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Probing the Structure of the Mitochondrial Channel, VDAC, by Site-Directed Mutagenesis: A Progress Report

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Abstract

The voltage-dependent anion-selective channel (VDAC) of the mitochondrial outer membrane is formed by a small (~30 kDa) polypeptide, but shares with more complex channels the properties of voltage-dependent gating and ion selectivity. Thus, it is a useful model for studying these properties. The molecular biology techniques available in yeast allow us to construct mutant versions of the cloned yeast VDAC gene *in vitro*, using oligonucleotide-directed mutagenesis, and to express the mutant genes in yeast cells in the absence of wild-type VDAC. We find that one substitution mutation (lys 61 to glu) alters the selectivity of VDAC.

Key Words: Yeast VDAC; oligonucleotide-directed mutagenesis; ion selectivity; voltage gating; mitochondrial outer membrane; anion channel; ion channel.

Introduction

The property of voltage gating of ion flow, which many ion channels possess, is central to many important biological processes. Voltage-gated ion channels are quite common to animal cells. They are involved in generating or modulating a variety of cellular functions, most notably in the production of action potentials in neurons and muscle cells but including the regulation of egg fertilization and hormone release. At present, two types of voltage-gated channels are known to exist: (1) the high-molecular-weight channel formers

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that produce highly selective, narrow aqueous pores, e.g., the sodium channel (Noda *et al.*, 1984); and (2) the low-molecular-weight channel formers that produce wide aqueous pores which are poorly selective for small ions, e.g., the VDAC channel of the mitochondrial outer membrane (Schein *et al.*, 1976). The molecular basis for voltage gating in both of these is unclear despite much speculation.

The channel-forming protein, VDAC (voltage-dependent anion channel), has been isolated from the mitochondria of organisms in each eukaryotic kingdom (Schein *et al.*, 1976; Colombini, 1979; Zalman *et al.*, 1980; Frietag *et al.*, 1982b; Smack and Colombini, 1985; Forte *et al.*, 1987a). It has been localized to the outer membrane (Mannella and Colombini, 1984) and seems to be the pathway by which molecules and ions travel from the cytoplasm to the intermembrane space (Colombini, 1979). The functional properties of VDAC are highly conserved (Colombini, 1989) and include the formation of a highly conductive pathway in membranes (4 nS in 1 M KCl) with weak anion selectivity and steep voltage dependence at both positive and negative potentials.

The nuclear gene encoding VDAC in the yeast *Saccharomyces cerevisiae* has been cloned, and its sequence determined (Mihara and Sato, 1985; Forte *et al.*, 1987b). We have made use of the well-developed molecular genetics of this yeast to probe the structure of the VDAC ion channel. With these techniques it is a simple matter to delete a gene from the yeast genome if one has cloned DNA for that gene. In addition, vectors are available in which genes can easily be introduced into yeast, either on single-copy or multicopy plasmids, or by integration into the yeast genome. We have used these methods to construct yeast strains lacking the gene for VDAC, and then to reintroduce the original wild-type gene or altered versions of the gene back into the yeast cell. Thus, we can study mutant versions of the VDAC protein in the absence of the wild-type protein. Using these methods, we hope to be able to identify specific domains within the VDAC protein that are responsible for its selectivity and its voltage dependence.

From Sequence to Model

The VDAC genes from *S. cerevisiae* and *Neurospora crassa* encode proteins of 283 amino acids (Mihara and Sato, 1985; Forte *et al.*, 1987a; Kleene *et al.*, 1987). Each protein contains a short amino-terminal segment that could form an amphipathic α-helix, similar in structure to mitochondrial targeting sequences found on other nuclearly encoded mitochondrial proteins (Roise *et al.*, 1986; Von Heijne, 1986). These amino-terminal segments are not cleaved from the mature VDAC molecules (Freitag *et al.*, 1982a;

Mihara *et al.*, 1982; Gasser and Schatz, 1983). Surprisingly, for an integral membrane protein, VDAC contains no long stretches of hydrophobic amino acid residues. Instead, it contains many segments of alternating hydrophilic and hydrophobic residues. Nevertheless, the protein behaves in solution as if it were extremely hydrophobic.

