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Evidence for Titratable Gating Charges Controlling the Voltage Dependence of the Outer Mitochondrial Membrane Channel, VDAC

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Summary. A voltage-dependent anion-selective channel, VDAC, is found in outer mitochondrial membranes. VDAC's conductance is known to decrease as the transmembrane voltage is increased in either the positive or negative direction. Charged groups on the channel may be responsible for this voltage dependence by allowing the channel to respond to an applied electric field. If so, then neutralization of these charges would eliminate the voltage dependence. Channels in planar lipid bilayers which behaved normally at pH 6 lost much of their voltage dependence at high pH. Raising the pH reduced the steepness of the voltage dependence and raised the voltage needed to close half the channels. In contrast, the energy difference between the open and closed state in the absence of a field was changed very little by the elevated pH. The groups being titrated had an apparent pK of 10.6. From the pK and chemical modification, lysine epsilon amino groups are the most likely candidates responsible for VDAC's ability to respond to an applied electric field.

Key Words membrane protein · pore · protein modification · gating mechanism · planar bilayer membrane · protein titration

Introduction

The mechanism of cell membrane permeability control is of considerable interest. One way to achieve this control is for the transport system, under the influence of different environmental conditions, to be able to translocate molecules or ions across membranes at different rates. Conductance measurements indicate that most channel-forming molecules can exist in at least two states: a high conductance, or "open state," and a low conductance, "closed," state. If the voltage difference across the membrane influences which conductance state the channel will occupy, that channel is referred to as "voltage-dependent." Thus, voltage-dependent channels are an effective way to control membrane permeability.

VDAC, a voltage-dependent anion-selective channel, is found in outer mitochondrial membranes (Parsons et al., 1966; Mannella & Bonner, 1975; Colombini, 1979, 1980*b*; Zalman et al., 1980; Freitag et al., 1982; Linden et al., 1982; Roos et al., 1982). The properties of VDAC can be studied after its insertion into planar phospholipid bilayers (Schein et al., 1976). The channel formed by VDAC has an estimated radius of 20 Å (Colombini, 1980*a*), although smaller estimates have been reported (Mannella, 1982; Freitag et al., 1982). VDAC's conductance decreases as the transmembrane voltage is increased in either the positive or negative direction (Schein et al., 1976). This conductance decrease results from channel closure. Thus VDAC's voltage dependence stems from the fact that as the electric field increases, the probability of finding the channel in the open state decreases. VDAC is also somewhat selective for anions over cations of comparable size and charge (Colombini, 1980*b*) suggesting that fixed positive charges are present in or near the channel.

In theory, voltage dependence could arise from the movement of charges (referred to as gating charges) along an electric field or the alignment of dipoles with an electric field (Hodgkin & Huxley, 1952). This movement is relative to the field and may result in whole or part from movement of the electric field (Colombini, 1984). Coupled to this movement (simultaneous or subsequent) is the insertion of a barrier (steric or electrostatic) within the channel which reduces ion flux. The channel is open in the absence of the barrier and closed in its presence. The field, through charges and/or dipoles on the molecule, changes the potential energy of one or both of the states. This can either stabilize or destabilize a particular conformational state causing the probability of finding the channel in a given state to be changed (Ehrenstein et al., 1970). The molecular events underlying this gating mechanism are not known.

As VDAC is a protein (Colombini, 1980; Linden et al., 1982; Mannella, 1982; Roos et al., 1982), the

physical nature of the gating charges would probably be charged amino acid residues comprising the channel molecule. By changing the environmental pH, gating charges could be titrated, thereby reducing or eliminating the voltage-dependent component by neutralizing the structure that responded to the field. If, on the other hand, an uncharged dipole were responsible for the voltage dependence (as postulated for alamethicin by Hall and co-workers, 1984), pH changes would probably be ineffective in eliminating the channel's ability to respond to voltage.

The purpose of the present research was to examine the molecular nature of VDAC's gating mechanism by focusing on the possible existence of titratable gating charges. In this study, the pH of the solutions surrounding a planar phospholipid bilayer containing VDAC was increased in order to neutralize the proposed positive gating charges. The voltage dependence of VDAC was measured at control and high pH to determine if high pH altered the gating mechanism. More specifically, two parameters were calculated to determine if the change in pH altered VDAC's voltage-dependent nature: the steepness of the voltage dependence n and the voltage at which half of the channels are open V_o.

