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The 3D structures of VDAC represent a native conformation

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Abstract

The most abundant protein of the mitochondrial outer membrane is the voltage-dependent anion channel (VDAC), which facilitates the exchange of ions and molecules between mitochondria and cytosol and is regulated by interactions with other proteins and small molecules. VDAC has been extensively studied for more than three decades, and last year three independent investigations revealed a structure of VDAC-1 exhibiting 19 transmembrane β -strands, constituting a unique structural class of β -barrel membrane proteins. Here, we provide a historical perspective on VDAC research and give an overview of the experimental design used to obtain these structures. Furthermore, we validate the protein refolding approach and summarize biochemical and biophysical evidence that links the 19-stranded structure to the native form of VDAC.

The discovery of VDAC and early structural work

In 1966, Chance and colleagues identified protein patches in negatively stained electron microscopy (EM) images from the mitochondrial outer membranes (MOM) of plants [1]. Ten years later, an enriched mitochondrial membrane fraction from *Paramecium aurelia* was incorporated into planar lipid bilayers and a voltage-dependent anion-selective channel (VDAC) was first identified [2]. Another decade passed before immuno-EM images from the MOMs of *Neurospora crassa* revealed that the protein features, originally identified by Chance, were in fact VDAC molecules and the stain densities defined actual transmembrane “holes” [3,4]. Shortly after this milestone discovery, the importance of VDAC as the major channel in mitochondrial outer membranes became evident with the isolation and characterization of VDACs from many diverse eukaryotic sources [5,6].

Since its initial discovery in 1976, extensive structure/function analyses of VDAC proteins were conducted on material isolated from native mitochondria as well as recombinantly produced VDAC proteins showing many conserved structural and functional properties (Figure

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1) [7-13]. A prominent feature of the pore emerged: when reconstituted into planar lipid bilayers, there is a voltage dependent switch between an anion-selective high-conductance state with high metabolite flux and a cation-selective low-conductance state with limited passage of metabolites. The consistency of experimental data from different VDAC isoforms, as well as protein sources, established a general model of structure and function, although a few exceptions have been reported (e.g. yeast VDAC-2) [14,15]. This view was strengthened by a number of other observations: *i*) the VDAC polypeptide is highly similar in total length (e.g. 283 amino acid residues in yeast and human VDAC-1) and composition between distantly related organisms (e.g. similarity/identity values of 67%/24% between yeast and human VDAC-1). *ii*) Both atomic force microscopy (AFM) and EM studies on native as well as reconstituted mitochondrial outer membranes from multiple sources revealed a channel with a wall-to-wall diameter of 34–38 Å (Figure 1 and Table 1).

Topology models of VDAC

Based on its similarities with bacterial outer membrane proteins, it was generally accepted that VDAC predominantly adopts a β -barrel fold. However, the number and relative orientation of the β -strands was extensively debated. Topology predictions for VDAC varied dramatically with strand numbers ranging from 12 to 19. Two principal approaches were used to derive topological arrangement of VDAC: (i) Bioinformatic analysis of the VDAC amino acid sequence and (ii) experimental techniques, often utilizing the voltage gating behavior of the protein, as well as various microscopic modalities.

As early as 1987, Forte et al. used the Delphi computer algorithm for a topology prediction, resulting in a 19-stranded β -barrel model which very closely resembles the recently determined 3D structures [16] (Fig. 2A). Later, Blachly-Dyson et al. used mutational scanning of the sequence and voltage-gating experiments in planar lipid bilayers to suggest a pore composed of 12 β -strands and an N-terminal helix as part of the barrel wall [8]. This model was further refined by Song et al. using biotin-binding in conjunction with voltage-gating experiments to include an additional β -strand (13 β -strands) and still maintained the N-terminal helix as part of the barrel wall [17] (Fig. 2B). Casadio et al. applied neural network-based predictors, resulting in a 16-stranded model [18] whereas Runke et al. used truncation and deletion experiments, voltage-gating experiments and tryptophan fluorescence resulting in a different 16-stranded model [19]. A third 16-stranded model was proposed by Engelhardt et al. based on protease digestion and theoretical considerations [20]. Finally in 2007, Young et al. carried out an extensive analysis of VDAC sequences including intron and exon evolution, resulting in a 16 stranded model common for all VDAC isoforms [15]. In spite of these extensive structure, function and bioinformatic studies performed by a number of different groups using a plethora of techniques, there was no clear consensus on the overall topology of VDAC.

