Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 288–292, January 1990
Biochemistry

Identification of a noncatalytic cGMP-binding domain conserved in both the cGMP-stimulated and photoreceptor cyclic nucleotide phosphodiesterases

(visual transduction/sequence homology)

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Communicated by William A. Catterall, September 15, 1989 (received for review August 23, 1989)

ABSTRACT **Partial amino acid sequence has been determined for the cone, α′ subunit of the bovine photoreceptor cyclic nucleotide phosphodiesterase (PDE) and deduced from nucleotide sequences of a partial cDNA clone. These sequences identify the α′ subunit as the product of a gene that is distinct from those encoding the α or β subunits of the membrane-associated rod photoreceptor PDE. Comparisons between the recently determined cGMP-stimulated-PDE sequence and those of the α and α′ photoreceptor PDE subunits reveal an unexpected sequence similarity. In addition to the catalytic domain conserved in eukaryotic PDEs, all three PDEs possess a second conserved segment of ≈340 residues that contains two internally homologous repeats. Limited proteolysis and direct photolabeling studies indicate that the noncatalytic, cGMP-binding site(s) in the cGMP-stimulated PDE is located within this conserved domain, suggesting that it also may serve this function in the photoreceptor PDEs. Moreover, other PDEs that do not bind cGMP at noncatalytic sites do not contain this conserved domain. The function of the conserved segment in the photoreceptor PDEs is not known, but the homology to allosteric sites of the cGMP-stimulated PDE suggests a role in cGMP binding and modulation of enzyme activity.**

Multiple isozymes of cyclic nucleotide phosphodiesterases (PDEs) are found in many eukaryotic species; in some cases, distinct isozymes exist within a single cell type (1, 2). Within several PDE families, additional diversity is created by apparent alternative mRNA splicing (3, 4). Tissue and cell-type-specific expression of the isozymes has been demonstrated for several of the isozymes and is suspected in others (1–6). As part of an effort to analyze structure–function relationships among the PDE isozymes, we are determining the sequence of the bovine cone photoreceptor PDE (5) and the cGMP-stimulated (cGS) PDE (7, 8). Both PDEs have distinct kinetic parameters, substrate preferences, and apparent cellular functions but share the ability to bind cGMP at high-affinity, noncatalytic sites (5, 9–11).

Cone and rod cGMP-stimulated PDEs hydrolyze cGMP in a light-dependent manner and have a key role in the cascade that transduces a light signal into a neural response (for review, see ref. 12). Rod and cone PDEs differ significantly in subunit composition, sensitivity to transducin, and cGMP-binding properties (5, 13). Each enzyme has two noncatalytic, cGMP-binding sites; the affinity (5, 13) of the sites on the cone PDE (K_d = 11 nM) is significantly lower than those of the rod PDE (K_d < 1 nM). The bovine rod PDE oligomer is comprised of α (88 kDa) and β (84 kDa) subunits, which appear to be catalytic, and an inhibitory γ subunit (11 kDa), whereas the cone PDE (5) has two α′ subunits (93.5 kDa) and three small subunits (11, 13, and 15 kDa).

The cGS-PDE is a homodimer of two identical (105 kDa) subunits and can hydrolyze both cAMP and cGMP with nearly equal efficiency (7, 8). cGMP is a positive allosteric regulator of this enzyme, and micromolar concentrations produce a 10-fold stimulation of cAMP hydrolysis (7–10). A cGMP-specific, allosteric site has been demonstrated in several types of cyclic nucleotide binding experiments (7, 9–11).

In this report, we describe the partial amino acid sequence of the cone α′ subunit and nucleotide sequences from a partial cDNA clone encoding it.§ The protein sequence data confirms the identity of the full-length cDNA clone described in the companion paper by Li *et al.* (14) and shows that the α, α′, and β subunits of photoreceptor PDEs are distinctive gene products. The α and α′ subunits and the cGS-PDE have extensive sequence similarity within single segments that are not part of their conserved, catalytic domains (15, 16). Each segment displays internal homology and appears to comprise a regulatory, cGMP-binding domain.

MATERIALS AND METHODS

Partial Amino Acid Sequence Analysis of the Cone PDE α′ Subunit. The bovine cone photoreceptor PDE was purified from dark-adapted frozen retinas (Hormel, Austin, MN) by using procedures described by Gillespie *et al.* (5, 17). The α′ subunit of the cone PDE was resolved from the smaller 15-kDa, 13-kDa, and 11-kDa subunits using reverse-phase HPLC chromatography on a narrow-bore Brownlee, BU-300 column (C4, 2.1 × 30 mm) with the linear trifluoroacetic acid/acetonitrile gradient system described by Gillespie *et al.* (17) for the separation of rod PDE subunits.

