Cyclic GMP Contact Points within the 63-kDa Subunit and a 240-kDa Associated Protein of Retinal Rod cGMP-Activated Channels†

R. Lane Brown,‡ Robert Gramling, Robert J. Bert, and Jeffrey W. Karpen*

Department of Physiology, C-240, University of Colorado School of Medicine, Denver, Colorado 80262

Received January 17, 1995; Revised Manuscript Received March 22, 1995⊗

ABSTRACT: Ion channels from retinal rods and a variety of other cells are directly gated by cyclic nucleotides. The rod channel is known to contain a 63-kDa subunit, and there is molecular genetic evidence for the existence, in human retina, of a second subunit with a deduced molecular mass of about 100 kDa. When purified from bovine rods, the channel consists of the 63-kDa subunit and a 240-kDa associated protein that has been shown recently to contain a version of the cloned second subunit as part of a larger complex. We had previously shown that a photoaffinity analog of cGMP, 8-(*p*-azidophenacylthio)-[^{32}P]cGMP, specifically labels both the 63- and 240-kDa proteins. Here the analog was used to identify cGMP-binding regions and amino acid contact points within these proteins. The specific labeling of the 63-kDa subunit was localized to a 66 amino acid fragment (Tyr-515−Met-580) that is contained entirely within a 110 amino acid region proposed to be the cGMP-binding site on the basis of homology with other cyclic nucleotide-binding proteins. Within this fragment, amino acid residues Val-524, Val-525, and Ala-526 were found to contain label. These residues are part of a larger hydrophobic cluster that appears to line the binding pocket. The results also indicate that the 240-kDa protein contains a similar cGMP-binding site. Sequencing of a specifically labeled 8-kDa fragment through 16 amino acid residues indicated that the fragment was derived from the portion of the 240-kDa complex that contains the second subunit. Alignment of the amino acid sequences of the bovine 63-kDa subunit and the human second subunit based on homology indicates that the specifically labeled peptide from the 240-kDa protein is the corresponding fragment to that labeled from the 63-kDa subunit.

In retinal rods, channels directly gated by cGMP[1] close in response to a light-induced decrease in the cGMP concentration (Yau & Baylor, 1989; McNaughton, 1990; Kaupp, 1991; Stryer, 1991; Lagnado & Baylor, 1992; Yarfitz & Hurley, 1994). The resulting hyperpolarization decreases the rate of transmitter release onto second-order cells in the retina (Dowling & Ripps, 1973; Baylor & Fettiplace, 1977). Cyclic nucleotides are now known to exert their effects through direct actions on ion channels in a number of other systems as well (Yau, 1994). Activation of the rod channel requires the binding of at least three molecules of cGMP (Haynes et al., 1986; Zimmerman & Baylor, 1986; Karpen et al., 1988). A 63-kDa subunit has been purified from bovine rods and its cDNA (bRCNC1) has been cloned and expressed in *Xenopus* oocytes (Cook et al., 1987; Kaupp et al., 1989). This protein contains a 110 amino acid region that shows some homology with cyclic nucleotide-dependent protein kinases and the catabolite gene activator protein (CAP) from *Escherichia coli*. Previous work has shown that Thr-560 in this region (Altenhofen et al., 1991), as well as a proposed α-helical stretch from residues 588 to 609 (Goulding et al., 1994), affect the channel's preference for cGMP over cAMP. There is little direct structural information, however, about the cGMP-binding site.

Although injection of oocytes with cRNA encoding only the 63-kDa subunit is sufficient for the expression of a cGMP-activated conductance, several lines of evidence suggest that this channel may not be identical to that found in the rod. For example, the expressed channel is much less sensitive to block by *l-cis*-diltiazem and exhibits dramatically slower single-channel gating kinetics. In 1993, a second cDNA was obtained from a human retinal library on the basis of sequence homology with the 63-kDa subunit (Chen et al., 1993). This new cDNA (hRCNC2a) and a larger alternatively spliced version (hRCNC2b) are expected to encode proteins of molecular mass 70 843 and 102 330 Da. These proteins show 30% overall amino acid identity with the 63-kDa subunit from human retina and 50% identity in the proposed cyclic nucleotide-binding region. Although neither cDNA encoded a functional channel on its own, when coexpressed with the 63-kDa subunit either one created a channel that more faithfully reproduced the properties of the native channel. The biochemical identity of the second subunit was initially a puzzle because highly purified channel preparations contain only the 63-kDa subunit and an associated 240-kDa protein. Recently, however, the 240-kDa protein was shown to contain a bovine version of hRCNC2b (Chen et al., 1994), as well as another gene product of predicted molecular mass 65 kDa (Illing et al., 1994). The molecular nature of the linkage between these two proteins, as well as the functional role of the 65-kDa component, is not yet known.

