ENZYME HISTOCHEMICAL–IMMUNOCHEMICAL CHARACTERIZATION OF CYCLIC-ADENOSINE MONOPHOSPHATE PHOSPHODIESTERASE IN *DICTYOSTELIUM DISCOIDEUM* IN GEL MEDIUM

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1. Introduction

When *Dictyostelium discoideum* cells are starved, they undergo aggregating and differentiating processes to produce a multicellular organism. At the aggregation stage, cells interact with each other by chemotaxis to form multicellular associations. In this system, cyclic adenosine monophosphate(cAMP) is excreted rhythmically and plays the role of a chemotactic hormone [1]. These cells excrete also a cyclic adenosine monophosphate phosphodiesterase (cAMP-PDE) which regulates the cAMP signal-to-noise ratio [2] by hydrolyzing the message [3]. Two kinetically different forms of the enzyme have been described [4]. Enzyme activity has been reported to be inhibited by an excreted protein inhibitor which is produced early in the transition from growth to cell aggregation [5].

Although evidence that a membrane bound enzyme regulates the extracellular cAMP level has been presented [6], the extracellular phosphodiesterase also appears to play an important role: Alcantara and Bazill [7] described a secreted factor which accelerates the aggregation of starved cells. It behaves as if it was identical with extracellular phosphodiesterase when compared on DEAE chromotography, gel filtration and electrophoresis. Furthermore, a morphogenetic mutant has been described whose aggregationless phenotype can be completely reversed by addition of exogenous cAMP-PDE [8].

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It is apparent from these studies that the isolation, purification and characterization of the molecular forms of phosphodiesterase would be an interesting avenue of research for understanding the physiological role of these molecules during aggregation of previously independant cells.

Quantitative immunoelectrophoresis [9] combined with histochemical staining designed to demonstrate specific enzyme activity has proved to be particularly helpful in characterization of an enzyme in mixture of antigens [10]. It offers the possibility of measuring changes in biological activity in terms of changes in protein quantity affording a tool for the monitoring of fractionation procedures. Therefore, with this technique it is possible to distinguish between activation of previously synthesized molecules and de novo synthesized molecules [11].

Until now the method used for the location of cAMP-PDE in gel media was based on the loss of fluorescence under ultraviolet light as NADH is oxidized [12]. By this method the enzyme activity shows up as a dark band on a fluorescent background and photographs have to be taken in long wave ultraviolet light. We found this technique not satisfactory and sought a method where cAMP-PDE activity could be revealed by the precipitation of formazan salts in situ [13].

Breckenridge set forth the principles of a technique where cAMP is converted proportionally to glucose-6-phosphate by an enzymic cyclic system. The yield of glucose-6-phosphate can be several thousand-fold greater than the adenylate hydrolyzed [14]. In the present method glucose-6-phosphate is then dehydro-

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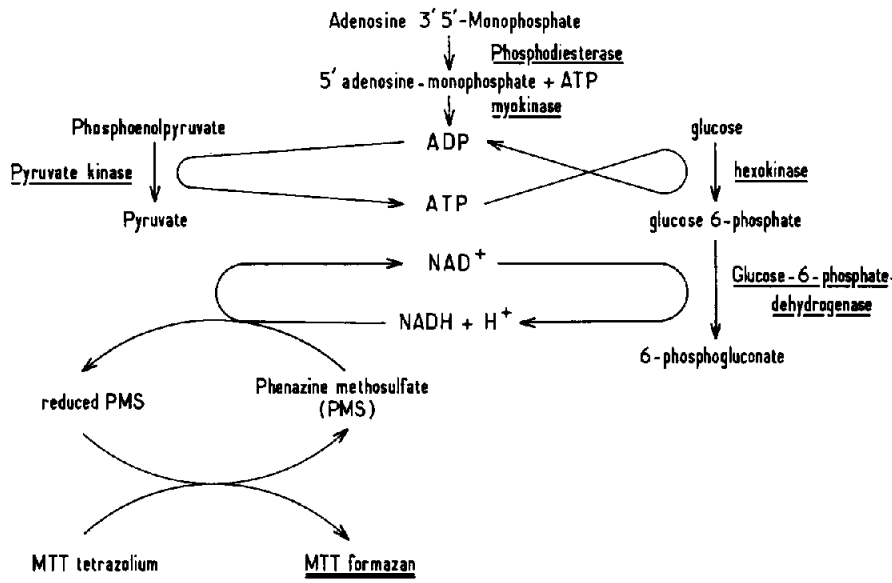

