The Ca²⁺-ATPase SERCA1a (sarcoplasmic–endoplasmic reticulum Ca²⁺-ATPase isofrom 1a) from rabbit has been overexpressed in *Saccharomyces cerevisiae*. This membrane protein was purified by avidin agarose affinity chromatography after biotinylation, which selects for properly folded protein. Finally, we show, with its discovery, rabbit SR Ca²⁺-ATPase (SERCA1a, sarcoplasmic–endoplasmic reticulum Ca²⁺-ATPase isofrom 1a) has been a favorite subject for membrane studies, because vesicles predominantly consisting of Ca²⁺-ATPase and with intact Ca²⁺-transporting properties can be prepared in large yield from skeletal muscle. Moreover, this protein is a paradigm of P-type ATPases, which constitute the major cation transporter family. P-type ATPases are characterized by the formation of a covalent aspartylphosphorylated intermediate during their catalytic cycle. Crystalization attempts of SERCA1a started a long time ago from detergent-solubilized membranes, which provided valuable initial structural information as to how to proceed with crystallization of this difficult-to-handle membrane protein (2, 3). Recently, several 3D structures corresponding to various conformations of the catalytic cycle have been described, all of them obtained from the native protein of SR (4–10). With the object of studying structural and functional properties of mutants of this transporter, we here outline a procedure for purification of Ca²⁺-ATPase after heterologous expression in yeast (11). Our aim was to increase the quantity and, most importantly, the quality of our purified protein to be used for 3D crystallization. For this, we have chosen to use a recombinant protein: the SERCA1a fused to a biotin acceptor domain (SERCA1a-BAD). This procedure allows us to specifically purify the fusion protein by affinity chromatography after *in vivo* biotinylation, which selects for properly folded protein. Finally, we show, with wild-type SERCA1a as an example, that the purified protein can be used for crystallization and functional studies, including transport, and that its structure is isomorphous to the native enzyme from rabbit.

**Methods**

**Protein Purification.** Expression in yeast was performed as described in ref. 11. Starting from a 4-liter culture, 75 ml of a light membrane fraction (protein concentration ~15 mg/ml) were solubilized with dodecyl maltoside (DDM) (detergent/protein ratio of 3:1) in solubilization buffer (0.05 M Tris, pH 7.0/1 M NaCl/20% glycerol/1 mM MgCl₂/1 mM 2-mercaptoethanol/1 mM PMSF) at room temperature for 2 h. After centrifugation at 120,000 × *g* for 10°C for 30 min, the supernatant from the solubilization step was mixed with ~9 ml of avidin resin (Softlink Soft Release Avidin Resin from Promega) and gently stirred at 4°C overnight. The suspension was then loaded onto a column. The resin was initially washed with 12 resin volumes of high-salt buffer [0.05 M Tris, pH 7/1 M NaCl/20% (wt/vol) glycerol/1 mM MgCl₂/1 mM 2-mercaptoethanol/0.05% (wt/vol) DDM] and then with 12 resin volumes of low-salt buffer (0.05 M Tris, pH 7/0.15 M NaCl/20% glycerol/2.5 mM MgCl₂/1 mM 2-mercaptoethanol/0.05% DDM) at a flow rate of 1 ml/min. The encoded protein contains a thrombin cleavage site (LEVPNRS motif) separating the biotinylation domain from the C terminus of SERCA1a. Thus, the resin was resuspended with ~6.4 ml of low-salt buffer, and 350 units of thrombin (Sigma-Aldrich) was added. The mixture was gently stirred at room temperature for 30 min, and another 250 units of thrombin was added to the suspension. After an additional 30 min, the thrombin cleavage was stopped by adding 2.5 mM PMSF, and the resin, reformed into a column, was placed in the cold room. The protein was eluted from the column by adding low-salt buffer, 1 ml at the time. The SERCA1a-containing fractions were identified by ATPase activity assays or Western blot and appeared after elution in ~25 ml. SERCA1a fractions were pooled and concentrated by Centricon-30 (Amicon) to <500 μl. The concentrate was applied at 0.5 ml/min to a gel-filtration column (a 30-cm TosoHaas TSK-gel G3000SWXL column), using a System Gold from Beckman in the following buffer: 100 mM 3-(N-morpholino)propanesulfonic acid (Mops), pH 6.8/80 mM KCl/1 mM MgCl₂/1 mM CaCl₂/20% (wt/wt) glycerol/0.5 mg/ml C₁₂Es (dodecyl octaethylene glycol monoether). The fractions containing SERCA1a were concentrated by Centricon-30 until reaching a volume of ~50 μl.

