Autophosphorylation of cGMP-dependent protein kinase is stimulated only by occupancy of one of the two cGMP binding sites

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cGMP-Dependent protein kinase contains, per subunit, 2 binding sites for cGMP. The apparent K_D values for site 1 and 2 were 12 and 55 nM. The analogues 8-benzyl-amino-cAMP and N^2-monobutyryl-cGMP bind preferentially to site 1 and 2, respectively. Both analogues stimulate autophosphorylation of the enzyme at concentrations at which only half of the phosphotransferase activity of the enzyme is expressed. Complete expression of the phosphotransferase activity requires a high concentration of each analogue and is accompanied by inhibition of the autophosphorylation reactions. It is concluded that occupancy of site 1 or 2 stimulates autophosphorylation while occupancy of both sites prevents autophosphorylation.

Autophosphorylation *cGMP-dependent protein kinase* *8-Benzyl-amino-cAMP* N^2-*Monobutyryl-cGMP*

1. INTRODUCTION

cGMP-Dependent protein kinase (cGMP-kinase) is a dimer of two identical polypeptide chains of 75 kDa (see [1]). The enzyme binds 2 mol cGMP/mol subunit with a slightly different affinity [2]. Several analogues of cGMP and cAMP bind to each site with a different affinity and allow, therefore, activation of the enzyme by occupancy of one specific binding site [1,3]. The affinity of cGMP for site 1 is lowered by Mg·ATP [4,5].

The enzyme incorporates up to 4 mol phosphate/mol subunit in an autocatalytic process [5]. An optimal rate of this reaction is observed in the presence of cAMP, while phosphate incorporation is only slightly enhanced by cGMP [5,6]. cAMP stimulates autophosphorylation of cGMP-kinase by binding to the enzyme [7]. The reason for the marked difference in the efficiency of cGMP and cAMP to stimulate autophosphorylation has not been elucidated so far. Recently, it was suggested that cAMP binds slightly tighter to site 2 than to site 1 [3]. This finding raised the possibility that the autophosphorylation reaction of cGMP-kinase is promoted by occupancy of site 2. We have investigated this possibility and used analogues of cyclic nucleotides which select preferentially for site 1 or site 2. These experiments suggest that the autophosphorylation reaction of cGMP-kinase is stimulated by occupancy of either site 1 or site 2, while occupancy of both sites inhibits autophosphorylation completely.

2. MATERIALS AND METHODS

Phosphotransferase reactions were carried out at pH 7.0 and 30°C in 50 mM Mes buffer containing 150 mM NaCl, 0.3 mM EGTA, 10 mM magnesium acetate, 0.5 mg/ml bovine serum albumin, 0.05 mM [γ-^{32}P]ATP, cyclic nucleotides at the indicated concentrations and, if present, 0.1 mg/ml or 1.0 mg/ml substrate peptide. Reactions were started by addition of aliquots of purified cGMP-kinase and terminated after 2 min when peptide phosphorylation was measured, and 15 min when autophosphorylation was measured. Separation of phosphorylated products was carried out as in [5].

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Binding of cGMP or cAMP to the purified enzyme was performed at 2°C as in [2]. [^{3}H]cGMP and [^{3}H]cAMP were always repurified on a poly(ethyleneimine)-cellulose column [5]. cGMP-Kinase was purified as in [5]. For further methods see [5]. Each experiment was carried out several times with 2–3 different enzyme preparations.

[^{3}H]cGMP and [^{3}H]cAMP were purchased from NEN Chemicals. ATP, cAMP, cGMP and 8-Br-cGMP were from Boehringer, Mannheim. 8-Benzylamino-cAMP, N^2-monobutyryl-cGMP, and the substrate peptide termed 'Kemptide' (Leu-Arg-Arg-Ala-Ser-Leu-Gly) were from Sigma.

3. RESULTS AND DISCUSSION

3.1. *Number of binding sites*

As observed in [2], the dissociation of [^{3}H]cGMP from cGMP-kinase follows two different time constants indicating the presence of two distinct binding sites (fig.1). The $t_{½}$ for site 1 and site 2 was 257 ± 23 and 147 ± 18 min at 4°C (table 1). ATP·Mg increased the dissociation rate from site 1 2–3-fold and had only a small effect on the dissociation rate from site 2. The dissociation rate from both sites increased with temperature. ATP still doubled the dissociation rate from site 1 at 22°C (table 1). The presence of two distinct binding sites for cGMP was further confirmed by

