PC–stained cells showed that this analog remains on the cell surface during the experiment.

19. With our present setup, we find the divergence of the laser beam to be no greater than 0.4 μm for 1 μm either side of the plane of focus. Assuming the leading lamella is 1 μm thick, a bleached line of width 4 μm focused on the dorsal surface may be expected to broaden by 10% (4.4 μm) on the ventral surface.
20. Computer simulation has revealed that a broadening of the bleached line recovery profile (2.7 s after photobleaching) of no greater than 6% would be expected for cells moving at speeds between 30 to 60 μm/min, where one surface was assumed to be stationary with respect to the other. In the experimental situation the majority of cells are moving slower than 30 μm/min and so line broadening would be less than 6%. Such a small amount of broadening would be very difficult to detect above experimental variation or noise. A greater broadening of simulated bleached lines occurs at a later time after photobleaching but this corresponds to the virtual completion of diffusional recovery.
21. Particles and concanavalin A attached to the dorsal surface of motile fibroblasts have been observed to move rearward at about twice the velocity of forward protrusion (*1, 12*). Thus if the velocity of membrane lipid flow (V_f) = −2 and the velocity of cell advancement (V_c) = +1, then the normalized relative velocity $R = (V_c - V_f)/V_c = +3$.
22. As in (*21*), if $V_f = +2$ and $V_c = +1$, then the normalized relative velocity, $R = -1$.
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28. Cells were stained with diI, washed with warm PBS (as described in Fig. 1), then placed in culture medium with serum and incubated in the dark at 37°C for increasing times after staining (from 10 to 90 min). Cells were then mounted on slides in fresh cooled medium (4°C) and kept on ice until ready for use. Cells were cooled to halt locomotion and to keep further internalization of diI to a minimum. Cells were viewed with a Zeiss laser scanning confocal microscope, at 488 nm with a ×63 oil immersion objective. Digitized images were collected using a Matrox IP512 imaging system and recorded on 35-mm film with a Polaroid freeze-frame color recording system. Each image is an average of four to eight frames.
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Selectivity Changes in Site-Directed Mutants of the VDAC Ion Channel: Structural Implications

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The gene encoding the yeast mitochondrial outer membrane channel VDAC was subjected to site-directed mutagenesis to change amino acids at 29 positions to residues differing in charge from the wild-type sequence. The mutant genes were then expressed in yeast, and the physiological consequences of single and multiple amino acid changes were assessed after isolation and insertion of mutant channels into phospholipid bilayers. Selectivity changes were observed at 14 sites distributed throughout the length of the molecule. These sites are likely to define the position of the protein walls lining the aqueous pore and hence, the transmembrane segments. These results have been used to develop a model of the open state of the channel in which each polypeptide contributes 12 β strands and one α helix to form the aqueous transmembrane pathway.

VOLTAGE-GATED ION CHANNELS are a class of transmembrane proteins that form aqueous pores in cell membranes—pores that open and close in response to changes in transmembrane voltage and change the ionic permeability of the membrane. Although such channels have been studied primarily in neurons and muscle cells, they are involved in a wide variety of biological processes in many cell types (*1, 2*). Whereas the more extensively studied voltage-gated channels, Na^+ and K^+ channels for example, are high molecular weight protein complexes that form narrow aqueous pores, the mitochondrial voltage-dependent anion-selective channel (VDAC, also known as mitochondrial porin), forms a large voltage-gated pore (*3, 4*) with a relatively small amount of protein (*5*), thus simplifying molecular studies and their interpretation. In addition, since VDAC is found in the yeast *Saccharomyces cerevisiae* (*6*), as well as in the outer mitochondrial membranes of organisms from all eukaryotic kingdoms (*7*), it can be studied with the sophisticated molecular genetic techniques available in this unicellular eukaryote. We have used these techniques to probe the structure of the VDAC molecule and examine the molecular correlates of its biophysical properties.

