Predicted Structures of the cGMP Binding Domains of the cGMP-Dependent Protein Kinase: A Key Alanine/Threonine Difference in Evolutionary Divergence of cAMP and cGMP Binding Sites†

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ABSTRACT: Mammalian cGMP- and cAMP-dependent protein kinases show considerable similarity in amino acid sequence, although they specifically bind different cyclic nucleotides. Results of cGMP analogue binding experiments, combined with modeling of the cGMP binding sites by analogy to the structure of the homologous catabolite gene activator protein, suggest that a threonine residue forms a hydrogen bond with the 2-NH_2 of cGMP. This threonine is invariant in all cGMP binding domains, but the corresponding residue in 23 out of 24 cAMP binding sites of protein kinases is alanine, which cannot form the same hydrogen bond. This alanine/threonine difference has the potential for discriminating between cAMP and cGMP and may be important in the evolutionary divergence of cyclic nucleotide binding sites.

The two prominent cyclic nucleotides in eucaryotic cells, cGMP and cAMP, are believed to be second messengers for different physiological functions. Although other receptors may exist, many effects of cGMP and cAMP are mediated by the cGMP- and cAMP-dependent protein kinases (cAPK and cGPK), which presumably recognize different cellular protein substrates (Beebe & Corbin, 1986). The cGPK is composed of two identical subunits of 76 331 daltons, each of which contains a regulatory and a catalytic component (Lincoln & Corbin, 1983), and binds two molecules of cGMP (Corbin & Doskeland, 1983; MacKenzie, 1982). The cAPK also binds two molecules of cyclic nucleotide on each of two regulatory subunits (Beebe & Corbin, 1986). For both cyclic nucleotide dependent protein kinases, the two sites differ in cyclic nucleotide dissociation rate and analogue specificity (Rannels & Corbin, 1980; Corbin et al., 1986).

Other than possible differences in conformation or electron distribution, there are two structural features that distinguish cAMP and cGMP. The cAMP molecule has an NH_2 group at the 6-position and a hydrogen at the 2-position of the purine ring, while cGMP has a keto group at the 6-position and an NH_2 group at the 2-position. The difference in relative specificity of cAMP and cGMP for the cAPK and cGPK approaches a factor of 100 (Lincoln & Corbin, 1983), despite the similarities in amino acid sequence of the two protein kinases. It might therefore be expected that differences in the cyclic nucleotide specificities of the respective kinases would be due to different amino acid contacts at the 2- or 6-position of the purine rings. Thus, a search has been carried out to find a structural difference that could discriminate between cAMP and cGMP binding.

Each of the cyclic nucleotide binding sites of cGPK and cAPK shares amino acid sequence homology with the cAMP binding domain of the bacterial catabolite gene activator protein (CAP) (Weber et al., 1982; Takio et al., 1984b). CAP senses the level of cAMP and regulates transcription from several operons in *Escherichia coli* (Zubay et al., 1970; Anderson et al., 1972), including lactose, galactose, and ara C [for reviews, see deCrombrugghe et al. (1984) and deCrombrugghe and Pastan (1978)]. The amino-terminal domain of CAP binds cAMP, while the smaller carboxy-terminal domain forms the DNA binding site (Weber & Steitz, 1984, 1987). The crystal structure of the CAP dimer with two bound molecules of cAMP has been determined (McKay & Steitz, 1981; McKay et al., 1982), which permits modeling of cyclic nucleotide binding sites, as was done previously for cAPK (Weber et al., 1987). The models of the cAMP binding sites have been useful in studies of the cAPK, such as for predictions of specific amino acid residues that could be modified by site-specific mutagenesis (Bubis et al., 1988). It is expected that the models of the cGMP binding sites of cGPK will also be useful for these purposes.

