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Oriented Channel Insertion Reveals the Motion of a Transmembrane Beta Strand During Voltage Gating of VDAC

M. Zizi, L. Thomas*, E. Blachly-Dyson[2], M. Forte[2], M. Colombini[1]**

[1]Laboratory of Cell Biology, Department of Zoology, University of Maryland, College Park, MD 20742
[2]Vollum Institute, Oregon Health Sciences University, Portland, OR 97201

Abstract. Yeast VDAC channels (isolated from the mitochondrial outer membrane) form large aqueous pores whose walls are believed to consist of 1 α helix and 12 β strands. Each channel has two voltage-gating processes: one closes the channels at positive potentials, the other at negative. When VDAC is reconstituted into phospholipid (soybean) membranes, the two gating processes have virtually the same steepness of voltage dependence and the same midpoint voltage. Substituting lysine for glutamate at either end of one putative β strand (E145K or E152K) made the channels behave asymmetrically, increasing the voltage dependence of one gating process but not the other. The asymmetry was the same whether 1 or 100 channels were in the membrane, indicating oriented channel insertion. However, the direction of insertion varied from membrane to membrane, indicating that the insertion of the first channel was random and subsequent insertions were directed by the previously inserted channel(s). This raises the prospect of an auto-directed insertion with possible implications to protein targeting in cells. Each of the mutations affected a different gating process because the double mutant increased voltage dependence of both processes. Thus this strand may slide through the membrane in one direction or the other depending on the gating process. We propose that the model of folding for VDAC be altered to move this strand into the sensor region of the protein where it may act as a tether and guide/restrict the motion of the sensor.

* *Present address:* Department of Physiology, 6811 Med. Sciences Bldg 2, University of Michigan, Ann Arbor, MI 48109

** *Present address:* Department of Physiology, K.U. Leuven Medical School, Gasthuijsberg, 3000 Leuven, Belgium

Correspondence to: M. Colombini

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Introduction

The mitochondrial outer membrane channel, VDAC, forms large pores in the outer membrane of mitochondria from eukaryotic species of all kingdoms (*see* Colombini, 1994 for review). The pores are somewhat anion selective and are thought to be the main passageway for ions and metabolites crossing this membrane. An accumulating body of evidence points to a complex regulation of VDAC permeability, compatible with an important role for the outer membrane in control of cellular respiration (Colombini, 1994).

When purified from mitochondria and incorporated into planar phospholipid membranes, VDAC forms voltage-dependent channels which close to lower conductance states when positive or negative potentials are applied to the membrane. This "closed" state is actually a lower conducting state, but of reversed selectivity, which may account for the large reduction in outer membrane permeability to (negatively charged) adenine nucleotides when intact mitochondria are treated with agents that close VDAC (Benz, Kottke & Brdiczka, 1990; Liu & Colombini, 1992). Available evidence (Thomas et al., 1991; Peng et al., 1992*b*) also indicates that each channel is formed by a single 30 kDa polypeptide chain without internal repeats. Computer modeling of the primary amino acid sequence (Forte, Guy & Mannella, 1987; Blachly-Dyson et al., 1989) and studies on site-directed mutant forms of the channel have led to the development of models of the transmembrane organization of VDAC consisting of a single transmembrane alpha helix and a 12-strand beta sheet (Blachly-Dyson et al., 1990). Thus, although gating at positive and negative potentials appear to be symmetrical, there must actually be two different gating processes operating at opposite potentials.

Voltage-dependent gating of channels is thought to result from the movement of a charged moiety through or reorientation of a dipole with respect to the applied transmembrane potential, resulting in a conformational change in the molecule. The charged domain or dipole is referred to as the "voltage sensor." Changing the charge on the voltage sensor of a channel by site-directed mutagenesis should change the gating properties of the channel in a predictable manner. This approach has led to the identification of a number of residues which contribute to the voltage sensor of yeast VDAC (Thomas et al., 1993). Residues throughout the amino-terminal one-third of the molecule and at the extreme carboxy-terminus were found to affect the gating processes. Mutations which increased the net positive charge in these regions increased the steepness of the voltage dependence (reflected in the estimated effective charge on the voltage sensor), while mutations that decreased the net positive charge decreased the voltage dependence. These residues are among those previously identified that affect ion selectivity in the open, but not or by less than the expected amount, the closed state of the channel (Peng et al., 1992*a*).

In the previous work, the effects of these mutations were investigated in multichannel membranes, and gating at positive and negative potentials were averaged, since channels were assumed to insert into the planar membranes in random orientation (i.e., one channel opening did not preferentially face one aqueous compartment). Thus, these studies did not distinguish the voltage sensors acting at positive potentials and negative potentials. While some sites in the molecule are likely to move through the membrane potential during both gating processes, others may contribute only to one process. In this report, we describe mutations (E145K and E152K) which identify two such sites in yeast VDAC. We find that each of these mutations affects only one of the two gating processes when single channels are examined, and furthermore, that membranes containing large numbers of these asymmetrical channels behave as if all the channels insert in the same orientation. This "autodirected insertion" behavior has allowed us to demonstrate that residues E145 and E152 each affect only one of the two different gating processes. From this we conclude that these residues lie at opposite ends of a polypeptide strand that traverses the membrane and moves in opposite direction during the two gating processes.

