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Reconstitution of the *Neurospora crassa* plasma membrane H^+-adenosine triphosphatase

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Abstract

The purified H^+-ATPase of the *Neurospora crassa* plasma membrane has been reconstituted by a gel filtration method into lipidic vesicles using sodium deoxycholate as the detergent. Reconstitution was performed for lipid/ATPase ratios ranging from 1000:1 to 5:1 (w/w). Whatever the lipid/ATPase ratio, the ATPase molecules completely associate with the lipid vesicles. The ATPase specific activity is identical for all proteoliposomes regardless of the lipid/ATPase ratio, but the H^+ transport decreases at high protein/lipid ratios, suggesting that the proteoliposomes are more leaky to H^+ as the amount of protein inserted into the lipidic membrane increases. Analysis of the fragments generated by trypsin proteolysis in the presence and in the absence of MgATP + vanadate indicate that most of the reconstituted ATPase molecules are able to assume the transition state of the enzyme dephosphorylation reaction, and are therefore functional. The orientation (inside-out or rightside-out) of the ATPase molecules in the vesicles is independent of the lipid/ATPase ratio chosen for the reconstitution. For all the lipid/ATPase ratios tested, most of the ATPase molecules (> 99%) expose their cytoplasmic side to the outside of the reconstituted proteoliposomes. The size of the vesicles increases parallel to the ATPase amount. Although the H^+ leakiness of our preparation at low lipid/protein ratios prevents proton pumping measurements, the reconstitution procedure described here has the main advantage on other procedures to allow the obtention of vesicles at high protein-to-lipid ratios, facilitating further structural characterization of the ATPase by biochemical and biophysical techniques. Therefore, the procedure described here could be of general interest in the field of membrane protein study.

Keywords: ATPase; Reconstitution; Plasma membrane; (*N. crassa*)

1. Introduction

The H^+-ATPase of the *Neurospora crassa* plasma membrane is an electrogenic pump which couples H^+ transport across the membrane to ATP hydrolysis [1,2]. This enzyme belongs to the aspartyl phosphate intermediate family of transport ATPases which includes the Na^+/K^+-, H^+/K^+-, and Ca^{2+}-ATPases of animal cell plasma membranes, the Ca^{2+}-ATPase of sarcoplasmic reticulum, the *E. coli* plasma membrane K^+-ATPase, and the H^+-ATPases of fungal and plant plasma membranes [3–7]. This integral membrane protein was first purified from *Neurospora crassa* cells by Addison and Scarborough [8], Bowman et al. [9] and the purification was scaled up and improved by Smith and Scarborough [10]. As isolated by the Smith and Scarborough method [10], the neurospora H^+-ATPase exists as a homogeneous hexameric complex of six 105 kDa monomers [11]. The purified H^+-ATPase has been reconstituted into lipid vesicles by a freeze-thawing procedure in which sonicated asolectin SUV are mixed with the purified H^+-ATPase and the whole mixture is then frozen in liquid N_2 and thawed in a 30° C bath [12]. In the presence of a large excess of lipid, this reconstitution of the H^+-ATPase leads to proteoliposomes containing ATPase monomers able to translocate H^+ ions and to hydrolyse ATP [13]. However, the freeze-thawing procedure does not allow the insertion of large amounts of protein into tightly coupled proteoliposomes (minimum

Abbreviations: ATPase, adenosine triphosphatase; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; SUV, small unilamellar vesicle; DOC, (Na)deoxycholate; ACMA, 9-amino-6-chloro-2-methoxyacridine; FF1, Folch Fraction 1.

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lipid/protein ratios of 770:1 (w/w) [14]). This high lipid content impedes subsequent analysis of the ATPase by biochemical and biophysical techniques.

In the present paper, we characterize proteoliposomes with much higher protein to lipid ratios obtained by a gel filtration method. The distribution of the protein and the orientation of the ATPase (inside-out or rightside-out) in the reconstituted vesicles, the size of the reconstituted vesicles and the ATPase specific activity and functionality are analyzed as a function of the lipid/protein ratio used for the reconstitution.

2. Materials and methods

2.1. Chemicals

The H^+-ATPase was purified from *Neurospora crassa* cell wall-less mutant cells, designated fz;sg;os-1 V (Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City, KS 66103) as described by Smith and Scarborough [10]. The purified ATPase (about 400 μg of protein/ml) is stored at −20° C in a solution containing 35% (w/v) glycerol, 2 mM ATP, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.2% DOC, 2 μg/ml chymostatin, 154 μg/ml dithiothreitol (DTT) pH adjusted to 6.8 with Tris. The phospholipids used in this study were crude soybean phospholipids ('L-α-phosphatidylcholine type IIS' from Sigma), washed by the procedure of Kagawa and Racker [15]. The phospholipid composition of such preparation was determined by Casey et al. [16] as 22% phosphatidylcholine, 40% phosphatidylethanolamine, 20% phosphatidylserine, 5% phosphatidic acid, 11% lysophosphatidyl compounds and 2–3% other acidic phospholipids. The G-50 medium Sephadex was from Pharmacia. Trypsin was from Boehringer-Mannheim. The ATP, the sodium orthovanadate (Na_3VO_4), the 9-amino-6-chloro-2-methoxyacridine (ACMA), the nigericin and the FF1 ('Folch Fraction 1', a lipid extract from bovine brain) were purchased from Sigma. All other reagents were of the highest purity grade commercially available.

