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Crystallization of the membrane protein hVDAC1 produced in cell-free system

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ABSTRACT

Structural studies of membrane proteins are in constant evolution with the development of new improvements for their expression, purification, stabilization and crystallization. However, none of these methods still provides a universal approach to solve the structure of membrane proteins. Here we describe the crystallization of the human voltage-dependent anion channel-1 produced by a bacterial cell-free expression system. While VDAC structures have been recently solved, we propose an alternative strategy for producing the recombinant protein, which can be applied to other membrane proteins reluctant to expression, purification and crystallization by classical approaches. Despite a lot of efforts to crystallize a cell-free expressed membrane protein, this study is to our knowledge one of the first reports of a successful crystallization. Focusing on expression in a soluble and functional state, in a detergent environment, is the key to get crystals. Although the diffraction of VDAC crystals is limited, the simplicity and the rapidity to set-up and optimize this technology are drastic advantages in comparison to other methods.

1. Introduction

Despite important efforts made in the last ten years, solving a membrane protein structure still remains a challenge. A universal and efficient method for the production of membrane proteins dedicated to structural studies does not exist. To overcome this severe bottleneck, progresses have resided in building up robust and rationale strategies that i) facilitate the exploration of large number of expression systems (including eukaryotic cells) [1], ii) improve their efficiency [2–4] iii) introduce novel surfactants and iv) extend crystallization to innovative methods (i.e. lipidic phases) [5,6]. In parallel, automation and miniaturization introduced by the structural genomic programs help in screening large number of conditions from expression to crystallization with limited amounts of proteins. Such tools contributed to recent successes in membrane proteins structures. Cell-free expression introduced in the eighties as an alternative approach to bacterial expression [7] is an attractive way for obtaining high yield protein production. The method is interesting as any additional compounds such as lipids or surfactants can easily be supplemented to the reaction medium. Several groups have already optimized the cell-free systems for different types of membrane protein: channels, G protein-coupled receptors, transporters, … [8–10]. Currently, selenomethionine EmrE used to determine phases is the only published example of crystallization for a cell-free expressed membrane protein [11]. Therefore, *in vitro* protein synthesis is promising for membrane protein structures, but no report has yet described the way from gene to crystals so far.

VDAC is the main outer mitochondrial membrane protein. It is involved in the exchange of metabolites between the cytosol and the inter-membrane space but a better understanding of its molecular mechanism requires detailed structural information. Mannella's team was the first to study the structure of VDAC at low resolution by electron microscopy [12]. Then, two-dimensional crystals [13] or atomic force microscopy [14,15] provided low-resolution structural information of the protein and its arrangement in the native membrane. High-resolution structures of mouse and human VDAC have only been solved by the end of 2008 [16–18], and revealed an unpredicted 19 strands β-barrel. In these three studies, VDAC has been overexpressed in inclusion bodies in *Escherichia coli* and then refolded after solubilization in guanidinium. As described in the literature the optimization from refolding to exploitable-crystals of VDAC in detergent took more than ten years [13,19], demonstrating the difficulty of crystallizing VDAC.

The expression of VDAC by a cell-free method described herein, is an innovative approach in membrane protein crystallization extendable to other membrane proteins. Our strategy is to directly express the protein in the presence of a detergent compatible with the crystallization in order to obtain a soluble, functional and crystallizable protein. We obtained VDAC in nearly mg quantities per 3 ml of cell-free synthesis reaction. Combining a one-step purification, nanodrop crystallization and in-drop diffraction screens led to X-ray diffracting-VDAC crystals in six different detergents, therefore demonstrating the potential use of the cell-free method for other membrane proteins.

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2. Material and methods

2.1. Materials

All chemicals, except the detergents, were from Sigma. Detergents were from Anatrace at anagrade quality level.

2.2. hVDAC1 expression and purification

Human VDAC-1 sequence was cloned in the pIVEX2.4b from Roche by using NdeI and XhoI restriction enzymes. The following primers were designed to clone hVDAC1: hVDAC1*for* GGAATTCCATATGG-CTGTGCCACCCACGT, hVDAC1*rev* TAGGACTGGAATTTCAAGCATAACT-CGAGTAACGC. Expression was performed with the Roche RTS 100 (small volume batch system) and RTS 500 (medium scale with continuous exchange by dialysis) kits and the mixture was incubated in the Eppendorf Thermomixer comfort device. Initial screens were done in 50 µl volume RTS 100 *E. coli* lysate, substrate mix, amino acids mix, Roche reconstitution buffer were mixed with 1 µg of plasmidic DNA and the desired detergent. The mixture is incubated for 6 h and then centrifuged 10 min at 15,000 *g*. Supernatant and pellet proteins were loaded and separated on 10% SDS-PAGE gel, transferred to nitrocellulose membrane and revealed with HRP conjugated anti-His antibody (Sigma) on a Kodak 4000MM image station. Quantification was done with the molecular imaging software (Kodak). RTS500 expression has been done in 1 to 5 ml reaction mix. In the reaction and feeding mix, EDTA-free protease inhibitors (Roche) and detergents were added. Typically, 0.2% of β-DDM was used. In the reaction mix, 15 µg of DNA was also added per ml of reaction mix. The reaction was started for 48 h at 20 °C with shaking of 900 rpm. At the end of the expression, the reaction mix solution was centrifuged for 30 min at 100,000 *g* (Beckman Coulter-TLA100.3 rotor). The supernatant was recovered, diluted four times in 50 mM Tris pH 7.5, 0.5 M NaCl, 0.1% β-DDM, 10 mM imidazole, and mixed with 1 ml of Ni-NTA resin (Qiagen) preequilibrated in this last buffer. After a 2 h incubation at 4 °C of this mixture, the resin was washed three times with 20 mM Tris pH 7.5, 0.5 M NaCl, 0.05% β-DDM (or the new detergent when exchanged was performed) and increasing imidazole concentration (10, 20 and 50 mM). hVDAC1 was then eluted in the same buffer with 500 mM imidazole. Influence of cholesterol on the diffraction power of the crystals was assayed by addition of 0.1 to 1% of cholesterol (w:v) in all buffers from expression mix to crystallization buffer.

