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Overproduction in yeast and rapid and efficient purification of the rabbit SERCA1a Ca^{2+}-ATPase

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Abstract

Large amounts of heterologous C-terminally his-tagged SERCA1a Ca^{2+}-ATPase were expressed in yeast using a galactose-regulated promoter and purified by Ni^{2+} affinity chromatography followed by Reactive red chromatography. Optimizing the number of galactose inductions and increasing the amount of Gal4p transcription factor improved expression. Lowering the temperature from 28°C to 18°C during expression enhanced the recovery of solubilized and active Ca^{2+}-ATPase. In these conditions, a 4 l yeast culture produced 100 mg of Ca^{2+}-ATPase, 60 and 22 mg being pelleted with the heavy and light membrane fractions respectively, representing 7 and 1.7% of total proteins. The Ca^{2+}-ATPase expressed in light membranes was 100% solubilized with L-α-lysophosphatidylcholine (LPC), 50% with *n*-dodecyl β-D-maltoside (DM) and 25% with octaethylene glycol mono-*n*-dodecyl ether ($C_{12}E_8$). Compared to LPC, DM preserved specific activity of the solubilized Ca^{2+}-ATPase during the chromatographic steps. Starting from 1/6 (3.8 mg) of the total amount of Ca^{2+}-ATPase expressed in light membranes, 800 μg could be routinely purified to 50% purity by metal affinity chromatography and then 200 μg to 70% with Reactive red chromatography. The purified Ca^{2+}-ATPase displayed the same K_m for calcium and ATP as the native enzyme but a reduced specific activity ranging from 4.5 to 7.3 μmol ATP hydrolyzed/min/mg Ca^{2+}-ATPase. It was stable and active for several days at 4°C or after removal of DM with Bio-beads and storage at −80°C.

Abbreviations: SR, sarcoplasmic reticulum; SERCA, sarco/endoplasmic reticulum calcium ATPase; SERCA1a, gene encoding the SR calcium-transporting ATPase of fast twitch skeletal muscle; 6HCSERCA1a, SERCA1a gene with addition of a part at the 5′-end encoding a Gly_2His_6 tag; HMb, heavy membrane(s); LMb, light membrane(s); 6HC-Ca^{2+}-ATPase, C-terminal Gly_2His_6-tagged Ca^{2+}-ATPase ($M^1 \rightarrow G^{994}G_2H_6$); LPC, L-α-lysophosphatidylcholine; $C_{12}E_8$, octaethylene glycol mono-*n*-dodecyl ether; DM, *n*-dodecyl β-D-maltoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EYPC, egg yolk L-α-phosphatidylcholine; EYPA, egg yolk phosphatidic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; DTT, DL-dithiothreitol; NTA, Ni^{2+}-nitrilotriacetic acid; RR120, Reactive red 120 agarose beads

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1. Introduction

Sarcoplasmic reticulum (SR) Ca^{2+}-ATPase SERCA1a is a large membrane protein of 110 kDa that plays a critical role in muscle relaxation by depleting calcium present in the cytosol of a muscle cell and accumulating it in the SR. This membrane pump belongs to the family of P-type ATPases that actively transport cations such as H^+, Na^+, K^+ or Ca^{2+} across biological membranes [1]. The mature form of Ca^{2+}-ATPase present in skeletal muscle contains 994 residues as deduced from cloning and sequencing of the SERCA1a gene by MacLennan and coworkers [2]. Sequence-derived structure predictions and experimental data have suggested that the protein has a large cytosolic domain connected by a stalk of putative helices to a membrane-embedded region organized in 10 putative transmembrane spans M1–10 and linked by small luminal loops (see [3] for a review). Such a predicted topology has been confirmed by the recent 3D structure of the protein, resolved at 2.6 Å by Toyoshima and coworkers [4]. The 3D structure shows that the cytosolic part of the protein is organized in a transduction domain A, a phosphorylation domain P and a nucleotide-binding domain N. The membrane part is indeed organized in 10 helix spans. Three of them are unwounded, allowing the formation of two calcium-binding sites. This 3D structure offers the opportunity to analyze previous kinetic and functional data on a structural basis, as recently carried out by Lee and East [5].

The natural abundance of Ca^{2+}-ATPase in SR, about 70–80%, and the availability of the material, rabbit or chicken, has allowed a biophysical and biochemical characterization of the native protein (for a review see [6,7]). A lot of efforts have been made to achieve functional expression of this large membrane protein. Various systems' host cells have been used for heterologous expression including COS cells [8–10], *Spodoptera frugiperda Sf*9 [11,12] and *Sf*21 [13] cells via baculovirus, and yeast [14–16]. These have been a successful approach in identifying residues by site-directed and/or specific regions essential for calcium binding and transport (see e.g. [8,17–21]). Expression of SERCA1a Ca^{2+}-ATPase can be very high, particularly in COS-1 cells, as recently reported by Inesi and colleagues [10]. Replacing the SV40 promoter by the CMV one in a recombinant adenovirus vector, the level of expression increased to 10–15% and they could carry out a direct measurement of calcium binding with a reduced noise/data ratio. Despite this high level of expression, it remains difficult and rather expensive to use COS-1 cells or baculovirus systems to produce large amounts of recombinant SERCA1a Ca^{2+}-ATPase. The previously described yeast systems are easier and cheaper to use but produced a functional protein at a rather low level of 0.1–0.3% [14–16].

We describe here a yeast expression system that has been optimized for the expression of large amounts of recombinant SERCA1a Ca^{2+}-ATPase with respect to the type of plasmid, yeast strain and expression conditions. Fusion of a short Gly_2His_6 peptide to the C-terminus of the protein allowed its efficient purification by Ni^{2+}-nitrilotriacetic acid affinity chromatography to more than 50% homogeneity. A second affinity chromatography using a Reactive red 120 resin increased purity to about 70%. This purification protocol allowed preparation of mg amounts of pure and active Ca^{2+}-ATPase in 1 day.

2. Materials and methods

2.1. Materials

Biochemical products were from Sigma (Saint Quentin-Fallavier, France) unless specified. Restriction and modification enzymes were purchased from New England Biolabs (Beverly, MA, USA) or Eurogentec (Seraing, Belgium). Products for yeast and bacteria cultures were from Difco (Detroit, MI, USA). Ni^{2+}-nitriloacetic acid-agarose was from Qiagen (Hilden, Germany). Purified egg yolk L-α-phosphatidylcholine (EYPC), egg yolk phosphatidic acid (EYPA) and plant L-α-lysophosphatidylcholine (LPC) were from Avanti Polar Lipids (Alabaster,

AL, USA). Pure *n*-dodecyl β-D-maltoside (DM) was from Anatrace (Maumee, OH, USA), and the ^{14}C radioactively labeled derivative was from Saclay (France). Reactive red 120 agarose, type 3000-CL, was from Sigma. PD-10 columns were from Amersham Pharmacia Biotech (Uppsala, Sweden). Biobeads SM-2 absorbent (20–50 mesh), precision protein standards and kaleidoscope prestained standards were from Bio-Rad (Hercules, CA, USA). Octaethylene glycol mono-*n*-dodecyl ether ($C_{12}E_8$) was from Nikkol Chemical (Tokyo, Japan). Immobilon-P membranes were from Millipore (Bedford, MA, USA). Sarcoplasmic reticulum membranes were prepared as in [21] and provided by Philippe Champeil (CEA Saclay, France).

2.2. Strains, plasmids and constructions

2.2.1. Strains

Saccharomyces cerevisiae W303.1b (*a*, *leu2*, *his3*, *trp1*, *ura3*, *ade2-1*, *can*^{r}, *cir*$^+$) was from Denis Pompon (CGM, Gif-sur-Yvette, France) and is described in [22]. A W303.1b (*a*, *leu2*, *his3*, *trp1::TRP1-GAL10-GAL4*, *ura3*, *ade2-1*, *can*^{r}, *cir*$^+$) strain (W3031.b/Gal4) was generated in this study to overexpress Gal4p upon galactose addition. The GAL4 gene was integrated in the W303.1b genome with a *Bgl*II-linearized pPAP1488 plasmid [23] by in vivo crossing-over using the TRP1 marker. Competent yeast was transformed with this plasmid and selected for tryptophan autotrophy on minimal plates. The additional copy of the GAL4 gene is placed under the control of a galactose-regulated promoter, GAL10.

2.2.2. Plasmids

Expression plasmid pYeDP1/8-10-SERCA1a described in [14] was used for the constructions described in this work. Plasmid pYeDP60 described in [22] was generously given by Denis Pompon (CGM).