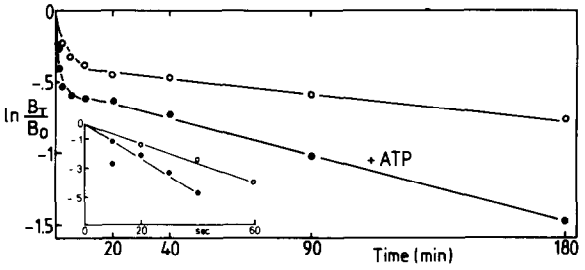

Fig.1. Dissociation of [^{3}H]cGMP from cGMP-kinase. cGMP-kinase at a subunit concentration of 33 nM was incubated at 2°C for 60 min in the presence of 0.5 μM [^{3}H]cGMP. The dissociation was initiated at zero time by the addition of cGMP (final concentration 1 mM) (○), or cGMP (1 mM), ATP (0.2 mM) and magnesium acetate (10 mM) (●). Dissociation of [^{3}H]cGMP was stopped at the indicated time points by dilution of the sample with ice-cold 2.6 M $(NH_4)_2SO_4$ solution. Binding to site 1 and 2 is defined by the slow and fast (inset) part of the dissociation curve.

Table 1

ATP affects the apparent half times of dissociation from site 1 and 2

ATP (μM)	$t_{½}$ (min)	
	Site 1	Site 2
Experiment A		
0	257 ± 23 (3)	0.47 ± 0.18 (3)
200	135 ± 28 (3)	0.33
Experiment B		
0	13.3 ± 0.4 (4)	0.2 (4)
50	6.0 ± 0.6 (3)	0.2 (3)

Experiment A was carried out as described in legend to fig.1. In experiment B a preincubation period with [^{3}H]cGMP of 20 min at 20°C and the buffer system of the autophosphorylation reaction was used. Dissociation was followed at 4°C (A) or 20°C (B). The values are $\bar{x}$ ± SEM with the number of individual experiments in parentheses

equilibrium binding experiments (fig.2). The enzyme bound a total of 1.66 mol cGMP/mol subunit. Binding to site 1 was measured 3 min after the addition of 1 mM unlabelled cGMP, at which time [^{3}H]cGMP bound to site 2 was completely displaced by unlabelled cGMP (fig.1). Scatchard analysis of these data indicated that site 1 and 2 bound 0.77 and 0.81 mol cGMP/mol subunit with apparent K_D values of 12 and 55 nM. The addition of Mg·ATP did not affect the total amount of cGMP bound to either site but increased the apparent K_D for site 1 and 2 to 37 and 89 nM. Equilibrium binding experiments in which [^{3}H]cGMP was replaced by [^{3}H]cAMP gave a total binding capacity of 1.7 mol cAMP/mol subunit and an apparent K_D value of 3 μM. Due to the low affinity of cAMP for these sites no differentiation was attempted. Mg·ATP (0.2 mM) apparently lowered the maximal amount of [^{3}H]cAMP bound by the enzyme to 0.83 mol/mol subunit and decreased slightly the apparent K_D value. The results of these experiments confirm the presence of two distinct cGMP binding sites, which also bind cAMP. Cyclic AMP presumably binds in the presence of ATP to site 2, since ATP lowered the affinity of cGMP for site 1 and had only a minimal effect on site 2. A preferential binding of cAMP to site 2 has been reported [3].

3.2. *Site specificity of autophosphorylation*

The binding experiments suggested that cAMP might stimulate the autophosphorylation reaction of cGMP-kinase by a selective interaction with site 2 in the presence of ATP. This possibility was further probed by testing the ability of several 'site specific' [1,3] compounds to stimulate autophosphorylation and the phosphorylation of an exogenous substrate. The cAMP analogue 8-benzylamino-cAMP and 8-Br-cGMP bind preferentially to site 1, whereas N^2-monobutyryl-cGMP selects preferentially for site 2 (fig.3). This apparent site selectivity was not changed by variation of the temperature from 4 to 22°C or by the presence of ATP·Mg. Autophosphorylation of cGMP kinase, which was determined at a subunit concentration of 0.7 μM was stimulated by each compound. cGMP and 8-Br-cGMP stimulated phosphate incorporation maximally at a total concentration of 1 μM – the free concentration being considerably lower – to 0.2 mol phosphate/mol subunit and suppressed the phosphotransferase activity completely at higher concentrations. The other analogues stimulated the autophosphorylation at a higher total and free concentration to about a mol phosphate/mol subunit, but again the autocatalytic activity was inhibited at higher concentrations of compounds. Comparison of the concentration dependency of the autophosphorylation with that of substrate phosphorylation reveals that cGMP and 8-Br-cGMP were maximally active at a concentration of 1 μM and did not inhibit the peptide phosphorylation reaction at higher concentrations. N^2-Monobutyryl and 8-benzylamino-cAMP stimulated peptide phosphorylation in a biphasic manner. A half maximal enzyme activity was observed at the lower concentrations,