The VDAC forms large, voltage-gated channels when incorporated into planar phospholipid membranes. It is believed to form the pathway through which metabolic intermediates [for example, adenosine triphosphate (ATP), adenosine diphosphate (ADP), and succinate] enter and leave the mitochondrion (*4*). Measurements of the pore size of VDAC by the Stokes-Einstein radius of the largest nonelectrolyte able to pass through the channel (*8*) and by electron microscopy of negatively stained channels (*9*) indicate that the pore is about 3 nm in diameter. The yeast protein that forms this channel consists of 283 amino acid residues (molecular weight, 29,883) (*10, 11*). The sequence is rather hydrophilic (45.5% charged and polar residues), which is consistent with it being a low molecular weight protein that forms a large aqueous pore with thin walls. It contains no hydrophobic region long enough to span the bilayer as an α helix. The sequence contains many stretches of alternating hydrophobic and hydrophilic residues, which could form a β sheet with hydrophobic residues protruding on one side and hydrophilic residues on the other. This has led to a model of the VDAC channel as a β barrel consisting of a single layer of such a β sheet (*11*). By searching for the most likely transmembrane β strands, 12 probable membrane-spanning stretches have been identified (*12*). The NH_2-terminal 20 residues of VDAC could form an amphipathic α helix typical of mitochondrial tar-

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geting sequences (*13*). These residues are not cleaved in the mature VDAC protein.

We constructed a yeast strain lacking the chromosomal copy of the VDAC gene by gene transplacement (*14*) (Fig. 1). The resulting strain (M22-2) could grow on nonfermentable carbon sources (glycerol) at 30°C but not at 37°C. Others have also found that strains lacking the VDAC can grow on glycerol in a temperature-sensitive manner or after a delay period, perhaps due to the presence or induction of an alternate pathway for metabolites to enter and leave the mitochondrion (*15, 16*). Oligonucleotide-directed site-specific mutagenesis was used to introduce defined mutations into 29 codons throughout the cloned VDAC gene both singly and in combinations (*17, 18*). The resulting mutant *VDAC* genes were introduced into the *VDAC*-deleted strain (M22-2) and mutant VDAC proteins were purified (*19*) from mitochondrial membranes. The protein preparations contained a single band of approximately 30 kD (Fig. 2) (*20*). All the mutant genes (except K234E), when introduced into the *VDAC*-deleted strain, restored the ability of cells to grow on glycerol at 37°C. Purified wild-type and mutant VDACs were introduced into planar phospholipid bilayers for biophysical analysis (*21*).

The large pore size of VDAC indicates that small ions probably cross the membrane without intimate interaction with the walls of the channel. Thus, the weak anion selectivity of the channel (*3, 7*) is likely to be controlled by charges lining the channel's walls. The replacement of some of the positive charges lining these walls by negative charges should reduce or even reverse VDAC's selectivity. However, a change in

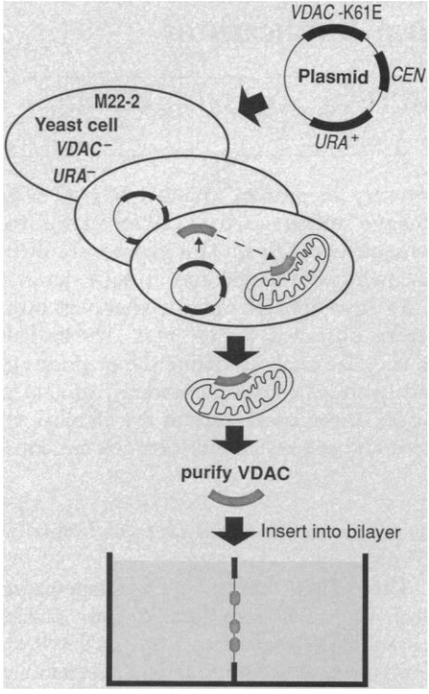

Fig. 1. A schematic of the steps for producing mutated VDAC for electrophysiological analysis. As an example, a plasmid containing the VDAC gene with a K61E mutation is shown. *URA* is used as a selection marker. *CEN*, centromere.

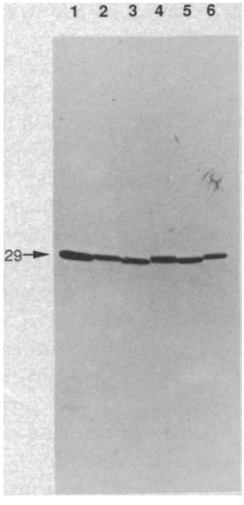

Fig. 2. Mutant VDAC proteins. VDAC was prepared (*19*) from yeast cells containing mutant VDAC genes and subjected to electrophoresis on a 10% polyacrylamide SDS gel, followed by silver staining. Lane 1, wild-type VDAC; lane 2, VDAC-K84E; lane 3, VDAC-K205E; lane 4, VDAC-K19E; lane 5, VDAC-K267E; and lane 6, VDAC-K274E.

