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Research paper

Conservation of the oligomeric state of native VDAC1 in detergent micelles

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ABSTRACT

The voltage-dependent anion-selective channel (VDAC) is an intrinsic β-barrel membrane protein located within the mitochondrial outer membrane where it serves as a pore, connecting the mitochondria to the cytosol. The high-resolution structures of both the human and murine VDACs have been resolved by X-ray diffraction and nuclear magnetic resonance spectroscopy (NMR) in 2008. However, the structural data are not completely in line with the findings that were obtained after decades of research on biochemical and functional analysis of VDAC. This discrepancy may be related to the fact that structural biology studies of membrane proteins reveal specific static conformations that may not necessarily represent the physiological state. For example, overexpression of membrane proteins in bacterial inclusion bodies or simply the extraction from the native lipid environment using harsh purification methods (*i.e.* chaotropic agents) can disturb the physiological conformations and the supramolecular assemblies. To address these potential issues, we have developed a method, allowing rapid one step purification of endogenous VDAC expressed in the native mitochondrial membrane without overexpression of recombinant protein or usage of harsh chaotropic extraction procedures. Using the *Saccharomyces cerevisiae* isoform 1 of VDAC as a model, this method yields efficient purification, preserving VDAC in a more physiological, native state following extraction from mitochondria. Single particle analysis using transmission electron microscopy (TEM) demonstrated conservation of oligomeric assembly after purification. Maintenance of the native state was evaluated using functional assessment that involves an ATP-binding assay by micro-scale thermophoresis (MST). Using this approach, we were able to determine for the first time the apparent K_D for ATP of 1.2 mM.

1. Introduction

The voltage-dependent anion channel (VDAC) is an outer mitochondrial membrane (OMM) protein present in plant, fungi and mammals. It is relatively small (~30 kDa) and abundantly expressed. Initially described as a molecular filter similar to bacterial porins, VDAC is now recognized as being a fairly selective channel. VDAC serves as the communication link between the mitochondrial matrix and the cytosolic environment by controlling the constituents of the mitochondrial inter-membrane space. While VDAC can be permeable to molecules under 5 kDa, which includes most cellular ions and vital metabolites (succinate, citrate, NADH, ATP, etc.), there is strong evidence of direct substrate binding and selective transport. Of particular relevance are the nucleotide binding sites (NBS) and the ability to transport ATP generated by the mitochondria. Thus, VDAC, in tandem with the selective inner mitochondrial ADP/ATP carrier, can serve as a regulated supply gate for energy delivery throughout the cell. The direct binding and transport of ATP to VDAC was first demonstrated through binding studies on ATP agarose columns and later confirmed using radiolabeled and photo-activated cross-linking studies using nucleotide analogues [1]. The purification method

Abbreviations: Anapoe-C_8E_4, Tetra ethylene glycol monooctyl ether; Anapoe-$C_{10}E_6$, Hexa ethylen glycol monodecyl ether; HECAMEG®, 6-*O*-(*N*-heptylcarbamoyl)-methyl-α-D-glucopyranoside; HRP, horseradish peroxidase; HA, Hydroxyapatite; BSA, bovine serum albumin; ScVDAC1 or Por1p, isoform 1 of the yeast mitochondrial voltage-dependent anion-selective channel; TEM, transmission electron microscopy.

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used refers to the review article of Varda Shoshan-Barmatz and Dan Gincel [2] where hydroxyapatite (HA) chromatography was employed to extract the ADP/ATP carrier and VDAC from isolated mitochondria. In order to remove the large portion of the ADP/ATP carrier present in the eluted fraction, the HA was combined with Celite chromatography. A second step of purification was then performed using a reactive red-agarose (RRA) column to concentrate the protein content and to exchange the detergent.

VDAC activity is mediated by interactions with various proteins such as hexokinase, ADP/ATP carrier, mitochondrial creatine kinase (MtCK), pro-apoptotic proteins belonging to the BCL2 family, tubulin and also the IP_3 receptor [3]. It plays an important role in the metabolism and apoptosis of eukaryotic cells and it was recently proposed to be a therapeutic target for the treatment of Alzheimer's disease [4], diabetes [5] and, most promisingly, cancer [6].

Following the discovery of mitochondrial VDAC nearly forty years ago by Mannella and Bronner [7], the structure and membrane topology has been the subject of active debate and controversy in spite of the 2008 publications of three independent high-resolution 3D structures of mammalian homologues [8–10]. The structures revealed a β-barrel pore containing 19 anti-parallel β-strands. The reported structural findings sharply contrast the information gathered after decades of intense biochemical analysis, probing the functional organization of the protein. Interestingly, the flexible *N*-terminal domain of about 20 amino acids is located inside the pore in all three published structures, while previous topological analysis demonstrated 13 β-strands with the *N*-terminal domain residing within the bilayer [11].

In these three above mentioned structural studies, human or mouse VDAC were overexpressed in *E. coli*. Solubilization from inclusion bodies and subsequent refolding were mandatory steps prior to purification, leading to potential complications and unnatural conformations. The difficulty in producing a physiologically relevant VDAC in an exogenous environment has been demonstrated using the purified protein in functional assays. According to Colombini, only a fraction of the purified VDAC in Triton X-100 micelles was functional after reconstitution in cholesterol loaded artificial liposomes. In this particular condition, following their purification procedure, the presence of sterol lipids seems to be essential to recover functional protein. Such a protein-detergent-lipid complex was not suitable for crystallization by vapor diffusion methods employed for VDAC during the determination of the three published structures. Thus, Colombini argued that the protein used for crystallization was not in a functional configuration. This highlights the important role of lipid molecules, particularly cholesterol and analogues, in the structure determination of VDAC and their need to keep the protein within its native environment. Moreover, the topology of the high-resolution structures has not yet been fully correlated with previous surface accessibly data such as protease treatment, peptide-specific antibodies and FLAG labeling [12–14]. Also, the overwhelming incongruity between functional biochemical and structural data has led many, including Colombini, to propose that the 3D structures correspond to a non-functional states of VDAC [11].

In response to Colombini's critique [11], the authors of the 3D structural work of VDAC argued as follows: Prior to structure determination, different models were proposed for VDAC on the basis of biochemical, biophysical and computational approaches, with drastic variations in overall topology, containing 12 to 16 β-strands [15]. On the other hand, the recently resolved mammalian structures consistently revealed 19 β-strands in three independent investigations, using two distinct structural technologies, X-ray crystallography and nuclear magnetic resonance spectroscopy. Also, these authors note, that all three structures showed residue E73 to be oriented toward the hydrophobic membrane environment, as originally suggested by De Pinto et al. by who determined the specific interaction of this residue with the highly hydrophobic compound dicyclohexylcarbodiimide (DCCD) [16]. The structural investigations furthermore point out that the refolding technique has been validated extensively and that, from 60,000 structures deposited in RCSB Protein Data Bank, at least 1000 were structurally solved following proper refolding. To this end, it should be noted, however, that only about 2.5% of these are integral membrane proteins which potentially weakens this argument. Then, looking at previous TEM and AFM images, these authors furthermore indicate that the dimension of the pore of the new 3D-structures of 3.4–3.8 nm is similar to that revealed by those images. However, numerous studies revealed smaller pore-sizes of 1.3–2.5 nm. Thus, in spite of all these arguments in favor of the 3D structures, there are still important questions to be addressed with regard to the size of the pore and the real number of β-strands, the localization of the *N*-terminus and, as highlighted below, the native conformation of VDAC used for structural determination.