Purification of aggregated forms of VDAC was done by solubilizing the pellet from ultracentrifugation by 1% of β-DDM during 12 h. Then, the protein was purified in the same way as for the soluble form of cell-free expressed VDAC.

VDAC expressed in cell-free wheat germ extract was produced using RTS100 and RTS500 kits from Roche. Both are continuous dialysis exchange systems. Detergent choice and concentration optimization were done with the RTS100 kit in 50 µl reaction volume and 500 µl feeding volume. Large-scale experiments were done with the RTS500 kit in similar conditions as the ones used for bacterial extract except that only 0.04% of β-DDM were used during expression. The purification procedure is the same as the one used for the bacterial extract.

For crystallization the buffer is exchanged on a PD10 column (GE-healthcare) for 20 mM Tris pH 7.5, 50 mM NaCl, 0.05% β-DDM (or the exchanged detergent at the desired concentration). Then, the protein was concentrated up to 5 to 15 mg/ml in amicon concentrator (30 kDa cut-off), concentration determined by UV absorbance ($\varepsilon_{280} = 39{,}000\ M^{-1}\ cm^{-1}$). Finally, the tag was cleaved by using 1 U of factor Xa per 150 µg of pure hVDAC1. Gel filtration of Ni-NTA purified hVDAC1 was performed by loading 250 µl of sample on a 20 ml superdex-200 column (GE-healthcare). The chromatography was performed at 0.4 ml/min in 20 mM Tris pH 7.5, 50 mM NaCl and 0.05% β-DDM.

2.3. SDS-PAGE analysis

All steps were carried at 20 °C. 10 µl of protein samples were mixed with 2 µl of SDS-PAGE loading buffer (0.35 M Tris–HCl pH 6.8, 30% glycerol (w/v), 10% SDS (w/v), 0.6 M β-mercaptoethanol, 0.01% bromophenol blue) just before running the gels. Proteins were separated on 10% acrylamide SDS-PAGE gels and transferred onto nitrocellulose membrane (Biorad) when necessary. Protein gels were stained by Coomassie blue R250.

2.4. Electrophysiology

Giant proteoliposomes were prepared as described in [8] with few modifications. 1 µg of pure hVDAC1 was added to 2 ml of 100 mM KCl, 1 mg/ml asolectin, 10 mM Hepes (pH 7.4). After 20 min equilibration at room temperature, 320 µg of SM2 Bio-Beads (Bio-Rad) were added to the solution and incubated overnight at 4 °C to remove the detergent. The Bio-Beads having settled to the bottom, the supernatant was removed and centrifuged 25 min at 90,000 rpm (Beckman centrifuge, rotor TLA 100.3). The pellet was resuspended in 15 µl 10 mM Hepes (pH 7.4). To form giant liposomes, a single cycle of dehydratation/rehydratation was performed by drying 7 µl of sample under vacuum and resuspending in 15 µl of 100 mM KCl, 10 mM Hepes (pH 7.4).

For patch-clamp recordings, 7 µl sample of giant liposomes were deposited in a Petri dish used as a patch-clamp chamber. The chamber was then filled with 7 ml of 100 mM KCl, 10 mM Hepes (pH 7.4). Channel activity was recorded using the patch-clamp technique in the inside-out conformation [20]. Patch pipettes were pulled from borosilicate capillaries to a resistance of about 5–10 MΩ and were filled in with a solution containing 100 mM KCl, 5 mM $MgCl_2$, 2 mM $CaCl_2$ and 10 mM Hepes (pH 7.4). The currents were recorded using a Biologic RK-300 amplifier, filtered at 300 Hz, sampled at 1 kHz and analysed with Clampfit 10 (Axon) [21].

