What family of ATPases does the vacuolar H^+-ATPase belong to?

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Polyacrylamide gel electrophoresis (PAGE) of partially purified ATPase from vacuoles of *Saccharomyces carlsbergensis* under non-dissociating conditions revealed 3 bands with ATPase activity. Further PAGE in dissociating conditions showed the similarity in composition of these 3 ATPase preparations. They were assumed to contain the same vacuolar ATPase exhibiting, however, different electrophoretic mobility due to the formation of enzyme complexes with different proteins and phospholipids. The ATPase preparation with the highest electrophoretic mobility contained 6 subunits of 75, 62, 16, 14, 12 and 9 kDa. Inhibitors of vacuolar ATPase [^{14}C]DCCD and [^{14}C]NEM bound to a 9 kDa polypeptide, while [^{14}C]DES associated with a polypeptide of 75 kDa. A partially purified preparation of the vacuolar ATPase was not phosphorylated by [γ-^{32}P]ATP under conditions when plasma membrane ATPase formed a phosphorylated intermediate. Our results show that vacuolar H^+-ATPase consists of several polypeptides, does not form the phosphorylated intermediate, and evidently represents a new type of H^+-ATPase of yeast.

ATPase *Subunit* *Phosphorylated intermediate* *Yeast vacuole*

1. INTRODUCTION

Yeast and fungal cells are known to possess at least 3 types of H^+-ATPases: mitochondrial ATPase, ATPase of the plasma membrane and ATPase of the vacuolar membrane. The first 2 have been purified and well characterised [1–5]. Mitochondrial H^+-ATPase belongs to the family of F_1F_0-ATPases which includes also ATPases of bacteria, chloroplasts and probably chromaffin granules [6]. It consists of 8–12 subunits [4,5] forming a water-soluble catalytic complex F_1 and a hydrophobic part F_0 performing proton translocation. The H^+-ATPase of the plasma membrane is similar to $(Na^+ + K^+)$-ATPase [7] and Ca^{2+}-ATPase of animal cells [8] and bacteria [9] as well as K^+-ATPase of bacteria [9]. It consists of one subunit of 105 kDa and forms a phosphorylated intermediate during the reaction [1,10–13]. Unlike this class of ATPases, the F_1F_0-ATPases do not form a phosphorylated intermediate [14].

The vacuolar H^+-ATPase differs from the mitochondrial one in the pH optimum [15] and insensitivity to the mitochondrial ATPase inhibitors, azide and oligomycin [15,16]. Unlike the plasmalemma ATPase, it is insensitive to orthovanadate [15,16] and exhibits probably a lower substrate specificity [15]. In addition it is specifically inhibited by NO_3^- [16,17]. The question arises as to whether the vacuolar H^+-ATPase belongs to one of the known types of membrane ATPases or represents a new one.

This paper reports that the vacuolar H^+-ATPase of *Saccharomyces carlsbergensis* consists of different subunits, does not form a phosphorylated intermediate during the reaction and contains a DCCD and NEM-binding polypeptide of 9 kDa.

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; DES, diethylstilbestrol; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis

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2. MATERIALS AND METHODS

The yeast *S. carlsbergensis* IBPM-366 was grown in a medium with peptone [16]. Spheroplasts and vacuoles were isolated and the ATPase was solubilized and purified as described [16].

Different electrophoretic procedures were used for this study. Electrophoresis in non-dissociating conditions was performed according to Davis [18] on slabs of separating gel (14 × 10 × 0.8) with 7.5% acrylamide and 0.2% *N,N'*-methylenebisacrylamide (w/v). Electrophoresis was carried out at 4°C in 20 mM Tris/0.2 M glycine buffer, pH 8.5, for 4–5 h at a constant current of 30 mA. Proteins were stained with Coomassie blue, and the ATPase activity in the gel was determined by substrate-dependent staining of the enzyme with lead salts [19].

SDS-PAGE was carried out on 15% polyacrylamide gel, pH 8.5 [20] or 10% polyacrylamide gel, pH 2.4 [21]. The location and intensity of the protein bands stained with Coomassie blue or lead were revealed by spectrophotometric analysis at 550 or 360 nm, respectively, in an Opton spectrophotometer.

