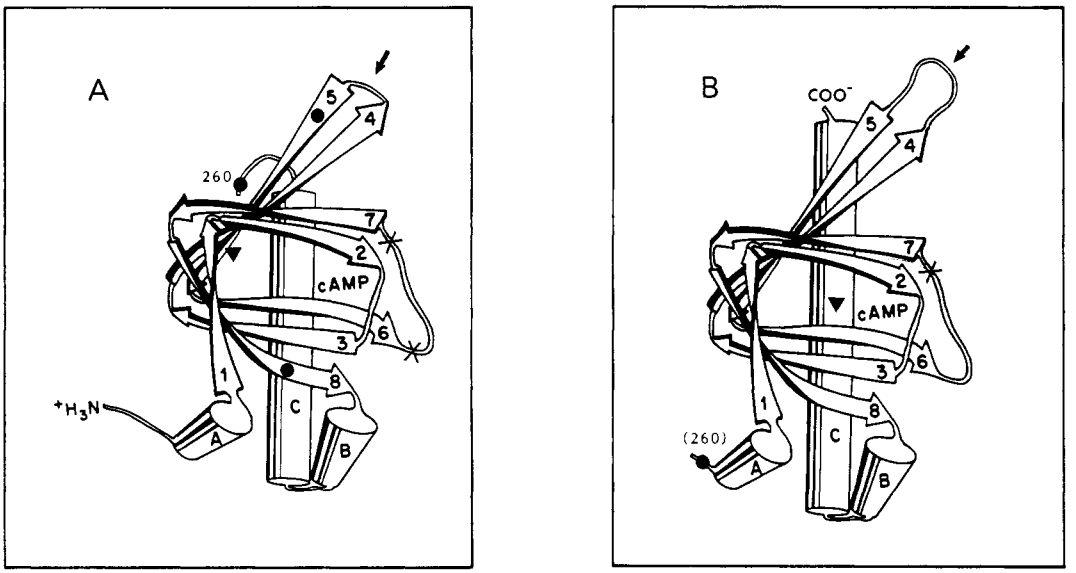

FIGURE 6: Structures predicted for the cAMP binding domains of RIA (panel A) and RIIB (panel B). The sites of single amino acid deletions relative to the CAP structure are indicated by crosses. The arrows mark a four-residue deletion in RIA and a seven-residue insertion in RIIB at the loop connecting β-strands 4 and 5. The helical segment corresponding to αC in CAP is shown as a shorter helix in RIA. The positions of tryptophan residues are indicated by black circles. Trp-260, which is photolabeled in RI, is located at the end of a segment of unknown structure that connects the C helix of domain A with the A helix of domain B and is indicated in both domains. The photolabeled Tyr-196 (panel A) and Tyr-381 (panel B) in RII are indicated by black triangles. The two cAMP binding sites are indicated. The cAMP in domain A is near the photolabeled Trp-260 in RI only and the Tyr-196 of RII that has the amino-terminal section removed by proteolysis. The cAMP in domain B lies near the photolabeled Tyr-371 in RI and Tyr-381 in RII.

Photoaffinity labeling of RI has provided chemical evidence for a specific contact point between the cAMP binding domains. 8-N_3cAMP bound to domain A leads to covalent modification of Trp-260, and 8-N_3cAMP bound to domain B covalently modifies Tyr-371 (Bubis & Taylor, 1986). Trp-260 is potentially important for specifying an interdomain contact, since it lies at the end of the segment which links the two domains (Figure 6), yet is labeled by cyclic nucleotide bound to domain A. The two domains can be aligned easily to make this contact, which also brings the C helix of domain A into close contact with the A helix of domain B. Such an alignment predicts that the two cAMP binding sites must lie at least 20 Å apart, although it is not easy to predict the precise orientation of the two domains.