Materials and Methods

VDAC Mutants

Site-directed mutations in the yeast VDAC gene were created and mutant proteins expressed in and purified from VDAC-deficient yeast as described in Blachly-Dyson et al., 1990.

Planar Phospholipid Membranes

The planar membranes were built according to the method of Montal and Mueller (1972) as modified (Schein et al., 1976). In short, 20 μl of a phospholipid-containing solution (1% asolectin, 0.2% cholesterol in hexane) was layered on the surface of the 5 ml aqueous phase in each compartment of a two-sided experimental chamber. Following hexane evaporation, the bilayer was built one monolayer at a time by raising the fluid level in each side of the chamber.

Electrophysiological Recordings

The state of the channels was monitored by recording the net ion flow through the membrane as a current while the voltage across the membrane was controlled (voltage-clamped as previously described, Schein, Colombini & Finkelstein, 1976). Calomel electrodes were used to interface the electronics with the aqueous phase. Individual insertion events were followed until a sufficient number of channel molecules had inserted into the membrane. The voltage-dependence of the inserted channels was then probed by varying the voltage linearly with time (0.4 mV/sec). Current recordings from several such voltage ramps were digitized to obtain the probability of the channel being open as a function of voltage. Only those records made when the channels went from a closed to an open state were analyzed because of the fast rate of opening. If dV/dt were reduced by a factor of 2 it would not change the conductance/voltage relationship. VDAC voltage gating is usually analyzed by fitting to a two-states (open-closed) model. This model yields a sigmoid distribution curve of the open probability of the channel as a function of the voltage. This curve can be linearized and yields the values of the two parameters defining the gating process: n, the calculated gating valence, and "V_o", the midpoint potential for which the probability of being open equals the probability of being closed.

$$Ln\left(\frac{closed}{open}\right) = Ln\left(\frac{G_{MAX} - G}{G - G_{MIN}}\right) = n\left(\frac{F}{RT}\right)V - \frac{nFV_o}{RT} \qquad (1)$$

G_{max}, G_{min}, and G are the conductance when all the channels are open, when they are all closed (the closed state is still conductive to KCl), and at any voltage (V), respectively F, R, and T have their usual meaning.

n is the parameter, measured for gating at positive and negative potentials, that provides information on orientation of the mutant channel(s) in the phospholipid membrane.

Experimental Conditions

Both compartments of the chamber contained: 1 M LiCl, 5 mM $CaCl_2$, 5 mM MES, pH 5.8. Each compartment also contained 5.75×10^{-2} mg/ml of Dextran sulfate 500 kDa. This magnifies the steepness in voltage dependence of VDAC by a factor of 10 while decreasing the V_o values by the same factor (normal VDAC gating values, $n = 2.5$ and $V_o = 25$ mV, become $n = 25$ and $V_o = 2.5$). These conditions give us more resolution and confidence in the n values and do not change the energetics of the gating processes. *See also* (Mangan & Colombini, 1987; Thomas et al., 1993).

Results

When pure, detergent-solubilized VDAC channels are added to the aqueous phase bathing a phospholipid membrane, individual channels insert spontaneously. The voltage dependence of the gating process can be mea-

sured either for a single channel or for a population resulting from the insertion of many channels into a membrane. For wild-type channels reconstituted into soybean phospholipid membranes, the two gating processes (one responding to positive and the other to negative potentials) have identical parameters (same steepness of the voltage dependence and same magnitude of potential needed to close half the channels) and therefore the orientation of channels in the membrane cannot be determined. This symmetry was seen both in studies of membranes containing one channel and those containing many (50 to 100) channels (Fig. 1). It was observed regardless of the side of the membrane into which the channels inserted and regardless of the polarity of the applied potential during the insertion period (*see* Fig. 4).

In contrast, VDAC containing either of two point mutations, with lysine replacing glutamate at position 145 or 152 (E145K and E152K), displayed asymmetrical voltage-gating behavior (Fig. 2), in that each of the mutations changed the gating processes at one polarity only. The steepness of the voltage dependence, as measured by the parameter n (the gating charge) increased by ≈50% for the affected gating process (from 26 for wild type to 36 for E152K and to 40 to E145K). We used the asymmetric behavior of these molecules to probe the directionality of channel insertion into phospholipid membranes. In typical single-channel experiments (Figs. 2*A* and *C*), the open state probability of the mutant VDAC declined much more steeply at one potential than at the opposite. In some experiments, the steeper voltage dependence was seen at negative potentials, while in others it was seen at positive potentials. This behavior presumably reflects the differing orientations of the single channels inserted into the bilayers in each experiment.

