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# CHARACTERIZATION OF A 5'-AMP BINDING SITE IN PURIFIED MEMBRANES FROM *DICTYOSTELIUM DISCOIDEUM*

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SUMMARY

Although *D.discoideum* amoebae do not bind AMP at their surface if they are not disrupted, total cell lysates display high levels of AMP binding activity specifically associated with the plasma membrane. The binding of AMP is not competed by adenosine and only poorly by ADP and ATP. The AMP binding sites have a single affinity of 0.6 µM for AMP; the association and dissociation rate constants are respectively $8 \times 10^3$ $sec^{-1}M^{-1}$ and $4.8 \times 10^{-3} sec^{-1}$. The AMP binding occurs at a site distinct from the cAMP binding site and from the catalytic site of a membrane bound enzyme.

The first part of the developmental program of *Dictyostelium discoideum* amoebae can be triggered by starving the cells in liquid suspension (1,2). Among various phenomena it involves the apparition of membrane-bound cAMP receptor sites and of a membrane associated phosphodiesterase activity (3,4). cAMP which plays a dual role as chemoattractant (5,6) and as an inducer of the differenciation (7,8) is periodically synthetized by adenylate cyclase (9); a complex system of phosphodiesterases both intra- and extra-cellular controls the degradation of this signal molecule in AMP. This paper describes the investigation of the binding of AMP to whole *D.discoideum* cells and to their purified plasma membranes.

## MATERIAL AND METHODS

Membrane preparation. Exponentially growing cells of *Dictyostelium discoideum* strain AX2 grown at 22°C in HL5-glu broth (10) were washed and starved at the concentration of $10^7$ cells/ml in phosphate buffer 17mM pH 6.2 Plasma membranes were prepared by

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a slight modification of the procedure of Siu et al (11): The cells (usually competent for agregation), were resuspended in Tris acetate buffer 25mM pH 7.6 containing 1mM EDTA (buffer A) and disrupted with 50 strokes from a B pestle in a Dounce homogenizer at 0°C. The cell lysate ("total crude extract") was centrifuged one hour at 100,000g and the supernatant ("soluble crude extract") was collected. The pellet refered to as "particulate crude extract" was resuspended in a mixture of polyethylene glycol and Dextran T500 in aqueous solution (12). After 10 min centrifugation at 12,000 g the membrane fraction was collected at the interphase. This procedure was repeated two or three times, until the amount of material in the pellet was negligible; purified membranes were washed with buffer A and stored frozen at -20°C. Their purity was checked by electron microscopy.

Binding of AMP to whole *Dictyostelium* amoebae and to purified membranes: Binding of [$^3$H] AMP to undisrupted cells was measured as described (13): cell bound ligand was separated from free ligand by quickly spinning the cells through a layer of silicone oil; the pellet was cut out, disolved and counted.

Binding of labelled nucleotides to purified membranes was measured by a filter assay (14): 20-50 µg of extract were incubated at 0°C in 50 mM potassium phosphate buffer pH 6.4 (buffer B) in the presence of [$^3$H] AMP. After 20 min, the samples were diluted in 2ml ice cold buffer B and immediately filtered through Selectron filters (type BA 85). The filters were quickly washed three times with 2ml of buffer, dried and counted. The entire filtration procedure took no more than 10-15 sec. Background binding of [$^3$H]AMP to the filters was determined for each series of assays and always substracted from binding values. It never exceeded 20% of the radioactivity bound in the presence of the extracts. Every assay was run in duplicate. Protein concentration was measured by the method of Lowry (15).

The analysis by thin layer chromatography of the labelled molecules present in the assay at the end of the incubation showed that [$^3$H]AMP was not degraded during the incubation (less than 0.5%). It was also shown that the radioactivity bound to the filter was due to [$^3$H]AMP itself: for this, the filter was soaked in 2ml buffer B containing 0.1% SDS just after the final wash in the assay procedure; after lyophilization, the powder was resuspended in 0.2ml water and the labelled molecule identified by thin layer chromatography.

