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Expression of a chimeric, cGMP-sensitive regulatory subunit of the cAMP-depedent protein kinase type Iα

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Abstract To study the fluctuations of cGMP in living cells through changes of energy transfer of dissociable fluorescence labeled subunits, we constructed a cGMP-sensitive probe by combining the N-terminus of the type I regulatory subunit of cAMP-dependent protein kinase (PKA) with the cGMP binding sites of cGMP-dependent protein kinase Iα (PKG). This chimeric regulatory subunit retained PKA-like dimerization and PKG-compatible cGMP binding constants (K_d = 53 nM) for both binding sites. High affinity interaction with the PKA catalytic subunit was verified by Surface Plasmon Resonance (K_d = 3.15 nM). Additionally, the chimera inhibits the formation of wild-type holoenzyme with an apparent K_i of 1.05 nM. Furthermore, cGMP dissociated the mutant holoenzyme with an apparent activation constant of 146 nM. Thus, our construct provides all the requirements needed to investigate changes in intracellular cGMP concentrations.

Key words: cAMP-dependent protein kinase; cGMP-dependent protein kinase; Site-directed mutagenesis; Protein expression; Protein folding

1. Introduction

cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) are the major cellular receptors for cyclic nucleotides. While PKA and PKG share many structural features, they differ remarkably in their mechanism of kinase activation (for review see [1–3]). The PKA holoenzyme complex (R_2C_2) consists of a regulatory subunit dimer (R_2) and two catalytic subunit (C) monomers. Binding of two molecules of cAMP to each regulatory subunit causes the complex to dissociate and subsequently to release the active C-subunits. In contrast, in the PKG R- and C-domains are fused in a single polypeptide chain and activation of this enzyme involves major conformational changes [4]. The unique property of PKA, that is, the dissociation of R- and C-subunits during activation proved valuable for investigating the subcellular function and localization of cAMP inside living cells. After labeling the R- and C-subunits with rhodamin and fluorescein, respectively, the so formed holoenzyme ((Rh)-R_2C_2-(Fl)) showed strong energy transfer between the chromophores and changes of the energy transfer upon activation [5]. After microinjecting the labeled holoenzyme in living cells this technique allowed to measure free cAMP concentrations as a response to extracellular stimulation by the use of confocal fluorescence microscopy. Adams et al. [5] showed that the change in energy transfer is completely reversible, therefore not only rising cAMP concentrations could be measured but also the decrease of the allosteric regulator. In addition, Bacskai et al. [6] found with fluorescence-labeled PKA that cAMP synthesis after stimulation with serotonin is much higher in the processes of *Aplysia* neurons than in the cell bodies. Alternatively, the localisation of the dissociated subunits could be determined as has been shown for the catalytic subunit [7]. The second messenger cGMP plays, like cAMP, an important role in mediating a large number of extracellular signals and with the steadily increasing number of cellular cGMP-receptors such as cation channels and phosphodiesterases [8], its function in regulatory processes is less clear than that for cAMP. Therefore, we constructed a cGMP-sensitive receptor protein, which can be used as a probe to monitor specifically the fluctuation of the cGMP concentration inside cells. We describe here the mutagenesis, expression and functional analysis of a chimeric regulatory subunit that contained the N-terminal 147 amino acids of PKA Iα, including the hinge region, and the two cGMP binding sites (aa 115–353) of PKG Iα.

2. Materials and methods

2.1. Reagents and materials

Polyethylenimine cellulose and cAMP-agarose were from Sigma. [γ-^{32}P]ATP (4500 Ci/mmol) was from Amersham, whereas [α-^{35}S]dATP (1150 Ci/mmol), [8,5′-^{3}H]cGMP (30 Ci/mmol) and [2,8-^{3}H]cAMP (28 Ci/mmol) were obtained from DuPont. [^{3}H]Cyclic nucleotides were purified before use [9]. Nitrocellulose filters (0.45 μM) were from Schleicher & Schuell. 8-Br-cGMP was purchased from BioLog. Phosphotransferase assays were performed with Kemptide (Sigma) or the synthetic peptide (Ac-RAERRASI-NH_2) [10]. Oligonucleotides were synthesized on the ABI Syntheziser, model 392 and purified with Quiagen kits. Sucrose density gradient centrifugation was performed with a Beckman ultracentrifuge (L8-70M) and a VTi 65.1 rotor. Enzymes for DNA manipulation were obtained from Boehringer-Mannheim, USB, New England Biolabs, or Gibco-BRL. All reagents for cloning were of molecular biology grade. All other chemicals, including media, protease inhibitors and reagents used in protein purification, were of analytical grade.

