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Evidence for a membrane-bound pyrophosphatase in *Dictyostelium discoideum*

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Plasma membrane enriched fractions of *Dictyostelium discoideum* contain a Des-insensitive ATPase activity that can be fractionated by DEAE-Sephacel into a major vanadate-sensitive activity and a minor vanadate-insensitive activity. The vanadate-insensitive activity hydrolyzed pyrophosphate considerably more rapidly than ATP or any other substrate tested, and the enzyme was therefore designated a pyrophosphatase. The enzyme had no activity on AMP or *p*-nitrophenyl phosphate. The pyrophosphatase activity was maximal at alkaline pH values and stimulated by Mg^{2+} but not by Ca^{2+}, properties of the enzyme that are very similar to those of the previously characterized pyrophosphatases of the plant tonoplast membrane. The pyrophosphatase activity of total membrane extracts changed very little during *Dictyostelium* differentiation.

Pyrophosphatase; Vanadate; Des; (*Dictyostelium discoideum*)

1. INTRODUCTION

Membrane bound, Mg^{2+} stimulated pyrophosphatases have been described in a variety of plant tissues [1–6]. These enzymes are localized in the tonoplast membrane and there is evidence that they function as proton pumps [4–6]. Despite the apparent wide-spread occurrence of the enzyme in plants, there have been no reports of membrane bound pyrophosphatase activity in animal cells or in lower eucaryotes.

During the course of our studies on the membrane bound ATPases of *Dictyostelium discoideum* [7,8] we detected the presence of a minor membrane bound activity that was insensitive to Des and vanadate [8]. In this report we provide evidence that this activity is a pyrophosphatase, and show that some of its properties are similar to those of the well characterized plant enzymes.

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2. MATERIALS AND METHODS

2.1. *Materials*

Bacteriological peptone and yeast extract were from Oxoid. DEAE-Sephacel was purchased as pre-swollen beads from Pharmacia. All other chemicals were the best available grade from Fisher Scientific or Sigma Chemical Co.

2.2. *Organism and culture conditions*

The axenic mutant of *D. discoideum*, Ax-2, was grown in HL-5 media [9] at 22°C on a gyratory shaker to a density of 5×10^6–10^7 cells/ml. The cells were harvested by centrifugation at $700 \times g$ and washed twice with Bonners salts [10]. The wild type strain V12-M2 was grown in association with *Enterobacter aerogenes* and differentiation was initiated by depositing 10^8 washed cells on a Millipore filter saturated with 20 mM phosphate buffer, pH 6.5 [11].

2.3. *Membrane preparation and solubilization*

Total membrane and plasma membrane enriched fractions were prepared as described [7] with the exception that the buffers contained 1 mM *p*-aminobenzamidine and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and were freshly prepared. The plasma membranes were resuspended at a protein concentration of 1.5–2.0 mg/ml in 10 mM Tris-Cl, pH 7.5, 20% (v/v) glycerol, 1.0 mM *p*-aminobenzamidine and 0.1 mM PMSF. $C_{12}E_9$ was added from a 10% (w/v) stock solution to give a final concentration of 1% and the suspension was incubated, with stirring, at 0°C for 15 min. The suspension was

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centrifuged at 100000 × *g* for 30 min and the supernatant containing the solubilized enzyme activity fraction was removed.

2.4. *DEAE-Sephacel chromatography*

The $C_{12}E_9$ extracts were loaded onto a DEAE-Sephacel column (1 × 10 cm) that had been previously equilibrated with 10 mM Tris-Cl, pH 7.5, 0.5% Chaps, 1 mM *p*-aminobenzamidine and 1 mM PMSF. The column was washed with the equilibration buffer until all 280 mm absorbing material had been eluted and then eluted in three 18 ml steps with equilibration buffer containing 0.1 M NaCl, 0.3 M NaCl and finally 0.5 M NaCl.

2.5. *Enzyme assays and protein determination*

Enzyme activity was assayed at 30°C by the release of inorganic phosphate. Unless indicated otherwise, the reaction mixtures contained 10 mM Mes-Tris, pH 6.8, 10 mM $MgCl_2$ and cell free extract in a final volume of 1.0 ml. After a 10 min pre-incubation, reactions were initiated by the addition of substrate, at concentrations indicated in the text. Reactions were terminated after 15–60 min by the addition of 0.1 ml 10% (w/v) trichloroacetic acid and 1% (w/v) SDS and inorganic phosphate was measured as described [7], a modification of the method of Ames [12]. Protein determinations were by the method of Sanderman and Strominger [13] unless otherwise indicated.

