Inhibitory action of certain cyclophosphate derivatives of cAMP on cAMP-dependent protein kinases

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A series cAMP derivatives with modifications in the adenine, ribose and cyclophosphate moiety were screened for their binding affinity for the two types of cAMP-binding sites in mammalian protein kinase type I. In addition, the activation of the kinase by these analogs was monitored. The binding data indicate that cAMP is bound to both sites in a comparable manner: the adenine appears to have no hydrogen-bond interactions with the binding sites, whereas the ribose may be bound by three hydrogen bonds involving the 2', 3' and 5' positions of cAMP. The binding data are not conclusive about the nature of the interaction with the exocyclic oxygen atoms on phosphorus, though a charge interaction seems to be absent. The cAMP molecule seems to be bound in the syn conformation.

The results of activation experiments show that modifications in the adenine and ribose moiety do not affect the maximal activation level, while alteration of the two exocyclic oxygen atoms may result in a reduced maximal activation level and in one case, (R,)-adenosine 3', 5'-monophosphorothioate [R,-cAMPS], in total absence of activation even at concentrations at which the analog saturates both binding sites. Since occupancy of the cAMP-binding sites by this derivative apparently did not lead to activation of the enzyme, we examined whether this compound could antagonize the activation by cAMP. Indeed (R,)-cAMPS was found to inhibit cAMP stimulated kinase activity at concentrations compatible to its binding affinity. Also with mammalian protein kinase type II (R,)-cAMPS showed antagonistic activity, while with a cAMP-dependent protein kinase from Dictyostelium discoideum partial agonistic activity was observed.

Previously a mechanism for activation of protein kinase type I was proposed involving a charge interaction between the equatorial exocyclic oxygen atom and the binding site [De Wit et al. (1982) Eur. J. Biochem. 122, 95–99]. This was based on measurements with impure preparations of (R,)-cAMPS and the R, and S, isomers adenosine 3', 5'-monophosphodimethylamidate, cAMPN(CH),. The present work using highly purified compounds suggests the absence of a charge interaction, since the uncharged analog (S,)-cAMPN(CH), activates the kinase effectively. The data seem compatible with an activation model involving the formation of a covalent bond with phosphorus in both cAMP binding sites.

It is well established that mammalian cAMP-dependent protein kinases are activated by a cAMP-induced release of free (active) catalytic subunits from a tetrameric holoenzyme [1]. The holoenzyme consists of two catalytic and two regulatory subunits and each regulatory subunit binds two molecules of cAMP to distinct sites. In the cellular slime mold Dictyostelium discoideum a cAMP-dependent protein kinase has been reported which showed different properties. The holoenzyme apparently consists of more than four subunits, the 40-kDa regulatory subunit contains one type of cAMP-binding site and the catalytic subunits often form large aggregates [2–5]. Mammalian cAMP-dependent protein kinases have been studied using over 200 derivatives of cAMP. Recently several derivatives were observed to bind with clearly distinct affinity to the two types of binding sites, suggesting a difference in structure of the binding sites [6–9]. The use of these selective analogs led to the conclusion that both sites are involved in the activation of the kinase [10–12].

Our approach for studying the binding sites and activation mechanism is different : a selected series of 16 cAMP analogs is used, of which each one is modified in order to probe one possibly essential interaction with the binding site [13,14]. Thus a scheme of the interactions of cAMP with its binding proteins could be obtained [15].

Recently, one of the cAMP binding sites (‘stable’, B, or 1) of protein kinase type I was mapped using this approach [16]. It was observed that one compound, (R,)-cAMPS, was defective in stimulating kinase activity, though binding experiments revealed that this compound bound competitively with cAMP. At concentrations of (R,)-cAMPS saturating the ‘stable’ site, only a slight activation was observed. A similar observation was reported for type II protein kinase [17]. In addition, binding studies revealed that all derivatives had a lower affinity for the ‘stable’ binding site of the holoenzyme than of the free regulatory subunit, except (R,)-cAMPS, (R,)-cAMPN(CH), and (S,)-cAMPN(CH),. It was concluded that these compounds failed to establish a charge interaction with the ‘stable’ binding site. In the case of cAMP this leads to an increased binding affinity and activation by inducing a conformational change in the regulatory subunits and subsequent dissociation of the holoenzyme. In other words: there

Abbreviations: cAMPS, adenosine 3',5'-monophosphorothioate; cAMPN(CH),, adenosine 3',5'-monophosphodimethylamidate; HPLC, high-performance liquid chromatography.