Surprisingly, the same degree of asymmetry was observed in membranes containing many channels (between 50 and 100, Fig. 2*B* and *D*). Consequently, in these membranes, the entire channel population behaved as if all the inserted channels had the same orientation in the membrane. The polarity of the potential that yielded the steeper voltage dependence varied from membrane to membrane in the multichannel experiments, as it did in the single channel experiments (Fig. 3). This behavior would be expected if the first channel in a membrane were to insert randomly, and all subsequent insertions into that membrane were in the same orientation. In one experiment (indicated by the asterisk in Fig. 3), the difference in gating between the two gating processes was less than that seen in single channel experiments. In this case, there may have been initial insertion events in both orientations.

To investigate the possibility that the mutant channels behave as dipoles, whose direction of insertion depends on the membrane potential, mutant VDAC protein was added to either the positive or negative side of the membrane. The channel orientation varied from experiment to experiment in these multichannel experiments as it did in single-channel experiments, and did not correlate with the sign of the voltage applied during the insertion process (Fig. 4). In the absence of other explanations, we conclude that the first channel insertion into a bilayer is random and independent of membrane polarity, and that the orientation of the first channel guides or catalyzes the insertion of later channels into the same orientation. Consistent with this explanation is the long-standing observation that the insertion of the first channel is a slow process (up to one hour after sample addition) whereas subsequent insertions occur much more rapidly.

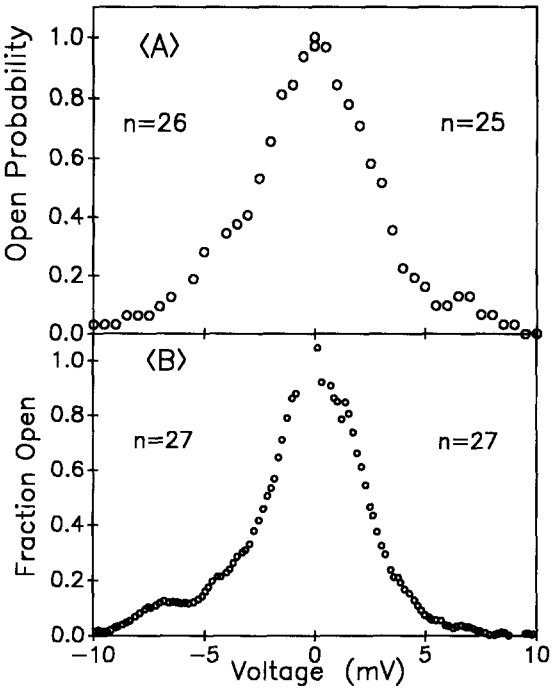

Fig. 1. The voltage dependence of the conductance of the wild-type form of the yeast VDAC channel reconstituted in asolectin planar phospholipid membranes. (*A*) The probability for a single channel molecule to be open or closed as a function of the voltage was obtained by analyzing the response of a single channel to a series of voltage ramps. (*B*) The fraction of channels in the open state as a function of voltage was obtained by analyzing the response of multichannel membrane (>50 channels) to a series of voltage ramps. The experimental conditions were as described in the methods. The values of n are the measure of the steepness of voltage dependence (*see* text). The experiments were performed in the presence of dextran sulfate to amplify the voltage dependence.

Since much more data can be obtained from the analysis of populations of channels than single channels, the fact that multichannel membranes containing E145K and E152K mutant VDAC molecules exhibit the same asymmetry as membranes containing single channels enabled us to investigate in more detail the effects of the mutations on VDAC gating. A reasonable explanation for the effect of the mutation E145K on the gating charge

