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Cyclic nucleotide specificity of the activator and catalytic sites of a cGMP-stimulated cGMP phosphodiesterase from *Dictyostelium discoideum*

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The cellular slime mold *Dictyostelium discoideum* has an intracellular phosphodiesterase which specifically hydrolyzes cGMP. The enzyme is activated by low cGMP concentrations, and is involved in the reduction of chemoattractant-mediated elevations of cGMP levels. The interaction of 20 cGMP derivatives with the activator site and with the catalytic site of the enzyme has been investigated. Binding of cGMP to the activator site is strongly reduced (more than 80-fold) if cGMP is no longer able to form a hydrogen bond at N^2H_2 or $O^{2'}H$. Modifications at N^7, C^8, $O^{3'}$ and $O^{5'}$ induce only a small reduction of binding affinity. A cyclic phosphate structure, as well as a negatively charged oxygen atom at phosphorus, are essential to obtain activation of the enzyme. Substitution of the axial exocyclic oxygen atom by sulphur is tolerated; modification of the equatorial oxygen atom reduces the binding activity of cGMP to the activator site by 90-fold.

Binding of cGMP to the catalytic site is strongly reduced if cGMP is modified at N^1H, C^6O, C^8 and $O^{3'}$, while modifications at N^2H_2, N^3, N^7, $O^{2'}H$, and $O^{5'}$ have minor effects. Both exocyclic oxygen atoms are important to obtain binding of cGMP to the catalytic site. The results indicate that activation of the enzyme by cGMP and hydrolysis of cGMP occur at different sites of the enzyme. cGMP is recognized at these sites by different types of molecular interaction between cGMP and the protein.

cGMP derivatives at concentrations which saturate the activator site do not induce the same degree of activation of the enzyme (activation 2.3–6.6-fold). The binding affinities of the analogues for the activator site and their maximal activation are not correlated. Our results suggest that the enzyme is activated because cGMP bound to the activator site stabilizes a state of the enzyme which has a higher affinity for cGMP at the catalytic site.

Cyclic nucleotides have important functions in the cellular slime mold *Dictyostelium discoideum*. This organism lives in the soil where it feeds on bacteria. When the food supply is exhausted the single cells aggregate to form a multicellular slug, which finally differentiates into a fruiting body. Cell aggregation is mediated by chemotaxis to cAMP, which is detected by cell surface receptors. Extracellular cAMP induces a rapid transient accumulation of intracellular cGMP levels, which reach a maximal concentration after about 10 s (for reviews see [1–3]).

D. discoideum cells contain two classes of cyclic nucleotide phosphodiesterase activity. One class of enzymes hydrolyzes cAMP and cGMP with similar rates; these enzymes are located extracellularly, intracellularly and on the cell surface [4–6]. A second class of enzymes hydrolyzes only cGMP and is localized only intracellularly [7–9]. Non-specific phosphodiesterase is present in large excess over the cGMP-specific enzyme. These enzyme activities can be easily separated by concanavalin-A–Sepharose column chromatography, since all cAMP-hydrolyzing activity binds to the column, while the cGMP-specific enzyme passes through the column [8].

Mutant cells have been isolated which are totally devoid of the cGMP-specific enzyme [10, 11]. Transduction of chemotactic signals in these mutant cells indicate that cGMP is involved in the chemotactic reaction and that in wild-type cells cGMP is degraded mainly by the cGMP-specific enzyme [12].

Detailed kinetic studies have shown that the cGMP-specific enzyme is activated about threefold by low cGMP concentrations, which is due to a decrease of K_m, while V_{max} is unaltered. Half-maximal activation occurs at about 0.1 μM cGMP; it is a second-order process with a half-life of about 20 s at 0.1 μM cGMP [13]. The use of cGMP derivatives has revealed that the enzyme has two distinct cGMP sites, one for activation of the enzyme and another for hydrolysis by the enzyme; e.g. cAMP binds to neither site, cIMP binds only to the catalytic site, and 8-bromoguanosine 3′,5′-monophosphate binds preferentially to the activator site [8, 14].

cGMP-stimulated phosphodiesterases have been observed in many organisms and tissues [15–17]. They differ from the slime mold enzyme in molecular mass and in cyclic nucleotide specificity; the mammalian enzyme also hydrolyzes cAMP [16, 17]. In this report the interaction of 20 cGMP derivatives

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Enzyme. cGMP phosphodiesterase (EC 3.1.4.35).