2.5. Limited proteolysis assays and analysis

Pure hVDAC1 at 1 mg/ml was mixed with proteases (chymotrypsin, trypsin, elastase, subtilysin, thermolysin or papain) at a weight-to-weight ratio between 1:100 and 1:1000. The limited proteolysis was started by adding protease to VDAC solution and kinetics were stopped by adding 3 µl of SDS-PAGE loading buffer. Limited proteolysis results were analysed by SDS-PAGE gels and N-terminal sequencing was done after transfer of the protein on PVDF membrane. The protein bands were cut from the membrane and sequenced by automated Edman degradation on an Applied Biosystems gas-phase sequencer, model 477A, with on-line analysis of the phenylthiohydantion derivatives.

2.6. Crystallization–crystallography

Initial screens, salt, and additive screens were performed in 96 well-plate hanging-nanodrops (Greiner). For a 100 µl reservoir, the drops were made of 100 nl of protein supplemented with 100 nl of reservoir. QIAgen commercial screens were initially used (The classics, CS Lite, PEG/Ion, MembFac, Natrix, Quickscreen, Grid screens, The classics II). Salt and additive screens from Hampton were added directly to the reservoir in 96 well-plates. Manual optimizations were carried out with drops made of 1 µl of protein plus 1 µl of reservoir, for a 500 µl reservoir. Lipids assayed as additives were added to the protein just before setting-up the crystallization drops [22]. Best crystals of hVDAC-1 in β-DDM were obtained in 17–18% PEG550MME, 100 mM Tris pH8.5, 200 mM KNitrate and in α-DDM were obtained in 22–23% PEG550MME, 100 mM Tris pH 8.5, 100–200 mM KNitrate.

Dehydration and cross-linking post-crystallization treatments have been done according to the different procedures developed and described in [23].

In situ diffraction tests were done by putting the nanodrop crystallization plates directly into the beam on the BM30A beamline at the ESRF [24]. For diffraction screens and data collections, crystals were harvested, plunged into mother liquor containing 10% of glycerol as cryoprotectant if necessary and flash frozen in liquid nitrogen. Diffraction data were collected at 100 K on ID14eh1, ID14eh2, ID14eh3, ID14eh4, ID23eh1, ID23eh2 and ID29 beamlines at the ESRF Grenoble.

3. Results

3.1. Expression–purification

The human VDAC1 (hVDAC1) was expressed in a commercial cell-free *E. coli* extract (Roche). The construct contains a cleavable N-terminal hexahistidine tag. hVDAC1 expression was assayed in the absence or in the presence of different detergents in order to directly obtain a soluble, folded and functional form of the protein. The detergents were chosen among those which have already proven to be successful in crystallization (http://www.mpdb.ul.ie/ [25]). However, detergents already known to strongly inhibit the protein synthesis, such as β-OG [8], were excluded. In a first attempt, expression conditions were optimized in small volumes (25–50 μL) with 6 h expression time and analysed by Western-blotting. Two criteria have been taken into account: the yields of total expression and of the soluble fraction (Fig. 1a). In the absence of detergent the expression yield exceeded 1 mg/ml of aggregated hVDAC1 (data not shown). By exploring the temperature and different detergents, the quantity of protein expressed in a soluble form varied. The temperature was optimized with the most commonly used detergent β-DDM. Decreasing the temperature from 30 °C to 20 °C allowed an important decrease of the aggregation of hVDAC1 during the expression (data not shown). Then, the choice of the best detergent compatible with the expression of a high amount of protein in a soluble form was done. Seven different detergents have been tested with concentrations ranging from 0.1 to 0.2% for low cmc detergents ($C_{12}E_8$, β-DDM, α-DDM, LDAO, foscholine-12), 0.4% for cymal-5 and 1% for C_8E_4. In LDAO and cymal-5, hVDAC1 was not or almost not expressed, respectively (Fig. 1a). Expression was low in the presence of C_8E_4 or α-DDM. In $C_{12}E_8$, large expression of VDAC was detectable but in an aggregated form (Fig. 1a). Only two detergents gave interesting results: β-DDM and foscholine-12, in which hVDAC1 was highly expressed with a large amount of soluble protein (Fig. 1a). Because there is little crystallization experience with foscholine-12, β-DDM has been chosen for the scale-up experiments.

Expression time and detergent quantity were optimized in a 1 ml reaction chamber with continuous exchange by membrane dialysis. Although large aggregates of hVDAC1 started to appear after 36 to 48 h expression in the presence of 0.1–0.2% β-DDM, the yield of expression still increased after 48 h (data not shown). The best expression conditions of soluble hVDAC1 were 48 h at 20 °C in the presence of 0.2% β-DDM. The aggregates of hVDAC1 were removed by ultracentrifugation at the end of the expression reaction. hVDAC1 was purified in one-step Ni-NTA chromatography (Fig. 1b) with a fold similar to the native protein, as shown by circular dichroism (data not shown). The functionality of pure hVDAC1 was assayed by patch-clamp after reconstitution in giant liposomes. Single channel currents could be observed at both positive and negative membrane potentials (Fig. S1a). Recordings were characterized by the presence of multiple conductance levels, the most frequently being around 225pS and the largest around 450pS (Fig. S1b). These values match those described previously in the literature for native VDAC [26]. Crystallization assays of hVDAC1 were done with a nanodrop crystallization robot by testing 288 conditions from Qiagen screens. Although initial crystals were