Materials and Methods

Experimental

Planar lipid bilayers were made according to the method of Montal and Mueller (1972) as modified by Schein et al. (1976). In short, a 1% (wt/vol) solution of soybean phospholipid in hexane was used to make a bilayer across a 0.15-mm diameter hole in a sheet of saran that separated two compartments, called *cis* and *trans*. Before use, the soybean phospholipids were purified as described by Kagawa and Racker (1971). Both compartments were filled with a known volume (approximately 5 ml) of an aqueous solution consisting of 1 M LiCl, 5 mM $CaCl_2$ and 5 mM 2-(N-morpholino)ethane sulfonic acid (MES; pK_a 6.15), pH approximately 6.

VDAC was purified from rat liver mitochondria according to the method of Colombini (1983). Fraction one was used which consisted of partially purified VDAC in 0.7% Triton X-100, several salts, and 20% DMSO. Addition of the VDAC solution to the *cis* side of the chamber while stirring resulted in the spontaneous insertion of VDAC channels monitored as an increase in current in the presence of a 10-mV driving force.

Current flow was measured under voltage-clamp conditions using a pair of calomel electrodes such that the voltage on the *trans* side of the chamber was controlled with respect to the *cis* side. Current flow was recorded with a Kipp & Zonen BD41 dual-pen chart recorder. Stimuli in the form of asymmetric triangular voltage waves were generated by a model 184 Wavetek sweep generator and applied across the membrane via the same electrodes. The rate at which the voltage was increased was much faster than the rate at which it was decreased. It had previously been shown that current records obtained while the voltage was increasing were distorted by VDAC's slow closing kinetics (Schein et al., 1976). Therefore the current recorded as the voltage was increasing was not used. Only the current recorded as the voltage was decreasing was analyzed. We also found that changing the rate at which the voltage was decreasing (between 15 and 45 mV/min) did not significantly change the current record.

VDAC's voltage-dependent properties were characterized at pH values around 6, referred to as control pH, by means of the above-mentioned voltage waves. The voltage was changed at a constant rate while the current flowing through the membrane was recorded. The voltage-dependent parameters were calculated from these recordings as described below. The pH was then changed by adding 1.0 M 3-(cyclohexylamino)-1-propane-sulfonic acid (CAPS; pK_a 10.4) to both the *cis* and *trans* compartments. Once the pH had been increased the voltage dependence of the channels was again examined as above.

During an experiment, the pH of the *cis* side was measured using an Orion Research Model 231 pH meter and a fine-tipped combination electrode. At the conclusion of an experiment, the pH of the *trans* compartment was also measured.

Raising the pH of the solutions surrounding an unmodified bilayer, i.e. a bilayer not containing VDAC channels, did not change its low conductance. All experiments were performed at room temperature. The reagents used were purchased from Sigma Chemical Co., St. Louis, Mo., or were reagent grade.

Data Analysis

The measurements of current as a function of voltage (Fig. 1) were converted to conductance as a function of voltage (Fig. 2) using a Hewlett-Packard digitizer operated by a Hewlett-Packard 85 computer. Analysis of the channels' voltage dependence was performed using a modification of the methods described for excitability inducing material channels (EIM) by Ehrenstein et al. (1970) and for VDAC by Schein et al. (1976). For this analysis, it was assumed that the channels were in equilibrium at any voltage V and that VDAC could exist only in either an "open" or "closed" conductance state.

Equation (1) was used to analyze VDAC's voltage dependence. This equation is based on the Boltzmann distribution and is a modification of the equation used by Schein et al. (1976):

$$\ln (G\text{-}G\text{min}/G\text{max}\text{-}G) = (-nFV + nFV_o)/RT. \quad (1)$$

G is the conductance at any voltage (V), Gmax is the maximum conductance (when all the channels are open), and Gmin is the minimum conductance (when all the channels are closed). F, R and T are Faraday's constant, the gas constant, and the temperature in degrees Kelvin, respectively. n is a measure of the steepness of the voltage dependence and V_o is the voltage at which half the channels are open. From a plot of $\ln(G\text{-}G\text{min}/G\text{max}\text{-}G)$ *vs.* V, the n and V_o values were obtained from the slope and the voltage-axis intercept of the resulting straight line.

When the applied voltage is equal to V_o, the energy levels of the open and closed states are equal. For voltages lower than V_o, the energy level of the open state is lower than that of the closed state (i.e. the open state is more probable); while for higher voltages, the opposite is true. When $V = 0$, the energy difference between the two states is the intrinsic conformational energy difference. The energy needed to compensate for this energy difference is nFV_o.

