Structural and functional characterization of *Solanum tuberosum* VDAC36

Running Title: Functional and structural study of VDAC

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ABSTRACT

As it forms water-filled channel in the mitochondria outer membrane and diffuses essential metabolites such as NADH and ATP, the VDAC (voltage-dependent anion channel) protein family plays a central role in all eukaryotic cells. In comparison with their mammalian homologues, little is known about the structural and functional properties of plant VDACs. In the present contribution, one of the two VDACs isoforms of *Solanum tuberosum*, stVDAC36, has been successfully overexpressed and refolded by an in-house method, as demonstrated by the information on its secondary and tertiary structure gathered from circular dichroism and intrinsic fluorescence. Cross-linking and molecular modelling studies have evidenced the presence of dimers and tetramers, and they suggest the formation of an intermolecular disulphide bond between two stVDAC36 monomers. The pore-forming activity was also assessed by liposome swelling assays, indicating a typical pore diameter between 2.0 and 2.7 nm. Finally, insights about the ATP binding inside the pore are given by docking studies and electrostatic calculations.

Keywords

Voltage-dependent anion channel, protein structure, oligomeric states, ATP binding, circular dichroism

1. Introduction

Voltage Dependent Anion Channels (VDACs) are pore-forming proteins found in the outer mitochondrial membrane of all eukaryotic cells. The function of those proteins is associated with the permeability of mitochondria. Indeed, they regulate the diffusion of ions and metabolites such as nicotinamide adenine dinucleotide (NAD) or adenosine triphosphate (ATP). In addition, VDAC proteins are involved in apoptosis, as well as in DNA and tRNA transport.[1–4] Their structural properties are highly conserved and they adopt a β-barrel structure generally composed of 19 antiparallel β-strands. This conformation leads to the formation of an inner channel restricted by a N-terminal α-helix that folds into the pore.[5] VDAC proteins usually show poor selectivity and are consequently described as general diffusion pore. Diffusion is mainly controlled by the size of the pore, which may change with the conformation of the locking α-helix.[6] In addition, the chemical nature of the inner cavity can also affect the selectivity as preferential diffusion of anions over cations was observed.[7]

Up to now, only ten VDAC 3D-structures have been described experimentally. The Protein Data Bank (PDB) contains seven VDAC1 from human, two VDAC1 from mouse, and one VDAC2 from fish. In contrast to VDAC1, the structural and functional characterizations of the other VDAC isoforms (2 and 3) are rather limited. Moreover, no consensus has been reached regarding the oligomeric state of VDAC proteins, and their actual role in nucleotides transport and apoptosis also remains unclear. Indeed, VDACs being integral membrane proteins, their overexpression and characterization are still challenging. Particularly, the structure and properties of plant VDACs are less described than their mammalian homologues.[3-8–13] In this context, we have studied POM36 (here termed stVDAC36), a VDAC isoform of the *Solanum tuberosum* outer mitochondrial membrane, which shows voltage-dependent and channel properties similar to mammalian isoforms.[14] This protein has recently been reported to be associated with the tRNA mitochondrial transport system.[8]

To gain insights into stVDAC36 structure and function, it was first expressed and refolded by following a protocol based on bacterial expression into inclusion bodies completed by an in-house SDS-MPD refolding method. The latter procedure has already been proven efficient for membrane proteins, including bacterial porins.[15–17] The stVDAC36 was then reconstituted into liposomes, allowing to assess its inner channel size by swelling assay. The oligomeric states of stVDAC36 were investigated by cross-linking experiments. A molecular model was also built to investigate the stVDAC36 structure at the atomic scale. Comparisons to experimental data were successfully carried out, whereas the residues forming the inner channel and potentially binding ATP were identified by docking studies and electrostatic calculations.

2. Methods

2.1 Expression and purification of stVDAC36. The cDNA encoding stVDAC36 was cloned into pQE60 vector by Salinas *et al,*[9] while *Escherichia coli* BL21 (DE3) were transformed with the vector to overproduce 6-His-tagged stVDAC36 proteins in inclusion bodies. Bacteria were grown in 800 mL of lysogeny broth **(**LB**)** medium at 37°C under constant agitation. When the absorbance at 600 nm reached 0.7, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added (final concentration of 2 mM) to stimulate the protein expression during 3h. Bacteria were then pelleted by centrifugation at 4000 g for 30 minutes at 4°C. The pellets were then suspended in the lysis buffer (50 mM Tris-HCl pH 8, 5 mM $MgCl_2$, 17 mM NaCl, 1% (w/v) Triton X-100, 1 mg/mL lysozyme, 1 mM PMSF). Sodium deoxycholate and DNAse I were added (final concentrations of 0.2% (w/v) and 0.125 mg/mL, respectively). The lysed solution was agitated at 37°C for two hours and then centrifuged at 9000 g for 30 minutes at 4°C. The supernatant was discarded and the pellet was washed twice with the washing buffer (20 mM Tris-HCl pH 8, 500 mM NaCl, 2% (w/v) Triton X-100). The resulting solution was

centrifuged at 5000 g for 10 minutes at 10°C. The final pellet incorporating the inclusion bodies was solubilized in the solubilizing buffer (20 mM phosphate pH 8, 1% (w/v) SDS).

The solubilized proteins were then loaded on a 5 mL HiTrap IMAC FF (GE Healthcare) previously prepared with Ni^{2+}. The column was previously equilibrated with buffer A (20 mM phosphate pH 8, 100 mM NaCl, 0.1% (w/v) SDS), and stVDAC36 was then eluted with a gradient of buffer B (20 mM phosphate pH 8, 100 mM NaCl, 0.1% (w/v) SDS, 250 mM imidazole). The protein buffer was exchanged using a PD-10 desalting column (GE Healthcare), depending on the subsequent treatments.