Experimental Procedures

The amino acid sequence of bovine cGPK was aligned with the sequences of CAP and the cAMP binding domains of bovine RI and RII, as shown in the structural alignment of Weber et al. (1987). In addition, amino acid sequences of 11 other R subunits and 1 other cGPK, that have recently been deduced by using molecular cloning techniques, have been aligned maintaining essentially the same gap locations. The starting coordinates for modeling the cGPK domains were taken from the RI model structure that had been built by using the CAP coordinates from the crystal structure refined to 2.5-Å resolution (Weber & Steitz, 1987). The RI model structure was taken as a starting point since this protein is more similar to cGPK than is RII in amino acid sequence and kinetic properties (Doskeland et al., 1987). The deletions and insertions relative to the CAP structure had previously been determined (Weber et al., 1987). The amino acid side chains of the cGMP binding domains of cGPK were substituted for those of RI 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, a small adjustment in the position

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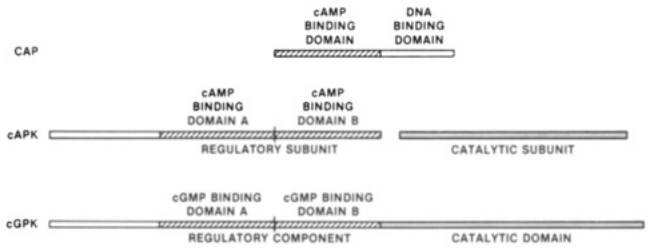

FIGURE 1: Summary of the domain structures of CAP, cAPK, and cGPK. Each polypeptide chain is indicated as a linear structure from the amino- to the carboxy-terminal direction. Homologous cyclic nucleotide binding domains are indicated by hatching; the catalytic portion is stippled.

of the amino acid side chain was required in order to avoid collisions with neighboring atoms. Cyclic GMP was substituted for cAMP, and both syn and anti conformations were examined in the two cGMP binding sites.

Results and Discussion

Conserved Domain Structure. General structural features of CAP, cAPK, and cGPK are summarized in Figure 1. All of the published amino acid sequences of the cyclic nucleotide binding domains of CAP, bovine cGPK, 13 different regulatory subunits (RI and RII), and *Drosophila* cGPK are compared in Figure 2. The alignment of these 30 cyclic nucleotide binding domains shows a pattern of conserved residues, which can be analyzed with respect to the structure of the cAMP binding domain of CAP. There are only four positions where there are insertions or deletions relative to the CAP sequence, and these lie between elements of secondary structure. A region of variable length is observed between β4 and β5, single amino acid deletions occur at the positions corresponding to CAP 70 in all A domains and at CAP 79 in both A and B domains. Single amino acids are inserted in the *Drosophila* cGPK A and B domains between CAP 88 and 89. There are conserved differences between the A and B domains. For example, Tyr-77 is invariant in all A domains, but no B domain contains Tyr at residue 77; residue 70 which is phenylalanine in the B domains is deleted in all A domains; and residue 95 is tryptophan in the A domains and valine or leucine in the B domains. The region corresponding to the helices αB and αC differs in the A and B domains.

Glycines-45 and -71 are invariant in all of the sequences, and Gly-33 is conserved in 29 out of 30 of the domains. These glycines also are conserved in two other bacterial proteins, the