Table 1. Single-channel conductances and reversal potentials of VDAC point mutations. (**A**) Mutations affecting selectivity. (**B**) Mutations not affecting selectivity. VDAC proteins with the indicated mutations were isolated from yeast mitochondrial membranes and reversal potentials were determined as in Fig. 3. Multiple estimates of the reversal potential were made on each channel-containing membrane and these were averaged and considered as one observation. The number of observations refers to the number of different channel-containing membranes. Δ, The difference between the mutant and wild-type reversal potentials; Δ (mV) expected, for multiple mutations, the sums of the changes observed in the corresponding single mutations; and Δ per unit charge, the changes in reversal potential between mutant and wild type were divided by the change in charge induced by the mutation. Values are the means ± SD, with the number of observations given in parentheses.

Species	Single-channel conductance (nS)	Reversal potential (mV)	Δ (mV)	Δ (mV) expected	Δ per unit charge
A					
Wild type	1.80 ± 0.06 (4)	10.2 ± 0.2 (3)			
D15K	2.08 ± 0.08 (5)	15.7 ± 1.1 (6)	5.5		2.8
K19E	1.73 ± 0.14 (6)	−2.1 ± 0.3 (3)	−12.3		6.2
D30K	1.90 ± 0.10 (3)	14.6 ± 0.3 (4)	4.4		2.2
K46E	1.61 ± 0.13 (4)	4.2 ± 1.9 (4)	−6.0		3.0
K61T	1.67 ± 0.14 (2)	4.9 ± 0.5 (2)	−5.3		5.3
K61E	1.80 ± 0.17 (3)	1.8 ± 0.5 (7)	−8.4		4.2
K65E	1.70 ± 0.04 (7)	5.7 ± 0.3 (6)	−4.5		2.3
K84E	1.75 ± 0.06 (7)	2.8 ± 1.1 (4)	−7.4		3.7
K95E	1.91 ± 0.14 (3)	3.0 ± 1.0 (3)	−7.2		3.6
R124E	1.82 ± 0.03 (3)	5.2 ± 0.3 (3)	−5.0		2.5
G179D	1.78 ± 0.09 (4)	7.0 ± 0.4 (3)	−3.2		3.2
K234Q	1.68 ± 0.02 (3)	6.1 ± 0.4 (3)	−4.1		4.1
K248E	1.84 ± 0.05 (6)	6.0 ± 0.6 (4)	−4.2		2.1
T256K	1.93 ± 0.11 (3)	12.2 ± 0.5 (4)	2.0		2.0
D282K	1.80 ± 0.05 (3)	15.4 ± 0.8 (5)	5.2		2.6
K19E,K61E	1.94 ± 0.05 (8)	−12.5 ± 1.2 (6)	−22.7	−20.7	5.7
K19E,K95E	1.95 ± 0.05 (3)	−10.8 ± 0.6 (6)	−21.0	−19.5	5.3
K19E,K248E	1.93 ± 0.06 (4)	−4.8 ± 0.7 (4)	−15.0	−16.5	3.8
K46E,K61E	1.83 ± 0.02 (2)	−2.6 (1)	−12.8	−14.4	3.2
K46E,K95E	1.90 ± 0.00 (2)	−2.5 ± 0.5 (3)	−12.7	−13.2	3.2
K61T,K65A	1.81 ± 0.07 (4)	3.4 ± 1.6 (3)	−6.8		3.4
K61T,K65E	1.96 ± 0.15 (4)	1.0 ± 1.1 (4)	−9.2	−9.8	3.1
K61E,K65E	1.66 ± 0.08 (3)	−2.4 ± 1.0 (4)	−12.6	−12.9	3.2
K46E,K61E K65E	2.09 ± 0.05 (2)	−6.1 ± 0.1 (2)	−16.3	−18.9	2.7
B					
D51K	1.70 ± 0.13 (2)	10.1 ± 0.5 (2)	−0.1		−0.1
K108E	1.85 ± 0.07 (2)	9.4 ± 0.0 (2)	−0.8		0.4
K132E	1.76 ± 0.06 (3)	10.1 ± 0.4 (3)	−0.1		0.1
D156K	1.76 ± 0.08 (3)	9.8 ± 0.2 (3)	−0.4		−0.2
K174E	1.80 ± 0.00 (2)	9.4 ± 0.4 (2)	−0.8		0.4
K205E	1.70 ± 0.05 (4)	9.2 ± 1.5 (4)	−1.0		0.5
K205Q	1.51 ± 0.0 (2)	9.0 ± 0.0 (2)	−1.2		1.2
K211E	1.83 ± 0.09 (5)	11.0 ± 0.3 (3)	0.8		−0.4
E220K	1.71 ± 0.08 (4)	10.9 ± 0.1 (2)	0.7		0.4
R252E	1.90 ± 0.10 (3)	10.1 ± 1.3 (3)	−0.1		0.1
K267E	1.85 ± 0.09 (6)	10.6 ± 0.5 (4)	0.4		0.2
K274E	1.83 ± 0.08 (4)	10.1 ± 0.5 (3)	−0.1		0.1