Table 1. Percent of channels closing after arginine-modifying reagent addition[a]

Time after reagent addition (min)	Arginine-modifying reagent	
	p-hydroxy-phenylglyoxal	camphorquinone-10-sulfonate
10	105 ± 10	—
20	108 ± 7	—
30	105 ± 6	—
40	106 ± 6	99
50	103 ± 3	—
60	98 ± 8	—
70	104	—
80	91	99
120	—	100
160	—	105
200	—	99
220	—	97

[a] The percentage of channels closing was determined by stepping the voltage to 40 mV and measuring the decrement in conductance after 100 sec. This was done before and after modifier addition. The conductance change after modification, expressed as a percent of that before modification, is the value quoted as percentage of channels closing. The error estimates are standard deviations of from 3 to 6 estimates. All but one of the values quoted without error estimates are means of two determinations.

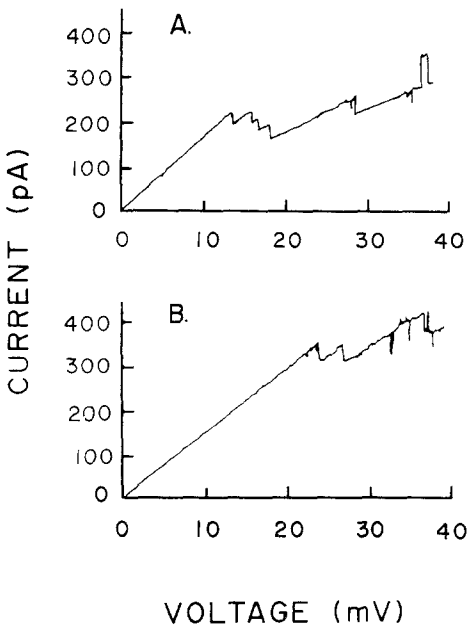

Fig. 1. Current flowing through VDAC channels as a function of transmembrane voltage at pH 6.2 (*A*) and 10.6 (*B*). The current was measured across a single membrane containing five channels. The applied voltage was decreasing with time at a rate of 18.6 mV/min

Although it has been found that VDAC channels exist in several different conductance states (Colombini, 1980*b*) the above voltage-dependent analysis was used for the following reasons: (1) the results of the analysis are consistent with a two-state system; (2) since these channels exist in many different closed states with different conductance levels, a mathematical analysis of such a system would prove to be extremely difficult; (3) the exact meaning of the voltage-dependent parameter values can be questioned, but these values are a valid measure of the steepness of the voltage dependence irrespective of the number of closed states.

The analysis of VDAC's voltage dependence at the elevated pH raised a serious problem. Elevating the pH reduced VDAC's voltage dependence and thus made it necessary to apply higher than normal voltages in order to close all of the channels and so measure the *G*min. This was very difficult because at the elevated pH the membranes were quite unstable at high voltages. Thus it became necessary to estimate *G*min indirectly. Single-channel experiments showed that at the elevated pH the open-channel conductance and the percent drop in conductance upon channel closure did not differ appreciably from the values observed at control pH. Thus it was possible to estimate *G*min from the value of *G*max. The percent closure before pH modification was calculated and used with the *G*max value obtained after pH modification to calculate the *G*min. Once the *G*min was determined, the voltage-dependent parameters of n and V_n could be obtained.

Chemical Modification Experiments

Planar phospholipid membranes were made as described above and VDAC channels were inserted. The voltage dependence of these channels was tested prior to and after the addition of an arginine-modifying reagent. The ability of the channels to close was estimated by measuring the membrane conductance at a voltage at which the channels were open (10 mV) and at a voltage at which they were closed (after a 100-sec exposure to a 40-mV potential). The percent drop in conductance, at various times after the addition of modifier, was expressed as a percent of that before modifier addition (Table).

The arginine reagents used were *p*-hydroxyphenylglyoxal (Yamasaki et al., 1980) and camphorquinone-10-sulfonate (Pande et al., 1980). They were dissolved in DMSO but, once added to the solution bathing the membrane, the final DMSO concentration did not exceed 2% (vol/vol). The aqueous solutions bathing the membranes were chosen to be compatible with the reagent, to allow us to make the membranes, and to facilitate the current measurements. For the phenylglyoxal reactions we used 1 M NaCl, 5 mM $MgCl_2$, 0.1 M phosphate buffer adjusted to pH 7.5 or pH 8.1 with NaOH. The final phenylglyoxal concentration in the aqueous solution was between 0.8 to 1.1 mg/ml. For the camphorquinone experiments we used 1 M LiCl, 5 mM $CaCl_2$, 0.2 M borate, Na^+ salt, pH 8.0. The final camphorquinone concentration was 2.7 mg/ml. All reactions were run at room temperature.

