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A COMPARISON OF THE MEMBRANE-BOUND AND EXTRACELLULAR CYCLIC AMP PHOSPHODIESTERASES OF *DICTYOSTELIUM DISCOIDEUM*

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The *Dictyostelium discoideum* membrane-bound and extracellular cyclic nucleotide phosphodiesterases (EC 3.1.4.17) share several properties including the ability to react with a specific glycoprotein inhibitor and small inhibitory molecules. We have partially purified the membrane-bound enzyme and compared its properties to those of the extracellular form. The kinetic properties of the two forms were similar except that, while associated with membrane particles, the membrane-bound form exhibited non-linear kinetics when assayed over a broad substrate range. The isoelectric point of the membrane-bound phosphodiesterase was identical to that of the extracellular enzyme when isoelectrofocusing was done in the presence of 6 M urea. The molecular weights of membrane-bound and extracellular enzyme, determined by gel filtration, were the same following isoelectrofocusing in the presence of 6 M urea. When precipitated with an antiserum prepared against purified extracellular phosphodiesterase, the partially purified membrane-bound enzyme preparation was shown to contain a M_r 50000 polypeptide comigrating with the extracellular enzyme during SDS polyacrylamide gel electrophoresis. When the iodinated extracellular enzyme and the iodinated M_r 50000 polypeptide from membrane-bound enzyme were subjected to partial proteolytic digestion, similar profiles were obtained indicating extensive regions of homology.

Introduction

Dictyostelium amoebae aggregate by chemotaxis toward sources of cAMP. Cells release cAMP in pulses and the resulting transient gradients are detected by cAMP receptors on the surfaces of neighboring cells. Because the K_d of the receptors is 10–100 nM [1] and a group of signalling cells can produce micromolar concentrations of cAMP [2], the cAMP must be degraded during the few minutes between pulses or the receptors will remain insensitive to an ensuing signal. The degradation of cAMP is accomplished by cAMP phosphodiesterases which can be found bound to the outer surface of the cell [3] and in the extracellular space (see Orlow et al. [4], for references).

Several efforts have been made to compare the membrane-bound and extracellular forms of the enzyme. In the absence of detailed physical characterization, comparisons of the two forms have been indirect. The extracellular enzyme is inhibited by a glycoprotein secreted by starving cells in the absence of cAMP. Membrane-bound cAMP phosphodiesterase is not inhibited by the inhibitor

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glycoprotein, but when solubilized the membrane-bound form becomes more susceptible to inactivation by the inhibitor [3,5]. Both forms of the enzyme are equally affected by various cAMP analogues [6].

Evidence suggesting that the two phosphodiesterase activities are different comes from studies on their induction by cAMP. Neither form is present in large amounts in growing cells. During starvation of the cells in phosphate buffer both the extracellular and the membrane-bound enzyme accumulate. The membrane-bound form appears rapidly if pulses of cAMP are applied to starving cells but there is no acceleration with a steady application of cAMP. The appearance of the extracellular form is accelerated whether the cAMP is applied in pulses or continuously [7]. Another observation which suggests that the two forms are different are the non-linear kinetics observed with the membrane-bound, but not with the purified extracellular form [1,5]. Attempts to compare the two forms by charge transfer electrophoresis have produced contradictory results [8,9].

Recently the kinetic properties and susceptibilities to various agents of extracellular phosphodiesterase and cell associated enzyme were compared [10]. The results demonstrated similar characteristics but a somewhat different affinity of the two forms for the substrate. We describe here the most extensive purification of the membrane-bound enzyme so far and further comparisons of this enzyme with the extracellular form.

Methods and Materials

Conditions of growth and development. The axenic strain AX3 was grown and allowed to develop under conditions described previously [4] except that the cells were collected by centrifugation ($1000 \times g$; 1 min) after 8 h starvation, washed twice with 50 mM Tris-HCl, pH 8.2 at 4°C, and frozen at −20°C. Unless otherwise noted Tris refers to this buffer. During starvation cAMP was added at the rate of 50 μmol per h/l.