† This work was supported by NIH Grant EY09275. R.L.B. was the recipient of National Research Service Award EY06425. R.J.B. was supported by NIH Training Grant T32NS07083.

‡ Present address: R.S. Dow Neurological Sciences Institute, 1120 N.W. 20th Ave., Portland, OR 97209.

⊗ Abstract published in *Advance ACS Abstracts,* June 1, 1995.

[1] Abbreviations: cGMP, guanosine 3′,5′-cyclic monophosphate; APT-[^{32}P]cGMP, 8-(*p*-azidophenacylthio)-[^{32}P]cGMP.

0006-2960/95/0434-8365$09.00/0

In an effort to identify the cGMP-binding components of the channel, we developed a photoaffinity analog of cGMP, 8-(*p*-azidophenacylthio)-[^{32}P]cGMP (APT-[^{32}P]cGMP), that specifically labeled both the 63-kDa channel subunit and the 240-kDa associated protein in a partially purified preparation from bovine rods (Brown et al., 1993a). At that time, however, it was unclear whether the 240-kDa protein was labeled because it contains a cGMP-binding site or because it is in extreme proximity to the cGMP-binding site of the 63-kDa subunit. In the present study we isolated peptides from both proteins that were specifically labeled by APT-[^{32}P]cGMP. The results indicate that the 240-kDa protein does indeed bind cGMP in a corresponding homologous region to the 63-kDa subunit. In addition, sequence analysis allowed us to identify amino acids that contact cGMP in the 63-kDa subunit.

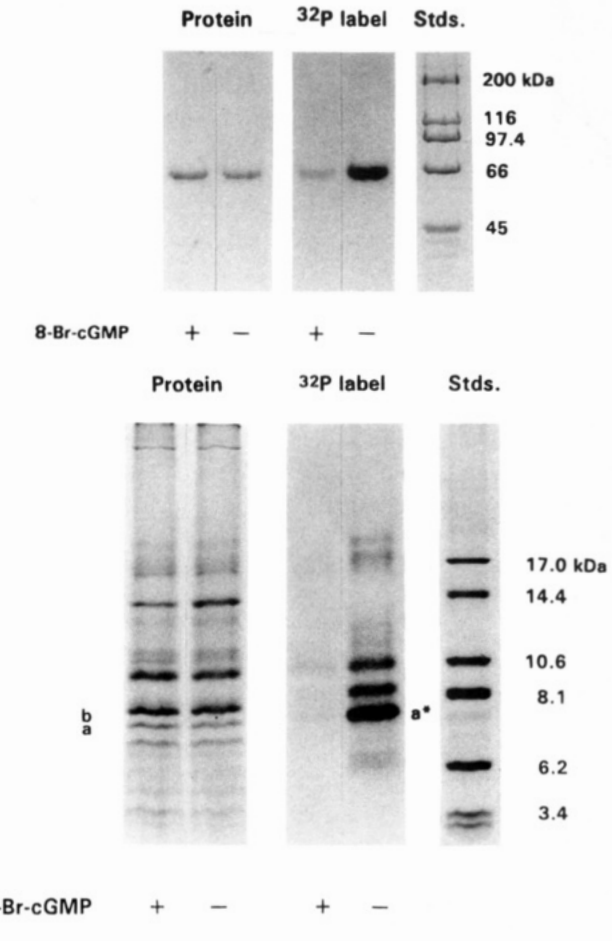
EXPERIMENTAL PROCEDURES

Synthesis of APT-[^{32}P]cGMP. APT-[^{32}P]cGMP was synthesized as previously described (Brown et al., 1993a), with the following modifications. [^{32}P]cGMP was synthesized from α-[^{32}P]GTP using guanylate cyclase partially purified from sea urchin sperm (*Strongylocentrotus purpuratus*) by Lubrol extraction and GTP affinity chromatography (Garbers, 1976). α-[^{32}P]GTP (5 mCi; 25−100 Ci/mmol) was dissolved in 0.6 mL of 50 mM triethanolamine, pH 7.9, and 5 mM $MnCl_2$. The reaction was initiated by the addition of 50 μL of a 0.25 mg/mL preparation of guanylate cyclase and incubated at room temperature for 3 h. At this time conversion was nearly complete as estimated by PEI−cellulose TLC (Brown et al., 1993a). After bromination of [^{32}P]cGMP, the mixture was treated with 12 units of 5′-nucleotidase from *Crotalus atrox* venom (Sigma) for 24 h at 37 °C in 250 mM glycine, pH 9, prior to anion-exchange HPLC purification. A typical yield of purified APT-[^{32}P]-cGMP was 1−1.5 mCi.

