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Daming Huang

**Molecular Determinants of cGMP-binding to Chicken
Cone Photoreceptor Phosphodiesterase**

Daming Huang

A dissertation submitted in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy

University of Washington

2004

Program Authorized to Offer Degree: Molecular and Cellular Biology

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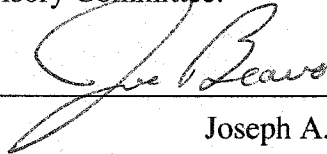
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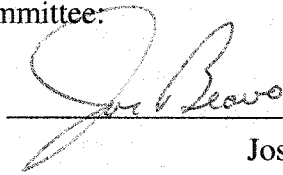
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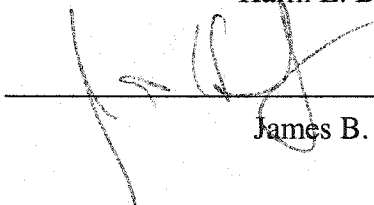
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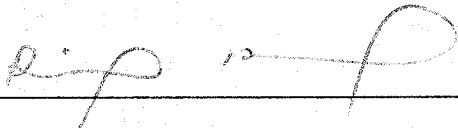
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Abstract

Molecular Determinants of cGMP-binding to Chicken
Cone Photoreceptor Phosphodiesterase

Daming Huang

Chair of the Supervisory Committee:
Professor Joseph A. Beavo
Pharmacology

The photoreceptor phosphodiesterase (PDE6) is a key regulator in the signaling of photo-transduction. It possesses two GAF domains, GAF-A and GAF-B. Sequence homology alignment between the GAF domains of PDE2, 5 and 6 suggests that GAF-A is the non-catalytic cGMP binding site of PDE6. Based on 3D-modeling of the GAF-A domain of chicken cone PDE6 with the crystal structure of mouse PDE2A GAF-B domain, residues making side-chain contact with cGMP were identified. Mutagenesis of these residues indicated that the overall architecture of the GAF cGMP-binding pocket is largely conserved between PDE2, 5 and 6. Residues F123, D169, T172, T176 are critical for the binding of cGMP and their corresponding alanine mutants lost the ability to bind cGMP completely. Three of the 4 crucial residues map to the H-4 helical structure of GAF domain. It is speculated that this

region is a necessary structure unit for cGMP binding. The GAF-A domain was demonstrated to be the non-catalytic cGMP binding site of PDE6. In addition, we have characterized the photoreceptor PDEs of chicken retina. Two histone-activated PDE6 peaks were separated by ion-exchange chromatograph. The first and second peaks were identified as chicken cone and rod photoreceptor PDEs respectively by mass spectrometry. Chicken photoreceptor PDEs have an ion-exchange profile, cGMP-binding and enzyme kinetics similar to the bovine photoreceptor PDEs. Our characterization of chicken photoreceptor PDEs suggests that the cGMP-binding and enzymatic characteristics are well conserved between birds and mammals.

Furthermore, the cGMP-binding of chicken cone PDE6 holo-enzyme was shown to have very similar cGMP-binding kinetics to bacteria expressed individual GAF-A or GAF-A/B domains.

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LIST OF ABBREVIATIONS

cAMP	adenosine 3':5' - cyclic monophosphate
cGMP	guanosine 3':5' - cyclic monophosphate
EDTA	ethylenediamine-N.N.N',N' -tetraacetic acid
GAF	cGMP-regulated PDEs, <i>Anabaena</i> adenylyl cyclase, <i>Escherichia coli</i> protein Fh1A
[³ H]	tritium
IBMX	3-isobutyl-methylxanthine
IC ₅₀	unlabeled ligand concentration at 50% inhibition of radioligand binding
IPTG	isopropyl-β-D-thiogalactopyranoside
K _d	dissociation constant
L	liter
M	molar
mL	milliliter
mg	milligram
min	minute

nm	nanometer
nM	nanomolar
pmol	picomole
PBS	phosphate buffered saline
PDE	3':5'-cyclic nucleotide phosphodiesterase
PDE6	photoreceptor PDE
pH	- log of hydrogen ion concentration
PMSF	phenylmethylsulfonyl fluoride
rpm	revolution per minute
SDS	sodium dodecyl sulfate
sec	second
V_{max}	velocity at saturating levels of substrate
ug	microgram
uL	microliter
uM	micromolar
umol	micromole

ACKNOWLEDGEMENTS

I would like to express my gratitude to my mentor, Dr. Joseph Beavo, for training and guiding me with patience and kindness. I also would like to thank the members of my reading committee, Dr. Karin Bornfeldt and Dr. James Hurley, for reading this manuscript and providing valuable input. The encouragement and helpful advice from the other members of my thesis committee, Dr. Nelson Fausto, Dr. David Kimelman, Dr. Neil Nathanson and Dr. Daniel Storm, are sincerely appreciated as well.

The support and encouragement of my mother, Guo-Rong Xiao, has been invaluable during this and all other periods of my education. Finally, the enthusiasm, support and love of my husband, Yung-Jen Tsai, make the dissertation research an accompanied and joyous journey.

DEDICATION

I would like to dedicate my dissertation to my dear mother, Guo-Rong Xiao, to my dear husband, Yung-Jen Tsai and to my darling daughter, Thalia, the joy of my life.

CHAPTER I

INTRODUCTION

Vision requires a precise and immediate transmission of surrounding visual information to the central nervous system. Evolution has meticulously refined the photo-transduction system into an extremely sensitive and discriminatory signaling pathway. The regulation of guanosine 3':5'-cyclic monophosphate (cGMP) plays an essential role in the process of visual transduction. The intracellular level of cGMP within the photoreceptor cells is tightly controlled and modulated by photoreceptor cyclic nucleotide phosphodiesterases (PDE6s).

Cyclic nucleotide phosphodiesterases

Cyclic nucleotides such as cAMP and cGMP serve as second messengers in signal transduction and participate as an essential part in fulfilling various cellular functions. The cyclic nucleotide levels in the cytosol or other compartments within the cell are regulated delicately by their generation by adenylyl and guanylyl cyclases and their degradation by cyclic nucleotide phosphodiesterases (PDEs). PDEs eliminate the cyclic nucleotide signal by cleaving the cyclic nucleotide

phosphodiester bond between the phosphorus and oxygen atoms at the 3'-position, and converting 3', 5'-cAMP or -cGMP to 5'-AMP or -GMP.

There are 11 families of PDEs distinguished by their differential expression, hydrolytic selectivity, inhibitor profiles and sequence homology (Beavo, 1995; Soderling and Beavo, 2000). PDEs are composed of a highly conserved catalytic domain toward the carboxyl terminus and differential regulatory domains toward the amino terminus (Figure 1). Various signaling pathways are involved in the regulation of the catalytic activity of PDEs. It has been shown that PDEs are regulated by intracellular cAMP and cGMP levels, calcium/calmodulin, phosphorylation by kinase such as PKA, PKB, binding of regulatory proteins and subcellular localization. The duration, intensity and location of cyclic nucleotide signals are affected profoundly by these parameters. A large number of different PDEs from multiple families facilitate the precise, cell-type specific regulation of cyclic nucleotide flux and its subsequent physiological output in any given cell.

Structures of 11 PDE Gene Families

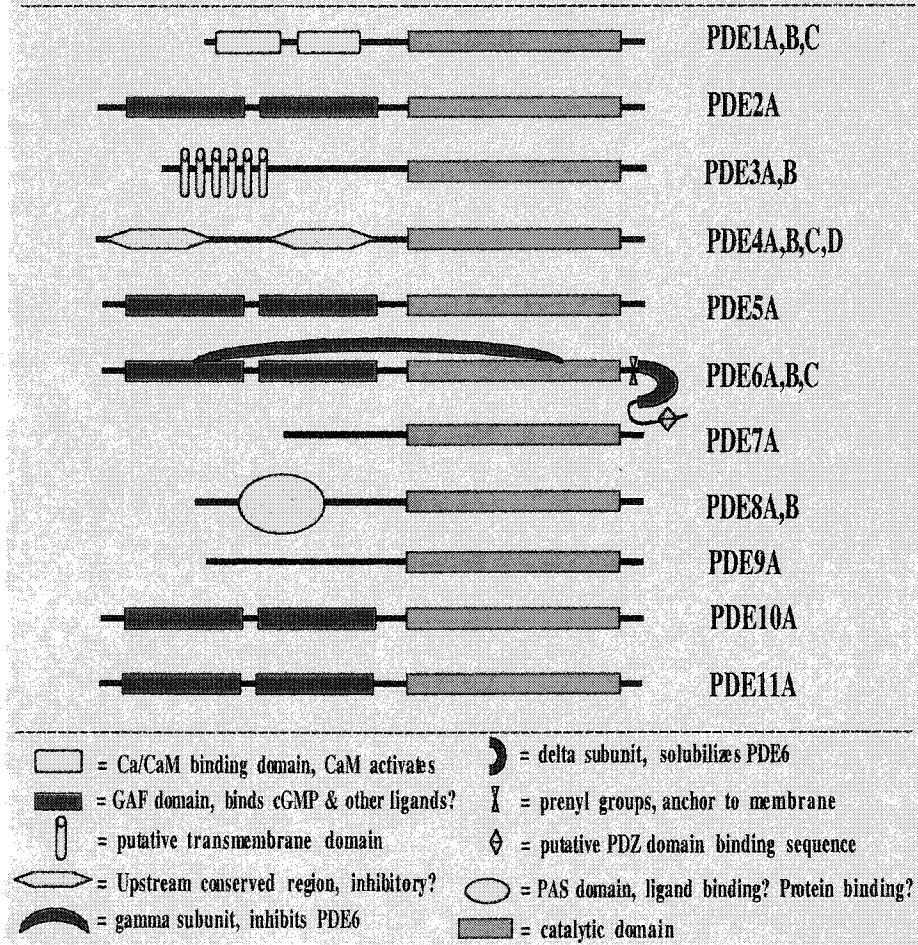


Figure 1. Structures of eleven phosphodiesterase gene families

Adapted from (Soderling and Beavo, 2000)

Photoreceptor signaling and PDEs

Photoreceptor cells can sense and respond to changes of light very rapidly through a G-protein mediated signaling cascade. Photoreceptor cyclic nucleotide phosphodiesterases (PDEs) are key regulators in this signal transduction pathway. When a photon hits the transmembrane G-protein coupled receptor, rhodopsin, 11-*cis*-retinal embedded in the protein is isomerized to all-*trans*-retinal. This *cis-trans* isomerization upon photon absorption causes a conformational change in rhodopsin that allows it to interact with the G protein transducin. Transducin can then exchange guanosine diphosphate (GDP) for guanosine 5'-triphosphate (GTP) and convert to the active form. Activated transducin interacts with the inhibitory P-gamma subunit of PDE6 and subsequently releases PDE6 from P-gamma inhibition. Activated PDE6 rapidly decreases the cytoplasmic cGMP level which then leads to the closure of cGMP-gated cation channels, reduced influx of sodium and calcium, and eventually hyperpolarization of the photoreceptor cells (Figure 2).

In the dark, the cGMP-gated cation channels actively transport sodium and calcium ions into the cell, while other channels maintain a continuous efflux of

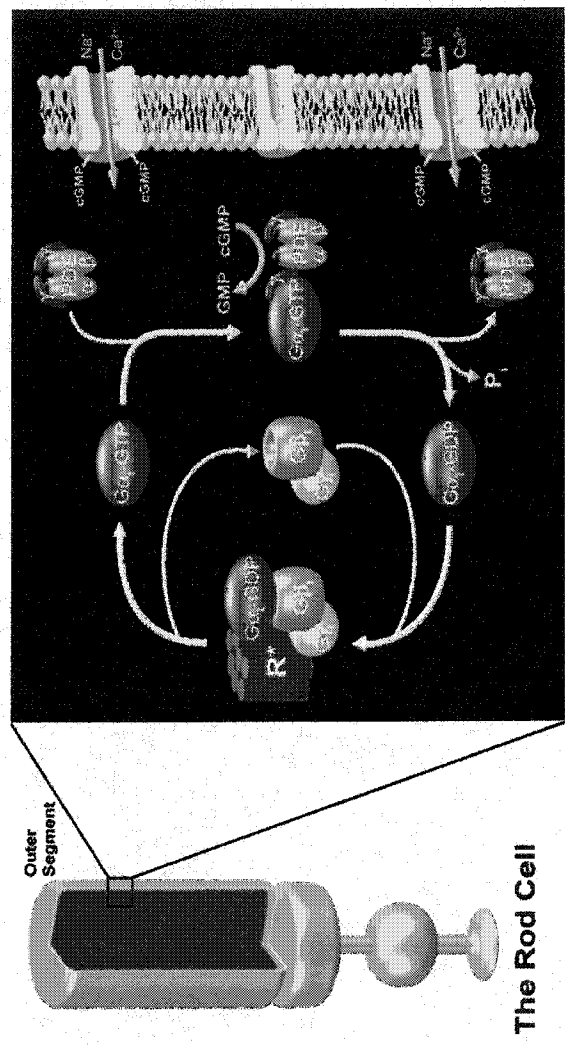


Figure 2. The signaling cascade of photo-transduction.

sodium. Closure of the channels, in response to light, disrupts the influx, while the efflux of sodium and calcium continues undisturbed. As with all neuronal synapses, depolarization of the synaptic terminal elicits the release of neurotransmitter. Thus, light exposure reduces the release of neurotransmitter due to hyperpolarization of photoreceptor cells. Furthermore, the signal generated by the hyperpolarized cells is processed by the retinal neuronal cells. Ultimately, the signal is transmitted to the brain (Palczewski et al, 1997; Helmreich and Hofmann, 1996).

In addition, PDE6s participate in the termination of visual transduction cascade. The hydrolysis of GTP to GDP leads to the inactivation of transducin. Inactivated transducin no longer can interact with the inhibitory P-gamma subunit and the photoreceptor PDEs become much less active. The intracellular level of cGMP is largely increased due to the re-synthesis by guanylate cyclase and decreased activity of PDE6. Thus, the cGMP gated channels open again and the photoreceptor cells return to a depolarized status. Therefore, PDE6 plays a crucial role in both the turn-on and the turn-off of photo-transduction pathway.

Rods and Cones

The two classes of photoreceptors, rods and cones, are named for the morphology of the outer segment of each cell. Rods and cones are highly polarized cells composed of several distinct regions. The outer segment is the region responsible for visual transduction (Figure 3). The inner segment contains the synaptic terminal and the organelles essential for the normal functional of the photoreceptor cells. The synapse is located in the distal region of the inner segment. The visual signal initiated at the outer segment is transmitted through the synapse to bipolar cells, which process and eventually present the signal to the central nervous system.

Rod and cone photoreceptors differ substantially in their response to light (Pugh and Cobbs, 1986). Rods are very sensitive to low levels of light and can respond to a single photon. Cone cells are about 100 times less sensitive to light than are rod cells (Baylor, 1987). The light response of the cone outer segment may be faster and is terminated more rapidly than the rod signal. Furthermore, cones have significantly more pronounced light adaptation than rods (Pugh and Cobbs, 1986).

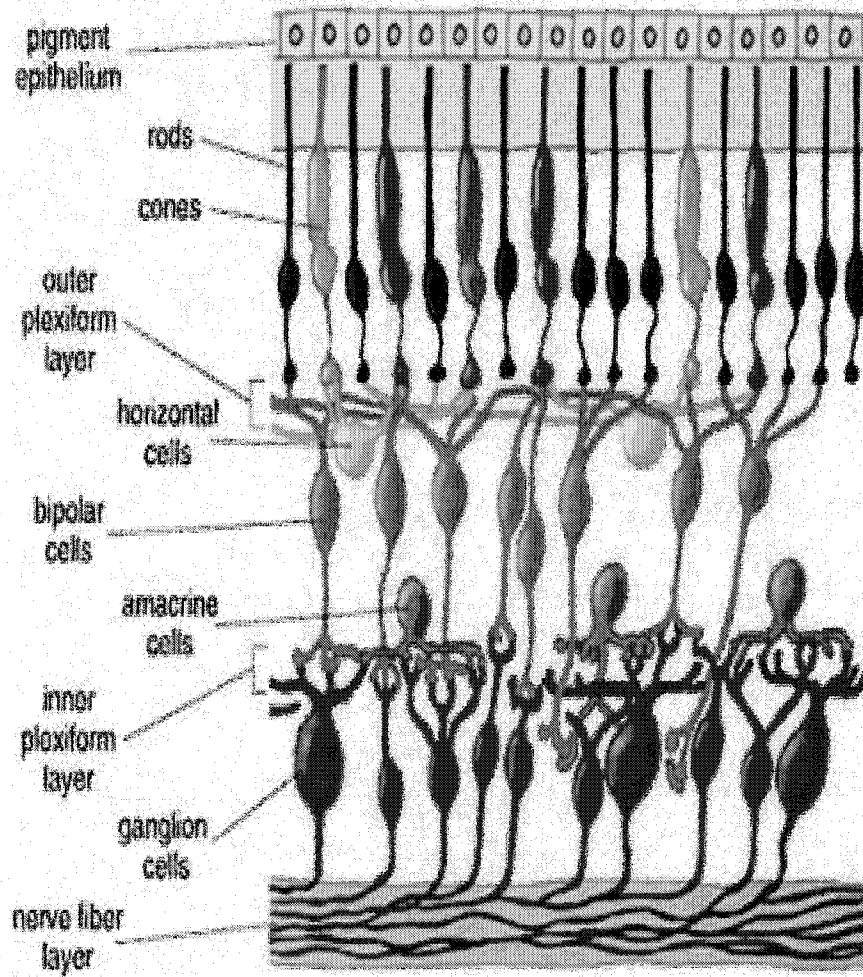


Figure 3. Schematic diagram of multiple cell layers of retina

In most mammals, rods outnumber cones by approximately 20-fold. However, in birds, cones are dominant and make up about 80% of the photoreceptor cells.