## RESULTS AND DISCUSSION

Binding of AMP to *D.discoideum* cells and to purified membranes:

Table 1 shows a comparison of the binding of AMP and cAMP to whole, undisrupted cells at various ligand concentrations. The values for cAMP are in agreement with previous data obtained with agregation competent cells (3,13,16). In contrast to cAMP, there is very little binding of AMP to the cells: even when the concentration was raised to 0.5 µM, no more than 0.06 pmol/$10^7$ cells were bound,

TABLE 1- [$^3$H]AMP and [$^3$H]cAMP binding to aggregation competent *D.discoideum* cells.

| Free Ligand (AMP or cAMP) concentration (μM) | 0.025 | 0.05 | 0.25 | 0.5 |
|---|---|---|---|---|
| AMP bound (pmol/$10^7$ cells) | 0.004 | 0.01 | 0.02 | 0.06 |
| cAMP bound (pmol/$10^7$ cells) | 0.45 | 0.67 | 1.84 | 2.46 |

Binding was measured by centrifugation of the cells through silicone oil (see Methods). The number reported are mean values of at least two measurements. Non specific binding measured in the presence of cold ligand in excess (1mM) has been substracted. Specific radioactivity were 15000 cpm/pmol and 12000 cmp/pmol for [$^3$H]cAMP and [$^3$H]AMP respectively.

which represents more than 50 times less than the cAMP binding capacity. Since the binding test involves short incubation times to prevent cAMP degradation by cell-associated phosphodiesterase (13), it could be that the absence of AMP binding was due to an insuffisant time of incubation of the cells with the ligand. This possibility was eliminated since no better binding activity was obtained when the incubation of the cells with AMP was extended up to 10 min.

In contrast, the AMP binding activity is high in cell lysates, as shown in table 2: The "total crude extract" binds 3.7 pmol/mg which is about 50 times more than the 0.06 pmol/$10^7$ cells measured at the same ligand concentration with undisrupted cells (see table 1). Table 2 also shows a comparison between AMP and adenosine binding to various fractions of the membrane purification procedure: whereas more than 90% of adenosine binding is soluble, 80% of the AMP binding activity is particulate. Purified membranes, which represents about 15% of the "particulate crude extract", are enriched five times in AMP binding activity.

The amount of AMP receptors present in purified membranes is not dependent on the starvation time of the cells from which the

Table 2 - AMP and adenosine binding to crude extracts and purified membranes.

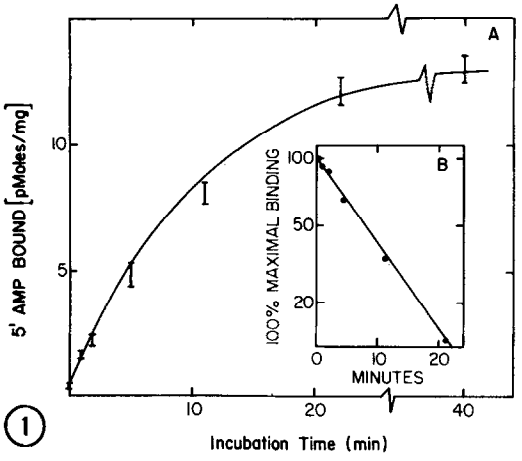
| Fraction | [3]AMP binding (total pmol) | [3H]AMP binding (pmol/mg protein) | [3H]adenosine binding (total pmol) |
|---|---|---|---|
| Total crude extract | 3500 | 3.7 | 3200 |
| Soluble crude extract | 540 | 1.8 | 2970 |
| Particulate crude extract | 3000 | 6 | 280 |
| Purified membranes | 400 | 19 | 27 |

The extracts were incubated at 0°C for 20 min with 0.5 µM [3H]AMP (9000 cpm/pmol) or 0.5 µM [3H] adenosine (15300 cmp/pmol). The assays usually contained 10 µl of extracts in a final volume of 50 µl. Numbers are mean values of duplicate measurements. Background binding has been substracted.

membranes have been prepared; in particular there was no increase at the time of aggregation competence over the vegetative stage (results not shown) in contrast with what is observed with cell-surface cAMP receptor sites (3).

Characterization of AMP binding to purified membranes: Figure 1A shows the kinetics of AMP binding as a function of time. The binding is relatively slow and follows a single exponential, as expected for a simple process if the ligand concentration is high compared to that of the binding sites. Under these conditions, the slope of the line representing the data in semilogarithmic coordinates (part B) is equal to $Lxk_{+1}$ where L is the ligand concentration and $k_{+1}$ the association rate constant of the binding reaction. The data of fig 1 give a value of $K_{+1}=8.3 \times 10^3 sec^{-1}M^{-1}$.

