Efficient solubilization and purification of the gastric H⁺,K⁺-ATPase for functional and structural studies

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When gastric H⁺,K⁺-ATPase-containing microsomes are solubilized by detergents, a rapid loss of ATPase activity is generally observed. In this article, SDS/PAGE of octa(ethylene glycol)-dodecyl monoether (C₁₂E₈)- and *n*-dodecyl β -D-maltoside-solubilized microsomes and their purifications by affinity chromatography on Reactive Red column reveal that inactivation is due to two main effects. (i) Solubilization activates an aspartic protease that cleaves down the α -subunit of the H⁺,K⁺-ATPase. Addition of pepstatin A at slightly acidic pH and at low temperature prevents the proteolysis. (ii) A too-harsh delipidation inactivates the ATPase. When *n*-dodecyl- β -D-maltoside is the detergent, the soluble H⁺,K⁺-ATPase is highly active (2.5 μ mol/mg per h at pH 6.0 and 5 °C) as long as ATP is added.

When C₁₂E₈ is used, the detergent induces an inactivation due to delipidation, since addition of lipids restores activity. The two subunits of the H⁺,K⁺-ATPase are present in equimolar ratio in the *n*-dodecyl β -D-maltoside-purified complex. Moreover, two main types of complex (330 and 660 kDa) were resolved in non-denaturing gels and should be the dimeric ($\alpha\beta$)₂ and tetrameric ($\alpha\beta$)₄ heterodimers respectively. In conclusion, purification of active, stable, soluble complexes of H⁺,K⁺-ATPase with few lipids (a lipid/protein ratio of 0.25, w/w) has been achieved. This material should be useful for further structural studies.

Key words: affinity chromatography, detergent solubilization, P-type ATPase.

INTRODUCTION

The enzyme H⁺,K⁺-ATPase (EC 3.6.1.36) from pig gastric mucosa is a proton pump that secretes acid into the gastric lumen by catalysing an electroneutral exchange of H⁺ for K⁺ at the expense of ATP [1,2]. This H⁺,K⁺-ATPase belongs to the P-type family, which shares sequence and functional homologies [3]. The gastric H⁺,K⁺-ATPase is composed of two subunits, a catalytic α -subunit and a heavily glycosylated β -subunit, which are closely interacting to give conformational stability to the functional holoenzyme [4]. Biochemical and structural studies suggest that the H⁺,K⁺-ATPase is a dimeric ($\alpha\beta$)₂ heterodimer [5–9]. The function of the β -subunit is not clear yet. It has been proposed that inhibition of acid secretion is accompanied by sialylation of parietal cell-membrane glycoproteins [10] and the β -subunit of H⁺,K⁺-ATPase was identified as the most abundant glycoprotein in gastric tubulovesicles [11].

One of the most important factors limiting our understanding of functional mechanisms of the H⁺,K⁺-ATPase is the lack of pieces of high-resolution structural information. A pre-requisite for structural and functional studies is availability of a large amount of highly purified and active H⁺,K⁺-ATPase. Moreover, crystallization experiments require a stable delipidated enzyme [12,13]. An active gastric protein has been prepared in membrane-bound form from the gastric mucosa of a variety of species by glycerol- and/or sucrose-gradient centrifugation [5,14–16]. These preparations are enriched in $\alpha\beta$ -subunits of H⁺,K⁺-ATPase, making up to 60–80 % of the total protein content. However, further purification has proved difficult, largely because of loss of enzyme activity during the detergent solubilization and storage. Several classes of detergent, cationic (dodecyltrimethylammonium bromide [17]), anionic (SDS and cholate [16,18,19]) and non-ionic {octylglucoside, Tween, Emulgen, octa(ethylene

glycol)dodecyl monoether (C₁₂E₈), Triton X-100, Nonidet P-40 and *n*-dodecyl- β -D-maltoside (DOM) [6,11,17–22]} have been tested. Several types of biochemical technique (centrifugation, chromatography, etc.) were also used to purify the protein [11,18–20]. None of these procedures provided a fully active enzyme and/or a delipidated complex ready for crystallization studies.

Several studies have explored the process of either liposome or proteoliposome solubilization by detergents. It has been shown that detergents interact differently with proteins and lipids [23]. In particular, ultrastructural investigation with cryotransmission electron microscopy has revealed that intermediates between the non-soluble and the soluble states of both pure liposomes and proteoliposomes vary with detergents [24–28]. The composition of lipid–protein–detergent micelles differ from one detergent to another for different membrane proteins [29].

This work reports the solubilization and purification of a fully active and stable H⁺,K⁺-ATPase enzyme. We compare two detergents (C₁₂E₈ and DOM) and demonstrate that protease inhibitor, protective conditions and low temperatures are essential to maintain activity during solubilization. Finally, we show that affinity chromatography in the presence of DOM allows the purification of H⁺,K⁺-ATPase with enough lipids to conserve a fully functional enzyme.