The purified α′ subunits were cleaved at lysyl residues using *Achromobacter* protease I as described (18). The resulting peptides were first separated by size-exclusion HPLC chromatography on two tandem TSK-3000 PW columns equilibrated in 0.1% trifluoroacetic acid/40% (vol/vol) acetonitrile. Pooled fractions containing peptides of similar size were resolved by reverse-phase HPLC chromatography on Brownlee RP-300 (C8, 2.1 × 100 mm) or RP-18 (C18, 2.1 × 100 mm) using linear gradients of 0.09% trifluoroacetic acid/80% acetonitrile at a flow rate of 300 μl/min. Selected peptides obtained in good yield were subjected to automated Edman degradation on an Applied Biosystems model 470A

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Abbreviations: PDE, cyclic nucleotide phosphodiesterase; cGS-PDE, cGMP-stimulated PDE.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M29465).

gas-phase sequencer equipped with an on-line HPLC system (model 120A).

Isolation of a Bovine Retinal cDNA Encoding the Cone α′ Subunit. Degenerate oligonucleotides corresponding to sequences from the cone α′ subunit (TPPMDHWT and DEYFTFQK; single-letter code) having minimal sequence similarity with the rod α subunit were synthesized and ^{32}P end-labeled. A bovine retinal cDNA library (19) in λgt10 (provided by Jeremy Nathans, The Johns Hopkins University) was screened by hybridization to both labeled oligonucleotides. Hybridization was done in 6× SSC (1× SSC is 0.15 M sodium chloride/0.015 M sodium citrate, pH 7)/2 × Denhardt's solution (1× Denhardt's solution is 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin)/0.05% sodium pyrophosphate/20 mM sodium phosphate buffer, pH 7 at 50°C (10°C below the calculated t_m of the oligonucleotides). Two independent positive phage were plaque purified, and their *Eco*RI inserts were subcloned into the Bluescript plasmid vector (Stratagene). A series of nested deletions were prepared from the *Eco*RI inserts and subcloned into the Bluescript vector by using Stratagene procedures. Nucleotide sequences were determined with a modification (20) of the dideoxynucleotide chain-termination procedure of Sanger *et al.* (21) by using deoxyadenosine 5-[α-[^{35}S]thio]triphosphate and Sequenase.

Analysis of Structural Relationships. Structural relationships among PDEs were analyzed by using the ALIGN and RELATE programs described by Dayhoff *et al.* (22) and supplied by the National Biomedical Research Foundation. The RELATE program and a dot-matrix plotting routine included in the GENEPRO software package (Riverside Scientific, Seattle) were used to detect possible homology between sequences of different lengths.

RESULTS AND DISCUSSION

Partial Amino Acid Sequence Analysis. Lysyl peptides from the α′ subunit of the bovine cone PDE were purified by using a combination of size-exclusion and reverse-phase HPLC chromatography. Twenty-one distinct peptides were recovered in good yield and sequenced with the use of automated gas-phase techniques. A total of 393 residues were assigned and are listed in Table 1 or designated by underlining in Fig. 1. This partial sequence data shows that the α′ subunit of the cone PDE is similar, but not identical, to the α subunit of the rod PDE (16). It should be noted that this sequence analysis provided no evidence for the existence of closely related, yet different, subunits in the cone PDE preparations. Neither HPLC chromatography nor SDS/PAGE indicated the presence of more than one type of subunit (5), and it is reasonable to conclude that the cone PDE is a homodimer of α′ subunits.