2.2. Reconstitution of the H^+-ATPase

DOC/phospholipid micelles (1.3:1 (w/w) ratio) were prepared by dispersion of a dry film containing 20 mg crude soybean phospholipids in 67 μl of 10% (w/v) sodium deoxycholate + 4 μl of 100 mM ethylenediaminetetraacetic acid (EDTA) + 4 μl of buffer (100 mM Mes, 500 mM potassium acetate adjusted to pH 6.8 with Tris). The 0.4 mg/ml purified ATPase solution was added (5 μg to 1 mg) to the mixed phospholipids-DOC micelles and this mixture was eluted on a 1.5 × 27 cm G-50 medium Sephadex column at a flow rate of 1 ml/min in 10 mM Mes, 50 mM potassium acetate adjusted to pH 6.8 with Tris (reconstitution buffer).

For control experiments, liposomes were prepared according to the same procedure but the ATPase solution was replaced by a 35% (w/v) glycerol solution in the reconstitution buffer.

2.3. Discontinuous glycerol gradients

Separation of proteoliposomes from pure lipid vesicles

Glycerol density gradients are able to separate vesicles which contain a single copy of the ATPase monomer from pure lipid vesicles [13]. Here, 200 μl of the vesicle preparation were layered on 3 ml of a 17% glycerol solution in the reconstitution buffer, itself layered on 500 μl of a 50% glycerol solution. An overnight 100 000 × *g* centrifugation at 4° C in a Beckman sw60Ti rotor led to the separation of ATPase-free liposomes at the top of the gradient, from the ATPase-bearing liposomes (more dense) which migrate to the bottom of the gradient. After centrifugation, 0.2 ml fractions were collected from the bottom of the tube.

Separation of proteoliposomes from pure protein aggregates

200 μl of the proteoliposomes were centrifuged overnight at 100 000 × *g* at 4° C after layering on a 29% glycerol solution (with a 500 μl cushion of 50% glycerol) in a Beckman sw60Ti rotor. 0.2 ml fractions were collected from the bottom of the tube. In this gradient, the free (non-reconstituted) ATPase migrates at the bottom of the tube while the proteoliposomes stay on top.

2.4. Limited trypsinolysis of H^+-ATPase reconstituted in the proteoliposomes

The ATPase-bearing vesicles were concentrated in the reconstitution buffer to about 25 mg phospholipids/ml within a CF-ConFilt hollow-fibers bundle (M_r cutoff 6000, Bio-Molecular dynamics, USA). The trypsin treatment was performed essentially as described by Hennessey and Scarborough [14]. Briefly, 50 μl aliquots containing the proteoliposomes were mixed with 3 μl H_2O (controls) or 3 μl of a mixture containing MgATP (200 mM, pH 6.8 with Tris) and sodium orthovanadate (10 mM), and then incubated at 30° C for 10 min minimum (preincubation). The final MgATP and vanadate concentrations are respectively 10 mM and 100 μM. A freshly prepared stock solution of trypsin (1 mg/ml in the reconstitution buffer) was then added to the proteoliposome preparation (the proteinase/ATPase ratio was 1:10 (w/w)) and the mixture was incubated at 30° C for 0 to 60 min with occasional mixing. The total incubation time (sum of the preincubation time plus time of incubation with trypsin) at 30° C was 70 min for all samples. The incubation was stopped by adding 1/4 volume of acidic disaggregation buffer containing 100 mM Tris, 8% (w/v) sodium dodecylsulfate (SDS), 100 mM 2-mercaptoethanol, 40 μg/ml chymo-

statin, 0.00125% (w/v) bromophenol blue (adjusted to pH 2 with phosphoric acid (H_3PO_4)).

Controls incubated without trypsin were prepared for each series of samples, as well as 'zero time controls' obtained by adding a premixed solution of acidic disaggregation buffer and the proteinase to the mixtures after the preincubation period in presence of the ligands. All the samples were then kept on ice until the SDS-PAGE was carried out.

2.5. SDS-PAGE analysis

The samples were mixed with 1/4 volume of acidic disaggregation buffer (100 mM Tris, 8% (w/v) sodium dodecylsulfate (SDS), 100 mM 2-mercaptoethanol, 40 μg/ml chymostatin, 0.00125% (w/v) bromophenol blue, pH adjusted to 2 with phosphoric acid (H_3PO_4)) and run on a $7 \times 9 \times 0.15$ cm gel (7.5% polyacrylamide in the resolving gel and 4% polyacrylamide in the stacking gel [17]). High molecular weight standards (Sigma) were used in the same disaggregation buffer. The gels were stained with Coomassie brilliant blue 250 [18] or with silver nitrate [19].

Quantitative densitometric analyses of Coomassie brilliant blue 250 stained gels were carried out with a UC630 Color Scanner linked to a Macintosh computer equipped with the NIH image software. The width of the scanned lane was adjusted in order to contain either the entire band width or a thin region in the middle of the lane. Agreement between the data obtained by both methods was always better than 5%. The baseline for integration was drawn between the 150000 and 70000 Da positions on the scan.

2.6. Size distribution of the proteoliposomes

The proteoliposome size was determined by photon correlation spectroscopy (dynamic light scattering) using a Malvern Zetasizer 3 with the 633 nm line of a helium neon laser focused onto the sample in a glass cell maintained at constant temperature (20° C). After calibration with polystyrene beads (with a diameter from 30 nm to 1000 nm), the light scattered by liposomes and proteoliposomes (500 μg/ml lipids) with various lipid/ATPase ratios (5:1 to 80:1 (w/w)) in a 10 mM Mes 50 mM potassium acetate 7% glycerol pH 6.8 buffer, was measured with an angle of 90° in the photon correlation mode. The refractive index of the preparation was 1.344. The diffusion coefficient of the particles was calculated from the correlation function generated by the fluctuating intensity of the light scattered. Mass distribution and number distribution of the vesicles sizes were determined for each population by the exponential sampling method [20,21].

