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(R_P)-cAMPS inhibits the cAMP-dependent protein kinase by blocking the cAMP-induced conformational transition

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Abstract **(R_P)-cAMPS is known to inhibit competitively the cAMP-induced activation of cAMP-dependent protein kinase (PKA). The molecular nature of this inhibition, however, is unknown. By monitoring the intrinsic tryptophan fluorescence of recombinant type I regulatory subunit of PKA under unfolding conditions, a free energy value ($\Delta G_D^{H_2O}$) of 8.23 ± 0.22 kcal/mol was calculated. The cAMP-free form of the regulatory subunit was less stable with $\Delta G_D^{H_2O}$ = 6.04 ± 0.05 kcal/mol. Native stability was recovered by treatment of the cAMP-free protein with either cAMP or (S_P)-cAMPS but not with (R_P)-cAMPS. Thus, (R_P)-cAMPS binding to the regulatory subunit keeps the protein in a locked conformation, unable to release the catalytic subunit. This finding was further supported by demonstrating that holoenzyme formation was greatly accelerated only when bound cAMP was replaced with (R_P)-cAMPS but not with cAMP or (S_P)-cAMPS.**

Key words: cAMP-dependent protein kinase; (R_P)-cAMPS; Protein folding; Fluorescence spectroscopy

1. Introduction

The activation of cAMP-dependent protein kinase (PKA) appears to be a rather distinct mechanism within the protein kinase family since it involves dissociation of its subunits. The holoenzyme of PKA is a tetrameric protein which consists of a regulatory subunit dimer (R_2) and two catalytic subunits (C). Activation of the complex R_2C_2 is mediated by cAMP binding to two in-tandem cAMP-binding sites per regulatory subunit, which induces a conformational change and subsequently the dissociation of the active catalytic subunits (for reviews see refs. [1,2]).

$$R_2C_2 + 4\ \text{cAMP} = R_2(\text{cAMP})_4 + 2\ \text{C}.$$

Each monomer of the R-subunit contains two distinct cAMP-binding sites designated A and B. The two sites are similar with respect to their primary amino acid sequence [3] but differ regarding their cAMP-analog selectivity [4] and overall kinetics [2]. The molecular events that eventually lead to holoenzyme dissociation require a well defined step-wise occupation of the cAMP-binding sites [5,6]. cAMP binds first to site B of each monomer. Since cAMP binding is cooperative [7,8], occupation of site B induces a conformational change that allows cAMP-binding to site A which mediates dissociation of the C-subunit. In this model site A provides the major contact with the C-subunit which was further supported by studies with PKA mutants [9–11] and cAMP-analogs [12].

Holoenzyme dissociation is competitively inhibited by (R_P)-cAMPS, a cAMP-analog with a sulfur substitution at the equatorial exocyclic phosphate-oxygen [13,14] as shown in Fig. 1. In contrast, the diasteriomeric (S_P)-cAMPS acts as a cAMP agonist [13]. So far, (R_P)-cAMPS and its nucleo-base modified derivatives [15,16] are the only known potent antagonists of the type I and II kinases.

The recent solution of a 2.8 Å resolution crystal structure of a deletion form of the type I R-subunit [17] revealed the architecture of the two cAMP-binding sites and structural details of the amino acid residues involved in cAMP binding, particularly the cyclic phosphate moiety. In both binding sites the equatorial exocyclic phosphate-oxygens of cAMP interact specifically with the guanidinium side chain of an invariant arginine at positions 209 and 333 known to be important for cAMP binding [10,11]. Thus, the structure explains the low binding-affinities observed for (R_P)-cAMPS and its derivatives [15,16]. However, the molecular nature of the holoenzyme inhibition cannot be deduced from the structure alone.

Fluorescence-spectroscopy was employed in order to probe the changes in conformational stability that take place upon cAMP binding to the R-subunit. In addition, the changes in protein stability when switching from the natural ligand to (R_P)-cAMPS and (S_P)-cAMPS were determined. The free-energy changes ($\Delta G_D^{H_2O}$) for the wild-type regulatory subunit were compared to a mutant form ($Arg^{209}Lys$) which abolishes high affinity cAMP binding to site A [10,11].

