Binding of cGMP to both allosteric sites of cGMP-binding cGMP-specific phosphodiesterase (PDE5) is required for its phosphorylation

Illarion V. TURKO, Sharron H. FRANCIS and Jackie D. CORBIN¹

Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN 37232-0615, U.S.A.

cGMP-binding phosphodiesterases contain two homologous allosteric cGMP-binding sites (sites *a* and *b*) that are arranged in tandem; they constitute a superfamily of mammalian cyclic nucleotide receptors distinct from the cyclic nucleotide-dependent protein kinases/cation channels family. The functional role of each of these two sites in the phosphodiesterases is not known. The cGMP-binding sites of one of these phosphodiesterases, the cGMP-binding cGMP-specific phosphodiesterase (cGB-PDE, PDE5), have been analysed by using site-directed mutagenesis. Mutations that affect cGMP binding to either one or both allosteric sites do not influence cGMP hydrolysis in the catalytic site under the conditions used. However, compared with wild-type enzyme, the D289A, D478A and D289A/D478A mutants,

which are defective in cGMP binding to either site *a* or site *b*, or both allosteric sites, require much higher cGMP concentrations for the allosteric stimulation of phosphorylation by the catalytic subunit of cAMP-dependent protein kinase. The cGMP effect is on the cGB-PDE rather than on the catalytic subunit of the protein kinase because the latter enzyme does not require cGMP for activity. The D289N mutant, which has higher binding affinity for cGMP than does the wild-type enzyme, is phosphorylated at lower concentrations of cGMP than is the wild-type enzyme. It is concluded that cGMP binding to the allosteric sites of cGB-PDE does not directly affect catalysis, but binding to both of these sites regulates phosphorylation of this enzyme.

INTRODUCTION

The superfamily of cyclic nucleotide phosphodiesterases (PDEs) catalyses the hydrolysis of 3':5'-cyclic nucleotides to the corresponding nucleoside 5'-monophosphates, and the catalytic functions of these enzymes are regulated by myriad mechanisms. Three of the seven PDE families (PDE2, PDE5 and PDE6) contain two homologous allosteric cGMP-binding sites that are arranged in tandem in the N-terminal regulatory portion of these proteins [1–4]. These allosteric cGMP-binding sites are distinct from the sites of cyclic nucleotide hydrolysis, and they are not homologous with the well-described members of the superfamily of cyclic nucleotide-binding sites that are found in catabolite gene activator protein, cAMP-dependent protein kinase (cAK), cGMP-dependent protein kinase (cGK) and cyclic nucleotide-gated ion channels [5]. The protein kinases contain two different intrasubunit cyclic nucleotide-binding sites; the functional roles of each, although not completely understood, have been thoroughly investigated. Although cGMP-binding PDEs form a second major superfamily of mammalian intracellular receptors for cyclic nucleotides [6], the roles of each of the allosteric cGMP-binding sites (sites *a* and *b*) for PDE regulation have not been studied. The reported regulatory effects of cGMP-binding include: (1) binding of cGMP to the allosteric sites of PDE2 stimulates the hydrolysis of cAMP in the catalytic site [4]; (2) cGMP binding to the allosteric sites of the frog rod outer segment PDE6 regulates interaction of the enzyme with an inhibitory subunit and with the G-protein transducin [7]; and (3) cGMP binding to the allosteric sites of the cGMP-binding cGMP-specific phosphodiesterase (cGB-PDE) enhances phosphorylation of a single serine residue, Ser-92, in the enzyme by either cAK or cGK [8]. However, the role of individual binding sites in eliciting functional changes has not been determined for any of these enzymes.

Recently we have shown [9] that an invariant aspartic acid residue in each of the two allosteric cGMP-binding sites of cGB-PDE is important for cGMP binding. Replacement of Asp-289 in site *a* or Asp-478 in site *b* with alanine markedly diminishes the affinity for cGMP in the respective sites [6,9]. We proceeded in the present study to determine that the double D289A/D478A mutant has only trace levels of cGMP binding to the allosteric sites, and another mutant, D289N, has improved cGMP-binding affinity. All of these individual mutants and the double mutant were used to determine whether cGMP binding to either of the allosteric sites affects catalytic activity, and to evaluate the role of each of the allosteric cGMP-binding sites in the effect of cGMP on the phosphorylation of the cGB-PDE by the catalytic subunit of cAK.

