Bacterial Expression and Characterization of the Mitochondrial Outer Membrane Channel

EFFECTS OF N-TERMIAL MODIFICATIONS

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Several forms of the voltage-dependent anion-selective channel (VDAC) have been expressed at high yield in Escherichia coli. Full-length constructs of the proteins of Neurospora crassa and Saccharomyces cerevisiae (ncVDAC and scVDAC) have been made with 20-residue-long, thrombin-cleavable, His$_6$-containing N-terminal extensions. ncVDAC purified from bacteria or mitochondria displays a far-UV CD spectrum (in 1% lauryl dimethylamine oxide at pH 6–8) similar to that of bacterial porins, indicating extensive β-sheet structure. Under the same conditions, the CD spectrum of bacterially expressed scVDAC indicates lower β-sheet content, albeit higher than that of mitochondrial scVDAC under the same conditions. In phospholipid bilayers, the bacterially expressed proteins (with or without N-terminal extensions) form typical VDAC-like channels with stable, large conductance open states (4–4.5 nano siemens in 1 M KCl) and voltage-dependent transitions to a predominant substate (about 2 nano siemens). A variant of scVDAC missing the first eight residues and having no N-terminal extension also has been expressed in E. coli. The truncated protein has a CD spectrum similar to that of mitochondrial scVDAC, but its channel activity is abnormal, exhibiting an unstable open state and rapid transitions between multiple subconductance levels.

VDAC or mitochondrial porin is the most abundant transport protein in the mitochondrial outer membrane. Although VDAC proteins from different species have only weak sequence homology, their functional properties are highly conserved (1, 2). In artificial lipid bilayers, the channel occupies a high conductance, anion-selective open state (4–4.5 nS in 1 M KCl) at small membrane potentials and switches, at potential amplitudes above 30–40 mV, to lower conductance substates (2–2.5 nS) that are cation-selective and less permeable to ATP and ADP (3–5). The critical potential at which gating occurs is decreased in the presence of polyanions and a soluble mitochondrial protein fraction (6, 7). VDAC sequences from a number of species contain numerous stretches of alternating hydrophobic and hydrophilic residues (e.g. see Refs. 8 and 9), suggesting an amphipathic β-barrel motif like that of the bacterial porins (10, 11). Analysis of VDAC sequences with the Gibbs sampler indicates the presence of numerous matches to a residue-frequency motif associated with transmembrane β-strands in bacterial porins (12). Circular dichroism studies of VDAC purified from Neurospora crassa in both lipids and non-denaturing detergents (13) suggest that ncVDAC has a high β-sheet content, consistent with a bacterial porin-like β-barrel structure (e.g. see Ref. 14).

The main focus of our research is to determine the molecular basis for VDAC's functional properties, in particular, its permeability and mechanism of gating. In this report, we describe the bacterial expression of functional VDAC from Saccharomyces cerevisiae and N. crassa and compare the effects of N-terminal extension and truncation on the properties of this channel protein.

EXPERIMENTAL PROCEDURES

Isolation of Mitochondria—Mitochondria were isolated from liquid cultures of wall-less N. crassa (FGSC 326) and from blocks of dry S. cerevisiae (Red Star) (15, 16). Chemicals were obtained from Sigma unless indicated otherwise, and solutions were made with reagent-grade deionized water (Milli-Q system, Millipore Corp., Bedford, MA).

Mitochondrial VDAC Isolation—VDAC was purified using a procedure modified from that of Shao et al. (13). Mitochondrial suspensions were solubilized at room temperature in 20 ml of 2% Buffer A (4% LDAO (Calbiochem, La Jolla, CA), 2 mM EDTA, pH 7.0) and centrifuged (27,000 x g, 30 min). (This and subsequent steps were performed at 4 °C.) The supernatant was diluted to a final concentration of 2% LDAO with TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.0) and loaded onto a prewashed (Buffer A) ceramic hydroxylapatite column (Bio-Rad). VDAC was eluted with Buffer A containing 50 mM KCl and 5 mM potassium phosphate (pH 6.8), and pooled fractions were concentrated by centrifugation in Centricon 30 tubes (Amicon, Beverly, MA) to 100–200 μg of protein/ml and stored at −70 °C.

