Large scale rearrangement of protein domains is associated with voltage gating of the VDAC channel

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ABSTRACT The VDAC channel of the mitochondrial outer membrane is voltage-gated like the larger, more complex voltage-gated channels of the plasma membrane. However, VDAC is a low molecular weight (30 kDa), abundant protein, which is readily purified and reconstituted, making it an ideal system for analyzing the molecular basis for ion selectivity and voltage-gating. We have probed the VDAC channel by subjecting the cloned yeast (*S. cerevisiae*) VDAC gene to site-directed mutagenesis and introducing the resulting mutant channels into planar bilayers to detect the effects of specific sequence changes on channel properties. This approach has allowed us to formulate and test a model of the open state structure of the VDAC channel. Now we have applied the same approach to analyzing the structure of the channel's low-conducting "closed state" (essentially closed to important metabolites). We have identified protein domains forming the wall of the closed conformation and domains that seem to be removed from the wall of the pore during channel closure. The latter can explain the reduction in pore diameter and volume and the dramatically altered channel selectivity resulting from channel closure. This process would make a natural coupling between motion of the sensor and channel gating.

INTRODUCTION

Voltage-gated ion channels have been the subject of biophysical investigation for the past 50 years. Until recently, however, molecular details of how these proteins form aqueous pores through membranes and the nature of the voltage-driven conformational changes that take place within these molecules have been the subject only of theoretical speculation. Over the past few years, the application of molecular genetic approaches has allowed the manipulation of many of the proteins forming narrow voltage-gated channels in ways that have led to insights into the structure and function of this family of molecules. In the case of the best studied plasma membrane channel, the voltage-sensitive K^+ channel, these studies have provided a biochemical picture of the mechanisms underlying inactivation, defined regions of the protein forming the aqueous pore, and quantified the number of polypeptide subunits that form a functional channel (for review see reference 1). Despite these recent developments, a true molecular picture of the transitions that occur in response to voltage changes to cause the channels to open and close has not been developed and will be difficult to define for such plasma membrane channels because they are formed by large, oligomeric protein complexes. Smaller voltage-gated channels, such as the mitochondrial channel VDAC, that form large voltage-gated pores with much less protein are likely to provide significant and relevant examples of how these molecular transitions can take place in response to voltage.

VDAC has been found in species of all eukaryotic kingdoms where it is believed to provide the major permeability pathway for metabolites passing through the outer mitochondrial membrane (2–4). VDAC channels have been observed following reconstitution of purified protein into planar phospholipid bilayers. Measurement of the pore size by the Stokes-Einstein radius of the largest permeant nonelectrolyte and by electron microscopy of negatively stained channels indicate that the pore is ~3 nm in diameter (5, 6). Open VDAC channels have a weak anion selectivity (2:1, Cl^-:K^+) which is probably due to the overall charge within the pore since the pore is large and admits a wide variety of molecular ions (4, 7). The effects of covalent modification (8, 9) indicate that multiple amino groups contribute to the selectivity of VDAC channels. VDAC channels close to a family of lower conductance states when a positive or negative voltage is applied to the bilayer (4, 10). This closure occurs at lower potentials in the presence of a protein factor found in mitochondria (the VDAC modulator) (11), or in the presence of a synthetic polyanion known as König's polyanion (12). Both the diameter and the internal volume of the channel are reduced during channel closure (12–14). The closed states are still permeable to KCl, so their selectivity can be measured in a manner analogous to that for the open state. Unlike the open channels, the closed channels show a weak cation selectivity (15, 16). Examination of

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freeze-dried VDAC arrays by scanning transmission electron microscopy indicates that each channel is formed by a single 30 kDa polypeptide (17, 18). Thus, given the small size of the VDAC protein and the relatively large size of the pore, it is likely that most of the VDAC molecule contributes to the channel's walls and that the walls are formed from a single layer of protein.

The primary amino acid sequence of the VDAC protein from yeast, *Neurospora,* and humans has been determined (19–22). Each consists of 282 or 283 amino acids. Analysis of the yeast VDAC sequence suggested that the protein consisted primarily of a "sided" β-sheet with a hydrophobic side facing the lipid bilayer and a hydrophilic side facing the interior of the pore (20). In addition, the 20 amino-terminal residues could form an amphiphilic α helix, similar to mitochondrial targeting "signal sequences" found on other mitochondrial proteins (23). A computer algorithm, designed to detect stretches of 10 or more alternating hydrophobic and hydrophilic residues that could form sided β strands, detected twelve potential transmembrane β segments (23). This information led to a model in which the wall of the channel was formed by 13 strands: 12 β strands and 1 α helix. Computer analysis of Neurospora and human VDAC suggests that this overall structure has been conserved (23).

If this model is correct, the overall charge within the pore should determine the selectivity of the channel because VDAC's large pore size would permit small ions to cross the membrane without interacting with the walls of the channel. We predicted therefore that changing the charge of amino acid residues at positions predicted to line the channel should change the channel's selectivity. To test this idea, mutations that changed the charge of specific residues were generated by site-directed mutagenesis (24). In most cases, charged amino acid residues were changed to residues of the opposite charge. When no charged residues were present in a region of interest, charged residues were introduced at positions predicted to line the channel in the alternating hydrophobic/hydrophilic pattern. The proteins produced by mutant genes were analyzed as outlined in Fig. 1 following introduction into yeast strains lacking the endogenous VDAC gene. This analysis has allowed us to define protein domains forming the walls of the open VDAC channel by identifying the location of residues that alter the selectivity of the channel when their charge is changed by site-directed mutagenesis (24). This same notion, applied to VDAC's low conducting or closed state has now allowed the identification of protein domains forming the wall of the closed channel and, therefore, domains that seem to be removed from the wall of the pore during channel closure. These results indicate that large scale rearrangements of protein domains are associated with voltage gating of the VDAC ion channel.

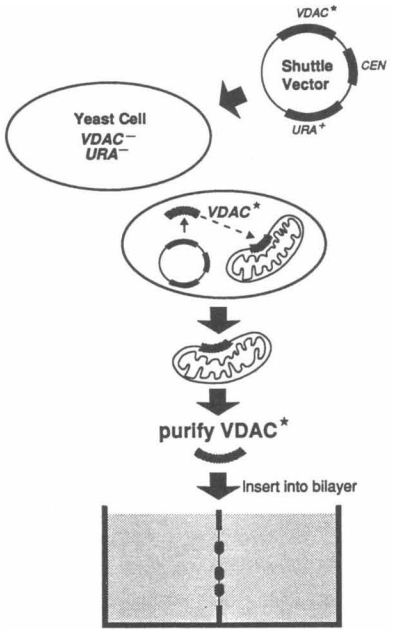

FIGURE 1 A schematic of the steps for producing VDAC proteins containing site-directed mutations for electrophysiological analysis. VDAC* indicates a VDAC gene in which a specific mutation has been engineered. URA is used as a selection marker. CEN, centromere.