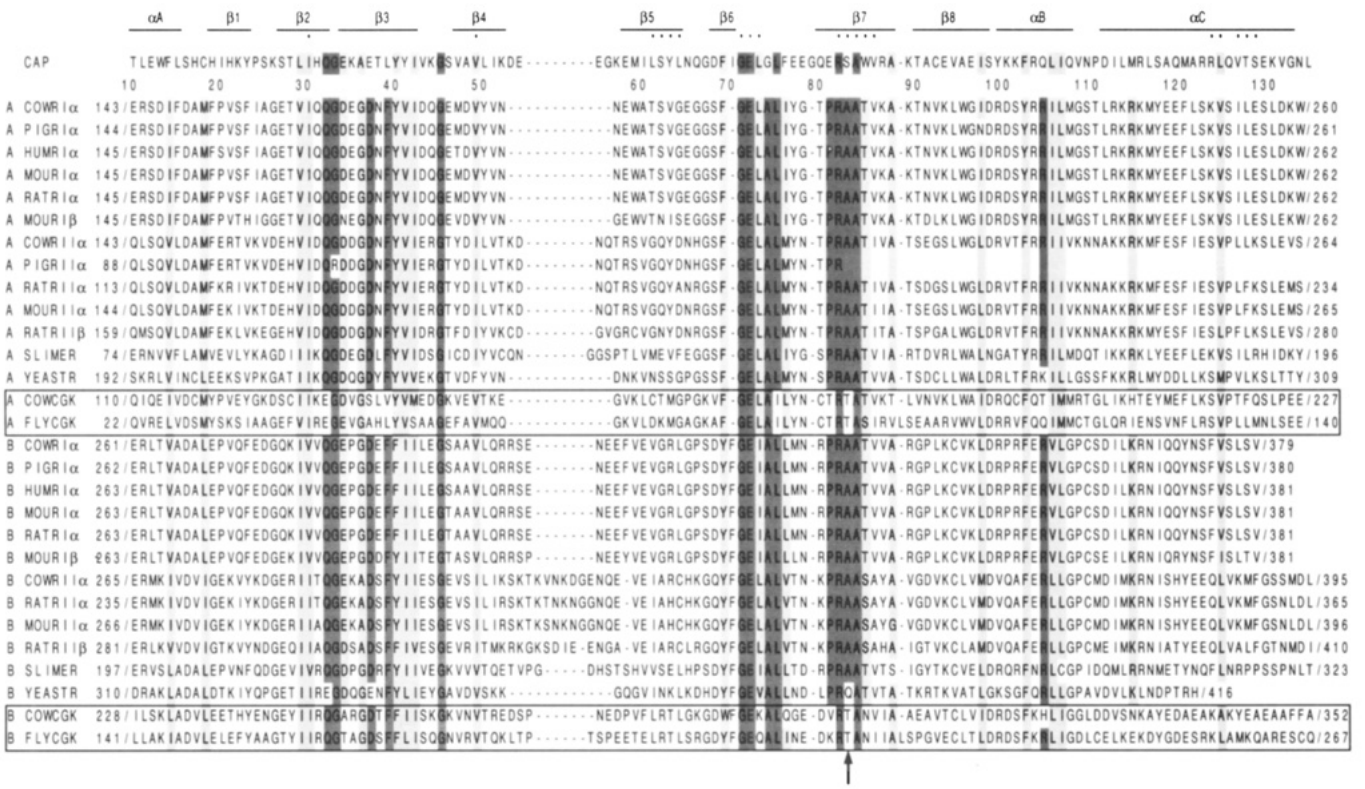

FIGURE 2: Amino acid sequence comparison of CAP, cAPK, and cGPK cyclic nucleotide binding domains. CAP is the first alignment represented and is used for orientation and numbering purposes. RI and RII represent different isozymes, and α and β are different subclasses of the isozymes. The A and B cyclic nucleotide binding domains of all R subunits and cGPKs are continuous. Secondary structural elements of CAP are indicated by lines labeled α for α helices and β for β strands. Asterisks mark amino acids that lie close to cAMP in the CAP structure. For ease of location, cGMP binding domains are boxed. The shaded columns (light and dark) represent amino acids that are conserved in at least 24 of 25 R-subunit cAMP binding domains. The darker shaded columns represent identical amino acids, and the lighter shaded columns represent similar amino acid functional groups. Functional group designations are as follows: proline (P), disulfide forming (C), histidine (H), acidic (D and E), uncharged polar (N and Q), basic (K and R), small aliphatic (A and G), hydroxyl (S and T), hydrophobic (I, L, M, and V), and aromatic (F, W, and Y). Amino acid sequences represented are CAP (Aiba et al., 1982; Cossart & Gicquel-Sanzey, 1982), COWRIα [bovine skeletal muscle RIα (Titani et al., 1984)], PIGRIα [porcine RIα from LLC-PK$_1$ cells (Nowak et al., 1987)], HUMRIα [human testis RIα (Sandberg et al., (1987)], MOURIα [mouse RIα (Clegg et al., 1988)], RATRIα [rat brain RIα (Kuno et al., 1987)], MOURIβ [mouse brain RIβ (Clegg et al., 1988)], COWRIIα [bovine heart RIIα (Takio et al., 1984a)], PIGRIIα [partial sequence of domain A of porcine RIIα from LLC-PK$_1$ cells and porcine skeletal muscle (Hemmings et al., 1986; Bubis & Taylor, 1987)], RATRIIα [rat skeletal muscle RIIα (Scott et al., 1987)], MOURIIα [mouse brain RIIα (Scott et al., 1987)], RATRIIβ [rat granulosa cell RIIβ (Jahnsen et al., 1986)], SLIMER [*Dictyostelium discoideum* R (Mutzel et al., 1987)], YEASTR [*Saccharomyces cerevisiae* R (Cannon & Tatchell, 1987; Kunisawa et al., 1987; Toda et al., 1987)], COWCGK [bovine lung cGPK (Takio et al., 1984)], and FLYCGK [*Drosphila melanogaster* cGPK (Foster et al., 1988)]. The cDNA for human testis RIIβ has recently been cloned and sequenced (Levy et al., 1988) but is not included in this figure. Two amino acids in domain A and six amino acids in domain B differ from rat RIIβ, but none of the changes occur at conserved positions.