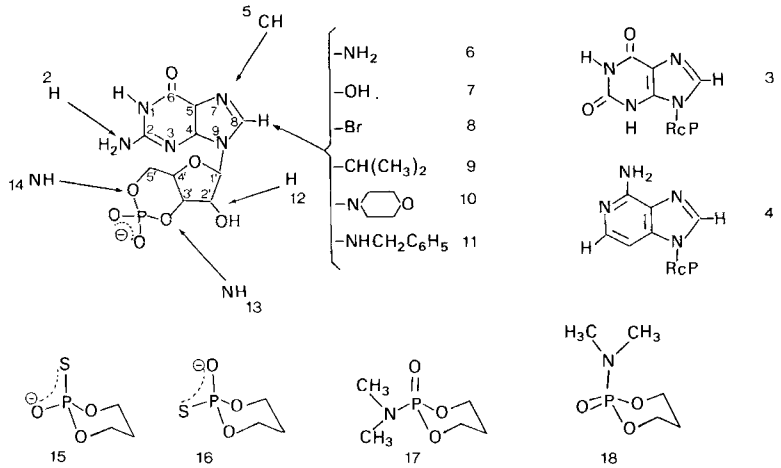

Fig. 1. *Structures of cGMP derivatives.* RcP in compounds 3 and 4 represents the ribosecyclophosphate moiety. Formulae 15–18 are partial structures of the cyclophosphate moiety

with the activator site and the catalytic site of the cGMP-specific phosphodiesterase from *D. discoideum* is described. The results show that the derivatives may have very different affinities for both sites. Furthermore, we show that each derivative may activate the enzyme to a different extent. A mechanism on the activation process is proposed, which is based on the differential stabilization of an activated and non-activated enzyme species by cGMP derivatives.

MATERIALS AND METHODS

Materials

[8-^{3}H]cAMP (1.5 TBq/mmol) and [8-^{3}H]cGMP (0.6 TBq/mmol) were purchased from Amersham International. [8-^{3}H]cIMP was synthesized from [^{3}H]cAMP by a slight modification [13] of the method of Casnellie et al. [18]. The reversed-phase packing material, μBondapak C_{18}/Porasil B_1 35–75 μm, was obtained from Waters; concanavalin-A–Sepharose was from Pharmacia, and Dowex AG1X2 was from Serva. DEAE-Sepharose and snake venom (*Ophiophagus hannah*) were purchased from Sigma. Compounds 1, 2, 3, 4, 12, 19 and 20 (see Fig. 1 and Table 1) were obtained from Boehringer Mannheim; compounds 6, 7, 8, 10 and 11 were generous gifts from Dr Muhlegger, Boehringer Mannheim [19]; compound *9* was kindly supplied by Dr Shugar (Polish Academy of Science, Warsaw). Compounds 5 and 13 were synthesized as described [20, 21]. The synthesis of compound 14 will be described elsewhere; the syntheses of compounds 15, 16, 17 and 18 are given below.

Synthesis of guanosine 3′,5′-mono[thio]phosphates (PS) and (PR) isomers (15 and 16)

Compounds 15 and 16 (P*S*) and (P*R*) isomers respectively of guanosine 3′,5′-mono[thio]phosphate, were synthesized according to the procedure for the synthesis of the analogous derivatives of cAMP [22] with some essential modifications. The 2′-OH and 2-NH_2 functions of cGMP were protected by isobutyryl groups in a reaction of cGMP (tri-*n*-butylammonium salt, 0.53 g, 1 mmol) with isobutyric anhydride (3.3 ml, 20 mmol) and 4-dimethylaminopyridine (0.25 g, 2 mmol) in 30 ml dimethylformamide (reaction time: 24 h; isolation: flash chromatography; yield: 75%). Then $N^2,O^{2'}$-diisobutyrylguanosine 3′,5′-monophosphate (0.41 g, 0.6 mmol) was converted to the diastereoisomeric mixture of $N^2,O^{2'}$-diisobutyrylguanosine 3′,5′-anilidophosphates (P*R* and P*S*) by means of triphenylphosphine (0.48 g, 1.83 mmol), carbon tetrachloride (0.28 g, 1.83 mmol) and aniline (0.34 g, 3.66 mmol) in pyridine solution (isolation: flash chromatography; yield: 24%). An introduction of [^{15}N]aniline and the subsequent determination of the $^1J_{^{31}P-^{15}N}$ coupling constant allowed assignment of the absolute configuration at phosphorus in these diostereoisomeric anilidates $^1J_{^{31}P-^{15}N}$ = 53.7 Hz for the (P*R*) isomer and $^1J_{^{31}P-^{15}N}$ = 39.2 Hz for the (P*S*) isomer. The guanosine 3′,5′-mono[thio]phosphates (15 and 16) were synthesized from the correspondent anilidates in stereospecific reaction with sodium hydride and carbon disulphide [22]: $\delta_{^{31}P(D_2O)}$ = 54.9 ppm for (P*S*) isomer (15) and $\delta_{^{31}P(D_2O)}$ = 56.6 ppm for (P*R*) isomer (16).