Determination of the 3D structures of VDAC

In 2008, more than 30 years after its initial discovery, three independent structural projects of VDAC-1 were completed by the laboratories of four of the presenting authors [21-23]. The first of the three structures was solved by multi-dimensional NMR spectroscopy using human VDAC-1 (hVDAC-1) solubilized in LDAO micelles [21]. The second applied a hybrid approach using crystallographic data to 4.1 Å resolution in combination with NMR restraints again of hVDAC-1 in the detergent Cymal-5 [23]. The third structure was for mouse VDAC-1 (mVDAC-1; differs from hVDAC-1 by 4 conservative single amino acid substitutions) crystals grown in a lipidic medium by X-ray crystallographic techniques at a resolution of 2.3 Å [22].

The three structures were determined by two high-resolution techniques (NMR and X-ray diffraction) from proteins maintained in multiple detergents (LDAO and cymal-5) as well as in DMPC lipid bicelles, yet the overall structures of the pore domain are very similar yielding

an RMSD of 1.5 Å over 171 C α atoms between the NMR and X-ray structures (Figure 3). The 3D structures of VDAC-1 revealed some exciting structural features. First, VDAC-1 represents a new structural class of outer membrane β -barrel proteins with an odd number of strands. The 19-stranded β -barrel has a shear number of 20 as the main architectural unit, where strands β 1 and β 19 form parallel β -sheet pairing (Fig. 3). In addition, the N-terminal segment of 25 residues forms a partial α -helical structure that transverses the pore but is not part of the barrel wall. Another surprising aspect is that the negatively charged side chain of residue E73 is oriented towards the hydrophobic membrane environment.

The existence of this rare side chain orientation in VDAC had previously been revealed by the De Pinto group. Their experiments were based on an earlier discovery that the hydrophobic compound dicyclohexylcarbodiimide (DCCD) can form covalent cross links to individual carboxyl side chains of proteins [24]. Because DCCD is very hydrophobic, it reacts only with few, if any, carboxyl groups of a given protein, namely those that are embedded in a hydrophobic environment. When incubated with low concentrations of DCCD only three members of the mitochondrial proteome are found to react: two components of the F₀F₁ ATPase and VDAC [25]. By using protease digestion, mass spectrometry and polypeptide sequencing methods, De Pinto and coworkers could unambiguously identify that for VDAC just a single residue, glutamic acid 73, is affected by DCCD, leading to the conclusion that this side chain is oriented facing the hydrophobic membrane interior [9]. This biochemical result is now perfectly confirmed with our structures (Fig. 3G), and this feature is further of particular interest, because it allows a validation for alternative structural models.

Does the 3D structure reflect the “native state” of VDAC?

Because the identical fold has been obtained by three different technical approaches, and from proteins maintained in LDAO and Cymal 5 micelles as well as in DMPC/CHAPSO bicelles, there is no doubt that these structures describe a low-energy conformation of VDAC-1 [26]. However, in a recent opinion paper in this journal, Marco Colombini – a renowned VDAC researcher – raised the question whether the reported VDAC-1 3D structures represent the protein in its native environment and came to the conclusion that it does not, in part because the VDAC-1 proteins used in these structural studies were refolded from inclusion bodies [26]. Instead, the author favors a structural model derived by his own group, in which 13 β -strands and a single α -helix form the wall of the VDAC barrel. As stated above, this topology model was one of the many predictions based on biochemical experiments in artificial lipid planar bilayers reconstituted with VDAC isolated in detergent from mitochondria. Thus, like the 3D structures, the experimental data for this 2D topology model was also not obtained in the protein's native environment (Fig. 1).

General validity of using refolded protein for structural studies

For both NMR and X-ray diffraction techniques, milligram quantities of pure and homogeneous protein are required. To overcome the limitations of low endogenous expression as well as the difficulties resulting from purifying proteins, recombinant techniques are commonly employed where a host organism is exploited to express the protein of interest. Frequently, the expressed protein can not be correctly folded by the host organism and inclusion bodies are formed [27]. After the inclusion bodies have been isolated from the host organism, these macroscopic aggregates require that the protein be denatured and refolded to adopt its correct fold. This technique is frequently used for structure-function studies and was utilized for all three-structure determination projects of VDAC-1. It was also used for biochemical characterization of VDAC-1 with additional techniques, such as circular dichroism (CD)-spectroscopy and functional assays in lipid planar bilayers, prior to 3D structure determination [22,28,29].