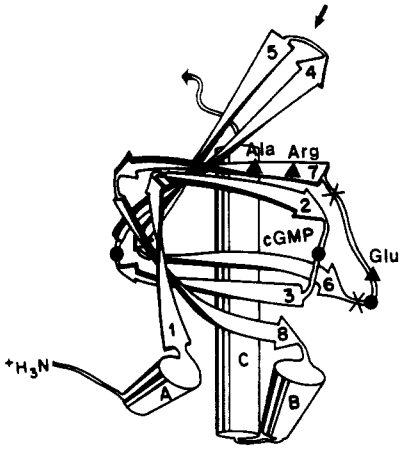

FIGURE 3: Schematic representation of the structure of the cGMP binding domain A of cGPK, modeled by analogy to the crystal structure of CAP with cAMP. Helices are shown as lettered cylinders and β strands as numbered arrows. cGMP is indicated in the cyclic nucleotide binding pocket. The two single amino acid deletions (CAP 70 and 79) are indicated by crosses. The β4-5 loop of variable length is indicated by an arrow. Black dots indicate the conserved glycines (33, 45, and 71 in CAP). The black triangles show the positions of the invariant residues, Glu-72, Arg-82, and Ala-84, in CAP numbering.

fnr gene product (Shaw et al., 1983) and the *fixK* gene product (Batut et al., 1989), that are homologous to CAP, although there is no evidence that they bind either cAMP or cGMP. In CAP, these glycines occur in turns between two β strands and may be required for correct formation of the antiparallel β-roll structure (Figure 3). The region corresponding to the C helix (residues 111–132) shows less similarity among the sequences of CAP, cGPK, and cAPK. Helix αC of the A domains must be followed by a linkage to the αA helix of the B domains in cGPK, RI, and RII (Figure 1). In the A domains of RII and cGPK, there is a proline at the position corresponding to CAP 125 which is expected to terminate the helix. It is not obvious where the corresponding αC helices terminate in the B domains; this is the carboxy terminus of the protein in the case of RI and RII and is connected to the catalytic domain in cGPK.

The predicted structure of the cGMP binding domain A of cGPK is illustrated in Figures 3 and 4. Conservation of tertiary interactions in the model structure is indicated by the identical glycines at turns between β strands, and by the presence of homologous aromatic residues in an internal hydrophobic pocket. In CAP, four aromatic residues, Phe-69, Phe-102, Tyr-41, and Phe-14, occupy a space between helices αA, αB, and αC. The same site is occupied by aromatic side chains both in modeled domains of cGPK and in cAPK. Positions 69 and 102 are conserved aromatic residues in all 30 cyclic nucleotide binding domains. This hydrophobic pocket in the A domain of cGPK is occupied by Phe-165, Phe-196, and Trp-189, while the B domain is occupied by Trp-288, Phe-320, and Phe-257. Trp-189 in cGPK is conserved in all A domains, and Phe-257 of cGPK is conserved in all the domains of cAPK and the two B domains of cGPK. This group of conserved aromatic side chains indicates that the three helices, αA, αB, and αC, are packed together in the same manner in all of these cyclic nucleotide binding domains. Therefore, structural modeling combined with sequence analysis suggests that the overall folding of all the cyclic nucleotide binding domains is conserved; however, no differences in the overall structure are indicated which could discriminate between cAMP and cGMP binding.

