Supramolecular Assembly of VDAC in Native Mitochondrial Outer Membranes

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The voltage-dependent anion channel (VDAC) is the most abundant protein in the mitochondrial outer membrane (MOM). Due to its localization, VDAC is involved in a wide range of processes, such as passage of ATP out of mitochondria, and particularly plays a central role in apoptosis. Importantly, the assembly of VDAC provides interaction with a wide range of proteins, some implying oligomerization. However, many questions remain as to the VDAC structure, its supramolecular assembly, packing density, and oligomerization in the MOM is unknown. Here we report the so far highest resolution view of VDAC and its native supramolecular assembly. We have studied yeast MOM by high-resolution atomic force microscopy (AFM) in physiological buffer and found VDAC in two distinct types of membrane domains. We found regions where VDAC was packed at high density (~80%), rendering the membrane a voltage-dependent molecular sieve. In other domains, VDAC has a low surface density (~20%) and the pore assembly ranges from single molecules to groups of up to 20. We assume that these groups are mobile in the lipid bilayer and allow association and dissociation with the large assemblies. VDAC has no preferred oligomeric state and no long-range order was observed in densely packed domains. High-resolution topographs show an eye-shaped VDAC with 3.8 nm × 2.7 nm pore dimensions. Based on the observed VDAC structure and the pair correlation function (PCF) analysis of the domain architectures, we propose a simple model that could explain the phase behavior of VDAC, and illustrates the sensitivity of the molecular organization to conditions in the cell, and the possibility for modulation of its assembly. The implication of VDAC in cytochrome c release from the mitochondria during cell apoptosis has made it a target in cancer research.

Introduction

It is generally accepted that mitochondria evolved from cell-enclosed symbiotic prokaryotes, therefore mitochondria and prokaryotes share several characteristics.¹ Both possess outer membranes with densely packed porins that allow the passage of relatively large molecules. Bacterial porins are structurally well described,² in contrast, many questions remain as the voltage-dependent anion channel (VDAC) structure, its supramolecular assembly, packing density, and oligomerization in the mitochondrial outer membrane (MOM) are unknown. VDAC is a 30 kDa protein found in the MOM of all eukaryotes.³ Sequence, biochemical and computational analyses indicate a beta-barrel architecture with a fold of 12⁴ to 17⁵ beta-strands and an N-terminal domain that is probably alpha-helical.⁵ Electron microscopy (EM) of 2D-crystals, readily found in MOM preparations,⁶ or formed through lipid withdrawal from MOM,⁷ or through VDAC reconstitution,⁸ revealed the overall shape of the molecule, a pore with an inner diameter of ~3 nm,
from which a beta-barrel C\(^n\)-diameter of 3.6–3.8 nm was estimated.

The sequence and function of VDAC are well preserved among eukaryotes. It forms a single pore with voltage dependence and anion selectivity.\(^7\) VDAC is the most abundant protein in the MOM (>50% of total protein) and is implicated in a large number of processes beyond that of a simple anion channel function: ATP transport,\(^9\) superoxide anion release,\(^10\) and apoptosis\(^11,13\) among others. It has been shown that interactions with cations (Ca\(^{2+}\)) control the gating properties of MOM and VDAC.\(^14\) Furthermore, VDAC interacts with several mitochondrial and cytoplasmic proteins including kinases,\(^15,17\) cytochrome \(c,\)\(^11,13\) and actin,\(^18\) at different binding sites. VDAC is believed to act as an anchor point to proteins, which therefore have an easier access to ATP produced by the mitochondrion.\(^16\) In cell apoptosis,\(^12\) the function of VDAC can be regulated by interacting with the members of the Bcl-2 (B-cell leukemia/lymphoma 2) family,\(^13,19\) therefore VDAC has become a drug target for cancer therapy.\(^20,21\) Recently, no less than 55 novel functional interaction partners of VDAC were found.\(^22\) The full extent and understanding of VDACs importance in the cells life and death is being extensively studied.\(^23\)

