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Structural Features of the Noncatalytic cGMP Binding Sites of Frog Photoreceptor Phosphodiesterase Using cGMP Analogs*

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Marcia C. Hebert‡, Frank Schwede§, Bernd Jastorff§, and Rick H. Cote‡¶

From the ‡Department of Biochemistry and Molecular Biology, University of New Hampshire, Durham, New Hampshire 03824 and §Zentrum für Umweltforschung und Umwelttechnologie (UFT), Abt. III Bioorganische Chemie, Universität Bremen, D-28359 Bremen, Federal Republic of Germany

The cGMP-specific phosphodiesterase (PDE) of retinal photoreceptors is a central regulatory enzyme in the visual transduction pathway of vertebrate vision. Although the mechanism of activation of PDE by transducin is well understood, the role of the noncatalytic cGMP binding sites located on the catalytic subunits of PDE remains obscure. We report here for the first time the molecular basis of the noncovalent interactions between cGMP and the high affinity, noncatalytic cGMP binding sites of frog photoreceptor PDE.

None of the tested cGMP analogs were able to bind with greater affinity than cGMP itself, and the noncatalytic sites were unable to bind cAMP. The major determinant for discrimination of cGMP over cAMP is in the N-1/C-6 region of the purine ring of cGMP where hydrogen bonding probably stabilizes the selective binding of cGMP. Substitutions at the C-2 position demonstrate that this region of the molecule plays a secondary but significant role in stabilizing cGMP binding to PDE through hydrogen bond interactions. The unaltered hydrogen at the C-8 position is also important for high affinity binding. A significant interaction between the binding pocket and the ribose ring of cGMP occurs at the 2′-hydroxyl position. Steric constraints were greatest in the C-8 and possibly the C-6/N-1 regions, whereas the C-2/N-3 and C-2′ regions tolerated bulky substituents better. Several lines of evidence indicate that the noncatalytic site binds cGMP in the *anti*-conformation. The numerous noncovalent interactions between cGMP and the noncatalytic binding pocket of the photoreceptor PDE described in this study account for both the high affinity for cGMP and the high level of discrimination of cGMP from other cyclic nucleotides at the noncatalytic site.

Visual excitation of vertebrate retinal photoreceptors begins with the absorption of light by the visual pigment (opsin) which is followed by activation of a G-protein, transducin. This results in activation of a photoreceptor-specific cGMP phosphodiesterase (PDE).[1] Increased cGMP hydrolysis lowers the cytoplasmic cGMP concentration which is believed to cause closure of the cGMP-gated ion channel in the plasma membrane and the generation of an electrical response (for reviews, see Refs. 1–5).

The rod photoreceptor PDE holoenzyme is a heterotetramer consisting of two non-identical catalytic subunits (α and β) and two small subunits (γ) that serve to inhibit catalytic activity. Cone photoreceptors have a highly homologous PDE that consists of two identical catalytic subunits (α') and two cone-specific γ' subunits. In addition to containing the active site for cGMP hydrolysis, the catalytic subunits of rod and cone PDEs also possess noncatalytic cGMP binding sites (6). Two other classes of PDEs, namely the cGMP-stimulated PDE (PDE2) and the cGMP-specific PDE (PDE5), are also known to contain noncatalytic binding sites for cGMP. In the case of PDE2, binding of cGMP at the noncatalytic site allosterically activates cyclic nucleotide hydrolysis at the active site (7, 8). For PDE5, the noncatalytic cGMP binding site has been proposed to indirectly regulate catalytic activity of the enzyme via protein phosphorylation (for review, see Ref. 9).

The function of the noncatalytic cGMP binding sites on the photoreceptor PDE (classified as PDE6) is currently not well understood. Recent evidence has supported the idea that binding of cGMP to noncatalytic sites on amphibian rod PDE enhances the affinity of the inhibitory γ subunits for binding to the catalytic subunits (10). Conversely, displacement or removal of the γ subunits from PDE by G-protein activation causes a marked decrease in the binding affinity of cGMP with these binding sites (11, 12). These results suggest that the noncatalytic cGMP binding sites may be the locus for an additional type of regulation of PDE during visual transduction.

To better understand the functional significance of these noncatalytic binding sites, we have examined the structural requirements for the binding of cGMP to the binding pocket of the noncatalytic sites on rod PDE. We have employed a series of derivatives of cGMP modified in particular positions of the cGMP molecule to identify the types of interactions that stabilize the high affinity binding of cGMP to noncatalytic sites on PDE. Cyclic nucleotide analogs have been successfully employed to define the topology of the nucleotide binding site in several proteins, including the cAMP-dependent protein kinase (13, 14), the cGMP-dependent protein kinase (15), the cyclic nucleotide-gated ion channels (16–19), as well both catalytic (20, 21) and noncatalytic (9, 22–24) binding sites on the various classes of PDEs.

Our results with the noncatalytic cGMP binding site of frog

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¶ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of New Hampshire, 46 College Rd., Durham, NH 03824-2617. Tel.: 603-862-2458; E-mail: Rick.Cote@unh.edu.

[1] The abbreviations used are: PDE, phosphodiesterase; ROS, rod outer segments; (R_p)-cGMPS, guanosine 3′,5′-cyclic monophosphorothioate (R_p isomer); (S_p)-cGMPS, guanosine 3′,5′-cyclic monophosphorothioate (S_p isomer); cPUMP, purine 3′,5′-cyclic monophosphate; TEAF, triethylammonium formate; PDE2, cGMP-stimulated PDE; PDE5, cGMP-binding, cGMP-specific PDE; HPLC, high pressure liquid chromatography; FAB-MS, fast atom bombardment mass spectroscopy.

rod PDE demonstrate that the high affinity binding of cGMP is a consequence of multiple sites of interaction between the cGMP molecule and the binding pocket of the enzyme. In particular, the C-6, N-1, C-2, and C-8 positions of the purine ring, the cyclic phosphate moiety, and the 2′-hydroxyl group of the ribose ring all contribute to stabilizing specific binding to cGMP. Furthermore, the noncatalytic site of the rod PDE shows the greatest ability to discriminate cGMP from cAMP and the highest binding affinity of any cyclic nucleotide binding protein studied to date.

EXPERIMENTAL PROCEDURES

Materials—Frogs were obtained from Charles Sullivan, Inc. Multiscreen filtration plates (MAHA N45) were purchased from Millipore. Scintillation fluid (Ultima Gold) was obtained from Packard Instrument Co. [^{3}H]cGMP was obtained from NEN Life Science Products. All chemicals other than cyclic nucleotide analogs were obtained from Sigma.

Cyclic Nucleotides and Derivatives—cGMP, cIMP (**8**), cXMP (**19**), cAMP (**7**), thymidine 3′,5′-cyclic monophosphate, uridine 3′,5′-cyclic monophosphate, cytidine 3′,5′-cyclic monophosphate, 8-Br-cGMP (**29**), 2′-*O*-monobutyryl-cGMP (**47**), 2′-deoxy-cGMP (**46**), and 5′-GMP (**43**) were purchased from Sigma. (R_p)-cGMPS (**37**) and (S_p)-cGMPS (**38**) were purchased from Biolog Life Science Institute, Bremen. We also acknowledge the kind gifts of the following cGMP analogs: compound **27** (F. Seela, Osnabrück); compounds **30**, **31**, and **36** (G. Weimann, Boehringer Mannheim); compounds **32** and **33** (S. H. Francis, Vanderbilt University); and compound **44** (M. Morr, Braunschweig). All nucleotides in this study showed a purity of >98–99.5% and less than 0.1% cGMP contamination, as determined by analytical HPLC using a 250 × 4-mm, 10-μm LiChrosorb RP 18 column (Merck).

Synthesis—Previously reported methods were used to synthesize compounds **1** (25), **2**, **4**, **5**, **28**, **29** (26), **39**, **40**, **41** (27), **35** (28), **42** and **48**,[2] and **45** (29). Compounds **9**, **11–18**, **20-26**, **34** were prepared with modifications to the method of Michal *et al.* (30).