2.2. Mutagenesis

A *Bgl*II restriction site was introduced into pUC 18 R[1], containing the coding sequence of the PKA Iα regulatory subunit [11], by mutating nt 433 (A→T) and nt 434 (G→C) using PCR overlap extension [12]. The PCR reaction mixture (100 μl) contained 10 mM Tris pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.25 mM dNTPs, 1 ng template DNA, 0.25 mM of primer A: 5′-GACTCCATCGTGCAGCTGTGC-3′, and B: 5′-GTCAAAAATGTCAGATCTCTCGTTATCAT-3′, or primer C: 5′-GATAACGAGAGATCTGACATTTTTGA-3′, and D: 5′-TTCTGTAGCTGTCCCGGT-3′ and 2.5 U Taq-polymerase. The flanking primers A and D correspond to nt 91-111 and nt 674-691, whereas the mutagenesis primers B and C were complementary to nt 419-447. The amplified DNA fragments were purified by 6% PAGE and subsequently electroeluted. A second PCR reaction with primers A and D

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and the two primary PCR products as template gave the final DNA-fragment which was cut with *Sfi*I and *Xho*I, ligated into pUC 18 R^1 and electroporated into *E. coli* XL1 blue (Stratagene). *Bgl*II positive clones (pUC 18 R^1/*Bgl*II) were selected and sequenced according to the method described by Sanger [13]. The sequence of PKG Iα [14] coding for the cGMP binding sites (nt 344-1059) was amplified by PCR using primer I: 5′-AACGAGAGATCTGACATTGTGGATTGTATGT-ACC-3′ corresponding to nt 344-363 (containing a *Bgl*II site) and primer II: 5′-CTAGTCTAGATTAGGCGAAGAAAGCAGC$^{3'}$ complementary to nt 1045-1059 (introducing a stop codon and a *Xba*I site). The PCR product was cut with *Bgl*II and *Xba*I and ligated into pUC 18 R^1/*Bgl*II to yield pUC 18 AG R^1. Transformation was performed by electroporation into *E. coli* XL1 blue and positive clones were sequenced. For expression of the mutant protein, subsequently referred to as AG R^1, the gene was subcloned into the T_7-RNA-Polymerase dependent pET 3d vector [15]. pUC 18 AG R^1 was digested with *Nco*I and *Sal*I, while pET 3d was cut with *Nco*I and *Bam*HI. After ligation of the *Nco*I site, ends were refilled with T_4-DNA-polymerase, ligated and sequenced.

2.3. Expression and purification of AG R^I

Expression of AG R^1 was performed in *E. coli* BL21 (DE3)(pLysE) at 37°C in LB-broth (100 μg/ml ampicillin, 34 μg/ml chloramphenicol) [15]. Cells were induced at an $OD_{620} = 0.5$ with 0.5 mM IPTG and incubated for 2 h. The bacterial pellet was subjected to a French Pressure Cell and centrifuged. The expressed protein was found in the pellet fraction with a yield of approximately 40 mg/l. The inclusion bodies were solubilized in 100 mM KPO_4-buffer, pH 7.0, 8 M urea, 0.3 M β-mercaptoethanol and 100 μM cAMP for 45 min at room temperature. In order to remove the urea, the supernatant of subsequent centrifugation was extensively dialyzed against 100 mM KPO_4-buffer, pH 7.0, 15 mM β-mercapthoethanol, 1 mM EDTA, 0.2 mM PMSF and 100 μM cAMP at room temperature and at 4°C against cAMP free buffer. The protein solution was centrifuged and the supernatant loaded on a cAMP-agarose column equilibrated with 20 mM KPO_4-buffer, pH 7.0, 100 mM NaCl, 15 mM β-mercaptoethanol, 2 mM benzamidine, 1 mM EDTA, 1 mM PMSF, 43 μM TPCK, 20 μM TLCK. After binding over night at 4°C, the column was washed with buffer containing 100 mM NaCl, then 0.5 M NaCl and again with 100 mM NaCl. The protein was eluted at room temperature with 1 mM cAMP in running buffer. The pooled fractions contained 16 mg purified protein (= 8 mg/l culture). Protein concentrations were measured according to Bradford [16].

2.4. Enzyme assays

Equillibrium binding (K_d) was determined by a modified method described by Døskeland and Øgreid [17]. The chimeric protein was incubated at a concentration of 40 nM for cGMP binding (respectively 200 nM for cAMP binding) in 200 μl binding buffer (50 mM MES, pH 6.9, 10 mM NaCl, 10 mM DTT, 1 mM Mg-acetate, 0.4 mM EGTA and 0.5 mg/ml BSA) with varying concentrations of [^{3}H]cGMP or [^{3}H]cAMP at 0°C. After 90 min of incubation, the protein was precipitated with ice-cold 95% ammonium sulfate, filtered over 0.45 μm pore size filters, washed with ice-cold 95% ammonium sulfate and counted in Triton X-100/toluene scintillation mix.

cGMP dissociation rates (k_d) were determined by saturating 50 nM AG R^1 with 250 nM [^{3}H]cGMP during 90 min at 0°C in a final volume of 200 μl binding buffer. Dissociation was started by adding 20 μl of 1 mM cGMP. After designated time points, the reaction was stopped with ice-cold 95% ammonium sulfate and treated as described above. 8-Br-cGMP which selectively binds to the slow dissociating site B [18] was added to a final concentration of 10 μM in experiments designated to measure the fast dissociating binding site A.

Kinase activity of holoenzyme preparations was determined by [γ-^{32}P]ATP phosphotransferase assay. The holoenzyme (10 ng/assay) was incubated with 50 μM kemptide, 100 μM [γ-^{32}P]ATP and various cGMP concentrations. The phosphotransferase reaction was carried out for 3 min at 30°C, according to a method described previously [19].

2.5. Holoenzyme formation

Holoenzyme was formed in the presence of 100 μM Mg-ATP. The regulatory chimera and the PKA catalytic subunit were dialysed with an 2.5 molar excess of chimeric subunit against 40 mM KPO_4, pH 6.6, 15 mM β-mercaptoethanol, 100 μM ATP, 500 μM $MgCl_2$ and 5% glycerol for 24 h [20]. Holoenzyme formation was verified by Surface Plasmon Resonance spectroscopy, native PAGE and FPLC chromatography (see below).

