Mini review on the structure and supramolecular assembly of VDAC

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Abstract The Voltage Dependent Anion Channel (VDAC) is the most abundant protein in the outer membrane of mitochondria. This strategic localization puts it at the heart of a great number of phenomena. Its recent implication in apoptosis is an example of the major importance of this protein and has created a surge of interest in VDAC. There is no atomic-resolution structure allowing a better understanding of the function of VDAC, so alternative techniques to X-ray diffraction have been used to study VDAC. Here we discuss structural models from folding predictions and review data acquired by Atomic Force Microscopy (AFM) imaging that allowed to observe VDAC's structure and supramolecular organization in the mitochondrial outer membrane.

Keywords Atomic force microscopy · Membrane protein · Mitochondrion · Porin · Voltage dependent anion channel

Introduction

Scientists discovered the voltage dependent anion channel (VDAC) about 30 years ago in the outer mitochondrial membrane of paramecia, a channel protein that was seven times more permeable to Cl⁻ than to K⁺ (Schein et al.

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1976). Later, it was shown that VDAC was a single channel with ion selectivity that reacts to membrane potentials (Colombini 1980). In the open state, in reconstituted phospholipidic membranes, it has a conductance of 3.3+/− 0.1 nS. A voltage of 50 mV applied across the membrane causes channel conductance for anions to fall and greatly reduces membrane permeability to metabolites in general. VDAC has a complex gating behavior that has given birth to a wealth of sometimes-contradictory literature. For extensive reviews on VDAC function see (Colombini et al. 1996; Rostovtseva et al. 2005).

Discovery of the implication of VDAC in apoptosis has created a gain of interest for this protein in the scientific community. As studies progress, VDAC was shown to play a role in several important functions in mitochondria and also, indirectly, in the cell. Its localization in the external membrane of mitochondria, where it is the most abundant protein, makes it a major gate for molecules that need access to and exit from the intermembrane spaces and a functional anchor point to molecules that interact with mitochondria.

Therefore it's not surprising to see that VDAC not only transports ATP in its open state but also that it interacts with molecules which need to be in the proximity of ATP, gaining an energetic advantage (Rostovtseva and Colombini 1996). Interactions with members of the Bcl (B-cell leukemia) family have also been extensively studied (Shimizu et al. 1999; Shimizu et al. 2001; Vander Heiden et al. 2001), and two different models causing subsequent apoptosis were proposed. In one, VDAC closes, causing mitochondrial intermembrane space swelling and membrane rupture, followed by the release of cytochrome c (cyt$_c$). In the other model, VDAC is in the open state and by interacting with Bax it would suffer structural modifications, enlarging and allowing cyt$_c$ to exit the mitochondria and cause apoptosis.

Such large changes in structure seem quite unlikely if we take into account that the function of VDAC is conserved among species and several studies contradict the assumption that Bax interacts with VDAC (for a review see (Rostovtseva et al. 2005)). AFM imaging of MOM exposed to a Bax solution confirmed this hypothesis (unpublished results). The full understanding of VDAC's part in apoptosis is still under study and very recently novel pathways interacting with VDAC and controlling cell death in cancer cells were discovered (Tajeddine et al. 2008)

Other relevant VDAC functions include superoxide anion release (Han et al. 2003) and influence on membrane lipid composition (Rostovtseva et al. 2006). Other relevant molecules interacting with VDAC are hexokinases (Mathupala et al. 2006) and creatine kinases (Schlattner et al. 2001). A total of more than fifty proteins have been identified as possibly interacting with VDAC (Roman et al. 2006). The full range of VDAC's influence on the life and death of the cell is continuously investigated.

This 'ubi-functionality' has led to a wealth of sometimes-contradictory data. Recently VDAC has been excluded from being part of the permeability transition pore (PTP), in experiments where deletion of the VDAC genes did not interfere with the apparition of the mitochondrial permeability transition state (Baines et al. 2007). This shows that there is a need to be cautious with assumptions regarding the VDAC pore function.

