The crystal structure of mouse VDAC1 at 2.3 Å resolution reveals mechanistic insights into metabolite gating

Rachna Ujwal, Dulio Cascio, Jacques-Philippe Colletier, Salem Faham, Jun Zhang, Ligia Toro, Peipei Ping, and Jeff Abramson

*Department of Physiology, Cardiovascular Research Laboratories, Department of Anesthesiology, Division of Molecular Medicine, David Geffen School of Medicine, and Department of Energy Institute of Genomics and Proteomics, University of California, Los Angeles, CA 90095

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The voltage-dependent anion channel (VDAC) constitutes the major pathway for the entry and exit of metabolites across the outer membrane of the mitochondria and can serve as a scaffold for molecules that modulate the organelle. We report the crystal structure of a β-barrel eukaryotic membrane protein, the murine VDAC1 (mVDAC1) at 2.3 Å resolution, revealing a high-resolution passing through the VDAC pore. This segment is ideally positioned to regulate the conductance of ions and metabolites passing through the VDAC pore.

β-barrel | mitochondria | outer membrane protein | ATP flux

All eukaryotic cells require efficient exchange of metabolites between the cytoplasm and the mitochondria. This exchange is mediated by the most abundant protein in the outer mitochondrial membrane, the voltage-dependent anion channel (VDAC), which facilitates movement of ions and metabolites between the cytoplasm and the intermembrane space of the mitochondria. VDAC was first discovered in 1976 (1) and has since been extensively studied by a number of biochemical and biophysical techniques demonstrating its conserved properties of voltage gating and ion selectivity and its ability to act as a scaffold for modulator proteins from both sides of the outer membrane (2, 3).

Single-channel conductance experiments on VDAC1 at low membrane potential (10 mV) show a high conductance indicative of a large pore, often referred to as the open state of the channel (2). As voltage is increased (>30 mV) in either a positive or negative direction, a lower conductance, ostensibly the closed state, is obtained. Endogenous potentials caused by chemical gradients across the outer membrane [Donnan potentials (4)] may thus be sufficient to regulate this channel. Although the nature of either of these states is unknown in the absence of structural data, transition between them presumably involves conformational changes constituting a gating action that hinders the passage of metabolites such as adenine nucleotides. Furthermore, this transition is associated with altered ion selectivity because the channel shifts from weakly anion selective to weakly cation selective as it moves from open to closed. This complex gating behavior has driven numerous investigations that have provided sometimes contradictory findings; however, the role of VDAC to regulate metabolite traffic across the outer membrane is firmly established.

As the major pathway into and out of mitochondria, VDAC mediates an intimate dichotomy between metabolism and death in all cells (5). Mitochondrial-dependent cell death involves numerous proteins [including hexokinase (6) and the Bel-2 family of proteins (7), in particular] that alternatively promote or prevent mitochondrial dysfunction through interaction with, and potentially modification of, VDAC. Alterations in mitochondrial permeability have been implicated in the metabolic stresses of cancer (8) and cardiovascular disease (9). Therefore, the ability to modulate VDAC function in a rational manner will have important implications for novel therapeutics to modulate cell survival in different diseases (10). We have determined the structure of the murine VDAC1 (mVDAC1) at 2.3 Å resolution crystallized in a lipidic environment. In addition to providing concise structural details that can aid drug design, the high-resolution structure of mVDAC1 reveals insights into the gating mechanism for metabolites and ions entering and exiting mitochondria.

Results

We have expressed and purified mVDAC1 based on a procedure by Koppel et al. (11) with extensive modifications. After purification, the protein was shown to be folded by circular dichroism (data not shown). mVDAC1 was functionally characterized in planar lipid bilayers where it exhibited single-channel conductance of 3.7 ± 0.4 nS at 10 mV in 1 M KCl [open state; supporting information (SI) Fig. S1]. As the applied voltage was increased to ±30 mV, the channel shifted to a predominant conductance of 1.7 ± 0.2 nS (closed state; Fig. S1). These values are in good agreement with those recorded for endogenous VDAC1 isolated from rat (12). To understand functional aspects of ion and metabolite trafficking and the complex gating pattern observed electrophysiologically, we pursued the task of obtaining a high-resolution structure of mVDAC1.