The sequences of the C helices in the B domains are not homologous to those in the A domains or to CAP, although both the A and B domains are internally homologous (Figure 4). The C helix in domain B contains the photolabeled Tyr at a position corresponding to 120 in CAP. In the predicted structures, this Tyr points toward the cAMP, so that one side of this C helix interacts with cAMP and the other is on the surface of the domain. The high conservation of residues in RIB and RIIB in this region suggests that the C helix has an important function and is not merely exposed to solvent. This

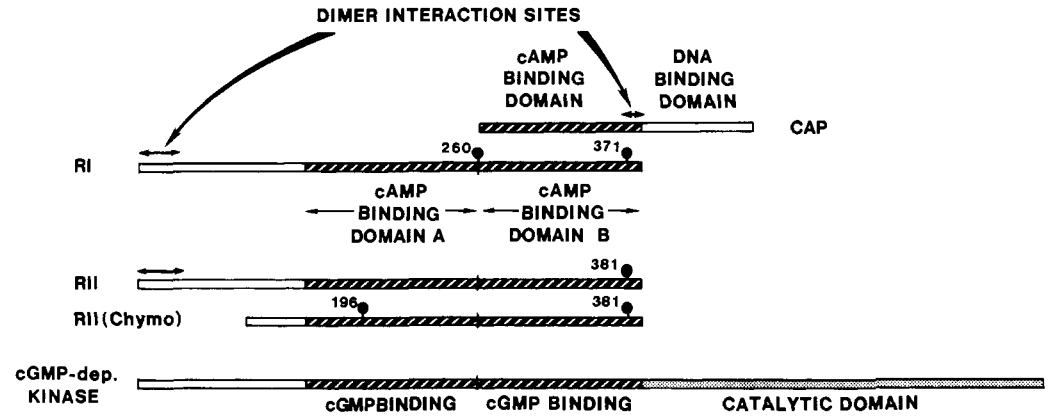

FIGURE 7: Summary of domain structures of CAP, R, and cGMP-dependent protein kinase. Each polypeptide chain is indicated as a linear structure from the amino- to the carboxy-terminal direction. Homologous cyclic nucleotide binding domains are indicated by cross-hatching; the catalytic domain is stippled. The dimer interaction sites in R and CAP are indicated by arrows. The proteolytic fragment of RII is designated RII(Chymo). Residues that are covalently modified with 8-N_3cAMP are numbered and marked with circles.

C helix most likely interacts with the catalytic subunit in the holoenzyme, which would be consistent with the domain structure of cGMP-dependent protein kinase (Takio et al., 1984b). Although the regulatory and catalytic segments of this enzyme are homologous to cAMP-dependent protein kinase, they are part of a single contiguous polypeptide chain with the catalytic region following immediately after the second cGMP binding domain (Figure 7). Thus, the C helix in domain B would be in close proximity to the amino-terminal segment of the C subunit. The surface loop between β-strands 4 and 5, which differs in all the R domains (Figure 1), lies close to the C helix and may participate in interdomain contacts as seen in CAP (McKay et al., 1982).

cAMP Binding Sites. The cAMP binding sites of the R domains and of CAP are illustrated in Figure 8. The interactions between the ribose–phosphate region of cAMP and the protein are similar although not identical for each structure. The ionic interaction between the equatorial phosphate oxygen and an arginine side chain (Arg-82) appears to be conserved. Hydrogen bonds also can form between the axial oxygen and the NH group and/or the Ser-OH of residue 83 in CAP and between the ribose 2′-OH and the side chain of Glu-72 (I. T. Weber and T. A. Steitz, unpublished results). Arg-82 and Ala-84 are invariant in all structures, whereas Ser-83 is consistently Ala in the R subunits. Residues 71–73 form a pocket around the ribose 2′-OH and also are conserved in R and CAP. Glu-72 interacts with Arg-123 from the C helix in CAP, which is not conserved in the R subunits, and it is difficult to predict which residue would complete this ion pair in the R subunit. The two regions of lower homology in the cAMP binding site are residues 61–64, which in CAP have interdomain hydrogen bonds, and the C helix that in CAP is also involved in intersubunit contacts.