Results

Examples of the type of data which were collected are shown in Figure 1. Panel *A* shows a current-voltage relationship derived from a bilayer at pH 6.2 containing five channels. The number of channels can be determined by counting the number of transi-

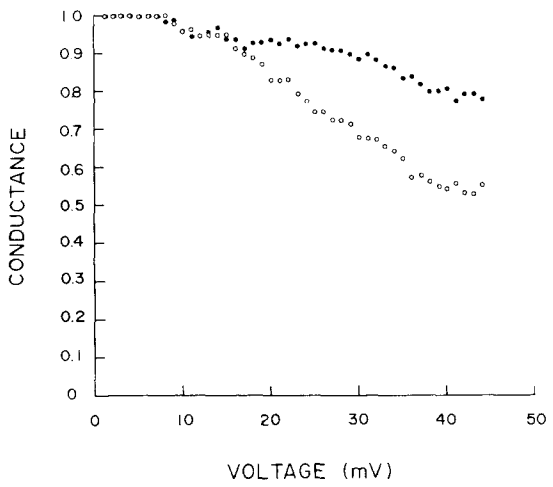

Fig. 2. Normalized conductance of a multi-channel membrane as a function of voltage at pH 6.2 (open circles) and pH 10.7 (closed circles). The data were taken from current records made when the voltage was decreasing with time at a rate of 43.2 mV/min. The conductance values for each pH were normalized relative to the maximum conductance found at low voltages. The control pH points (open circles) are averages of five recordings taken prior to the pH change while the high pH points (closed circles) are the results of one recording after the pH was raised. The maximum conductance at the control pH was 200 nS (average for the five records) while that at the high pH was 170 nS. This conductance drop was not typical

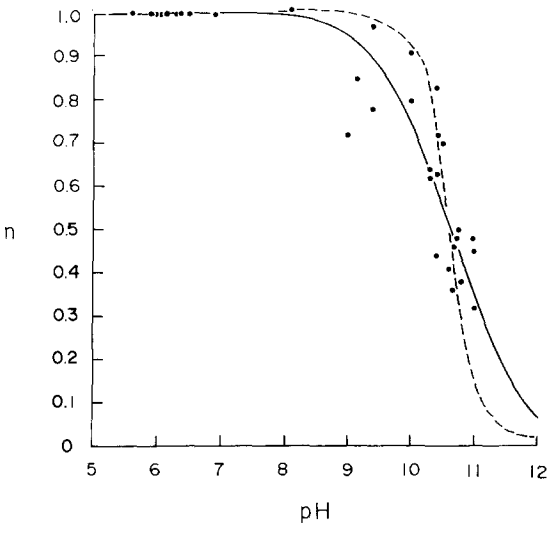

Fig. 3. The steepness of the voltage dependence n as a function of increasing pH. n was calculated as described in Materials and Methods (*see* Data Analysis) and normalized to its value prior to raising the pH. The mean and standard deviation for n at the control pH's (pH 5.5 to 6.5) is 4.3 ± 0.6 (23 estimates). The dashed line represents a pK curve of 10.6 generated by assuming five dissociable groups all having the same equilibrium constants (*see* Appendix, Eq. 2). The solid line represents a pK curve generated for five dissociable groups with equilibrium constants equally spaced between pK 9.6 and 1.16 (*see* Appendix, Eq. 10)

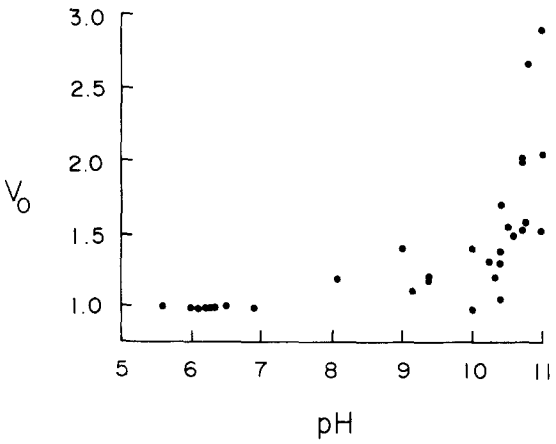

Fig. 4. The voltage at which half of the channels are open V_0 as a function of pH. The values of V_0 were calculated as described in Materials and Methods (*see* Data Analysis) and then normalized. The mean and standard deviation of the V_0 at the control pH's (pH 5.5 to 6.5) is 20.8 ± 2.8 mV (23 estimates)

tions between conducting levels. Figure 1*B* shows the same membrane after the pH was increased to 10.6. Only three of the five channels closed in this example. It can be seen that an increase in pH resulted in altered channel properties.