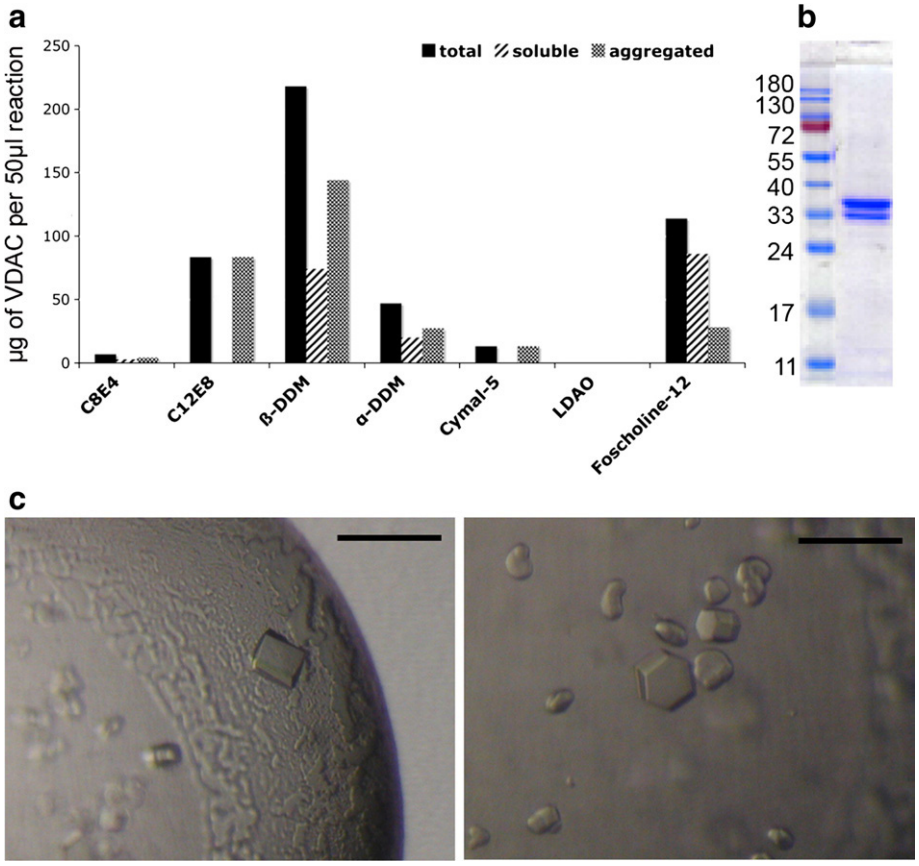

Fig. 1. Expression and crystallization screenings. Panel a, quantification of the total, soluble and aggregated expression of hVDAC1 in different detergents. Data are means of 2 to 4 independent experiments. Panel b, SDS-Page gel of pure hVDAC1 expressed without protease inhibitor. Panel c, initial hVDAC1 crystals in nanodrops. Scale bar: 100 μm.

rapidly obtained (Fig. 1c), biochemical optimizations of the protein solution have been required to circumvent the non-reproducibility of these crystals.

3.2. Biochemical optimizations for the crystallization

Obtaining good-quality membrane protein crystals crucially depends on the purity, conformational homogeneity and monodispersity of the protein. These three points were optimized to reproduce initial crystals.

hVDAC1 purified in a single step in the presence of β-DDM sometimes appeared as a double band on SDS-PAGE gels (Fig. 1b). The lower band is the consequence of proteolysis during expression, because the addition of protease inhibitors to the cell-free extract at the beginning of the production resulted in its disappearance. However, this pure and homogenous VDAC did not crystallize.

Conformational heterogeneity can be induced by flexible parts of the protein that might hamper crystallization [27]. Limited proteolysis assays were then used to identify a rigid core of hVDAC1, favourable to crystallization. Thermolysin, trypsin and chymotrypsin principally led to the cleavage of the tag just after the factor Xa site (Fig. 2a and S2), as revealed by N-terminal sequencing. Other proteolysis products of hVDAC1, around 20 kDa, were obtained with trypsin (Fig. 2a, lane 3–5 and Fig. S2) and in a lower extent with elastase (data not shown), starting at residues 114 and 107, respectively (Fig. S2). Thus, limited proteolysis demonstrated that, apart from the tag, cell-free expressed hVDAC1 has a rigid core. Cleavage of the tag with factor Xa led to reproducible crystals. The presence in the crystals of hVDAC1 without histidine tag was confirmed by N-terminal sequencing.

Size exclusion chromatography after cleavage increased the sample purity by removing the factor Xa, the cleaved histidine tag, as well as few aggregates and other contaminants (Fig. 2b and c). hVDAC1 was eluted as a well-shaped peak with an elution volume around 12 ml (Fig. 2c) indicative of monodispersity (Fig. 2c), which is favourable for crystallization. Nonetheless, the use of a desalting column instead of a superdex-200 gave similar results (Fig. 2d), and both purification procedures led to crystals with similar diffraction powers. Therefore, desalting column was chosen for the limited loss of protein and time in comparison to gel filtration.