Binding of cAMP analogues to CAP (Anderson et al., 1972; Scholubbers et al., 1984), RI (de Wit et al., 1984), and RII (Yagura & Miller, 1981) shows that the ribose O-3′, O-5′, and 2′-OH of cAMP provide important interactions and that a negative charge on the exocyclic phosphate oxygen is required. This is consistent with the crystal structure of CAP where the ribose O-3′ and O-5′ positions are deeply buried within the protein so that alterations of these atoms would be difficult. The model structures for the R domains have similar interactions.

In contrast to the conserved structure surrounding the ribose–phosphate moiety, mapping with cAMP analogues predicts that the specific protein interactions with the adenine ring differ for CAP and R. In the crystal structure, cAMP is bound to CAP in an anti conformation and forms hydrogen bonds between N^6 and Thr-127 and Ser-128 of the adjacent subunit.

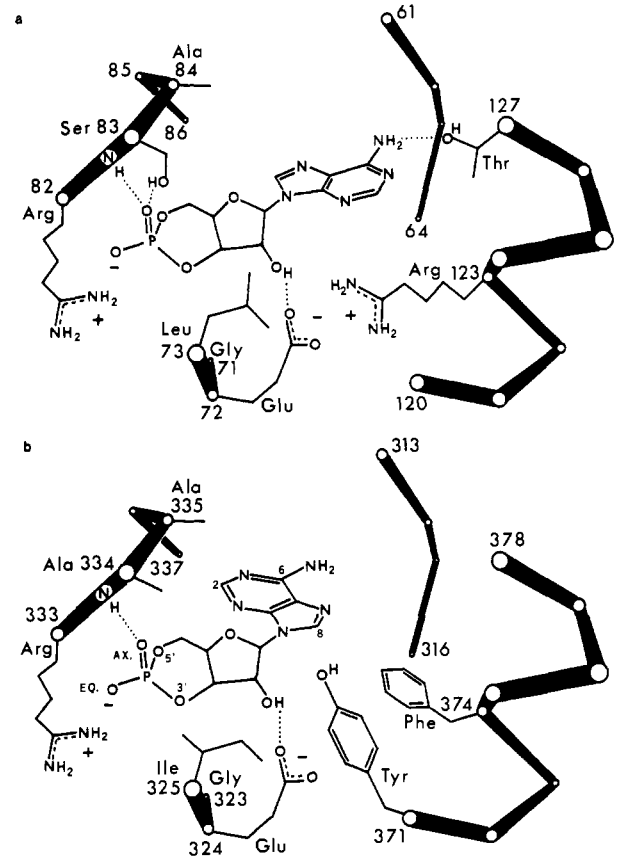

FIGURE 8: (a) Representation of cAMP and closest amino acids in one cAMP binding domain in the CAP structure. The residues forming the binding pocket are indicated by circles at the αC positions. The size of the circles shows the distance from the viewer. Side chains are shown for conserved residues or amino acids that interact with cAMP that is in the anti conformation. Charged residues are indicated by plus and minus signs and hydrogen bonds by dotted lines. Note that, in the CAP dimer, an additional hydrogen bond interaction that is not shown here occurs between N^6 of cAMP and Ser-128 from the adjacent subunit. Residues 120 to 127 are part of the C helix; residues 61–64 are in β5 and residues 82–86 in β7. Tyr-63 forms a hydrogen-bond interaction with amino acid 171 of the carboxy-terminal domain in one subunit of the CAP dimer. (b) Cyclic AMP and closest amino acids in the RIB domain in a representation similar to (a). The cAMP is in the syn conformation and may interact with the nearby Tyr-371 and Phe-374 of the C helix. The interactions of cAMP in the other R domains are similar except for interactions with the C helix.

If the cAMP is moved into the syn conformation, the hydroxyl of Ser-83 is too close to the adenine ring that lies between residues 83 and 84. The syn conformation is possible in the R domains since the homologous residue is the smaller alanine (Figure 8b). In contrast, analogue binding studies with R consistently have indicated that hydrogen bonding to the adenine ring does not contribute to binding (de Wit et al., 1984; Jastorff et al., 1979; Doskeland et al., 1983). The specific protein interactions differ for domains A and B and are discussed separately.