Preparation of 0^6-Benzyl-guanosine and O^6-Benzyl-cGMP (3)—O^6-Benzyl-cGMP (**3**) was synthesized according to the methods of Chae *et al.* (31) and Genieser *et al.* (26) with significant modifications. In brief, O^6-benzyl-guanosine was prepared by reacting 2-NH_2-6-Cl-purine riboside (1.7 g, 5.63 mmol) with sodium benzoxide (6.21 mmol) in 20 ml of dry benzyl alcohol. The alkylating reagent was generated by pretreatment of the alcohol with NaH (149 mg, 6.2 mmol) at 0–25 °C for 2 h. This mixture was stirred at room temperature for 30 h and then evaporated to dryness. The residue was resuspended in 200 ml of water, neutralized with 1 N HCl, and the volume reduced to approximately 100 ml by evaporation. Upon cooling, a white precipitate of crude product was filtered off, washed twice with 20 ml of cold water, and dried to give analytically pure O^6-benzyl-guanosine (1.971 g, 5.24 mmol, 93%) as a white solid.

^{1}H NMR (Me_2SO-d_6), δ 3.53 (dd, 1*H*, *J* = 3.5, 12.0 Hz, H5′b), 3.63 (dd, 1*H*, *J* = 3.5, 12.0 Hz, H5′a), 3.89 (ddd, 1*H*, *J* = 3.5, 3.5, 3.5 Hz, H4′), 4.10 (dd, 1*H*, *J* = 3.5, 5.5 Hz, H3′), 4.46 (dd 1*H*, *J* = 5.5, 6.0 Hz, H2′), 5.50 (d, 2*H*, *J* = 1.0 Hz, —CH_2—), 6.28 (d, 1*H*, *J* = 6.0 Hz, H1′), 6.50 (broad s, 2*H*, −NH_2), 7.30–7.55 (m, 5*H*, phenyl ring), 8.10 (s, 1*H*, H8); FAB-MS (glycerol), positive mode: *m/z* 374 $(M + H)^+$, 242 $(B + H)^+$, negative mode: *m/z* 372 $(M - H)^-$, 240 $(B - H)^-$, 745 $(M_2 - H)^-$, UV (pH 7.0), λ_{max} = 282 nm (ε = 10,700).

O^6-Benzyl-guanosine (655 mg, 1.76 mmol) was phosphorylated in the 5′-position and cyclized to the 3′,5′-cyclic monophosphate essentially as described (26). The crude product was purified by preparative HPLC with 45% methanol, 55% water, 40 mM triethylammonium formate (TEAF) (pH 6.5); the stationary phase was a 250 × 25 mm LiChrosorb RP 18 (either 7- or 10-μm particle size; Merck). Fractions containing the product were pooled and freeze-dried eight times to remove residual TEAF. The resulting white foam was neutralized with 0.1 N NaOH and dried to obtain 556.6 μmol (32%) of compound **3** as the sodium salt. The purity was higher than 99.3% as verified by analytical HPLC and no cGMP was detectable.

^{1}H NMR (Me_2SO-d_6), δ 3.86–4.10 (m, 3*H*, H4′, H5′a, H5′b), 4.40–4.46 (m, 2*H*, H2′, H3′), 5.49 (s, 2*H*, —CH_2—), 5.78 (s, 1*H*, H1′), 5.79 (d, 1*H*, *J* = 4.0 Hz, 2′-OH), 6.58 (broad s, 2H, —NH_2), 7.31–7.52 (m, 5*H*, phenyl ring), 7.96 (s, 1*H*, H8); ^{31}P NMR (Me_2SO-d_6), δ −2.58 ppm, FAB-MS (glycerol), positive mode: *m/z* 436 $(M + H)^+$, negative mode: *m/z* 434 $(M - H)^-$, UV (pH 7.0), λ_{max} = 281 nm (ε = 10,700).

Preparation of N^2,3-Etheno-O^6-benzyl-GMP (6)—Syntheses of compounds **6** and **10** were based on the method of Kusmierek *et al.* (32). In brief, O^6-benzyl-cGMP (**3**) (137.1 mg, 300 μmol) was mixed with sodium acetate, bromoacetaldehyde, and ethanol and stirred in darkness at 37 °C for 7 days. Analytical HPLC indicated 95% conversion of the starting material and a major product signal (70%) of a compound with enhanced hydrophobicity. Evaporation of all volatile components under vacuum gave a yellow residue, which was purified using preparative HPLC (solvent: 30% methanol, 70% water, 40 mM TEAF (pH 7.0)). The sample was freeze-dried three times and neutralized with 0.1 N NaOH to yield 116 μmol (37%) analytically pure N^2,3-etheno-O^6-benzyl-GMP (**6**) (>98.5% purity).

^{1}H NMR (Me_2SO-d_6), δ 4.08–4.17 (m, 3*H*, H4′, H5′a, H5′b), 4.24 (m, 1*H*, H3′), 4.62 (dd, 1*H*, *J* = 4.0, 4.5 Hz, H2′), 5.58 (s, 2*H*, —CH_2—), 6.24 (d, 1*H*, *J* = 4.0 Hz, 2′-OH), 6.35 (s, 1*H*, H1′), 7.51 (s, 1*H*, H6), 7.34–7.56 (m, 5*H*, phenyl ring), 7.94 (s, 1*H*, H5), 8.23 (s, 1*H*, H2); ^{31}P NMR (Me_2SO-d_6), δ −2.80 ppm, FAB-MS (nitrobenzyl alcohol), positive mode: *m/z* 460 $(M + H)^+$, negative mode: *m/z* 458 $(M - H)^-$, UV (pH 7.0), λ_{max} = 226 nm (ε = 32,900), 266 nm (ε = 6800).

Preparation of N^2,3-Etheno-cGMP (10)—Compound **6** (80 μmol) was added to 10 ml of 70% methanol (in water) in the presence of 10% palladium on charcoal (50 mg); the sample was completely hydrogenated after 40 min at room temperature, as observed by analytical HPLC. The catalyst was filtered off, extracted 3 times with 70% methanol, and then extracted 3 times with toluene/methanol/water (5:5:1, v/v/v). The solvents were evaporated under vacuum at 20 °C and purified by preparative HPLC (solvent: 15% methanol, 85% water, 20 mM TEAF (pH 7.0)) to yield 52 μmol of **10** (97% purity). A purity higher than 99.3% with no detectable cGMP contamination was obtained after two additional chromatographic separations under identical conditions. Freeze-drying and neutralization with 0.01 N NaOH gave 27.5 μmol (34%) product.

^{1}H NMR (Me_2SO-d_6), δ 4.06–4.17 (m, 3*H*, H4′, H5′a, H5′b), 4.21 (m, 1*H*, H3′), 4.57 (dd, 1*H*, *J* = 4.0, 4.0 Hz, H2′), 6.19 (s, 1*H*, H1′), 6.22 (d, 1*H*, *J* = 4.0 Hz, 2′-OH), 7.14 (d, 1*H*, *J* = 2.0 Hz, H6), 7.62 (d, 1*H*, *J* = 2.0 Hz, H5), 8.06 (s, 1*H*, H2); ^{31}P NMR (D_2O/CD_3OD, 1/1), δ − 1.30 ppm, FAB-MS (nitrobenzyl alcohol) positive mode: *m/z* 370 $(M + H)^+$, negative mode: *m/z* 368 $(M - H)^-$, UV (pH 7.0), λ_{max} = 225 nm (ε = 29,700), 258 nm (ε = 10,700).

Analytic Section—^{1}H and ^{31}P NMR, as well as FAB-MS analyses, were used to confirm the structure of each cGMP analog. ^{1}H and ^{31}P NMR spectra (internal standard Me_4Si and external H_3PO_4 85%, respectively) were recorded with a Bruker WH-360 spectrometer. Mass spectra were obtained with a Finnigan MAT spectrometer (model 8222) in the FAB mode and glycerol or nitrobenzyl alcohol as matrix. UV spectra were recorded with a Milton Roy Spectronic 1204 photometer in aqueous buffer at pH 7.0.