Conductance-voltage relationships from a membrane containing many channels before and after pH change are shown in Figure 2. The conductance values were normalized relative to the maximum conductance value. The maximum conductance usually changed very little as a result of raising the pH. However, an increase in pH caused a marked loss of the channels' voltage-dependent closure. The loss of voltage-dependent conductance was quantified (*see* Data Analysis) by determining n, the steepness of the voltage-dependent change in conductance, and V_0, the voltage at which half the channels are open. At the control pH, the average values of n and V_0 were 4.3 and 21 mV, respectively. With an increase in pH, n decreased (Fig. 3) and V_0 increased (Fig. 4) about 2.5-fold. Normalized values for n and V_0 were plotted to reduce the variability that is present between different membranes. The normalized values were calculated, for each experiment, by dividing the value at high pH by the value at the low pH. In the absence of an electric field, the energy difference between the two conductance states nFV_0 decreased by a small amount with the same change in pH (Fig. 5).

Experiments using membranes with only a few channels indicated what may be occurring with VDAC at the molecular level. Figure 1 shows that at high pH only some of the channels closed at the

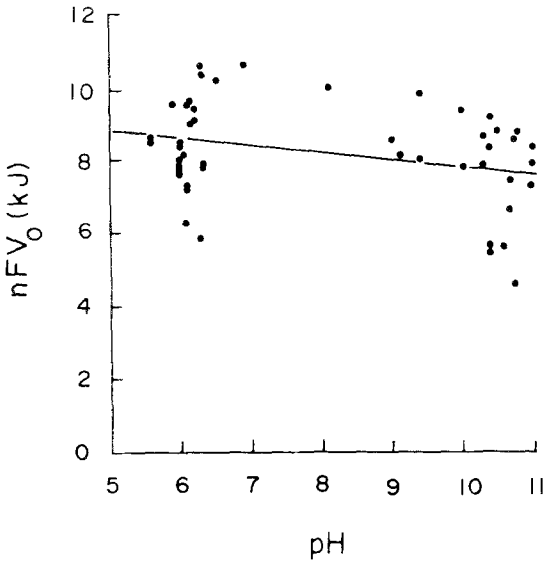

Fig. 5. The energy difference between the open and closed conductance states nFV_o as a function of pH. The line, generated using least-squares regression, was found to have a correlation coefficient of −0.30. This was a significant decrease at the 95% confidence level. The mean and standard deviation at the control pH's (pH 5.5 to 6.5) is 8.4 ± 1.1 kJ (23 estimates)

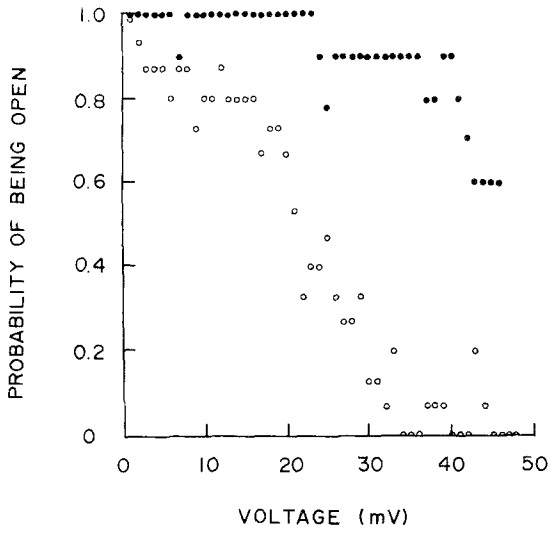

Fig. 6. The probability of a channel being in the open conductance state versus the transmembrane voltage a pH of 6.1 (open circles) and pH 10.7 (closed circles). This figure was generated from many current/voltage records obtained with three different membranes, each containing one channel. The probability of the channel being open at a particular voltage was obtained by summing the number of times it was observed to be open at that potential, divided by the number of observations (15 at low pH and 10 at high pH). The current-voltage records were obtained in the same way as experiments with multi-channel membranes

high voltages where normally all of the channels are closed. This figure also shows that the single-channel conductance remained about the same at high pH. By analyzing many single-channel current/voltage records, the probability of a channel being open as a function of transmembrane voltage was determined at both low and high pH (Fig. 6). At low pH, the probability of the channel being open decreased steeply with increasing voltage. At high pH, the probability did not decrease as rapidly with increasing voltage.

The possibility that the gating charges are arginine groups was tested by adding specific arginine-modifying reagents directly to the aqueous phase bathing the channel-containing membrane. The channels were exposed to the reagents for extended periods of time and periodically tested for their ability to close at elevated potentials. The results show that voltage-dependent channel closure was not significantly affected by either of the two reagents.

