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THE HYDROPHOBIC CHARACTER OF THE MEMBRANE-BOUND PHOSPHODIESTERASE FROM *DICTYOSTELIUM DISCOIDEUM*

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Summary

A phosphodiesterase activity is shown to copurify with the plasma membrane fraction prepared by the two-phase partition method. The enrichment in phosphodiesterase parallels that of alkaline phosphatase, which is thought to be a typical membranous enzyme. Up to 66% of the phosphodiesterase activity can be solubilized by a treatment with 0.2% Triton X-100. Higher doses were ineffective in solubilizing more activity. Analysis by native gel electrophoresis showed that an activity extracted by 2 M NaCl migrated at the same position as 'soluble' phosphodiesterase of cytosolic or extracellular origin. In contrast, the Triton-solubilized enzyme had an apparently higher molecular weight. When subjected to charge shift electrophoresis on agarose gels in the presence of an ionic detergent, the Triton-solubilized phosphodiesterase displayed a hydrophobic character. This behaviour contrasts with that of 'soluble' phosphodiesterases, the electrophoretic mobility of which is unaffected by the presence of an anionic detergent. The hydrophobic character of the membranous enzyme was lost after gentle hydrolysis by papain.

Introduction

The developmental program of *Dictyostelium discoideum* amoebae is triggered by food deprivation. The first stage of this differentiation cycle consists of the aggregation of the population toward specific centers. Successive cell movements result from periodic emissions and receptions of cyclic AMP, a chemotactic mediator [1]. These signals are generated by periodic activations of adenylate cyclase activity [2,3]. Phosphodiesterase plays an important role in the process of cell communication since continuous hydrolysis of cyclic

AMP is required to keep the cells sensitive to successive signals [4—6].

High phosphodiesterase activities are found during the first hours of starvation as well as during aggregation [7]. The enzyme is in part excreted into the extracellular medium [8,9]. This activity, however, may be depressed, due to the excretion of a specific macromolecular inhibitor [10]. The cell-associated phosphodiesterase is found both as a 'soluble' cytosolic form and as a particle-bound enzyme [7,9]. Cytochemical and biochemical data have shown that some of this latter activity is closely associated with the plasma membrane and may be attached to the outer face of the membrane [4,11,12].

This membrane-bound phosphodiesterase exhibits properties different from the extracellular phosphodiesterase. For example, it displays non-Michaelian kinetics with respect to cyclic AMP, and a greater resistance to the inhibitor [4,7].

In an attempt to characterize the membrane-bound phosphodiesterase as a membrane-anchored protein, we have investigated the copurification of the activity with plasma membranes and, afterwards, compared its hydrophobic nature with the nature of the extracellular enzyme.

Materials and Methods

1. Chemicals

The materials used and their sources are listed: cyclic AMP (Calbiochem); cyclic [^{3}H]AMP, specific activity 25 Ci/mmol, from CEA (Saclay); Dextran T500 (Pharmacia); poly(ethylene glycol) Carbowax 6000 from Union Carbide Corp.; Magnesium-Titriplex (ethylenediaminetetraacetic acid dipotassium-magnesium salt) and Triton X-100 (octylphenoxy polyethoxyethanol) from Sigma; deoxycholic acid and sodium salt (Sigma); Agarose (Indubiose) (Industrie Biologique Française); papain (Boehringer, Mannheim); *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, trypsin from hog pancreas (Sigma); pancreas (Calbiochem); *p*-nitrophenylphosphate, *p*-nitrophenyl-α-D-mannopyranoside from Calbiochem.

2. Culture conditions

Exponentially growing cells of axenic strain AX2 were harvested from HL5 * medium [13] by centrifugation at 1000 × *g* for 5 min and washed with 17 mM potassium phosphate buffer, pH 6.2. Unless otherwise indicated, the amoebae were starved as a spinner suspension in the buffer at a density of 10^7 cells/ml until they had developed aggregative properties. In order to promote aggregation and to obtain maximum phosphodiesterase activity during starvation, cells were pulsed with exogenous cyclic AMP as described previously [14,15].

