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ENRICHMENT OF 5'-NUCLEOTIDASE IN MEMBRANE FRAGMENTS ISOLATED FROM ACANTHAMOEBA sp.

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SUMMARY

A purified membrane fraction enriched in 5'-nucleotidase by 12–19-fold relative to homogenate on a specific activity basis was isolated from amoeboid cells of Acanthamoeba sp. The purified preparation also showed an enrichment in ATPase activity but membranes possessing glucose-6-phosphatase and succinic dehydrogenase activities were relatively absent. From electron micrographs it was apparent that the isolated fraction was a clean preparation of membranous strands and vesicles. The increased specific activity of 5'-nucleotidase, which has been shown to be a marker enzyme for plasma membrane in several mammalian tissues, strongly suggests that the preparation from Acanthamoeba sp. is purified plasma membrane. Moreover, the enzyme appears to be located primarily on the inner surface of the plasma membrane, since its activity was markedly latent in intact cells.

INTRODUCTION

Acanthamoeba castellanii, a free living soil amoeba, is quite suitable for biochemical developmental studies in that it has a simple two-phase life cycle consisting of amoeboid and cyst stages. Furthermore, the organism will encyst synchronously and can be cultured axenically in liquid medium[1].

Several biochemical studies related to membrane phenomena have been carried out with this organism. Experiments reported by KLEIN[2] indicate that the amoebae cells are capable of actively accumulating K^+ against a concentration gradient, whereas Na^+ permeates the membrane by passive means. In addition an ATP hydrolyzing activity has been detected on whole amoeba cells and in a microsomal fraction, but the enzyme seems to be characteristic of an apyrase rather than an ATPase[3]. In a further communication KLEIN AND BRELAND[4] reported evidence implicating the involvement of this apyrase in the mechanism for active uptake of K^+. They found, for example, that introduction of 2,4-dinitrophenol inhibited both uptake of K^+ by the cells and ATP hydrolysis by a microsomal fraction. However, ouabain, the classical inhibitor of active cation transport, had no effect on either active K^+ transport or apyrase activity[5]. It was concluded that the ATP hydrolyzing activity probably does provide energy for active transport of K^+, but that the system is quite different from the typical Na^+–K^+ transport and its associated ATPase activity found on mammalian cell membranes. Biochemical and electron microscopic studies

performed by KORN AND WEISMAN[6,7] have demonstrated that Acanthamoeba cells ara capable of ingesting polystyrene and polyvinyl latex beads by phagocytosis. Their observations indicated that the endocytotic process is energy dependent. Thus it seems apparent that at least some of the phenomena generally characteristic of surface membrane are present on the plasma membrane of Acanthamoeba cells.

As a prelude to an investigation of membrane structure and function during encystment of Acanthamoeba, various types of membrane present at the amoeboid stage of development are being isolated and characterized. This communication describes the preparation and properties of membrane fragments isolated from the amoebae cells by a method originally developed for obtaining purified plasma membrane from rat liver[8]. A detailed examination of these isolated membranes has indicated that they possess many of the features of isolated rat-liver surface membrane.

MATERIALS AND METHODS

Culturing

Amoeboid cells of *Acanthamoeba castellanii* (Neff strain) were cultured in the optimal growth medium described by NEFF *et al.*[1]. 2-l erlenmeyer flasks containing 1 l of growth medium were used and aeration was achieved by shaking at 100 rev./min on an Eberbach rotating shaker. The temperature was maintained at 28°. Growth was allowed to continue until the cells had reached a population density of $20 \cdot 10^4$–$40 \cdot 10^4$ cells per ml of growing suspension. The cells were counted with a Bright-Line hemacytometer. At this stage of growth cysts were normally absent, but on the odd occasions when they were present the cultures were not used if the proportion of cysts was greater than 2 %. Wet mounts of all cultures were examined with a light microscope just prior to use in order to monitor for bacterial contamination.