Photoreceptor PDE Preparation—Rod outer segments (ROS) from bullfrog (*Rana catesbiana*) retinas were purified on a Percoll gradient in total darkness as described previously (33). Briefly, ROS were isolated from frog retinas by gentle shaking and purified on a discontinuous gradient containing 60, 30, and 5% Percoll in a Ringer's solution consisting of (in mM): 105 NaCl, 2.0 $MgCl_2$, 2.0 KCl, 1.0 $CaCl_2$, and 10 HEPES, pH of 7.5. Purified ROS were washed once in Ringer's to remove Percoll and resuspended in Buffer A, containing (in mM): 77 KCl, 35 NaCl, 2.0 $MgCl_2$, 1.0 $CaCl_2$, 10 HEPES, and 1.2 EGTA (pCa 300 nM), pH 7.5; the ionic composition of Buffer A partially mimicked that of frog ROS cytosol. The ROS suspension was homogenized 12 times with a Potter-Elvehjem tissue grinder to completely disrupt cellular morphology. Endogenous cGMP initially bound to PDE would interfere with the cGMP binding assay; this problem was avoided by incubating homogenized ROS suspensions at room temperature for 30 min, during which time >95% of the bound cGMP dissociated from PDE and was hydrolyzed by the PDE activity in the homogenate (33). The ROS extract was stored in the dark at 4 °C until use. Binding of cGMP to photoreceptor PDE was determined without further purification of the protein, since it has been previously demonstrated that PDE is the only high affinity cGMP binding protein in frog ROS extracts (33). The concentration of PDE holoenzyme was determined by measuring the rhodopsin concentration by difference spectrophotometry (34) and knowledge of the molar ratio of PDE to rhodopsin (0.003 mol of PDE holoenzyme per mol of rhodopsin). The subunit stoichiometry of the PDE holoenzyme in our frog ROS homogenates is assumed to be $\alpha\beta\gamma_2$ based on the following considerations. 1) When saturating levels of [^{3}H]cGMP (1 μM) were used in binding assays as controls, we routinely observed 1.8–2.1 mol of cGMP bound per mol of PDE holoenzyme, in agreement with our previous measurements of the binding site density of frog PDE (11, 33). Since PDE which lacks Pγ subunits cannot bind

[2] B. Jastorff, unpublished results.

cGMP at detectable levels (11, 12), we infer that ≥90% of the PDE molecules must have bound Pγ subunits. 2) Measurements of the PDE hydrolytic activity of frog ROS homogenates (12 nM PDE) show <3% of the activity of fully activated PDE (induced by either transducin activation or limited trypsin proteolysis of the Pγ subunit). This too indicates that >97% of the PDE catalytic subunits is associated with Pγ.

The PDE catalytic site inhibitor, zaprinast, was added 5 min prior to addition of cyclic nucleotides to prevent cyclic nucleotide hydrolysis; direct measurements confirm that <10% hydrolysis of cGMP had occurred at the time when samples were filtered. Zaprinast had little effect on the ability of cGMP to bind to the noncatalytic sites. In contrast to the ability of catalytic site inhibitors to stimulate cGMP binding to noncatalytic sites on PDE2 (23), PDE5 (35), or the cone photoreceptor PDE (36), zaprinast shows no ability to stimulate cGMP binding to frog photoreceptor PDE.[3] We find rather that 1 mM zaprinast causes a <15% decrease in cGMP binding to the noncatalytic sites of PDE when assayed under the exact conditions of our displacement experiments.

Filter Binding Assay—The extent of equilibrium binding of [^{3}H]cGMP in the presence of varying levels of cyclic nucleotide analogs was determined with a modification of a previously used filter binding assay (33). Portions of homogenized ROS (4 μM rhodopsin, 12 nM PDE holoenzyme) were mixed with 60 nM [^{3}H]cGMP (6.64×10^{16} dpm/mol; prepared in Buffer A) which had been premixed with the indicated concentration of a cyclic nucleotide analog. This concentration of [^{3}H]cGMP was chosen such that ~50% of the high affinity noncatalytic sites on PDE would be occupied in the absence of competing nucleotides. Within 5 min after the addition of 60 nM [^{3}H]cGMP, ~90% of the maximum extent of binding was observed; no change in the extent of binding was observed between 10 and 60 min of incubation. The approach to equilibrium was not delayed when the competing nucleotide analog was added at the same time as cGMP.

After incubation at 24 °C for 15 min, samples were applied to a 96-well Multiscreen filter plate prewet with Buffer A, and the filters were rinsed with 300 μl of the same solution. The filters were punched out directly into scintillation vials, mixed with 100 μl of water, vortexed, and then mixed with 3 ml of scintillation fluid, and radioactivity was determined in a scintillation counter. To determine the extent of nonspecific entrapment of [^{3}H]cGMP by the filters, ROS were mixed with [^{3}H]cGMP containing 1 mM nonradioactive cGMP and treated as above. Less than 1% of the radioactivity was nonspecifically retained in the presence of ROS membranes; similar levels of nonspecific binding were observed when [^{3}H]cGMP alone was passed through the filter and rinsed.

Data analysis of the nucleotide displacement curves was performed assuming a single class of noninteracting sites to which cGMP and the nucleotide analogs both bound in a competitive fashion. The data analysis programs Sigmaplot and RADLIG (37) each provided similar values for the binding parameters. The K_i value was calculated from the IC_{50} value obtained from the displacement curve using the equation (38), $K_i = IC_{50}(1 + K_D/[cGMP])$, where the dissociation constant, K_D, for cGMP binding to PDE is 60 nM (11, 33). The K_i values presented in Tables I–V represent the mean ± S.D. for three or four independent experiments for each analog. For all pairwise comparisons of cGMP analogs discussed in the text, the K_i values were determined to be statistically significant at the $p < 0.005$ level of significance using a paired *t* test.

RESULTS AND DISCUSSION

We chose a set of chemically modified cGMP analogs (Fig. 1) to probe in detail the structural requirements for cGMP binding to the noncatalytic cGMP binding sites of frog rod PDE. Since the rod PDE consists of two non-identical α and β catalytic subunits, we first addressed the question of whether we could distinguish two distinct noncatalytic binding sites in our experiments. Previous equilibrium binding data at sub-micromolar cGMP concentrations have provided no evidence for two distinct classes of high affinity sites in nonactivated PDE (11, 33). In addition, the determination of the K_i for cIMP was carried out at several different [^{3}H]cGMP concentrations that resulted in 0.2, 0.4, 1.0, or >1.8 mol cGMP bound per mol of PDE holoenzyme in the absence of the cGMP analog; no significant difference was found in the K_i value as a function of the extent of occupancy by cGMP.[4] Furthermore, in those instances where the [^{3}H]cGMP concentration was high enough to saturate all high affinity sites on PDE, addition of cGMP analogs never displayed complex displacement behavior. These observations support the notion that the two noncatalytic binding sites on non-activated PDE holoenzyme cannot be distinguished by the equilibrium binding measurements performed in this study. In addition, binding of [^{3}H]cGMP to the catalytic sites of PDE can been ruled out for two reasons as follows: 1) the low concentration of [^{3}H]cGMP used was 1000-fold lower than the K_M of the enzyme, and 2) the inclusion of 1 mM zaprinast (a competitive inhibitor of cGMP hydrolysis) effectively blocked [^{3}H]cGMP binding and catalysis at the active site of PDE.

