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Contribution of Ion Binding Affinity to Ion Selectivity and Permeation in KcsA, a Model Potassium Channel

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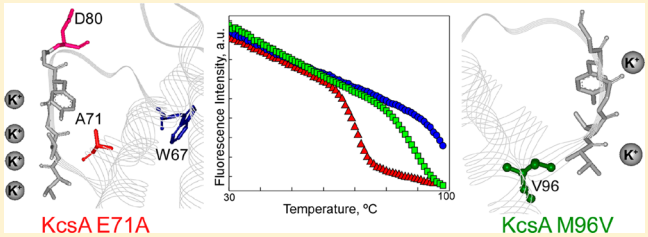
ABSTRACT: Ion permeation and selectivity, key features in ion channel function, are believed to arise from a complex ensemble of energetic and kinetic variables. Here we evaluate the contribution of pore cation binding to ion permeation and selectivity features of KcsA, a model potassium channel. For this, we used E71A and M96V KcsA mutants in which the equilibrium between conductive and nonconductive conformations of the channel is differently shifted. E71A KcsA is a noninactivating channel mutant. Binding of K^+ to this mutant reveals a single set of low-affinity K^+ binding sites, similar to that seen in the binding of K^+ to wild-type KcsA that produces a conductive, low-affinity complex. This seems consistent with the observed K^+ permeation in E71A. Nonetheless, the E71A mutant retains K^+ selectivity, which cannot be explained on the basis of just its low affinity for this ion. At variance, M96V KcsA is a rapidly inactivating mutant that has lost selectivity for K^+ and also conducts Na^+. Here, low-affinity binding and high-affinity binding of both cations are detected, seemingly in agreement with both being permeating species in this mutant channel. In conclusion, binding of the ion to the channel protein seemingly explains certain gating, ion selectivity, and permeation properties. Ion binding stabilizes greatly the channel and, depending upon ion type and concentration, leads to different conformations and ion binding affinities. High-affinity states guarantee binding of specific ions and mediate ion selectivity but are nonconductive. Conversely, low-affinity states would not discriminate well among different ions but allow permeation to occur.



Potassium channels are complex membrane proteins that conduct K^+ at high rates with a marked selectivity over Na^+, the biologically relevant competitor.[1] Early electrophysiological experiments show that the concentration of permeating and/or blocking ions modulates the selectivity and gating properties of many of these channels,[2] suggesting a role for the ions as channel "effectors" that goes beyond their passive passage through the ion-conducting pores. For instance, the absence of extracellular K^+ causes changes in the rates of deactivation or C-type inactivation.[3−7] Moreover, K^+-free medium causes Na^+ permeation in some instances[8−11] or even irreversible "collapse" of potassium conductances.[12,13]

KcsA, a potassium channel from *Streptomyces lividans*,[14] was the first ion channel to be identified using X-ray crystallography.[15] KcsA is a homotetramer in which each subunit defines two transmembrane segments connected by a pore region that contains a tilted short helix (pore helix) and an ion selectivity filter with the sequence TVGYG unmistakably homologous to the more complex eukaryotic K^+ channels. The backbone carbonyl oxygens of such residues create a stack of multiple, nonequivalent binding sites at which K^+ may bind in a dehydrated form. Indeed, two of such bound K^+ ions can be seen, single file, in the KcsA crystal structure [Protein Data Bank (PDB) entries 1BL8 and 1K4C].[15,16] The ion conduction pathway also has a lower and wider water-filled region called the cavity, which opens to the cytoplasm. According to the current understanding of the process, selectivity for K^+ over other physiologically relevant cations is exerted both at the cavity but mostly at the selectivity filter.[16,17]