EXPERIMENTAL

Materials

Detergents (C₁₂E₈ and DOM), buffers (Mes, Hepes), Reactive Red Agarose, pepstatin A, inhibitor cocktail and phosphoenolpyruvate were purchased from Sigma. Lactate dehydrogenase,

Abbreviations used: C₁₂E₈, octa(ethylene glycol)dodecyl monoether; DOM, *n*-dodecyl- β -D-maltoside; DTT, dithiothreitol; CDTA, trans-1,2-diaminocyclohexane-*N,N,N',N'*-tetra-acetic acid; NBD, 7-nitrobenz-2-oxa-1,3-diazole-4-yl.

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pyruvate kinase, NADH and endoglycosidase F were from Boehringer Mannheim. All other reagents were reagent grade.

Preparation of gastric microsomes

The enriched H^+, K^+ -ATPase-containing gastric microsomes were isolated from pig stomachs as described previously [30]. Fresh stomachs were obtained from a local slaughterhouse (Truffaut Abattoirs, Mantes la Jolie, France). All steps were performed at 4 °C. The mucosa of gastric fundus was cut off, minced with scissors and homogenized in 250 mM sucrose/40 mM Hepes solution adjusted to pH 7.2 with Tris powder. Homogenate was first centrifuged for 10 min at 800 *g*, and then the supernatant was centrifuged at 27000 *g* for 7 min (rotor SS34, Sorvall RC5C centrifuge). The microsomes were pelleted from the latter supernatant at 100000 *g* for 30 min (rotor 70 Ti, Beckman LS65 centrifuge). The resulting crude pellet was suspended in 43 % (w/w) sucrose Hepes/Tris solution at pH 7.2 plus 150 mM NaCl and equilibrated by running a 35–30–8 % (w/w) stepped sucrose gradient at 27000 *g* for 16 h. The enriched H^+, K^+ -ATPase membranes were collected at the 8–30 % interface. Proteins were measured using a Bio-Rad kit and BSA as standard.

Assay procedures

ATPase activity was determined by a coupled assay in which ADP production was linked to NADH oxidation. The reaction was started by adding 0.05–0.1 mg of soluble proteins to a reaction mixture (2.0 ml) containing 20 mM Mes/Tris (pH 6.0), 0.4 mg/ml detergent, 2 mM magnesium chloride, 2 mM phosphoenolpyruvate, 2 mM ATP, 20 mM KCl, 0.2 mM NADH, 30 units/ml lactate dehydrogenase and 30 units/ml pyruvate kinase. Oxidation of NADH at 5 °C was monitored at 340 nm with an UV2 UNICAM spectrophotometer.

The amounts of lipids in the gastric microsomes and purified H^+, K^+ -ATPase were determined as described previously [31]. Intrinsic fluorescences of tryptophan and 7-nitrobenz-2-oxa-1,3-diazole-4-yl (NBD)-lipid tracer fluorescence were used to analyse protein and lipid contents of chromatographic fractions, respectively. Aliquots of 4–8 μ l were added to a reaction mixture (2.0 ml) containing 20 mM Mes/Tris (pH 6.0) and 1 mg/ml detergent. Fluorescence changes were monitored using a LS50B Perkin–Elmer spectrofluorimeter with excitation and emission wavelengths at 290 or 340 nm and 330 or 450 nm for tryptophan or NBD, respectively.

Solubilization of gastric microsomes was achieved by stepwise additions of various detergents and monitored for changes in turbidity of the solution at 550 nm as described previously [27,28].

SDS/PAGE and Western blots

SDS/polyacrylamide gels (10 % acrylamide) were stained with Coomassie Brilliant Blue or blotted on to nitrocellulose sheets and stained with Ponceau Red S [32].

Antigen–antibody characterization

Monoclonal antibody 95-A3 was produced in our laboratory as ascitic fluid and used in standard conditions with PBS buffer [30]. After electroblotting, nitrocellulose sheets were saturated by three successive incubations in PBS (pH 8) with 0.2 % Tween 20/0.5 % BSA for 10 min. Then monoclonal antibody 95-A3 (1/5000 dilution) was incubated for 60 min. After nitrocellulose-sheet rinsing, the antigen–antibody complex was detected with

the mouse peroxidase Vectastain ABC kit distributed by Biosys. Staining was developed with 20 mg of 4-chloronaphthol dissolved in 10 ml of ethanol, diluted into 40 ml of a Tris/HCl solution (pH 7.5) and 50 μ l of 30 % H_2O_2 was added. Monoclonal antibody 95-A3 epitope is located in the N-terminal intracytoplasmic sequence of the H^+, K^+ -ATPase (J.-C. Robert and A. Thomas-Soumarmon, unpublished work).