In domain B there are several residues on the C helix that potentially interact with the adenine ring of cAMP. These are Tyr-371 and Phe-374 in RI and the homologous Tyr-381 in RII (Figure 5). The model is consistent with photolabeling with 8-N_3cAMP, which leads to nearly stoichiometric modification of both tyrosines. The hydroxyl of Tyr-371 is 4.3–4.4 Å from the C8 position of the adenine ring whether the bound cAMP is in the syn or the anti conformation. It may be possible to form stacking interactions between the aromatic rings of Phe-374, Tyr-371, and cAMP, and dipole–dipole interactions may contribute to binding. In both syn and anti conformations of cAMP, the C2 and 6-amino group would be quite inaccessible. Since the opportunities for hydrogen bonding to N^6 are minimized in the syn conformation, this prediction is most consistent with analogue studies.

In domain A the role of the C helix with respect to cAMP interactions is less clear, although the helix is likely to be shorter as indicated earlier. The position corresponding to Tyr-371 in domain B (RI) is Phe in both A domains and is not photolabeled by 8-N_3cAMP. Instead, cAMP bound to domain A of RI causes the covalent modification of Trp-260. When the two in-tandem cAMP binding domains are covalently joined, it is relatively easy to rotate domain B so that Trp-260 is positioned in close proximity to the adenine ring of cAMP bound to domain A (Figure 6). The N^6 group of adenine would be exposed to solvent in either syn or anti conformation due to the shorter C helix, but the C8 and C2 positions would be more shielded in the syn conformation. Opportunities for hydrogen bonding to adenine are significantly less in the syn conformation, and photolabeling of Trp-260 is more favorable.

Domain A of RII must be considered separately from RI since the two proteins differ. Photolabeling of native RII with 8-N_3cAMP leads to the covalent modification of a single residue, Tyr-381 (Kerlavage & Taylor, 1980). The corresponding position in domain A, Phe-251, is not labeled nor is the residue corresponding to Trp-260 in RI, which is serine. Photolabeling of a proteolytic fragment of RII (residues 92–400) results in covalent modification of Tyr-196 (J. Bubis and S. S. Taylor, unpublished results). In CAP the homologous residue, Tyr-63, is directed away from cAMP and forms an interdomain hydrogen bond with Glu-171 of the DNA binding domain, while the adjacent residues, Ser-62 and Leu-64, lie close to the adenine ring of cAMP. Tyr-196 of RII can be rotated toward the cAMP if the adjacent Gln-195 and Asp-197 are rotated toward the solvent. Then Tyr-196 is close to the C8 of adenine when cAMP is in the syn conformation. This implies that cAMP tends to be in the syn conformation in the absence of the amino-terminal region of RII.

The predicted structures for domains A and B correlate well with studies of the binding of cAMP analogues and in particular with the ability of specific analogues to selectively displace bound cAMP. Several laboratories have shown that cAMP analogues that are substituted at the N^6 position preferentially displace cAMP from the fast dissociation site. cAMP bound to the slow dissociation site, also referred to as the "stable" cAMP binding site, is preferentially displaced by C8-substituted analogues. The model proposed here predicts that the C8 position would be more accessible in the B domain, whereas in the A domain the shorter C helix would result in a more exposed N^6 position. This would identify the B domain as the slow dissociation site and the A domain as the fast dissociation site. This also is consistent with the results of Bubis and Taylor (1986), who have correlated analogue specificity with photoaffinity labeling in RI. The enhanced stability of the slow dissociation site in domain B may be conferred in part by an interaction of the adenine ring of cAMP with Tyr-371 in RI or -381 in RII. Finally, the model is consistent with cAMP binding in the syn conformation, which provides a better basis for the observed photolabeling and also minimizes the potential for hydrogen bonding to the adenine ring.