MATERIALS AND METHODS

All experiments were performed on VDAC channels purified from mitochondria isolated from the yeast, *S. cerevisiae,* as previously described (24). To isolate VDAC proteins with amino-acid substitutions at desired locations, a VDAC gene modified by site-directed mutation was introduced into a yeast strain lacking the chromosomal copy of the VDAC gene (see reference 24 for details). 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 the 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 a planar phospholipid bilayer made from soybean phospholipids by the monolayer method of Montal and Mueller as modified (4). All experiments were performed in the presence of a 10-fold KCl gradient across the membrane and channel selectivity was estimated by measuring the reversal potential. A Saran partition containing a 150 micrometer hole (for the membrane) separated the two aqueous compartments (*cis* and *trans*). The *cis* compartment contained 1.0 M KCl, 5.0 mM $CaCl_2$, and 1.0 mM MES buffer pH 5.8, whereas the *trans* contained the same substances except that the KCl concentration was 0.10 M. Calomel electrodes were used to interface the electronics with the solutions, and all reported voltages were corrected for any asymmetry. All experiments were performed under voltage-clamp conditions (4).

VDAC channels were inserted by adding (while stirring) a 2 to 10

microliter aliquot of VDAC channels solubilized in 1% Triton X100 to the 4 ml of solution on the *cis* side. The amount added was adjusted to achieve the insertion of only 1 or a few channels.

König's polyanion (a copolymer of methacrylate, maleate, and styrene (1:2:3), Mav = 10,000 (12) was a gift of Tamás König.

RESULTS AND DISCUSSION

Open Channel

Site-directed mutations were engineered into over 30 positions of the yeast VDAC gene, some in predicted transmembrane strands, others in predicted loop regions (24). These mutant genes were then reintroduced into a yeast strain lacking an endogenous VDAC gene, mutant proteins were purified, reconstituted into planar lipid bilayers and tested for their effect on the selectivity of the open VDAC channel (Fig. 1). Many of the mutations altered the open-channel selectivity. The changes were in the direction expected if these charges were in the lining of the pore and the overall charge in the pore determined selectivity. The mutations did not significantly alter the single-channel conductance, indicating that there were no gross changes in the channel's protein structure. While most of the residues predicted to lie in the transmembrane strands affected selectivity, and most of the residues predicted to lie in loop regions had no effect, the placement of a few strands had to be altered to make the model fit the data. In addition, it was found that mutations in the predicted NH_2-terminal α helix affected selectivity, indicating that the helix forms part of the pore. These data were used to constrain the location of transmembrane segments forming the wall of the pore. This information was then used to develop the model of the transmembrane organization of the open VDAC channel shown in Fig. 2.

Teorell (25) developed a theory which seems to fit our data for the open state remarkably well. The theory describes ion flux through pathways in a membrane containing uniformly distributed fixed charges. In the special case of zero current, the potential across the

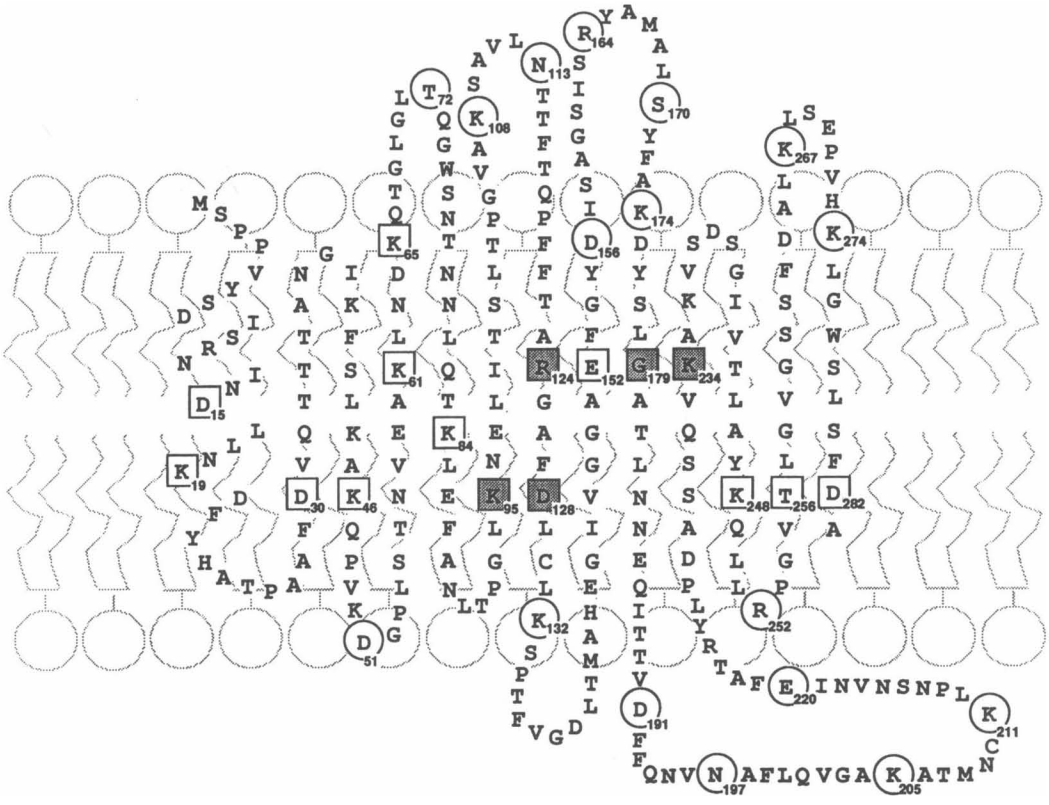

FIGURE 2 A schematic model of the VDAC molecule in the membrane. An NH_2-terminal α helix (*left*) is flanked by 12 strands of antiparallel β sheet. Residues for which mutations altered selectivity in the open state are boxed; residues in which the mutation left the selectivity unchanged are circled. Reversal potentials not previously published: T72D, 9.7 ± 0.2 (3); N113D, 9.6 ± 0.3 (4); S170D, 10.1 ± .5 (3) (mean ± SD [number of estimates]). Shaded residues indicate positions that appear to remain in the wall of the closed-channel pore.