Analysis of α′ Subunit cDNA Clones. Two different clones were isolated from a retinal cDNA library by using oligonucleotide probes based upon α′ subunit sequences. Restriction enzyme analysis and partial nucleotide sequencing showed that neither clone was full length and that both were structurally related because they were truncated near the same site at the 3′ end. The shorter of the two clones, BCα-1, contains an insert [≈1300 base pair (bp)] encompassing much of the coding region of the protein. The complete nucleotide sequence¶ of an *Eco*RI fragment derived from the 5′ end of BCα-1 was deduced from the analysis of both strands (Fig. 1). As illustrated in Fig. 1, nine different lysyl peptide sequences (145 residues) from the α′ subunit are absolutely identical to the amino acid sequence predicted from the nucleotide sequence, establishing that BCα-1 encodes part of the α′ subunit of the cone PDE. The polypeptide sequence encoded by BCα-1 is similar (65% sequence identity) but not identical to that of the α subunit of the rod PDE, demonstrating that the α and α′ PDE subunits are distinct gene products. Both the protein sequences and the DNA sequence correspond to segments encoded by a full-length cDNA clone p21-1, isolated and described by Li *et al.* (14) in this issue. The sequence identity demonstrates that the cDNA clone of Li *et al.* (14) codes for the α′ subunit of the cone PDE. There are at least three distinct genes coding for the large subunits of the rod and cone PDEs because Li *et al.* (14) have shown that the protein encoded by a full-length α′ clone is not identical to a partial β subunit sequence.

Table 1. Lysyl peptide sequences from the cone PDE α′ subunit

No.	Cone peptide sequence	Residue no. in α subunit*
1	YLEANPQFAK	11–20
2	LQVEVPSGGAQAPASASFPGRTLAEEAALY-E-L-VLLEAGSVELAA	27–74
3	LLDVTPtSKFEDNLVVPDREAVFPLDVGIVG WVAHTKK	111–148
4	TFNVPDVK	149–156
5	QTGYVTRNLLATPIVMGKEVLAVFMAVN KV-A-eF	168–202
6	VFEGLTDVERQFHK	247–260
7	ALYTVRTYLNCERYSIGLLDMTK	261–283
8†	TPDGR	306–310
9	STSPLARLHGSSILERHHLEYSK	613–635
10	TLLQdESLNIFQNLSK	636–651
11	METEEEAIK	690–698
12	EITPMLNGLQNNRVEWK	793–809
13	SLADEYDEK	810–818

Peptide sequences are given in single-letter code with lowercase letters indicating residues that were tentatively assigned and hyphens denoting unidentified residues.

*Residue numbers indicate the position of corresponding sequences in the rod α subunit (16).

†The remaining sequence of peptide 8 and those of several other peptides are given in Fig. 1.

Sequence Homology Among the Photoreceptor PDE Large Subunits and the cGMP-stimulated PDEs. A conserved catalytic domain (of ≈250 residues) has been identified near the C terminus of all eukaryotic PDE isozymes, including the α and β subunits of the rod PDE (16) and the cGS-PDE (11, 15). Sequences of four peptides of the cone α′ subunit (nos. 9, 10, 11, and 12 in Table 1) can be aligned with 57 residues within the catalytic domain of the α subunit (16). The high degree of sequence similarity within these segments (72% sequence identity) clearly establishes that the α′ subunit also possesses the catalytic domain that is conserved among eukaryotic PDEs. The full-length α′ cDNA clone of Li *et al.* (14) confirms this finding.

The α and α′ subunits of the photoreceptor PDEs (14, 16) as well as the cGS-PDE‖ have ≈500–600 contiguous residues located on the N-terminal side of the conserved catalytic domain (Fig. 3). As suggested previously (15), these sequences probably provide the binding site(s) for various regulatory ligands (e.g., transducin, cyclic nucleotides). Comparisons of these N-terminal segments from the cGS-PDE and the α and α′ subunits of the photoreceptor PDEs have revealed an unexpected structural relationship (Table

¶After obtaining a partial cDNA clone for the α′ subunit, we learned that a full-length clone of Li *et al.* (14) had been isolated; therefore, efforts to obtain extensive nucleotide sequence and to isolate a complete cDNA clone in our laboratory were not continued.

‖The primary structure of the cGS-PDE from bovine heart has been deduced in our laboratories by sequence analysis of the protein and of a cDNA clone. The proof of sequence will be published elsewhere.

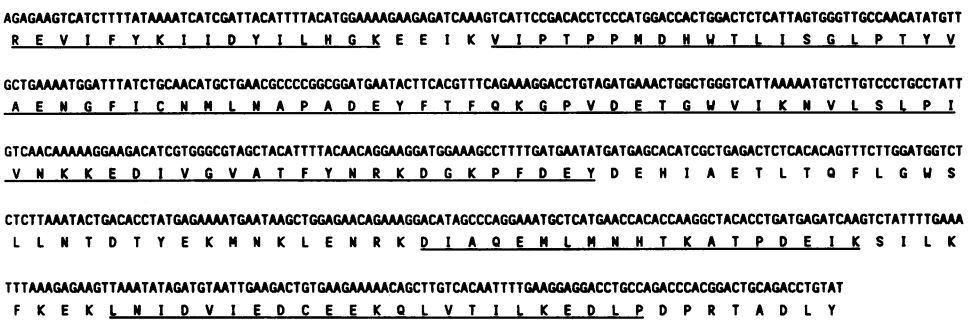

FIG. 1. Comparison of cone α' subunit sequences with a partial cDNA clone. The nucleotide sequences from the 5' *Eco*RI fragment of the α' subunit cDNA clone, BCα-1, is given on the top line with the predicted sequence (single-letter code) below. The underlined sequences were also directly determined by amino acid sequencing.