VDAC forms a large aqueous pore (about 3 nm in diameter and 5 nm long), perhaps by using two 30-kDa polypeptide chains. The amount of protein used to form this large pore is rather small and, therefore, the walls of the channel must be thin, i.e., one layer of protein. It is widely accepted that protein embedded in a membrane has a high degree of secondary structure in order to satisfy the backbone hydrogen bonds. In the case of VDAC, preliminary CD measurements indicate a high degree of β structure (Mangan and Colombini, unpublished). Thus, a β barrel seems to be a likely candidate (such a structure for the VDAC channel was proposed by Forte *et al.*, 1987b). The transmembrane strands forming this barrel must have an alternating polar–nonpolar pattern as the side chains alternately face the lipid and aqueous phases. To look for good candidates for transmembrane β strands, a simple program was written to search for stretches of the sequence that have alternating polar–nonpolar side chains. By considering stretches of 10 amino acids and taking the hydropathy value (Kyte and Doolittle, 1982) of the first amino acid side chain, subtracting the value of the second, adding to the third, and so on, one obtains a large number if the residues alternate between polar and nonpolar. If the residues do not alternate, the number is small. This summation was performed for every group of 10 continuous amino acids starting with the N-terminal residue and plotted in Fig. 1a. Note that peaks are formed representing the best alternating sequences and, therefore, the most likely transmembrane β strands.

The pattern seems to be fundamental to the structure and not the result of the parameters used in the calculations. The choice of 10 residues was based on a 3.5-nm hydrophobic region within the membrane. If 8 or 12 residues were used in the calculations, the results changed little. The use of other hydropathy tables did not change the basic pattern very much but did raise or lower some of the peaks. Virtually the same pattern was evident with both available VDAC sequences: *S. cerevisiae* and *N. crassa* (Fig. 1a, b). Despite the fact that the strict homology between these two sequences is only 43% (Kleene *et al.*, 1987), the conservation of the β pattern indicates the importance of this structural feature.

The amount of protein needed to form the walls of the pore is constrained both by the nature of the structural units used to form the pore and the available information on the size of the aqueous pore. Using an interchain distance of 0.47 nm (from silk) and a mean side-chain length of 0.5 nm, one can easily show that in order for a β barrel to form a 3-nm-diameter pore

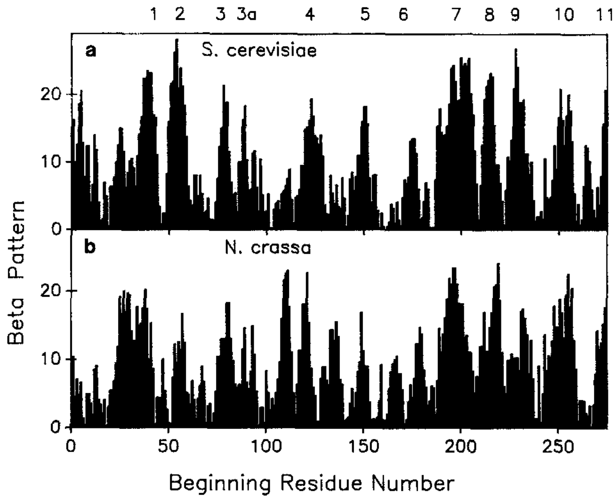

Fig. 1. An evaluation of the potential of stretches of amino acids in the VDAC sequences from *S. cerevisiae* and *N. crassa* to form β strands lining the walls of a water-filled pore. The hydropathy value (Kyte and Doolittle, 1982) of each group of the 10 adjacent amino acids was combined as follows: $\Sigma_{i=1}^{10}(-1)^{i+1}v(i)$, where $v(i)$ is the hydropathy value of the ith amino acid. The value of these sums was plotted against the number of the first amino acid in the summation. For the upper plot (a) the sequence of VDAC from *S. cerevisiae* was used while that from *N. crassa* was used for (b).

(the most likely diameter of the channel in the open state), 27 strands are needed (assuming that the chains are perpendicular to the plane of the membrane).

