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Mapping of residues forming the voltage sensor of the voltage-dependent anion-selective channel

(voltage gating/β barrel/mitochondrion/outer membrane/yeast)

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ABSTRACT **Voltage-gated ion-channel proteins contain "voltage-sensing" domains that drive the conformational transitions between open and closed states in response to changes in transmembrane voltage. We have used site-directed mutagenesis to identify residues affecting the voltage sensitivity of a mitochondrial channel, the voltage-dependent anion-selective channel (VDAC). Although charge changes at many sites had no effect, at other sites substitutions that increased positive charge also increased the steepness of voltage dependance and substitutions that decreased positive charge decreased voltage dependance by an appropriate amount. In contrast to the plasma membrane K^+ and Na^+ channels, these residues are distributed over large parts of the VDAC protein. These results have been used to define the conformational transitions that accompany voltage gating of an ion channel. This gating mechanism requires the movement of large portions of the VDAC protein through the membrane.**

Voltage-dependent membrane channels change their ability to conduct ions in response to small changes in membrane electric potential. This sensitivity to voltage is thought to result from the linkage of the conformational change responsible for the change in ion conductance and the movement of a charged domain through the membrane potential or the effective alignment of a large dipole with respect to the electric field. The charged domain or region containing the large dipole would then correspond to the "voltage sensor." For the best studied plasma membrane channels, the voltage-sensitive K^+ and Na^+ channels, a localized region of the protein, the S4 domain, has been identified by site-directed mutagenesis as containing residues forming the voltage sensor of these channels (1–6). Despite this information, a true molecular picture of the transitions that occur in response to voltage changes to cause these channels to open and close has been and will be difficult to define experimentally since the channels are formed by large oligomeric proteins. Smaller channels that form voltage-gated pores with much less protein are likely to provide insights into how an electric field can induce these molecular transitions. We have used site-directed mutations to identify the sensor region in one such channel, a mitochondrial channel, the voltage-dependent anion-selective channel (VDAC). In contrast to the S4 domain identified in Na^+ and K^+ channels, the voltage-sensing domain in VDAC is distributed over a relatively large region of the protein. Our data support a mechanism for voltage gating of this channel that requires the movement of large amounts of protein mass through the membrane.

MATERIALS AND METHODS

VDAC genes were modified by site-directed mutagenesis and introduced into a *Saccharomyces cerevisiae* strain lacking the chromosomal copy of the VDAC gene, and the VDAC channels were purified as described (7, 8). Mutants are designated by the letter–number–letter notation. The number is the location of the amino acid in the primary sequence starting at the amino terminus and leading and tailing letters represent the amino acid at the numeric location in the wild-type and mutant, respectively. The purified protein was reconstituted into planar phospholipid bilayers made from soybean phospholipids by the monolayer method of Montal and Mueller (9) as modified (10). Soybean phospholipid membranes separated two compartments, each containing either 1 M LiCl, 5 mM $CaCl_2$, dextran sulfate (500 kDa) at 58 μg/ml, and 5 mM Mes (pH 5.8), for experiments done in the presence of dextran sulfate, or 1 M KCl, 5 mM $CaCl_2$, and 5 mM Mes (pH 5.8), for experiments performed in the absence of dextran sulfate. A 5- to 10-ml aliquot of a sample of the indicated yeast VDAC, dissolved in 1% Triton X-100, was added to one compartment. A symmetrical 3-mHz triangular voltage wave was applied to the VDAC-containing membrane, and the resulting current was recorded. The sign of the voltage refers to the side of the membrane to which VDAC was added. The current records were digitized and converted to conductance values. Only the part of the wave during which the electric field was decreasing with time was used for analysis. Conductance–voltage relationships of mutants were fit to a two-state model (10) to determine the steepness of the voltage dependence (n) and the voltage at which half the channels are closed (V_0). Thus,

$$\ln[(G_{max} - G)/(G - G_{min})] = (nFV - nFV_0)/RT,$$

where G, G_{max}, and G_{min} are the conductance at any voltage V, the maximum conductance, and the minimum conductance, respectively, and F, R, and T are the Faraday constant, the gas constant, and the absolute temperature, respectively.