2.7. ACMA fluorescence assay

Proton transport catalyzed by the ATPase was measured by fluorescence quenching of ACMA in reconstituted vesicles (lipid/ATPase ratio from 1000:1 to 50:1 (w/w)). 5 μl of ACMA (0.125 mM stock solution in dimethylsulfoxide) was added to 1.2 ml of the proteoliposomes preparation (5 mg/ml lipids in the reconstitution buffer). The preparation was incubated for 5 min in the cuvette of the spectrophotofluorometer at 30° C. The chamber was closed and the recorder adjusted to 100% fluorescence by varying the emission slit width. Mixing was accomplished magnetically. An aliquot of 36 μl of 0.2 M MgATP (disodium ATP/$MgSO_4$ adjusted to pH 6.8 with concentrated Tris) was added to start the reaction. The other additions were 5 μl valinomycin (0.1 mg/ml in ethanol), 5 μl nigericin (1 mg/ml in ethanol) and 24 μl vanadate (10 mM Na_3VO_4 in H_2O). ACMA fluorescence measurements were recorded in a SLM-Aminco spectrophotofluorometer with excitation and emission wavelengths of 415 and 485 nm, respectively.

2.8. Other procedures

The ATPase activity of proteoliposomes was measured according to the following procedure: reactions were initiated by the addition of 50 μl of a reaction mixture (containing 77 mM MgATP, 23 mM EGTA, 19 mM potassium acetate in a 3.8 mM Mes buffer adjusted to pH 6.8 with KOH) to 300 μl of the proteoliposomes preparation. To estimate the H^+-ATPase activity in the proteoliposomes in the absence of back-inhibition by the protonmotive force [12], 1 μl nigericin (5 mg/ml in dimethylsulfoxide) was added to the reaction mixture before incubation. The samples were incubated at 30° C for 30 min and the reaction stopped by adding 500 μl of 7% (w/v) sodium dodecylsulfate. The inorganic phosphate content was measured as described by Stanton [22] and evaluated by comparison with phosphate standard samples (0 to 0.2 μmol of KH_2PO_4), which were incubated in parallel with the reaction mixture containing MgATP and EGTA.

The protein content of the samples was determined by the method of Lowry et al. [23] as modified by Bensadoun and Weinstein [24] with bovine serum albumin as a standard. The lipid content was evaluated with a 'Test-combination Phospholipids Kit' (Boehringer). The lipid/ATPase (w/w) ratio was confirmed by Fourier Transform Infrared Spectroscopy, using an average calibration curve as described by Goormaghtigh et al. [25].

3. Results

3.1. Reconstitution procedure

When mixed phospholipids-DOC-ATPase micelles, prepared as described in materials and methods, are run at room temperature on a gel filtration resin (G-50 medium Sephadex) to remove the detergent, a turbid band becomes visible in the column as soon as the sample has progressed

approx. 1.5 cm through the resin bed, indicating the early formation of the proteoliposomes and the efficiency of the detergent removal. The turbid band elutes in the excluded volume of the column (peak 1, Fig. 1A), well separated from the absorbing material of the ATPase storage solution which elutes in the included volume (peak 2, Fig. 1A). Lipid content analysis of the peaks 1 and 2 indicates a 95% recovery of the lipids in the first peak (Fig. 1B). In addition, all the ATPase activity is associated with the first vesicle-containing peak. Fourier Transform infrared spectroscopy was used to estimate the final lipid/ATPase (w/w) ratio in the proteoliposomes from the lipid ν(C=O) absorption band (1735 cm^{-1}) and the protein amide I absorption band (1655 cm^{-1}) as described by Goormaghtigh et al. [25] (not shown). The analysis showed that the lipid/ATPase (w/w) ratio is identical before and after the gel filtration step, indicating that no loss of protein occurred during the reconstitution procedure.

Efficient reconstitution occurs only for DOC/phospholipid (w/w) ratios higher than 1:1, which corresponds to the detergent concentration required to solubilize the lipids into micelles as judged from the disappearance of turbidity. Ratios higher than 2:1 are efficient for reconstitution but slightly decrease the specific activity of the ATPase after reconstitution (not shown). We routinely used a 1.3:1 DOC/phospholipid (w/w) ratio.

3.2. Characterization of the reconstituted ATPase vesicles

Homogeneity

To characterize the distribution of the ATPase molecules in the lipid vesicles present in peak 1 (Fig. 1) for lipid/ATPase ratios ranging from 5:1 to 80:1 (w/w), the proteoliposomes were separated from the pure liposomes

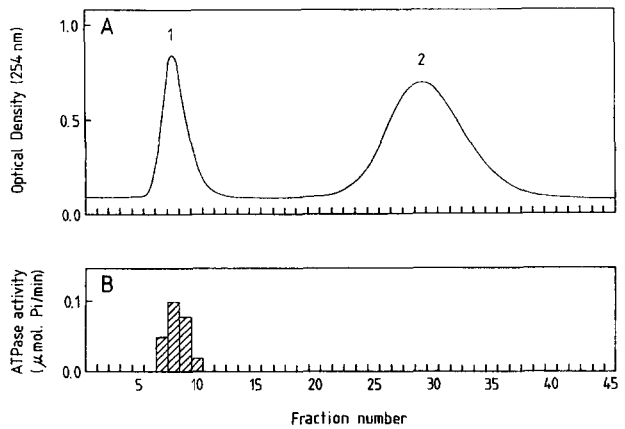

Fig. 1. Gel filtration chromatography profile during the reconstitution on a Sephadex G-50 column. The mixed micelles (DOC/soybean phospholipid/ATPase, 1.3:1:0.2 (w/w)) were eluted on a 1.5×27 cm G-50 (medium) Sephadex column equilibrated with the reconstitution buffer. The column was eluted at a flow rate of 1 ml/min at room temperature. The total elution time was 50 min. The upper profile (A) shows the optical density at 254 nm and the lower (B) represents the ATPase activity assay on the collected fractions (μmol of inorganic phosphate released per min).