Circular dichroism (CD) studies have shown that human VDAC1 (hVDAC1) exhibits different contents of secondary structure in detergent micelles as compared with phospholipid bilayers, with the latter displaying a higher content of β-sheet and a lower content of α-helix (13). Because a majority of the functional studies have been performed in phospholipid bilayers (2, 14) and it is assumed that under lipidic conditions VDAC would more closely resemble a “native” conformation, we crystallized mVDAC1 in 1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine (DMPC)/3-[3-Cholamidopropyl]dimethylammonium]-2-hydroxy-1-propanesulfonate (CHAPS) bicelles (15) using 2-methyl-2,4-pentanediol (MPD) as a precipitant. In contrast to crystallization in detergent micelles, bicelles are small bilayer...

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The authors declare no conflict of interest.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 3EMN).

1To whom correspondence should be addressed. E-mail: jabramson@mednet.ucla.edu.

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like discs that more closely mimic the native lipid environment. As a result, the structure we present here likely represents that of the endogenous channel.

**Structural Overview.** Optimized crystals of mVDAC1 belong to the monoclinic space group C2 with 1 molecule per asymmetric unit. The structure was solved by the single isomorphous replacement with anomalous scattering (SIRAS) technique by using an engineered cysteine to incorporate a single mercury atom, a method that was successfully used for LacY (16) and vSGLT (17). The model was refined from merged data to a resolution of 2.3 Å with $R_{free}$ of 27.7% and $R_{work}$ of 24.2% (Table S1). All 283 amino acids of mVDAC1 were built along with 47 water molecules. Thus, all structural features represented in this study, including the location of the N-terminal segment, were assigned on the basis of high-resolution data. Further details regarding the crystallization and structure determination can be found in Methods. Experimental and refined electron density maps are shown in Fig. S2.

Predictions regarding the number of strands contained in the VDAC $\beta$-barrel varied widely with 2 values in particular, 13 (18) and 16 (19, 20), emerging as the predicted number of $\beta$-strands. The structure of the mVDAC1 protomer forms a $\beta$-barrel composed of 19 $\beta$-strands with a shear number of 23 (Figs. 1 and 2). All of the strands exhibit an antiparallel pattern with the exception of strands 1 and 19 that associate in a parallel manner to close the barrel (Fig. 2A). The maximal height and width of the $\beta$-barrel is 35 and 40 Å, respectively. This report illustrates the high-resolution crystal structure of a eukaryotic $\beta$-barrel membrane protein with an odd number of strands (all known

![Fig. 1. Secondary structure schematic of mVDAC1. The mVDAC1 protein structure is colored from the N terminus in blue to the C terminus in red. The $\beta$-strands and $\alpha$-helix are depicted as arrows and cylinders, respectively, and are colored by using single-letter amino acid code. $\beta$-Strand residues facing the inside of the pore are shown with a black border. Residues in red form the hinge region.](image1)

![Fig. 2. Overall structure of mVDAC1. (A) Cartoon representation of mVDAC1 viewed parallel to the plane of the membrane. The mVDAC1 protein structure is colored from the N terminus in blue to the C terminus in red. (B) Cross-section view of A rotated 90° clockwise. $\beta$-Strands 3–7 are removed to illustrate positioning of the N-terminal segment. (C) Cross-section view of A with $\beta$-strands 19 and 1–4 removed. The interior surface of the mVDAC1 channel (cyan), created using the program HOLLOW (http://hollow.sourceforge.net), illustrates the contour of the pore. Dimensions at the entrance and along the narrowest point in the center of the pore are displayed. (D) Cartoon representation of mVDAC1 viewed perpendicular to the membrane plane (same coloring as in A).](image2)
prokaryotic β-barrels have an even number of strands), confirming the architecture observed in the recent NMR study on hVDAC1 (21).

The correct orientation of the protein within the outer membrane of the mitochondria cannot be ascertained from the structure presented herein, because there have been multiple biochemical studies producing conflicting results on the endogenous position of the N and C termini (22–24) (i.e., whether they are facing the cytosol or intermembrane space). The hydrophilic N terminus of the protein forms a distorted α-helix that is nestled within the pore and tethered to the first β-strand. Both the N and C termini reside on the same side of the membrane and clearly demonstrate that the N terminus is not embedded in the membrane as has been suggested (18).