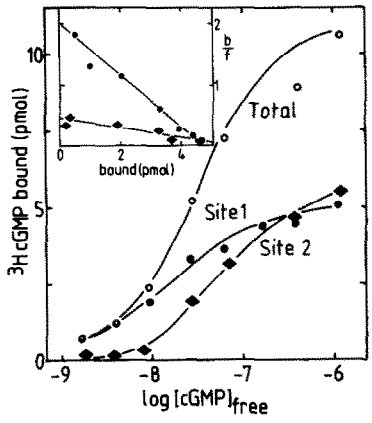

Fig.2. Equilibrium binding of cGMP by cGMP-kinase. cGMP-kinase (33 nM subunit) was incubated for 60 min at 2°C with [^{3}H]cGMP. Total binding of cGMP was determined with one half of the tubes (○), binding of cGMP to site 1 (●) was determined 3 min after the addition of non-radioactive cGMP (1 mM). Binding to site 2 (◆) was calculated by subtracting values for site 1 from total binding. The inset shows Scatchard plots for the binding to site 1 and 2.

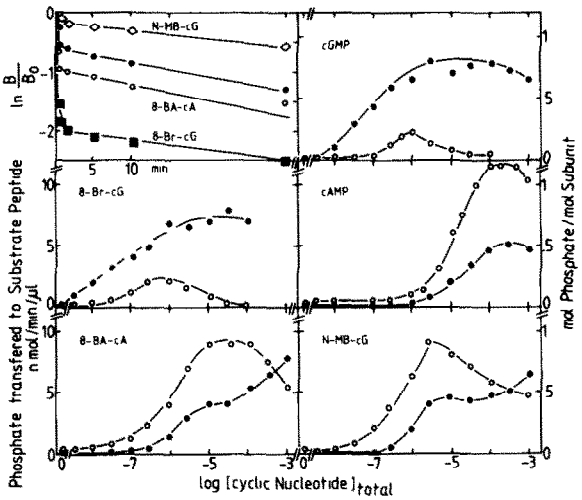

Fig.3. Site-specific stimulation of the autophosphorylation. Top, left: the apparent site specificity of various compounds was determined as in fig.1. cGMP kinase (17 nM subunit) was incubated at 2°C for 60 min with 0.1 μM [^{3}H]GMP in the absence (●) or presence of 10 μM N^2-monobutyryl-cGMP (*N*-MB-cG, ◇) or 30 μM 8-benzylamino-cAMP (8-BA-cA, ○), or 2 μM 8-bromo-cGMP (8-Br-cG, ■) or 100 μM cAMP. Dissociation in the presence of cAMP followed that shown in the absence of compound. Note that 8-BA-cA and 8-Br-cG bind preferentially to site 1 whereas N-MB-cG selects for site 2. The stimulatory efficiency of the compounds to stimulate phosphorylation is shown in the remaining part of the figure (left: middle and lower; right: top, middle and lower). Autophosphorylation (○) was measured at an enzyme subunit concentration of 0.7 μM. Phosphorylation in the absence of compound (0.2 mol/mol subunit) has been subtracted. Phosphorylation of substrate peptide (0.1 mg/ml, ●) was measured at a subunit concentration of 5 nM (2 ng/ml). Phosphorylation in the absence of compounds has been subtracted (1 nmol·μl^{-1}·min^{-1}).

which stimulated autophosphorylation maximally. A further increase of the compound concentration resulted in doubling of the enzyme activity at concentrations which inhibited autophosphorylation strongly. These data suggested that compounds which bind preferentially to site 1 or site 2 stimulate autophosphorylation and that the autophosphorylation reaction is optimal at those concentrations at which half of the enzyme activity is expressed. These experiments implied further that complete activation of the enzyme which is achieved by occupancy of both sites [2] suppressed the autophosphorylation reaction. This suggestion is supported by the experiment shown in fig.4, in which complete activation of the enzyme by 8-Br-cGMP suppressed immediately further autophosphorylation of the enzyme. The low efficiency of cGMP to stimulate autophosphorylation is explained by the finding that cGMP does not bind preferentially to one site (fig.2) and saturates both sites at similar concentrations of cGMP.

