Opinion

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The published 3D structure of the VDAC channel: native or not?

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The recently published 3D structures of the mitochondrial voltage-dependent anion-selective channel (VDAC) are almost identical to each other. However, they are in conflict with the results of biochemical and functional studies published in the past 18 years. Transmembrane folding patterns based on many biochemical and functional studies differ from the 3D structures in the exclusion of distinct transmembrane strands. These differences might be the consequence of changes observed *in vitro* that result in the formation of channels with the characteristic functional properties of VDAC. Is it possible to reconcile the discrepancies between the 3D structures and earlier models? As it was refolded from inclusion bodies, the protein used to obtain the 3D structures might not be in the native conformation. Here, I propose structural rearrangements that could occur spontaneously as a possible path to convert the 3D structure to my preferred biochemically determined native structure.

Would the native VDAC structure please come forth.... High-resolution 3D structures of the mitochondrial outer membrane channel VDAC (voltage-dependent anion selective channel) have been determined and published in three high-profile papers [1–3], including one for which I was a co-author [1]. To obtain enough protein for the characterization, the authors of each paper expressed VDAC in *Escherichia coli* and then refolded the protein from inclusion bodies. They obtained a highly homogeneous preparation and the structure was solved both by NMR methods and X-ray crystallography. All three structures are almost the same, featuring a 19-stranded β barrel and an α helical region within the pore. The folding pattern corresponding to one of these is shown in Figure 1a. There is no dispute that these publications are correct in the sense that each accurately reflects the 3D structure of the protein studied. However, the fly in the ointment is that these 3D structures are in apparent conflict with many of the results of biochemical and functional studies published over the last eighteen years. Therefore, what is at issue is whether the refolded protein is in the native conformation or some other conformational state easily achieved by the refolding process. Functional studies on the refolded protein used in reference [1] and performed in my laboratory showed that it lacked the characteristic properties of VDAC, known for over thirty years. These properties could be restored by treating with a combination of cholesterol and Triton X100 but the treatments interfered with the ability to solve the structure. Therefore, there was a discrepancy between the conditions used to solve the structure and to show native activity. The supplemental information supplied with the publication by Ujwal and coworkers [3] does show beautiful records of VDAC with its characteristic properties. The discrepancy between my observations and these are not explained but the fact remains that only an extremely small fraction of the added protein actually inserts into the membrane (one part in 100 million) and, thus, might not reflect the properties of the population. The structural studies measure the structure of the greater population, whereas the functional studies measure the properties of the channels that have inserted. This difference could be crucial to the discussion at hand. In this manuscript I summarize the experimental constraints on the structure of VDAC from a variety of biochemical studies so that the extent of the conflict between these and the 3D structure becomes clear. In addition, I will propose a hypothetical conformational change that would convert the refolded structure into what I believe to be the native structure.

The metabolite channel in the mitochondrial outer membrane: VDAC

VDAC channels are found in the mitochondrial outer membrane of cells from all eukaryotic kingdoms [4]. The properties of the archetypal form of VDAC from vastly different species (e.g. potato and human) are highly conserved. These include single-channel conductance (rate of ion flow), ion selectivity and voltage-dependent channel closure to metabolites. VDAC channels are the major pathways by which metabolites, such as ATP, pass through the outer membrane and, therefore, are likely to have important roles in the regulation of mitochondrial functions.

A single 30 kDa polypeptide [5,6] forms a channel with an open diameter of ~3 nm [7,8]. When reconstituted into planar phospholipid membranes, VDAC channels respond to elevated transmembrane electrical potentials by undergoing a large conformational change whereby a positively charged region of the channel wall translocates to the membrane surface, reducing the size of the pore (from 3 to 1.8 nm in diameter) and inverting its selectivity (from one that prefers anions to one that prefers cations) [9–13]. The resulting closed state is essentially impermeable to ATP and has a reduced permeability to organic anions [14,15]. Electrophysiological measurements identify a large family of low-conducting states [12] referred to as closed states so as to be in harmony with the role of VDAC

