Biochimica et Biophysica Acta 1788 (2009) 303–313

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

V H$^+$-ATPase along the yeast secretory pathway: Energization of the ER and Golgi membranes

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ARTICLE INFO

Article history:
Received 28 February 2008
Received in revised form 3 November 2008
Accepted 10 November 2008
Available online 19 November 2008

Keywords:
V H$^+$-ATPase
H$^+$ transport
Yeast
Endoplasmic reticulum
Golgi

ABSTRACT

H$^+$ transport driven by V H$^+$-ATPase was found in membrane fractions enriched with ER/PM and Golgi/Golgi-like membranes of *Saccharomyces cerevisiae* efficiently purified in sucrose density gradient from the vacuolar membranes according to the determination of the respective markers including vacuolar Ca^{2+}-ATPase, Pmc1::HA. Purification of ER from PM by a removal of PM modified with concanavalin A reduced H$^+$ transport activity of P H$^+$-ATPase by more than 75% while that of V H$^+$-ATPase remained unchanged. ER H$^+$ ATPase exhibits higher resistance to bafilomycin (I_{50} = 38.4 nM) than Golgi and vacuole pumps (I_{50} = 0.18 nM). The ratio between a coupling efficiency of the pumps in ER, membranes heavier than ER, vacuoles and Golgi is 1.0, 2.1, 8.5 and 14 with the highest coupling in the Golgi. The comparative analysis of the initial velocities of H$^+$ transport mediated by V H$^+$-ATPases in the ER, Golgi and vacuole membrane vesicles, and immunoreactivity of the catalytic subunit A and regulatory subunit B further supported the conclusion that V H$^+$-ATPase is the intrinsic enzyme of the yeast ER and Golgi and likely presented by distinct forms and/or selectively regulated.

1. Introduction

The vacuolar H$^+$-ATPases constitute a family of ATP-dependent proton pumps responsible for acidification of intracellular compartments in all eukaryotic cells. They function in the lysosomes, clathrin-coated vesicles, endosomes and Golgi apparatus in animal cells, plant and yeast/fungal vacuoles and plant Golgi [1–6]. V H$^+$-ATPases participate in key physiological processes such as H$^+$ homeostasis, secondary transport of ions and nutrients, protein sorting, fusion/fission of membrane vesicles and establishment of left–right asymmetry of vertebrates [1–7]. The enzyme has an important role in proliferation of tumor cells [8], cell–cell fusion [9], hyphal growth and virulence of the human fungal pathogen *Candida albicans* [10]. The endosomal V H$^+$-ATPase of the proximal tubule epithelial cells can regulate endocytic degradative pathway and modulate membrane trafficking by recruiting and interacting with cytosolic GTPase Arf6 and GDP/GTP exchange factor ARNO [11]. These data together with others point to the V H$^+$-ATPase as a pH sensor that may couple the intra-endosomal pH to the formation of the endocytic transport vesicles [12].

It has been widely accepted that the enzyme of plants and animal cells is assembled in the ER but becomes active only in the organelles of secretory pathway such as Golgi, endosomes and vacuoles/lysosomes[1–6]. It was initially assumed that yeast V H$^+$-ATPase is functionally active only in vacuolar membrane [4,13,14]. The assumption of localization of its Stv1p containing form to the Golgi/endosomes in budding yeast [15] was proved by indirect immunofluorescence microscopy showing its presence in the late Golgi [16]. The existence of functional V H$^+$-ATPase in the Golgi/endosomes was supported by the genetic experiments showing that only deletion of both VPH1 and STV1 caused the phenotypes identical to that of the mutants with deletion of the other single copy V H$^+$-ATPase subunits. However, there were no reports on the direct measuring of the H$^+$ transport in the Golgi/endosome enriched membrane vesicles. The indirect biochemical evidence of the localization of the functionally active enzyme in the ER and Golgi besides the vacuole came from the determination of the bafilomycin A_1 sensitive Ca^{2+}/H^+ exchange and ATPase activities in membranes of *S. cerevisiae* fractionated in sucrose density gradient [17,18]. This localization was later supported by the demonstration of the functionally active and immunochemically distinct V H$^+$-ATPases in the secretory pathway organelles of fission yeast as well as of the Ca^{2+}/H^+ exchanger, which used the ΔpH formed by these V H$^+$-ATPases [19]. An indirect evidence of the localization of

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doi:10.1016/j.bbamem.2008.11.006

the functionally active enzyme to the yeast endosomes was also reported [20]. Here we show that the membrane vesicles derived from the ER, Golgi and Golgi-like membranes and efficiently purified from vacuole membranes are endowed with H^+ transport activity mediated by V H^+-ATPase and that this pump exhibits different properties depending on the organelle of secretory pathway. The results presented in this report significantly advance further investigation and understanding of the physiological role of V H^+-ATPase. Together with a previous finding [19] they call attention to the participation of this key H^+ pump in the functioning of the whole secretory pathway of yeast and probably fungi and other eukaryotic cells.

2. Materials and methods

2.1. Yeast strains

Wild type yeast *Saccharomyces cerevisiae* strains AA255 (*MATα ade2 his3Δ200 leu2-3 112 lys2Δ201 ura3-52*) and X2180 (*MATα SUC2 mal mel gal2 CUP1*) were gifts from Dr. L. Lehle (Regensburg University, Germany). Wild type strain SEY 6210 (*MAT leu2-3, 112 ura3-52 his3200 trp1-901 lys2-801suc2-9*) was a gift from Dr. W.Tanner (Regensburg University, Germany). The strain K699 was kindly provided by Dr. K. Cunningham (*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 PMC1*::*HA*). All strains were grown at 30 °C in YPD medium containing 1% yeast extract, 2% bactopeptone and 2% glucose.

2.2. Membrane isolation and fractionation

Yeast membranes were isolated and fractionated according to [19,21,22]. Briefly, the middle logarithmic phase cells were transformed to the spheroplasts by incubation at 37 °C in buffer containing 1.2 M sorbitol, 10 mM Tris–HCl, pH 7.4, 30 mM β-mercaptoethanol and 1 mg lyticase (Sigma)/1 g of wet cells. After 50 min the incubation mixture of spheroplasts, old cells and cell walls was rapidly cooled and received EDTA, benzamidine and PMSF at 1.2 M sorbitol and 10 mM Tris–HCl, pH 7.4 to give final concentrations 1 mM of each protease inhibitor. The obtained mixture was loaded on the solution of 1.4 M sorbitol in 10 mM Tris–HCl, pH 7.4, centrifuged for 5 min at 3000× *g* and then resuspended and homogenized in a Potter glass homogenizer using a lysis buffer (12.5% sucrose, 20 mM MOPS-Na pH7.4, 1 mM DTT, 1 mM benzamidine, 1 mM PMSF and a cocktail of the polypeptide protease inhibitors). Total membranes were precipitated for 45 min at 120,000× *g*, resuspended in lysis buffer and loaded onto a step gradient formed of 56, 52, 48, 45, 42, 39, 36, 33, 30, 25 and 20% sucrose (w/w) prepared in 10 mM MOPS-Na pH 7.2. The cocktail of the polypeptide protease inhibitors was applied to each step of gradient. After centrifugation for 2 h 45 min at 140,000× *g* membrane fractions were collected from the bottom and frozen.

To evaluate a coupling efficiency of the V H^+-ATPase in different compartments, the total membranes (1.2 mL) were applied to a simplified sucrose gradient (50, 38 and 25% sucrose), and membrane fractions enriched with vacuole, Golgi and ER membranes were collected from the respective interfaces after centrifugation in standard conditions. Membranes of the higher density than ER were found in pellets and were considered here as those which are enriched with nuclear envelope (NE). They were resuspended in 50% sucrose prepared in 10 mM MOPS-Na pH 7.2.

To purify intracellular membranes from PM, the modification of PM of spheroplasts by concanavalin A (ConA) with a subsequent sedimentation of the PM-ConA sheets at low speed was performed according to [23,24].