Construction of Expression Plasmids—Four different VDAC constructs were generated using two different IPTG-inducible expression systems. Two constructs were made with the pMAL system (New England Biolabs, Beverly, MA), which yields fusion proteins containing maltose-binding protein (MBP) at the N terminus, followed by a Factor Xa cleavage site. scVDAC cDNA was partially digested to make an EcoRV-HindIII fragment that was incorporated into the pMAL-c expression plasmid between StuI and HindIII sites (nucleases obtained from New England Biolabs). Factor Xa cleavage would yield scVDAC lacking the first 8 residues. Polymerase chain reaction was used to generate a 165-base pair fragment of ncVDAC cDNA encompassing residues 3–51, and silent mutations were made at nucleotide 9 to create a SnaBI site and at nucleotide 132 to remove a second BsrEI site. This cDNA fragment and a BsrEI-NsiI fragment containing residues 52–283 were cloned between the XmnI and PstI sites of pMAL-c2 (New
England Biolabs). Factor Xa cleavage of the resulting protein would be missing the first 2 residues. The pMAL-based plasmids were transformed into a competent Escherichia coli host strain, NM522, and grown in the presence of 100 µg/ml ampicillin. Full-length constructs of ncVDAC and scVDAC were made by polymerase chain reaction amplification and in-frame insertion of the pET-15b expression vector (Novagen, Milwaukee, WI). These proteins contain a 20-residue N-terminal extension MGSSHHHHHHSSGLVPRGSH that ends with a thrombin cleavage site. DNA sequences of the entire reading frames were determined and found to be identical to published sequences (8, 17). The pET-15b-based plasmids were transformed into the E. coli host strain BL21(DE3) and grown in the presence of 100 µg/ml ampicillin.

**VDAC Expression and Purification from Inclusion Bodies—**Bacterial growth and induction were carried out essentially according to plasmid manufacturers’ instructions, and inclusion bodies were collected using a method based on that of Kaplan et al. (18). Briefly, overnight cultures were diluted 50-fold (1 liter final volume) into LB media (containing 100 µg/ml ampicillin) and grown to an A600 of 0.5–0.6. After addition of 0.5 mM IPTG, the cells were grown for an additional 2.5 h, placed on ice for 5 min, and centrifuged (4000 x g, 10 min). The pellets were resuspended with 100 ml of 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 20% sucrose, and incubated with lysozyme (12.5 µg/ml, 22 °C, 10 min) and Triton X-100 (0.6%, 4 °C, 10 min). This lysate was sonicated (two 30-s pulses at a setting of 4 using a model 50 sonic dismembrator, Fisher Scientific, Pittsburgh, PA), and centrifuged (12,000 x g, 15 min). Pellets were washed with 20 mM Tris-HCl (pH 8.0) containing 2 mM CaCl2 for the pET-based constructs, resuspended (12,000 x g, 15 min), and dissolved in 75 ml of solubilization buffer (100 mM NaCl, 20 mM Tris-HCl, pH 8.0, and 6 mM GdnHCl). After stirring for 1 h, insoluble material was removed by centrifugation (12,000 x g, 15 min).

**Purification of MBP Fusion Proteins—**DAO (2% final concentration) was added to the solubilized inclusion bodies containing MBP-VDAC fusion proteins and GdnHCl was removed by dialysis against loading buffer (2% DAO, 20 mM Tris-HCl, pH 8.0). The protein suspension was loaded onto a prewashed ceramic hydroxylapatite column, and bacterial porins were eluted with a KCl gradient (100–500 mM) containing 5 mM potassium phosphate (pH 6.8). MBP-VDAC was eluted with 300 mM potassium phosphate (pH 6.8) and treated with factor Xa (20 µg of enzyme/mg of protein in loading buffer containing 100 mM NaCl and 2 mM CaCl2) to cleave the MBP and VDAC polypeptides. Following dialysis against loading buffer, the sample was loaded onto a second hydroxylapatite column and VDAC was eluted with 100 mM KCl, 5 mM potassium phosphate (pH 6.8). Foiled fractions were concentrated to 2 mg/ml by centrifugation in Centricon 30 tubes.