Crystal structures of wild-type KcsA obtained under different ionic conditions indicate that the selectivity filter adopts distinct conformations associated with the presence of low or high concentrations of K^+ ions.[18−20] At 5 mM K^+, the filter goes into a predictably nonconductive conformation in which ions bind at the ends of the selectivity filter (the so-called sites S1 and S4), with an average occupancy of just one K^+ distributed between those two sites. As the K^+ concentration is increased, a second ion goes into the middle of the filter (site S2 or S3) and a change in conformation to a conductive state occurs, which has an average occupation of two K^+ ions per channel.[18,21] Simulation studies predict that binding of K^+ to the S2 site is required for the conformational change to occur,[22] while both S2 and S3 sites seem to undergo substantial changes in their architecture when in the absence or presence of K^+.[23]

In the context of such crystallographical and "in silico" simulation data, we reported studies of binding of ions to wild-

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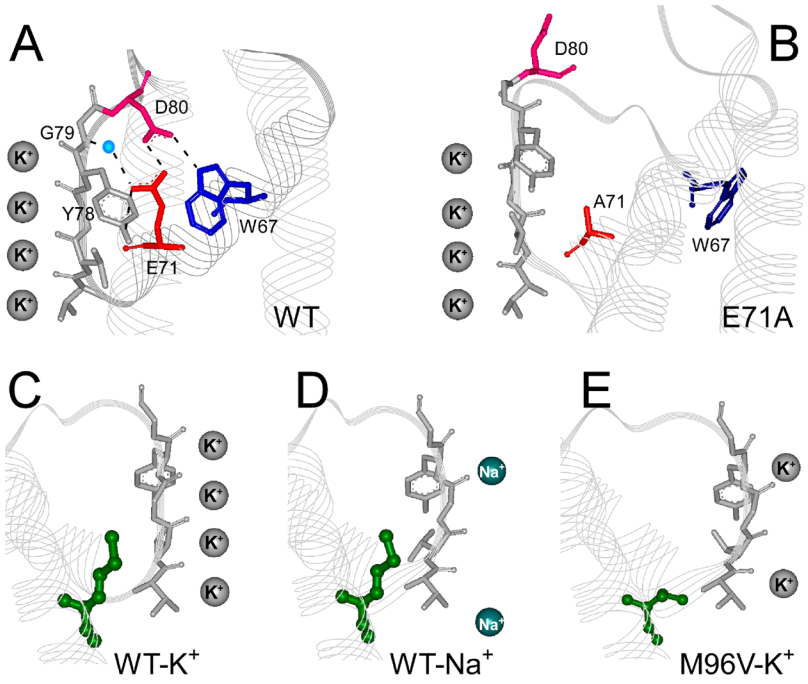
Scheme 1. Structures of KcsA Mutants[a]



[a]Panels A and B illustrate the effects of the E71A mutation on the structure of KcsA. Panel A (PDB entry 1K4C) highlights the amino acid residues involved in the stabilizing interaction network in the inactivated state of wild-type KcsA. Panel B shows that substitution of glutamate 71 with alanine disrupts the interaction with D80 and W67 among others (PDB entry 2ATK). Panels C and D focused on the conformation of the selectivity filter of wild-type KcsA (labeled WT in the scheme) in the presence of a high concentration of K^+ (panel C, PDB entry 1K4C) or Na^+ (panel D, PDB entry 2ITC). Only one of the four subunits is shown in these schemes for the sake of clarity. Panel E shows the conformation of the selectivity filter resulting from the M96V mutation in the presence of a high concentration of K^+ (PDB entry 2NLJ). It should be noticed that the latter structure resembles closely that of wild-type KcsA in the presence of Na^+, including the absence of S2 and S3 K^+ binding sites due to a reorientation of the carbonyls of G77 and V76.

type KcsA that strongly argued for the existence of two sets (high- and low-affinity) of K^+ binding sites.[24] Binding to the high-affinity sites for K^+ is related to the formation of a nonconductive KcsA*·K^+ complex. Likewise, as the concentration of K^+ is increased, binding of an additional ion takes place, giving rise to the lower-affinity, conductive form of the complex according to

$$\mathrm{KcsA} + \mathrm{K^+} \leftrightarrow \underset{\text{(nonconductive)}}{\mathrm{KcsA^*{\cdot}K^+}} + \mathrm{K^+} \leftrightarrow \underset{\text{(conductive)}}{\mathrm{KcsA{\cdot}2K^+}}$$

Thus, the nonconductive form of the channel would have a high affinity (and, thus, be highly selective) for K^+, while the open channel form would decrease the affinity for K^+ to facilitate permeation at a high rate.