Although rods far outnumber cones in most mammalian retinas, many more rod axons converge upon a single bipolar neuronal cell than cone axons upon the cone bipolar cells. There is only one kind of rod photoreceptor that stimulates only a single type of bipolar cell. This circuit design ensures the rod system is highly sensitive to even a single photon. Therefore, rod photoreceptors are able to be responsive for night and dim light vision (Bloomfield and Dacheux, 2001).

Cone photoreceptors serve as the primary sensor for daylight and color hues.

In mammalian species, three different types of cones exist as the key elements of color vision. The differences of these three types of cones are their unique opsins that are called blue-, green- and red-sensitive opsin with λ_{\max} at 419, 531 and 558 nm respectively (Dartnall et al., 1983). Photons of different wavelength have different likelihoods of being absorbed by the three cone classes: short-(S), middle-(M), and long-wavelength-sensitive-(L)cones corresponding to their unique blue-, green- and red-sensitive opsin respectively. Once absorbed, the information obtained is the photon count in each cone but not the wavelength of the absorbed photons (Rushton, 1972). The photon count can be elevated by either an increase in light intensity, or a

change to a more favorable wavelength. To distinguish the color of an object, the output of the three cone types is processed via the neuronal circuitry. The synaptic connections of the circuitry are arranged so that the responses of the blue cones are compared with those of the red or green cones. By comparing their outputs, the neuronal circuitry can create signals that reflect the spectral composition of the stimulus (Jacobs, 1993).

Photoreceptor PDEs

Photoreceptor PDEs (PDE6s) are highly concentrated in the outer segments of photoreceptor cells. Bovine rod PDE6 and its signal transduction mechanism have been thoroughly studied because bovine rod outer segments can be quickly purified with retained ability to respond to light.

Since PDE6s are the key modulator of cGMP in the visual transduction cascade, the kinetics, structure and function of these enzymes are well investigated and understood. The catalytic subunits of PDE6s have the domain structure typical of many PDEs studied thus far, which composed of the catalytic domain close to the C-terminus and regulatory GAF domains close to the N-terminus. The photoreceptor PDE catalytic subunits are expressed almost exclusively by the photoreceptor cells

with very little expression in other tissues. The catalytic subunits of PDE6s have the highest K_{cat} values of any known PDE. Trypsin activated PDE6s have K_m values of about 15-20 μ M for cGMP.

Photoreceptor PDEs are usually purified as an oligomer which consist of two catalytic subunits and numerous accessory subunits. Rod PDE appears to be a heterotetramer containing one α -subunit (PDE6A), one β -subunit (PDE6B), and two γ -subunits (Deterre et al., 1988). Cone PDE is composed of two identical α' -subunits (PDE6C) and two γ -subunits (Gillespie and Beavo, 1988). The α , β , and α' subunits contain the catalytic regions which hydrolyze cGMP. The γ -subunit is the inhibitory subunit that binds to and inhibits phosphodiesterase activity of catalytic subunits. In addition, the γ -subunit also can increase the rate of GTP hydrolysis by transducin and is required for the correct termination of the visual transduction cascade (Pages et al., 1993; Tsang et al., 1998).

Besides the inhibitory γ -subunit, one or more δ subunits are also co-purified with the catalytic subunits of PDE6s. It has been shown that the δ subunit solubilize the membrane-bound PDE6s by binding to the isoprenylated C-termini of the PDE6 catalytic subunits (Florio et al., 1996).

GAF domains

The GAF domain, one of the regulatory small-molecule-binding domains (SMBDs), is named for its presence in cGMP-regulated PDEs, certain adenylyl cyclases and the transcription factor FhlA of bacteria (Aravind and Ponting, 1997). The presence of GAF domain has been found among a wide range of species (Figure 4) (Aravind and Ponting, 1997). Certain GAF domains of PDEs constitute cGMP-binding modules. Cyclic GMP binds to one of two GAF domains of PDE2, PDE5 and PDE6 (Martins et al., 1982; Gillespie and Beavo, 1988).

To date, more than 1600 GAF-containing proteins have been identified (Aravind and Ponting, 1997; Anantharaman et al., 2001). The ligands for most GAF binding domains remain unknown. The first members having GAF domains was discovered in the cGMP-regulated phosphodiesterases, such as PDE2, PDE5 and PDE6. All have high affinity binding sites for cGMP. Recently, the GAF-B domain of

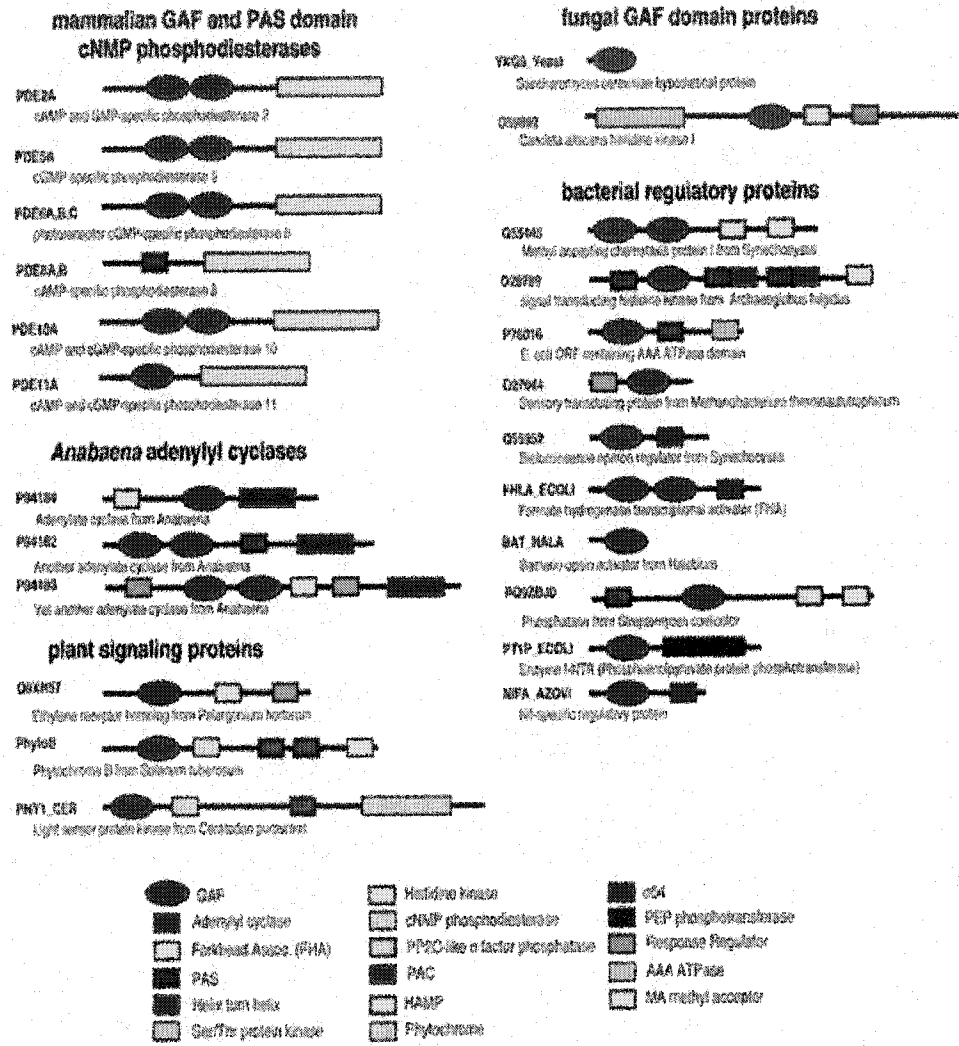


Figure 4. GAF domain-containing proteins

Domain structure of mammalian GAF and PAS domain-containing phosphodiesterases and other representative GAF domain proteins.

Adapted from (Ho et al., 2000)

cyanobacterial adenylyl cyclase (*cyaB1*) has been reported to bind cAMP specifically (Kanacher et al., 2002).

Among cGMP-binding PDEs, PDE2 is the first one with a well-determined role for the GAF domain in regulation of its catalytic activity. The cAMP hydrolytic activity of PDE2 is greatly stimulated by cGMP binding to its GAF domain (Martins et al., 1982; Stroop and Beavo, 1992). In addition, a recent report on PDE5 has shown that PDE5 is activated directly upon cGMP binding to its GAF-A domain (Rybalkin et al., 2003). Similarly, the activity of cyanobacterial adenylyl cyclase (*cyaB1*) is greatly increased upon cAMP binding to its GAF-B domain. The cAMP occupation of the *cyaB1* GAF-B domain results in rapid exponential activation of its adenylyl cyclase catalytic activity since cAMP is the product of *cyaB1* adenylyl cyclase catalytic reaction (Kanacher et al., 2002). Interestingly, when the cAMP-binding GAF-A/B domain of *cyaB1* is replaced by the cGMP-binding GAF-A/B domain of rat PDE2, the adenylyl cyclase activity of this chimeric protein was stimulated by cGMP binding. This result suggests that the basic mechanism of cyclic nucleotide binding to GAF domains and subsequent stimulation of catalytic activity is conserved through evolution. Furthermore, this mechanism of GAF domain regulation of

adjacent catalytic domains may be applicable to many GAF domain-containing proteins.

The GAF domain and the equally ubiquitous PAS domain are clearly linked in their evolution. Both classes of domains bind a diverse set of regulatory small molecules (Aravind and Ponting, 1997). Various signaling and sensory proteins contain PAS domain. In addition, a considerable number of proteins contain both PAS and GAF as their regulatory domain.

PDEs and GAF domain

Except for PDE4 and PDE8, all other PDE families can hydrolyze cGMP. Five of these cGMP-hydrolyzing PDE families, PDE2, 5, 6, 10 and 11, contain one or two complete GAF domains at the N-terminal regulatory region (Beavo, 1995; Soderling and Beavo, 2000).

cGMP binding to a GAF domain is known to regulate the PDE enzyme activity. For example, the catalytic activity of PDE2A is allosterically stimulated by cGMP binding to its GAF domain (Martins et al., 1982). A conformational change induced by cGMP occupation at the GAF-B of PDE2, transforms PDE2 to a more active form that is more susceptible to modification and proteolysis. Recently, the 3D

structure of the GAF domains of mouse PDE2A was revealed by a crystallography study in our lab (Martinez et al., 2002). GAF-B was shown to be the cGMP-binding domain and GAF-A was demonstrated to form the dimer interface.

In PDE5, cGMP binding to GAF domains increases PDE5 catalytic activity and facilitates phosphorylation at N-terminal Ser92 (Rybalkin et al., 2003). The phosphorylation of PDE5 by cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) has been reported to increase PDE5 catalytic activity (Burns et al., 1992; Corbin et al., 2000). The occupation of the cGMP-binding sites appears to cause an increase in the Stokes radius of the enzyme, implying that PDE5 undergoes a conformational change (Francis et al., 1998).

In PDEs 2, 5, and 6, the GAF domains strongly prefer cGMP over cAMP and are intolerant of modification at almost any group on cGMP (Zoraghi et al, 2004). In PDEs 5 and 6, cAMP shows little competition with cGMP. In PDE2, cAMP binding to its GAF domain is considerably weaker compared to cGMP binding (Erneux et al., 1985; Martinez et al., 2002; Wu et al., 2004). The cGMP selectivity of PDEs 5 and 6 is the most restrictive of any known cyclic nucleotide-binding proteins (Francis et al., 1990; Hebert et al., 1998).

Crystal structure of mouse PDE2A GAF-A/B domain

Recently, the 3D-structure of mouse PDE2A GAF-A/B (mmPDE2A GAF-A/B) domains was determined by X-ray diffraction crystallography at 2.9Å resolution (Figure 5)(Martinez et al., 2002). The regulatory GAF-A/B domains form a dimer in which only GAF-B binds cGMP. GAF-A and GAF-B are connected by a short linker helix. Cyclic GMP apparently binds to the GAF-B in an extended anti conformation. The cGMP appears to be deeply buried within the binding pocket with little solvent accessible surface (Figure5) (Martinez et al., 2002).

There are 11 amino acid residues that make contact with cGMP and form the binding pocket. In PDE5, 10 of these 11 cGMP contact residues are conserved in GAF-A, which is the cGMP-binding domain of this PDE. A consensus cGMP-binding motif, based on the similarity between PDE2A GAF-B and PDE5 GAF-A domains, has been proposed (Martinez et al., 2002):

S X(13-18) **FD** X(18-22) **IA** X(21) [Y/N] X(2) **VD** X(2) **T** X(3) **T** X(19) [E/Q]
1 2,3 4,5 6 7,8 9 10 11

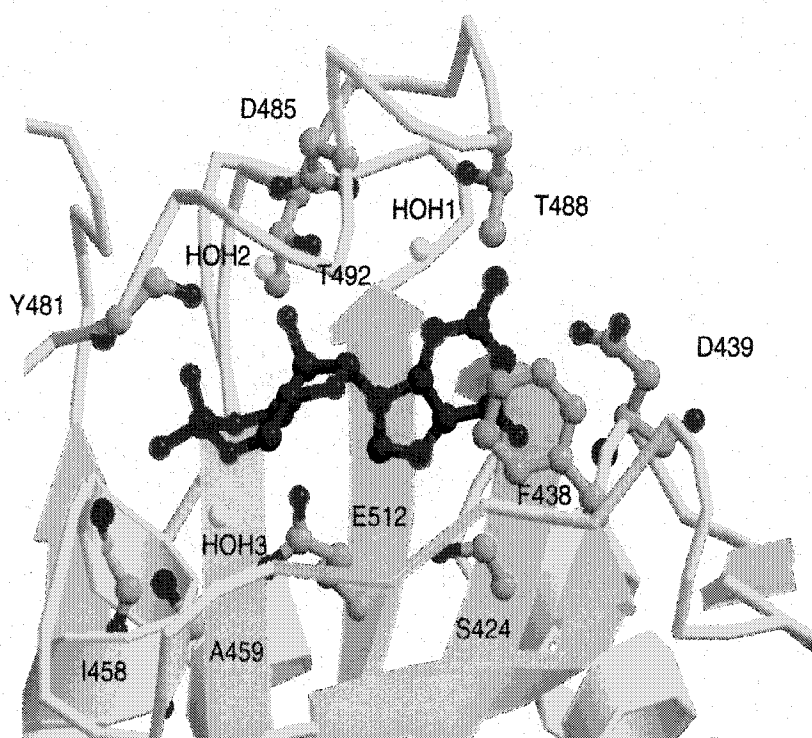


Figure 5. Crystal structure of the cGMP-binding site in PDE2A GAF-B

Shown are ten residues that interact with cGMP in the binding site: six make hydrogen bonds, one base stacks with the guanine ring, and three make backbone amide contacts. Adapted from (Martinez et al., 2002)

Eight of the 11 mouse PDE2A residues, i.e. S424, F438, D439, V484, D485, T488, T492 and E512 contact cGMP via side chains. The crystal structure of PDE2 GAF-B suggests that its ability to discriminate cGMP versus cAMP is partially determined by D439, which provides positive specificity for cGMP-binding via hydrogen bonds between its main chain NH, side chain carboxyl and O6, N1 guanine base of cGMP (Martinez et al., 2002).

Compared to the GAF domains of PDE2 and PDE5, the PDE6 GAF domain has both higher binding affinity and specificity for cGMP (Gillespie et al., 1988, Herbert et al., 1998). Two of the three PDE6 family members, PDE6A and PDE6B form a heterodimer in bovine, frog, and human rods, while PDE6C forms a homodimer in cones. Bovine rod PDE binds cGMP with a K_d of 25-500 nM at the low-affinity binding site and <500pM at the high-affinity site. Cone PDE has one cGMP binding site with a K_d of about 10nM (Gillespie et al., 1989). A functional chimeric bovine PDE6C/PDE5 enzyme also has been reported to contain a single class of non-catalytic cGMP binding sites with a K_d of about 450nM (Granovsky et al., 1998).

Based on the cGMP-binding motif mentioned above and sequence homology alignment between PDE2 and PDE6 GAF domains, the GAF-A but not GAF-B

domain of PDE6s is most likely to be the cGMP binding site. There are 8 residues in the PDE2 GAF-B domain contacting the cGMP molecule through their side chains. In the GAF-A domain of cone PDE6, 6 of these 8 residues are conserved based on the sequence homology alignment between PDE2 GAF-B and PDE6 GAF-A domain.

The conserved residues are shown below:

mouse PDE2A GAF-B:	S424, F438, D439, V484, D485, T488, T492, E512
chicken PDE6C GAF-A:	S97, F123, E124, D169, T172, T176
bovine PDE6C GAF-A:	S92, F118, E119, D164, T167, T171
bovine PDE6A GAF-A:	S95, E117, D167, T170, T174
bovine PDE6B GAF-A:	S93, E120, D165, T168, T172

Thus, these conserved amino acid residues of PDE6C GAF-A domain are located such that they could play crucial roles in non-catalytic cGMP-binding.