Beyond the structure of the individual subunits, the VDAC supramolecular assembly has been demonstrated by atomic force microscopy (AFM) from freshly isolated OMMs. The results revealed the existence of monomers to tetramers, hexamers and higher oligomers, based on experiments without chemical modification or detergent solubilization [17]. Previous studies focused on the structural organization of VDAC based on observations from isolated VDAC protein from different sources after cross-linking [18,19]. Interestingly, the group of Mannella showed, based on low-dose electron microscopy images analysis from 2D-crystal of VDAC, a unit cell composed of six channels [20]. Moreover, the oligomerization of VDAC into the unit cell seems to be regulated. Indeed, VDAC oligomerization is involved in the release of pro-apototic factors such as cytochrome *c* from mitochondria, inducing subsequent apoptosis [21].

Given the above statements, our aim was to develop an efficient protocol to isolate non-recombinant VDAC in a conserved native form for subsequent physiological and structural evaluation.

In general, membrane protein (MP) purification represents a major challenge in protein biochemistry. MPs usually exist at low concentrations in biological membranes and hence they need to be overexpressed in a heterologous system. VDAC, on the contrary, is naturally highly expressed in the OMM and it can easily be isolated from yeast mitochondria. Starting with isolated mitochondria is an advantage in order to maintain VDAC in its native lipid environment as much as possible prior to purification. Therefore, we developed a VDAC purification procedure, allowing us to obtain pure and functional non-recombinant VDAC1 directly from isolated yeast mitochondria. This avoids the risky and cumbersome step of protein re-naturation after solubilization from inclusion bodies that is necessary when human or mouse VDAC is over-expressed in *E. coli*.

Saccharomyces cerevisiae VDAC1 (ScVDAC1) corresponds to isoform 1 of the two existing isoforms in yeast. It shares 58% similarity with the human VDAC and 71% with the mouse homologue [13], suggesting that the results obtained with the yeast protein will be highly correlative to the human orthologue. In our study, we successfully purified, using a one-step protocol, ScVDAC1 in non-denaturing conditions, using HECAMEG® as detergent. Then we determined the oligomeric structure of solubilized proteins reflecting its native functional state, by negative staining transmission electron microscopy. HECAMEG® is non-ionic, glucose based very mild detergent for membrane protein purification known to be suitable for structural biology approaches. Following this simple and fast method, we present structural data at low resolution of isolated ScVDAC1 and discuss the supra-molecular

organization.

To assess the integrity and functional state of the purified ScVDAC1 protein, micro-scale thermophoresis (MST) of solubilized ScVDAC1 was performed measuring binding to ATP.

2. Material and methods

2.1. Chemicals and immunochemical reagents

Hydroxyapatite (HA) chromatography was purchased from Bio-Rad (Biogel #130-0420, Hercules, CA, USA). The detergents octyl tetraethylene glycol ether (Anapoe-C_8E_4), polyoxyethylene (6) decyl ether (Anapoe-$C_{10}E_6$), 3-Laurylamido-*N*,*N*′-dimethylpropyl amine oxide (LAPAO), α-[4-(1,1,3,3-tetramethylbutyl)phenyl]-ω-hydroxy-poly(oxy-1,2-ethanediyl) (Triton X-100) were purchased from Affymetrix® (St. Clara, CA, USA) and 6-*O*-(*N*-heptylcarbamoyl)-methyl-β-D-*gluco*-pyranoside (HECAMEG®) from VEGATEC SARL (Rungis, France). Bovine serum albumin (BSA) was from Sigma-Aldrich® (St. Louis, MO, USA). Rabbit polyclonal antibodies against yeast SDS-treated ScVDAC1 were provided by Guy Lauquin (IBGC, Bordeaux, France).

2.2. Bacterial and yeast strains, media

Strains were cultivated and transformed as previously described [22]. A full description of the compositions of all media used, including YPL (lactate-containing rich medium), can be found in the same article. The *E. coli* strain used for plasmid propagation was *DH5α* (Invitrogen) (*F- φ80dlacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17(rk-,mk+) phoA supE44 λ-thi-1 gyrA96 relA1*). The yeast strain used in this study was *W303* (MATa{leu2-3112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15}), provided by Patrice Catty (CEA, Grenoble, France).

2.3. Large scale growth

Large-scale growth of *W303* was performed using a Scientific FS 305 fermenter from New Brunswick (the cylindrical glass reactor having a 12-l culture volume capacity). The dissolved oxygen level in the YPL medium was regulated by sterile air pressure. The fermenter was inoculated by diluting a saturated culture (1:60) in growth medium. It was subsequently agitated at 300 rpm in a water bath at 30 °C. Antifoam agent was previously added to avoid emulsion.

2.4. Isolation of mitochondria

The protocol for mitochondria isolation was adapted from Daum et al. (1982) [23] and has been previously described [24]. The crude mitochondrial pellet was carefully re-suspended in homogenization buffer (10 mM Tris-HCl pH7.4, 0.6 M mannitol, 0.1% bovine serum albumin and 1 mM PMSF) and centrifuged for 5 min at 1,000*g* to remove any residual cell debris. The supernatant was centrifuged at 8,000*g* for 10 min. Mitochondria were stored frozen in liquid nitrogen.

2.5. SDS-PAGE and western blotting

The protein concentrations were determined using a BCA assay kit (Sigma-Aldrich®, St. Louis, MO, USA) using bovine serum albumin (BSA) as a standard. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 15% polyacrylamide running gel [25], and resolved protein was visualized by staining with Coomassie Brilliant Blue R250 or by silver staining [26]. Antibodies directed against SDS-treated-ScVDAC (α-ScVDAC1) were used at a 1/1000 final dilution. Immunodetection was performed using horseradish peroxidase (HRP)-coupled protein A and the enhanced chemiluminescence (ECL) system (Amersham Biosciences®, GE, Healthcare Europe, Glattbrugg, Switzerland).

2.6. UV-VIS absorbance spectra

The presence of ergosterol was assessed by UV-VIS absorption spectra analysis from each collected fraction of purified ScVDAC1 (Shimadzu® 1605 Spectrophotometer; 200–350 nm scan range). For data acquisition, experiments utilized a microcomputer and Suprazil® quartz cell from Hellma® (Plainview, NY, USA).