As for Na^+, the physiologically blocking cation counterpart, only one set of Na^+ binding sites has been detected in wild-type KcsA, whose occupancy is ascribed to the formation of a nonconductive KcsA**·Na^+ complex different from the KcsA*·K^+ complex described above:

$$\mathrm{KcsA} + \mathrm{Na^+} \leftrightarrow \underset{\text{(nonconductive)}}{\mathrm{KcsA^{**}{\cdot}Na^+}}$$

Interestingly, a "B" binding site for Na^+ (and Li^+), different from the S1−S4 K^+ binding sites, has been inferred from in silico and structural studies,[25] which could account for the binding of Na^+ described above.

For this study, we have chosen specific point mutations of KcsA with known X-ray structures in which the selectivity filter is presumably locked into conductive or nonconductive conformations. Our goal in this report is to explore whether the altered permeation features resulting from changing the conformation of the selectivity filter in such mutant channels can be correlated with specific ion binding properties. As a model for a conductive channel, we chose the pore helix mutant E71A KcsA, which behaves as a noninactivating channel with a high opening probability.[26] The X-ray structure shows that the E71A mutation disrupts the carboxyl−carboxylate bridge between E71 and D80, which seems to be responsible for C-type inactivation in the wild-type channel[26,27] (Scheme 1A,B). Our second choice has been the M96V KcsA mutant,[21] which was originally predicted to be a "collapsed" form of the KcsA channel on the basis of the similarity of its X-ray structure with that of wild-type KcsA in presence of a high concentration of Na^+ (Scheme 1C−E) and presumably serves as a model for a nonconductive, inactivated channel.[28]

MATERIALS AND METHODS

Mutagenesis and Purification of KcsA. E71A and M96V KcsA mutants were obtained through site-directed mutagenesis, using the wild-type gene inserted into the pQE30 plasmid (Qiagen) as a template. Mutants were created by using the following synthetic oligonucleotides (Invitrogen): E71A, 5′-TGGTGGTCCGTGGCGACCGCGACG-3′ (sense) and 5′-CGTCGCGGTCGCCACGGACCACCA-3′ (antisense); M96V, 5′-GCCGTGGTGGTGGTGGTCGCCGGGATC-3′ (sense) and 5′-GATCCCGGCGACCACCACCACCACGGC-3′ (antisense). The mutations were confirmed by dideoxynucleotide sequencing.