Solubilization and partial purification of membrane-bound cAMP phosphodiesterase. The frozen cells ($8 \cdot 10^{10}$ cells per preparation) were thawed, resuspended in Tris and centrifuged at $30000 \times g$ for 20 min. The pellet was resuspended in Tris and centrifuged again. The crude membranes were resuspended to the equivalent of $3 \cdot 10^8$ cells/ml in Tris. *n*-Butanol was added in a ratio of 7.5 ml butanol to 10 ml of membrane suspension [11]. After vigorous agitation, the butanol suspension was left at 4°C for 15 min and was then centrifuged at $12000 \times g$ for 10 min. The aqueous layer was recovered and centrifuged again to remove residual particles.

Membrane-bound phosphodiesterase remained in the aqueous phase and was applied to a column of Concanavalin A-Sepharose 4B (50 ml bed volume) which was equilibrated with Tris. The column was washed with two bed volumes of 1 M NaCl and 0.2% Trition X-100 in Tris followed by one bed volume of 0.2% Triton X-100 in Tris, and was eluted with several bed volumes of 20% α-methylmannoside and 0.2% Trition in Tris, over a period of 3–4 days.

The eluate was concentrated 10-fold by ultrafiltration through an Amicon PM-10 membrane and was applied to a DE-52 column (15 ml bed volume). The column was washed with four bed volumes of Tris after which the membrane-bound phosphodiesterase activity was eluted with 50 ml of 1 M NaCl in Tris. The eluate was concentrated and dialysed against Tris in preparation for isoelectrofocusing in the presence of 6 M urea by a procedure described previously [4]. The isoelectrofocusing gels were sliced and the slices eluted in 2 changes of 0.3 M KCl in Tris. The eluates containing enzyme activity were pooled, concentrated, and applied to an Ultrogel AcA-34 gel filtration column equilibrated with 50 mM Tris, 0.2 M NaCl, 0.02% sodium azide.

Membrane-bound phosphodiesterase purified through the gel filtration step and a sample of purified extracellular phosphodiesterase were dialyzed against 50 mM sodium phosphate (pH 7.5) and lyophilized in preparation for iodination. The iodination was done using chloramine-T according to the method of Greenwood et al. [12]. The phosphodiesterase samples were dissolved in 150 μl of water. 1 mCi of $Na^{125}I$ in 10 μl was added to each sample followed by 25 μl of freshly prepared chloramine-T (4 mg/ml). The reaction was terminated after 5 min by the addition of 100 μl of sodium metabisulfite (2.4 mg/ml) and 200 μl of sodium iodide (18 mg/ml). The iodinated proteins

were separated from free iodide by gel filtration on a Biogel P6 column (25 ml bed volume), equilibrated with Tris containing 0.2 M NaCl, 0.02% sodium azide, 0.01% bovine serum albumin, and eluted with the same buffer. Fractions containing iodinated protein were pooled, and antiserum against purified extracellular phosphodiesterase was added. The antiserum had been purified 2-fold by heating for 20 min at 60°C, precipitation with ammonium sulfate at 40% of saturation, and extensive dialysis against Tris containing 0.1 M NaCl. After 12 h at 4°C, Pansorbin was added and the samples were centrifuged (2000 $\times g$ for 20 min). The pellets were washed twice with 0.1 M NaCl in Tris and were resuspended in 0.0025% Bromphenol blue, 12.5% glycerol, 6.25% 2-mercaptoethanol, and 2.5% SDS, boiled for 2 min, and subjected to SDS-polyacrylamide gel electrophoresis (10% acrylamide) as described previously [13]. ^{125}I-labelled bands were located by autoradiography, and cut out. Peptide maps were performed as described by Cleveland et al. [14] with slight modifications (see legend to Figure 3).

Assays of enzyme activity. All assays were performed as described [4]. 1 unit of enzyme activity is defined as the amount of enzyme which hydrolyses 1 nmol of cAMP per min.