by centrifugation on a glycerol step gradient containing 0%, 17% and 50% (w/v) (Fig. 2). After a 100 000 × g spin for 12 h, the ATPase-free liposomes migrate in fractions 5–10 at the 0%–17% glycerol interface (bottom panel with no ATPase), while the ATPase-bearing proteoliposomes are concentrated at the bottom of the gradient at the 17%–50% glycerol interface (fractions 1–3), where we recover most of the ATPase activity. While the shape of the lipid profile where no ATPase activity is detected (fractions 5–10) is somewhat different from one plot to another, analysis of Fig. 2 indicates that, for otherwise identical conditions, the amount of lipid participating in the formation of proteoliposomes remains almost constant when the protein amount is increased from 30 to 480 μg: 52% of the lipids are associated with ATPase-containing vesicles for a 80:1 lipid/ATPase (w/w) ratio reconstitution, 60% for a 5:1 ratio.

Reconstitution efficiency

In order to check whether all the ATPases collected at the 17%–50% glycerol interface were inserted in the proteoliposomes or whether pure protein aggregates were present, the vesicles (reconstitution at a 20:1 lipid/ATPase (w/w) ratio) were layered on a 29% (w/v) glycerol solution in the reconstitution buffer (with a 50% glycerol cushion at the bottom of the tube), and centrifuged overnight (100 000 × g spin). A pure ATPase preparation was also centrifuged in the same conditions. In the absence of lipids, the ATPase migrates to the 29%–50% glycerol interface (see also [10]) while the ATPase-bearing vesicles stay on the top of the 29% glycerol solution (data not shown) according to lipid assay as well as to ATPase activity assay in the presence of 'Folch Fraction 1' (FF1). FF1 is a crude lipid extract from bovine brain (containing predominantly phosphatidylinositides, phosphatidylserine and cerebrosides) which has been shown to stimulate the ATPase activity of non-inserted ATPase [8,12]. This assay reveals that the total ATPase activity is concentrated at the top of the gradient and that no activity is detected at the bottom of the 29% glycerol solution.

Size of the vesicles

The mean size and size distribution of the vesicles were determined by photon correlation spectroscopy for proteoliposomes with lipid/ATPase ratios of 5:1, 10:1, 20:1, 40:1, and 80:1 (w/w) and for liposomes prepared without ATPase. For all proteoliposomes preparations, the size distributions were characterized by a standard deviation between 9.7 (80:1 proteoliposomes) to 16 (5:1 proteoliposomes). The average diameter of the liposomes prepared without ATPase is 30 ± 2 nm while for ATPase-containing vesicles it varies monotonically as a function of the protein content from 40 ± 2 nm (80:1 lipid/ATPase (w/w) ratio) to 70 ± 2 nm (5:1 lipid/ATPase (w/w) ratio).

Measurements performed on freshly prepared and on liquid N_2 frozen – 30° C thawed vesicles (in the reconsti-

tution buffer adjusted to 7% (w/v) glycerol) indicated that the freezing-storage-thawing cycle did not affect the size of the vesicles.

Orientation of the ATPase in the vesicles

The orientation 'inside-out' or 'rightside-out' of the ATPase molecules in the proteoliposomes can be tested by