Since its first usage, many researchers have been skeptical of protein refolded from inclusion bodies, in particular for membrane proteins. However, the technique has been validated extensively through both structure and functional analysis. There are currently over 60,000 structures deposited in the PDB (November 2009), of which at least 1000 were determined from refolded protein. This proportion is significantly larger for β -barrel membrane proteins, where 55% of the 44 bacterial β -barrel membrane protein structures were derived for proteins refolded from inclusion bodies [30]. Furthermore, in at least seven structural studies of β -barrel membrane proteins, the structures have been determined both from refolded inclusion bodies and with protein isolated from natural source, and the pairs of structures have turned out to be essentially identical (Table 2).

Characteristics of the 13-stranded topology model

In contrast to topology predictions and the three 3D structures, the 13 β -strands+1 α -helix model favored by Colombini is unprecedented, and unsupported by 3D structural data and energetic considerations. The model consists of 13 β -strands and an α -helix that is supposed to be part of the wall of the barrel. To date, there are more than 200 unique structures of integral membrane proteins [31] forming two distinct classes: β -barrels and α -helical membrane proteins. No fold even remotely similar to the suggested hybrid α -helix/ β -stranded barrel protein has been observed. Furthermore, such a structure would be energetically unfavorable: In a β -barrel, all backbone amide protons and carbonyl groups are involved in hydrogen bonds. Thus, to incorporate an α -helix as part of the wall, the helix would have to disrupt this favorable hydrogen bonding pattern and itself form hydrogen bonds by connecting acceptors and donors from two flanking β -strands solely through side chain interactions. Side chains suitable for such action are clearly not present at appropriate positions in the N-terminal helix of VDAC and it is not even clear if such a scenario would be possible at all. Moreover, the number and location of the 13 β -strands is contradicted by state-of-the-art secondary structure prediction methods, which indicate 19 β -strands for all VDAC representatives [32,33]. Finally, in the 13 β /1 α -model residue E73 is not located in a transmembrane strand, in disagreement with the results from the De Pinto group, as described above[SC1]. Taken together, it seems highly unlikely that an energetically stable hybrid structure of α -helical and β -barrel secondary structure could be formed for VDAC-1.

Linking the 3D structures to AFM and EM data

There are several experimental findings that establish a convincing link between the refolded recombinant protein and the native state of VDAC-1 in the MOM. (i) One of the strongest correlations is provided through comparison with the architecture observed from EM and AFM measurements performed on both reconstituted protein as well as native MOM (Fig. 3 and Table 1). All of the reconstructions define a backbone diameter in the range of 34–38 Å which matches the values observed in the 3D structures. A 19-stranded β -barrel with a shear number of 20 forms a pore with a 34 Å diameter, facilitating the large conductance values observed in VDAC. At the same time, these EM and AFM data provide yet another strong argument against the 13 β /1 α -model. To achieve the required diameter of 34 Å with a 13-stranded barrel, an unusually large tilt angle of 58° would be needed, which is clearly outside the accessible range for β -barrel proteins (Figure 4). Such a large angle would also result in substantially longer β -strands which are not consistent with the amino acid sequence of VDAC. (ii) Refolded VDAC has the same electrophysiological characteristics as natively isolated VDAC [28,29]. This is highlighted by the mVDAC-1 protein, which readily forms the characteristic channel properties in lipid planar bilayers [22]. (iii) The mVDAC-1 structure was determined in bicelles which are composed from DMPC lipids (dimyristoyl PC, 14:0) and are very similar to the DiPHPC lipids (diphytanoyl PC, 16:0) used for generating lipid planar bilayers in which functional assays are formed [22]. With this approach, the protein used for functional characterization

and crystallization will have the same structure. (iv) Binding of β -NADH is observed both for isolated VDAC in voltage gating experiments and for recombinantly refolded VDAC in LDAO micelles [10,21], further strengthening the likelihood that the two forms of VDAC feature the same structure.