A partially purified ATPase preparation was phosphorylated by incubation with 0.2 mM [γ-^{32}P]ATP as described [13]. After electrophoresis at pH 2.4 and drying of gel plates, the location of ^{32}P-labeled proteins was determined using fluorography.

A partially purified ATPase preparation was labeled either with [^{14}C]DCCD (20 nmol/mg protein, 0°C, 16 h), or [^{14}C]DES (20 nmol/mg protein, 0°C, 2 min) or [^{14}C]NEM (150 nmol/mg protein, 0°C, 2 min). Labeled proteins were precipitated with 6% trichloroacetic acid at 5000 × *g* for 15 min, electrophoresed on 15% polyacrylamide gel with 0.1% SDS, and subjected to fluorography.

3. RESULTS AND DISCUSSION

After solubilization of vacuolar proteins and further ATPase purification by centrifugation in a glycerol density gradient, the ATPase preparation was almost completely purified from alkaline phosphatase, UTPase, pyro- and polyphosphatase and partially (70–90%) from GTPase [15]. According to inhibitor analysis there were no other known membrane H^+-ATPases, except for the vacuolar ATPase, in the partially purified vacuolar ATPase preparation [16].

PAGE of the partially purified vacuolar ATPase preparation in non-dissociating conditions reveals 8 protein zones (fig.1). The substrate-dependent ATPase staining with lead acetate reveals 3 zones (A–C) exhibiting ATPase activity (fig.1). Zones A–C were cut out from gel plates, and proteins were eluted by 20 h shaking of gel with 60% formic acid at 37°C. After gel separation by centrifugation, the eluate was lyophilized and used for electrophoresis on 15% polyacrylamide gel with 0.1% SDS.

The ATPase preparation from zone A contained 9 polypeptides (fig.2a). SDS-PAGE of the initial preparation of partially purified ATPase revealed a similar picture (not shown). The ATPase

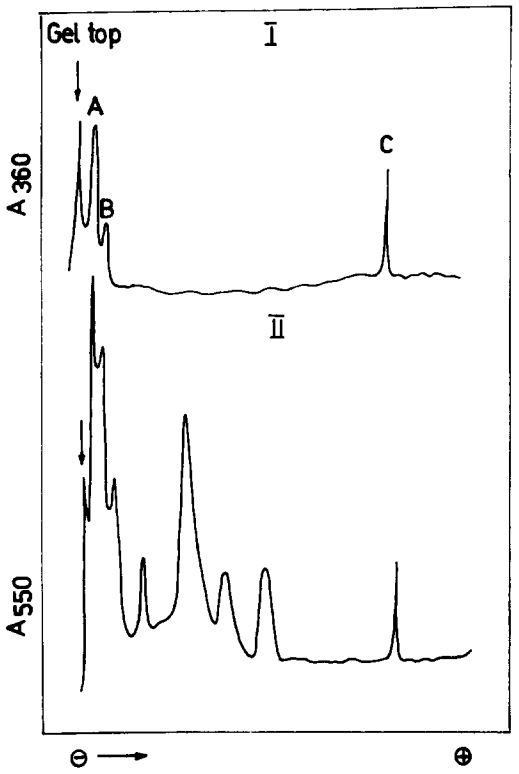

Fig.1. PAGE of the partially purified vacuolar ATPase preparation in non-dissociating conditions. (I) Identification of ATPase by substrate-depending gel staining with lead acetate; (II) protein staining by Coomassie blue.