Discussion

Literature reports on the effects of pH changes on voltage-dependent channels yield a mixed message. In some cases pH simply changes the channels' conductance, while in others there are indications that groups associated with the gating mechanism have been titrated. Most of the work has centered on the sodium and potassium channels of nerve and muscle.

Lowering the pH outside *Myxicola* axons resulted in a reduction in the gating current associated with the activation of sodium channels (Schauf, 1983), but this was not accompanied by a shift in the current's voltage-dependent properties. Moreover, the voltage-dependent properties of the sodium conductance did not change appreciably at the lower pH (Schauf & Davis, 1976). Similar experiments using axons from *Loligo* and *Rana* were interpreted as titration of surface charges not directly related to the gating mechanism (Hille, 1968; Carbone et al., 1978). The potassium channels in *Myxicola* axons experienced a shift in V_o but no change in n when the pH was lowered (Schauf & Davis, 1976). Thus a simple titration of gating charges is unlikely.

Increasing the intracellular pH in *Loligo* axons resulted in an inhibition of the inactivation process associated with sodium channels (Carbone et al., 1981). The elevated pH reduced n and increased V_o. These investigators concluded that positive charges (pK 10.4) associated with the inactivation process were neutralized at the elevated pH. However, the elevated pH increased the gating current measured after long depolarizations (Wanke et al., 1983). This increase in gating current at high pH was attributed

to the activation gating charges. The authors postulated that at control pH the positive charges associated with the inactivation gating process immobilize some of the activation gating charges. There is evidence that these positive charges may be arginine or lysine residues. Investigators have found that inactivation was removed by a specific protease for arginine and lysine residues (Rojas & Rudy, 1976) and by specific reagents for arginine (Eaton & Brodwick, 1978).

In contrast with the above discussion, our results seem to be consistent and straightforward. An increase in pH reduced the steepness of the voltage dependence (as measured by the parameter n) and simultaneously increased the voltage needed to induce the transition (as measured by the parameter V_o). The parameter n can be interpreted as a measure of the minimum number of charged groups on the protein which detect the applied electric field and allow the protein to respond to the field. A decrease in this parameter is consistent with the neutralization of some of these charges with the increased pH. As a result of this neutralization, there would be a reduction in the channel's ability to respond to the electric field.

The energy needed to close half the channels is nFV_o. If the number of gating charges n decreased with increasing pH, the voltage needed to generate the same amount of energy must be increased. The decrease of n with increased pH was matched by an increase in V_o so that the value of nFV_o changed very little. [This is in contrast to results of the chloride channel from the *Torpedo electroplax* membrane (Hanke & Miller, 1983) in which the V_o value changed markedly with the pH change, while the n value remained virtually unchanged.] The small decrease in nFV_o could either indicate that the gating charges are located in a higher dielectric constant environment in the open as compared to the closed state or simply that other charges have been titrated that influence the conformational energy difference between the open and closed states.

Another possible interpretation of the change in the conductance-voltage relationship with the increase in pH (Fig. 2) could be that the minimum conductance of the channels (*G*min) was increased after pH modification, therefore causing the conductance-voltage relationship to appear less voltage dependent. However, this interpretation is probably incorrect. By using membranes containing only one channel we demonstrated that after a pH increase VDAC can still close to the same extent. In these membranes, the percent drop in conductance was: 69.4 ± 2.5%, at control pH, and 72.4 ± 2.3%, at a pH of 10.7. Moreover, single-channel experiments showed that, at high pH, the closures were much briefer, i.e. the closed state was a less probable conductance state. Therefore the elevated pH did not significantly affect the channels' ability to conduct ions[1]. At any given voltage, the channel was spending more time in the open state (Fig. 6).

Single-channel experiments yielded results which were consistent with those of multi-channel experiments. The probability of finding the channel open decreased less rapidly with voltage at high pH than it did at low pH. In multi-channel experiments, the conductance decreased less rapidly with voltage at high pH as compared to low pH. Hence the conductance changes observed in multi-channel membranes reflect changes in the probability of finding a channel open.

The possibility that kinetic delays distorted the results was investigated. At elevated pH, the rates of opening and closing do slow down but not sufficiently to affect the results. When the frequency of the voltage wave was doubled from 18.6 to 43.2 mV/min there was no significant change in the conductance-voltage plot or in the voltage-dependent parameters n and V_o. Furthermore, values for an experiment run at slower frequencies of 7.4 mV/min did not seem to be significantly different from the experiments run at 18.6 or 43.2 mV/min. Most of the experiments were performed using the higher frequencies because of the time factor involved when using an unstable bilayer.