membrane is a combination of three terms:

$$\text{Potential difference} = \pi 2 + \pi 1 + (\phi 2 - \phi 1), \quad (1)$$

where $\pi 1$ and $\pi 2$ are the Donnan potentials at the two channel openings, and $(\phi 2 - \phi 1)$ is the Hendserson-Planck diffusion potential within the channel due to unequal ion mobilities. Because these terms depend on the concentration of fixed charges within the pore, a relation between the number of charges within the pore and the measured reversal potential can be calculated from this theory and compared with the experimental findings (24). Because the theory assumes a uniform distribution of fixed charge and the VDAC channel is expected to have its charges located on the protein forming the walls of the pore, an attempt was made to bridge the gap by dividing the channel into two portions: a cylindrical shell, the thickness of one Debye length, with ion-conducting properties as described by Teorell's theory, and a central cylinder of fluid devoid of fixed charge that acts as a shunt (in KCl). This shunt acts to reduce the potential at zero current. The shell generates the potential, V_1:

$$V_1 = \frac{u - v}{u + v} \cdot 58 \log \left(\frac{c_1(r_1 u + v/r_1)}{c_2(r_2 u + v/r_2)} \right) + 58 \log \left(\frac{r_2}{r_1} \right), \quad (2)$$

where the Donnan ratio,

$$r = \sqrt{1 + \left(\frac{X}{2c} \right)^2} - \frac{X}{2c}. \quad (3)$$

The central cylinder generates potential V_2:

$$V_2 = \frac{u - v}{u + v} \cdot 58 \log \left(\frac{c_1}{c_2} \right) \quad (4)$$

The resultant potential, E, is a weighted sum (weighted by the cross-sectional area):

$$E = V_2 - (V_2 - V_1)(1 - (a - K)^2/a^2), \quad (5)$$

u and v are the bulk-phase mobilities of K^+ and Cl^-, respectively; c_1, c_2, r_1, and r_2 are the ion activities and the Donnan ratios in side 1 and 2 respectively; X is the effective fixed-charge concentration in a cylindrical shell adjacent to the wall of the pore (pore length taken as 5 nm plus 2.4 nm for access resistance); K is the thickness of this shell, taken as 0.4 nm thick (about the thickness of a Debye length in the salts used); a is the pore radius, 1.5 nm for the open state and 0.9 nm for the closed state (9, 10, 11). Attempts to refine the theory further to take into account such things as graded change in Debye length with distance and cylindrical geometry were judged to be premature.

As it is, the theory can account for the large change in reversal potential between the published sizes of the open and closed states of VDAC if the difference between the published sizes of the open and closed states of the channel is explained by removal of a portion of the channel wall, with its associated charge, during channel closure (see below). Although the net charge in the open and closed state is a fitted parameter, its value is consistent with values obtained by simply adding charges in the proposed transmembrane regions in the two states and consistent with the charge translocation required by the voltage-gating process.

The solid curve in Fig. 3 is the result of the theoretical calculations. The value of the reversal potential of the wild-type channel was plotted (*filled diamond*) assuming a net charge in the pore of 2.5. All other values were plotted with the net charge shifted by the engineered mutation(s). For the open state, sites judged to be within the pore (*triangles and squares*) follow the theoretical curve rather well. Deviations are likely to arise due to the different degrees of effectiveness of charge changes at different sites. For example, the displaced nature of the points indicated by the triangles is due to the presence of K19E. The more pronounced effect of charge change at this location holds true even when a milder mutation was made. K19Q had exactly half the effect on the selectivity of the open state (reversal potential = 3.3 + 0.3 [S.D.], 10 values). The remarkable fit to Teorell's theory suggests that, as predicted by the model shown in Fig. 2, the open VDAC channel can be viewed on a molecular scale as a cylindrical pore whose walls contain rather uniformly distributed fixed charges.

The consistency of the results obtained for the open

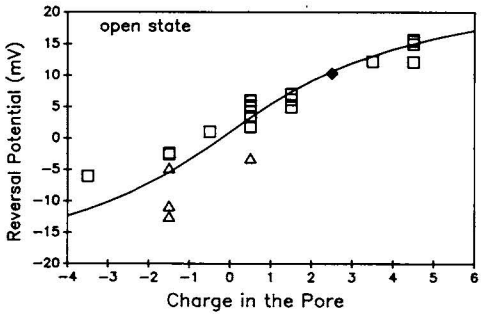

FIGURE 3 The reversal potential of VDAC channels in the open state as a function of net charge within the pore. The solid curves are calculations from the modified theory of Teorell (see text). The data for wild-type VDAC was plotted as a solid diamond with a net charge within the pore assumed to be 2.5 for the open state. For the mutant channels (*squares, triangles*), the charge was shifted from the wild-type by the engineered mutation. Only mutations judged to be in the pore were plotted. Triangles were used to designate mutant channels containing K19E. The plot includes some double and one triple mutant (data from reference 24).

state are partly due to the choice of the amino acid substitution. We chose to substitute amino acids at sites expected to face the aqueous environment with residues of comparable polarity. Clearly, sites facing this environment are less likely to result in undefined, large scale conformational changes as compared to sites responsible for interactions between protein domains that are essential for proper conformation (probably rare in VDAC but common in other proteins). Despite this strategy, two mutations did result in radical phenotypes. VDAC genes in which K234 is changed to *E* cause a dominant lethal phenotype. The biological basis for this effect is unknown. A milder mutation, K234Q, produced well-behaved channels with interpretable changes in properties. When mitochondria from yeast containing VDAC genes with K236E mutations were subjected to VDAC protein purification, no protein or channels were obtained, although outer mitochondrial membranes purified from these mitochondria contained a 29 kDa protein that cross reacted with a VDAC antibody. It is likely then that the region of the protein defined by these two mutations is particularly sensitive to charge changes since such changes appear to result in major disruptions of the protein's structure. One amino acid substitution (FI27R) was purposely generated to see if VDAC's structure would tolerate a major disrupting influence such as the substitution of a non-polar residue, thought to be facing the lipid bilayer, with a charged residue (24). Unexpectedly, this mutation did not produce a totally inactive product. While the majority of reconstitution attempts failed, on two occasions a single channel was detected. These two single channels had wild-type selectivity despite the fact that the mutation is located in a transmembrane strand between residues that influence channel selectivity. It is likely that the arginine pointed toward the bilayer actually extends up to the surface of the membrane. It certainly does not face into the channel; otherwise it would influence the ion selectivity. The proximity of position 127 to the surface is indicated by the weak effect on selectivity of charge change at position 128.

Closed Channel

An electric field can drive VDAC to low-conducting states, which are referred to as closed states because there are indications that at least some of these are essentially impermeable to important metabolites (15, 16, 26). The large number of closed states observed in vitro (4, 10) may be due to the absence of a controlling factor present in vivo (the VDAC modulator, 11). By using a synthetic polymer known as König's polyanion (12), the number of closed states can be markedly reduced. In the state selected by this polyanion, VDAC is still permeable to K^+ and Cl^-. Therefore, its selectivity for these ions can be determined by measuring the reversal potential of the channels by methods used previously for the open channel. In addition, the relatively large pore size of the closed state ($\approx$1.8 nm in diameter) (12, 13) indicates that charges on the wall of the pore are very likely to dominate the selectivity as is the case for the open channel (27). Thus, VDAC proteins with single amino acid substitutions were selected for study so as to include each transmembrane segment and connecting loop proposed in the model of the open channel (Fig. 2). By assessing the reversal potentials of the closed state of these mutant channels, we can get information about the location of the proposed β strands, α helix and loops in this state.