2. Materials and methods

2.1. *Adenosine cyclic-3′,5′-phosphorothioates*

The purities of (R_P)- and (S_P)-cAMPS were analyzed by Reversed-Phase-HPLC. Removal of cAMP contamination was achieved by incubating 10 mM (R_P)-cAMPS in buffer A (10 mM Tris-HCl, pH 7.6, 2 mM $MgCl_2$) and 300 μg/ml 3′,5′-cyclic nucleotide phosphodiesterase (Boehringer), since the enzyme cannot hydrolyze (R_P)-cAMPS [18,19]. (S_P)-cAMPS was free of cAMP. A control experiment showed that untreated (R_P)-cAMPS samples at concentrations >50 μM activated the holoenzyme type I, commensurate with the low levels of cAMP contamination that were observed. In contrast, the PDE-treated sample did not activate up to 1 mM (R_p)-cAMPS. Before use, (R_p)- and (S_p)-cAMPS were checked routinely by HPLC to verify that no contaminating cAMP was present.

2.2. *Protein preparations*

The recombinant type I regulatory subunit was expressed and purified according to Saraswat et al. [20]. The mutant type I regulatory subunit R209K was obtained as described previously [10]. cAMP free R-subunit was prepared by incubating 1–2 μM protein in 5 M urea, 50 mM MES, pH 6.5, 5 mM 2-mercaptoethanol for 3 h. This urea concentration was sufficient to abolish cAMP binding but not high enough to totally denature the protein [21]. The precaution was indispensable to assure proper refolding of the protein. The mixture was then passed

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over a G-25 gel filtration column equilibrated with 5 M urea, 50 mM MES, pH 6.5, 5 mM 2-mercaptoethanol to remove cAMP. Removal of urea and refolding of stripped R-subunit was accomplished by a second G-25 gel filtration run without urea. Protein concentrations (0.25–0.5 μM) were determined according to Bradford [22] and the fraction of unoccupied cAMP binding sites determined by measuring the amount of bound [^{3}H]cAMP at 0°C using an ammoniumsulfat precipitation assay as described previously [23]. Under these conditions bound cAMP will not exchange with [^{3}H]cAMP rapidly. On average 95% of the cAMP binding sites were free of cAMP for the urea stripped proteins. Reloading of the stripped wild type and Arg[209]Lys mutant R-subunits with cyclic nucleotides was accomplished by a 3 h incubation with a 3-fold excess of cAMP, 15-fold (S_P)-cAMPS and 60-fold (R_P)-cAMPS.

2.3. *Fluorescence measurements*

The protein solutions were incubated in various concentrations of denaturant (0–8 M urea) to give a final protein concentration of 0.25 μM. The mixtures were then allowed to come to equilibrium for 3 h at room temperature. All fluorescence measurements were performed at least in dublicate on a Spectrofluorometer (SLM Aminco SPF-500). The protein samples were excited at 293 nm, and the tryptophan emission was monitored at 347 nm. The resulting emission spectra were then followed from 300 nm to 400 nm. A fluorescence intensity ratio (353 nm/340 nm) was used to follow the shift in wavelength since the native form and the denatured form of the wild type protein displayed maxima at 340 and 353 nm, respectively. The data was then used to construct an unfolding curve (Fig. 2) using the following equation:

$$F_u = 1-[(I_O-I_D)/I_N-I_D)] \quad (1)$$

where F_u is the fraction of unfolding, I_O is the observed intensity ratio at various urea concentrations, and I_N and I_D are the fluorescence intensity ratios of the fully folded und unfolded proteins, respectively [24]. A two-state model, represented by $F_N + F_U = 1$ where F_N is the fraction of native protein and F_U is the fraction of unfolded protein, was then used to calculate the free energy of denaturation ΔG_D [25] according to equation (2).

$$F_N/F_U = K_D$$

$$K_D = e^{-\Delta G_D/RT} \quad (2)$$

$$-RT \ln (F_U/F_N) = \Delta G_D \Delta$$

$G_D{}^{H_2O}$ in the absence of denaturant [25] was obtained according to equation (3), (inset of Fig. 2):

$$\Delta G_D = \Delta G_D{}^{H_2O} - m\,[\text{denaturant}] \quad (3)$$

2.4. *Holoenzyme formation*

In order to determine the rate of holoenzyme formation a dialysis technique has been used as described previously [12]. Urea stripped wild type R-subunit (0.25 μM) which had been recovered with either cAMP, (S_P)-cAMPS or (R_P)-cAMPS was dialyzed with C-subunit at a 1:1.1 molar ratio at room temperature. At designated time points aliquots were removed and the remaining catalytic activity monitored by the coupled photo-spectrometric assay according to Cook et al. [26].