2.7. Negative staining transmission electron microscopy (TEM)

After purification, HECAMEG® solubilized ScVDAC1 was adsorbed for 10 s to parlodion carbon-coated copper grids (400 mesh) rendered hydrophilic by glow discharge for approximately 10 s at low-pressure air. Grids were washed with three drops of double-distilled water and negatively stained with two drops of 2% (w/v) uranyl formate. Electron micrographs were recorded at a magnification of 110,000× on a Morada CCD camera from OLYMPUS. The Philips CM-12 electron microscope operated at 80-kV acceleration voltage.

Micrograph of reconstituted protein was taken in same conditions than described up but under Philips CM-10 equipped with a LaB6 filament at a magnification of 94,000× at CiNA (Basel, Switzerland).

2.8. Lipids extraction and thin layer chromatography (TLC)

The lipid extraction protocol presented in this paper is adapted from Bligh and Dyer [27]. First, 200 µl of ScVDAC purified at 0.35 mg/ml is mixed with 750 µl of 2:1 (v:v) $CHCl_3$ and finally 250 µl of water is added. The sample is centrifuged at 1,000*g* during 15 min at RT. The upper band of the biphasic phase corresponds to the soluble hydrophilic part containing than interface where protein are precipitate and bottom part hydrophobic with lipids.

Lipids were transferred to a glass tube and solvent was evaporated under constant flow N_2 gas. Dry lipids were solubilized into 1:9 (v:v) methanol:dichloromethane.

In parallel, silica layer (#1.05724.0001 Silica gel 60, Merck, Whitehouse station, USA) was dried into Pasteur sterilization incubator for 45 min at 160 °C. 50 nmol of cholesterol (CHO) (#C8667, Sigma-Aldrich®, St. Louis, MO, USA) was loaded on line 1 and ScVDAC lipid extract on line 2. Samples were ran in a saturated chamber containing 1:9 (v:v) methanol:dichloromethane. Iodine (I_2) staining and UV light were used to visualize the presence of lipid bands.

2.9. Micro-dialysis

Reconstituted protein was obtained by dialysis using a home-built dialysis button (40 µl chamber volume) associated with a 14 kDa MWCO membrane. Membrane protein solution (1 mg/ml in 0.2% HECAMEG®) and detergent-solubilized lipids (DMPC) at (10 mg/ml in 2% HECAMEG®) were mixed at lipid-protein ratios of 0.1 (w/w) and incubated on ice for 1 h. The ternary mixture was dialyzed against a continuously exchanged 1 L buffer containing 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 15 mM $MgCl_2$, 2 mM DTT and 2 mM NaN_3. A specific temperature profile was applied during the four days of dialysis (two days at 34 °C). The resulting vesicles were evaluated by negative stain transmission electron microscopy.

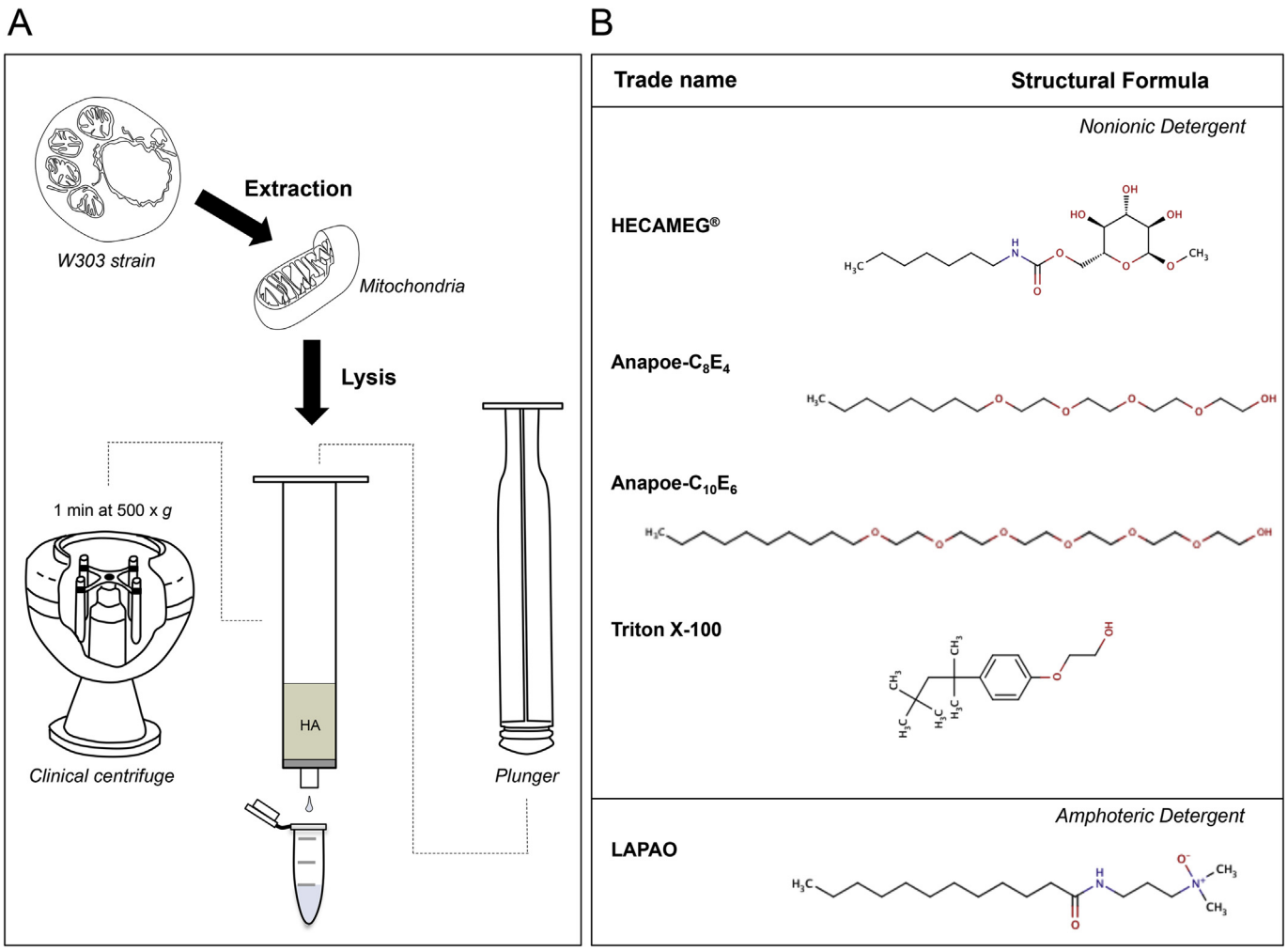

Fig. 1. **(A)** Workflow of ScVDAC1 purification. Mitochondria were isolated from *W303* strain and solubilized in detergent. The lysate was loaded on a hydroxyapatite (HA) column. The protein was eluted by centrifugation or by application of air pressure via a plunger. **(B)** Detergent structure used for purification of ScVDAC1. Detergents are classified according to their charge properties. Structural formulas were generated with MarvinView program from ChemAxon Suite where oxygen and nitrogen atoms are colored in red and blue, respectively.