RESULTS AND DISCUSSION

In planar lipid bilayers, VDAC channels are open at zero and low membrane potentials and close to low-conducting states at higher (≈30 mV) positive and negative potentials (11, 12, 25). Several lines of evidence indicate that the voltage sensor in VDAC responsible for these conformational changes consists of positively charged residues. (*i*) Titration of the channel to high pH neutralizes the sensor with an apparent pK value of 10.6 (13). (*ii*) Low levels of succinic anhydride, which converts amino groups to carboxyl groups, result in the elimination of voltage dependence (14). (*iii*) Lowering the pH to levels that would result in the neutralization of carboxyl groups increases the voltage dependence of the channel (15). The apparent pK of the titratable groups and the succinic anhydride modification suggest that positive

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Abbreviation: VDAC, voltage-dependent anion-selective channel.

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charges, probably lysine residues, are involved in voltage sensing. If this is correct, increasing the positive charge in the sensing region should increase the channel's voltage dependence, whereas decreasing the charge in the same region should decrease the voltage dependence. If a region of the protein is not involved in voltage sensing, then charge changes in this region should not affect voltage dependence.

Site-directed mutations were made in the VDAC gene as described (7). These mutations resulted in charge changes at specific locations allowing us to determine the distribution of residues contributing to the voltage sensor throughout the VDAC polypeptide. To allow data collection at lower potentials, some experiments were performed in the presence of dextran sulfate (57 μg/ml, 500 kDa). Dextran sulfate does not bind to VDAC but acts to increase the voltage dependance of VDAC in bilayers 10-fold by partitioning into the access resistance region of the channel, thereby inducing channel closure by interacting electrostatically with the sensor (16). Fig. 1 *A* and *B* illustrates the voltage dependence of a protein containing the Lys-211 → Glu substitution (K211E) and a wild-type control. The results in Fig. 1*A* were fitted to a simple two-state model to quantitatively estimate the voltage-dependence parameters (n and V_0) (10, 16). The fact that the transformed data fit very well to a straight line justifies considering the different closed states observed as one closed state. Decreasing the positive charge by two at position 211 resulted in no significant change in the voltage dependence (n) or in the voltage that is required to close half the channels (V_0) (Fig. 1*B*). Other mutations that did not affect voltage dependence are listed in Table 1.

In contrast, increasing the positive charge by two at position 15 (Fig. 1 *C* and *D*) by substituting lysine for aspartate (D15K), resulted in an increase in voltage dependence compared to the wild-type control. Other mutations affecting voltage dependence are listed in Table 1. Since the membranes contained large numbers of channels, the channels were assumed to insert randomly and the response to positive and negative voltages were averaged. In all cases, increasing the positive charge increases the voltage dependence and decreasing the positive charge reduced the voltage dependence.

Our criteria for determining whether charge on the sensor had been changed were (*i*) that there be a change in parameter n, (*ii*) that the change in n agree in both sign and magnitude with the charge change introduced into the protein, and (*iii*) that there be no significant structural perturbation of the mutant channel as assessed by calculating nFV_0, the difference in the intrinsic conformational energy between the open and closed states. The mutations listed in Table 1 meet the first two criteria, and the changes in nFV_0 are small (0.3 kcal/mol on the average and a maximal change of 0.6 kcal/mol; 1 cal = 4.184 J). In addition, all of the mutant channels had open-state single-channel conductances indistinguishable from wild type (7), another indication that the overall protein structure was not altered by the mutations. The discrete reduction in the steepness of the voltage dependance observed in site-directed mutant forms of VDAC must result from a decrease in the effective translocation of charge across the membrane that is coupled to channel gating. Although alterations in the steepness of the voltage dependance of K^+ channels have been reported as the result of the substitution of nonpolar residues (2), one reasonable interpretation of these observations is that part of the sensor is immobilized by such changes. In the present work, the change in charge on the sensor induced by mutation was, in all cases, of a magnitude and sign entirely consistent with the engineered mutation. It is unlikely that this correlation is coincidental.