Closed-state selectivity was estimated by measuring the reversal potential of single or few channels in this state, in the presence of a transmembrane KCl gradient. After insertion into a planar phospholipid membrane, the single-channel conductance was measured, and selectivity in the open state determined by finding the potential needed to bring the current through the channel(s) to zero (the reversal potential). Having verified the open-state properties, we added König's polyanion to the high-salt side (0.6 μg/ml final concentration) and varied the potential continuously in the form of a triangular voltage wave to find the reversal potential of the closed state. Under these conditions, the slope of the current trace yields the conductance. Channel closure is indicated by an abrupt transition to a trace with shallower slope.

As illustrated in Fig. 4, the polyanion induced VDAC to close at lower potentials resulting in the current trace crossing zero (the reversal potential) while the channel was in the closed state. With time, the polyanion induced channels to enter a more closed state with even lower conductance. We focused our analysis on closed states whose conductance ranged between 40 and 60% of that of the open state. Within this range, there is no significant correlation between conductivity and the measured selectivity of wild-type channels. For the purpose of this analysis therefore, we assumed that there is one closed state within this conductance range.

In Fig. 4, two VDAC channels in which lysine at position 108 was replaced by glutamate (K108E) inserted into a phospholipid membrane. Their open-state reversal potential (E_o = 9.6 mV) and conductance (1.8 nS for each) were measured to verify the channel's open-state characteristics. A triangular voltage wave (0.35 mV/sec) was applied and König's polyanion (0.6 μg/ml final conclusion) was added to the high salt side. The linear increase in negative current reflects the linearly increasing negative potential. Its slope represents the combined conductance of the channels in the

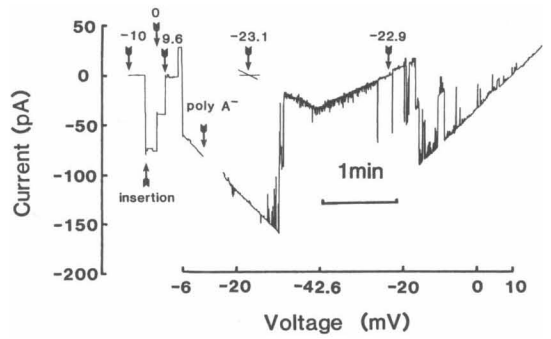

FIGURE 4 Example of the approach used and data recorded to measure the closed-state reversal potentials for yeast VDAC channels. To a planar phospholipid bilayer, 3 μl aliquot of mutant (K108E) VDAC channel solubilized in 1% Triton X-100 was added to the 1M KCl side. Two mutant channels inserted where indicated. The beginnings of the first three voltage steps are indicated by downward arrows. The voltage scale at the bottom shows the voltage values applied as a triangular wave. The time scale is indicated and applies to the entire record (the break in the trace occurred when the pen was lifted from the paper during polyanion addition to avoid the stirring artefact. König's polyanion was added where indicated (poly A−) to a final concentration of 0.6 μg/ml).

open state (3.6 nS). Then two aburpt upward transitions occurred as the two channels closed. The conductance dropped to 1.61 nS (from the slope of the subsequent recording), a 55% closure. Estimates of the reversal potential of the closed state were obtained both by extrapolation back to zero current (E_c = 23.1 mV) and by actual crossing of I = 0 by the recording (E_c = −22.9 mV). Fig. 5 illustrates experiments with other mutants.

Table 1 summarizes reversal potential estimates for the wild-type and mutant channels in both the open and

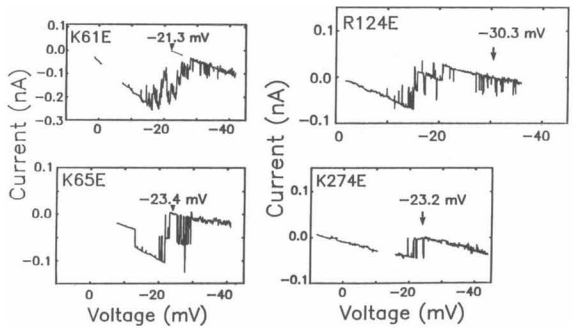

FIGURE 5 Examples of recordings of closed-state reversal potentials of mutants assigned to groups 1, 2, 3, and 5. The recordings were obtained by essentially the same method as illustrated in Fig. 4. In these current records, time proceeds from left to right. In each panel, the value of the closed-state reversal potential and its location on the record are illustrated. In the case of K61E, an extrapolation was required in order to measure the reversal potential.

TABLE 1 **Reversal potentials of wild-type and mutant channels in their open and closed state**

Channel type	E_o ± S.E. (n)	E_c ± S.E. (n)	δz	$\delta E_o/\delta z$	$\delta E_c/\delta z$
	(mV)	(mV)			
Wild	10.3 ± .1 (6)	−22.6 ± 6 (6)	0	—	—
		Group 1			
D15K	15.6 ± .3 (13)	−21.0 ± .3 (4)	2	2.7	0.8_
K61E	1.8 ± .2 (7)	−22.4 ± .5 (4)	−2	4.3	−0.1_
E152K	12.1 ± .1 (7)	−23.9 ± .3 (5)	2	0.9	−0.7_
K248E	6.0 ± .2 (12)	−24.1 ± .5 (22)	−2	2.2	0.8_
T256K	12.2 ± .2 (4)	−23.1 ± .7 (4)	1	1.9	−0.5_
D282K	15.4 ± .2 (8)	−21.4 ± .7 (6)	2	2.6	0.6_
		Group 2			
D30K	14.9 ± .2 (16)	−18.7 ± .6 (12)	2	2.3	2.0**
K95E	3.4 ± .1 (18)	−28.6 ± .3 (9)	−2	3.5	3.0***
R124E	5.2 ± .1 (3)	−29.1 ± .6 (3)	−2	2.6	3.3***
D128R	12.0 ± .3 (32)	−19.5 ± .2 (4)	2	0.9	1.6**
G179D	6.9 ± .2 (5)	−25.2 ± .3 (4)	−1	3.4	2.6**
K234Q	6.1 ± .2 (3)	−26.9 ± .3 (3)	−1	4.2	4.3**
		Group 3			
K108E	9.7 ± .04 (20)	−22.0 ± .6 (4)	−2	0.3	−0.3_
K132E	10.2 ± .1 (11)	−22.6 ± .6 (4)	−2	0.1	0.1_
R164D	9.2 ± .1 (3)	−23.6 ± .5 (3)	−2	0.6	0.5_
D191K	9.9 ± .2 (11)	−23.8 ± 1.3 (4)	2	−0.2	−0.6_
K205E	9.4 ± .3 (5)	−23.8 ± .4 (5)	−2	0.5	0.6_
K274E	9.9 ± .1 (14)	−23.9 ± 1.0 (5)	−2	0.2	0.7_
		Group 4			
R252E	9.8 ± .2 (13)	−26.1 ± .8 (6)	−2	0.3	1.8*
		Group 5			
K19E	−3.2 ± .2 (14)	−29.8 ± .4 (13)	−2	6.8	3.6***
K46E	4.9 ± .4 (9)	−25.1 ± .6 (10)	−2	2.7	1.3*
K65E	5.7 ± .1 (6)	−25.3 ± .5 (6)	−2	2.3	1.4**
K84E	3.4 ± .1 (22)	−24.5 ± .5 (11)	−2	3.5	1.0*