Statement of problem

The sequence homology between the GAF domain of PDE6C and PDE2A provides a clue for delineating the structural components of the cGMP-binding pocket of PDE6C GAF. These structural components within the binding pocket contribute to the affinity and selectivity of cGMP and cAMP binding. Therefore we wanted to test by mutagenesis which of these conserved residues of the PDE6 GAF-a domain might be important for cGMP binding and cAMP/cGMP selectivity. In our study, we used

chicken PDE6C-GAF (ggPDE6C-GAF) domains because they have robust expression in *E.Coli* and retain cGMP-binding capability similar to cone PDE6 holoenzyme whereas this was not the case for PDE6-GAF domain protein constructs made from cDNAs of other species. Since little has been reported on the cGMP-binding and enzyme kinetics properties of chicken photoreceptor PDEs, to ensure the bacterial expressed GAF domain of PDE6C represent the cGMP-binding properties of the holoenzyme, we also characterized the photoreceptor PDEs of chicken retina. The main specific aims fulfilled in this thesis research were as follow:

I. Characterize chicken photoreceptor PDEs.

- 1. Isolate and identify rod and cone PDEs.**
- 2. Determine the cGMP-binding kinetics of rod and cone PDEs.**
- 3. Determine the enzymatic kinetics of rod and cone PDEs.**

II. Determine the structural components contributing to cGMP-binding of the PDE6 GAF domain.

In order to fulfill above goal, questions addressed were shown below:

- 1. Which GAF domain binds cGMP?**

- 2. What are the cGMP-binding kinetics characteristics including the off-rate and K_d ?**
- 3. Which amino acid residues are essential for binding to cGMP?**
- 4. Which amino acid residues determine the cGMP/cAMP selectivity?**

CHAPTER 2**CHARACTERIZATION OF CHICKEN PHOTORECEPTOR****PHOSPHODIESTERASES****SUMMARY**

The photoreceptor phosphodiesterase (PDE6) is a key regulator of photo-transduction. Structural studies on this PDE have been hampered for many years by an inability to express and purify substantial amount of active enzyme. This thesis research describes the expression of large amounts of the chicken cone PDE6 GAF domains. As little data is available on the kinetics and binding properties of the chicken cone PDE6, we have also characterized the photoreceptor PDEs of chicken retina. Two histone-activated PDE6 peaks were separated by ion-exchange chromatography. The first and second peaks were identified as chicken cone and rod photoreceptor PDEs respectively by mass spectrometry. Chicken photoreceptor PDEs have an ion-exchange profile, cGMP-binding and enzyme kinetics similar to the bovine photoreceptor PDEs. Our characterization of chicken photoreceptor PDEs suggests that the cGMP-binding and enzymatic characteristics are well conserved between birds and mammals. Furthermore, the cGMP-binding of chicken cone PDE6

holo-enzyme was shown to have very similar cGMP-binding kinetics to bacterially expressed individual GAF-A or GAF-A/B domains.

INTRODUCTION

Chicken rod and cone

Unlike the retinas of most mammalian species, the retinas of chickens are cone dominant. Approximately 85% of the photoreceptors in retina are cone cells (Morris and Shorey, 1967). The cone-dominant character of the avian retina contrasts with the retinas of mice, rats, and dogs, in which cones comprise less than 5% of the photoreceptor cell population. The abundance of cone photoreceptors in chicken retina has made it a valuable model system for studies of cone cell-specific proteins, development and physiological functions.

The structure of the chicken retina is similar to that found in many vertebrate species. All cellular and fiber layers present in mammalian retina exist in chicken retina. Nevertheless, chicken retina exhibits several distinguishing features that are unique for the avian retina. First, there are two morphological types of cone photoreceptors in chicken retina: single cone and double cone cells. Second, chicken

cone cells contain colored oil droplets. Third, chicken cones possess four different types of visual pigments that are red, green, blue and violet opsins in addition to the rod pigment rhodopsin. In combination with the presence of the colored oil droplets, the absorption spectra of the visual pigments allow the production of the tetrachromatic color vision in chickens and other avian animals (Okano et al., 1992; Okano et al., 1995).

In spite of the unique features of chicken retina, the organization and patterns of development of the chicken retina are similar to those observed in other species. The dominance of cone photoreceptors in the chicken retina makes it an excellent model system for studying the function and physiology of cone cells. Moreover, some of the most devastating human retinal dystrophies affect the cone cells that are responsible for color perception and high visual acuity in humans. Therefore, avian species, and in particular the domestic chicken, provide a valuable model system for studies of cone cell response to retinal diseases (Semple-Rowland and Lee, 2000). The value of the chicken as a model system for studies of cone cell function is strengthened by the fact that the development, morphology, and electrophysiology of the chicken retina are fairly well characterized.

Our previous understanding of cone PDE6 was based largely on the characterization of the bovine cone enzyme. However, because of the limited supply of cone photoreceptor cells from bovine eyes, the studies on cone photoreceptor PDEs have been hindered for many years. Therefore, it is expected that our characterization of chicken PDE6 enzymes should facilitate further investigations and insight on the molecular mechanism and regulation of cone PDE6 in photo-transduction.

RESULTS

Immuno-cytochemistry study on chicken rod and cone PDE6

It has been shown previously that the ROS-1 and ROS-3 monoclonal antibodies recognize rod and cone photoreceptor PDEs differentially by immuno-cytochemistry (Hurwitz et al., 1984). In bovine photoreceptors, ROS-1 monoclonal can only identify rod PDE6 whereas ROS-3 recognizes both rod and cone PDE6. Initially, we thought we might be able to use the specificity of ROS-1 and ROS-3 antibodies to distinguish rod or cone photoreceptor PDEs of chicken. However, our immuno-cytochemistry studies on chicken photoreceptors suggested that ROS-3

could detect both rod and cone PDE6s whereas ROS-1 did not recognize either the chicken rod or cone photoreceptor PDEs (Figure 6).

Separation and identification of chicken cone and rod PDE6

In order to characterize chicken photoreceptor PDEs, we first needed to isolate and identify rod and cone PDE6. Two histone-activated PDE6 peaks of chicken retina were separated by DE52 anion-exchange chromatograph (Figure 7). Comparison of the DE52 chromatograph profiles showed that the first and second peak of PDE activity super-imposes with the cone and rod PDE6 peaks of bovine retina respectively (Figure 7).

The immuno-cytochemical study on chicken photoreceptor PDEs using ROS-1 and ROS-3 monoclonal antibodies could not discern the rod PDE from the cone one as shown in Figure 6. Although the two PDE6 peaks were separated by ion-exchange chromatography corresponded to bovine rod and cone PDE6, additional purification and analysis are required to unambiguously identify which was rod and which was

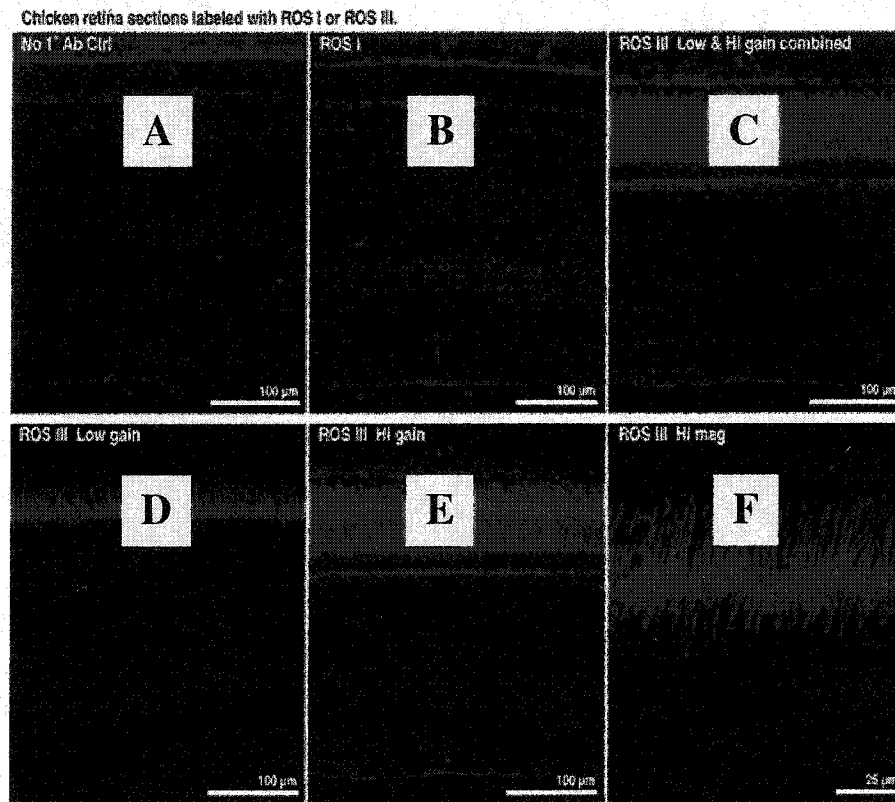
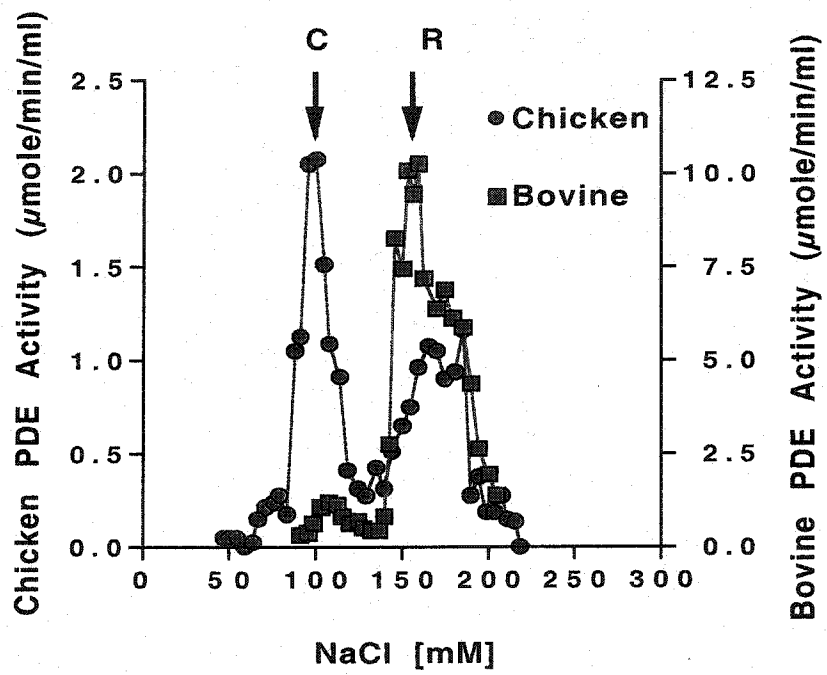


Figure 6. Immuno-staining of chicken cone and rod photoreceptors with ROS-I and ROS-3 antibodies.

A. Without primary antibody. B. Immuno-staining with ROS-I antibody. C-F. Immuno-staining with ROS-3 antibody.

Figure 7. DE52 ion-exchange column fractionation of the hypotonic extracts of chicken retina.

Methods similar to those described for isolation of bovine PDE6 were used for the chicken PDE6s (Cook et al., 2000). 100 frozen chicken retinas were extracted with hypotonic buffer (10 mM Tris, pH 7.5, 1 mM MgCl₂, 1 mM DTT and 0.2 mM PMSF). After 100,000 g centrifugation, the retinal supernatant was applied to a 50 ml DE52 ion-exchange column equilibrated in the hypotonic buffer plus 20 mM NaCl and eluted with a linear NaCl gradient (20-300 mM) run at 1 ml/min at 4°C. A total of 60 fractions of 5 ml each were collected and assayed for PDE activity in the presence of 2.5 mg/ml Histone. There are two histone-activated PDE6 activity peaks: rod (R) or cone (C) PDE6 peak.



cone PDE6. Therefore, the putative chicken rod and cone PDE6 peaks were further purified by cGMP-affinity column or immunoprecipitation. The presence and purity of chicken cone and rod PDE6 in either the eluate off the cGMP-affinity column or the immunoprecipitated pellets were determined by silver or Coomassie stain (Figure 8; Figure 9).

The eluate off the cGMP-affinity column containing the purified putative cone PDE6 was trypsin digested and analyzed by mass spectrometry. The Mascot search result indicates that 13 peptides from MS/MS spectra are exact matches with chicken cone PDE6 alpha' subunit. Thus, we identified the first histone-activated PDE6 activity peak off DE52 ion-exchange column as the chicken cone PDE6 (Figure 10).

However, the putative rod PDE6 did not bind to the cGMP-affinity column and could not be purified by this method. Therefore, the putative rod and cone PDE6 peaks off the DE52 column were further purified by immunoprecipitation with ROS-3 monoclonal antibody. The proteins of the immunoprecipitation pellets were separated by SDS-PAGE and stained with coomassie blue (Figure 9). The protein bands corresponding to the molecular weight of PDE6 were sliced out, trypsin-digested and analyzed by mass spectrometry.

Figure 8. SDS-PAGE analysis chicken cone PDE6 purified by cGMP affinity chromatography.

Seven fractions from the first peak of the DE52 ion-exchange chromatography (35 ml) were pooled together and loaded on an epoxy-Sepharose cGMP affinity column (Martins et al., 1982). After washing with 15 ml (5x bed volume) wash buffer (20 mM Tris, pH 7.5, 200 mM NaCl, 2 mM EDTA, 10 mM DTT, 1 mM PMSF and 1 mM IBMX). The cGMP-binding proteins including chicken cone PDE6 were eluted with 1mM cGMP at the presence of 2mM EDTA and 1mM IBMX. The purity of the eluted protein fractions was analyzed by silver stain of the SDS-PAGE gels (M: marker, E: eluate, W: wash, F: flow through). The arrow head indicates the protein band of chicken cone PDE6 alpha' subunit.

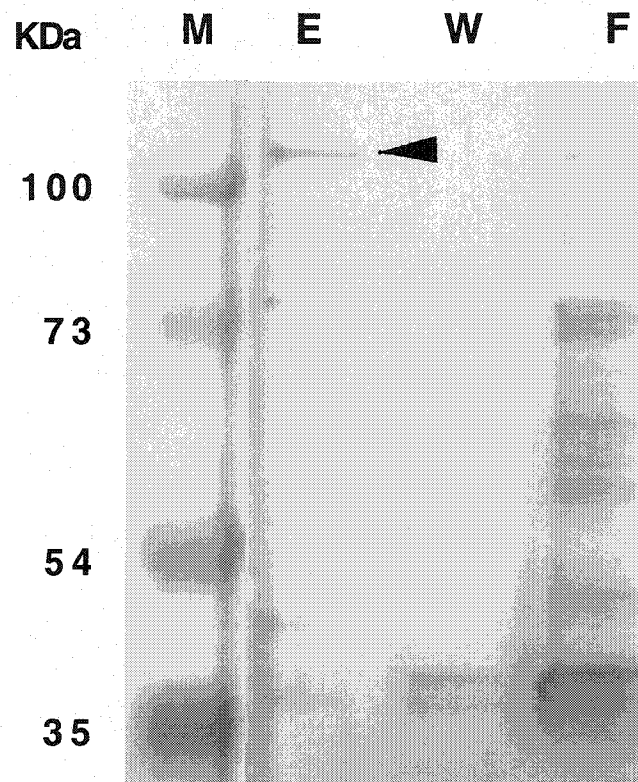
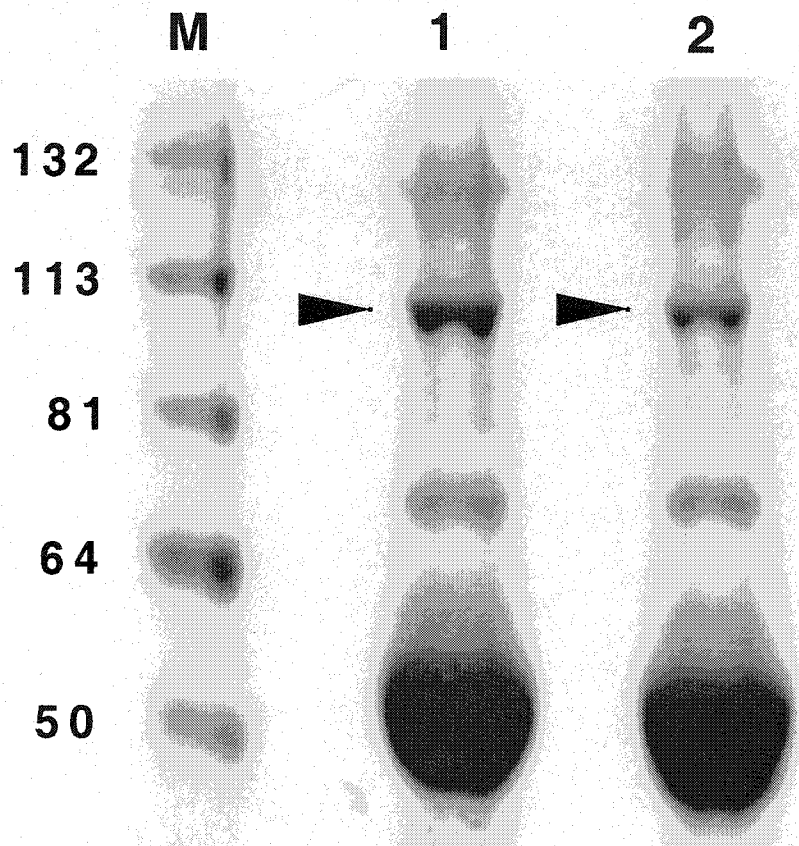


Figure 9. SDS-PAGE analysis of immunoprecipitated chicken rod and cone PDE6 holoenzyme.