Partial Purification and Photoaffinity Labeling of the Bovine cGMP-Activated Channel. The cGMP-activated channel was purified from bovine rod outer segments by the procedure of Cook et al. (1987) through the ion-exchange chromatography step. A typical preparation started with 120 mg of rhodopsin. Photoaffinity labeling was carried out in a 6-mL volume as previously described (Brown et al., 1993a), except that APT-[^{32}P]cGMP was used at a concentration of 5 μM and a specific activity of 25−100 Ci/mmol.

Gel Purification of Labeled Channel Subunits. After photoaffinity labeling, a channel preparation (derived from 120 mg of rhodopsin) was diluted 40-fold in 1% CHAPS and concentrated to a volume of 0.5 mL in a Centriprep-30 (Amicon). The sample was then diluted with 2 volumes of gel solubilization buffer containing 10% SDS and 7 M urea (Brown et al., 1993a) prior to loading on a 20- × 20- × 0.15-cm SDS−9% polyacrylamide gel. This method of sample concentration provided higher yields of channel proteins than TCA precipitation, possibly because the channel was difficult to resolubilize. Proteins were visualized and electroeluted as before (Brown et al., 1993a).

Western Blot Analysis of Channel Subunits. Proteins separated on SDS−polyacrylamide gels were electrophoretically transferred to nitrocellulose membrane and probed with monoclonal antibodies using previously described techniques (Molday et al., 1990). Immobilized antibodies were detected using a chemiluminescent method (ECL, Amersham).



***

Peptide "a": 515 YIIKEGKLAVVADDGITQF . . . 580

Peptide "b": 214 FVRTRTGYLEQGLLVKEER . . . 279

FIGURE 1: (Top) Electrophoresis of APT-[^{32}P]cGMP-labeled 63-kDa channel subunit purified by electroelution. The left-hand panel shows a Coomassie-stained SDS−9% polyacrylamide gel of the purified subunit. The center panel shows the corresponding autoradiogram. Molecular mass standards are shown at right. Labeling of a partially purified channel preparation with APT-[^{32}P]-cGMP was carried out in the presence or absence of 1 mM 8-Br-cGMP as indicated. Each lane contains protein purified from rod outer segments containing 5 mg of rhodopsin. (Middle) Identification of a 7.5-kDa peptide fragment from the 63-kDa channel subunit specifically labeled by APT-[^{32}P]cGMP. Fragments generated by CNBr cleavage of the 63-kDa subunit were separated by tricine−SDS−16.5% PAGE. The left-hand panel shows the resulting peptide pattern after staining with Coomassie Brilliant Blue; an autoradiogram of the same gel is shown in the center panel. The intact channel was labeled in the presence or absence of 8-Br-cGMP (1 mM) as indicated. Each lane contains 63-kDa subunit peptides derived from rod outer segments containing 25 mg of rhodopsin. The peptides designated "a" and "b" were electroblotted from a duplicate gel onto PVDF membrane and subjected to sequence analysis. The labeled fragment designated "a*" was electroblotted from a different gel and subjected to the analysis shown in Figure 2. Molecular mass standards were myoglobin CNBr cleavage fragments and glucagon. (Bottom) Partial amino acid sequences of peptides "a" and "b". The underscored residues were identified by sequence analysis of the purified peptides; the numbers indicate the position of each peptide within the deduced primary structure of the 63-kDa subunit. Asterisks designate the amino acids to which APT-[^{32}P]cGMP covalently attached (see text and Figure 2).

Cleavage, Electrophoresis, and Sequencing of Peptides. Purified, labeled channel subunits were concentrated in 0.1% SDS to a volume of 50 μL in a Centricon microconcentrator

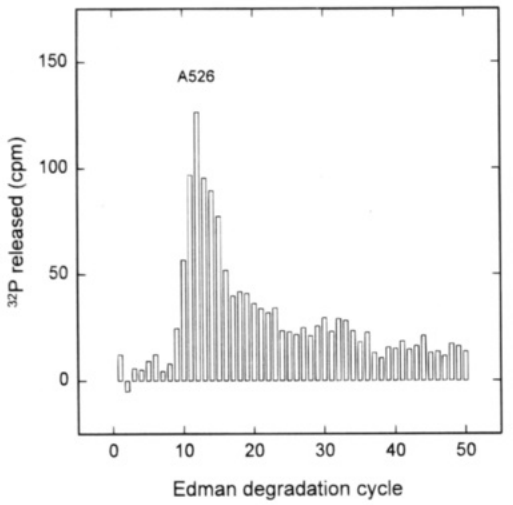


FIGURE 2: Release of ^{32}P label at each cycle of Edman degradation of the 7.5-kDa labeled fragment from the 63-kDa channel subunit. The peak of released radioactivity was in cycle 12, which corresponds to alanine 526 in the sequence of peptide "a". The blot contained a total of about 10 000 dpm (estimated by Cerenkov counting) prior to analysis. Approximately 600 dpm was detected in cycles 1−50 and 5000 dpm remained on the blot (all values were corrected for decay from the date of electroblotting). A duplicate blot in which photoaffinity labeling of the channel was carried out in the presence of an excess of 8-Br-cGMP contained only about 500 dpm (estimated by Cerenkov counting). This indicates that, in the blot subjected to Edman degradation, 95% of the radioactivity was associated with specifically labeled sites.