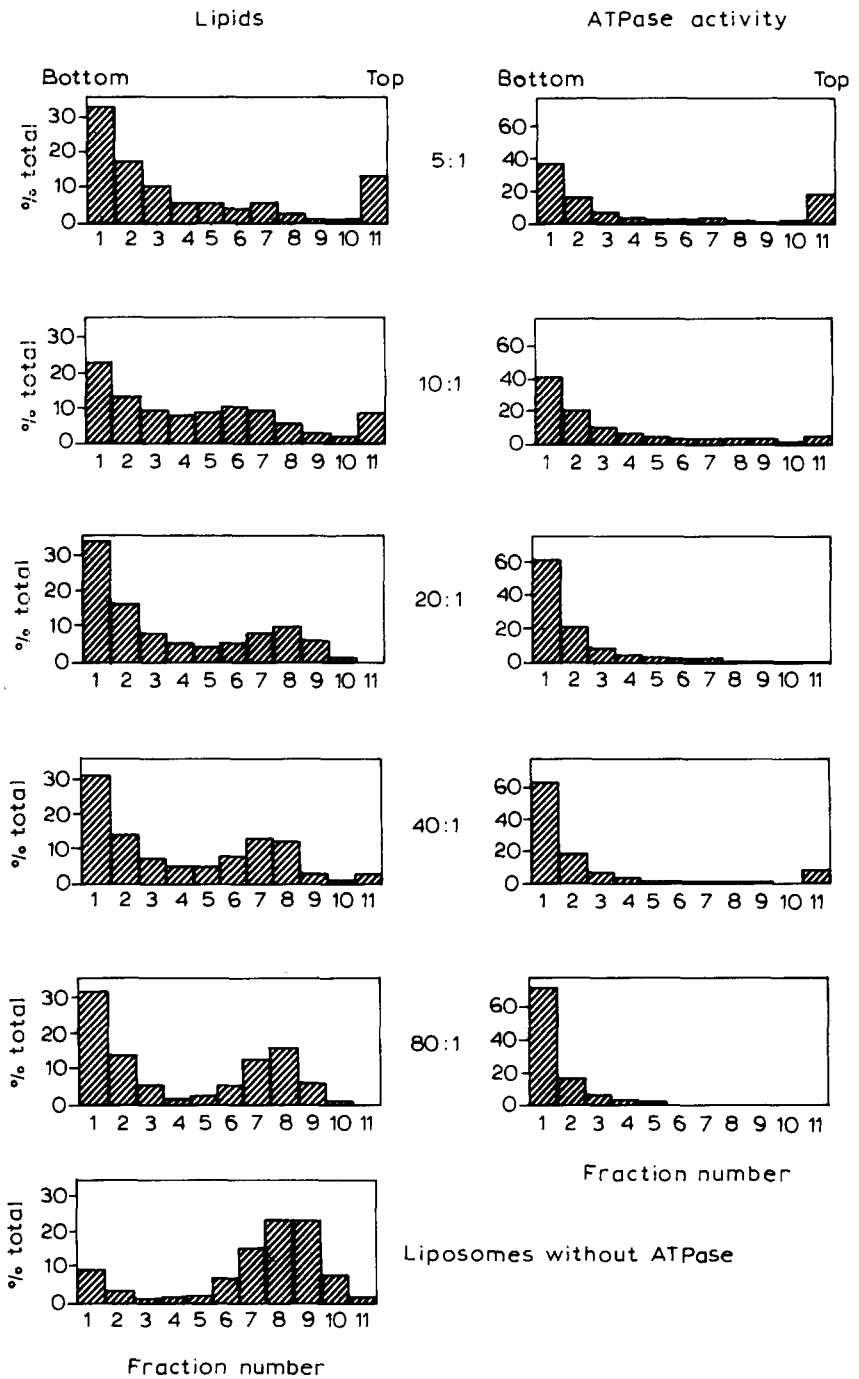

Fig. 2. ATPase activity and phospholipid in discontinuous 0%–17%–50% (w/v) glycerol density gradients. A 200 μl vesicles preparation was layered on 3 ml of a 17% glycerol solution in the reconstitution buffer, itself layered on 500 μl of a 50% glycerol solution in the same buffer. The amount of phospholipids is constant (2.4 mg) and the amount of protein varies from 0 μg to 480 μg in order to obtain the lipid/protein ratios (w/w) indicated on the figure (from 5:1 to 80:1). After a 100000 × *g* centrifugation at 4° C overnight, phospholipid and ATPase activity assays were performed as described in Materials and methods. The activity and lipid content are expressed as percentage of the total amount applied. Fractions were collected from the bottom of the gradient.

trypsin proteolysis. In the 'inside-out' orientation (with respect to cellular orientation), the large cytoplasmic ATPase domain is exposed to the outside of the vesicle and is readily cleaved by trypsin, while in the 'rightside-out' orientation, the small extracytoplasmic domain is exposed, which is not cleaved by trypsin [14]. The disappearance of the ATPase band at about 105 kDa followed by SDS-PAGE can thus be correlated with the proteolysis of trypsin accessible (inside-out) ATPase molecules (Fig. 3A). The proportion of ATPase molecules which are accessible to trypsin was determined for several lipid/ATPase ratios (5:1, 10:1, 20:1, 40:1 and 80:1 (w/w)) by densitometry of SDS-PAGE gels stained with Coomassie brilliant blue of trypsin digested proteoliposomes preparations (trypsin/ATPase (w/w) ratio of 1:10). A calibration curve relating the amount of protein layered on the gel lane (from 0.2 to 20 μg) to the surface of the corresponding peak, was constructed for each gel. 'Zero time controls' (incubated with inactivated trypsin, see Materials and methods) showed the same intensity of the 105 kDa band as the control samples (proteoliposomes incubated without trypsin), indicating that no ATPase digestion occurred during the gel electrophoresis. For all lipid/ATPase ratios tested, the ATPase band disappeared almost completely after 2 min of proteinase treatment; this pattern did not change much for a longer digestion time (1 h digestion) as illustrated in Fig. 3A. It can be estimated from the gel scanning that about 99% of the ATPase molecules are accessible to trypsin for all the lipid/ATPase ratios tested.

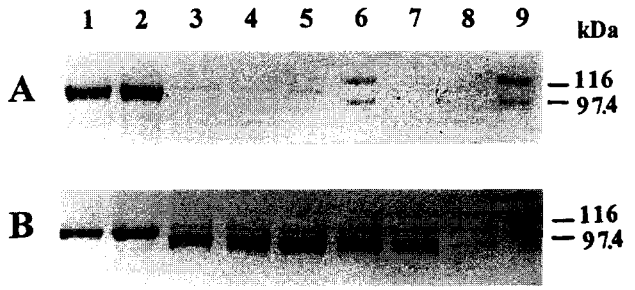

Fig. 3. Proteolysis of the reconstituted ATPase by trypsin. After preincubation in the presence or absence of MgATP + vanadate, reconstituted ATPase samples (lipid/ATPase 5:1 (w/w)) were exposed to trypsin at 30° C during 0, 2, 5, 10, 20, 30 and 60 min. The trypsin/ATPase ratio was 1:10 (w/w). The incubation was stopped by adding 1/4 volume of acidic disaggregation buffer (see Materials and methods). They were then run on a 7.5% polyacrylamide gel. 'Zero time controls' were prepared by addition of the acidic disaggregation buffer prior to trypsin. (A) Samples incubated without MgATP + vanadate (15 μg of protein per lane), (B) samples incubated with MgATP (10 mM) + sodium orthovanadate (100 μM) (15 μg of protein per lane). Lane 1, control; lane 2, zero time control (acidic disaggregation buffer added to the proteoliposomes before trypsin treatment); lanes 3, 4, 5, 6 (B), lanes 7 and 8: 2, 5, 10, 20, 30 and 60 min of digestion by trypsin, respectively; lanes 6 (A) and 9 (A and B): high molecular mass standards (3.5 μg per lane) (phosphorylase B (97.4 kDa), *E. coli* β-galactosidase (116 kDa)). The gel was stained with the Coomassie brilliant blue method [18].