2.3. Enzyme activities

To measure the H^+ transport membrane vesicles (fraction aliquots of the same volume, 40–80 μL, ~20–70 μg) were added to incubation medium containing 50 mM KCL, 2.5 mM $MgSO_4$, 12.5% sucrose, 20 mM Tris–Cl pH 7.4 and 1 μM 9-amino-6-cloro-2-methoxyacridine (ACMA). H^+ transport was initiated by the addition of 1 mM ATP-Na and monitored by fluorescence quenching of ACMA [14,19] using fluorimeter Shimadzu RF-530 1PC. Pre-incubation of membranes with 200 μM sodium vanadate and the inhibition of H^+ transport by bafilomycin A_1 or concanamycin A were used to show that H^+ gradient was formed by V H^+-ATPase. Subsequent addition of 20 mM NH_4Cl or 1 μM FCCP was used to demonstrate a fluorescence recovery which indicates a collapse of the preliminary formed H^+ gradient. F_{max} reflects a steady-state amplitude of the ΔpH formation achieved after 10 min of H^+-transport; it was calculated as $\Delta F/F$ and expressed as percentage. Initial velocity of H^+ transport was determined by an extrapolation of the fluorescence quenching curve for 1 min. When the signals were out of the proportionality, that is a fluorescent quenching for the steady state was higher than 50%, smaller fraction volumes were used. The obtained values were then normalized for total fraction volume (~0.3 mL) and further normalized for 1 mg of the membrane protein loaded on the gradient in order to compare different experiments and evaluate a contribution of each compartment and each fraction to the enzyme activity of all membranes.

To evaluate a coupling efficiency of the pump, the initial velocity of its H^+-translocase activity and ATP hydrolytic activity were determined as concanamycin sensitive processes for the same membrane fractions. Concanamycin A concentrations of 22, 110 and 1000 nM were tested and a similar inhibition was found with these different concentrations of the antibiotic using at least 5 replicates for each concentration and control (without inhibitor). In parallel determinations, the P-type H^+ pump was blocked with 200 μM orthovanadate and concanamycin-sensitive ATPase was also determined. Both type of the detecting of V ATPase (in the presence or absence of vanadate) gave similar results. ATP hydrolysis was measured both in the presence of ATP regenerating system and without it. In the first case it was done according to [25] with some modifications. 6–12 μg of the membrane protein were pre-incubated with 22 nM concanamycin for 4 min at 30 °C in the medium containing 100 mM sorbitol, 50 mM KCl, 20 mM NaCl, 2.5 mM $MgSO_4$, 0.2 mM EGTA, 20 mM MOPS-K pH7.2 and ATP regenerating system (2 mM phosphoenolpyruvate, 0.35 mM NADH, 23 U/mL pyruvate kinase and 11 U/mL lactate dehydrogenase). The ATP hydrolysis was initiated at final concentration of 1 mM ATP-Na pH 7.2 and was indirectly monitored by a decrease of NADH content at 340 nm (Schimadzu UV-1203 spectrophotometer). In order to prevent a possible side effect of the regenerating system, it was omitted from two protocols of ATP hydrolysis determined by the P_i release performed according to [26] with some modifications. In these assays the linearity of the P_i release was verified for 30 min by incubation at 30 °C with 5 mM ATP-Na and 7.5 mM $MgSO_4$ or for 10 min when the concentrations of those reagents were 1 mM and 2.5 mM, respectively. To stop the ATP hydrolysis the assay tubes were placed in ice and reaction medium received cold water to give a final volume of 1 mL and then 2 mL of solution C prepared by a mixture (100:1) of solution A (0.5% ammonium molibdate, 2% H_2SO_4 and 0.5% sodium dodecylsulphate (free of P_i)) and solution B (10% ascorbic acid). The assay mixture was incubated at 30 °C and the P_i released was estimated by measuring the blue phosphomolybdenic complex after 10 min at 750 nm. All these protocols used for determination of ATP hydrolysis by V H^+-ATPase gave similar results, but the activities obtained with 1 mM ATP in the absence of the ATP regenerating system were lower (values did not differ by more than 30%), and a relative ratio between the coupling of the pump in different organelles were preserved. The coupling ratio of the pump in each compartment was calculated by dividing V_0 of H^+ transport with ATPase activity. The data presented in Table 2 were obtained using the regenerating system.

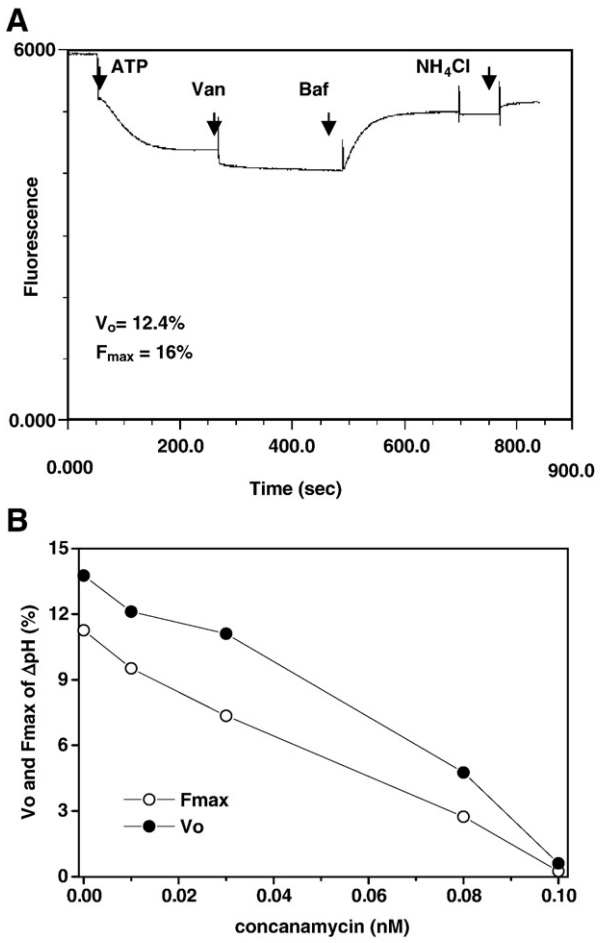

Fig. 1. ATP dependent H^+ transport mediated by V H^+-ATPase in total membranes of *S. cerevisiae* AA255. (A) Membranes were pre-incubated for 3 min with 100 μM vanadate before the addition of 1 mM ATP. The second addition of vanadate had no effect on ΔpH but 80 nM bafilomycin collapsed it completely. (B) Formation of ΔpH is prevented by low concentrations of concanamycin added 3 min before 1 mM ATP. One representative experiment of at least ten independent isolations is shown.

Determination of the activities of organellar marker enzymes as well as protein content followed published procedures [19,21,27–30]. Sucrose concentration was determined using a refractometer.

2.4. Immunoblot

The proteins of the selected membrane fractions, each of 10 or 20 μL, were separated by 7.5% SDS-PAGE, transferred to nitrocellulose membranes and probed with monoclonal antibodies specific for either subunit A or subunit B of yeast V H^+-ATPase (Molecular Probes). SDS-PAGEs were run separately for detection of subunits A and B except for that shown in Fig. 4B. Dot blots performed for the Pmc1 Ca^{2+}-ATPase detection in membrane fractions (2–10 μL in different experiments) were probed with anti-HA antibody (dilution 1:5000). Cross-reacting proteins were revealed using peroxidase-conjugated secondary antibody (GE Healthcare) and then quantified according to [31].

3. Results and discussion

3.1. Separation and characterization of yeast membranes

At first the activity of V H^+-ATPase was evaluated in crude membranes of *S. cerevisiae* AA255. The chemical gradient of H^+ ions, ΔpH, was formed in the presence of ATP and 100–200 μM orthovanadate, the inhibitor of P-type H^+-ATPase (Fig. 1A). The gradient was rapidly dissipated by the addition of bafilomycin A_1 (Fig. 1A) or concanamycin A (not shown). When 0.05–0.07 nM concanamycin A was added before ATP, it reduced vanadate insensitive H^+ transport by 50% (Fig. 1B), and at concentration of 0.1 nM the H^+ transport was blocked by more than 90% (Fig. 1B). Similar results were obtained with total membranes isolated from *S. cerevisiae* X2180, SEY6210 and K699.