2). All three PDEs have a second conserved segment of ≈340 residues (Figs. 2 and 3) that is not present in other PDEs. As discussed below, there is evidence that this second conserved region is the location for the cGMP-binding sites that exist on all three enzymes. Although this conserved segment includes a region of relatively high sequence similarity between the α and α' subunits, its recognition as a conserved element of functional importance required comparisons with the more distantly related cGS-PDE sequence. For ease of discussion, this second conserved region will be designated herein as the conserved cGMP-binding domain to distinguish it from the conserved catalytic domain, although the function of this segment has not been unequivocally established.

The conserved cGMP-binding domain encompasses residues 125–468, 124–467, and 261–564 of the α subunit, α' subunit, and cGS-PDE, respectively. The sequence alignments of Fig. 2 illustrate their homology, and alignment scores (22) of 13.9 and 13.8 establish the significance of the relationship of cGS-PDE to α and α' subunits, respectively. As might be expected, the sequence similarity is greatest between segments from the two photoreceptor isozymes (67% sequence identity). The sequence identity between the cGS-PDE segment and those of the α and α' subunits are 27% and 30%, respectively. If chemically conservative substitutions are considered, then 43% and 44% of residues from the cGS-PDE are similar to those of the α and α' subunits, respectively.

A striking feature of the conserved cGMP-binding domain is the presence of two internally homologous, 120-residue repeats. The percent sequence identities between the internal repeats of cGS-PDE, α subunit, and α' subunit (with corresponding alignment scores in parentheses) are 19% (4.8), 29% (6.6), and 29% (5.5), respectively. As illustrated in Figs. 2 and 3, the internal repeats are located near the N- and C-termini of the conserved cGMP-binding domain and are separated by a central segment that has no significant sequence similarity to either internal repeat. The intervening segments of the three proteins are homologous to one another, although that of cGS-PDE domain requires a gap of 25 residues for optimal alignment. The presence of internal homology indicates that this conserved domain evolved by a gene duplication mechanism.

Table 2. Structural relationship among PDEs and other cyclic nucleotide binding proteins

Protein	Source	Segment comparison score: α Subunit*	Segment comparison score: RII cAK
cGK	Bovine	−1.6	19.0
cGS-PDE	Bovine	11.8*	−0.6
α' Subunit	Bovine	69.5*	−1.6
Dunce PDE	*Drosophila*	−0.6*	−0.3
PDE 2	*Saccharomyes cerevisae*	2.5*	0.1

Segment comparison scores were generated for sequences of different size using the RELATE program (22) with a segment length of 25. Segment comparison scores > 5 indicate that a statistically significant structural relationship exists between two sequences. Sequences for the bovine type II regulatory subunit of the cAMP-dependent protein kinase (RII cAK) and the bovine cGMP-dependent protein kinase (cGK) were taken from the Protein Identification Resource protein data base.

*Only N-terminal segments lying outside of the catalytic domain were used for these comparisons. Segments examined were as follows: rod α subunit, residues 1–535 (16); cGS-PDE 1–617[‖]; dunce PDE 1-223 (23); and yeast PDE 2, 1–241 (24).

Localization of the High-Affinity cGMP-binding Sites from the cGS-PDE and α' Subunit. The combined photolabeling and limited proteolysis studies of Stroop *et al.* (11) have shown that the cGS-PDE can be separated into two functional domains, one retaining catalytic activity but unregulated and the other an allosteric, cGMP-binding site(s). Two structurally related limited proteolytic fragments (60 kDa and 57 kDa) are derived from the N-terminal end of the cGS-PDE molecule and encompass the second conserved segment that is homologous to corresponding segments of the photoreceptor PDEs (11). Direct photolabeling experiments show that both fragments possess a high affinity, cGMP-specific binding site(s) with properties matching those expected for the allosteric sites responsible for the 10-fold activation of the enzyme by cGMP. As illustrated in Fig. 3, the cGMP-binding site(s) has been more precisely localized to a 28-kDa fragment produced by cleaving the photolabeled protein with CNBr. This fragment spans residue 314 to residue 553 of the cGS-PDE and is totally encompassed within the N-terminal, conserved segment. This location strongly implicates the homologous sequences of the photoreceptor PDEs in the formation of a noncatalytic cGMP-binding site.