2.10. Micro-scale thermophoresis

The micro-scale thermophoresis (MST) uses infrared sources to create localized microscopic temperature gradients in a capillary with a very small volume of 4 μl. The motion of macromolecules such as proteins in temperature fields is sensitive to changes in its size, charge and solvation shell. Based on this biophysical properties, protein-small molecule interaction can be measured and a titration can be perform to access to affinity constant (K_D) at steady state. MST is equipped with a fluorescence detector able to measure thermophoresis of labeled proteins in capillaries containing different concentration of the substrate. This method has been described in detail elsewhere [28]. K_D of ATP was measured using the Monolith NT.115 from Nanotemper Technologies. ScVDAC1 was fluorescently labeled according to the manufacturer's protocol using the L003 Monolith™ Protein Labeling kit RED-NHS (amine reactive) without or pre-incubation with 10 mM ATP. The labeling procedure and the subsequent removal of free dye were performed within 1 h. Solutions of substrates were serially diluted from 50 mM to 1.5 μM for ATP in presence of 20 nM labeled ScVDAC1. The samples were loaded into capillaries (NanoTemper Technologies GmbH, München, Germany). Measurements were performed at 25 °C in 10 mM Tris-HCl pH 7.3, 100 mM Na_2SO_4 and 0.07% HECAMEG®, by using 85% LED power and 80% IR-laser power using a NT.115 instrument (NanoTemper Technologies GmbH, München, Germany). Data analysis was performed using Nanotemper Analysis software, v.1.2.101 and KaleidaGraph software (Synergy Software, PA, USA).

3. Results

3.1. Detergent screening for ScVDAC1 purification

The method used to purify non-recombinant ScVDAC1 was adapted from Brandolin et *al.* [29] and further developed in this study. The principle of the method is based on the retention of all proteins from a mitochondrial lysate on a hydroxyapatite (HA) column, except ScVDAC1, the mitochondrial ADP/ATP and phosphate carriers, which are not being retained given the detergent structure used. The chemical nature, the combination and the concentrations of the detergents used to solubilize mitochondrial membranes are critical parameters [30]. Indeed, the actual method to purify VDAC is based on using of Triton X-100 as extraction detergent after HA whereby the ADP/ATP carrier is also co-purified in excess (section results on this study and [31]). Interestingly, the authors show that the combination of HA with Celite

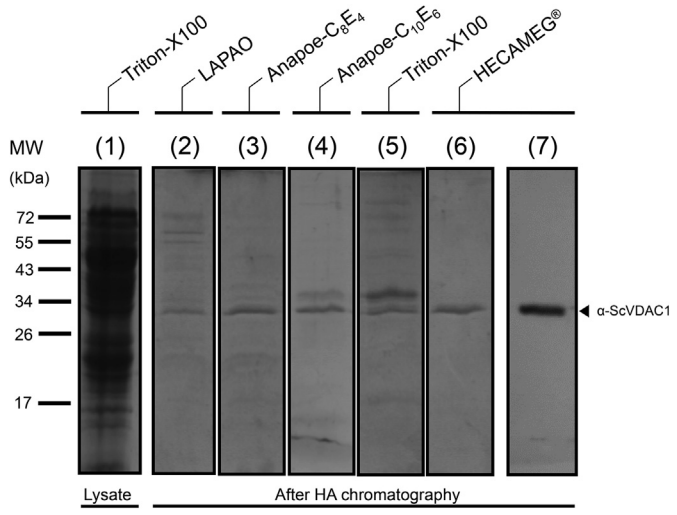

Fig. 2. Screening of detergents for one-step purification ScVDAC1. The experiments were performed as described in paragraph 3.1. Eluates obtained after centrifugation of the mitochondrial lysate on the HTP column were analyzed by SDS-PAGE (15% acrylamide) Coomassie blue staining. Five detergents were tested: LAPAO (lane 2), Anapoe-C_8E_4 (lane 3), Anapoe-$C_{10}E_6$ (lane 4), Triton X-100 (lane 1 and 5) and HECAMEG® (lane 6 and 7). The purified protein was ScVDAC1 as shown by immuno-staining with an antibody directed against ScVDAC1 (lane 7).

chromatography permits to removal of the contaminating ADP/ATP carrier from eluted fraction. A second step of purification was mandatory to exchange detergent and to concentrate the protein. Conversely, to prevent co-purification of VDAC with ADP/ATP carrier, some investigators used alternative protocols with mitoplasts as a starting material and they employed another detergent, $C_{12}E_8$, instead of Triton X-100 [32].

In this study, we tried to determine which detergent after HA allows a one-step purification to extract pure VDAC, whereby the purity was accessed by Coomassie Blue staining after SDS-PAGE. Freshly thawed mitochondria isolated from the *W303* strain were solubilized at 10 mg.ml^{-1} in the presence of a final detergent/protein ratio of 10:1 (w/w), in lysis buffer composed of 10 mM Tris-HCl pH 7.3, 100 mM Na_2SO_4, 1 mM EDTA, 1 mM DFP, and a "cOmplete, mini, EDTA-free" antiprotease cocktail (25 tablets #11836170001, Roche, Basel, Switzerland), according to the manufacturer's instructions. After 10 min of gentle shaking at 4 °C, the solubilized protein was purified by short centrifugation of the hydroxyapatite column (2:1 column volume to lysate volume ratio) during 1 min at 500*g* or in few seconds by air pressure of the lysate on the column with the aid of a plunger (Fig. 1A). The obtained flow-through fraction was containing the purified ScVDAC1.

The solubilization assay was performed comparing 5 detergents: Nonionic detergents such as HECAMEG® (glucoside), Triton-X100 (polyethylene oxide), Anapoe-$C_{10}E_6$ and Anapoe-C_8E_4 (polyoxyethylene glycol) were used and one zwitterionic detergent, *i.e.* LAPAO (3-Laurylamido-*N*,*N*′-Dimethylpropyl Amine Oxide) (Fig. 1B). The analysis of the flow-through fractions based on Coomassie Blue staining after SDS-PAGE is shown in Fig. 2. Many proteins co-eluted with VDAC when mitochondria were lyzed with LAPAO, Triton X-100 or Anapoe-C_8E_4. Two major proteins where eluted with Anapoe-$C_{10}E_6$. Pure VDAC1 was obtained only with HECAMEG® and the identity of the eluted protein was confirmed by immunoblotting (Fig. 2). The final decision with regard to the correct detergent that yields extraction of pure VDAC was made using a detergent condition screen. In order to extract the maximal amount of protein necessary for structural analysis with minimal quantity of detergent that often impairs subsequent biophysical and biochemical analysis, a further step of optimization was essential, as describe below.