the charge of residues located outside the pore might not affect selectivity. To look for residues affecting VDAC's ion selectivity, we began by site-directed mutagenesis of lysine residues, since previous studies had shown that lysine residues are important for channel selectivity (*22*). In addition, the positions of 13 of the 19 lysine residues in the yeast VDAC are exactly conserved in *Neurospora crassa* VDAC (*23*), indicating that they are likely to perform conserved functions. Finally, by converting these positive residues to negative residues one can produce large changes in the local electrostatic environment. Accordingly, each of the conserved lysine codons, as well as a number of other codons throughout the molecule, was mutated to produce a charge change, and the mutant genes were transformed into M22-2. Transformants were obtained for all mutations except K234E (*24*), which appears to encode a protein producing dominant lethality.

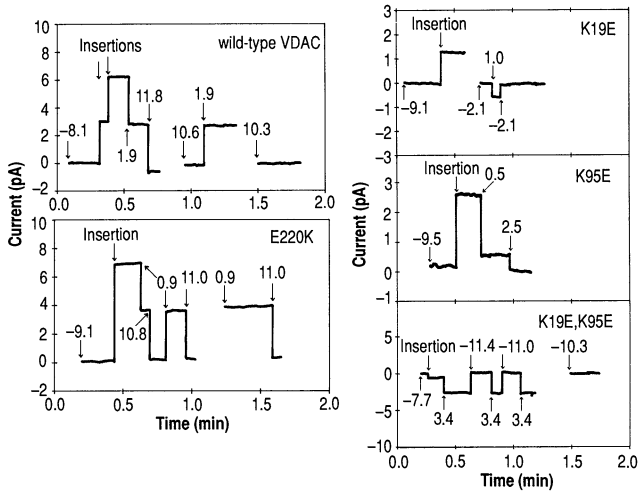

Fig. 3. Measurements of the reversal potentials in wild-type and mutant yeast VDAC (mutant code, Table 1). The membranes were made with soybean phospholipids and separated 1*M* KCl from 0.1*M* KCl [each side also contained 5 m*M* $CaCl_2$ and 1 m*M* morpholinoethanesulfonic acid (Mes), *p*H 5.8]. A 2- to 4-μl aliquot of a 1% Triton X-100 extract of a VDAC preparation (as indicated in the upper right of each panel) was added to the high salt side of the membrane. The applied potential is indicated in the figures at the point of application and was held constant until a new value is indicated (the sign refers to the high salt side). Insertion of channels is indicated by an abrupt change in current with no change in applied potential. Potentials were applied to bring the current back to 0. This is the reversal potential for that particular channel. All potentials were corrected for electrode asymmetry. Gaps in the traces represent editing introduced to simplify presentation of the data.

When introduced into planar phospholipid bilayers, the mutant VDAC proteins inserted spontaneously, producing discrete changes in current. The selectivity of the mutant channels was estimated by measuring the potential needed to bring the current to zero (reversal potential) (Fig. 3). All of the results were obtained from membranes containing a single or few channels in order to have high confidence that all observations were made on channels in their open conformation (7). The first current change in all the records represents the insertion of one or two channels. Shortly after the insertion, the membrane potential was changed to determine the membrane conductance [Δ current (I)/Δ voltage (V)] and the reversal potential. The reversal potentials of the wild type and, for example, VDAC-E220K, were between 10 and 11 mV. By contrast, changing the charge at position 19 (K19E) or 95 (K95E) resulted in pronounced decreases in anion selectivity. The double mutant, K19E, K95E, resulted in a change in reversal potential that approximated the sum of the changes produced by the two mutations individually.