Note: δE_o and δE_c are the difference of the reversal potential between the mutant and wild-type channels in their open and closed states, respectively. The data are presented as means ± standard error with the number of experiments in parenthesis (one experiment per membrane). The δz is the charge change engineered by the mutation. A student's t test was used to determine whether the numbers in the last column were significantly different from zero: "_" not significant; "*" significant at 95% level; "**" at 99%; "***" at 99.9%.

closed states (column 2 and 3). In addition, the change in reversal potential from the wild type was calculated and normalized by the charge change engineered by the mutation (columns 5 and 6). As was found for open state channels, mutations at some positions (such as K95E, K124E) altered the selectivity of the closed state channel, whereas mutations at other positions had little or no effect (D15K, K248E). As a result of the analysis, the majority of the mutant channels could clearly be placed into one of five groups. In group 1, the change in reversal potential per charge change in the open state, dE_o/dz, is significant, while that of the closed state, dE_c/dz, is not significantly different from zero. In group 2, dE_o/dz and

dE_c/dz are comparable and large. In group 3, dE_o/dz and dE_c/dz are both close to zero. In group 4, $dE_o/dz < dE_c/dz$ by at least 1 mV. In group 5, dE_c/dz is significantly different from zero but is only approximately half as large as dE_o/dc.

Mutations at four positions (group 2) had strong effects ($dE_o/dz > 2.0$) on the selectivity of the closed state. In addition, these mutations all affect the selectivity of the open state, and were deduced to lie in the walls of the open state pore. Thus, these residues are likely to be located in positions that remain in the pore when the channel closes. The mutation at position 128 has a weak effect in the open state and thus a proportionally weaker effect in the closed state. When compared with theoretical expectations (see below) this site was also judged to be in the channel in both the open and closed states. This is also consistent with the location of another residue on that strand, 124. Position 30 has been placed in this group as well although it has a weaker than expected effect on closed state selectivity based on theoretical considerations (see below).

Mutations at the six positions in group 3, have no significant effect on selectivity of the channels in either the open or the closed state, and therefore these residues probably lie outside the channel in both the open and close states.

Of greatest interest are the mutations in groups 1 and 5. Changes of the amino acid residues in group 1 alter the selectivity of the open state but have no significant effect on the selectivity of the closed state. These mutations are likely to identify domains that form part of the open state pore and are removed from the pore wall on channel closure. Those in group 5 also altered the open-state selectivity but had effects on the closed state much weaker that expected, but still significant, when compared to group 2 mutants.

The predictions of our revision of Teorell's theory can also be applied in the analysis of residues that effect the closed-channel selectivity. Fig. 6 shows that for the closed channel, the wild-type value matches the theoretical curve at a value of net charge in the pore of −2.5. In an effort to compensate for differential effects of different sites on open-state selectivity, the reversal potentials of mutant channels in the closed state were corrected by their effects on the open state. The mutations in group 2 (K95E, R124E, D128R, G179D, and K234Q; Fig. 6, *filled squares*), all fall near the theoretical curve. Thus, we conclude that the residues changed by these mutations define domains of the protein that remain in the wall of the channel in the closed state. One of the group 2 mutants, D30K (*inverted triangle*), lies far away from the theoretical curve and thus may have been shifted upon channel closure to a location still influencing but farther away from the ionic current. The high

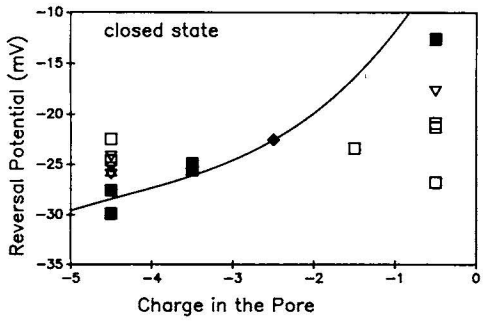

FIGURE 6 The reversal potential of VDAC channels in the closed state as a calculated function of net charge within the pore. The solid curve is calculated from the modified theory of Teorell (as in Fig. 3). Here, the wild-type reversal potential was fitted to the theory by assuming a net charge in the closed state of −2.5. The closed-state reversal potentials for the mutants were adjusted as follows:

$$E_c^{\text{mutant}} = [\delta E_c/\delta E_0] \cdot [\text{mean of } \delta E_0/\delta z] \cdot \delta z + E_c^{\text{wild-type}},$$

where [mean of $\delta E_o/\delta z$] includes all mutants in groups 1, 2, and 5. Only mutations judged to be in the pore in the open state were plotted here. The filled squares (K95E, R124E, G179D, and K234Q) and the filled inverted triangle (D128R) are locations judged to be in the pore in the closed state. Mutants in group 1 had no significant effect on closed-state selectivity and were plotted as open squares. Inverted triangles (*open*) are locations that resulted in less than expected changes in closed-state selectivity (i.e., D30K, K46E, K65E, K84E). K19E is designated by an open triangle.

variability of the results obtained with channels containing mutations at this site suggests that this position is displaced on channel closure. In contrast, the mutations in group 1 (D15K, K61E, E152K, K248E, T256K, and D282K) that affect the selectivity of the open state had no significant effect on the closed-state selectivity (Fig. 6, *open squares*). These residues are likely to define protein domains that are removed from the pore in the process of forming the closed state channel and thus, as expected, fall away from the theoretical curve for the closed state channel.

Our revision of Teorell's theory agrees well with the observations made of the open state and, significantly, with the closed-state results described above. Both the open and closed state can be viewed to some degree then as cylinders with uniformly distributed charges. Charges that are removed from the channel on closure no longer line the pore and thus do not contribute to the distribution of charges present in the walls of the closed state pore. However, five mutations including group 5 (K19E, D30K, K46E, K65E, and K84E) all affected the open-channel selectivity and had closed-state selectivities significantly different from the wild-type value but the difference was not as great as predicted by the theory (Fig. 6, *all triangles*). These sites may remain in the pore

during channel closure and be partially shielded so that the charge changes have less effect on selectivity, or they may be removed to a position, such as at the rim of the pore, where the charge produces a weaker effect. Thus, like those sites in group 1, these sites (group 5) may move out of the pore upon channel closure but not completely. It is also possible that the closed state in this conductance range is not a single state, but a population of states that cannot be resolved by current techniques, and that some of the residues with small effects on closed state selectivity are in the channel in some sub-states but not in others, producing an average effect less than that of residues that are in the channel in all closed sub-states (group 2 mutants).