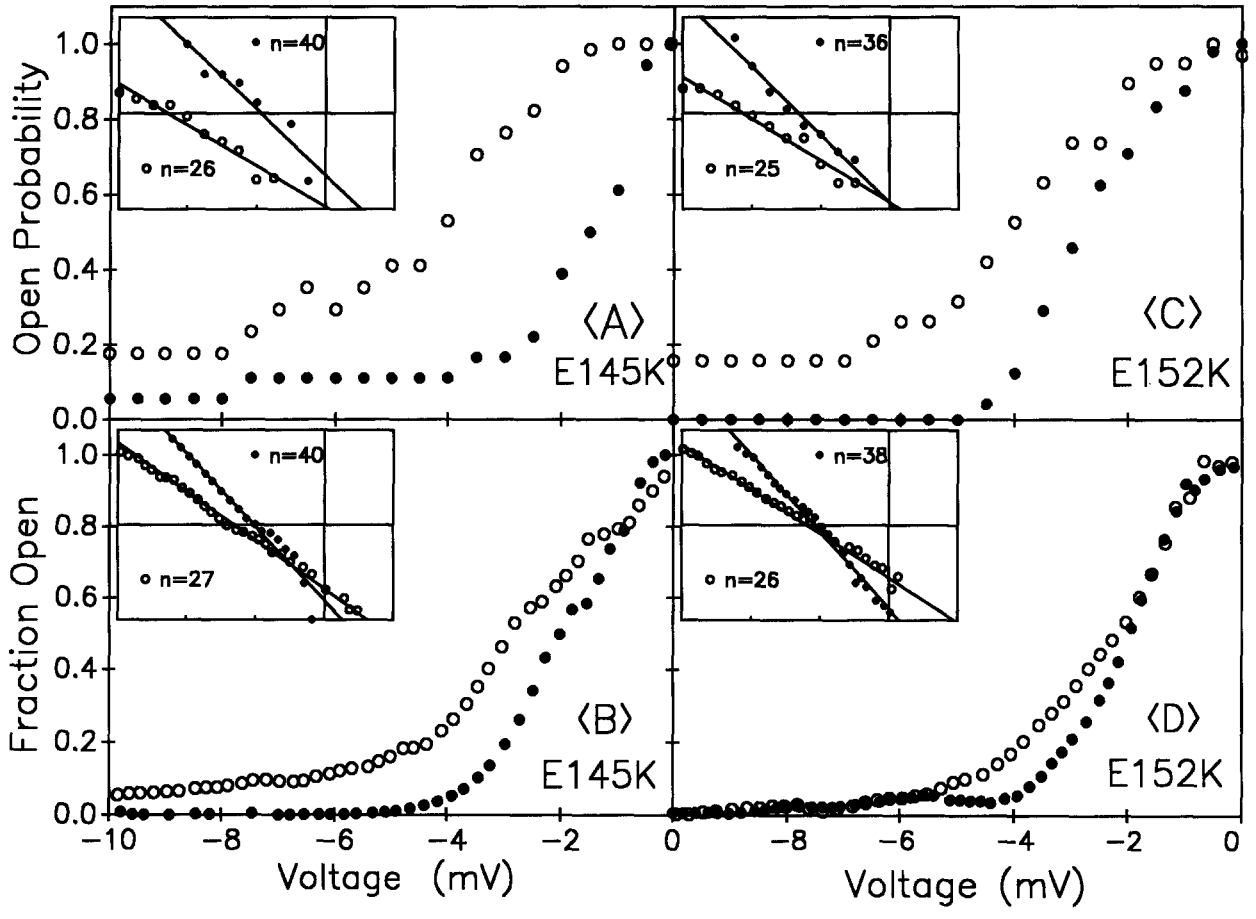

Fig. 2. The mutant channels (glutamate replaced by lysine at positions 152, "E152K", and 145, "E145K") display an asymmetric voltage gating behavior in their two gating processes. This asymmetry is shown here at the single channel level (*A* and *C*) and at the multichannel level (*B* and *D*). Data obtained at positive potentials was plotted in the second quadrant for comparison to data obtained at negative potentials (open and filled symbols were used to distinguish the two sets of data). In the *C* experiment, there were between 1 and 3 channels in the membrane. The multichannel membranes contained between 50 and 100 VDAC molecules. The data were fitted to a two-state model (insets) by plotting $ln\{(G_{max} - G)/(G - G_{min})\}$ *vs.* *V*, as in the equation in the text, and the steepness of the voltage dependence, *n*, for voltage gating at each potential is indicated.

n is that this residue forms part of a "voltage sensor" that moves through the membrane in response to applied potential during one gating process, but does not traverse the membrane potential during the other gating process. Likewise, E152 moves through the membrane in only one of the two gating processes. Thus, the charge increase of +2 introduced by each mutation increases the *n* value for only the affected gating process. If both mutations affected the same gating process, then combining the two mutations in a single molecule should produce a channel with one wild-type gating process and the other should be changed by twice the amount of change caused by either mutation alone. If the two mutations affected the two different gating processes, a doubly mutant VDAC molecule should form a channel with increased *n* for both gating processes.

Since the channels insert into the membrane all in the same orientation, we were able to use multichannel membranes to obtain sufficient data to distinguish between these possibilities. The doubly mutant VDAC (E145K/E152K) was constructed and introduced into bilayers. The resulting channels had *n* values for both gating processes that were increased to the same level seen for the single altered gating process in the singly mutant channels (Fig. 5 and Table 1). This result was seen both in single channel and multichannel experiments. This supports the hypothesis that each mutation affects only one of the two gating processes. When comparing the free energy differences between the channel in the open and closed states for the wild type and the three mutants, it can be seen that the double mutation results in a strictly additive alteration. This argues that the two mutations, although near to each other within the primary sequence, are functionally independent of each other. The additive nature of the mutations argues strongly that the effects were local and primarily related to the charge change.