Concluding remarks[SC2]

Protein refolding for structure-function studies is a routine and well-accepted technique for isolating β -barrel membrane proteins. Structures using refolding methods have been validated on both a functional, as well as a structural level, to form native-like folds. Furthermore, refolded VDAC-1 (from both mouse and human) has been well characterized by MS, CD, EM and functional assays in lipid planar bilayers. We are aware that, within the large body of published biochemical and electrophysiological results on VDAC reconstituted into lipid bilayers, some data exist that at first sight seem to be inconsistent with these structures (as pointed out by Colombini [26]). We are, however, not deeply concerned about such discrepancies, which are intrinsic to the scientific process [34]. We expect that the explanations for the discrepancies will be provided by future scientific studies, some of which may already be in progress. The 19-stranded 3D structure, obtained under different experimental sources from 3 different labs, fits EM and AFM data from native membrane sources and represents a biologically relevant state of native VDAC-1.

Box 1

The function of VDAC

The VDAC pore allows diffusive passage of different substances across the outer mitochondrial membrane. In its open state, ions and small molecules such as ATP, ADP, water, metabolites and cofactors can pass through VDAC. An upper limit for the substrate size is given by the dimensions of the VDAC pore. Small proteins up to about 5 kDa can diffuse through VDAC, and it has also been suggested that linear DNA molecules could use VDAC to cross the membrane [35]. A well-known example of a protein that cannot diffuse through VDAC is cytochrome c, which is too large to cross VDAC in its folded form.

The most prominent functional feature of VDAC is its voltage gating activity, which has been extensively studied *in vitro* [8,17,36]. The channel has a conductance of about 4 nS in the open state, and at a membrane potential above +30 mV or below -30 mV, the channel switches into a partially closed state, with about 2 nS conductance. In the partially closed state, the channel is cation selective, whereas it is anion selective in the open state. The precise location of the voltage sensor in the protein is not known. In connection with the 13 β /1 α -structural model it has been suggested that the mobile voltage sensor comprises residues 1–23, 39–48, 55–65, 79–88, 144–153, 275–283 and that these residues move collectively in- and outside the barrel upon voltage gating (Figs. 2B & D) [37]. If this gating model was true, it would need to explain how the stability of the β -barrel is maintained while parts of its wall are absent. Rather, the 3D structures at hand make it plausible that just the N-terminal domain (residues 1–23) is involved in gating, and that its motion may be the primary gating mechanism [22,38]. Although the bilayer voltage-gating feature is highly conserved among VDACs from different organisms and among VDAC isoforms, it has not yet been unambiguously correlated with a biological function. Possibilities include regulation of cellular energy production by control of metabolite flux across the MOM and an involvement in apoptosis [39,40]. Other intriguing functional features of VDAC include its site-specific interactions with the small molecules NADH and cholesterol and the cations gadolinium and calcium, which may have individual regulatory purposes [21,41–43]. VDAC also interacts with other proteins, such as hexokinase, and possibly also with creatine

kinase and adenosine nucleotide transporter [44,45]. Of particular interest are the interactions with BCL-2 [SC3]family proteins, which play a role in apoptosis [46,47]. These interactions are dependent on the isoforms of VDAC as well as on those of the BCL-2 proteins [46] and could thus provide an additional assay to confirm the functioning of refolded VDAC samples.

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Figure 1. Experimental approaches for VDAC characterization

The two primary production sources of VDAC protein are indicated by gray rectangles (native expression and inclusion bodies). Black rectangles designate selected preparative states of VDAC together with characterization techniques that have been used in these states (AFM: atomic force microscopy; EM: electron microscopy; NMR: nuclear magnetic resonance; VG: voltage gating; X-ray: X-ray crystallography). The small figures illustrate the experimental findings, reproduced with permission from [4,11,13,22,48,49][SC4]. The black arrows designate the preparative paths leading to individual experimental states and the procedures along these paths are given in magenta. Blue arrows highlight consistent experimental findings from the same technique applied to protein samples prepared by native expression in mitochondria and heterologous expression in inclusion bodies. All figures were prepared using PYMOL [50].

Figure 2. Predicted and experimentally determined topologies of VDAC
(A) Structural model of VDAC by Forte et al. as determined by the Delphi algorithm in 1987 (reproduced with permission from [16]). Bold letters indicate amino acid residues lining the aqueous pore, small letters interact with the hydrophobic membrane. The 19 β -strands as observed in the 3D X-ray and NMR structure are indicated by red rectangles. (B) Model developed by the Colombini and coworkers, suggesting the VDAC pore is formed by 13 β -strands and 1 α -helix (reproduced with permission from [26]). Residues in black circles denote the edges of the β -strands. Residues marked red were proposed by Colombini and coworkers to comprise the voltage sensor [37]. (C) Topology of VDAC-1 with 19 β -strands as determined by 3D NMR and X-ray structures [21-23]. Bold residues point towards the hydrophobic phase,

the other residues into the aqueous pore. The blue cylinders indicate the alpha helix which extends into the pore but is not part of the wall. (D) 3D structure of VDAC-1 (blue; PDB-entry: 3EMN). The voltage sensor proposed by Colombini and coworkers is indicated in red (compare also to panel B) [37].