Deglycosylation of the β -subunit of H^+, K^+ -ATPase

This was achieved as described previously [17]. H^+, K^+ -ATPase-solubilized microsomes (24 μ g) or purified complex were incubated with 37.5 m-units of endoglycosidase F at 37 °C for 8 h in a 100 mM sodium acetate/10 mM EDTA/1 % Triton X-100/1 % mercaptoethanol/10 μ M pepstatin A buffer (pH 6.0). The reaction was stopped by adding the sample buffer used to run SDS/PAGE. Blue native electrophoresis was performed as described previously [33] and later modified [7].

Chromatographic procedure

Purification of the soluble proteins was performed by affinity chromatography on Reactive Red agarose (Sigma). A 1-ml syringe was filled to 0.8 ml with the resin. The resin was washed with 5 ml of water and equilibrated with 4.8 ml of the following buffer: 50 mM Mes/Tris (pH 6.0)/2 mM dithiothreitol (DTT)/0.3 mM *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetra-acetic acid (CDTA)/2 mM ATP/10 % (w/v) glycerol/1.5 mg/ml detergent/10 μ M pepstatin A. Microsomes (2–3 mg/ml) were solubilized at 5 °C for 2 h with DOM or for 30 min with $C_{12}E_8$. Detergent concentrations were 5–7.5 mg/ml in a solution containing 10 % glycerol/2 mM ATP, 2 mM CDTA and 20 mM Mes/Tris (pH 6.0). The material was then centrifuged at 120000 *g* for 20 min at 5 °C in a Beckman TL100 centrifuge. The supernatant was applied to the column. The unbound material was washed with 4 ml of buffer and the purified H^+, K^+ -ATPase eluted with a buffer containing DOM or $C_{12}E_8$ as detergent, 550 mM KCl or 1 M NaCl, 50 mM Mes/Tris (pH 6.0), 2 mM DTT, 2 mM ATP, 0.3 mM CDTA and 10 % glycerol.

RESULTS AND DISCUSSION

Solubilization

Several detergents were used previously to solubilize the H^+, K^+ -ATPase [6,11,18–20,22] with low success in retaining activity. Up to now the most efficient detergent was the non-ionic detergent $C_{12}E_8$, which was also successful for several other P-type ATPases, monomers, such as the Ca^{2+} -ATPase [34], and oligomers, like the Na^+, K^+ -ATPase [35]. DOM was shown recently to efficiently maintain an oligomeric structure of the H^+, K^+ -ATPase [7]. We started with those two detergents and compared their effects on the H^+, K^+ -ATPase.

The solubilization of gastric microsomes at 25 °C by DOM can be analysed as a three-step procedure: step I, saturation of vesicles with detergent; step II, solubilization intermediates, and step III, complete solubilization (Figure 1A). Solubilization by $C_{12}E_8$ (Figure 1B) is mostly similar but a discontinuity is observed within the second step: two intermediate stages, IIa and IIb, are observed [36]. Repeating the titration with different protein concentrations (0.5, 1.0 and 1.5 mg/ml) indicated that the $C_{12}E_8$ /protein and DOM/protein ratios at the transition between phases I and II were 0.2 and 0.4 (w/w) respectively (Figures 1A and 1B, insets). The concentration ratios required to step from phase II to III and reach complete solubilization were 1.8 and 1.2 (w/w) respectively.

Figure 1 Solubilization of gastric microsomes by DOM (A) and C₁₂E₈ (B)

Changes of the absorbance of microsomes (1.5 mg/ml) upon addition of detergents were measured at 550 nm and 25 °C. Phase transitions are indicated by the vertical bars: phase I is the onset of saturation, phase II (IIa and IIb for C₁₂E₈) is the solubilization process and phase III is at complete solubilization. (Insets) Protein-detergent phase diagrams were obtained by using different concentrations of proteins. The total concentrations of C₁₂E₈ and DOM required to reach the different phase transitions are plotted as functions of protein concentration. The slopes from the linear relationships observed [28] enabled us to determinate the detergent/protein ratio needed to saturate and to solubilize proteoliposomes (phases I/II and II/III, respectively). The intercepts (arrows) are the critical micellar concentrations, i.e. 0.05 and 0.1 mg/ml for DOM and C₁₂E₈ respectively.

The final absorbances reached after complete solubilization by both detergents were similar and increased with vesicle concentrations. We attributed this to a non-soluble material that was removed by centrifugation. The non-soluble material contained H⁺,K⁺-ATPase plus other proteins. Analysis of SDS/PAGE supports the hypothesis that DOM extracts more ATPase than C₁₂E₈ (Figure 2, lanes 2 and 7). The soluble material accounted for 60–90% of the intrinsic fluorescence of initial microsomes (Table 1).