None of the single or multiple amino acid substitutions resulted in a significant change in single-channel conductance, indicating that no extensive conformational changes took place as a result of these engineered alterations (Table 1). For residues that affected the selectivity of VDAC (as measured by the reversal potential) (Table 1A), the observed change in reversal potential depended on both the magnitude and sign of the charge change. A change by one unit of charge (for example, K61T) produced roughly half as much change as a two-unit change at the same position (K61E). Although a charge change at certain sites, especially position 19, had a more pronounced effect, in general the change in reversal potential was 2 to 4 mV per unit change in charge. Many, but not all of the conserved lysine residues affected selectivity. In addition, changes in some nonlysine residues, both charged and uncharged (for example, D15K, R124D, G179D, T256K, and D282K), altered channel selectivity. When two or more mutations were present in the same molecule, the effects on selectivity were essentially additive, suggesting that each residue contributes independently to the overall selectivity of the channel. (We would not expect the reversal potentials to be strictly additive, but our results indicate that the mutations have a cumulative effect.) When multiple mutations were introduced into the VDAC protein, it was possible to completely reverse the normal channel selectivity characteristics: the normally anion-selective channel became cation-selective. There was a high degree of correlation between the sign and magnitude of the selectivity change and the sign and magni-

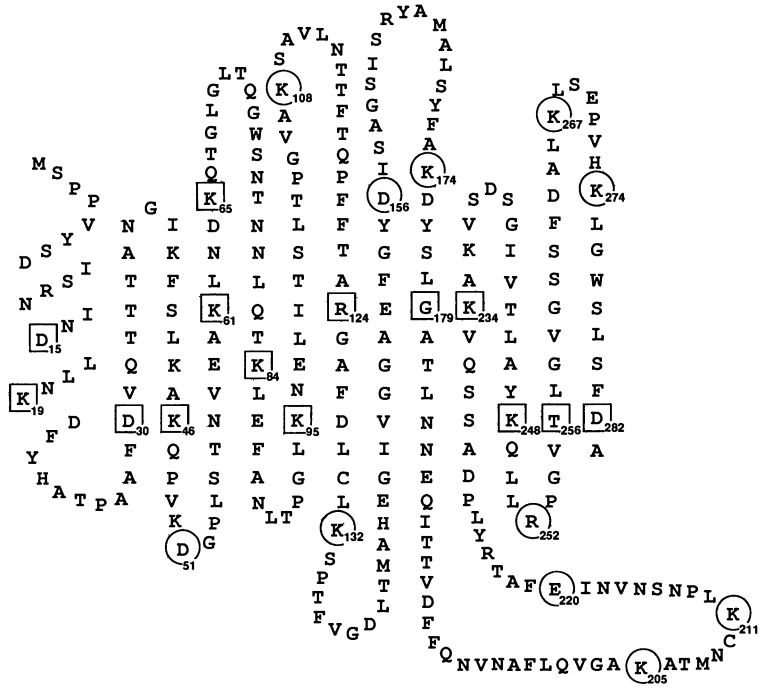

Fig. 4. A schematic model of the VDAC molecule in the membrane. An NH_2-terminal α helix (left) is flanked by 12 strands of antiparallel β sheet. Residues for which mutation altered selectivity are boxed; residues in which the mutation left the selectivity unchanged are circled.

tude of the change in charge at the site of the mutation. Thus, the mutations' effects are most likely local.

Given the size of the VDAC molecule and the large diameter of the channel it forms, it is likely that most of the molecule contributes to the formation of the channel walls. Thus, mutations that affect channel selectivity probably identify transmembrane segments and can be used to position portions of the molecule forming the walls of the channel relative to the primary amino acid sequence. On the basis of the results in Table 1 and an analysis of the primary sequence (*11, 12*), we developed a model of the open-channel state of the VDAC molecule (Fig. 4). Each polypeptide is proposed to form 12 transmembrane β strands and one transmembrane α helix. These transmembrane segments contain all the residues that affect selectivity and lack the residues that do not (*25*). The α helix is amphipathic with the nonpolar residues on one surface facing the lipid bilayer. The β strands have the alternating hydrophobic-hydrophilic pattern of a sided β sheet (*26*). Residues and sequences that would be expected to break this pattern (for example, prolines and adjacent charged residues) are often located at the ends of transmembrane segments. Using reasonable estimates for spacing between transmembrane strands (0.47 nm for β strands) and for the size of the side chain residues (0.5 nm), we conclude that two monomers (folded as shown in Fig. 4) would form a hole ~3 nm in diameter. This model is also consistent with available evidence that suggests that the VDAC channel is composed of two identical subunits (*27*).