MATERIALS AND METHODS

Materials

[³H]cGMP and [γ -³²P]ATP were purchased from Amersham Life Science (Cleveland, OH, U.S.A.). cGMP, histone VIII-S, *Crotalus atrox* snake venom and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Hydroxyapatite was from Bio-Rad Laboratories (Hercules, CA, U.S.A.) and Sephadex G-200 was from Pharmacia Biotech Inc (Piscataway, NJ, U.S.A.).

Site-directed mutagenesis and construction of expression vectors

Oligonucleotide-directed mutagenesis was performed as described in detail previously [9]. Briefly, a *Hind*III fragment of cGB-8, encoding the entire cGMP-binding region, was subcloned into the pBluescript SK(–) vector to produce a cGB-8-H3f-

Abbreviations used: cAK, cAMP-dependent protein kinase; cGB-PDE, cGMP-binding cGMP-specific phosphodiesterase; cGK, cGMP-dependent protein kinase; IBMX, 3-isobutyl-1-methylxanthine; PDE, 3':5'-cyclic nucleotide phosphodiesterase.

¹ To whom correspondence should be addressed.

pBSSK(–) construct, and a uracil-containing single-stranded cGB-8-H3F-pBSSK(–) was prepared for use as a template. The following mutagenic oligonucleotides were used: (1) D289A, 5'-GGG ACA TTC ACT GAA AAA GCC GAA AAG GAC TTT GCT GCT-3'; (2) D289N, 5'-GGG ACA TTC ACT GAA AAA AAC GAA AAG GAC TTT GCT GCT-3'; (3) D478A, 5'-AAG GCT TTC AAC CGC AAC GCT GAA CAG TTT CTG GAA GCT-3'. The altered bases are underlined. After the mutagenesis reaction with T4 DNA polymerase and T4 DNA ligase, the resulting products were used to transform *Escherichia coli* XL1-blue (*dur*⁺, *ung*⁺). The construction of expression vectors was performed in accordance with the procedure previously described [6]. To create the double D289A/D478A mutant, the 631 bp fragment was excised from pBacPAK9-cGB-PDE(D289A) vector by *NheI/KpnI* digestion, and replaced with a *NheI/KpnI* fragment containing the D478A mutation.

E. coli XL1-blue cells were used for all transformations. DNA fragments were purified by the freeze-squeeze method from agarose slices with SPIN-X® centrifuge filter units (Corning Costar, Cambridge, MA, U.S.A.). DNA was purified from large-scale vector preparations with Qiagen Plasmid Maxi kit in accordance with the manufacturer's protocol (Qiagen, Chatsworth, CA, U.S.A.). All DNA segments subjected to mutagenesis and subcloning reactions were sequenced in their entirety with a Sequenase® Version 2.0 kit (U.S. Biochemical, Cleveland, OH, U.S.A.) to ensure the presence of the desired mutation and proper in-frame subcloning.

Expression and purification of wild-type and mutant enzymes

Sf9 cells were co-transfected with *Bsu*36I-digested BacPAK6 viral DNA (Clontech, Palo Alto, CA, U.S.A.) and one of the cGB-8/14 clones in pBacPAK9 expression vector by the lipofection method, in accordance with the protocol from Clontech. At 3 days after infection, the co-transfection supernatant was collected, amplified twice in Sf9 cells and then used as virus stock directly for expression without additional purification of recombinant viruses. High Five cells (Invitrogen, Carlsbad, CA, U.S.A.), grown at 27 °C in complete Grace's insect medium (Invitrogen) with 10% (v/v) fetal bovine serum (Intergen, Purchase, NY, U.S.A.) and 10 µg/ml gentamycin (Life Technologies, Grand Island, NY, U.S.A.), were infected by 5 ml of virus stock per 30 ml of culture medium. The culture medium was harvested at 90 h after infection, and wild-type or mutant cGB-PDE was purified from culture medium as described previously [6]. Briefly, fractionation by (NH₄)₂SO₄ gel filtration on Sephadex G-200 and chromatography on hydroxyapatite and mono-Q columns were used.

cGB-PDE assays

Phosphodiesterase activity, with [³H]cGMP as the substrate, was measured as described previously [10]. In all studies, less than 15% of the total [³H]cGMP was hydrolysed during the reaction. The apparent *K_m* values for cGMP were determined by assaying phosphodiesterase activity in triplicate at 1–40 µM cGMP.