2.6. Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) was used to study the interaction between the C-subunit and wild type or mutant R-subunits using a BIAcore instrument (Pharmacia/Biosensor). SPR detects mass changes in real time on a sensor chip surface which was prepared by direct coupling of the C-subunit by primary amines to the CM-dextran (Biosensor Amine Coupling Kit). All proteins were purified by gel filtration or affinity chromatography. The experiments were performed as outlined previously [21]. Kinetic constants were calculated by linear regression of data, using the BIAcore pseudo-first-order rate equation:

$$dR/dt = k_{ass} \times CR_{max} - (k_{ass} \times C + k_{diss}) \times R_t$$

The association rate is k_{ass}, k_{diss} the dissociation rate, C is the concentration of the injected analyte and R is the response. Plots of dR/dt vs. R_t

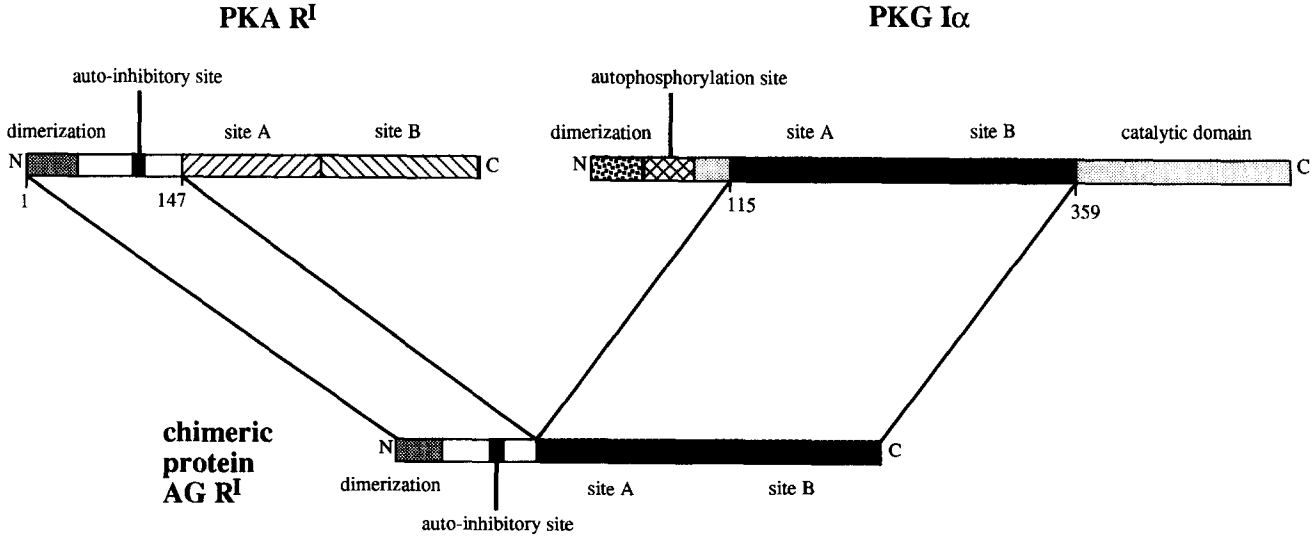

Fig. 1. Scheme for the construction of AG R^I. The mutant protein contains the N-terminal amino acids 1–147 of the regulatory subunit R^1 of PKA and residues 115–359 of PKG Iα. The N-terminus of PKA includes the auto-inhibitory site ^{94}RRGAI^{98}, whereas the sequence of PKG codes for the cGMP-binding sites.

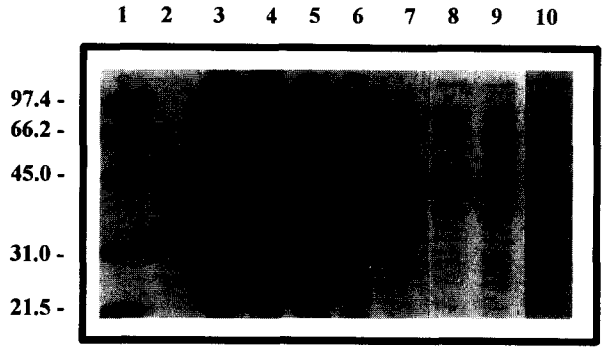

Fig. 2. Expression of the chimeric AG R^I-subunit in *E. coli* BL21 (DE3) (pLysE). Lane 1 = molecular weight marker, lane 2 = purified AG R^I (2 μg), lane 3 = cells prior to induction (approx. 25 μg), lane 4 = cells 2 h after induction (approx. 25 μg), lane 5 = French press homogenate, lane 6 = supernatant (15 μg), lane 7 = pellet, lane 8 = refolded protein (3 μg), lane 9 = cAMP-agarose flow through (3 μg), lane 10 = cAMP-agarose eluate (3 μg). In order to show the distribution of AG R^I in pellet and supernatant, the same fraction of total volume as in lane 6 was loaded in lanes 5 and 7.

have a slope of k_s. When k_s is plotted against C the resulting slope is equal to k_{ass}. The k_{diss} was calculated by integrating the rate equation when $C = 0$, yielding:

$$\ln(R_{t1}/R_{tn}) = k_{diss} \times (t_n - t_1)$$

Affinity constants were calculated from the equation: $K_d = k_{diss}/k_{ass}$. Association and dissociation rate calculations were performed using concentrations between 75 nM and 500 nM for wild type R and AG R^I subunit.