Materials. Cyclic-[2,8-^{3}H]AMP (32.3 Ci/mmol) and Na^{125}I (17 Ci/mg) were obtained from New England Nuclear. Papain (type III), cGMP (sodium salt), and Triton X-100 were purchased from Sigma. Pansorbin was purchased from Calbiochem-Behring. *Staphylococcus aureus* V8 protease was obtained from Miles Laboratories. The sources of all other reagents are listed elsewhere [13].

Results

Membrane-bound phosphodiesterase on whole cells and isolated membranes

Membrane-bound phosphodiesterase was produced by starving $8 \cdot 10^{10}$ cells as described in Methods. Crude membranes were prepared according to published methods [3]. In order to be sure that the phosphodiesterase activity associated with the crude membranes was the same as that found on the outer surface of the cells we performed the following experiment. Whole cells were assayed for cAMP phosphodiesterase activity as described by Malchow et al. [3]. An aliquot of these cells was harvested and frozen. Crude membranes were prepared from the frozen cells by centrifugation of thawed homogenates. The pellets were washed once and then resuspended in the same volume as the original aliquot of live cells. Both preparations were assayed under similar conditions and the results are shown in Table IA. The amount of cAMP phosphodiesterase activity associated with the membrane particles is the same as that found in vivo on the external portion of the cells. The specific cAMP phosphodiesterase inhibitor was included in the assay to suppress extracellular enzyme activity produced by the cells [3]. However, assays without the addition of inhibitor gave nearly identical results (data not shown).

TABLE I

DISTRIBUTION OF CELLULAR cAMP PHOSPHODIESTERASE ACTIVITY

(A) Time harvested after onset of starvation (h)	In vivo [a] (units/10^7 cells)	Crude membranes [a] (units/10^7 cells)
4 h	0.26	0.24
6 h	0.64	0.67
(B) Homogenate [b] (units/10^7 cells)	**Crude membranes (units/10^7 cells)**	**Cytoplasmic (units/10^7 cells)**
12.9	5.5	6.3
10.6	4.7	4.8

[a] The assays were done in 17 mM phosphate buffer at 22°C [3].

[b] Cells were harvested 8 h after the beginning of starvation, washed once with Tris, and frozen at −20°C. Homogenate was prepared by the addition of 1 ml of 1% Triton X-100 in Tris to 10^8 cells. Crude membranes were prepared, and solubilized with 1% Triton X-100. The cytoplasmic fraction is the supernatant following removal of crude membranes by centrifugation. The activities were determined by the spectrophotometric assay method [4].

Table IB shows that all the cellular activity in the homogenate can be accounted for by membrane-bound activity in the crude membrane pellet plus cytoplasmic activity in the supernatant. Differences in activity between parts A and B of Table I are due mainly to the assay conditions. In part A, cells and membranes were assayed in phosphate buffer, pH 6.0 at 22°C. These conditions do not disturb the living cells. Assays for

part B were done on Triton X-100 solubilized material in 50 mM Tris-HCl, pH 7.4 at 35°C. There is about a five-fold increase in activity when the assay is carried out in Tris at 35°C. Triton X-100 solubilization causes an additional 25% activation, presumably by the unmasking of activity. Purified extracellular cAMP phosphodiesterase is not activated by Triton X-100 (unpublished data).

Partial purification of membrane-bound phosphodiesterase

The membrane-bound phosphodiesterase was purified from crude membranes as described in Methods and in Table II. Triton solubilization was replaced by butanol extraction in spite of lower yield, since greater purification was achieved. When small aliquots of washed crude membranes were extracted with butanol the recovery of cAMP phosphodiesterase activity in the aqueous phase was often greater than 70%. Extracellular cAMP phosphodiesterase is extracted by butanol with approximately the same efficiency as membrane bound cAMP phosphodiesterase. When the particulate fraction remaining after the butanol extraction (interphase) was extracted with Tris-buffer containing 1% Triton X-100, less than 10% of the original activity was recovered. This indicates that the activity not recovered in the aqueous phase had been lost by denaturation. No evidence exists to indicate that there is a preferential extraction of one molecular species. When the butanol extractions were carried out with increased volumes the efficiency of the extraction procedure decreased.