The orifice of the pore resembles a slightly elliptical cylinder with a maximal inner dimension of 27 × 24 Å (Fig. 2C). The pore forms a large pathway that transcends the entire length of the protein, likely representing the open conformation of the channel. These dimensions are in good agreement with those reported by electron microscopy (25), atomic force microscopy (26), and the recently published NMR structure of hVDAC1 (21). Structural alignments between the crystal structure of mVDAC1 and the 20 deposited NMR structures of hVDAC1 (Protein Data Bank ID code 2K4T) showed an rmsd that varied from 1.72 to 4.68 Å (the average rmsd among all 20 NMR structures is 4.79 Å) (Fig. S3). In the NMR study, only 5 residues were assigned in the voltage-sensing N-terminal segment (residues 6–10). These studies were performed on hVDAC1 solubilized in detergent micelles, which may be responsible for the high degree of mobility. The structure presented here at 2.3 Å is that of mVDAC1 crystallized in lipidic bicelles. This high-resolution image reveals structural insights into the conduction pathway and moreover the precise localization and structural features of the voltage-sensing N-terminal segment. The assignment of the N-terminal segment is essential, because this helix plays a crucial role in gating the pore.

**Characteristics of the Pore.** The exterior of the β-barrel primarily consists of hydrophobic residues that are exposed to the lipid environment, whereas the interior is extensively hydrophilic, which is conducive for the efficient passage of ions and large metabolites (Fig. 3). Analysis of the interior walls of the pore reveals 15 positively-charged residues and 11 negatively-charged residues (Fig. 3). These charged residues are not uniformly distributed along the β-strands of the barrel, but are clustered primarily along strands 1–8. In contrast, there are fewer charged residues present on β-strands 9–19 (Fig. 3B and D). The pore spanning N-terminal α-helical segment contains 3 positive (Lys-12, Lys-20, and Arg-15) and 2 negative (Asp-9 and Asp-16) charges facing the interior of the pore. Electrostatic calculations (Fig. 3C and D) show that the interior of the pore has a higher density of positive versus negative charges, a fact that could account for the channel’s preference for transporting anions over cations in the open conformation (2). The α-helix of the N-terminal segment is located approximately halfway down the pore, resulting in a partial narrowing of the cavity to 27 × 14 Å (Fig. 2C), yet there is still ample space for the passage of metabolites. Thus the structure presented here likely represents the open conformation. This charged α-helix is ideally positioned to regulate the conductance of ions and metabolites passing through the VDAC pore.

**Arrangement and Coordination of the N-Terminal Helix.** Numerous studies have focused on the importance of the N-terminal α-helical segment’s role in channel function. There has been a wide range of predictions as to the functional disposition of this domain, ranging from forming a segment of the channel wall (22, 27) to acting as the voltage sensor (2, 11). In 2007, De Pinto et al. (28) used NMR to resolve a peptide fragment of the N terminus (Prn2–20) corresponding to amino acids 2–20 of hVDAC1. Their findings show a complex behavior of the N-terminal domain where the peptide is unstructured in aqueous solvent and forms a well-ordered α-helix from residues 5–16 in SDS. In the recently reported NMR structure of hVDAC1, an accurate analysis of the structure of the N-terminal segment and its localization with respect to the rest of the protein was not possible because of the incomplete assignment of this region (residues 1–5 and 11–20 unassigned) (21).