**Purification of His-containing VDAC Constructs—**Solubilized inclusion bodies (75 ml) containing Hiscontaining VDAC proteins were diluted with 25 ml of solubilization buffer without GdnHCl and added to 20 ml (bed volume) of prepared TALON™ metal affinity resin (CLONTECH, Palo Alto, CA). After 30 min, the matrix was poured into a column (16 mm diameter), and washed with three volumes of column buffer (4.5 mM GdnHCl, 100 mM NaCl, 20 mM Tris-HCl, pH 8.0) containing 50 mM imidazole. Hiscontaining VDAC was eluted with two volumes of column buffer containing 50 mM imidazole. Protein fractions were concentrated to 6–8 mg/ml by centrifugation in Centricon 30 tubes. DAO was added to a final concentration of 2% and GdnHCl was removed by dialysis against loading buffer. Proteins were stored at −70 °C at a concentration of 1–5 mg/ml.

For some experiments, the His-containing VDAC constructs were treated with trituration according to manufacturer’s instructions (Novagen) and dialyzed overnight (Spectrapor membrane, 25,000 molecular weight cutoff) to remove N-terminal fragments. The extent of cleavage was monitored by SDS-PAGE (see below).

**Gel Electrophoresis and Western Blots—**Proteins in DAO-containing fractions were precipitated with cold acetone and solubilized in 1% DAO-solubilization buffer prior to electrophoresis in either 10% or 12% polyacrylamide slab gels (Mini-PROtein II, Bio-Rad) as described by Stanley et al. (19). The proteins were either visualized by Coomassie staining or electrotransferred (Mini-Trans-Blot, Bio-Rad) to nitrocellulose membranes (0.45 mm, Bio-Rad). Electrotransfection and immunoblotting were done as described previously (19) using polyclonal antipeptide antibodies with the N- and C-terminal regions of ncVDAC and scVDAC (19), an anti-MBP antibody (New England Biolabs), or an anti-His antibody (San Diego Bio, San Diego, CA).

**Mass Spectroscopy—**MALDI-TOF mass spectroscopic data were obtained with a Bruker (Billerica, MA) REFLEX II instrument using a matrix of 2,5-dihydroxybenzoic acid (Aldrich Chemical, Milwaukee, WI). Spectra, typically the average of 50 shots, were acquired in linear mode and calibrated with carbonic anhydrate (molecular mass: 28,834 daltons) as both an external and internal standard.

**Planar Lipid Bilayer Experiments—**Electrophysiological measurements were made using planar bilayers composed of soybean 1-α-lecithin phosphatidylcholine (Sigma) or synthetic diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL). The method of Labarca and Latorre (20) was used to generate bilayers across a 0.15-mm hole separating two chambers containing 1 mM KCl, 5 mM CaCl2, 5 mM Hepes, pH 7.4. Bacterially expressed proteins were incubated 30 min on ice with 10 volumes of an ergosterol suspension (1 mg/ml chamber buffer containing 2% DAO) before being added to the bilayer system (21). Channel activity was observed after adding 6–13 pmol of VDAC to one chamber, and resulting current traces were recorded and analyzed as described previously (13). Bilayers were voltage-clamped using a Dagan 3900A amplifier (Dagan, Minneapolis, MN) in the “inside-out” mode. Mean open times were determined at −25 mV from current traces usually 40–60 s in duration, which were low pass-filtered to 2 kHz and stored at 5 kHz using Strathclyde Electrophysiological Data Analysis Software (courtesy of J. Dempster, University of Strathclyde, Strathclyde, United Kingdom).

**Far-UV Circular Dichroism Spectropolarimetry—**CD measurements were carried out on a J-720 spectropolarimeter (Jasco, Easton, MD). Measurements were taken from VDAC suspensions (approximately 1 mg/ml) at 20 °C using path lengths of 0.5–0.05 mm. Spectra were recorded from 260 to 180 nm in 0.2-nm increments with a 1-s time constant. Each spectrum shown is the average of eight scans corrected for background by subtraction of the spectrum corresponding to the appropriate solvent minus protein. Mean residue molar ellipticities, θ (degree•cm2•dmol−1), were based on protein concentrations measured using the bicinchoninic acid assay (Sigma); a mean residue molecular weight of 106 was used (13). Solutions were buffered with either 20 mM Tris-HCl (pH 7.0–8.0) or 20 mM sodium citrate (pH 3.8–7.0).