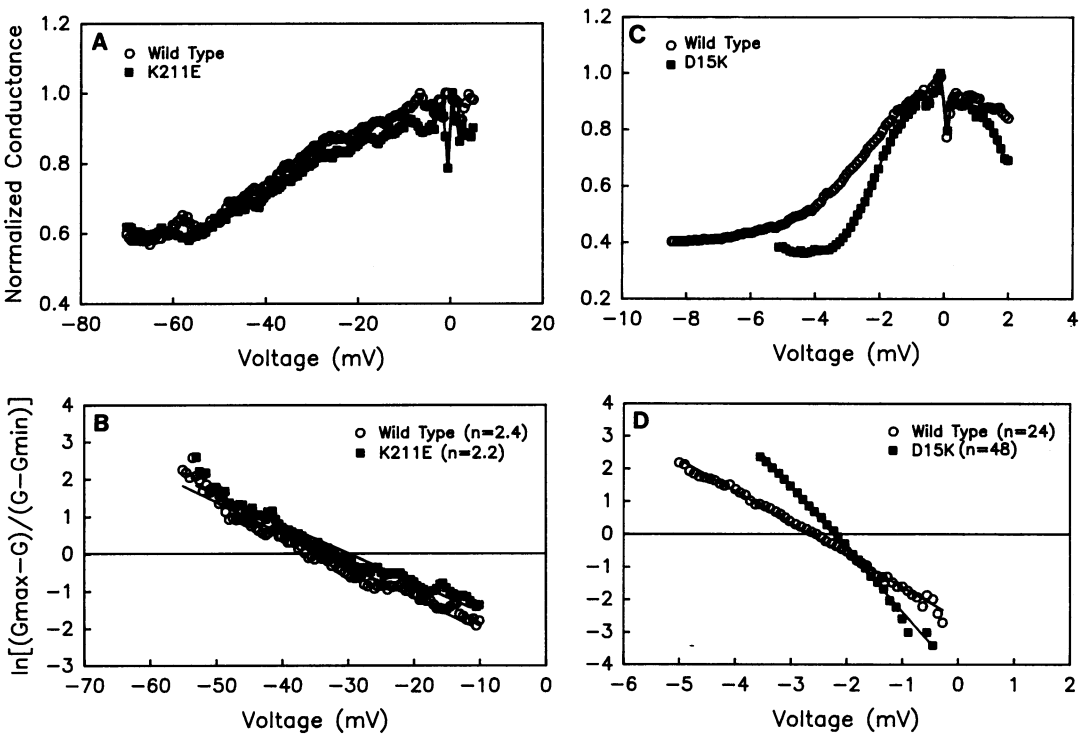

FIG. 1. Voltage dependence of the conductance of wild-type and mutant VDAC channels in yeast. A symmetrical 3-mHz triangular voltage wave (±10 mV for *C* and *D* and ±75 mV for *A* and *B*) was applied to the VDAC-containing membrane (≈100 channels in the membrane), and the resulting current was recorded. Experiments in *A* and *B* were performed in the absence of dextran sulfate, whereas those in *C* and *D* were performed in the presence of dextran sulfate. The sign of the voltage refers to the side of the membrane to which VDAC was added. *A* and *C* are conductance–voltage relationships of mutants K211E and D15K and their wild-type controls, respectively. These curves were fit to a two-state model to determine the steepness of the voltage dependence (n) and the voltage at which half the channels are closed (V_0). These transformations for the data in *A* and *C* are shown in *B* and *D*.

Table 1. Summary of voltage-dependence parameters for the site-directed mutants

Species	− dextran sulfate		+ dextran sulfate		Charge change
	n	V_0, mV	n	V_0, mV	
Wild type	2.5 ± 0.4	30 ± 4	24 ± 2	2.4 ± 0.4	
No effect on n					
D30K	2.7 ± 0.2	32 ± 3			0 (+2)
D51K			26 ± 2	2.2 ± 0.2	0 (+2)
K95E			23 ± 2	2.1 ± 0.2	0 (−2)
R124E			25 ± 5	2.6 ± 0.6	0 (−2)
K132E	2.3 ± 0.3	23 ± 3	23 ± 3	2.2 ± 0.4	0 (−2)
D156K	2.1	−27	25 ± 3	2.6 ± 1.2	0 (+2)
K205E	2.3 ± 0.4	29 ± 2			0 (−2)
K211E	2.6 ± 0.6	31 ± 7			0 (−2)
K234Q			23 ± 2	2.5 ± 0.3	0 (−1)
K248E	2.6 ± 0.2	19 ± 2			0 (−2)
R252E	2.6 ± 0.2	33 ± 3			0 (−2)
K267E	2.2 ± 0.4	29 ± 5			0 (−2)
Effect on n					
D15K			48 ± 9†	1.5 ± 0.5	+2 (+2)
K19E			10.2 ± 0.9†	−5 ± 1	−1 (−2)
K46E			17 ± 2†	3.0 ± 0.9	−1 (−2)
K61E			15 ± 3†	3.6 ± 0.8	−1 (−2)
K65E	1.6 ± 0.4†	32 ± 8			−1 (−2)
K84E			17 ± 3*	3.1 ± 0.6	−1 (−2)
E152K			34 ± 7†	2.1 ± 0.6	+1 (+2)
D282K			34 ± 2†	1.9 ± 0.2	+1 (+2)