2.2.3. Insertion of a Gly_2His_6 coding sequence at the 3′-end of SERCA1a

Oligonucleotides 2835 (5′-CCT CCA CTT CCT CAT CCT CTA-3′) and 2974C (3′-A GCC TTG ATG GAC CTC CCT CCA CCA GTG GTG GTG GTG GTG GTG ATT *CTC GAG* TGA C-5′) were used to amplify by PCR a DNA fragment of 209 bp containing the last 172 nucleotides of the SERCA1a gene followed by eight codons (double underlined) encoding the sequence Gly_2His_6 placed before the stop codon (underlined) and followed by a *Sac*I restriction site. This fragment was inserted in frame into the plasmid pYeDP1/8-10-SERCA1a described in [14] using the *Sal*I (positions 2857–2862) and *Sac*I restriction sites (*Sac*I is indicated with italics). The region generated by PCR was checked by DNA sequencing.

2.2.4. SERCA1a gene transfer from pYeDP1/8-10 to pYeDP60

Wild-type (WT) and modified cDNAs of SERCA1a were transferred by gap repair from pYeDP1/8-10-SERCA1a into pYeDP60. To achieve this a *Hin*dIII fragment, including the promoter, SERCA1a, the polylinker and the terminator, was isolated from each construct of pYeDP1-8/10-SERCA1a and co-transformed in *S. cerevisiae* W303.1b with a *Bam*HI-linearized pYeDP60. Clones were selected using *URA3* complementation as described [14]. Positive clones were identified by restriction analysis.

2.3. Expression of WT and modified Ca^{2+}-ATPases and preparation of membrane fractions

2.3.1. Culture and expression

Growth conditions and criteria for expression of the Ca^{2+}-ATPase were as in [14] for the test of individual clones and for the expression in minimal medium. Initial conditions for large scale expression were as in [22]. Briefly, yeast cells were maintained in minimal medium (2% glucose, 0.1% bactocasamino, 0.7% yeast nitrogen base) and amplified for 36 h at 28°C in YPGE-1 (1% yeast extract, 0.5% glucose, 1% bactopeptone, 2.5% ethanol) or YPGE-2 (2% yeast extract, 1% glucose, 2% bactopeptone, 2.7% ethanol). Before induction and depending on the experiment, the temperature of the medium was either maintained at 28°C or chilled to 10–12°C with water+ice. Induction of expression was initiated by addition of 2% galactose and continued for the indicated time. Optimization of Ca^{2+}-ATPase expression was generally carried out in 200 ml culture medium. Preparative cultures were carried out with 1–4 l in 1 l Fern-

bach flasks (having a deformed bottom that favors efficient ventilation) filled with 0.5 l of medium and shaken at 130 rpm. Expression of the Ca^{2+}-ATPase was checked by Western analysis from a crude extract of yeast prepared in the presence of 2% trichloroacetic acid as described in [24].

2.3.2. Preparation of membrane fractions

After expression, the yeast culture was placed in JA10 centrifuge tubes (Beckman) and rapidly chilled to 4°C in water+ice and left at 4°C overnight. Harvest and breakage were conducted as in [22] to give the starting crude extract (CE in Fig. 3). Starting from a 4 l culture, unbroken yeast and very heavy material were first pelleted at $1000\times g_{av}$ (2400 rpm) in a JA10 Beckman rotor with a J2-MC Beckman centrifuge, for 15 min at 4°C to give the $S_{1.1}$ supernatant and $P_{1.1}$ pellet. $P_{1.1}$ pellet was suspended in the initial volume of breakage buffer (50 mM Tris–Cl, pH 7.5, 1 mM EDTA, 0.6 M sorbitol), supplemented with 0.5 mM PMSF and centrifuged again to give $P_{1.2}$ (P1 in Fig. 3) pellet and $S_{1.2}$ supernatant. Heavy membranes (HMb) were isolated from supernatant $S_{1.1}$ by low speed centrifugation at $10\,000\times g_{av}$ (10 000 rpm) in a JA10 Beckman rotor with a J2-MC Beckman centrifuge, for 15 min at 4°C. The $P_{2.1}$ pellet was washed with the $S_{1.2}$ supernatant and centrifuged again to give the $P_{2.2}$ (P2 in Fig. 3) pellet and $S_{2.2}$ supernatant. $S_{2.1}$ and $S_{2.2}$ were pooled to give S2 and centrifuged at $120\,000\times g_{av}$ (40 000 rpm) in a Ti45 Beckman rotor with a L8-M Beckman centrifuge, for 60 min at 10°C to give the P3 pellet, enriched in light membranes (LMb), and the S3 supernatant containing the soluble material. The HMb fraction was suspended in breakage buffer and the LMb fraction was suspended in 20 mM HEPES–Tris pH 7.4, 0.3 M sucrose, 0.1 mM $CaCl_2$ (HSC buffer), both at a protein concentration of about 20 mg/ml. Membranes were frozen in liquid nitrogen and stored at −80°C.

2.4. Stripping of membranes

Two hundred and thirty milligrams of proteins of light membrane fraction containing 4 mg of expressed 6HC-Ca^{2+}-ATPase were rapidly thawed to 4°C and diluted six times in a cold stripping buffer containing 60 mM Tris–Cl pH 8.0, 12% glycerol, 0.72 M KCl, 1.2 mM $CaCl_2$, supplemented with 0.1 mM PMSF. The suspension was then centrifuged for 45 min at 10°C at $120\,000\times g_{av}$ (40 000 rpm) in a Ti45 Beckman rotor with a L8-M Beckman centrifuge. The pellet was suspended in the solubilization buffer which contains 50 mM Tris–Cl, pH 8.0, 0.1 M NaCl, 20% glycerol and 1 mM $CaCl_2$, at about 2 mg proteins/ml, taking into account that the stripping step (see below) removes about 56 ± 8% of proteins from light membranes and 28 ± 3% from heavy membranes.

2.5. Solubilization of membrane proteins

2.5.1. Membrane solubilization tests

Solubilization of light membranes by either LPC, DM or $C_{12}E_8$ was tested at a protein concentration of 2 mg/ml and with detergent/protein ratios of 1, 3 and 5. Solubilization was carried out for 30 min at 4°C followed by a centrifugation at 10°C for 30 min at $100\,000\times g$ (50 000 rpm with a TLA100 rotor in a TL100 Beckman centrifuge).

2.5.2. Preparative solubilization

The membrane suspension obtained after the stripping step and suspended in the solubilization buffer at about 2 mg proteins/ml was supplemented with DM or LPC at a detergent/protein ratio of 3. The suspension was incubated at 10°C (DM) or 24°C (LPC) for 30 min and then centrifuged for 30 min, 10°C, at $120\,000\times g_{av}$ (40 000 rpm) in a Ti45 Beckman rotor with a L8-M Beckman centrifuge.

2.6. Purification of 6HC-Ca^{2+}-ATPase

All steps were carried out in a cold room.

2.6.1. Ni^{2+}-nitrilotriacetic acid (NTA)-agarose chromatography

NTA-agarose beads (1.5 ml) were washed before use with 7.5 ml water and then equilibrated for 1 h with 13.5 ml of NTA buffer containing 50 mM Tris–Cl pH 8.0, 0.1 M NaCl, 20% glycerol, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5 mg/ml DM. The solubilized material was mixed and gently stirred with the resin at 4°C overnight. The suspension was then loaded on a 11×100 mm column (BioSepra, MA, USA) and the run-through was loaded a second time on the resin.

The agarose beads were washed at 2 ml/min, first with 90 ml of NTA buffer containing in addition 1 M NaCl, 0.25 mg/ml of a 10:1 mix of EYPC–EYPA and 10 mM imidazole, followed by a second washing step with 30 ml of the same buffer but with 0.1 M NaCl. The proteins were eluted from the resin by applying seven times 1 ml of NTA buffer containing in addition 0.1 M NaCl, 0.25 mg/ml of a 10:1 mix of EYPC–EYPA and 150 mM imidazole. Each addition of buffer was followed by a 5 min incubation with gentle stirring. Recovery of 6HC-Ca^{2+}-ATPase was followed by Ca^{2+}-dependent ATPase activity. The buffer of the pool was changed by size exclusion chromatography with a PD-10 column (Sephadex G25 M) equilibrated with 25 ml of Reactive red 120 (RR120) buffer containing 25 mM MOPS–Tris pH 7.0, 0.025 M NaCl, 20% glycerol, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5 mg/ml DM, 1 mM DTT.

2.6.2. Reactive red 120 agarose chromatography

One milliliter of RR120 suspension (500 μl resin) was withdrawn and loaded on a 1 ml syringe. The resin was washed with 5 ml water and then equilibrated at 1 ml/min with 5 ml of RR120 buffer. The pool collected from the PD-10-NTA step was loaded on the resin at 0.2 ml/min. Ca^{2+}-dependent ATPase activity was tested on 0.5 ml aliquots of flow-through to check the binding of 6HC-Ca^{2+}-ATPase to the resin. The resin was then washed with 5 ml of RR120 buffer at 0.2 ml/min and the 6HC-Ca^{2+}-ATPase was then eluted from the resin with the same buffer containing 0.7 M KCl [25]. Recovery of 6HC-Ca^{2+}-ATPase was followed by Ca^{2+}-dependent ATPase activity. The pool was subsequently submitted to a PD-10 column equilibrated with the RR120 buffer containing 40% glycerol and 0.1 M KCl. EYPC–EYPA (10:1 mix) were added to the pool at a final concentration of 1 mg/ml.