2.2 Circular Dichroism. Measurements were performed with a Jasco J-810 spectropolarimeter in the far-UV region (260 – 190 nm) at 20°C, using a 1 mm pathlength quartz Suprasil cell (Hellma), with protein concentrations of *ca.* 0.1 mg/ml. Four scans (50 nm/min, 1 nm bandwidth, 0.1 nm data pitch and 1 s DIT) were averaged, base lines were subtracted and no smoothing was applied. Data are presented as the molar residue ellipticity ($[\Theta]_{MRW}$) calculated using the molar concentration of protein and number of residues. Secondary structure analyses using the CDSSTR [18-19] algorithm were performed on the CD data with the Dichroweb analysis server [20-21] using the SMP180 reference set.[22]

2.3 Steady-state Fluorescence. Steady-state measurements were performed with a Varian Cary Eclipse spectrophotometer with a 2-mm path length cell. The excitation wavelength was 280 nm and the emission spectra were recorded from 290 to 450 nm with a 1 nm-step.

2.4 Reconstitution of stVDAC36 in liposome and swelling assay. Pure 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) was dissolved in chloroform, and the latter was evaporated using a rotary evaporator to obtain a thin homogenous film. The flask was then left one hour in a desiccator, and the lipid film was suspended in a buffer (20 mM phosphate

pH 7.4, 1 mM PEG6000) to obtain a final concentration of 2 mg/mL. The turbid solution was subsequently loaded on a mini Extruder (AvantiLipids) with 100 nm filter and extruded 30 times, resulting in a clear final solution.

The stVDAC36 was transferred in a buffer containing 20 mM phosphate pH 7.4, 0.2% (w/v) Triton X-100 by using a desalting PD10 column and then added to the liposomes. The final concentrations of protein and DOPC were 0.27 mg/mL and 1.4 mg/mL, respectively. The mixture was agitated for one hour. After that, 0.2 g/mL of BioBeads SM-2 (Bio-Rad) were added and the mixture was agitated two more hours to adsorb the detergent. The solution was then filtered for the beads removal.

Swelling assays were performed by adding 10 µL of proteoliposomes to 190 µL of test solution, *i.e.* 20 mM phosphate pH 7.4 and 1.05 mM of solute such as glycine, sugars or PEG. After addition of the proteoliposomes, the absorbance at 450 nm was recorded for five minutes. The absorbance values were normalized and the diffusion rate was calculated from the starting and final absorbance values.

2.5 Cross-linking. Three cross-linkers were used: dimethyl suberimidate (DMS, 11.0 Å), dimethyl adipimidate (DMA, 8.6 Å), and 1,5-difluoro-2,4-dinitrobenzene (DFDNB, 3.0 Å), that were dissolved in 20 mM phosphate buffer pH 8.5. Equal volumes of cross-linker solution and protein (~0.8 mg/mL) were mixed and incubated for 1.5 hours at 20°C under gentle agitation. The reaction was stopped by adding SDS-PAGE loading buffer (10% (w/v) SDS, 20% (w/v) glycerol, 0.2 M Tris-HCl pH 6.8, 0.05% (w/v) bromophenol blue). A 12% acrylamide SDS-PAGE was run for 50 minutes at 200V, and the gels were stained by Coomassie Blue.

2.6 Transmission electron microscopy. Transmission electron microscopy (TEM) pictures were obtained with a Philips TECNAI 10 microscope operating at 100 kV. Carbon-coated grids were hydrophilized by glow discharge. A 5 μL drop was deposited on the grid for 60 seconds. The grid was then rinced with water and 0.5% (w/v) of uranyl acetate was added for 120 seconds for a negative staining.

2.7 Model prediction and analysis. The RaptorX webserver (http://raptorx.uchicago.edu) was used to predict the 3D-model of stVDAC36 by using zebrafish VDAC2 (PDB ID: 4BUM) as main template.[23] The secondary structure content was evaluated by the DSSP algorithm.[24] The software Visual Molecular Dynamics (VMD) was used for visualizing the models and creating images,[25] while the electrostatic potential was calculated using the APBS software,[26] and docking studies of ATP inside the channel of stVDAC36 were performed using the GOLD program.[27] The binding region was defined as a 30 Å radius sphere centred on Arg-16, one of the residues in the α-helical N-terminal. For each of the 10 runs, a total of 100,000 genetic operations were carried out on five islands, each containing 100 individuals. The solutions were ranked by GOLD score.

3. Results and discussion

3.1 stVDAC36 refolding

Since the extraction and the purification of endogenous proteins from their native membranes may be both time and resources-consuming, a recombinant C-terminal His-tag version of stVDAC36 was first used to easily obtain pure protein with a high yield. After its production as inclusion bodies in *E. coli* and its further purification under denaturing conditions, the protein was refolded by dilution in a buffer containing sodium dodecylsulfate (SDS) and 2-methyl-2,4-pentanediol (MPD). We demonstrated that this peculiar detergent-alcohol association is able to successfully refold β-barrel proteins.[16-17] Indeed, MPD partially prevents SDS and water to interact with the protein, hence providing the suitable amphiphilic environment favoring its refolding. To determine the optimal operating conditions, a large panel of buffers has been considered, made of 20 mM phosphate pH 8, 66 mM NaCl and several concentrations of SDS and MPD. The protein was characterized by both circular dichroism (CD) and intrinsic fluorescence, as VDAC proteins have a high β-strand content with typical spectral features.[28] In the presence of SDS only (10-60 mM), a strong negative band near 205 nm, as well as weaker bands between 220 and 230 nm are observed (Figure 1A), which are typical of mainly unordered proteins, though α-helix structure may also be detected (Table 1).[29–32]

By adding an increasing amount of MPD, the CD spectra change to finally reach a signal corresponding to β-sheets and more particularly to a β-barrel structure (Figure 1B).[33–35] With a MPD concentration of 0.5 M, the minimum at ~205 nm is still present and it is concluded that this concentration is not sufficient to fully achieve the protein refolding. The CD spectrum corresponding to 30 mM SDS and 1 M MPD misses the minimum at ~205 nm and represents the transition between the unfolded state and the β-barrel structure. A suitable environment for the refolding is reached for the ratio 60 mM SDS and 1.5 M MPD, and the

CD spectrum in Figure 1B suggests that a β-barrel is indeed obtained. Interestingly, a similar ratio detergent:cosolvent is also required to obtain the highest refolding yield for Omp2a, a bacterial porin also forming β-barrel.[17] This is a new evidence of the effectiveness of the SDS-MPD refolding method.