Synthesis of guanosine 3′,5′-dimethylamidophosphates (PS) and (PR) isomers (17 and 18)

$N^2,O^{2'}$-Diisobutyrylguanosine 3′,5′-monophosphate (0.33 g, 0.48 mmol), obtained from cGMP and isobutyric anhydride, was converted to the diastereoisomeric mixture of $N^2,O^{2'}$-diisobutyrylguanosine 3′,5′-dimethylamidophosphate (P*R* and P*S*) in the reaction mixture containing 5 ml pyridine, triphenylphosphine (0.39 g, 1.5 mmol), carbon tetrachloride (0.23 g, 1.5 mmol) and [^{15}N]dimethylamine (0.23 g, 3 mmol). The (P*R*) and (P*S*) isomers were purified and separated by flash chromatography. The coupling constant is $^1J_{^{31}P-^{15}N}$ = 46.5 Hz for the (*P*R) isomer and $^1J_{^{31}P-^{15}N}$ = 57 Hz for the (*P*S) isomer. After removal of the isobutyryl protecting groups by means of 2M NaOH and methanol saturated with ammonia, pure diastereoisomers 17 and 18 were obtained [$\delta_{^{31}P(CDCl_3)}$ = 8.3 ppm for (P*S*) isomer (17), $\delta_{^{31}P(CDCl_3)}$ = 6.9 ppm for (P*R*) isomer (18)].

Isolation of the cGMP-specific phosphodiesterase

D. discoideum NC_4H cells were grown as described in [13]. Cells were starved in suspension for 2 h. All subsequent steps were carried out at 0–4°C. Cells were homogenized by sonication. The homogenate was centrifuged at 23000 × *g* for 10 min and the supernatant at 42000 × *g* for 1 h. The supernatant (8 ml derived from 8×10^8 cells) was applied to a concanavalin-A–Sepharose column (160 mm × 16 mm diam-

eter) and the column was eluted with 10 mM sodium/potassium phosphate buffer, pH 7.2. The fractions eluting between 18 ml and 43 ml were combined and applied to a DEAE-Sepharose column (120 mm × 9 mm diameter) at a flow rate of 25 ml h^{-1}. Then the column was eluted at a flow rate of 16 ml h^{-1} with 50 ml 10 mM phosphate buffer pH 7.2, followed by 85 ml 50 mM NaCl in the same buffer, and with 100 ml of a linear gradient of NaCl (0.05 – 0.2 M) in buffer. Fractions of 4 ml were collected and assayed for phosphodiesterase activity and protein concentrations. The fractions which contained the highest specific activity were combined and used in the experiments. In some experiments the enzyme was concentrated using Minicon B15 (Amicon).

Protein concentrations were determined according to the method of Lowry et al. [23]. Each experiment was performed with duplicate or triplicate incubations on at least three different enzyme preparations.

Phosphodiesterase assays

Three assays to quantify the enzymatic hydrolysis of cyclic nucleotides have been used. Assay I, which has been used to map the activator site, was essentially identical to the procedure described in [8, 13]. Briefly, the incubations (200 µl) contained 10 nM [^{3}H]cIMP (about 2 kBq), cyclic nucleotides, 10 mM phosphate buffer pH 7.5, and 150 µl enzyme. After 30 min at 22 °C the incubation was terminated by boiling the samples during 2 min. The product, [^{3}H]5′IMP was converted to [^{3}H]inosine by snake venom, and the substrate, [^{3}H]cIMP, removed by addition of 1 ml ion-exchanger. After centrifugation the radioactivity in the supernatant was determined.

Assay II, which has been used to map the catalytic site, is based on a separation technique by reversed-phase chromatography as described in [24]. Briefly, the incubation (100 µl) contained 20 µM [^{3}H]cIMP (1.5 kBq), cyclic nucleotides, buffer, and 30 µl enzyme. The incubation was terminated after 60 min at 22 °C by the addition of 25 µl 40 mM H_3PO_4 which reduces the pH to 3.0. Then 125 µl was applied to small reversed-phase columns, which were eluted at atmospheric pressure with 1.2 ml 1% methanol in phosphate buffer pH 3.0 (yielding [^{3}H]5′IMP) followed by 1.2 ml 50% methanol in buffer (yielding [^{3}H]cIMP).

Assay III, which has been used to measure the hydrolysis of all cGMP derivatives, was essentially identical to a procedure described previously [25]. Briefly, the incubations (20 µl) contained 50 µM cGMP or cGMP drivative, 10 mM phosphate buffer, pH 7.2, and 10 µl enzyme (which was concentrated 50-fold by Minicon B15, Amicon). The reaction was terminated after 60 min at 22 °C by adding 25 µl 4 mM H_3PO_4 which reduces the pH to 4.0. Samples were centrifuged at 8000 × *g* for 2 min, and 20 µl of the supernatant were analyzed by high-performance liquid chromatography by using the anion-exchanger Partisil PXS, 10/25 SAX eluted with 25 mM KH_2PO_4, 5% methanol, pH 4.5.

The accumulation of product was linear with time for all assays, provided that not more than 40% of the substrate was hydrolysed.