2.6.3. Detergent removal with Bio-beads

Bio-beads SM2 were prepared as described in [26] and stored at 4°C in sterile water supplemented with 5 mM NaN_3. Detergent was removed by adding Bio-beads to the last pool at a Bio-beads:detergent ratio of 200:1 (w/w) and gentle stirring at 4°C for 3 h. Bio-beads were then removed and the pool was either stored at 4°C or frozen in liquid N_2 and stored at −80°C.

2.7. SDS–PAGE

Aliquots of 20 μl containing 0.1–120 μg of proteins were rapidly thawed and mixed with an equal volume of denaturing buffer containing 100 mM Tris–Cl pH 8.0, 1.4 M β-Me, 4% SDS, 5 mM EDTA, 8 M urea, 0.05% bromophenol blue. Samples were heated at 100°C for 1 min, cooled and then loaded on a Laemmli-type 9% polyacrylamide gel as described in [27] and with a Bio-Rad 3 apparatus.

2.8. Protein estimation

Protein concentrations were measured by the bicinchoninic acid procedure [28] in the presence of 0.5% SDS.

2.9. Ca^{2+}-ATPase quantification and blotting

The expressed Ca^{2+}-ATPase was quantified by Western blotting followed by an immunodetection with the polyclonal antibody 79B (a gift of A.-M. Lompré, Université Paris XI, France). Blots were revealed with the ECL kit (Amersham) as described in [20]. Quantification was carried out with a GS-700 imaging densitometer (Bio-Rad) and the Molecular Analyst software (Bio-Rad) using as reference native SR membranes in which 75% of the protein content is assumed to be Ca^{2+}-ATPase. When indicated, the Ca^{2+}-ATPase was also blotted with antibodies $Ab_{577-588}$ and $Ab_{877-888}$ raised against SERCA1a peptides, 577–588 and 877–888 respectively. Both antibodies were previously described in [42] and were generously given by Prof. J.V. Møller (Aarhus, Denmark).

2.10. ATPase assay

ATP hydrolysis was assayed spectrophotometrically at 30°C as described [19], with about 200 μg of yeast membranes in 2 ml of medium containing a final Ca^{2+} concentration of 0.1 mM. The reaction was started after 5 min by addition of 1 mM Na_2ATP and measured for at least 100 s. The reaction was stopped by addition of 2 μg of thapsigargin [29] or with 5 mM EGTA. The Ca^{2+}-ATPase activity was calculated as the difference between the slopes obtained in the presence and in the absence of thapsi-

gargin or EGTA and using SR Ca^{2+}-ATPase as standard with a specific activity of 13 μmol/min/mg under these conditions. K_m for Ca^{2+} was estimated with the same enzymatic system but carried out at 20°C and pH 7.0 in 10 mM TES–Tris buffer, conditions which allow to get more accurate estimates of free calcium concentrations after addition of EGTA. Reactions were initiated with a starting Ca^{2+} concentration of 100 μM and increasing amounts of EGTA were then added sequentially to give successive decreasing free Ca^{2+} concentrations as established in [41,44]. K_m for ATP was estimated in the same conditions and using a Ca^{2+} concentration of 20 μM.

2.11. Electron microscopy

Yeast cells were grown to mid-log phase and formaldehyde and glutaraldehyde were added to a final concentration of 3.7% and 0.5%, respectively. Yeast cells were pelleted after 1 night at 4°C and suspended in 10 vols. of cold buffer (18 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 138 mM NaCl, pH 7.4) supplied with 3.7% formaldehyde and 0.5% glutaraldehyde. Immunolabeling of the expressed Ca^{2+}-ATPase and visualization by electron microscopy were carried out as described in [30] using the 79B antibody.

3. Results and discussion

3.1. Construction and evaluation of the C-terminally His-tagged Ca^{2+}-ATPase

The aim of the present work was to obtain as large amounts as possible of purified and functional wild-type or mutated Ca^{2+}-ATPase. To achieve this, we planned (i) to improve the expression system by changing the plasmid and optimizing various param-

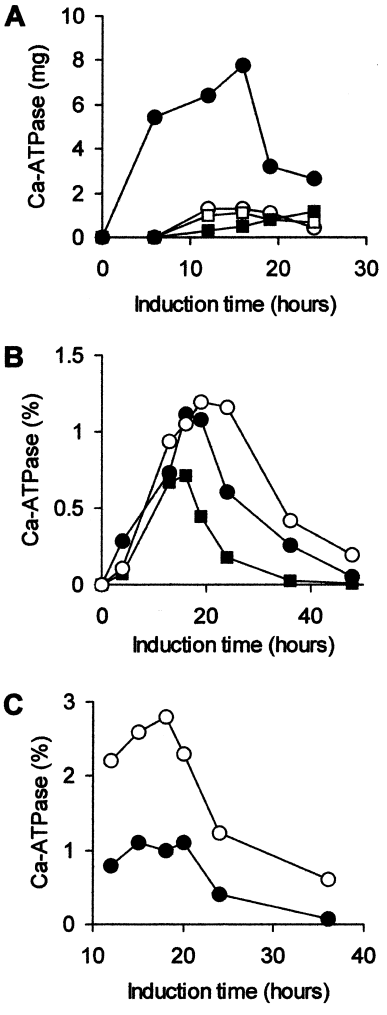

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Fig. 1. Increase of Ca^{2+}-ATPase expression. (A) Comparative expression of Ca^{2+}-ATPase with pYeDP1/8-10 and pYeDP60 plasmids in yeast growing in minimal or rich medium. The W303.1b yeast strain transformed with pYeDP1/8-10-6HCSERCA1a (squares) or pYeDP60-6HCSERCA1a (circles) was grown at 28°C in 200 ml of minimal medium (open symbols) or YPGE-1 medium (closed symbols). At time zero, 2% galactose was added and aliquots of each culture were withdrawn at the indicated times. Yeast cells were broken and the amounts of expressed Ca^{2+}-ATPase and total proteins were estimated as described in Section 2. Amounts of expressed Ca^{2+}-ATPase are given for 1 l of culture. (B) Effects of multiple galactose additions and medium composition. W303.1b yeast transformed with pYeDP60-6HCSERCA1a was grown at 28°C in 200 ml of YPGE-1 medium (closed symbols), or YPGE-2 medium (open circles). Galactose was added at time 0 (closed symbols, open circles) and 13 h later (circles). Aliquots were withdrawn at the indicated times and the amounts of proteins and Ca^{2+}-ATPase were estimated (see Section 2). (C) Effects of genetic modification of the yeast. The W303.1b yeast strain was genetically modified to increase the number of Gal4p copies (see Section 2). Modified (W303.1b/Gal4, open circles) or unmodified (W303.1b, closed circles) yeast strains were transformed with pYeDP60-6HCSERCA1a and grown in YPGE-2 medium after selection. Galactose was added a first time at time 0 and a second time 13 h later. Aliquots were withdrawn at the indicated times and the amounts of total protein and Ca^{2+}-ATPase were estimated (see Section 2). The amount of produced Ca^{2+}-ATPase is given as percentage of total protein content.

eters of expression, and (ii) to affinity purify the Ca^{2+}-ATPase produced initially at a rather low amount corresponding to 0.1–0.3% of the total yeast proteins. A hexahistidine tag was introduced at the C-terminus of the Ca^{2+}-ATPase as described in Section 2. Two additional glycine residues were inserted between the histidine tail residues and the C-terminal residue of Ca^{2+}-ATPase to increase the flexibility and/or accessibility of the tag. The final primary structure of the modified Ca^{2+}-ATPase, termed 6HC-Ca^{2+}-ATPase, was NH_2-M^1—$G^{994}G_2H_6$-COOH. The 6HC-Ca^{2+}-ATPase was produced from vector pYeDP1-8/10 using the expression system initially set up by our team and described in [14]. The level of expression was 1.1 mg/l (Fig. 1A, open squares), which is in the same range as the 0.9 mg/l reported for the wild-type, non-tagged enzyme [14]. We have also generated a construction in which the His tag was inserted at the N-terminus of the protein. The construction with the C-terminally located histidine tag was, however, favored in this study as it gave the highest expression level (data not shown).