The tryptophan (Trp) fluorescence is commonly used to study the protein refolding as it complements the CD analysis. The fluorescence of aromatic residues, mainly Trp and also Tyrosine (Tyr), is strongly affected by the polarity of the surroundings, thereby giving insights into the local environment of the residues (*e.g.* pointing towards the hydrophilic inner channel, or towards the lipid membrane). As stVDAC36 has only 1 Trp but 10 Tyr in its sequence, a 280-nm (for both Trp and Tyr) excitation wavelength was used to increase the sensitivity. In SDS-containing buffers, the stVDAC36 λ_{max} is 309 nm with a decreasing intensity when SDS concentration increases (Figure 2). This trend has also been observed for peptides denatured in SDS.[36] When adding MPD, a bathochromic shift towards a λ_{max} of 320 nm was observed, which corresponds to a more polar environment. This supports the recovery of the β-barrel conformation by addition of the MPD, as demonstrated by the CD analysis. Note that the red-shift between the SDS-unfolded and the native state was also pointed out for peptides.[37] The increasing of the fluorescence intensity also indicates that the Trp residue is less quenched after refolding. To sum up, the fluorescence results are in accordance with the information obtained from the CD analysis and support the evidences of the successful stVDAC36 refolding, which further expands the refolding-efficiency domain of the SDS/MPD method.

3.2 Structural studies of stVDAC36: secondary and tertiary structure

The CD data were analyzed in order to extract the percentage of each secondary structure pattern and thus improve our understanding of the conformational changes underwent by the protein during refolding (Table 1).

As illustrated by the high α-helix content for MPD-free conditions, SDS favors the formation of helix-like structures, as it was already observed for another plant porin.[38] When MPD is added, the micelles organization (CMC=2.6mM) is perturbed and the protein accessible surface for detergents is reduced,[39] and as a consequence, the α-helix content significantly drops. The turn content remains almost unchanged, independently of the SDS/MPD ratio. With 0.5 M and 1 M of MPD concentrations, the unordered structure contents are similar to what was observed for the denatured protein, whereas the β-strands formation is largely intensified by MPD. Finally, concentrations of 60 mM SDS and 1.5 M MPD give the highest amount of β-structures and the smallest of unordered structures, which perfectly matches the structural data found in the Protein Data Bank (PDB) for other VDAC proteins.

Membrane proteins remain a challenge for crystallographic studies and no plant VDAC structure has been resolved yet, leaving stVDAC36 exact structure still undescribed. To meet the need in structural information, molecular modelling is a cost and time effective alternative. As no VDAC homologue structure of stVDAC36 (sequence identity >25%) is available in the PDB to allow performing homology modelling, a 3D model of the protein was built by threading. The zebrafish VDAC2 structure (PDB: 4BUM) was the only template proposed by Raptor X, having the minimum required similarity of sequence with VDAC36.[40] As observed for experimental VDAC structures,[41] the resulting stVDAC36 model is composed of 19 β-strands separated by (i) short loops on the cellular side and (ii) turns in the intermembrane space. The β-strands contribute the most to the secondary content with a

percentage of 64% (Table 1), accordingly to the previously described CD spectra. Similarly to other β-barrels like porins, our stVDAC36 model presents two girdles of aromatic residues.[42] The N-terminal chain folds inside the barrel into an α-helix that forms a bottleneck restricting the size of the channel (Figure 3A). The resulting pore diameter (D_{pore}) is 1.56 nm, while the diameter of the channel, where the N-terminal extremity folds outside the barrel, is 2.16 nm (Figure 3B). The D_{pore} calculated from the model are in good accordance with other published results.[43–45]

3.3 Structural studies of stVDAC36: oligomerization studies by cross-linking

Structural and functional studies of some VDAC proteins have already been reported but their oligomeric state is still a matter of discussion. Indeed, previous works mention that monomers, dimers, trimers, tetramers and even hexamers have been hypothesized for this protein family,[46–48] ant it turns out that VDAC proteins retain their activity whatever its aggregation state, which makes difficult to establish any solid structure-property correlation. Nevertheless, evidencing the nature of the stVDAC36 oligomeric states would certainly help understanding the structural organization of the mitochondria outer membrane and its components, as well as the role of oligomerization in biological processes.[48–50] Indeed, a correlation between VDAC oligomerization and apoptosis has previously been reported [4]. In that context, cross-linking is a method of choice to characterize the quaternary structure of proteins. Three homobifunctional and irreversible cross-linkers were selected with several spacer lengths: DMS (11.0 Å), DMA (8.6 Å) and DFDNB (3.0 Å). DMS and DMA have an amine-reactive imidoester groups, and at pH 7-10, they all react with both N-terminal α-amines and lysine ε-amines. Though such cross-linkers leave unchanged the charge and native structure of the proteins and do not overreact with other nucleophilic groups, they have to be carefully handled as can lead to wide range of poorly defined oligomers, as well as large

aggregates of polymerized proteins. DFDNB contains active fluorine atoms reacting with amines, but it is less selective than DMS or DMA. This drawback can be partially circumvent by using a very low concentration of cross-linker combined to a short incubation time.

The tests were carried out with/without the β-mercaptoethanol reducing agent and the optimal refolding buffer (*i.e.* giving the highest β-sheets content) was selected for these experiments (60 mM SDS, 1.5 M MPD, 20 mM phosphate pH 8, 66 mM NaCl). Without β-mercaptoethanol, both DMA and DMS give strong bands at ~120 kDa, suggesting the presence of tetramers (Figure 4A). Interestingly, the intensity of this band is stronger than the one corresponding to the monomer. Note that even larger oligomers (> 250 kDa) could also be observed at the top of the gels. With DFDNB, weaker bands appear at ~30 and ~90 kDa, which corresponds to monomer and trimers, respectively, and the large majority of the protein has been aggregated.