Although it is possible that the observed changes in n were due to gross structural changes of the protein induced by increasing the pH, there are reasons to think otherwise. First, the conductance of the open and closed channel did not change appreciably, indicating that the channel could still conduct ions properly. Secondly, the pH experiment was reversible. When the increased pH was returned to its initial control value, the channels regained their original voltage-dependent properties. Finally, the voltage-independent energy difference between the states (nFV_o) changed only slightly with increase in pH, indicating that the channels' energy levels had been altered only slightly at elevated pH.

Since a change in the voltage dependence was measured while the pH was increasing from 6 to 11 the possible identities of the gating charges associated with the protein channel could be limited to the side chains of the amino acids: histidine, lysine and

[1] The results at high pH were recorded shortly after the pH was elevated. On occasion, the membrane would survive for some time after reaching the high pH. With time the conductance seemed to decrease. It is not clear whether this decrease was due to loss of channels from the membrane or a slow conformational change induced by the high pH.

arginine as well as the terminal amino groups. The pK ranges for these groups within a protein have been found to be approximately: 5.4 to 8.1, 9.1 to 10.7, 11.6 to 12.6 and 6.7 to 8.1, respectively (Fasman, 1976). Since the loss of voltage dependence occurred at a pH above 9, the most probable groups responsible for the gating charges are lysine and arginine.

Estimates of the pK (or pK range) of the groups titrated in these experiments were obtained by fitting a theoretical curve to the data of n *vs.* pH (Fig. 3). The following equation was derived (*see* Appendix) to describe the effective number of gating charges found on a channel (n) as a function of pH. The equation assumes that, there are five gating charges available for deprotonation per channel (this is consistent with a measured n value between four and five at control pH) and the equilibrium constants for each of the gating charges are identical.

$$n = \frac{5[H^+]^5 + 4(K[H^+]^4) + 3(K^2[H^+]^3) + 2(K^3[H^+]^2) + K^4[H^+]}{[H^+]^5 + (K[H^+]^4) + (K^2[H^+]^3) + (K^3[H^+]^2) + K^4[H^+] + K^5}. \quad (2)$$

This equation was used to generate "pK curves" for different pK values between 9 and 12, and a plot of n versus pH was generated. These curves were compared to the data and the best fit was determined by calculating the variance between the pK curve and the data. The pK with the lowest variance was 10.6. This pK curve is shown in Fig. 3 as the dashed line.

Equation (2) was derived assuming an n of five. The voltage-dependent energy change nFV induced by the electric field implicates five charges only if the charges experience all the applied field. If less than the entire field is felt by the gating charges, then the number of charges must be increased so that the product nFV remains unchanged. When fits to the data were made with equations based on n = 5, 7, 9 and 11, the optimal pK remained 10.6 and the variance between the theoretical line and the data increased with increasing n. Thus n = 5 seems to be the optimal value.

The possibility that the pK's of the gating charges are not identical was considered. Heterogeneity in the charged groups or close proximity of the charged groups could result in a spread of the pK values. For simplicity the spreads were assumed to be uniform. The lowest variance was obtained with a spread of plus and minus one pH unit from pH 10.6. This was true whether n was assumed to be 5, 7, 9 or 11, but the lowest variance was obtained with n = 5. Hence, the optimal fit to the data is a group of five charges whose pK ranges from 9.6 to 11.6. This pK curve is the continuous line in Fig. 3.

The results are consistent with either lysine or arginine side chains as candidates for the gating charges. The amino acid composition of the purified channel contained 9.44% lysine, 2.27% arginine and 1.35% histidine (Linden et al., 1982). These investigators concluded that two polypeptides form the VDAC channel with a total molecular weight of 60 kD (Linden & Gellerfors, 1983). Therefore the numbers of the above amino acids per channel is approximately 44, 9 and 6, respectively. The high lysine content suggests that the amino acid lysine is a prime candidate for the gating charges.

Chemical modifying agents have revealed information on the nature of the gating charges. Arginine-modifying reagents had no detectable effect on VDAC's voltage dependence. Therefore arginine is probably not involved in the gating mechanism. On the other hand, succinic anhydride, a chemical specific for amino groups, has been shown to modify VDAC's voltage dependence and alter its normal anion selectivity (Doring & Colombini, 1983, 1985). Therefore, all the results are consistent with the hypothesis that lysine is probably the major amino acid involved in VDAC's gating mechanism.