Trivial name: Kemptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly.

Enzyme: cAMP-dependent protein kinase (EC 2.7.1.37).
is no coupling between binding of these cAMP derivatives and activation of the enzyme.

In the present work the relation between binding of cAMP derivatives and activation is elaborated to the 'labile' site (site A or 2). Since the previously used preparations [16] of (R,)-cAMP and (S,)-cAMP(CH3)2 contained 2–6% of the respective S, isomers and up to 10% of cAMP, and (S,)-cAMP(CH3)2 was found to be degraded to a large extent, these preparations were repurified and now contained less than 0.1% contaminants. As a result the new data for binding and activation readjust the data presented earlier. Furthermore it is demonstrated that analogs defective in activating protein kinase type I are able to antagonize cAMP-induced activation.

The agonistic and antagonistic potencies of the four derivatives with modified exocyclic oxygen atoms were also investigated in two other types of protein kinases: type II from rabbit skeletal muscle and a cAMP-dependent kinase from D. discoideum.

EXPERIMENTAL PROCEDURE

Chemicals

[32P]ATP was purchased from New England Nuclear (Dreieich, FRG) and [2,8-3H]cAMP from Amersham International (Buckinghamshire, UK). cAMP, ATP, cGMP, cGMP, 6-chloropurine-ribose 3',5'-monophosphate, 8-bromoadenosine 3',5'-monophosphate and 2'-deoxyadenosine 3',5'-monophosphate were obtained from Boehringer (Mannheim, FRG). Adenosine N-oxide 3',5'-monophosphate, 3'-amino-3'-deoxyadenosine 3',5'-monophosphate, 5'-amino-5'-deoxyadenosine 3',5'-monophosphate, the S, and R, isomers of adenosine 3',5'-monophosphorothioate, benzimidazole-ribose 3',5'-monophosphate and purine-ribose 3',5'-monophosphate were synthesized as published previously [18–22]. The synthesis of the S, and R, isomers of adenosine 3',5'-monophosphorothioylaminemidate will be published elsewhere. Dr R. Hanze (Upjohns) kindly supplied 7-deazaadenosine 3',5'-monophosphate and Dr J. Miller (SRI International Life Sciences Division, Menlo Park, CA, USA) supplied 2-phenyladenosine 3',5'-monophosphate. Sephacryl S-300 was purchased from Pharmacia (Uppsala, Sweden), kemptide (Leu-Arg-Arg-Ala-Ser-Leu) was from Sigma (St Louis, MO, USA). The analogues (R,)-cAMPS, (S,)-cAMPS, (R,)-cAMP(CH3)2 and (S,)-cAMP(N(CH3)2) were checked for the absence of cAMP or other impurities by high-performance liquid chromatography (HPLC) and, if necessary, purified by HPLC.

Enzymes

cAMP-dependent protein kinase type I from rabbit skeletal muscle was isolated and purified to homogeneity as in [23] and [24]; kinase type II from rabbit skeletal muscle was obtained from Sigma. cAMP-dependent protein kinase holoenzyme from Dictyostelium discoideum strain AX-2 was isolated from aggregative cells as described in [4]. After homogenization, the cytosolic fraction was chromatographed over Sephacryl S-300 and fractions containing most of the cAMP-dependent kinase activity were pooled.

Binding assay

Ligand bound to cAMP-dependent protein kinase was determined by a filtration assay using cellulose-nitrate filters (Sartorius, Göttingen, FRG). The concentrations of [3H]cAMP (10000 cpm/pmol) were 30 nM, 100 nM and 300 nM and in the presence of 0.2 mM ATP tenfold higher. The incubation was at 25 °C for 1 h in 25 mM 4-morpholineethanesulfonic acid (Mes) pH 6.9, 4 mM magnesium acetate, 0.125 mM EGTA, 1 mg/ml bovine serum albumin and various concentrations of competing cAMP derivative. After cooling on ice for 10 min, 50 μl of each 100-μl sample was filtered directly for determination of binding to both sites, while 20 μM unlabeled cAMP was added to the remaining 50 μl. At 0 °C in the presence of excess unlabeled cAMP the two types of binding sites release bound [3H]cAMP at different rates. For the 'stable' site a rate constant of 0.029 h⁻¹ was obtained (0.23 h⁻¹ in the presence of 0.2 mM ATP) and for the 'labile' site 0.49 h⁻¹ (3.3 h⁻¹ with ATP present). Both binding types contributed 50±5% of the total binding. After 7 h of dissociation at 0 °C (or 1 h in case 0.2 mM ATP was present) the samples were filtered, yielding 82% of the original binding to the slowly dissociating 'stable' (B or 1) site and about 3.0% of the original binding to the other site. After correction for the loss in binding to the 'stable' site this value was subtracted from the total binding, yielding binding to the 'labile' site. The data were plotted according to Dixon [25]; all analogs were competitive inhibitors of [3H]cAMP binding.