3.2. Plasmid exchange of SERCA1a

One main improvement of Ca^{2+}-ATPase expression in yeast was to use pYeDP60, a plasmid designed by Pompon and colleagues [22] and similar to pYeDP1-8/10 except for the addition of the *ADE2* gene. In contrast to pYeDP1-8/10, pYeDP60 allows transformed *Ade2* cells to grow in rich medium in which the cell density is 10-fold higher than in minimal medium. We took advantage of the compatibility of pYeDP1-8/10 and pYeDP60 (see Section 2) to transfer by gap repair the SERCA1a gene from the former to the latter plasmid. As expected, the level of expression in minimal medium of the 6HC-Ca^{2+}-ATPase with pYeDP60 was similar to that with pYeDP1-8/10 (Fig. 1A, compare open symbols). In rich medium, on the contrary, the difference in 6HC-Ca^{2+}-ATPase expression was striking (Fig. 1A, compare closed symbols). The level of expression decreased in yeast transformed with pYeDP1-8/10-6HCSERCA1a (Fig. 1A, closed squares), the plasmid probably being slowly lost since it was no longer required for growth. On the contrary, the level of expression markedly increased to a maximal value of about 8 mg 6HC-Ca^{2+}-ATPase/l with pYeDP60-6HCSERCA1a (Fig. 1A, closed circles), due to the increase in biomass and to the maintenance of the plasmid in yeast. The best level of expression of 6HC-Ca^{2+}-ATPase obtained with pYeDP60 was 0.7% of total proteins as shown in Fig. 1B (time 16 h, closed squares), which is considerably below the level of 5% reported by Pompon and colleagues [22] for the expression of cytochrome *P*-450 with the same plasmid. This difference probably reflects the fact that cytochrome *P*-450 has only one putative transmembrane span whereas 10 are present in the Ca^{2+}-ATPase.

3.3. Optimization of the conditions of induction and growth

Increasing the number of galactose inductions was found to increase the level of expression of 6HC-Ca^{2+}-ATPase as shown in Fig. 1B. Two successive additions of galactose, one at time 0 followed by a second 13 h later caused a 1.5-fold increase in material (closed circles) when compared to the same culture induced at time 0 only (closed squares). Adding galactose more than twice was found to be useful for maintaining the expression at the highest level for a longer period of time but did not increase the level of expression further (not shown). Increasing the concentration of nutrients mainly stabilized the level of expression (Fig. 1B, compare open and closed circles).

3.4. Modification of the host strain

Expression of 6HC-Ca^{2+}-ATPase was improved further by increasing the level of Gal4p, the protein that interacts with Gal-type promoters such as the hybrid GAL10-CYC1 promoter that controls transcription of SERCA1a in pYeDP60. The natural level of this protein in yeast is very low since Gal4p is estimated to be present at 1–2 copies, which limits expression but guarantees tight control of the exogenous protein production [31]. We generated a W303.1b/Gal4 strain by integration of an additional copy of the Gal4 gene placed under the control of a Gal-type promoter into the TRP locus of the host yeast W303.1b genome (see Section 2). This modification favored overexpression of Gal4p protein only after galactose addition and concomitantly to expres-

sion of the 6HC-Ca^{2+}-ATPase. Five independent recombinant strains W303.1b/Gal4-(1 to 5) were transformed with pYeDP60-6HCSERCA1a and tested for their capacity to overexpress the Ca^{2+}-ATPase. Increased expression was obtained in each case when compared to the parental strain. Fig. 1C displays the expression of 6HC-Ca^{2+}-ATPase obtained with clone W303.1b-Gal4-5 (open circles) that is 2.4-fold higher than expression in the parental W303.1b strain (closed circles). Despite this result, the 2.4-fold increase observed here for the expression of 6HC-Ca^{2+}-ATPase remains limited when compared to the 10-fold increase reported for the expression of the (Na^++K^+)-ATPase [23]. The yield of 6HC-Ca^{2+}-ATPase expression obtained after the above modifications reached almost 3% (see Fig. 1C, open symbols) corresponding to 30 mg 6HC-Ca^{2+}-ATPase/l of culture, thus 10 times more than initially produced [14].

3.5. Subcellular localization of the expressed 6HC-Ca^{2+}-ATPase

3.5.1. Visualization of Ca^{2+}-ATPase in yeast by electron microscopy

Due to the high level of expression, the expressed 6HC-Ca^{2+}-ATPase could easily be detected by electron microscopy. As shown in Fig. 2A, accumulation of gold particles was observed in typical structures of double smooth membranes (see the magnification in Fig. 2B and membranes indicated by a white arrow) clearly related to *perinuclear cisternae*. A few gold particles could also be observed at the plasma membrane (Fig. 2B, black arrows). Such structures were never observed in control yeast transformed with the native plasmid and submitted to identical culture conditions (not shown). The same type of membranes was also observed when the Ca^{2+}-ATPase

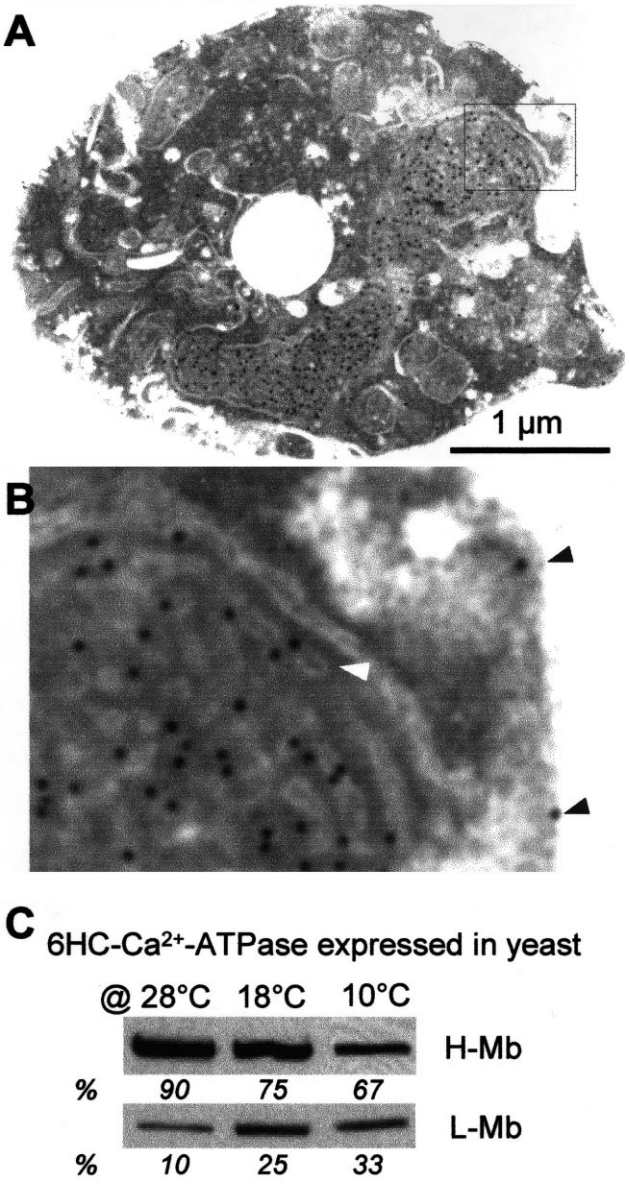

Fig. 2. Subcellular localization of Ca^{2+}-ATPase. (A) W303.1b/Gal4 [pYeDP60-6HCSERCA1a] was grown in YPGE-2 medium for 36 h at 28°C and expression was then induced with galactose at time 0 and again 13 h later. Yeast cells were harvested after 19 h of expression, fixed and immunolabeled as described in Section 2. Immunogold electron microscopy was carried out as described in Section 2. (B) 4-Fold magnification of the upper right corner of panel A. (C) Effect of temperature during expression on the membrane distribution of Ca^{2+}-ATPase. W303.1b/Gal4 [pYeDP60-6HCSERCA1a] was grown in YPGE-2 medium for 36 h at 28°C and then equilibrated to 28, 18 or 10°C before induction with galactose that was added initially and 13 h later. Cultures were incubated at 28, 18 and 10°C during induction and harvested 19 h later. Heavy (H-Mb) and light (L-Mb) membranes were prepared as described in Section 2 but using a centrifuge speed of $20\,000 \times g_{av}$ for the sedimentation of the heavy membranes. Amounts of total proteins were estimated as described in Section 2 and 10 µg of each fraction were separated by SDS–PAGE in an 8% gel followed by Western blotting for estimation of expressed 6HC-Ca^{2+}-ATPase. Values indicate the relative percentage of 6HC-Ca^{2+}-ATPase estimated for each membrane fraction, 100% corresponding to the cumulated values estimated for heavy+light membranes.

was expressed in yeast by using a strong constitutive promoter and attributed to periendoplasmic reticulum [15]. These membranes can be differently ordered such as the karmellaes observed for the expression of the plant H^+-ATPase in yeast [32]. Whatever the type of membrane arrangement, the function of the expressed protein was maintained since both expression of rabbit Ca^{2+}-ATPase [15] and that of plant H^+-ATPase [32] were described to complement the corresponding deficient yeast strain.