(Amicon). For cyanogen bromide (CNBr) digestion, the protein samples were added to 500 μL of 0.25 M CNBr in 70% formic acid. The digestion mixture was incubated at room temperature in the dark for 24 h. After lyophilization, the CNBr digest of each labeled protein was separated by tricine−SDS−PAGE (Schägger & von Jagow, 1987; Brown et al., 1993a). In order to obtain a good separation of CNBr peptides derived from the 63-kDa subunit, electrophoresis was performed on a 20-cm-long gel, a "spacer" gel [10% T and 3% C, as defined in Schägger and von Jagow (1987)] was included between the stacking and resolving gels, and the resolving gel had a high ratio of cross-linker (16.5% T and 6% C). CNBr peptides derived from the 240-kDa protein were separated on a similar system (13% T and 3% C in the resolving gel). For peptide sequencing, gels were blotted to PVDF (Immobilon) as previously described (Brown et al., 1993a). After localization of the desired bands by either Coomassie staining or autoradiography, they were excised and analyzed on an Applied Biosystems 470A protein sequencer. For identification of labeled residues, the product of each Edman degradation cycle was collected and analyzed by liquid scintillation counting. Gels run exclusively for the purpose of visualizing labeled peptides were Coomassie-stained, soaked in Novex Gel-Dry, and air-dried between cellophane membranes prior to autoradiography. Kodak XAR film was exposed for 3−12 h at −80 °C with an intensifying screen.

Subcleavage of CNBr Peptides. Cleavage of CNBr peptides at arginine residues was performed as follows: a lyophilized CNBr digest of the labeled 63-kDa subunit was dissolved in 2 mL of 100 mM ammonium bicarbonate, pH 8, 2 M urea, 5 mM $CaCl_2$, and 10 mM DTT. The cleavage was initiated by the addition of 2 μg each of clostripain (Promega) and endoproteinase Arg-C (Boehringer-Mannheim). Incubation was continued for 48 h at room temperature with subsequent protease additions at 12-h intervals to ensure complete digestion.