Chicken rod or cone PDE6 holoenzymes were immunoprecipitated using ROS-3 monoclonal antibody bound to Protein G-Sepharose resin. The pellets were analyzed by Coomassie stain of SDS-PAGE (M: marker, 1: first, 2: second PDE6 activity peak off DE52 column. The arrow heads indicate the protein bands of chicken cone and rod PDE6.



MGEVNDKAVEK_YLENNPQFAK_EYFDRK_MR_AEVLGSIQVSPGDVK_EGVSF 50
 K_DMSR_LEECNILELLTEIQDEAGSMEK_IVHK_TLQR_LSOLLAR_DR_CSM 98
 FICR_SR_NGIPEVATR_LLNVTPTSK_FEDNLVNPDK_ETVFPLDIGIAGWVAH 148
 TK_K_FFNIPDVK_K_NNHFSYLDK_K_TGYTTVNMMAIPITQK_EVLAVVMA 196
 LNK_LNASEFSK_EDEEVFK_K_YLNFI_SLVLR_NHHTSYLYNIESR_R_SQMLL 244
 WSANK_VFEELTDIER_QFHK_ALYTIR_MYLNCER_YSVGLLDMTK_EK_EFYD 292
 EWPIR_LGEAEPYK_GPK_TPDGR_EVNFYK_IIDYILLHGK_EEIK_VIPTPPAD 340
 HWCLISGLPTYVAENGFICNMMNAPADEYFTFQK_GPVDETWGVK_NVLSLPV 393
 NK_K_EEIVGVATFYNR_K_DGK_PFDEYDEQIIEITLQFLGWSVLNTDITYDK_M 442
 NK_LENK_K_DIAQEMLMYQTK_ATPTEVESILK_YK_EK_LNVK_SIEECDEK_ 488
 DLIRILK_EELPDPK_DLELYEFR_FSDFPVTEHGLITCGIR_LFFEINVVEK_F 538
 K_VPAEVLTR_WMYTVR_K_GYR_DITYHNRW_HGFNVGQTMFTLLMTGR_IK_K 585
 _YYTDLEAFAMVAAAFCHDIDHR_GTNNLYQMK_SAAPLAK_LHGSSILERHHLE 636
 YSK_TLLQDESLNIFQNLNK_RQFETVLHLFEVAIIATDLALYFK_K_R_TMFQK 686
 _IVDAIEK_METEEEAIAK_YISIDPTK_K_EVIMAMMMTGCDLSAITK_PWEVQS 735
 K_VALMVANEFWEQGDLER_TVLQQQPIPMMDR_NK_GDELPK_LQVGFIDFVCT 785
 FVYK_EFSRFHK_EITPMFDGLQNNR_VEWK_TR_ADEYEEK_MK_VIEEQK_K_ 831
 K_EEEAAAK_K_AENAAAGGGGGEDGK_SK_TCIIVL

Figure 10. MS/MS coverage of chicken cone PDE6 alpha' subunit.

Out of 862 amino acids from the protein sequence (GenBank accession number, I50186),

156 amino acids (shown in red) were verified by MS/MS, indicating an 18% protein coverage.

“ ” indicates the probable trypsin cleavage site.

In order to identify the putative rod PDE6 protein band, 70 MS/MS spectra were acquired during a 45 min LC/MS/MS run using data dependent selection of doubly, triply and quadruply charged peptide precursors. Upon inspection of these spectra, 37 low quality MS/MS spectra with ion intensities below 20 counts were discarded. The remaining 33 MS/MS spectra were searched against the non-redundant NCBI protein database using the MASCOT search engine. The search produced 12 matches to tryptic fragments of chicken PDE6 beta-subunit and 9 matches to protein contaminants (keratins, trypsin). In the end, the last 12 good quality MS/MS spectra (with ion intensities of at least 20 counts) were subjected to manual de-novo sequencing and all of them were assigned to chicken rod PDE6 beta peptides. These peptides were not identified by the initial database search because four of them contained oxidized methionines, two were non-tryptic fragments and the other three had more than 1 missed cleavage. In addition, one peptide contained an acrylamide modified cysteine and the N-terminal peptide was shown to be acetylated by the corresponding MS/MS spectra recorded for the doubly and triply charged ions of this peptide. Based on these data we can conclude that chicken rod PDE6 beta was the only major protein contained in the gel band. The protein coverage obtained by MS/MS is shown in Figure 11.

In addition, the Mascot search result indicates that 16 peptides from the putative cone PDE6 protein band are exact matches with chicken cone alpha' subunit (Figure 12). Therefore, we confirmed our earlier observation that the first histone-activated PDE6 activity peak as the chicken cone PDE6.

cGMP-binding kinetics of chicken rod and cone PDE6 holoenzyme

In order to study the cGMP-binding property of bacterially expressed chicken cone PDE6C-GAF domain, we wanted to understand the characteristics of non-catalytic cGMP-binding of chicken PDE6s holoenzymes. We examined cGMP-binding to chicken rod and cone holoenzyme in the rod and cone fractions from the DE52 ion exchange separation of hypotonic extract of chicken retina (Figure 7). cGMP binding to rod or cone PDE6 was measured after immunoprecipitation with an excess amount of ROS-3 monoclonal antibody bound to Protein G agarose beads.

In order to study the binding kinetics, the concentration dependence of cGMP binding to immunoprecipitated rod and cone PDE was measured. [³H]cGMP binds to

MSISEDDVEK_FLDGNPAFAK_QYFEK_K_LK_TESCDDNESEIILFELIQDMQES 50
 INMEK_VVFK_TLR_R_IR_SLIHADR_CSLFMYR_QR_NGTPELATR_LFNIQE 96
 GSTLEECLVSPDCEIIVYPLDLGIVGYVAQTK_K_TMNIK_DVSECPQFSSFVDEL 148
 TDYTTK_SILATPILNGK_DLVAVILAINK_LNGPFFTNSDETLFLK_YLNFASL 199
 NLK_IYHLSYLHNCETR_R_GVLLWSANK_VFEELTDIER_QFHK_AFYTVR_A 247
 YLNCDR_YSVGLLDMTK_QK_EFFDLWPVLLGEVPPYSGPR_TPDGR_EIVFYK_ 296
 VIDYILHGK_EDIK_VIPNPTPDHWALVTGLPTYVAESGFICNIMNAAADEMNF 349
 QEGPLDESGWTIK_NVLSMPIVNK_K_EEIVGVVTTFFNR_K_DGK_PFDEQDETL 398
 MESLTQFLGWSVLNTDITYDK_MNK_LENK_K_DIAQDMVLYHVK_CDK_DEIQEI 447
 LPTR_EK_LGK_EPSECEEEELASILK_EQLPGPTK_FEIYEFR_FSDFDCTELE 496
 LVK_CGIQMYEELGVVK_K_FQIPQEVLR_FVYSVK_GYR_K_ITYHNWR_HG 543
 FNVAQTMFTLLMTGK_LK_R_YYTDLEAFAMVTAALCHDIDHR_GTNNLYQMK_S 593
 QNPLAK_LHGSSILER_HHLDFGK_FLLSEESLNICQNLNR_R_QHEHMIHLMDI 643
 AIIATDLALYFK_K_R_TMFQK_IVDESK_TYDNTTAWTDYLSLETTK_K_EVVM 691
 AMMMTACDLSAITK_PWEVQSK_VALLVAAEFWEQGDLEISVLQQQP

Figure 11. MS/MS coverage of chicken rod PDE6 beta subunit.

Out of 736 amino acids from the protein sequence, 249 amino acids (shown in red) were verified by MS/MS, indicating a 34% protein coverage. “_” indicates the probable trypsin cleavage site.

1 MGEVNKDAVE KYLENNPQFA KEYFDRKMRA **EVLGSIFQVS** PGDVKEGVSF
51 KDMSRLEECN ILFELLTEIQ DEAGSMEKIV HKTLQRLSQL LARDRCSMFI
101 CRSRNGIPEV ATRLNVTPT SKFEDNLVNP DKETVFPDI GIAGWVAHTK
151 **KFFNIPDVKK** NNHFSDYLDK KTYTTVNMN AIPITQGKEV LAVVMALNKL
201 NASEFSKED E VFKKYLNFI SLVLRNHHTS YLYNIESRRS **QMLLWSANKV**
251 **FEELTDIERQ** FHKALYTIRM YLNCERYSVG LLDMTKEKEF YDEWPIRLGE
301 AEPYKGPKP DGREVN FYKI IDYILHGKEE IKVIPTPPAD HWCLISGLPT
351 YVAENGFICN MNAPADEYF TFQKGPVDET **GWVKNVLSL** PIVNKKEEIV
401 **GVATFYNRKD** GKPFDEYDEQ I IETLTQFLG WSVLNTD TYD KMNKLENRKD
451 IAQEMLMYQT **KATPTEVESI** LKYKEKLNVK SIEECDEKDL IRI LKEELPD
501 **PKDLELYEFR** FSDFPVTEHG LITCGIRLFF **EINNVKEFKV** PAEVLTRWY
551 TVRKG YRDIT YHNWRHGENV GQTMFTLLMT GRIKKYYTDL EAFAMVAAAF
601 CHDIDHRGTN NLYQMKSAAP LAKLHGSSIL ERHHLEYSKT **LLODESINIF**
651 **QNLNKRQFET** VLHLFEVAII ATDLALYFKK RTMFQKIVDA IEKMETEEEA
701 IKYISIDPTK KEVIMAMMMT GCDLSAITKP WEVQSKVALM **VANEFWEQGD**
751 **LERTVLQQQP** IPMMDRKNKGD ELPKIQVGF I DFVCTFVYKE FSRFHKEITP
801 MFDGLQNNRV EWKTRADEYE EKMKVIEEQK KKEEEAAAKK AENAAGGGGG
851 GEDGKSKTCI VL

Figure 12. Mass-spec analysis of chicken cone PDE6(IP). 19% coverage.

a single class of high affinity sites on chicken cone PDE6 with a K_d of 25 ± 5 nM (Figure 13). The apparent cGMP-binding affinity to the rod PDE6 is higher than that of cone, with a K_d value of 7 ± 3 nM (Figure 14). To prevent hydrolysis of cGMP by the holoenzyme, the immunoprecipitated PDE was incubated with [3 H]cGMP in the presence of IBMX and EDTA.

Bovine cone PDE6 holoenzyme possesses a single class of high affinity cGMP binding sites with a K_d of 11 nM (Gillespie and Beavo, 1988). Thus, chicken cone PDE6 has very similar non-catalytic cGMP-binding characteristics to bovine cone PDE6. In addition, bacterially expressed chicken cone PDE6 GAF-A/B and GAF-A domains bind cGMP with K_d values around 10-20 nM which is in good accordance with the K_d of the holoenzyme suggesting that the isolated GAF domain proteins are a reasonable model system for studying the cGMP binding properties of the holoenzyme.

Cyclic nucleotide hydrolyzing kinetics of chicken rod and cone PDE6

Using the DE52 rod and cone fractions, both cGMP- and cAMP-hydrolyzing kinetics of chicken rod and cone PDE6 were determined as shown in table 1. The K_m

Figure 13. cGMP-binding to chicken cone PDE6 holoenzyme.

Chicken cone PDE6 holoenzyme was immunoprecipitated using ROS-3 monoclonal antibody bound to Protein G PLUS Agarose resin as described in Methods. The immunoprecipitated enzyme was incubated with cGMP binding buffer in the presence of 2 mM EDTA and 1 mM IBMX, 2 to 600 nM (7.5 Ci/mmol) of ^3H -cGMP in a total volume of 200 μl for 2 hours at room temperature. After incubation, samples were subjected to filter-binding assay. The data shown here are from a single representative experiment that was repeated 3 times. The mean value was 25 ± 5 nM for cone PDE6 (mean \pm SE).

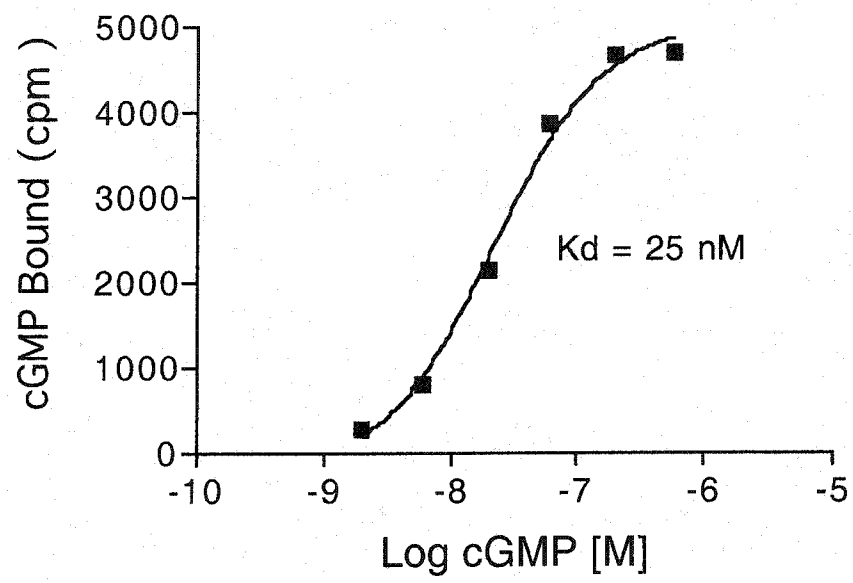


Figure 14. cGMP-binding to chicken rod PDE6 holoenzyme.

Chicken rod PDE6 holoenzyme was immunoprecipitated using ROS-3 monoclonal antibody bound to Protein G PLUS Agarose resin as described in Methods. The immunoprecipitated enzyme was incubated with cGMP binding buffer in the presence of 2 mM EDTA and 1 mM IBMX, 2 to 600 nM (7.5 Ci/mmol) of ^3H -cGMP in a total volume of 200 μl for 2 hours at room temperature. After incubation, samples were subjected to filter-binding assay. The data shown here are from a single representative experiment that was repeated 3 times. The mean value was 7 ± 3 nM for rod PDE6 (mean \pm SE).

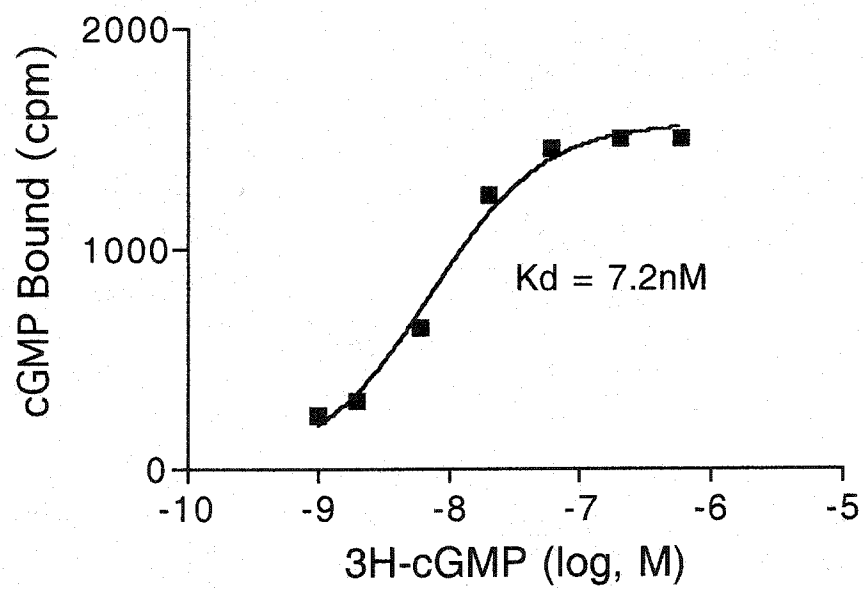


Table1**Apparent Km values of chicken and bovine PDE6 holo-enzymes**

	Chicken		Bovine*	
	Cone	Rod	Cone	Rod
K _{mc} GMP(μ M)	26	29	17	19
K _{mc} AMP(μ M)	717	820	610	ND

* Data from Gillespie et al., 1988

ND = Not Determined

values of histone activated chicken rod and cone PDEs are 29 ± 9 μM and 26 ± 5 μM for cGMP, and 820 ± 34 μM and 717 ± 44 μM for camp, respectively. Both cGMP- and cAMP-hydrolysis kinetics of chicken PDE6s are very similar to that of bovine PDE6s as shown in table 1.

DISCUSSION

Chicken rod and cone PDEs

Chickens are known to have cone-dominated retinas with up to 85% of the photoreceptors as cones (Morris, 1970), whereas most mammals have rod-dominated retinas. The histone-activation and kinetic properties of chicken photoreceptor PDEs suggests that it has similar enzyme characteristics to the photoreceptor PDEs of other species such as bovine, human and frog. In addition, the chicken rod and cone PDE6s separated by ion-exchange column were eluted at the same ionic strength, corresponding to rod and cone PDE6 from bovine retina. Furthermore, compared to bovine rod and cone PDE6s, chicken photoreceptor PDEs have very similar non-catalytic cGMP-binding kinetics and enzyme catalytic kinetics. These results suggest that the basic enzymatic and cGMP-binding characteristic have been greatly conserved between chicken and bovine PDE6s during evolution and that the

regulation and properties of the chicken PDE6 GAF domains are likely relevant to those of mammalian species.