**RESULTS**

**Purification of Bacterially Expressed VDAC—**First attempts to overexpress VDAC in E. coli employed the pMAL expression system, which offered, in principle, easy purification. Proteins are expressed as fusion proteins with MBP, fractionated over an amylose-containing matrix, and cleaved with factor Xa. Both MBP-scVDAC and MBP-ncVDAC were obtained in large quantities (20–30 mg/liter of cell culture) in inclusion body fractions. The MBP-scVDAC fraction yielded two prominent bands with SDS-PAGE at Mr ~ 70,000 and 66,000 (Fig. 1, lane 2). Both bands reacted with antibodies against both the N- and C termini of VDAC (data not shown), indicating that any significant truncation due to proteolysis probably occurred in the MBP part of the protein. Expression of MBP-scVDAC yielded a
characteristics of bacterially expressed VDAC

Detergent Solubility of Bacterially Expressed Proteins—Of several non-denaturing detergents screened, including octyl β-glucoside, the only one in which the bacterially expressed VDAC proteins are soluble at concentrations above 1 mg/ml is LDAO. ncVDAC(His6) is soluble at 4–20 °C in 1% LDAO at concentrations above 5 mg/ml, while scVDAC(His6) has a solubility limit of around 2 mg/ml.

Circular Dichroism and Channel Characteristics of Mitochondrial VDAC—Far-UV CD spectra of VDAC isolated from N. crassa and S. cerevisiae mitochondria are shown in Fig. 3. The spectrum of ncVDAC in 1% LDAO at pH 6–8 (Fig. 3A, solid curve) closely resembles those of the same protein (13) and of bacterial porins like OmpF (14) in nondenaturing detergents. The CD spectrum of ncVDAC, containing a single broad minimum at 214 nm and a crossover to positive ellipticity at 200 nm, is consistent with high β-sheet content (45%) and low α-helical content (12%) (Table I). The far-UV CD spectrum of mitochondrial scVDAC in 1% LDAO (Fig. 3A, dashed curve) is considerably different, displaying two minima, at 220 and 208 nm, and a crossover to positive ellipticity at 200 nm. This CD spectrum is similar to that of ncVDAC in 2% octyl β-glucoside at pH < 5 (13) and corresponds to lower β-sheet content (30%) and higher α-helical content (27%) relative to ncVDAC at pH 6–8 (Table I).

When inserted from 1% LDAO into planar phospholipid bilayers, both mitochondrially isolated proteins display classic VDAC-like single-channel characteristics in terms of transition sizes, mean open times, and voltage dependence (Fig. 6 and Table II). Therefore, the different secondary structures of the two types of VDAC in non-denaturing detergents are not associated with obvious differences in activity of the ion channels formed by the proteins in bilayers.

Circular Dichroism of Bacterially Expressed VDAC—The far-UV CD spectra of ncVDAC(His6) (Fig. 3B, dashed curve) and of scVDACΔ1–8 (Fig. 3B, dotted curve) in 1% LDAO at pH 7 were found to be very similar to those of the corresponding mitochondrial VDAC proteins. In contrast, the CD spectrum of scVDAC(His6) in 1% LDAO (Fig. 3B, dotted curve) differs significantly from that of mitochondrial scVDAC under the same conditions. In the region above 205 nm, the spectrum of scVDAC(His6) more closely resembles that of ncVDAC, having a single broad minimum at 216 nm. However, the crossover to positive ellipticity occurs at 202 nm, in between that of ncVDAC and scVDAC, and the ellipticity maximum is somewhat reduced compared with mitochondrial ncVDAC. The secondary structure content predicted from this spectrum is 35% β-sheet and 20% α-helix (Table I), indicating secondary structure intermediate between that of mitochondrial scVDAC and ncVDAC.
The CD spectra of scVDAC(His₆) and ncVDAC(His₆) were unchanged after cleavage of the N-terminal extensions with thrombin and overnight dialysis (Fig. 4).