However, our high-resolution structure presented herein shows the N-terminal segment (amino acids 1–26) forming a hydrogen-bonding pattern that facilitates its orientation against the interior wall of the pore (Fig. 4A) adjacent to β-strands 8–19. The first 5 N-terminal amino acids extend down the pore where 2 hydrogen bonds are observed between the carbonyl oxygens of Ala-2 and Pro-4 and the main-chain nitrogen of His-122 and ND2 of Asn-124, which are located on the wall of the pore. These interactions stabilize the N terminus to β-strand 8. Continuing in a C-terminal direction down the protein, a distorted helix is formed from amino acids 6–20 where the hydrogen-bonding pattern is broken at Leu-10 and Gly-11, separating the helix into 2 segments. Although the stable helical hydrogen-bonding pattern is disrupted, these 2 segments are capable of maintaining a rigid structure because Arg-15 forms bridging bidentate hydrogen bonds with the carbonyl oxygens from Ala-8 and Leu-10 (Fig. 4B). The helical portion of the N-terminal segment has 2 hydrogen bonds to β-strands 12 and 16, stabilizing its interaction with the wall of the pore. Connecting the soluble N-terminal domain to the first β-strand of the barrel is a highly conserved
between Arg-15 and the carbonyl oxygens of Ala-8 and Leu-10.

Physiological Modulators of VDAC. As predicted, VDAC has a large hydrophilic pore capable of facilitating the passage of ions and large metabolites such as ATP. There have been a number of reports suggesting the channel may have specific nucleotide binding sites (2, 30). To address this issue, we attempted to identify an ATP binding site in the structure of mVDAC1. We resolved the structures of mVDAC1 in the presence of 5, 10, 50, and 100 mM ATP, but the corresponding structures revealed no additional electron density that would correspond to ATP. Similarly, we have crystallized mVDAC1 in the presence of other known nucleotide modulators (ADP and AMP-PNP) and observed no changes in the mVDAC1 structure, as determined by inspection of $F_{\text{o(nucleotide)}} - F_{\text{o(native)}}$ maps. We therefore conclude that, at least in this conformation, there is likely no stable or specific binding site for these nucleotides.

Previous studies have shown that Ca$^{2+}$ binds and regulates VDAC (31, 32), a result with clear implications for the role of the protein in mitochondrial-dependent cell death. Under initial crystallization conditions (where trace Ca$^{2+}$ may be present), we were unable to identify electron density or a coordination pattern that resembles Ca$^{2+}$ ligation. To further address this issue, we solved the structure of mVDAC1 in the presence of 25 mM EGTA (a calcium-chelating agent that should bind all Ca$^{2+}$ ions in solution and from the protein) and separately in the presence of 25 mM CaCl$_2$. Crystals formed in the presence of EGTA, and separate successful crystallization trials in the presence of AlCl$_3$ or InCl$_3$ were indistinguishable from the initial conditions with trace Ca$^{2+}$. When Ca$^{2+}$ is present in abundance, we observe electron density near Glu-73 that is indicative of metal ion coordination between 2 antiparallel mVDAC1 monomers (Fig. S4). De Pinto et al. (33) had shown that Glu-73 is buried in a hydrophobic environment, an observation that is explained by the current structure, which demonstrates its localization within the membrane. Furthermore, mutagenesis and functional analyses have implicated this residue in Ca$^{2+}$ binding (32) and hexokinase-mediated protection against mitochondrial-dependent cell death (34). Based on the data presented here, Ca$^{2+}$ must be present at excessive concentrations for stable binding to the protein. The only way this could happen endogenously would be with enormous levels of Ca$^{2+}$ present and/or other gross disruption of the lipid interface with the outside of the pore, events that may occur during mitochondrial-dependent cell death.

Interestingly, crystallization in the presence of Mg$^{2+}$–ATP results in a very similar localization pattern of the Mg$^{2+}$ as was observed for Ca$^{2+}$ at position Glu-73 of the crystal antiparallel dimer (Fig. S4). As was the case in the presence of ATP alone, this crystallization scenario does not reveal stable or specific binding site for the nucleotide. As mentioned above, Glu-73 is essential for the binding of the antiapoptotic protein hexokinase to VDAC (34). It is well established that the hydrophobic N terminus of hexokinase is required for its interactions with mitochondria (35), presumably through insertion into the membrane. Furthermore, high concentrations of Mg$^{2+}$ are required for hexokinase to associate with the mitochondria (36). Thus, Mg$^{2+}$ coordination to Glu-73 may reveal a structural feature that facilitates association of the N-terminal segment of hexokinase with the external surface of the VDAC pore, a process likely to be concomitant with inhibition of mitochondrial-dependent cell death.

