Substrate Specificity of Cyclic Nucleotide Phosphodiesterase from Beef Heart and from Dictyostelium discoideum

Peter J. M. VAN HAASTERT, Peter A. M. DIJKGRAAF, Theo M. KONIJN, Emilio Garcia ABBAD, Georg PETRIDIS, and Bernd JASTORFF

Cell Biology and Morphogenesis Unit, Zoological Laboratory, University of Leiden; and Department of Chemistry, University of Bremen

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The substrate specificity of beef heart phosphodiesterase activity and of the phosphodiesterase activity at the cell surface of the cellular slime mold Dictyostelium discoideum has been investigated by measuring the apparent \( K_m \) and maximal velocity \( (V) \) of 24 derivatives of adenosine 3',5'-monophosphate (cAMP). Several analogs have increased \( K_m \) values, but unaltered \( V \) values if compared to cAMP; also the contrary (unaltered \( K_m \) and reduced \( V \)) has been observed, indicating that binding of the substrate to the enzyme and ring opening are two separate steps in the hydrolysis of cAMP.

CAMP is bound to the cell surface of the cellular slime mold via dipole-induced dipole interactions between the adenine moiety and an aromatic amino acid, and possibly by a hydrogen bond between the enzyme and one of the exocyclic oxygen atoms; a cyclic phosphate ring is not required to obtain binding. cAMP is bound to the slime mold enzyme via a hydrogen bond at the 3'-oxygen atom, and probably via a hydrogen bond with one of the exocyclic oxygen atoms. A cyclic phosphate ring is necessary to obtain binding to the enzyme. A specific interaction (polar or hydrophobic) between the base moiety and the enzyme has not been demonstrated. A negative charge on the phosphate moiety is not required for binding of cAMP to either enzyme.

The catalytic reaction in both enzymes is restricted to the phosphorus atom and to the exocyclic oxygen atoms. Substitution of the negatively charged oxygen atom by an uncharged dimethylamino group in axial or equatorial position renders the compound non-hydrolyzable. Substitution of an exocyclic oxygen by a sulphur atom reduces the rate of the catalytic reaction about 100-fold if sulphur is placed in axial and more than 10000-fold if sulphur is placed in equatorial position. A reaction mechanism for the enzymatic hydrolysis of cAMP is proposed.

Materials and methods

Materials

[8-\textsuperscript{3}H]cAMP (0.9 TBq/mmol) was obtained from Amersham, snake venom (Ophiophaga hannah) was from Sigma,
The analogs of cAMP derivatives were determined by incubating at 30°C in a volume of 100 μl containing 50 mM Tris/HCl, pH 7.5, 2 mM MgCl₂, 1 μM [³H]cAMP (ca. 2 kBq) 0.25 μg beef heart phosphodiesterase and different concentrations cAMP or cAMP analogs (0.1 μM – 1 mM).

The reaction was terminated after 45 min by boiling the samples during 2 min. After cooling, 50 μg snake venom (Ophiophaga hannah) was added. Non-hydrolyzed cAMP was removed after 30 min by the addition of 250 μl cold perchloric acid (3.5%, v/v). The lysates were neutralized by adding 115 μl KHCO₃ (50% saturated solution at 20°C), and centrifuged at 8000 × g during 2 min. The supernatant (350 μl) was incubated with 50 μg snake venom during 30 min, and non-hydrolyzed cAMP was removed by the addition of the ion-exchange slurry as described above.

For both enzymes product accumulation was linear with time and enzyme concentration. The interference of the analogs or their degradation products with the 5'-nucleotidase step was investigated by replacing [³H]cAMP with [³H]-
5'AMP in the above-described incubation procedure. Inhibition of the hydrolysis of [3H]5'AMP by the cAMP analogs or their degradation products has not been observed.