Fig.1. Flow diagram of cascade reaction for staining for AMP-PDE (see experimental procedure). A blue formazan is deposited on immunoprecipitate(s) possessing cAMP-PDE activity.

genated by glucose-6-phosphate dehydrogenase, the NADH formed being reoxidized by a chromogenic system (tetrazolium salt) which involves electron transfer to yield a reduced, insoluble colored product (formazan) (fig.1).

2. Experimental procedure

2.1. *Culture of* Dictyostelium discoideum

To obtain phosphodiesterase the wild type amoebae A X 2 was cultivated in HL5 axenic media [15].

Exponentially growing cultures were harvested, centrifuged and washed twice in 17 mM phosphate buffer, pH 6.2, then incubated in this buffer at a density of 2×10^7 cells/ml in spinner suspensions.

2.2. *Crude preparation containing phosphodiesterase activity*

Supernatant with phosphodiesterase activity was prepared from the starved cell suspensions described above. Cells were removed by centrifugation 6 h after the beginning of the starvation. The supernatant was concentrated 10-fold on XM50 membranes (Amicon, Waltam, MA), sterilized by filtration through 0.45 μm Millipore filters and frozen.

2.3. *Immunizations*

Rabbits were inoculated intradermally with 1 ml of the above 10-fold concentrated supernatant (1 mg/ml protein content) mixed with 1 ml Freund adjuvant. The dose was divided into 5–6 sites on the back skin. Animals were boosted every month with the same amount of supernatant in the ear-vein. Blood was drawn from an ear vein 7 and 10 days after the secondary immunization.

2.4. *Electrophoresis*

Agarose gel (Litex, HSA) was prepared as 1% solutions in Tris–veronal buffer, pH 8.7 (73 mM Tris, 24.5 mM veronal, 0.36 mM calcium lactate and 0.2 mM sodium azide). One-dimensional agarose gel electrophoresis was carried out at 15°C with 40 μl the supernatant described above above for 30 min at 10 V/cm. Crossed immunoelectrophoresis was carried out with the same procedure in the first dimension, and at 8 V/cm for 3.5 h in the second dimension. The second-dimension gel contained 100 μl antiserum/ml 1% agarose layered 1 mm thick on a 5 X 7 cm plate.

After electrophoresis, the gel was pressed, washed, dried and stained for protein with Coomassie brilliant blue R [16].

2.5. *Cyclic AMP phosphodiesterase characterization in gel medium*

After immunoelectrophoresis, the immunoelectropherogram was overlaid with a 1% agarose layer (1 mm thick) containing the reactants for cAMP phosphodiesterase assay, Tris–HCl 0.05 M, pH 7.9, cAMP 1.4 mM, $MgCl_2$ 10 mM, KCl 66 mM, phosphoenol pyruvate 2.8 mM, ATP 4×10^{-6} mM (added to initiate the sequence, as a small fraction of the cAMP hydrolyzed by the cAMP-PDE), glucose 20 mM, hexokinase 10 units/ml, pyruvate kinase 6 units/ml, and myokinase 30 units/ml (fig.1).

Because high salt concentration is very inhibitory to the cyclic reactions [17], the $(NH_4)_2SO_4$ concentration in the commercial enzymes must be minimized by centrifugation or dialysis.

After overnight incubation at room temperature, the upper gel was removed and the immunoelectropherogram covered with the following mixture for 2 h: 0.5 mg/ml NAD, 0.025 mg/ml methyl phenazonium methosulphate, 0.15 mg/ml 2,5-diphenyl-3-(4,5 dimethylthiazolyl-2)-tetrazolium bromide and 2.5 units/ml glucose-6-phosphate dehydrogenase. The gel was then washed, pressed and dried as above.

2.6. *Phosphodiesterase assay*

After one-dimensional agarose gel electrophoresis, the gel was cut into 2 mm pieces which were frozen to break gel structure. After thawing, the enzyme was eluted with 50 mM Tris buffer for cAMP-PDE assay. The enzyme activity was measured by the method based on enzymatic radioisotopic displacement described by Brooker et al. [18] and was expressed in nmol cAMP hydrolyzed/min/piece.