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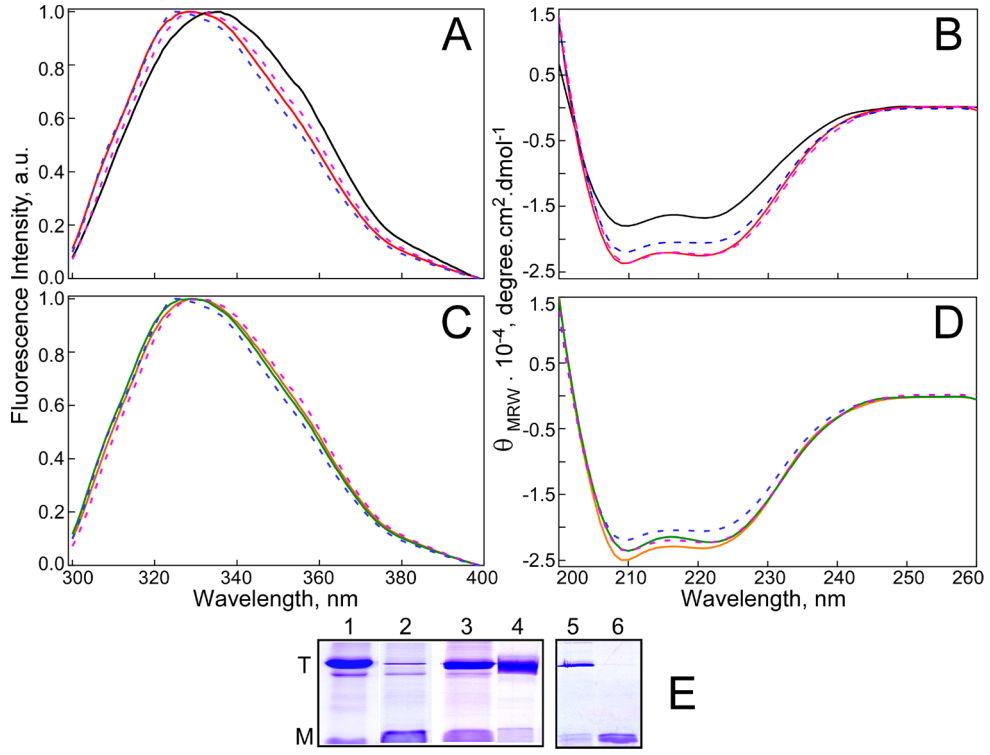

Figure 1. Structural characterization of E71A and M96V KcsA channel proteins in detergent solution (5 mM DDM). (A and B) E71A KcsA intrinsic fluorescence emission spectra (A) (1 μM protein) and far-UV CD spectra (B) (5 μM protein) of the E71A KcsA mutant in the presence of 100 mM KCl (red) or 100 mM NaCl (black). The spectra of wild-type KcsA (blue for K^+ and magenta for Na^+) are also included in these and all other panels for comparison. (C and D) Intrinsic fluorescence emission spectra and CD spectra, respectively, of M96V KcsA in 100 mM KCl (green) and 100 mM NaCl (orange). (E) SDS–PAGE (13.5% acrylamide) of DDM-solubilized E71A KcsA purified in the presence of 100 mM KCl (lane 1) or 100 mM NaCl (lane 2). Notice that under the latter conditions the E71A protein appears mostly in the form of monomers. Lanes 3 and 4 show the results of purifying the M96V KcsA mutant in 100 mM KCl or 100 mM NaCl. To confirm monomerization of the E71A mutant in the presence of NaCl, an aliquot of the mutant protein purified in the presence of 100 mM KCl (lane 5) was dialyzed against 100 mM NaCl for 48 h. After this period, only the monomeric form could be detected in the sample (lane 6). T and M within the figure stand for the tetrameric and monomeric forms of the protein, respectively.

Expression of the wild-type KcsA protein and mutants, all with an added N-terminal hexahistidine tag, was performed in *Escherichia coli* M15 (pRep4) cells, and its purification by affinity chromatography on a Ni^{2+}-Sepharose (GE Healthcare) column was conducted as reported previously.[29] The final protein stock was in 20 mM HEPES (Sigma-Aldrich) (pH 7.0), 5 mM DDM (Calbiochem), and 100 mM NaCl or 100 mM KCl (Merck). Protein concentrations were routinely determined from the absorbance at 280 nm, using a value of 34950 M^{-1} cm^{-1} as the molar extinction coefficient for the KcsA monomer, estimated from the extinction coefficients of model compounds.[30]

SDS–PAGE. Aliquots of wild-type or mutant KcsA channels were mixed with electrophoresis sample buffer[31] and loaded into a 13.5% polyacrylamide gel to check the tetramer integrity in each case. Protein bands were visualized after Coomassie Brilliant Blue staining.