Effects of SDS and pH on CD Spectra—As shown in Fig. 5A, the far-UV CD spectra of the three bacterially expressed VDAC proteins in 1% SDS are very similar, closely resembling that of mitochondrial ncVDAC in this detergent (13). The spectra, containing two minima (206–207 and 224 nm) and a crossover to positive ellipticity at 200 nm, are similar (but not identical) to those of ncVDAC in 1% LDAO at pH < 5 (13) and scVDAC in 1% LDAO at pH 7 (Fig. 3A). Analysis of the CD spectrum of ncVDAC(His₆) in SDS indicates a correspondingly low β-sheet content (22%, Table I).

The pH dependence of the CD spectrum of ncVDAC(His₆) in 1% LDAO also was determined. As with mitochondrial ncVDAC (13), the spectrum of ncVDAC(His₆) does not change significantly over the range pH 6–8. At lower pH values, the crossover in ellipticity shifts somewhat, from 205 nm at pH 6.0 to 203 nm at pH 5.0 and 3.8 (Fig. 5B), but the large changes observed with mitochondrial ncVDAC (13) do not occur. Instead, the low pH CD spectrum of ncVDAC(His₆) is similar to that of scVDAC(His₆) at pH 8.0.

Electrophysiological Characterizations of Bacterially Expressed VDAC—Both His₆-containing proteins insert from LDAO suspension into phospholipid bilayers and form stable channels when the protein suspensions were preincubated with 0.1% ergosterol (see “Experimental Procedures”). Differences in channel-forming activities per mg of bacterially expressed and mitochondrial proteins fall within the normal variability of the bilayer reconstitution experiments. Typical current traces and amplitude histograms at −25 mV are shown in Fig. 6 and single-channel parameters are summarized in Table II. The results indicate that the ion channels formed by the full-length bacterially expressed VDAC proteins are comparable to those formed by the mitochondrial proteins in terms of conductance levels (around 4.3 and 2.1 nS in 1 M KCl) and mean open times (around 100 ms).

In contrast, the bacterially expressed truncated variant, scVDACΔ₁₋₈, behaves atypically in bilayer experiments. Rather than forming stable open channels with discrete transitions to a lower subconductance level, scVDACΔ₁₋₈ induces channels that flicker rapidly. The characteristic maximum conductance level corresponding to VDAC’s fully open state (4–4.5 nS) is rarely observed at any voltage. Instead, the rapid current transitions appear to involve multiple states between two subconductance levels (S₁ and S₂ in Fig. 6), giving rise to two broad peaks in the current amplitude histogram. The larger subconductance level (S₂) is similar to the partially closed state of full-length VDAC (2–2.5 nS), and its mean dwell time is only 1.6 ms (Table II).

The relative open probabilities (Gₒ/Gₑ) of the mitochondrial and bacterially expressed VDAC channels as a function of transmembrane potential are plotted in Fig. 7. Both full-length, His₆-containing bacterially expressed proteins display voltage-dependent closure like that of the mitochondrial proteins. The situation is very different for the truncated variant scVDACΔ₁₋₈. The fully open state does not occur often enough to calculate its mean probability. Instead, the “open” probability plotted reflects the occupancy of the larger of the two subconductance states (S₂), which appears to be somewhat favored at larger amplitude transmembrane potentials.

DISCUSSION

Using the inducible pET expression system and metal affinity chromatography, we have been able to obtain and purify multi-milligram quantities of VDAC protein from bacterial inclusion bodies. In the case of ncVDAC, CD spectra and channel properties of the mitochondrial protein and His₆-containing

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TABLE I

| VDAC type | pH | Secondary structure content |
|-----------|----|-----------------------------|
|           |    | β-Sheet | α-Helix | β-Turn | Random coil |
| ncVDAC    | 8  | 45      | 12      | 13     | 24          |
| ncVDAC(His₆) | 8 | 35      | 20      | 17     | 28          |
| scVDAC    | 8  | 30      | 27      | 17     | 28          |
| scVDAC(His₆) | 5 | 22      | 28      | 22     | 29          |

* VDAC variants are grouped according to characteristic CD spectra as described under “Results.”

* Percent content of secondary structural elements were computed from the CD spectrum of the first protein in each group using the SELCON program (22).