From the change in charge required to just eliminate VDAC's selectivity for Cl^- over K^+ (to bring the reversal potential to zero) one would expect that each monomer would contribute an excess of three charges to the walls of the pore. The model in Fig. 4 has 14 positive and 13 negative charges in transmembrane regions resulting in one net positive charge. However, some residues (such as Lys^{19}) may have a disproportionately greater effect.

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17. A 1.8-kb DNA fragment containing the yeast VDAC gene was subcloned into M13mp9 and subjected to site-directed mutagenesis with synthetic oligonucleotides by the "gapped duplex" method [W. Kramer *et al.*, *Nucleic Acids Res.* **12**, 9441 (1984)]. After DNA sequencing to verify the mutations, the mutant genes were subcloned into a yeast shuttle vector containing a yeast selectable marker gene (*URA3*), centromere (*CEN4*), and replication origin (*ARS1*). Yeast cells lacking the wild-type VDAC gene were transformed [H. Ito, Y. Kukuda, K. Murata, A. Kimura, *J. Bacteriol.* **153**, 163 (1983)] with the resulting plasmids. The nucleotide changes corresponding to each mutation are as follows (double mutations contain the changes listed for both single mutations): E15K, A 43, G 45; K19E, G 55; D30K, A 88, G 90; K46E, G 136; D51K, A 151, G 153; K61E, G 181; K61T, C 182; K65E, G 193; K65A, G 193, C 194; K84E, G 250; K95E, G 283; K108E, G 322; R124E, G 370, A 371; K132E, G 394; E152K, A 454; D156K, A 466, G 468; R164D, G 490, A 491; K174E, G 520; G179D, A 536; D191K, A 571, G 573; K205E, G 613; K205Q, C 613; K211E, G 631; E220K, A 658; K234E, G 700; K234Q, C 700; K236E, G 706; K248E, G742; R252E, G 754, A 755; T256K, A 767, G 768; K267E, G 799; K274E, G 820; and D282K, A 844, C 846.
18. Single-letter amino acid abbreviations are A, Ala; D, Asp; E, Glu; G, Gly; K, Lys; Q, Gln; R, Arg; and T, Thr. Mutations are indicated by the single-letter amino acid code for the amino acid changed and the new residue at the corresponding position. For example, E15K indicates that E at position 15 was changed to K.
19. Yeast mitochondria were prepared essentially as by G. Daum, P. C. Böhni, and G. Schatz [*J. Biol. Chem.* **257**, 13028 (1982)], except that cells were disrupted in 0.3*M* sucrose, 10 m*M* tris-HCl, *p*H 7.5, 0.3% bovine serum albumin, and 1 m*M* EDTA; this buffer was used for subsequent washes. The mitochondria were lysed in 1 m*M* KCl and 1 m*M* tris-HCl, *p*H 7.5, and the membranes were collected by centrifugation a 27,000*g* for 20 min. The membranes were solubilized and chromatographed on mixed hydroxyapatite-Celite columns as by H. Freitag, R. Benz, and W. Neupert [*Methods Enzymol.* **97**, 286 (1983)], except that 2.5% Triton X-100 was used in place of the Genapol and the buffer also included 15% dimethylsulfoxide.
20. When mitochondria from yeast containing the VDAC-E236K gene were subjected to VDAC protein purification (*17*), no protein was obtained, although outer membranes purified from these mitochondria contained a 29-kD protein that cross-reacted with VDAC antibody. When these outer membranes were solubilized and exposed to planar phospholipid bilayers, no channels formed. It is likely that this mutation causes a major disruption of the protein's structure.
21. The membranes were made and studied under voltage-clamp conditions (*3*). Yeast VDAC channels were inserted by adding a portion of purified VDAC (solubilized in 1% Triton X-100) (Fig. 3).
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24. The K234E gene was inserted into several yeast plasmids and attempts were made to introduce it into yeast strains with wild-type or deleted VDAC genes. No transformants that expressed the mutant gene were recovered.
25. Lys^{65} is a possible exception, but it is close to the end of a transmembrane strand.
26. Asp^{64} is an exception, but since it is located at the end of a transmembrane-spanning region, presumably the COOH group extends into the aqueous phase.
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