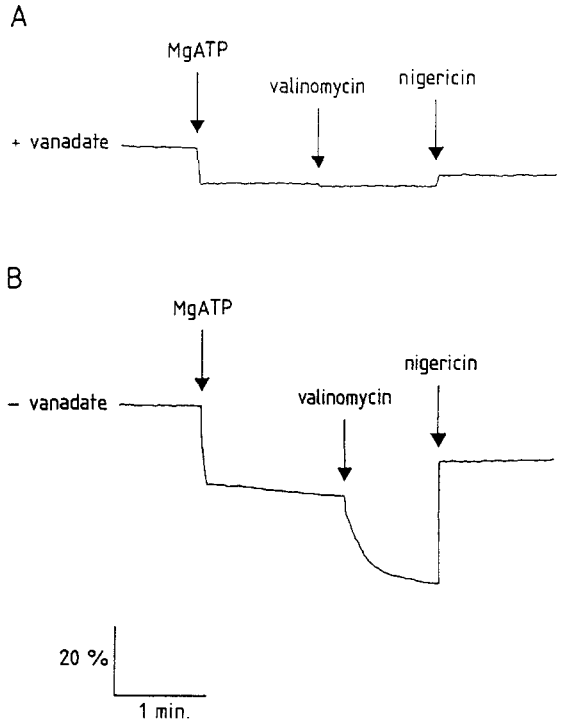

Fig. 4. ACMA fluorescence quenching by the reconstituted H^+-ATPase (lipid/ATPase ratio of 1000:1 (w/w)). See Materials and methods for details. Where indicated, the following additions were made: 36 μl of 0.2 M MgATP (pH 6.8 with Tris); 5 μl of valinomycin (0.1 mg/ml in ethanol); 5 μl of nigericin (1 mg/ml in ethanol). In A, vanadate (24 μl of 10 mM in H_2O) was added before starting the recording. In B, no vanadate was added. In both traces, a non-specific decrease in the fluorescence is seen when MgATP is added, which is attributable to the interaction of nucleotides with acridines.

ATPase activity and proton pumping

In the presence of nigericin, the specific ATPase activity measured after reconstitution is 6 μmol of phosphate liberated per mg of protein per min, with no significant variations for the different lipid/ATPase ratios. Furthermore, this ATPase activity is coupled to H^+ transport through the membrane. H^+ pumping towards the inside of the vesicles (lipid/ATPase (w/w) ratios from 1000:1 to 50:1) was demonstrated by measuring the quenching of fluorescence of ACMA (Fig. 4). Upon addition of MgATP to the ATPase-containing vesicles, a rapid decay in fluorescence was observed. This effect, already observed by other authors [26,43,12] is due to non specific interaction of the nucleotide with the acridine. The fluorescence decay is irreversible and its amplitude is related to the MgATP concentration [26]. This rapid decay of fluorescence is observed for a vesicle preparation with pumping ability (Fig. 4B) or in a non-pumping state (inhibited by vanadate) (Fig. 4A). For the pumping system only (Fig. 4B), it is followed by a slower specific decay of fluorescence corresponding to protonation of ACMA. The addition of valinomycin, which is a K^+-carrier, converts the $\Delta\psi$ component of the gradient, into a ΔpH as indicated by the ACMA fluorescence profile. The H^+ gradient (positive inside the

vesicles) was completely cancelled by the addition of nigericin which dissipates the pH gradient. (The effect of valinomycin on the pH gradient and on the specific ATPase activity has been discussed elsewhere [12].) The supplementary ATPase activity obtained by dissipating the proton gradient upon addition of a proton carrier is another indication of proton transport. The supplementary ATPase activity induced by the presence of nigericin for several lipid/ATPase ratios is shown in Table 1. Because the activity seen in the presence of nigericin is similar for all lipid/protein ratios, the level of back-inhibition of the ATPase activity by the pH gradient decreases as the amount of protein in the vesicles increases.