Kinase assay

Protein kinase activity was determined in 60-μl samples containing 50 mM Mes pH 6.5, 5 mM magnesium chloride, 0.5 mM EGTA, 10 mM sodium fluoride, 2.5 mM dithiothreitol, 20 μM kemptide, 0.2 mM [32P]ATP (50 cpm/pmol), 1 mg/ml bovine serum albumin, about 0.5 nM enzyme and various concentrations of cyclic nucleotides. The reaction was started by addition of 30 μl enzyme solution and terminated after a 10-min incubation at 30 °C by addition of 600 μl of a 50% slurry of Dowex 1X2 in 30% acetic acid. After equilibration for 1 h, samples were centrifuged for 2 min at 10000 × g. Radioactivity (in 175 μl supernatant) was determined by measuring Cerenkov irradiation.

Standardization

cAMP binding to the holoenzyme is strongly dependent on, for example, enzyme concentration and salt concentration [26–28]. In order to compare results in different systems, the following standardization is used [29]:

\[ \delta AG = -RT \ln \frac{K(cAMP)}{K(derivative)} \]

K is either K, (the inhibition constant derived from Dixon plots) or K, (the concentration of analog yielding half-maximal activation of kinase activity). The increment in binding energy (δAG) is 5.70 or 5.80 kJ mol⁻¹ for a tenfold increase in K value measured at 25 °C or 30 °C, respectively.

RESULTS

Binding to the 'stable' and 'labile' sites

As is shown in Table 1, the binding data of most of the derivatives were very similar for the two distinct sites. It thus appears that the interactions between cAMP and both binding sites are basically identical: no hydrogen bonding with the adenine moiety, but still a similar specificity for derivatives
The potencies of CAMP derivatives as activators increase in 6dC for both binding sites as a result of the presence of 0.2 mM ATP (Table 1). This is also expected when the gain in binding energy

<table>
<thead>
<tr>
<th>Analog</th>
<th>Binding increment, ΔG (kJ mol⁻¹)</th>
<th>Activation increment, ΔG (kJ mol⁻¹)</th>
<th>Level of maximal activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine 3',5'-monophosphate</td>
<td>0</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>Adenosine N-oxide 3',5'-monophosphate</td>
<td>6.3</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>6-Chloropurine-ribose 3',5'-monophosphate</td>
<td>-0.1</td>
<td>-0.1</td>
<td>1.1</td>
</tr>
<tr>
<td>7-Deazaadenosine 3',5'-monophosphate</td>
<td>4.0</td>
<td>-0.3</td>
<td>1.1</td>
</tr>
<tr>
<td>Benzimidazole-ribose 3',5'-monophosphate</td>
<td>5.2</td>
<td>6.7</td>
<td>1.1</td>
</tr>
<tr>
<td>Purine-ribose 3',5'-monophosphate</td>
<td>3.7</td>
<td>2.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Inosine 3',5'-monophosphate</td>
<td>8.8</td>
<td>10.2</td>
<td>1.1</td>
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<td>Guanosine 3',5'-monophosphate</td>
<td>12.7</td>
<td>11.9</td>
<td>1.1</td>
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<td>8.0</td>
<td>10.3</td>
<td>1.1</td>
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<td>-0.9</td>
<td>1.1</td>
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<tr>
<td>2'-Deoxyadenosine 3',5'-monophosphate</td>
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<td>20.2</td>
<td>1.1</td>
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<tr>
<td>3'-Amino-3'-deoxyadenosine 3',5'-monophosphate</td>
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<td>1.1</td>
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<tr>
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<td>16.8</td>
<td>1.1</td>
</tr>
<tr>
<td>Adenosine 3',5'-monophosphorothioate (S₉)</td>
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<td>9.4</td>
<td>1.1</td>
</tr>
<tr>
<td>Adenosine 3',5'-monophosphorothioate (R₉)</td>
<td>15.2</td>
<td>15.9</td>
<td>&lt;0.03</td>
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<tr>
<td>Adenosine 3',5'-monophospho-dimethylamide (S₉)</td>
<td>11.0</td>
<td>12.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Adenosine 3',5'-monophospho-dimethylamide (R₉)</td>
<td>21.3</td>
<td>24.0</td>
<td>≥21.9</td>
</tr>
</tbody>
</table>