Mutations at positions 19 and 152 deserve special consideration. K19E is in the proposed α helix-like D15K, however, unlike D15K, it still has an effect on closed-state selectivity. Like other mutants in group 5, its effect is much weaker than in the open state. Thus it seems that the α helix moves out of the pore upon channel closure but is close enough to the current flow so that position 19 still effects ion selectivity. Position 152 is in the center of the molecule flanked on both sides by strands that contribute to the wall of the channel in the closed state. It seems unlikely that the strand that includes this site moves out of the pore upon channel closure. It may be that this strand slides in a direction normal to the membrane resulting in position 152 moving out of the channel but the strand itself remaining in the channel.

Group 4 contains a single mutation, K252E, which has no effect on open-state selectivity and has a reproducibly small effect on closed-state selectivity. This residue is the only example so far of the expectation that channel closure can result in residues moving into the pore, or near the rim, as well as out of the pore (group 1 mutations). Perhaps the site did not actually move much but rather the reduced pore diameter resulted in a significant contribution of that site to channel selectivity.

Based on our results, we can propose a specific model for the gating process of the VDAC channel. Fig. 2 shows the proposed transmembrane orientation of VDAC in the open state. Our data clearly indicate that a subset of the transmembrane strands are removed from the lining of the channel upon voltage-dependant closure. Our measurements of closed-state selectivities are in general consistent with a model in which the strands that remain in the channel on closure are in the center of the primary amino-acid sequence whereas those close to the amino and carboxyl terminus seem to be moved out of the pore. Removal of these strands would result in a smaller channel, reduced in circumference, cross-sectional area, and volume. Consistent with this notion is the marked change in selectivity of the wild-type channel upon closure, indicating that the net charge within the pore changes by removal of protein domains containing net positive charges. This contrasts with a closure mechanism in which protein domains enter the pore, resulting in a mechanical obstruction. Zimmerberg and Parsegian (14) measured the change in the volume of water within the VDAC pore upon channel closure. The large volume change (~ 30 nm^3) could not be explained by a local constriction or block but rather by a uniform reduction in channel radius. Thus, our results and conclusions agree well with theirs. In addition, the fit to the predictions of Teorell's theory suggests that both the open and closed channel can be viewed on a molecular scale as cylinders with walls that contain rather uniformly positioned charges. In this model, strands that are moved out during closure by this mechanism could lie on the surface of the membrane with their nonpolar sides facing the bilayer, embedded between the phospholipid head groups, or could interact with portions of the helix that have been removed from the pore. Charged residues that move through the membrane potential during this rearrangement should correspond to gating charges, and mutations that change the charge of these residues should alter the closure of the channel in response to applied potential. It is interesting to note that the fit to Toerell's theory indicates a net reduction of five positive charges upon channel closure. If these charges moved an average of half way throught the field, they would account for a net of 2.5 gating charges. This is exactly what is observed for the voltage dependence of VDAC in yeast. We are currently investigating the effects of charge-changing mutations on the voltage-dependant gating of VDAC to see if alterations induced by these mutations fit the predictions of our model.

Our results show that site-directed mutagenesis can be used in combination with electrophysiological techniques to systematically identify domains of an ion-channel protein required for channel selectivity and domains that move during channel closure. Using this approach, we have been able to test a model of channel structure and develop new information about voltage-induced conformational transitions within the channel that could not be obtained by other approaches. We expect that an extension of this approach will allow us to confirm and refine the boundaries of the domains in this protein that move during voltage dependent channel closure. A limitation of this approach is that it does not provide direct information about the orientation of the protein in the membrane. However, if the information acquired through site-directed mutagenisis can be combined with that obtained by biochemical techniques such as protease sensitivity and accessibility to peptide-specific antibodies, then a very complete description of the structure and function of this simple ion channel will emerge.

This work was supported by National Institutes of Health grant GM 35759 to Michael Forte and Marco Colombini, and Office of Naval Research grant N00014-90-J-1024 to Marco Colombini.

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DISCUSSION

Session Chairman: Adrian Parsegian *Scribes:* Claire L. Careaga and Mingyao Liu

RON KABACK: Is there any homology between the bacterial porin, whose structure is known, and the VDAC channel?

MARCO COLOMBINI: There is no homology in the primary sequence or in the beta pattern. There are also large differences in selectivity and voltage dependence. There is similarity in that both are low molecular weight polypeptides that happen to be located in an outer membrane.

KABACK: Is there strong evidence that VDAC is a monomer versus porin that is a trimer?

COLOMBINI: Yes, actually in the porin trimers each monomer forms its own pore. In the case of VDAC, the evidence that one polypeptide forms a channel is very strong. This is based on stem mass measurements on two-dimensional crystals. Also, we've looked for hybrid channels from yeast that contain a copy of the gene for both wild-type and mutant VDACs. No channels with intermediate selectivity between wild-type and mutant channels were found using two different constructs. So we feel strongly that one polypeptide forms the actual pore.

KABACK: What is the physiological significance of voltage gating in outer membrane channels?

COLOMBINI: I believe that a very conserved VDAC in potatoes, protists, and mammals must have some function in the cell. I believe the function is to regulate the flux of metabolites between the cytoplasm and the mitochondria. There is, however, no evidence for that.

KABACK: What is the electric field across the mitochondrial outer membrane? Is it low?

COLOMBINI: Very good question. Because the channels are large, you simply can't have an ion pump or an ionic gradient. Unless the charges on the proteins in the intermembrane space and cytoplasm (the effective colloidal charge) are identical, there must be a Donnan potential across the membrane. It is essential. The question is, is it large enough? Secondly, is it controlled?

In principle, control can occur, for instance, by protein phosphorylation that would change the net colloidal charge and thus change the potential. The problem is monitoring it, measuring it. If you measure it in isolated mitochondria, you would only have half of the system, that contributes to the potential: the proteins in the intermembrane space.

The potential to close the channel is small, ~20 mV, and we've been working for a few years on a soluble protein localized in the intermembrane space, called the VDAC modulator. This modulator amplifies the voltage dependence of these channels. The channels can close at, as little as, 5 mV in the presence of this protein.

OLAF ANDERSEN: The Donnan potential across the plasma membrane is ~10 mV. So the voltage dependence may be a safety mechanism, which protects the cell against deleterious effects that would occur if errors in processing and targeting led to the incorporation of a porin into the plasma membrane?

COLOMBINI: Yes, it is possible. It is not true that the maximum access is the ideal access. Clearly there are a number of regulatory mechanisms that reduce the rate of respiration or rate of ATP production under different conditions. It might be an advantage for the cell to be able to tune the system up or down.

So regulation at a particular point in the outer membrane seems rational. Also, if you just wanted a safety device, it is surprising that the safety device is so finely tuned that it is often difficult to distinguish between a VDAC channel from a potato and a cow. Their properties look identical. The system is much more finely tuned than one would expect from a coarse sieve.