Cyclic Nucleotide Binding Sites. Amino acids that are in close proximity to the bound cyclic nucleotide can also be examined for features that might explain the specificity of binding. Studies of the binding of cAMP analogues to CAP (Anderson et al., 1972; Scholubbers et al., 1984), RI (Doskeland et al., 1983; de Wit et al., 1984), and RII (Yagura & Miller, 1981; Corbin et al., 1982), and the binding of cGMP analogues to cGPK (Corbin et al., 1986), all agree that the ribose O3′, O5′, and 2′-OH of the cyclic nucleotide provide important interactions, and a negative charge on the exocyclic phosphate oxygen is required. The CAP crystal structure shows specific interactions with cAMP in agreement with the analogue binding studies (Figure 5A). The ribose and phosphate groups of the cyclic nucleotides are bound within

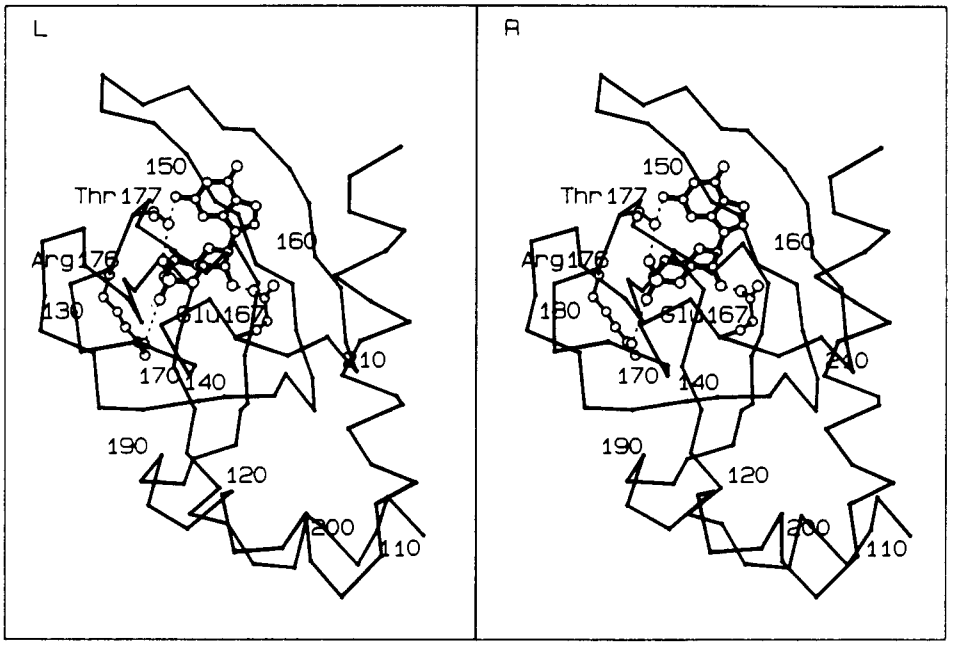

FIGURE 4: Stereoview of the model structure of the cGPK domain A. The Cα backbone of the domain is labeled every 10 residues from 110 to Pro-219 which is predicted to terminate the Cα helix. Cyclic GMP is shown in the syn conformation and the residues Glu-167, Arg-176, and Thr-177. Some of the interactions between these residues and cGMP are indicated by dashed lines. Figure 5B shows all of the possible interactions between cGMP and domain A of cGPK. The other amino acids forming the cGMP binding pocket have been omitted for clarity.