Figure 3. Structural features of VDAC

(A) Examples of data from each of the three experimental approaches for the VDAC-1 structure determination, from left to right: [21-23]. (B) Resulting 3D structures of VDAC. (C) Overlay of the three structures in top and side view, yielding an RMSD of 1.5 Å over 171 C $^{\alpha}$ atoms between the NMR-only and X-ray only structure. (D–G) Structural features of VDAC. (D, E) Cartoon representation of VDAC-1 viewed parallel (D) and perpendicular (E) to the plane of the membrane. The VDAC-1 protein structure is colored from the N-terminus in blue to the C-terminus in red. VDAC forms a large pore conducive to high metabolite flux representing the open conformation. The N-terminal helix contacts the inner barrel wall and is not part of the pore. (F) The unique parallel strand arrangement between strand 1 and 19 revealing a new class of β -barrel proteins. (G) Location of Glu 73 residing in the lipidic environment. (H) The 3D structure overlaid onto EM [4] (left panel) and AFM images [49] (right panel). The

arrangement of a VDAC hexamer is putative, based on the observed 2D crystal packing of VDAC-1. In the AFM images the hexamer overlap is poor because of a tilt of the AFM image.

Figure 4.

Geometrical constraints for β -barrel membrane proteins. The wall-to-wall diameter d of ideal, circular β -barrels is plotted versus the tilt angle α of the β -strands against the membrane normal, following the approach by Schulz [51]. For a given number of strands, N , the accessible d/α values are located on hyperbolic curves. A 19-stranded barrel with a shear number of 20, corresponding to VDAC-1, is indicated by a yellow circle. Blue circles denote all known monotopic integral β -barrel membrane protein structures. The range 37° – 45° of the tilt angles α , which all known such structures are limited to is underlined grey. A hypothetical 13-stranded barrel with a diameter of $34 \text{ \AA}$ is shown red, resulting in a tilt angle of 58° .

Table 1
[SC5] Diameters of VDAC as determined by various methods^a

Method	pore diameter	backbone diameter	Refs
Electron microscopy		38 Å	[4,7]
Atomic force microscopy	27 Å		[13]
Atomic force microscopy		33±5 Å	[49]
Theoretical calculations: 19-stranded barrel with shear 20[SC6]		34 Å	[51]
NMR / X-ray VDAC-1 3D structures		35 Å	[21-23]

^a Because of the average side chain length of 5 Å, the pore diameter is expected to be 10 Å smaller than the backbone diameter.[SC7]

Table 2
Comparison of integral β -barrel membrane proteins, of which native as well as refolded samples were studied.

case #	Protein name	Organism	Number of strands	Preparation type	PDB code	X-ray resolution	rmsd ^a [Å]	Refs
1	porin	<i>R. blasticca</i>	16	native	1PRN	1.96	0.2 ^b	[52]
				refolded	---	2.2		[53]
2	OmpG	<i>E. coli</i>	14	native	2FIC	2.3	0.52 ^c	[54]
				refolded	2IWW	2.7		[55]
3	OpcA	<i>N. meningitidis</i>	10	native	1K24	2.02	--- ^d	[56]
				refolded	---	2.01		[57]
4	NalP	<i>N. meningitidis</i>	12	refolded	1UYN	2.6	1.7 ^b	[58]
				native	2QOM	2.66		[59]
5	PagP	<i>E. coli</i>	8	native	1MM4	NMR	1.8 ^b	[60]
				refolded	1THQ	1.9		[61]
6	TbuX	<i>R. pickettii</i>	14	native	3BRY	3.2	1.45 ^c	[62]
				refolded	3BS0	2.6		
7	FepA	<i>E. coli</i>	22	native	1FEP	2.4	--- ^d	[63]
				refolded	---	---		[64]

^a root mean square deviation of the C α atoms in the two structures
^b taken from the respective references
^c calculated using PYMOL
^d data sets from native and refolded samples were judged by the authors to be essentially identical