3. Results and discussion

Prior to studying the supernatant from the starving cells by immunoelectrophoresis and enzymatic identification reactions, quantitative precipitin tests were performed in order to find out whether the enzyme retained its biological activity when precipitated with the antibodies, when the 10-fold concentrated supernatant was tested by precipitation with the antiserum (results not shown) up to 95% enzyme activity was found in the precipitate. This indicates that the active enzyme can be totally precipitated by the antibodies and is still active after precipitation with antibodies, suggesting that binding with such macromolecules does not take place at the enzymatic site and does not greatly change the configuration of the enzyme molecule.

The cAMP-PDE activity measured by the radioisotopic displacement technique on the sliced one-dimensional electropherogram is located between slices No. 1 and 7 (fig.2). Enzymatic activity starts just over the well, increasing rather slowly, reaching a maximum toward slices No. 5 and 6, and decreasing sharply at slice No. 7.

After crossed-immunoelectrophoreses and protein staining, the supernatant was shown to be an antigen mixture of several anodically moving proteins (fig.3A). The precipitation line No. 1 and the bell-shaped precipitate No. 2 lay between slices No. 1 and 7 where enzymatic activity was found on the sliced one-dimensional electropherogram. The immunoprecipitate No. 2 exhibited a foot on the side of the ascendent slope. Further from the cathode there was another group of precipitation lines not corresponding to any cAMP-PDE activity.

The same plate stained for cAMP-PDE activity (fig.3B) showed showed only one peak which was located exactly at the same place as the enzyme activity determined by the radioisotopic displacement method on the sliced one-dimensional electropherogram (slices

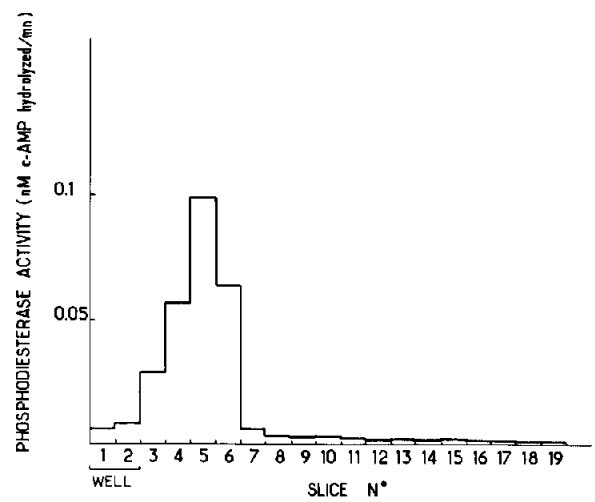

Fig.2. Phosphodiesterase activity of the sliced first-dimension electropherogram by the radioisotopic displacement method.

Fig.3A

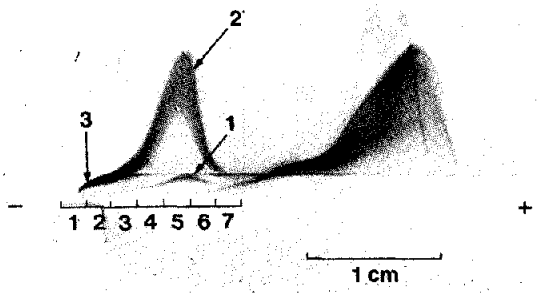

Fig.3B

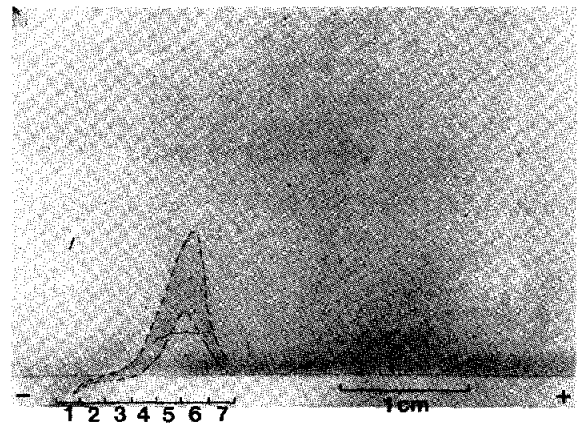

Fig.3. Enzymic characterization of immunoprecipitate obtained on crossed immunoelectrophoresis of excreted proteins from *Dictyostelium discoideum* starving cells. Analysis performed with antisera elicited against the crude extract. The gel was stained with Coomassie brilliant blue (A) and for cAMP-PDE activity (B). (The bar represents 1 cm.)