2.7. Determination of the apparent inhibition constant for holoenzyme formation

Recombinant catalytic subunit (0.05 nM in 0.1 ml kinase assay buffer containing 50 mM MES, pH 6.9, 10 mM DTT, 10 mM NaCl, 1 mM Mg-acetate, 0.4 mM EGTA, 100 μM [γ-^{32}P]ATP and 1 mg/ml BSA) was mixed with various concentrations of wild-type regulatory subunit and incubated for 15 min at 30°C. After preincubation, holoenzyme formation was monitored by determining the remaining catalytic activity for 15 min as described above (section 2.4). The apparent association constant ($K_{d,app}$) was obtained from plotting the % enzyme activity versus log[R-subunit]. The experiment was repeated in the presence of increasing concentrations of mutant regulatory subunit (2–20 nM). The apparent K_i was derived from the replot IC_{50} versus [AG R^I] as shown in Fig. 6.

2.8. Determination of activation constants by analytical FPLC-gel filtration

Analytical gel filtration was carried out, using a Superdex 200 PS 3.2/30 column on a SMART system (Pharmacia) [22]. All runs were done at 22°C in a buffer containing 20 mM MOPS, pH 7.0, 150 mM KCl and 1 mM DTT. 560 nM FITC-labeled C-subunit was mixed with a 1.5 molar excess of AG R^I and incubated with different concentrations of cyclic nucleotides, ranging from 20 nM to 10 μM, for 1 h. For each run the column was equilibrated with the appropriate cyclic nucleotide concentration in the gel filtration buffer. 20 μl of the samples were injected onto the gel filtration column and the percentage of holoenzyme at various cyclic nucleotide concentrations was calculated based on the peak areas corresponding to both holoenzyme and C-subunit. C-subunit (10 mg/ml) was labeled with fluorescein-5-isothiocyanate (FITC) using a 35-fold excess of label [22].

2.9. Sucrose density gradient centrifugation

5–15% sucrose density gradients were used to determine the $s_{w,20}$ values. Centrifugation was carried out at 65 000 rpm for 108 min. Proteins (45–300 μg) were dissolved in 20 mM potassium phosphate, pH 7.0, 2 mM benzamidine and 2 mM EGTA. Runs with holoenzyme contained 100 μM ATP and 500 μM $MgCl_2$ but no EGTA. The gradients were fractionated into 23 aliquots, analysed for protein concentrations by either the Bradford assay, cGMP binding or kinase activity. The following internal marker proteins were used: phosphorylase *b* ($s_{w,20} = 8.2$), γ-globulin (rabbit, $s_{w,20} = 6.36$), horseradish peroxidase ($s_{w,20} = 3.67$) and lysozyme ($s_{w,20} = 1.9$). PKA regulatory subunit I was run as a control ($s_{w,20} = 4.7$) [23,24].

2.10. Polyacrylamid gel electophoresis (PAGE)

12% SDS-PAGE was done according to Lämmli [25]. Native PAGE was performed on 6% gels with the following buffer coditions: stacking gel = 62.5 mM Tris-HCl, pH 6.8; separating gel = 375 mM Tris-HCl, pH 8.9; running buffer = 25 mM Tris-HCl, pH 8.8, 52 mM glycine; 5 × sample buffer = 312 mM Tris-HCl, pH 6.8, 50% glycerol, 0.05% bromphenol blue. For the electrophoresis of holoenzyme the gel and running buffer contained 100 μM ATP and 500 μM $MgCl_2$. Native gels