3. RESULTS AND DISCUSSION

We recently described the purification and characterization of a Des-insensitive, Mg^{2+} or Ca^{2+}-ATPase from membranes of *D. discoideum* [8]. During the course of the purification procedure this enzyme was separated by DEAE-Sephacel chromatography from a less active Des-insensitive ATPase, that was distinguished from the Mg^{2+} or Ca^{2+}-ATPase by its lack of sensitivity to vanadate [8]. Fig.1 shows that when freshly solubilized extracts were applied directly to a DEAE-Sephacel column, the recovery of the minor

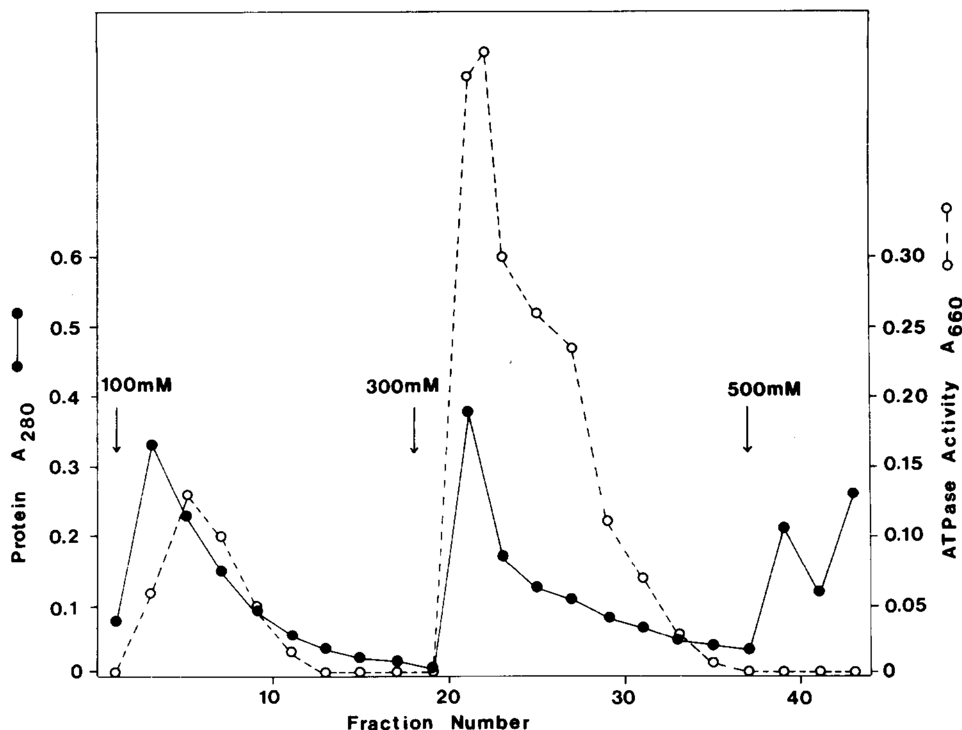

Fig.1. DEAE-Sephacel chromatography of detergent solubilized plasma membranes. Plasma membrane enriched fractions from *D. discoideum*, strain Ax-2, were extracted with 1% $C_{12}E_9$ and the extract (6 ml, 5 mg protein) was loaded onto a DEAE-Sephacel column (1.0 × 10 cm) pre-equilibrated with 10 mM Tris-Cl, pH 7.5; 0.5% Chaps; 1 mM *p*-aminobenzamidine and 0.1 mM PMSF. The column was washed with equilibration buffer to remove unbound protein and then eluted step-wise at the arrows with equilibration buffer containing the indicated concentrations of NaCl. Eluted fractions were assayed for protein by measuring absorbance at 280 nm (●), and for ATPase activity (○). The ATPase activity is expressed as the absorbance at 660 nm that resulted when 0.2 ml aliquots of the fractions were assayed using 3 mM ATP as substrate and an incubation time of 1 h. The unbound protein that eluted directly with equilibration buffer, had no ATPase activity (data not shown).

vanadate-insensitive species was considerably improved relative to that reported previously.

When the fractions containing the vanadate-insensitive activity were pooled and assayed using other phosphorylated compounds as substrates, it was found that pyrophosphate was hydrolyzed at the highest rate (table 1). UTP was hydrolyzed preferentially among the nucleoside triphosphates tested, but the activity was less than 20% of the activity with pyrophosphate as substrate. The preference for pyrophosphate as substrate further distinguishes the enzyme from the Des-insensitive Mg^{2+} or Ca^{2+}-ATPase activity, since the latter enzyme was without activity on pyrophosphate [8]. The enzyme had low activity with ADP as substrate and no activity with AMP or *p*-nitrophenyl phosphate (table 1). The hydrolysis of pyrophosphate was markedly stimulated by Mg^{2+} but not by Ca^{2+}, although no marked divalent cation preference was noted when nucleoside triphosphates were hydrolyzed (table 1). With pyrophosphate as substrate, the specific activity of the pyrophosphatase was comparable to that of the DEAE-Sephacel purified, Des-insensitive Ca^{2+} or Mg^{2+}-ATPase (table 1 and fig.1).