Substitutions in the N-1/C-6 Region of the Purine Ring—We first tested the ability of cGMP analogs with alterations in the C-6 and/or the N-1 positions of the cGMP molecule (Fig. 1*B*) to bind to the noncatalytic sites of photoreceptor PDE, since these atoms differ between cGMP and cAMP. Purified frog ROS extracts were incubated with 60 nM [^{3}H]cGMP and various concentrations of a cGMP analog. After the binding reaction had reached equilibrium, the extent to which [^{3}H]cGMP was displaced by the analog was quantitated by a filter binding assay. Fig. 2 shows typical results for several analogs substituted at the N-1 or the C-6 positions of the purine ring; displacement of [^{3}H]cGMP by nonradioactive cGMP is also included for comparison. We found that 6-mercapto-cGMP (**1**) was able to bind to the noncatalytic sites nearly as well as cGMP itself, as judged by its ability to prevent [^{3}H]cGMP binding at low concentrations (Table I). This suggests that the electronegativity of the oxygen attached to position 6 in cGMP stabilizes binding to the binding pocket of PDE and demonstrates that the mercapto group retains the ability to interact with the binding pocket. Although compound **1** can potentially exist in both a keto and enol tautomeric form (Fig. 1*B, inset*), existing evidence suggests that thio-substituted nucleosides exist almost exclusively in the keto (thio-oxo) form (39).

Replacement of the oxygen atom at the C-6 position with H (2-NH_2-cPUMP; **4**) or an O^6-benzyl group (**3**) reduced binding affinity 700–800-fold (Table I), suggesting that the oxo group provides an important stabilizing interaction between cGMP and the binding pocket. However, these substitutions also involve a deprotonation at N-1 due to formal rearrangement of the double bonds in the purine ring (Fig. 1), and so an interaction between the noncatalytic site and the N-1 hydrogen may also be important. This idea is supported by the 290-fold reduction in binding affinity that results when H bonding capacity at the N-1 position is eliminated (N^1-methyl-cGMP; **2**). The lowered binding affinity of compound **2** may also be due to steric constraints at the N-1 position.

The fact that the O^6-benzyl-cGMP (**3**) binds somewhat better than 2-amino-cPUMP (**4**) suggests the following: 1) the remaining electron withdrawing effect on the purine ring system of O^6-benzyl-cGMP may still stabilize binding to some extent, and 2) the spatial constraints of the binding pocket cannot be a major factor since it does not preclude binding of the bulky benzyl group. However, we cannot rule out the possible artifact that would result if the benzyl group of **3** favorably interacted with a hydrophobic amino acid residue in the binding pocket.

Although no definitive conclusion about the type of interaction with the binding pocket can be drawn from comparison of analogs **1–4**, it is likely that the electronegativity of the 6-oxo group stabilizes binding by serving as an hydrogen bond acceptor, whereas the N-1 hydrogen atom serves as a hydrogen bond

[3] M. R. D'Amours and R. H. Cote, manuscript in preparation.

[4] S. Thapar and R. H. Cote, unpublished observations.

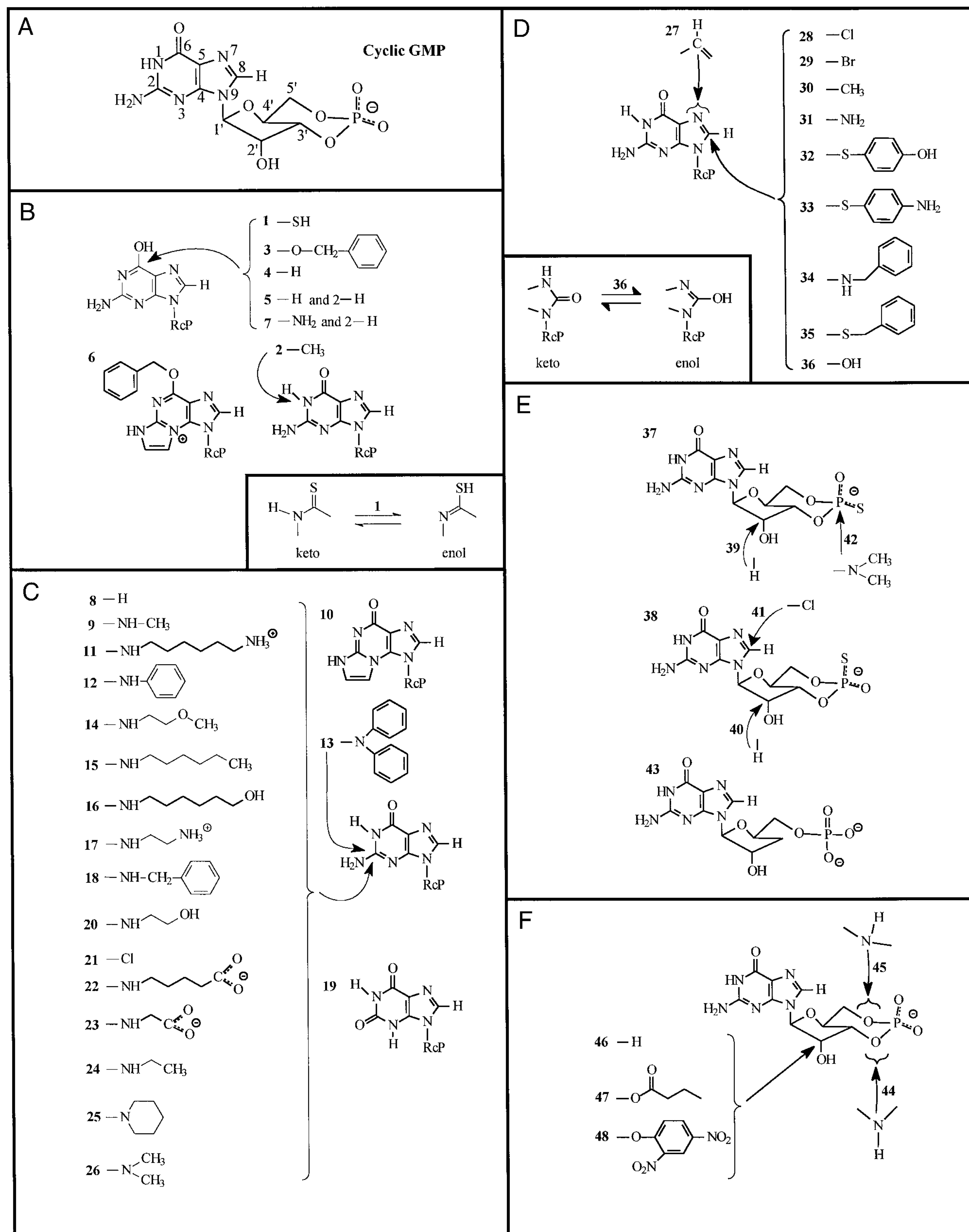

FIG. 1. **Structures of cGMP and the derivatives used in this study.** The name of each numbered compound is listed in Tables I–V. *A*, cGMP with purine and ribose ring atoms numbered; note that cGMP is drawn in the *anti*-conformation. *B*, analogs substituted at the N-1 and C-6 positions. *RcP*, ribose cyclic phosphate moiety. *Inset*, compound **1** can exist in both keto and enol tautomeric forms. *C*, cGMP analogs substituted at the C-2 or N-3 position. *D*, derivatives substituted at the N-7 and C-8 position. *Inset*, **36** is shown undergoing keto-enol tautomerism. *E*, analogs substituted at the cyclic phosphate moiety along with several di-substituted derivatives. *F*, analogs substituted in the ribose ring.

donor. However, the relative importance of these two interactions in the stabilization of cGMP binding to PDE cannot be quantitated at present.