TABLE II

| VDAC type      | Open conductance | Transition size | Mean open time | Gₒ/Gₑ |
|----------------|------------------|----------------|----------------|--------|
|                |                  | ns             |                |        |
| ncVDAC         | 4.3 ± 0.6 (11)   | 2.2 ± 0.2 (26) | 106 (707)      | 0.65 ± 0.2 |
| ncVDAC(His₆)  | 4.4 ± 0.4 (16)   | 2.1 ± 0.3 (25) | 105 (698)      | 0.7 ± 0.1 |
| scVDAC         | 4.3 ± 0.3 (10)   | 2.2 ± 0.6 (29) | 103 (553)      | 0.6 ± 0.1 |
| scVDAC(His₆)  | 4.2 ± 0.2 (10)   | 2.0 ± 0.1 (26) | 103 (953)      | 0.6 ± 0.1 |
| scVDACΔ₁₋₈    | 4.4 ± 0.6 (13)   | 1–2 (55)       | 1.6 (2959)     | 1.1 ± 0.2 |
bacterial construct are virtually indistinguishable, with one exception. The latter appears to resist the low pH-induced conformational change in non-denaturing detergent previously reported for ncVDAC isolated from mitochondria (13). This bacterially expressed protein is a suitable candidate for large scale crystallization trials, which are in progress.

Full-length scVDAC, both mitochondrial and bacterially expressed, forms typical voltage-dependent ion channels in phospholipid bilayers, although the CD spectra in non-denaturing detergents indicate considerably reduced β-sheet structure relative to ncVDAC at pH 7. In fact, the CD spectrum of mitochondrial scVDAC in LDAO at pH 7 is like that of ncVDAC at pH < 5. Shao et al. (13) have shown that the high β-sheet conformation of ncVDAC at pH 7 corresponds to an “open” state in liposomes and that lowering the pH below 5 causes full (but reversible) loss of permeability to sucrose.

The CD spectrum of the His6-containing scVDAC construct in LDAO at pH 7 is intermediate between that of mitochondrial ncVDAC and scVDAC. This is borne out by the predicted β-sheet content for scVDAC(His6) of 35%, which is between that of mitochondrial ncVDAC (45%) and scVDAC (30%). This raises the possibility that the scVDAC(His6) fraction may contain a mixture of high β and low β conformers of VDAC. The same appears to be true for ncVDAC(His6) at low pH, for which the CD spectrum is very similar to that of scVDAC(His6) at pH 7. After thrombin-cleavage of the His6-containing N-terminal extensions, bacterially expressed ncVDAC and scVDAC still do not assume the low β conformation of their mitochondrial counterparts (at pH 4 and 7, respectively). It may be that the N-terminal extensions induce folding differences in VDAC that persist even after their removal, or that the N-terminal extensions are not released by the proteins after thrombin cleavage.

That ncVDAC and scVDAC assume distinctly different secondary structures in non-denaturing detergents at pH 7 is an unexpected and potentially useful finding. Xu and Colombini (25) have shown that ncVDAC channels form more rapidly in phospholipid bilayers in the presence of urea and GdnHCl, suggesting that partial unfolding of the polypeptide may expedite the insertion process. Shao et al. (13) have speculated that the low β form of VDAC (reversibly induced by low pH in ncVDAC) might represent a folding intermediate in the membrane insertion process. It should be possible to test the hypothesis of Shao et al. by comparing the insertion kinetics of scVDAC and ncVDAC under conditions (e.g., non-denaturing detergent at pH 7) in which the former is in the low β conformation and the latter is in the high β conformation. (In the bilayer experiments reported in this paper, no attempt was made to compare initial rates of channel insertion for the different proteins.)

The N-terminal domain of VDAC has been implicated as a voltage-sensing region (26), and as a region that undergoes large scale motion in the course of channel gating (27–30). It may form part of the lumen wall in one or more states of the channel (30, 31), as does the N-terminal domain of bacterial porins that H-bonds to the C-terminal transmembrane β-strand (10, 11). There is evidence that the N-terminal region of VDAC tends to fold as an amphipathic α-helix (32) and not as a transmembrane β-strand as it does in the bacterial porins. Table III is a composite of the N-terminal sequences and channel characteristics of the three bacterially expressed constructs of scVDAC and ncVDAC reported in this paper and those of three other ncVDAC constructs previously reported by Popp et al. (21). Several important inferences may be drawn from the table.