3.2. Optimization of purification

Detergents are necessary to isolate and solubilize MP to facilitate the desired studies. In contrast, an excess of detergent is usually incompatible with biophysical approaches and thus needs to be minimized thoroughly. The detergent to protein ratio is always a critical parameter during the extraction process in order to solubilize the maximal amount of protein. In our particular affinity chromatography approach, the sample to column volume ratio is most critical. Different ratios of column to lysate volume were screened in order to obtain the highest possible protein concentration. As shown in Fig. 3A, the best results were obtained for a 1:1 ratio using a detergent/protein ratio of 10:1 (w/w). ScVDAC1 was purified in this one step protocol at a concentration of 0.35 mg ml^{-1}. The results revealed that, for a 1:2 ratio, contaminant proteins were eluted along with ScVDAC1.

Detergents are indispensible to solubilize MPs, but their concentration and the detergent to protein ratio is a key parameter maintaining MP in a close to native state and in keeping it functional. Furthermore, depending on the characteristics of the detergent, it can concentrate along with the protein during the MP concentration process and thus become deleterious to the MP. Therefore, it is desirable to purify ScVDAC1 with a minimal amount of detergent. Different detergent/protein ratios were evaluated while keeping a maximum effectiveness of extraction (Fig. 3B). A 1:1 detergent/protein ratio was inefficient in extracting ScVDAC1 from mitochondria after the HA gel. For efficient extraction of ScVDAC1 the ratios 5:1 and 10:1 were suitable. The best ratio appeared to be 5:1 as it efficiently permits extraction of ScVDAC1 with minimal amount of detergent.

However, the yield for purification of ScVDAC1 using this method was not optimal, primarily due to retention of part of ScVDAC1 in the HA gel which is proportional to the dead volume of the column (Fig. 3C). Given a 2:1 ratio of column to lysate volume, the elution volume obtained after the first centrifugation roughly corresponds to the dead volume of the column (Fraction 1), in which the concentration of purified ScVDAC1 was very low. An additional volume of elution buffer corresponding to that of the lysate gave rise to elution of the main component of ScVDAC1 in the flow-through fraction (not shown). As can be seen in Fig. 3C, ScVDAC1 mainly eluted in the first 3 fractions after successive additions of the lysis buffer containing 0.7% HECAMEG®, corresponding each time to one fifth of the lysate volume. The ScVDAC1 concentration in fractions 1 to 3 (3/5 of the lysate volume) was ~0.7 mg.ml^{-1}.

Taken together, we defined the optimal ratio of column to lysate volumes and detergent/protein ratio to purify VDAC in one centrifugation step, in order to minimize the time of purification from minutes to few seconds. Our main interest was to extract VDAC as fast as possible from isolated mitochondria, in order to reduce potential denaturation issues. Interestingly, we observed that, for a 2:1 ratio of column to lysate volumes, the protein was not eluted after the first passage. However, VDAC could be eluted after 3 successive small washing steps, as shown in Fig. 3C. This observation demonstrates that VDAC in HECAMEG® micelles is not interacting with the HA matrix and, therefore, resides in the dead volume of the column. Thus, the best conditions for maximal extraction of VDAC corresponds to a 2:1 ratio of column to lysate volumes by successive washing steps, resulting in a final protein concentration at approximately 0.7 mg.ml^{-1}.

The estimated yield of ScVDAC1 purification in HECAMEG® from a 12-l culture was 15 ml at a concentration of 0.7 mg.ml^{-1}. The