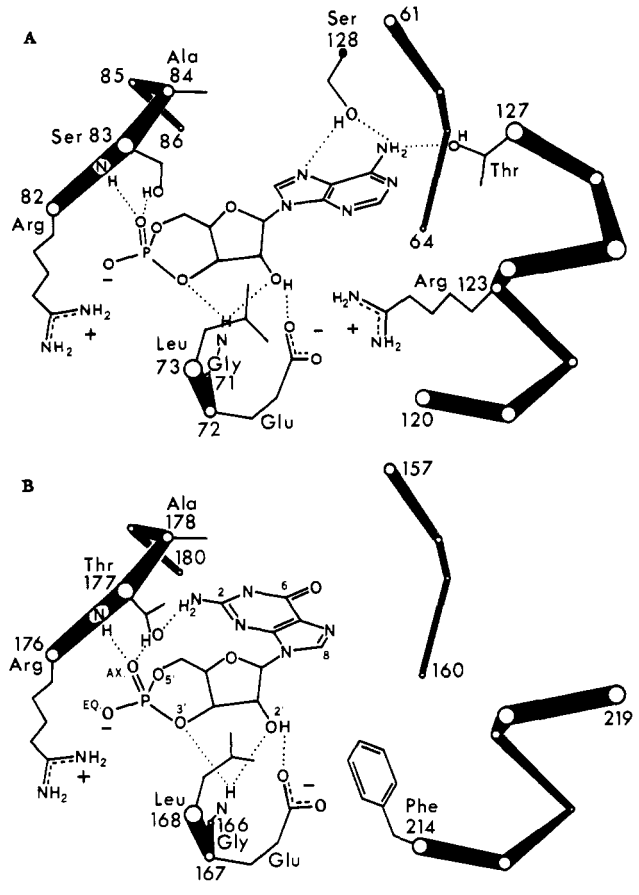

FIGURE 5: (A) Representation of cAMP and the closest amino acids in one cAMP binding domain in the crystal structure of the CAP dimer. The residues forming the binding pocket are indicated by circles at the αC positions. The size of the circles shows the distance from the viewer. Side chains are shown for conserved residues or amino acids that interact with cAMP which is in the anti conformation. Charged residues are indicated by plus and minus signs and hydrogen bonds by dotted lines. Ser-128 from the adjacent subunit in the CAP dimer is shown as a black dot. Residues 120–127 are part of the C helix; residues 61–64 are in β5 and 82–86 in β7. Gly-71, Glu-72, Arg-82, and Ala-84 are invariant in all the cyclic nucleotide binding domains surveyed in Figure 2. (B) Amino acid sequence of bovine cGPK was built into the crystal structure of CAP in order to model the two cGMP binding sites. Cyclic GMP and the closest amino acids in domain A of cGPK are shown in a representation similar to (A). The C helix is presumed to terminate at Pro-219. Cyclic GMP is shown in the syn conformation. The interactions of the ribose and phosphate moieties of the cyclic nucleotide with cGPK are similar to those seen in CAP. The interactions with the bases differ. There is no interaction observed at the 6-position, whereas the 2-NH_2 of cGMP is predicted to form a hydrogen bond with the Thr-177 OH (or the Thr-301 OH in the B domain).

the β-roll structure which shows the highest conservation of residues. Three invariant residues, Glu-72, Arg-82, and Ala-84, form part of the cAMP binding pocket in CAP, and the corresponding residues are expected to be important for cGMP binding to cGPK (Figure 5B). The ionic interaction between the equatorial phosphate oxygen and Arg-82 is conserved. Hydrogen bonds can form between the axial phosphate oxygen of the cyclic nucleotide and the NH and OH of Ser-83 in CAP, or of the corresponding Thr in cGPK (Figures 4 and 5B). A CAP variant with Ala-83, instead of Ser, still binds cAMP; however, the hydrogen-bond interaction of the 83 NH with the axial phosphate oxygen is still possible (Gronenborn et al., 1988). The ribose 2′-OH forms hydrogen bonds with the conserved Glu-72 and Gly-71 (Weber & Steitz, 1987). Mutations at the invariant residues corresponding to Gly-71, Glu-72, and Arg-82 in CAP appear to prevent cAMP binding to the altered site of cAPK (Ogreid et al., 1988; Bubis et al., 1988). CAP variants containing alterations at Thr-127 or Glu-72 as part of different double mutations also reduce the dependence of CAP on cAMP for activation (Harman et al., 1986; Garges & Adhya, 1985). A CAP mutation of Arg-82 to Leu does not bind cAMP (Gronenborn et al., 1988). These results indicate the importance of these invariant residues for binding of cyclic nucleotide. The two regions of lower homology in the cyclic nucleotide binding pockets are residues 61–64, which in CAP have interdomain hydrogen bonds, and the C helix, which in CAP is involved in intersubunit contacts.