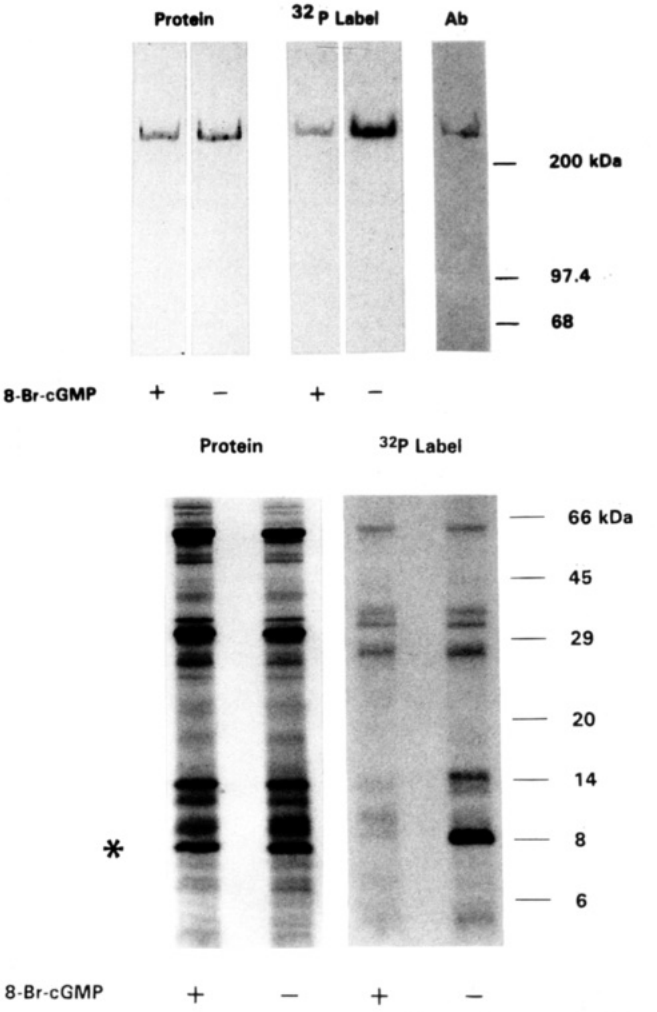


FIGURE 3: (Top) Electrophoresis of APT-[^{32}P]cGMP-labeled 240-kDa protein purified by electroelution. The left-hand panel is a Coomassie-stained SDS−5% polyacrylamide gel of the purified protein, and the center panel is the corresponding autoradiogram. Labeling of a partially purified channel preparation with APT-[^{32}P]-cGMP was carried out in the presence or absence of 1 mM 8-Br-cGMP as indicated. The right-hand panel is a western blot of a duplicate gel probed with monoclonal antibody PMs 4B2 (see text). Each lane contains 240-kDa protein purified from rod outer segments containing 10 mg of rhodopsin. (Bottom) Identification of an 8.2-kDa peptide fragment from the 240-kDa protein specifically labeled by APT-[^{32}P]cGMP. Shown is a Coomassie-stained tricine−SDS−13% polyacrylamide gel separation of the mixture of CNBr fragments (left-hand panel) and the corresponding autoradiogram (right-hand panel), with molecular mass markers on the right. The channel preparation was labeled in the presence or absence of 8-Br-cGMP (5 mM) as indicated. Each lane contains peptides derived from rod outer segments containing 45 mg of rhodopsin. The Coomassie-stained peptide indicated by the asterisk was subjected to sequence analysis (see text and Figure 4).

RESULTS

Following labeling of a partially purified channel preparation with APT-[^{32}P]cGMP, the 63-kDa subunit contains more ^{32}P label than the 240-kDa protein (Brown et al., 1993a), so we chose to locate the sites of covalent attachment in the 63-kDa protein first. It was purified by SDS−PAGE and electroelution (Brown et al., 1993a). Figure 1 (top) shows that the intact 63-kDa subunit remains specifically labeled following electroelution; the ratio of label incorporated in

```
hRCNC2   IVFQLLNYFTGVFAFSVMIGQMRDVVGAATAGQTYYRSCMDSTVKYMNFYKIPKSVQNRVKTWYEYTWHSQGMLDES
bRCNC1   YFFVVADFLIGVLIFATIVGNIGSMISNMNAARAEFQARIDAIKQYMHFRNVSKDMEKRVIKWFDYLWTNKKTVDER
         ──────── H5 ────────

hRCNC2   ELMVQLPDKMRLDLAIDVNYNIVSKVALFQGCDRQMIFDMLKRLRSVVYLPNDYVCKKGEIGREMYIIQAGQVQVLG
bRCNC1   EVLKYLPDKLRAEIAINVHLDTLKKVRIFADCEAGLLVELVLKLQPQVYSPGDYICKKGDIGREMYIIKEGKLAVVA

         ──────────────── cGMP-binding peptide ────────────────────────────────────────
hRCNC2   GPDGKSVLVTLKAGSVFGEISLLAVGG---GNRRTANVVAHGFTNLFILDKKDLNEILVHYPESQKLLRKKARRMLR
bRCNC1   D-DGITQFVVLSDGSYFGEISILNIKGSKAGNRRTANIKSIGYSDLFCLSKDDLMEALTEYPDAKGMLEEKGKQILM
         ──────── cGMP-binding peptide ──────────────────────────

hRCNC2   SNNKPKEEKSVLILPPRAGTPKLFNAALAMTGKMGGKGAKGGKLAHLRARLKELAALEAAAKQ---------QELVE
bRCNC1   -----KDGLLDINIANAGSDPKDLEEKV--TRMESSVDLLQTRFARI---LAEYESMQQKLKQRLTKVEKFLKPLID

hRCNC2   QAKSSQDVKGEEGSAAPDQHTHPKEAATDPPAPRTPPEPPGSPPSSPPPASLGRPEGEEEGPAEPEEHSVRICMSPG
bRCNC1   TEFSAIEGSGTESGPTDSTQD

hRCNC2   PEPGEQILSVKMPEEREEKAE
```

FIGURE 4: Amino acid sequence comparison of the carboxy-terminal portions of the bovine 63-kDa channel subunit (bRCNC1) and human channel subunit 2 (hRCNC2), showing the location of cGMP-binding-site peptides. The sequences begin at amino acids 373 for bRCNC1 (Kaupp et al., 1989) and 230 for hRCNC2a (Chen et al., 1993). Colons indicate identical residues. The proposed location of the final transmembrane segment, H5, is indicated (Bönigk et al., 1993; Chen et al., 1993).

the absence of competing nucleotide to that incorporated in the presence of 8-Br-cGMP was about 4:1. This subunit was then fragmented with CNBr and the labeled peptides were analyzed by tricine−SDS−PAGE. As shown in Figure 1 (middle), most of the radioactivity was contained in a 7.5-kDa peptide. At the peptide level, the ratio of specific to nonspecific labeling was nearly 20:1. This result suggests that the specific labeling was highly localized, while the nonspecific labeling was randomly distributed throughout the protein. Although the mobility of the labeled 7.5-kDa band did not precisely coincide with any of the Coomassie-stained peptides in the left-hand panel, the results presented below indicate that incorporation of APT-[^{32}P]cGMP into peptide "a" generated the labeled band, which we term "a*". The initial observation that the mobility of "a*" was slightly less than that of "a" but always greater than that of "b" is consistent with this interpretation. Attachment of the probe should increase the apparent molecular mass of the labeled peptide by about 500 Da; because labeling occurs at a low stoichiometry (Brown et al., 1993a), the labeled species would not be detected by Coomassie staining.