To measure the removal of DDM, the protocol was the same, except that we added 0.2 μCi (1 Ci = 37 GBq) ¹⁴C-labeled DDM [synthesized by the Centre d’Études de Saclay, France (12)] to the injected sample. Twenty-five microliters of each of the collected fractions was counted for detergent radioactivity.

**Protein Crystallization, Data Collection, and Refinement.** Before crystallization experiments, the concentrated SERCA1a sample (~50 μl) was carefully loaded into the membrane reservoir of a membrane protein crystallization screen (13). The reservoir was prepared with 3% MOPs (pH 7.0), 30 mM MgCl₂, and 5 mM CaCl₂ in a 1:1 ratio. The reservoir solution was adjusted to pH 7.0 with MOPS buffer (30% glycerol, 200 mM MgCl₂, and 200 mM CaCl₂) and placed in a cold room for 3 days. After this period, a few crystals appeared (13). The crystals were then directly transferred to a cryo-protectant solution containing 200 mM MOPS buffer, 200 mM MgCl₂, 200 mM CaCl₂, and 20% glycerol.

**Note**

1. to whom correspondence should be addressed. E-mail: jaxel@dsvdif.cea.fr.
μl at 12 mg/ml) was supplemented with 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Avanti (~0.4 mg), 10 mM Ca²⁺, and 1 mM AMPPCP (β,γ-methyleneadenosine 5’-triphosphate) and then subjected to ultracentrifugation (180,000 × g for 10 min in a Beckman TLSA10 rotor) to remove aggregated material. Crystallization experiments were performed in 2 + 2-μl hanging drops equilibrating against 450 μl of well solution [200 mM sodium acetate/10–14% (v/v) polyethylene glycol 4000/10% glycerol/14% tert-butanol]. Crystals formed after 1–3 days at 19°C and grew over 2 weeks to 100 μm × 100 μm × 50 μm. The crystals were cryoprotected by increasing the glycerol concentration to 20% in the well solution and moving the crystallization tray to 4°C for overnight equilibration by vapor diffusion. Crystals were mounted in nylon loops (Hampton Research, Riverside, CA) from the mother liquor and were flash-frozen and stored in liquid nitrogen. Data were collected at 100 K at the BESSY synchrotron (Berlin), beamline BL14.1. The crystals diffracted anisotropically beyond 3.1 Å, which is comparable (at this beamline) to crystals of similar size of the native protein. Crystallographic data were processed and scaled by using the HKL package (13). The data were isomorphous with data from crystals of native protein from rabbit fast-twitch muscle SR membranes. An unbiased difference Fourier map using \( F_{obs} \) (native) – \( F_{obs} \) (recombinant) coefficients based on experimental phases (7) was calculated to reveal any structural differences between the native and the yeast-expressed protein. The structure was represented by a model obtained by rigid-body and group B-factor refinement of PDB entry 1T5S (7) (with bound AMPPCP and metal atoms omitted) using CNS (14). The refinement yielded a free R-factor of 31.4% (40–3.3 Å, all data). A \( \sigma_A \)-weighted \( F_{obs} \) – \( F_{calc} \) omit map was derived showing unambiguous electron density for the omitted ligands (Mg:AMPPCP, two Ca²⁺ ions, and one K⁺). Maps and structures were displayed and analyzed by using the program o (15).

