Ligand binding properties of the cytoplasmic cAMP-binding protein of *Dictyostelium discoideum*

René J.W. de Wit*, Jos C. Arents and Roel van Driel

* *Cell Biology and Morphogenesis Unit, Zoological Laboratory, University of Leiden, Kaiserstraat 63, 2311 GP Leiden, The Netherlands* and *Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, P.O. Box 20151, 1000 HD Amsterdam, The Netherlands*

Received 9 June 1982

cAMP *Derivatives* *Binding site* *Cytoplasmic protein* D. discoideum

1. INTRODUCTION

In the cellular slime mold *Dictyostelium discoideum* cyclic AMP (cAMP) mediates chemotaxis [1,2] and differentiation [3,4]. Extracellular cAMP is detected by membrane-bound receptors after which an adenylate cyclase is transiently activated [5]. Part of the newly synthesized cAMP is secreted, part of it probably acts intracellularly. Intracellular cAMP is detected by a high-affinity cAMP binding protein, which has a M_r of 40 000 [6–12]. This protein is present in much higher concentrations in differentiating than in vegetative cells [9,12].

The 40 K cAMP binding protein is related to the regulatory subunits of mammalian protein kinase, since it is able to regulate mammalian kinase activity in a cAMP dependent manner [9]. In *D. discoideum* a cAMP dependent protein kinase was claimed [13,14], of which the regulatory subunit corresponds to the 40 K cAMP binding protein [14].

In the present paper we describe equilibrium and kinetic cAMP binding properties of the 40 K protein. The approach of Jastorff et al. [30,31] was applied to probe the structure of the cAMP binding site using 16 selected cAMP analogues. The structure is compared to the *D. discoideum* cell surface receptor for cAMP and rabbit muscle cAMP dependent protein kinase I.

* To whom correspondence should be sent.

2. EXPERIMENTAL

2.1. D. Discoideum *cyclic AMP binding protein*

D. discoideum cells (strain AX 2) were grown axenically and starved for 2 h as described earlier [15]. The soluble cell fraction contained only one high-affinity cAMP binding protein of 40 000 daltons as demonstrated by photoaffinity labelling ([14], U. Walter and R. Van Driel, unpublished). Also a low-affinity cAMP binding protein was detected. The high-affinity binding protein was purified about 25 times using Blue Sepharose (Pharmacia) [12]. The cAMP binding fractions were pooled and dialyzed against binding assay buffer. This preparation still contained some of the low-affinity binding protein, which did not affect cAMP binding data up to $3 \cdot 10^{-8}$ M cAMP. The total protein concentration was about mg/ml; the high-affinity cAMP binding activity was 5 nM.

2.2. *Materials*

[8-^{3}H]cAMP was purchased from Amersham Radiochemical Centre (U.K.). ATP, cAMP, cGMP, cIMP and compounds 3, 10 and 11 (table 1) were from Boehringer. All other cyclic nucleotide derivatives were kindly supplied by Dr B. Jastorff, University of Bremen, FRG. MES (2-(*N*-morpholino) ethane sulfonic acid) and EGTA (ethyleneglycol-bis-(β-amino ethyl ether) *N,N,N',N'*-tetraacetic acid) were purchased from Sigma. All analogues were checked for the absence of cAMP and other impurities by anion-exchange HPLC using various mobile phase compositions.

Published by Elsevier Biomedical Press

00145793/82/0000–0000/$2.75

Table 1

Binding and activity of cAMP analogues

No.	Analogue	cAMP-binding protein *D. discoideum* $\delta\Delta G$ (kJ . mol^{-1})	cAMP dependent protein kinase rabbit-muscle [a] $\delta\Delta G$ (kJ . mol^{-1})	cAMP chemotaxis receptor *D. discoideum*[b] $\delta\Delta G$ (kJ . mol^{-1})
1	Adenosine 3′,5′-monophosphate	0	0	0
2	Adenosine *N*-oxide 3′,5′-monophosphate	4.7	6.0	6.5 (21)
3	6-Chloropurine 3′,5′-monophosphate	1.8	1.9	19.5 (21)
4	7-Deazaadenosine 3′,5′-monophosphate	0.7	3.4	13–19.5 (21)
5	Benzimidazole 3′,5′-monophosphate	6.0	4.3	26.0 *
6	Purine 3′,5′-monophosphate	3.9	6.5	26.0 *
7	Inosine 3′,5′-monophosphate	3.9	8.9	26.0 (22)
8	Guanosine 3′,5′-monophosphate	13.9	13.0	19.5 (22)
9	2-Phenyladenosine 3′,5′-monophosphate	12.0	7.8	13.0 *
10	8-Bromoadenosine 3′,5′-monophosphate	−2.6	−0.6	19.5 (21)
11	2′-Deoxyadenosine 3′,5′-monophosphate	22.0	20.4	6.5 (21)
12	3′-Amino-3′-deoxyadenosine 3′,5′-monophosphate	13.0	17.5	26.0 (21)
13	5′-Amino-5′-deoxyadenosine-3′,5′-monophosphate	17.5	18.1	0–6.5 (21)
14	Adenosine 3′,5′-monothionophosphate (S_p)	4.5	9.6	13.0 (21)
15	Adenosine 3′,5′-monothionophosphate (R_p)	12.0	16.8	26.0 (21)
16	Adenosine 3′,5′-monophospho-dimethylamidate (R_p)	27.3	24.0	26.0 (21)
17	Adenosine 3′,5′-monophospho-dimethylamidate (S_p)	17.5	20.3	13.0 *