Steepness of the voltage dependence n and the voltage needed to close half the channels (V_0) were estimated as in Fig. 1. Experiments were performed in the presence or absence of dextran sulfate (as in Fig. 1). The results (mean ± SD) were grouped into mutants that had no significant effect on the n value (t test, $\alpha < 0.05$) and mutants that had a significant effect on n. (*, $0.01 > \alpha > 0.001$; †, $0.001 > \alpha$.) n and V_0 values for closure at positive and negative potentials were pooled, except for D156K and K19E where a sign is indicated in the V_0 value. The results for K19E were obtained under somewhat different conditions and should be compared to wild-type values: $n = 20 \pm 2$ and $V_0 = -3.4 \pm 0.3$. Charge change refers to an estimate of the change in charge on the sensor (n) in the absence of dextran sulfate. Since dextran sulfate amplifies the voltage dependence by a factor of 10, estimates of n obtained in the presence of dextran sulfate have been divided by this factor and rounded to the nearest whole integer. The number in parentheses is the engineered charge change.

The mutations that affected the voltage dependence identify regions of the protein that move in response to the membrane potential. The nearby residues, some of which are negatively charged, are likely to move as well. Thus, as pointed out by Ermishkin and Mirzabekov (15), the sensor contains both positive and negative charges. The net positive charge on the sensor accounts for the voltage dependence.

Wild-type VDAC channels are formed by a single 283-aa polypeptide that can close symmetrically in response to both positive and negative potentials (17–19). For a single asymmetric protein to behave in this manner, closure at opposite potentials might be mediated by motion of two separate sensors or by movement of a single sensing region in opposite directions. The experiments presented in Table 1 do not address this question, since results for positive and negative potentials were averaged from membranes containing many channels. Single-channel results for mutations at at least one position (K46E, Fig. 2) indicate that there is overlap between the sensors that respond to positive and negative potentials.

A model for the structure of open VDAC has been proposed based on secondary structure predictions from the protein sequence and the effects of site-directed mutations on open-channel selectivity (7, 20). In this model, the open channel is formed by a single VDAC polypeptide containing an amino-terminal α-helix and 12 transmembrane β-strands connected by loops that contact the aqueous medium on either side of the membrane (Fig. 3). Amino acid substitutions changing the charge of residues in the putative α-helix or transmembrane β-strands change the selectivity of the open channel in the direction expected if the selectivity is determined by the overall charge within the pore (7). Similar substitutions in the proposed loop regions have no effect on selectivity.

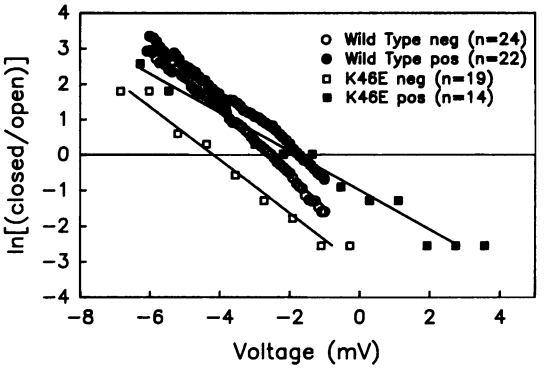

FIG. 2. Natural logarithmic transformations (as in Fig. 1 *B* and *D*) of measurements of the voltage dependence of the conductance of wild-type VDAC and the K46E mutant VDAC. The data for the wild-type VDAC were obtained from a multichannel membrane; data for the mutant are an average of 14 recordings made on the same single channel. The applied voltage for the data obtained at positive potentials was multiplied by −1 to facilitate comparison with the data obtained at negative potentials.