A comparison of the freeze-fracture images of VDAC arrays to the negatively stained images indicates (Thomas *et al.*, 1988) that the pore may be off-center from the mass of the protein. This fact coupled with the likely formation of an amphipathic α helix at the N-terminus leads to the proposal that, in addition to the β sheet, an α helix also forms the wall of the pore. Thus, a total of 22 to 24 β strands and two α helices would provide sufficient circumference to form a 3-nm pore. It is not yet clear whether the channel is formed by one or two subunits. If the amount of protein forming the barrel is maximized, the β barrel of the appropriate dimensions (if on the low side of estimated diameters) could be formed (Forte *et al.*, 1987b). However, from Fig. 1, each subunit is likely to form 11 or 12 transmembrane β strands. Thus, a dimer seems more probable. Figure 2 shows the most likely membrane-spanning portions of one polypeptide. At the very left, an amphipathic α helix spans the membrane followed by 12 transmembrane β strands. Note that the proline residues (boxed in) do not appear in the membrane-spanning segments.

Fig. 2. A model of the protein folding pattern for yeast VDAC based primarily on the pattern in Fig. 1a. Long stretches of amino acids in regions outside the membrane are indicated simply as a connecting line. The proline residues are boxed in.

Little information is available on the relative orientation of the putative dimer in the membrane. The most compelling comes from the voltage-gating properties. Each VDAC channel has two gating mechanisms: one closes the channels at positive potentials and the other at negative potentials (Colombini, 1986). Thus, a symmetrical protein in which the monomers are oriented in opposite directions seems favored despite the problems of inserting polypeptide chains in opposite directions in the membrane. The symmetrical structure would naturally yield symmetrical properties and the adjacent helices would have their strong dipoles oriented in the favorable antiparallel direction. An alternative is a dimer composed of subunits in different conformations.

Experimental Approaches

Constructing a Chromosomal Deletion of the VDAC Gene

In yeast, a mutant copy of a gene can be substituted for the chromosomal copy by homologous recombination (Rothstein, 1983). The technique is illustrated schematically in Fig. 3. A selectable marker gene (in this case, the *LEU2* gene, which allows cells to grow in the absence of leucine when they lack a functional *LEU2* gene in their genome) is inserted into the cloned gene of interest. To construct a deletion of the protein-coding sequences, the marker gene is substituted for all or part of those sequences. The resulting DNA fragment is introduced into yeast cells by a standard transformation

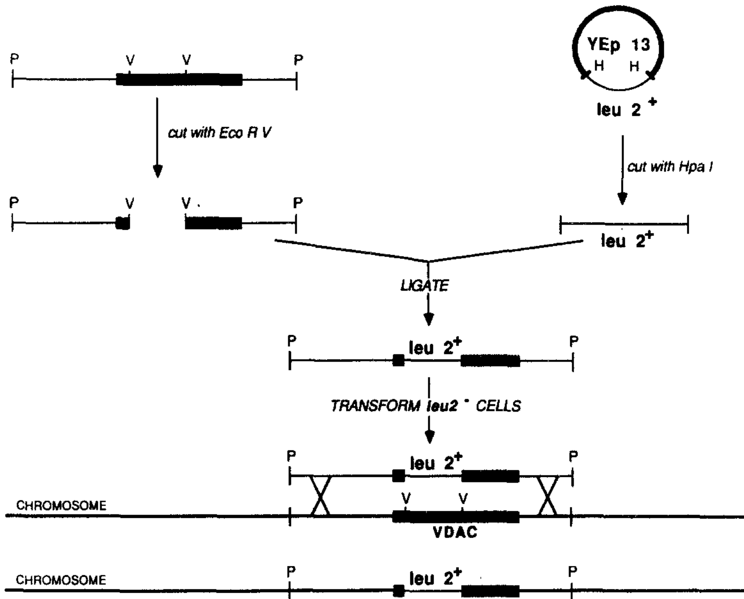

Fig. 3. Deleting VDAC gene sequences from the yeast chromosome. The cloned VDAC gene (upper left) is cut with a restriction endonuclease to remove a large segment of the protein coding region. The selectable marker gene, *LEU2*, is then inserted into the gap using DNA ligase. The resulting construct contains the *LEU2* gene flanked by the DNA sequences adjacent to the VDAC gene. This DNA is introduced into yeast cells lacking the *LEU2* gene, and Leu^+ cells are selected. In yeast, the cut ends of DNA recombine with homologous sequences at high frequency, so a majority of the Leu^+ transformants will be cells that have substituted the marked construct in place of the chromosomal VDAC gene.

procedure. Transformations are selected which grow on medium lacking leucine, the only cells capable of growth being those that have taken up the mutant gene. Southern blots can be performed on genomic DNA from the transformed yeast to verify that the expected substitution has taken place.