The dissociation rate constant of the AMP receptor complex was measured directly by diluting for increasing periods of time the membranes previously equilibrated with [3H]AMP (figure 2A). The



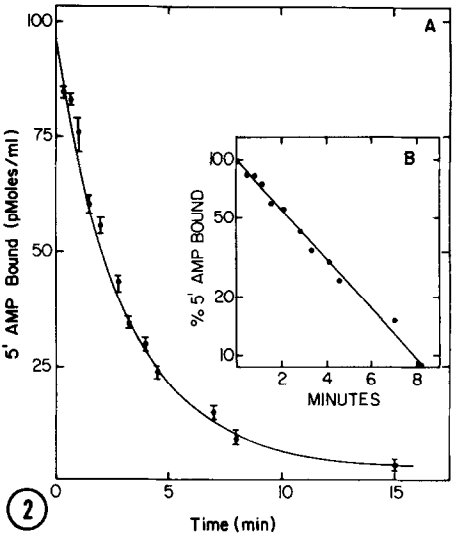




Figure 1 - AMP binding to purified membranes as a function of incubation time.

Part A. 10 µl of purified membranes (3mg/ml) were incubated with 10 µl of [$^3$H] AMP (1µM) at 0°C in a total volume of 50 µl of buffer B for increasing time periods. The incubation was stopped by dilution with 2ml buffer B and immediate filtration. Part B is the semi-logarithmic representation of the data of part A. The ordinate is the difference between the binding of AMP measured at equilibrium (40 min) with the value measured at time t, expressed in percent of value at $t_o$.

Figure 2 - Dissociation of the AMP-receptor complex:

Part A. 0.2ml of purified membrane (3mg/ml) were incubated 30 min at 0°C with 0.8ml AMP (1µM) in buffer B. Samples (50 µl) were withdrawn and diluted in 2ml ice cold buffer B. Dissociation of the complex was allowed to proceed at 0°C for the indicated time periods; the residual amount of AMP bound was measured as usually: the sample of 2ml was filtered, and the filter was counted after washing. Part B. Semi-logarithmic representation of the data of part A.

data give a straight line in semilogarithmic representation (part B) whose slope is equal to the dissociation rate constant $k_{-1}=4.8\times10^{-3}sec^{-1}$. It is important to note that the half life of the AMP-binding site complex is about 2 min; this justifies the use of the filter procedure in the binding assay since the total washing time of the filters never exceeds 15 sec.

Consequently, no more than 10% of bound AMP dissociates during the time of filtration, a value which is within the experimental error of the assay.

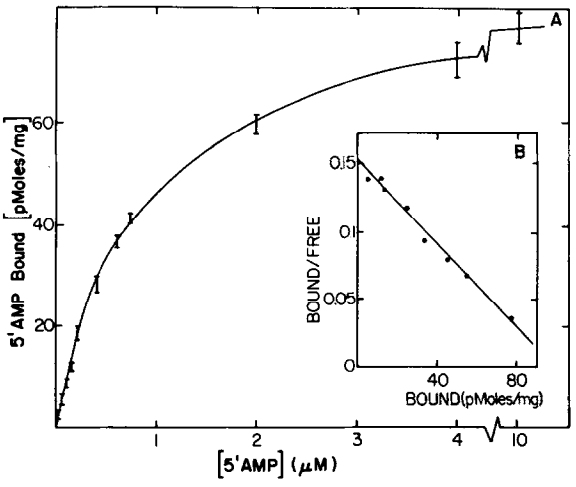



Fig.3. Equilibrium binding curve of AMP to purified membranes

Part A . 10 µl of membrane suspension (2mg/ml) were incubated 30 min at 0°C with increasing amounts of [$^3$H]AMP (8000 cpm/pMole) in a final volume of 50µl of buffer B and filtered. The background value i.e. the amount of radioactivity retained on the filter in the absence of extract was determined for each [$^3$H]AMP concentration and substracted from the total binding at the corresponding concentration. Part B. Scatchard plot of the data of part A. The dissociation constant derived from the slope is $K_D$=0,6µM.