TABLE II

PARTIAL PURIFICATION OF MEMBRANE-BOUND PHOSPHODIESTERASE

Purification step	Recovery	Units/mg protein	Purification
Crude membranes	100%	26	1×
Butanol extract	32%	100	4×
Con-A + DE 52	4.6%	1300	50×
Isoelectrofocusing + AcA-34	1.5%	4200	160×

The butanol-extracted aqueous phase was applied to a concanavalin A column which removed residual butanol and provided slight purification. The elution process took several days, and the inclusion of 0.2% Triton with the 20% α-methyl-mannoside was necessary. The purification scheme for extracellular cAMP phosphodiesterase also utilizes a concanavalin A column, but the extracellular enzyme can be eluted with α-methyl-mannoside more easily than the solubilized membrane form [4]. After elution from concanavalin A the material was applied to a DE-52 column. The DE-52 column, while not effective in purification, was useful for the removal of Triton. An aliquot of the DE-52 eluate was applied to an Ultrogel AcA-34 gel filtration column. The sample was heterogeneous, fractionating in three activity peaks. One peak was in the void volume, the second peak had a molecular weight of 150000–200000 and corre-

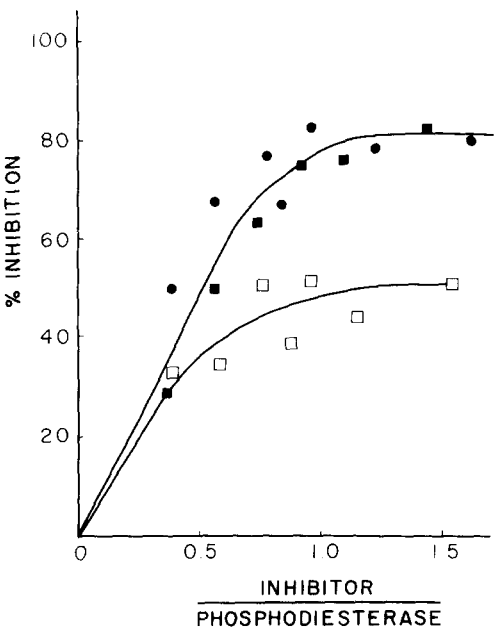

Fig. 1. Reaction of extracellular cAMP phosphodiesterase and membrane-bound cAMP phosphodiesterase with the inhibitory glycoprotein. 5.0 units of phosphodiesterase were incubated for 30 min at 35°C with various amounts of inhibitor (1.6–4.0 units) in a total volume of 1.32 ml Tris containing 0.05% Triton. 0.93 ml of sample was assayed spectrophotometrically. □, membrane-bound phosphodiesterase before urea isoelectrofocusing; ■, membrane-bound phosphodiesterase after urea isoelectrofocusing; ●, extracellular phosphodiesterase.

sponded to high M_r extracellular phosphodiesterase [4]. The elution volume of the third peak was that of low M_r extracellular phosphodiesterase (M_r 50000; results not shown).

When the DE-52 eluate was subjected to isoelectrofocusing in the presence of 6 M urea, the isoelectric point of the membrane-bound phosphodiesterase was 8.1–8.3 compared to an isoelectric point of 6.9–7.7 when isoelectrofocusing was done in the absence of urea (results not shown). This shift implies that a dissociation similar to that seen with the high M_r form of the extracellular phosphodiesterase [4] occurs with the membrane-bound forms. The purified low M_r extracellular cAMP phosphodiesterase has a pI of 8.1–8.3 [4].