3. Preparation of membranes

Amoebae were resuspended in potassium phosphate buffer at a density of 10^8 cells/ml and disrupted at 4°C in a Dounce homogenizer by 50 strokes of a tight-fitting pestle. Efficiency was monitored by means of phase-contrast microscopy. Unbroken cells were removed from the lysate by centrifugation at

* Composition of medium: 10 g proteose peptone (Difco), 5 g yeast extract (Oxoid), 0.35 g Na_2HPO_4, 0.35 g KH_2PO_4, 10 g glucose and 1 l water. pH = 6.4—6.6

500 × *g* for 5 min. The suspension of disrupted cells was next centrifuged for 15 min at 7800 × *g*. The resulting pellet was washed with potassium phosphate buffer. Phosphodiesterase activity assayed in these disrupted cell particles was called 'particle-bound' phosphodiesterase. From this material plasma membranes were purified according to the method of Brunette and Till [16] as modified by Siu et al. [17].

After centrifugation at 10 000 × *g* for 10 min, plasma membranes were found at the interface of the two-phase system formed by Dextran and poly-(ethylene glycol). New extractions were made until the pellet was negligible. The plasma membranes were then washed twice in potassium phosphate buffer. Each step of the preparation was controlled by electron microscopy. The pure plasma membranes obtained showed regular vesicular structures with rare dense material and no recognizable contaminant.

4. Extraction of membrane-bound phosphodiesterase activity with NaCl or Triton X-100

Membranes were resuspended in potassium phosphate buffer supplemented with NaCl or/and Triton X-100 at the desired concentrations (see text), incubated at room temperature for 30 min and then centrifuged at 20 000 × *g* for 30 min. The supernatants were dialysed with three changes for 24 h at 4°C against 1000 volumes of potassium phosphate buffer. The crude preparations of extracellular or cytosolic phosphodiesterases were treated as above in control experiments involving these activities.

5. Preparations of extracellular and cytosolic phosphodiesterases

The starvation medium of aggregation phase cells, pulsed with cyclic AMP as described in section 2, was used as a crude preparation of extracellular phosphodiesterase.

The cytosolic phosphodiesterase came from cells starved for 1.5 h without pulses. Such preparations gave maximal specific activities. The crude supernatant of the 7800 × *g* centrifugation (see section 3) was recentrifuged for 1 h at 20 000 × *g* and called cytosolic phosphodiesterase.

6. Sample conservation

Extracellular phosphodiesterase, cytosolic phosphodiesterase and membrane-bound phosphodiesterase extracts were sterilised by filtration through Millipore filters (0.45 μm) and kept at 4°C. When used for gel electrophoresis, samples were concentrated by freeze-drying.

7. Polyacrylamide gel electrophoresis

Samples were adjusted to 0.8% Triton X-100 and allowed to equilibrate overnight. The migration was performed according to Davis [18] in a separating gel of 6% acrylamide containing 0.2% Triton X-100. After electrophoresis at 2 mA/gel, tube gels were cut into 2-mm fractions. Phosphodiesterase was eluted from the slices by incubation in potassium phosphate buffer for 60 h at 4°C.

8. Charge shift electrophoresis in agarose gels

Comparison was made between the migration of samples in the presence of

0.8% Triton X-100 alone or in the presence of 0.8 Triton X-100 plus 0.4% deoxycholate as described by Helenius and Simons [19]. Electrophoresis was carried out on glass plates overlaid with 1% agarose [20] with a running buffer composed of 73 mM Tris-HCl, pH 8.7/25 mM sodium barbital/0.2 mM sodium azide and a detergent concentration 4-fold less than in the samples. Gels were run at 8 V/cm during 6 h, then cut in 2-mm fractions which were eluted for 24 h at 4°C in 1 ml of potassium phosphate buffer.

9. Treatment with proteolytic enzymes

The conditions of gentle hydrolysis were essentially as described by Parish et al. [21]. Samples containing the desired concentrations of detergent(s) used for the charge shift electrophoresis were treated with 70 μg/ml papain, 1.5 mg/ml trypsin or 1.5 mg/ml pronase. After a 30 min incubation at 22°C, they were immediately electrophoresed in agarose gels as described above.

10. Enzyme assays

Cyclic AMP phosphodiesterase was assayed by the radioisotopic procedure of Brooker et al [22] modified by Malkinson and Ashworth [23]. The incubation mixture contained 10^{-4} M unlabeled cyclic AMP and 0.5 μCi cyclic [^{3}H]-AMP.