DAVID BUSATH: The last suggestion neglects that the closed state of VDAC is very permeant, which short circuits the plasma membrane.

COLOMBINI: Good point.

ALAN FINKELSTEIN: You pointed out that the channel goes into the closed state for both positive and negative voltage, could you elaborate on this? Are the same groups moving either to one surface or another? What is the current model?

COLOMBINI: There are at least two mutants that address this. At least in one case it is clear that the same mutation will affect voltage-dependent closure at both positive and negative potentials. It's hard to do because you have to look at one channel over a long period of time to be able to obtain enough information. Another point mutation only affects voltage dependence at potentials of one sign and not the other. A similar, but not identical region, is moving upon channel closure. From the folding pattern, one would expect some difference in the region that moves because of the way the protein chain is folded in the membrane. Translocation in one direction would have to be somewhat different than in the other direction.

FINKELSTEIN: You characterized the ion permeability of the closed state, are they the same for closed states induced by positive verses negative voltage? Do they look identical?

COLOMBINI: The measurements were done on one or a few channels at a time, ignoring insertion orientation. Therefore, all measurements were taken with the same applied potentials. This pools together the properties of a variety of structures and is a little more muddled than I would like.

FINKELSTEIN: This leads to the question of how you know the channel is a monomer. One of the characteristics of the system is that the voltage dependence is fairly symmetric. Is this true? The obvious way to achieve that would be with an antisymmetric dimer.

COLOMBINI: For many years VDAC was thought to be a head-to-tail dimer even though that posed a problem in terms of insertion. Lorie Thomas did very nice work in collaboration with Alasdair Steven that showed unambiguously that, in channels forming two dimensional crystals, there is insufficient mass per unit channel for a dimer. It has to be a monomer.

ROGER KOEPPE II: You argue that if a mutation causes a change in selectivity, whether of the open or the closed channel state, then the result implies that the mutated residue is within the lining of the pore of, respectively, the open state and/or the closed state. This interpretation seems supported by the K19Q and K19E mutations, for which K19Q exhibits one-half of the effect on the selectivity of the open state. What is the effect of K19Q on the closed channel selectivity?

COLOMBINI: I do not have that number.

JOE FALKE: Is it possible to obtain a sufficient quantity of VDAC to begin to test specific features of the structural model. For example, have you used a spectroscopic approach like CD to test for beta sheet? Also, could you employ biochemical approaches such as cysteine engineering and sulfhydryl chemistry?

COLOMBINI: Yes, there are some CD measurements that are unpublished and they show typical beta structure. That was done, however, with outer membranes from *Neurospora crassa* that are predominantly, but not totally, VDAC. I feel the problem with doing CD on channels is that it is hard to know when you purify the channels how many are in the native versus the denatured state. Unlike an enzyme you can't look at specific activity easily. From two pounds of yeast you can easily get a couple of milligrams of pure VDAC.

RICHARD HORN: Have you made any conservative charge changes

from glutamate to aspartate, or lysine to arginine to see if it is consistent with your model? I know from some of my work on muconotoxin derivatives that you can make charge conserving changes and get very different effects on binding affinity.

COLOMBINI: At particular sites we have either changed positive charge to a negative charge, or visa versa, or a charge to a neutral species. So we've done graded changes at the same residue. Where we have changed the charge by one or two units, the results are consistent with our model. I also want to point out that in all cases that we've seen, selectivity changes when we have changed the charge, the changes have gone in the right direction. The only situation where we see changes in the wrong direction is when there is no significant change in the selectivity so we're in the noise.

There are some positions in the sequence where a charge change from a positive to a negative residue resulted in dramatic changes in the properties of the channel. The protein's behavior in the purification process was very different from the wild-type and we saw no channel formers in the membrane. The conformational effects were so drastic that we couldn't get insertion into the membrane.

ROBERT FARLEY: You had two mutations at sites 234 and 236 that seemed to be polar–polar instead of polar–nonpolar and that might not significantly distort the structure, yet no channels appear upon purification. Can you describe what changes are expected in the structure to explain this? Have you made other changes at those positions and found similar results?

COLOMBINI: There is nothing obvious or special about this region that would cause a protein to misfold. When site 234 was changed from a positive to a negative residue, marked changes in the structure was observed. We couldn't get the channel to insert. A milder change from a positive to a neutral residue resulted in channels we could make measurements on.

ROBERT GUY: At the risk of appearing critical of work I admire, I have doubts about the basic assumption you use to interpret your mutant data. You assume that if a charge change mutation alters the reversal potential then that residue must be in the transmembrane region, and if the change does not alter the reversal potential then the residue must be in a loop region between transmembrane strands. While this is the simplest interpretation, it is easy to envision models of VDAC for which the assumptions would not be valid. For example, VDAC could be a beta barrel with more than twelve strands and the NH_2-terminal amphipathic alpha helix could fit inside the barrel. This is like the loop region in porin fitting inside the beta barrel.

Permeant ions would probably pass between the polar face of the alpha helix and wall of the beta barrel opposite the helix. In this case, mutations of residues on transmembrane strands that are covered by the helix might not affect selectivity. Also, with this model strands would not have to move out of the membrane when the channel closed; your results could be explained by a model in which the ion pathway simply becomes narrower and more strands are covered by the alpha helix in the low conductance state. Conversely, mutations of charge residues at the entrances of the nicotinic acetylcholine receptor alter selectivity. This result suggests that charges in the loop regions could affect the reversal potential. Do you agree with me that yours is only the simplest explanation of the results and that other types of experiments need to be performed to determine the protein's transmembrane topology?

COLOMBINI: I agree we should all use other methods to test, and perhaps change, this model. My preference as to the alpha helix fitting inside the channel and taking up some of the space inside the channel, is not to agree. The alpha helix, like the beta strand, has a nonpolar and a polar surface. If I put it inside the channel I'd have to put the nonpolar against another nonpolar surface, and it's more natural to have it facing the bilayer. That doesn't mean that it isn't there. Aesthetically, I'd like to have a solid beta pore hydrogen bonded and next to the alpha helix.

It may be important not to make the pore too stable, if one does the channel may not respond. This channel has to undergo a large conformational change and result in a structure that has an almost identical energy to the open state. The energy difference between the open and closed states is ~8 kJ, which is about one-third of a hydrogen bond. This is remarkable and probably certain regions of the protein are selectively destabilized in order to achieve this. We'd like to investigate these destabilized regions and make a channel that can't close.

With regard to the charges on the loops, the difference between this and the other studies is that this is a large channel and the loops may not be close enough to the ion stream to influence selectivity. For a narrower channel where the edges around the mouth are within a Debye length of the ion stream, the influence might be expected in the absence of another rate-limiting step. Therefore, the loop regions that don't have an effect on ion selectivity must be far enough away from the ion stream.