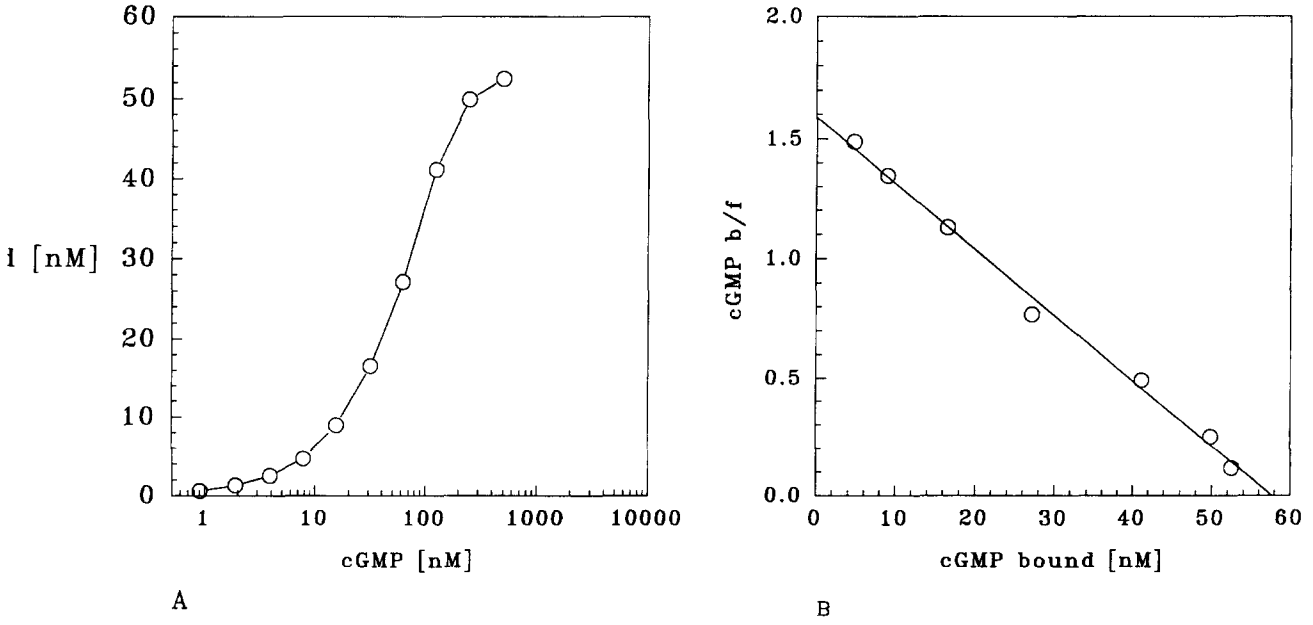

Fig. 3. Equillibrium cGMP binding to the recombinant AG R^I-subunit. The protein was incubated at a concentration of 40 nM as described in section 2. (A) Saturation curve showing high affinity binding. (B) Scatchard plot of cGMP binding.

were run at 8 mA for 16 h. The gels were stained with Coomassie blue R 250.

3. Results

3.1. Mutagenesis, expression and purification

In order to construct the chimera, a *Bgl*II site (nt 431-434) was introduced into the sequence of the regulatory subunit of PKA Iα. Flanking *Bgl*II and *Xba*I sites, which were added to the sequence of the cGMP binding sites (nt 344-1059) through PCR primers, allowed to substitute the DNA fragment coding for the cAMP binding sites by the cGMP binding sites of PKG Iα. Thus the mutant protein contains aa 1–147 of PKA R^1 and aa 115–359 of PKG Iα (Fig. 1).

The chimera was subcloned into the T_7-RNA-polymerase-dependent vector pET 3d and expressed at 37°C in *E. coli* BL21. High yields of mutant protein could only be obtained if the host strain contained the additional plasmid pLysE, coding for T_7-lysozyme. Apparently the lysozyme is necessary to stabilize the toxic mutant by reducing the rate of protein synthesis. Expression without or low intracellular concentrations of T_7-lysozyme, as in *E. coli* strains containing no pLys plasmid or the weakly expressed plasmid pLysD, gave only poor yields of mutant protein (data not shown). Incubation for 2 h after induction with IPTG yielded approximately 40 mg/l insoluble protein. Lower growth temperatures (30°C) did not increase the amount of soluble protein. The inclusion bodies were readily solubilized in urea and the protein refolded by removing the denaturing agent via dialysis. Addition of 100 μM cAMP to the renaturation buffer improved the efficiency of refolding (data not shown). Dialysis against cAMP free buffer allowed subsequent purification on cAMP-agarose with a final yield of approximately 8 mg renatured protein per liter culture. As jugded by SDS-PAGE the chimeric protein was purified to apparent homogeneity and had an apparent mass of 46 kDa (Fig. 2). A similar discrepancy between the calculated mass of 43.9 kDa and the apparent mass of 48 kDa has been reported for the wild-type R^1-subunit [21].

3.2. Dimerization

Since AG R^1 still retains the dimerization region of PKA at the N-terminus, we determined the Svedberg constants ($s_{w,20}$) of the regulatory subunit of PKA and of AG R^1 by sucrose density gradient ultracentrifugation. Both proteins comigrated in the same fraction ($s_{w,20}$ = 4.7), indicating that the conformations are very similar (Table 1), hence dimerization is not impaired by the cGMP binding sites. It has been shown that R-subunit dimerization occurs through two antiparallel disulfid bonds between residues 16 and 37 of each monomere [26]. Thus non-reducing conditions in SDS-PAGE gave a native like dimere with an apparent molecular weight of 86 kDa (data not shown).

3.3. Cyclic nucleotide binding properties

In order to determine the ligand binding characteristics of AG R^1, we studied cGMP and cAMP binding under equilibrium dissociation conditions. As shown in Fig. 3 and Table 1 equilibrium cGMP binding studies revealed that the AG R^1 was still capable to bind at least 1.5 mol cGMP/mol subunit with high affinity (apparent K_d = 53 nM). Scatchard analysis showed that unlike native PKG the two binding sites displayed no difference in their affinity for cGMP. Furthermore, the two cGMP sites showed no signs of a definite positive cooperativity (Hill coefficient = 1.1). In contrast to native PKG, which binds cAMP at micromolar concentrations [27], cAMP binding under equilibrium conditions allowed to measure only one binding site with an apparent K_d of 216 nM (Table 1). The second cyclic nucleotide binding site was not detecTable, presumably since it had a low affinity for cAMP.