Therefore, because of this conservation of the properties and regulation, the studies on chicken PDE seem likely to be applicable to the PDEs of other species including mammals. Moreover, since the domestic chicken is established as an excellent model system for the study on inherited retinal disease (Semple-Rowland and Lee, 2000), our characterization of chicken PDE may contribute to the investigation of retinal diseases.

Subunit composition of chicken rod PDE6

Rod photoreceptor PDE6s of various species are thought to be composed of a heterodimer containing two distinct major catalytic subunits: the alpha- and beta-subunits. However, mass spectrometry analysis of the enzyme from the second DE52 peak only detected the beta-subunit of rod PDE6. Initially we thought that this was due to the fact that only the alpha' subunit of chicken cone PDE6 and the beta-subunit of chicken rod PDE6 was in the database (Semple-Rowland & Green, 1994; Morin et al., 2001). However, when all of the undefined peaks from the mass spectrometer were sequenced, no peptide homologous only to rod alpha subunit from any other

species was found. Therefore, one must consider the possibility that the rod enzyme in chicken does not contain an alpha subunit and its absence may be a unique characteristic of chicken rod photoreceptor PDE. It is probably worth noting that among all the EST cDNAs in the chicken database, there are none that are most homologous to alpha subunit of any other species. Both observations taken together strongly suggest that the chicken rod enzyme is a homodimer of two beta subunits. The functional significance of this difference in structure remains to be determined.

MATERIALS AND METHODS

Materials

Chicken eyes were obtained from Tyson Company (Little Rock, AK). Bovine eyes were purchased from Schenk Packing Company (Stanwood, WA). [³H]cGMP was obtained from Amersham Pharmacia Biotech. CGMP and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma.

Immuno-cytochemistry study on chicken photoreceptor PDEs

Immediately after sacrifice of the animal, the eyes were removed, and eye cups were immersed and fixed in 4% paraformaldehyde/0.13 M sodium phosphate

(pH 7.4) at 4°C for 6 hr. After fixation, the eye cups were transferred from fixative to 30% sucrose/ 0.13 M sodium phosphate (pH 7.4), and stored overnight at 4°C. The tissues were cryo-sectioned at 12 μ m thickness. The sections were treated with blocking solution (2% horse serum, 1% BSA, 0.3% Triton x-100 in PBS) for 30 minutes at RT. Primary monoclonal antibody ROS-1 and ROS-3 were diluted to 1 μ g/ml with 0.3% Triton x-100 in PBS and sections were incubated overnight at 4°C. Sections were washed three times for 5 minutes each with PBS. The secondary antibody (anti-mouse Cy3) was applied to the sections for 30 minutes at RT. After incubation with secondary antibody, the sections were rinsed in PBS and mounted with 2% DABCO in glycerol.

Isolation and initial purification of chicken rod and cone PDE6 holoenzyme

Chicken retinas were dissected and homogenized in hypotonic buffer (containing 10 mM Tris PH 7.5, 1 mM MgCl₂, 1 mM DTT, and 0.2 mM PMSF) using a dozen motor-driven strokes of a Teflon pestle in a glass homogenizer (Potter-Elvehjem tissue grinder). The homogenate was centrifuged (Beckman) at 100,000 g for an hour. The retinal supernatant was applied to a 75 ml DE52 anion-exchange column and eluted with a linear NaCl gradient (20-300 mM) run at a flow rate of 1

ml/min at 4°C. Sixty 5ml fractions were collected at 4°C and assayed for Histone (type VIII-S)-activated (2.5 mg/ml) phosphodiesterase activity by measuring the release of phosphate (Gillespie et. al., 1988).

cGMP affinity column purification of chicken PDE6 holoenzyme

Two histone-activated PDE6 activity peaks were separated and partially purified by the DE52 anion-exchange chromatography. 35 ml of the first histone-activated PDE6 peak off the ion-exchange column were pooled and loaded on an epoxy-Sepharose cGMP affinity column (Gillespie et al., 1988). The cGMP-binding proteins including chicken photoreceptor PDE were eluted with 1mM cGMP in the presence of 2 mM EDTA and 1 mM IBMX. The presence and purity of chicken photoreceptor PDE was analyzed by silver stain.

Immunoprecipitation of Chicken photoreceptor PDEs

It has been shown that the ROS-3 monoclonal antibody recognizes both chicken rod and cone photoreceptor PDEs by the aforementioned immunocytochemistry study (Figure 6). The putative chicken cone or rod PDE6 holoenzymes corresponding to the first or the second histone-activated PDE6 peaks were

immunoprecipitated using ROS-3 monoclonal antibody bound to Protein G-Sepharose resin. 15 ml of DE52 fraction of either chicken rod or cone PDE was mixed with 500 μ l antibody resin (500 μ g antibody) in the presence of 150 mM NaCl. After mixing in the cold room overnight, the resin was pelleted and washed 3 times with 1 ml of 10 mM Tris pH 7.5, 1 mM $MgCl_2$, 300 mM NaCl.

Identification of chicken rod and cone PDE6 using mass spectrometry

On-line nano-LC/ESI-MS/MS experiments were performed on an API-US QTOF mass spectrometer (Micromass, UK) equipped with the CapLC system (Waters, Milford, MA). The stream select module was configured with an OPTI-PAK Symmetry300 C_{18} trap column (Waters, Milford, MA) connected in series with a nanoscale analytical column (75 μ m i.d. x 15 cm, packed with 3.5 μ m, XTerra MS C_{18} particles (Waters)).

The eluate off the cGMP-affinity column was concentrated to 100 μ g/ml by Centriprep centrifugal filter (Millipore) and then digested with 5 μ g/ml trypsin. The immunoprecipitated proteins were separated by SDS-PAGE and stained with Coomassie blue. The protein bands corresponding to the molecular weight of rod and cone PDE6 were sliced out and digested with trypsin (5 μ g/ml) for analysis by mass

spectrometry (Shevchenko et al., 1996). Protein digests (5 μ L) were injected onto the trap column (Waters, Milford MA) at 10 μ L/min, desalted and back-flushed to the analytical column at 0.5 μ L/min using gradient elution. The gradient consisted of 5-50% solvent B in 30 min, followed by 50% B for 15 min and 50-90% B in 5 min (A= 5% acetonitrile, 0.1% formic acid; B= 95 % acetonitrile, 0.1% formic acid).

QTOF parameters were set as follows: the electrospray potential was set to 3.5 kV, the cone voltage set to 60 V, the extraction cone set to 2V, the source temperature set to 80°C. The MS survey scan was m/z 400-1600 with a scan time of 1 sec and the collision energy set to 10 eV. When the intensity of a peptide peak rose above a threshold of 20 counts, tandem mass spectra were acquired using the data-dependent algorithm implemented in the MassLynx acquisition software. For operation in the MS/MS-mode, the scan time was increased to 2 s, the isolation width was set to include the full isotopic distribution of each peak (3 Da) and the collision energy was set to 15-25 eV. MS/MS spectra were recorded for the doubly and triply charged molecular ions of tryptic peptides. All MS/MS spectra were searched against the non-redundant NCBI protein database by using MASCOT (Perkins et al., 1999) assuming a mass tolerance of 0.3 Da for both the precursor and the fragment ions.

cGMP-binding Kinetics study on chicken rod and cone PDE6 holoenzyme

Chicken cone PDE6 holoenzyme was immunoprecipitated using ROS-3 monoclonal antibody bound to Protein G-Sepharose resin. 1 to 2 ml of DE52 fraction of chicken rod or cone PDE was mixed with 100-200 μ l antibody resin (100-200 μ g antibody) in the presence of 150 mM NaCl. After mixing in the cold room overnight, the resin was pelleted and washed 3 times with 1 ml of 10 mM Tris pH 7.5, 1 mM $MgCl_2$, 300 mM NaCl.

PDE immunoprecipitates were incubated with 5 mM Tris pH 7.5, 25 mM NaCl, 2 mM EDTA, 1 mM IBMX, 0.1 mg/ml bovine serum albumin, 2-600 nM $7.5Ci/mmol$ 3H -cGMP (200 μ l total volume) for 2 hours at room temperature. 3H -cGMP bound to immunoprecipitated PDE was separated from free ligand by filtration on a Millipore Filter as described above. The Millipore filters were dissolved in Filter-count complete LSC-cocktail (Packard BioScience) over night and counted in a Packard 1600 TR liquid scintillation analyzer.

Enzyme catalytic activity study on chicken rod and cone PDE6 holoenzyme

Both cGMP- and cAMP-hydrolysis kinetics of rod and cone PDE6 were determined using rod and cone fractions from the ion-exchange chromatograph. The

enzyme kinetics were determined using ^3H -cGMP (1 - 250 μM) and ^3H -cAMP (50 - 2500 μM) as hydrolysis substrate of PDE6.

CHAPTER 3**MOLECULAR DETERMINANTS FOR CYCLIC NUCLEOTIDE BINDING
TO THE GAF DOMAINS OF PHOSPHODIESTERASE 6****SUMMARY**

Like other PDE6s, the chicken cone enzyme possesses two GAF domains, GAF-A and GAF-B. Sequence homology alignment between the GAF domains of PDE2, 5 and 6 suggests that GAF-A is the non-catalytic cGMP binding site of PDE6. Based on 3D-modeling of the GAF-A domain of chicken cone PDE6 with the crystal structure of mouse PDE2A GAF-B domain, residues making side-chain contact with cGMP were identified. Mutagenesis of these residues indicated that the overall architecture of the GAF cGMP-binding pocket is largely conserved between PDE2, 5 and 6. Residues F123, D169, T172, T176 are critical for the binding of cGMP and their corresponding alanine mutants lost the ability to bind cGMP completely. Three of the 4 crucial residues map to the H-4 helical structure of the GAF-a domain. It is speculated that this region is a necessary structure unit for cGMP binding. Furthermore, residue E124 of ggPDE6 GAF-A corresponds to D439 of mmPDE2A GAF-B based on the homology model. Residue E124 appears to contribute to the

cGMP/cAMP selectivity of PDE6 GAF domain as residue D439 does to the nucleotide specificity of PDE2.

INTRODUCTION

GAF domains

Although more than 1600 GAF-containing proteins have been identified so far (Aravind and Ponting, 1997; Anantharaman et al., 2001), the ligands for most GAF binding domains remain unknown. The first members of GAF domains discovered in the cGMP-regulated phosphodiesterases, such as PDE2, PDE5 and PDE6, have high affinity binding sites for cGMP. Recently, the GAF-B domain of cyanobacterial adenylyl cyclase (cybB1) has been reported to bind cAMP specifically (Kanacher et al., 2002).

Among cGMP-binding PDEs, PDE2 is the first one with a well-determined role for the GAF domain in regulation of its catalytic activity. The cAMP hydrolytic activity of PDE2 is greatly stimulated by cGMP binding to its GAF domain (Martins et al., 1982; Stroop and Beavo, 1992). In addition, recent reports on PDE5 have shown that cGMP binding to its GAFa domain increases PDE5 catalytic activity and potentiates phosphorylation at an N-terminal serine (Rybalkin et al., 2003; Corbin et

al., 2003). Similarly, the activity of cyanobacterial adenylyl cyclase (cyaB1) is greatly increased upon cAMP binding to its GAFb domain. The cAMP occupation of the cyaB1 GAFb domain results in a rapid exponential activation of its adenylyl cyclase catalytic activity since cAMP is the product of cyaB1 adenylyl cyclase catalytic reaction (Kanacher et al., 2002). Interestingly, when the cAMP-binding GAF-A/B domain of cyaB1 is replaced by the cGMP-binding GAF-A/B domain of rat PDE2, the adenylyl cyclase activity of this chimeric protein was stimulated by cGMP binding. This result suggests that the basic mechanism of cyclic nucleotide binding to GAF domains and subsequent stimulation of catalytic activity are conserved through evolution. Furthermore, this mechanism of GAF domain regulation of adjacent catalytic domains may be applicable to many GAF domain-containing proteins.

GAF domain of PDEs (cGMP affinity and specificity)

Among GAF-domain-containing PDEs, PDE2, 5 and 6 share a conserved “**S X(13-18) FD X(18-22) IA X(21) [Y/N] X(2) VD X(2) T X(3) T X(19) [E/Q]**” motif that has been proposed to be the main feature of a cGMP-binding domain (Martinez et al., 2002). In PDE2, it appeared that the residues that interact with the phosphate-ribose ring are essential for cGMP binding, and the residues that interact with the

position where cAMP and cGMP are different should control cyclic nucleotide specificity. Many of these residues are conserved between the GAF domains of PDE2, 5 and 6. Mutagenesis in this motif in PDE5 suggested that the Phe in the FD dyad of PDE5 GAF-A domain is essential for cGMP binding (Sopory et al., 2003). A similar finding has also been shown in the PDE2A GAF-B domain (Wu et al., 2004). Eight of the 11 residues in mouse PDE2A, (i.e. S424, F438, D439, V484, D485, T488, T492 and E512) contact cGMP via side chains. Based on the sequence homology alignment, we predict that the conserved residues of PDE6 within the cGMP-binding motif are the key residues coordinating the high affinity binding to cGMP.

cGMP binding to GAF domains of PDE2 and 5 were demonstrated to be able to stimulate their own phosphodiesterase catalytic activity (Martins et al., 1982; Rybalkin et al., 2003, Corbin et al., 2003). However, so far, no data suggests direct activation of PDE6 catalytic activity by cGMP binding to its GAF domain. Among GAF domain-containing PDEs, PDE6 has the highest catalytic activity among cGMP-hydrolyzing PDEs. In addition, the PDE6 GAF domain has high binding affinity and specificity for cGMP (Martins et al., 1982; Gillespie et al., 1988; Gillespie and Beavo, 1989). By using cGMP analogs, Cote's group suggested that interaction

between GAF domain residues and the N-1/O-6 region of the purine ring of cGMP determine the specificity of cGMP binding of PDE6 (Hebert et al., 1998). This proposition is confirmed by the crystal structure of the GAF domain of mouse PDE2A. Indeed, Asp439 of mouse PDE2A, located in the GAF-B cGMP-binding pocket, forms hydrogen bonds between its main chain NH and O6, and between the side chain carboxylate and the N1 guanine base of cGMP (Martinez et al., 2002). The interaction between Asp439 of PDE2A and cGMP molecule provides positive selectivity for cGMP versus cAMP. It has been shown that cAMP has very little if any binding affinity to the non-catalytic cGMP-binding site of PDE6 (Hebert *et al.*, 1998). Based on the sequence homology alignment between the GAF-B of mouse PDE2A and GAF-A of chicken PDE6C, residue E124 of PDE6C GAF-A corresponds to D439 of PDE2A GAF-B and was predicted to contribute to cGMP binding specificity.

RESULTS

3D-modeling of chicken cone PDE GAF-A domain (GAF domain homology between PDE6C and PDE2A)

The crystal structure of mouse PDE2A GAF domains showed that GAF-B but not GAF-A binds cGMP (Martinez et al., 2002). The sequence alignment between GAF-A and GAF-B domains of chicken PDE6C to GAF-B of mouse PDE2A suggests that the GAF-A of PDE6 has much better homology and is most likely to be the cGMP binding site (Figure 15).

A homology alignment between GAF-A of chicken PDE6C and GAF-B of mouse PDE2A indicates that the basic architecture of the cGMP-binding pocket is conserved. In GAF-A of PDE6C, out of eight predicted contacting side chains, five are identical to that of GAF-B of PDE2A (Figure 15). In addition, D439 of mouse PDE2A GAF-B is conserved as E124 in chicken PDE6C GAF-A.

3-D structure modeling of chicken cone PDE6 GAF-A was based upon the crystal structure of PDE2A GAF-B domain (Figure 16). Because of the gaps generated by the homology alignment of the two GAF sequences, two extra insertions

Figure 15. Sequence homology alignment between chicken cone PDE6 GAF-A domain and mouse PDE2A GAF-B domain.

The homology alignment was performed using ClustalW (Thompson et al., 1994). The black arrows indicate the 6 conserved residues in close contact with cGMP. Two gaps between the homology sequence are underlined in black. The conserved residues of the H4 helix are underlined in red.

Figure 16. Three-dimensional model of chicken cone PDE6 GAF-A cyclic GMP binding pocket based on the crystal structure of mouse PDE2A GAF-B.

The model was produced using the Swiss-Model program (Guex et al., 1997).