When the reducing agent is added, stVDAC36 monomers still appear at the same apparent molecular weight of 30 kDa whereas the band at ~120 kDa disappears (Figure 4B). This result suggests that β-mercaptoethanol can prevent the formation of stable tetramers even though it does not lower the abundance of larger aggregates. Interestingly, a weak band shows up at ~60 kDa, indicating the possible degradation of the tetramers into dimers. The cross-linking experiments suggest that stVDAC36 can form dimers, trimers and tetramers, the latter being predominant but sensible to chemical conditions. Trimeric structures are associated to the weakest band which suggests a less probable state; they have therefore been discarded in the following discussion.

β-mercaptoethanol is known to reduce disulphide bonds, and we may therefore expect the tetramers are linked through such a bond. The single cysteine residue of stVDAC36 being

located on the external part of the β-barrel, it is indeed available for intermolecular disulphide bridge. On the basis of our stVDAC36 model, a dimer with a disulphide bond formed between the Cys158 residues of each monomer was therefore constructed (Figure 5). Its geometrical parameters were successfully optimized, which support its structural reliability and the model credibility. Interestingly, the zebrafish VDAC2 used as a template for the stVDAC36 model has also been proved to form dimers, though its single Cys residue does not participate in oligomerization.[40] This can be explained by the relative position of Cys, which is located at middle height of the barrel in zebrafish VDAC2, in comparison to stVDAC36 where it is found at the top of the β-barrel on the cytoplasmic side.

Previous reports on mammalian VDAC indicate the involvement of cysteine in the oligomerization but this has only been demonstrated for proteins with high content (up to nine) in cysteine residues.[51] On the other hand, it was proved that for human VDAC2 cysteines mainly exist in the reduced form, and that they are more likely to stabilize the β -barrel in an apolar environment [52]. Actually, the number of cysteines is variable across the mammalian isoforms and their role still need to be elucidated. In the case of plants, no study on the role of cysteines was performed yet, before our joint cross-linking and modelling study, which draw new hypotheses on the relation between cysteines and oligomerization.

3.4 Functional studies: pore-forming activity

As long as the electrophysiology of the VDAC channel is not concerned by the present study, it was not necessary to consider removing the C-terminal 6his-tag. Moreover, several studies on C-terminal 6His-tagged version of VDACs have shown pore properties similar to their native counterparts, and our results can therefore be confidently interpreted[53-54]

The purified stVDAC36 has consequently been reconstituted into large unilamellar vesicles (LUV) of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) prepared by extrusion. Phosphatidylcholine lipids are the main constituent of the mitochondria outer membrane where VDAC proteins are located.[55] DLS measurements were carried out and a hydrodynamic diameter of 148.4 ± 15.8 nm was recorded, whereas TEM images delivered an average diameter of 202.84 ± 31.35 nm (Figure S1). Micrographs were similar to those reported in literature.[56] The apparent bigger size of the liposomes by TEM is usually explained by the flattening of the vesicles after deposition onto the surface. The equation $d_{max} = \frac{\pi d}{2}$ gives the maximum diameter that a spherical object with diameter d can have after flattening.[57] Considering the latter equation, both DLS and TEM provided consistent sizes for DOPC liposomes.

Prior to functional assays, structural studies of stVDAC36 were conducted by CD to check the protein's conformational state after reconstitution. The spectrum of the proteoliposome is very similar to the one of the refolded protein (60 mM SDS-1.5 M MPD) (Figure 1B), and the calculated β-sheet content of 59% consistently confirms a correct folding of the protein inserted in DOPC liposomes. When compared to the spectrum of the SDS-MPD refolded protein, the slight changes observed in the CD signal can be attributed to the modifications of

the physical properties of the amphiphilic environment generated either by the SDS-MPD association or the DOPC liposomes themselves.[58]

Using liposome swelling assays, we evaluated the rate of penetration through the VDAC pore of several sugars as well as polyethylene glycol (PEG) chains of varying sizes (Figure 6). By following the evolution over time of the absorbance at 450 nm, which reflects the swelling of the liposome, we were able to evaluate the pore radius of stVDAC36. Such technique has demonstrated its full relevance to deal with very similar systems.[59–62] The selected solutes were classified according to their diameter (Table 2) considering that they adopt a spherical shape in water,[59-61–67] of course this approximation becomes less accurate as PEG length increases.

Protein-free liposomes were added to the solutions as negative controls, and no change in absorbance was observed. Consistently with previously reported results for reconstituted pore-forming proteins,[68] we noticed a more important swelling for smaller solutes (Figure 6), while no swelling was observed for PEG with a molecular weight equal or above 3500 Da. Consequently, we can conclude that the reconstituted stVDAC36 forms a channel where solutes with a molecular weight below 3500 Da are able to diffuse. The results also reveal a pore diameter of at least 2.0 nm, but smaller than 2.7 nm (Table 2).

The swelling assay results are fully consistent with our 3D model, as well as with other VDAC structures originating from other organisms.[44-45-69] The residual discrepancies may be explained by the elliptical shape of the VDAC pore, and by the spherical approximation used for the long tail PEG molecules as they could orientate along the main axis of the pore. In addition, the N-terminal locking α-helix, responsible of the channel gating, may have different conformations.[70-71] Due to equal concentrations of ions in the internal and external media, the

swelling data correspond to an open state.[10] However, the presence of high molecular weight PEG can induce ions partitioning in the vicinity of the proteins, hence resulting in local changes of the electrical potential.[72] This phenomenon can play a sensible role in the gating of the reconstituted stVDAC36, consequently altering the diffusion of the solutes.