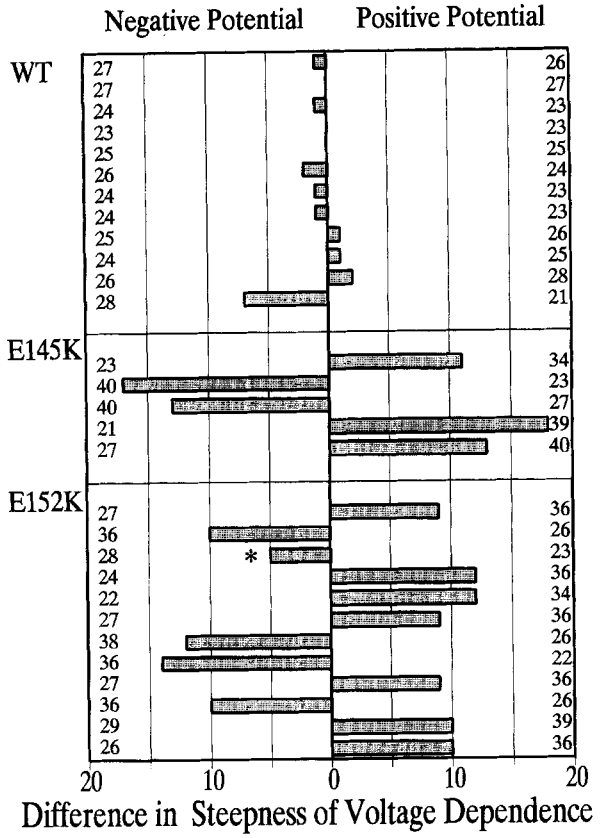

Fig. 3. The direction of the measured asymmetry varies from membrane to membrane. Experiments such as those illustrated in Fig. 2 are summarized here. All experiments were multichannel membranes except for one of the E145K. The horizontal bars represent differences in steepness of the voltage dependence at positive and negative potentials for 12 wild-type, 5 E145K, and 12 E152K experiments. The exact numerical values are also listed for clarity. The asterisk highlights the only experiment with a less pronounced asymmetry (*see* text).

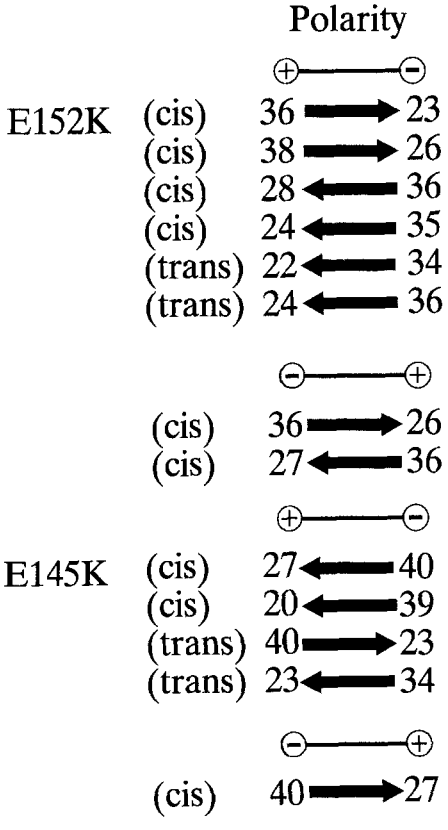

Fig. 4. The field polarity (*see* dipoles in the figure) during channel insertion does not correlate with the direction of this insertion. The voltage during insertion was kept constant in the direction indicated in the figure. The mutant channels were added to the cis or the trans side of the chamber, i.e., of the phospholipid membrane, and the asymmetry of the voltage gating processes was determined by analyzing the voltage dependence of the current recorded after a sufficient number of channels (between 50 and 100) had inserted. Arrows indicate the direction of asymmetry of the steepness of voltage dependence from high to low values.

However, there is also a change in the free energy difference between the states as estimated by nFV_0 indicating a change in the free energy of either the open or closed state or both (Fig. 6). This could result from interactions between the charged residue and its neighbors or a small structural change. In any event, the effect seems small.

Another indication that the mutations do not cause structural changes of any importance comes from measurements of the single-channel conductance (Table 2). There is very little difference between the conductance of the wild-type channel and either the single mutants or the double mutant. Thus, the structure of the channel seems to remain virtually unaltered by the mutations.

Discussion

We have identified two different positions in the VDAC protein where charge changes affect only one of the two gating processes. The fact that the resulting mutant channels have asymmetrical gating characteristics has allowed us to observe that the VDAC channels insert into bilayers in an oriented fashion, such that in most cases multichannel membranes behave as if all the channels in the membrane have the same orientation. The orientation of the channels is random with respect to the side of the membrane from which the protein was added, and with respect to the polarity of the membrane at the time of insertion.