RESULTS

Isolation and partial purification of the cGMP-specific phosphodiesterase

In a crude homogenate of *D. discoideum* cells, cAMP and cGMP are hydrolyzed at approximately equal rates, since the nonspecific enzyme, which hydrolyzes both cAMP and cGMP, is in large excess over the cGMP-specific enzyme. The separation of the cGMP-specific enzyme from the nonspecific enzyme by concanavalin-A – Sepharose column chromatography has been described previously [8]. It has been shown that the cGMP-specific phosphodiesterase isolated by this procedure is activated by low cGMP concentrations and that activation of the enzyme by cGMP and hydrolysis of cGMP occur at different sites of the enzyme.

Both the catalytic activity and the activation property of the enzyme were measured during the purification procedure. Ammonium sulphate precipitation can not be used, due to the loss of catalytic activity; DEAE-cellulose column chromatography, which has been used by others to isolate the cGMP-specific enzyme from *D. discoideum* [7, 9], is not useful in this experiment, since the activation property is largely lost. Affinity chromatography on a matrix in which the ligand 8-$NH_2(CH_2)_2$-S-cGMP is coupled to CNBr-activated Sepharose is not successful, because the described method for the affinity ligand [26] does not yield 8-NH_2-$(CH_2)_2$-S-cGMP in our hands. Affinity chromatography on a matrix in which cGMP is immobilized on epoxy-activated Sepharose [17] can not be used, since the cGMP-specific enzyme elutes from the column with a wash step containing 10 mM NaCl. This can be expected since cGMP is bound to the Sepharose column via its 2-amino or 2′-hydroxy groups [17] and both groups are required to obtain binding of cGMP to the activator site of the *Dictyostelium* cGMP-specific enzyme [8, 14].

DEAE-Sepharose column chromatography has been found to be the only procedure so far to purify the enzyme without losing activatability or catalytic properties. cGMP-hydrolyzing activity elutes as a single peak at about 0.12 M NaCl (Fig. 2). Elution with NaCl up to 0.4 M does not result in another fraction containing cGMP-hydrolyzing activity. The fractions with the highest specific activity are purified about 10-fold compared to the crude homogenate; the yield is about 25%. The activation of the enzyme by cGMP is completely preserved in this chromatography step. The enzyme is stable at 2 °C for at least four days, without losing catalytic or activating properties.

Selection of cGMP derivatives

cGMP can form several interactions with its surrounding medium (water or protein), such as hydrogen bonds, ionic bonds, and hydrophobic interactions. The derivatives were selected to reduce or remove one interaction potential. A possible hydrogen bond is prevented in compound 2 at N^2H_2, in compound 3 at N^2H_2 and N^3, in compound 4 at N^2H_2, N^1H and C^6O, in compound 5 at N^7, in compound 12 at $O^{2'}H$, in compound 13 at $O^{3'}$, and in compound 14 at $O^{5'}$ (see Fig. 1). The activity of these compounds may reveal the involvement of one of the mentioned hydrogen bonds in the binding of cGMP to the protein. Compounds 6 – 11 are all modified at the 8-position of cGMP. The activity of these compounds may provide information on the space at this position in the binding site of the protein and the preferential conformation of the base moiety. Compounds 15 – 18 have stereospecific modifications at the exocyclic oxygen atoms. Their activity may provide information on the stereoselective recognition of the phosphate moiety by ionic bonds, covalent bonds, or by hydrogen bonds. Additionally, in the case of cAMP it has been shown that antagonists may be present among these derivatives [27 – 32].