Figure 2 SDS/PAGE of solubilized gastric microsomes by C₁₂E₈ and DOM at 25 (A) and 5 °C (B)

(A) Lanes 1 and 6, gastric microsomes; lanes 2 and 3, and lanes 7 and 8, pellets and supernatants obtained with C₁₂E₈ and DOM respectively, at a detergent/protein ratio of 2. Centrifugations were 120 000 *g* for 20 min. Lanes 4 and 5, and lanes 9 and 10 are C₁₂E₈ and DOM supernatants respectively, incubated for 180 min at 25 °C without (lanes 4 and 9) and with (lanes 5 and 10) 10 μM pepstatin A. (B) Lane 1, gastric microsomes; lanes 2 and 3, and lanes 4 and 5 are C₁₂E₈- and DOM-solubilized microsomes respectively incubated for 20 h at 5 °C with (lanes 2 and 4) and without (lanes 3 and 5) 10 μM pepstatin A. Arrows indicate subfragments of the 95-kDa band.

Protection by ATP

When a detergent/protein ratio (w/w) of 3 was used with no ATPase-protective agent, the activity of the soluble H⁺,K⁺-ATPase clearly decreased with both detergents. This inactivation was slower with DOM than with C₁₂E₈ (Figure 3). As described previously by Rabon et al. [6] using other detergents, ATP protects against inactivation. Further solubilizations were thus performed in the presence of ATP.

Effect of temperature

Solubilization at low temperature has been reported to reduce protein aggregation [23]. We switched to low temperatures of solubilization (5 °C). Under these conditions, the stability of the C₁₂E₈ and DOM preparations were improved and were sufficient for further purification. The rate of inactivation of the H⁺,K⁺-ATPase was, however, still faster for C₁₂E₈ than for DOM (Figure

Table 1 Recapitulative recovery of proteins, lipids and ATPase-specific activities during the different steps of solubilization and purification

The $C_{12}E_8$ - and DOM-solubilized fractions are the supernatants of microsomes treated with a detergent/protein ratio (w/w) of 3 and centrifuged for 20 min at 120 000 *g*. The percentages of protein of the void fraction and of the detergent-purified fractions are the material recovered in the whole-load and elution volumes (Figure 5). The $C_{12}E_8$ - and DOM-purified fractions are from the 0.55 M KCl elution. Proteins are estimated from tryptophan fluorescence, lipids from NBD-lipid tracer fluorescence. The lipid/protein ratios are normalized with reference to microsomes. The ATPase-specific activities of the most enriched fractions are given.

Preparation	Protein (%)	Lipid/protein ratio (w/w)	ATPase-specific activity
Microsomes	100%	1	100%
$C_{12}E_8$ -solubilized	60–80%	≈ 1	80–100%
DOM-solubilized	80–90%	≈ 1	100–110%
Void fraction	30%	3–5	15–20%
$C_{12}E_8$ -purified	30%	0.1	20%
DOM-purified	30%	0.25 ± 0.05	140%

3C as compared with 3D). In those conditions, solubilization by DOM results in a transient activation of the ATPase.

Proteolysis

Solubilization unmasks a protease which cleaves the H^+,K^+ -ATPase, as revealed by the disappearance of the 95-kDa band

(Figure 2A, lanes 4 and 9) on the SDS/PAGE. Almost complete proteolysis of the α -subunit was observed in the presence of both detergents at 25 °C as little as 2 h after the addition of detergent. This proteolysis was highly reduced at lower temperature and only partial cleavage was observed clearly at 5 °C (Figure 2B, lanes 2 and 4). To prevent degradation, several protease inhibitors were tested: pepstatin A, an aspartic protease inhibitor, was the most efficient. Pepstatin A (10 μ M) almost completely inhibited the ATPase proteolysis, as observed on SDS/PAGE (Figure 2A, lanes 5 and 10, and Figure 2B, lanes 3 and 5, at 25 and 5 °C, respectively).

Aspartic proteases are highly sensitive to pH. They are more efficient at acidic than alkaline pH. We solubilized the H^+,K^+ -ATPase at pH 4.0, 6.0 and 8.0 and checked the remaining ATPase activity in the absence of pepstatin A (Figure 4A). The best conditions for solubilization with DOM were at pH 6.0, as described previously for $C_{12}E_8$ [6]. Solubilization at pH 4.0 led to a rapid loss of ATPase activity, probably due to an increased protease activity (Figure 4B, lane 3). At pH 8.0, the protease cleavage was not observed (Figure 4B, lane 7) but the ATPase activity was strongly decreased. The best conditions were at pH 6.0 (Figure 4A). This optimum is characteristic of the H^+,K^+ -ATPase since the Ca^{2+} -ATPase, another P-type ATPase, solubilizes optimally at pH 8.0 [37]. In the following, we added pepstatin A as protease inhibitor in all media.