[a] Reported previously [19] as determined by competitive binding to isolate regulatory subunits of mammalian protein kinase type I.

[b] Reported previously as determined by chemotactic activity of the cyclic nucleotide derivatives. References are given in brackets.

* Unpublished data from Dr Th.M. Konijn.

$\delta\Delta G$ is defined in the text.

2.3. *Nucleotide binding assay*

Binding assay buffer composition: 25 mM MES, 4 mM magnesium acetate, 10 mM sodium phosphate, 0.125 mM EGTA, adjusted with NaOH to pH 6.9. For determination of the inhibition constant (K_I) of an analogue, 0.5 nM cAMP binding activity was incubated with two [^{3}H]cAMP concentrations (3 and 10 nM) and varying concentrations of unlabeled cAMP or of a derivative. Results were plotted according to Dixon [16] which method allows determination of the K_I and distinguishes between competitive and non-competitive inhibition. All analogues were strictly competitive. Samples were equilibrated at 0°C for at least 1 h. No degradation of cyclic nucleotides was observed up to 2 h, as was determined using anion-exchange HPLC. Membrane filtration was done as reported previously [12].

2.4. *Standardization*

In order to compare the K_I values of analogues for the cAMP-binding protein of *D. discoideum* to results obtained with other proteins and using other methods, the following standardization was used

$$\delta\Delta G = -RT \ln \frac{K_I \text{ (cAMP)}}{K_I \text{ (derivative)}} \qquad [17–19]$$

Thus, the affinity of a derivative relative to cAMP

is transformed into the free enthalpy scale. A ten-fold increase in the K_I of a derivative corresponds to a $\delta\Delta G$ of 6.5 kJ . mol^{-1}.

3. RESULTS

The binding curve for the partial purified preparation was linear up to 3 . 10^{-8} M cAMP with a K_d of 2.2 nM (fig.1A). At higher concentrations binding to a low-affinity component was observed. Addition of 0.2 mM ATP had no effect on the high-affinity binding for cAMP. Binding to the low-affinity component was about two-fold reduced. Association and dissociation velocity measurements resulted in first order kinetics (fig.1B,C). The association rate constant was 8.0 . 10^5 M^{-1}s^{-1}, the dissociation rate constant 1.0 . 10^{-3} s^{-1} as measured using two methods (dilution of free ligand and addition of excess unlabeled cAMP). Above results suggest that in the range up to 3 . 10^{-8} M cAMP, binding to only one type of binding site is measured. Calculation of the K_d from the rate constants yields 1.3 nM, which is similar to the value obtained by measuring at equilibrium.

Table 1 shows the results of the binding competition by 17 analogues of cAMP. None of the analogues 2–7, altered in the base moiety with respect to the potency to form hydrogen bonds, shows a significant drop in binding affinity. This indicates the absence of hydrogen bond interactions between the adenine base and the protein. If the adenine moiety is recognized and bound by stacking-like interactions with an aromatic amino acid side chain, the binding affinity of derivatives should increase with the polarizing power of the base. However, a series of analogues with increasing polarizing power, analogue no. 5<6<1<7<8 [20] shows no increase in binding affinity.