Within two days, 200 to 300 µg of pure and "ready to crystallize" hVDAC1 solubilized in β-DDM at a concentration of 10 mg/ml (Fig. 2b), were obtained per ml of cell-free extract reaction.

3.3. Crystallization optimizations

hVDAC1 crystals are not well-ordered as seen from the rapid decrease in intensity as a function of resolution (Fig. 3a, left panel) and diffracted anisotropically to a maximum of about 12 Å (Fig. 3a). To improve the crystal quality additives including salts and detergents were screened in nanodrop crystallization assays. Diffraction quality was assayed directly from crystals in the nanodrops in which they grew by using a specific device designed on the BM30A beamline at the ESRF [24]. These crystallization and diffraction technologies allowed to screen hundred crystallization conditions by assessing the diffraction power of the crystals and circumvented the limited quantity of pure hVDAC1. Additive screens highlighted the use of potassium nitrate and spermidine or methanol to improve crystal quality. Detergent screened as additives led to crystals with almost all maltosides and with some other detergents without any significative improvement in diffraction. Scaling up the optimized conditions gave well-shaped β-DDM-hVDAC1 crystals and their size was increased by seeding (Fig. 3b). Other post-crystallization treatments such as dehydration or cross-linking importantly decreased the diffraction power. Addition of lipids, especially cholesterol, either in the crystallization drops or during all the process from expression to crystallization never improved the crystal quality. Finally, the best hVDAC1 crystals in β-DDM were cryoprotected with 15% of glycerol or with paraffin oil diffracted X-rays isotropically down to 8 Å (Fig. 3c).

Several recent successes in membrane protein crystallization have been obtained by exchanging the detergent to another in order to obtain new crystal forms or better crystal packing, with improved diffraction [28]. Exchange of β-DDM for α-DDM, β-undecylmaltoside (UDM), LDAO, C_8E_4, $C_{12}E_8$, cymal-5, cymal-6 and cymal-7 was performed on Ni-NTA resin. Several crystallization conditions (192 sparse and around 20 derived from the crystallization in β-DDM) were assayed for each detergent. Crystals were obtained with 5 other detergents: C_8E_4 (Fig. 4a), cymal-6 (Fig. 4b — left panel), UDM (Fig. 4b — right panel), cymal-7 (not shown) and α-DDM (Fig. 4c). Although C_8E_4 led to crystals in several conditions,

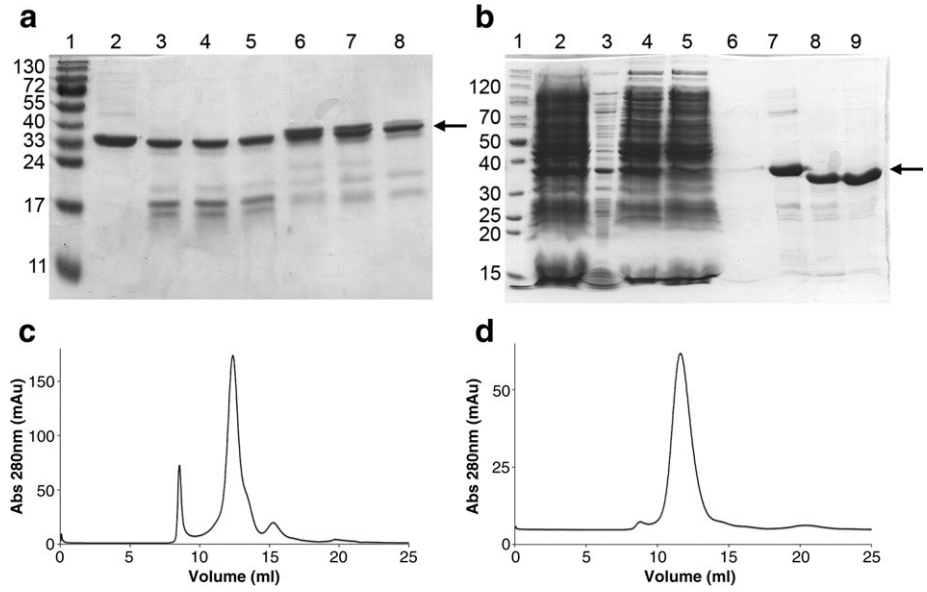

Fig. 2. Purification and characterization of cell-free expressed VDAC. Panel a, Limited proteolysis of pure hVDAC1. Lane 1: molecular markers, lane 2: pure hVDAC1, lane 3 to 5: 15 min, 30 min and 2 h time-point of the kinetic of hVDAC1 trypsinolysis, lane 6 to 8: 15 min, 30 min and 2 h time-point of the kinetic of hVDAC1 chymotrypsinolysis. Panel b, Analysis of hVDAC1 purification. Lane 1: molecular markers, lane 2: total expression, lane 3: aggregated proteins expressed, lane 4: soluble proteins expressed, lane 5: Ni-NTA flow-through, lane 6: 50 mM imidazole wash, lane 7: Ni-NTA elution, lane 8: Factor Xa cleavage, lane 9: pure hVDAC1 after superdex-200 gel filtration. Panel c, Size exclusion chromatography of Ni-NTA eluted proteins on superdex-200. Panel d, Size exclusion chromatography of desalted proteins on superdex-200.