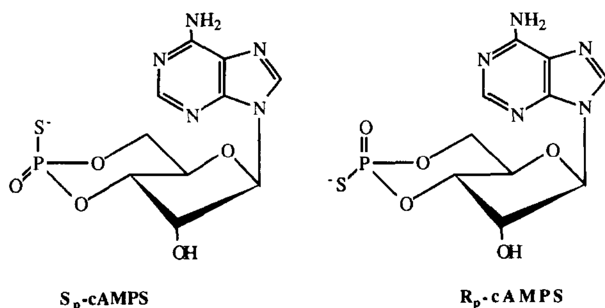


Fig. 1. Chemical structures of (R_P)-cAMPS and (S_P)-cAMPS.

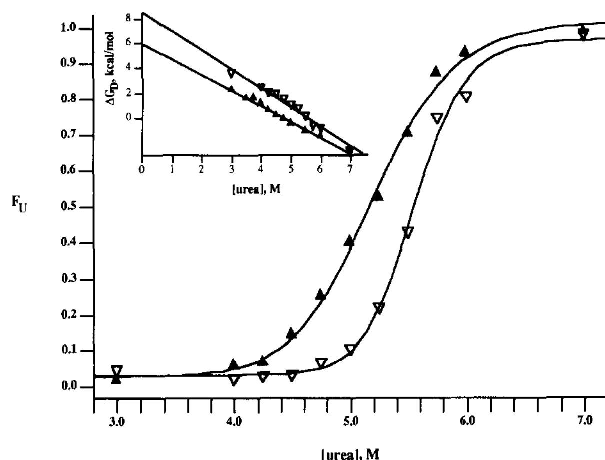


Fig. 2. Urea induced unfolding of the regularory subunit in the presence (▽) and absence (▲) of excess cAMP. The fraction of unfolded protein (F_U) was determined according to equation (1). The inset shows the equilibrium unfolding transition as a function of denaturant. The ΔG_D values were calculated (equation (2)) from the unfolding curves in the presence (▽) and absence (▲) of excess cAMP.

3. Results

3.1. *Fluorescence measurements under unfolding conditions*

Unfolding of the regulatory subunit of PKA is accompanied by a shift of the tryptophan fluorescence emission maximum from 340 nm (native form) to 353 nm (unfolded form) (data not shown). Thus, the unfolding process was followed by monitoring the fluorescence intensity ratio, I(353 nm/340 nm) as shown in Fig. 2. When native R-subunit was urea denatured the concentration midpoint of unfolding C_m was 5.55 M. In contrast, denaturation of cAMP-free R-subunit resulted in a shift to the left (C_m = 5.10 M urea), which is indicative of an increased sensitivity towards denaturation. This experiment demonstrated that bound cAMP stabilizes the R-subunit as has been described by Leon et al. [21]. The transition region of the unfolding curves is linear if one assumes a two-state model [25] and allows calculation of the free energy of denaturation (ΔG_D) for this unfolding curve. By linear extrapolation of the ΔG_D values, the free energy of denaturation at zero concentration of denaturant ($\Delta G_D{}^{H_2O}$) was calculated (inset of Fig. 2, Fig. 3).