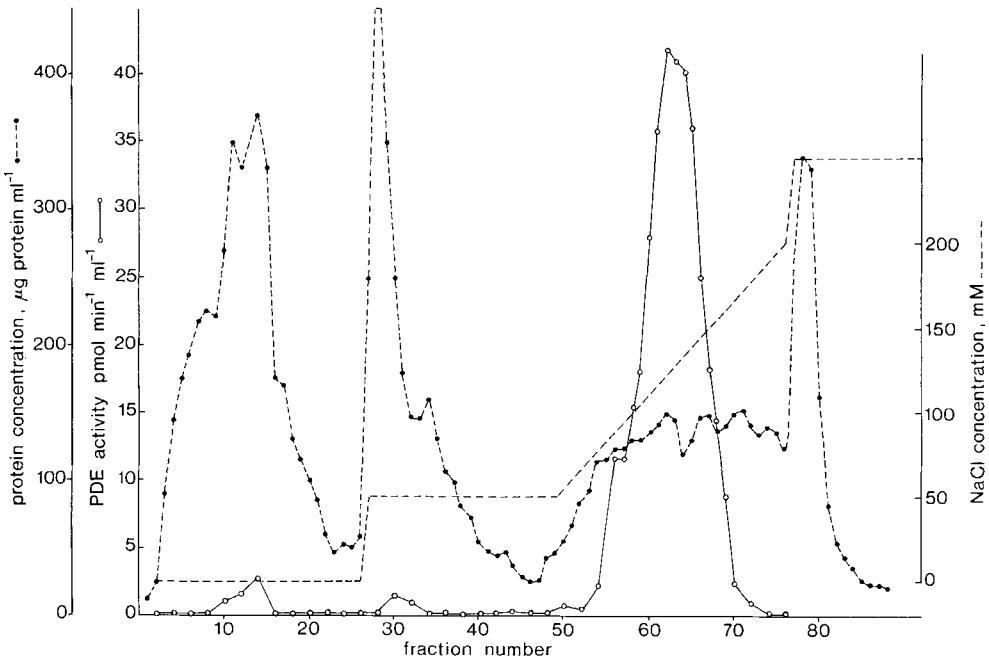

Fig. 2. *Purification of cGMP-stimulated cGMP phosphodiesterase by DEAE-Sepharose column chromatography.* cGMP-specific phosphodiesterase was isolated from 8×10^8 *D. discoideum* cells by concanavalin-A – Sepharose and applied to a column of DEAE-Sepharose as described in Materials and Methods. Fractions of 4 ml were collected and assayed for phosphodiesterase (PDE) activity at 1 μM cGMP (○) and for protein concentration (●). The concentration of NaCl is indicated by the dashed line

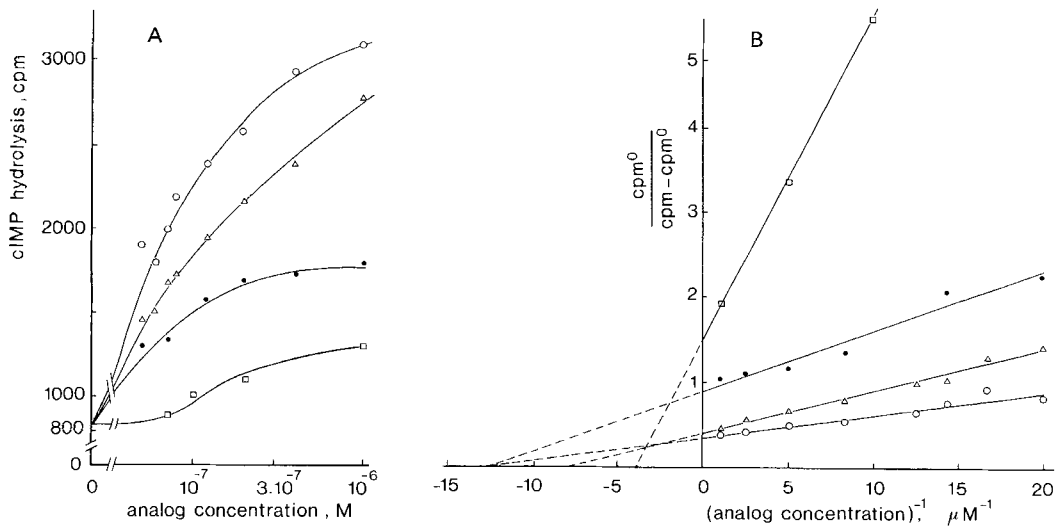

Fig. 3. *Activation of the hydrolysis of cIMP by cGMP derivatives.* The hydrolysis of 0.01 μM [^{3}H]cIMP (40000 cpm) by the DEAE-Sepharose-purified enzyme was measured in the presence of different concentrations of cGMP (●), b^{8}cGMP (○), compound 5 (□), and compound 15 (△). (A) Hydrolysis is presented as radioactivity formed after an incubation period of 30 min. The assay blank (850 cpm, assay I) has been subtracted from all values. (B) The data from A are transformed to a linear plot [13]. cpm^0 is the amount of substrate hydrolyzed in the absence of any derivative, and cpm that in the presence of the mentioned cGMP derivatives. The intercept on the abscissa equals $-K_a^{-1}$, while the intersection with the ordinate equals $(\alpha\text{-}1)^{-1}$, where α is the maximal-fold activation

Specificity of the activator site

Previously [13] it has been shown that cIMP is a good substrate of the cGMP-stimulated cGMP-specific phosphodiesterase from *D. discoideum,* but that it does not activate the enzyme. Therefore we used [^{3}H]cIMP as the substrate of the enzyme.

Activation of the hydrolysis of 10 nM [^{3}H]cIMP by compounds 1 (cGMP), 5, 8 and 15 is shown in Fig. 3A. These data were then transformed to a plot [13] shown in Fig. 3B. The intersection with the abscissa equals $-K_a^{-1}$; K_a is the apparent activation constant (concentration of derivative which induces half-maximal activation). The intersection with the ordinate equals $(\alpha\text{-}1)^{-1}$ where α is the maximal-fold activation. The result with all derivatives are summarized in Table 1. It appears that some analogs can activate the enzyme 6.6-fold, while others activate only 2.3-fold.

A few derivatives do not activate the enzyme. This could imply that they do not bind to the activator site, or that they do bind to the activator site, but are unable to activate (i.e.