The material eluted from the urea isoelectrofocusing gels were applied to the AcA-34 column and eluted as a single peak in the same position as the low M_r extracellular phosphodiesterase (K_{av} = 0.51, corresponding to a molecular weight of about 50000, assuming a globular protein).

Fig. 1 shows the interaction of membrane-bound phosphodiesterase before and after urea isoelectrofocusing with purified cAMP phosphodiesterase inhibitor protein. The interaction of the extracellular phosphodiesterase with the inhibitory protein is shown for comparison. Membrane-bound phosphodiesterase in vivo is unaffected by inhibitor. After urea treatment the membrane-bound phosphodiesterase can be inhibited up to 80%, which is comparable to the inhibition of extracellular phosphodiesterase. The inhibition of purified extracellular and partially purified membrane-bound cAMP phosphodiesterase by dithiothreitol and cGMP were compared by the radiometric assay method [4]. 1 mM dithiothreitol gave 60% inhibition for both forms of the enzyme, while 10 mM dithiothreitol inhibited each form by 96% as had been reported before [10]. When assayed in the presence of 1 mM cGMP both enzyme preparations were inhibited 80%, while 10 mM cGMP caused 99% inhibition.

Kinetic studies

Using membrane-bound enzyme which was purified through the AcA-34 step, Lineweaver-Burk plots were linear over the substrate range of 0.5–133 μM cAMP when assays were done in Tris at 35°C or in phosphate buffer at pH 6.0 at 22°C (see Table III). K_m values for membrane-bound phosphodiesterase associated with crude membranes and for partially purified enzyme were similar to extracellular phosphodiesterase values at all substrate ranges when assays were done in Tris at 35°C. The plots were also linear under these conditions. The substrate concentrations used in these experiments extended to a lower range than in previous work. Without introducing any obvious non-linearity in the Lineweaver-Burk plot, the K_m values found under these conditions were approximately half those reported by Kessin et al. [15]. Non-linear Lineweaver-Burk plots similar to

TABLE III

KINETICS

Phosphodiesterase was diluted in either Tris or 17 mM Na_2/K-phosphate buffer, pH 6.0, with 0.1 mg/ml of bovine serum albumin. Assays were done radiometrically using various concentrations of [^{3}H] cAMP [4]. Dual K_m values in lines 1 and 2 are extrapolations of different parts of the substrate range.

Phosphodiesterase preparation	Substrate range (μM)	K_m; assays in Tris at 35°C (μM)	K_m; assays in phosphate buffer at 22°C (μM)
Whole cells	0.5–500	—	0.7 and 30
Crude membranes	0.5–500	4	0.5 and 8
Solubilized post AcA-34	0.5–133	3	1.7
Extracellular (purified)	0.5–133 0.5–500	3.6 3	1.5 1.3

those found by Malchow et al. [5], and Green and Newell [1] were obtained when whole cells and crude membranes were assayed in phosphate buffer (pH 6.0) at 22°C. K_m values as low as 0.5 μM were obtained when a substrate range of 0.5 to 10 μM was employed. K_m-values ranging from 10 to 30 μM were obtained when only the 25–800 μM substrate range was considered.

Comparisons by iodination and partial proteolysis. The membrane-bound phosphodiesterase purified through the AcA-34 column was iodinated and/or immunoprecipitated in parallel with samples of purified extracellular phosphodiesterase. The precipitation of membrane-bound phosphodiesterase with antiserum raised against the extracellular form is as efficient as for the extracellular form. Many *Dictyostelium* proteins contain a highly immunogenic post-translational modification called 'the common antigen' [16]. Both the membrane-bound and the extracellular phosphodiesterases contain this modification, as does