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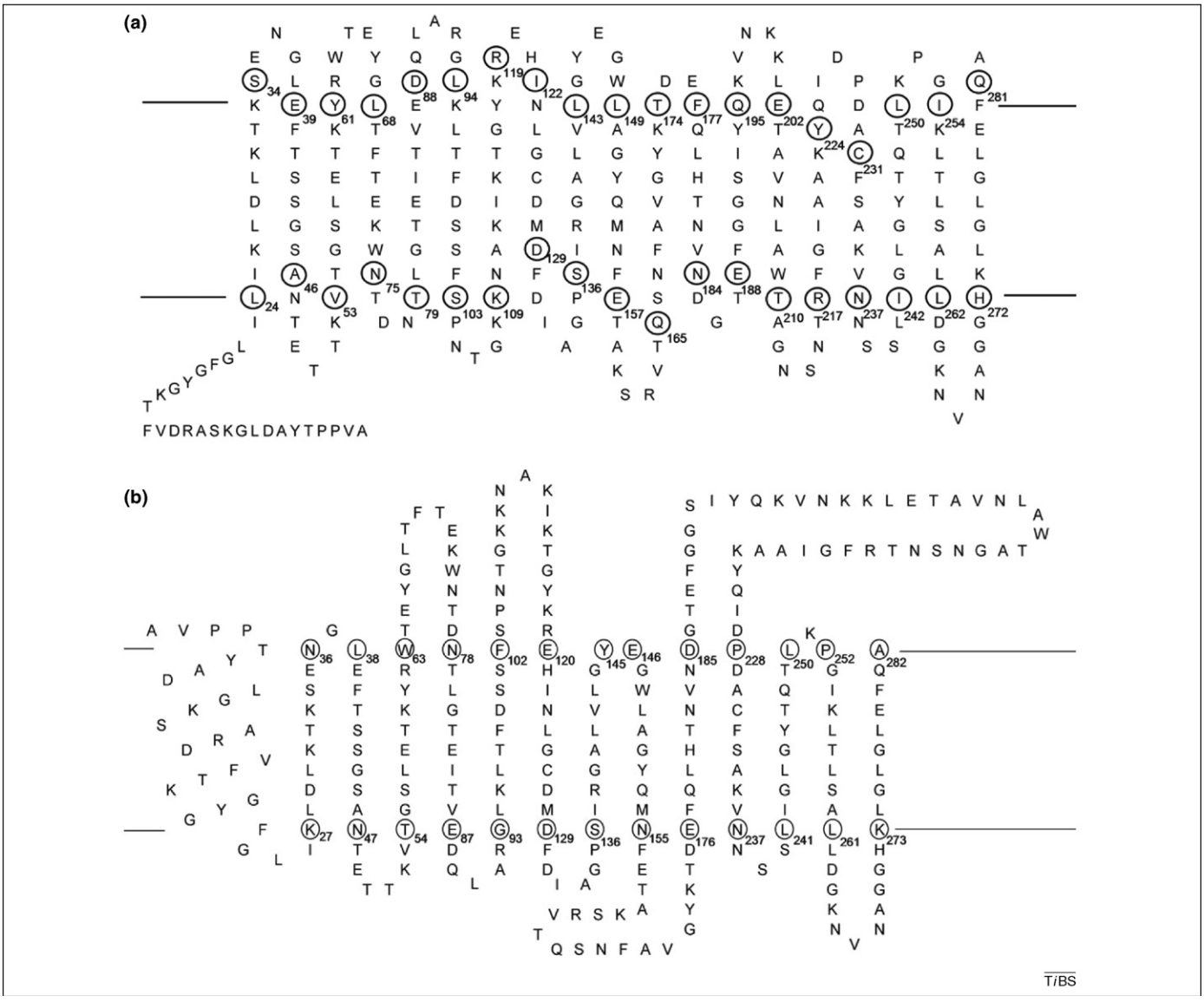

Figure 1. Folding patterns of human VDAC1 determined by NMR **(a)** [1] and a folding pattern deduced from functional studies **(b)** [4,16]. (a) A simplified drawing of the published folding pattern [1]. (b) The same pattern as that published previously in Ref. [4] but redrawn for clarity. The circled residues are ends of the β strands and the numbers are the amino acid number starting at the N terminus. Note that the N-terminal methionine has been removed.

to control the flux of metabolites. There are two distinct gating processes, one responding to positive and the other to negative membrane potentials, each with its set of closed states. Thus, within the experimental environment, this protein can adopt a variety of states.

A great deal of information about the structure and dynamics of VDAC was deduced from biochemical and functional studies. Among the conclusions was a transmembrane folding pattern for the open state of VDAC, which consists of a single α helix and thirteen β strands (Figure 1b) [16]. Several other similar structures have been proposed based on a combination of theoretical and experimental considerations [17–20]. There is no clear consensus among these folding patters but each differs from the folding pattern presented in the 3D structures. One might conclude that the cruder approach used in the biochemical studies just did not arrive at the correct structure. However, many of these studies provide solid constraints on the native structure and some of these are in conflict with the 3D structure. If the 3D structure is not fundamentally correct, this is not due to a failure to solve the structure correctly, rather the structure that was solved simply might not have been the native structure.

How reliable are the findings of the biochemical and functional experiments?

The experimental constraints on the VDAC structure deduced from functional studies vary in their solidity or reliability. Some of these provide a level of confidence that makes the constraint virtually unassailable. Others provide only weak support and are open to alternative interpretations. A detailed description is necessary to allow the reader to make an informed judgment.

First of all, it is necessary to realize that the functional studies were performed on VDAC channels from various sources, especially from the fungi *Saccharomyces cerevisiae* and *Neurospora crassa*. However, there is strong evidence that the secondary structure of VDAC is highly

conserved [9,21] and, thus, pooling the results obtained from VDAC from different sources seems reasonable. The experiments will be grouped by an experimental or theoretical approach and more emphasis will be placed on experiments that provide the more solid constraints.