**Activation in relation to binding**

The majority of the analogs was able to stimulate the kinase activity to the same extent as CAMP (Table 1). Clearly, modifications of the adenine and ribose moieties, including the endocyclic oxygen atoms of the cyclophosphate ring, do not affect the activating potency of CAMP. The four analogs that are modified in the two exocyclic oxygen atoms are important tools in the elucidation of the activation mechanism, since two of these show a clearly impaired maximal activation level, while one did not elicit any detectable activation. Thus, as proposed before [16], the phosphate region is not involved in binding of CAMP, but also in the process of activation, i.e., induced dissociation of holoenzyme. This is also observed when binding data of these compounds obtained in the presence of ATP are compared to those obtained without ATP. As ATP stabilizes the holoenzyme, which has a lower affinity for CAMP than free regulatory subunits [30], the binding affinity should be lower in the presence of ATP. This was indeed found for the majority of the derivatives including CAMP and for both binding sites, but not for (R₉)-cAMP and to a minor extent for (R₉)-cAMP-CH₃₂ (Table 1). It is a remarkable observation that these derivatives also show a reduced level of maximal activation (though for (R₉)-cAMP and CH₃₂ this is less certain, since the maximal level was not reached at the highest concentration of this compound that could be achieved). (R₉)-cAMPs did not stimulate the kinase activity to a detectable extent (<3% of CAMP), while (S₉)-cAMP-CH₃₂ activated the kinase to 75% of the level of CAMP (Fig. 2). A correlation exists between the extent of lowering of the binding affinity for both sites due to ATP and the maximal activation level (Table 1). This is also expected when the gain in binding energy

Table 1. Binding and activation by CAMP derivatives for protein kinase type I

The potencies of CAMP derivatives as activators of protein kinase type I (Kᵥ values) were related to that of CAMP and transformed into the free enthalpy scale as described under Experimental Procedures. The maximal activation levels of the analogs were also related to that of CAMP, and Kᵥ values were related to the Kᵥ values of CAMP (53 nM for the "stable" site; 25 nM for the "labile" site) and transformed to ΔG values. The increase in ΔG for both binding sites as a result of the presence of 0.2 mM ATP is shown in columns 2 and 4. The analog (R₉)-cAMP-N(CH₃)₂ did not reach the level of maximal activation; therefore minimum estimates of this level and of the ΔG value are shown. All determinations were done at least in triplicate. The standard deviation for binding data of the "stable" site (CAMP, n = 10) is about 1.5 kJ mol⁻¹; of the "labile" site (CAMP, n = 10) about 1.5 kJ mol⁻¹ and for activation (CAMP, n = 5) about 1.5 kJ mol⁻¹.
of cAMP for both binding sites upon dissociation of holoenzyme is the driving force for the dissociation of holoenzyme, according to a closed thermodynamic system:

\[
R_2C_2 + 4\text{cAMP} \rightleftharpoons K_2 R_2\text{cAMP}_2C_2
\]

\[
K_2 \rightleftharpoons 2\text{C} + 4\text{cAMP} \rightleftharpoons K_1 R_2\text{cAMP}_4 + 2\text{C}
\]

Scheme 1

where \(K_1\) is the dissociation constant for the binding of cAMP (or a derivative) to free regulatory subunits and \(K_2\) for the binding between regulatory and catalytic subunits. It is assumed that activation is only effected by the release of free catalytic subunits, i.e. neither holoenzyme nor ternary complex \([R_2\text{cAMP}]C_2, n = 1 - 4\) possess detectable activity. For cAMPz > 1, since the affinity for the holoenzyme was estimated to be 1000-fold lower than for free regulatory subunits [30]. In this way cAMP strongly shifts the binding equilibrium of the subunits to dissociation. However, for \((R_\theta)\text{-cAMPS}\) the apparent difference in binding affinity is smaller than for cAMP, in fact almost no difference is observed when binding without ATP (mainly free regulatory subunits) or with ATP (less free regulatory subunits and more holoenzyme) is monitored (Table 1). Thus \(\alpha\) is close to 1, implying that binding of \((R_\theta)\text{-cAMP}\) will not induce holoenzyme dissociation. In short, \(\alpha\) is the factor that determines the maximal activation level and the difference between the binding affinities measured in the absence of ATP and in the presence of ATP.