N-Acetyl glucosaminidase, alkaline phosphatase and α-mannosidase activities were tested as described previously [24—26] by the colorimetric measure of the *p*-nitrophenol formed.

For all these assays, 1 unit of activity represents the amount of enzyme hydrolysing 1 nmol substrate/min of incubation at 30°C. Protein determinations were performed as described by Lowry et al. [27] using bovine serum albumin as the standard. Specific activities are expressed as units/mg protein.

Results

1. Activity assayed in membrane preparation

Comparison between particle-bound phosphodiesterase activity and phosphodiesterase activity assayed on purified plasma-membranes showed that 86% was lost during the procedure. However, the purification of membranes allowed a 5—10-fold enrichment in specific activity. Identical results were found for alkaline phosphatase. Some *N*-acetyl glucosaminidase activity was found in the suspension of Dounce-disrupted cells: 99% of it was lost during membrane purification. We noticed phosphodiesterase activity in the supernatants of membrane washings. It varied with the incubation time of membranes in the buffer, but never exceeded 10% of the activity measured in the membrane suspension.

2. Solubilization of membrane-bound phosphodiesterase with Triton X-100

A maximum of 66% of the phosphodiesterase activity associated with the membranes could be solubilized by 0.2% Triton X-100. Treatment of the membranes with higher concentrations (up to 1%) of detergent, whether or not preceded by a sonication of the preparation, did not liberate the remaining 34%. The unsolubilized membrane pellet, as seen by electron microscopy, contained

few or no vesicles. In contrast to the membrane-bound phosphodiesterase, *N*-acetyl glucosamidase and α-mannosidase activities were entirely solubilized by 0.2% detergent.

3. Differential extraction of membrane-bound phosphodiesterase activity

The stepwise extraction of purified membranes is shown in Table I. Unlike membrane-bound phosphodiesterase or alkaline phosphatase, almost all the *N*-acetyl glucosaminidase activity is removed with 2 M NaCl plus 0.05% Triton X-100. The total amount of membrane-bound phosphodiesterase solubilized (66%) is comparable to that obtained with a single treatment with 0.2% detergent.

4. Electrophoretic mobility of the solubilized membrane-bound phosphodiesterase activities

The membrane-bound phosphodiesterase was solubilized by three successive extractions: 2 M NaCl, 0.05% Triton X-100 and 0.2% Triton X-100. The three resulting extracts were adjusted to 0.8% Triton X-100 and subjected to native polyacrylamide gel electrophoresis in order to compare the migration pattern of the solubilized phosphodiesterases. Two 'soluble' phosphodiesterases, the extracellular and cytosolic forms, were used as controls.

Both 'soluble' activities migrated as a single peak identical in position (Fig. 1A). The electrophoretic profiles of extracted plasma-membrane activities resolved into two peaks (Fig. 1B). The activity extracted by high salt strength (2 M NaCl) migrated principally as a peak at fraction 12, which corresponds approximately to the position of 'soluble' phosphodiesterase, and a minor peak at fraction 5. The relative size of these two peaks was altered as the concentration of Triton X-100 was increased and the extract became enriched in the slow-moving form of phosphodiesterase (Fig. 1B).

TABLE I

DISTRIBUTION OF SOME ENZYME ACTIVITIES EXTRACTED BY SUCCESSIVE TREATMENTS OF PURIFIED PLASMA MEMBRANES

The sample of membranes contained initially 4 mg/protein per ml. Incubation was done as described in Materials and Methods. In this experiment, 100% of activity corresponds to 70 units of phosphodiesterase, 17 units of alkaline phosphatase, 6 units of *N*-acetyl glucosaminidase/mg protein.