The interactions of these proteins with the base moiety of the cyclic nucleotide are expected to differ significantly. Cyclic GMP differs from cAMP in two positions on the base, the 6-position is O instead of NH_2, and at the 2-position, NH_2 replaces a hydrogen. In the crystal structure, cAMP is bound to CAP in an anti conformation and forms hydrogen bonds between 6-NH_2 and Thr-127 of the same subunit and Ser-128 of the adjacent subunit in the dimer (Figure 5A). This is consistent with analogue binding experiments showing that an important interaction occurs between CAP and the 6-NH_2 of cAMP, which is essential for activation of CAP (Anderson et al., 1972; Scholubbers et al., 1984). cGMP binds to CAP and prevents the binding of cAMP, but cGMP does not stimulate CAP-dependent transcription (Anderson et al., 1972). In contrast, analogue binding studies with RI and RII consistently have shown that hydrogen bonding to the adenine ring is not required for binding and cAMP is probably bound in the syn conformation (de Wit et al., 1984; Jastorff et al., 1979; Doskeland et al., 1983). Modeling of cAMP bound to RI and RII also placed the cyclic nucleotide in the syn conformation (Weber et al., 1987). However, cGPK requires the 2-NH_2 group of cGMP for tight binding (Corbin et al., 1986). Four analogues of cGMP in which 2-NH_2 was replaced with various substituents showed >100-fold lower affinity for either of the two binding domains. Three analogues that were modified by replacement of one hydrogen atom of 2-NH_2 exhibited a smaller loss of affinity.

The model structures for the cGMP binding domains of cGPK have been examined for potential interactions between the protein and the base moiety of cGMP. If cGMP is bound in the syn conformation, which seems likely from cGMP analogue studies (Corbin et al., 1986), a hydrogen bond can potentially form between the 2-NH_2 group and Thr OH in both binding sites (Thr-177 and Thr-301) (Figures 4 and 5B). This is the same threonine which is predicted to form hydrogen-bond interactions with the axial exocyclic phosphate oxygen of cGMP, as does Ser-83 with cAMP in CAP. Analogue binding studies show that the 6-position can be =0, or =S, and not $-NH_2$, but there is probably no hydrogen bonding at this position due to the short C helix predicted for the model structures. This is an important consideration since the 6-position could potentially serve to distinguish between cAMP and cGMP. The analogue β-phenyl-1-N^2-etheno-cGMP binds to domain A with 10 times the affinity of cGMP and binds to domain B with about the same affinity as cGMP. There is space for this large substitution in domain A, and it is possible that hydrophobic interactions with Phe-214 or Cys-157 contribute to the increased affinity for this analogue. Domain B has amino acids blocking the N-2 position for the anti conformation; however, there is space for the syn conformation, although there are several charged residues nearby which may inhibit binding of this hydrophobic analogue.

The binding of cAMP to CAP does not involve stacking interactions between the adenine ring and aromatic amino acid

side chains, although the binding pocket is hydrophobic. Domain B of cGPK also does not have aromatic side chains in the cGMP binding site. Domain A has the potential for some aromatic–aromatic interaction between the guanine ring and Phe-214 which is equivalent to Tyr-371 in RI and Tyr-381 in RII which are photolabeled by 8-N_3-cAMP (Kerlavage & Taylor, 1980; Bubis & Taylor, 1987). The cGMP binding site of domain B contains a high proportion of charged residues, including Glu-270, Asp-271, Arg-281, Asp-287, Asp-337, and Lys-341, in contrast to the more hydrophobic site in domain A. This difference in hydrophobicity of the two cGMP binding sites is expected to alter the affinities for cGMP analogues. The model is consistent with results from cGMP analogues, which suggest that cGMP binds to both sites in the syn conformation, and provides the potential for hydrogen bonding to the 2-NH_2 group.