Regulation of VDAC Permeability. It is well established that VDAC is the principal governor for the exchange of metabolites between the intermembrane space of the mitochondria and the cytosol. The structure presented here reveals a number of features that are consistent with the open conformation of the channel and provides a possible mechanism for regulating the flow of metabolites. The arrangement of charges in the wall of the $\beta$-barrel are clustered primarily on the first half of the pore (strands 1–8), whereas the second half is less hydrophilic ($\beta$-strands 9–19). In this second half of the pore, the soluble N-terminal segment descends into the cavity forming a distorted
a-helix (amino acids 6–20) containing 5 charged residues that face toward the aqueous environment. In this configuration the helix, rather than the beta-barrel, contributes to a symmetrical charge distribution (Fig. 3).

There is a general consensus that the N-terminal segment of VDAC is involved in voltage gating and therefore it may adopt different conformations depending on factors external to the protein (e.g., protein–protein interactions, ion interactions, lipid environment). In our crystals, the a-helix portion of the N-terminal segment is positioned halfway down the pore, causing a narrowing of the cavity (27 × 14 Å) (Fig. 2C). This constriction site is ideally situated to regulate the flow of metabolites. The connection from the a-helix to the first beta-strand is a flexible and highly conserved sequence (Gly-21–Tyr-22–Gly-23–Phe-24–Gly-25). The a-helix in red represents the proposed model of a closed state. The model was made by 10° rotation along the horizontal axis of the protein at the hinge region and would produce a conformation sufficient to perturb metabolite flux. As detailed in Discussion, the structure indicates that this hypothetical occluded state could be stabilized by interactions of the soluble N-terminal domain with the opposite wall of the pore.

What Are the Structural Rearrangements Underlying Such a Movement of the N-Terminal Gating Helix? There are a number of stimuli known to favor the closed state of the channel: voltage potentials in excess of ±30 mV (37), protein interactions (38), and chemical modulators like NADH (2). These stimuli may disrupt the hydrogen bonds that position the N-terminal segment along the interior wall of the beta-barrel. Once interactions with the wall of the pore are disrupted, the N-terminal segment would be free to reorient within the channel lumen aided by the flexibility in the hinge region. At this point, the repositioning of the a-helix may be facilitated by the protein’s inherent chemical potential: electrostatic calculations reveal a cluster of positive charges along the a-helix and a patch of negative charges located on the opposing wall (beta-strands 2, 3, 5, 6, and 8) in the region where the helix could possibly interact if VDAC would adopt the more occlusive conformation of the pore. Mutational data from the Colombini group (39) support this hypothesis, wherein substitutions of residues forming the N-terminal a-helix alter voltage-dependent gating in a charge-dependent manner. Alternatively, the 2 segments of the a-helix portion are joined by bridging bidentate hydrogen bonds from Arg-15 (Fig. 4B). Perturbation of this interaction could also impair metabolite flux by repositioning of amino acids 1–10.

Further evidence in support of alterations in the position of the a-helix came from the recent NMR structure of hVDAC1. Hiller and colleagues (21) report an NADH interaction site on beta-strands 17 and 18 involving residues at position Leu-242, Ile-243, Gly-244, Ala-261, Leu-263, and Asp-264 (residues from hVDAC1) (Fig. 5). NADH has been shown to favor the closed state of the channel (2), and the interaction site described above flanks the hinge region of the N-terminal segment as determined from the data in the present study. Our structure clearly demonstrates that there is insufficient space for NADH binding in this region. Hence, the N-terminal segment would have to be repositioned to accommodate NADH and in doing so would close the channel.

As is the case with most high-resolution structures, many critical features of the protein’s anatomy have been identified, explaining several key features of its physiological behavior. However, many new questions also arise that will require additional structure and functional studies to answer. The structure and hypothesized mechanism of mVDAC1 presented herein establish a template to be tested by biochemical/biophysical techniques and provides critical information for our understanding of mitochondrial biology.