In Fig. 3, the positions of residues where charge changes altered or had no effect on voltage gating are indicated. A large portion of the protein, particularly the amino-terminal region, contains residues that affect the gating properties of the resulting channels when their charge is changed. These residues identify regions of the protein that are translocated through the field in response to voltage changes. In studies on the effects of charge changes on the selectivity of closed VDAC channels (8), residues were identified that affected the selectivity of the open state but had reduced or no effect on the closed-state selectivity. These are presumably residues that are removed from the wall of the pore during channel closure. This group includes all of the residues identified in this study as affecting voltage gating. Thus, two lines of evidence indicate that these residues move out of the channel during channel closure. A working model for the molecular rearrangements associated with voltage gating and channel closure is that the amino-terminal α-helix and four adjacent β-strands move out of the membrane during channel closure, perhaps to lie on the surface of the membrane with the hydrophobic side in contact with the membrane and the hydrophilic side facing the aqueous environment. In this model, charged residues move out of the pore wall either by motion perpendicular to the membrane (in which case they would participate in the voltage dependence of channel opening and closure) or may move out of the channel without moving through enough of the electric field to affect the voltage dependence. Residues such as K248 that affect the selectivity of the open, but not the closed state (8), and do not alter the voltage dependence may belong to the latter category.

An unresolved problem with this model is that some charged residues that would be expected to move (Asp-30 and Asp-51) have no effect on gating when their charge is reversed. Similarly, mutation of Glu-152 affects gating, although it is surrounded by residues that do not. Thus the rearrangements associated with closure are no doubt more complex than this working model would indicate. However, our results clearly show that residues contributing to the voltage sensor in VDAC are distributed throughout the

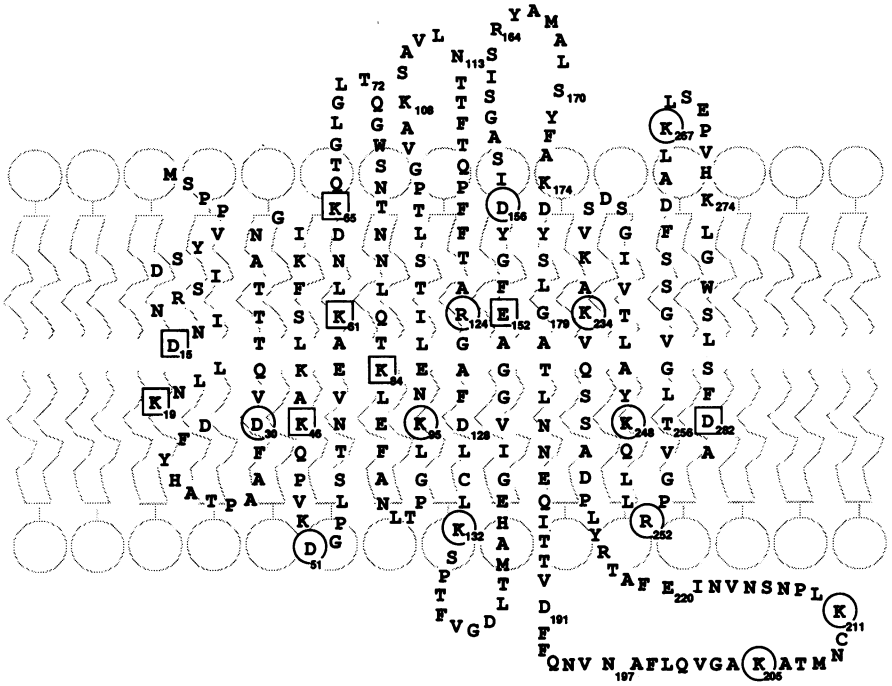

FIG. 3. Location and motion of the voltage sensor. The proposed secondary structure of the VDAC protein in the membrane consists of an amino-terminal α-helix flanked by 12 strands of antiparallel β-sheet. Residues for which a mutation altered the voltage dependence are boxed; residues in which the mutation left the voltage dependence unchanged are circled.

polypeptide, in contrast to current models of the structure of Na^+ and K^+ channels. Further, while the details of the conformational changes driven by voltage need to be carefully elucidated, our results indicate that changes in voltage result in the removal of large portions of the protein forming the wall of the open pore. This results in the observed reduction in pore radius (21), pore volume (22), and channel selectivity (12, 23, 24) that occur on voltage-dependent closure of VDAC. While the magnitude of the proposed structural change may be surprising, the results presented here eliminate a variety of other possibilities.

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