Sequence analysis through six residues of peptide "a" (Figure 1, bottom) revealed that it was derived from CNBr cleavage at methionine 514 (Kaupp et al., 1989). The molecular mass suggests that it extends to the next methionine at position 580. This peptide is contained entirely within the 110 amino acid region of the 63-kDa subunit that shows homology with the cGMP-dependent protein kinase and is thought to be the cGMP-binding site. In contrast, sequence analysis of peptide "b" (Figure 1, bottom) indicated that it was generated by CNBr cleavage at methionine 214 and contains regions thought to lie within the channel's transmembrane domain (Kaupp et al., 1989).

We next set out to identify the specific amino acid residues which contacted the probe and were covalently modified. A CNBr digest of the 63-kDa subunit was separated by electrophoresis and blotted as for sequence analysis. The radiolabeled band at 7.5 kDa, "a*", was excised and subjected to automated Edman degradation, and the product of each cycle was collected for scintillation counting. Chromatographic analysis was not possible due to the small amount of material contained in "a*" (<100 fmol). Figure 2 shows that measurable radioactivity began to emerge at cycle 10 and peaked at cycle 12; these cycles correspond to amino acids valine 524, valine 525, and alanine 526 in the channel sequence (see Figure 1, bottom). A tail of decreasing radioactivity was released over at least four more cycles. One possible explanation for this is that subsequent residues also incorporated the probe. Alternatively, the tail may have resulted from a low efficiency of cleavage at the modified residues and/or inefficient elution of the products from the PVDF membrane (Brzeska et al., 1989; Galzi et al., 1990). The recovery of counts during Edman degradation was low (about 6%—see Figure 2 legend) and was variable among preparations. A similar recovery has been observed in other studies (Brzeska et al., 1989; Galzi et al., 1990). The major contributing factor was probably blockage of the N-termini during electrophoresis (Allen, 1989).

To verify that most of the ^{32}P label was incorporated in the region of "a*" subjected to sequence analysis (the first 50 residues), "a*" was subcleaved with the arginine-specific endoproteases clostripain and Arg-C. This produced a radiolabeled fragment with an apparent molecular mass of 5.5 kDa (data not shown). This result agrees with the predicted 5.2-kDa fragment (44 amino acids) that should be produced if most of the label were located on the stretch of amino acids from Val-524 to Ala-526 and cleavage occurred at the subsequent arginine (558). The result also provides additional evidence that labeled peptide "a*" derives from peptide "a", since the CNBr/Arg-C fragment from peptide "b" that would contain amino acids 10−12 from the N-terminus is expected to be only 14 amino acids long.

The same experimental strategy was used to identify the specifically-labeled region of the 240-kDa protein. It was purified by SDS−PAGE and electroelution and retained its original pattern of labeling (Figure 3, top). Also shown in the figure is a Western analysis of the gel-purified labeled protein using monoclonal antibody PMs 4B2 (kindly provided by Dr. Robert S. Molday), which indicated that it was the same as the 240-kDa protein previously shown to copurify with the 63-kDa channel subunit (Molday et al., 1990; Hsu & Molday, 1994). In rod outer segments (Molday et al., 1990) and in our partially purified channel preparation containing about 30 Coomassie-stained proteins (Brown et al., 1993a), the antibody stains only this protein. Figure 3 (bottom) shows an electrophoretic analysis of fragments generated by CNBr cleavage which revealed the striking specific labeling of an 8.2-kDa peptide. The corresponding Coomassie-stained band was sequenced, and the first 16 residues were found to be identical to amino acids 372−387 of the deduced primary structure of hRCNC2a, the human second subunit recently cloned by Chen et al. (1993). [This agrees with the recent study showing that the 240-kDa protein contains a bovine version of hRCNC2 (Chen et al., 1994).] The specifically labeled peptide from the 240-kDa protein is the corresponding fragment to that labeled from the 63-kDa subunit. Figure 4 is a comparison of the C-terminal amino acid sequences of the bovine 63-kDa subunit (bRCNC1) and hRCNC2; the locations of the peptides labeled by APT-[^{32}P]cGMP are indicated. The N-termini of both peptides (sequence Tyr-Ile-Ile) were generated by cleavage at a conserved methionine. This result indicates that the 240-kDa protein is also a cGMP-binding protein. It would be very difficult, given the limitations of the current methods, to obtain enough labeled protein for the purpose of identifying individual residues that incorporated the probe (as was done for the 63-kDa subunit in Figure 2). However, the overall sequence similarity between the labeled peptides does suggest that the 240-kDa protein binds cGMP in a manner similar to the 63-kDa subunit.