The green ribbon indicates the location of the H4-helix of chicken cone PDE6 GAF-

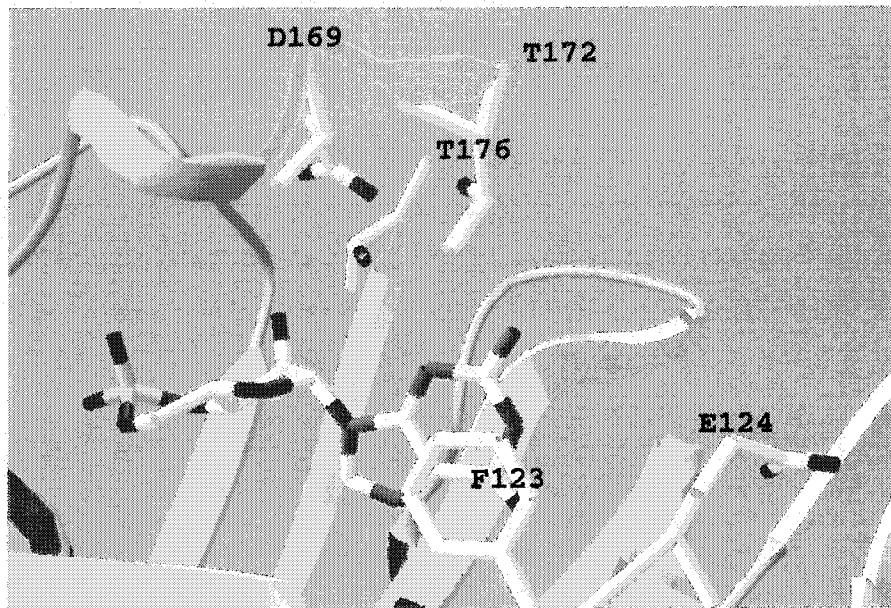
A. The model illustrates the position of the residues altered by mutagenesis

including, the three conserved cGMP-contacting residues located on Helix 4 (D169,

T172 and T176). These residues are oriented towards cGMP and could potentially

form hydrogen bonds directly or through a water molecule with cGMP. The phenyl

ring of F123 base-stacks with the guanine ring of cGMP.



are introduced as solvent-exposed loops in the 3D modeling structure of chicken cone PDE6 GAF-A. The first insertion resides between the first α -helical and the first β -sheet strand. The second insertion locates between the first β and second β strand. Neither of the two insertions interrupts the secondary structure of the 3D model. The overall folds of the modeling structure of chicken cone PDE6 GAF-A domain are very similar to that of mouse PDE2A GAF-B. Furthermore, the locations of the cGMP-contacting residues are highly conserved within the cGMP-binding pocket of chicken cone PDE6 GAF-A. Based on the 3D model structure of GAF-A, potential hydrogen bonding between GAF-A and cGMP has been proposed (Figure 17).

GAF-A domain is the cGMP-binding site

Three chicken cone PDE6 GAF domains, GAF-A, GAF-B and GAF-A/B, were successfully expressed as soluble proteins in *E.coli* (Figure 18). These GAF domain proteins could be purified by Talon resin and gel filtration HPLC to achieve >98% purity (Figure 19 & 20). The cGMP-binding kinetics studies on purified GAF domain proteins showed that both GAF-A/B and GAF-A possess high binding affinity to cGMP with K_d values of 10-20 nM (Figure 21 & 22). However, as

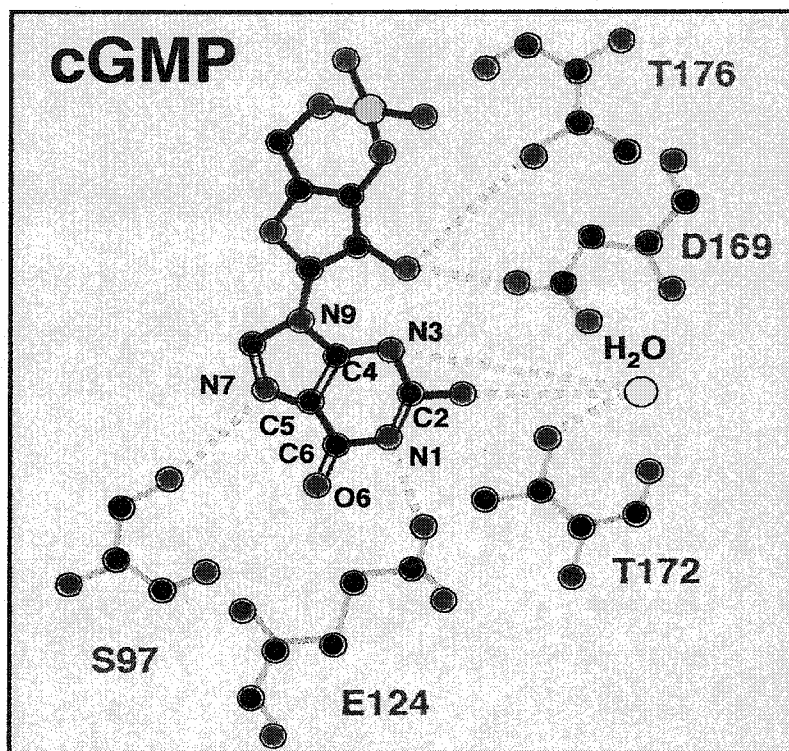


Figure 17. LigPlot showing probable hydrogen bond interactions with cGMP.

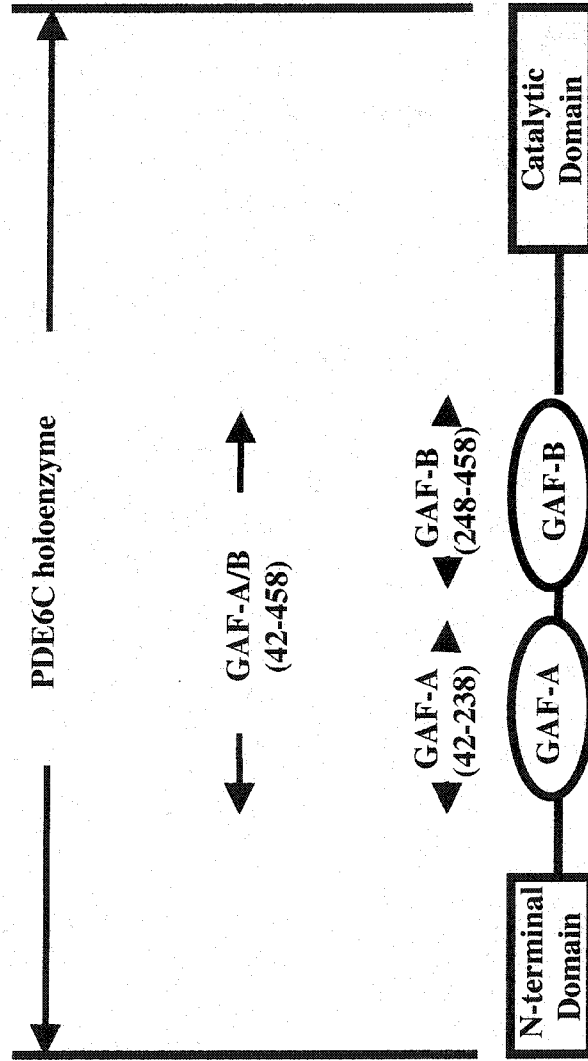


Figure 18. Schematic diagram of PDE6C holoenzyme and GAF domain proteins.

Schematic diagram showing the various constructs generated in this study. The numbers in parentheses are the residue boundaries of the individual PDE6C GAF domain constructs.

Figure 19. Superose-12 HPLC purification of chicken cone PDE6 GAF-A.

Partially purified chicken cone PDE6 GAF-A domain protein (from the Talon affinity resin) was applied to a Superose-12 HPLC sizing column. The column was run at 0.5 ml/min and 0.5 ml fractions were collected. The major protein peak eluted at an apparent molecular weight of about 50KDa corresponding to a GAF-A dimer (assuming a globular shape).

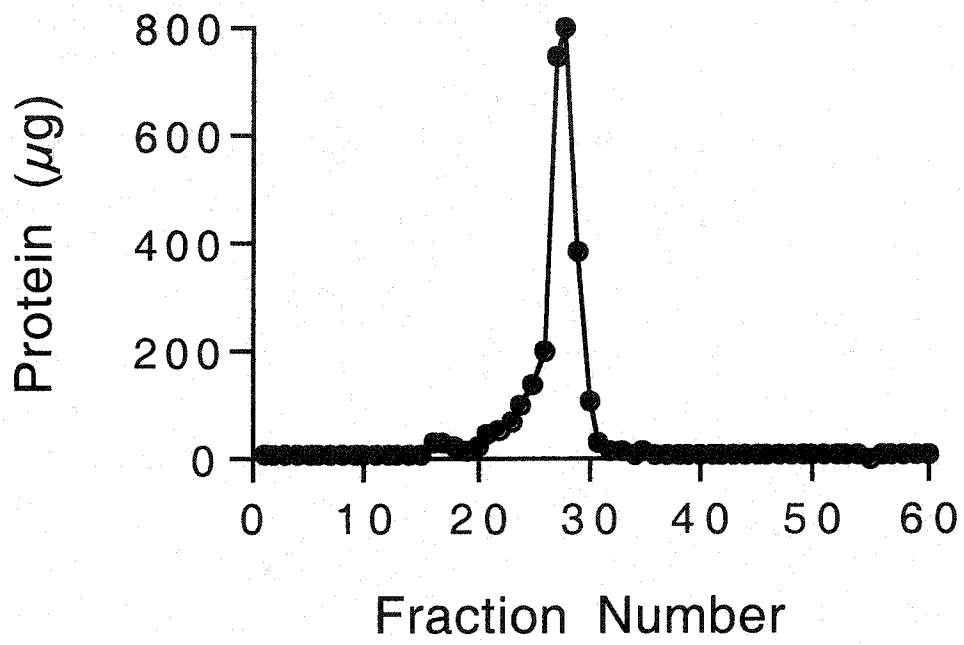


Figure 20. SDS-PAGE of Superose-12 purified chicken cone PDE6 GAF-A.

Chicken cone PDE GAF-A domain protein was purified by Talon resin and subsequent Superose-12 sizing column. Superose-12 fractions 20 to 32 corresponding to the GAF-A protein peak were analyzed by SDS-PAGE and Coomassie Blue stain. The purity of GAF domain proteins were > 98% as quantified using NIH Image.

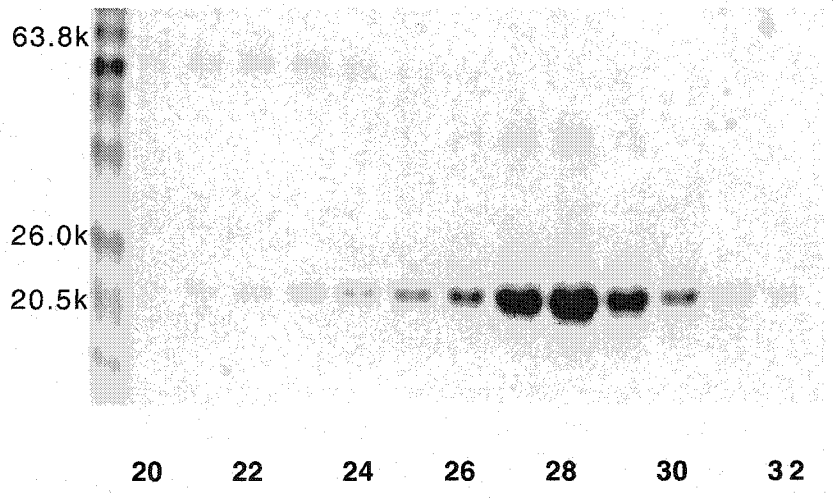


Figure 21. cGMP binding to chicken cone PDE6 GAF-A.

cGMP-binding competition assays were carried out on chicken cone PDE6 GAF-A protein. 1 nM GAF protein was incubated with 1 nM ^3H -cGMP and various amounts of unlabeled cGMP ranging from 2 nM to 600 nM. The K_d value (determined as IC_{50} s) for cGMP-binding to GAF-A was 21 ± 4 nM ($n=7$). Value is given as the mean \pm SE.

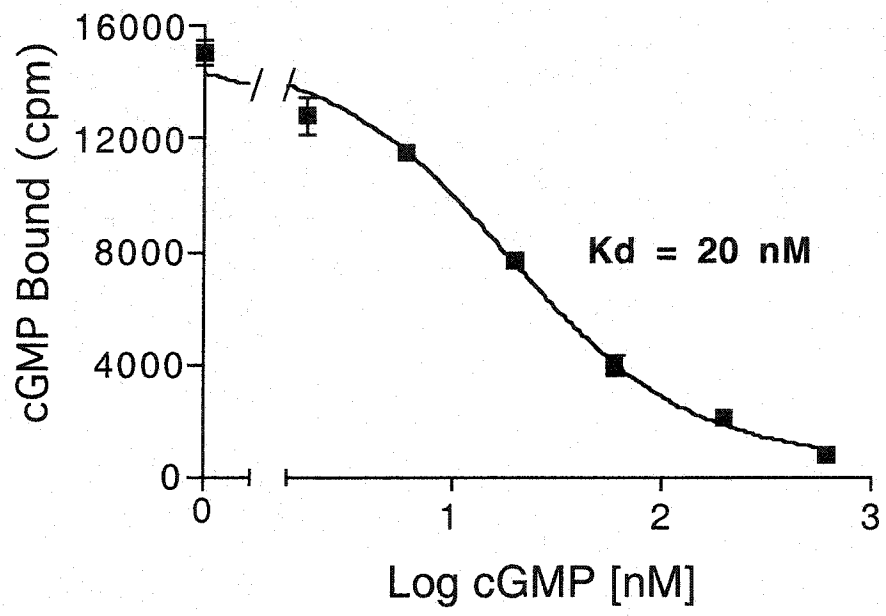
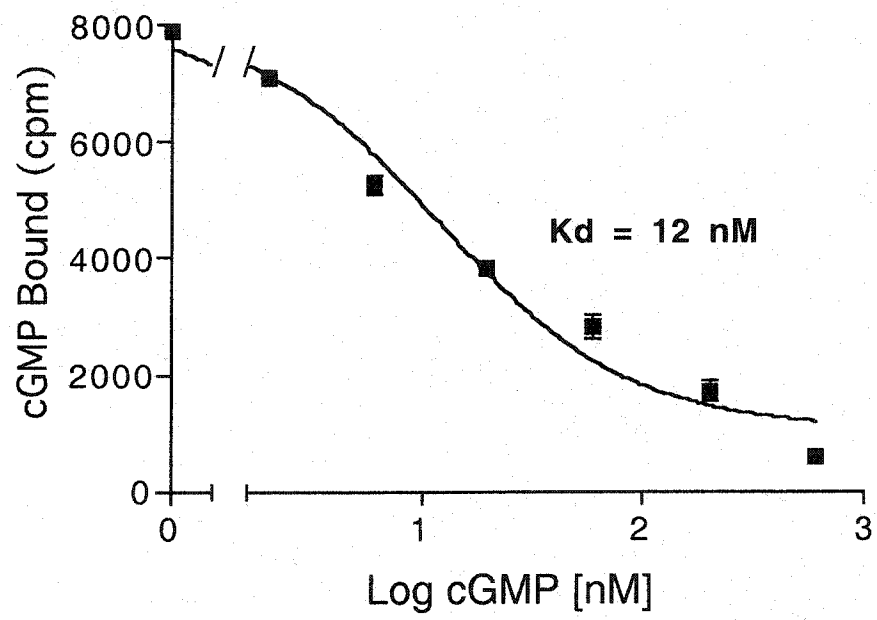


Figure 22. cGMP binding to chicken cone PDE6 GAF-A/B.

cGMP-binding competition assays were carried out on chicken cone PDE6C GAF-A/B proteins. 1 nM GAF protein was incubated with 1 nM ^3H -cGMP and various amounts of unlabeled cGMP ranging from 2 nM to 600 nM. The K_d value (determined as IC_{50} s) for cGMP-binding to GAF-A/B was 10 ± 3 nM ($n=5$, as the mean \pm SE).



predicted, the GAF-B domain does not bind to cGMP (data not shown). Thus, GAF-A domain appears to be the only high affinity cGMP-binding site of chicken cone photoreceptor PDE.

cGMP dissociation rate of chicken cone PDE6C GAF-A domain

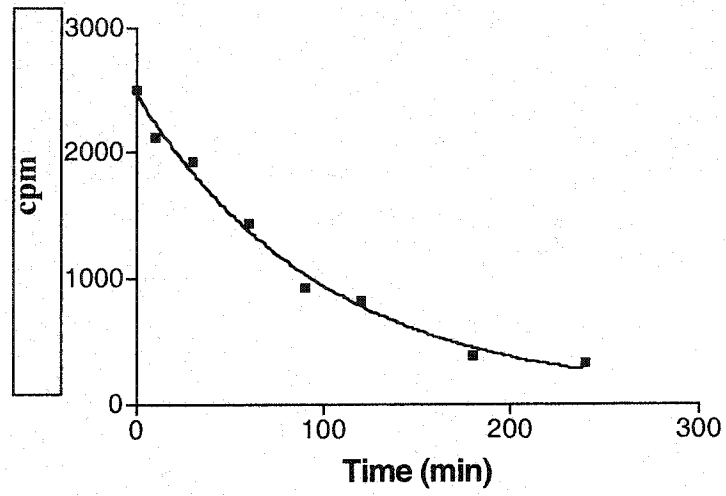
It has been reported that the cGMP dissociation rate for bovine cone PDE6 is very slow. The time required for half of the cGMP to dissociate ($t_{1/2}$) at 4°C and 37°C was 10 hours and 21 minutes, respectively (Gillespie, 1998). In order to characterize cGMP binding properties of chicken cone PDE6C GAF domain, the cGMP dissociation rates of GAF A domain were studied at both room temperature and 0°C. Similar to bovine cone PDE6, the off-rate of cGMP-binding to chicken cone PDE6 GAF-A is extremely slow at 0°C with $t_{1/2}$ as 12 hours (Figure 23B). At room temperature, $t_{1/2}$ is 69 minutes (Figure 23A). Because of the exceptionally slow cGMP dissociation rate at 0°C of GAF-A, all the cGMP-binding experiments were carried out at room temperature for more than 2 hours to reach ligand-binding equilibrium.

Figure 23. cGMP dissociation rate for chicken PDE6C GAF-A.