Table 1. *Properties of the cGMP derivatives*
K_a is the apparent activation constant of the derivative [concentration which induces half-maximal activation of the hydrolysis of [^{3}H]cIMP]. α is the maximal-fold activation; data between parentheses mean that the derivatives do not bind to the activator site. IC_{50} is the concentration of derivative which induces a 50% inhibition of the hydrolysis of 20 μM cIMP as determined in Fig. 5. The hydrolysis of all derivatives was measured by HPLC; data are presented relative to the hydrolysis of cGMP

Analog	Activator site		Catalytic site	
	K_a	α	IC_{50}	hydrolysis
	μM		μM	%
1. Guanosine 3′,5′-monophosphate (cGMP)	0.12	3.8	14	100
2. Inosine 3′,5′-monophosphate (cIMP)	≥10	(1)	45	69
3. Xanthine 3′,5′-monophosphate	≥10	(1)	25	54
4. Adenosine 3′,5′-monophosphate (cAMP)	≥300	(1)	1800	<2
5. 7-Deazaguanosine 3′,5′-monophosphate	0.57	2.3	16	89
6. 8-Aminoguanosine 3′,5′-monophosphate	0.10	5.1	25	97
7. 8-Hydroxyguanosine 3′,5′-monophosphate	1.7	4.1	≥1000	3
8. 8-Bromoguanosine 3′,5′-monophosphate (b^{8}cGMP)	0.07	6.6	350	35
9. 8-Isopropylguanosine 3′,5′-monophosphate	1.55	5.1	500	<2
10. 8-Morpholinoguanosine 3′,5′-monophosphate	9.9	3.9	≥3000	<2
11. 8-Benzylaminoguanosine 3′,5′-monophosphate	1.72	5.8	2800	3
12. 2′-Deoxyguanosine 3′,5′-monophosphate	≥10	(1)	250	<2
13. 3′-Deoxy-3′-aminoguanosine 3′,5′-monophosphate	2.3	2.3	3000	<2
14. 5′-Deoxy-5′-aminoguanosine 3′,5′-monophosphate	2.5	2.4	150	<2
15. Guanosine 3′,5′-mono[thio]phosphate, (P*S*) isomer	0.11	5.4	500	<2
16. Guanosine 3′,5′-mono[thio]phosphate, (P*R*) isomer	11	3.2	1000	<2
17. Guanosine 3′,5′-monophosphodimethylamidate, (P*S*) isomer	≥100	(1)	300	<2
18. Guanosine 3′,5′-monophosphodimethylamidate, (P*R*) isomer	≥100	(1)	2000	<2
19. Guanosine 5′-monophosphate	≥100	(1)	3000	<2
20. Guanosine	≥100	(1)	≥10000	<2

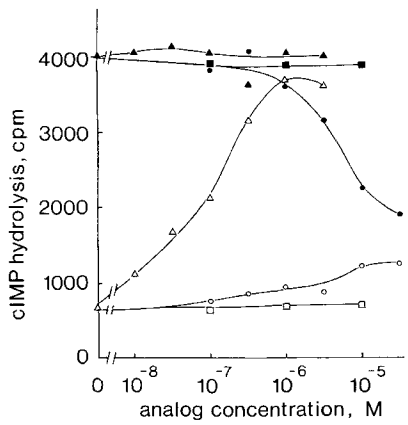

Fig. 4. *Inhibition of the b^{8}cGMP-induced activation of cIMP hydrolysis by cGMP derivatives which do not bind to the catalytic site.* Phosphodiesterase activity was measured by assay I which contained 0.01 μM [^{3}H]cIMP, DEAE-Sepharose-purified enzyme, different concentrations of cGMP-derivatives in the absence (open symbols) or presence (closed symbols) of 0.3 μM b^{8}GMP. The cGMP derivatives are compound 6 (▲, △), compound 14 (●, ○) and compound 17 (■, □)

they are full antagonists). Discrimination beween these possibilities can be made by investigating whether the derivatives can inhibit the activation induced by b^{8}cGMP (compound 8). The experiment is shown in Fig. 4 for three derivatives. Compound 6 activates the enzyme almost to the same extent as b^{8}cGMP does. By addition of increasing concentrations of compound 6 to an incubation containing b^{8}cGMP, an exchange between b^{8}cGMP and derivative 6 will occur at the activator site. Because compound 6 and b^{8}cGMP activate the enzyme to the same extent, no alteration of enzyme activity occurs. Compound 14 activates the enzyme only twofold. The fivefold activation by b^{8}cGMP is reduced by compound 14 to a threefold activation. Thus compound 14 exchanges with b^{8}cGMP at the activator site. Compound 17 does not activate the enzyme. Since it does not reduce the activation by b^{8}cGMP it appears that compound 17 does not bind to the activator site. Similar experiments were done with compounds 2, 3, 4, 12, 19 and 20 which indicate that none of them binds to the activator site.