We therefore suggest from the overall data that, in the presence of pepstatin A and ATP, solubilization of the H^+,K^+ -ATPase results in an activation that appears transiently in DOM (Figure 3D) and a slow time-dependent inactivation clearly obtained in

Figure 3 Time course of ATPase activity of solubilized microsomes at 25 (A, B) and 5 °C (C, D)

Solubilization was performed with $C_{12}E_8$ (A and C) and DOM (B and D). Microsomes (3 mg/ml) were solubilized with detergents (9 mg/ml) in 20 mM Mes/Tris (pH 6.0)/0.3 mM CDTA/10% glycerol/2 mM DTT without (○, □) and with (●, ■) 2 mM ATP and with (◆) 10 μ M pepstatin A. Aliquots of 0.24 mg were removed at various times to measure the ATPase activity. The native enzyme was incubated in the same buffer without detergent (▽). Each point is the percentage of ATPase activity remaining after incubation at the indicated time and temperature. The 100%, i.e. the initial microsome activity, was 2.5 μ mol/mg per h at pH 6.0 and 5 °C.

Figure 4 pH effects on ATPase activity and subunit stability at 5 °C in the presence of DOM

(A) Time course of ATPase activity of solubilized microsomes at pH 4 (○), 6 (●) and 8 (▽). Microsomes (3 mg/ml) were solubilized with DOM (9 mg/ml) in 0.3 mM CDTA/10% glycerol/2 mM DTT/2 mM ATP/20 mM acetate at pH 4.0, Mes/Tris at pH 6.0 or Tris/HCl at pH 8.0, respectively. Aliquots of 0.24 mg were removed at various times to measure the ATPase activity. The native enzyme was incubated in the same buffers without detergent (▼). ATPase activities were measured at pH 6 as described in the Experimental section. (B) SDS/PAGE of DOM-solubilized gastric microsomes at 5 °C. Lane 1, gastric microsomes; solubilized microsomes were in lanes 2 and 3 (pH 4), lanes 4 and 5 (pH 6) and lanes 6 and 7 (pH 8). They were incubated for 20 h with (lanes 2, 4 and 6) and without (lanes 3, 5 and 7) 10 μM pepstatin A. Lanes 3 and 5 clearly show three major subfragments of the 95 kDa band (at about 66, 38 and 29 kDa).

all conditions. Inactivation could be due to a residual proteolysis and a delipidation by detergent, as explained below.

Purification of soluble H^+,K^+ -ATPase

Purification of soluble membrane proteins can be obtained by different biochemical techniques, such as gel-filtration and affinity chromatography. With the H^+,K^+ -ATPase, we observed that, during gel chromatography on Sephadex or Sepharose, the elution of detergent-solubilized proteins of microsomes was masked by a massive peak of lipids and detergent, as described previously with other membrane proteins [29].

Purification of the Ca^{2+} -ATPase of sarcoplasmic reticulum by affinity chromatography has been used to prepare material for functional and structural studies [31,38,39]. The elution profiles of Reactive Red agarose columns used for the purification of gastric H^+,K^+ -ATPase solubilized either in DOM (Figure 5A) or

Figure 5 Elution patterns obtained from detergent-solubilized microsomes using Reactive Red agarose column at 5 °C

Supernatant (15 mg) of DOM (A)- or $C_{12}E_8$ (B)-solubilized and centrifuged microsomes were loaded on to a 1-ml resin. The plots show the three steps of purification: column load (L), wash (W) and elution (E). Fractions (0.4 ml) were collected and protein and lipid contents were measured by intrinsic fluorescence of tryptophan (○, □) and NBD-lipid tracer fluorescence (●, ■) respectively. The arrows indicate when 0.55 and 1 M KCl buffers were added.

$C_{12}E_8$ (Figure 5B) can be divided into three parts: the load, the wash and the elution of bound material. During the load (L in Figure 5), one third of the total proteins was recovered in the void volume (Table 1). The lipid-detergent micelles of DOM and $C_{12}E_8$ were there, as revealed by the large peak of fluorescent lipids (Figure 5, closed symbols). The SDS/PAGE analysis of void volumes indicated the presence of some ATPase together with lower-molecular-mass proteins (Figure 6A, lane 3). As analysed previously by immunoprecipitation of gastric microsomes, the 53–55, 50, 32 and 43 kDa bands should correspond to the α -, β -, γ -subunits of F_1 -ATPase that are contaminating microsomes and to actin, respectively [40].

During the wash (W in Figure 5), no protein was detected, whereas lipids were present, especially in the $C_{12}E_8$ wash (Figure 5B). For the DOM column, the amount of lipids was proportional to the detergent concentration in the washing buffer, with a threshold concentration corresponding to the critical micellar concentration of DOM (≈ 0.05 mg/ml). This supports the idea that both detergents extract lipids from the lipid-protein-detergent micelles that are bound to the column. Thus increasing the concentration of detergent during the wash decreases the amount of lipids eluting later with the H^+,K^+ -ATPase (Figure 5).