Next, total membranes isolated from four analyzed strains were submitted to subcellular fractionation in sucrose density gradient. To improve the efficiency of the membrane separation and reduce a partial contamination of one membrane population by neighboring ones, more detailed membrane fractionation was carried out by increasing the number of fractions from commonly used 11–15 to 48–53.

The membrane population showing the higher activity of a Golgi membrane marker, GDPase [27] (Fig. 2A, fractions 27–43) was separated from membrane population enriched with the ER marker [28], NADPH cytochrome *c* oxidoreductase (fractions 10–27). The localization of this ER marker in membrane fractions 10–27 migrated at 37–48% sucrose (w:w) is in good agreement with other studies [19,21,22,28,32,33] and with our previous data on the migration of other ER marker, BiP, in the same sucrose density when fission yeast membranes were fractionated in identical conditions [22]. Third ER marker enzyme, mannosyl protein transferase [34], displayed 85% of its activity in membrane fractions containing 39–49% sucrose and was efficiently separated from the Golgi enriched membranes of strain SEY6210 (not shown). Membrane fractions 1–10 (49–56% sucrose) were likely derived from the nuclear envelope/ER and partly from PM. The latter co-migrated mainly with the ER membranes according to determination of the vanadate sensitive H^+ transport mediated by P H^+-ATPase (see Fig. 7 below) and vanadate sensitive ATPase activity (not shown). These data are consistent with previous findings showing co-migration of yeast PM and ER membranes under similar conditions of fractionation [32,33].

The migration of the Golgi membrane vesicles (Fig. 2A, fractions 27–43) at 27–38% sucrose (w:w) was found for different organisms [19,21,22,27,35] and was earlier proved by measurement of the latent KEX2 protease as well as GDPase in fission yeast [19]. It is of note that endosomes can co-migrate with the Golgi membranes [36,37] and here we use “Golgi” as equivalent to “Golgi and Golgi-like membranes” [32].

Since the finding of the H^+-translocase activity of V H^+-ATPase in the ER and Golgi enriched membranes (see below) raises the question on their possible contamination with the vacuolar membrane vesicles, a distribution of the conventional vacuolar marker, alkaline phosphatase [30], was analyzed. Fig. 3A shows that the main peak of the alkaline phosphatase activity co-migrated in the lightest membranes with the principal peak of the Ca^{2+}/H^+ antiport activity, which represents an activity of the V H^+-ATPase, which provides ΔpH for the antiporters. Notably, low alkaline phosphatase activity found in the ER and Golgi enriched membranes did not precisely coincide with the antiport activity of those membranes suggesting their low contamination with vacuolar membranes.

Given the importance of the evaluation of the ER and Golgi contamination by vacuolar membranes, distribution of vacuole Ca^{2+}-ATPase Pmc1p [38] was further determined. The highest Ca^{2+}-transport activity insensitive to protonophore FCCP was detected in the lightest membranes (Fig. 3B). As expected from earlier reports [18,19,21,22,32,39] the Ca^{2+}-ATPase activity was also found in several membrane populations derived from different organelles of secretory pathway. In order to distinguish Pmc1p from Ca^{2+}-ATPases of other compartments including Pmr1 Ca^{2+}-ATPase of the Golgi [17 (b),18,21,22,24,32,39] and evaluate a possible contamination by vacuolar membranes, a distribution of Pmc1p::HA along the gradient was determined (Fig. 3B) The single peak of Pmc1::HA clearly coincides with the main peak of Ca^{2+}-ATPase activity in the lightest

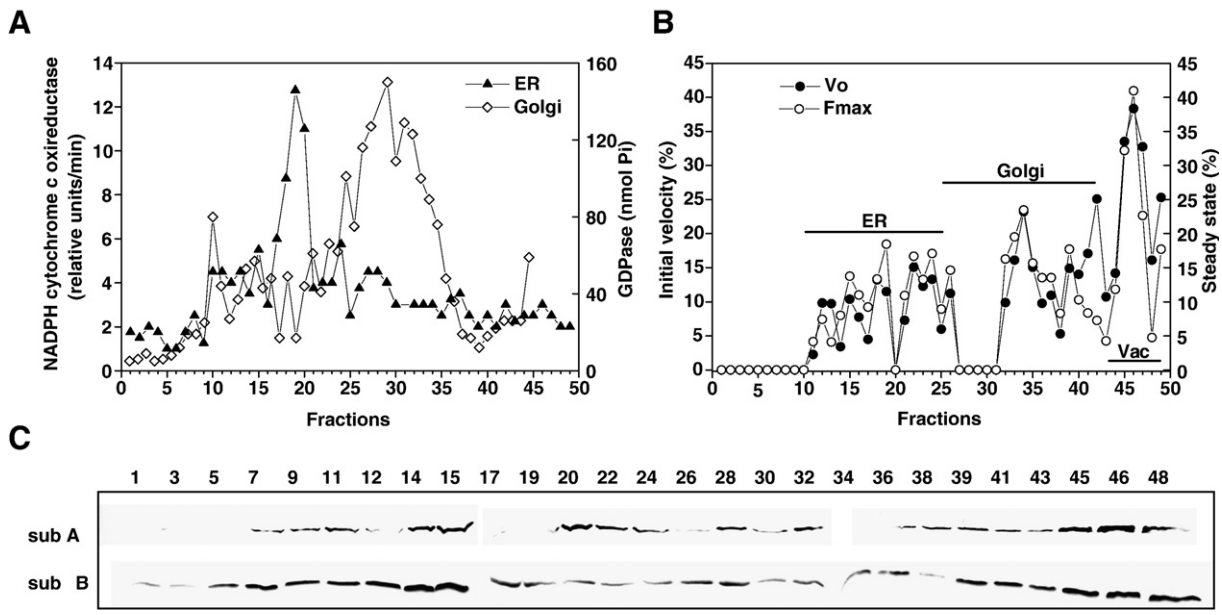

Fig. 2. Different populations of the yeast membranes separated in sucrose density gradient show H^+ transport mediated by V H^+-ATPase. (A) Activity of GDPase and NADPH cytochrome "*c*" oxidoreductase, marker enzymes of Golgi and endoplasmic reticulum, respectively, in membrane fractions of *S. cerevisiae* AA255. (B) The ATP dependent H^+ transport sensitive to 80 nM bafilomycin A_1 across yeast membranes. Initial velocity,V_0 and "steady state" (F_{max}) of ΔpH formation for each membrane fraction are presented as % of the ACMA fluorescent quenching after 1 min and 10 min of the ATP addition, respectively, and normalized for 1 mg of the membrane protein loaded onto the gradient. (C) Western blotting of the selected membrane fractions (numbers shown). One representative experiment of at least seven independent is shown. Aliquots of membrane fractions (20 μL) were subjected to SDS-PAGE and immunoblotted with antibody to subunit A or B (69 kD and 60 kD, respectively).

membranes which also show the highest activity of other vacuole membrane markers, namely of α-mannosidase [29] and alkaline phosphatase (Figs. 3, 4A). Other smaller peaks of Ca^{2+}-ATPase activity did not exhibit detectable content of Pmc1p when the analyses were made under conditions of linear/proportional response of the signals. Very weak cross-reactivity was only found in fractions 8,11,24 and 36. It is noteworthy that total immunoresponse of the fifteen selected fractions from number 3 to 42 represented only 1.3% of the total signal detected in fractions 44, 46, 47 and 49 (Fig. 3B). However, their Ca^{2+}/H^+ antiport activity, which reflects a steady state of the H^+ transport catalyzed by V H^+-ATPase, was 1.4 times higher than in the vacuolar membrane fractions 44,46,47 and 49 taken together (Fig. 3A, B). Notably, the increase of protein content in the membrane samples resulted in the saturation of immunosignals in vacuole membranes while only a slight increase in immunoresponse was observed for ER and Golgi membrane fractions (Fig. 3B and not shown). It is of note that even in conditions of the underestimation of Pmc1p in vacuole membranes, its content in ER and Golgi is relatively very low (Fig. 3B).

Therefore, our data on the distribution of the vacuolar markers between different membrane populations provide the strong evidence that under our experimental conditions the ER and Golgi enriched membranes displayed a low contamination by vacuolar membranes or even are not contaminated by those membranes at all.