High-resolution structural information on VDAC and continuous studies of its interactions with other molecules will allow the elucidation of the mechanisms underlying such different aspects as apoptosis, energy production and communication of mitochondria. Despite the effort of the scientific community, there are still no three-dimensional (3D) crystals amenable for X-Ray crystallography and no atomic-resolution structure of VDAC. Therefore other techniques have been privileged to study the molecule's structure and its supramolecular organization in the membrane. Here we review data on structural models derived from sequence based folding prediction, electron microscopy (EM) and atomic force microscopy imaging of VDAC and the mitochondrial outer membrane. Indeed, the AFM has to date provided the highest-resolution view of VDAC and additionally reported about its native assembly in the outer mitochondrial membrane, the focus of this mini-review.

Sequence analysis and fold prediction

Mitochondria are believed to be the result of an endosymbiotic event (Wallin 1922), where bacteria were internalized by other cells, later developing their energy generation specificity. This hypothesis accounts for the existence of mitochondrial DNA (Schatz 1963; Rabinowitz et al. 1965)

and the double membrane structure of mitochondria. Therefore, sequence and functional similarity between VDAC and bacterial outer membrane beta-barrel porins has been taken into account for insight on the study of these proteins. No beta-barrel porins were found in cytoplasmic membranes. Since bacterial membrane protein structures are easier to obtain than eukaryotic, because of the convenience of protein production in large quantities, several structures of bacterial beta-barrel porins at atomic resolution are available. This knowledge has been used for several structure predictions concerning VDAC, even though there is no sequence homology between bacterial porins and VDAC, and large structural differences are predicted at the constriction site and in the N-terminal region. Circular dichroism studies, EM structures, along with bacterial porin similarity and secondary structure predictions indicate that VDAC is a beta-barrel protein (Mannella et al. 1992; Shao et al. 1996; Rostovtseva et al. 2005). Unfortunately there is no consensus as to the number of beta strands composing the barrel, the number ranges in different studies from 13 to 18 and therefore the need for a high resolution structure is evident to provide concise structural insights. This has a major relevance when trying to understand functional aspects such as the gating mechanism, predicted to be assured by the N terminus segment.

Colombini and collaborators have contributed pioneering work on VDAC (Colombini 1980). They predict a 14 segment membrane protein, barrel shaped, made of 13 beta-strands and 1 alpha-helix (Song et al. 1998). They support their claim with circular dichroism experiments and polar-nonpolar pattern recognition of the amino acids in the sequence. Also site directed mutagenesis and double cysteine bonds are taken into account for this folding prediction. They confirm their approach by making steric considerations and comparing with the low-resolution EM structures.

Court and collaborators also studied extensively VDAC folding, they reviewed and proposed several models, the most recent using a genome sequence analysis approach of the increasing numbers of genomes that are now available (Young et al. 2007). They also integrate knowledge from point mutation and deletion studies and site specific biotynilation (Bay and Court 2002). They predict a 16-strand protein with a N terminus strand located in the intermembrane space.

We used VDAC's pore dimensions obtained by AFM (Fig. 1a, (Gonçalves et al. 2007)), and compare them to the pore dimensions of the known bacterial beta-barrel porin structures. We plotted protein circumference against the number of beta-strands present in the barrel, using protein data bank entries for published structures of beta-barrel proteins. A general linear fit can be applied to the data,
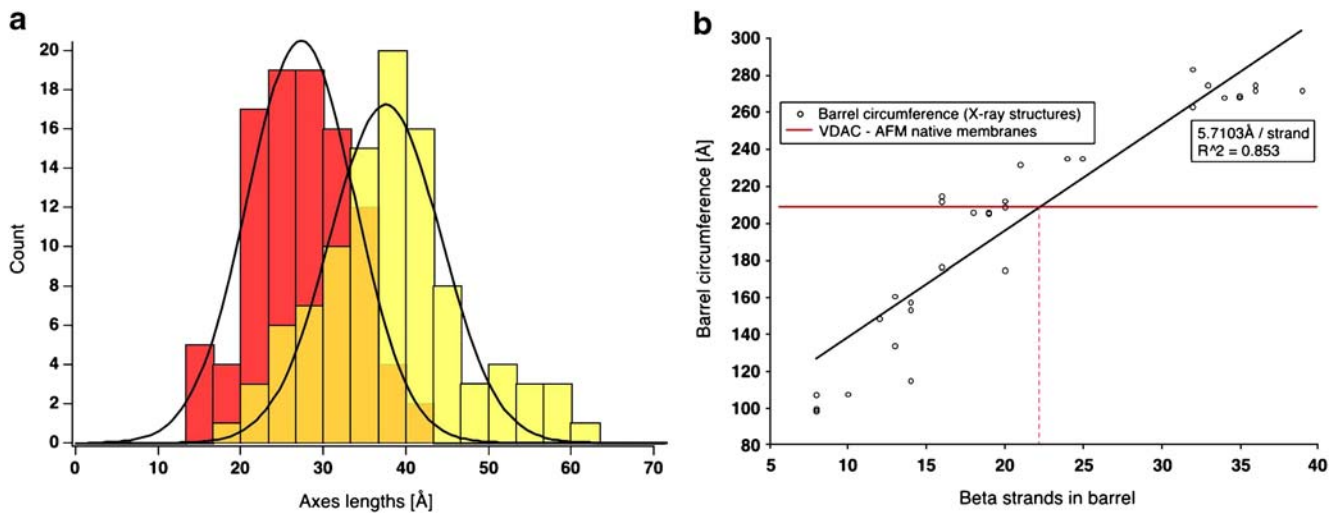
Fig. 1 Structural characteristics of VDAC and comparison with other beta-barrel membrane proteins. **a** Histogram representing long and short axis lengths measured on high-resolution AFM topographs. **b** Barrel circumference versus beta strands in barrel for membrane proteins of known atomic structure. A general tendency of 5.7 Å per strand can be deduced but variations according to tilt and protein structure must be taken into account. Structures used for the graph are