The cGMP-saturation binding assay was done by Millipore filtration as described previously for soluble extracts of transfected COS-7 cells [9]. This procedure was used with small modifications: (1) cAMP and 8-bromo-cGMP were omitted from the reaction mixture because purified cGB-PDEs were used for assay, (2) 0.5 mg/ml histone VIII-S (Sigma), which enhances cGMP binding to cGB-PDE, was added to the reaction mixture and (3) [³H]cGMP was used instead of [³²P]cGMP. The data were corrected by subtraction of non-specific binding, which was defined as either the [³H]cGMP bound in the absence of cGB-

PDE or the [³H]cGMP bound in the presence of a 100-fold excess of unlabelled cGMP. A similar 2–4% non-specific binding was obtained with each method. The data were subjected to non-linear least-squares analysis with the program MINSQ II (Micro-math Scientific Software, Salt Lake City, UT, U.S.A.) to obtain the dissociation constant (*K_d*). The observed free energies of cGMP binding were calculated with the equation:

$$\Delta G = RT \ln K_d$$

Phosphorylation of wild-type and mutant enzymes

Wild-type enzyme and mutants were phosphorylated at 30 °C for 5 min in 10 mM sodium phosphate (pH 7.2)/0.2 nM IBMX/10 mM MgCl₂/100 µM [^γ-³²P]ATP (0.2 µCi/nmol)/125 nM cGB-PDE/0.2 µM catalytic subunit of cAK containing various concentrations of cyclic nucleotide in a final volume of 20 µl. The augmented phosphorylation of D289A, D478A and D289A/D478A mutants was done under the same conditions with 10 mM cGMP, 2.5 µM catalytic subunit of cAK and a 30 min reaction. The phosphorylation reactions were terminated by the addition of 5 µl of a mixture of 10% (w/v) SDS and 25% (v/v) 2-mercaptoethanol, boiled for 2 min and subjected to SDS/PAGE [10% (w/v) gel]. The gels were stained with Coomassie Blue and dried. The bands corresponding to the cGB-PDE were excised and counted by Čerenkov radiation in a scintillation counter. The stoichiometry of the phosphorylation obtained was calculated from the specific radioactivity of [^γ-³²P]ATP and the amount of cGB-PDE applied to the gel.

Peptide mapping of proteolysed cGB-PDE

Wild-type or mutant cGB-PDE (24 µg) was phosphorylated by the catalytic subunit of cAK under the augmented phosphorylation conditions described above (10 mM cGMP, 2.5 µM purified bovine heart catalytic subunit of cAK and a 30 min reaction at 30 °C). For removal of free [³²P]ATP, the phosphorylated products were desalted on a Sephadex G-25 column (1.5 cm × 20 cm) equilibrated in 0.2 M ammonium acetate, pH 8.0. Fractions from the Sephadex G-25 column containing the peak of protein-bound radioactivity were pooled and concentrated with a Centriplus concentrator (Amicon, Danvers, MA, U.S.A.). The ³²P-labelled cGB-PDE was then digested with 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one-treated trypsin, at a 1:10 (w/w) ratio of trypsin to cGB-PDE, for 2 h at 30 °C. The digest was chromatographed on a HPLC Aquapore RP-300 (30 mm × 2.1 mm) column with a linear gradient of CH₃CN (0–40%) in 0.08% trifluoroacetic acid over 60 min at a flow rate of 1 ml/min. Fractions of 1 ml were collected; radioactivity was determined by liquid-scintillation counting.

Other methods

SDS/PAGE [10% (w/v) gel] and Western blot analysis were done as described previously [9]. Protein concentrations were determined by the method of Bradford with BSA as the standard.