3.2. Biochemical and immunochemical detection of V H^+-ATPase in the secretory pathway organelles

The determination of the ATP dependent and bafilomycin A_1/concanamycin A sensitive H^+ transport in different membrane populations revealed the highest activity in fractions enriched with vacuolar membrane vesicles (Fig. 2B, fractions 43–49). It is noteworthy that this activity was also found in membrane fractions 11–26 and 32–43 which were enriched with the ER/PM and Golgi membrane vesicles, respectively (Fig. 2B). Importantly, the antibody against the catalytic subunit A of the yeast V H^+-ATPase selectively recognized the epitopes in different membrane fractions (Fig. 2C). As expected, stronger cross-reactivity was found in fractions 45–47 endowed with highest H^+ translocase activity. However, such correlation between the subunit A immunoreactivity and initial velocity of H^+ transport was not observed for some other membrane fractions. For example, fraction 48 which exhibited the initial velocity of H^+ transport similar to that of membrane fractions 24, 34–36 and 41, was characterized by significantly higher immunoreactivity than these fractions (Fig. 2C). Also, H^+ pumping was found in membrane fractions 12, 17, 26 and 34 displaying very weak affinity to anti-subunit A antibody.

Similar results were obtained for membranes of the wild type strain X-2180. The highest H^+-transport activity of V H^+-ATPase was found in the vacuolar membrane fractions which also exhibited a peak of α-mannosidase activity, a vacuolar marker [29] (Fig. 4A). The ratio of the H^+-transport activity to α-mannosidase activity in membranes distinct of the vacuolar membranes differs from that of vacuolar membranes, being higher in fractions 1–31 and lower in fractions 36–43. It is also of note that α-mannosidase activity, which co-migrated with the ER membranes is due to 1,2 α-mannosidase, the ER resident enzyme [40]. The data suggest that H^+ transport activity found in membrane fractions 1–43 is not due to the contamination by vacuolar membranes. Interestingly, the ER and Golgi enriched membrane vesicles displayed very similar total initial velocity of H^+ transport (fractions 10–24 and 28–40, 103 and 99 relative units, respectively; Fig. 3A). Therefore, both the ER and Golgi membranes possess twice higher total V_0 comparing to the vacuolar membranes (Fig. 4A, 54 relative units for fractions 44–48). Given that membrane fractions 3–9 also contribute in the enzyme activity the data suggest that less than 20% of the functionally active V H^+-ATPase molecules of the yeast cell are localized to the vacuolar membranes if all forms have identical kinetic properties. Another data interpretation is that the ER and Golgi enzyme forms exhibit stronger coupling of the ATP hydrolysis and H^+ transport than a vacuole pump.

Similarly to the AA255 strain, the strongest cross-reactivity of the subunit A antibody was detected in the lightest membrane fractions enriched with vacuolar membrane vesicles of the X2180 strain (Fig. 4B, C, fractions 48–53). Noteworthy, similar or even higher initial velocities of H^+ transport in several ER derived fractions in comparison with those of vacuole were in apparent contradiction

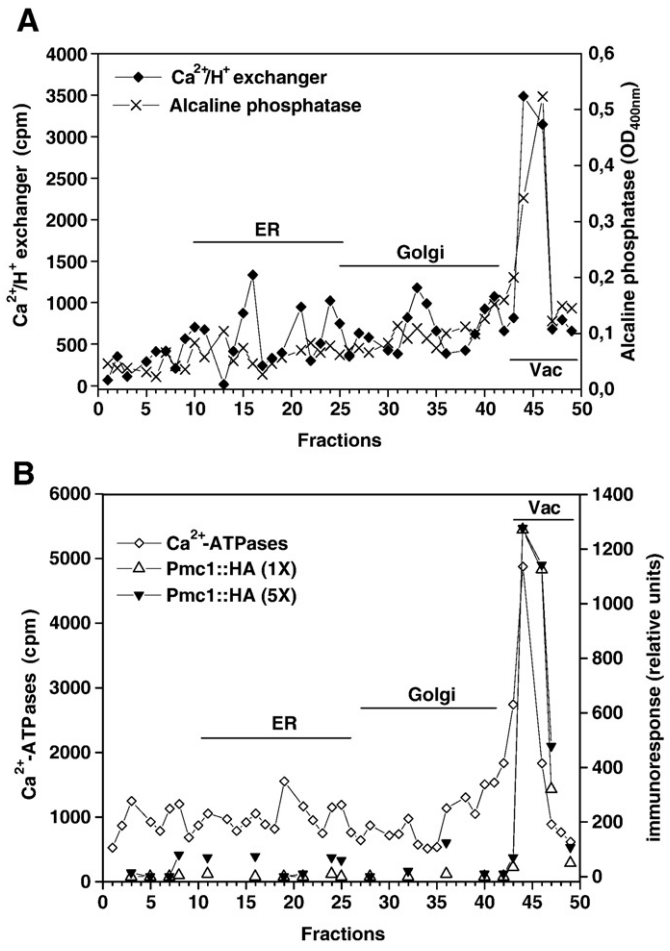

Fig. 3. Membrane fractions enriched with the ER or Golgi are free from vacuole membranes. Membranes were isolated from spheroplasts of K699 strain, which were pre-incubated 10 min with 100 mM glucose and then fractionated in sucrose density gradient. (A) Main peaks of the V H^+-ATPase dependent Ca^{2+}/H^+ exchange activity and the alkaline phosphatase activity coincide with vacuolar membrane fractions. (B) The major peak of the Ca^{2+} transport mediated by Ca^{2+} ATPase correlates with that one of the Pmc1 Ca^{2+}-ATPase immunoresponse (Pmc1::HA). Please note a very low immunoreactivity for Pmc1p in all membranes distinct of the vacuolar membranes including the Golgi (open triangles). The use of the higher membrane protein (5×) displayed an increase of the immunosignals (closed triangles) of non vacuole membranes, while the vacuole ones clearly show a saturation of the signal.

with a relatively low immunoreactivity of subunit A in ER enriched membranes when compared to vacuolar membranes (Fig. 4B, C fractions 17–28 and 48–53, respectively).

Immunoblot analysis with the antibody against the subunit B of yeast vacuolar H^+-ATPase revealed the presence of cross-reactive protein in the tested membrane fractions of *S. cerevisiae* AA255 and X2180 (Figs. 2C and 4C). In general, no direct correlation was found between the immunoreactivity of the subunit B and H^+ translocase activity in ER and Golgi enriched membranes comparing to those of vacuolar vesicles (Figs. 2B, C and 4B, C). For example, immunoreactivity of subunit B in membrane fractions 21–28 is weaker than that of the vacuolar fractions 48–53 contrasting with higher initial velocity of the H^+ transport found in those ER enriched membranes (Fig. 4B, C).

Detection of H^+ transport mediated by V H^+-ATPase in membrane vesicles migrated at sucrose density typical for the ER and PM derived vesicles makes it possible also to assume that the enzyme can be localized to each of those membranes or even only to PM. The localization of the functionally active enzyme to PM of some specialized animal and insect cells was shown previously [2,41]. In plant cells the presence of the enzyme in PM was detected by electron immunomicroscopy [42]. However, the pump is not considered up to now as a good candidate for resident protein of the PM, probably due to a lack of data showing the H^+ transport mediated by the enzyme in the plant PM vesicles [43]. To our knowledge there are no data on the detection of V H^+-ATPase in yeast or fungi PM. Even more, the enzyme was not found in the yeast secretory vesicles transporting P H^+-ATPase to PM [44]. However, we verified a possibility of the localization of the functionally active enzyme to the yeast PM. To this end we have used the specific modification of the PM by concanavalin A (ConA) which results in the formation of large heavy sheets of PM-ConA rather than vesicles [23,24]. These sheets can be removed by centrifugation at low speed together with two-thirds of chitin synthetase activity, while the ER, Golgi and vacuole vesicles were retained in supernatant [23]. The labeling of the PM proteins of