We also examined three analogs that were modified at both the 2- and 6-positions of the purine ring; these di-substituted analogs also resulted in deprotonation at N-1 (**5–7**). In all three

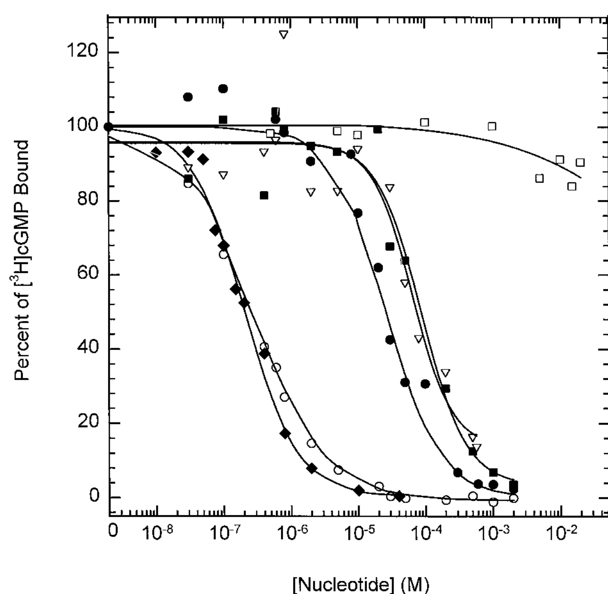

FIG. 2. **Displacement of [^{3}H]cGMP binding by cGMP analogs and by cGMP and cAMP.** Photoreceptor PDE was incubated with 60 nM [^{3}H]cGMP and the indicated concentration of cGMP (◆), 6-SH-cGMP (**1**, ○) N^1-CH$_3$-cGMP (**2**, ●), O^6-benzyl-cGMP (**3**, ■), 2-NH$_2$-cPUMP (**4**, △), and cAMP (**7**, □). The amount of radioactivity bound to PDE was quantitated with a filter binding assay (see "Experimental Procedures"). Binding was normalized in each experiment to the value obtained in the presence of 60 nM [^{3}H]cGMP alone (1.0 ± 0.2 mol of cGMP bound per mol of PDE holoenzyme). Each data point represent the average of three or four experiments, where the coefficient of variation was typically ≤10%.

TABLE I
Substitutions in the N-1/C-6 region of cGMP

Results in this table are calculated from the displacement curves in Fig. 2, as described in detail under "Experimental Procedures."

No.[a]	Nucleotide	K_i[b]	K_i/K_D[c]
		μM	
1.	6-SH-cGMP	0.15 ± 0.04	2.5
2.	N^1-CH$_3$-cGMP	17.4 ± 0.2	290
3.	O^6-Benzyl-cGMP	40.2 ± 0.1	670
4.	2-NH$_2$-cPUMP	47.3 ± 0.2	788
5.	cPUMP	>4 mM	$>7 \times 10^4$
6.	N^2,3-Etheno-O^6-benzyl-cGMP	>5 mM	$>8 \times 10^4$
7.	cAMP	>40 mM	$>7 \times 10^5$

[a] Compound numbers refer to the structures in Fig. 1.
[b] The inhibition constant (K_i) was determined from the fitted curves in Fig. 2, using the equation $K_i = \mathrm{IC}_{50}(1 + K_D/[\mathrm{cGMP}])$. The values represent the mean ± S.D. for n = 3 or 4 experiments.
[c] The ratio of the inhibition constant to the cGMP dissociation constant (K_D) was calculated using a K_D = 60 nM.

cases, no detectable affinity for the noncatalytic binding sites could be discerned. Indeed, no significant displacement of 60 nM [^{3}H]cGMP was observed when 20 mM cAMP was added.

This result represents a $>10^5$-fold level of discrimination between the two cyclic nucleotides, implying that the noncatalytic binding pocket on photoreceptor PDE has evolved to have an absolute preference for cGMP over cAMP. This preference is defined by interactions between the cyclic nucleotide and the region of the purine ring encompassing the N-1, C-2, and C-6 positions. In addition, it is likely that an altered charge transfer interaction potential as well as a different amount and direction of the dipole moment in the adenine base compared with the guanine base are of significance in discriminating cGMP from cAMP (40).

Comparison of analogs substituted in the N-1/C-6 region with C-2 substituted analogs (Table II) indicates that the C-6 oxo group and/or the N-1 hydrogen atom are of primary importance in stabilizing cGMP binding to the noncatalytic binding pocket. For example, replacement of the C-6 oxygen with a hydrogen atom (concomitant with deprotonation at N-1) to form 2-NH$_2$-cPUMP (**4**) results in an 800-fold reduction in binding affinity, whereas substituting the C-2 amino group for a hydrogen atom (cIMP, **8**) reduces binding affinity only about 10-fold. As will be described in the next section, the C-2 position of cGMP tolerates a variety of substitutions, whereas the binding pocket is more stringent around the C-6 or N-1 positions. We conclude that the major determinant of specificity for cGMP binding resides in the N-1/C-6 region of the cGMP molecule.

Pyrimidine Nucleotides—We examined whether cyclic nucleotides that contained a pyrimidine ring instead of a purine ring could occupy the binding pocket of the photoreceptor PDE. Neither thymidine 3′,5′-cyclic monophosphate, uridine 3′,5′-cyclic monophosphate, nor cytidine 3′,5′-cyclic monophosphate were found to compete with [^{3}H]cGMP for binding to PDE at concentrations up to 5 mM (data not shown). We conclude that there is an absolute requirement for the purine ring structure in order for a cyclic nucleotide to bind to the noncatalytic site of photoreceptor PDE.

TABLE II
Substitutions in the C-2/N-3 region of the purine ring

See Table I for definitions for this and the following tables.

No.	Nucleotide	K_i	K_i/K_D
		μM	
8.	cIMP	0.68 ± 0.11	11
9.	N^2-CH$_3$-cGMP	1.9 ± 0.4	32
10.	N^2,3-Etheno-cGMP	4.8 ± 1.4	80
11.	N^2-(6-Aminohexyl)-cGMP	5.6 ± 0.3	93
12.	N^2-Phenyl-cGMP	6.5 ± 0.2	108
13.	N^2-Biphenyl-cGMP	6.7 ± 0.1	111
14.	N^2-(2-Methoxyethyl)-cGMP	8.0 ± 0.01	130
15.	N^2-Hexyl-cGMP	8.0 ± 0.1	130
16.	N^2-(6-OH-hexyl)-cGMP	8.4 ± 0.3	140
17.	N^2-(2-Aminoethyl)-cGMP	9.6 ± 0.2	160
18.	N^2-Benzyl-cGMP	11.2 ± 0.2	190
19.	cXMP	13.1 ± 0.2	220
20.	N^2-(2-OH-ethyl)-cGMP	15.3 ± 0.1	260
21.	2-Cl-cIMP	16.8 ± 0.2	280
22.	N^2-(5-Carboxypentyl)-cGMP	17.9 ± 0.1	300
23.	N^2-(2-Carboxyethyl)-cGMP	20.1 ± 0.2	340
24.	N^2-Ethyl-cGMP	30.9 ± 0.3	515
25.	2-Piperidino-cIMP	>2 mM	$>3 \times 10^4$
26.	N^2-(CH$_3$)$_2$-cGMP	>4 mM	$>7 \times 10^4$

Substitutions in the C-2/N-3 Region of the Purine Ring—Since the amino group at the C-2 position is of primary importance in discriminating cAMP from cGMP in other binding proteins, it was important to determine the factors involved in stabilizing the preference for cGMP binding over cAMP in photoreceptor PDE. Nineteen cGMP derivatives modified at the C-2 position (Fig. 1*C*) were tested; of these, compounds **10** and **19** also resulted in changes at the N-3 position. Analysis of the binding results of cGMP analogs substituted at the C-2 position can most easily be done by categorizing the compounds according to H bonding potential, bulkiness, and the functional group of the substituent.

In the first group, several compounds were tested, having a formal loss of H bonding donor potential in position 2. Replacement of the 2-NH$_2$ group with hydrogen (cIMP; **8**) caused the least (11-fold) decrease in binding (Table II). This indicates that the interaction between the amino group and the binding pocket is not essential for high affinity binding and is consistent with other regions of the cGMP molecule providing the crucial stabilizing interactions with the binding pocket. Note that the dipole size and the angle of cGMP compared with cIMP are extremely similar; the angle differs less than 2° (41). Therefore, cIMP mimics cGMP with respect to electron density and

localization within the purine ring.