OmpK36 (1OSM), Omp32 (1E54), OmpF (2OMF), OmpC (2J1N), OmpG (2F1C), PhoE (1PHO), ScrY (1A0T), MspA (1UUN), OprD (2ODJ), TolC (1EK9), VceC (1YC9), OprM (1WP1), BtuB (1NQF), Cir (2HDI), OmpA (1BXW), OmpT (1I78), OmpW (2F1V), OmpX (1QJ8), OmpLA (1QD5), OpcA (1K24), NspA (1P4T), NalP (1UYN), Hia (2GR8), EspP (2QOM), PagP (1MM4), FadL (1T16), Tsx (1TLY), FhuA (2FCP), FepA (1FEP), FecA (1KMP), FptA (1XKW)

resulting in average a circumference of 5.7 Å times the number of beta-strands (Fig. 1b). This would give 22 beta-strands for VDAC, but we have to take into account differences in the tilt angle of the beta strands (46° for VDAC (Abrecht et al. 2000) and structural particularities in the proteins. For example, there are two 16 beta-strand proteins that have the same circumference as VDAC. Although it is only qualitative, the large dimensions of the VDAC pore seen by AFM seem to indicate that Court and coworkers should be closer to the truth than the predictions from the Colombini group.

Atomic force microscopy studies of VDAC in the native membrane

AFM is becoming a tool of increasing importance in modern biology, bridging the resolution-gap between optical microscopy techniques and high-resolution structure determination techniques. It's high signal to noise ratio in imaging mode combined with the force spectroscopy mode allow high-resolution visualization of biological samples and acquisition of rheological parameters at the same time. Recently, imaging of supramolecular assemblies of proteins in native photosynthetic membranes (Scheuring and Sturgis 2005) and in healthy and pathological eye lens membranes (Buzhynskyy et al. 2007a; Buzhynskyy et al. 2007b) demonstrated how AFM can be used as a high precision imaging and diagnosis tool. Also, force spectroscopy has numerous applications in biology, allowing measurement of for example: reversible unfolding of individual titin (Rief et

al. 1997), gradient of rigidity on lamellipodia (Laurent et al. 2005), physical properties of microbial surfaces (Dufrêne 2001), elasticity and rupture forces of unsupported membranes (Gonçalves et al. 2006) and intermolecular forces in bacterial surface layers (Müller et al. 1999).

VDAC can be directly studied in the native outer mitochondrial membrane by AFM. This technique allowed the acquisition of the highest resolution structural data of VDAC to date, to our knowledge, and importantly, the AFM observation was performed in conditions very close to native (Gonçalves et al. 2007). Outer membranes were prepared as previously described through mild extraction from *Saccharomyces cerevisae* mitochondria (Mannella 1998). Images are taken in buffer solution (150 mM KCl, 25 mM trisHCl) under normal temperature and pressure conditions. Furthermore it was possible to gain information on the supramolecular organization of the protein showing high- and low-density regions and different oligomerization states. Results were first obtained in yeast (Gonçalves et al. 2007) and confirmed in potato tubers mitochondria (Hoogenboom et al. 2007).