3.5 Functional studies: ATP binding to plant VDAC

A closer look into the channel topology provides essential information about the mechanism of selectivity of transport proteins. Whilst ATP permeation through plant VDAC is still unknown, some new insights can be gained from our model.

VDACs were shown to have a high-conductance open-state, and lower-conductance partially closed-states, in which the flux of ATP molecules drops to zero.[53-73-74] Based on previous modelling results as well as the crystal structure of mVDAC1 bound to ATP, our model is supposed to be in the open state, with the N-terminal α-helix inside the pore and still allowing the permeation of ATP.[73] As for mammalian VDAC1, the electrostatic potential analysis shows that the stVDAC36 pore inner-face has a higher positive charged density, which is partly due to the N-terminal α-helix, and explains the preference for transporting anions, (such as ATP), over cations in the open conformation (Figure 7).[75]

A low-affinity binding site for ATP was identified in mVDAC1 in the N-terminal α-helical segment containing 3 positive (Lys-12, Lys-20, and Arg-15) and 2 negative (Asp-9 and Asp-16) charges facing the pore inner face.[75-76] Interestingly these residues are conserved in stVDAC36 (Lys-13, Arg-16, Asp-10, Asp-17), except for Lys-20 which is replaced by an arginine (Arg-21). Furthermore, an additional lysine (a serine in mVDAC) is present in the α-helix of stVDAC36, reinforcing the positive charge of the helix. Based on a docking study, several binding modes of ATP were identified inside the pore and interacting with the positive

residues of the N-terminal, supporting the hypothesis of a low-binding affinity. One of these binding modes involves the specific Arg-21 found in stVDAC36 and Lys-28 from the β1 sheet, both interacting with the ATP phosphate group (Figure 8).

4. Conclusion

In this work, one of the two VDACs isoforms of *Solanum tuberosum*, stVDAC36, has been successfully overexpressed and subsequently refolded in one step by the SDS/MPD method. The highest β-sheet content was reached with SDS and MPD refolding concentrations of 60 mM and 1.5 M, respectively. Supported by circular dichroism data, a stVDAC36 model was built and gave information on the structural properties of the channel protein, including the diameter of its pore. VDACs being known to adopt various oligomeric states in many different environments, the formation of oligomers was then considered by cross-linking experiments. Coupled with molecular modelling studies, they evidence the presence of dimers and tetramers, and they also suggest the involvement of cysteine residues to form an intermolecular disulphide bond between two stVDAC36 monomers.

Once the protein reconstituted into lipid vesicles, the pore-forming activity of stVDAC36 was also assessed by liposome swelling assays. It was deduced the protein could not diffuse solutes bigger than 3500 Da, resulting from a pore diameter of at least 2.0 nm but smaller than 2.7 nm. This is in agreement with the size of the pore calculated from our model and other known similar channels.

Finally, docking studies and electrostatic calculations have highlighted the higher positive charged density of the pore inner-face, partly due to the N-terminal α-helix. We were also able to identify low-affinity binding sites of ATP in stVDAC36.

All these data give new insights about the structure and function of plant VDACs, which are less described than their mammalian homologues. Further investigations on the endogenous

VDAC36 extracted from the native *solanum tuberos*um mitochondrial membrane will confirm the present results.

References

1. Shoshan-Barmatz V, Ben-Hail D, VDAC, a multi-functional mitochondrial protein as a pharmacological target, Mitochondrion, 2012, 12, 1, 24–34.
2. Salinas T, Duchêne A-M, Maréchal-Drouard L, Recent advances in tRNA mitochondrial import, Trends Biochem Sci, 2008, 33, 7, 320–329.
3. Homblé F, Krammer E-M, Prévost M, Plant VDAC: Facts and speculations, Biochim Biophys Acta - Biomembr, 2012, 1818, 6, 1486–1501.
4. Keinan N, Tyomkin D, Shoshan-Barmatz V, Oligomerization of the mitochondrial protein voltage-dependent anion channel is coupled to the induction of apoptosis., Mol Cell Biol, 2010, 30, 24, 5698–709.
5. Hiller S, Garces RG, Malia TJ, Orekhov VY, Colombini M, Wagner G, Solution Structure of the Integral Human Membrane Protein VDAC-1 in Detergent Micelles, Science (80-), 2008, 321, 5893, 1206–1210.
6. Teijido O, Ujwal R, Hillerdal C-O, Kullman L, Rostovtseva TK, Abramson J, Affixing N-terminal α-Helix to the Wall of the Voltage-dependent Anion Channel Does Not Prevent Its Voltage Gating, J Biol Chem, 2012, 287, 14, 11437–11445.
7. Colombini M, VDAC structure, selectivity, and dynamics., Biochim Biophys Acta, 2012, 1818, 6, 1457–65.
8. Salinas T, El Farouk-Ameqrane S, Ubrig E, Sauter C, Duchêne A-MM, Maréchal-Drouard L, Molecular basis for the differential interaction of plant mitochondrial VDAC proteins with tRNAs, Nucleic Acids Res, 2014, 42, 15, 9937–9948.
9. Salinas T, Duchene A-M, Delage L, Nilsson S, Glaser E, Zaepfel M, Marechal-Drouard L, The voltage-dependent anion channel, a major component of the tRNA import machinery in plant mitochondria, Proc Natl Acad Sci, 2006, 103, 48, 18362–18367.
10. Saidani H, Krammer E-M, Prevost M, Homble F, Plant VDAC Selectivity and Voltage-Dependence are Uncoupled, Biophys J, 2014, 106, 2, 791a.
11. Fischer K, Weber A, Brink S, Arbinger B, Schünemann D, Borchert S, Heldt HW, Popp B, Benz R, Link TA, Porins from plants. Molecular cloning and functional characterization of two new members of the porin family., J Biol Chem, 1994, 269, 41, 25754–60.
12. Carbonara F, Popp B, Schmid A, Iacobazzi V, Genchi G, Palmieri F, Benz R, The role of sterols in the functional reconstitution of water-soluble mitochondrial porins from plants, J Bioenerg Biomembr, 1996, 28, 2, 181–189.
13. Popp B, Schmid A, Benz R, Role of Sterols in the Functional Reconstitution of Water-Soluble Mitochondrial Porins from Different Organisms, Biochemistry, 1995, 34, 10, 3352–3361.
14. Heins L, Mentzel H, Schmid A, Benz R, Schmitz UK, Biochemical, molecular, and functional characterization of porin isoforms from potato mitochondria., J Biol Chem, 1994, 269, 42, 26402–10.
15. Kinzel J, Sauer DF, Bocola M, Arlt M, Mirzaei Garakani T, Thiel A, Beckerle K, Polen T, Okuda J, Schwaneberg U, 2-Methyl-2,4-pentanediol (MPD) boosts as detergent-substitute the performance of ß-barrel hybrid catalyst for phenylacetylene polymerization, Beilstein J Org Chem, 2017, 13, 1, 1498–1506.
16. Michaux C, Pomroy NC, Privé GG, Refolding SDS-Denatured Proteins by the Addition of Amphipathic Cosolvents, J Mol Biol, 2008, 375, 5, 1477–1488.
17. Roussel G, Perpète EA, Matagne A, Tinti E, Michaux C, Towards a universal method for protein refolding: The trimeric beta barrel membrane Omp2a as a test case, Biotechnol Bioeng, 2013, 110, 2, 417–423.
18. Manavalan P, Johnson WC, Variable selection method improves the prediction of protein secondary structure from circular dichroism spectra, Anal Biochem, 1987, 167, 1, 76–85.
19. Sreerama N, Woody RW, Estimation of Protein Secondary Structure from Circular Dichroism Spectra: Comparison of CONTIN, SELCON, and CDSSTR Methods with an Expanded Reference Set, Anal Biochem, 2000, 287, 2, 252–260.
20. Whitmore L, Wallace BA, DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data, Nucleic Acids Res, 2004, 32, Web Server,