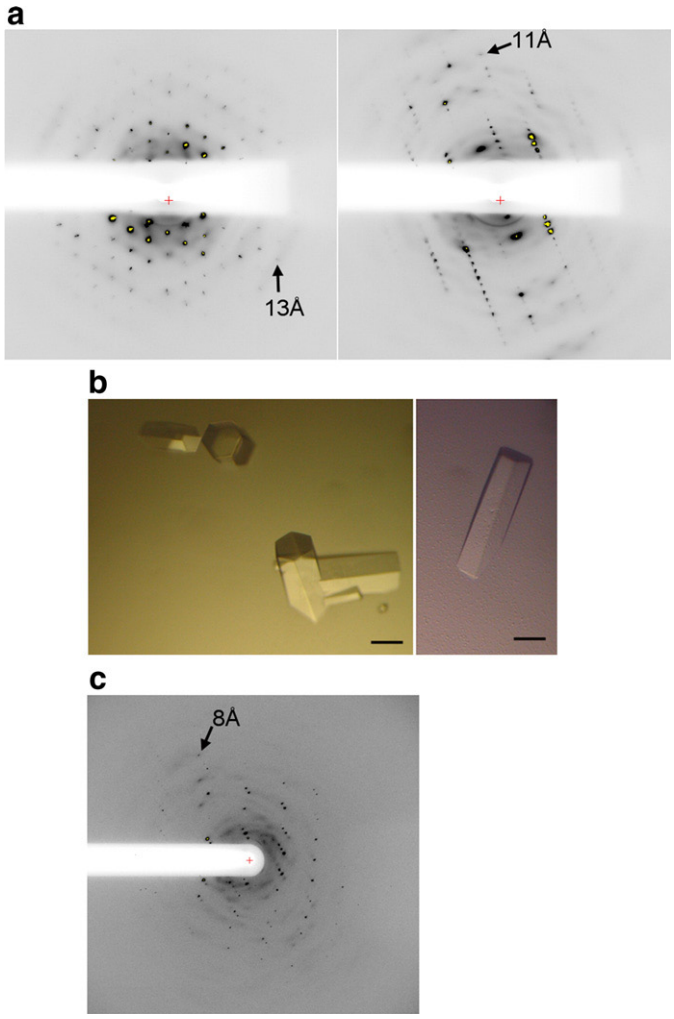

Fig. 3. Optimized crystals and diffraction of crystals of hVDAC1 in β-DDM. Panel a, diffraction images at 0° and 90° of an initial crystal of hVDAC1 in β-DDM. Panel b, crystals of cleaved hVDAC1 in β-DDM after optimization of the conditions. Scale bars: 100 µm. Panel c, diffraction image of optimized crystals of hVDAC1 in β-DDM.

none of these crystals diffracted. UDM led to thin plates in similar conditions than C_8E_4, while cymal-6, cymal-7 and α-DDM crystallized in conditions deriving from that of β-DDM one. In UDM, hVDAC1 crystals diffracted to less than 20 Å. In cymal-6, hVDAC1 crystallized as very small rods, which after optimization diffracted only at very low resolution (30 Å). In cymal-7, hVDAC1 crystallized in the same form and in the same conditions than in β-DDM but diffracted poorly (30 Å). After optimization, hVDAC1 crystals obtained with α-DDM, diffracted slightly better than with β-DDM in term of resolution and isotropy (Fig. 4d). Space group and cell parameters derived from the diffraction data (Table 1) suggest that hVDAC1 in α-DDM and in β-DDM crystallizes in the same space group as in [16], but with larger unit cell parameters, mainly along the *c*-axis.

4. Discussion

Although VDAC structures have recently been described [16–18], the use of cell-free expressed membrane protein in the presence of detergent has never been reported for crystallization. Thus, the successful crystallization of human VDAC1 described herein, even with limited diffraction, proves that a large majority of the produced protein is well folded and demonstrates that detergent cell-free expression of membrane proteins is useful for structural studies. It is therefore noteworthy to highlight the advantages and the capabilities of this approach and to compare cell-free hVDAC1 crystals with the previously published ones.