cGMP dissociation showed a biphasic pattern, indicative of

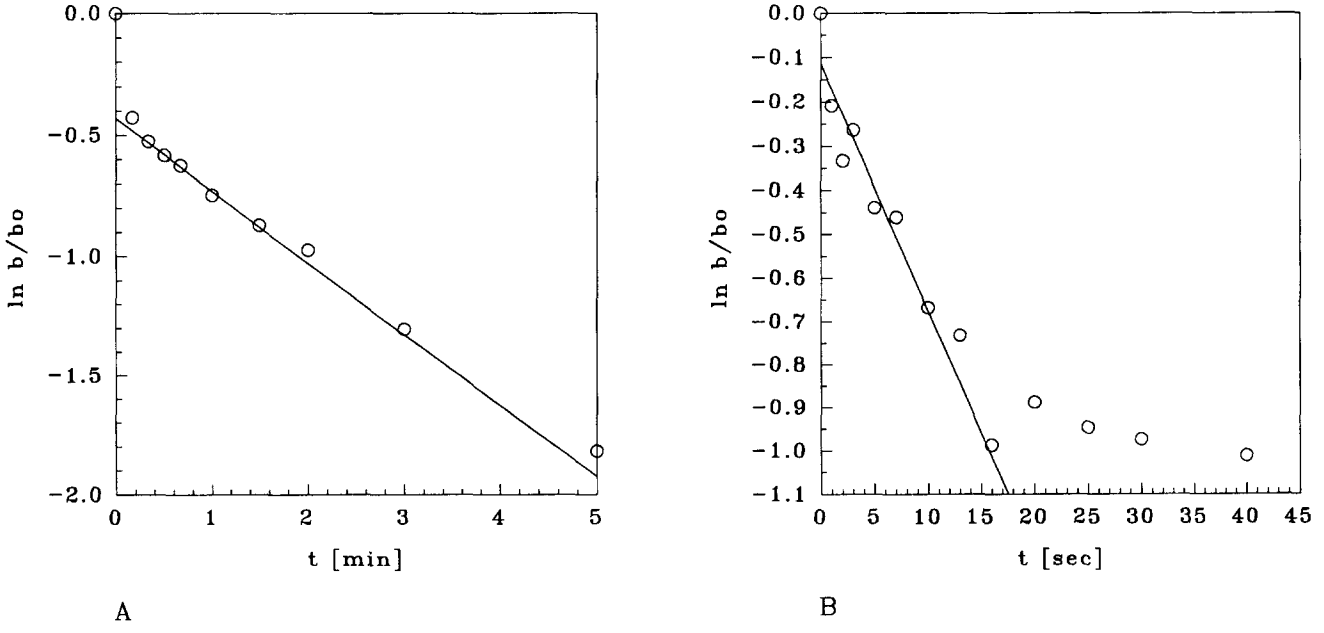

Fig. 4. Dissociation rates (k_{off}) of bound [^{3}H]cGMP from cGMP-binding sites A and B of the AG R^1-subunit. (A) After saturation of 50 nM AG R^1 with 250 nM [^{3}H]cGMP for 90 min at 0°C, dissociation was started by adding a 350-fold excess of cold cGMP. (B) Analysis of the fast cGMP binding site A by preincubating 50 nM AG R^1 in the presence of 10 μM 8-Br-cGMP. Dissociation was started with a 350-fold excess of cold cGMP.

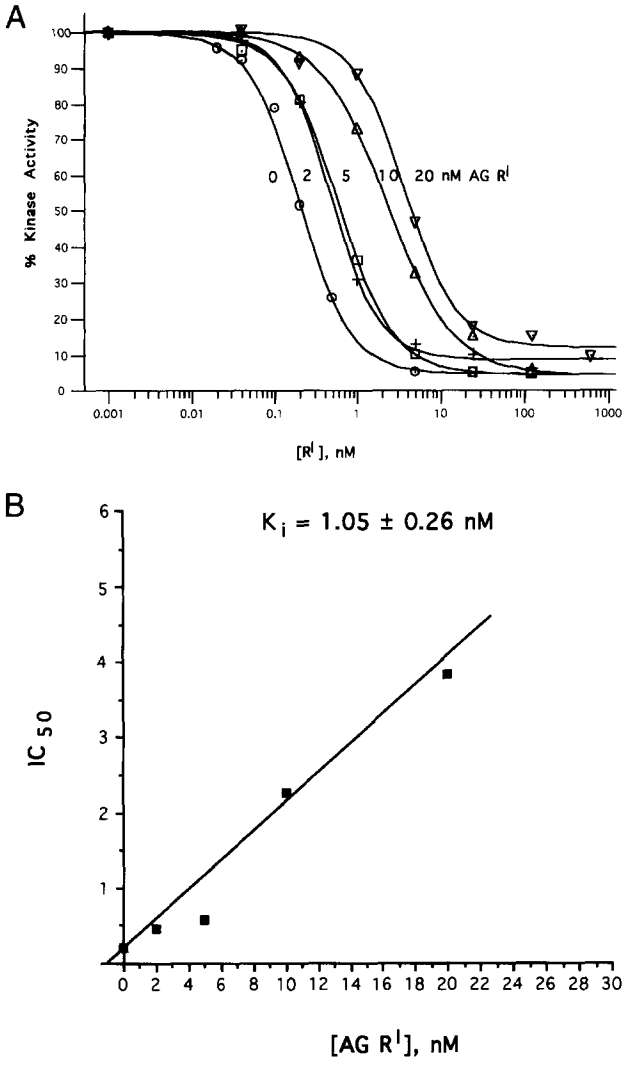

Fig. 5. AG R^I inhibits holoenzyme formation competitively. (A) Catalytic subunit (0.05 nM) was incubated with various concentrations of regulatory subunit R^I in the presence of increasing concentrations of mutant AG R^I (○ = 0 nM, + = 2 nM, □ = 5 nM, △ = 10 nM, ▽ = 20 nM). (B) Replot of the apparent IC_{50} from Fig. 5A versus the concentration of AG R^I-subunit gave an apparent K_i of 1.05 ± 0.26 nM.

