SOLUBLE, CYTOPLASMIC CYCLIC AMP-BINDING PROTEINS OF *DICTYOSTELIUM DISCOIDEUM*

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1. Introduction

Cyclic AMP plays an important role in the life cycle of the cellular slime mold *Dictyostelium discoideum* (review [1,2]). Stimulation of starving *D. discoideum* cells with extracellular cyclic AMP, which is a chemoattractant, results in a steep, transient rise of the intracellular cyclic AMP level [4]. This is due to an activation of adenylate cyclase [3]. Part of the nascent intracellular cyclic AMP is secreted, part is hydrolyzed inside the cell [5]. In addition to these rapid changes *D. discoideum* cells show a slower continuous increase in intracellular cyclic AMP concentration during starvation, in the aggregation phase of their developmental cycle [6,7]. There is evidence that intracellular cyclic AMP is involved in the control of *D. discoideum* cell differentiation [8,9]. This cyclic AMP effect is presumably mediated by one or more cyclic AMP-binding proteins in the cell. Photo-affinity labelling, using 8-azido-cyclic [^{32}P]AMP [10], and fractionation of cell extracts on DEAE-cellulose [11–13], have shown that differentiating cells contain only one major cytoplasmic cyclic AMP-binding protein with a high affinity for cyclic AMP (K_d in the nM range). In addition a protein with lower affinity for cyclic AMP has been found (K_d in the μM range) [14].

Here, we describe some properties and the purification of a soluble, cytoplasmic cyclic AMP-binding protein of *D. discoideum* cells.

2. Experimental

2.1. *Preparation of cell extracts*

Dictyostelium discoideum cells (strain AX2) were grown and starved for 2 h as in [15]. Subsequently, the cells were washed 2 times, resuspended in 10 mM Tris–HCl, 2.5 mM $MgCl_2$, 5 mM mercaptoethanol, 0.25 M sucrose (pH 7.5) containing 0.25 mM phenylmethylsulfonylfluoride (homogenization buffer), at a final density of 2×10^8 cells/ml, and ruptured in a Dounce homogenizer at 0°C. The homogenate was centrifuged for 1.5 h at $10^5 \times g$ at 4°C. The supernatant was used immediately or stored at −70°C. Its protein concentration was ~10 mg/ml.

2.2. *Cyclic AMP-binding assay*

Protein samples were incubated in 50 mM potassium phosphate, 50 mM KCl, 10 mM EDTA, 2 mM dithiothreitol (pH 5.5) containing 10 nM cyclic [^{3}H]AMP (The Radiochemical Centre, Amersham; 45 Ci/mmol) and equilibrated 15 min at 0°C. Subsequently, 1.0 ml was forced through a Millipore membrane filter (type HAWP, 0.45 μM pore size) by suction. The filter was washed once with 3 ml ice-cold buffer without cyclic nucleotide. The amount of non-competable cyclic [^{3}H]AMP retained by the filter was determined by repeating the binding assay in the presence of excess (2×10^{-5} M) unlabelled cyclic AMP.

3. Results

Fig.1 shows an equilibrium cyclic AMP-binding curve of the soluble cytoplasmic fraction of *D. discoideum* cells, that had been starved for 2 h. The binding curve can be described with a single dissociation constant: $K_d = 0.8 \times 10^{-9}$ M. The result indicates that $\sim 5 \times 10^4$ cyclic AMP molecules can be bound per cell. Little or no binding activity was found in the particulate cell fraction. Fig.2 shows that cyclic AMP is bound specifically. Competition experiments show

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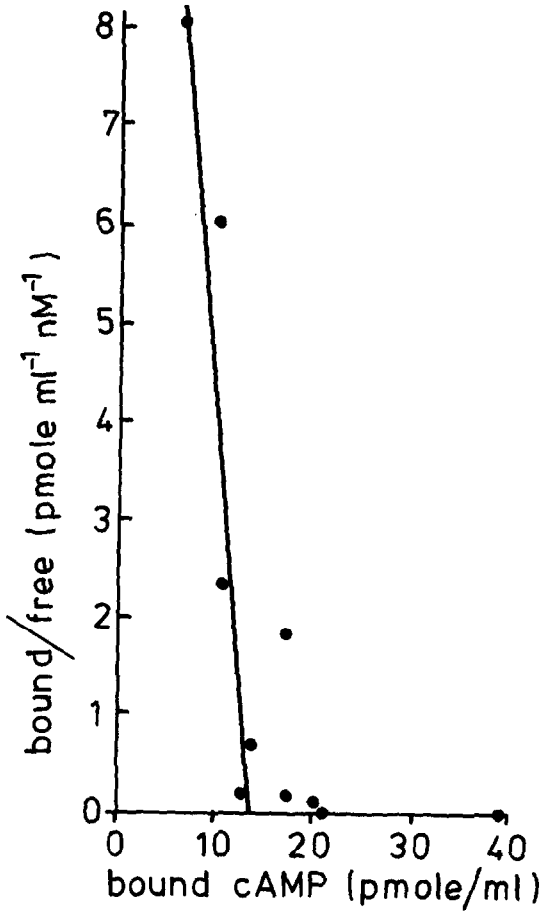

Fig.1. Equilibrium cyclic AMP-binding curve of the soluble cytoplasmic cell fraction (Scatchard plot). The line corresponds to a binding isotherm characterized by a K_d of 0.8×10^{-9} M. One ml extract was derived from 2×10^8 cells.