**Protein Purity Analysis.** For SDS/PAGE, aliquots were diluted in the corresponding purification buffer and mixed with an equal volume of denaturing buffer (100 mM Tris-Cl, pH 8/1.4 M 2-mercaptoethanol/4% SDS/5 mM EDTA/8 M urea/0.05%/bromophenol blue). Samples were boiled for 90 sec, cooled, and loaded on a Laemmli-type 8% polyacrylamide gel as described in ref. 16. Proteins were stained by Coomassie brilliant blue.

**Protein Reconstitution in Proteoliposomes.** For reconstitution, 20 mg of lipid (DOPC) was dissolved into 1 ml of cholate buffer [30 mM Tris, pH 7/400 mM NaCl/400 mM sucrose/1 mM MgCl₂/1 mM Na₂SO₄/1% (v/v) sodium cholate/50 mM DTT] and stirred until clear. Before reconstitution, HPLC fractions containing yeast-expressed SERCA1a were concentrated until reaching a concentration of >1 mg/ml, whereas an SR membrane preparation (17) (kindly provided by P. Champeil, Commissariat à l’Énergie Atomique de Saclay) was solubilized with C₁₂E₈ (detergent/protein ratio, 2:1) in HPLC buffer and centrifuged at 180,000 × g for 35 min. In both cases, at least 100 μg of protein was added to 5 ml of the clear lipid mixture. In this procedure, the lipid-to-protein ratio was ~700:1 and thus much higher than previously described (18). Proteoliposomes were formed by dialyzing the mixture at 10°C against a phosphate buffer (0.1 M NaH₂PO₄, pH 7.0/1 mM MgCl₂/5 mM DTT/1 mM Na₂SO₄) containing Bio-Beads resin (Bio-Rad). This resin absorbs detergents, and thus cholate and C₁₂E₈. The dialysis buffer was exchanged four times over a period of 30 h; the last two changes did not contain DTT. After a moderate withdrawal of water by treatment of the cellophane bags with PEG 20,000, the reconstituted vesicles were stored at 4°C. For specific activity calculation, we assumed that all of the protein used was reconstituted into the proteoliposomes.

**Calcium Transport.** For Ca²⁺-uptake measurement, vesicles were diluted in reaction buffer (0.03 M imidazole, pH 7/1 mM MgCl₂/1 mM NaCl/150 mM NaCl) with 0.33 mM ⁵²Ca²⁺ and 0.5 mM EGTA. The reaction was initiated by addition of 5 mM MgATP, and the separation of proteoliposomes from the reaction medium was carried out by centrifugation through a gel column (Sephadex G-50, Amersham Biosciences), according to the method of Penefsky (19). Centrifuged effluents were collected in counting vials, and the radioactivity was determined by scintillation counting.

**Results**

**Overexpression of SERCA1a-BAD in S. cerevisiae.** For heterologous expression, we have chosen the yeast system, which is an attractive tool for producing mammalian proteins (for a review, see ref. 20). To perform a fast and efficient purification, we have added to the SERCA1a gene a DNA fragment coding for the Klebsiella pneumoniae C-terminal oxaloacetate decarboxylase BAD (21) (kindly provided by R. Kaback and D. Hardy, University of California, Los Angeles). Inserted after a thrombin cleavage site at the C-terminal part of the protein, this tag is chosen for the very-high-affinity binding of its biotinylated end to avidin, allowing efficient purification of the protein. The BAD (from oxaloacetate decarboxylase) is naturally biotinylated in yeast (22). Thus, the SERCA1a-BAD gene was inserted into a shuttle vector (23) with a galactose inducible promoter, allowing expression of the fusion protein at the end of yeast growth (11). Under these conditions, SERCA1a-BAD represents ~0.6% of the total membrane protein, and, from a 4-liter yeast culture, 75 ml of a membrane “light” fraction is obtained after differential centrifugation at a protein concentration of 15 mg/ml. At this step, SERCA1a-BAD represents 1% of the total protein (corresponding to 100 pmol/mg of membrane protein).