Methods

Expression, Purification, and Refolding. The expression and purification protocol was adapted from Koppel et al. (11) with extensive modifications. Briefly, histidine-tagged mVDAC1 was expressed in Escherichia coli and the inclusion bodies (IB) were isolated. Solubilized IB were purified by using a Talon affinity column and refolded by using n-dodecyl-β-N,N-dimethylamino-N-oxide. Refolded protein was applied to a Superdex 75 column for isolation of a homogenous protein population. More details can be found in SI Text.

Single-Channel Conductance Measurements. The single-channel conductance across black lipid bilayers was recorded as described (13). More details can be found in the SI Text.

Crystallization and Structure Determination. Bicelles were prepared as described (15). Purified mVDAC1 at a concentration of 15 mg/ml was mixed in a 4:1 protein/bicelle ratio with a 35% (2.8:1) DMPC/CHAPSO bicellar solution, giving 12 mg/ml mVDAC1 in 7% bicelles. Our best crystals grew at 20°C in 18–20% MPD, 0.1 M Tris HCl (pH 8.5) with 10% PEG400 added to the protein drop only. Heavy atom labeling of single cysteine constructs was done by using methyl mercurl acetate. Native data were collected at the Advanced Light Source (Berkeley, CA) at beamline 5.0.2, and Hg-derivative data were collected at the Advanced Photon Source (Chicago) at the microfocal beamline 24-ID-E. Both native and derivative data were processed by using the program DENZO (40).

One heavy atom site was found for Cys-127, and SIRAS phases were calculated in PHENIX and MLPHARE (41). Density modification was done by using DM from the CCP4 package (42). Diffraction data from 20–2.3 Å were used for refinement and electron-density map calculations. Graphic operations and model building were performed with COOT (43). Refinement and map calculations were performed by using PHENIX version 1.3 (44) and CNS version 1.2 (45). At the end of the refinement, the Rfree was virtually featureless at ≤3 sigma. Figures were produced with PyMOL (www.pymol.org). Molecular topologies and parameters of DMPC were created by using ELBOW in PHENIX. The quality of the structure was checked and validated by using PROCHECK. Refinement statistics are shown in Table S1.

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Supporting Information

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Expression, Purification, and Refolding.

mVDAC1 construct with 6× histidine tag at the amino terminus was generated by subcloning mVDAC1 encoding gene into pQE9 vector and subsequently transformed into M15 E. coli for protein expression. Cells were grown at 37°C for ~2 h in LB medium to A600 = 0.6 and induced for 4 h with 1 mM IPTG. Cells were harvested and resuspended in 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 20% sucrose. The resuspended pellet was sonicated and centrifuged (12,000 × g, 15 min) to obtain inclusion bodies. The inclusion body pellet was washed with 2 column volumes of wash buffer [20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 2 mM CaCl2], repelleted and solubilized in equilibration buffer [20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 6 M guanidinium hydrochloride (GdnHCl)]. Solubilized inclusion bodies were applied to a Talon metal affinity column, washed with equilibration buffer, and eluted with equilibration buffer containing 150 mM imidazole. Affinity-purified protein was concentrated to 5 mg/ml by using Amicon Ultra-10 concentrators and transferred to a 6- to 8-kDa dialysis bag. Refolding dialysis was carried out in three steps: (i) 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 1 mM DTT, 3M GdnHCl; (ii) 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 1 mM DTT, 2% LDAO; and (iii) 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM DTT, 2% LDAO. Refolded protein was concentrated by using Amicon Ultra-30 concentrators and ultracentrifuged (355,000 × g, 30 min) to remove aggregated protein.

The refolded protein sample was applied to a Superdex 75 column and eluted with SEC buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM DTT, 0.1% LDAO] to obtain a homogenous protein population. The eluted peak was washed with 3 volumes of crystal buffer [20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.1% LDAO], concentrated by using Amicon Ultra-30 (Millipore) and ultracentrifuged (355,000 × g, 30 min). Protein purity and identity were verified by SDS/PAGE, Western blotting, ESI-MS, and tandem mass spectrometry.