3.5.2. Reduction of temperature during expression

When expressed at 28°C, 90% of the 6HC-Ca^{2+}-ATPase was pelleted with the heavy membrane fraction and 10% remained in the light membrane fraction, as shown by Western blotting in Fig. 2C. As observed previously [20], 6HC-Ca^{2+}-ATPase present in heavy membranes showed reduced enzymatic activity compared to that contained in light membranes, and was poorly solubilized with a mild detergent (not shown). Addition of 5–10% glycerol to the culture medium did not change this pattern (not shown), suggesting that glycerol does not act as a chemical chaperone for the expressed Ca^{2+}-ATPase, in contrast to what was described for production of human MDR1 in yeast [33]. As shown in Fig. 2C, decreasing the expression temperature from 28 to 18, and even 10°C, increased the density of 6HC-Ca^{2+}-ATPase from 10 to 33% in light membranes and decreased the density in heavy membranes proportionally. The cellular distribution of 6HC-Ca^{2+}-ATPase was found to be independent of expression tem-

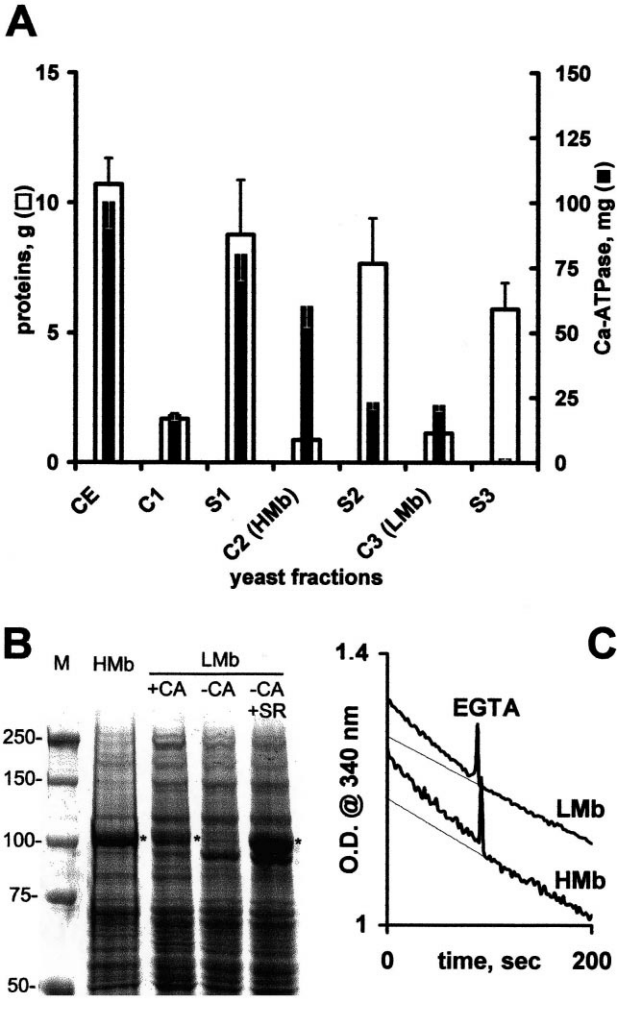

Fig. 3. Preparative expression of 6HC-Ca^{2+}-ATPase. W303.1b/Gal4 [pYeDP60-6HCSERCA1a] was grown in 8×0.5 l of YPGE-2 medium for 36 h at 28°C and then equilibrated to 18°C before induction. Galactose was added after thermal equilibration and 13 h later. Cultures were incubated at 18°C during induction and harvested 19 h later. (A) Yeast fractionation pattern. Protein (open area) and Ca^{2+}-ATPase (closed area) were estimated as described in Section 2. The figure corresponds to the mean of three experiments and standard errors are indicated in black for proteins and in white for Ca^{2+}-ATPase. CE corresponds to the crude extract; C1 and S1 correspond to the pellet and supernatant from centrifugation of CE at 1000×*g* for 15 min; C2 and S2 correspond to the pellet and supernatant from centrifugation of S1 at 10 000×*g* for 15 min; C3 and S3 correspond to the pellet and supernatant from centrifugation of S2 at 100 000×*g* for 60 min. (B) SDS–PAGE of yeast fractions. Proteins were loaded on a 9% SDS–PAGE as described in Section 2. The electrophoretic migration was stopped when the 50 kDa protein standard reached the bottom of the gel and the gel was then stained with Coomassie blue. Lanes: M, precision protein standards (Bio-Rad); HMb, 30 μg of heavy membranes of the C2 pellet containing the expressed 6HC-Ca^{2+}-ATPase; LMb+CA, 30 μg of light membranes (LMb) of the C3 pellet containing the expressed 6HC-Ca^{2+}-ATPase; LMb−CA, 30 μg of LMb from yeast transformed with the expression plasmid without the SERCA1a gene; LMb−CA+SR, as in the preceding lane but with 4 μg of SR added. Stars indicate the position of native and expressed Ca^{2+}-ATPase. (C) Ca^{2+}-dependent ATPase activity in HMb and LMb fractions. ATPase activity was measured as described in Section 2 after addition of 200 μg proteins of heavy or light membrane fractions to the cuvette, corresponding to 13.6 and 3.5 μg of expressed 6HC-Ca^{2+}-ATPase respectively. The reaction was started by addition of ATP and stopped after addition of EGTA. Dotted lines correspond to the basal activity measured in the presence of EGTA.

perature as judged from electron microscopy of yeast expressing 6HC-Ca^{2+}-ATPase at 18 or 10°C (not shown).

Interestingly, reduction of temperature also increased the efficiency of 6HC-Ca^{2+}-ATPase solubilization from heavy and light membranes. Complete solubilization was even observed with membranes isolated from yeast grown at 10°C (not shown). However, lowering the temperature to 10°C during expression dramatically reduced the yield of expression to less than 1 mg 6HC-Ca^{2+}-ATPase/l of culture instead of 30 mg/l at 28°C while it remained high (about 25 mg/l) when the expression was carried out at 18°C (not shown).

3.6. *Preparative expression of 6HC-Ca^{2+}-ATPase and membrane fractionation*

We routinely expressed 6HC-Ca^{2+}-ATPase at 18°C and produced about 200 g of yeast, in portions of 4 l culture as described in Section 2. Fig. 3A displays a mean pattern of fractionation of yeast proteins and expressed 6HC-Ca^{2+}-ATPase from such a culture. The amount of produced 6HC-Ca^{2+}-ATPase was estimated in the crude extract to be 100 ± 10 mg equivalent to 0.9% of the protein content. About 80 mg was recovered in the first low speed supernatant (S1 in Fig. 3A) that removes unbroken yeast and very heavy material such as nuclei. The heavy and light membranes contain about 60 and 22 mg respec-