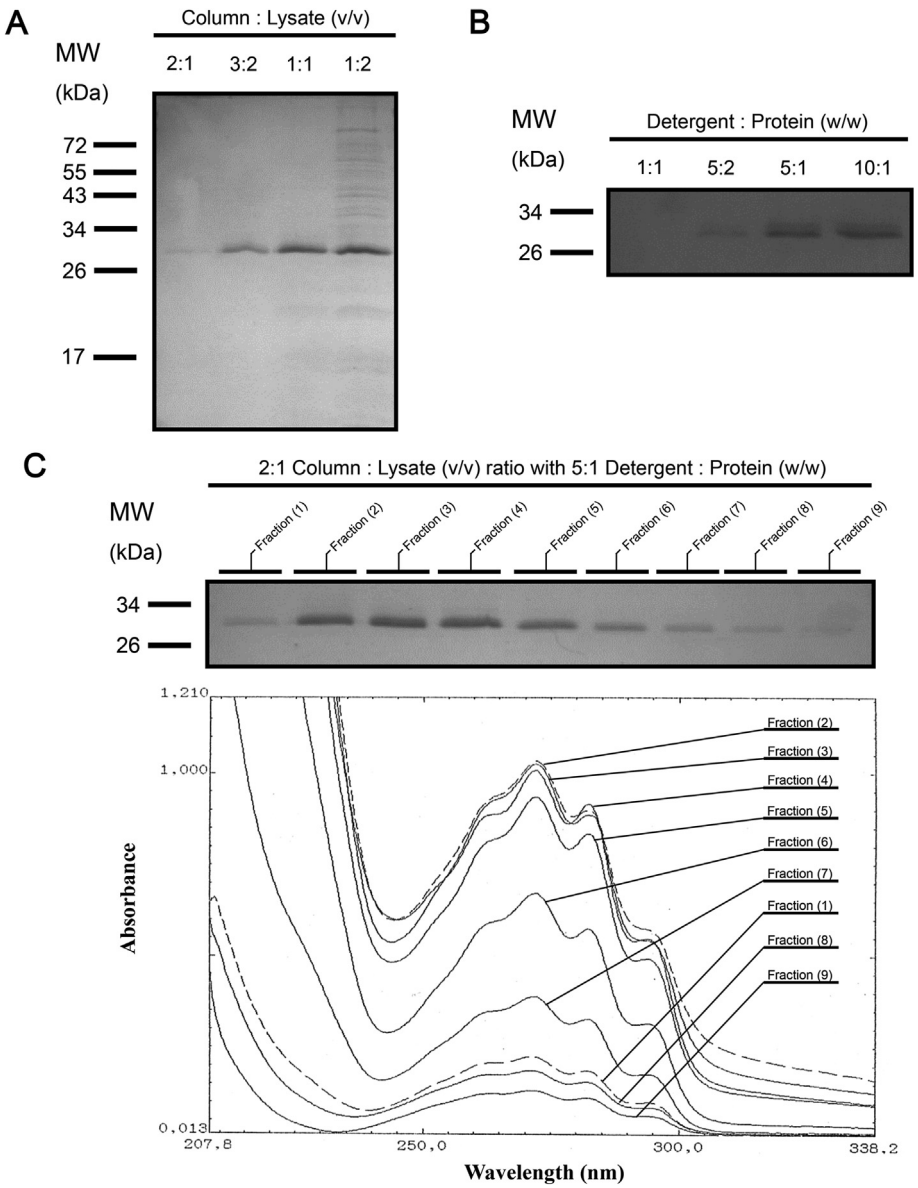

Fig. 3. Further improvements of HECAMEG® solubilized of ScVDAC1 purification **(A)** Different ratios of column volume to lysate volume (v/v) were tested. The purification was carried out by centrifugation of the lysate through the HA column, according the protocol described in paragraph 3.1. Protein fractions were analyzed by SDS-PAGE (15% gel) and Coomassie Blue staining. Pure ScVDAC1 was obtained with a 1:1 ratio (v/v) using a final detergent/protein ratio of 10:1 (w/w). **(B)** Determination of the minimal amount of HECAMEG® necessary to purify mitochondrial ScVDAC1 by HA chromatography. The experiments were performed with a 1:1 column to lysate volume ratio, as described in A. Different detergent/protein ratios (w/w) were tested. **(C)** Optimization of the purification protocol of ScVDAC1 HECAMEG® detergent by HA chromatography. 5:1 detergent/protein ratio (w/w) was chosen (see B) with a 2:1 column to lysate volume ratio (v/v) (see A). Top: Analyses by SDS-PAGE (15% gel) of the eluted fractions followed by Coomassie Brilliant Blue staining. (1) Centrifugation of the lysate on the HA column, (2) to (9) successive elutions (by centrifugation) from the HA column with a volume of elution buffer corresponding to one fifth of the lysate volume. The protein was concentrated in 2–4 fractions, with a protein concentration of 0.7 mg/ml. Bottom: Absorption spectra the eluted fractions of the column of HA showing the presence of ergosterol.

protein was concentrated down to ~5 mg.ml^{-1} with an Amicon Ultra-15 Centrifugal filter unit (30 K) (Merck Millipore) and stored at −20 °C for up to two months.

Previous purification of VDAC particularly from *Neurospora crassa* revealed, based on UV absorption spectroscopy, the presence of ergosterol [33]. Given this finding, we decided to analyze all of the eluted fractions, in order to access ScVDAC purity, as described below.

3.3. Characterization of the ScVDAC1 fractions

To monitor the purity of the eluate, an absorption spectrum was determined for each eluted fraction (Fig. 3C). Although a protein absorption peak at 280 nm was not observed, interestingly, we detected a characteristic absorption peak for ergosterol [33,34]. According to SDS-PAGE, following Coomassie Blue staining, the absorbance of the different peaks increased in proportion to the amount of purified ScVDAC1 (Fig. 3C). Ergosterol is present together with the ADP/ATP carrier in the HA flow-through fraction, when mitochondria are lyzed with Emulphogen, but it is absent when lyzed in DDM [34,36]. Therefore, ergosterol binding is not a unique feature of ScVDAC1. Ergosterol is present in all yeast and fungus membranes and it may be solubilized by numerous detergents. However, as seen for the ADP/ATP carrier, ergosterol does not

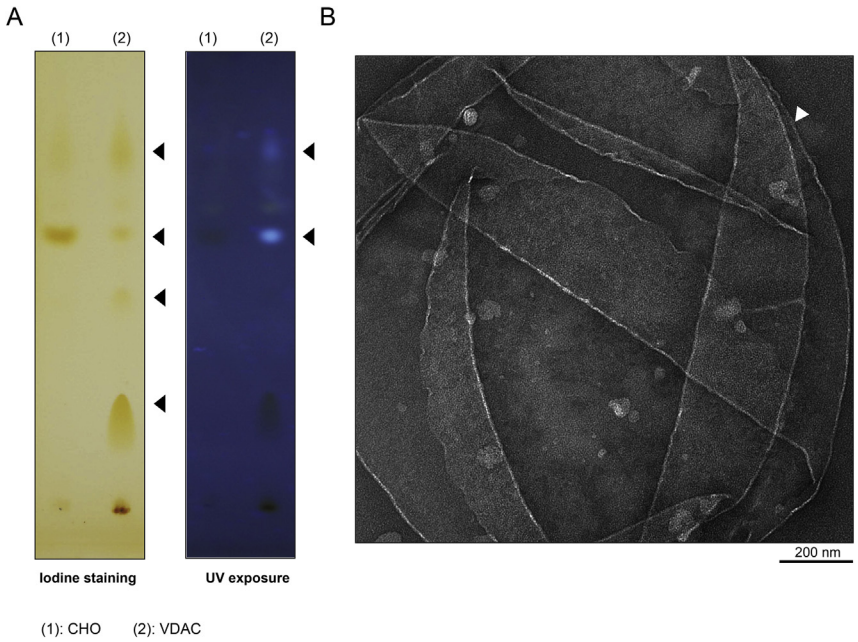

Fig. 4. Associated VDAC-lipids and reconstitution **(A)**. Thin Layer Chromatography (TLC) of lipids extract from ScVDAC purified visualized after iodine staining and UV exposure. **(B)** Micrograph of reconstitute ScVDAC in liposomes after negative staining. The ScVDAC is incorporated into endogenous lipids as well as into supplemented DMPC.

compromise further functional studies nor does it impair MP activity, because ergosterol is a natural and reasonably abundant component of OMM [34–36]. The presence of ergosterol was confirmed from lipids extracted from the ScVDAC1 eluted fraction by separation and subsequent analysis using thin layer chromatography (TLC), following iodine staining and UV-detection, with cholesterol (CHO) as a marker (Fig. 4A). For TLC, CHO was used as structural analogues of ergosterol which has the same migration propriety, but, in contrast to ergosterol, exhibits no fluorescence under UV light. The TLC of ScVDAC sample revealed the presence of lipids other than ergosterol, which are more polar and possibly correspond to phospholipids. The observed upper band, which is also fluorescent, may correspond to an aggregate of ergosterol.

In order to demonstrate the high amount of endogenous co-purified lipid with our protein preparation, we tried to reconstitute ScVDAC into liposome without addition of lipids. Indeed, complete reconstitution of ScVDAC as proteoliposomes was observed, when using a very low lipid to protein ratio (LPR) of 0.1, using DMPC, as shown by TEM, following negative staining (Fig. 4B).

3.4. Supramolecular assembly of purified ScVDAC1

Usually, membrane protein function depends highly on the proper supramolecular and oligomeric assembly, in order to warrant full cellular activity. According to the literature, this also holds true for VDAC. Thus, we employed TEM, following negative staining of purified and solubilized ScVDAC, to access its oligomeric state. The oligomeric state was assessed by TEM with sufficient resolution to distinguish the supramolecular assembly, following the procedure of Dolder et al. [37]. The level of oligomerization is an important indicator of the correct native state of membrane proteins such as ScVDAC1 [38] and it needs to be carefully considered before undertaking structural studies.

TEM experiments require low concentrations of proteins (~15 $\mu g.ml^{-1}$). Therefore, we diluted the purified VDAC sample to the appropriate concentration after one HA chromatography passage (Fig. 3C). The eluate was further analyzed by SDS-PAGE (15% gel), followed by silver staining. As shown in Fig. 5A, ScVDAC1 migrated as one band at 31 kDa, but a contaminant band of approximately 14–15 kDa was also detected. Nonetheless, since the molecular weight of this contaminant is much lower than that of ScVDAC1, it was expected not to interfere with TEM analysis.