two binding sites. The dissociation rates (k_{off}) for both sites were relatively fast (site A: 6.1 min^{-1}, site B: 0.31 min^{-1} (Fig. 4, Table 1)). In order to measure the fast dissociating site A more correctly, we incubated the protein with 8-Br-cGMP which binds to site B over site A with a 3.7-fold preference [18]. While the fast site A is quite similar to site A of the native PKG (3,7 min^{-1}) [28], the slow site shows a 60-fold increased dissociation rate (0.31 min^{-1} vs. 0.005 min^{-1})[28].

3.4. Holoenzyme formation and dissociation

In order to study the interaction of AG R^I with the catalytic subunit of PKA, we employed surface plasmon resonance (SPR) as the most sensitive method. Native R^I of PKA was used as a control in the experiments. Recombinant C-subunit could be immobilized readily by amine coupling onto the carboxymethyl surface of a sensorship [21]. First we showed that wild-type and mutant R-subunits bound with a stoichiometric relationship of 1:1 to the C-subunit surface. Next we verified that the complexes were fully dissociated by treatment with 10 μM cAMP or 10 μM cGMP respectively, thus completely regenerating the C-subunit surface. The association rate constants (k_{ass}) were determined using protein concentrations ranging from 75–500 nM. Wild-type and mutant association rate constants were 1×10^5 $M^{-1} \cdot s^{-1}$ and $1.9 \times 10^5 M^{-1} \cdot s^{-1}$, respectively (Table 1). The dissociation rates (k_{diss}) were calculated after the end of an injection. Rates of 2.3×10^{-5} s^{-1} and 6.06×10^{-4} s^{-1} were determined for the wild-type and the chimeric complex, respectively. From the association and dissociation rate constants apparent K_d's of 0.23 nM and 3.15 nM for the wild-type R-subunit and AG R^I were calculated (Table 1).

Once we had verified that the chimera still retained the capability to form a stable high affinity complex with the catalytic subunit, we obtained larger amounts of mutant holoenzyme by dialyzing AG R^I and C subunit in a 1:1.1 molar ratio according to Bubis et al. [26]. When assaying the remaining catalytic activity by [^{32}P]phosphorylation of kemptide, as an indicator for the degree of holoenzyme formation, we found that AG R^I showed almost no inhibition of the C-subunit. In contrast the catalytic activity was completely inhibited within 24 h at 4°C by native R^I. When AG R^I/C holoenzyme was electroeluted from a non-denaturing gel, it was still catalyticaly active. Hence complex fomation of mutant R and C-subunit does not inhibit the catalytic activity. Therefore, we tested whether or not AG R^I competed with the wild-type R-subunit for the catalytic subunit. Wild-type holoenzyme formation was accomplished at 30°C for 15 min, which is sufficient to completely form the complex [29]. Incubation of 0.05 nM catalytic sububit with increasing concentrations of R^I-subunit was performed as decribed in section 2. The apparent association constant was determined as K_d = 0.20 nM and is in accordance with the published value [29]. The measurement was repeated with increasing concentrations of mutant R-subunit and the IC_{50} replotted to determine the apparent K_i of 1.05 ± 0.26 nM as shown in Fig. 5. In addition, holoenzyme formation was confirmed by non-denaturing gel electrophoresis (data not shown) and sucrose gradient ultracentrifugation. The Svedberg constants ($s_{w,20}$) of the mutant holoenzyme differed with 6.83 slightly from that for PKA holoenzyme ($s_{w,20}$ = 7.25) (Table 1). It appeared that the complex was only stable when 100 μM Mg-ATP were present.

In order to determine the holoenzyme dissociation constants for cGMP and cAMP, we used analytical FPLC gel filtration as described in section 2. As shown in Fig. 6, the mutant holoenzyme could be dissociated by the addition of cGMP or cAMP with apparent activation constants of $K_{a,cGMP}$ = 146 nM and $K_{a,cAMP}$ = 640 nM. Gel filtration experiments also proved a strong tendency of AG R^I to rebind the catalytic subunit, since the experiments were only successful when the column was equilibrated with running buffer containing the appropriate concentration of cyclic nucleotide.

4. Discussion

PKA and PKG display a remarkable similar subdomain organisation. Both regulatory domains consist of a N-terminal dimerization region, followed by a hinge region which contains the primary auto-phosphorylation or inhibition site. C-Terminal to the hinge, two highly homologues cyclic nucleotide binding sites are fused. Apart from the cAMP/cGMP binding do-