DISCUSSION

A modeling study of the cGMP-binding site of the 63-kDa subunit (Kumar & Weber, 1992), loosely based on the crystal structure of the catabolite gene activator protein (CAP) from *E. coli* (McKay et al., 1982), predicted that the labeled residues, Val-524, Val-525, and Ala-526, would be located on a segment of β strand in close proximity to the C_8 position of the guanine ring system. All three residues are predicted to be within reach of the photolyzed azide moiety, which we estimate can reside more than 10 Å from the C_8 position. This model is therefore consistent with our photoaffinity labeling result, suggesting that the extension of limited sequence homology between the CAP protein and the cGMP-activated channel to structural homology is valid over regions of this cyclic nucleotide binding site. In conjunction with neighboring amino acids Leu-522 and Ala-523, as well as amino acids Phe-533, Val-534, Val-535, and Leu-536 from the proposed adjacent β strand (Kumar & Weber, 1992), the stretch of labeled amino acids may form a hydrophobic pocket on one wall of the binding site. Such a pocket could explain the high-affinity binding of several derivatives of cGMP that contain hydrophobic substituents at the C_8 position of the guanine ring, including 8-Br-cGMP, 8-[[(fluoresceinylcarbamoyl)methyl]thio]-cGMP, 8-(benzylthio)-cGMP, APT-cGMP, and 8-(*n*-propylthio)-cGMP (*n*PT-cGMP) (Zimmerman et al., 1985; Koch & Kaupp, 1985; Caretta et al., 1985; Tanaka et al., 1989; Brown et al., 1993a,b). In agreement with this idea, the positively charged derivatives 8-[(aminoethyl)thio]-cGMP and 8-[[(trimethylamino)ethyl]thio]-cGMP were much less effective in activating the channel than *n*PT-cGMP (Brown et al., 1993b).

In previous photoaffinity labeling studies of another cyclic-nucleotide binding protein, 8-azido-[^{32}P]cAMP was used to identify amino acid contact points in both isotypes of cAMP-dependent protein kinase regulatory subunits (RI and RII) (Kerlavage & Taylor, 1980; Bubis & Taylor, 1985). In these studies a conserved tyrosine residue (Y371 in RI and Y381 in RII) was labeled in each protein. These residues lie far downstream in the primary structure from the residues corresponding to the three labeled in the current study. The conserved tyrosine residues are thought to exist on an extended α-helix (Kerlavage & Taylor, 1980; Bubis & Taylor, 1985), that corresponds to the C-helix of the CAP protein (McKay et al., 1982). In a recent study, the corresponding region of the bovine rod channel (residues 588−609) was shown to affect cyclic nucleotide selectivity (Goulding et al., 1994). In light of these results, it is interesting to note that 8-azido-[^{32}P]cGMP failed to label the channel under conditions in which APT-[^{32}P]cGMP labeled the channel subunit strongly (data not shown). Although the proposed C-helical region of the channel is likely to make contact with some part of the guanine ring system (Goulding et al., 1994), these residues may not be in close proximity to the C_8 position. The difference in labeling results on the kinase and channel suggests at least two possibilities: (1) the two proteins have a somewhat different structure in this region or (2) cGMP binds to the channel in a different conformation than cAMP assumes when it binds to the kinase.

The data presented here provide biochemical evidence that the 240-kDa protein that copurifies with the 63-kDa channel subunit binds cGMP. Taken together with other studies, the current results support the conclusion that the 240-kDa protein is an integral component or subunit of rod cGMP-activated channels. As described earlier, the 240-kDa protein contains a version of the cloned protein that confers on expressed channels several functional properties exhibited by the native channel (Chen et al., 1993, 1994). It has also been found that the complex of Ca^{2+} and calmodulin decreases the native channel's sensitivity to cGMP and that this complex binds to the 240-kDa protein (Hsu & Molday, 1993, 1994). Based on the work described here, an important goal for the future will be to discover what role cGMP binding to the 240-kDa protein plays in channel activation. In this regard, it is interesting to note that the cloned second subunit of olfactory cyclic nucleotide-gated channels appears to be largely responsible for the native channel's sensitivity to cAMP (Bradley et al., 1994; Liman & Buck, 1994).

ACKNOWLEDGMENT

We thank Dr. MariaLuisa Ruiz and Dr. Arthur Polans for their comments on the manuscript.

REFERENCES

Allen, G. (1989) *Sequencing of Proteins and Peptides*, 2nd ed., Chapter 4, Elsevier, New York.