**Purification of SERCA1a.** After solubilization of the light membrane fraction with DDM (see protein pattern in Fig. 2a, lane 1) the solution was applied to a monomeric avidin resin. The SERCA1a protein without the BAD was then eluted from the column by the use of thrombin protease. The advantage of thrombin cleavage is first to remove the BAD, and secondly to increase considerably the yield of protein elution relatively to the biotin elution previously used (21, 24, 25). This procedure also prevents the elution of host biotinylated proteins. To increase protein purity, the eluate was concentrated on Centricon-30, applied onto a HPLC gel-filtration column, equilibrated, and eluted with buffer containing 0.5 mg/ml C₁₂E₈, thus exchanging DDM. This chromatography allowed us to separate SERCA1a from a small amount of aggregated protein, the thrombin protease, and other contaminants (see Fig. 1). To follow the removal of DDM, we added ¹⁴C-labeled DDM to the injected sample. The absence of radioactivity in fractions containing SERCA1a (see Fig. 1) demonstrates that all of the bound DDM was replaced at this step by C₁₂E₈.

The SERCA1a-containing fractions were selected (see Fig. 1) and pooled. The protein pattern of this sample is analyzed and presented in Fig. 2a (lane 2). On this Coomassie blue-stained SDS/PAGE, protein purity is estimated to be nearly equivalent to that of protein prepared from rabbit SR (Fig. 2a, lane 3). We have measured the Ca²⁺-dependent ATPase activity of our purified protein and obtained a value of ~3 nmol/min/mg at 25°C, consistent with the usual activity obtained for the native ATPase prepared from SR. We have also reconstituted the recombinant protein in DOPC using a standard method (18), allowing for calcium transport assays. As shown in Fig. 2b, the vesicles are able to transport Ca²⁺ at approximately the same rate as native SR Ca²⁺-ATPase, reconstituted by the same method. Yields of SERCA1a per 4 liters of yeast culture were ~1.2 mg of delipidated protein after affinity chromatography.
Perform crystallization attempts.

C12E8 detergent present in the sample at a final weight ratio of concentrated sample. For this, DOPC was solubilized by the for crystallization trials by relipidation of the purified and Jidenko

Fig. 2. Properties of yeast-expressed rabbit SERCA1a. (a) Coomassie blue-stained SDS/PAGE. Lane 1, DDM-solubilized yeast light membrane fraction before purification; lane 2, pool of HPLC-purified SERCA1a; lane 3, 0.5 μg of SR Ca2+ -ATPase; lane M, standard proteins, with the indicated molecular masses. (b) Ca2+ -uptake by SERCA1a proteoliposomes. Filled triangles, proteoliposomes containing SERCA1a solubilized from rabbit SR; open squares, proteoliposomes containing purified yeast-expressed SERCA1a.

and 0.8 mg after gel filtration. Selected HPLC fractions containing SERCA1a were pooled and concentrated by Centricon-30 until reaching a final volume of about 50 ml and a final protein concentration of ~15 mg/ml was obtained, allowing us to perform crystallization attempts.

Crystallography of SERCA1a. We prepared stable SERCA1a samples for crystallization trials by elipsoid of the purified and concentrated sample. For this, DOPC was solubilized by the C12E8 detergent present in the sample at a final weight ratio of ~1:3 (DOPC/C12E8) followed by addition of 10 mM Ca2+ and 1 mM AMPPCP. The sample was eventually diluted to reach a protein concentration of 12 mg/ml. A general crystallization screen did not yield any crystals, but a specific screen focusing protein concentration of 12 mg/ml. A general crystallization screen did not yield any crystals, but a specific screen focusing