W668–W673.
21. Whitmore L, Wallace BA, Protein secondary structure analyses from circular dichroism spectroscopy: Methods and reference databases, Biopolymers, 2008, 89, 5, 392–400.
22. Abdul-Gader A, Miles AJ, Wallace BA, A reference dataset for the analyses of membrane protein secondary structures and transmembrane residues using circular dichroism spectroscopy, Bioinformatics, 2011, 27, 12, 1630–1636.
23. Källberg M, Wang H, Wang S, Peng J, Wang Z, Lu H, Xu J, Template-based protein structure modeling using the RaptorX web server., Nat Protoc, 2012, 7, 8, 1511–22.
24. Kabsch W, Sander C, Dictionary of protein secondary structure: Pattern recognition of hydrogen-bonded and geometrical features, Biopolymers, 1983, 22, 12, 2577–2637.
25. Humphrey W, Dalke A, Schulten K, VMD: Visual molecular dynamics, J Mol Graph, 1996, 14, 1, 33–38.
26. Jurrus E, Engel D, Star K, Monson K, Brandi J, Felberg LE, Brookes DH, Wilson L, Chen J, Liles K, Chun M, Li P, Gohara DW, Dolinsky T, Konecny R, Koes DR, Nielsen JE, *et al.*, Improvements to the APBS biomolecular solvation software suite, Protein Sci, 2018, 27, 1, 112–128.
27. Jones G, Willett P, Glen RC, Leach AR, Taylor R, Development and validation of a genetic algorithm for flexible docking 1 1Edited by F. E. Cohen, J Mol Biol, 1997, 267, 3, 727–748.
28. Bayrhuber M, Meins T, Habeck M, Becker S, Giller K, Villinger S, Vonrhein C, Griesinger C, Zweckstetter M, Zeth K, Structure of the human voltage-dependent anion channel., Proc Natl Acad Sci, 2008, 105, 40, 15370–5.
29. Otzen D, Protein-surfactant interactions: a tale of many states., Biochim Biophys Acta, 2011, 1814, 5, 562–91.
30. Andersen KK, Oliveira CL, Larsen KL, Poulsen FM, Callisen TH, Westh P, Pedersen JS, Otzen D, Vej GW, C D-A, C D-A, The Role of Decorated SDS Micelles in Sub-CMC Protein Denaturation and Association, J Mol Biol, 2009, 391, 1, 207–226.
31. Otzen DE, Sehgal P, Westh P, α-Lactalbumin is unfolded by all classes of surfactants but by different mechanisms, J Colloid Interface Sci, 2009, 329, 2, 273–283.
32. Venyaminov SY, Yang JT, Determination of Protein Secondary Structure. Springer, Boston, MA, 1996.
33. Wallace BA, Lees JG, Orry AJW, Lobley A, Janes RW, Analyses of circular dichroism spectra of membrane proteins., Protein Sci, 2003, 12, 4, 875–84.
34. Roussel G, Matagne A, De Bolle X, Perpète EA a., Michaux C, Purification, refolding and characterization of the trimeric Omp2a outer membrane porin from Brucella melitensis, Protein Expr Purif, 2012, 83, 2, 198–204.
35. Tamm LK, Hong H, Liang B, Folding and assembly of β-barrel membrane proteins, Biochim Biophys Acta - Biomembr, 2004, 1666, 1–2, 250–263.
36. Roussel G, Caudano Y, Matagne A, Sansom MS, Perpète EA, Michaux C, Peptide-surfactant interactions: A combined spectroscopic and molecular dynamics simulation approach, Spectrochim Acta Part A Mol Biomol Spectrosc, 2018, 190, 464–470.
37. Michaux C, Roussel G, Lopes-Rodrigues M, Matagne A, Perpète EA, Unravelling the mechanisms of a protein refolding process based on the association of detergents and co-solvents, J Pept Sci, 2016, March, 485–491.
38. Popp B, Gebauer S, Fischer K, Flügge UI, Benz R, Study of Structure and Function of Recombinant Pea Root Plastid Porin by Biophysical Methods, Biochemistry, 1997, 36, 10, 2844–2852.
39. Roussel G, Rouse SL, Sansom MSP, Michaux C, Perpète EA, The role of 2-methyl-2, 4-pentanediol in sodium dodecyl sulfate micelle dissociation unveiled by dynamic light scattering and molecular dynamics simulations, Colloids Surfaces B Biointerfaces, 2014, 114, 357–362.
40. Schredelseker J, Paz A, López CJ, Altenbach C, Leung CS, Drexler MK, Chen J-N, Hubbell WL, Abramson J, High resolution structure and double electron-electron resonance of the zebrafish voltage-dependent anion channel 2 reveal an oligomeric population., J Biol Chem, 2014, 289, 18, 12566–77.
41. Hiller S, Abramson J, Mannella C, Wagner G, Zeth K, The 3D structures of VDAC represent a native conformation., Trends Biochem Sci, 2010, 35, 9, 514–21.