ATPase functionality

The specific activities measured here for the reconstituted ATPase are lower than values previously reported [12,27]. It was then a concern that part of the ATPase could have been denaturated during the reconstitution process. It has been shown previously that the binding of specific ATPase ligands to functional ATPase molecules changes their conformation [28,14,29] to forms that are markedly resistant to degradation by trypsin. Therefore, the effects of ATPase ligands on trypsin degradation of the reconstituted H^+-ATPase molecules were assessed. Proteoliposomes with lipid/ATPase ratios of 5:1, 10:1, 20:1, 40:1 and 80:1 (w/w) were treated with trypsin in the presence of MgATP + vanadate. After preincubation in the presence or in the absence of the ligands, trypsin was added and the incubation allowed to proceed for 0 to 60 min, after which the reactions were stopped by the addition of acidic disaggregation buffer (see Materials and methods). For trypsin-treated proteoliposomes (lipid/ATPase (w/w) ratio of 5:1) in the presence of MgATP and vanadate, 105, 97, and 88 kDa bands appear after SDS-PAGE analysis (Fig. 3B). As previously reported [28,14], SDS-PAGE gels stained with Coomassie brilliant blue were analyzed by densitometry. Fig. 5 shows that after 2 min of trypsin treatment of the proteoliposomes in the presence of the ligands, the intensity corresponding to the sum of the three 105, 97 and 88 kDa bands was about identical to that of the ATPase band of the control (99% of the control for proteoliposomes with all lipid/ATPase (w/w) ratios tested) while in the experiment carried out in the absence of MgATP + vanadate, only 9% of the original intensity was left over in the 105–88 kDa region after 2 min incubation for 5:1 (w/w) proteoliposomes. Less than 2% remained after 2 min for the proteoliposomes with higher lipid/ATPase ratios. The intensity of the ATPase band in the samples (5:1 proteoliposomes) treated by trypsin without ligands decreases to 4.5% after 5 min and to less than 1% of the control after 30 min or 1 h of trypsin treatment while it is still of 90%, 43% and 11% of the control for samples treated for, respectively 5, 30 min and 1 h with trypsin in presence of MgATP + vanadate. From the 2 min data, we may therefore estimate that, for 5:1 proteoliposomes, at least 90% of the exposed ATPase molecules are protected against trypsin proteolysis by ligand binding, and at least 97% are protected for the other lipid/ATPase compositions. Therefore, the great majority of the reconstituted ATPase molecules are functional as previously explained [28].

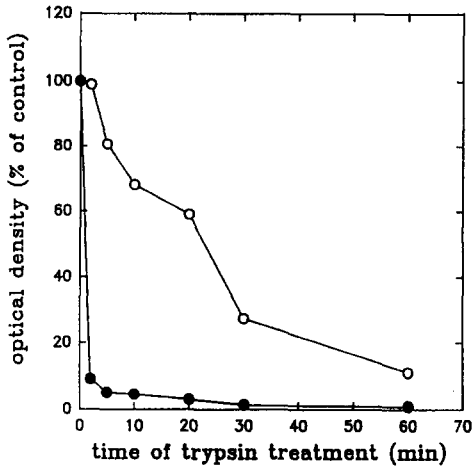

Fig. 5. Densitometric scans of the data from Fig. 3. Proteoliposomes with a lipid/ATPase of 5:1 (w/w) were treated with trypsin as described in Materials and methods in the presence (○-○) or in absence (●-●) of 20 mM $MgSO_4$, 20 mM Tris-ATP, 0.1 mM Na_3VO_4. In both cases, the data points show the measured amounts of the polypeptides (scans from 105 kDa to 88 kDa) expressed as percent of the initial 105 kDa band of the control. Individual points are the average values obtained from three scannings.

Table 1
Effect of the different lipid/ATPase (w/w) ratios on the ATPase activity of crude soybean phospholipids proteoliposomes prepared by gel filtration

Lipid/ATPase ratio (w/w)	Percentage of supplementary activity
10:1	7
40:1	33
100:1	80
500:1	77
700:1	170
1000:1	222

Proteoliposomes with decreasing amount of protein (240 μg to 2.4 μg) but constant amount of phospholipids (2400 μg) were prepared as described in Materials and methods. For each lipid/ATPase (w/w) ratio reported the percentage of supplementary ATPase activity in the presence of nigericin was measured.

Storage of the vesicles

The ATPase activity, the supplementary ATPase activity upon nigericin addition, and the size of the vesicles remain unchanged when the ATPase-bearing proteoliposomes in reconstitution buffer adjusted to 7% glycerol are frozen in liquid N_2 and kept at −20° C for several months.

4. Discussion

Detailed structural investigations of membrane proteins by biophysical means usually require the purification of the protein and its reconstitution into lipid vesicles. Among the methods of reconstitution used for membrane proteins such as Ca^{2+}-, Na^+/K^+-ATPases or bacteriorhodopsin, the detergent removal procedure is one of the most commonly described. Elimination of the detergent by a variety of means, including centrifugation on hollow-fibers [30,31], slow dialysis [32–34], centrifugation in a sucrose gradient [35], gel filtration [36], specific adsorption of the detergent on hydrophilic polystyrene beads [37–39] or dilution with a detergent-free media [40] induces the spontaneous formation of proteoliposomes.

Biophysical studies such as UV spectroscopy, circular dichroism and infrared spectroscopy require a high protein/lipid ratio in the reconstituted vesicles since lipids interfere with most spectroscopic techniques. Previously, the purified *Neurospora crassa* plasma membrane H^+-ATPase had been reconstituted into preformed sonicated asolectin vesicles by freeze-thawing but the lipid/protein ratio of 4800:1 (w/w) [12], or more recently 770:1 [14], is too high for spectroscopic approaches. The procedure described here allows the high yield (close to 100%) incorporation of the purified H^+-ATPase into lipid vesicles at substantially lower lipid/ATPase ratios. The procedure described here is also extremely useful since it guarantees the absence of significant amounts of unreconstituted protein after the gel filtration step, making unnecessary the time consuming gradient purification of the proteoliposomes.

Although the lipid/protein ratio is considerably lowered compared to the usual preparations [14], a supplementary 2-fold enrichment in ATPase can be obtained by density gradient purification of the proteoliposomes at any lipid/protein ratio, if needed.

Before undertaking extensive biophysical characterization of the reconstituted ATPase, three questions must be answered:

(1) What is the orientation of the reconstituted ATPase with respect with its native cellular orientation?
(2) Are the reconstituted ATPase molecules functional?
(3) What is the stability of the reconstituted system?