Activity assayed in:	Percentage of activity		
	Phospho-diesterase	Alkaline phosphatase	*N*-Acetyl glucosamidase
Plasma membranes before treatment	100	100	100
Supernatant of the treatment by 2 M NaCl mixed with 0.05% Triton	21	14	85
Supernatant of the wash with potassium phosphate buffer	9	7	1
Supernatant of the treatment by 0.2% Triton	36	31	7
Total extracted	66	52	93

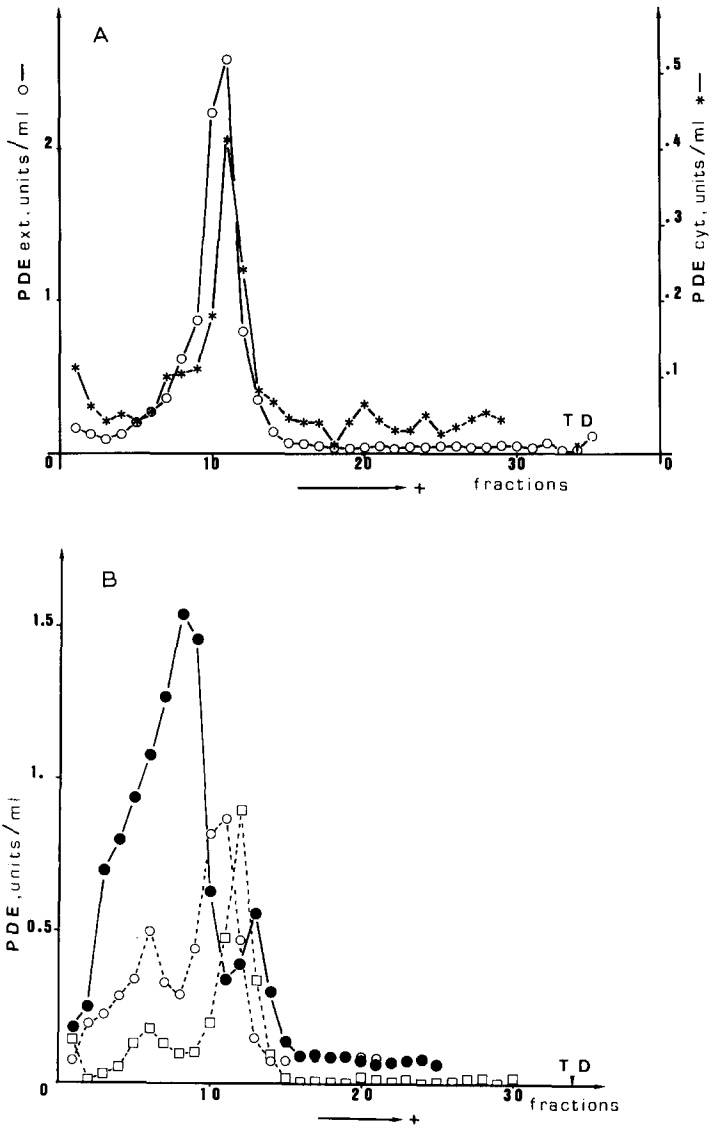

Fig. 1. Polyacrylamide gel electrophoresis of crude 'soluble' samples and extracted supernatants of purified plasma membranes. A. ○, extracellular activity (PDE ext); *, cytosolic activity (PDE cyt). B. Membrane-bound phosphodiesterase activities: the purified plasma membranes were submitted to three successive extractions by 2 M NaCl 0.05% Triton, 0.2% Triton, and washed twice with potassium phosphate buffer between these treatments. The supernatants were treated as described in Materials and Methods. □, Activity of the 2 M NaCl extract; ○, activity of the 0.05% Triton extract; ●, activity of the 0.2% Triton extract. TD, tracking dye.

5. The hydrophobic nature of membrane-bound phosphodiesterase

The method of Helenius and Simons [19] takes advantage of the fact that amphiphilic-membrane proteins can bind large amounts of ionic detergents which in turn modify their net charge.