The pyrophosphatase exhibited a marked preference for alkaline pH values (fig.2). This pH curve is similar to that for the *D. discoideum* alkaline phosphatase that is also localized in plasma membrane enriched preparations [14,15]. However, since purified alkaline phosphatase does not catalyze the hydrolysis of ATP [14] and the pyrophosphatase described here is without activity on AMP and *p*-nitrophenyl phosphate (table 1), it is clear that the two activities are not due to the same enzyme.

In order to determine if there was evidence for more than one membrane-bound pyrophosphatase

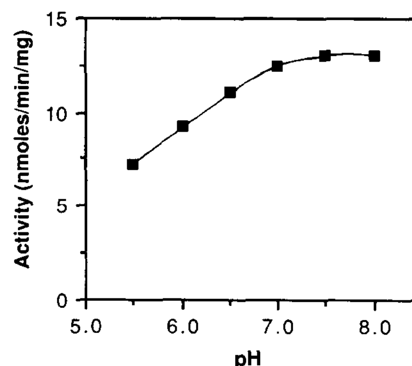

Fig.2. Effect of pH on pyrophosphatase activity. The pooled vanadate insensitive activity that eluted from the DEAE-Sephacel column with 100 mM NaCl was assayed using 1 mM pyrophosphate as substrate and a 15 min incubation time as described in section 2. The pH of the reaction was varied using buffer obtained by titrating 10 mM Mes with 10 mM Tris base.

Table 1

Substrate specificity and the effect of divalent cations on the vanadate insensitive activity

Substrate[a]	Activity (nmol P_i/min per mg protein)	
	Ca^{2+}	Mg^{2+}
3 mM ATP	13.6 ± 1.2	12.8 ± 0.6
3 mM GTP	12.0 ± 2.3	9.3 ± 1.1
3 mM CTP	10.6 ± 0.5	10.9 ± 0.3
3 mM UTP	17.8 ± 0.7	16.0 ± 1.9
3 mM ADP	–	7.4 ± 1.2
3 mM AMP	–	0
3 mM *p*-nitrophenyl phosphate	–	0
1 mM pyrophosphate	10.0 ± 1.1	82.1 ± 7.8

[a] Reactions were carried out using the pooled vanadate insensitive activity from the DEAE-Sephacel column with the indicated concentrations of substrates in the presence of either 10 mM $MgCl_2$ or 10 mM $CaSO_4$. Since 1 mol of pyrophosphate yields 2 mol of phosphate, the activity with pyrophosphate as substrate was calculated as half the rate of phosphate liberation. The data shown are the means of four independent experiments ± the standard deviation

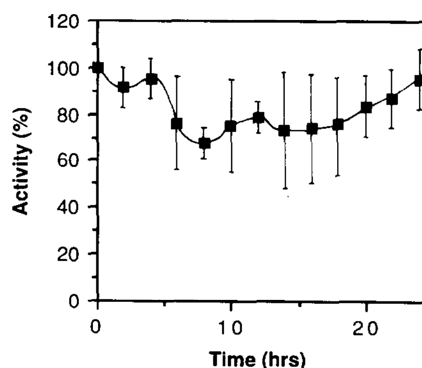

Fig.3. Developmental time course of pyrophosphatase activity. Membranes were isolated from V12-M2 cells at the indicated times after the onset of development and pyrophosphatase activity was assayed using 1 mM pyrophosphate as substrate. The results are the means ± standard deviation for three experiments. Since the specific activity of the enzyme in total membrane preparations was slightly variable from experiment to experiment, activities of 0 h cells were normalized to 100%. The mean specific activity of the 0 h samples was 5.9 ± 1.4.

in *Dictyostelium*, total membrane extracts were assayed for pyrophosphatase activity. The activity obtained exhibited almost exactly the same divalent cation and pH responses as the partially purified enzyme (not shown). These responses to divalent cations and pH are similar to those of the plant pyrophosphatases [1–4]. In an attempt to extend the correlation further, total membranes were assayed for pyrophosphatase activity in the presence of potassium at concentrations that markedly stimulate the plant enzymes [1–6]. The *Dictyostelium* enzyme was not, however, stimulated at any concentration of potassium (not shown).

The availability of an assay for pyrophosphatase activity in total membrane preparations of *Dictyostelium* provided an opportunity to determine the possible developmental regulation of the activity. The data shown in fig.3 indicate that the enzyme activity changes only slightly during differentiation.

Since the enriched plasma membrane preparations used in these studies may also contain vacuolar membranes, the similarity in properties between the *Dictyostelium* and plant pyrophosphatases raises the possibility that the *Dictyostelium* enzyme is also a vacuolar proton pump. If the pyrophosphatase is a vacuolar membrane enzyme rather than a plasma membrane enzyme, it might prove useful as a marker for vacuolar membrane isolation. Gross and co-workers have recently proposed a model for cell type determination in *Dictyostelium* that involves a vacuolar proton pump [16]. Although their model specifies an ATPase, the pyrophosphatase described in this report might be vacuolar and therefore constitutes a potential candidate for this putative activity.

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