The saturation curve of AMP binding to purified membranes is depicted in figure 3. The binding measurements are done at equilibrium which allows to make a Scatchard plot of the data. Fig 3B shows that this representation gives a straight line yielding to the dissociation constant $K_D$ = 0.6 µM. This parameter could also be calculated directly from the independent measure of $k_{+1}$ and $k_{-1}$ since $K_D = k_{-1}/k_{+1}$ by definition. The data of figures 1 and 2 give a value of $K_D$ = 0.58µM, which is in excellent agreement with the value measured at equilibrium.

The kinetic data as well as the michaelian binding curve are in favor of the presence of only one class of AMP binding sites, in contrast to what has been observed for cAMP receptors (3). AMP is probably not binding at the catalytic site of a membrane bound enzyme since (i) bound AMP is not degraded (ii) the high

affinity of AMP for its receptor complex (2min) would prevent the putative enzyme to have any significant turn over. In itself this argument makes it very unlikely that AMP would bind in the membrane at the product site of phosphodiesterase. This possibility is further eliminated by the observation that the same level of AMP binding activity could be detected in membranes prepared from a phosphodiesterase deffective mutant (results not shown).

The specificity of AMP binding to purified membranes was evaluated by competing [$^3$H] AMP binding with cold competitors. A twenty fold excess of ADP was necessary to reduce by 50% the binding of AMP. ATP was ten times less efficient and adenosine was without effect up to concentrations in the millimolar range. Competition of [$^3$H]AMP binding by cold cAMP was difficult to evaluate since the high level of phosphodiesterase present in the extracts resulted in the production of cold AMP during the incubation of the assay. However, results of assays done under conditions where cAMP hydrolysis was reduced indicate that cAMP is not a good competitor of the AMP binding.

Incubation of membranes with Triton X100 resulted in the solubilization of only a minor part of the binding activity which is still associated to the membrane to the extend of 80% in the presence of as high as 0.5% Triton. Other non-anionic detergents like lubrol and deoxycholate were not more efficient to solubilize the AMP binding activity.

In conclusion, the AMP binding site that has been described appears to be specific and strongly associated with the plasma membrane of the amoebae. Whether it is an authentic receptor protein

or a regulatory site of a membrane bound enzyme will only be decided upon further purification of the AMP binding activity.

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REFERENCES

1. Newell,P.C. (1977) Microbial Interaction 3,3-57.
2. Gerisch,G. and Malchow,D. (1976) in "Advances in Cyclic Nucleotide Research". P.Greengard and G.A.Robison,eds.), vol.7,pp 49-68.
3. Green,A.A. and Newell,P.C. (1975) Cell,6, 129-136.
4. Malchow,D., Nagele,B., Schwartz,H. and G. (1972) Eur.J.Biochem. 28,136-142.
5. Konijn,T.M., Van de Meene,J.G.C., Bonner,J.T. and Barkley,D.S. (1967). Proc.Natl.Acad.Sci.USA 58,1152-1154.
6. Robertson,A.,Drage,D.J. and Cohen,M.M. (1972) Science,N.Y. 175,333-334.
7. Darmon,M., Brachet,P. and Pereira da Silva,L.H. (1975) Proc.Natl.Acad.Sci.USA 72,3163-3166.
8. Gerisch,G., Fromm,M., Huesgen,A. and Wick,U. (1975) Nature 155,547-549.
9. Klein,C., Brachet,P. and Darmon,M. (1977) Febs Letters 76,145-147.
10. Watts,D.J. and Ashworth,J.M. (1970) Biochem.J. 119,171-174.
11. Siu,C.H., Lerner,R.A. and Loomis,W.F. (1977) J.Mol.Biol.116,469-488.
12. Brunette,D.M. and Till,J.E. (1971) J.Membrane Biol.5,215-224.
13. Klein,C. and Juliani,M.H. (1977) Cell,10,329-335.
14. Gilman,A.G. (1970) Proc.Natl.Acad.Sci.USA 67,305-312.
15. Lowry,O.H.,Rosebrough,N.J.Farr,A.K. and Randall,R.J. (1951) Biol.Chem.193,265-275.
16. Malchow,D. and Gerisch,G. (1974) Proc.Natl.Acad.Sci.USA 71,2423-2427.