RESULTS

Purification of wild-type and mutants

The recombinant proteins were expressed in High Five cells and purified from culture medium. Each protein migrated as a 93 kDa band and exhibited a high degree of purity as determined by

Figure 1 SDS/PAGE of wild-type and mutant cGB-PDEs expressed in High Five cells

A 2 μ g sample of each cGB-PDE was loaded onto an SDS/10% (w/v) polyacrylamide gel. The gel was stained with Coomassie Blue. The molecular masses (in kDa) of the standards are shown at the left.

Figure 2 Binding of [3 H]cGMP to wild-type and mutant cGB-PDEs

Purified wild-type enzyme ($\blacktriangle$), D289N mutant ($\blacksquare$) or D289A/D478A mutant ($\bullet$) was incubated in 10 mM sodium phosphate buffer (pH 6.8)/1 mM EDTA/0.5 mg/ml histone VIII-S/0.2 mM IBMX for 3 h at 4 $^{\circ}$ C with increasing concentrations of [3 H]cGMP. After this incubation, aliquots (10 pmol of enzyme) were subjected to the filter binding assay [7]. Results are given as percentages of maximum, in which the maximum equals [3 H]cGMP bound/mg of wild-type cGB-PDE achieved at 14 μ M [3 H]cGMP. Results are means $\pm$ S.D. for $n = 3$ separate experiments.

SDS/PAGE [10% (w/v) gel] and Coomassie Blue staining (Figure 1). The identity of the recombinant proteins was verified by Western blot analysis (results not shown).

cGMP-binding activity

cGMP binding to wild-type and mutant cGB-PDEs as a function of [3 H]cGMP concentration is shown in Figure 2. It can be seen that the D289A/D478A mutant displayed a much lower cGMP-

Table 1 Kinetic parameters

PDE activity was measured under standard assay conditions [9] [40 mM Mops (pH 7.5)/0.5 mM EGTA/15 mM magnesium acetate/0.15 mg/ml BSA containing 1–40 μ M cGMP, [3 H]cGMP (100 000 c.p.m. per assay tube) and one of the cGB-PDE samples] in a total volume of 250 μ l. The incubation time was 10 min at 30 $^{\circ}$ C. Total [3 H]cGMP degradation was less than 15%. The apparent K_m values for cGMP were determined from Lineweaver–Burk plots. Results are means $\pm$ S.D. for $n = 3$ separate experiments.

cGB-PDE	Specific activity (μ mol of cGMP hydrolysed/min per mg)	K_m (μ M)
Wild-type	2.5 ± 0.1	2.0 ± 0.4
D289A/D478A	2.3 ± 0.1	7.5 ± 1.0
D289N	2.5 ± 0.1	6.0 ± 1.0

binding affinity than the wild-type enzyme. Because of the limitation of the maximum [3 H]cGMP concentration that could be used in this assay, it was not possible to determine an accurate K_d value for this mutant; however, extrapolation of the D289A/D478A curve to saturation produced an approximation of $K_d > 200$ μ M. In comparison with wild-type cGB-PDE ($K_d = 1.3$ μ M), the cGMP-binding affinity of this mutant was at most 1/150. However, the D289N mutant ($K_d = 0.25$ μ M) displayed an approx. 5-fold higher binding affinity for cGMP than did the wild-type cGB-PDE. Calculation of $\Delta\Delta G_{\text{binding}}$ for this mutant showed that the mutation caused an increase in binding energy of 0.8 kcal/mol (3.3 kJ/mol). Although this value was too low to indicate the introduction of an additional hydrogen bond or ionic interaction [common values for these interactions are 2–5 kcal/mol (8.4–21 kJ/mol)], the replacement of Asp-289 with asparagine apparently promotes some type of interaction that is not found in the wild-type structure.

cGB-PDE activity

The specific enzyme activity of each of the purified enzymes was 2.3–2.5 μ mol of cGMP hydrolysed/min per mg of cGB-PDE (Table 1), which approached that of native cGB-PDE purified from lung [10]. The K_m for cGMP was not significantly different between these mutant proteins and the wild-type enzyme. These results suggested that changes in the allosteric cGMP-binding affinity, either negative (D289A/D478A mutant) or positive (D289N mutant), do not directly affect the catalytic site with the conditions used.