GUY: You said one reason you didn't like the alpha helix sitting inside the beta barrel is that the beta strands have alternating hydrophobic and hydrophilic residues and you need a hydrophobic surface. The reason you think that is because that is the segment you search for. There are segments that have consecutive hydrophobic residues, and those could be the segments that are forming the part of the barrel the helix contacts.

COLOMBINI: That is possible.

GUY: When you pull several strands out with the alpha helix, and you've only started with twelve strands, then you only have an eight or nine stranded beta barrel. That would be smaller than what Carmen Mannella sees with electron microscopy.

COLOMBINI: The tilt we are proposing is 55° which is a very severe tilt, so that the pores would have the right diameter. I agree that the actual magnitude of the conformational change seems rather dramatic. The channel, however, changes from weakly anion to weakly cation selective. The ratio of cation to anion selectivity changes by a factor of ten. The region that pulls out has a net positive charge, so that not only do we gain the positive charge moving though the field as required by the voltage dependence, but in addition resulting in a channel that now has a net negative charge that can account for the change in selectivity.

Simpler models that don't involve a large conformational change do not account for the voltage dependence of the channel or the selectivity. They might account for the volume change by blocking the channel in some way, but the voltage dependence is there. It is also voltage dependent in both positive and negative fields so that this region also has to be able to move in both directions as would be accounted for by our model.

Also, the best fit to the theory of Teorell says the net effective charge is +2.5 in the open state and −2.5 in the closed state. Net change is 5 charges that would be ~2.5 charges moving halfway through the field, exactly the n value for these VDACs. This does need to be tested further.

ALFRED VILLARROEL: If I think in molecular terms, I cannot understand a change in selectivity without a change in conductance. If

you change a charge would you expect a change in the local potential that will affect conductance?

COLOMBINI: The conductance only changes by a small amount. When one changes a positive to a negative residue, one is now swapping a negative for a positive counterion. The charge carrying capacity is the same, just the selectivity is changing.

We model the channel as having two regions, one that forms a cylindrical shell with a fixed charge environment that behaves one way, and a central neutral region that behaves in a neutral way so that we have two channels in one. There are situations that one would predict in which ion flow in the shell region is opposite from ion flow in the center.

VILLARROEL: If this is true would you expect differences if you do the experiment not with KCl but with a salt in which anions and cations have different mobilities?

COLOMBINI: We have looked at the reversal potentials of ions with different mobilities. We haven't looked closely at the conductance, but the effects on the reversal potential are consistent with our model.

JOSHUA ZIMMERBERG: If you change the ionic strength of your medium, according to your model you change the Debye length and thus the portion of the conductive pathway that is bulk versus charged. Have you done any experiments to test the model based on changing ionic strength?

COLOMBINI: Yes, with the reversal potential. The results are consistent with expectations.

ZIMMERBERG: In terms of the symmetry of the channel opening and closing, it is my understanding that for any single channel there is not an exact symmetry between the response to positive and negative voltages. Do you agree?

COLOMBINI: Depending on how the membranes are made, one can get asymmetries. Normally we use mixed lipids and we're probably making the membrane asymmetrical, causing shifts in the switching region. The voltage dependence doesn't seem to change.

ZIMMERBERG: How do you account for the variability of the subconductance states and your previous chemical modification studies?

COLOMBINI: All I can say is the model proposes that part of the wall is moving out of the channel. Whether the same part moves out all the time, and if the strands are shifting perpendicular to the membrane, we have no evidence. All these would cause changes in the properties of the channel. There is enough flexibility in the model to give different closed states.

ZIMMERBERG: Could more than one loop come out at a time as you get to further closed states?

COLOMBINI: Maybe a different set might come out under different conditions. When one channel closes and opens repeatedly you find that the channel enters different states. Chemical modification data fit well. Succinic anhydride reacted with lysine residues and caused the channel to become anion selective. When there is intermediate succinylation of the channel you lose voltage dependence. Higher amounts restore the sensor. The net charge is regenerated. If there is enough stress due to the field and net charge the sensor can move. If it has net negative charge it is still able to respond.

ANDERSEN: In your Fig. 2, shouldn't No. 30 be shaded?

COLOMBINI: The mutation of residue 30 resulted in a change in the selectivity of the closed state of the channel but this change was less than expected by our theoretical calculations. In Fig. 6, D30K is indicated by the inverted triangle at a charge in the pore of -0.5. The weaker than normal effect indicates that position 30 is also moved, at least partially, out of the pore.

ANDERSEN: To follow up on Alan Finkelstein's question, your model seems to imply that the beta-loops can exit at either side of the membrane in order to account for the symmetrical gating. That is bothersome.

COLOMBINI: A region of the wall carrying a net positive charge would feel a force when an electric field is applied no matter what its direction might be. Clearly, the same set of strands cannot translocate in both directions. We propose that most of the strands that are translocated are the same, but the strands that are at the interface between those that move and those that remain static will likely be different.

ANDERSEN: Also, how do you envision the unzippering/rezippering of the beta-barrel as part of the barrel is removed/reinserted.

COLOMBINI: The hydrogen bonds that link adjacent strands can certainly be broken one at a time, the backbone forming hydrogen bonds with water. The hydrophobic residues on the side of the wall facing the lipids would still face the lipids even when located on the surface of the membrane. Reopening would involve sliding of the mobile region back into the membrane and the reforming of the hydrogen bonds.

HARVEY POLLARD: I wish to follow up on the question of subconductance states. If your fixed charge model is correct, and if subconductance states are due to changes in energetics of interaction of ions with charges in the walls of the channel, then one might anticipate that some conductance states would be missing. Alternatively the values of some conductances might be changed, leaving others unmodified. The mutations having effects on subconductance states might thus be useful in delineating residues in the conductance pathway. So, what then are the consequences of the mutations on subconductance behavior?

COLOMBINI: We haven't looked at that. We have many complicated states, something like twenty different states.

POLLARD: Can't you just look for missing states?

COLOMBINI: Yes, that is an excellent suggestion.

MARK BRAIMAN: Have you tried CD on the channel? Perhaps a change in the position of the alpha helix can be detected by infrared/linear dichroism?

COLOMBINI: We have preliminary CD data on open and closed states. When we closed the channel with chemical agents we saw no change. Perhaps linear dichroism could pick up the change.

BRAIMAN: What is the lowest lipid to protein ratio?

COLOMBINI: When we did CD on VDAC in *Neurospora crassa* outer membrane it was 60–80% pure. The protein to lipid ratio was about one to one by mass.

MICHAEL GREEN: Do you have a ball park estimate of the activation energy to go from the open to closed state?

COLOMBINI: No, I don't have a good estimate. The channel opening is fast and not voltage dependent; it takes less than a millisecond to open. In contrast, closure is voltage dependent and slow; the time scale is on the order of 1 s. The kinetics fit a slow, voltage dependent tearing out of the barrel wall.