Antagonism

Since \((R_\theta)\text{-cAMPS}\) and \((R_\theta)\text{-cAMPN(CH}_2)_2\) showed reduced agonistic activity, even at concentrations which saturate the binding sites, we examined whether these compounds replace bound cAMP and thus inhibit a CAMP-induced stimulation of kinase activity. As shown in Fig. 3, \((R_\theta)\text{-cAMPS}\) antagonized the stimulation by 0.1 pM cAMP, while \((R_\theta)\text{-cAMPN(CH}_2)_2\) showed no significant effect, which may have been caused by the extremely low affinity or by its partial agonistic activity (Fig. 2). According to the equation:

\[
K_i = \frac{I_{50}}{[\text{cAMP}]} + \frac{K_s}{[\text{cAMP}]} = \frac{K_s}{[\text{cAMP}]} \]

the \(K_i\) value for \((R_\theta)\text{-cAMPS}\) may be calculated from the concentration of \((R_\theta)\text{-cAMPS}\) that inhibits 50% of the kinase activity \((I_{50} = 16 \mu M)\) using the \(K_i\) for cAMP (0.034 \mu M) and the cAMP concentration (0.1 \mu M). The obtained \(K_i\) value of 12 \mu M may then be related to the \(K_s\) of cAMP, yielding a \(\Delta G\) value of 14.8 kJ mol\(^{-1}\) which agrees with the value that was obtained from binding data: 15.2 kJ mol\(^{-1}\) for the 'stable' sites (B or 1) and 15.9 kJ mol\(^{-1}\) for the 'labile' sites. The observed antagonistic behavior of \((R_\theta)\text{-cAMPS}\) was not caused by competition of this compound for the ATP binding site, since a higher cAMP concentration (1.0 \mu M) could overcome the inhibition by 100 \mu M \((R_\theta)\text{-cAMPS}\); the inhibition decreased from 82% to 15%. Furthermore, \((R_\theta)\text{-cAMPS}\) apparently is a competitive antagonist of cAMP (data not shown).

Other cAMP-dependent protein kinases

Table 2 summarizes the results from activation measurements with the four analogs modified in the exocyclic oxygen...
DISCUSSION

cAMP-dependent protein kinases have been extensively studied using derivatives of cAMP. Recently evidence has been accumulating that the two types of cAMP binding sites differ in kinetic properties [5] and specificity for certain derivatives [6-12]. The use of these site-specific analogs led to the proposal that both cAMP binding sites play a role in activation of the mammalian kinase [10-12]. Using a limited set of derivatives it was possible to obtain information about the interactions between cAMP and the ‘stable’ (B or 1) binding site [16]. In addition, a derivative modified in one of the exocyclic oxygen atoms on phosphorus, \((R_1)-cAMP\), was observed to activate the kinase only partially [16].

\[ K_i \]

\[ (S_1)-cAMP \quad 0.034 \quad 0.4 \quad 0.15 \]
\[ (S_2)-cAMP \quad 1.1 \quad 1.8 \quad 1.9 \]
\[ (R_1)-cAMP \quad <0.03 \quad <0.03 \quad 0.52 \]
\[ (S_1)-cAMP(\text{CH}_3)_2 \quad 0.75 \quad 1.0 \quad 1.0 \]
\[ (R_1)-cAMP(\text{CH}_3)_2 \quad \geq 0.4 \quad 0.03 \quad -^e \]

\[ K_i \]

\[ (R_1)-cAMP \quad 1.0 \quad 1.0 \quad 1.0 \]
\[ (R_1)-cAMP(\text{CH}_3)_2 \quad 1.0 \quad 0.85 \quad 1.0 \]
\[ (S_1)-cAMP \quad <0.03 \quad <0.03 \quad 0.52 \]
\[ (S_1)-cAMP(\text{CH}_3)_2 \quad 0.75 \quad 1.0 \quad 1.0 \]
\[ (R_1)-cAMP(\text{CH}_3)_2 \quad \geq 0.4 \quad 0.03 \quad -^e \]