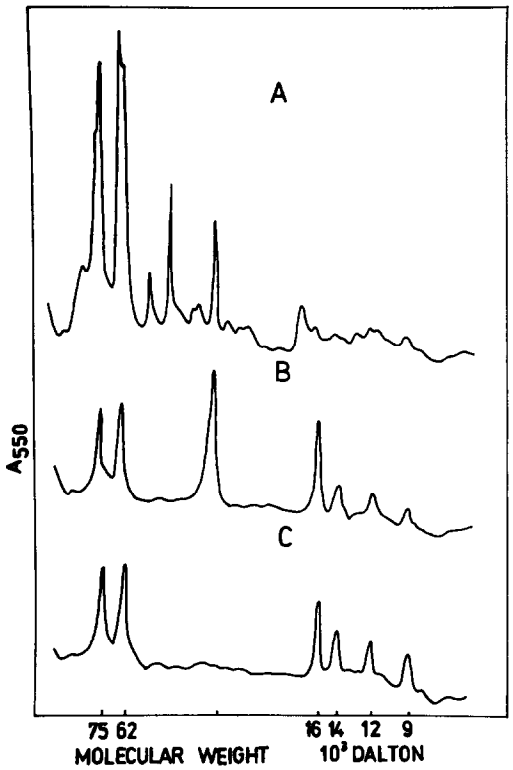

Fig.2. SDS-PAGE analysis of proteins from zones A–C obtained by electrophoresis under non-dissociating conditions (fig.1).

preparation from zone B contained 7 polypeptides, and 4 of them (16, 14, 12 and 9 kDa) were minor in preparation A (fig.2). The ATPase preparation from zone C contained the same polypeptides as that from B except for a polypeptide of 30 kDa (fig.2c).

The analysis of the data in fig.2 assumes that the 3 ATPase zones obtained by electrophoresis under non-dissociating conditions (fig.1) represent the complex of the vacuolar ATPase with the other vacuolar proteins. This is confirmed by the fact that the electropherograms of the preparation from zone A (fig.2a) and the initial partially purified ATPase preparation (not shown) are identical.

The preparation from zone C, having the highest electrophoretic mobility in non-dissociating conditions and exhibiting ATPase activity, is the most pure ATPase preparation and contains 6 protein bands of 75, 62, 16, 14, 12 and 9 kDa. The data obtained indicate that the vacuolar H^+-ATPase has a subunit structure, though there is no reliable evidence that all the 6 proteins represent the polypeptide composition of the enzyme (or that the enzyme consists only of these 6 polypeptides).

The preparation of partially purified ATPase also possesses GTPase activity [16] which cannot be completely explained by the nonspecific GTP hydrolysis by ATPase (not shown). To differentiate these 2 enzyme activities, we have studied the effect of the ATPase inhibitors, DCCD, DES and NEM, on ATPase and GTPase activities in preparations of these enzymes obtained after gradient centrifugation of solubilized vacuolar proteins (table 1). At the concentrations employed, DCCD, DES and NEM inhibited the ATPase activity by more than 80%, leaving the GTPase activity practically unaltered.

The insensitivity of GTPase to the inhibitors listed was used to specify the subunit composition of the vacuolar ATPase in experiments with ^{14}C-labeled inhibitors. Incubation of the partially purified vacuolar ATPase preparation with [^{14}C]DCCD or [^{14}C]NEM resulted in the incorporation of the label mainly into a polypeptide of 9 kDa (fig.3). In some experiments the label from [^{14}C]DCCD incorporated equally into 9 and 16 kDa proteins. The polypeptide of 9 kDa is evidently similar to a DCCD-binding protein of mitochondrial ATPase of *Neurospora crassa* and *S. cerevisiae* [22] as well as ATPase of *E. coli* plasma membrane [23], which is known to form a proton channel. It is significant that NEM and DCCD block the proton transport into isolated vacuoles, and NEM (10–30 μM) does not inhibit the ATP hydrolysis (not shown).

Table 1

Effect of inhibitors on the activity of partially purified vacuolar ATPase and GTPase preparations

Inhibitors	ATPase activity (%)	GTPase activity (%)
None	100[a]	100[a]
50 μM DES	17	91
10 μM DCCD	14	110
100 μM NEM	13	104

[a] Specific activity of ATPase 2 μmol P_i/mg protein per min, of GTPase 0.5 μmol P_i/mg protein per min