Elution of the H^+,K^+ -ATPase was obtained in few fractions (1–5 ml) with either KCl or NaCl concentrations higher than 0.55 and 1 M, respectively. Lipid contents of these fractions were very different in the DOM and $C_{12}E_8$ experiments, the former having a higher lipid/protein ratio than the latter [0.25 as compared with 0.1 (w/w); Table 1]. The DOM-eluted fractions exhibited a 7-fold higher ATPase-specific activity than the $C_{12}E_8$ fractions (Table 1). The requirement for a minimum amount of lipids within the lipid-protein-detergent micelles was demonstrated by adding lipids in the washing solution and the $C_{12}E_8$ elution mixtures. This increased the ATPase activity of the purified fractions up to 50%. This is in agreement with previous studies where delipidation followed by reconstitution were shown to decrease and then restore some ATPase activity [19–21].

The Ca^{2+} -ATPase was purified previously using the same kind of resin. The ATP site was implicated in the binding since (i) the protein was eluted with a substrate and (ii) a FITC-modified enzyme, known to have a 'blocked-occupied' catalytic site, was not able to bind [37]. Conversely to sarcoplasmic reticulum Ca^{2+} -ATPase [37], the H^+,K^+ -ATPase was not eluted from the resin

Figure 6 SDS/PAGE and Western-blot analyses of the major fractions obtained during the purification of the H^+,K^+ -ATPase

(A) Coomassie Brilliant Blue staining of SDS/PAGE. All lanes were loaded with equal amounts of protein: gastric microsomes (lane 1), supernatant of DOM-solubilized microsomes (lane 2), void fractions corresponding to L of Figure 5 (lane 3), 0.55 M KCl- and 1 M NaCl-eluted fractions (lanes 4 and 5 respectively), and 0.55 M KCl-eluted fraction of $C_{12}E_8$ -solubilized microsomes (lane 6). (B) SDS/PAGE of deglycosylated fractions; lanes are the same as in (A). (C) Western blot of the fractions; lanes are as in (A). A monoclonal antibody anti- α -subunit was used (95-A3).

with nucleotides whatever the detergent used for solubilization. Moreover, ATP did not prevent the binding of the H^+,K^+ -ATPase to the resin. An alternative binding could come from sugars on the H^+,K^+ -ATPase [4,41]. Binding on the resin was nevertheless selective of the ATPase, as revealed by SDS/PAGE (Figure 6A). There was a clear enrichment of the H^+,K^+ -ATPase as compared with microsomes. The protein recovery (Table 1) and the ATPase purification (Figure 6A, lanes 4–6) were almost equivalent whatever the detergents and salts of the elution buffer. However, the recovered ATPase activity was clearly higher with DOM compared with $C_{12}E_8$. The DOM-purified fraction accounted for 42% of the initial activity and had 140% of the initial specific activity. Figure 6(B) demonstrates the presence of the β -subunit at 32 kDa after deglycosylation. Indeed, the β -subunit is a glycoprotein, which migrates as a broad 60–80-kDa band in SDS/PAGE, whereas a sharp 32-kDa band is seen after deglycosylation with endoglycosidase F [17]. The α -to- β subunit stoichiometry (1:1) of the purified fractions was confirmed by scanning the gels. Immunoblots reveal that the main band at 95 kDa is the α -subunit of the H^+,K^+ -ATPase. Immunoreactivity is also present in the 190 kDa band (Figure 6C).

Figure 7 Blue native PAGE analysis of the fractions obtained during the purification in DOM of H^+,K^+ -ATPase and of sarcoplasmic reticulum Ca^{2+} -ATPase

DOM-solubilized sarcoplasmic reticulum vesicles (lanes 1 and 5), gastric microsomes (lane 2) and KCl- and NaCl-eluted H^+,K^+ -ATPase fractions (lanes 3 and 4 respectively). Standards are a mixture of aldolase (158 kDa) and ferritin (440 and 880 kDa).

Soluble ATPase

Non-denaturing gels were run to check the oligomeric state, and the gastric ATPase behaviour was compared with that of the sarcoplasmic reticulum Ca^{2+} -ATPase (Figure 7). The supernatants of solubilized gastric microsomes had a main band around 330 kDa with an additional weaker band at 660 kDa, whereas the sarcoplasmic reticulum Ca^{2+} -ATPase had a major band at 115 kDa. The 330- and 660-kDa bands should correspond to a $(\alpha\beta)_2$ dimer and a $(\alpha\beta)_4$ tetramer, respectively [22]. Those bands were obtained after purification with both detergents (results not shown with $C_{12}E_8$) and were found whenever KCl or NaCl were used in the DOM elution procedure (Figure 7, lanes 3 and 4).