Using this approach, we used the cloned VDAC gene (Forte *et al.*, 1987b) to construct deletion mutations of the VDAC genes of several yeast strains. Southern blot analysis confirmed that the expected substitutions had occurred, and Western blotting with antibody to VDAC protein (Forte *et al.*, 1987a) showed that the mutant cells contained no detectable VDAC protein. Since VDAC is thought to be the major pathway for metabolites entering and leaving the mitochondrion, we expected that cells lacking the VDAC gene would be respiration deficient. If this were the case, they should be able to grow on fermentable carbon sources such as glucose, but not on nonfermentable carbon sources such as glycerol. As expected, yeast lacking VDAC grew well on glucose-based media. Surprisingly, the cells were also able to grow on

glycerol-based medium (YPG) at 30°C (the normal growth temperature for yeast), but failed to grow on the same medium at 37°C (the parent strain grew well on glycerol at both temperatures). The permissive temperature for growth on glycerol after deletion of the VDAC gene varied among different yeast strains, with some growing poorly even at 30°C. Other laboratories have also found that yeast lacking the VDAC gene grow on glycerol in a temperature-sensitive manner (Guo and Lauquin, 1986), or after a delay of several days (Dihanich *et al.*, 1987). Thus, an alternate pathway for the movement of metabolites into and out of the mitochondria may exist or is induced by the absence of VDAC. Evidence exists which suggests the presence of a novel channel in yeast strains which are deleted for VDAC (Benz *et al.*, 1989).

Since yeast cells lacking the VDAC gene are viable, we have been able to subject the VDAC gene to *in vitro* mutagenesis, introduce modified genes into these cells, and study mutant forms of the VDAC protein in the absence of the wild-type protein. Several techniques are available for random *in vitro* mutagenesis of a closed gene, including chemical mutagenesis with such agents as hydroxylamine, or enzymatic mutagenesis by incorporation of mismatched thionucleotides during repair of a gapped plasmid (Shortle *et al.*, 1982). We have chosen to focus our efforts on site-directed mutagenesis, using oligionucleotides to change the sequence of the VDAC gene at specific codons. Several techniques for oligonucleotide directed site-specific mutagenesis have been described (for example, Zoller and Smith, 1984; Kramer *et al.*, 1984), and commercial kits are available. After a mutation had been constructed in an M13 vector (using the technique of Kramer *et al.*, 1987), it was subcloned into a yeast vector and introduced into yeast cells lacking the wild-type VDAC gene. This general scheme is outlined in Fig. 4. The yeast vector contained yeast centromere sequences, so the plasmid was maintained at approximately one copy per cell, allowing the mutant gene to be expressed at approximately the same level as wild-type VDAC.

Reconstruction of Mutant VDAC Protein into Planar Phospholipid Bilayers

VDAC protein can be purified from detergent-solubilized mitochondrial membranes by a simple chromatographic procedure (Freitag *et al.*, 1983). The same procedure was used to prepare mutant VDAC protein from cells containing mutant plasmids. The procedure for reconstruction of VDAC into planar lipid bilayers has been described by Schein *et al.* (1976). Briefly, a phospholipid bilayer is formed between two chambers containing salt solutions. The membrane potential is clamped, and the current across the membrane is monitored. The solubilized VDAC protein is introduced into one of the chambers (defined as the *cis* side). The protein then spontaneously