VDAC has a very high density in isolated mitochondrial outer membrane (MOM), more than 50% of the total protein content (Mannella 1998), as confirmed by SDS-PAGE (Gonçalves et al. 2007). Low-resolution AFM topographs show a typical adsorption of MOM preparations on mica (Fig. 2). Three distinct zones are distinguished in the MOM. First, flat, lipid only regions, second, intermediate, slightly corrugated zones, and third, compact and dense regions. In intermediate zones, VDAC represents in
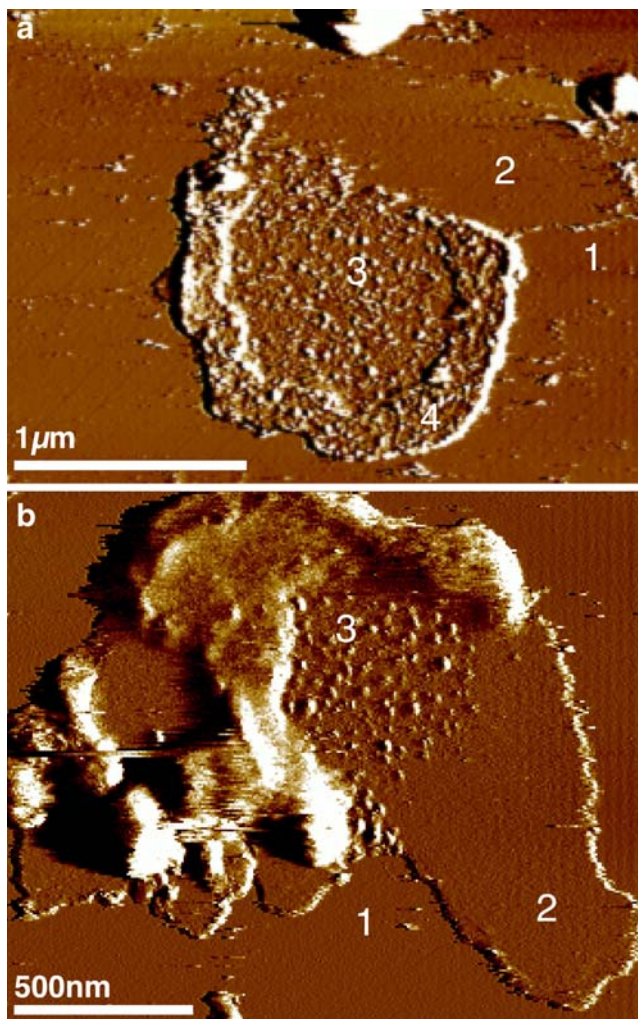
Fig. 2 AFM topographs of native mitochondrial outer membrane. **a** Vesicle adsorbed on atomically flat mica (*1*). Lipid-only regions (*2*) are distinguished from two main regions of interest, a central one with low protein density (*3*), surrounded by a high-density membrane region (*4*). **b** Typical vesicle adsorption on mica. Three zones are distinguished: atomically flat mica (*1*), lipid bilayer (*2*) and corrugated membrane, with low protein density (*3*)

average 20% of the surface density, this percentage rises to about 80% in other regions. In these most dense regions, VDAC seems to gain some level of self-organization due to the high concentration of protein. These zones could form the nucleation cores for the formation of VDAC 2D crystals, readily found in MOM preparations (Mannella 1982) or in preparations after application of lipid withdrawal procedures (Mannella 1998) that were studied by electron microscopy. These 2D-crystals were poorly ordered and yielded low-resolution maps. In contrast, in the native membrane, pair correlation function analysis of high resolution AFM topographs showed that VDAC does not form crystalline arrays in native membranes but that VDAC pores are assembled in a loose hexagonal packing (Fig. 4c).