cGB-PDE phosphorylation

It has been reported [8,11] that cGB-PDE from bovine lung is rapidly phosphorylated by cGK or cAK *in vitro* when cGMP is bound to the allosteric cGMP-binding sites of the cGB-PDE. Both kinases phosphorylate the same site (Ser-92). When cGK is used to study the phosphorylation of cGB-PDE, the interpretation of the results is complicated by the fact that cGK requires the presence of either cGMP or a cGMP analogue for expression of catalytic activity, and the presence of cGMP is also required for the substrate-directed stimulation of phosphorylation of the cGB-PDE. Therefore the free catalytic subunit of cAK was used for subsequent studies because it phosphorylates the same site and its activity is cyclic nucleotide-independent, so that effects produced by the addition of cGMP can be assumed to be exclusively substrate-directed. The cGMP-stimulated phosphory-

Figure 3 cGMP-stimulated phosphorylation of wild-type and mutant cGB-PDEs by the catalytic subunit of cAK

Wild-type or mutant cGB-PDE (2.5 pmol) was incubated at 30 °C for 5 min in 10 mM sodium phosphate (pH 7.2)/0.2 mM IBMX/10 mM MgCl₂/100 μM [γ-³²P]ATP (0.2 μCi/nmol)/0.2 μM catalytic subunit of cAK, with various concentrations of cGMP in a final volume of 20 μl. The phosphorylation reactions were terminated by the addition of 5 μl of a mixture of 10% (w/v) SDS and 25% (v/v) 2-mercaptoethanol; they were then subjected to SDS/PAGE [10% (w/v) gel]. The resulting autoradiographs are shown in (A,B). (C) After SDS/PAGE the gels were stained with Coomassie Blue and dried. The bands corresponding to the cGB-PDE were cut out of the gel and counted in a scintillation counter. The stoichiometry of phosphorylation was calculated from the specific activity of [γ-³²P]ATP and the amount of cGB-PDE used. The cGB-PDE concentration was determined by the method of Bradford. These experiments were repeated three times with less than 15% variation.

lation of wild-type cGB-PDE and the D289A/D478A, D289N, D289A and D478A mutants by the catalytic subunit of cAK is shown in Figure 3. Consistent with previous studies of the phosphorylation of native cGB-PDE from lung [8], the wild-type cGB-PDE was a very good substrate for phosphorylation but only in the presence of cGMP. Phosphorylation of cGB-PDE was dependent on cGMP concentration and saturable for the wild-type enzyme and the D289N mutant (Figures 3A and 3C); the D289N mutant was phosphorylated at a lower cGMP concentration, consistent with its higher affinity for the nucleotide. The concentration of cGMP required for half-phosphorylation in a 5 min reaction was 6 μM for D289N and 33 μM for the wild-type enzyme. The D289A/D478A mutant was phosphorylated only slightly; cGMP did not stimulate the reaction even at 1 mM concentration (Figure 3A).

The role of cGMP binding to each of the two cGMP-binding sites was addressed by using the enzymes mutated in the individual binding sites. Figure 3(B) shows that phosphorylation of either of the single-site mutants (D289A in site a and D478A in site b) was not stimulated appreciably in the presence of low concentrations of cGMP. The results suggested a crucial role of both sites in the regulation of phosphorylation. Clearly the substitutions that produced increases (D289N) or decreases (D289A, D478A and D289A/D478A) in cGMP-binding affinity produced the corresponding effects on phosphorylation of the cGB-PDE.

Table 2 Phosphorylation of the mutants defective in cGMP binding under augmented phosphorylation conditions

Wild-type or mutant cGB-PDE (2.5 pmol) was incubated at 30 °C for 30 min in 10 mM sodium phosphate (pH 7.2)/0.2 mM IBMX/10 mM MgCl₂/100 μM [γ-³²P]ATP (0.2 μCi/nmol)/2.5 μM catalytic subunit of cAK/10 mM cGMP in a final volume of 20 μl. The phosphorylation reactions were terminated by the addition of 5 μl of a mixture of 10% (w/v) SDS and 25% (v/v) 2-mercaptoethanol, then subjected to SDS/PAGE [10% (w/v) gel]. After SDS/PAGE the gels were stained with Coomassie Blue and dried. The bands corresponding to cGB-PDE were cut out of the gel and counted in a scintillation counter. Results are means ± S.D. for *n* = 3 separate experiments and are presented as percentages of wild-type phosphorylation.