First, lengthening the VDAC polypeptide by 12–20 residues at the N terminus has no obvious effect on channel stability, pore size, or voltage dependence. This finding is not inconsistent with the purported involvement of the N-terminal domain in forming part of the open-state lumen, or in serving as a voltage sensor, since the extensions carry little or no net charge at the pH (7) at which the determinations were made. However, it may be useful to compare the effects of varying pH and multivalent metal ion concentration on the gating characteristics of VDACs with and without the His6-containing N-terminal extension. Additionally, since movement of the N terminus has been implicated in the mechanism of gating of VDAC, the
gating kinetics of the channels with and without N-terminal extensions should be compared.

Second, while lengthening the N-terminal domain of VDAC has no significant effects on channel properties, other types of alterations in this region have marked effects. For example, substituting 11 of the first 20 amino acids of ncVDAC and extending the overall length of the N-terminal region by 1 residue (variant nc ΔN(2–12) of Popp et al. (Ref. 21)) destabilizes the open state of the channel. The same degree of channel instability is exhibited by nc ΔN(3–20) (21), which retains only two of the first 20 residues and is 7 residues shorter than wild-type VDAC. However, the variant that forms the most atypical channels is the one with the shortest N-terminal domain, scVDACΔ1–8. This protein retains more N-terminal residues (12) than either truncated variant of Popp et al. but is 8 residues shorter than wild-type VDAC, 9 residues shorter than nc ΔN(2–12), and 2 residues shorter than nc ΔN(3–20). Unlike the longer VDAC variants, which display voltage-induced partial closures from a long-lived open state (21), scVDACΔ1–8 has a seldom-observed fully open state and displays rapid transitions between multiple lower conductance substates. Apparently shortening of the VDAC polypeptide at the N terminus by more than 6–7 residues makes the normal fully open state of the pore essentially inaccessible to the polypeptide. This observation strongly supports those structural models that have the N-terminal domain forming an integral part of the lumen wall in VDAC’s open state (30, 31). It is possible that the highest

![Fig. 6. Single-channel behavior of mitochondrial and bacterially expressed VDAC. Left side, total current amplitude histograms of bilayers containing one or a few of each type of channel, showing the relative occupancy of the open state (O) and closed substate (S) or, for scVDACΔ1–8, of 2 substates (S1 and S2) during a 60-s recording with voltage clamped at −25 mV. Right side, typical current traces in the same experiments.](image)

| VDAC type       | N-terminal sequence | L<sup>a</sup> | Stable open state | Normal current transitions |
|-----------------|---------------------|--------------|------------------|---------------------------|
| scVDAC          | MSPPVYSDISRINDLLNKD | 20           | +                | +                         |
| scVDAC(His<sub>6</sub>) | MGSSHHHHHHSSGLVPRGSHMSPPVYSDISRINDLLNKD | 40           | +                | +                         |
| seVDACΔ1–8     | ISRNINDLLNKD        | 12           | −                | −                         |
| ncVDAC         | MAVPAFSDIAKSANDLLNKD | 20           | +                | +                         |
| ncVDAC(His<sub>6</sub>) | MGSSHHHHHHSSGLVPRGSHMAVPAFSDIAKSANDLLNKD | 40           | +                | +                         |
| nc (His<sub>6</sub>)<sup>c</sup> | MRGSHHHHGSMAVPAFSDIAKSANDLLNKD | 32           | +                | +                         |
| nc ΔN(2–12)<sup>c</sup> | MRGSHHHHGSMANDLLNKD | 21           | −                | −                         |
| nc ΔN(9–20)<sup>c</sup> | MRGSHHHHGSM | 14           | −                | −                         |

<sup>a</sup> His<sub>6</sub>-containing extensions are underlined.

<sup>b</sup> Length of the N-terminal domain.

<sup>c</sup> ncVDAC variants constructed and characterized by Popp et al. (21).
conductance substate of scVDAC$_{1–8}$ ($S_2$ in Fig. 6) may correspond to the partially closed substate (S in Fig. 6) occupied by wild-type VDAC at large transmembrane potentials, both of which are 2–2.5 nS. If so, the fact that the open probability of substate $S_2$ increases with voltage (Fig. 7) would explain why occupancy of this particular substate in wild-type VDAC normally predominates at high voltages.