Theoretical predictions

That the fundamental structure of the channel was a β barrel was evident from an examination of the primary sequence and the analogy to the family of proteins referred to as porins, located in the bacterial outer membrane. The high degree of β structure from circular dichroism studies [22] and the thin channel wall from electron microscopic studies [23] also indicated a β barrel. However, identification of the strands that are truly transmembrane is the key to knowing the native structure of VDAC. Some attempts were made to identify the transmembrane β strands by assuming homology with some of the porins from bacteria [17,20]. The obvious problem with this approach is that there is no significant sequence homology between the sequences of VDAC and porins. There is also a tremendous evolutionary distance and the two families of channels are subject to very different environmental selection pressures. Thus predictions based on homology with porins are inherently suspect.

Theoretical predictions were also made based on biophysical constraints [21,24] (Box 1). If all transmembrane strands predicted by this method were indeed transmembrane, rather than forming a surface domain, the number of β strands predicted to form the channel would be essentially the same as those identified in the 3D structure of VDAC.

Identification of surface domains by protease treatment, peptide-specific antibodies, and after FLAG labeling

By using antibodies raised against peptides representing different regions of the VDAC protein, Stanley and coworker [25] sought to obtain constraints on the transmembrane folding pattern of VDAC in isolated mitochondria. DePinto and coworkers [26] sought the same goal by treating mitochondria with proteases. McDonald and coworkers [27] sought to determine the orientation of VDAC in mitochondria by placing the FLAG epitope at various locations. The results from these studies can be used to constrain the folding pattern of VDAC once the orientation of VDAC in the mitochondrial outer membrane

Box 1. Probing the secondary structure of channel-forming membrane proteins

The process of obtaining reliable insights on the structure of channel-forming proteins is greatly aided by their location in the membrane because the protein is divided into two separate surfaces that can be accessed independently. A third surface, the part of the protein imbedded in the membrane and facing the lipid environment, can be probed separately with lipid-soluble probes. A fourth surface is the polar lining of the pore. It can be probed by making changes that influence the flow of ions through the channel. VDAC has an additional advantage in that a large aqueous pore is formed using only 30 kDa worth of protein. This means that there must be only one layer of protein forming the complete wall of the aqueous pore as more layers would require more protein mass. Furthermore, analysis of VDAC by circular dichroism and direct analysis of the primary sequence both showed a high degree of β structure [22,24]. As a result, strong biophysical constrains exist on the nature of the protein surfaces forming the wall of the pore and these require that each β strand have a polar surface facing the water and an apolar surface facing the lipids. Thus, in the protein sequence the side chains must alternate between an apolar side chain and a polar or charged side chain. This alternating pattern should extend for a sequence that is about ten amino acids long, which is enough to span the non-polar portion of the membrane. A simple algorithm can be used to search for segments that are good candidates for being transmembrane strands based of these biophysical characteristics [9]. This produces peaks in the 'β pattern' (Figure I). Each peak is the beginning of a 10-amino-acid segment that has an appropriate alternating polar/non-polar pattern. Other, more sophisticated methods have been used to predict the secondary structure of VDAC (discussed in a recent review [20]). Regardless of the theory, the predicted transmembrane segments have to be tested experimentally to determine whether they are, indeed, transmembrane strands. A precise approach is to use site-directed mutagenesis. Amino-acid substitutions are made in residues predicted to face the aqueous phase as these can cause changes that influence the rate of ion flow through the channel. Making substitutions that change the charge of the residue is particularly effective because, if they are located within the pore as predicted, they will change the net charge within the pore itself and thus the selectivity of the channel. Changing residues at the protein–water interface is less likely to influence the packing of the protein because rather than interacting with another region of the protein they are interacting with a fluid, water. To increase the likelihood that the mutant proteins are in a native conformation, they were expressed in yeast lacking VDAC and purified from isolated mitochondria in standard ways [28]. The proteins were never denatured. In addition, their functional properties were characterized.