Reconstitution of KcsA into Asolectin Lipid Vesicles. DDM-solubilized KcsA protein was mixed with asolectin [soybean lipids, type II-S (Sigma)] vesicles[32] previously resolubilized in 3 mM DDM at a lipid:tetrameric KcsA molar ratio of 500:1, for 2 h. Proteoliposomes were formed via removal of the detergent by gel filtration on Sephadex G-50 (medium, 15–20 mL bed volume) previously swollen overnight in buffer without detergent.[29]

Measurements of Fluorescence Spectra. The intrinsic fluorescence emission spectra were recorded on an SLM 8000 spectrofluorometer using 0.5 cm path-length quartz cuvettes, as described previously.[33] The samples were excited at 280 nm, and the emission was recorded between 300 and 400 nm in 1 nm increments. The intrinsic fluorescence in KcsA arises exclusively from five tryptophan residues present in each of the four channel subunits and at both ends of the transmembrane helical segments of the protein. Two such residues are positioned at the intracellular membrane interface (W26 and W113) and three at the opposite side of the channel (W67, W68, and W87).[24,33]

Fluorescence Monitoring of Thermal Denaturation. Thermal denaturation of DDM-solubilized KcsA was monitored in a Varian Cary Eclipse or a PTI QuantaMaster spectrofluorometer by recording the temperature dependence of the protein intrinsic emission at 340 nm after excitation at 280 nm. The temperature up-scan rate was set to 0.6 °C/min in all the experiments, matching the conditions of previously reported studies.[24] Experiments were performed under noncompetitive conditions, at a final KcsA concentration of 1 μM solubilized in a buffer containing 20 mM HEPES (pH 7.0), 5 mM DDM, and 5 mM NMDG. The initial Na^+ concentration, coming from the protein stock, was 1.5 mM, except for the E71A channel, which was characterized in 1.5 mM K^+ as the initial condition. Increasing amounts of either NaCl or KCl

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were then added to the samples, and the binding of each cation was analyzed separately. In both approaches, the midpoint temperature of dissociation and unfolding of the tetramer (T_m) was calculated from the thermal denaturation curve by fitting the data (changes in fluorescence intensity at 340 nm with an increase in temperature) to a two-state unfolding model, assuming a linear dependence of the pre- and post-transition baselines on temperature.[34]

The increase in the T_m observed at increasing concentrations of the ligand cations is directly related to the degree of ion occupancy of the selectivity filter[24,33,35,36] and can be used to estimate the dissociation constant of the protein−ligand complex using the following equation

$$\frac{\Delta T_m}{T_m} = \frac{T_m - (T_m)_0}{T_m} = \frac{R(T_m)_0}{\Delta H_0} \ln\left(1 + \frac{[L]}{K_D}\right) \quad (1)$$

where T_m and $(T_m)_0$ refer to the denaturation temperature (in kelvin) for the protein in the presence and absence of ligand, respectively (we use the term t_m to refer to the midpoint denaturation temperature in degrees Celsius), R is the gas constant, and ΔH_0 is the enthalpy change upon protein denaturation in the absence of ligand.[24] Monitoring the change in the melting temperature of a protein as a result of ligand binding has been the method of choice for characterizing the binding energetics in many instances.[37−41]

Circular Dichroism. Far-UV circular dichroism (CD) spectra were recorded on a Jasco J810 spectropolarimeter at a scan rate of 100 nm/min using a 0.5 nm resolution. The mean residue ellipticity, θ_{MRW}, was calculated as described in ref 42. Aliquots of KcsA were diluted with 20 mM Tris (pH 7.0), 5 mM DDM, 50 mM Na_2SO_4, or K_2SO_4 to a final concentration of 5 μM to obtain the average of six spectra.