Massive insertion of channels from preoriented structures such as membrane vesicles or two-dimensional crystals (Mannella, Colombini & Frank, 1983) could, in principle, account for this result. This is unlikely, however, as we used pure detergent-solubilized proteins. The hydrodynamic properties of such VDAC-Triton X-100 preparation are only compatible with the existence of monomers or dimers (Linden & Gellerfors, 1983). The use of detergent (Triton X-100) allows us to

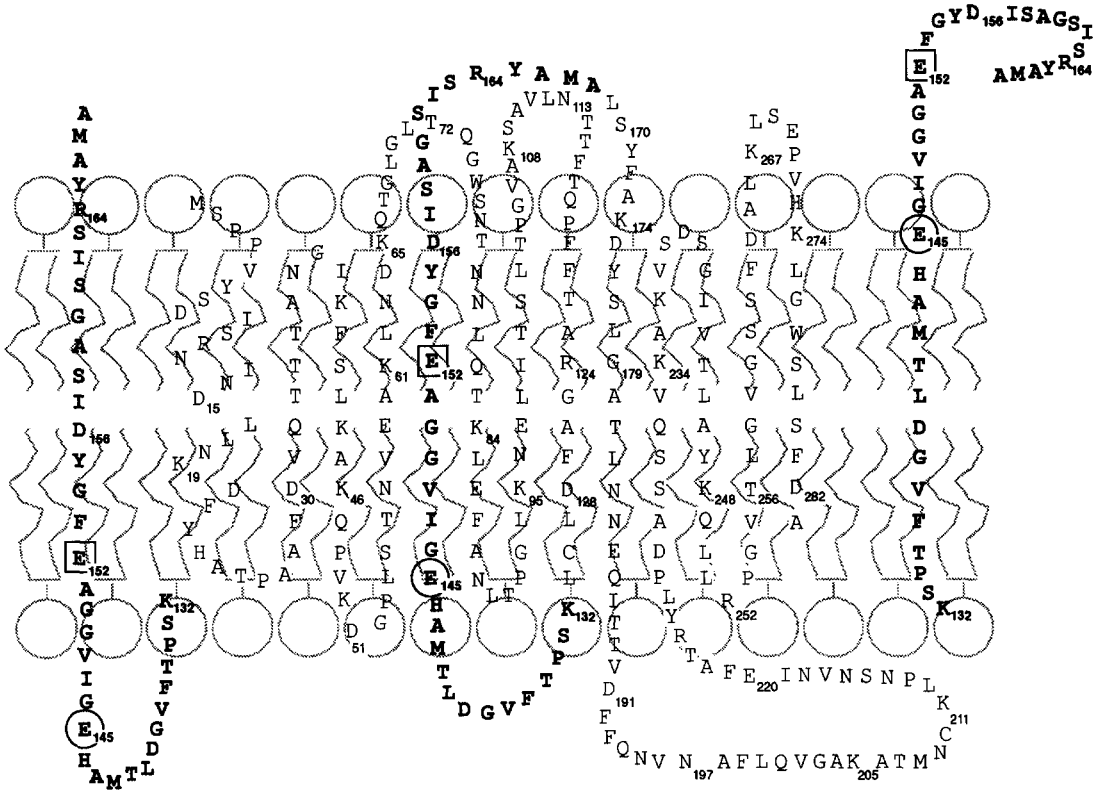

Fig. 5. Proposed motion of the β-strand studied in this paper. The proposed folding pattern of yeast VDAC in the membrane (center drawing) is illustrated using the single letter code for amino acids and starting from the N-terminus on the left to the C-terminus on the right. The strand containing residues 145 and 152 is proposed to be located closer to the N-terminus in the region containing the bulk of the voltage sensor. To form the barrel, the strands are tilted and rolled into a cylinder. The illustrations on the sides show the proposed motion of this strand during channel gating at either positive or negative potentials (the strand moves toward the negative side of the membrane). The numbered residues are sites where substitutions have been engineered in the molecule in either these or previous experiments.

dissociate the channel molecules into the aqueous phase of the experimental chamber. The high dilution after addition of the Triton-containing solution to the chamber decreases the detergent concentration to trivial amounts (less than 0.002%), well below the critical micellar concentration of 0.016% for Triton. Thus, it is likely that individual purified channels are able to interact with preinserted channels. More importantly, each insertion event occurred on a relatively slow time scale. We could see that channels inserted individually, not in groups, and usually we waited until many channels had inserted to check the voltage gating.

A built-in asymmetry in the phospholipid membranes also does not account for our results. The planar phospholipid membranes used in these experiments are built one monolayer at a time (Montal & Mueller, 1972; Schein et al., 1976) using mixed lipids that contain some charged moieties. Thus, differences in the densities of surface charges in the two monolayers could result in an asymmetric membrane. But such an asymmetry should only shift the midpoint of the gating process (V_o), without affecting the steepness of the voltage dependence (n) (Muller & Finkelstein, 1972). In fact, we observed asymmetries in n, leading us to reject this explanation.