The analogies between CAP and the R subunits of cAMP-dependent protein kinase provide a framework for the overall folding of the polypeptide chain and the structure of the cAMP binding sites. However, the detailed environment surrounding the bound cAMP in each structure has important differences. For example, the R subunits bind cAMP with a K_d in the range of 10^{-8}–10^{-9} M, whereas CAP has a much lower affinity for cAMP ($K_d \sim 10^{-5}$ M). The binding of cAMP analogues also differs for the two proteins. The most striking example of this is the specificity of these proteins for the chiral analogues (R_P)-cAMPγS and (S_P)-cAMPγS. In the R subunit, S_P is an agonist behaving similarly to cAMP while R_P is an antagonist that appears to lock the R subunit into a configuration that has high affinity for the C subunit, thus preventing activation of the holoenzyme (Bolen et al., 1980; Rothermel et al., 1983; de Wit et al., 1984; O'Brian et al., 1982). The effect of these analogues is reversed in CAP where S_P is an antagonist and R_P is an agonist (Scholubbers et al., 1984). It is not clear why the two analogues differ in their effects on CAP and the R subunits.

Fluorescence techniques also have been used to correlate structural changes with cAMP binding. The three tryptophan residues in RI (188, 222, and 260) are all in domain A (Figure 6A) and the single Trp-226 in RII is homologous to Trp-222 in RI. RI and RII show distinctly different fluorescence properties. RI shows significant quenching of fluorescence in conjunction with cAMP binding, while RII fluorescence is unaltered by cAMP binding. Since the single conserved Trp-222 is well segregated (16 Å) from the proposed cAMP binding sites, the quenching observed in RI would be predicted to relate to Trp-260, which is photolabeled by 8-N_3cAMP bound to domain A. The modeled cAMP binding sites also are consistent with the results of fluorescence measurements with N^6-etheno-cAMP (LaPorte et al., 1980). The B site of RI contains Tyr-371 near cAMP (Figure 8b), which would be expected to affect the fluorescence of N^6-etheno-cAMP. The biphasic quenching of N^6-etheno-cAMP bound to RII may be due to the presence of Tyr-196 near site A and Tyr-381 and possibly Cys-326 in cAMP binding site B.

Considerable attention has focused recently on the potential role that the regulatory subunit may play in the regulation of gene expression in eukaryotic cells. A number of genes, such as those coding for PEP carboxykinase (Loose et al., 1985) and plasminogen activator (Nagamine et al., 1983), are known to be regulated by cAMP, and a consensus sequence has been identified (Wynshaw-Boris et al., 1986) that is apparently responsible for the cAMP responsiveness of these genes.

Because of the homology of the R subunit with CAP, it has been tempting to speculate that the R subunit may play an analogous role. Although Nagamine and Reich (1985) speculated that the R subunit may be homologous to the DNA binding domain of CAP and cited a sequence in R that may correspond to the α-helices E and F in CAP that recognize specific DNA sequences, there is no compelling homology. The sequence that Nagamine and Reich cite includes residues 347–357 in the cAMP binding domain B of RII. This is a highly conserved region that in CAP forms part of β-strands 7 and 8, and this β-structure is almost certainly conserved in both the R subunits. If the R subunit does interact directly and specifically with DNA, which is not yet experimentally verified, there is no evidence that the interaction is structurally analogous to that of CAP.

The modeled structures described here for the cAMP binding domains A and B of RI and RII are consistent with experimental observations of photoaffinity labeling, the binding of analogues of cAMP, and fluorescence properties. The models, derived from the crystal structure of CAP, show the interactions of the proteins with cAMP and predict several interdomain contact regions. Obviously, it is essential to determine the crystal structure of R in order to obtain the precise interactions. This model, however, provides a valuable framework for the predicted interactions and can be tested directly by other experimental approaches such as site-directed mutagenesis.

Registry No. cAMP, 60-92-4; EC 2.7.1.37, 9026-43-1.