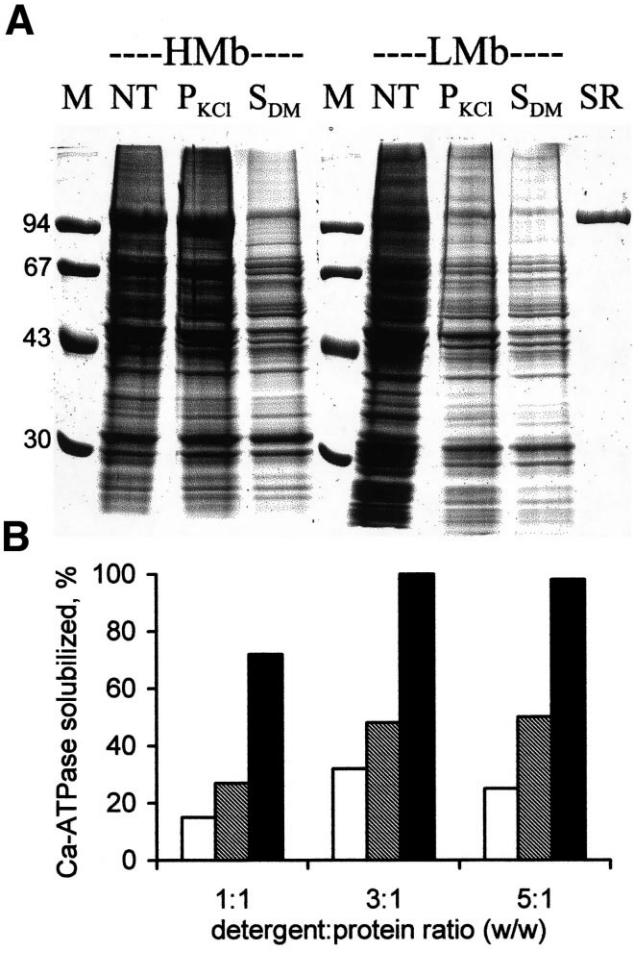

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Fig. 4. Enrichment of 6HC-Ca^{2+}-ATPase by KCl stripping and recovery after solubilization. (A) Effect of KCl stripping of heavy and light membranes and solubilization of 6HC-Ca^{2+}-ATPase. Heavy and light membranes were prepared as described in Fig. 3 and stripped as described in Section 2. Each suspension was centrifuged and the pellet suspended at about 2 mg/ml in solubilization buffer as described in Section 2. Solubilization was carried out by addition of DM as described in Section 2. The protein content of each fraction was estimated and aliquots were loaded on a 9% SDS–PAGE, stained with Coomassie blue after migration. Lanes of non-treated (NT) membranes correspond to the protein content (40 μg, 100%) of heavy or light membranes before stripping. Lanes P_{KCl} correspond to the protein content of each fraction after KCl stripping: 28 μg (70% of starting material) of stripped heavy membranes and 17.6 μg (44% of starting material) of stripped light membranes. Lanes S_{DM} correspond to supernatant fractions resulting from solubilization with DM at a DM:protein ratio of 3:1, followed by centrifugation of KCl-stripped heavy and light membranes as described in Section 2. The same volume of sample as that loaded for stripped fractions has been loaded allowing a direct comparison of P_{KCl} and S_{DM} lanes. They correspond to 16 and 14 μg of solubilized material from KCl-stripped heavy and light fractions respectively. Lane SR corresponds to 2.5 μg of native SR Ca^{2+}-ATPase. Lanes M correspond to molecular mass markers (Pharmacia): phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa). (B) Compared solubilization of 6HC-Ca^{2+}-ATPase with $C_{12}E_8$, β-dodecyl maltoside and lysophosphatidylcholine. Light membrane proteins (2 mg/100 μl HSC buffer) were thawed and suspended in 1 ml of 25 mM Tris–Cl pH 8.0, 20% glycerol, 0.05 M NaCl, 1.4 mM β-Me and 5 mM imidazole. Aliquots of 400 μg proteins/200 μl were withdrawn and supplemented with either $C_{12}E_8$, DM or LPC at a detergent:protein ratio (w:w) of 1, 3 and 5. Samples were prepared as described in Section 2. The amount of 6HC-Ca^{2+}-ATPase was estimated by immunodetection from aliquots withdrawn before and after centrifugation. Values are given in % of the total amount of 6HC-Ca^{2+}-ATPase. White, dashed and black symbols correspond to 6HC-Ca^{2+}-ATPase solubilized with $C_{12}E_8$, DM and LPC respectively.

tively of the expressed ATPase (C2 and C3 in Fig. 3A), corresponding to 6.8 and 1.7% of the protein content. Such amounts of expressed 6HC-Ca^{2+}-ATPase could be observed on a Coomassie blue stained SDS–PAGE as shown in Fig. 3B, for heavy membranes (HMb lane) and even for light membranes ('LMb+CA' lane) when compared to control lanes, 'LMb−CA' and 'LMb−CA+SR'. The present amounts are higher than those reported in the literature for this type of multi-membrane span protein expressed in yeast such as Ca^{2+}-ATPase (e.g. [15,16]), (Na^++K^+)-ATPase [23] or H^+-ATPase (e.g. [32,34,35]).

As shown in Fig. 3C, a Ca^{2+}-dependent ATPase activity could be detected in the light membrane fraction, corresponding to specific ATPase activity of 4.4 μmol ATP hydrolyzed/min/mg Ca^{2+}-ATPase. This value is a little lower than the values of 5.0 or 6.2 reported by us before [19,20], but although they remain in the same range, the difference can also be due to the insertion at the C-terminus of the His tag or to the conditions of overexpression. We observed a specific ATPase activity of 1.1 μmol ATP hydrolyzed/min/mg Ca^{2+}-ATPase in the heavy membranes fraction, which indicates that only a fraction of the expressed Ca^{2+}-ATPase has activity in such membranes. Both heavy and light membrane fractions could be frozen in N_2 liquid and stored at −80°C without any loss of Ca^{2+}-dependent ATPase activity.

3.7. Enrichment of 6HC-Ca^{2+}-ATPase by KCl stripping and recovery after solubilization

As the 1.7% purity of 6HC-Ca^{2+}-ATPase in light membranes is rather low we added a stripping step with KCl at high ionic strength before solubilization. Such a stripping was tested with light and heavy membranes. As shown in Fig. 4A, the incubation of membranes with 0.6 M KCl was very efficient for light membranes compared to the heavy ones since 60% of the protein content of light membranes was removed while not more than 30% was lost in the heavy membranes (compare P_{KCl} lanes in Fig. 4A). No more than 5% of the expressed ATPase was lost during this step (see Table 1), which increased the density of the ATPase to 4% of the protein content in light membranes. A further solubilization step with DM of stripped light membranes, at a 3:1 detergent:protein (w/w) ratio, showed that 50% of 6HC-Ca^{2+}-ATPase was solubilized together with 75% of the proteins and represented 2.9% of the solubilized proteins.

In addition to DM, we also tested the solubilization of the 6HC-Ca^{2+}-ATPase expressed in light membranes with other mild detergents such as $C_{12}E_8$ and LPC at detergent:protein ratios of 1, 3 and 5. The results are displayed in Fig. 4B which shows that the maximal solubilizing effect was reached at a 3:1 ratio and was not more than 25%

Table 1
Purification of 6HC-Ca^{2+}-ATPase[a]

Fraction	Protein (mg)	Ca^{2+}-ATPase (mg)	Ca^{2+}-ATPase/protein (%)	Specific activity (μmol/min)		Yield[e] (%)
				/mg protein	/mg Ca^{2+}-ATPase	
LMb	230	3.8[c]	1.7	0.07	4.1	100
P^b_{KCl}	92	3.6[c]	4.0	0.09	2.4	95
S^b_{DM}	69	2.0[c]	2.9	0.19	6.5	53
NTA FT[b]	–	0.40[c]	–	–	–	–
NTA pool	1.6	0.81[d]	50	1.6	3.2	21
RR120 pool	0.26	0.20[d]	70	5	4.9–7.3[f]	5.3

[a]In this batch, we started with 230 mg of light membrane proteins, the equivalent of 700 ml of yeast culture.
[b]P_{KCl} corresponds to the pellet of KCl-stripped membranes and S_{DM} corresponds to the supernatant of DM-solubilized fraction as displayed in Fig. 4A. NTA FT, NTA flow-through.
[c]Amounts of Ca^{2+}-ATPase determined by Western analysis as described in Section 2.
[d]Amounts of Ca^{2+}-ATPase determined by densitometry of the SDS–PAGE displayed in Fig. 5A,B with native SR Ca^{2+}-ATPase as reference.
[e]100% corresponds to the amount of starting material, 3.8 mg of Ca^{2+}-ATPase.
[f]4.9 and 7.3 correspond to specific activities measured immediately or after 5 days at 4°C respectively.

with $C_{12}E_8$ and 50% with DM while LPC fully solubilized the protein. LPC thus appeared as the best solubilizing detergent but further steps of purification showed that DM was better than LPC for the following reasons. (i) At low temperature and in the presence of 1 mM Ca^{2+} – two conditions that are essential to preserve activity of the solubilized Ca^{2+}-ATPase during purification – we observed that the supernatant of LPC-solubilized material became cloudy during incubation with NTA-agarose beads. This was not the case with DM and we found that this was due to the presence of calcium in the solution as an excess of EGTA clarified the solution. (ii) LPC solubilized 100% of the light membrane proteins compared with 75% observed with DM and this resulted in a lower level of purification than that obtained with DM after NTA chromatography. (iii) The expressed 6HC-Ca^{2+}-ATPase was less active after solubilization with LPC compared to that with DM and the activity could not be restored after exchange of detergents. (iv) The protein solubilized with LPC was less efficiently bound to NTA agarose than DM solubilized protein.

3.8. Purification of the 6HC-Ca^{2+}-ATPase by Ni^{2+}-NTA and Reactive red affinity chromatography

We routinely worked with about 250 mg of LMb proteins (230 mg in the batch shown in Table 1) containing about 4.0 mg of 6HC-Ca^{2+}-ATPase. After KCl stripping, membranes were suspended at 2 mg/ml and solubilized by addition of DM to a detergent:protein ratio of 3 as described in Section 2, which solubilized 2 mg of 6HC-Ca^{2+}-ATPase. The result of a typical purification procedure is summarized in Table 1.