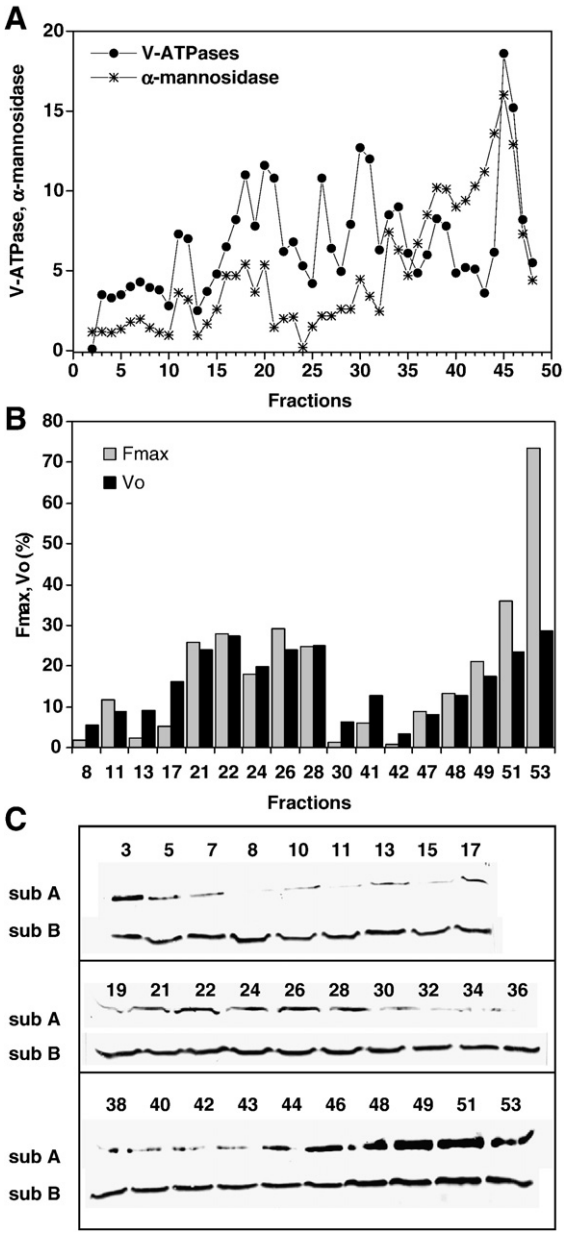

Fig. 4. V H^+-ATPase detected in the ER and Golgi membranes is not due to vacuolar membranes. (A) Initial velocity (V_0) of H^+ transport mediated by V H^+-ATPase and α-mannosidase activity of membrane fractions of *S. cerevisiae* X2180 separated in sucrose density gradient. (B) H^+ transport and (C) Western blotting for the subunits A and B of V H^+-ATPase in the membranes of *S. cerevisiae* X2180, separated in sucrose density gradient. Membranes were isolated without pre-incubation of spheroplasts with glucose.

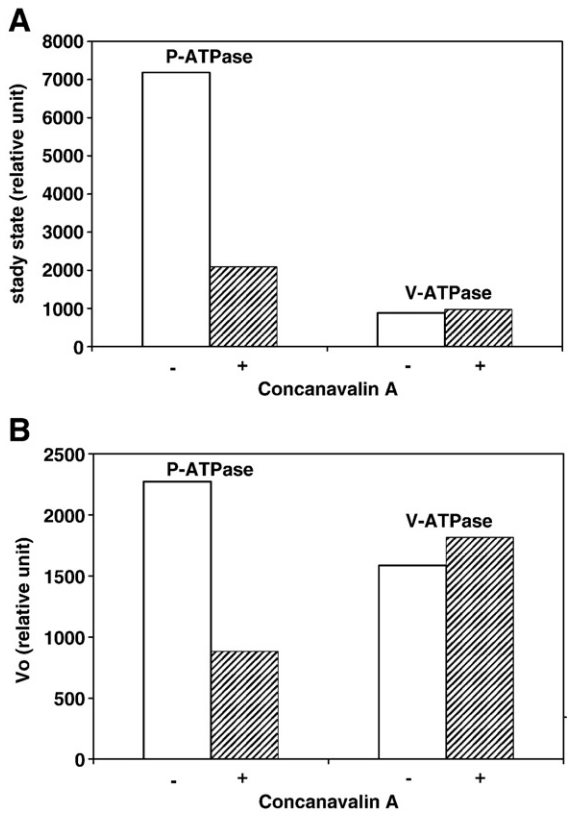

Fig. 5. Removal of the PM did not decrease H^+ transport mediated by V ATPase in PM and ER enriched fractions. Modification of the PM of spheroplasts with concanavalin A and subsequent removal of PM reduced H^+ transport activity of P H^+-ATPase of membrane population enriched with the ER and PM (combined fractions 12–32 of 50) more than 75% without any decrease of V H^+-ATPase activity (one representative experiment of two is shown). Membranes were isolated after 10 min pre-incubation of spheroplasts with 100 mM glucose. Steady state and V_0 of H^+ transport mediated by P and V ATPases before (−) and after (+) the ConA removal of PM is shown in A and B, respectively.

spheroplasts with ^{125}I and the following demonstration of the absence of the ^{125}I-marked proteins in these membranes indicated that they were free from the PM [23]. Using this delicate procedure of the intracellular membranes purification from PM and subsequent fractionation of intracellular membranes in sucrose density gradient we found that V_0 of the H^+ transport mediated by P H^+-ATPase activity was decreased in ER and PM enriched fractions by 60% (Fig. 5). Given that 50% of PM vesicles are right side out (detectable activity) and 50% are right side in (the enzyme has not available ATP), one can calculate that 75% of P H^+-ATPase activity of those fractions was removed together with the PM-ConA complexes. In terms of the steady state of H^+ transport 83% of the activity was lost as the result of PM elimination. It is noteworthy, that V H^+-ATPase activity was not decreased at all by this procedure, showing even increase of the V_0 and the steady state by 15% and 11%, respectively. Our data, therefore, collaborate with published results on the very low content of V H^+-ATPase in the yeast secretory vesicles which transport P H^+-ATPase to the PM [44] and support the conclusion on the low content of V H^+-ATPase in PM and its small contribution (if any) to the total H^+ transport catalysed by this pump. Finally, they indicate that the activity of V H^+-ATPase detected in the ER and PM enriched fractions is mainly (if not exclusively) due to the ER enzyme.

Since the ER and Golgi membranes are almost free of the vacuolar membranes (see Figs. 3 and 4A), our data point to the existence of functionally active V H^+-ATPase localized to the yeast ER and Golgi membranes additionally to its known localization to vacuoles. However, the absence of the direct correlation between the initial velocity of H^+ transport and the immunoreactivity of subunits A and B within the secretory pathway (Figs. 2, 4) supposes the presence of distinct/modified forms of the pump in yeast secretory pathway organelles.

3.3. Distinct forms of the V H^+-ATPase along the secretory pathway

Additional differences between V H^+-ATPases of the ER, Golgi and vacuolar membranes can be observed when the apparent stoichiometry B:A is taken into account. Since two different antibodies were used in this study to detect these subunits we did not expect that they would show similar affinity. However, the ratio between immunoresponses (signals) revealed for subunits B and A was close to 1:1 in the vacuolar membrane vesicles of both strains, AA255 and X2180 (fractions 45 and 46, Fig. 2C and fractions 46–53, Fig. 4C). This is in line with the determination of the stoichiometry B:A of V H^+-ATPase in vacuoles of different organisms which is used in the widely accepted model of the enzyme structure [1–5,41,45–47]. Interestingly, in both strains the ratio of signals for the subunits B and A was considerably higher in membranes denser than vacuolar ones (fractions 1–19; 26–43 Figs. 2C, 4C and 6C, fractions 10–47 and 49–52).

Further experiments are necessary to investigate the reasons of the apparent variations of stoichiometry B/A in membranes enriched with ER or Golgi and membranes heavier than ER. We assume that the post-translational modification of the catalytic subunit A and regulatory subunit B can alter their immunoreactivity promoting both the seeming increase of the stoichiometry B:A and the apparent contradiction between V_0 of H^+ transport and the content of subunits A and B in different compartments of secretory pathway. Such modification seems more likely for the catalytic subunit A since its immunoresponse was more irregular and weaker in the ER and Golgi than that detected for subunit B. A possibility of the phosphorylation of different enzyme subunits by WNK protein kinases was recently shown for *Arabidopsis thaliana* [48]. The apparent deviation from the expected properties of the enzyme in compartments different from vacuoles could be due to some other factors which also do not change the real 1:1 stoichiometry of subunits A and B in the enzyme molecule. There are several reports showing the binding of different subunits of the V_1 complex with microtubules [41,49–51], glycolysis enzymes like aldolase [2,41,52,53] and proteins of the RAVE complex [2,53,55]. For example, if microtubules could bind free subunits B as well as the enzyme itself, all subunits B would dissociate in the presence of SDS from those complexes and therefore increase the total content of subunit B in comparison with that one of subunit A in some membrane fractions.