These experiments were performed with a membrane-bound phosphodi-

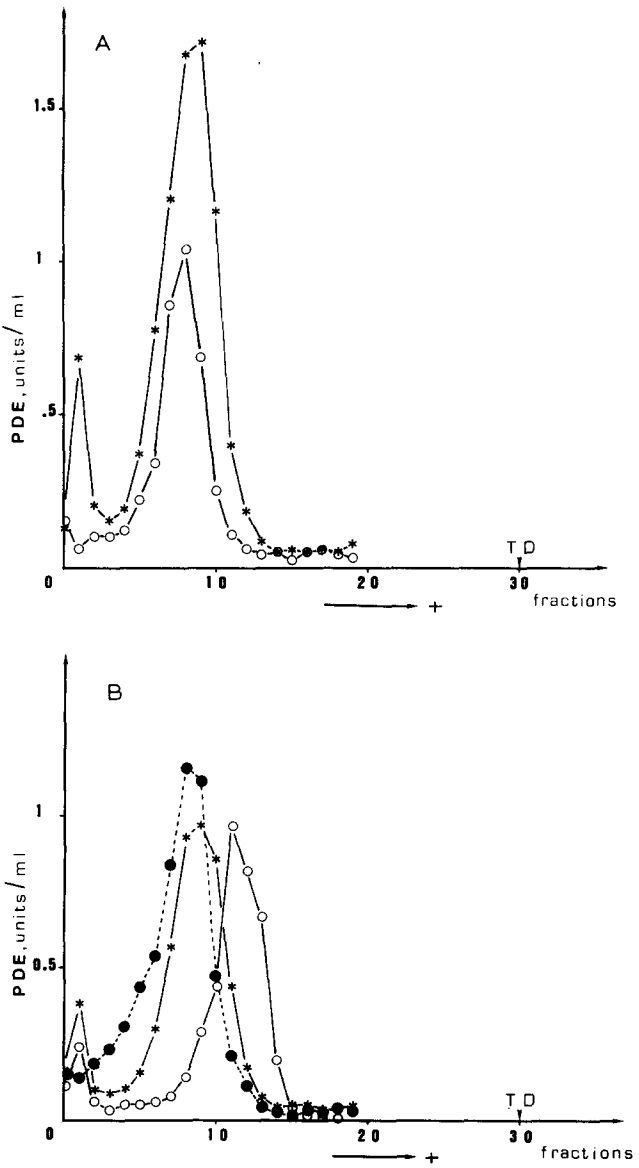

Fig. 2. Agarose gel electrophoresis of: A, Extracellular phosphodiesterase (PDE) activity. ★, migration profile in Triton X-100; ○, migration profile in deoxycholate. B, Membrane-bound phosphodiesterase (PDE) activity extracted by 0.2% Triton X-100 (after treatment with 0.05% Triton supplemented with 2 M NaCl). ★, migration profile in Triton X-100; ○, migration profile in deoxycholate; ●, migration profile in deoxycholate after a partial hydrolysis of the sample by papain. TD, tracking dye.

esterase extracted by 0.2% Triton X-100 after a treatment of the membranes with 2 M NaCl supplemented with 0.05% Triton X-100. The phosphodiesterase preparations were electrophoresed on an agarose support in the presence either of the neutral detergent Triton X-100 or the anionic detergent, deoxycholate. Comparison of the migration patterns presented in Fig. 2A showed that deoxy-

cholate did not affect the electrophoretic mobility of either of the 'soluble' phosphodiesterases.

In contrast, membrane-bound phosphodiesterase showed a shift toward the anode when migrated in deoxycholate (Fig. 2B). This shift was consistently reproduced but the size of the displacement varied slightly from one membrane preparation to another.

Since these experiments were performed with crude membrane extracts the migration pattern of the extracellular phosphodiesterase was examined in an environment similar to that of the membrane-bound phosphodiesterase. Addition of a Triton extract from vegetative cell membranes, which contain very little membrane-bound phosphodiesterase, did not affect the electrophoretic mobility of the extracellular phosphodiesterase.

6. Loss of the hydrophobic character of solubilized membrane-bound phosphodiesterase

The samples of membrane-bound phosphodiesterase and of extracellular phosphodiesterase were gently treated by proteolytic enzymes. Treatment of the preparations by trypsin or pronase slightly increased phosphodiesterase activity. On agarose gels, membrane-bound phosphodiesterase and extracellular phosphodiesterase activities resolved as one or several peaks the position of which varied from one experiment to another.

The action of papain, did not affect the phosphodiesterase activity. The proteolytic enzyme has no noticeable effect on the electrophoretic mobility of the extracellular phosphodiesterase. In contrast, after a partial hydrolysis by papain, the membrane-bound phosphodiesterase adopted the same behaviour as the extracellular phosphodiesterase: no shift in the migration was observed when deoxycholate was added (Fig. 2B). Therefore, partial proteolysis of the membrane-bound phosphodiesterase sample by papain suppressed the hydrophobic character observed without this treatment.