Table 1. Crystallographic data

<table>
<thead>
<tr>
<th>Rabbit SERCA1a expressed in S. cerevisiae, CαE1-AMPPCP form</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group C2</td>
<td></td>
</tr>
<tr>
<td>Unit cell a = 163.1 Å, b = 75.97 Å, c = 151.1 Å, β = 108.9°</td>
<td></td>
</tr>
<tr>
<td>Wavelength, Å 0.9754</td>
<td></td>
</tr>
<tr>
<td>Resolution, Å 40–3.3 (3.4–3.3)</td>
<td></td>
</tr>
<tr>
<td>I/σI 17.1 (3.2)</td>
<td></td>
</tr>
<tr>
<td>Completeness, % 90.0 (85.8)</td>
<td></td>
</tr>
<tr>
<td>Redundancy 2.7 (2.7)</td>
<td></td>
</tr>
<tr>
<td>Rsym, % 5.8 (30.2)</td>
<td></td>
</tr>
<tr>
<td>Re to native, % 11.4 (23.6)</td>
<td></td>
</tr>
<tr>
<td>Model Starting model PDB entry 1T5S, AMPPCP and bound cations omitted</td>
<td></td>
</tr>
<tr>
<td>Refinement Rigid body refinement of domains Grouped B-factor refinement from single, overall B-factor</td>
<td></td>
</tr>
<tr>
<td>R/Re, % 0.304/0.318 (0.341/0.351)</td>
<td></td>
</tr>
</tbody>
</table>

Days, and batch-dependent variations in the crystallization properties were larger compared with the native enzyme. A lower success rate in forming large, single crystals was observed compared with the native enzyme (10–15% of crystallization drops, versus ~33% for native protein), and crystals appeared within a narrow range of PEG 6000 concentration compared with the native protein (5% wt/vol window versus 6% wt/vol, respectively). This narrow range is probably the reason that no crystals were observed in the general crystallization screen. We believe that the differences are mainly due to variations in the protein/lipid/detergent ratios of the purified, relipidated, and concentrated protein. The C12E8 concentrations were routinely estimated from the increase in protein concentration, resulting from the Centricon filtration, on the assumption that micellar C12E8, like the protein, would not pass the filter. Although the validity of this assumption could be confirmed with radiolabeled detergent, improved control of the protein/lipid/detergent parameters might be a key to reproducing in full the crystallization properties of the native SERCA1a protein from rabbit. It is well known that the ratio detergent/protein is crucial for membrane protein crystallization (26, 27).

A crystal of recombinant SERCA1a was cryo-protected and flash-frozen at 100 K as described in ref. 7. Diffraction extended anisotropically beyond a 3.1-Å resolution, and a full data set was collected at 3.3-Å resolution (Table 1). The crystal form is similar to that of the native enzyme as indicated by the space group and unit cell dimensions. Scaling to a data set of the native enzyme exhibits a merging R-value of 11.4% (30- to 3.3-Å resolution), indicative of a high level of isomorphism. Indeed, the unbiased Fobs(native) – Fobs(recombinant) difference Fourier map shows no significant features (Fig. 3). Furthermore, an Fobs – Fcalc omit map shows that the functional ligands such as Mg:AMPPCP and Ca2+ are present at their respective binding sites as previously identified from studies of the native protein (5, 7, 8) (Fig. 3). Thus, even at a detailed level, there are no structural differences, either in the cytoplasmic or the transmembrane regions, and the structure of the yeast-expressed protein represents in full the native protein.