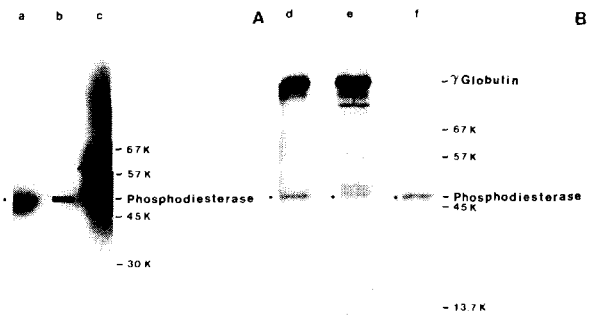

Fig. 2. SDS-polyacrylamide gel electrophoresis of immunoprecipitated membrane-bound and extracellular cAMP phosphodiesterases. (A) Samples were ^{125}I-labelled and immunoprecipitated as described in the text. Following electrophoresis, fixing, and staining, the wet gel (in 7% acetic acid) was wrapped in Saran Wrap, and exposed at 4°C between 2 Cronex screens (Dupont) with preflashed Kodak XAR-5 film. Lane a: ^{125}I-labelled and immunoprecipitated extracellular enzyme, lane b: position of unlabelled extracellular enzyme; lane c: ^{125}I-labelled and immunoprecipitated membrane-bound enzyme. (B) Aliquots containing an equal number of units of extracellular and partially purified membrane-bound enzyme were incubated with antiphosphodiesterase gamma globulin. The gamma globulin was purified by ammonium sulfate precipitation and ionic exchange on DE-52 in 10 mM Tris-HCl, pH 7.7. The immunecomplexes were collected by centrifugation and prepared for SDS-gel electrophoresis as described, except that no mercaptoethanol was used. The gel was stained with Coomassie brilliant blue. Lane d: immunoprecipitated extracellular cAMP phosphodiesterase; lane e: immunoprecipitated membrane-bound cAMP phosphodiesterase; lane f: extracellular cAMP phosphodiesterase. The molecular weight markers used were (K = kDa): bovine serum albumin (67 kDa), pyruvate kinase (57 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and ribonuclease A (13.7 kDa).

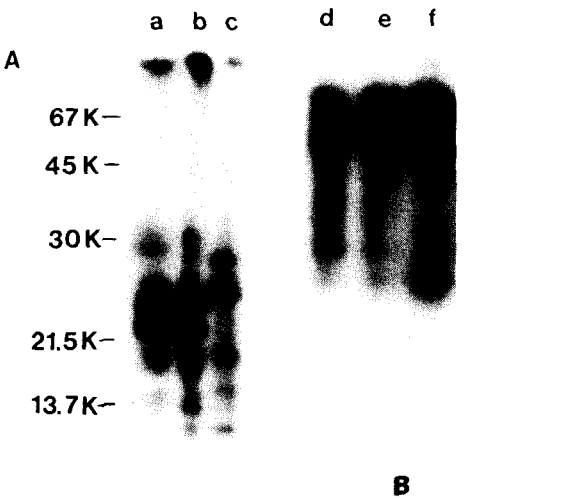

Fig. 3. Peptide maps obtained by limited proteolysis in SDS polyacrylamide gels. (A) Gel slices containing ^{125}I-labelled proteins from the gel shown in Fig. 2A were soaked with several changes of buffer containing 125 mM Tris-HCl, 0.1% SDS, 1 mM EDTA (pH 6.8). The presoaked slices were transferred to wells of 0.15 × 14 cm slab gels (11.5 cm running gels with 18% acrylamide, 0.08% Bis, 0.1% SDS, 1 mM EDTA, 0.37 M Tris-HCl, 0.03% ammonium persulfate, 0.025% Temed, pH 8.8; 4.5 cm spacer gels with 4.8% acrylamide, 0.15% Bis, 0.1% SDS, 1 mM EDTA, 125 mM Tris-HCl, 0.1% ammonium persulfate, 0.05% Temed, pH 6.8). To each cell were added 30 μl of solution containing 125 mM Tris-HCl, 0.5% SDS, 1 mM EDTA, 15% sucrose (pH 6.8) and 25 μl of solution containing 125 mM Tris-HCl, 0.5% SDS, 1 mM EDTA, 10% sucrose, 10 μg bovine serum albumin, and 4 μg *S. aureus* V8 protease (pH 6.8). Electrophoresis was carried out for 14 h at 10 mA, and 1.5 h at 25 mA, with the electrode buffers described by Laemmli [19]. a. ^{125}I-labelled extracellular enzyme; b. ^{125}I-labelled membrane-bound enzyme; c. ^{125}I-labelled 60 kDa peptide. The molecular weight markers are described in the legend of Fig. 2, except soybean trypsin inhibitor (21.5 kDa). (B) Stained slices from the gel shown in Fig. 2B were iodinated and treated with *S. aureus* V8 protease in 18% polyacrylamide gels [18]. d. soluble extracellular enzyme; e. immunoprecipitated extracellular enzyme; f. immunoprecipitated membrane-bound enzyme.