**Results and Discussion**

Atomic force microscopy (AFM)\(^24\) has developed into a powerful tool in membrane research. Recently, topographs at \(~10\) Å resolution of multi-component native membranes from bacteria\(^7\) and eukaryotes\(^25\) were acquired. Here, we used AFM to study yeast MOM in physiological buffer, under close-to-native conditions, reporting on the structure and the supramolecular organization of VDAC in native membranes. MOM were purified from yeast essentially as previously described.\(^7\) The majority of the membranes had vesicular shape (Figure 1(a)) with an area of \(~1\) \(\mu\)m\(^2\) (Figure 1(b)). These flattened vesicles contained featureless, presumably lipid-only domains with an average thickness of 3.8 nm, and other domains densely packed with pores whose diameters were consistent with those of the mitochondrial porin VDAC. VDAC is the by far most abundant protein in our preparation as analyzed by Coomassie brilliant blue stained SDS-PAGE and mass spectrometry (Figure 1(c)). In early studies, poorly ordered 2D-crystals of VDAC were readily found in MOM preparations.\(^6\)

In medium resolution topographs, corrugated pore-containing membrane areas were readily distinguished from the smoother, lipid-only regions, allowing the estimation of pore packing densities. Mixed domains contained pores at low (~20%) density, in other regions the pores were packed at high (~80%) density (Figure 2(a)). Often the pore topography was accompanied by large protrusions of \(~4\) nm height (Figure 2(a)). Since VDAC is the only outer membrane protein present at sufficient quantity to account for this high density of pores, it is safe to presume that the pores represent VDAC, and so the protrusions are likely proteins that bind to VDAC on the mica-facing side of the membrane. Due to the large number of molecules that interact with VDAC,\(^10\) it is difficult to ascertain which one it might be. As it was suggested that VDAC’s channel behavior depends on its surface density,\(^7\) both the high-density and the low-density domains are of significant functional interest. In mixed domains, VDAC monomers, dimers, trimers, hexamers, and arrays of up to 20 molecules were found (Figure 2(b) and (c)). These molecules are highly mobile and difficult to image by AFM. High mobility of VDAC groups in the mixed domains could trigger VDAC association in the densely packed porous domains and thus act as a regulator of VDAC activity. Such an association–dissociation equilibrium is a simple way of modulating channeling cooperativity\(^7\) and possible interactions that demand oligomerization.\(^15,27\)

Little is known on VDACs structure. EM of poorly ordered 2D-crystals allowed the calculation of low-resolution maps, showing an approximately circular pore with dimension \(~3.7\) nm (beta-barrel backbone distance).\(^7\) Sequence analysis, analogy considerations with bacterial outer membrane porins and circular dichroism studies all indicate that VDAC would span the membrane as a beta-barrel (for a review see Bay & Court\(^5\)). The arrangement of the
beta-strands, the loops and the N terminus, that may form an alpha-helical central plug, is still a matter of controversy. The proposed number of beta-strands in the barrel ranges from 12\(^4\) to 17.\(^7\) High-resolution AFM imaging of the densely packed membranes allowed us to obtain further structural information (Figure 3(a)). VDAC is an eye-shaped pore with dimensions of 3.8(±0.8) nm×2.7(±0.6) nm (\(n=98\)) (Figure 3(b)). Some VDAC exhibited variability in pore dimension that we assign to the protein’s intrinsic flexibility. This flexibility may have hampered the growth of highly ordered crystals so far, and could functionally be related to different conductance states. The AFM tip could enter up to 2 nm inside some pores, indicating that the membranes probably exposed their cytoplasmic face, that does not feature the N-terminal, possibly pore-filling domain, and that such a plug remained rather in the inter-membrane-space half side of the channel. Unlike in EM reconstructions,\(^6\) only one side of the protein could be imaged. Electrostatic interactions with the negatively charged mica surface may favor unidirectional adsorption of MOM. The VDAC cytoplasmic surface is irregular with maximal protrusion of ~1 nm over the bilayer surface, corresponding to the beta-turns connecting the beta-strands as was seen by EM and predicted by sequence analysis.

Pair correlation function (PCF) analysis revealed interesting details about VDAC assembly in native

![Figure 2](image2.png)

Figure 2. Low-density (~20%) and high-density (~80%) VDAC domains in MOM. (a) Deflection image of a membrane patch containing low-density (L) and high-density (H) domains. Protein corrugation (white outline) is easily distinguishable from smooth lipid areas. Large protrusions underlie VDAC in both low-density and high-density domains (yellow outlines). (b) and (c) High-resolution topographs of low-density domains. Outlines: monomeric (m), dimeric (d), trimeric (t), hexameric (h), and arrays of up to 20 VDAC (x) were found. The hexameric (h) VDAC assembly resembles the unit cell arrangement found in 2D crystals.\(^7\)

![Figure 3](image3.png)