These interpretations were not supported by the results obtained with cAMP. This nucleotide maximally stimulated auto- and peptide-phosphorylation at 0.1 mM (fig.3). At this concentration only half of the total enzyme activity was obtained indicating that cAMP occupied only site 2. However, a further increase in the cAMP concentration inhibited auto- as well as peptide phosphorylation. Additional experiments showed that cAMP com-

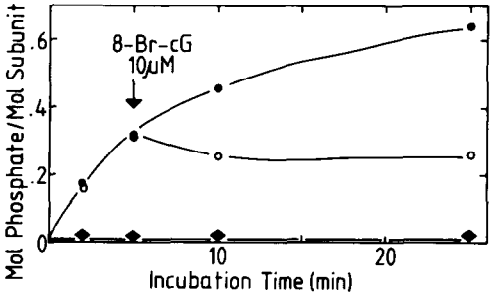

Fig.4. Inhibition of autophosphorylation by complete activation of the enzyme. Autophosphorylation of cGMP-kinase (0.7 μM subunit) was stimulated by 10 μM 8-Br-cGMP (♦); or by 1 μM *N*-MB-cGMP (●) or by 1 μM *N*-MB-cGMP followed at 5 min by 10 μM Br-cGMP (○). Identical results were obtained if 0.3 μM 8-Br-cGMP was used or if *N*-MB-cGMP was replaced by 10 μM 8-BA-cAMP. Phosphorylation in the absence of compounds has been subtracted.

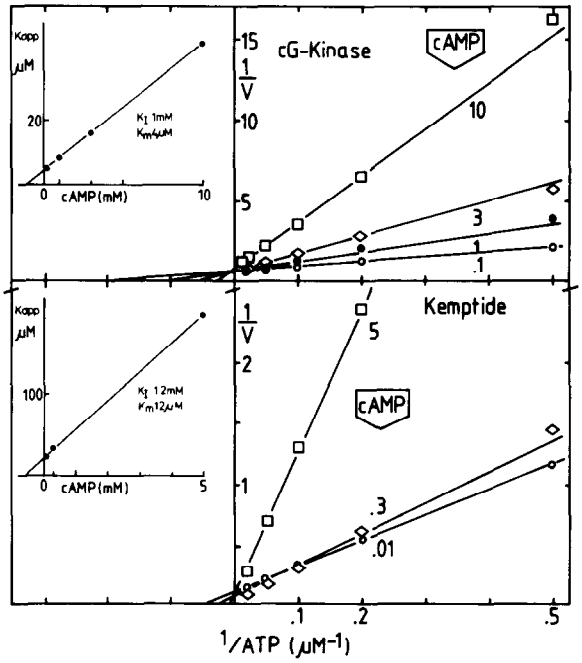

Fig.5. cAMP inhibits competitively ATP binding. The $1/V$ *vs* 1/ATP plots of the original values are shown with a replot of the app. K_m *vs* cAMP as inset. Autophosphorylation of cGMP-kinase (1.2 μM subunit; upper panel) and phosphorylation of substrate peptide (1 mg/ml, pH 7.5, lower panel) was determined in the presence of various concentrations of ATP and cAMP. V_{max}, $K_{I(cAMP)}$ and $K_{m(ATP)}$ values are 1.66 mol phosphate·mol subunit^{-1}·15 min^{-1}, 1.08 mM and 4 μM for the autophosphorylation reaction and 7.7 μmol phosphate·min^{-1}·mg^{-1}, 1.2 mM and 12 μM for the peptide phosphorylation.

peted competitively with ATP at the ATP substrate site (fig.5). Secondary plots of the inhibition curves for auto- and peptide-phosphorylation yielded K_I values for cAMP of 1.08 and 1.2 mM, respectively. From these plots K_m values of 3.6 and 12 μM were obtained for auto- and peptide-phosphorylation, respectively. The similarity of these values suggests that cAMP inhibited both phosphorylation reactions by the same mechanism, namely by binding competitively to the ATP-substrate site of the enzyme. This inhibition mechanism made it impossible to demonstrate complete activation of the enzyme by cAMP.

The results obtained with cAMP, therefore, support again the above interpretation that the autophosphorylation reaction is stimulated by oc-

cupancy of either one of the two cGMP binding sites. A direct proof of this interpretation is at present not possible since the low affinity of these compounds for either site prevents an accurate determination of the binding stoichiometry under the conditions used for autophosphorylation. The physiological significance of the autophosphorylation mechanism is unclear. The purified enzyme contains covalent-bound phosphate [5,7] suggesting that cGMP-kinase is phosphorylated in vivo. The enzyme(s) and the mechanism involved in this modification are unknown, but it is interesting to speculate that this reaction may occur if the enzyme is activated half maximally in vivo.

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