Soluble ATPase stability

Functional and structural studies require large amounts of active soluble H^+,K^+ -ATPase. Both detergents were successful in producing large amounts of soluble protein, but only DOM gave a largely active preparation. Purification on the Reactive Red agarose column can give very concentrated fractions, up to 10 mg of ATPase/ml. There was a linear relationship between the amount of load and the concentration of the purified H^+,K^+ -ATPase in the elution (Figure 8A). This relation existed whatever the eluting salt was, as long as its concentration was over 0.55 and 1 M for KCl and NaCl, respectively. It is important to notice here that the stability of purified ATPase activity was dependent upon salt concentration. Inactivation of H^+,K^+ -ATPase occurred within 2–3 days in high salt concentrations (Figure 8B). If the salts were diluted down to 0.2 M after purification, ATPase stabilization was obtained. When 30% glycerol was added, the purified fraction was as stable as the whole solubilized microsomes (Figure 8B): 50% of the activity was maintained after 3 days in the cold room. When the purified fractions were stored in liquid nitrogen, the glycerol effect was even better, since less than 10–20% of the activity was lost after a freeze–thaw cycle.

In conclusion, we observed first that the solubilization of gastric microsomes enables a protease to efficiently cleave H^+,K^+ -ATPase. Most H^+,K^+ -ATPase was cleaved within a few hours at room temperature or 24 h in a cold room, whereas the membraneous H^+,K^+ -ATPase was stable for days in similar con-

Figure 8 Characterization of the purified H^+,K^+ -ATPase fraction

(A) Yield of purification. The concentration of eluted ATPase is plotted against the amount of loaded gastric microsomes. The salt concentrations in the elution buffer were 0.55 M KCl (■) and 1 M NaCl (○). (B) Stability of the purified fractions at 5 °C. The activities of 0.55 M KCl (□) and 1 M NaCl (▽)-purified fractions are compared with native (○) and solubilized (◇) microsomes. ▼ and ■, respective 0.55 M KCl- and 1 M NaCl-purified fractions when salts were diluted down to 0.2 M just after purification. ●, KCl-eluted and diluted fractions when glycerol concentration was adjusted to 30%.

ditions. It suggests that detergents solubilize a protease or that ATPase cleavage sites are made accessible by solubilization. In previous studies, among the different detergents used [6,11,17–20,22], $C_{12}E_8$ was the best at protecting the ATPase activity [6,11]. In our hands, DOM was even better since more than 80 % of the activity was conserved for 24 h after solubilization as compared with less than 50 % with $C_{12}E_8$ in the same conditions.

The importance of the lipid/protein ratio for the activity of the H^+,K^+ -ATPase is observed clearly by comparing the remaining activity after purification in the presence of DOM or $C_{12}E_8$ and the effect of lipid addition. Could this be required to maintain the α - β interactions? It is unlikely since Rabon et al. [6] have shown that α - β interactions within the ATPase complex are almost independent of detergents, potassium and ATP.

We also observed that, in our active soluble fractions, the oligomeric state ($\alpha\beta$)₂ dimer was conserved whenever DOM or $C_{12}E_8$ had been used.

Several attempts were already made to solubilize and purify the gastric proton pump. The main problem encountered was ATPase activity. Takaya et al. [20] described a multi-step procedure requiring about 4 days; the time was probably too long. Later, Okamoto et al. [17] and Callaghan et al. [11] fractionated detergent-solubilized pig membrane by lectin affinity chromatography in a single-step procedure performed within

hours. However, the detergents, Triton X-100, Nonidet P-40 and $C_{12}E_8$ were probably too efficient to delipidate the soluble H^+,K^+ -ATPase complex.