In order to investigate the effects of bound (R_P)- and (S_P)-cAMPS on the thermodynamics of R-subunit unfolding, urea stripped, cAMP-free protein was incubated with an excess of (R_P)- or (S_P)-cAMPS. Incubation of the urea stripped R-subunit with cAMP served as a control experiment to assure that the protein was capable of regaining native stability. As shown in Fig. 3, $\Delta G_D{}^{H_2O}$ of the R-subunit, in its native form was determined as 8.23 ± 0.22 kcal/mol. The protein has cAMP bound with high affinity [27]. Treatment of the regulatory subunit in 5 M urea is sufficient to obliterate cAMP binding, while the protein is still to a large extent in its natural folded state [21]. This cAMP free form of the regulatory subunit was significantly less stable as indicated in a $\Delta G_D{}^{H_2O}$ value of 6.04 ± 0.05 kcal/mol. By adding cAMP or (S_P)-cAMPS back to the cAMP free form of the protein the native stability could be regained (Fig. 3, panels 3 and 4). However, addition of (R_P)-cAMPS to the cAMP free protein was not capable of recovering the native stability (Fig. 3, panel 5). Since (S_P)- and (R_P)-cAMPS have

lower affinities to the cAMP binding sites the concentrations of added cAMP analog were adjusted according to their binding affinities [15].

The regulatory subunit type Iα contains three tryptophan residues at positions 188, 222 and 260, all of which are located within cAMP binding site A. In order to prove that the observed changes in free energy ($\Delta G_D^{H_2O}$) are only associated with site A, we studied a mutant form of the regulatory subunit in which residue Arg[209] of the cAMP binding site A was changed to Lys. This mutant, Arg[209]Lys, abolishes high affinity binding of cAMP to site A with an K_D of >1 μM [10,11]. When denaturation experiments were repeated with the Arg[209]Lys R-subunit mutant no changes in free energy were observed (data not shown). $\Delta G_D^{H_2O}$ values of 5.0 ± 0.8 kcal/mol were obtained regardless whether the mutant was stripped off bound cAMP or not. Addition of (R_P)- or (S_P)-cAMPS had no effect, too. Moreover, $\Delta G_D^{H_2O}$ for the Arg[209]Lys-mutant equaled that for the cAMP-free wild type. Thus, the stability of the cAMP free wild type protein and the mutant form of the regulatory subunit are remarkable similar.

3.2. Holoenzyme formation

When urea stripped wild type R-subunit was incubated with a 3-fold excess of cAMP at room temperature, more than 97% of the cAMP-binding sites were occupied within 15 min as was determined by [^{3}H]cAMP exchange (see section 2.2.). Fig. 4 shows the reassociation of regulatory and catalytic subunits under equilibrium dialysis conditions. The R-subunits were stripped of cAMP and subsequently reloaded with either cAMP, (R_P)- or (S_P)-cAMPS as described in section 2. Holoenzyme formation was notably increased when the R-subunit had (R_P)-cAMPS bound prior to the dialysis with a half-life of holoenzyme formation ($\tau_{1/2}$) of 30 min. Similarly, the Arg[209]Lys-mutant R-subunit displayed an enhanced rate of holoenzyme formation as described recently [10]. In contrast, (S_P)-cAMPS or cAMP treated protein samples showed a 6-fold shift with $\tau_{1/2}$ values of 180 min. Thus, (R_P)-cAMPS bound R-subunit is able to complex the C-subunit more efficiently, than native cAMP bound R-subunit does.

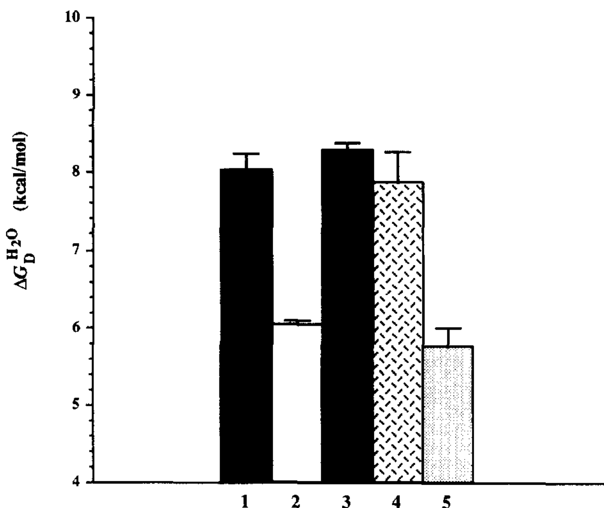


Fig. 3. Free energy changes ($\Delta G_D^{H_2O}$) were calculated by linear regression of the unfolding transition data (inset of Fig. 2) according to equation (3). Panel 1: native R-subunit; panel 2: urea stripped, cAMP-free R-subunit; panel 3: urea stripped R-subunit + excess cAMP; panel 4: urea stripped R-subunit + excess (S_P)-cAMPS; panel 5: urea stripped R-subunit + excess (R_P)-cAMPS. In the experiments described in panel 3–5, the cyclic nucleotides were added prior to denaturation.