Nonetheless, there remains strong evidence that the presence of a hydrogen atom at the N^2 position helps to stabilize binding of cGMP analogs to the noncatalytic site. Substitution of both hydrogens at the N^2 position with methyl groups (N^2-$(CH_3)_2$-cGMP; **26**) results in a compound that is not tolerated by the binding pocket. However, N^2-monomethyl-cGMP (**9**) retains H bonding potential and binds only 30-fold less well than the parent cGMP molecule. The 70,000-fold difference in binding between compounds **26** and **9** can be attributed to two factors as follows: 1) the total loss of H bonding donor capacity, and/or 2) the possibility that steric constraints from a second methyl group cannot be tolerated. The fact that the dimethyl analog (**26**) and 2-piperidino-cIMP (**25**) are both completely unable to bind (Table II) is also likely to be due to a combination of the following: (1) spatial constraints by the binding pocket in the immediate vicinity of the amino group of cGMP, and (2) a lack of hydrogen bond formation between the binding pocket and an N^2 hydrogen.

Both cXMP (**19**) and 2-Cl-cIMP (**21**) bound relatively poorly to the noncatalytic binding pocket of PDE (Table II). In both cases, the replacement of the NH_2 group with an electronegative atom results in a 220- or 280-fold reduction in binding affinity, respectively. We conclude from these results that the presence of one hydrogen atom at the N^2 position permits a favorable interaction with the binding pocket. In the absence of this interaction cyclic nucleotide binding can still occur, albeit with reduced affinity.

Another group of 2-substituted cGMP analogs varying in the chain length and the type of functional group were synthesized to determine whether spatial factors, polarity, or the type of charge of the cGMP derivative affected the ability of these analogs to bind to the PDE noncatalytic binding pocket. For the case of three comparisons of short *versus* longer chain length substituents at the C-2 position (**24** *versus* **15**, **17** *versus* **11**, and **20** *versus* **16**), the longer alkyl group demonstrated a statistically significant higher binding affinity than the shorter alkyl chain. The moderate binding of N^2-phenyl-cGMP (**12**), N^2-biphenyl-cGMP (**13**), and N^2-benzyl-cGMP (**18**) demonstrates that overall bulkiness is not the limiting factor for tight binding at the C-2 position; these three compounds are bulky but still retain their capacity for H bonding. The fact that methyl, ethyl, phenyl, biphenyl, and hexyl substituents at position 2 all bound with moderate affinity indicates that this region of the binding pocket is not spatially constrained, at least in one of the directions occupied by the two N^2 hydrogen atoms in the cGMP molecule.

Different functional groups were also introduced at the N^2 position to test the effects of changing the charge and/or polarity in this region of the binding pocket. Replacing a hydrogen atom at N^2 with a methyl group (**9**) caused only a 30-fold decrease in binding affinity. In contrast, N^2-(5-carboxy-pentyl)-cGMP (**22**) and N^2-(2-carboxy-ethyl)-cGMP (**23**) bound with a 300–340-fold reduction in affinity compared with cGMP. Carboxyl-containing derivatives **22** and **23** behaved similarly to electronegative analogs (*i.e.* **19** and **21**) in terms of their relative binding affinity. In contrast, cGMP analogs containing a positively charged amino group (**11** and **17**) or a methoxy group (**14**) were better tolerated than other functional groups tested. It appears that either a negative charge or an electronegative atom in this region of the binding pocket destabilizes binding.

Two derivatives were tested that altered the N-3 position in addition to changing the C-2 substituent. N^2,3-etheno-cGMP (**10**) contains a five-member ring joining positions C-2 and N-3 yet binds relatively well to the binding pocket of PDE. The reduction in binding affinity of this compound is comparable in magnitude to other analogs with similar alterations only at C-2, suggesting that the N-3 position does not specifically interact with the binding pocket. cXMP (**19**) results in protonation of N-3 in addition to replacement of the 2-NH_2 group with an oxo group, but in this case it is likely that the introduction of the electronegative oxygen, resulting in the loss of H bonding donor potential, may be the primary factor in the binding behavior of this analog. In summary, there is no evidence to indicate that the N-3 position forms an interaction with the noncatalytic binding pocket of PDE.

TABLE III
Substitutions in the C-8/N-7 region of cGMP

No.	Nucleotide	K_i	K_i/K_D
		μM	
27.	7-Deaza-cGMP	0.56 ± 0.2	9.3
28.	8-Cl-cGMP	5.2 ± 0.2	87
29.	8-Br-cGMP	13.4 ± 0.4	220
30.	8-CH_3-cGMP	21.4 ± 0.1	360
31.	8-NH_2-cGMP	33.3 ± 0.1	555
32.	8-(4-OH-phenylthio)-cGMP	>10 mM	$>2 \times 10^5$
33.	8-(4-NH_2-phenylthio)-cGMP	>8 mM	$>1 \times 10^5$
34.	8-Benzylamino-cGMP	>2 mM	3×10^4
35.	8-Benzylthio-cGMP	>2 mM	3×10^4
36.	8-OH-cGMP	>4 mM	7×10^4

We conclude that the C-2 position of the purine ring is a significant but secondary determinant for binding specificity of cGMP to noncatalytic sites on photoreceptor PDE. The presence of at least one hydrogen atom at N^2 can stabilize cyclic nucleotide binding but is not an absolute requirement. The binding pocket tolerates a variety of substitutions reasonably well, which suggests that this region of the binding site is not spatially constrained. The neighboring N-3 position does not play a role in stabilizing nucleotide binding to the noncatalytic site of PDE.

Substitutions to the N-7 and C-8 Positions of the Purine Ring—In contrast to some other cGMP binding proteins in which substitutions at the C-8 position result in a higher binding affinity than cGMP itself (*e.g.* cGMP-dependent protein kinases (15, 42, 43) and the cGMP-gated ion channel (17, 19)), almost all substitutions to this position of the purine ring significantly destabilize cGMP binding to photoreceptor PDE noncatalytic sites (Table III).

All substitutions at the C-8 position of cGMP (Fig. 1*D*) caused a significant decrease in binding affinity. The 90- and 220-fold reductions in binding of 8-Cl-cGMP (**28**) and 8-Br-cGMP (**29**), respectively, demonstrate that a single electronegative atom at C-8 can induce a major destabilization of binding interactions (Table III). However, the fact that 8-CH_3-GMP (**30**) and 8-NH_2-cGMP (**31**) bind even more poorly than the halide derivatives indicates that the electronegativity of the Cl or Br atoms may be secondary to spatial constraints of the binding pocket near the C-8 position of the purine ring. Supporting this notion is the very low binding affinity of 8-substituted cGMP analogs containing a bulky aromatic ring (**32**-**35**).

In addition to the potential importance of spatial constraints near C-8, the chemical nature of the C-8 substitution is in some cases crucial in determining binding affinity. Another cGMP derivative, 8-OH-cGMP (**36**), demonstrates a complete inability to compete with [^{3}H]cGMP at the noncatalytic binding site of PDE. This result is further evidence that spatial constraints alone cannot explain why some 8-substituted analogs show the behavior reported in Table III. We considered the possibility that the inability of 8-OH-cGMP to bind might result in part from protonation at N-7 that is a consequence of the keto-enol tautomerism of this analog (Fig. 1*D*, *inset*). However, the relatively high binding affinity of 7-deaza-cGMP (**27**; K_i/K_D = 9) which is also protonated at this position does not support this

TABLE IV
Substitutions in the cyclic phosphate moiety of cGMP

No.	Nucleotide	K_i	K_i/K_D
		μM	
37.	(R_p)-cGMPS	1.4 ± 0.2	23
38.	(S_p)-cGMPS	3.9 ± 0.3	65
39.	2′-Deoxy-(R_p)-cGMPS	5.6 ± 0.2	93
40.	2′-Deoxy-(S_p)-cGMPS	12.1 ± 0.1	200
41.	8-Cl-(S_p)-cGMPS	21.2 ± 0.3	350
42.	guanosine-3′,5′-cyclic monophosphorodimethylamidate (R_p isomer)	>4 mM	$>7 \times 10^4$
43.	5′-GMP	>10 mM	$>2 \times 10^5$

TABLE V
Substitutions in the ribose moiety of cGMP

No.	Nucleotide	K_i	K_i/K_D
		μM	
44.	3′-NH-cGMP	0.07 ± 0.01	1.2
45.	5′-NH-cGMP	0.68 ± 0.18	11
46.	2′-Deoxy-cGMP	1.6 ± 0.2	27
47.	2′-*O*-Monobutyryl-cGMP	3.6 ± 0.1	60
48.	2′-*O*-(2,4-Dinitrophenyl)-cGMP	5.6 ± 0.1	93

notion. Rather, the destabilization of cGMP by introduction of modifications at the C-8 position appears to be localized to the C-8 substituent itself. The nitrogen atom at N-7 of the purine ring does not appear to be important for stabilizing the interaction of cGMP with the binding pocket.