The V of the analogs was determined by measuring their degradation with HPLC. The equipment consisted of a Beckman 100A pump and a Laboratory Data Control UVIII monitor at 254 nm. The column used was the anion-exchanger Partisil 10-SAX (Whatman) and the mobile phase composition was 50 mM KH2PO4, 15% propan-1-ol, 5% methanol, pH 5. This composition excludes most of the hydrophobic interactions between solutes and the stationary phase [34], thus separating almost exclusively on the charge, which is the main difference between cyclic nucleotides and nucleotides. For the beef heart phosphodiesterase the incubation took place at 30°C in a total volume of 50 μl containing 50 mM Tris/HCl, pH 7.5, 2 mM MgCl2, 2.5 mM cAMP or cAMP analogs and 30 μg phosphodiesterase. At 5 min or longer intervals 1 μl of this mixture was injected in the separation system described above. The degree of degradation was quantified by measuring the decrease of the peak area of the reaction products, occurring in the supernatant of the assay. The half-life of cAMP was 12 min. If required, analysis was continued up till 24 h.

For the slime mold phosphodiesterase the incubation at 20°C contained in a total volume of 50 μl: 10 mM phosphate buffer, pH 7.5, 0.2 mM cAMP or cAMP analogs and 107 cells. The reactions were terminated after 0, 2, 5, 7.5, 10, 20 and 30 min by centrifugation for 5 s at 8000g and adding 25 μl of the supernatant to 75 μl cold ethanol. After centrifugation for 2 min at 8000g 15 μl of the supernatant was analysed on the separation system described above. The half-life of cAMP varied between 5 and 10 min.

RESULTS AND DISCUSSION

Selection of cAMP Derivatives

The interaction of a small molecule with a protein is mainly electrostatic [35]. The types of interaction include: ion-ion interactions, dipole interactions such as hydrogen bonds, hydrophobic interactions such as π-electron stacking, and changes of free energy due to solvophobic effects. cAMP can form several hydrogen bonds with its surrounding medium (e.g. water, protein). One hydrogen bond cannot form any longer in each of the derivatives 2-4, 7-10 (Fig. 1 a). The derivatives 5 and 6 were used to change the syn-anti equilibrium, which is 1:1 in cAMP [36], to the syn conformation [37]. Compound 8 was used to introduce a bulky substituent close to the site of the catalytic reaction.

In derivatives 11 and 12 the electron density distribution of the negative charge is no longer symmetrically located but preferentially on oxygen. Derivatives 13 and 14 were used to remove the negative charge; thus the double bond oxygen is regio-selectively fixed. These derivatives (11-14) may reveal the stereochemical orientation and involvement of the exocyclic oxygen atoms during the hydrolytic reaction.

The derivatives 1, 15-18 (Fig. 1 c) form a sequence of decreasing polarizability (15, 1, 16, 18, 17 [38]) and have decreasing polarizing power (18, 17, 1, 16, 15 [38]). The derivatives 19-25 are not cyclic nucleotides. Some of them (19-21) can mimic the ribose-cyclic phosphate moiety of cAMP and bind to their receptor proteins [14, 39] (Fig. 1 d).

The polarity of all derivatives has been measured by high-performance liquid reversed phase chromatography. These data might reveal hydrophobic interactions between cAMP and phosphodiesterases.
Specificity of the Cell-Surface Phosphodiesterase of D. discoideum

Table 2 reveals that most of the cAMP analogs modified in the base or ribose moiety (2–10) have a $V$ comparable to cAMP. Exceptions are compound 6, which has a bulky substituent on the 8-position, compound 9, for which $V$ could not be calculated, and compound 10. This indicates that the base moiety and 2'-hydroxy are not directly involved in the catalytic reaction. The $K_m$ values of these analogs (2–10) suggest...
Table 2. Specificity of phosphodiesterase and cAMP receptors relative to cAMP

$K_m$ is the apparent $K_m$ of the analog relative to cAMP; $K_m = K_m$ (analog)/$K_m$ (cAMP). $V'$ is the maximal velocity of the hydrolysis of the analog relative to cAMP; $V' = V$ (analog)/$V$ (cAMP). The polarity of the analogs is presented as selectivity to cAMP; $\alpha = k'/(cAMP)/k'$ (analog) (see Materials and Methods). sm, slime mold; bh, beef heart.