cGB-PDE	Phosphorylation (%)
Wild-type	100 ± 12
D289A	98 ± 12
D478A	93 ± 12
D289A/D478A	60 ± 7

The results shown in Figure 3 suggested that the loss in cGMP-stimulated phosphorylation of cGB-PDE could be a function of the loss in affinity of the cGMP-binding allosteric sites rather than a total abrogation of this process. In fact a slight stimulation of phosphorylation of the D289A mutant occurred at the highest

Figure 4 Peptide mapping of proteolysed cGB-PDEs

Wild-type cGB-PDE and D289A/D478A mutant were phosphorylated by the catalytic subunit of cAK with [32 P]ATP using the augmented phosphorylation conditions described in the caption of Table 2 and digested with trypsin; the peptides were separated on a HPLC Aquapore RP-300 (30 mm $\times$ 2.1 mm) column as described in the Materials and methods section.

concentrations (100–1000 μ M) of cGMP tested in the experiment shown in Figure 3(B). The phosphorylation of the D289A, D478A and D289A/D478A mutants was therefore attempted by using 10 mM cGMP and 2.5 μ M catalytic subunit of cAK in a 30 min reaction. As shown in Table 2, these augmented conditions overcame the decreased cGMP-binding affinity and produced a level of phosphorylation of the D289A, D478A and D289A/D478A mutants comparable to that of the wild-type enzyme. In the absence of cGMP the incorporation of phosphate under these conditions was 20–30 % of the extent of the phosphorylation of the wild-type cGB-PDE in the presence of cGMP. The results in Table 2 implied that differences in phosphorylation *in vitro* of mutant and wild-type enzymes (Figure 3) were not due to differences in endogenous phosphate content of the enzymes or to non-specific conformational effects induced by the mutations.

The phosphorylation sites of wild-type and mutant enzymes were examined by performing phosphopeptide mapping. PDEs were phosphorylated under the augmented phosphorylation conditions (10 mM cGMP, 2.5 μ M catalytic subunit of cAK and a 30 min reaction at 30 $^{\circ}$ C). Phosphorylated enzymes were subjected to digestion by trypsin under the same conditions described earlier for the native lung cGB-PDE [8]. Peptides were purified as described in the Materials and methods section. Figure 4 shows results for the wild-type enzyme and the D289A/D478A mutant. Liquid-scintillation counting of the collected fractions identified a single radiolabelled peak. The elution

position of this peak was similar for both proteins shown and was consistent with that of a purified phosphopeptide from native cGB-PDE [8]. This peptide was shown to contain only two serine residues (no threonines); only one of these (Ser-92) is a consensus site for phosphorylation by cyclic nucleotide-dependent protein kinases, and this site was identified as the phosphorylated residue in the native lung enzyme [8]. Taken together, these results suggested that both mutant and wild-type enzymes are phosphorylated at the same site.

DISCUSSION

The role of the two allosteric cGMP-binding sites in one family of cGMP-binding PDEs has been investigated by generating site-directed mutations at these sites in the cGB-PDE (PDE5). Recent work [6,9] has shown that an invariant aspartic acid residue in each of the homologous binding sites is critical for cGMP binding: Asp-289 in the higher-affinity site, *a*, and Asp-478 in the lower-affinity site, *b*. We report here that the double mutant (D289A/D478A) is almost completely devoid of cGMP-binding activity under standard assay conditions, whereas the D289N mutation in site *a* increases the cGMP-binding affinity of cGB-PDE. None of these mutations appeared to change the catalytic properties of cGB-PDE, suggesting that cGMP hydrolysis is not under direct regulation by the cGMP-binding allosteric sites. This is in contrast with the effect of cGMP binding to stimulate catalysis in another cGMP-binding PDE, i.e. PDE2 [4], and suggests that cGMP binding might elicit different functional effects in the three PDE families that exhibit this property.