Specificity of the catalytic site

The interaction of cyclic nucleotide derivatives with the catalytic site of a cGMP-stimulated phosphodiesterase from rat liver has been determined by detecting the inhibition of the hydrolysis of substrate by the completely activated enzyme [16]. For the present enzyme this would imply that the enzyme is activated by b^{8}cGMP, and that the inhibition of the hydrolysis of [^{3}H]cIMP by the derivatives is measured. However, the derivatives activate the enzyme to a different extent. Thus the addition of, for instance, cGMP to the incubation mixture may result in an exchange with b^{8}cGMP at the activator site (which reduces the hydrolysis of [^{3}H]cIMP, because cGMP does not activate the enzyme as strongly as b^{8}cGMP), or cGMP may exchange with [^{3}H]cIMP at the catalytic site (which also reduces the hydrolysis of [^{3}H]cIMP).

The procedure we use to establish the interaction of the cGMP derivatives with the catalytic site is shown for cGMP, b^{8}cGMP, and compound 12 in Fig. 5. Enzyme activity is measured at 20 μM [^{3}H]cIMP, because activation at higher substrate concentrations is small (this is due to the fact that V_{max} is not altered by the derivatives; e.g. b^{8}cGMP activates the enzyme only 2.5-fold). The data at low analog concentrations are transformed to the plot (Fig. 5, top) from which

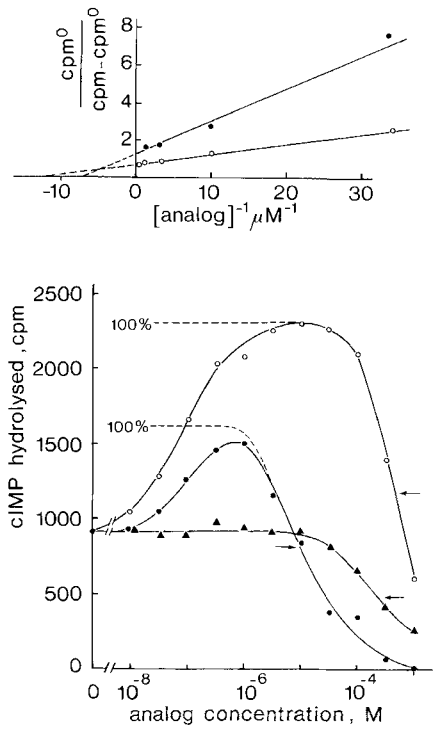

Fig. 5. *Inhibition of the hydrolysis of cIMP by cGMP derivatives.* Phosphodiesterase activity was measured by assay II which contained 20 μM [^{3}H]cIMP (≈40000 cpm), DEAE-Sepharose-purified enzyme and different concentrations of cGMP (●), b^{8}cGMP (○), and compound 12 (▲). The assay blank (50 cpm) has been subtracted from all data. Top: the data at low derivative concentrations are transformed to a plot from which the maximal-fold activation is obtained. This value is used in the main figure to indicate the maximal activity (100%, which is different for each derivative); then the derivative concentration yielding half-maximal inhibition (IC_{50}) is calculated (indicated by arrows)

the maximal-fold activation is obtained. With this value the activity of the enzyme without inhibition at the catalytic site is known for the derivative (100%, Fig. 5). Then the analog concentration which inhibits this value by 50% is called IC_{50}.

IC_{50} values (Table 1) range from 14 μM to concentrations above 10 mM. Some derivatives do not inhibit the hydrolysis of cIMP at even the highest derivative concentrations (which is generally 1 mM); apparently, these derivatives do not bind to the catalytic site.

Degradation of cyclic nucleotides

A second characteristic of the catalytic site, besides the binding of a substrate molecule, is the enzymatic conversion of the substrate. Degradation of all cGMP derivatives was measured by high-performance liquid chromatography. Only eight derivatives were found to be hydrolyzed; the other derivatives were hydrolyzed at least 50-fold slower than cGMP (see Table 1).

DISCUSSION

In this report the apparent activation constant, the maximal-fold activation, the apparent inhibition constant and the rate of enzymatic hydrolysis of 20 cGMP derivatives by a cGMP-stimulated cGMP-specific phosphodiesterase have been measured.

The activator site

Binding of cGMP to the activator site is strongly diminished (more than 80-fold) if a hydrogen bond at N^2H_2 or $O^{2'}H$ cannot be formed. Binding of cGMP to the activator site is diminished 20-fold if a hydrogen bond at $O^{3'}$ or $O^{5'}$ cannot be formed, while binding is reduced only slightly if a putative hydrogen bond at N^7 is impaired. Some of the analogs modified at the 8 position bind better to the activator site than cGMP, and some of them bind worse. The energy contribution of a hydrogen bond to the binding energy of a ligand to a protein is above 10 kJ/mol [33] which is proportional to a 60-fold reduction of binding affinity. Therefore, we tentatively propose that cGMP is bound to the activator site via hydrogen bonds at N^2H_2 and $O^{2'}H$. Hydrogen bonds at N^7, $O^{3'}$ and $O^{5'}$ are absent. The involvement of hydrogen bonds at N^1H, N^3, and C^6O in the binding of cGMP to the activator site remains unknown, because analogs specifically modified at these atom groups are not available.