Discussion

We have used the previously described heterologous system S. cerevisiae to overexpress the membrane protein SERCA1a (11). The yeast offers more advantages than Escherichia coli, because exogenous proteins may be targeted to membrane-bound compartments, based on the conservation of a basic cellular machinery and signal transducing pathways similar to that of higher eukaryotes. Yeast membranes have a lipid composition (see, e.g.,
Many different tags, including polyhistidine, have been used for purification (36, 37). Previously, the purification of in vivo-biotinylated membrane proteins has been successfully applied in a few cases, like a plant sucrose carrier from yeast (24), the lactose permease from E. coli (21), and the human P-glycoprotein expressed in yeast (25). The comparison between polyhistidine tag and BAD is in favor of the biotin, in particular for the protein activity (unpublished work). In our case, even if the BAD domain is relatively large (≈10 kDa), a potential advantage is that, in vivo, the biotinylation reaction only takes place with properly folded fusion protein, as previously described in the case of GFP-fused membrane proteins expressed in E. coli (38). Thus, it is likely that inactive Ca^{2+}-ATPases proteins, improperly folded, could be eliminated at this step. In the case of lactose permease, Pouny et al. (21) have shown that an in vitro biotinylation by BirA biotin ligase increases significantly the amount of protein obtained by in vivo biotinylation. In our hands, we have obtained enough in vivo-biotinylated material to perform the affinity chromatography on an avidin monomeric column. Moreover, we have introduced a thrombin cleavage and elution, giving rise to a relatively purified protein, instead of using the classical method of elution with biotin (21, 24, 25). The following HPLC gel-filtration step worked well because the size of Ca^{2+}-ATPase is very different from the contaminants (see Fig. 1). Both in terms of purity and enzymatic activity, the purified, overexpressed protein is nearly equivalent to that of ATPase prepared from SR. This is a great advantage relative to some other expression-purification methods for this protein (11, 39).

Finally, as a most prominent point in terms of protein purity and integrity, we were able to obtain well diffracting crystals of this overexpressed membrane protein, leading to a structure identical to that of the native protein (7). To our knowledge, this result has not been obtained before with a mammalian overexpressed membrane protein. However, we also observe that the overexpressed protein crystallizes within a narrow window of precipitant concentrations compared with the native protein, and crystallization screens should therefore encompass a finer sampling of this parameter.

To summarize, it has been possible after repurification with DOPC to recover the functional and structural properties characteristic of SR Ca^{2+}-ATPase based on heterologously expressed and chromatographically purified SERCA1a. This result provides us with a powerful tool to study, in detail, the structure of SERCA1a mutants with interesting functional properties (see, e.g., ref. 40 for a review and ref. 41 for one recent example). It also emphasizes the applicability of a heterologous expression system in the crystallization of mammalian membrane proteins.

Concluding Remarks

The amount of purified protein obtained here is within the range of what was obtained in yeast or other expression systems for mammalian membrane proteins (for reviews, see refs. 36 and 37 and references cited therein): Starting from 1 liter of culture, we routinely obtain 2.8 mg of SERCA1a protein after its expression and differential centrifugation, 1 mg after its solubilization, and 0.3 mg after avidin chromatography, and 0.2 mg of purified protein after the gel-filtration HPLC step. In some cases, higher yields than ours have been obtained (36, 37). Although low expression levels would certainly impair the efficiency of the purification procedure, one should be aware that very high production levels are likely to be problematic for correct membrane insertion and folding. It would seem that the most important property of an overexpression system would be the ability to provide active protein rather than high yields as a goal by itself. The polyhistidine tag is very popular for affinity chromatography. Our choice of using as a tag a large cleavable biotin domain, together with the elution by a protease, allows, after selective in vivo biotinylation, both efficient binding to the avidin resin and specific elution. This procedure constitutes an alternative to polyhistidine and other affinity purification strategies (36). It is a procedure that could be seriously considered for future works, in particular when the expression levels are moderate. New crystallization technologies are less protein-consuming and this will make the final yield a less important issue. In our experience, for 3D crystallization, the high level of specific activity and purity of the enzyme have been the most crucial factors.
We thank Claus Olesen and Anne-Marie Lund Jensen for help with data collection and data processing and Bitten Holm for expert technical assistance. This work was supported by the Danish Medical Research Foundation, the Novo-Nordic Foundation (Denmark), and the Commissariat à l’Energie Atomique and Centre National de la Recherche Scientifique (France). R.C.N. is supported by a stipend from the Lundbeck Foundation.