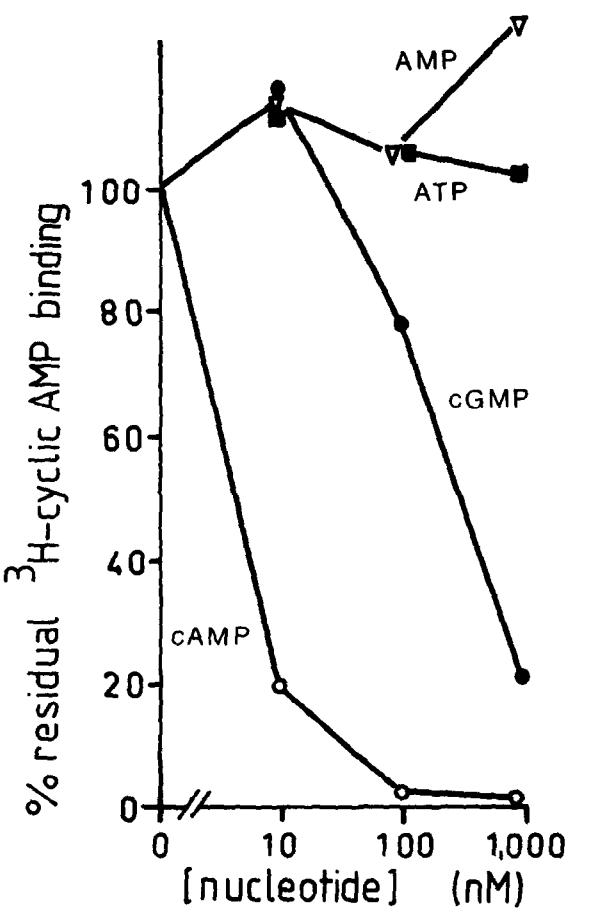

Fig.2. Specificity of cyclic AMP-binding. Cell extract samples were incubated with 10^{-9} M cyclic [^{3}H]AMP plus varying amounts of various nucleotides. The percentage residual binding is plotted against the nucleotide concentration.

that the affinity for cyclic GMP is ~2 orders of magnitude lower than that for cyclic AMP. ATP and 5′-AMP do not compete, even if present in a thousand-fold excess over cyclic AMP (not shown).

All cyclic AMP-binding activity in a cell extract was retained by blue Sepharose (Pharmacia Fine Chemicals, Uppsala), equilibrated with homogenization buffer. The binding activity could be eluted with a salt gradient as a single peak, at 0.6 M NaCl (not shown). This gave ~30-fold purification, the yield being 50–80%. The cyclic AMP-binding activity that was eluted from blue Sepharose was quantitatively bound by 8-(2-aminoethyl)-amino-cyclic AMP–Sepharose (a gift from Dr H. de Jonge, prepared according to [16]). After extensive washing of the affinity gel with buffer containing 1 M NaCl, 2 polypeptides could be specifically eluted with buffer containing 10 mM cyclic AMP. Their app. M_r-values were ~40 000 and 175 000 (fig.3). In some experiments 1–3 additional bands in the 36–40 kM_r range have been observed. We assume that these are degradation products of the 40 kM_r polypeptide. The 40 kM_r polypeptide is completely eluted, the 175 kM_r one only partially. The eluted proteins were separated from free cyclic AMP by gel filtration on Sephacryl-S200 (fig.4). Cyclic AMP-binding activity eluted as a single peak. Gel electrophoresis of the eluted fractions showed a close correlation between cyclic AMP-binding activity and the presence of the 40 kM_r polypeptide. The overall yield of the procedure is 5–10%.