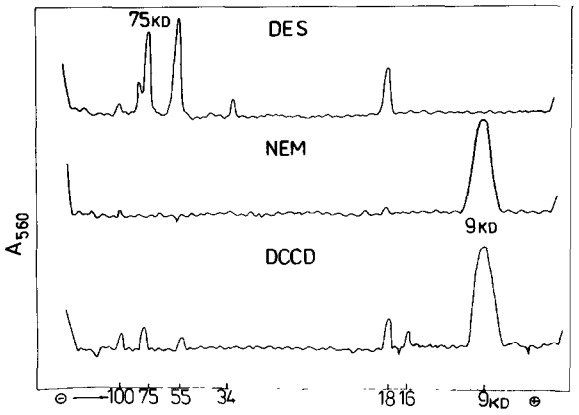

Fig.3. Fluorography of gel obtained after separation of proteins of partially purified ATPase preparations labeled with ^{14}C-labeled inhibitors (incubation conditions are described in section 2). SDS electrophoresis was carried out on 15% polyacrylamide gel, pH 8.5.

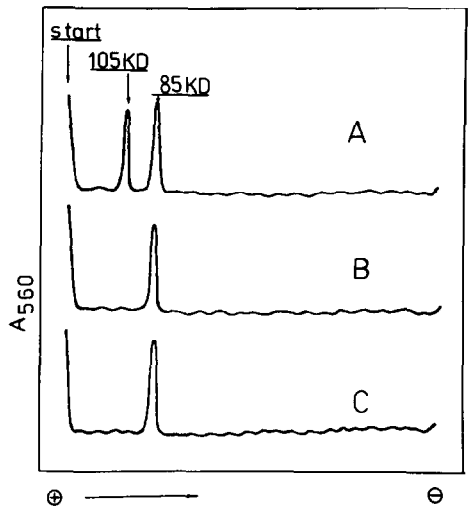

Fig.4. Labeling of plasma membrane proteins (A,B) and proteins of partially purified vacuolar ATPase (C) by [γ-^{32}P]ATP (1000 Ci/mmol). (A) 50 μg of plasmalemma proteins were incubated for 30 s at 0°C with 0.5 mM [γ-^{32}P]ATP, precipitated with perchloric acid and processed for acid electrophoresis; (B) perchloric acid was added before the [γ-^{32}P]ATP. The same picture was observed when phosphorylated plasma membranes were incubated with hydroxylamine as reported [12] or plasma membranes were preincubated with 100 μM vanadate; (C) 30 μg vacuolar ATPase preparation were incubated for 30 s at 0°C with 0.2 mM [γ-^{32}P]ATP. The same picture was observed when perchloric acid was added before the [γ-^{32}P]ATP. After acid electrophoresis the gels were submitted to fluorography.

Among several proteins in the partially purified enzyme preparation reacting with [^{14}C]DES, only that of 75 kDa belongs to the ATPase complex (cf. figs 3 and 2c).

The determination of the subunit composition of vacuolar ATPase and the inhibitor analysis indicate that the enzyme, similarly to mitochondrial ATPase, consists of several polypeptides and possesses a proton channel with binding sites for DCCD and NEM. However, it differs essentially from the mitochondrial one not only in the structure composition but also in its sensitivity towards inhibitors. For instance, the ATPase of intact vacuoles and the partially purified enzyme preparation were not inhibited by such mitochondrial ATPase inhibitors as oligomycin, azide, and Dio-9 [1,15,16]. On the contrary, DES and NO_3^-, at concentrations decreasing the vacuolar ATPase activity, did not affect the mitochondrial one [1,16,17].

An important criterion for determination of an ATPase belonging to either category of known membrane ATPases is to determine its ability to form a phosphorylated intermediate. The insensitivity of the vacuolar ATPase to vanadate [16] suggests its inability to form the intermediate. This was confirmed in experiments with [γ-^{32}P]ATP. Incubation of the isolated vacuoles or partially purified ATPase preparation with [γ-^{32}P]ATP under conditions appropriate for phosphorylated intermediate formation by plasma membrane ATPase [12,13] did not result in labeling of the vacuolar ATPase (fig.4).

Taken together, the data obtained suggest that the vacuolar membrane H^+-ATPase may not belong to the known categories of membrane ATPases but may represent a new type.

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