Discussion

The phase-partition method was reported by several investigators to provide a valuable tool for the purification of plasma membranes of *D. discoideum* [17, 28]. Use of this technique on cells which had developed aggregative properties has demonstrated a copurification of phosphodiesterase activity with the membranes. Purified membranes were markedly enriched in enzymatic activity. The specific activity of the phosphodiesterase was increased by a factor of 5—10 over the starting homogenate. This copurification of the phosphodiesterase with plasma membrane parallels strikingly that of alkaline phosphatase, which is considered a typical membrane enzyme [17,29—31]. In agreement with Ono et al. [28] we have detected, in purified plasma membranes, the presence of α-mannosidase and *N*-acetyl glucosaminidase which may indicate some disruption of lysosomal vesicles [26,32].

The similar behaviour of membrane-bound phosphodiesterase and alkaline phosphatase is not only observed during the purification of membranes. Comparable amounts are liberated during different procedures of extraction. In order to remove extrinsic proteins [33], the purified membranes were incu-

bated with 2 M NaCl supplemented with 0.05% Triton X-100. This treatment caused solubilization of almost all of the *N*-acetylglucosaminidase or α-mannosidase activities. Only 20% of the membrane-bound phosphodiesterase or alkaline phosphatase activities were solubilized under these conditions, and a little more enzyme was liberated during the wash with potassium phosphate buffer (Table I). A maximum amount of 66% of the initial phosphodiesterase activity was extracted by 0.2% Triton with or without previous incubation with 2 M NaCl. Higher doses of detergent as well as sonication of the preparation were unable to detach the remaining 34% of the phosphodiesterase activity. These data indicate that both activities are more or less firmly associated with the membranes.

Heterogeneity of the phosphodiesterase was directly demonstrated by fractionating the solubilized proteins with native polyacrylamide gel electrophoresis. In these crude extracts, the presence of the enzyme in the gel can only be observed by its activity. The membrane-bound phosphodiesterase activity extracted by 2 M NaCl showed a major peak with an electrophoretic mobility comparable to that of the crude extracellular phosphodiesterase sample. The enzyme extracted with 0.05% Triton resolved in two peaks. The slow-moving material was further enriched and largely prominent when the enzyme solubilized with 0.2% Triton was run on the gel. This heavier form may be a distinct molecular species more firmly associated with the membrane. Alternatively, the high Triton concentration might solubilize aggregates of the same molecular entity or of several different proteins. Partially interconvertible low and high molecular weight phosphodiesterase species were described previously for the extracellular enzyme [34].

The study of the hydrophobic character by charge shift electrophoresis as described by Helenius and Simons [19] was performed with a membrane-bound phosphodiesterase extracted by 0.2% Triton X-100 and the fractionation was achieved on agarose gels. It seems that the two forms observed on polyacrylamide gels bear the same net charge because only one peak of activity is detected with agarose gels. The peak observed in the presence of Triton X-100 is displaced towards the anode when deoxycholate is added. According to the principle of the method this shift is interpreted as the result of detergent binding on a hydrophobic domain, a domain which does not seem to be present on the 'soluble' extracellular phosphodiesterase molecule (Fig. 2A). Compared with that of the T4 membranous complex of *D. discoideum* (Bordier et al. [33]) the shift obtained here is small.

The charge modification due to the binding of the anionic detergent is only proportional to the amount of the hydrophobic region accessible to deoxycholate. The small binding can be due to the configuration of the protein or to the procedure of extraction. If solubilization has caused formation of aggregates, hydrophobic sequences could be at the inside of the structure associated to other hydrophobic molecules and thus poorly accessible to the detergent.

One can imagine that the membrane-bound phosphodiesterase, similarly to cytochrome b_5 [35], bears a short hydrophobic sequence permitting its anchorage into the membrane. This configuration could be consistent with the preservation of the phosphodiesterase activity and with the loss of the hydrophobic character following partial hydrolysis by papain, since the junction with

the hydrophobic sequence is preferentially the point of cleavage by proteolytic enzyme [36]. Loss of this hydrophobic sequence would minimize the binding of deoxycholate, thus preventing an alteration of the net charge of the molecule in the presence of this detergent. Preliminary experiments support to some extent this hypothesis, since limited papain digestion of membranes results in the extraction of some phosphodiesterase activity.