Further imaging of MOM yielded information on VDAC's supramolecular organization. Topographs show how high density zones are clearly separated from the low-density domains (Fig. 3a). Membrane areas where protein density is low, and imaging is difficult, indicate high mobility of VDAC in these zones, as opposed to the proteins that are captured in dense zones and are immobile over time. These highly mobile molecules could act as a functional regulator, by increasing and decreasing the area of the conglomerated zone and altering the protein's environment, as it is known that this channel's behavior depends strongly on it's surface density (Mannella 1998) and membrane lipid composition (Rostovtseva et al. 2006).
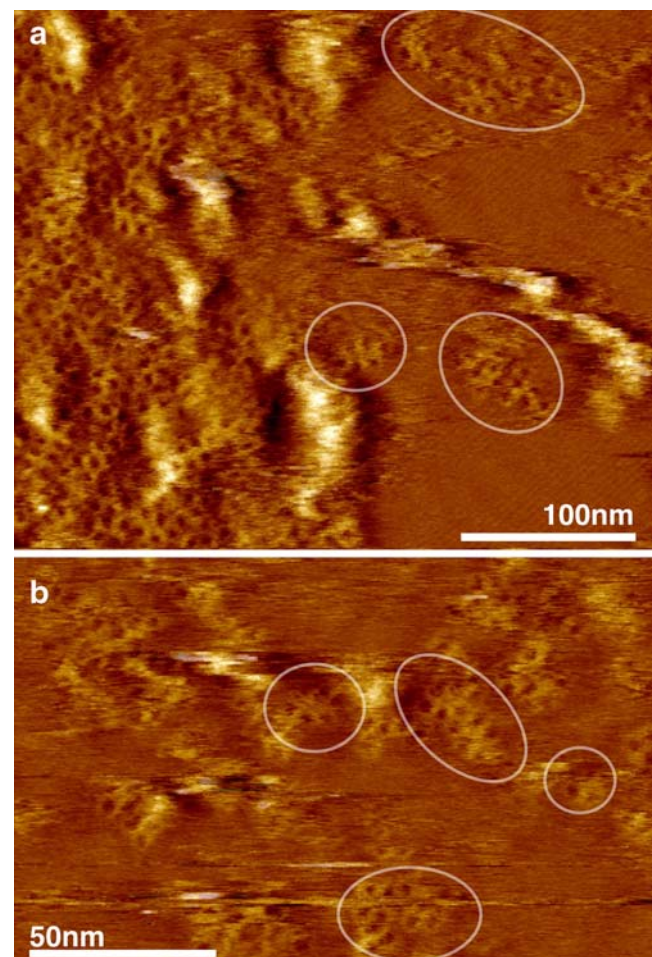

Fig. 3 Supramolecular organization of VDAC in native Mitochondrial outer membranes. **a** High resolution AFM topograph giving an overview of a membrane region with two distinguishable protein density contents. On the left, individual VDAC molecules can be seen clustering and forming very dense regions (about 80% in surface density). On the right part of the membrane, molecules can be seen clustering in groups of variable size (*insets*). Surface density in these areas is about 20%. **b** High resolution image of the low-density zone. VDAC can be seen clustering in groups ranging from two to 20 molecules (*insets*). No particular repetitive arrangement can be seen in this area. (Modified from Figure 2 in Gonçalves et al. 2007)
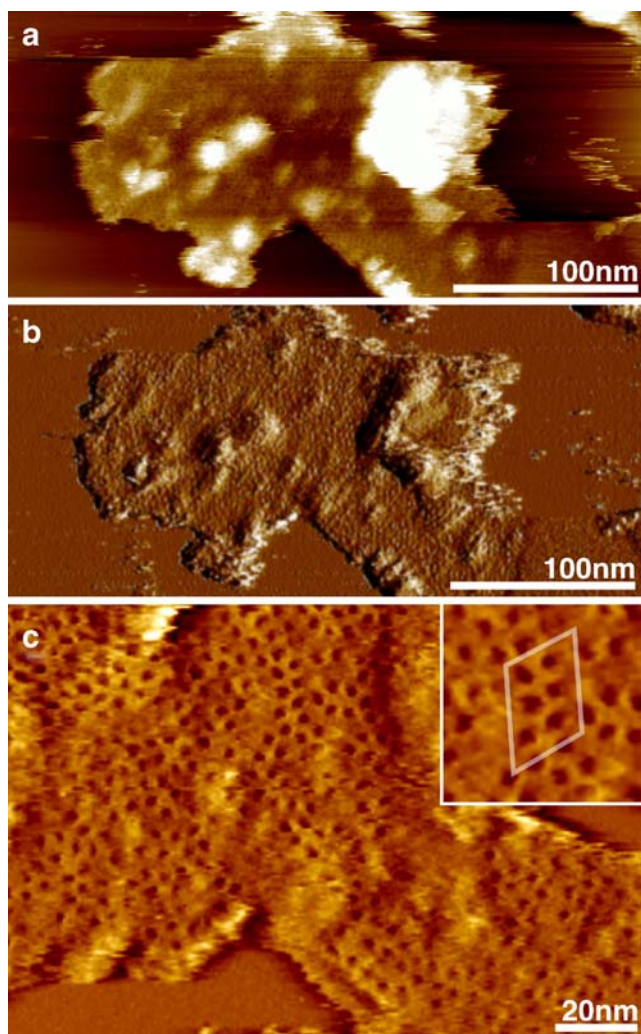
Fig. 4 High protein density regions in mitochondrial outer membranes. Height image (**a**) and deflection image (**b**) of highly corrugated MOM fragment. Membrane height, 3.7 nm. **c** High resolution image of the supramolecular organization of VDAC in high-density membranes. Although no long-range order can be measured, local spatial arrangements are similar to the crystalline arrays observed by electron microscopy (*inset*) (Inset modified from Figure 3b in Gonçalves et al. 2007)