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Yeast Cytochrome *c* Peroxidase: Mutagenesis and Expression in *Escherichia coli* Show Tryptophan-51 Is Not the Radical Site in Compound I†

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ABSTRACT: Using oligonucleotide-directed site-specific mutagenesis, we have constructed a system for the mutation and expression of yeast cytochrome *c* peroxidase (CCP, EC 1.11.1.5) in *Escherichia coli* and applied it to test the hypothesis that Trp-51 is the locus of the free radical observed in compound I of CCP [Poulos, T. L., & Kraut, J. (1980) *J. Biol. Chem. 255*, 8199–8205]. The system was created by substituting a CCP gene modified by site-directed mutagenesis, CCP(MI), for the *fol* gene in a vector previously used for mutagenesis and overexpression of dihydrofolate reductase. *E. coli* transformed with the resulting plasmid produced the CCP(MI) enzyme in large quantities, more than 15 mg/L of cell culture, of which 10% is holo- and 90% is apo-CCP(MI). The apoenzyme was easily converted to holoenzyme by the addition of bovine hemin. Purified CCP(MI) has the same catalytic activity and spectra as bakers' yeast CCP. A mutation has been made in CCP(MI), Trp-51 to Phe. The Phe-51 mutant protein CCP(MI,F51) is fully active, and the electron paramagnetic resonance (EPR) spectrum, at 89 K, of its oxidized intermediate, compound I, displays a strong sharp resonance at $g = 2.004$, which is very similar to the signal observed for compound I of both bakers' yeast CCP and CCP(MI). However, UV–visible and EPR spectroscopy revealed that the half-life of CCP(MI,F51) compound I at 23 °C is only 1.4% of that observed for the compound I forms of CCP(MI) or bakers' yeast CCP. Thus, Trp-51 is not necessary for the formation of the free radical observed in compound I but appears to exert a significant influence on its stability.

Yeast cytochrome *c* peroxidase (ferrocytochrome *c*:H_2O_2 oxidoreductase, EC 1.11.1.5) is a monomeric 294-residue enzyme (Kaput et al., 1982; Takio et al., 1980) that resides in the mitochondrial intermembrane space and contains a single noncovalently bound ferric protoporphyrin IX as a prosthetic group. It catalyzes the two-electron reduction of hydroperoxides by cytochrome *c*:

$$2H^+ + ROOH + 2\text{cyt } c(Fe^{2+}) \rightarrow 2\text{cyt } c(Fe^{3+}) + ROH + H_2O$$

The study of CCP[1] and its macromolecular redox partner, cytochrome *c*, affords insight into the enzymic mechanism of O–O bond cleavage, as well as the nature of macromolecular recognition and long-distance interprotein electron transfer. It is easily isolated and crystallized and has many similarities to more complex enzyme systems such as the membrane-bound cytochrome oxidases (Malmström, 1982; Hatefi, 1985). CCP is well suited for investigations of the relationship between the three-dimensional structure and the enzymic function since a large body of information already exists concerning its physicochemical properties (Yonetani, 1976), including the amino acid sequence (Takio et al., 1980) and the high-resolution X-ray crystallographic structure (Poulos et al., 1978, 1980; Finzel et al., 1984) of CCP from bakers' yeast.

The development of oligodeoxynucleotide-directed site-specific mutagenesis [reviewed by Smith (1985)] has made possible the selective replacement of specific amino acid residues of proteins by manipulation of the nucleotide sequences of their genes. The isolation (Goltz et al., 1982) and nucleotide sequence determination (Kaput et al., 1982) of the CCP gene from a standard strain of *Saccharomyces cerevisiae* opened the door to structure–function studies of CCP using directed

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[1] Abbreviations: CCP, cytochrome *c* peroxidase; DHFR, dihydrofolate reductase; EPR, electron paramagnetic resonance; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; IPTG, isopropyl β-D-thiogalactopyranoside; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; ENDOR, electron nuclear double resonance; DEAE-cellulose, (diethylaminoethyl)cellulose; kb, kilobase(s); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; YT, 8 g of tryptone/5 g of yeast extract/5 g of NaCl per liter; LB, 10 g of tryptone/5 g of yeast extract/10 g of NaCl per liter.

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