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REFERENCES

1. De Pinto, V., Ludwig, O., Krause, J., Benz, R., and Palmieri, F. (1987) *Biochim. Biophys. Acta* 894, 109–119
2. Colombini, M. (1989) *J. Membr. Biol.* 111, 103–111
3. Benz, R., Kotke, M., and Brdiczka, D. (1990) *Biochim. Biophys. Acta* 1022, 311–318
4. Liu, M. Y., and Colombini, M. (1992) *Biochim. Biophys. Acta* 1098, 255–260
5. Röstovtseva, T., and Colombini, M. (1996) *J. Biol. Chem.* 271, 28006–28008
6. Colombini, M., Yeung, C. L., Tung, J., and König, T. (1987) *Biochim. Biophys. Acta* 905, 279–286
7. Heldren, M. J., and Colombini, M. (1993) *Biochim. Biophys. Acta* 1144, 396–402
8. Forte, M., Guy, H. R., and Mannella, C. A. (1987) *J. Bioenerg. Biomembr.* 19, 341–350
9. Song, J., and Colombini, M. (1996) *J. Bioenerg. Biomembr.* 28, 153–161
10. Weiss, M. S., Kreusch, A., Schiltz, E., Nestel, U., Welte, W., Weckesser, J., and Schulz, G. E. (1991) *FEBS Lett.* 280, 379–382
11. Cowan, S. W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Paupit, R. A., Janssenius, J. N., and Rosenbusch, J. P. (1992) *Nature* 358, 727–733
12. Mannella, C. A., Neuwald, A. F., and Lawrence, C. E. (1996) *J. Bioenerg. Biomembr.* 28, 161–167
13. Shao, L., Kinnally, K. W., and Mannella, C. A. (1996) *Biophys. J.* 71, 778–786
14. Markovic-Housley, Z., and Garavito, R. M. (1986) *Biochim. Biophys. Acta* 868, 158–170
15. Mannella, C. A. (1982) *J. Cell Biol.* 94, 680–687
16. Trembath, M. K., and Tzagoloff, A. (1979) *Methods Enzymol.* 55, 160–163
17. Kleene, R., Panner, N., Pfeller, R., Link, T., Sebald, W., Neupert, W., and Tropschug, M. (1987) *EMBO J.* 6, 2627–2633
18. Kaplan, R. S., Mayor, J. A., Gremse, D. A., and Wood, D. O. (1995) *J. Biol. Chem.* 270, 4108–4114
19. Stanley, S., Dias, J. A., D’Arcangelis, D., and Mannella, C. A. (1995) *J. Biol. Chem.* 270, 16694–16700
20. Labarca, P., and Latorre, R. (1992) *Methods Enzymol.* 207, 447–448
21. Popp, B., Court, D. A., Benz, R., Neupert, W., and Lill, H. (1996) *J. Biol. Chem.* 271, 13593–13599
22. Sreerama, N., and Woody, R. W. (1994) *J. Mol. Biol.* 242, 497–507
23. Manavalan, P., and Johnson, W. C., Jr. (1987) *Anal. Biochem.* 167, 76–85
24. Johnson, W. C., Jr. (1990) *Proteins* 7, 205–214
25. Mannella, C. A., and Kinnally, K. W. (1996) in *Biomembranes* (Lee, A. G., ed) Vol. 6, pp. 377–410, JAI Press, London
26. Thomas, L., Blachly-Dyson, E., Colombini, M., and Forte, M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 5446–5449
27. Peng, S., Blachly-Dyson, E., Forte, M., and Colombini, M. (1992) *Biophys. J.* 62, 123–135
28. Mannella, C. A. (1990) *Experientia* (Basel) 46, 137–145
29. Guo, X. W., and Mannella, C. A. (1993) *Biophys. J.* 64, 545–549
30. Mannella, C. A. (1998) *J. Bioenerg. Biomembr.* 29, 525–531
31. Blachly-Dyson, E., Peng, S. Z., Colombini, M., and Forte, M. (1990) *Science* 247, 1235–1236
32. Guo, X. W., Smith, P. R., Cognon, B., D’Arcangelis, D., Delgino, E., and Mannella, C. A. (1995) *J. Struct. Biol.* 114, 41–59