42. Lopes-Rodrigues M, Zanuy D, Alemán C, Michaux C, Perpète EA, 3D structure of a Brucella melitensis porin: molecular modelling in lipid membranes, J Biomol Struct Dyn, 2018, 1–13.
43. Colombini M, VDAC structure, selectivity, and dynamics, Biochim Biophys Acta - Biomembr, 2012, 1818, 6, 1457–1465.
44. Zimmerberg J, Parsegian VA, Polymer inaccessible volume changes during opening and closing of a voltage-dependent ionic channel, Nature, 1986, 323, 6083, 36–39.
45. Colombini M, Yeung CL, Tung J, König T, The mitochondrial outer membrane channel, VDAC, is regulated by a synthetic polyanion, Biochim Biophys Acta - Biomembr, 1987, 905, 2, 279–286.
46. Betaneli V, Petrov EP, Schwille P, The role of lipids in VDAC oligomerization., Biophys J, 2012, 102, 3, 523–31.
47. Hoogenboom BW, Suda K, Engel A, Fotiadis D, The Supramolecular Assemblies of Voltage-dependent Anion Channels in the Native Membrane, J Mol Biol, 2007, 370, 2, 246–255.
48. Zalk R, Israelson A, Garty ES, Azoulay-Zohar H, Shoshan-Barmatz V, Oligomeric states of the voltage-dependent anion channel and cytochrome c release from mitochondria., Biochem J, 2005, 386, Pt 1, 73–83.
49. Krause J, Hay R, Kowollik C, Brdiczka D, Cross-linking analysis of yeast mitochondrial outer membrane., Biochim Biophys Acta, 1986, 860, 3, 690–8.
50. Peng S, Blachly-Dyson E, Colombini M, Forte M, Determination of the number of polypeptide subunits in a functional VDAC channel from Saccharomyces cerevisiae., J Bioenerg Biomembr, 1992, 24, 1, 27–31.
51. De Pinto V, Reina S, Gupta A, Messina A, Mahalakshmi R, Role of cysteines in mammalian VDAC isoforms' function, Biochim Biophys Acta - Bioenerg, 2016, 1857, 8, 1219–1227.
52. Maurya SR, Mahalakshmi R, Cysteine Residues Impact the Stability and Micelle Interaction Dynamics of the Human Mitochondrial β-Barrel Anion Channel hVDAC-2, PLoS One, 2014, 9, 3, e92183.
53. Shuvo SR, Ferens FG, Court DA, The N-terminus of VDAC: Structure, mutational analysis, and a potential role in regulating barrel shape, Biochim Biophys Acta - Biomembr, 2016, 1858, 6, 1350–1361.
54. Engelhardt H, Meins T, Poynor M, Adams V, Nussberger S, Welte W, Zeth K, High-level expression, refolding and probing the natural fold of the human voltage-dependent anion channel isoforms I and II, J Membr Biol, 2007, 216, 2–3, 93–105.
55. de Kroon AIP., Dolis D, Mayer A, Lill R, de Kruijff B, Phospholipid composition of highly purified mitochondrial outer membranes of rat liver and Neurospora crassa. Is cardiolipin present in the mitochondrial outer membrane?, Biochim Biophys Acta - Biomembr, 1997, 1325, 1, 108–116.
56. Walsh P, Vanderlee G, Yau J, Campeau J, Sim VL, Yip CM, Sharpe S, The mechanism of membrane disruption by cytotoxic amyloid oligomers formed by prion protein(106-126) is dependent on bilayer composition., J Biol Chem, 2014, 289, 15, 10419–30.
57. Baxa U, Imaging of Liposomes by Transmission Electron Microscopy, in *Characterization of Nanoparticles Intended for Drug Delivery*, Humana Press, New York, NY, 2018, 73–88.
58. Miles AJ, Wallace BA, Circular dichroism spectroscopy of membrane proteins, Chem Soc Rev, 2016, 45, 18, 4859–4872.
59. Godbole A, Mitra R, Dubey AK, Reddy PS, Mathew MK, Bacterial expression, purification and characterization of a rice voltage-dependent, anion-selective channel isoform, OsVDAC4, J Membr Biol, 2011, 244, 2, 67–80.
60. Jacob-Dubuisson F, Channel Formation by FhaC, the Outer Membrane Protein Involved in the Secretion of the Bordetella pertussis Filamentous Hemagglutinin, J Biol Chem, 1999, 274, 53, 37731–37735.
61. Nikaido H, Rosenberg EY, Porin channels in Escherichia coli: studies with liposomes reconstituted from purified proteins., J Bacteriol, 1983, 153, 1, 241–52.
62. Robertson JWF, Rodrigues CG, Stanford VM, Rubinson KA, Krasilnikov O V., Kasianowicz JJ, Single-molecule mass spectrometry in solution using a solitary nanopore, Proc Natl Acad Sci, 2007, 104, 20, 8207–8211.
63. Tam CM, Tremblay AY, Membrane pore characterization—comparison between single and