Derivatives 8 and 9 show a more pronounced decrease in affinity, which may be due to steric

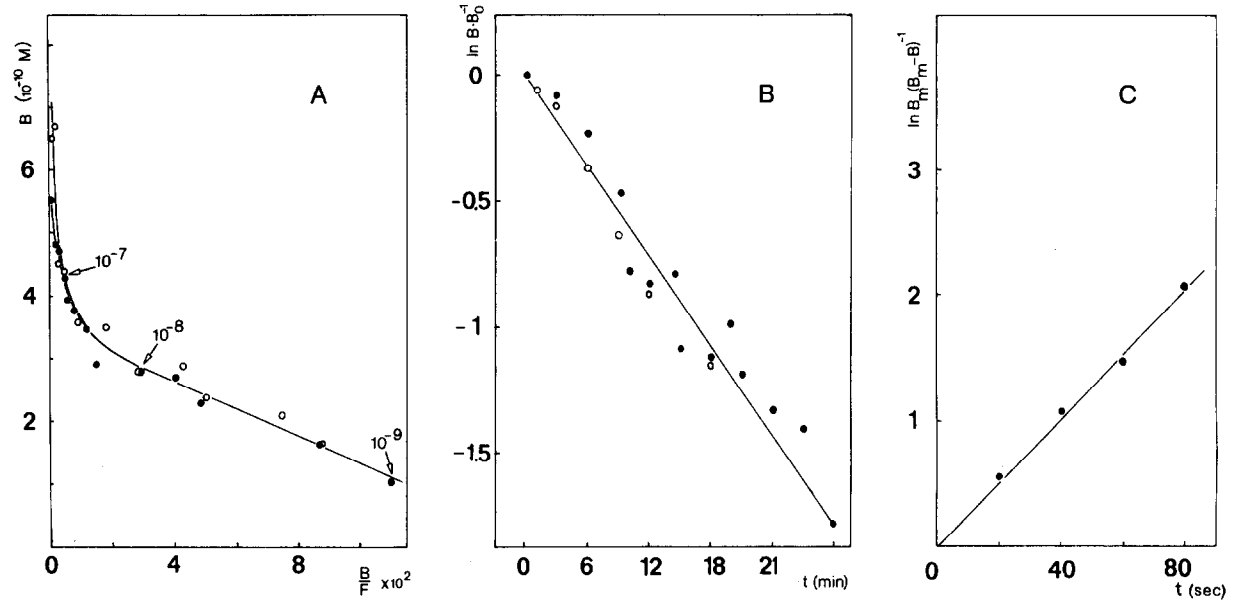

Fig.1. (A) Scatchard plot for binding of cAMP to 0.35 nM binding protein. Samples were equilibrated at 0°C for 1 h before filtration. (-○-) Binding in the absence of ATP, (-●-) in the presence of 0.2 mM ATP. The arrows indicate free cAMP concentrations (F) in mol/l. (B) Dissociation kinetics at 0°C. Samples were equilibrated with 3 . 10^{-8} M cAMP for 15 min at 0°C. (-●-) At t_0 10^{-4} M unlabeled cAMP was added and at various times samples were withdrawn and filtrated. (-○-) At t_0 the incubation mixture was diluted 100-fold and at various times samples were withdrawn and filtrated. The bound radioactivity of all samples was corrected for the residual radioactivity after 1 h of dissociation. B_0 = bound cAMP at time zero. (C) Association kinetics at 0°C. At t_0 3 . 10^{-8} M cAMP was added to 0.5 nM cAMP-binding protein. At several times samples were taken and filtrated. B_m maximal bound cAMP, determined after 10 min incubation.

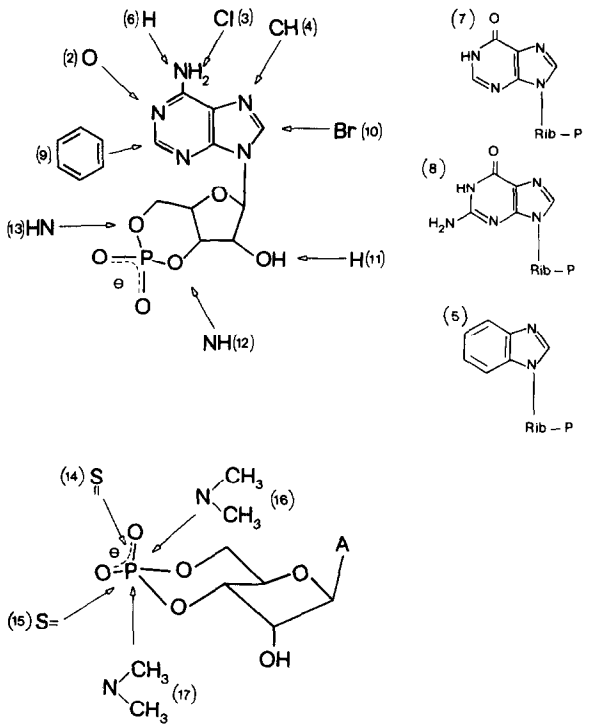

Fig.2. Structures of the cAMP derivatives used. Rib–P = ribose 3′,5′-monophosphate. Analogue numbers are defined in table 1.

limitations at the 2-position of the base. Analogue 10 has a higher affinity for the binding protein than has cAMP. As this derivative renders the cAMP molecule almost totally in the *syn*-conformation, it is very likely that the cyclic nucleotide is bound in that conformation.