3.8.1. Ni^{2+}-NTA chromatographic step

Final conditions are detailed in Section 2 but as a starting point to carry out the first trials of purification of the 6HC-Ca^{2+}-ATPase by NTA-agarose, we used conditions close to those of Palmgren and colleagues described for the purification of a His-tagged plant H^+-ATPase expressed in yeast [35]. However, in contrast to them, we could not reach a purification yield higher than 25% (not shown), probably because we used a cruder membrane fraction. We observed that the best yield of binding of 6HC-Ca^{2+}-ATPase on the NTA resin was obtained with an incubation time longer than 3 h and under gentle stirring of the resin. Typically, after 1 night of incubation, about 80% of the solubilized 6HC-Ca^{2+}-ATPase was bound to the NTA resin. The level of purification was also increased up to 50% (Western vs. protein estimation) by adding as described in Section 2 a washing step with a high ionic strength buffer (1 M NaCl), and also by increasing the volume of washing buffers from 4 to 60 times the volume of resin. However, we observed that this extensive washing step leads to the purification of a poorly active Ca^{2+}-ATPase ($\sim$1 μmol ATP/min/mg Ca^{2+}-ATPase, not shown). This is probably due to the fact that using such large volumes of detergent-containing buffers delipidates the Ca^{2+}-ATPase too efficiently, which contributes to its inactivation [25]. The addition of asolectin or mixed soybean phospholipids [35] partially restored the ATPase activity but the best and reproducible results were obtained with the addition of 0.25 mg/ml of highly pure EYPC–EYPA, mixed at a 10:1 ratio as previously used for reconstitution experiments [43]. As indicated in Table 1, about 0.8 mg of the 6HC-Ca^{2+}-ATPase was eluted after incubating the resin seven times with 0.75 vol. of elution buffer containing 150 mM imidazole. A picture of the elution profile obtained under these conditions is displayed in Fig. 5A. It shows that a major band of approx. 110 kDa was eluted in the first six fractions. No 6HC-Ca^{2+}-ATPase was detected in the seventh elution (not shown). Additional bands could be observed at higher and lower molecular masses than that of SERCA1a. A Western blot carried out with three different antibodies raised against the N-terminal region, the C-terminal region and the middle of SERCA1a (lanes 1 of Fig. 5C) clearly showed that they do not correspond to either aggregates or proteolyzed fragments of the purified protein. At this step, the specific Ca^{2+}-dependent ATPase activity of the pool was found to be 3.2 μmol ATP hydrolyzed/min/mg Ca^{2+}-ATPase.

3.8.2. Reactive red chromatographic step

In order to obtain a highly purified and functional material, the most concentrated fractions (1–5) of the NTA elution were pooled and subjected to RR120 affinity chromatography as initially described for the purification of native Ca^{2+}-ATPase from solubilized

SR [36], and more recently for that of the gastric (H^++K^+)-ATPase [25]. Before this step, it was necessary to change the buffer of the NTA pool by passing through a PD-10 column since imidazole dramatically reduced the binding capacity of the RR120 resin. In such conditions, the adsorption of the 6HC-Ca^{2+}-ATPase to the RR120 resin was complete and no ATPase activity was found in the flow-through. According to [25], elution was achieved with a buffer containing 0.7 M KCl rather than with ADP. As shown in Fig. 5B, the ATPase was eluted after the first milliliter and with 2 ml of elution buffer (fractions 3–6). About 200 μg of 6HC-Ca^{2+}-ATPase was recovered at this step with a purity estimated to be 70% (Table 1). The few remaining bands detected by Coomassie blue staining at higher and lower molecular masses than that of SERCA1a does not correspond to either aggregates or proteolyzed fragments of the purified protein as checked by immunodetection carried out with three different antibodies (lanes 2 of Fig. 5C). The kinetics of Ca^{2+}-dependent ATPase activity carried out with the PD-10(RR120) pool corresponded to a specific activity of 4.9 μmol ATP hydrolyzed/min/mg Ca^{2+}-ATPase. A lower value of 3.6 μmol ATP hydrolyzed/min/mg Ca^{2+}-ATPase was also found with another batch of purification. After purification and contrarily to the kinetics displayed in Fig. 3C, no contaminating activity remained in the pool after addition of EGTA as shown in Fig. 6A.

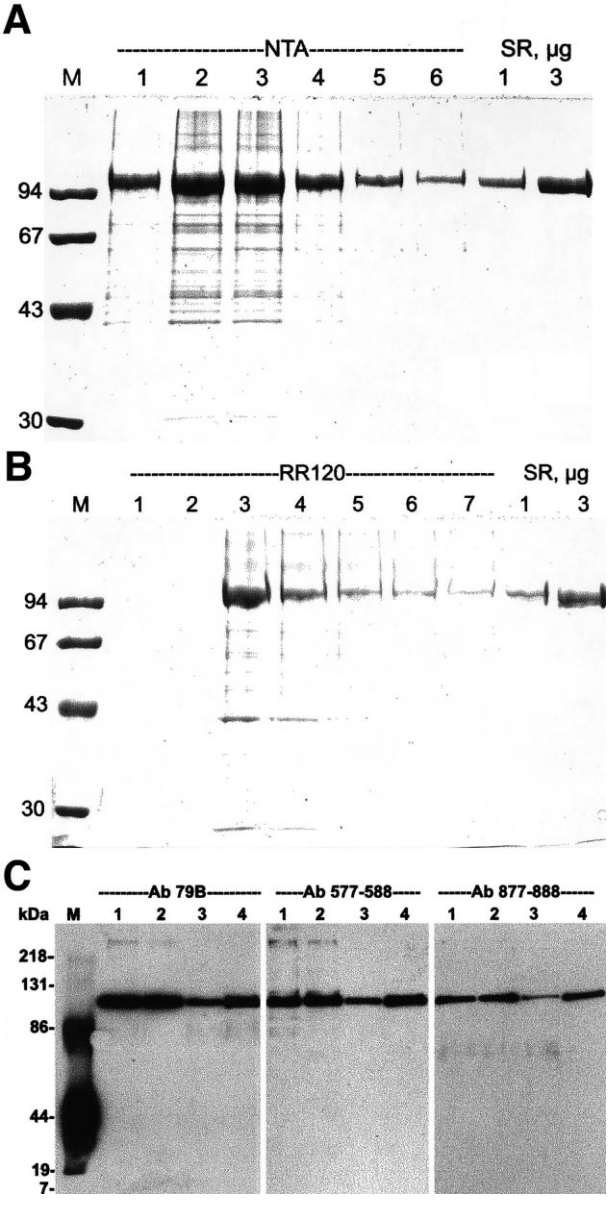

Fig. 5. Coomassie blue-stained SDS–PAGE and Western blot analysis of the purification steps of 6HC-Ca^{2+}-ATPase. (A) Ni^{2+}-NTA purification step. Proteins were loaded on a 9% SDS–PAGE and stained with Coomassie blue after migration as described in Section 2. Lane M corresponds to molecular mass markers (Pharmacia) as in Fig. 4. Lanes labeled NTA, 1–6, correspond to the first six fractions eluted with 150 mM imidazole as described in Section 2. Lanes labeled SR correspond to 1 and 3 μg of native SR Ca^{2+}-ATPase. (B) Reactive red 120 purification step. Proteins were loaded on a 9% SDS–PAGE and stained with Coomassie blue after migration as described in Section 2. Lane M corresponds to molecular mass markers (Pharmacia) as in Fig. 4. Lanes labeled RR120, 1–7, correspond to the seven fractions eluted with KCl 0.7 M as described in Section 2. Lanes labeled SR correspond to 1 and 3 μg of native SR Ca^{2+}-ATPase. (C) Western blot of the purified 6HC-Ca^{2+}-ATPase with antibodies raised against N-terminus, C-terminus and cytoplasmic parts. Purified 6HC-Ca^{2+}-ATPase (lanes 1 and 2) and native SR (lanes 3 and 4) were loaded on a 8% SDS–PAGE and blotted after migration to Immobilon-P membranes. Membranes were probed with 79B, $Ab_{577-588}$ and $Ab_{877-888}$ antibodies and revealed as described in Section 2. 79B antibody mainly recognizes the region 1–198 while the two others are specific for SERCA1a sequences 577–588 (located in the nucleotide binding domain) and 877–888 (located in the L7-8 loop) as described in [41]. Lane M corresponds to prestained kaleidoscope standards (Bio-Rad). Lanes 1 and 2 correspond to 0.3 μg of 6HC-Ca^{2+}-ATPase purified by Ni-NTA chromatography (pool of fractions 1–5) and Ni-NTA chromatography followed by Reactive red 120 chromatography (pool of fractions 3–7) respectively. Lanes 3 and 4 correspond to 10 and 60 ng of native SR respectively.