multicomponent solute probe techniques, J Memb Sci, 1991, 57, 2–3, 271–287.
64. Schultz SG, Solomon AK, Determination of the effective hydrodynamic radii of small molecules by viscometry., J Gen Physiol, 1961, 44, 6, 1189–99.
65. Fee CJ, Van Alstine JM, Prediction of the Viscosity Radius and the Size Exclusion Chromatography Behavior of PEGylated Proteins, Bioconjug Chem, 2004, 15, 6, 1304–1313.
66. Kuga S, Pore size distribution analysis of gel substances by size exclusion chromatography, J Chromatogr A, 1981, 206, 3, 449–461.
67. Ma Z, LeBard DN, Loverde SM, Sharp KA, Klein ML, Discher DE, Finkel TH, TCR Triggering by pMHC Ligands Tethered on Surfaces via Poly(Ethylene Glycol) Depends on Polymer Length, PLoS One, 2014, 9, 11, e112292.
68. Senaratne RH, Mobasheri H, Jenner P, Lea EJA, Papavinasasundaram KG, Expression of a Gene for a Porin-Like Protein of the OmpA Family from Mycobacterium tuberculosis H37Rv Expression of a Gene for a Porin-Like Protein of the OmpA Family from Mycobacterium tuberculosis H37Rv, 1998, 180, 14, 3541–3547.
69. Colombini M, The VDAC channel: Molecular basis for selectivity, Biochim Biophys Acta - Mol Cell Res, 2016, 1863, 10, 2498–2502.
70. Koppel DA, Kinnally KW, Masters P, Forte M, Blachly-Dyson E, Mannella CA, Bacterial expression and characterization of the mitochondrial outer membrane channel. Effects of n-terminal modifications., J Biol Chem, 1998, 273, 22, 13794–800.
71. Guardiani C, Scorciapino MA, Amodeo GF, Grdadolnik J, Pappalardo G, De Pinto V, Ceccarelli M, Casu M, The N-Terminal Peptides of the Three Human Isoforms of the Mitochondrial Voltage-Dependent Anion Channel Have Different Helical Propensities, Biochemistry, 2015, 54, 36, 5646–5656.
72. Aksoyoglu MA, Podgornik R, Bezrukov SM, Gurnev PA, Muthukumar M, Parsegian VA, Size-dependent forced PEG partitioning into channels: VDAC, OmpC, and α-hemolysin., Proc Natl Acad Sci U S A, 2016, 113, 32, 9003–8.
73. Choudhary OP, Ujwal R, Kowallis W, Coalson R, Abramson J, Grabe M, The Electrostatics of VDAC: Implications for Selectivity and Gating, J Mol Biol, 2010, 396, 3, 580–592.
74. Rostovtseva T, Colombini M, VDAC channels mediate and gate the flow of ATP: implications for the regulation of mitochondrial function, Biophys J, 1997, 72, 5, 1954–1962.
75. Ujwal R, Cascio D, Colletier J-P, Faham S, Zhang J, Toro L, Ping P, Abramson J, The crystal structure of mouse VDAC1 at 2.3 A resolution reveals mechanistic insights into metabolite gating, Proc Natl Acad Sci, 2008, 105, 46, 17742–17747.
76. Choudhary OP, Paz A, Adelman JL, Colletier J-P, Abramson J, Grabe M, Structure-guided simulations illuminate the mechanism of ATP transport through VDAC1, Nat Struct Mol Biol, 2014, 21, 7, 626–632.

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Table 1: Calculated secondary structure content of stVDAC36 in different refolding conditions, obtained from far-UV CD data.

Table 2: Diameter and diffusion rate of the solutes used for the liposome swelling assay. The diffusion rate was calculated from the initial and last absorbance values (after 300 seconds).

Figure 1: Far UV circular dichroism spectra of stVDAC36. Following purification, the protein was incubated in different refolding buffers composed of 20 mM phosphate, pH 8 and 66 mM NaCl, and containing various concentrations of sodium dodecylsulfate (SDS) and 2-methyl-2,4-pentanediol (MPD). A) The protein is unfolded regardless the concentration of detergent. B) Spectra show a distinct β-barrel signature in the presence of MPD.

Figure 2: Steady-state intrinsic fluorescence spectra of stVDAC36 in buffers composed of 20 mM phosphate pH 8 and 66 mM NaCl, and with different concentrations of SDS and MPD. The λ_{max} is shifted from 309 to 320 nm upon addition of MPD.

Figure 3: stVDAC36 model built by threading. Secondary structure is displayed with the following colors: β-strands (yellow), α-helix (red), turns (cyan) and loops (white). A) Side views with the maximum diameter of the channel in blue. B) Side view without the N-terminal α-helix.

Figure 4: SDS-PAGE of stVDAC36 after incubation with three cross-linkers of several space lengths: dimethyl suberimidate (DMS, 11.0 Å), dimethyl adipimidate (DMA, 8.6 Å) and 1,5-difluoro-2,4-dinitrobenzene (DFDNB, 3.0 Å). The buffer is composed of phosphate pH 8, 66 mM NaCl, 60 mM SDS and 1.5 M MPD. The tests were carried out without (A)/with (B) the β-mercaptoethanol reducing agent

Figure 5: A) Side view and B) top view representation of the modelled stVDAC36 dimer with the intermolecular disulphide bond formed by the Cys158 residues of each monomer. Secondary structure is displayed with the following colors: β-strands (yellow), α-helix (red), turns (cyan) and loops (white).

Figure 6: Liposome swelling assay of stVDAC36 reconstituted in DOPC liposomes. The absorbance was recorded at 450 nm after dilution of the proteo-liposomes into the test solutions containing glycine, saccharides or poly(ethyleneglycol) (PEG) of various sizes.

Figure 7: Electrostatic potential (units: k_bT/e) displayed on the stVDAC36 accessible surface.

Figure 8: Proposed ATP (carbon atoms are in orange) binding mode inside stVDAC36.