Single-Channel Conductance Measurements.

diPhPC bilayers were generated across a 0.1-mm hole separating the cis and trans chambers containing 10 mM Tris-HCl (pH 7.4), 1 M KCl, 5 mM CaCl2. One-microliter aliquots of 0.1 μg/ml mVDAC1 were added to the cis compartment and stirred. When channel activity appeared, voltages of ±10 ± 60 mV in steps of 10 mV were applied and currents recorded for at least 3 min at each voltage. From the single-channel measurements, the total point histogram at each applied voltage was plotted, and the most frequent conductance was calculated at that voltage. The relative conductance (G0/GU) was plotted as a function of the applied voltage, where G0 is the conductance at 10 mV and GU is the conductance at voltage U. All experiments were performed at room temperature. The cis chamber was the voltage-controlled side, whereas the trans chamber was connected to ground. The amplified signal was filtered at 0.5 kHz with an 8-pole Bessel filter viewed with an analog/digital oscilloscope and recorded online. A custom-made software (GpatchM) was used for data collection and analysis.

Crystallography.

Purified mVDAC1 protein at 15 mg/mL was mixed with up to 7% NEM solution, giving 12 mg/mL mVDAC1 in 7% bicine. Crystallization trials, using up to 700 commercially available conditions, were performed by using the hanging drop vapor diffusion method at 20°C with the Mosquito crystallization robot (TTP Labtech). Initial crystals grew in 0.1 M Tris-HCl (pH 8.5), 15% MPD; 0.1 M Tris-HCl (pH 7.5), 15% ethanol, 0.3 M NaCl; and 0.1 M Tris-HCl (pH 7.5), 9% isopropanol, 0.3 M NaCl. The proteinaceous nature of the crystals was confirmed by UV microscopy (Korima). Our best crystals grew in 18–20% MPD, 0.1 M Tris-HCl (pH 8.5) with 10% PEG400 added to the protein drop only (and not in the reservoir). A technical advantage of bicelle mixtures over other lipidic media is the ability to use robotics for crystallization; bicelle mixtures have lower viscosity at low temperature (≈4°C), but as the temperature increases (to ≈30°C), the bicelles form a gel that provides a scaffold for type I crystals.

 Mercury Labeling. All cysteine constructs were labeled by using a 5-fold molar excess of methyl mercuric acetate (MMA) for 2 h at room temperature before crystallization. Excess MMA was removed by repeated washing with crystal buffer in an Amicon Ultra-30 concentrator. The wild-type sequence, which contains two cysteines at position 127 and 232, did not produce any crystals after MMA labeling. Single-cysteine constructs, mVDAC1-C127A and mVDAC1-C232A, were prepared by using the site-directed mutagenesis kit (Stratagene) by mutating the native cysteines to alanines. The mVDAC1-C232A construct, having the single Cys-127 residue, was successfully labeled and used for structure determination.

Electrostatic Potentials. Electrostatic potentials were calculated by using the nonlinear Poisson–Boltzmann equation, as implemented in APBS version 4.0 (1), at a temperature of 310 K and a salt (NaCl) concentration of 150 mM. Hydrogens were added and optimized at pH 7.4 by using PDB2PQR (2) before performing the APBS calculation.

Identification of Metal Binding Sites.

To assess the binding of ions and nucleotides, difference Fourier maps were computed by using the observed structure factor amplitudes for (i) Ca2+/mVDAC1 co-crystal, (ii) Mg2+/ATP mVDAC1 co-crystal, and (iii) EGTA-soaked mVDAC1 crystal and both the observed structure factor amplitudes and the calculated phases of native mVDAC1. Structure factor amplitude difference maps were Q-weighted to improve the signal-to-noise ratios of the Fourier difference maps (3).