10 nM PDE6C GAF-A protein was incubated with 100 nM ^3H -cGMP for 2 hours either at room temperature (A) or 0°C (B). After incubation, samples were filtered at the indicated times after adding in excess (200 μM) unlabeled cGMP.

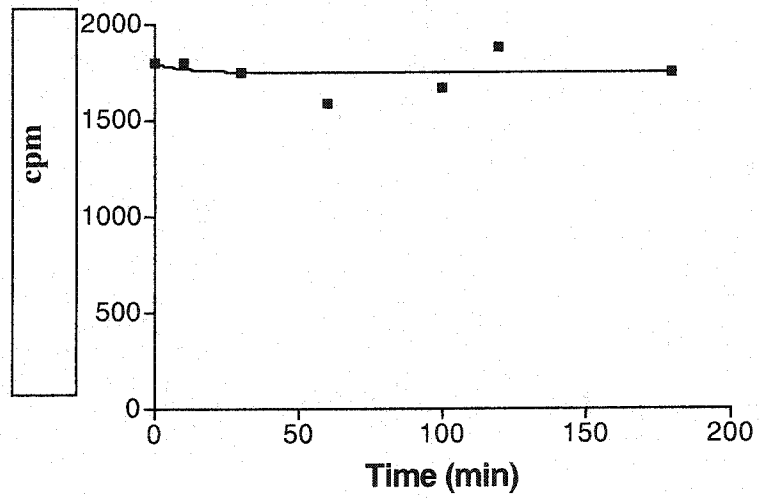
A

Off-rate of cGMP-binding to gg6C-GAFa



B

off-rate of cGMP-binding to gg6C-GAFa (0°C)



Conserved architecture between the cGMP-binding pockets of PDE GAF domains

There are 8 residues in the mouse PDE2 GAF-B domain making side chain contact with the cGMP molecule. In the GAF-A domain of chicken cone PDE6, 6 of these 8 residues are conserved as S97, F123, E124, D169, T172 and T176 (Figure 15). All three cGMP-contacting residues D169, T172 and T176 located at the helix α -4 (H4) are identical between PDE2 GAF-B and PDE6 GAF-A domains (Figure 15).

Mutagenesis studies were carried out on all 6 conserved amino acid residues predicted to make contact with cGMP at the cGMP-binding pocket. Point mutation to alanine was introduced into these 6 residues. All of these mutations have negative effects on cGMP-binding kinetics as predicted (Figure 24; Table 2). Both S97A and E124A mutants decrease the cGMP-binding affinity about 5 fold (Figure 24).

In mouse PDE2A, the side chain of F438 is base stacked with the guanine ring of the cGMP molecule through π - π interaction. F123 of chicken PDE6C corresponds to F438 of mouse PDE2A. The 3D-model structure suggests that the phenyl ring of F123 base-stacks upon the guanine ring of cGMP in close proximity (Figure 16). When F123 was mutated to alanine or tryptophan, the cGMP-binding capability was

Figure 24. cGMP-binding to chicken cone PDE6 GAF-A/B wild-type, S97A mutant and E124A mutant.

cGMP-binding competition assays were carried out as in figure 4: wild-type (closed squares) and two mutants, S97A (closed triangles) and E124A (closed circles). Figures are plotted using Prism4 constraining the 100% and 0% binding points. A representative experiment is shown. The experiment was repeated 3 times and the mean values \pm SE were 57 ± 9 nM for E124A, 63 ± 7 nM for S97A, and 10 ± 3 nM for WT.

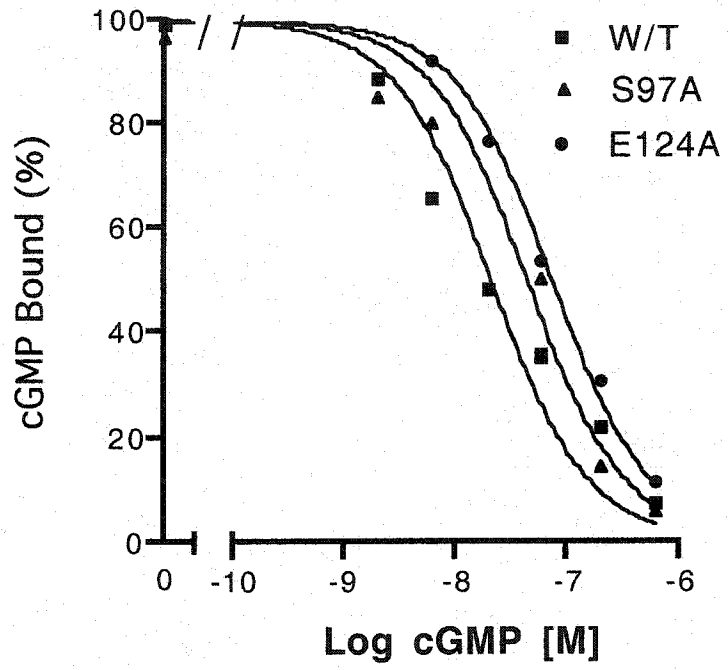


Table 2. Effects of mutation of residues lining the cGMP binding pocket on cGMP affinity.

Column 2. Apparent K_d value for each construct, including wild-type and mutants, was derived from at least 3 individual cGMP-binding experiments with duplicate points and expressed as Mean±SE. Column 3. Amino acid in corresponding position in the mouse PDE2A GAF-B crystal. CZ: Carbon-zeta; OD: Oxygen-delta; OG: Oxygen-gamma. Column 4. The distances between cGMP and the amino acid side-chains of mouse PDE2A GAF-B domain.

AA residue change	K _d for cGMP (nM)	Bond W/ cGMP	Distance (Å)
GgPDE6c-GAFab W/T	10±3		
Guanine Ring Interacting Residues			
F1 23 A	NB	CZ	3.2
F1 23 W	NB	CZ	3.2
F1 23 Y	38±5	CZ	3.2
E1 24 A	57±9	OD1 or 2	2.7
T172 A	NB	OG1	3.2
Phosphate/Ribose or Imidazole Ring Interacting Residues			
S97 A	63±7	OG	2.7
D169 A	NB	OD2	2.9
T176 A	NB	OG1	2.8

For W/T GAFab, n=5; For mutants, n=3.

NB = No Binding

undetectable up to 100 μM ^3H -cGMP (Table 2). However, when F123 was mutated to tyrosine, the cGMP-binding K_d was about 40 nM (Table 2), very similar to the wild-type protein. Thus, it demonstrated that a π - π interaction between the phenyl ring of F123 and the guanine ring of cGMP is likely to be one factor for stabilizing cGMP-binding to chicken cone PDE6 GAF domain since the tyrosine mutant could still form a π - π interaction.

Helix α -4 (H4) structural unit and cGMP-binding

So far, there are two published GAF domain structures elucidated by X-ray crystallography, mouse PDE2A and yeast YKG9 protein GAF domains (Ho et al., 2000). The GAF domain of YKG9 has no cGMP-binding capability. The overall folds of the GAF domains are very similar. However in PDE2A, between β strands 4 and 5, there is an additional helix structure (H4) which does not exist in the YKG9 GAF domain.

Three amino acid residues, D485, T488 and T492 located in the H4 helical region of mouse PDE2A GAF-B domain make side chain contact with bound cGMP. All three residues are conserved in the GAF-A domain of PDE6A, B, C and PDE5

ggPDE6C-GAFa H4 : SDYLDKKTGYTTVN
btPDE6A-GAFa H4 : CDFVDTLTEYQTKN
btPDE6B-GAFa H4 : SSFADELTDYVTRN
btPDE6C-GAFa H4 : SDFMDLQTGYVTRN
mmPDE2A-GAFb H4 : YRGVDDSTGFRTRN
* * *

Figure 25. Sequence alignment of the H4 helical strand of cGMP-binding GAF domains.

The H4 helical structure units, the GAF-A domains of chicken PDE6C, bovine PDE6A, 6B, 6C, and the GAF-B domain of mouse PDE2A, were aligned. The * indicates the conserved Asp and two Thr amino acid residues in the H4 structure unit.

(Figure 25). The corresponding residues in the chicken cone PDE6 GAF-A domain are D169, T172, T176. When these residues were mutated to alanine, they lost their ability to bind cGMP (Table 3). Alanine mutations were introduced into the residues, S165, D166, K170 and T175, that are also located in the H4 helical structure but are oriented away from the binding pocket, and can not make contact with cGMP. These alanine mutants, S165A, D166A, K170A, T175A, retain most of the binding activity of wild-type GAF protein (Table 3). Even though these residues are in close proximity to the cGMP interacting amino acids. Based on the above observations, we propose that the H-4 helical structural unit is essential for cGMP binding and the interaction between the 3 amino acid side chains and cGMP molecule is necessary for the binding.

Furthermore, when T172 and T176 were mutated to either serine or valine, both valine mutants, T172V and T176V, lost cGMP-binding completely. However, the serine mutants, T172S and T176S, retained the ability to bind cGMP comparable to the wild-type GAF domain protein (Table 2). These observations suggest that the hydroxyl group of T172 and T176 are essential for their interaction with cGMP, and the hydroxyl group might form a hydrogen bond with N2 or N3 of the cGMP guanine

Table 3. Binding of cGMP to mutants on the H4 helix of GAF-A.

Column 2. Apparent K_d value for each construct, including wild-type and mutants, was derived from at least 3 individual cGMP-binding experiments with duplicate points and expressed as Mean±SE.
 Column 3. Amino acid in corresponding position in the mouse PDE2A GAF-B crystal. CZ: Carbon-zeta; OD: Oxygen-delta; OG: Oxygen-gamma. Column 4. The distances between cGMP and the amino acid side-chains of mouse PDE2A GAF-B domain.

AA residue Change	K _d for cGMP (nM)	Bond W/ cGMP	Distance (Å)
GgPDE6C-GAFab W/T	10±3		
Residues Interacting Directly With cGMP			
D169A	NB	OD2	2.9
T172A	NB	OG1	3.2
T176A	NB	OG1	2.8
Residues Not Interacting Directly With cGMP			
S165A	103±11		
D166A	178±27		
K170A	70±12		
T175A	79±9		

NB = No Binding

ring similar to what has been demonstrated in mouse PDE2A GAF-B (Martinez et al., 2002).

Cyclic nucleotide selectivity of the GAF domain of chicken cone PDE6

In contrast to the high affinity binding to cGMP, the chicken cone PDE6 GAF domain has extremely low affinity for cAMP with a K_d value of 30 ± 7 mM (Figure 27). cGMP and cAMP are composed of phosphate-ribose and purine base moieties. cGMP and cAMP differ at their purine base and have different hydrogen bonding potential in the C-2 and C-6 positions of the purine ring. We speculate that the residues of the PDE6 GAF domain that determines cyclic nucleotide specificity work at least in part by binding to the guanine ring of cGMP. Based on the 3D-model structure of chicken PDE6C GAF-A domain, three residues, F123, E124 and T172, are predicted to make contact with the guanine ring of cGMP.

The crystal structure of mouse PDE2A GAF-A/B suggests that Asp439 provides a positive specificity for cGMP binding due to the hydrogen bonds between its main chain NH, side chain carboxylate of aspartic acid and the O6, N1 of the guanine base of cGMP (Martinez et al., 2002). Residue E124 of chicken PDE6C

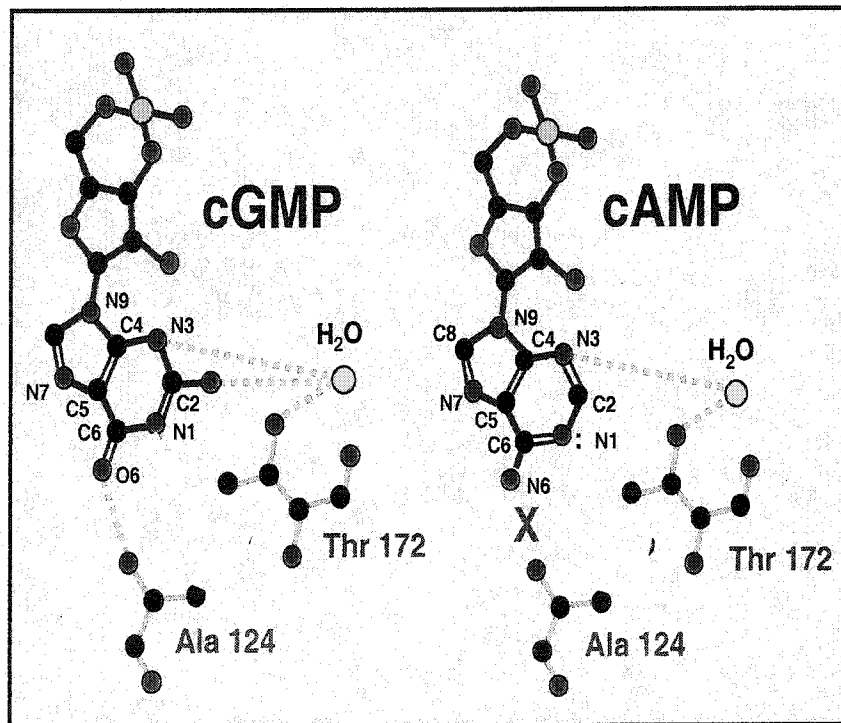


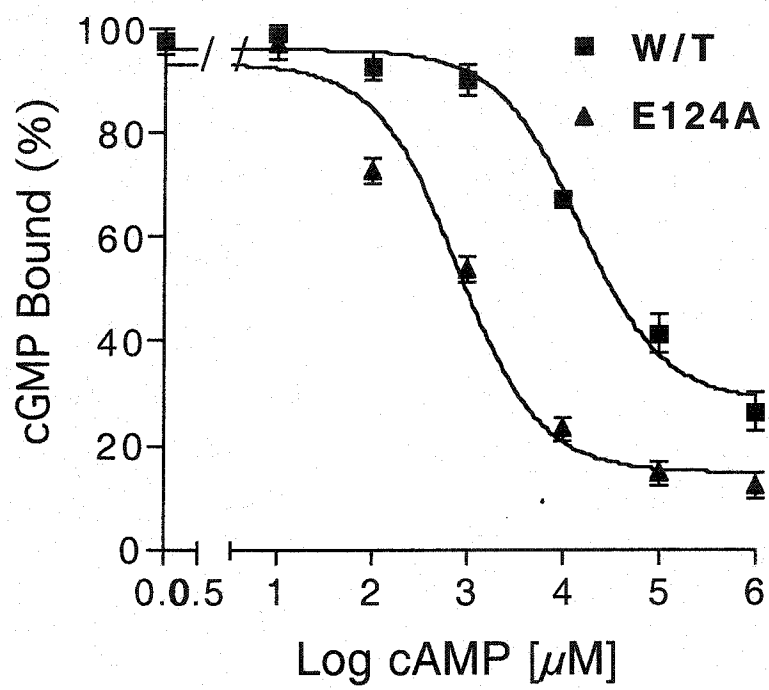
Figure 26. Hypothesis for purine selectivity by the GAF-A domain on PDE6C.

cGMP and cAMP are modeled into the binding site of PDE6 GAF-A with potential hydrogen bonds. Two panels with a proposed hydrogen binding of E124A showing the loss of hydrogen bonds and steric clashes with the amino group of C6.

Figure 27. cAMP-binding of GAF-A wild-type and E124A mutant.

cAMP-binding competition assays were carried out on chicken cone PDE6 GAF-A wild-type and E124A mutant proteins. 1 nM GAF protein was incubated with 1 nM ³H-cGMP and various amount of unlabeled cAMP ranging from 1 uM to 100 mM. The IC₅₀ values (mean ± SE) for cAMP-binding were 35 ± 11 mM (n=3) and 1.0 ± 0.3 mM (n=4) for the wild-type GAF-A and E124A mutants respectively.

Figures were plotted as described in figure 5.



GAF-A corresponds to D439 of mouse PDE2A GAF-B and conserves both the main chain NH group and the side chain carboxylate group. Thus, E124 of chicken PDE6C GAF-A was predicted to contribute to the cGMP-binding specificity of PDE6C.

Indeed, the alanine mutant E124A produces a 5-fold decrease in cGMP affinity and 30-fold increase in cAMP affinity. The changes in cGMP and cAMP affinity of E124A are compatible with the 3D-model structure of ggPDE6C GAF-A based on its sequence homology alignment with mmPDE2A GAF-B. In this model of ggPDE6C GAF-A, the hydrogen bonding between the side chain carboxylate of E124 and the N1 of the guanine base of cGMP was eliminated by alanine mutation (Figure 26). Meanwhile, the E124A mutation removed a clash between the polar side chain of E124 and the C-6 amino group of cAMP (Figure 26). Thus, the removal of negative interaction with cAMP increases the binding-affinity of E124A mutant to cAMP.

DISCUSSION

cGMP and cAMP are composed of phosphate-ribose and purine base moieties. cGMP and cAMP differ at their purine base and have different hydrogen bonding potential in the C-2 and C-6 positions of the purine ring. We speculate that the residues of PDE6 GAF domain that determine cyclic nucleotide specificity bind to

the guanine ring of cGMP. Based on the 3D-model structure of chicken PDE6C GAF-A domain, three residues, F123, E124 and T172, are predicted to make contact with the guanine ring of cGMP.