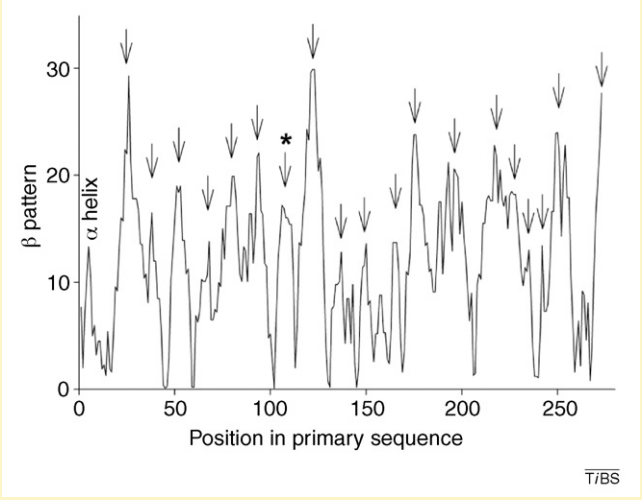

Figure I. The theoretical prediction of the location of transmembrane β strands in VDAC by calculating the β pattern. The primary sequence of human VDAC1 was processed through a simple algorithm to locate regions of the protein with biophysical characteristics compatible with forming the lining of the wall of a β barrel membrane channel. This wall separates the polar aqueous environment from the lipid phase of the membrane. Thus, one surface must be largely non-polar and the other polar. The side chains of β strands alternatively face one and then the other side of the β barrel. Thus, an alternating pattern of polar and non-polar residues would be ideal. This pattern should be long enough for the β strand to cross the membrane. With tilted strands [34], 10 residues are needed to span the membrane's lipid environment. Thus, the algorithm looks for stretches of 10 residues with an alternating polar/non-polar pattern. The Kyte-Doolittle hydropathy scale [39] was convenient because hydrophobic residues have positive numbers and hydrophilic residues have negative numbers. Thus, starting with the N terminus, the hydropathy values of every 10 amino acids was summed as follows: $\sum_{i-1}^{10}(-1)^{i+1} v_i$. The results are plotted in the figure as a function of the position in the primary sequence of the first amino acid. High values indicate a good alternating pattern and thus the location of the peaks indicate a possible transmembrane β strand. The arrows show the location of possible strands.

is established. The FLAG study clearly demonstrates that the C terminus faces the cytosol. This is the orientation that will be adopted.

The peptide-specific antibody study provides some useful constraints. According to the 3D structures, the α helix (residues 1–20) should be in the pore and not accessible to antibodies whether the mitochondrial outer membrane is intact or torn by hypotonic shock. Yet antibodies detect this region. Region 195–210 should be facing the intermembrane space, unlike the expectation from the 3D structure. Region 129–145 was determined to face the cytosol and this also disagrees with the 3D structure.

Protease treatment indicated that the following positions face the cytosol: 108–119, 117 (different protease), 172 and 227–229. However, according to the 3D structure, the first three sites should be within the membrane (Figure 1a) and not accessible to proteases. The 227–229 position does agree with the 3D structure in facing the cytosol. The points of disagreement could be explained by the fact that protease cleavage could expose new areas previously hidden. Yet the level of disagreement is striking.

The FLAG experiments were designed based on the published 3D structure. In agreement with the 3D structure, FLAG inserted in the middle of the α helix region was not accessible to immunoprecipitation until after detergent solubilization. Also as expected, a FLAG in the turn between β strands 2 and 3 from the N terminus was localized to the intermembrane space. However, a FLAG between strands 5 and 6 was localized to face the intermembrane space in disagreement with the 3D structure. Finally, a FLAG located in the middle of strand 14 showed ready access to the cytosol, in conflict with its location in the 3D structure. This is a site that site-directed mutations (below) indicate is not within a transmembrane strand. The authors explain their results by speculating that the discrepancies could indicate flexibility in the VDAC protein. Alternatively, as is my view, this could be explained if the 3D structure is not the native structure. Interestingly, all the results of these experiments are consistent with the folding pattern shown in Figure 1b because this thirteen transmembrane β strand structure has the strands in the appropriate orientation.

Identification of transmembrane strands by site-directed mutagenesis

Of the roughly twenty possible transmembrane regions identified by peaks in the β pattern (Box 1) twelve were identify.ed as transmembrane strands [28] by monitoring selectivity changes following charge-altering amino-acid substitutions. Remarkably, all these agree with the 3D structure. A thirteenth β strand was identified from topological studies [16] (see below) and this agrees as well (Table 1). For the other potential transmembrane strands, there is compelling evidence that these are not part of the β barrel. In the region spanning three of these strands (residues 188–224) four separate charge-change mutations had no significant effect on the selectivity of the channel [28,29]. Based on the 3D structure and theoretical considerations, the sites of these mutations should have placed them facing the ion stream. This is hard evidence that these sites do not face the stream of ions flowing through the channel. Thus, the location of these strands in the 3D structure is incompatible with this experimental evidence. Similarly for the remaining three regions: three mutations in region 164–174, two mutations in region 108–119 and one for region 68–77 [30]. The region 108–119 is of particular interest (Box 1; Figure I, arrow with asterisk) because it contains six cationic residues. This pattern would, in theory, make an outstanding voltage sensor but experiments indicate otherwise.