Figure 3. VDAC structure and supramolecular organization in densely packed domains. (a) High-resolution AFM topograph of densely packed VDAC in a native MOM. (b) High-resolution view of eye-shaped VDAC (pore dimensions 3.8 nm×2.7 nm; pore depth up to 2 nm). (c) PCF analysis of VDAC in densely packed domains. A peak at 53 Å represents the most frequent pore-to-pore distance. A minor broad peak at ~92 Å represents rough hexagonal packing; no long-range order peak was found (sketches represent corresponding pore-pore assemblies).
MOM (Figure 3(c)): the most frequently found assembly distance is 53 Å, corresponding to two neighboring pores. This value is slightly larger than 43 Å to 50 Å, found in EM reconstructions. Importantly, the 53 Å distance represents the assembly in the native membrane, whereas the shorter distances were obtained on 2D-crystals. A minor and broad signal was found around 92 Å. This is $\sqrt{3}$ times the pore–pore neighbor distance, indicating that VDAC is roughly in hexagonal packing. The broadness of the 92 Å PCF peak is probably caused by the elliptical shape of VDAC and a lack of rotational alignment inducing distance dispersion between molecules separated by one VDAC. No long-range order peaks were found. In agreement, calculated power spectra of these membrane regions did not reveal distinct diffraction peaks corresponding to crystalline order. Lack of crystallinity is not contradictory with reports that predict VDAC association with oligomeric kinases and Bax, because the very high local density allows this kind of binding. Indeed, only a non-crystalline fluid assembly can account for assembly dynamics that are demanded for the manifold interactions and functional alterations of VDAC.

In an attempt to model the supramolecular structure of VDAC with a minimum of parameters, we have examined, using a Monte-Carlo approach, potentials capable of driving the formation of membrane domains resembling those that we have observed. The presence of many small aggregates indicates the absence of a macroscopic phase separation and a very dynamic system, strongly influenced by fluctuations and probably close to the critical point. We used triangular potentials (hard-core diameter 53 Å) with long-range attractive forces (diameter 106 Å) and potential well depths for VDAC–VDAC interactions of 1.7 $k_B T$ to 2.0 $k_B T$ ($k_B$ is the Boltzmann constant and $T$ is the absolute temperature). The resulting model (Figure 4(a)) fits well many aspects of our analysis of the MOM. Notably, the differentiation of high-density and low-density domains, a supramolecular assembly that results in the same PCF peaks (Figure 4(b)) and the presence of several small dynamic aggregates, are reproduced. However, though this model is able to reproduce some aspects of the VDAC assembly in the MOM, it is very sensitive to the parameters used, in particular the interaction potential depth. Thus if this model is realistic it predicts also a strong sensitivity of VDAC organization to parameters that might modulate protein–protein interaction strength such as pH, membrane potential, ion concentration and interactions with other proteins. It is particularly noteworthy that in this case, as in our previous studies of bacterial photosynthetic membranes, a strong lateral organization and differentiation of distinct membrane regions appear to be driven by relatively weak (a few times $k_B T$) long-range forces. The origin of such long-range forces has not yet been investigated, but membrane elastic and solvation forces are probably of particular importance.

AFM imaging of MOM has allowed us to study the supramolecular organization of VDAC and to gain further insight into the protein structure. We show how the mitochondrial porin can associate in mobile groups of highly variable numbers, probably in a function-related manner that needs further

**Figure 4.** Modeling of the supramolecular assembly of VDAC in native MOM. (a) VDAC distribution model in MOM. The separation of VDAC in high-density and low-density domains and the existence of small aggregates ranging from single molecules up to about 20, are reproduced (compare to Figure 1). (b) PCF analysis of the modeled VDAC assembly. As in the experimental data, a peak at 53 Å represents the most frequent intermolecular distance, and a minor peak at ~92 Å represents rough hexagonal packing in high-density domains (compare to Figure 2(c)).
elaboration. VDAC also forms densely packed patches, which may facilitate docking of other oligomeric proteins.