the inhibitor protein (unpublished data). Therefore immunoprecipitation serves as a convenient way to recover enzyme from solution, but not as an indication of identity. The pellets resulting from the parallel immunoprecipitation of iodinated material were analyzed by SDS-gel electrophoresis and the results are shown in Fig. 2A.

Lane a shows the iodinated and immunoprecipitated extracellular cAMP phosphodiesterase. Lane c is the iodinated and immunoprecipitated membrane-bound cAMP phosphodiesterase. The origin of the high molecular weight material in lane c is not clear, since the starting sample contained proteins that fractionated with an average M_r of about 50000 on a gel filtration column.

The bands were cut from the gel and subjected to partial hydrolysis with *S. aureus* V8 protease during SDS-gel electrophoresis. The results are shown in Fig. 3A. Lane a (extracellular) and lane b (membrane-bound) share most of the visible peptides and are related, although there are a number of differences in intensity between corresponding bands. Lane c (M_r 60000 protein) has few pepetides in common with lane a and lane b, and is not closely related to cAMP phosphodiesterase. This protein may have been recovered because it contains the common antigen [16]. Similar results were obtained when the bands were partially hydrolyzed with papain or α-chymotrypsin (results not shown).

Fig. 2B shows the protein patterns obtained following immunoprecipitation with purified gamma globulin of an equal number of units of extracellular and membrane-bound enzyme. The bands indicated by dots were cut from the gel, iodinated, and subjected to partial hydrolysis with *S. aureus* V8 protease. The results are shown in Fig. 3B, and demonstrate that the extracellular and membrane-bound phosphodiesterases have several common peptides. When the gel bands shown in Fig. 2B were analyzed following complete tryptic digestion as described by Elder et al. [17], and modified by Luna et al. [18], no differences were detected (results not shown).

Discussion

In this paper we have shown that the membrane-bound phosphodiesterase is similar to the extracellular enzyme. Using a partially purified membrane-bound phosphodiesterase preparation it was possible to make direct physical comparisons with the purified extracellular form [4].

The evidence that we have partially purified membrane-bound cAMP phosphodiesterase and not contaminating extracellular enzyme consists of the following observations: (i) we have demonstrated that the enzyme activity recovered in crude membrane preparations corresponds exactly to the amount of membrane associated activity when whole cells are assayed (Table IA). If the original membrane preparation had been heavily contaminated with intracellular or extracellular enzyme, we would have expected to assay more activity in membrane preparations than with living cells. (ii) The final recovery of 1.5% (Table II) is low, but the extracellular enzyme, when purified through the same number of purification steps, shows a recovery of approx. 10% [4]. This means that the original contamination of the membrane-bound preparation with extracellular enzyme would have been more than 15%. This conflicts with the data shown in Table IA which indicate that extracellular contamination is less than 5%. Another observation is consistent with the supposition that washing the cells reduces extracellular enzyme contamination to much less than 15%. If cells are starved in the presence of 1 mM cAMP, extracellular activity is increased and membrane associated activity is suppressed to less than 10% of the levels measured in the absence of cAMP (unpublished data). The excess of extracellular enzyme does not interfere with measurement of the reduced amount of membrane-bound enzyme. If extracellular enzyme were contaminating at the level of 15% of peak activity, a suppression of membrane activity to less than 10% peak activity would not be observed. (iii) Throughout the purification procedure the membrane associated activity behaves differently from the extracellular enzyme. While the bulk of extracellular enzyme activity can be eluted from concanavalin A-Sepharose 4B with 2% (w/v) α-methylmannoside [4] in the absence of detergent, the membrane-bound enzyme activity can be eluted only slowly with 20% (w/v) α-methylmannoside, and the presence of detergent is necessary. In addition, the extracellular M_r 50000 enzyme does not bind to DE-52 [4] while the