One cannot exclude, however, that the membrane-bound phosphodiesterase is tightly associated with a hydrophobic molecule(s) which confers to the complex its hydrophobic character, as observed in the case of membrane-bound penicillinase [37]. Only studies with the purified enzyme could lead to an unambiguous answer.

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References

1 Konijn, T.M., Barkley, D.S., Chang, Y.Y. and Bonner, J.T. (1968) Am. Nat. 102, 225—233
2 Roos, W., Scheidegger, C. and Gerisch, G. (1977) Nature 266, 259—261
3 Klein, C., Brachet, P. and Darmon, M. (1977) FEBS Lett. 76, 145—147
4 Malchow, D., Fuchila, J. and Nanjundiah, V. (1975) Biochim. Biophys. Acta 385, 421—428
5 Alcantara, F. and Bazill, G.W. (1976) J. Gen. Microbiol. 92, 351—368
6 Darmon, M., Barra, J. and Brachet, P. (1978) J. Cell. Sci. 31, 233—243
7 Malchow, D., Nägele, B., Schwartz, H. and Gerisch, G. (1972) Eur. J. Biochem. 28, 136—142
8 Chang, Y.Y. (1978) Science 161, 57—59
9 Pannbaker, R.G. and Bravard, L.J. (1972) Science 175, 1014—1015
10 Riedel, V. and Gerisch, G. (1971) Biochem. Biophys. Res. Commun. 42, 119—124
11 Rossomando, E.F. and Cutler, L. (1975) Exptl. Cell. Res. 95, 67—87
12 Farnham, C.J.M. (1975) Exptl. Cell. Res. 91, 36—46
13 Watts, D.J., and Ashworth, J.M. (1970) Biochem. J. 119, 171—174
14 Darmon, M., Brachet, P. and Pereira da Silva, L.H. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3163—3166
15 Darmon, M., Barrand, P., Brachet, P., Klein, C. and Pereira da Silva, L. (1977) Dev. Biol. 58, 174—184
16 Brunette, D.A. and Till, J.E. (1971) J. Membrane Biol. 5, 215—224
17 Siu, C.-H., Lerner, R.A. and Loomis, W.F. (1977) J. Mol. Biol. 116, 469—488
18 Davis, B.J. (1964) Ann. New York Acad. Sci. 121, 404—427
19 Helenius, A. and Simons, K. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 529—532
20 Nivet, C. (1977) FEBS Lett. 84, 174-177
21 Parish, R.W., Schmidlin, S. and Müller, U. (1977) Exptl. Cell. Res. 110, 267—276
22 Brooker, G., Thomas, C.J. and Appleman, M.M. (1968) Biochemistry 7, 4177—4181
23 Malkinson, A.M. and Ashworth, J.M. (1973) Biochem. J. 134, 311—319
24 Loomis, W.F. (1969) J. Bacteriol. 97, 1149-1154
25 Loomis, W.F. (1969) J. Bacteriol. 100, 417—422
26 Loomis, W.F. (1970) J. Bacteriol. 103, 375—381
27 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
28 Ono, K.I., Ochiai, H. and Toda, K. (1978) Exp. Cell Res. 112, 175—185
29 Green, A. and Newell, P.C. (1974) Biochem. J. 140, 313—322
30 Lee, A., Chance, K., Weeks, C. and Weeks, G. (1975) Arch. Biochem. Biophys. 171, 407—417
31 Parish, R.W. amd Pelli, C. (1974) FEBS Lett. 48, 293—296
32 Wiener, E. and Ashworth, J.M. (1970) Biochem. J. 118, 505—512
33 Bordier, C., Loomis, W.F., Elder, J. and Lerner, R. (1978) J. Biol. Chem. 253, 5133—5139

34 Dicou, E.L. and Brachet, P. (1979). Biochim. Biophys. Acta 578, 232—242
35 Spatz, L. and Strittmatter, P. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 1042—1046
36 Springer, T.A. and Strominger, J.L. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 2481—2485
37 Sawai, T., Crane, L.J. and Lampen, J.O. (1973) Biochem. Biophys. Res. Commun. 53, 523—530