We conclude, therefore, that the difference between V H^+-ATPases of the secretory pathway organelles might be explained by a combination of several factors including a post-translational modification of the V_1 subunits and/or their binding with different proteins, which probably reflects the selective regulation of V H^+-ATPase within the secretory pathway.

The dissimilarity between V H^+-ATPases of the secretory pathway organelles was further confirmed by experiments where a weak H^+ pumping activity of the V H^+-ATPase was found in vacuolar membranes but the activity was still high enough in the ER and Golgi enriched membrane vesicles (Fig. 6A). It has been reported that in glucose-depleted medium the peripheral catalytic complex V_1 dissociates rapidly from the complex V_0 embedded into membrane [56]. Since the isolation of spheroplasts in our experiments was performed in the absence of extracellular glucose, we verified whether such dissociation resulted in the lower content of V_1 in vacuolar membranes in comparison with the Golgi and ER and therefore can explain a low H^+ pumping in vacuolar vesicles. It was found that the amount of subunit A in vacuolar fractions 49–52 (Fig. 6B) exhibiting no H^+ transport activity was similar or even higher in comparison to Golgi fractions 31–40 endowed with the highest activity. Additionally, the difference in immunoreactivity of subunit B in these vacuolar and

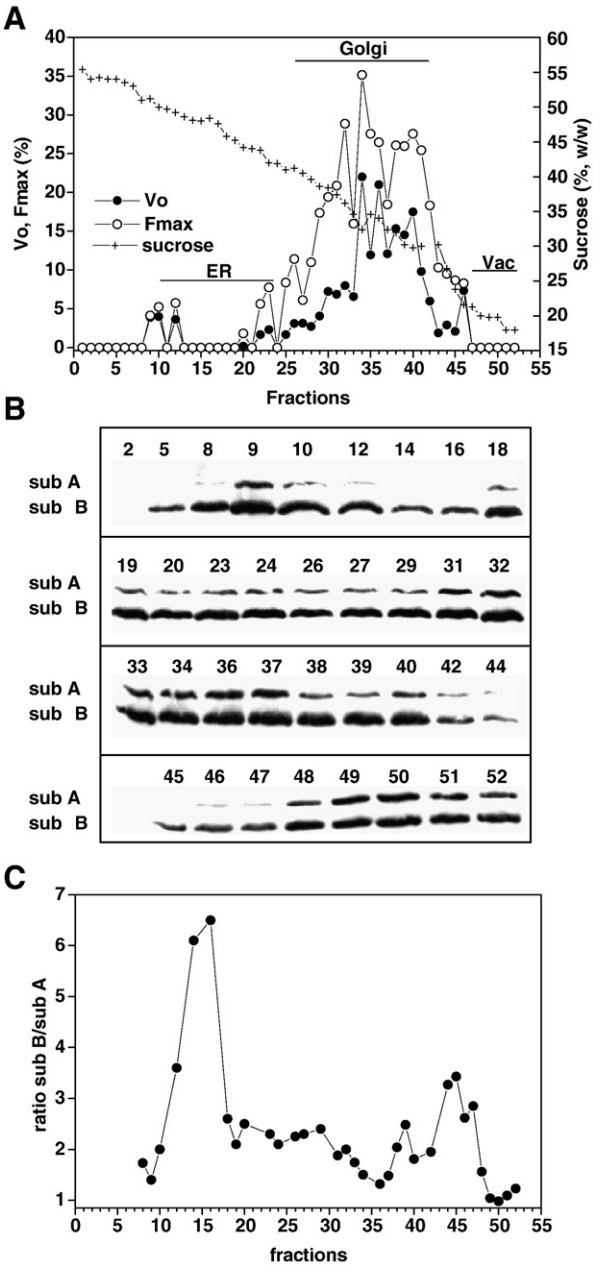

Fig. 6. H^+ transport activity of V H^+-ATPase of membranes derived from distinct organelles of *S. cerevisiae* AA255 does not correlate with immunoreactivity of its subunits A and B. (A) H^+ transport activity of V H^+-ATPase and (B) cross-reactivity of subunits A and B in membrane vesicles of *S. cerevisiae* AA255; (C) ratio between the immunoreactivity of subunit B and subunit A. In (B and C) the numbers represent selected fractions and the analysis of the band densities was done according to [31]. One representative experiment of four independent isolations is shown.

Golgi membranes was not sufficient enough to explain the weak activity or even absence of H^+ pumping in vacuolar membranes. The same is true when one compares the membrane fractions 49–52 with fractions 9, 10 and 12 (Fig. 6A, B). Identical results were obtained for strain X2180 (Fig. 7). The vacuolar membrane fractions 47–49 contained the same or even a higher amount of subunit A in comparison with the ER or Golgi enriched membranes contrasting with their weaker capacity of H^+ transport. (Fig. 7, fractions 9–25 and fractions 27–37, respectively) Furthermore, a low cross-reactivity of subunit A in Golgi membrane fractions 33–37 is in apparent contradiction with a relatively high H^+ transport activity of these fractions. Our data can be taken as evidence that the properties of the V H^+-ATPase of Golgi as well as of the ER are distinct from those of the vacuole enzyme. The vacuolar enriched membranes isolated from spheroplasts not pre-incubated with glucose and showing the weak H^+ transport activity or even its absence presented a high activity when spheroplasts were pre-incubated with glucose in parallel experiments (not shown, manuscript in preparation). These data rule out a possibility of the irreversible inactivation of the vacuolar enzyme when spheroplasts were not incubated with glucose. They also indicate that the H^+ pumps of the ER, Golgi and vacuoles are regulated selectively and/or have different sensitivity to conditions of the membrane isolation and fractionation. Future experiments may explain this distinction between the organellar enzyme forms. One possibility could be a different degree and kinetics of the V_1 dissociation along the secretory pathway under glucose deficit, which can start with a dissociation of subunit C only [56]. A difference between the Golgi and vacuolar H^+ pumps could be attributed to the isoforms of subunit a, Vph1p and Stv1p [1,2,5,15,16]. It was reported that Stv1p isoform is distinct from Vph1p and determines a low coupling of the H^+ transport and ATP hydrolysis as well as a low capacity of dissociation of V_1 from the V_0 complex when overexpressed and localized to vacuolar membrane. Since the V H^+-ATPase activity can be modulated by membrane lipids [57] future experiments should provide more insight whether these or other differences between two forms of the enzyme can be found when the Golgi enzyme would be localized to and analyzed in the native Golgi membrane. When our manuscript was under the revision, it has been reported that the Stv1p containing V H^+-ATPase has also a low capacity for the dissociation of the V_1 complexes from V_0 in the Stv1p containing membranes [58]. However, the H^+ transport activity in those Stv1p containing membranes was not demonstrated.