Another titratable group with a pK close to 10.6 is tyrosine. The titration of tyrosine would generate a negative charge and could thereby neutralize neighboring positive charges (the gating charges). Thus the pK that was estimated may not be the pK of the positive charges on VDAC, but that of tyrosine. A similar point was suggested by investigators studying the sodium channel inactivation mechanism (Brodwick & Eaton, 1978; Oxford et al., 1978; Carbone et al., 1981). This possibility cannot, at present, be ruled out.

Theoretically, the molecular mechanism underlying voltage dependence could rely on charges or dipoles. VDAC seems to sense the electric field by using positive charges and when some of these charges are neutralized, the channel becomes less sensitive to the field. A group of lysine residues probably form the sensor portion of the gating mechanism.

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Appendix

If it is assumed that there are five dissociable groups associated with a channel, each with it's own equilibrium constant (K), the dissociation of the protons from the channel can be expressed by the following proton-transfer reactions:

$$H_5P^{+5} \overset{K_5}{\leftrightarrow} H_4P^{+4} + H^+$$

$$H_4P^{+4} \overset{K_4}{\leftrightarrow} H_3P^{+3} + H^+$$

$$H_3P^{+3} \overset{K_3}{\leftrightarrow} H_2P^{+2} + H^-$$

$$H_2P^{+2} \overset{K_2}{\leftrightarrow} HP^{+} + H^-$$

$$HP^{+} \overset{K_1}{\leftrightarrow} P + H^+$$

where $[P]$ stands for the uncharged protein channel and each dissociation reaction is associated with an equilibrium constant numbered from K_5 to K_1. While the numbering of the equilibrium constants is not conventional, an equilibrium constant with a number equal to the number of protons available for the dissociation reaction was a desirable simplification for the sake of analysis.

An expression relating n as a function of the proton concentration ($[H^+]$) was derived from the following:

$$n = \frac{5[H_5P^{+5}] + 4[H_4P^{+4}] + 3[H_3P^{+3}] + 2[H_2P^{+2}] + [HP^+]}{[H_5P^{+5}] + [H_4P^{+4}] + [H_3P^{+3}] + [H_2P^{+2}] + [HP^+] + [P]} \quad (3)$$

where the numerator represents the total number of charges/unit volume and the denominator represents the total number of molecules/unit volume. Therefore, Eq. (3) is an expression of the effective number of charges per molecule, i.e. n. This does not imply that there are only five positive charges on the channel. We are only interested in the charges which respond to the electric field across the membrane, i.e. the gating charges. The other charges on the channel are not included in the derivation.

Each proton-transfer reaction, can be described by an equilibrium equation:

$$[H_5P^{+5}] = [H_4P^{+4}][H^+]/K_5 \quad (4)$$

$$[H_4P^{+4}] = [H_3P^{+3}][H^+]/K_4 \quad (5)$$

$$[H_3P^{+3}] = [H_2P^{+2}][H^+]/K_3 \quad (6)$$

$$[H_2P^{+2}] = [HP^+][H^+]/K_2 \quad (7)$$

$$[HP^+] = [P][H^+]/K_1. \quad (8)$$

Furthermore each charged form can be expressed in terms of the uncharged form $[P]$ and the equilibrium constants, and substituted into Eq. (3) yielding the following:

$$n = \frac{5([P][H^+]^5/K_1K_2K_3K_4K_5) + 4([P][H^+]^4/K_1K_2K_3K_4) + 3([P][H^+]^3/K_1K_2K_3) + 2([P][H^+]^2/K_1K_2) + ([P][H^+]/K_1)}{([P][H^+]^5/K_1K_2K_3K_4K_5) + ([P][H^+]^4/K_1K_2K_3K_4) + ([P][H^+]^3/K_1K_2K_3) + ([P][H^+]^2/K_1K_2) + ([P][H^+]/K_1) + [P]}. \quad (9)$$

This can be further simplified into:

$$n = \frac{5[H^+]^5 + 4(K_5[H^+]^4) + 3(K_4K_5[H^+]^3) + 2(K_3K_4K_5[H^+]^2) + (K_2K_3K_4K_5[H^+])}{[H^+]^5 + (K_5[H^+]^4) + (K_4K_5[H^+]^3) + (K_3K_4K_5[H^+]^2 + (K_2K_3K_4K_5[H^+]) + K_1K_2K_3K_4K_5}. \quad (10)$$

If we assume that all the equilibrium constants are equal to one another, then Eq. (10) becomes:

$$n = \frac{5[H^+]^5 + 4(K[H^+]^4) + 3(K^2[H^+]^3) + 2(K^3[H^+]^2) + K^4[H^+]}{[H^+]^5 + (K[H^+]^4) + (K^2[H^+]^3) + (K^3[H^+]^2) + K^4[H^+] + K^5}. \quad (2)$$