3.8.3. Stability and storage of the purified material

Several buffer conditions were tested to keep stable the purified 6HC-Ca^{2+}-ATPase either at 4°C for several days or after liquid N_2 freezing and storage at −80°C and the best conditions we found are detailed in Section 2. It was first necessary to change the buffer of the RR120 pool by another PD-10 step as described in Section 2, with a buffer containing 40% glycerol and then to add 1 mg/ml of the lipid mix. In such a buffer the protein remained markedly stable for at least the tested period of 5 days and was even more active since the specific ATPase activity increased to 7.3 μmol ATP hydrolyzed/min/mg Ca^{2+}-ATPase, maybe because of the slow removal of an inhibitory lipid or detergent. For a longer-term storage at −80°C and preservation of the best activity after thawing, it was necessary to remove the detergent. This was carried out using Bio-beads and following the conditions described in [26]. As displayed

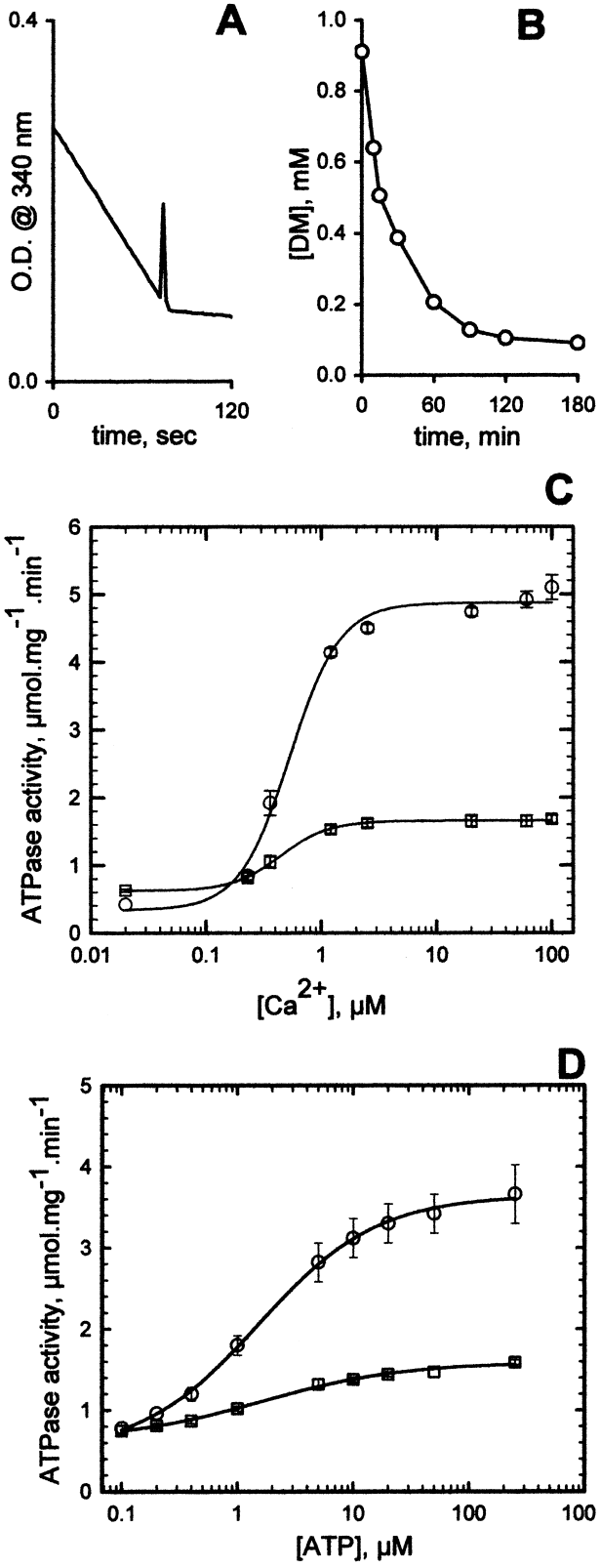

Fig. 6. Reconstitution and kinetic analysis of the purified 6HC-Ca^{2+}-ATPase. (A) Ca^{2+}-dependent ATPase activity after Reactive red 120 purification. ATPase activity was measured as described in Section 2 after addition of 7 μg of 6HC-Ca^{2+}-ATPase from the PD-10(RR120) pool as described in Section 2. The reaction was started by addition of ATP and stopped after addition of EGTA. The Ca^{2+}-ATPase activity was calculated as the difference between the slopes obtained in the presence and in the absence of EGTA. (B) [^{14}C]DM removal with Bio-beads. The PD-10(RR120) pool of purified 6HC Ca^{2+}-ATPase was supplemented with Bio-beads as detailed in Section 2 and incubated at 4°C for 3 h. Aliquots were withdrawn at the indicated times and radioactivity counted. Values are given in mM of residual [^{14}C]DM. (C) Calcium-dependent ATPase activity of $C_{12}E_8$ solubilized native SR and 6HC-Ca^{2+}-ATPase. ATPase activity was measured with 1 mM ATP, at pH 7.0 and 20°C as described in Section 2 after addition of 8 μg of 6HC-Ca^{2+}-ATPase (squares) or 4 μg of SR Ca^{2+}-ATPase (circles), preincubated for 30 min in the same buffer as that in which the purified enzyme is recovered after the Bio-beads step (25 mM MOPS–Tris, pH 7.0, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM DTT, 40% glycerol and 0.1 M KCl, 1 mg/ml EYPC–EYPA (10:1 mix), 0.1 mM DM). Increasing amounts of EGTA were added to the medium to reach the indicated concentrations of free calcium. Each point represents the mean ± S.D. of three experiments. Curves were fitted with the Sigmaplot software. (D) ATP-dependent ATPase activity of $C_{12}E_8$ solubilized native SR and 6HC-Ca^{2+}-ATPase. ATPase activity was measured as in panel C but with 20 μM calcium and increasing amounts of ATP added to the medium to reach the indicated concentrations.

in Fig. 6B, using [^{14}C]DM in such conditions we observed that the DM concentration decreased from 1 mM to 0.09 mM, thus 2 times less than the CMC of 0.18 mM reported for this detergent [37]. This incomplete removal of DM may be due to trapping of fragments of Bio-beads within the proteoliposomes as suggested previously [38] for $C_{12}E_8$ or, for DM [39], to a slow flip-flop rate of this detergent. For further fluorescence measurements and according to the work of de Foresta and colleagues [40], an additional 10 times reduction of the DM concentration will probably be necessary.

3.8.4. Kinetic properties of the purified His-tagged Ca^{2+}-ATPase

The K_m for calcium and ATP at 20°C and pH 7.0 and V_{max} either at 20 or 30°C of the purified enzyme were estimated and compared to that of the native Ca^{2+}-ATPase incubated in the same storage buffer. In these conditions, K_m values for calcium and ATP were estimated from the curves displayed in Fig. 6C,D to be 0.44 μM and 1.52 μM respectively for the purified enzyme. These values are close to those of the native enzyme measured here, 0.53 μM and 1.62 μM, and previously reported [44]. On the contrary, the V_{max} of ATP hydrolysis of the purified enzyme calculated from the values of Fig. 6C or D, 1.00 and 0.96 μmol/min/mg respectively, is lower than that of the native enzyme for which we found 4.54 and 3.10 μmol/min/mg. The V_{max} values were also estimated at pH 7.3 and 30°C and found to be 4.5 and 13.2 μmol/min/mg for the purified and native enzyme. Since we could not detect any degradation of the protein during purification, this difference suggests that some parameters of the purification–reconstitution procedure lead to partial inactivation of the protein, maybe the lipid content during reconstitution and/or the freezing step, since we measured a higher ATPase activity (7.3 μmol/min/mg) when the enzyme was stored at 4°C (see above).

3.9. Concluding remarks

As detailed in Table 1, the global yield of purification remains rather good after the first chromatographic step (21%) giving 0.8 mg of purified Ca^{2+}-ATPase. The fact that this value corresponds to 1/6 of the total amount of prepared LMb suggests that about 5 mg of protein, at least 50% purified, could be obtained with such a procedure. This is enough for functional studies of wild-type and mutated Ca^{2+}-ATPases requiring a large amount of material, such as Ca^{2+}-binding, intrinsic or extrinsic fluorescence measurements. However, if the procedure is stopped after the NTA chromatography, it will be necessary to exchange the NTA buffer of the pool by the buffer used for the PD-10 step which followed the RR120 chromatography, and which confers a better stability and specific activity to the enzyme approaching that of Ca^{2+}-ATPase in native SR.

The second chromatographic step is somewhat costly since we lost 75% of the material. However, it increases the purity and thus the specific activity of the protein. Therefore this step will be preferably used for structural studies of wild-type and mutated Ca^{2+}-ATPases. Although the amount of material purified at this step is rather low, 1.2 mg estimated for a 4 l culture, it is very easy and cheap to scale up the membrane preparation from yeast.

Just before the present work was submitted, Mirras and coworkers [12] published the purification of a His-tagged Ca^{2+}-ATPase expressed in *Sf*9 cells. Strikingly and contrary to the present work they did not succeed in purification of the His-tagged protein by NTA chromatography although we reach about 80% of binding at this step. The main difference between their construction and ours is that we have introduced two glycine residues between the last residue (a glycine) of the Ca^{2+}-ATPase and the histidine tail. The presence of three glycine residues may be critical for exposing the His tag and affect the flexibility and the required length of the His tag, and may therefore greatly improve the efficiency of binding to the NTA group. The authors could, however, use Reactive red chromatography to purify the His-tagged Ca^{2+}-ATPase and reach 26% homogeneity. This shows that a RR120 step alone remains less efficient than NTA chromatography when compared to the present work. By using both chromatographic steps, our procedure thus constitutes a substantial improvement for the purification of expressed His-tagged rabbit Ca^{2+}-ATPases. We are currently using this purification process for the purification of mutated Ca^{2+}-ATPases in order to obtain functional and structural information. Although structural data are difficult to obtain, the recent report of

Jahn et al. [45] is rather encouraging, showing that it is possible to get, at least 2D, crystals from a recombinant (plant) P-type ATPase overexpressed in yeast.

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