In contrast to the adenine base, the ribose moiety is bound by at least three hydrogen bonds at the positions 2′, 3′ and 5′, as indicated by the significant lower affinity of analogues 11–13. In compounds 14 and 15 the negative charge of the phosphate group is fixed on one of the exocyclic oxygen atoms. Compound 15 shows a more pronounced decrease than 14, which may indicate that a salt bridge is formed preferentially with the equatorial exocyclic oxygen atom. When no charge is present (compound 16,17) the binding affinity is still lower, which may also be due to steric effects of the bulky dimethylamino group.

The data in table 1 suggest that cAMP is bound in the same conformation and by the same interactions as to mammalian protein kinase [19]. In contrast, binding to the chemoreceptor is of a significantly different type. With this protein cAMP may form hydrogen bonds with N-6 and N-7 of the base moiety and only with 3′-O in the ribose. The salt bridge may be much alike. Another difference is cAMP being bound to the chemotactic receptor in the anti-conformation [21,22].

4. DISCUSSION

In the present report the structure of the cAMP binding site of the cytoplasmic cAMP binding protein of *D. discoideum* was probed and compared to the 'stable cAMP binding site' [27] of mammalian protein kinase and the cell surface chemoreceptor of *D. discoideum*. This approach reveals a close resemblance between the positions and interactions by which cAMP is bound to the cytoplasmic 40 K protein and the 'stable site' of rabbit muscle protein kinase type I. The binding to the chemotactic cAMP receptor is of an entirely different character, which may indicate the absence of a structural relationship. Also the catabolite repressor protein (CRP) from *Escherichia coli* binds cAMP with interactions different from the 40 K protein. The CRP seems to bind cAMP derivatives irrespective of the modifications in the base moiety, but the cyclic phosphate-ribose rings are specifically recognized [23,24].

Though the binding sites on the 40 K protein and mammalian protein kinase are similar, these proteins are rather different in several other aspects. The mammalian kinase forms tetramers of two catalytic and two regulatory subunits [25]. Each regulatory subunit has two non-identical cAMP binding sites, being different in dissociation kinetics [26–28]. In addition cAMP is bound in a cooperative way and the binding affinity for cAMP is strongly affected by ATP. Binding of cAMP to the kinase results in dissociation of the catalytic subunits from a regulatory subunit dimer. The isolated dimeric regulatory subunits bind cAMP to both types of sites in a cooperative way, but are unaffected by ATP. In contrast the cAMP binding protein of *D. discoideum* occurs as a monomer ([11] and our unpublished observations) and in later differentiation stages as a faster sedimenting complex [11]. Furthermore, kinetic results in this report indicate one type of binding site, binding cAMP non-cooperatively and unaffected

by ATP. A 37 K tryptic derivative of mammalian regulatory subunit was shown to exist in monomeric form, while binding to both cAMP sites was normal [29]. It is, however, unlikely that the studied 40 K protein is a proteolytic product, since various protease inhibitors did not lead to the detection of a cAMP-binding protein heavier than about 40 K [12,14]. Recently a 42 K regulatory subunit of a cAMP dependent protein kinase was reported, which may be more related to the 40 K protein of *D. discoideum* [32].

The similarity in cAMP binding to the 40 K protein and the mammalian protein kinase regulatory subunit may suggest a structural relationship, which supports previous claims that a functional relationship exists, i.e., cAMP-dependent regulation of protein kinase activity [9,14]. The relatively fast dissociation kinetics of the 40 K protein allow this protein to respond to the short-term changes in cAMP concentration that occur during oscillation in *D. discoideum* [33], whereas the kinetics of the mammalian kinase allow response to only long term changes.

ACKNOWLEDGEMENTS

We thank Dr B. Jastorff for generously providing the cAMP derivatives.