A valid concern is the possibility of 'site-directed mutilation'; the point mutation could alter the structure of VDAC. The experimental evidence argues strongly against this. First, the mutated proteins showed wild-type properties of single-channel conductance and voltage dependence. This cannot be said of the protein used to

Table 1. Location[a] and orientation of transmembrane strands as deduced from functional studies [16] and from the 3D structure [1]

	Location of strands			Orientation		
Strand number	Song et al.	3D structure	Comparison	Song et al.	3D structure	Comparison
1	α helix	α helix	Agreement[b]	↓	↓	Agreement
2	27–36	26–34	Agreement	↑	↑	Agreement
3	38–47	39–46	Agreement	↓	↓	Agreement
4	54–63	53–61	Agreement	↑	↑	Agreement
5	–	68–77	–	–	↓	–
6	78–87	79–88	Agreement	↓	↑	Conflict
7	93–102	94–103	Agreement	↑	↓	Conflict
8	–	109–119	–	–	↑	–
9	120–129	122–129	Agreement	↓	↓	Agreement
10	136–145	136–143	Agreement	↑	↑	Agreement
11	146–155	149–157	Agreement	↓	↓	Agreement
12	–	165–174	–	–	↑	–
13	176–185	177–184	Agreement	↑	↓	Conflict
14	–	188–195	–	–	↑	–
15	–	202–210	–	–	↓	–
16	–	217–224	–	–	↑	–
17	228–237	231–237	Agreement	↓	↓	Agreement
18	241–250	242–250	Agreement	↑	↑	Agreement
19	252–261	254–262	Agreement	↓	↓	Agreement
20	273–282	272–281	Agreement	↑	↑	Agreement

[a]The residue number indicated is from the N terminus and excludes the N-terminal methionine.
[b]Song et al. [16] placed the α helix in the wall of the channel, whereas the 3D structures have the helix within the aqueous pore. Both are transmembrane and, thus, these are judged to be in agreement.

determine one of the 3D structures [1]. It showed no voltage gating and displayed a wide range of single-channel conductance upon reconstitution into planar membranes. Second, the choice of mutations was conservative, replacing charged residues by other charged or polar residues. Confirmation that point mutations used did not result in detectable structural changes was evident from the finding that double and triple mutations showed additive changes in selectivity as expected from the engineered charge change [28].

Another concern could be that, if the α helix were located within the pore as in the 3D structures, it could cover residues and prevent them from influencing the ion flow. However, most of the residues mentioned above, where a charge substitution failed to influence the channel selectivity, are far from the location of the α helix as determined in the 3D structures [1–3].

Thus, the site-directed mutations effectively probed the transmembrane β strands and provide strong evidence that the extra strands are not forming the wall of the pore.

Topological constraints using streptavidin access to biotinylated sites

More complex experiments were performed to probe the dynamics and topology of the VDAC channel [16]. These studies were performed on VDAC from *N. crassa* because it lacks cysteine residues. Site-directed mutagenesis was used to introduce single cysteine residues at specific locations. The proteins were purified from yeast mitochondria under mild conditions [28] to obtain native proteins. The purified protein was biotinylated specifically on the cysteine and the protein reconstituted into planar membranes. After determining that it showed properties consistent with other VDAC channel studied (single-channel conductance and voltage dependence), streptavidin (a membrane-impermeable protein that binds specifically to biotin) was added to one or the other side of the membrane to probe the location and dynamics of the biotinylated site (Box 2). Two types of sites were identified: type 1 being on the static parts of VDAC and type 2 on the voltage sensor. These two types of sites were easily distinguished by the functional behavior of the channel. Type 1 resulted in a reduced conductance and slower kinetics of one of the gating processes but the channel could still show voltage-gating behavior. Type 2 locked the channel in a closed state. Once a set of sites were characterized as being either type 1 or type 2 sites, double cysteine mutant proteins were constructed using only the known sites. Experiments were performed to determine whether the sites were in the same or on opposite sides of the membrane. Single channels were examined so there was no possibility of misinterpretation. These studies were used to deduce the topology of the VDAC structure [30]. The relative orientation of the identified transmembrane strands determined in this way is compared with those in the 3D structures (Table 1). There are areas of agreement and islands of disagreement. The sites of disagreement flank the β strands found in the 3D structure but in conflict with the results of the biochemical studies. This pattern has given rise to my proposal for how the 3D structure could be converted into the native structure (see below).

Constraints from structural studies of 2D crystals

Images of VDAC channels in 2D crystals have been obtained by transmission electron microscopy of negatively stained [23,31], frozen hydrated [32] and freeze-dried/shadowed crystals [5] of VDAC in native membranes. Atomic force microscopy was also used [8,33] to obtain these images and to probe the pore. These all provide information on the size of the channel but are too low in resolution to provide information on the transmembrane folding pattern. All proposed folding patterns for VDAC would form channels with a structure consistent with these images. Even the folding pattern with only thirteen β strands would form a sufficiently large channel because it proposes that the N-terminal α helix is part of the wall of the pore. The measured tilt of the β strands, 46 degrees [34], allows for a large pore. The 1 nm protrusion detected on the cytoplasmic surface [33] is consistent with the large surface domains inherent in this model and others [20] where substantial loops are proposed to exist. The existence of these protrusions and their asymmetry (prominent only on one end of the channel) is not consistent with the 3D structures.