Preliminary limited proteolysis and photolabeling studies indicate that the cone α' subunit can be separated into two functional domains in a manner similar to that observed with the cGS-PDE (P. G. Gillespie, R.K.P., H.C., K.A.W. and J.A.B., unpublished results). As with the cGS-PDE, this experiment suggests that fragments containing the N-terminal conserved domain also have a high-affinity cGMP-binding site. All PDEs having this conserved domain have the ability to bind specifically cGMP at noncatalytic sites. It seems likely that other high molecular weight PDEs that may bind cGMP at noncatalytic sites, such as the cGMP-binding PDE of bovine lung (25) and possibly the low K_m, cGMP-inhibited-PDE (26, 27) will also contain a domain homologous to the one identified here in the cGS- and photoreceptor PDEs.

Possible Structural Relationship Between the Putative cGMP-Binding Domain and Other Cyclic Nucleotide-Binding Proteins. It is of interest to inquire whether PDE isozymes are structurally related to other proteins that bind cyclic nucleotides since knowledge of the structure and function of other well-characterized, cyclic nucleotide-binding proteins can be

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ROS: VAPDS---EIVFPLDMGVVGHVALSKKIVNVPNTEEDEH 160
CGS: VIGDKVLEEISFPLTTGRLGQVVEDKKSIQLKDLTSEDM 295
COS: VVPDR---EAVFPLDVGIVGWVAHTKKTFNVPDVKKNSH 159

ROS: FCDFVDTLTEYQTKNILASPIMN--GKDVVAIIMAVNKV 197
CGS: --QQLQSMLGCEVQAMLCVPVISRATDQVVALACAFNKL 332
COS: FSDFMDKQTGYVTRNLLATPIVM--GKEVLAVFMAVNKV 196

ROS: DGPHFTENDEEILLKYLNFANLIMKVFHLSYLHNCETRR 236
CGS: GGDLFTDQDEHVIQHCFHYTSTVLTS-TLAFQKDQKLKC 370
COS: DASEFSKQDEQVFSKYLSFVSIILKLHHTNYLYNIESRR 235

ROS: G-QILLWSGSKVFEELTDIERQFHKALYTVRAFLNCDRY 274
CGS: ECQALLQVAKNLFTHLDDVSVLLQEIITEARNLSNAEIC 409
COS: S-QILMWSANKVFEELTDVERQFHKALYTVRTYLNCERY 273

ROS: SVGLLDMTKQKEFF-DVWPVLMGEAPPYAGPRTPDGREI 312
CGS: SVFLLD---QNELVAKVFDGGVVEDESY---------- 434
COS: SIGLLDMTKEKEFY-DEWPVKLGEVEPYKGPKTPDGREV 311

ROS: NFYKVIDYILHGKEDIKVIPNPPPDHWALVSGLPTYVAQ 351
CGS: ----------------EIRI----PADQ-----GIAGHVAT 450
COS: IFYKIIDYILHGKEEIKVIPTPPMDHWTLISGLPTYVAE 350

ROS: NGLICNIMNAPSEDFFAFQKEPLDESGWMIKNVLSMPIV 390
CGS: TGQILNIPDAYAHPLFY--RGVDDSTGFQTRNILCFPIK 487
COS: NGFICNMLNAPADEYFTFQKGPVDETGWVIKNVLSLPIV 389

ROS: NKKEEIVGVATFYNRKDGKPFDEMDETLMESLAQFLGWS 429
CGS: NENQEVIGVAELVNKINGPWFSKFDEDLATAFSIYCGIS 526
COS: NKKEDIVGVATFYNRKDGKPFDEYDEHIAETLTQFLGWS 428