A stereoselective recognition of the phosphate moiety at the activator site is indicated by the data for compounds 15–18; modification of the apical exocyclic oxygen atom by sulphur (compound 15) does not change the binding affinity, while modification of the equatorial exocyclic oxygen atom (compound 16) reduces the binding affinity 90-fold. The uncharged compounds 17 and 18 do not bind to the activator site up to 0.1 mM. The nature of the interaction between the phosphorus moiety and the activator site of the enzyme are not completely resolved by the present data. A covalent bond, as was suggested for other cyclic nucleotide binding proteins [34], seems unlikely, because compound 15 would have reduced affinity, and compound 17 would not be inactive. A charge-charge interaction is possible if it is directed to the equatorial exocyclic oxygen atom (cf. compounds 15 and 16).

A similar influence on the biological activity by sulphur substitution has been observed for the correspondent cAMP[S] derivatives in *Escherichia coli* where the (P*R*) isomer has very high biological activity, while the (P*S*) isomer has only low activity [35]. In this case an H-bond-directed ion pair between an arginine in the cAMP-acceptor protein and the axial oxygen atom in cAMP was determined by X-ray analysis [36].

Recently it has been shown that some of the cAMP derivatives analogous to compounds 15–18 may have cAMP antagonizing activities in several cAMP-dependent reactions [27–32]. None of the cGMP derivatives 15–18 are antagonists for the present enzyme, since compounds which bind to the activator site also activate the enzyme.

The catalytic site

cGMP is bound to the catalytic site quite differently from its binding to the activator site of the enzyme (Table 1). Binding of cGMP to the catalytic site is stronlgy diminished if cGMP is modified at N^1H/C^6O, C^8, $O^{3'}$, and at the exocyclic oxygen atoms. Modifications at N^2H_2, N^3, N^7, $O^{2'}H$, and $O^{5'}$ have only minor effects on the binding affinity of cGMP to the catalytic site. Therefore, we propose that cGMP is bound to the catalytic site via hydrogen bonds at N^1H and/

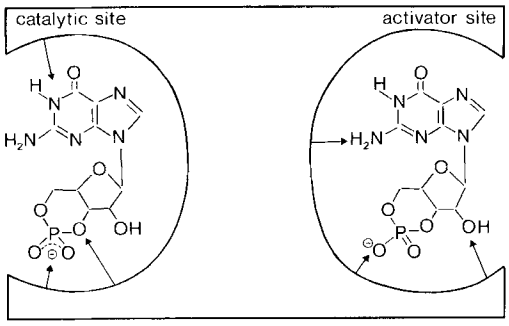

Fig. 6. *Tentative models of the cGMP-binding sites in the cGMP-stimulated cGMP phosphodiesterase from* D. discoideum. The atom or atom groups of cGMP directly involved in a binding interaction with the protein are indicated by an arrow

or C^6O, at $O^{3'}$, and possibly at one or both exocyclic oxygen atoms. Hydrogen bonds at N^2H_2, N^3, N^7, $O^{2'}H$, and $O^{5'}$ are absent. Bulky substituents at the 8-position strongly reduce the binding affinity of cGMP to the catalytic site.

Tentative models of the interaction of cGMP with the activator site and the catalytic site are shown in Fig. 6. Both sites have recognition points in the guanine moiety, the ribose cyclophosphate moiety, and at the exocyclic oxygen atoms, but the atoms which are recognized are different in both sites. The guanine moiety is recognized in the pyrimidine ring, but not in the imidazole ring; this may explain why cAMP is not recognized at either site.

Activation mechanism

Binding of cGMP to the activator site induces an alteration at the catalytic site by which substrate is bound more easily (reduction of K_m). We observed that the extent to which the cGMP derivatives activate the enzyme is different for each derivative. This may imply that many states of the catalytic site with different K_m values exist; the state depends on the molecular structure of the cGMP derivative at the activator site. On the other hand, the results are more easily explained by the assumption that the catalytic site can exist in only two states E and E* with respectively high and low K_m. Activation of the enzyme may then proceed via the cyclic scheme,

$$\begin{array}{ccc} E + A & \underset{}{\overset{K_d}{\rightleftharpoons}} & EA \\ K \Updownarrow & & \Updownarrow \gamma K \\ E^* + A & \underset{\beta K_d}{\rightleftharpoons} & E^*A \end{array}$$

where A is the activator (cGMP or a cGMP derivative), K is the equilibrium constant of E and E* ($K = [E]/[E^*]$), and K_d is the dissociation constant of EA. If this activation process is a closed thermodynamic system, then $\beta = \gamma$. In this scheme the extent of activation depends mainly on the values of K and β. β is the difference in affinity of the cGMP derivative between the non-activated enzyme species E and the activated enzyme species E*. Since these binding affinities depend on the molecular structure of cGMP, β will be different for each derivate.

The model predicts that a complete range of activation folds can be expected if the ratio of affinities of a derivative for E and E* are different. The term 'efficacy' used in pharmacology to explain the relationship between stimulus molecule and the maximal response evoked in a living cell is demonstrated here in a single molecular entity.

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