No. 1–7); it was at the same place and showed the same form as the protein-stained precipitate No. 2, the bell-shape form indicating an individual homogenous enzyme precipitate [19] and the foot could be enzyme aggregation of some kind (T. C. Bøg-Hansen personal communication).

In the conditions and with the techniques I have used, the study of cAMP-PDE activity, excreted after 6 h starvation by *Dictyostelium discoideum* amoebae wild strain AX2, leads to the conclusion that this supernatant contains at least two forms of active enzyme depending on the aggregation degree of the molecules.

In other species and in different tissues multiple forms of cAMP-PDE have been suggested by several lines of evidence: Some isoenzymes can be selectively inhibited by pharmacological agents [20] others have different abilities to combine with endogenous calcium dependent activators [21] some molecules have an allosteric site that can be influenced by GMP [22]. Furthermore, they exhibit different properties such as pH optimum, heat stability, cellular distribution, substrate affinity and so on.

We believe the method we have been developing for the analysis of cAMP-PDE of *Dictyostelium discoideum* to be applicable as well to the study of these isoenzymes.

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References

[1] Loomis, W. F. (1975) in: *Dictyostelium discoideum*, Academic Press, New York.
[2] Shaffer, B. M. (1956) J. Exptl. Biol. 33, 645–657.
[3] Chang, Y. Y. (1968) Science 160, 57–59.
[4] Chassy, B. M. (1972) Science 175, 1016–1018.
[5] Riedel, V. and Gerisch, G. (1971) Biochem. Biophys. Res. Commun. 42, 119–124.
[6] Malchow, D., Fuchila, J. and Nanjundiah, V. (1975) Biochem. Biophys. Acta 385, 421–428.

[7] Alcantara, F. and Bazill, G. W. (1976) J. Gen. Microbiol. 92, 351–368.
[8] Brachet, P., Barra, J., Darmon, M. and Barrand, P. (1977) in: Developments and Differentiation in the Cellular Slime Moulds (Cappuccinelli and Ashworth, eds) pp. 125–133, Elsevier/North-Holland, Amsterdam.
[9] Clarke, H. G. M. and Freeman, T. A. (1967) in: Prot. Biol. Fluids, 14 (Peeters, H. ed) Elsevier, Amsterdam.
[10] Owen, P. and Smyth, C. J. (1976) in: Immunochemistry of Enzymes and Their Antibodies (Salton, M. R. J. ed) John Wiley and Sons, New York.
[11] Bøg-Hansen, T. C. and Daussant, J. (1974) Anal. Biochem. 61, 522–527.
[12] Monn, E. and Christiansen, R. O. (1971) Science 173, 540–542.
[13] Markert, C. L. and Møller, F. (1959) Proc. Natl. Acad. Sci. USA 45, 753–763.
[14] Breckenridge, B. M. (1964) Proc. Natl. Acad. Sci. USA 52, 1581–1586.
[15] Watts, D. J. and Ashworth, J. M. (1970) Biochem. J. 119, 171.
[16] Weeke, B. (1973) Scand. J. Immunol. 2, Suppl. 1, 15–36.
[17] Goldberg, N. D., O'Toole, A. G. and Haddox, M. K. (1972) Adv. Cyclic N. Res. 2, 63–80.
[18] Brooker, G., Thomas, L. J. and Appleman, M. M. (1968) Biochemistry 7, 4177–4181.
[19] Brogren, C. H. and Bøg-Hansen, T. C. (1975) Scand. J. Immunol. 4, Suppl. 2, 37–51.
[20] Weiss, B., Fertel, R., Figlin, R. and Uzunov, P. (1974) Mol. Pharmacol. 10, 615–625.
[21] Uzunov, P. and Weiss, B. (1972) Biochim. Biophys. Acta 284, 220–226.
[22] Hait, W. N. and Weiss, B. (1977) Biochim. Biophys. Acta 497, 86–100.