membrane-bound enzyme does bind to DE-52.

There is no evidence to suggest that the 98.5% of membrane-bound enzyme lost during the purification procedure is different from the enzyme recovered. None of the column fractionations employed separates the enzyme in multiple peaks.

We cannot prove that the M_r 50000 polypeptide in the membrane-bound fraction is the only one associated with cAMP phosphodiesterase activity, but there is good reason to think that the major portion of the activity is associated with the M_r 50000 polypeptide. When an equal number of units of purified extracellular enzyme and partially purified membrane-bound enzyme were immunoprecipitated, the M_r 50 000 polypeptide stained about equally with Coomassie brilliant blue (Fig. 2B). Little activity (less than 10%) was left in the supernatants following immunoprecipitation.

The two forms are similar with regard to a number of other criteria. After isoelectrofocusing in the presence of 6 M urea the membrane bound enzyme has the same isoelectric point as the extracellular form, and will interact with the purified inhibitor glycoprotein to the same extent as the extracellular enzyme (Fig. 1). Dithiothreitol and cGMP inhibit both forms of the enzyme to the same extent, as do glutathione and cIMP [3,10]. Both purified forms exhibit identical molecular weights by gel filtration. When the kinetics of the membrane-bound phosphodiesterase purified through gel filtration were compared to those of the extracellular phosphodiesterase, similar K_m values were found. Both enzymes had linear kinetics. Non-linear kinetics are a characteristic of the membrane-bound enzyme prior to purification which distinguish it from the extracellular form, but this may be a property conferred by membrane association.

Partially purified membrane-bound phosphodiesterase can be precipitated with an antiserum raised against the extracellular enzyme. When both enzymes were iodinated, immunoprecipitated, and compared by SDS-gel electrophoresis, a band was present with a molecular weight identical to the extracellular enzyme (Fig. 2A). When the corresponding bands were removed from the gel and compared by partial proteolysis, the digest were similar but not identical. Blondelet and Brachet [8] suggested that the membrane-bound phosphodiesterase has a hydrophobic tail or is bound to hydrophobic material which attaches it to the membrane, while the extracellular phosphodiesterase lacks this hydrophobic characteristic. The presence of a hydrophobic portion of the enzyme was not observed by Yamasaki and Hayashi [9] using similar techniques. Okamoto [20] suggested that the appearance of specific insertion sites in the membrane might be responsible for the developmental regulation of the membrane-bound cAMP phosphodiesterase.

We have attempted to demonstrate the existence of receptor sites by extracting crude membranes with urea in the presence of salt as described by Luna et al. [21]. The extractions were followed by incubations of the extracted membranes with crude extracellular phosphodiesterase. While the extractions resulted in the removal of 75–80% of the membrane-bound phosphodiesterase, no significant restoration of membrane-bound activity occurred during the subsequent incubations with extracellular enzyme (unpublished data).

Available data demonstrate that the membrane and the extracellular enzymes have several common properties and similar amino acid sequences. The differences which were observed when partial proteolytic digests were compared indicate that the membrane-bound and extracellular enzyme are not identical. Recovering a clone of the cAMP phosphodiesterase gene should help to establish whether we are dealing with two related genes or one gene whose product can be delivered to the membrane or extracellular compartments depending on the conditions faced by the aggregating cells.

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