Influence of deletion mutants

Deletions of short segments (4 to 23 amino acids) of the VDAC sequence [35] result in changes in the electrophysiological properties of channels formed in phospholipids membranes. A large reduction in channel-forming ability was interpreted as '. . .indicating disruptions in key β-strands or β-turns' [35]. This not only leaves lots of room for subjective interpretation, but it also is not a necessary conclusion. Clearly the perturbations are large and conformational changes are likely. In addition, the proteins studied were refolded from inclusion bodies, adding another layer of uncertainty. However, the deletion of part or all of the N-terminal α helix (the first 19 residues) makes specific predictions that depend on the proposed structure of the channel. The 3D structures locate the α helix within the pore and thus removal of this region should result in channels with larger conductance. Some models place this region outside the channel [20] and thus one might expect no effect, at least on the open-state conductance. One model proposes that this helix is part of the wall of the pore [16] (Figure 1b) and thus its removal should result in a pore of lower conductance. Experiments by different investigators report the formation of pores with lower conductance [20,35,36].

Folding pattern consistent with biochemical/functional constraints

From the preceding it is clear that the results of numerous experiments are in conflict with the published 3D structures. Yet, there are also many proposed folding patterns from the biochemical and functional studies and, therefore, there is no consensus. A comparison of the regions proposed to form transmembrane β strands in the 3D structure and three other folding patterns (Table 2) shows that the 3D structure includes all the possible β strands whereas these others specify a subset of the nineteen strands of the 3D structure. Is there a transmembrane folding pattern that is consistent with all the available data, accounting for the degree of reliability? The pattern deduced by Song and coworkers [16] meets this criterion

Box 2. Defining the orientation of the transmembrane strands

The topology of the VDAC channel can be deduced from functional experiments by taking advantage of the natural dynamics of this protein. VDAC has a mobile domain that forms part of the wall of the channel (Figure I, black region). Being both mobile and positively charged, it can be driven out of the channel by applying a voltage across the membrane. Thus, this domain is the voltage sensor and the conformational change results in a channel with reduced pore size and inverted charge selectivity. The metabolic anions (such as ATP) that moved easily through the open state face a formidable barrier to flow through the closed states (Figure I). They face both a steric and electrostatic barrier because the movement of the positively charged sensor domain out of the channel leaves the walls of the channel with a net negative charge, repulsive to anions. When tags (biotin attached to cysteine residues) are placed in either the static (white region, tag 1) or mobile regions (black region, tag 2), addition of a membrane impermeant binding protein (streptavidin) to the aqueous phase has different affects on the voltage-gating properties of the channel [30]. When streptavidin binds to tag 1 (Figure I, top), the channel can still gate but gating when the streptavidin side is made positive is slowed down owing to steric effects. In addition, the ability of the channel to pass ions is reduced somewhat. Thus, especially in single-channel experiments, there are characteristic changes that can be categorized as type 1. However, when the tag is on the voltage sensor (tag 2), streptavidin could only bind when the channel closed in one of the two closed conformations. This binding locked the channel in that conformation and all voltage gating stopped [30]. This is the type 2 effect (Figure I, middle). When tags were placed at two sites, a tag1 and tag 2, single-channel experiments show when each binding event occurs. If both events can be observed by adding streptavidin only to one side of the membrane, then both sites are on the same side. If streptavidin causes a type 1 effect when added to one side and a type 2 effect when added to the opposite side then the sites must be on opposite sides of the membrane. A set of such experiments was used to define the topology of VDAC [16].