REFERENCES

[1] Robertson, A.D.J. and Grutsch, J.F. (1981) Cell 24, 603–611.
[2] Van Driel, R. (1982) in: The Handbook of Pharmacology; Physiology of Cyclic Nucleotides (Nathanson, J.A. and Kebabian, J.W., eds) Vol. 58/II, pp. 365–386, Springer Verlag, Heidelberg.
[3] Kessin, R.H. (1981) Cell 27, 241–243.
[4] Landfear, S.M. and Lodish, H.F. (1980) Proc. Natl. Acad. Sci. USA 77, 1044–1048.
[5] Roos, W., Malchow, D. and Gerisch, G. (1977) Cell Diff. 6, 229–239.
[6] Cooper, S., Chambers, D.A. and Scanlon, S. (1980) Biochim. Biophys. Acta 629, 235–242.
[7] Vernon, M. and Patte, J.-C. (1978) Develop. Biol. 63, 370–376.
[8] Rahmsdorf, H.J. and Gerisch, G. (1978) Cell Diff. 7, 249–257.
[9] Leichtling, B.H., Spitz, E. and Rickenberg, H.V. (1981) Biochem. Biophys. Res. Commun. 100, 515–522.
[10] Leichtling, B.H., Thion, C., Spitz, E. and Rickenberg, H.V. (1981) Develop. Biol. 82, 150–157.
[11] De Gunzburg, J. and Veron, M. (1981) Biochemistry 20, 4547–4554.
[12] Arents, J.C. and Van Driel, R. (1982) FEBS Lett. 137, 201–204.
[13] Sampson, J. (1977) Cell 11, 173–180.
[14] Leichtling, B.H., Majerfield, I.H., Coffman, D.S. and Rickenberg, H.V. (1982) Biochem. Biophys. Res. Commun. 105, 949–955.
[15] Van Driel, R. (1981) Eur. J. Biochem. 115, 391–395.
[16] Dixon, M. (1953) Biochem. J. 55, 170–171.
[17] Hoppe, J., Freist, W., Marutzky, R. and Shaltiel, S. (1978) Eur. J. Biochem. 90, 427–432.
[18] Jastorff, B., Hoppe, J. and Morr, M. (1979) Eur. J. Biochem. 101, 555–561.
[19] De Wit, R.J.W., Hoppe, J., Stec, W.J., Baraniak, J. and Jastorff, B. (1982) Eur. J. Biochem. 122, 95–99.
[20] Wagner, K.G., Arfmann, H.A., Lawaczeck, R., Opatz, K., Schomburg, I. and Wray, V. (1978) in: Nuclear Magnetic Resonance Spectroscopy in Molecular Biology (Pullman, B. ed) pp. 103–110.
[21] Van Haastert, P.J.M., Jastorff, B., Pinas, J.E. and Konijn, Th.M. (1982) J. Bacteriol. 149, 99–105.
[22] Mato, J.M., Jastorff, B., Morr, M. and Konijn, Th.M. (1978) Biochim. Biophys. Acta 544, 309–314.
[23] Ebright, R.H. and Wong, J.R. (1981) Proc. Natl. Acad. Sci. USA 78, 4011–4015.
[24] Anderson, W.B., Perlman, R.L. and Pastan, I. (1972) J. Biol. Chem. 247, 2717–2722.
[25] Builder, S.E., Beavo, J.A. and Krebs, E.G. (1980) J. Biol. Chem. 255, 2350–2354.
[26] Døskeland, S.O., Ueland, P.M. and Haga, H.J. (1977) Biochem. J. 161, 653–665.
[27] De Wit, R.J.W. and Hoppe, J. (1981) FEBS Lett. 127, 9–12.
[28] Rannels, S.R. and Corbin, J.D. (1980) J. Biol. Chem. 255, 7085–7088.
[29] Weber, W. and Hilz, H. (1979) Biochem. Biophys. Res. Commun. 90, 1073–1081.
[30] Jastorff, B., Garcia Abbod, E., Petridis, G., Tegge, W., De Wit, R.J.W., Erneux, C., Stec, W.J. and Morr, M. (1981) Nucleic Acids Res. 9, 219–223.
[31] Jastorff, B., Hoppe, J., Mato, J.M. and Konijn, T.M. (1979) in: Molecular Mechanisms of Biological Recognition (Balaban, M., ed). pp. 107–117, Elsevier Biomedical, Amsterdam, New York.
[32] Kudlow, J.E., Watson, R.K. and Gill, G.N. (1981) J. Cycl. Nucl. Res. 7, 151–159.
[33] Gerisch, G. and Wick, U. (1975) Biochem. Biophys. Res. Commun. 65, 364–370.