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* $V'$ could not be calculated due to the absence of binding of the analog to the phosphodiesterase.

* Hydrolysis and polarity of this compound has not been measured due to the absence of a chromophor.

In contrast to the relatively low specificity in the adenine moiety, 2'-hydroxy and 5'-oxygen also are not directly involved (e.g. via hydrogen binding) in the binding of cAMP to the enzyme. A possible explanation of the low $K_m$ value of compound 10 has been given in the previous section. Compound 8 has a very bulky substituent at the 2'-position, close to the catalytic site, and it has a high $V$ comparable to that of CAMP, which indicates that hydrogen bonds at the 3'-oxygen position, and one of the exocyclic oxygens (probably the double bond oxygen in axial position), but not by a charge-charge interaction. A distinct hydrophobic interaction between the base moiety and the enzyme seems unlikely. Hydrolysis requires a correct steric orientation of the exocyclic oxygen atoms.

Specificity of the Beef Heart Phosphodiesterase
cAMP analogs modified in the base or ribose moiety (compounds 2 – 10) have a $V$ comparable to cAMP, except the compounds with bulky substituents (5, 6 and 8). Most of these analogs (2 – 10) have a $K_m$ comparable to cAMP. The high $K_m$ of compound 2 may indicate a hydrogen bond at N$^3$ with the enzyme; however, this is not shown by cIMP (compound 17), cGMP (compound 18), and compound 15. Although cAMP is hydrolyzed at the 3'-oxygen atom, the results with compound 9 (3'-oxygen replaced by an amino group) show that a direct chemical interaction between the enzyme and the 3'-oxygen atom during binding and hydro-
lysis is absent. This observation is in strong contrast to the slime mold enzyme.

Modifications of the exocyclic oxygen atoms (compounds 11-14) have a strong effect on the rate of hydrolysis; compound 12 is hydrolyzed 70-times slower than CAMP, hydrolysis of the other compounds has not been observed (hydrolysis is at least 10,000-fold slower than the hydrolysis of CAMP). In contrast to the drastic effects on the hydrolyzability, these compounds (11-14) bind to the enzyme with apparently high affinity. However, these results should be interpreted very cautiously, since the relative proportioning of \( k_1 \) to \( k_2 \) is unknown. Also the non-cyclic nucleotides (compounds 19-25) bind to the enzyme but hydrolysis has not been observed.

Hydrogen bonds between the adenine moiety and the beef heart enzyme are unlikely. The results of the \( K_m \) values of compounds 1, 15-18 may suggest stacking interactions of the \( \pi \)-electrons of adenine via a polarization of the \( \pi \)-electrons of an aromatic amino acid, since the order of decreasing affinity (increasing \( K_m \) values) corresponds with the order of decreasing polarizing power \((18 > 17 > 1 > 16 > 15; [38])\). Fig.4C reveals that analogs which are modified in the base moiety have an increased affinity for the enzyme if they are more polar. This correlation between polarity of the analog and \( K_m \) value is absent in analogs not modified in the base moiety, which confirms the assumption that the adenine moiety is bound to the enzyme by dipole-induced dipole forces via stacking interactions due to polarization of an aromatic amino acid. Hydrophobic interactions do not seem to be involved in the catalytic reaction of this enzyme (Fig.4D).

The phosphodiesterase activities of slime molds and beef heart differ significantly in the following aspects: (a) their apparent \( K_m \) values for CAMP, (b) the involvement of the adenine moiety in the binding of CAMP to the enzyme, (c) the involvement of the 3'-oxygen atom in both the binding and the catalytic reaction, and (d) the binding of non-cyclic analogs. This last observation might explain why all the classical phosphodiesterase inhibitors (caffeine, theophylline) fail to inhibit the slime mold phosphodiesterase [40].

**Reaction Mechanism of Hydrolysis**

Several CAMP analogs have relatively unaltered \( V \) values, while the \( K_m \) is increased significantly (compound 8 with slime mold and compound 2 with beef heart); the opposite, unaltered \( K_m \) and reduced \( V \), has also been observed (compound 14). This demonstrates that hydrolysis of CAMP proceeds in at least two steps: binding of CAMP to the enzyme, and ring opening catalyzed by the enzyme.