Detailed examination of the low-density zones showed VDAC existed in a variety of oligomerization states (Fig. 3b, outlines) forming groups of 1 to 20 molecules. This showed the molecule's variability in participating in different oligomeric states, which we speculate to represent yet another means of diversifying its functional capacities.

In denser regions, protein-protein interactions confer rigidity to the membrane, facilitating AFM high-resolution imaging (Figs. 3 and 4) and revealing an eye-shaped porin, similar to bacterial porins (Cowan et al. 1992) with dimensions of 3.8×2.7 nm. The AFM tip could enter the pore constriction to a depth of 2 nm. These dimensions compare well with EM results (Mannella 1982). The slightly larger values found by AFM of native membranes

indicate that crystalline order at very high molecular density may have an influence on the molecule's structure, particularly it may mask pore flexibility preserved in non-ordered native assemblies. In AFM images, the pore shows a wide plasticity (measured pore width standard deviation, 0.8 nm (Gonçalves et al. 2007)), and further studies are needed to link these structural changes to functional aspects of VDAC, particularly regarding the pore opening mechanisms. The tip's possible influence on the sample can be informative, probing the conformational space of the proteins (Scheuring et al. 2002), or destructive, damaging the sample, and has to be kept in mind. Very large conformational changes that would cause mitochondrial leakage or could explain VDAC's role in the mitochondrial transition pore are unconvincing and difficult to imagine in light of the similarity of the beta-barrel structure to bacterial porins. Furthermore, recent results show that VDAC is not essential to obtain the mitochondrial transition states (Baines et al. 2007).

Conclusions

The case of VDAC is interesting in view of modern structural biology and specifically in the membrane protein domain. Identified about 30 years ago and recently demonstrated to be implicated in major phenomena like apoptosis and cancer, VDAC has resisted 3D crystallization efforts and no atomic-resolution structure is available to date. The difficulty to acquire 3D crystals and to solve the atomic structure encourages alternative techniques for studying membrane proteins, and shows the usefulness of medium resolution studies that will sooner or later be outdated by the X-ray structure. AFM has found its place in the study for several reasons, its ability to work in native liquid conditions, to image supramolecular assemblies in native membranes and to perform force spectroscopy. AFM has given the highest resolution image of VDAC, also showing the flexibility of this protein in the native membrane and the variability in its supramolecular arrangements. Although X-ray crystallography yields the highest resolution, the high order of the crystals that is required does not allow revealing molecular flexibility and supramolecular variability, and hampers drawing conclusions about potential functions that could arise from such properties. We believe that an atomic structure will provide a deep understanding on VDAC gating, however in parallel other techniques have to be pursued to fully understand the function of membrane proteins and membranes as a whole. This includes the ensemble behavior of the complexes of all molecules in a membrane. AFM appears to be a key technique for such analysis as demonstrated on MOM containing VDAC.

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