Fig. S1. Electrophysiology experiments on mVDAC1. To ensure that the protein used for crystallization was functional, we examined the electrophysiological properties of purified mVDAC1. (A) (Left) Total point conductance histograms of mVDAC1 incorporated in black lipid membranes at different applied voltages. The histograms show the $3.7 \pm 0.4 \text{nS}$ (S1) and the $1.7 \pm 0.2 \text{nS}$ (S2) preferred states during 3-min recordings. (Right) Representative current traces at the indicated voltages. At lower voltages ($< \pm 30 \text{mV}$), the channel primarily exists in a fully open state (S1) with a dominant conductance of $4 \text{nS}$. As the applied voltage is increased in positive ($> +30 \text{mV}$) or negative ($< -30 \text{mV}$) direction, the channel shifts to a predominant subconductance of $2 \text{nS}$ (S2). Baseline (B) for each trace is indicated by a dotted line. (B) Conductance profile (voltage vs. relative conductance plot) of mVDAC1 shows characteristic voltage-dependent behavior. Mean relative conductances ($G/G_0$) were calculated as a function of the bilayer voltage; $G$ is the dominant conductance at the applied voltage, and $G_0$ is the dominant conductance at $10 \text{mV}$. Bars indicate standard deviations.
Fig. S2. Electron density maps. (A) SIRAS experimental map displayed at 1.2 $\sigma$ showing the crystal packing. (B) Final 2 $F_o$ – $F_c$ map displayed at 1.2 $\sigma$ showing the crystal packing. (C) Stereoview of a close-up of the N-terminal helix. Arg-15 is shown interacting with the main-chain carbonyl oxygen of Ala-8 and Leu-10. The 2 $F_o$ – $F_c$ map displayed at 1.2 $\sigma$ is superimposed on the model.
Fig. S3. Overlay of mVDAC1 and hVDAC1. Structural alignment of mVDAC1 (blue) with hVDAC1 (Protein Data Bank ID code 2k4t) NMR models: model 2 (yellow), model 6 (orange), and model 8 (light orange). The average rmsd of the 20 deposited structures of hVDAC1 is 4.79 Å, whereas that between those structures and mVDAC1 ranges from 1.72 to 4.68 Å. Models 2, 6, and 8 are those with the lowest rmsd values (~2 Å). The N terminus of each model is numbered. (A) Viewed perpendicular to the plane of the membrane. (B) Cross-section view parallel to the plane of the membrane.
Fig. S4. \( F_o - F_c \) maps showing localization of Ca\(^{2+}\) and Mg\(^{2+}\) along the mVDAC1 dimer interface. The difference Fourier maps \((F_o - F_c)\) were computed by using the observed structure factor amplitudes for Ca\(^{2+}\)/mVDAC1 and Mg\(^{2+}\)/ATP/mVDAC1 cocrystals and both the observed structure factor amplitudes and the calculated phases of mVDAC1 in the absence of these compounds. Structure factor amplitude differences were Q-weighted to improve the signal-to-noise ratios of the Fourier difference maps. Maps for Ca\(^{2+}\) and Mg\(^{2+}\) are colored blue and pink, respectively. Both maps are contoured at 4.5 \( \sigma \).
<table>
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<tr>
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<th>Native</th>
<th>Hg derivative</th>
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<td>Unit Cell abc, Å</td>
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<td>98.95, 57.97, 66.81</td>
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<td>αβγ, °</td>
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<td>90, 97.94, 90</td>
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<td>Resolution, Å</td>
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<td>50–2.9 (3.0–2.9)</td>
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<td>Rmerge, %</td>
<td>2.9 (47.1)</td>
<td>9.6 (29.1)</td>
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<td>Completeness, %</td>
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<td>99.7 (98.9)</td>
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<td>Redundancy</td>
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<td>5.3 (3.4)</td>
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<tr>
<td>l/</td>
<td>θ</td>
<td>24.1 (2.6)</td>
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<tr>
<td>No. of unique reflections</td>
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<td>Rwork/Rfree</td>
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<td>rmsd bonds, Å</td>
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<td>SIRAS phasing statistics</td>
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<td>Phasing power (acentric/centric)</td>
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<tr>
<td>Figure of merit (after density modification)</td>
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The values in parentheses refer to the highest-resolution shell. $R_{merge} = \Sigma |I_i - \langle I\rangle|/\Sigma |I_i|$, where $I_i$ is the intensity of the $i$ reflection. $R_{work} = \Sigma |F_{o} - |F_{c}||/\Sigma |F_{o}|$. $R_{Cullis} = \Sigma |F_{PH} + F_{PH} - |F_{PH} (calc)||/\Sigma |F_{PH} - F_{PH}$. Phasing power $= |F_{PH (calc)}|/\Sigma |F_{PH} \pm |F_{PH} (calc)|$.  