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REFERENCES

- Pettitt, J. M., Humphris, D. C., Barrett, S. P., Toh, B. H., Van Driel, I. and Gleeson, P. A. (1995) *J. Cell Sci.* **108**, 1127–1141
- Van Driel, I. R. and Callaghan, J. M. (1995) *Clin. Exp. Pharmacol. Physiol.* **22**, 952–960
- Moller, J. V., Juul, B. and LeMaire, M. (1996) *Biochim. Biophys. Acta* **1286**, 1–51
- Tyagarajan, K., Chow, D. C., Smolka, A. and Forte, J. G. (1995) *Biochim. Biophys. Acta* **1236**, 105–113
- Rabon, E., Gunther, R. D., Bassilian, S. and Kempner, E. S. (1988) *J. Biol. Chem.* **263**, 16189–16194
- Rabon, E., Bassilian, S. and Jakobsen, L. J. (1990) *Biochim. Biophys. Acta* **1039**, 277–289
- Shin, J. M. and Sachs, G. (1996) *J. Biol. Chem.* **271**, 1904–1908
- Hebert, H., Xian, Y., Hacksell, I. and Mardh, S. (1992) *FEBS Lett.* **299**, 159–162
- Xian, J. and Hebert, H. (1997) *J. Struct. Biol.* **118**, 169–177
- Usumoto, R., Okada, Y. and Tsuji, T. (1994) *Digestive Dis. Sci.* **39**, 169–17
- Callaghan, J. M., Toh, B.-H., Simpson, R. J., Baldwin, G. S. and Gleeson, P. A. (1992) *Biochem. J.* **283**, 63–68
- Kühlbrandt, W. (1988) *Q. Rev. Biophys.* **21**, 429–477
- Kühlbrandt, W. (1992) *Q. Rev. Biophys.* **25**, 1–49
- Saccomani, G., Shah, G., Spenney, J. G. and Sachs, G. (1975) *J. Biol. Chem.* **250**, 4802–4809
- Soumarmon, A., Abastado, M., Bonfils, S. and Lewin, M. J. M. (1980) *J. Biol. Chem.* **255**, 11682–11687
- Nandi, J., Menh-Ai, Z. and Ray, T. K. (1987) *Biochemistry* **26**, 4264–4272
- Okamoto, C. T., Karpilow, J. M., Smolka, A. and Forte, J. G. (1990) *Biochim. Biophys. Acta* **1037**, 360–372
- Soumarmon, A., Robert, J.-C. and Lewin, M. J. M. (1986) *Biochim. Biophys. Acta* **860**, 109–117
- Soumarmon, A., Grelac, F. and Lewin, M. J. M. (1983) *Biochim. Biophys. Acta* **732**, 579–585
- Takaya, J., Omori, K., Taketani, S., Kobayashi, Y. and Tashiro, Y. (1987) *J. Biochem. (Tokyo)* **102**, 903–911
- Rabon, E., Gunther, R. D., Soumarmon, A., Bassilian, S., Lewin, M. J. M. and Sachs, G. (1985) *J. Biol. Chem.* **260**, 10200–10207
- Shin, J. M. and Sachs, G. (1994) *J. Biol. Chem.* **269**, 8642–8646
- Kragh-Hansen, U., LeMaire, M. and Moller, J. V. (1998) *Biophys. J.* **75**, 2932–2946
- Vinson, P. K., Talmon, Y. and Walter, A. (1989) *Biophys. J.* **56**, 669–681
- Walter, A., Vinson, P. K., Kaplun, A. and Talmon, Y. (1991) *Biophys. J.* **60**, 1315–1325
- Edwards, K. and Almgren, M. (1991) *J. Colloid Interface Sci.* **147**, 1–21
- Knol, J., Sjollem, K. and Poolman, B. (1998) *Biochemistry* **37**, 16410–16415
- Lambert, O., Levy, D., Ranck, J.-L., Leblanc, G. and Rigaud, J.-L. (1998) *Biophys. J.* **74**, 918–930
- Moller, J. V. and LeMaire, M. (1993) *J. Biol. Chem.* **268**, 18659–18672
- Benkouka, F., Peranzi, G., Robert, J.-C., Lewin, M. J. and Soumarmon, A. (1989) *Biochim. Biophys. Acta* **987**, 205–211
- Lacapère, J.-J., Stokes, D. L., Olofsson, A. and Rigaud, J.-L. (1998) *Biophys. J.* **75**, 1319–1329
- Robert, J.-C., Benkouka, F., Bayle, D., Hervatin, F. and Soumarmon, A. (1990) *Biochim. Biophys. Acta* **1024**, 167–172
- Schägger, H. and Von Jagow, G. (1991) *Anal. Biochem.* **199**, 223–231
- LeMaire, M., Moller, J. V. and Tandford, C. (1976) *Biochemistry* **15**, 2336–2342
- Cornelius, F. and Skou, J. C. (1984) *Biochim. Biophys. Acta* **772**, 357–373
- Kragh-Hansen, U., LeMaire, M., Noël, J.-P., Gulik-Krzywicki, T. and Moller, J. V. (1993) *Biochemistry* **32**, 1648–1656
- Lund, S., Orlowski, S., DeForesta, B., Champeil, P., LeMaire, M. and Moller, J. V. (1989) *J. Biol. Chem.* **264**, 4907–4915
- Coll, R. J. and Murphy, A. J. (1984) *J. Biol. Chem.* **259**, 14249–14254
- Young, H. S., Rigaud, J.-L., Lacapère, J.-J., Reddy, L. G. and Stokes, D. L. (1997) *Biophys. J.* **72**, 2545–2558
- Bayle, D., Benkouka, F., Robert, J.-C., Peranzi, G. and Soumarmon, A. (1992) *Comp. Biochem. Physiol.* **101**, 519–525
- Tai, M. M., Im, W. B., Davis, J. P., Blakeman, D. P., Zurcher-Neely, H. A. and Heinrikson, R. L. (1989) *Biochemistry* **28**, 3183–3187