We investigated the possibility that the mutant channels behave as dipoles whose direction of insertion depends on the membrane potential. Oriented insertion was observed regardless of whether the potential was kept constant or changed during the period of channel insertion. The direction of insertion varied from experiment to experiment and did not correlate with the sign of the voltage applied during the insertion process (Figure 4). This applied both to single-channel and multichannel membranes. Thus, we have not been able to identify any factor other than the random orientation of the initial insertion event, which affects the orientation of subsequent insertions. The ability of preinserted channels to determine the orientation of subsequent channel insertions, demonstrated by the results presented here with planar lipid bilayer, may reflect mechanisms used in the targeting and insertion of VDAC channels into the outer mitochondrial membrane.

This autodirected insertion resembles enzyme catalysis. Once in the membrane, a VDAC channel may re-

Table 1. Channel gating parameters for Yeast VDAC, Wild-type, E152K, E145K, and double-mutant E145K-E152K

Gating process 1	Gating process 2	Free energy difference
Wild type (15 exp)		
$n = 25 \pm 0.4$	25 ± 0.5	
$V_o = 2.6 \pm 0.1$ mV	2.7 ± 0.1 mV	
$\Delta G = 6.2 \pm 0.3$ kJ/mol	6.4 ± 0.4 kJ/mol	≈0.2 kJ/mol
E145K (5 exp)		
$n = 24 \pm 1$	39 ± 1	
$V_o = 3.5 \pm 0.4$ mV	2.4 ± 0.2 mV	
$\Delta G = 8.0 \pm 1$ kJ/mol	8.9 ± 1 kJ/mol	≈0.9 kJ/mol
E152K (12 exp)		
$n = 36 \pm 0.8$	26 ± 0.5	
$V_o = 2.2 \pm 0.1$ mV	2.2 ± 0.1 mV	
$\Delta G = 7.7 \pm 0.5$ kJ/mol	5.5 ± 0.4 kJ/mol	≈2.2 kJ/mol
Double mutant (E145K–E152K) (3 exp)		
$n = 37 \pm 0.4$	38 ± 0.1	
$V_o = 1.6 \pm 0.2$ mV	2.4 ± 0.1 mV	
$\Delta G = 5.7 \pm 0.7$ kJ/mol	8.8 ± 0.4 kJ/mol	≈3.1 kJ/mol
Double mutant (E145K–E152K) (3 exp, single channel recording)		
$n = 40 \pm 1$	41 ± 0.9	
$V_o = 2.0 \pm 0.2$ mV	2.7 ± 0.2 mV	
$\Delta G = 7.6 \pm 0.9$ kJ/mol	10.6 ± 0.9 kJ/mol	≈3.0 kJ/mol

Values are mean ± SEM. Conditions were as in Experimental. The "Free energy difference" column is the absolute difference between the energetics of the two voltage gating processes. The legend to Fig. 6 shows how the data were averaged.

duce the energy barrier to subsequent insertions. Comparing the potential target area of the planar membrane (18,000 μm^2) and that of one VDAC channel (20 nm^2, which is an overestimate of the real target site), one can estimate that the insertion process is increased locally by a factor of 10^9, comparable to the increase in rate of a reaction by typical enzymes (Jenks, 1987). This underestimates the true rate increase because there is a large preference for insertion at the VDAC site as compared to anywhere else on the membrane. An indication of this is the observation that the insertion of the second channel into the membrane occurred four times more rapidly on average than the first one (*data not shown*). That would put the estimated rate increase at 4×10^9. If this process occurs in vivo, it will have profound implications on protein targeting. If there were only one VDAC molecule in the outer membrane of one mitochondrion, the insertion of a second molecule would preferentially occur on this spot as opposed to anywhere else on that surface by at least a factor of 6,000.

We used the oriented multichannel membranes to determine more carefully the gating parameters of VDAC channels containing one or both of the mutations (E145K and E152K) that cause asymmetrical gating. We found that each mutation individually increased the steepness of the voltage dependence (reflected in an increase in the calculated gating valency, n) for one polarity, while leaving it unchanged for the other polarity. Channels containing both mutations had increased n values for gating at both polarities. This indicates that the two mutations are individually affecting each of the two gating processes.

A model for the structure of the VDAC channel proposes that it consists of a single transmembrane alpha helix and a 12-strand beta sheet forming a hollow cylinder across the membrane (Blachly-Dyson et al., 1990). In this model, residues E145 and E152 are located near opposite ends of a transmembrane beta strand. The fact that a net increase in positive charge by two elemental units at each location resulted in an increase in the voltage dependence of only one gating process (by 1.5 elemental units) is best explained by each residue translocating across the membrane only when one polarity of potential is applied and not the other. The fact that the double mutant resulted in an increased voltage dependence at both positive and negative potentials confirms that the residues are indeed located at opposite sides of the pore and that the strand most likely moves in a direction normal to the plane of the membrane during voltage gating.