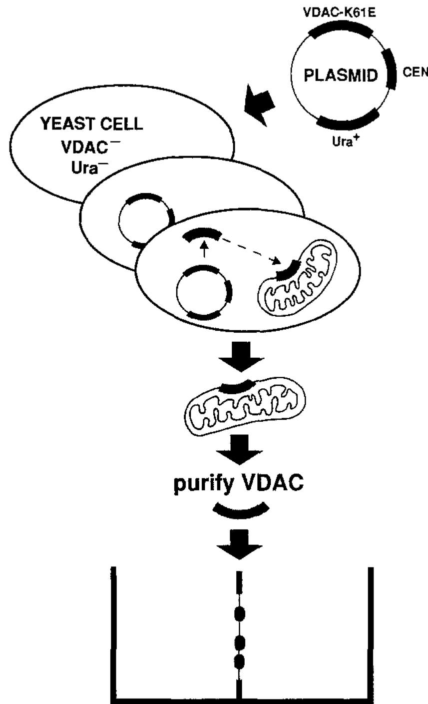

Fig. 4. Scheme for producing and analyzing mutant VDAC properties. Yeast cells lacking the VDAC gene (constructed as shown in Fig. 3) are transformed with a single-copy yeast plasmid containing a mutant VDAC gene. Cells that have taken up the plasmid produce the mutant VDAC protein and incorporate it into their mitochondrial outer membranes. The mutant protein is isolated from the membranes and introduced into planar phospholipid bilayers, where its electrophysiological properties are analyzed.

inserts into the membrane, forming channels that can be observed as increases in the current across the membrane (for example, see Fig. 5).

The selectivity of the channel is determined by examining the current in the the presence of a salt gradient (1.0 M KCl, 5 mM $CaCl_2$ on the *cis* side, 0.1 M KCl, 5 mM $CaCl_2$ on the *trans* side). If the channels were equally permeable to K^+ and Cl^- ions, no net current would flow under these conditions in the absence of an applied potential. However, VDAC is selectively permeable to Cl^- ions, so that a current is produced. For wild-type yeast VDAC channels, a potential of 10–11 mV must be applied to bring this current to zero (Forte *et al.*, 1987a and Fig. 5). This reversal potential is a measure of the selectivity of the channel, and we would expect that mutations in parts of the protein important for selectivity would change the reversal potential of the mutant channels.

The voltage dependence of VDAC channels is determined by measuring the conductivity as a function of applied voltage in bilayers that contain a

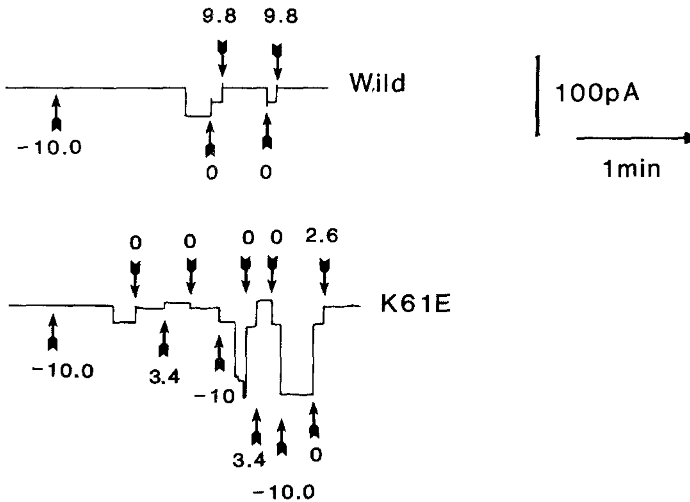

Fig. 5. Recordings from wild-type and mutant VDAC channels. The channels were inserted into planar phospholipid membranes (soybean phospholipids) under voltage-clamp conditions. The Triton-solubilized channels were added to the high-salt side of the membrane (1 M KCl, 5 mM $CaCl_2$); the other side contained 0.1 M KCl and 5 mM $CaCl_2$. The potentials indicated refer to the high-salt side. The insertion of the first channel in each record is indicated by the first downward current change. Prior to that event, the current level was essentially zero. The reversal potential was 9.8 mV for the wild type and 2.5 mV for the mutant, K61E (lysine 61 changed to glutamate).

large number of channels. VDAC channels are open at zero applied potential and close when either a positive or negative potential is applied (Schein *et al.*, 1976). The steepness of the voltage dependence can be used to calculate the number of gating charges n (Schein *et al.*, 1976). For yeast VDAC, $n = 4$, indicating that a minimum of four charged residues are needed to account for the voltage dependence (Forte *et al.*, 1987a). Mutations that change the charged residues involved in gating would be expected to change the voltage dependence of the channel.

If the model of VDAC structure presented above is correct, we would expect that any change in the charge of residues lining the aqueous pore should alter the selectivity of the channel. Thus, by selectively changing the charge at locations predicted to be inside or outside the channel, the model can be verified/modified.