In summary, the decreased binding affinity resulting from modification at the C-8 position can potentially be credited to many influences, including spatial constraints, *syn- versus anti*-conformation (see below), and/or hydrophobicity. These influences are difficult to distinguish experimentally, and a decrease in analog binding might involve more than one of these factors. With two significant exceptions, analogs substituted at the C-8 position show decreased binding as the size of the substituent increases. If the binding pocket was very constricted in this region, bulky substituents would not be accommodated. However, spatial restrictions in this region of the binding pocket cannot account for the behavior of 8-OH-cGMP, and further work will be needed to define the other factors (*e.g.* electron density distribution and/or dipole moment) that influence the interaction of this region of the purine ring with the noncatalytic binding pocket.

Substitutions in the Cyclic Phosphate Ring—Seven derivatives of cGMP with modifications in the cyclic phosphate portion of the molecule were tested for their interaction at the noncatalytic binding site of PDE (Table IV). Two compounds, guanosine-3′,5′-cyclic monophosphorodimethylamidate (R_p isomer) (**42**) and 5′-GMP (**43**), were completely unable to bind. For compound **42**, a combination of steric factors, loss of potential ionic or hydrogen bonding interactions, and/or an increase in hydrophobicity in this region may account for its inability to occupy the binding pocket. In the case of 5′-GMP, no displacement of [^{3}H]cGMP could be detected when the 5′-GMP was raised to 50 mM (10^6-fold discrimination), demonstrating that the cyclic phosphate structure is absolutely essential for nucleotide binding to the noncatalytic site.

Replacement of one of the two exocyclic oxygen atoms with sulfur in (R_p)-cGMPS and (S_p)-cGMPS (**37** and **38,** respectively) resulted in a 24- and 65-fold decrease in relative binding affinity, respectively. This suggests that both exocyclic oxygen atoms are involved in binding to the noncatalytic site by forming a not totally symmetric salt bridge and/or hydrogen bonding interaction to stabilize cGMP binding. The greater loss of binding affinity when the axial oxygen is replaced in S_p-cGMPS implies that the axial exocyclic oxygen may interact with the binding pocket to stabilize binding slightly more than the equatorial oxygen. Furthermore, this result shows that substituting sulfur for oxygen at the cyclic phosphate position has a 10-fold greater destabilizing effect than was observed at the C-6 position of the purine ring (**1**). The small extent of stereospecificity (<3-fold) exhibited by the R_p and S_p diastereomers distinguishes the photoreceptor PDE from other cGMP binding sites (22, 44). The small difference in binding affinities of the R_p and S_p analogs of cGMP suggest that either 1) there is a single residue in the binding pocket positioned nearly equidistant from the two exocyclic oxygens, or 2) there are two sites in the binding pocket, each one of which forms a similar interaction with the equatorial and axial exocyclic oxygens.

Three cGMPS analogs were tested that contained a modification either in the 2′-position of the ribose ring (**38** and **40**) or in the 8-position of the purine ring (**41**). The binding data confirm the above discussed stereoselectivity of the mono-substituted sulfur analogs (**37** and **38**). Furthermore, in all three instances, the reduction in binding affinity was ~10-fold less than expected if each modification acted independently to reduce the binding interaction.

Substitutions in the Ribose Moiety—Three different positions of the ribose ring were examined for the possibility of specific interactions with the noncatalytic binding pocket (Table V). Replacing an oxygen at the 3′-position of the ribose ring with a secondary amino group (3′-NH-cGMP, **44**) had no effect on the binding affinity, whereas a similar substitution at the 5′-position (5′-NH-cGMP, **45**) reduced binding affinity 11-fold. Introducing an amino group at the 5′-position may reduce the favorable ionic or hydrogen bonding interaction of the axial exocyclic oxygen with the binding pocket. This notion is consistent with the results of the previous section with thio-substituted analogs. This difference in binding affinity between **44** and **45** is also observed for the cGMP-stimulated PDE from bovine adrenal tissue (22), whereas the cGMP-dependent protein kinase type Iα (15) and the cGMP-stimulated PDE from *Dictyostelium discoideum* (44) bound both nucleotides with similar affinities.

Three analogs substituted at the 2′-position of the ribose ring show progressively lower binding affinity as the bulkiness of the substituent increases. The 27-fold decrease in binding affinity of 2-deoxy-cGMP (**46**) suggests an interference with a putative hydrogen bond between the 2′-hydroxyl group and the binding pocket. However, we cannot unambiguously determine whether the decreased binding affinity results from the loss of the oxygen as a potential H bond acceptor or the inability of the hydrogen atom to form an H bond with an acceptor in the binding pocket (Fig. 3). The ~30-fold decrease in binding affinity of **46** to the photoreceptor PDE contrasts markedly with the 250-fold reduction in binding of **46** to the cGMP-dependent protein kinase (15).

The binding pocket in this region tolerates bulky substituents relatively well. Introduction of a monobutyryl group (**47**) or a 2,4-dinitrophenyl group (**48**) results in a small additional decrease in binding affinity compared with 2′-deoxy-cGMP. This indicates that the region of the noncatalytic binding pocket near the ribose moiety is not spatially constrained.

Evidence Favoring cGMP Binding to the Noncatalytic Site in the Anti-conformation—The orientation of the purine base relative to the ribose sugar is determined by the torsion angle χ about the glycosidic bond, with the main conformations referred to as *syn* or *anti* (39). Both crystallographic (45) and computational studies (46) support the idea that cGMP prefers the *syn*-conformation over the *anti*-conformation. Current evidence suggests that the formation of an intramolecular H bond between the amino group on the C-2 position and the axial phosphate oxygen atom contributes to stabilizing the *syn*-form

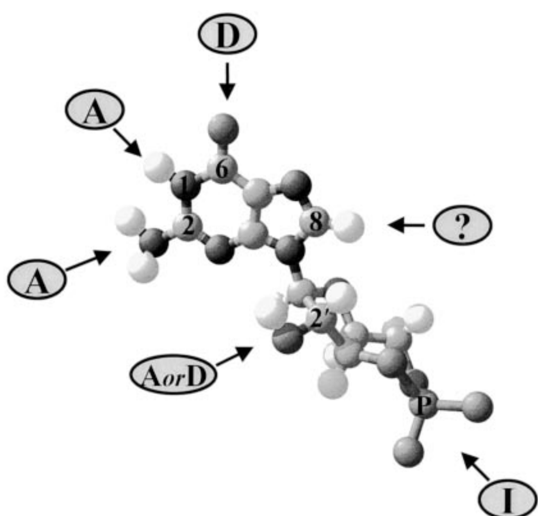

FIG. 3. **Model of the noncatalytic cGMP binding sites on frog rod photoreceptor PDE.** The cGMP molecule is shown occupying the binding pocket of PDE in its *anti*-conformation (see text for discussion). Potential noncovalent interactions between the binding pocket and cGMP are indicated based on the type of group present in the binding pocket. *A*, hydrogen bond acceptor; *D*, hydrogen bond donor; *I*, ionic interaction.

of cGMP compared with the *anti*-conformation (20, 39, 40).