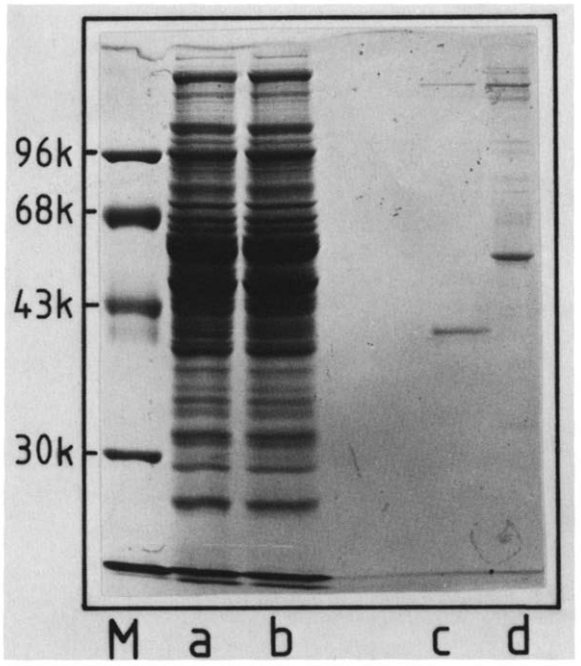

Fig.3. Affinity chromatography on cyclic AMP–Sepharose. Protein composition of: (a) cell extract before incubation with cyclic AMP–Sepharose; (b) cell extract after incubation with cyclic AMP–Sepharose; (c) polypeptides eluted with 1 mM cyclic AMP; (d) polypeptides that remain bound to the affinity gel after cyclic AMP elution; these proteins were solubilized in boiling 1% SDS. Polyacrylamide gel electrophoresis in the presence of SDS. The lane marked M contains marker proteins; their M_r-value is indicated.

Most 40 kM_r protein is lost in the gel filtration step of this procedure, which may be due to the low protein concentration.

4. Discussion

The 40 kM_r protein that is specifically eluted from cyclic AMP–Sepharose is most probably identical with the cytoplasmic 42 kM_r cyclic AMP-binding protein in [13], and the 39 kM_r protein that can be labelled by photo-affinity with 8-azido-cyclic AMP [10]. Photo-affinity labelling of cell extracts used in our experiments with 8-azido-cyclic IMP gave the same result, i.e., labelling of one 40 kM_r polypeptide (U. Walter, R. v. D., unpublished).

The 175 kM_r polypeptide that was only partially eluted from cyclic AMP–Sepharose may correspond to a less specific cyclic AMP-binding protein [13,14] of 185 000 M_r under non-denaturing conditions, and a low affinity for cyclic AMP (K_d ~10^{-6} M). Because cyclic AMP in our assay is only 10 nM, it is unlikely that this low affinity protein contributed significantly to the cyclic AMP-binding activity we measured.

The function of cyclic AMP-binding proteins is of considerable interest. It is expected that such proteins are involved in the control of *D. discoideum* cell development (reviews [1,2]). Many eukaryotic cells

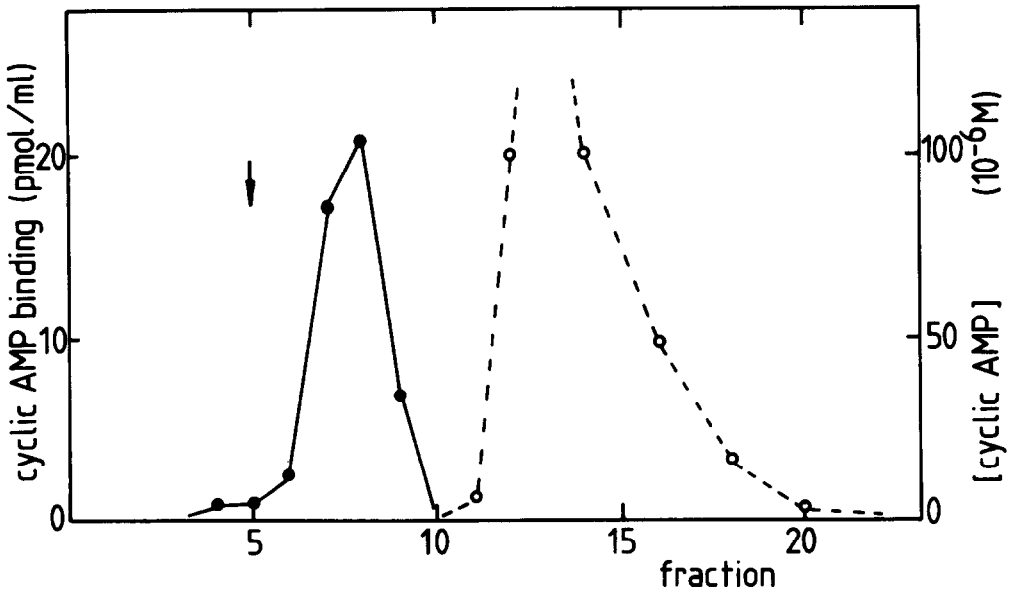

Fig.4. Gel filtration of the proteins that were specifically eluted from cyclic AMP–Sepharose (fig.3(c)): cyclic AMP binding (•——•); cyclic AMP concentration (○– – –○). (→) void volume of the column.

contain cyclic AMP-binding proteins that are regulatory subunits of a cyclic AMP-dependent protein kinase. In [17] cyclic AMP-dependent protein kinase activity was reported in *D. discoideum* extracts. However, this could not be reproduced in [12,18]. We also looked for cyclic AMP-dependent protein kinase activity in cell extracts and cell homogenates, using autoradiography after gel electrophoresis to detect phosphorylated polypeptides. The result was negative. Nevertheless, in [13] the 42 kM_r cyclic AMP-binding protein from *D. discoideum* was shown to control the activity of a mammalian protein kinase in a cyclic AMP-dependent manner. This suggests that the *D. discoideum* binding protein is involved in controlling a protein kinase activity in the cell, but that at present we are unable to detect its substrate.

Acknowledgement

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