Altenhofen, W., Ludwig, J., Eismann, E., Kraus, W., Bönigk, W., & Kaupp, U. B. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9868−9872.
Baylor, D. A., & Fettiplace, R. (1977) *J. Physiol. 271*, 391−424.
Bönigk, W., Altenhofen, W., Müller, F., Dose, A., Illing, M., Molday, R. S., & Kaupp, U. B. (1993) *Neuron 10*, 865−877.
Bradley, J., Li, J., Davidson, N., Lester, H. A., & Zinn, K. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 8890−8894.
Brown, R. L., Gerber, W. V., & Karpen, J. W. (1993a) *Proc. Natl. Acad. Sci. U.S.A. 90*, 5369−5373.
Brown, R. L., Bert, R. J., Evans, F. E., & Karpen, J. W. (1993b) *Biochemistry 32*, 10089−10095.
Brzeska, H., Lynch, T. J., Martin, B., & Korn, E. D. (1989) *J. Biol. Chem. 264*, 19340−19348.
Bubis, J., & Taylor, S. S. (1985) *Biochemistry 24*, 2163−2170.
Caretta, A., Cavaggioni, A., & Sorbi, R. T. (1985) *Eur. J. Biochem. 153*, 49−53.
Chen, T.-Y., Peng. Y.-W., Dhallan, R. S., Ahamed, B., Reed, R. R., & Yau, K.-W. (1993) *Nature 362*, 764−767.
Chen, T.-Y., Illing, M., Molday, L. L., Hsu, Y.-T., Yau, K.-W., & Molday, R. S. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11757−11761.
Cook, N. J., Hanke, W., & Kaupp, U. B. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 585−589.
Dowling, J., & Ripps, H. (1973) *Nature 242*, 101−103.
Galzi, J.-L., Revah, F., Black, D., Goeldner, M., Hirth, C., & Changeux, J.-P. (1990) *J. Biol. Chem. 265*, 10430−10437.
Garbers, D. L. (1976) *J. Biol. Chem. 251*, 4071−4077.
Goulding, E. H., Tibbs, G. R., & Siegelbaum, S. A. (1994) *Nature 372*, 369−374.
Haynes, L. W., Kay, A. R., & Yau, K.-W. (1986) *Nature 321*, 66−70.
Hsu, Y.-T., & Molday, R. S. (1993) *Nature 361*, 76−79.
Hsu, Y.-T., & Molday, R. S. (1994) *J. Biol. Chem. 269*, 29765−29770.
Illing, M., Colville, C. A., Williams, A. J., & Molday, R. S. (1994) *Invest. Ophthalmol. Vis. Sci. Suppl. 35*, 1474.
Karpen, J. W., Zimmerman, A. L., Stryer, L., & Baylor, D. A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1287−1291.
Kaupp, U. B. (1991) *Trends Neurosci. 14*, 150−157.
Kaupp, U. B., Niidome, T., Tanabe, T., Terada, S., Bönigk, W., Stühmer, W., Cook, N. J., Kangawa, K., Matsuo, H., Hirose, T., Miyata, T., & Numa, S. (1989) *Nature 342*, 762−766.
Kerlavage, A. R., & Taylor, S. S. (1980) *J. Biol. Chem. 255*, 8483−8488.
Koch, K.-W., & Kaupp, U. B. (1985) *J. Biol. Chem. 260*, 6788−6800.
Kumar, V. D., & Weber, I. T. (1992) *Biochemistry 31*, 4643−4649.
Lagnado, L., & Baylor, D. A. (1992) *Neuron 8*, 995−1002.
Liman, E. R., & Buck, L. B. (1994) *Neuron 13*, 611−621.
McKay, D. B., Weber, I. T., & Steitz, T. A. (1982) *J. Biol. Chem. 257*, 9518−9524.
McNaughton, P. A. (1990) *Physiol. Rev. 70*, 847−883.
Molday, L. L., Cook, N. J., Kaupp, U. B., & Molday, R. S. (1990) *J. Biol. Chem. 265*, 18690−18695.
Schägger, H., & von Jagow, G. (1987) *Anal. Biochem. 166*, 368−379.
Stryer, L. (1991) *J. Biol. Chem. 266*, 10711−10714.
Tanaka, J. C., Eccleston, J. F., & Furman, R. E. (1989) *Biochemistry 28*, 2776−2784.
Yarfitz, S., & Hurley, J. B. (1994) *J. Biol. Chem. 269*, 14329−14332.
Yau, K.-W. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3481−3483.
Yau, K.-W., & Baylor, D. A. (1989) *Annu. Rev. Neurosci. 12*, 289−327.
Zimmerman, A. L., & Baylor, D. A. (1986) *Nature 321*, 70−72.
Zimmerman, A. L., Yamanaka, G., Eckstein, F., Baylor, D. A., & Stryer, L. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8813−8817.

BI950100V