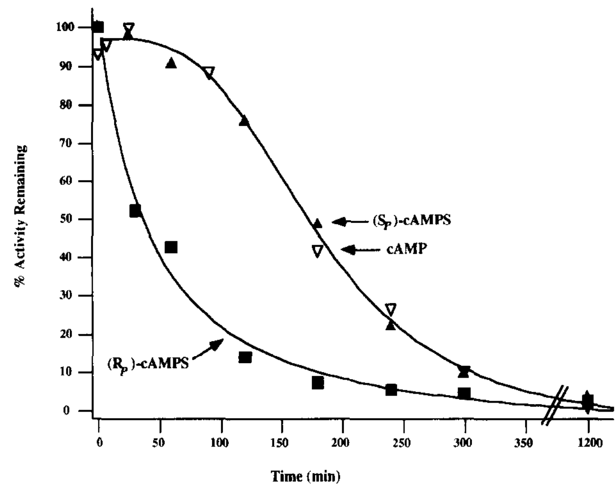
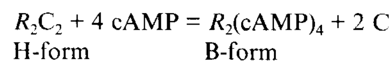


Fig. 4. Time course of holoenzyme formation in the presence of MgATP by equilibrium dialysis at room temperature. The remaining phosphotransferase ativity of unbound catalytic subunit was measured [26] and plotted against the time of dialysis. Prior to the dialysis regulatory subunit was stripped off cAMP as described in section 2, reloaded by either an excess of cAMP (▽), (R_P)-cAMPS (■) or (S_P)-cAMPS (▲) and mixed with C-subunit in a 1:1.1 molar ratio.

4. Discussion

The regulatory subunit of PKA exists in vivo of two stable conformations: the cAMP-bound conformation (B-form) in which the protein is stable in solution and an in the cAMP-free holoenzyme conformation (H-form). In the H-form the R-subunit can only exist when complexed with the catalytic subunit [12].



$$R_2C_2 + 4\ \text{cAMP} = R_2(\text{cAMP})_4 + 2\ \text{C}$$
H-form B-form

Obviously, cAMP binding induces a conformational shift from H-form to B-form. This change in conformation is the molecular basis for the allosteric regulation of the enzyme. The crystal structure of the R-subunit allowed to analyze the specific ligand-receptor interactions on an atomic scale [17]. In particular, the exocyclic oxygens of the phosphate moiety display a meticulous network of interactions for each site which are severely disturbed by either equatorial or axial sulfur substitution [17] and thus, explains the lower binding affinities observed for (R_P)-, and (S_P)-cAMPS [15] and the relative low inhibition constant for (R_P)-cAMPS (K_i = 7.9 μM (13]) (Fig. 1). Furthermore, the crystal structure revealed that in the cAMP-binding site A the side chain of residue Arg[209] which interacts specifically with the equatorial position of the phosphate, is also linked to Asp[170]. (R_P)-cAMPS will prevent this linkage that extends beyond the immediate cAMP-binding site by breaking the interaction with Arg[209] [17]. Whether the signaling by cAMP-binding involves Asp[170] remains to be seen. However, the results obtained from the fluorescence experiments presented in this work indicate that (R_P)-cAMPS binding to site A is sufficient to induce the observed effects.

In conclusion, (R_P)-cAMPS cannot induce a conformational

shift necessary for dissociating the PKA holoenzyme, which is also in accordance with earlier observations [14,28]. Therefore, (R_P)-cAMPS locks the regulatory subunit in the holoenzyme conformation (H-form). This 'H-form-(R_P]-cAMPS-bound R-subunit' reassociates faster with the C-subunit, since the protein is already in the suitable 'H-form' conformation as was shown with the dialysis experiments. These findings are in agreement with the results obtained with a mutant form of the R-subunit ($Arg^{209}Lys$) which abolishes high affinity cAMP to binding site A [10,11].

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