Selection of Sites to Mutagenize

When using the site-directed mutagensis to analyze a gene, it is necessary to make *a priori* decisions about what codons to change and how to change

them. With the VDAC gene, we are interested in ion selectivity and gating functions. Although both dipoles and charges can perform these functions, there is strong evidence implicating lysine residues. For rat-liver VDAC, lysine residues and not arginine have been linked to the voltage sensor by both titration and chemical modification (Bowen *et al.*, 1985; Doring and Colombini, 1985). Chemical modification of VDAC with succinic anhydride (which modifies the amino groups of lysine residues, converting them to carboxylic acids) can reduce or abolish the voltage dependence of VDAC channels, and can convert the channels from anion selectivity to cation selectivity (Doring and Colombini, 1985).

The important lysine residues in VDAC are likely to be conserved among the VDAC proteins of various species. Comparison of the VDAC sequences from yeast and *Neurospora* shows that 13 of 19 lysine residues are found in the same positions in both species. We have initially focused our attention on these residues as targets for site-directed mutagenesis. There may well be other residues important both for gating and for selectivity, but among these 13 lysine residues we should be able at least to find some of the ones responsible for the effects of succinic anhydride modification.

Lysine 61 Influences VDAC Selectivity

Yeast VDAC contains a lysine residue at position 61, which is found in *Neurospora* VDAC at the analogous position, 62. This residue is within a predicted β strand (peak 2 in Fig. 1), and is thus a good candidate for influencing selectivity. We used oligonucleotide directed site-specific mutagenesis (Kramer *et al.*, 1984) to change the AAG codon encoding lys 61 to GAG, encoding glutamic acid at position 61. Changing a positively charged residue to a negative one may seem to be a drastic change, but results of succinic anhydride modification indicated that multiple lysine residues could be converted to negatively charged groups without destroying the channel (Doring and Colombini, 1985).

The mutant VDAC gene was subcloned into a yeast vector, and the resulting plasmid was introduced into a yeast strain lacking the VDAC gene. Mitochondrial membranes were prepared from yeast cells containing the plasmid, and VDAC was prepared by the protocol used for the wild-type protein (Freitag *et al.*, 1983). When the mutant protein (K61E) was introduced into planar phospholipid membranes, channels formed (Fig. 5). These channels had a conductance similar to those formed by wild-type VDAC, indicating that the channel-forming capacity of the protein was not damaged by the mutation (note that a change in applied voltage of 10 mV resulted in the same current change in both K61E and the wild type channel). However,

the selectivity of the mutant channels was unlike the wild-type. The insertion event (Fig. 5, first downward current change) was smaller for the mutant due to the altered selectivity (the current is driven both by the applied potential, $-10\,mV$, and the salt gradient). While wild-type channels had a reversal potential of $10.2 \pm 0.2\,mV$ in a 0.1 M to 1.0 M salt gradient, the reversal potential of the mutant channels was only $1.8 \pm 0.5\,mV$ (Fig. 5). This indicates a large reduction in selectivity, pointing to the importance of lys 61 in determining the selectivity of the channel. This finding supports the transmembrane localization of the second β strand in Fig. 2.

Future Studies

We have found that it is possible to identify an amino acid residue important for the selectivity of the VDAC ion channel using site-directed mutagenesis in conjunction with techniques of yeast molecular biology. By altering the codons for charged residues in various parts of the molecule, we should be able to identify domains of the protein that constitute the selectivity filter. We expect that these will be the domains that form the lining of the channel's aqueous pore.

Mutations that alter the selectivity of the VDAC channel, such as the change of lys 61 to glutamic acid, can also be used to address the question of how many 29-kD subunits make up a single functional channel. This can be done by introducing a plasmid containing the mutant gene into yeast cells containing a wild-type chromosomal copy of the VDAC gene. If VDAC channels consist of a single subunit, then VDAC protein isolated from such cells should form two types of channel: wild-type channels and channels with the mutant level of selectivity. On the other hand, if the channels are made of two (or more) subunits, there should be both mutant and wild-type channels and also a third type of channel with intermediate selectivity.