Isolation of the membrane fragments

The membranes were isolated by a technique essentially similar to the method described by NEVILLE[9] and later modified by EMMELOT *et al.*[8] for preparation of plasma membrane from rat liver. Cells were harvested from the growth medium by centrifugation at $800 \times g$ for 10 min at 4°. All subsequent centrifugations were also carried out at 4° and average g forces are quoted throughout. The total wet weight of the sediment was determined and the cells resuspended in enough buffered water (1 mM $NaHCO_3$, pH 7.5) to make a 20 % suspension. The suspension was then homogenized by 15 strokes with a pestle-type homogenizer (clearance 0.13–0.18 mm) at 1700 rev./min. The resulting homogenate was diluted with 4 volumes of buffered water, strained through 6 layers of cheesecloth and centrifuged at $1500 \times g$ for 10 min. The supernatant was discarded and the sediment resuspended in buffered water (2.25 ml per original g of packed cells) by 3 strokes of homogenization and centrifuged in clear tubes in a Spinco 30 rotor at $1220 \times g$ for 10 min. The resulting pellet was double layered consisting of a well-packed bottom portion and a loose fluffy upper portion. The supernatant was syringed off but in so doing, special care was taken not to disturb the fluffy layer. The upper portion of the pellet was then resuspended in buffered water (1.5 ml per original g of packed cells) and centrifuged as before. These procedures of resuspension and centrifugation were repeated until a homogeneous pellet of fluffy material that showed no visible trace of the original bottom portion

was obtained. At this stage, the procedure according to NEVILLE[9] was modified in that this pellet was again resuspended in buffered water (1.5 ml per original g of packed cells) and incubated with stirring for 4 h in order to lyse the membranous vesicles and allow release of any trapped protein. At the end of this period the suspension was centrifuged at 1220 $\times g$ for 10 min and the sediment resuspended in enough buffered water to allow a proportion of the suspension to be used as payload (input of partially purified membranes to the sucrose gradient) and the remainder to be retained for chemical and biochemical determinations.

A final purification was achieved by density gradient centrifugation through the discontinuous sucrose gradient described by EMMELOT *et al.*[8] in which separation occurs by flotation. A Spinco SW-25.1 rotor was used. The bottom layer of the gradient consisted of 3.1 ml of payload mixed with 5.8 ml of sucrose, density 1.34, and upon this 7 ml each of sucrose solutions of densities 1.20, 1.18 and 1.16 were layered successively. After centrifugation for 2 h at 58750 $\times g$ a layer was present at each interface and there was a pellet at the bottom of the tube. The uppermost interface layer was the purified membrane fraction.

The interface layers were removed from the gradient with a syringe, diluted with approx. 5 volumes of buffered water, pelleted by centrifugation and resuspended in a small volume of buffered water for subsequent chemical and biochemical determinations. The pellet at the bottom of the tube was suspended directly. These together with aliquots of the homogenate, payload and the other fractions obtained during the preparation were stored at $-4°$ until required for the analyses, but storage was never for longer than 4 days.

Enzyme assays and protein determinations

The fractions were assayed for the following enzymatic activities: 5'-nucleotidase (EC 3.1.3.5)[10], glucose-6-phosphatase (EC 3.1.3.9)[11], succinate dehydrogenase (EC 1.3.99.1)[12], ATPase (EC 3.6.1.3)[3]. Prior to the ATPase assay the fractions were washed twice in 1 mM versene (pH 7.0) in order to chelate any endogenous Ca^{2+} or Mg^{2+}. The assay mixture contained Mg^{2+} as the stimulating ion[3]. Since the substrate was continuously being broken down under the conditions of the assay, samples were always read in a double-beam spectrophotometer against a blank containing substrate and no enzyme. A further blank containing enzyme but no substrate was also subtracted. Levels of P_i were determined by the method of KING[13].

For experiments in which 5'-nucleotidase activities were compared for whole and homogenized cells, the amoebae were harvested by centrifugation in an International clinical centrifuge. The cells were then washed twice by gentle resuspension in 0.3 M sucrose (pH 7.4 with $NaHCO_3$) and centrifugation in the clinical centrifuge. The final cell sediment was resuspended in enough sucrose–$NaHCO_3$ solution to make a 20% suspension. A portion of this was homogenized in the same manner as for preparation of the purified membrane fraction and the remainder was retained as a suspension of whole cells.

Protein determinations were routinely carried out by the method of LOWRY *et al.*[14].

Electron microscopy

Samples of the freshly prepared membrane fraction were fixed in 3% glutaraldehyde in 0.05 M phosphate buffer (pH 7.0), post-fixed in 1% OsO_4 (pH 7.0) and

embedded in vestopal[15]. Thin sections were post-stained with uranyl acetate (1 % aqueous solution) and lead citrate (0.1 % in 0.1 M NaOH).

RESULTS

Enzymatic properties of the purified membrane fraction

The fraction of purified membrane was enriched in 5′-nucleotidase by 12–19-fold with respect to homogenate on a specific activity basis (Table I). It is also apparent from the data in Table I that the density gradient centrifugation step contributed significantly to the purification. Enrichment of 5′-nucleotidase was found

TABLE I

ENZYME DATA FOR ISOLATED FRACTIONS FROM ACANTHAMOEBA sp.