Although cGMP binding to the allosteric binding sites does not seem to alter catalytic function, occupation of these sites is required for phosphorylation of the enzyme at Ser-92 by either cGK or cAK [8]. However, the importance of each of the cGMP-binding sites, site *a* and site *b*, in eliciting this conformational change in the cGB-PDE remains unknown because the contribution of each of these sites to changes in function has not been determined for any of the cGMP-binding PDEs.

Our finding that the D289A/D478A mutant, which has minimal cGMP binding, is not phosphorylated to a significant extent under the standard incubation conditions, even in the presence of 1 mM cGMP, suggests tight coupling of cGMP binding to the allosteric sites with phosphorylation of the enzyme. In support of this, the moderately increased cGMP-binding affinity of the D289N mutant is accompanied by a decrease in the cGMP concentration at which cGB-PDE phosphorylation occurs. The contribution of the individual cGMP-binding sites in the regulation of phosphorylation has been studied by using the D289A mutant in site *a* and the D478A mutant in site *b*, each of which exhibits markedly diminished cGMP binding to the respective site [6,9]. The catalytic activity of each of these mutants is kinetically indistinguishable from that of wild-type enzyme by the methods used here [6,9], but neither mutant is phosphorylated to a significant extent. The profound decrease in phosphorylation in each of these mutants suggests that occupation of the *a* and *b* sites acts in concert to elicit the conformational change that exposes the phosphorylation site. Phosphorylation of the mutants that are defective in cGMP binding to the allosteric sites can be achieved when high concentrations of cGMP and catalytic subunit of cAK are used. This finding confirms that the phosphorylation of cGB-PDE is a function of both cGMP-binding sites and of their respective cGMP-binding affinities.

Phosphorylation is a common mechanism of regulation of PDEs [12]. It has been demonstrated that phosphorylation and activation of the adipocyte cGMP-inhibited PDE (PDE3) [13] occurs after treatment of rat adipocytes with either insulin or the

cAMP-elevating agent isoprenaline. Phosphorylation also seems to be an important mechanism of regulation of the activity of cAMP-specific PDE (PDE4) isoforms [14–16]. It has also been reported that the phosphorylation of the inhibitory subunit (PDE_γ) of the photoreceptor cGMP PDE (PDE6) [17,18] increases the potency with which PDE_γ inhibits PDE6 catalytic activity. Phosphorylation of the calmodulin-stimulated PDE (PDE1) has not been shown to alter the catalytic properties of this enzyme directly, but it decreases the affinity of the enzyme for Ca²⁺/calmodulin [19–21]. For cGMP-stimulated PDE (PDE2) from brain [22] and for one recombinant PDE4 [23], phosphorylation that occurs *in vitro* has not been shown to alter the catalytic properties of the enzymes. It was suggested that these phosphorylations might have a role either in protein–protein interactions or in cellular localization of the enzymes. The role of phosphorylation of cGB-PDE (PDE5) is still unknown, but the precise allosteric regulation of phosphorylation by cGMP provides a strong argument for its biological importance. Although no effect of phosphorylation on the activity of the purified enzyme has been described [8], the phosphorylation of cGB-PDE occurs in primary cultures of rat aortic vascular smooth-muscle cells in response to an increased cGMP level elicited by atrial natriuretic factor (T. A. Wyatt, A. Naftilan, S. H. Francis and J. D. Corbin, unpublished work). Pyne and co-workers have reported that the phosphorylation of partly purified cGB-PDE from guinea pig lung by exogenous catalytic subunit of cAK causes increased PDE activity [24,25]. This suggests that the phosphorylated cGB-PDE interacts with cellular components that contribute to the regulation of its catalytic activity. Recently the same group reported the identification of two low-molecular-mass proteins that seem to interact with cGB-PDE to prevent its activation by cAK [26].

The current studies provide the first evidence that both allosteric cGMP-binding sites in a cGMP-binding PDE are required to effect functional changes in these enzymes. These results establish a foundation for advancing our understanding of the function of these sites in the PDE5 family as well as in other cGMP-binding PDEs.

We thank Linda M. McAllister-Lucas for assistance in the initial stages of mutagenesis. This work was supported by NIH Grants GM41269 and DK40029.