Evolutionary Significance. Cyclic GMP differs from cAMP in two positions on the base which could differentiate binding. The 6-keto group of cGMP has the potential for hydrogen bonding, but the predicted structures and the cGMP analogue studies do not support such an interaction. However, cGPK requires the 2-NH_2 group of cGMP for tight binding. If cGMP is bound in the syn conformation, as expected from cGMP analogue studies, the model suggests that a hydrogen bond can potentially form between the 2-NH_2 group and Thr OH in both binding sites (Thr-177 and Thr-301) (Figure 5). This cannot occur in the cAMP binding sites of cAPK, since all but one of the eukaryotic cAMP binding domains shown in Figure 2 have alanine at this position, while every cGPK binding domain has threonine (see arrow, Figure 2). This is the only conserved residue in the regulatory subunit sequences that is altered and invariant in the cGPK sequences. The predicted three-dimensional structures and amino acid sequence comparisons, in combination with cyclic nucleotide analogue studies, point to this Ala/Thr as a pivotal change in the evolutionary divergence of cGPK and cAPK.

Although it is difficult to ascertain the order of events in the course of evolution, sequence similarities suggest that either the cAMP or the cGMP binding domain may have evolved from a gene that had already duplicated to provide two intrasubunit cyclic nucleotide binding sites. The following arguments are valid regardless of which cyclic nucleotide binding site evolved first. If two intrasubunit cAMP binding sites were already present when cGMP binding emerged, it follows that each cAMP binding domain was mutated *independently* into a cGMP binding domain, implying the transient presence of a cAMP/cGMP hybrid. We are not aware of ligand binding domains or catalytic domains of any other proteins that are proposed to have evolved by this kind of mechanism. Both intrasubunit cyclic nucleotide binding sites are believed to be involved in activation of the respective protein kinase (Rannels & Corbin, 1980; Corbin & Doskeland, 1983). Thus, after the conversion of one of the cAMP binding domains into a cGMP binding domain, it is presumed that there was strong selective pressure to convert the other domain. Such an alteration would have been facilitated by the fact that an alanine to threonine mutation would require only a single base change. We propose that this was a key evolutionary development in the divergence of cAMP and cGMP binding domains. When coupled with slight alterations in the substrate recognition site of the kinase component, this could have produced separate functions for cAMP and cGMP in animal cells.

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Site-Directed Mutagenesis of Tyrosine Residues in the *lac* Permease of *Escherichia coli*

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ABSTRACT: By using oligonucleotide-directed, site-specific mutagenesis, each of the 14 Tyr residues in the *lac* permease of *Escherichia coli* was replaced with Phe, and the activity of each mutant was studied with respect to active transport, equilibrium exchange, and efflux. Ten of the mutations have no significant effect on permease activity. Of the four mutations that alter activity, replacement of Tyr26 or Tyr336 with Phe severely decreases all modes of translocation, and the binding affinity of the mutant permeases for *p*-nitrophenyl α-D-galactopyranoside is markedly decreased (i.e., K_D is increased). In addition, the Phe336 mutant permease is inserted into the membrane to a lesser extent than wild-type permease, as judged by immunoblot experiments. Permease containing Phe in place of Tyr236 catalyzes lactose exchange approximately 40% as well as wild-type permease but does not catalyze active transport or efflux. Finally, permease with Phe in place of Tyr382 catalyzes equilibrium exchange normally, but exhibits low rates of active transport and efflux without being uncoupled, thereby suggesting that replacement of Tyr382 with Phe alters a kinetic step involving translocation of the unloaded permease across the membrane.

The *lac* permease of *Escherichia coli* is a 46.5-kDa hydrophobic transmembrane protein that catalyzes the coupled translocation of a single β-galactoside molecule with a single H^+ (i.e., symport or cotransport) [cf. Kaback (1983, 1986) for reviews]. The polypeptide is encoded by the *lacY* gene, and it has been solubilized from the membrane, purified to homogeneity, reconstituted into proteoliposomes, and demonstrated to be fully functional as a monomer. On the basis of circular dichroism studies and hydropathy analysis (Foster et al., 1983), a secondary structure model for the permease has been proposed in which the polypeptide is organized into 12 hydrophobic α-helical domains that traverse the membrane in zigzag fashion, connected by more hydrophilic, charged segments (Figure 1). Evidence supporting some of the general features of the model has been obtained from laser Raman (Vogel et al., 1985) and Fourier transform infrared (P. D. Roepe, H. R. Kaback, and K. J. Rothschild, unpublished results) spectroscopy, from limited proteolysis (Goldkorn et al., 1983; Stochaj et al., 1986), from binding studies with

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