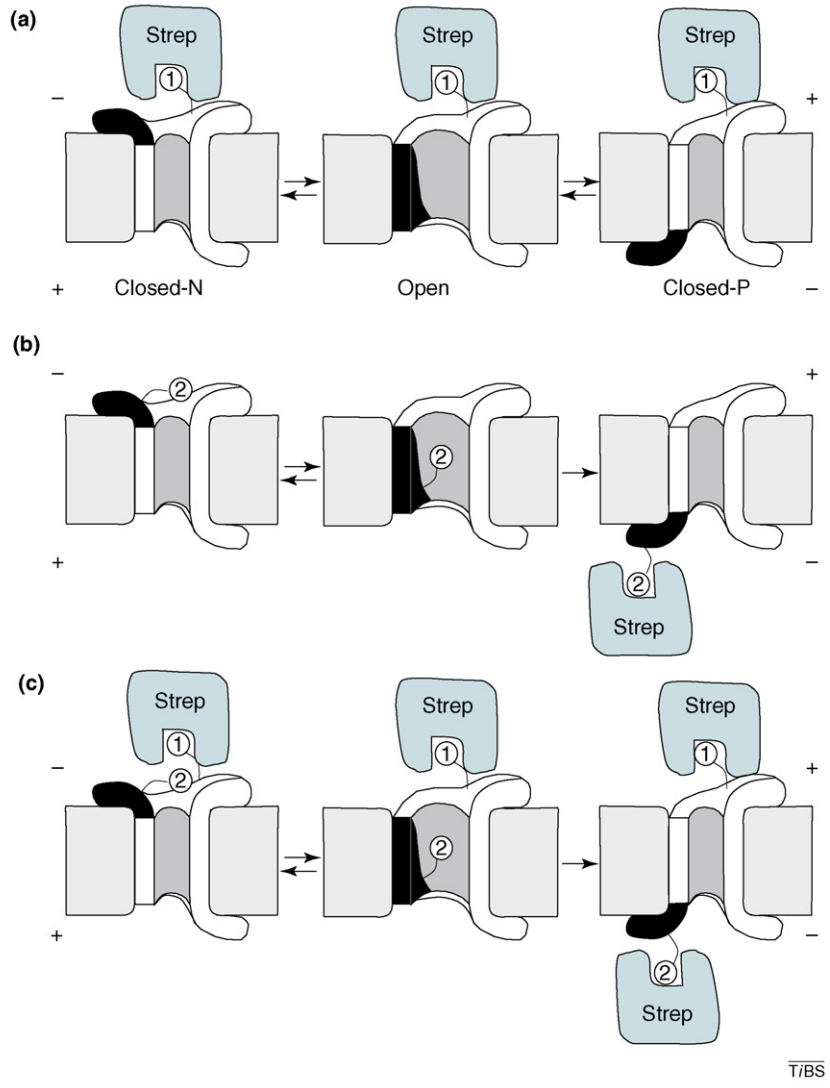

Figure I. Streptavidin (Strep) binding to a biotinylated site on VDAC alters the function of the channel in two ways and can be used to deduce the relative orientation of transmembrane strands. The VDAC channel is portrayed as a bisected cylinder to show the inner structure. In each of the three sets of reactions, the central image is of the open state of the channel and the side images are the closed states: the positive closed state (Closed-P) on the right and the negative closed state (Closed-N) on the left. The two closed states are achieved by applying opposite electric fields, indicated by the positive and negative signs. The white portions of the channel are static and the dark portion is positively charged and moves in response to the applied transmembrane voltage. This is the voltage sensor. The panels illustrate three typical experimental situations. **(a)** A cysteine was introduced in a region of the molecule that is static, resulting in a type 1 change in properties: reduction in ion flow through each state and reduction in the rate of voltage gating when the straptavidin side is made negative. These effects are due to some steric interference owing to the binding of streptavidin to the biotin covalently linked to the cysteine. **(b)** The biotinylated cysteine is on the voltage sensor resulting in a type 2 effect following streptavidin binding. The channel becomes locked in the closed state that allows the biotin to reach the surface and bind to streptavidin. In this case, voltage gating stops. **(c)** The channel has been labeled with biotin at two separate sites. These happen to be accessible on opposite sides of the membrane. Being of different types, by adding streptavidin first to the side accessible to the type 1 site and then to the other side of the membrane, first a type 1 effect and then a type 2 can be observed, telling the observer which site is reacting and that the sites are on opposite sides of the membrane.

Table 2. Comparison of the location of transmembrane β strands for the 3D structure and three published folding patters

β strand	3D structure	Song et al.	Casadio et al.	Runke et al.
1	26–34	27–36*	21–33	26–33*
2	39–46	38–47*	39–48*	39–46*
3	53–61	54–63*	53–61*	57–64
4	68–77	–	75–82	73–80
5	79–88	78–87*	87–92	84–91
6	94–103	93–102*	95–102*	94–101*
7	109–119	–	111–118*	105–112
8	122–129	120–129*	–	121–128*
9	136–143	136–145*	–	–
10	149–157	146–155*	146–155*	146–153
11	165–174	–	162–172*	166–173*
12	177–184	176–185*	–	179–186*
13	188–195	–	183–194	197–202
14	202–210	–	201–210*	206–211*
15	217–224	–	214–223*	219–226*
16	231–237	228–237*	229–237*	–
17	242–250	241–250*	243–250*	242–249*
18	254–262	252–261*	255–262*	252–259*
19	272–281	273–282*	270–279*	–

*Indicates essentially the same position of the β strand as in the 3D structure with total differences of the boundary positions of 5 or less.

(Figure 1b). Competing patterns [19,36] are consistent with most, but not all, of the experiments that provide strong constraints (single amino-acid substitutions and biotinylation experiments). Furthermore, the Song pattern is consistent with three out of four of the protease accessibility constraints, whereas the Casadio pattern is consistent with two and the Runke pattern with one. Recall that protease cleavage results in structural changes that could expose hidden sites so perfect agreement is not expected.

In addition to the location of the β strands, their orientation also needs to be considered. The three gaps in the Casadio pattern, as compared with the 3D structures, means that either the C- or N-terminal region must be in the opposite orientation as compared with the 3D structures. The wide separation of the two gaps on the Runke pattern shows that wide regions of the protein would be misoriented compared with the 3D structure. There are two small regions of apparent misorientation in the Song pattern. However the published data [16] would indicate that those regions are correctly oriented and it is the 3D structure that is misoriented in these regions. Thus, the Song pattern seems to be correct.