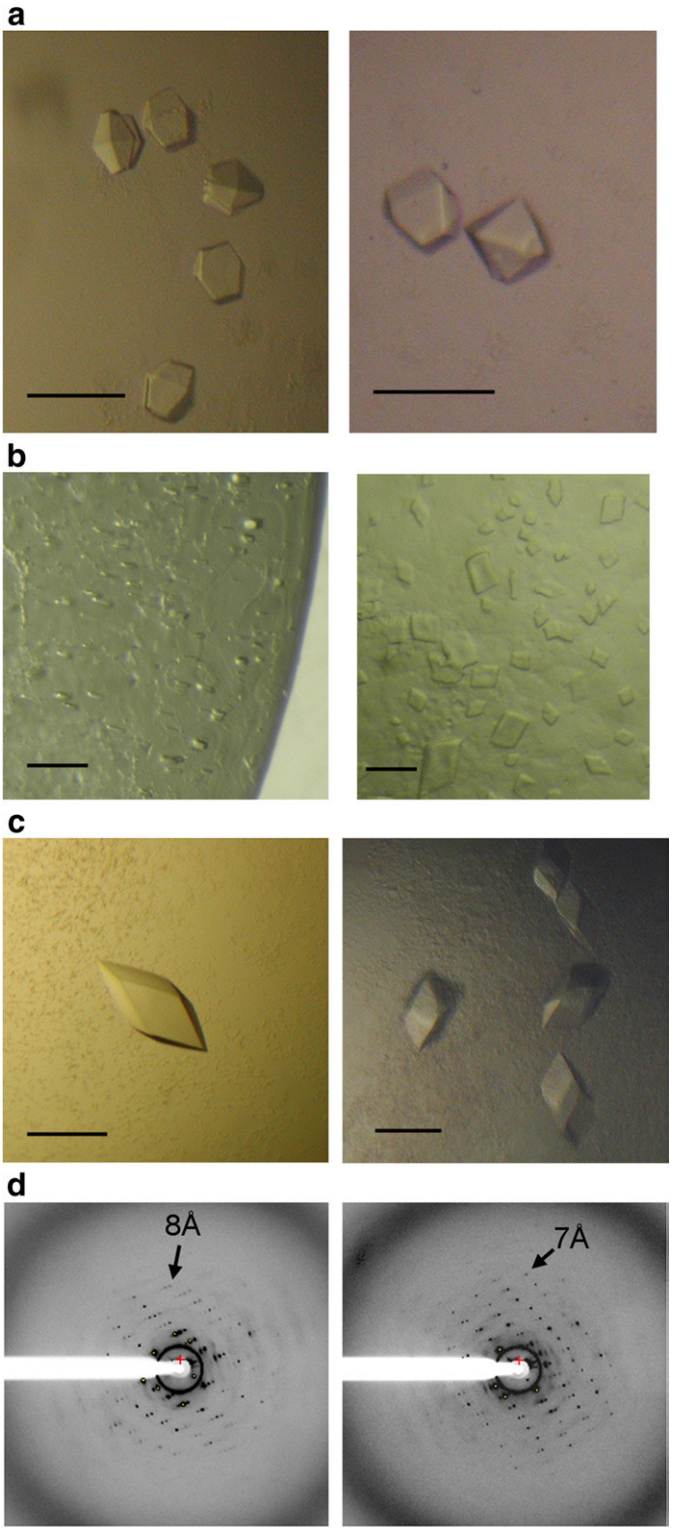

Fig. 4. Crystals of hVDAC1 in different detergents and diffraction of crystals in α-DDM. Panel a, crystals of hVDAC1 in C_8E_4. Panel b, microcrystals of hVDAC1 in cymal-6 (left picture) and plate crystals in UDM (right picture). Panel c, optimized crystal of hVDAC1 in α-DDM (left picture) and crystals of hVDAC1 in α-DDM in nanodrops (right picture). Panel d, diffraction images at 0° and 90° of an optimized crystal of hVDAC1 in α-DDM. Scale bars: 100 µm.

4.1. Advantages of the method

Cell-free expression of membrane proteins presents many benefits compared to all other expression systems. First of all, the simplicity

Table 1
Unit cell parameters of VDAC in β-DDM- or in α-DDM and parameters of the crystals obtained in cymal-5 [16]. In the three cases, the space group is P321.

Detergent	a (Å)	b (Å)	c (Å)	Diffraction limit (Å)
β-DDM	90	90	192	8
α-DDM	77	77	173	7
Cymal-5 from [16]	78	78	167	4.1

and the rapidity of the method for testing the expression of membrane protein along with a single purification step render this approach very attractive for crystallographic studies. Indeed, in the case of VDAC, only one to two months were required to go from the cloning to the pure protein, and diffracting crystals were obtained in less than six months. However, the time frame to go from gene to structure of any membrane protein would be different but always faster with cell-free expression than with any other expression method. Moreover, the strategy optimized for VDAC expression can be directly transferred to other membrane proteins. Indeed, for several membrane proteins tested in our laboratory, expression time between 24 and 48 h is optimal and reducing the temperature down to 20 °C is a prerequisite to obtain a good fraction of soluble protein in the presence of a surfactant. Due to residual bacterial proteases in the lysate, the use of protease inhibitors is also crucial to obtain a full-length protein after expression. Only the detergent has to be specifically chosen according to the level of expression and the quality of each protein. It is also noteworthy that cell-free expression allows to screen, for tens of different amphiphiles, the expression level and the solubility of a membrane protein in only two days. Similarly, several detergents can be explored by on-column exchange, in order to select the most appropriate for stabilizing and/or crystallizing the protein.