5′-Nucleotidase, ATPase and glucose-6-phosphatase activities are expressed as μg P_i per mg protein per h and succinate dehydrogenase as μmoles reduced indonitrotetrazolium chloride per mg protein per h. n.d. = not detectable.

Enzyme	*Expt.*	*Homogenate*	*Payload*	*Purified membrane fraction*
5′-Nucleotidase	A	56.6	435	765
	B	66.5	412	816
	C	62.0	553	765
	D	51.5	508	855
	E	57.0	815	1114
ATPase*	A	344	392	938
	B	121	386	1140
	C	238	266	594
	D	119	320	890
Succinate dehydrogenase	A	0.052	n.d.	n.d.
	B	0.137	0.015	0.024
	C**	0.092	n.d.	n.d.
Glucose-6-phosphatase	A	38.5	59.2	n.d.
	B	40.6	50.9	n.d.
	C**	30.6	18.3	17.3

* Prior to the assay the fractions were washed in 1 mM versene, pH 7.0.

** The culture contained $100 \cdot 10^4$ cells per ml. In all other cases, the cultures contained less than $50 \cdot 10^4$ cells per ml.

to depend quite critically upon the cell population of the cultures. In order to obtain 12–19-fold enrichment it was necessary to use cultures with cell counts of less than $50 \cdot 10^4$ cells per ml since only 5–7-fold purifications were manifest in the membrane fractions isolated from more heavily populated cultures (Table II). The purified membrane preparation was also enriched in Mg^{2+}-stimulated ATPase activity (Table I) but in these experiments the fractions were washed before the enzyme determinations were carried out and thus the homogenate in particular was modified in that all soluble protein had been removed. Glucose-6-phosphatase and succinate dehydrogenase activities in the purified fraction were very low and sometimes not detectable (Table I).

TABLE II

ENRICHMENT OF 5′-NUCLEOTIDASE IN PURIFIED MEMBRANE PREPARATIONS FROM DIFFERENTIALLY POPULATED CULTURES OF ACANTHAMOEBA sp.

Relative enrichment: ratio of the specific activity in the purified membrane fraction to that of the homogenate.

Number of cells ($\times 10^{-4}$ *per ml of culture*)	*Relative enrichment*
124	5.8
75	6.7
43	19.0
34	12.4
26	16.6
21	12.4

TABLE III

5′-NUCLEOTIDASE ACTIVITY OF WHOLE CELLS AND HOMOGENIZED CELLS OF ACANTHAMOEBA sp.

n.d. = not detectable. Activities are expressed as μg P_i per mg protein per h.

Expt.	*Whole cells*	*Homogenized cells*
A	3.04	28.4
B	6.24	35.2
C	n.d.	18.3

In experiments designed to assess the effectiveness of the incubation step in the preparative procedure, purified membrane fractions were prepared from both incubated and non-incubated payloads and the specific activities of 5′-nucleotidase in the purified fractions and their respective payloads were compared. In some experiments a relative increase in purity was apparent in incubated as compared with non-incubated preparations, but this was not consistently the case, for in other instances no difference between the two treatments was observed.

Data for comparative determinations of 5′-nucleotidase activity on whole and homogenized cells are set out in Table III. In all cases, whole cell activity was very low and sometimes not detectable, but homogenization caused a very significant increase in activity.

Recoveries with respect to homogenate for 5′-nucleotidase, glucose-6-phosphatase and succinate dehydrogenase activities ranged from 73 to 135 %, 64 to 90 % and 61 to 110 %, respectively. It was not possible to calculate recovered activities for ATPase because of the modification incurred by the homogenate as a result of the washing procedure.

Electron microscopy

Electron micrographs of the purified membrane preparation are shown in Fig. 1 and indicate that the fraction was a clean preparation consisting of both strands and vesicles of membrane. Remnants of organelles could not be distinguished and in high resolution micrographs (Fig. 1b) the trilamellar feature of the membranes was clearly visible.