ROS: VLNPDTYELMNKLENRKDIFQDMVKYHVKCDNEEIQTIL 468
CGS: IAHSLLYKKVNEAQYRSHLANEMMMYHMKVSDDEYTKLL 565
COS: LLNTDTYEKMNKLENRKDIAQEMLMNHTKATPDEIKSIL 467
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FIG. 2. Alignment of the putative cGMP-binding domains from the cGS-PDE (CGS), rod α subunit (ROS), and cone α' subunit (COS). Boxes enclose residues that are identical in all three sequences. Gaps (hyphens) have been introduced to optimize alignments. Underlined residues are encompassed within each of two internally homologous segments. Sequence data for the α subunit (16) and the α' subunits (14) include residues 125–468 and 124–467, respectively, with residue numbers indicated on the right. Residue numbers for the cGS-PDE are taken from the complete sequence that is to be published elsewhere.

applied to the PDEs. The regulatory subunits of the cAMP-dependent protein kinase, the regulatory domains of the cGMP-dependent protein kinase, and the *Escherichia coli* catabolite gene activator protein comprise a family of structurally related cyclic nucleotide-binding proteins (28). However, comparisons of the conserved catalytic domain from four different PDEs (15) with the sequences from this protein family have failed to demonstrate a statistically significant homologous relationship. Moreover, no significant *extended* relationship can be found between the putative cGMP-binding domain of the PDEs and members of this cyclic nucleotide-binding protein family, as exemplified by the comparisons listed in Table 2. Thus, neither catalytic nor noncatalytic cyclic nucleotide-binding domains of PDEs show homology to the catabolite gene activator protein family of cyclic nucleotide-binding proteins; two separate evolutionary families appear to be involved.

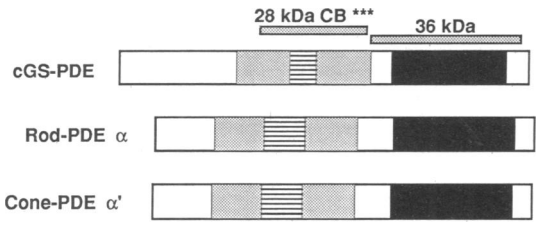

FIG. 3. Diagrammatic illustration of the structural relationship among the cGS-PDE, the rod α subunit, and cone α' subunit. Black boxes indicate the location of the conserved catalytic domains, whereas shaded boxes indicate the two internally homologous subdomains located within the putative cGMP-binding domain (Fig. 2). The boxes with horizontal lines designate intervening segments that are homologous to corresponding regions of each of the other PDEs but not to either repeat. The position of key limited proteolytic or chemical cleavage fragments are indicated above the sequence. Asterisks (***) designate a fragment bearing noncatalytic sites directly photolabeled with [^{32}P]cGMP (see text). Note the C-terminal positions of these fragments are estimated from molecular weights obtained by SDS/PAGE.

Nevertheless, several investigators (6, 14, 23) have noted that *short* sequences from both conserved PDE domains resemble segments in the catabolite gene activator protein family of cyclic nucleotide-binding proteins, and Li *et al.* (14) have noted "consensus sequences" derived from other nucleotide-binding proteins. Although such segments are intriguing, conclusions regarding possible similarities in the structure of PDE nucleotide-binding sites must be made with caution. It is difficult to establish statistically meaningful relationships with short sequences (29), and "consensus sequences" are often found in unrelated proteins (30). More importantly, many of the short sequences identified are not among those that are completely conserved within the PDE family of isozymes and, therefore, are not as likely to be essential residues within a cyclic nucleotide-binding site.

The function of the conserved segment from the photoreceptor PDE is not fully understood; however, preliminary limited proteolysis and photolabeling experiments suggest that this conserved section may also be the location for noncatalytic, cGMP-binding sites. The similarity in sequence and location (within the linear sequence) relative to the catalytic domain suggests the possibility that the noncatalytic, cGMP-binding sites of the photoreceptor PDE may modulate catalysis in a manner analogous to the corresponding domain of the cGS-PDE. Thus far, no such effects have been reported. Unlike in the cGS-PDE, action of the photoreceptor cGMP-binding sites may be much more complex due to additional modulating effects of transducin and other associated small subunits. The findings reported here emphasize the importance of reexamining the role of cGMP in the modulation of photoreceptor PDE function.

We are grateful to Santosh Kumar and Maria Harrylock for their assistance with the protein sequence analysis and to Drs. Peter Gillespie and Steve Stroop for many stimulating and helpful discussions. We thank Dr. Meredithe L. Applebury for freely communicating results from the α' subunit cDNA clone in advance of publication and for many helpful discussions. This work was supported by National Institutes of Health Grants DK21723 and EY 08197 (J.A.B.) and GM 15731 (K.A.W.).

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