As shown in Fig. 5B, ScVDAC1 was nicely solubilized without aggregation and it was mainly present in the form of monomers, dimers, and trimers which correspond to the previously reported functional states, although higher oligomeric states were also visible but to a much lower extent. Close-ups of the monomers, dimers and trimers are shown in Fig. 5C. Each monomer contains one round-shaped pore, independent of the degree of the oligomerization state. Different oligomerization states were also reported for ScVDAC1 based on atomic force microscopy [39], although we did not observe oligomerization degrees as high as those reported these investigators. Our results confirm data obtained by cross-linking experiments, which demonstrated naturally occurring dimers and trimers of VDAC [40] and also higher degrees of oligomerization [41]. The monomers are similar to the ones observed by Dolder et al. [37].

TEM analysis of purified fractions verified the conserved native oligomeric state of ScVDAC after extraction. In other words, the detergent is able to extract the protein from the lipid bilayer without disrupting its oligomeric states. The next step was to determine whether the purified ScVDAC solubilized using our conditions with HECAMEG® as detergent was still functional. One of the principal critiques of Colombini concerns the property of the purified VDAC used for crystallization which turned out not to be in a functional state. Indeed, even if the protein was reconstituted into liposomes for electrophysiological measurements, the conditions used were different from purified protein and they did not represent the overall property of the sample used for crystallization. In our studies, we preferred to determine the integrity of the channel directly in the purified fraction that was used for structural analysis and also, we did not focus on electrophysiological measurements for the following reason. In recent years, VDAC was found to be a rather selective channel particularly for ATP, rather than a simple,

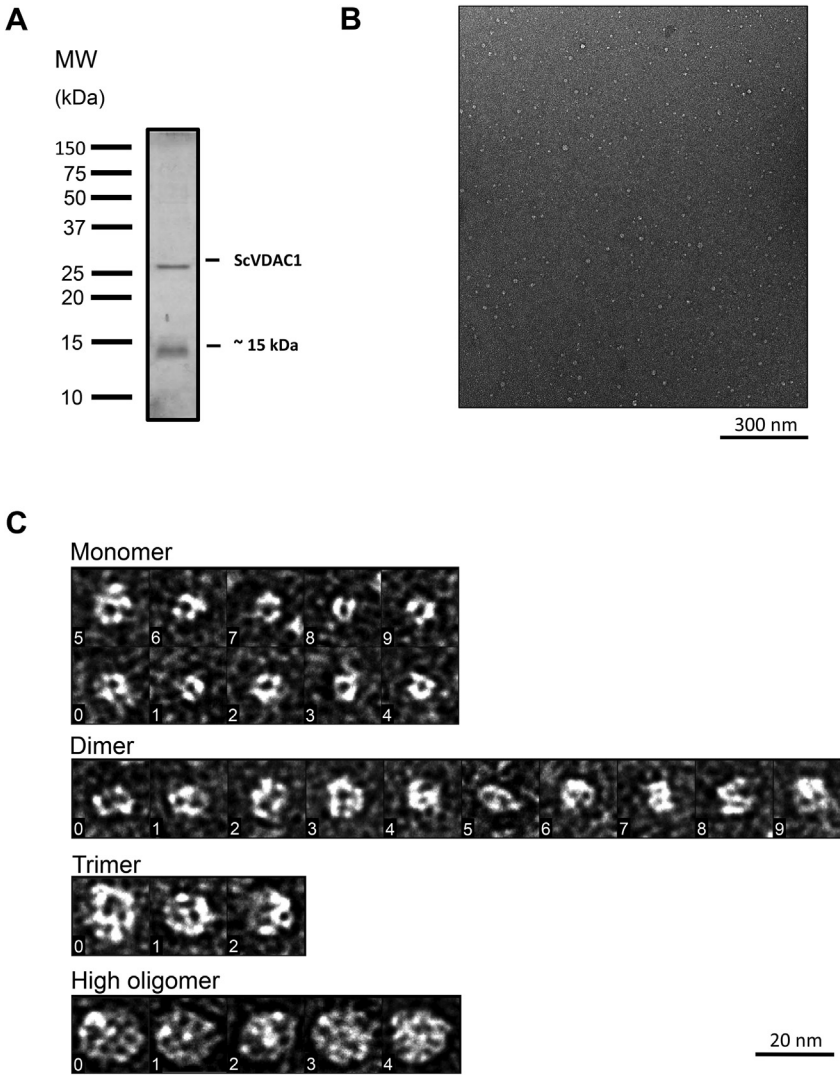

Fig. 5. TEM analyses of purified ScVDAC1. **(A)** SDS-PAGE (13% gel) of purified ScVDAC1 used for this study in silver staining. A 15 kDa-contaminant band is visible. **(B)** Micrograph obtained by TEM after negative staining of ScVDAC1 solubilized in HECAMEG® reveals the presence of oligomers in the preparation. The scale bar is 300 nm. **(C)** Organization of ScVDAC1 solubilized in HECAMEG®. Oligomers were manually classified using EMAN2 software (box size: 64 × 64 pixels). The various oligomeric states derived from the same micrograph were zoomed and classified in a gallery of images. Ten monomers, several dimers, three trimers and five higher oligomers are labeled.

non-specific pore. Given this finding and the hypothesis that this protein was misfolded and therefore was not able to bind to its natural substrate, we decide to use micro-scale thermophoresis (MST) to assess binding to ATP.

3.5. Measurements of ATP binding on ScVDAC1 solubilized in HECAMEG® by MST

Following membrane protein purification, determination of the specific substrate affinity of isolated protein to its ligand provides a direct molecular indication of the functional state and structural integrity of the purified protein. We decide to use a recently improved MST technique that is based on the migration proprieties of macromolecules in solution. In this technique, the property of the protein of interest is directly related to its hydration shell, the conformational changes and the increase in size induced by specific binding of substrates. ScVDAC labeling with dye was necessary in order to measure the displacement from the detector with increasing concentration of ATP from 1.5 μM to 50 mM. “Standard”, “hydrophilic”, “hydrophobic” and “premium” capillaries types were tested before titration to avoid potential aggregation of the protein due to the matrix support. The “premium” capillaries where surface is covalently coated with a dense brush of a specially designed polymer were selected for our study. We unequivocally show binding of ATP to solubilized ScVDAC1 by MST. The $K_{D\ (ATP)}$ measured corresponds to 1.2 mM ± 155 μM with $R^2 = 0.97$ using four-parameter logistic nonlinear regression curve fitting (Fig. 6A). To address the question of whether the protocol using the Nanotemper labeling kit may result in unwanted reactivity with amine groups of the protein that could affect K_D measurements, we pre-incubated VDAC with 10 mM ATP before labeling, in order to saturate and protect the ATP binding site(s) exposed during the procedure. Excessive reactive molecules and ATP were then removed using the standard gel filtration protocol from the kit and measurements were performed as described in material and methods. The K_D measured was 2.5 mM ± 642 μM with $R^2 = 0.96$. This experiment confirms our previous data showing that the K_D of VDAC for ATP is within the millimolar range.