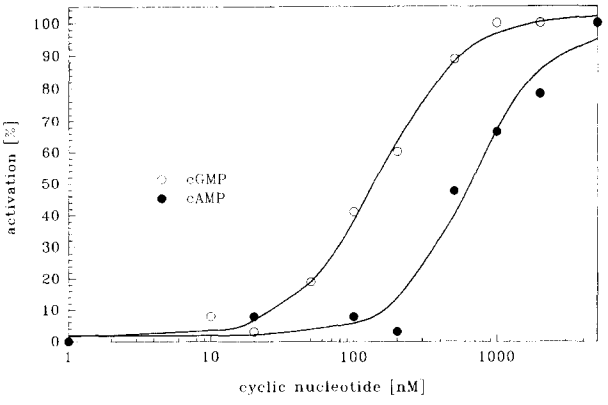

Fig. 6. K_a of AG R^1 for cGMP and cAMP, determined by analytical FPLC-gelfiltration of free catalytic subunit versus holoenzyme. Mutant holoenzyme, which contained fluorescence labelled C-subunit, was dissociated with increasing concentrations of cGMP or cAMP. The ratio of fluorescence of free to bound catalytic subunit, representing the percentage of activation, was plotted versus the log of cyclic nucleotide concentration to give an activation curve with apparent activation constants of 146 nM and 640 nM for cGMP and for cAMP, respectively.

mains the two subunits show particularly little homology at their N-termini including the hinge regions. While the R-subunit of PKA Iα dimerizes through two cysteine bonds [26], the type Iα PKG apparently dimerizes by the use of a leucine zipper interaction [4]. The differences regarding the hinge region of PKA and PKG are particularly eminent [1]. Furthermore, PKA has a well-defined autoinhibitory site which is indicative of a clear structured interaction with the catalytic subunit, whereas for PKG the structural nature of the R/C-interaction is less obvious. It has been shown that the PKA R-subunit primarily interacts with the C-subunit by residues around the consensus sequence in the hinge region [30,31]. As judged from the X-ray crystal structure of the 1–91 deletion form of the type Iα R-subunit of PKA [32], the two nucleotide binding sites form an elongated structure apart from the hinge region allowing for multiple interactions with the catalytic subunit.

When we started out to construct a cGMP-sensitive R-subunit it became apparent that the construct needed to contain as much of the type Iα R-subunit sequence of PKA as possible to account for most of the interactions responsible to reversibly bind the PKA catalytic subunit. The mutant protein (AG R^1-subunit) still bound cGMP with high affinity even though the cooperativity between the two sites was lost. Apparently, the cooperativity is influenced by kinase specific sequences N-terminal of the binding sites [19,21] and by PKG specific disulfide bonds [33]. Most importantly, the AG R^1-subunit retained the PKA R-subunit specific property to form a stable complex with the PKA catalytic subunit. However, the so formed mutant holoenzyme complex was still catalytically active. This implies that in the native holoenzyme additional interactions are responsible to fully inhibit the catalytic activity of the enzyme. It was recently suggested that cAMP binding to the PKA holoenzyme does not neccessarily induce dissociation of the subunits [34,35]. Instead, the cAMP·R_2C_2-complex is sufficient to generate enzyme activity. The active AG-R^1 holoenzyme was fully dissociated upon cGMP binding with an apparent activation constant of 146 nM as we could show by analytical FPLC gel-filtration (Fig. 6). Thus, the mutant holoenzyme was acivated by cGMP just as well as wild-type Iα PKG [28]. However, cAMP was also capable to activate the complex with an apparent activation constant of 640 nM. This suggests that the mutant holoenzyme has a diminished cGMP selectivity since the wild type Iα PKG has a micromolar activation constant for cAMP [36]. In addition we observed a strong Mg-ATP dependency to stabilize the mutant holoenzyme.

Mutant holoenzyme dissociation is the most crucial function

Table 1
Physical and kinetic constants of PKA type Iα, PKG type Iα and the chimera A/G

	AG R^1	R^1 PKA	PKG Iα
Physical constants			
MW calc. [kD]	42	43.9 [21]	76.4 [14]
MW SDS-PAGE [kD]	46	48 [21]	75 [28]
$S_{w,20}$ R-subunit	4.7	4.7	–
holoenzyme	6.83	7.25	8.0 [19]
Equilibrium nucleotide binding			
K_D (cAMP) [nM]	216 ± 5	site A: 60/site B: 15 [21]	site A: 39 800/site B: 1900 [27]
stoichiometry	1	2	2
K_D (cGMP) [nM]	site a and b: 53 ± 2.4	–	site A: 148 site B: 14 [28]
stoichiometry	2		2
cyclic nucleotide dissociation			
k_{off} (cAMP) [min^{-1}]	n.d.	site A: 0.6/site B: 0.02 [21]	–
k_{off} (cGMP) [min^{-1}]	site A: 6.1/site B: 0.31	–	site A: 3.7/site B: 0.005 [28]
R/C-interaction			
app. k_{ass} (BIAcore) [M^{-1} s^{-1}]	1.9×10^5	1.0×10^5	–
app. k_{diss} (BIAcore) [s^{-1}]	6.06×10^{-4}	2.3×10^{-5}	–
app. K_D (BIAcore) M	3.15×10^{-9}	2.3×10^{-10}	–
K_i holo. formation [nM]	$1.05 \pm 0.26 \times 10^{-9}$	–	–
Enzyme activation			
K_a (cAMP) [nM]	640 ± 76	105 [21]	19600 [36]
K_a (cGMP) [nM]	146 ± 6	4100 [37]	100 [28]

in vivo with regard to changes in fluorescence energy-transfer. Thus the AG R^1-subunit fulfills all the major requirements for a cGMP-sensitive probe which can be used to study intracellular cGMP concentrations in living cells by the use of confocal fluorescence spectroscopy. We are currently investigating methods to chemically inactivate the C-subunit in order to circumvent the observed catalytic activity of the AG-R^1 holoenzyme.

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