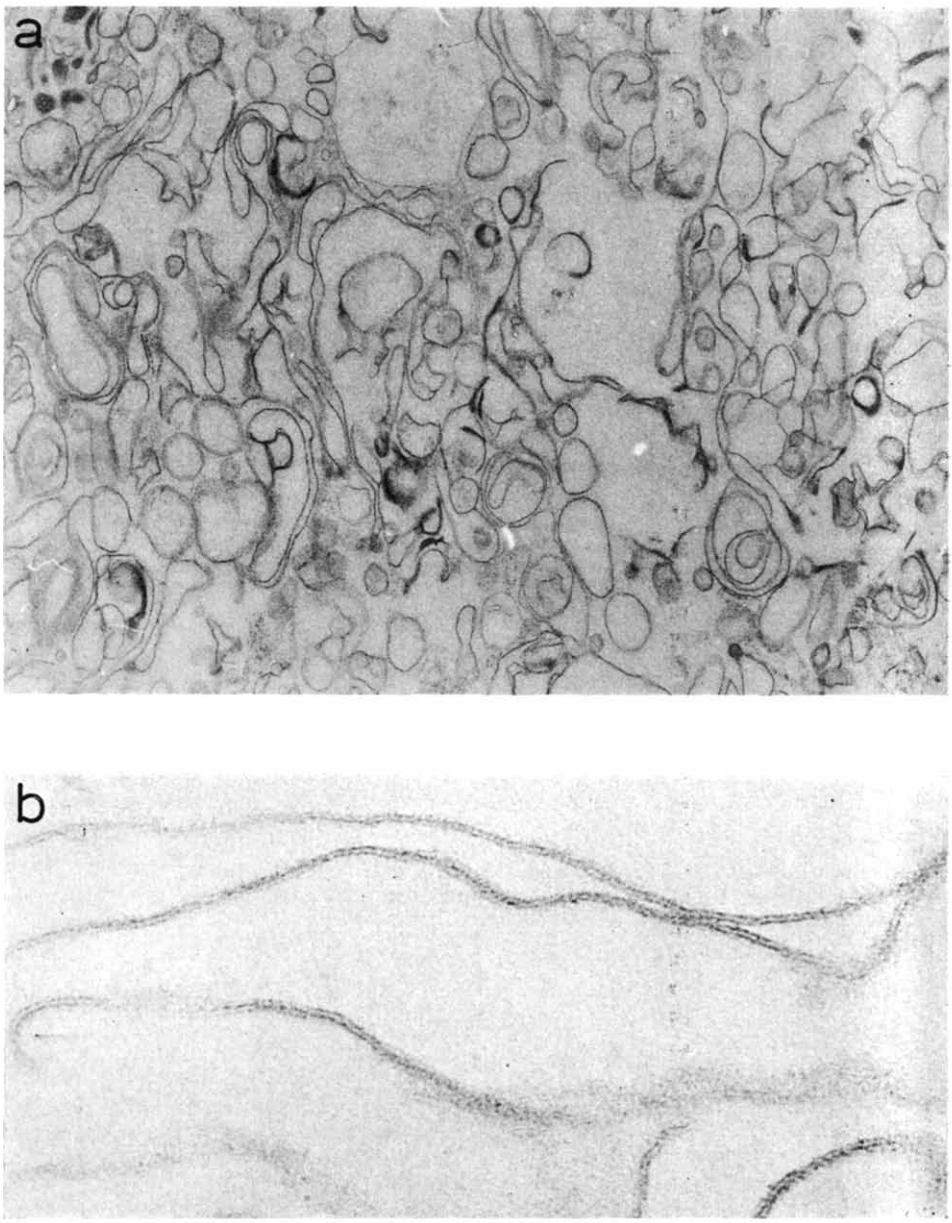

Fig. 1. Electron micrographs of an isolated membrane fraction from Acanthamoeba sp. (a) × 23430. (b) × 148500.

DISCUSSION

It has been quite clearly shown that 5′-nucleotidase is localized exclusively on the plasma membrane of cells from a variety of mammalian tissues such as liver, kidney, and intestine[3,16–18]. Hence at the outset it was postulated that this enzyme

might also be present on the surface membrane of Acanthamoeba cells and thus serve as a marker for this membrane. The evidence obtained in this study would seem to indicate that this is, in fact, the case. Furthermore, in view of the marked latency of 5′-nucleotidase observed in whole cells as compared with homogenates, it would seem that the enzyme is located primarily on the inner surface of the plasma membrane.