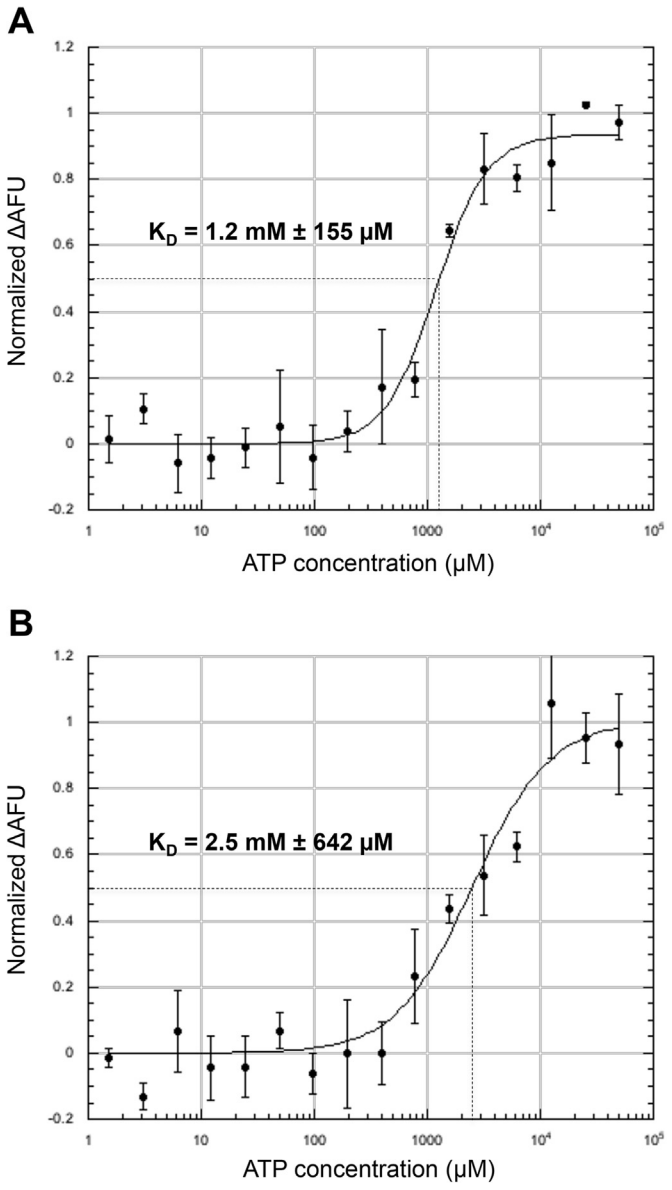

Fig. 6. Determination of the dissociation constant (K_D) for ATP of ScVDAC1, as measured by MST. **(A)** ATP (1.5 µM–50 mM) was titrated into a fixed concentration of labeled ScVDAC1 (20 nM). **(B)** ATP (1.5 µM–50 mM) was titrated into a fixed concentration of labeled ScVDAC1 (20 nM) saturated with 10 mM ATP. In both experiment, thermophoresis is represented as normalized fluorescence (F_{Norm}), multiplied by 1000, yielding a relative fluorescence change per thousand [‰]. The recording was performed at 20 °C using the red LED at 85% and the IR-laser at 80% of maximal power on the device. The results show the isotherm derived from the raw data and it was fitted according to the law of mass action to yield an apparent K_D (1.2 mM ± 155 µM) and (2.5 mM ± 642 µM) pre-incubated with ATP.

4. Discussion

The nature of the physiologically relevant, native structure of VDAC is still a puzzle, despite excellent progress through numerous NMR and high-resolution crystallographic structure determinations. Both of these structural approaches utilized non-functional recombinant and overexpressed protein from bacteria that was extracted from inclusion bodies, and thus these structures appear to correspond to VDAC refolded into a non-native state as described by Colombini.

The immediate goal of our study was therefore to implement a simple, fast and efficient purification protocol to isolate pure and functional, non-recombinant ScVDAC1 for structural studies. We estimate that this progress will be the key to the future high-resolution structural determinations of VDAC in its native functional state.

The herein reported approach for ScVDAC1 isolation is based on a simple purification procedure. Briefly, ScVDAC was purified from mitochondria by either centrifugation or application of air pressure to an HA column. This procedure assured maximal purification yields and minimized purification time, with least number of purification steps. Thus, the procedure is elegant, as most purification protocols require more than one step to achieve the desired level of purity. Unlike TEM which does not require high concentrations of purified protein, crystallography studies are more demanding in this regard. Based on our procedure, we were able to obtain pure ScVDAC1 at concentration ranges 0.7 to 3–5 mg/ml, thus making our protocol suitable for both types of structural studies.

Regardless of the approach used, the purification of MPs inevitably starts with extraction and solubilization using amphipathic molecules known as detergents. Efficient extraction of integral membrane proteins requires both the selection of the appropriate detergent and the use of suitable solubilization conditions to preserve the activity of the MP. The choice of the detergent is undoubtedly of crucial nature because it has a direct impact on the structure and activity of the protein. In our case, HECAMEG® was an effective detergent, capable of extracting and solubilizing ScVDAC1 while preserving its native oligomeric states observed in the membrane and its ability to bind substrates, as demonstrated by the measurement of K_D for ATP. Interestingly, we noticed that anapoe-C_8E_4 with an 8C-aliphatic chain can also purify VDAC, but with less efficiency (Fig. 2B) compared to HECAMEG® which has 7C-aliphatic chain (Fig. 1B). Based on these observations, it appears that for purification of these ScVDAC1, it is necessary to use a detergent with a small aliphatic chain.

Our results confirm the supramolecular assembly of ScVDAC1 observed previously by AFM studies [39]. Moreover, we were able to measure for the first time the K_D of ATP and reveal a value in the mM range from isolated ScVDAC1. Interestingly, unique binding was observed with an affinity constant ~ 1–2 mM, which makes sense, knowing that the ATP concentration inside the cell is typically 1–10 mM [42]. Indeed, VDAC is expected to work as a channel with low affinity (K_D in the mM range) and high capacity. This result is distinct from that of earlier studies [43], which suggested that there might be high and low affinity-binding sites within the micromolar range, similar to the ADP/ATP carrier.

Ergosterol was found to be associated with pure ScVDAC1. This steroid is the biological precursor of vitamin D2 (a vitamin D supplement, also known as ergocalciferol). Ergosterol is a component of yeast and fungal cell membranes and does not occur in plant or mammalian cells. It is considered as an equivalent of cholesterol in higher eukaryotes. This lipid likely facilitates insertion of ScVDAC1 into artificial membranes which is important for electron crystallography of two-dimensional crystals using cryo-electron microscopy.

In conclusion, our ScVDAC1 purification protocol is efficient and appears appropriate for future structural and functional studies because the purified ScVDAC1 exhibits the properties of its native functional conformation and oligomeric state.

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