Enzymatic hydrolysis of phosphate ester bonds generally takes place via nucleophilic substitution reactions with phosphate intermediates arranged in trigonal bipyramidal configuration [41]. The nucleophilic attack may take place in three directions (Fig.5): opposite the leaving group (in line), or opposite a ligand which is not the leaving group (endo-adjacent or exo-adjacent, Fig.5). There are two possibilities for the nature of the nucleophile, i.e. a water molecule, or a site of the enzyme. The product of the reaction is 5'SAMP if water is the nucleophile; however, if the enzyme is the nucleophile the product is an enzyme-5'SAMP complex. This complex is subsequently cleaved by a nucleophilic attack of a water molecule with three possibilities (in line, endo-adjacent or exo-adjacent). Therefore, twelve different routes are possible for the hydrolysis of CAMP by phosphodiesterase (Table 3).

Burger et al. [26] showed that beef heart phosphodiesterase hydrolyzes CAMP with inversion at phosphorus. This eliminates seven hydrolysis routes (Table 3).

Recently, Van Ool and Buck [27] published quantum chemical calculations on CAMP-enzyme intermediates with trigonal bipyramidal configurations. Their calculations show that (a) the trigonal bipyramidal configuration of CAMP with a diequatorial cyclophosphate ring is about 100 kJ/mol lower in energy than the equatorial-apical ring-positioned intermediate of CAMP; (b) the intermediate of compound 12 is about 525 kJ/mol lower in energy than the intermediate of compound 11 if they both have a diequatorially-positioned cyclophosphate ring; (c) the energy difference is only 53 kJ/mol in favour of compound 12 if the intermediates have an equatorial-apical-positioned cyclophosphate ring. Our observation that only compound 12 is hydrolyzed strongly suggests...
that the intermediate has a diquatorially-positioned cyclophosphate ring. This excludes another three hydrolysis routes (Table 3). Note that Van Ool and Buck conclude that cAMP is hydrolyzed via route c or d (Table 3). This result was based on the report that compounds 11 and 12 are hydrolyzed at the same rate [42]; therefore, they had to conclude that the intermediates of 11 and 12 have an equatorial-apical positioned cyclophosphate ring. Our observation that only the (Sp)-diasteroisomer is a substrate has been confirmed by others (personal communication of Dr Eckstein to Dr Jarvest, cited in [43] (p. 464). Note also that Jarvest et al. [43] proposed that cAMP is hydrolyzed via route a (Table 3), which is excluded in the present paper. The quantum-chemical calculations by Van Ool and Buck were not available at that time.

The two remaining routes (f and j) differ in an exo- or endo-attack of the enzymatic site in the first nucleophilic reaction. The only — not very strong — argument in favour of the route f (exo-attack) might be that an endo-attack is constrained spatially. This might be especially important if cAMP is bound to the catalytic site in the syn conformation [25]. Therefore, the most likely reaction mechanism for the hydrolysis of cAMP by beef heart phosphodiesterase consists of the binding of cAMP to the enzyme followed by two nucleophilic substitution reactions (Fig. 6). The first reaction is an exo-attack of a nucleophile of the enzyme (e.g. serine or threonine). This leads to an intermediate with the cyclophosphate ring in the favourable diquatorial conformation. After pseudorotation the 3'-phosphate bond is cleaved leading to an enzyme-5'AMP intermediate. The second reaction is an in line attack of a water molecule by which the enzyme-5'AMP bond is cleaved.

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P. J. M. Van Haastert, P. A. M. Dijkgraaf, and T. M. Konijn,
Celbiologie en Morphogenese, Zoologisch Laboratorium, Universiteit Leiden,
Kaiserstraat 63, NL Leiden, The Netherlands

E. G. Abbad, G. Petridis, and B. Jastorff, Institut für Chemie der Universität Bremen,
Loebenerstraße, D-2800 Bremen, Federal Republic of Germany