These results have allowed us to identify a polypeptide strand (the strand between E145 and E152) that crosses the membrane. One possible way for this strand to move normal to the membrane in response to changes in transmembrane voltage is for the strand to be pulled out of the beta sheet during gating. For this to occur, the free energy cost of breaking the interstrand hydrogen bonds might be overcome by formation of hydrogen bonds with water. In our previous model of VDAC structure (Thomas et al., 1993), this segment forms a beta strand in a region of the protein that appears not to move during gating by two criteria: (i) charge changes in this region affect selectivity of both the open and the closed channel, and (ii) these charge changes do not alter gating. The results presented here make it seem more likely that the strand containing residues 145 and 152 is found in another part of the overall structure. Thus, unlike the bacterial porins, the beta strands of VDAC may not be arranged in linear order from N-terminus to C-terminus (Fig. 5). The strand containing residues 145 and 152 has a net negative charge and yet is probably moving, along with the rest of the voltage sensor, toward the negative side of the membrane because mutations E145K and E152K that increase the positive charge increase the voltage dependence. Thus, this strand is probably moving in concert with another part of the molecule that has a net positive charge (most likely somewhere near the N-terminus, Fig. 5). Since the strand is linked to others that have been shown not to move during the gating processes, this strand can only slide normal to the membrane. It may then act as a tether, guiding the motion of the sensor.

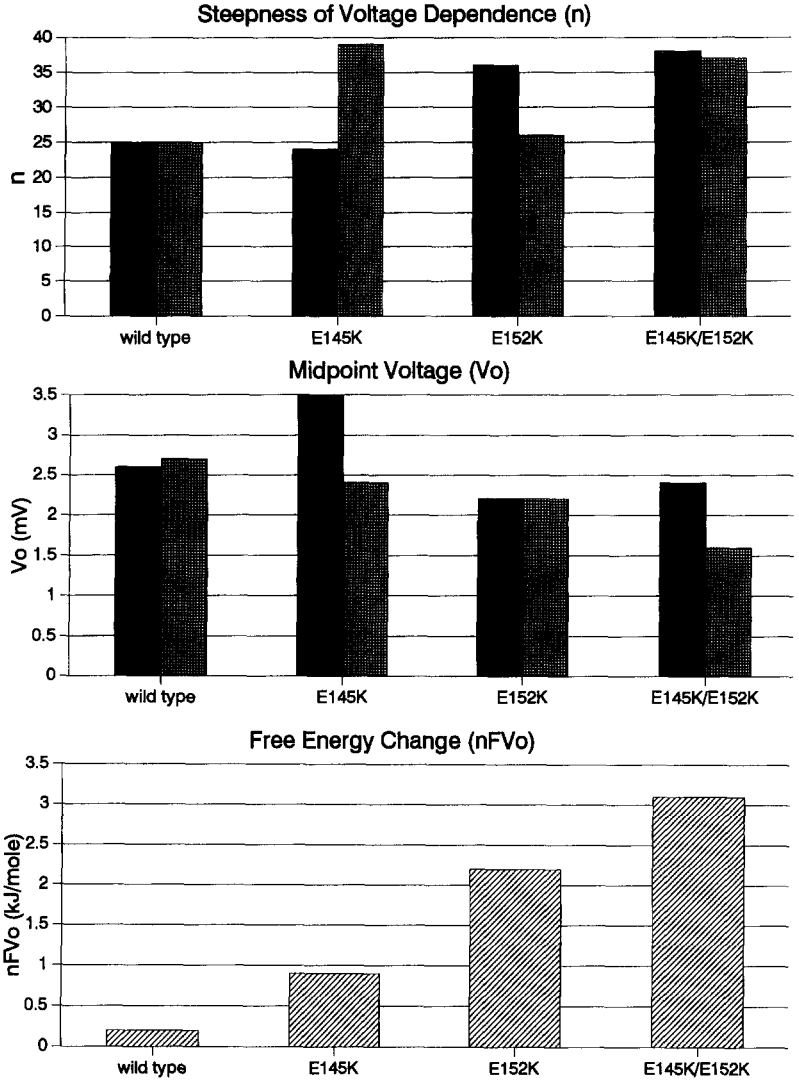

Fig. 6. The effect of glutamate to lysine substitution on the voltage dependence parameters of VDAC. The mean values of the steepness of the voltage dependence, *n*, the voltage at which half the channels were open, V_o, and the energy difference between the states, nFV_o, is illustrated for wild type, and three mutant channels: E145K, E152K, and the double mutant, E145K/E152K. The adjacent bars represent each of the two gating processes. For E145K and E152K, the gating processes with the high voltage dependence were averaged together irrespective of the applied potential. For the double mutant, the gating process with the higher V_o was averaged together. No such differentiation was made for the wild type since no significant difference was observed in the voltage dependence of the two gating processes.

Table 2. Single-channel conductance of wild-type and mutant channels

Species	Conductance[a] (NS)
Wild type	3.02 ± 0.07 (6)
E145K	3.01 ± 0.09 (5)
E152K	3.17 ± 0.08 (6)
E145K/E152K	2.91 ± 0.07 (5)

[a] Mean ± SD (number of measurements).

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