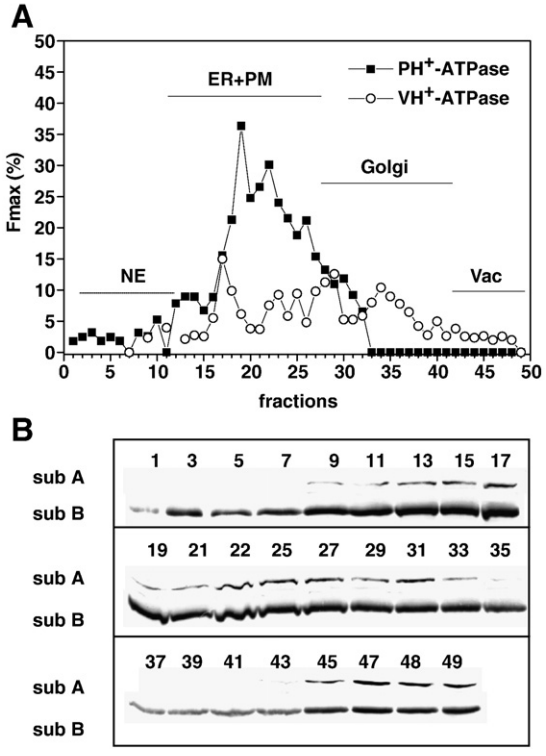

Fig. 7. H^+ transport activity of V H^+-ATPase of membranes derived from distinct organelles of *S. cerevisiae* X2180 does not correlate with immunoreactivity of its subunits A and B. (A) H^+ transport driven by V and P H^+-ATPases in different membrane fractions of *S. cerevisiae* X2180 separated in sucrose density gradient. (B) Western blotting of subunits A and B in the selected fractions (numbers are shown). A representative experiment of three independent isolations is shown.

Table 1
ER H^+-ATPase exhibits higher resistance towards bafilomycin than Golgi and vacuole pumps

Fractions	15	16	17	18	20	22	23	24	25	26	27	28	29	30
$IC_{50,}$ nM	27.4	28.8	38.7	29.2	26.1	37.2	57.5	31.8	36.7	68.5	1.67	0.94	0.27	0.072
Fractions	31	32	33	34	35	36	37	38	39	40	41	43	44	45
$IC_{50,}$ nM	0.152	0.082	0.220	0.165	0.198	0.189	0.188	0.155	0.160	0.075	0.169	0.175	0.240	0.4

Inhibitory analysis of the steady state of ΔpH formation was done using different membrane fractions (numbers are indicated) isolated from *S. cerevisiae* AA255. To find a respective IC_{50}, three-five different concentrations of bafilomycin were used. ER/PM enriched membranes correspond to fractions 10–26, while Golgi and vacuole enriched membranes are in fractions 29–38 and 39–45, respectively.

The ER H^+ pump also differs from the vacuolar V H^+-ATPase. For example, membrane fractions 23, 25–29 (Fig. 6) exhibited H^+ transport activity while the content of subunit A was at least 2–3 times less than in vacuolar fractions 49–51 which did not show H^+ transport activity at all. Also, the ER-derived membranes of *S. cerevisiae* X-2180 (fractions 17–28, Fig. 7A) which showed higher H^+ transport than vacuolar fractions 43–49, exhibited similar or even significantly less immunoreactivity of the subunit A of V H^+-ATPase (Fig. 7B).

The comparison of the subunit A cross-reactivity and H^+ transport revealed also the differences between the ER and Golgi enzymes (Figs. 6, 7).

Our findings can indicate that, firstly, the vacuolar enzyme form still has the higher immunoresponse for both A and B subunits even after partial dissociation of V_1 in the absence of extracellular glucose [2,56], showing, however, lower activity than the Golgi enzyme form which probably lost only a small part (if any) of its V_1 complexes [16,58]. Secondly, low activity of the H^+ pump in vacuolar vesicles is not due to the low content of the catalytic complexes V_1, determined by the presence of subunits A and B. There are several possibilities to interpret this fact: i) under our experimental conditions the first step of the dissociation of V_1 starts with a loose of subunit C by the vacuole enzyme form [56], while the main part of V_1 is still bound to membrane; ii) there is another regulatory mechanism different of the dissociation of V_1 and V_0. Future experiments should reveal a mechanism of the selective down regulation of the vacuole enzyme.

Thirdly, the Golgi and ER enzyme forms differ from that of the vacuolar membranes by their still significant H^+ pumping activity while the vacuolar enzyme lost it or possess a low activity. This fact is of special attention since it can be taken as additional evidence that V H^+-ATPase found in the ER and Golgi enriched membrane vesicles was not due to contamination by vacuolar membranes. It is not clear yet why the vacuolar enzyme form can retain H^+ translocase activity (Figs. 2B and 4B) or partly loose it (Figs. 6 and 7) when membranes were isolated without pre-incubation of spheroplasts with glucose. One possible explanation could be a different kinetics of the modulation of the enzyme activity depending of the secretory pathway organelle and a concentration of extracellular glucose.

Since all data taken together points to the presence of the different enzyme forms in the ER, Golgi and vacuoles, we further analyzed the effect of different inhibitors on their H^+ translocase activity. All forms were completely blocked by 50 mM nitrate (not shown).

We next compared their sensitivity to bafilomycin and concanamycin, expecting to find out additional difference between the Golgi and vacuole pumps. It is known that they have distinct isoforms of the subunit a, which is also a target of these inhibitors [59] additionally to the main target, namely the c subunit [60,61]. Surprisingly, H^+ transport detected in the Golgi and vacuole membranes displayed identical sensitivity to bafilomycin (I_{50} = 0.18 ± 0.02 nM, Table 1). This value is in a good agreement with the reported data on the inhibition of the hydrolytic activity of the vacuolar enzyme (I_{50} = 0.22 ± 0.03 nM [59]. It looks, therefore, likely that the Golgi and vacuolar enzyme forms of the *S. cerevisiae* are more sensitive to bafilomycin than those of tobacco cells, which were only slightly inhibited by 0.66 nM bafilomycin [62]. It is also of note that H^+-translocase activity of both enzyme forms from yeast was completely blocked at 0.5 nM of bafilomycin. Unexpectedly, the ER enzyme form displayed significantly higher resistance to the inhibitor (I_{50} = 38.4 ± 4.4 nM).

Interestingly, concanamycin did not reveal such distinct sensitivity to the antibiotic between the ER and Golgi or vacuole pumps, showing, however, its higher inhibitory capacity than bafilomycin. For example, I_{50} of the initial velocity of the H^+ transport mediated by the ER, Golgi and vacuolar enzyme forms was similar and varied between 0.01–0.08 nM depending on the antibiotic lot, while a full inhibition was commonly achieved at concentrations up to 1.0 nM (not shown). The data suggest a necessity to use even lower concentrations of the inhibitor to verify a differential sensitivity of the organelle enzyme forms or its incapacity to distinguish between those forms from the yeast.

Similar sensitivity of H^+ translocase activities of vacuole and Golgi pumps to bafilomycin might indicate that subunit c is a dominant factor in such sensitivity of these two forms and that the subunit a isoforms is a less critical determinant. This assumption presumes that a subunit composition of c-ring in vacuole and Golgi forms is similar, since all single and even double mutations which decreased the sensitivity of the vacuolar H^+-ATPase to bafilomycin and concanamycin are exclusively localized to subunit c [60,61]. However, more than 200 times higher resistance of the ER enzyme form to bafilomycin in comparison to vacuole and Golgi forms unintentionally reminds a similar difference found for vacuole enzyme from the bafilomycin resistant strains with mutations in subunit c and wild type strain of *Neurospora crassa* and *S. cerevisiae* [60,61]. Given this information we may interpret our data suggesting that the c-ring (rotor) of the ER enzyme form is somewhat different from that one of the Golgi and vacuole enzyme forms.

The fact that all mutations which significantly increased the resistance of vacuole enzyme to bafilomycin were localized to subunit c was used to assume that this subunit has higher affinity to antibiotic in comparison with the subunits c′ and c″ [60,61]. Therefore we further speculated that the stoichiometry 4c:1c′:1c″ reported for the c-ring of the vacuolar enzyme [1-3,5,41,47] is similar in the Golgi enzyme form but is somehow modified in favor of the c′ and/or c″ subunits in the ER pump. Such modification could increase the resistance of the ER enzyme to bafilomycin. This suggestion collaborates well with the very recent finding of the organelle specific composition of the proteolipid complex V_0 in *Arabidopsis thaliana* [69].

Another interesting difference of the organellar forms of V H^+-ATPases was found when their coupling efficiency between two catalyzed processes was compared (Table 2). The lowest coupling ratio of the V_0 of H^+ transport to ATP hydrolysis was detected in ER membranes, reflecting, probably, not completely assembled functional enzyme complex which presented the higher specific activity of ATPase but a relatively weak H^+ transport activity. However, a possibility that the ER enzyme form is more sensitive to conditions of the membranes isolation/fractionation and may loose its coupling efficiency/factor cannot be ruled out.