REFERENCES

- Francis, S. H., Lincoln, T. M. and Corbin, J. D. (1980) *J. Biol. Chem.* **255**, 620–626
- Coquil, J., Franks, D. J., Wells, J. N., Dupuis, M. and Hamet, P. (1980) *Biochim. Biophys. Acta* **631**, 148–165
- Yamazaki, A., Sen, I., Bitensky, M. W., Casnellie, J. E. and Greengard, P. (1980) *J. Biol. Chem.* **255**, 11619–11624
- Martins, T. J., Mumby, M. C. and Beavo, J. A. (1982) *J. Biol. Chem.* **257**, 1973–1979
- Shabb, J. B. and Corbin, J. D. (1992) *J. Biol. Chem.* **267**, 5723–5726
- Turko, I. V., Haik, T. L., McAllister-Lucas, L. M., Burns, F., Francis, S. H. and Corbin, J. D. (1996) *J. Biol. Chem.* **271**, 22240–22244
- Arshavsky, V. Y., Dumke, C. L. and Bownds, M. D. (1992) *J. Biol. Chem.* **267**, 24501–24507
- Thomas, M. K., Francis, S. H. and Corbin, J. D. (1990) *J. Biol. Chem.* **265**, 14971–14978
- McAllister-Lucas, L. M., Haik, T. L., Colbran, J. L., Sonnenburg, W. K., Seger, D., Turko, I. V., Beavo, J. A., Francis, S. H. and Corbin, J. D. (1995) *J. Biol. Chem.* **270**, 30671–30679
- Thomas, M. K., Francis, S. H. and Corbin, J. D. (1990) *J. Biol. Chem.* **265**, 14964–14970
- Colbran, J. L., Francis, S. H., Leach, A. B., Thomas, M. K., Jiang, H., McAllister, L. M. and Corbin, J. D. (1992) *J. Biol. Chem.* **267**, 9589–9594
- Conti, M., Nemoz, G., Sette, C. and Vicini, E. (1995) *Endocr. Rev.* **16**, 370–389
- Degerman, E., Smith, C. J., Tornqvist, H., Vasta, V., Belfrage, P. and Manganiello, V. C. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 533–537
- Sette, C., Iona, S. and Conti, M. (1994) *J. Biol. Chem.* **269**, 9245–9252
- Sette, C. and Conti, M. (1996) *J. Biol. Chem.* **271**, 16526–16534
- Houslay, M. D. and Kilgour, E. (1990) in *Cyclic Nucleotide Phosphodiesterases: Structure, Function, Regulation, and Drug Action* (Beavo, J. and Houslay, M. D., eds.), pp. 185–218, John Wiley, Chichester
- Hayashi, F., Lin, G. Y., Matsumoto, H. and Yamazaki, A. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4333–4337
- Udovichenko, I. P., Cunnick, J., Gonzalez, K. and Takemoto, D. J. (1994) *J. Biol. Chem.* **269**, 9850–9856
- Sharma, R. K. and Wang, J. H. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2603–2607
- Sharma, R. K. and Wang, J. H. (1986) *J. Biol. Chem.* **261**, 1322–1328
- Florio, V. A., Sonnenburg, W. K., Johnson, R., Kwak, K. S., Jensen, G. S., Walsh, K. A. and Beavo, J. A. (1994) *Biochemistry* **33**, 8948–8954
- Whalin, M. E., Strada, S. J. and Thompson, W. J. (1988) *Biochim. Biophys. Acta* **972**, 79–94
- Luther, M., Holmes, B., Kassel, D., Lenhard, J., Hamacher, L., Burkhart, W., Moyer, M., Patel, I., Overton, L., Hoffman, C. and Rogue, W. (1994) *FASEB J.* **2141**, A370
- Burns, F., Rodger, I. W. and Pyne, N. J. (1992) *Biochem. J.* **283**, 487–491
- Burns, F. and Pyne, N. J. (1992) *Biochem. Biophys. Res. Commun.* **189**, 1389–1396
- Lochhead, A., Nekrasova, E., Arshavsky, V. Y. and Pyne, N. J. (1997) *J. Biol. Chem.* **272**, 18397–18403