The use of site-directed mutagenesis to alter codons for charged residues should also make it possible to identify the residues that constitute the gating charge of VDAC. Measurements of voltage dependence indicate that approximately four charges are involved in closing the channel in response to a negative applied potential, and approximately the same number are involved in closing the channel in response to a positive potential (Forte *et al.*, 1987a). Site-directed mutagenesis should allow us to identify the charged residues that participate in gating and thereby to identify domains of the protein that move as the channel opens and closes. We should also be able to determine whether the same four charges move in response to both positive and negative potentials, or whether gating in response to opposite

potentials involves the movement of different domains of the protein, as is indicated by electrophysiological studies (Colombini, 1986).

We believe that this approach will allow us to construct a model of the structure and function of the VDAC channel that is more detailed than has previously been possible for any other ion channel, and we hope that the understanding we gain will provide a better understanding of how all ion channels operate.

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References

Benz, R., Schmid, A., and Dihanich, M. (1989) *J. Bioenerg. Biomembr.* **21**, 00–00.
Bowen, K. A., Tam, K., and Colombini, M. (1985) *J. Membr. Biol.* **86**, 51–59.
Colombini, M. (1979) *Nature* (*London*) **279**, 643–645.
Colombini, M. (1986) In: *Ion Channel Reconstruction* (C. Miller, ed.), Plenum Press, New York, pp. 533–552.
Colombini, M. (1989) *J. Membr. Biol.*, in press.
Dihanich, M., Suda, K., and Schatz, G. (1987) *EMBO J.* **6**, 723–728.
Doring, C., and Colombini, M. (1985) *J. Membr. Biol.* **83**, 81–85.
Forte, M., Adelsberger-Mangan, D., and Colombini, M. (1987a) *J. Membr. Biol.* **99**, 65–72.
Forte, M., Guy, H. R., and Mannella, C. (1987b) *J. Bioenerg. Biomembr.* **19**, 341–350.
Freitag, H., Janes, M., and Neupert, W. (1982a) *Eur. J. Biochem.* **126**, 197–202.
Freitag, H., Neupert, W., and Benz, R. (1982b) *Eur. J. Biochem.* **123**, 629–636.
Freitag, H., Benz, R., and Neupert, W. (1983) *Methods Enzymol.* **97**, 286–294.
Gasser, S. M., and Schatz, G. (1983) *J. Biol. Chem.* **258**, 3427–3430.
Guo, X. J., and Lauquin, G. J-M. (1986) *Yeast* **2** (Special Issue), S138.
Kleene, R., Pfanner, N., Pfaller, R., Link, T. A., Sebald, W., Neupert, W., and Tropschug, M. (1987) *EMBO J.* **6**, 2627–2633.
Kramer, W., Drutsa, V., Janse, H-W., Kramer, B., Pflugfelder, M., and Fritz, H-J. (1984) *Nucleic Acids Res.* **12**, 9441–9456.
Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132.
Mannella, C. A., and Colombini, M. (1984) *Biochim. Biophys. Acta* **774**, 206–214.
Mihara, K., and Sato, R. (1985) *EMBO J.* **4**, 769–774.
Mihara, K., Blobel, G., and Sato, R. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 7102–7106.
Noda, M., Shimuzu, S., Tanabe, T., Takai, T., Kayano, T., Ikeda, T., Takahashi, H., Nakayama, H., Kanaoka, Y., Minamino, N., Kangawa, K., Matsuo, H., Raftery, M. A., Hirose, T., Ingman, S., Hayashida, H., Miyata, T., and Numa, S. (1984) *Nature* (*London*) **312**, 121–127.
Roise, D., Horvath, S. J., Tomich, J. M., Richards, J. H., and Schatz, G. (1986) *EMBO J.* **5**, 1327–1334.
Rothstein, R. J. (1983) *Methods Enzymol.* **101**, 202–211.
Schein, S. J., Colombini, M., and Finkelstein, A. (1976) *J. Membr. Biol.* **30**, 99–120.
Shortle, D., Grisafi, P., Benkovic, S. J., and Botstein, D. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 1588–1592.

Smack, D. P., and Colombini, M. (1985) *Plant Physiol.* **79**, 1094–1097.
Thomas, L., Colombini, M., and Erbe, E. (1988) *Biophys. J.* **53**, 492a.
Von Heijne, G. (1986) *EMBO J.* **6**, 1335–1342.
Zalman, L. S., Nikaido, H., and Kagawa, Y. (1980) *J. Biol. Chem.* **255**, 1771–1774.
Zoller, M. J., and Smith, M. (1984) *DNA* **3**, 479–488.