The highest coupling efficiency was found in the Golgi enriched membranes even in comparison with vacuole membranes (Table 2). That is unexpected finding from the point of view that Stv1p may decrease a coupling efficiency four times when it is artificially directed to vacuolar membranes [16,25]. However, this property of the Stv1p was not yet verified in the Golgi native membranes. It is of note that the coupling

Table 2
H^+ transport (V_0), ATP hydrolysis and coupling efficiency of organelle forms of V H^+-ATPase of *S. cerevisiae**

Membranes	Initial velocity of H^+ transport, V_0 (% fluorescent quenching . mg^{-1} . min^{-1})	ATP hydrolysis (μmol P_i . mg^{-1} . min^{-1})	Coupling ratio[a] (V_0/ATPase)
Vacuoles	738±54	0.317±0.020	2350±248
Golgi	980±81	0.295±0.066	3906±833
ER	103±7	0.400±0.069	276±54
Pellet	114±22	0.201±0.044	580±106

[a] Data are mean values of three independent experiments±standard deviation. Coupling ratio is in % of fluorescence quenching/μmol ATP.

efficiency of Golgi V H^+-ATPase in selected membrane fractions of the full scale gradient is 1.65 times higher than that of vacuole enzyme (Fig. 4A fractions 29, 33–39, 40, 42 and 44, 46–48, respectively; not shown), while this value in the simplified gradient is 1.66 (Table 2). Therefore, our results show that the coupling efficiency of the Golgi enzyme form is at least not lower than that of the vacuole pump.

It is also of interest that the coupling efficiency of the Golgi pump in native membranes (Table 2) is six times higher than that one of vacuole membranes, which have artificially received the Stv1p enzyme form in the result of the overexpression of the respective gene (Table 1 in Ref. [25]). At the same time coupling efficiency of the vacuole pump detected in our work is only 13% lower than that reported for native vacuole membranes containing Vph1p [25]. Taken together these data point to the dominating role of the lipid membrane environment rather than the isoform of subunit a for the coupling efficiency of the V H^+-ATPase. Our findings are also in agreement with the important role of the membrane lipid environment for the coupling efficiency of the enzyme [2,57]. Further experiments should clarify more precisely the coupling ratio of these two forms in different physiological conditions, but importance of the Golgi apparatus in the formation of secretory vesicles even under conditions of nutrient limitation can dictate and explain a necessity for high coupling efficiency of the V H^+-ATPase in Golgi and secretory vesicles. The determination of the enzyme coupling efficiency in Golgi sub-compartments itself is one of the interesting questions, since the different coupling of H^+ and Ca^{2+} transport by Ca^{2+}/H^+ exchanger has been previously shown for different Golgi sub-compartments in the fission yeast *Sch. pombe* [19].

It was also found that a coupling efficiency of the V H^+-ATPase of membranes heavier than ER and derived probably from the nuclear envelope is two times higher than that of the ER membranes (Table 2). Therefore, considering all data on the coupling efficiency of V H^+-ATPase in different organelles, we speculate that i) any membrane equipped with V H^+-ATPase has a coupling efficiency of the pump higher than ER; ii) coupling efficiency is a relatively constant characteristic of the determined type of the organelle, and iii) all these membranes differ by their coupling efficiency of the pump.

Future experiments have to verify whether coupling efficiency of the pump is modulated for the same type of membrane under various physiological conditions but continue to be different between distinct organelle forms of the V H^+-ATPase.

Taken together our data provide convincing evidence that the V H^+-ATPase is an intrinsic enzyme of the ER, membranes heavier than the ER, Golgi and Golgi-like membranes besides the vacuole and very likely presented by distinct forms and/or selectively regulated.

Future studies are necessary to further characterize the V H^+-ATPases of different organelles of the yeast secretory pathway. Additional suppositions explaining different properties of the organellar V H^+ pumps can be made. These include divergent mechanisms of the enzyme regulation [1–3,43,46], diverse lipid environment of the enzyme in distinct compartments [2,57] and the possible modification of the enzyme composition by changing the isoform of subunit a Vph1p to Stv1p at least in the late Golgi [15,16]. The V H^+-ATPases of different secretory pathway organelles can vary one from the other by their resistance/sensitivity to the conditions of membrane isolation and fractionation, which probably reflects all possible factors indicated above as well as others.

The finding of the V H^+-ATPase in the yeast ER requires some commentaries. According to available information, the pH of the ER lumen in HeLa cells is close to that of cytosol [63,64] and therefore is in apparent contradiction with a possibility of the significant acidification of the ER lumen. In general, one should consider the importance of both the primary H^+ pump and of ion channels and secondary transporters. Their coordinate activities can contribute in the final pH of the organelle lumen as well as in a different manner of the energization of the membranes of the secretory pathway organelles. The finding of low coupling efficiency of the ER enzyme form (Table 2) can, probably, partly explain relatively high pH in the ER lumen. We assume additionally that *in vivo* the ER V H^+-ATPase together with Ca^{2+}/H^+ exchanger can create predominantly an electrochemical gradient of Ca^{2+} formed by the membrane potential of Ca^{2+}, E_M^{Ca2+} and ΔpCa, while ΔpH is small in comparison to Golgi. In other words, we assume that ΔpH increases from the ER to Golgi and then to vacuole in according to [63,64], but E_M^{Ca2+} decreases [65,66]. It has been shown that the energization of the vacuolar membrane by V H^+ ATPase can be modified by Ca^{2+} due to its exchange with H^+ and a conversion of the ΔpH to E_M^{Ca2+} when anion channel is closed by Ca^{2+} and V H^+-ATPase is activated (because it is released from proton control). As a result, ΔpH decreases and membrane potential formed by Ca^{2+} increases supporting a strong activation of the α-ketoglutarate or citrate uptake [65,66]. The presence of the Ca^{2+}/H^+ exchanger in the yeast ER membranes and the NE-like membranes was shown (References[17(a)–19], Fig. 3A and in preparation) but its possible role in the regulation of the magnitude of the E_M^{Ca2+} and ΔpH across the ER membranes needs to be investigated. One could suppose that the change of organellar lumenal pH would modify a ratio between a free and bound Ca^{2+} in the Ca^{2+} store organelles. From this point of view the free Ca^{2+}concentration has to decrease from vacuoles to Golgi and then to ER where a relatively low free Ca^{2+} concentration was determined [67]. However, the efficient Ca^{2+} release from the Ca^{2+} bound pool has been demonstrated [68].

Additional interesting questions follow from the finding of organelle forms of V H^+-ATPase in yeast. These included the presence of anion channel in the yeast ER and Golgi, its role in the regulation of the respective V H^+-ATPase forms and a level (degree) of the luminal acidification, a possible role of Ca^{2+} in the regulation of the channel itself and organelle V H^+-ATPases. It is interesting to note in this context the surprisingly high efficiency of Ca^{2+} to decrease the V_0 of the ΔpH formation in the ER in comparison with Golgi and vacuoles (not shown).

In conclusion, this study provides direct evidence that the functionally active V H^+-ATPase is an intrinsic enzyme of the ER and Golgi of *S. cerevisiae*. It can stimulate the investigations of the role of V H^+-ATPase in: i) energization of the secretory pathway membranes and its importance for a proper function of each organelle and entire secretory pathway, including fusion and fission of membrane vesicles and protein targeting and secretion; ii) ion homeostasis of each organelle and whole cell; and iii) Ca^{2+} and H^+ signaling.

Acknowledgments

This work was supported by CNPq (Conselho Nacional de Pesquisa e Desenvolvimento), FAPERJ (Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro) and UENF (Universidade Estadual do Norte Fluminense). The authors thank Prof. L. Lehle, W. Tanner and K. Cunningham for kindly providing yeast strains and Dr. M. Gentzch for the determination of protein mannosyltransferase activity. We are grateful to L.C. Dutra de Souza for technical assistance. S.S.S. and C.E.S.

T. were PhD fellows from UENF, F.E.S. and C.C.R. are recipients of a CAPES (Coordenação de Aperfeiçoamento de Pessoal no Nível Superior) PhD fellowship, T.M.G. and N.R.B. were recipients of fellowships from CNPq and UENF, respectively.

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