It is difficult to determine whether the reduction in binding affinity of a cGMP analog results from a change in the *syn*/*anti*-conformational equilibrium or to some other alteration in the molecular and electronic structure of the analog. Although no definitive statement can be made about the conformational preference of the noncatalytic binding sites for cGMP, the following evidence all supports the idea that cGMP binds to the photoreceptor PDE noncatalytic site in the *anti*-conformation. 1) Substitutions at the C-8 position of the purine ring increase bulkiness and shift the conformational equilibrium toward the *syn*-form (19, 39, 47, 48). If cGMP bound to the noncatalytic sites in the *syn*-conformation, it would be expected that 8-substituted analogs might show increased binding affinity compared with cGMP, as has been observed for the binding sites of the cyclic nucleotide-gated ion channel (16, 18, 19) and the cGMP-dependent protein kinase (15). In both of these proteins, cGMP is believed to occupy the binding site in the *syn*-conformation (18, 49, 50). Instead, almost all substitutions at the C-8 position substantially reduce the binding affinity of cGMP analogs to the PDE noncatalytic sites (Table III), consistent with an *anti*-conformation of cGMP in the binding pocket. 2) NMR proton chemical shift measurements as well as molecular modeling calculations and model building indicate that N^2,3-etheno-cGMP (**10**) exists primarily, if not exclusively, in the *anti*-conformation (40). This is likely due to the presence of the etheno ring structure preventing free rotation about the glycosidic bond (32). The fact that **10** binds moderately well to the noncatalytic cGMP binding site is consistent with the binding pocket preferring the *anti*-conformation. It should be noted that **10** binds poorly to the regulatory sites of the cGMP-dependent protein kinases type Iα, Iβ, and II (40); this is consistent with this site preferring to bind cGMP in the *syn*-conformation (see above). 3) Molecular modeling of 8-benzylthio-cGMP (**35**) suggests that this analog is constrained exclusively to the *syn*-conformation.[5] The complete inability of **35** to bind is also consistent with an *anti*-conformation of cGMP in the binding pocket.

Comparison of the Noncatalytic and Catalytic cGMP Sites on Photoreceptor PDE—A comparison of this study with the effects of cGMP analogs on the catalytic site of bovine rod photoreceptor PDE (20) clearly highlights the much greater degree of nucleotide discrimination at the noncatalytic site. Several analogs bind nearly as well as cGMP to the catalytic site, including 2′-deoxy-cGMP (**46**), cIMP (**8**), 8-Br-cGMP (**29**), 6-SH-cGMP (**1**), and 3′-NH-cGMP (**44**). Of this group of analogs, only **1**, **8**, and **44** show a similar relative affinity for the noncatalytic site. Compounds **46** and **29** bind with 27- and 220-fold lower affinity to the noncatalytic site than cGMP, respectively (20). In marked contrast to the inability of cAMP to bind at the noncatalytic cGMP binding sites on PDE (Table I), the K_M for cAMP hydrolysis is only 20-fold greater than the K_M for cGMP breakdown (51).

In terms of individual positions of the cGMP molecule, the catalytic (20) and noncatalytic sites of the photoreceptor PDE both appear to use the C-6 oxo group to interact with a hydrogen bond donor in the binding pocket. The N-1 position of the purine ring is also involved in stabilizing cGMP binding at both catalytic and noncatalytic sites. In other portions of the cGMP molecule, however, the two binding sites exhibit different properties. 1) Binding of cGMP to the catalytic site is not significantly stabilized by the amino group at the C-2 position (20), in contrast to the noncatalytic site where the majority of analogs tested bound $>$100-fold less well than cGMP (Table II). 2) Substitutions at the 8-position have little effect on the ability of analogs to bind to the catalytic site (20), except in the case of 8-OH-cGMP (**36**) which may be exerting its effects via protonation of the N-7 position (Fig. 1*D*, *inset*) or via changes in the electron density distribution of the purine ring. In contrast, almost all substitutions at the C-8 position adversely affect nucleotide binding to the noncatalytic site (Table III). 3) Substitutions at the 2′-position on the ribose ring have only minor ($<$2-fold) effects on catalytic site binding (20), whereas identical cGMP derivatives have 30–60-fold lowered affinity to the noncatalytic sites (Table V). In summary, the catalytic site of the bovine rod photoreceptor PDE stabilizes cGMP binding via favorable interactions at N-1, C-6, and N-7 on the purine ring. It may bind cGMP in the *syn*-conformation (20). The C-2, C-8, and C-2′ positions do not appear necessary for cGMP binding to the catalytic site, whereas binding to the noncatalytic site is stabilized by interactions of these positions of the cGMP molecule with the binding pocket.

cGMP Interactions with other Noncatalytic PDE Binding Sites—In addition to the photoreceptor PDE, both the cGMP-stimulated PDE (PDE2) and the cGMP-specific PDE (PDE5) have noncatalytic cGMP binding sites that have been probed with cGMP derivatives (9, 22, 24). Qualitatively, there is good agreement among the three different noncatalytic cGMP binding sites regarding structural requirements for cGMP binding. Specifically, PDE2, PDE5, and the photoreceptor PDE all share favorable interactions between the noncatalytic binding pocket and the C-6, N-1, C-2, and C′-2 positions, as well as poorly tolerating substitutions at the C-8 position. However, the photoreceptor PDE shows a great deal more discrimination for cGMP over other nucleotides than the other PDEs examined to date. For example, cAMP is able to bind approximately 50–100-fold less well to the noncatalytic sites of PDE2 (22, 23) and PDE5 (52, 53) compared with the $\sim 10^6$ level of discrimination of cAMP from cGMP at the photoreceptor noncatalytic binding site. In addition, cPUMP (**5**) (22), 8-OH-cGMP (**36**) (9), and 8-CH_3-cGMP (**30**) (24) all bind considerably less well to the photoreceptor PDE than to PDE2 or PDE5. There is also a notable lack of stereospecificity in the binding of (R_p)- (**37**) and (S_p)-cGMPS (**38**) to the photoreceptor enzyme (Table IV; see also Ref. 54) compared with PDE2 (22).

[5] F. Schwede, unpublished observations.

Because cGMP binding assays have been performed under a variety of conditions, direct comparisons of the intrinsic binding affinity of cGMP are difficult. Nonetheless, the photoreceptor PDE is likely to have more favorable binding interactions between the noncatalytic site and the cGMP molecule than PDE2 or PDE5. This can be inferred from the value of the dissociation constant for cGMP observed for amphibian rod photoreceptor PDE (60 nM (11)), mammalian cone (10 nM (54)), or rod photoreceptor PDE (sub-nanomolar (55)). In contrast, PDE2 and PDE5 have dissociation constants ≥100 nM at 4 °C (22, 52). It should be noted, however, that the association of the inhibitory γ subunit of PDE to the photoreceptor catalytic subunits can modulate the cGMP binding to the noncatalytic sites of amphibian PDE (6, 11, 12).

Conclusions—The principal finding of this paper is that the noncatalytic cGMP binding pocket on the photoreceptor PDE interacts in a highly specific manner with the purine ring (principally at C-6/N-1, as well as at C-2 and C-8), the ribose ring (at the 2′-position), and with the cyclic phosphate ring of cGMP (Fig. 3). Furthermore, current evidence favors binding of cGMP in the *anti*-conformation to the binding pocket. Although the general binding motifs are similar to those of the PDE2 and PDE5 noncatalytic binding sites, the photoreceptor enzyme discriminates cyclic nucleotides more precisely and binds cGMP with higher intrinsic affinity than do the other cGMP-binding PDEs. In comparison to the catalytic site of the photoreceptor PDE (Beltman *et al.* (20)), the noncatalytic binding site interacts at more distinct sites with the cGMP molecule, leading to higher affinity interactions and more precise discrimination of other cyclic nucleotides.

A limitation to this approach of determining the structural features of the noncatalytic binding pocket is that a particular cGMP analog might contain a chemical group that interacts favorably with the binding pocket in a way that does not occur with cGMP itself. In this case, the apparent binding affinity of the analog would not provide information about cGMP binding interactions with the noncatalytic binding pocket. However, our use of several chemical modifications at each tested position of the cGMP molecule reduces the likelihood of misinterpreting the effects of a single substitution in terms of cGMP binding interactions with the noncatalytic binding site on PDE. Ultimately, structural information on the identity of the individual amino acids in the noncatalytic binding pocket that interact with each distinct region of the cGMP molecule must await functional expression and site-directed mutagenesis of the photoreceptor PDE, as has been initiated with PDE5 (53, 56, 57).

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