The centrifugal behaviour of the membrane fragments during the preparative procedure very closely paralleled that of plasma membrane from rat liver when isolated by the same method[8,9]. This suggests that the initial disruptive process has a similar effect on the plasma membrane of both cell types in spite of their totally different properties of aggregation. In addition it would seem likely that the two membranes have similar densities. Since the surface membrane of Acanthamoeba lacks a distinguishing morphological feature by which it can be identified, it was not possible to conclude from electron micrographs that plasma membrane was the predominant species in the fraction. However, the preparations did show enrichments in 5′-nucleotidase of 12- to 19-fold with respect to homogenate. These are somewhat greater than the 6–8-fold purifications of this enzyme reported for preparations of plasma membrane from guinea-pig tissues by the same method[17]. It is possible that this difference is attributable to the 4-h incubation in hypotonic medium that was introduced as a modification of procedure, but it must be emphasized that no consistent increase in purity was noted as a result of this treatment. On the other hand, the lack of consistent response may simply reflect a variation among experiments in the amount of protein trapped within the vesicles of the payload fraction. The enrichments of 5′-nucleotidase in the membranes from Acanthamoeba are, however, quite comparable in magnitude to those reported recently by COLEMAN *et al.*[18] for surface membrane isolated from rat liver under isotonic conditions and by a different technique.

Enrichments of ATPase in the membrane preparation were not as great as those of 5′-nucleotidase when compared with their respective homogenates, but this is to be expected in view of the modified nature of the homogenate used in the ATPase assays. Furthermore, KLEIN[3] has reported that ATP hydrolyzing activity is present in both microsomal and mitochondrial fractions of this organism, although the two activities have differing pH optima. KLEIN's observation that an ATPase activity, with a pH optimum identical to that of the microsomal enzyme, is present in a suspension of whole cells is consistent with the enrichment of this enzyme in an isolated fraction of purified plasma membrane. Since it is well documented that a proportion of surface membrane forms vesicles of microsomal size upon homogenization[18], it seems reasonable that the ATPase activity detected by KLEIN in microsomal fractions can be attributed to the presence of plasma membrane in this fraction.

From a detailed study by electron microscopy, BOWERS AND KORN[19] concluded that Acanthamoeba cells are ultrastructurally similar to mammalian cells. They possess the major cytoplasmic membrane systems including smooth and rough endoplasmic reticulum, but in addition digestive and contractile vacuoles are quite conspicuous[19]. The essentially similar morphologies of virtually all these membranes in an isolated state, with the exception of rough endoplasmic reticulum, precluded an assessment by electron microscopy of the relative abundance of membranes that might be expected to contaminate a preparation of plasma membrane. However,

contamination by membranes containing glucose-6-phosphatase and succinate dehydrogenase activities was found to be minimal (Table I). Glucose-6-phosphatase is well documented as a marker for endoplasmic reticulum in mammalian cells[20,21], but it has not been determined whether this holds true for Acanthamoeba. It was found in the course of these studies, however, that centrifugation of homogenates of Acanthamoeba at 146000 × *g* for 3 h caused sedimentation of 95–100 % of the glucose-6-phosphatase activity and this, together with the presence and sometimes enrichment of this activity in the payload fraction (Table I), indicates that the enzyme is primarily membrane bound. The inclusion in the glucose-6-phosphatase assay of KF and ethylene diamine tetraacetic acid as inhibitors of alkaline and acid phosphatases helped to ensure that the substrate was being hydrolyzed only by glucose-6-phosphatase[11]. KLEIN[2] has shown that 90–98 % of the succinate dehydrogenase activity is localized in a mitochondrial fraction from Acanthamoeba and hence it seems likely that this enzyme serves adequately as a marker for mitochondrial membrane fragments. Vacuolar membranes are also a possible source of contamination, but these membranes, although certainly modified in this state, are for the most part derived from plasma membrane in this organism[6,7]. It is thought that the decreased specific activity of 5′-nucleotidase in preparations from heavily populated cultures may reflect a modification of the plasma membrane which has perhaps come about as a result of partial initiation of encystment due to a decreased availability of nutrient. No significant difference in specific activity between homogenates from light and heavy cultures was apparent. Moreover the lower enrichment did not appear to be due to decreased purity of the membrane preparation since contamination, as estimated from determinations of glucose-6-phosphatase and succinate dehydrogenase activities, had not changed. Variations in enzyme recoveries were encountered but this can presumably be attributed to the large volume of supernatant that accumulated during the preparative procedure and its attendant exaggeration of any small error in measurement of its enzyme activity.

The relative absence of markers for membrane species other than plasma membrane suggests that the isolated fraction is a preparation of purified plasma membrane. This conclusion is substantiated by the striking similarities between the amoeboid membranes and plasma membrane isolated from rat liver and other mammalian tissues. Not only are 5′-nucleotidase and ATPase common features, but the absolute specific activities of these enzymes in the preparation from Acanthamoeba are very comparable in magnitude to those reported for plasma membrane isolated from rat liver[3,18].

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