Materials and Methods

MOM purification

Mitochondria were purified from yeast (Saccharomyces cerevisiae strain W303) grown overnight on YP-glycerol (10 g/l yeast extract, 10 g/l peptone, 20 g/l glycerol) media at 30 °C. Cells were harvested and washed successively with water, Tris-SO4 buffer (100 mM Tris-H2SO4 (pH 9.4), 10 mM DTT) and sorbitol-buffer (1.2 M sorbitol, 20 mM KPO4 (pH 7.4)). The cell wall was then digested with 20,000 units of zymolase (Sigma) per gram fresh weight of cells for 2 h at 30 °C. Spheroplasts were collected and washed twice with fresh ice-cold sorbitol-buffer before being resuspended in breaking buffer (0.6 M sorbitol, 20 mM Hepes-KOH (pH 7.4), 1 mM PMSF) and lysed by 15 strokes with a Dounce homogenizer. Cells debris were sedimented by centrifugation at 1500 g for 5 min and mitochondria collected by centrifugation at 12,100 g for 10 min and then washed once in the same buffer. Mitochondria were quick frozen in liquid nitrogen and stored at –80 °C. Outer membranes were prepared essentially as described. Briefly, the mitochondrial pellet was resuspended in ice-cold lysis medium (0.25 mM EDTA, 0.25 mM EGTA, adjusted to pH 7.0 with NaOH), undispersed material was removed by centrifugation at 1000 g for 5 min, the solution was homogenized by three strokes of a Dounce homogenizer and stirred for 25 min on ice. Solid manitol was then added to a final concentration of 5% and the resulting solution layered onto a 0.55 M–0.9 M sucrose step gradient. After centrifugation at 60,000 g for 90 min in a Beckman SW28 rotor the outer membranes were recovered at the 0.55 M–0.9 M sucrose interface, diluted five times with 1 mM Tris-HEC (pH 7.4) and collected by centrifugation at 60,000 g for 45 min and washed with the same buffer. SDS-PAGE and mass spectrometry were used to characterize the MOM protein content.

Atomic force microscopy

The AFM24 was operated in contact mode at ambient temperature and pressure. Imaging was performed with a commercial Nanoscope-E AFM (Veeco, Santa Barbara, CA, USA) equipped with a vertical engagement 160 μm J-scanner and oxide-sharpened Si3N4 cantilevers (length 100 μm; k=0.2 N/m; determined by the thermal calibration method on a Picoforce AFM). For imaging minimal loading forces of ~100 pN were applied, at scan frequencies of 4–7 Hz using optimized feedback parameters. 3 μl of membrane solution (0.1 mg/ml of protein concentration) were injected into a 20 μl adsorption buffer drop (10 mM Tris-HEC (pH 7.2), 150 mM KCl, 25 mM MgCl2) on freshly cleaved mica. Subsequently, after ~2 h of adsorption, the sample was rinsed with recording buffer (10 mM Tris-HEC (pH 7.2), 150 mM KCl).

Data analysis

All image treatment and analysis of AFM topographs were performed using self written routines for the ImageJ image processing package28 and IGOR PRO (WaveMetrics, Lake Oswego, OR 97035 USA). Surface density calculations were made using the lipid surface height as threshold, and comparison of the surface occupied by VDAC with the protein free surface. Pore dimensions were measured by examining height profiles of VDAC topographs, setting the lipid bilayer height as pore delimiting threshold.

For pair correlation function analysis,29 pore center x and y coordinates were defined using cross-correlation routines.28 From this, separation vectors r, between pores were calculated. Hence, the probability p(r) of finding a complex (B) in a distance interval from r1 to r2 from a given complex (A) can be normalized, taking into account the surface area covered within the interval and the total number of complexes within the membrane. If the distribution is random, p(r) is always close to 1.27 For each complex, the distance to the closest membrane border was set as the maximum search radius, rmax.

Modeling

A Monte-Carlo approach was used to study the VDAC assembly in MOM. A 2D random mixture of discs at a density corresponding to the observed was used as starting condition. The modeling was performed over 108 Monte-Carlo steps in the NVT ensemble. Step sizes for displacements were updated regularly to maintain approximate 50% success rates. The interaction potentials used incorporated the observed hard-core diameters of 53 Å for VDAC, and an attractive long-range triangular potential well that falls to zero at a distance of 106 Å. This choice of potential distance is based on the observed lack of long-distance order in dense regions, and our knowledge of the phase diagram. The well depth was adjusted to approximate the observed PCF, paying particular attention to the relative heights of the successive peaks, with a well depth of 1.7 kBT to 2.0 kBT for VDAC–VDAC interactions providing kinetic trapping of the assembly and disassembly of associations into aggregates of a few molecules up to densely packed domains, in agreement with our measurements. With weaker attractive forces (e.g. 1.4 kBT) the dense regions were much less ordered and the second peak in the PCF was considerably too low, while stronger attractive forces (e.g. 2.2 kBT) resulted in multiple strong long-range oscillations in the PCF.

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