Secondly, the expression level of the system is insensitive to many factors. It is possible to express toxic proteins, which is a frequent characteristic of membrane proteins. Mutations, truncations and/or insertions have in general a minor effect on the expression level, as observed for three VDAC constructs with loop and β-strand deletions (data not shown). As long as they do not interfere with the transcription and/or translation process, it is possible to add various compounds in the expression mix. It can be a specific ligand or lipid that could help in the folding and/or could block a single conformation for the crystallization (*i.e.* addition of cholesterol was assayed in this study). This technique also allows to produce a labelled-protein, for NMR study [29] or crystallographic phasing, without important production decrease [30]. Moreover, seleno-methionine and/or -cysteine labelled protein expression is easily performed in this system and does not require the use of specific strains.

Finally, the cost of specifically-labelled protein production or the addition of ligands is reduced in cell-free system due to the small reaction volume (1 ml compared to 1 l).

4.2. Discussion of other cell-free expression concepts previously published

Frank Bernhard's group has intensively studied cell-free expression [10] of different membrane proteins, including G protein-coupled receptors (GPCR) [31]. Their constraints are different from ours, as they express proteins mainly for NMR. The idea developed in this manuscript is to obtain in one step the membrane protein in a soluble and functional form surrounded by a detergent compatible with crystallographic studies. NMR does not necessarily require homogeneous detergent belts around the protein. Therefore, among their favoured detergents they also consider not-chemically well-defined molecules (*i.e.* Brij). They also work on detergent-resolubilized aggregated protein synthesized in the absence of surfactants. It is known that such proteins display a conformational variability that hampers crystallization. Indeed, we have attempted the purification of hVDAC1-aggregated form. The pellet has been solubilized in different detergents. In the best cases, after purification the quantity of protein obtained was comparable to the one obtained after purification of the protein expressed in a soluble form. However, the crystallization of the resolubilized hVDAC1 only led to microcrystals that very poorly diffracted X-rays, illustrating the probable presence of slightly misfolded proteins.

Other cell lysates can also be explored for the cell-free expression. Kaiser et al. expressed functional GPCRs from wheat germ extracts [32]. Indeed, the folding of eukaryotic proteins in wheat germ extract could be facilitated by the presence of chaperones and co-factors not present in *E. coli*. This alternative expression has also been tested for hVDAC1. However, the expression level in wheat germ extract was found to be more sensitive to detergents than compared to the *E. coli* lysate. To circumvent this problem, the quantity of detergent such as β-DDM or cymal-5 has been decreased down to 2 to 4 cmc. But, this affected the quantity of soluble-expressed hVDAC1. Finally, pure hVDAC1 was obtained in low quantity, ten times less than from *E. coli* extracts. The crystallization of wheat germ expressed hVDAC1 led to microcrystals with poor diffraction.

4.3. Comparison with previously published VDAC crystallization

Three VDAC structures have recently been published [16–18], all based on VDAC expressed in *E. coli* inclusion bodies followed by a refolding procedure. The work done by the group of Zeth is the closest to ours, since VDAC has been crystallized from detergent solution [16]. The authors have tested many detergents [33] and obtained their best crystals in cymal-5 (4 Å). In DDM their crystals did not diffract better than 20 Å. It is interesting to notice that, in our study, VDAC did not crystallize in cymal-5, but led to crystals in five different maltoside detergents. The best crystals diffracted down to 7 Å in β-DDM and 6 Å in α-DDM. A comparison detergent by detergent shows that the proteins expressed in *E. coli* and refolded from inclusion bodies or expressed in the cell-free system behave very differently in crystallization. This demonstrates once again the interest of the method described herein as it opens a new field of exploration. The three crystal forms of VDAC, in cymal-5 (PDB: 2JK4, [16]) and in β- and α-DDM, have the same space group and similar unit cell dimensions (Table 1) suggesting comparable crystal packing. The VDAC structures published so far were based on proteins expressed in *E. coli* as inclusion bodies. The studies had to overcome several bottlenecks; the optimization of the refolding [19], two- and three-dimensional crystallization from refolded proteins [13], and obtention of X-ray diffracting crystals [34]. VDAC is one of the few cases of membrane protein crystallized after a refolding procedure [35]. Comparatively, our method has given interesting crystals in β-DDM, in more or less one year essentially due to the limited number of steps that have to be optimized. This difference in time scale is important to consider for future expression/purification/structural studies of other membrane proteins. Moreover, VDAC produced in cell-free expression system is highly stable over time and against proteases (Fig. 2a). This is consistent with the behavior of the native VDAC extracted from mitochondria and of porins in general. Therefore, our approach allowed the production of well-behaving membrane protein usable for crystallization and comparable to the native one, which is of high interest for structure/function relationships studies.

5. Conclusion

Cell-free expression system has been successfully used to solve structures of soluble proteins [36]. The strategy that we developed led to the successful crystallization of a cell-free expressed membrane protein: the human mitochondrial porin VDAC. Cell-free expression led to reproducible results in a short period of time, on a protein for which production and crystallization were shown to be a difficult task.

The strategy described herein should certainly be extended to other membrane proteins as an alternate approach.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2010.04.010.

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