Hypothesis for reconciling the structures

The Song structure is by far the most consistent with the biochemical/functional data. Can it be generated from the 3D structure by a reasonable conformational change? Table 1 compares the Song pattern and the 3D structure in detail. It is interesting to note that discrepancies in the relative orientation of transmembrane strands, between the Song pattern and the 3D structure, lie in two regions, the same regions in which there is disagreement in the transmembrane location of strands. More importantly, the extra strands flank both sides of the regions of conflict in strand orientation. The numbers of the flanking additional strands are odd, not even. In my opinion, it is easy to see how a simple rotation in a plane normal to the membrane of strand 177–184 could result in the expulsion of the additional flanking strands (Figure 2). Such a rotation would leave the hydrophobic surface of the rotated strands firmly imbedded in the lipid environment and thus could be a relatively low-energy transformation. In the case of the two strands in the region of 79–103, the linkage region (89–103) could translocate through the membrane concomitant with the rotation in opposite direction of the transmembrane strands simultaneously resulting in the correct orientation (according to the functional experiments) and the expulsion of the two adjacent strands. Again, this rotation would be of relatively low energy. Perhaps the refolded and detergent-solubilized VDAC protein undergoes this rearrangement upon exposure to sufficient quantities of cholesterol and Triton X-100.

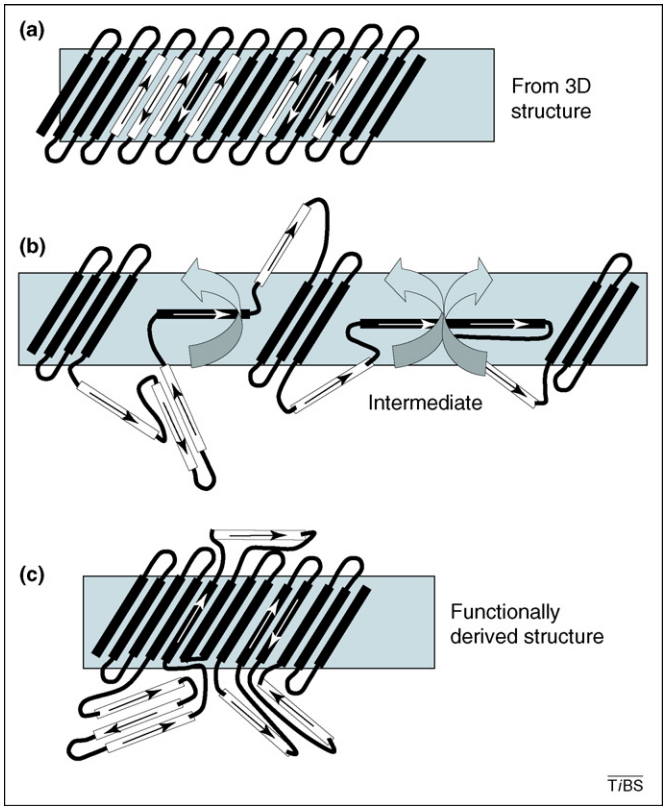

Figure 2. A possible mechanism by which the refolded structure of VDAC elucidated by NMR and X-ray crystallography **(a)** could convert to the structure deduced by Song *et al.* [16] from functional studies **(c)**. **(b)** The hypothesized transition state between (a) and (c). The straight arrows show the directions of the strands in the regions where the structures differ. The white arrows are on strands that both models agree are transmembrane but have opposite orientations in the two models. The black arrows are on strands that are transmembrane only in the 3D structure. The curved arrows indicate the proposed motion needed to convert the 3D structure into the functionally derived structure.

The 3D structure represents a stable conformation that includes all amphipathic β strands. The structure of VDAC with the functional properties that define the VDAC channel seems to be formed by a pair of relatively reasonable conformational changes. This type of motion, involving the rotation of β strands perpendicular to the plane of the membrane, could indicate a way in which other major structural changes occur during voltage gating.

Concluding remarks

There is no doubt that when the structure of a protein is determined to atomic resolution, its particular area of science is advanced dramatically. This is also true in the

case of VDAC. However, quoting Winston Churchill, '...this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning.' In a broader context, the VDAC structure is only the latest example of 'solved' 3D structures that are probably not native structures. For example, in the case of the mechanosensitive channels, a major domain in the crystal structure had to be drastically revised to make sense biophysically [37]. Perhaps the most dramatic controversy involved the structure and motion of the voltage sensor regions of channels specific for conducting alkali-metal ions. Well-constrained models based on clever functional studies seemed to be shattered by the published crystal structure but, in fact, the crystal structure was only one milestone in the long road to find the correct native structures. In his Nobel Prize lecture, Rod MacKinnon admitted that the Fab fragments used to achieve crystallization caused the protein to be in a non-native conformation (http://nobelprize.org/nobel_prizes/chemistry/laureates/2003/mackinnon-lecture.html). In a 2004 commentary responding to a publication presenting evidence in conflict with the crystal structure, MacKinnon concludes by stating: 'What we need next are new structures, additional biochemical and functional analyses, accessibility data on the closed conformation of the channel, and a better chemical understanding of the protein-lipid interface' [38]. He is exactly right; crystal structures and NMR structures alone are insufficient.

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