Trypsin proteolysis of proteoliposomes with lipid/protein ratios varying from 80:1 to 5:1 (w/w), led us to conclude that less than 1% of the ATPase molecules in the reconstituted proteoliposomes are oriented with their cytoplasmic domain towards the inside of the vesicles at all lipid/protein ratios employed. This sidedness determination is based on the assumption that the small extracytoplasmic part of the ATPase is not susceptible to trypsin hydrolysis as described previously [14] and that trypsin does not penetrate into the vesicles as indicated by others [41,42]. It must be noted that the few ATPases not accessible to trypsin hydrolysis could either have a right-side orientation or be embedded in internal lipid bilayer of multilamellar vesicles even though no multilamellar vesicles are visible by electron microscopy (not shown). In support of this data, we determined that about 99% of the reconstituted ATPase molecules have the ATP binding site facing the outside of the vesicles, as evidenced by the extent of ligand protection against tryptic degradation. The surprising homogenous orientation of the ATPase in the reconstituted vesicles may be related to the nature of the mixed micelles (lipid/ATPase/DOC) in which the ATPase must be anchored by its transmembraneous region and its smallest hydrophilic side. The removal of the detergent probably results in a lipidic bilayer formation including this preformed structure. Although it is not always the case [43], asymmetry has been observed in other detergent removal reconstitutions [16,31,36,44].

As to the specific ATPase activity (6 μmol of phosphate per mg of protein and per min) measured for all the proteoliposomes whatever the phospholipid/ATPase ratios, it is about 1/3 of the activity measured for the purified ATPase activated by the lipid brain extract (Folch Fraction I) (about 30 μmol of phosphate per mg of protein and per min). In the freeze-thaw procedure, the activity is around 20–23 μmol of phosphate per mg of protein and per min [13]. This relatively low specific activity of our preparation raised the potential problem of the presence of a population of reconstituted but denatured ATPase molecules. The functionality of the reconstituted ATPase molecules in these studies was assessed by the ability of the ATPase ligands MgATP and vanadate to lock the ATPase molecules in the transition state conformation of the enzyme dephosphorylation reaction. The spectacular resistance of this ATPase conformation with respect to the unliganded conformation to trypsin degradation allowed us to quantify the proportion of the functional ATPase molecules in this preparation [14,28,29,45]. These data indicate that for proteoliposomes with a 5:1 (w/w) ratio, 99% of the protein remains in the 80–105 kDa region of 7.5% SDS polyacrylamide gels in the presence of MgATP + vanadate versus 9% in the absence of ligands after 2 min of incubation in the presence of trypsin (2% for the 80:1 lipid/ATPase (w/w) ratio) indicating that at least 90% of the reconstituted ATPase molecules are functional with respect to ATP hydrolysis. The low specific activity is therefore not related to the denaturation of a fraction of the ATPase molecules but most probably to the lipid environment.

The second aspect of the reconstituted ATPase function is its ability to transport protons across the lipid bilayer. Assessing this property is important since it definitively proves the correct orientation of the different protein regions with respect to the lipid bilayer. ACMA fluorescence quenching indicated the formation of a pH gradient, acidic inside the vesicles, but does not allow quantification of the

proportion of the ATPases capable of pumping protons into the vesicles. On the contrary, the measurement of the supplementary ATPase activity induced by the addition of nigericin to the proteoliposomes provides a minimum estimate for the fraction of the total population of hydrolytically active ATPase molecules that are involved in proton gradient formation. As elaborated upon elsewhere [12], supposing one population of fully active ATPase molecules incapable of pumping protons to the vesicle interior and a second population completely back-inhibited by the proton gradient, for the 1000:1 lipid/protein ratio, the supplementary ATPase activity of 222% (Table 1) means that at least 69% (= 222/322) of the hydrolytically active ATPase molecules in the proteoliposomes are inhibited by the pH gradient but became fully active in the presence of nigericin. This proportion must in fact be higher because the crude soybean phospholipids vesicles are more leaky to protons (F. Homblé, personal communication, [12]) than many lipid preparations [46], preventing complete back-inhibition by the proton gradient. At 'high' lipid/protein ratios, the supplementary ATPase activity measured in the presence of nigericin is high with respect to coupling values usually obtained for proton P-type ATPases [12,27,26], but lower than for other ATPases. It must therefore be stressed that the entire pumping mechanism might not be fully functional even though functional conformational changes have been demonstrated for the large majority of the ATPase molecules. The percentage of supplementary activity measured upon addition of nigericin is reduced when the amount of ATPase increases. This increased leakiness of the membrane observed at low lipid/protein ratios might be due to a conserved hexameric assembling of the ATPase molecules in the membrane. Indeed, projection maps of H^+-ATPase hexamers crystals at 12 Å reveal a central hole, which could be the cause of the leakiness observed for proteoliposomes with a 10:1 or a 5:1 lipid/protein (w/w) ratio (G.A. Scarborough, personal communication). Our preparation is therefore not suitable for proton-pumping measurements.

Finally, by all criteria investigated here including ATPase activity, percentage of supplementary activity obtained upon nigericin addition and size of the vesicles measured by photon correlation, the proteoliposomes are identical when freshly prepared or when frozen in liquid nitrogen and stored at −20° C in the presence of 7% glycerol. This interesting feature allows us to form and characterize large amounts of proteoliposomes for subsequent studies. The size of the proteoliposomes is also unmodified when kept on ice overnight, implying the absence of fusion between the reconstituted vesicles. This absence of fusion was suggested in a similar system but with the Na^+/K^+-ATPase [34].

In conclusion, our results show that the gel filtration method allows the preparation of proteoliposomes with a large majority of active H^+-ATPase molecules oriented with their cytoplasmic side facing outward. This reconstitution procedure is rapid and efficient, and in comparison to the freeze-thaw procedure has the added advantage of a greatly reduced lipid content of the proteoliposomes.

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