By using cGMP analogs, Cote's group suggested that interactions between GAF domain residues and the N-1/O-6 region of the purine ring of cGMP determine the specificity of cGMP binding of PDE6 (Hebert et al., 1998). This proposition is confirmed in part by the crystal structure of the GAF domain of mouse PDE2A. Indeed, the Asp439 of mouse PDE2A, locating in the GAF-B cGMP-binding pocket, forms hydrogen bonds between its main chain NH and O6, and between the side chain carboxylate and the N1 guanine base of cGMP (Martinez et al., 2002). The interaction between Asp439 of PDE2A and the cGMP molecule provides positive selectivity for cGMP versus cAMP.

It has been shown that cAMP has very little if any binding affinity to the non-catalytic cGMP-binding site of PDE6 (Hebert et al., 1998). The same observation was obtained with our bacteria-expressed chicken cone PDE6 GAF proteins. Residue E124 of chicken PDE6C GAF-A corresponds to D439 of mouse PDE2A and was predicted to contribute to cGMP binding specificity. Indeed, alanine mutant of E124 had a 5-fold decrease in cGMP affinity and 30-fold increase in cAMP affinity. The

changes in cGMP and cAMP affinity of E124A are compatible with the 3D-model structure of ggPDE6C GAF-A based on its sequence homology alignment with mmPDE2A GAF-B. In this model of ggPDE6C GAF-A, the hydrogen bonding between the side chain carboxylate of E124 and the N1 of the guanine base of cGMP was eliminated by alanine mutation (Figure 26). Meanwhile, the E124A mutation removed the clash between the polar side chain of E124 and the C-6 amino group of cAMP (Figure 26). Thus, the removal of a negative interaction with cAMP increases the binding-affinity of the E124A mutant to cAMP.

Although the E124A mutant has about 30 fold increased affinity to cAMP, its binding affinity to cAMP is still more than thousand fold lower than that to cGMP. Therefore, we speculate that, besides the positive selectivity for cGMP and negative selectivity for cAMP of E124 at the binding pocket, certain other structural components of photoreceptor PDE GAF domain have negative selectivity for cAMP and can exclude cAMP binding to the GAF domain of PDE6. The D439A mutation of PDE2A significantly increases its cAMP binding resulting in similar binding affinity of the D439A mutant to both cAMP and cGMP (Wu et al., 2004). PDE2 and PDE5 GAF domains have about 20 and 1000 fold greater selectivity respectively for cGMP versus cAMP while PDE6 has more than 100,000 fold greater selectivity (Herbert et

al., 1998). Most likely, there are additional structural components confining negative selectivity for cAMP only for the photoreceptor PDEs.

Compared to that of PDE2 and PDE5, the isolated GAF domain of PDE6 has much greater discrimination between cGMP and cAMP. Nevertheless, the cGMP binding affinities of the individual bacteria-expressed GAF domains of PDE2, 5 and 6 are very similar to each other with K_d values around 10 nM (Wu et al., 2004). Many of the coordinating residues in PDE2A GAF-B are identical to or close in character with the predicted cGMP binding GAF domains of PDE5 and PDE6. In PDE6, when these conserved residues were mutated to alanine, cGMP-binding was negatively affected. This data supports the homology model of PDE6 GAF-A that suggests a high similarity of the overall binding pocket structure between PDE GAF domains.

The 3 cGMP contacts, D169, T172 and T176 of chicken cone PDE6, are conserved in all cGMP-binding GAF domains of PDEs. In mouse PDE2A GAF-B, alanine mutants of two cGMP-contacting residues located on H4, D485A, T492A, lose their cGMP-binding capability completely (Wu et al., 2004). In chicken PDE6C GAF-A, the corresponding cGMP-contacting residues located on H4 are D169, T172 and T176. Alanine mutation at any of these three residues totally abolishes the cGMP-binding capacity of the chicken PDE6C GAF domain. In addition, valine

substitution at T172 and T176 diminishes cGMP-binding to undetectable level.

However, serine substitution at these two residues retains most of the cGMP-binding ability of wild-type suggesting the importance of a hydroxyl group for the interaction with cGMP.

In addition, F123 of ggPDE6C corresponds to F 438 of mmPDE2A. The base-stacking force between the phenyl ring of F123 and guanine ring of cGMP appears to be essential for binding. Thus, both the similarity of cGMP binding kinetics and the conservation of cGMP-contacting residues suggest that the overall architectures of these cGMP-binding GAF domains of various PDE family members are quite similar and are greatly conserved through evolution.

However, the homology model does not explain the differences in cyclic nucleotide binding between PDE6 and PDE2. In PDE2, three residues including Phe438, Asp439 and Thr488 were demonstrated to contribute to the cAMP/cGMP selectivity (Wu et al., 2004). Only one corresponding residue, E124 in PDE6, has been shown to participate in nucleotide discrimination. In addition, the modeled structure of PDE6 GAF-A does not elucidate the one or more molecular determinants that produce the much greater cyclic nucleotide selectivity of PDE6 compared to PDE2 and PDE5. Nevertheless, understanding the difference in binding character

between GAF domains of different PDEs is essential for deciphering the differential regulation of their catalytic activity.

MATERIALS AND METHODS

Materials

[³H] cGMP was obtained from Amersham Pharmacia Biotech. cGMP, 3-isobutyl-1-methylxanthine (IBMX), isopropyl β-thio-glucopyranoside (IPTG) were from Sigma. Pfu DNA polymerase and QuickChange site-directed mutagenesis kits were obtained from Stratagene. Restriction enzymes were purchased from NEB.

Methods

3D structure modeling of chicken cone PDE6 GAF-A domain

3D modeling of chicken PDE6 GAF-A domain was based upon the crystal structure of PDE2A GAF-B domain using the Swiss-Model part of the Swiss-Pdb Viewer program (Guex and Peitsch, 1997). First, the protein sequence of chicken PDE6 GAF-A and mouse PDE2A GAF-B was aligned based on their homology. The

homology alignment lays the ground for deriving a 3D model structure of chicken PDE6C GAF-A from the crystal structure of mouse PDE2A GAF-B. For a single template, the backbone of the homology model is the same as the template, except for loops that are added or deleted. After adding side chains from a rotamer database, the model is energy minimized using Gromos 96.

Cloning, expression and purification of GAF domains of chicken cone PDE6

P^{RunH} plasmid (a derivative of the pMW172 vector (Way et al., 1990)) containing His6 tag was used as an expression vector for GAF-A, GAF-B and GAF-A/B domains of chicken PDE6C. Full-length chicken PDE6C cDNA was a generous gift from Dr. Semple-Rowland (University of Florida). The boundaries of GAF-A/B, GAF-A and GAF-B domains are amino acids 42-458, 42-238 and 248-458 respectively, based on homology alignment with other PDE GAF domains. DNA coding for these domains was PCR-amplified using full-length chicken PDE6C as template and primers containing BamHI and XhoI sites. The PCR products were ligated into the p^{RunH} vector with T7 promoter, and His6 located at the C-terminal of the inserted DNA.

Chicken cone PDE6 GAF domain constructs were transformed into C41 competent *E. Coli*. Protein expression was induced by IPTG at 16°C and grown overnight. The cell pellets were resuspended in lysis buffer (containing 100 mM NaCl, 20 mM Tris, PH 7.5, 1 mM MgCl₂, 10 ug/ml Dnase I, 1 ug/ml leupeptin, 1 ug/ml pepstatin) and disrupted through either a French Press or a Microfluidizer cell disrupter apparatus. The lysates were centrifuged at 10,000 g for 1 hour at 4°C and the supernatant was incubated with Talon resin (Clontech) for 2 hrs at 4°C. Proteins without the His6 tag do not bind to or have much less affinity to Talon resin. Thus, His6-tagged GAF domain proteins were separated from other proteins without the His6 tag. The bound His6-tagged proteins were eluted with buffer containing 150 mM imidazole, 50 mM sodium phosphate (pH 7.0) and 300 mM NaCl. The eluted protein was concentrated by Centriprep centrifugal filter (Millipore) and subjected to gel-filtration using a Superose 12 column (Pharmacia) to remove aggregated protein and determine the apparent MW of the GAF proteins. SDS gel electrophoresis was carried out to determine the purity of the purified GAF domain proteins.

Site-directed mutagenesis studies on chicken cone PDE6C GAF-A and GAF-A/B domains

The QuikChange site-directed mutagenesis kit (Stratagene) was used to make point mutations in chicken cone PDE6 GAF-A and GAF-A/B DNA constructs. *E. coli* XL-blue competent cells were used for transformations and the mutants DNAs were purified by QIAprep Spin Miniprep Kit (Qiagen). All mutant DNAs were sequenced to ensure the proper in-frame sub-cloning and the desired mutation.

cGMP-binding kinetics study on chicken cone PDE6 GAF domain proteins

The binding kinetics of cGMP to GAF domain proteins were analyzed by Millipore filter binding assay described previously (Yamazaki et al., 1980) or a competition assay developed in our lab (Wu et al., 2004). In the case of the cGMP-binding competition assay, 1 nM GAF domain proteins was incubated with a fixed amount of ^3H -cGMP (such as 1 nM) and various amounts of unlabeled cGMP from 2 nM to 600 nM for 2 hours at room temperature. This mixture was filtered through pre-moistened Millipore HAWP filters (pore size 0.45 μm) and rinsed twice with a total of 10ml 3.3M ammonium sulfate buffer (20 mM Tris, pH 7.5, 100 mM NaCl, and 5 mM EDTA). The filters were dissolved in Filter Count (Parkard) scintillation

cocktail and counted with a Packard 1600 TR liquid scintillation analyzer. The bound protein counts were corrected by subtraction of nonspecific binding, which was defined as the [³H]cGMP bound in the absence of GAF protein. The data was subjected to nonlinear least squares analysis using Prism (Graphpad Software) to obtain IC₅₀ values. Most K_d values reported in this dissertation were determined by the homologous or heterologous displacement methods. Care was taken to utilize concentrations of protein that were lower than the measured K_d so that the data represented true binding curves and not a titration analysis. Similarly, for all IC₅₀ determinations the concentrations of labeled radioligand was adjusted to be lower than the measured IC₅₀ value so that the IC₅₀ approached the K_i for the cold ligand (Cheng and Prusoff, 1973). Therefore, the affinity of the ligand can be calculated using the equation of Cheng and Prusoff which states that the equilibrium dissociation constant of the ligand, $K_i = IC_{50} / (1 + [Radioligand]/K_d]$. In the case of homologous displacement, eg ³[H]-cGMP being displaced by cGMP, the equation further simplifies to $K_i = IC_{50} - [Radioligand]$, since $K_i = K_d$. Curve fitting was done using GraphPad Prism4® constraining the 100% and 0% binding points, with a one site competition model. Better fits were not obtained with a multiple site model. Based on

IC₅₀ values, the K_d of nucleotide binding was derived using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

cAMP-binding kinetics study on chicken cone PDE6 GAF domain proteins

In the case of cAMP-binding competition assay, 1 nM GAF domain proteins were incubated with a fixed amount of ³H-cGMP such as 1 nM and various amounts of unlabeled cAMP from 1 μM to 100 μM for 2 hours at room temperature.

CHAPTER 4

CONCLUDING REMARKS AND PERSPECTIVE

Chicken photoreceptor PDEs

The retinas of birds including chicken are cone dominant whereas the retinas of most mammals are highly abundant with rod cells. In addition, because of the similarity in physiology and development with mammals, the domestic chicken retina has been established as a valuable model system for studying the function of photoreceptor cells, in particular of cone cells (Chader, 2002). Moreover, since the characterization of the chicken cone PDE6 holoenzymes suggested that the kinetics and binding properties are greatly conserved between chicken, bovine, and frog isoforms, our understanding of the catalytic and regulatory properties learned from chicken cone enzyme is likely to be widely applicable. Hopefully it will facilitate new insights into the function and regulation of cone photoreceptor.

Structural determinants of PDE6 GAF domain and its implication

This study of the cGMP-binding components of chicken cone PDE6 and others of PDE2 and PDE5 have shown the similarity of cGMP binding kinetics and

the conservation of cGMP-contacting residues. It suggests that the overall architectures of these cGMP-binding GAF domains of various PDE family members are quite similar and are greatly conserved through evolution.

To our knowledge there have been no previous reports of high yield bacterial expression of functional bovine PDE6, either in its entirety or of its individual domains. Therefore, the robust bacterial expression of chicken cone PDE6 GAF domains that show proper high affinity cGMP-binding properties has allowed these basic characterization studies to be carried out. The sequence homology between the PDE6 GAF domains of various species indicates the preservation of the molecular components of the cGMP-binding pocket. Furthermore, our results suggested that a similarity of molecular formation of the binding pocket exists among the GAF domains of different PDE family members. Since the characterization of the chicken PDE6 holoenzymes suggested that the kinetics and binding properties are greatly conserved between chicken and bovine isozymes, our understanding of the regulatory properties learned from analysis of these domains is likely to be widely applicable. Hopefully they will facilitate new insights into the function and evolution of this unique domain as an essential unit of various signaling and sensory transducers.

GAF domains of PDE6 and P- γ inhibitory subunit

The cGMP-hydrolyzing activity of photoreceptor PDEs are very high, much greater than any other class of PDEs (Beavo et al., 1995). In order for rod and cone photoreceptors to respond rapidly to changes in light stimulation, a very effective inactivation mechanism with inhibitory γ subunit (P- γ) has evolved. P- γ inhibits the catalytic activity of PDE6 by directly interacting with the cGMP-binding pocket of the catalytic site (Granovsky et al., 1997). It also increases the rate of GTP hydrolysis by transducin which is required for precise termination of the photo-transduction cascade (Pages et al., 1993, Tsang et al., 1998).

In addition, the P- γ subunit has been shown to bind the GAF-A domain of PDE6 directly (Muradov et al., 2002). In the frog rod photoreceptor, it has been demonstrated that occupancy of the GAF domains with cGMP increases the binding affinity of P- γ for P- $\alpha\beta$. Reciprocally, binding of P- γ to P- $\alpha\beta$ enhances high-affinity binding of cGMP to the non-catalytic cGMP-binding site (Cote et al., 1994; Yamazaki et al., 2002). Our finding of GAF-A as the non-catalytic cGMP binding site and the data of direct interaction between P- γ subunit and GAF-A suggests that GAF-A is the region for P- γ regulation of cGMP binding to photoreceptor PDEs. The cooperative regulation of PDE by its inhibitory γ subunit and by cGMP has been

proposed to contribute to light adaptation of amphibian rod photoreceptors (Cote et al., 1994). Furthermore, the binding of P- γ subunit to the non-catalytic cGMP-binding site (GAF domains) was proposed to induce a conformational change at the catalytic site and result in altered catalysis of phosphodiesterase (D'Amours and Cote, 1999).

Based on the aforementioned observations, a model has been proposed on how non-catalytic cGMP binding at GAF domain is involved in light adaptation of amphibian rod photoreceptors (Cote et al., 1994). When light activates rod PDE6 and induces a drop of cGMP levels significantly and rapidly, cGMP dissociates from the non-catalytic GAF sites. Without the occupancy of cGMP at the non-catalytic binding sites, rod PDE6 has lower affinity to inhibitory P- γ . Thus, dissociation of cGMP from GAF sites promotes the physical removal of P- γ by transducin from rod PDE6. Due to the accelerated GTPase activity in the complex of transducin α -subunit and P- γ , the catalytic activity of rod PDE6 is inactivated much faster and the intracellular cGMP concentration would not be depleted completely. Therefore, the photoreceptors could adapt to the prolonged exposure of light. Although based on the studies of amphibian rod photoreceptor PDEs, the model of light adaptation and cGMP-binding of GAF domains may be applicable to the rod PDEs of other species and the cone PDEs.

Recently, it has been reported that a point mutation at the regulatory GAF region of rod PDE6B causes congenital stationary night blindness (CSNB) (Muradov et al., 2003). The P γ inhibition of rod PDE6 catalytic activity is significantly impaired due to the altered binding between the inhibitory P γ subunits and the mutated GAF region of CSNB patients. This impairment leads to the incomplete inactivation of PDE6 by P- γ in the dark and subsequently, desensitization of rod photoreceptors.

Perspectives

Many questions remain to be answered on the structural details and regulation properties of the GAF domains of photoreceptor PDEs. From this study and other studies on the GAF domains of PDE2 and 5, we have learned the overall conservation of the architecture of the cGMP-binding GAF domain of PDEs (Martinez et al., 2002, Wu et al., 2004). However, the cGMP specificity of PDE6 needs to be addressed in a precise and defined way, probably with a crystal structure in the future.

Furthermore, does cGMP-binding at the GAF-A domain regulate the catalytic activity of PDE6? It has been shown that both in PDE2 and PDE5, occupancy of cGMP at GAF domain stimulates the catalytic activity of these enzymes. Vigorous

studies should be carried out to test for any regulatory characteristics of PDE6 GAF-A domain upon its catalytic properties.

Moreover, what are the molecular determinants that contribute to the interaction between P- γ and GAF domain of PDE6? Which residues located at PDE6 GAF domains are critical for inhibitory regulation of P- γ on its catalytic activity? The answer to these two questions might facilitate our in-depth understanding on certain retina degenerative diseases such as the aforementioned CSNB (Muradov et al., 2003).

In addition, most researchers in the photoreceptor PDE field are working with either bovine or frog PDE6. So far from our experience and many others, there is no sufficient or functional bacterial expression of bovine PDE6 in its entirety or its individual domains such as the GAF domains or the catalytic domain. Nevertheless, we obtained robust bacterial expression of GAF domains of chicken cone PDE6 which may pave the way for future x-ray crystallization study and other functional analysis of photoreceptor PDEs.

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Publications:

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