\[ ^a \]

\[ ^b \]

\[ ^c \]

\[ ^d \]

\[ ^e \]

\[ ^f \]

\[ ^g \]

\[ ^h \]

\[ ^i \]

\[ ^j \]

\[ ^k \]

\[ ^l \]

\[ ^m \]

\[ ^n \]

\[ ^o \]

\[ ^p \]

\[ ^q \]

\[ ^r \]

\[ ^s \]

\[ ^t \]

\[ ^u \]

\[ ^v \]

\[ ^w \]

\[ ^x \]

\[ ^y \]

\[ ^z \]

\[ ^{\text{not determined because of inactivity of the derivative.}} \]

\[ ^{\text{no antagonism was observed, probably as a result of the partial agonistic potency of the compounds.}} \]

\[ ^{\text{no antagonism was observed, probably as a result of extremely low affinity of the derivative [3].}} \]

\[ ^{\text{activator of the type I kinase than reported before, suggests a clearly different interaction. Two possibilities are (a) polar interactions, such as hydrogen bonds or dipole interaction, and (b) a covalent bond with the binding site yielding a pentacovalent phosphorus atom. The formation of such a bond between cAMP and its receptor proteins has been suggested before [31]. Based on quantum chemical calculations it was proposed that the cyclophosphate ring should be in diequatorial position, after nucleophile attack of an amino acid side chain in the binding site (Fig. 4). If one of the exocyclic oxygen atoms is replaced by a sulphur, the derivative with sulphur in equatorial position after endo attack of the enzyme [(S_1)-cAMP], should be energetically favored over the other isomer [32]; as a consequence the S_1 isomer should be a better ligand and activator. Such calculations have not yet been performed for the phosphodimethylamidates, but it would be interesting to know whether the theoretical predictions in the case of endo attack fit the experimental data.}} \]

As argued before, the absence of coupling between binding and activation results in identical binding affinities in the presence and absence of ATP (Scheme 1). Of the 16 derivatives tested, this was only found for the binding of (R_1)-cAMP and of (R_1)-cAMP(\text{CH}_3)_2 to a minor extent, to both sites of kinase type I. This implies that dissociation of holoenzyme coincides with the same change in binding of cAMP to both sites. It follows that the cyclophosphate region of cAMP in both binding sites is involved in activation of protein kinase type I and probably also the other types. This conclusion is in agreement with earlier reports [10-12]. According to Scheme 1, the coupling factor \( \alpha \) is the link between binding of a cAMP derivative and subsequent activation of the enzyme. However, a given value of \( \alpha = 1 \) may still effect activation levels between 0% and 100% depending on the enzyme concentration in relation to the binding affinity between the regulatory and the catalytic subunits. This may be easily understood from two extreme situations: (a) when \( \alpha = 1 \) and the enzyme concentration is much smaller than the dissociation constant \( (K_i) \) for binding between the subunits, the kinase will be fully activated as a result of spontaneous dissociation; (b) when \( \alpha = 100 \) and the enzyme concentration is much larger than 100 times the dissociation constant \( (K_i) \) for the subunits, there will be no activation upon addition of the cyclic nucleotide.
For the three investigated kinases similar relative activities of the $R_p$ and $S_p$ isomers were observed: $(S_p)$-cAMPS stimulates at lower concentrations than $(R_p)$-cAMPS (which is only partially agonistic for the mammalian kinases and inactive for the D. discoideum kinase). However, the levels of maximal activation by these compounds differ in these three kinases. According to the reasoning above, this does not necessarily imply that the activation mechanisms are different. More likely, the differences may be accounted for by different $K_r$ values (Scheme 1) and concentrations of the kinases. Thus, our data suggest a similar mechanism of activation for the three cAMP-dependent kinases.

Summarizing, we have shown that cAMP is bound similarly to both types of binding sites of the protein kinase type I and subsequently induces activation of the enzyme via interaction of the cyclophosphate regions of the cAMP molecules in two distinct binding sites with a functional group of the regulatory subunit. The formation of these bonds causes a conformational change, which should lead to a decreased affinity between catalytic and regulatory subunits and thus formation of free, active catalytic subunits.

The antagonistic activity of $(R_p)$-cAMPS opens the way to investigation of the functioning of cAMP and cAMP-dependent protein kinases in vitro. Recently, this compound was indeed reported to antagonize glycogenolysis in isolated hepatocytes [33].

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