Electrophysiological Recordings in Reconstituted Giant Liposomes. Inside-out patch clamp recordings[43] were conducted on excised patches from giant liposomes containing wild-type KcsA, as reported previously.[44] Recordings were obtained using an EPC-10 (Heka Electronic, Lambrecht/Pfalzt, Germany) patch clamp amplifier, at a gain of 50 mV/pA. The holding potential was applied to the interior of the patch pipet, and the bath was maintained at virtual ground. The recordings were filtered at 1 kHz, and the data were analyzed with Clampfit-9 (Axon Instruments). An Ag-AgCl wire was used as the reference electrode through an agar bridge. The measurements were taken at room temperature, and unless indicated otherwise, the pipet (extracellular side) solution contained 10 mM HEPES buffer (pH 7) and 100 mM KCl and the bath solution contained 10 mM MES buffer (pH 4) and 100 mM KCl for regular measurements. The solutions used for selectivity experiments under bi-ionic conditions are described in the legend of Figure 7. Voltage ramps or different holding potentials (see the legends of Figures 6 and 7) were routinely imposed on the membrane patches to record E71A and M96V KcsA currents. The inactivation rates for the M96V mutant and wild-type KcsA were estimated by measuring the current at 150 mV from voltage ramps taken at different times. Data were normalized to the initial current value and plotted versus time.

RESULTS

Ions as Effectors of the Structure and Stability of the E71A and M96V KcsA Mutant Channels. Both in detergent solution and in a membrane bilayer, the wild-type KcsA channel undergoes characteristic changes in its conformation and stability when in the presence of permeating (K^+) or nonpermeating (Na^+) ionic species. Indeed, the ion-induced shifts in the intrinsic fluorescence emission spectra of the protein have been used previously to describe ion binding.[24,33,35]

Like wild-type KcsA, the detergent-solubilized E71A and M96V mutant channels used here also respond to the presence of ions as effectors of the channel proteins (Figure 1). In the presence of K^+, the intrinsic fluorescence emission spectrum of the E71A mutant is positioned halfway between those of the K^+-saturated and Na^+-saturated wild-type KcsA, while in the presence of Na^+, the spectrum of the mutant channel is much more red-shifted (Figure 1A). This seems consistent with the observation that Na^+ causes partial unfolding of the E71A mutant, as revealed by circular dichroism (Figure 1B), as well as dissociation of the tetrameric channel into its constituent subunits (Figure 1E). Interestingly, a similar phenomenon of dissociation of the tetrameric E71A mutant channel is also observed at very low (submillimolar) K^+ concentrations (data not shown).

In the M96V mutant channel, the fluorescence spectrum of the protein is much less sensitive to the presence of either K^+ or Na^+ than that of the E71A mutant, indicating that the protein tertiary structure in M96V is less dependent on the type of monovalent cation used in the experiments (Figure 1D). Spectral shifts in M96V with respect to wild-type KcsA are also different from those observed with the E71A mutant: a red shift is observed in K^+ buffers, and a blue shift occurs in Na^+ (Figure 1C). In addition, the CD spectra of the M96V mutant channel reveal that neither K^+ nor Na^+ causes a significant departure in the secondary structure from that observed in wild-type KcsA (Figure 1D).

With regard to the quaternary structure of the channel proteins, it should be noted that as for the wild-type KcsA channel, the two mutants are generally expressed and purified as homotetramers that are stable enough to remain as such in SDS−PAGE (Figure 1E). The most noticeable exception to this is the E71A mutant in Na^+ medium, which appears almost exclusively as monomeric species in the electrophoretic analysis, in apparent agreement with the structural deterioration suggested from the fluorescence and circular dichroism studies.

Monitoring the ion-induced changes in the protein's intrinsic fluorescence as a function of temperature has been used to characterize binding of ions to the detergent-solubilized channels. The advantage of using this procedure versus previously explored monitoring of ion-induced fluorescence spectral shifts has been discussed previously.[24] KcsA undergoes an irreversible, cooperative process of thermal denaturation as the temperature is increased, which includes the dissociation of the tetrameric protein into individual monomers, which is concomitant with its partial unfolding.[42] Panels A and B of Figure 2 show examples of such thermal denaturation processes in the two mutant channels, in both K^+ and Na^+ media. As expected, thermal denaturation of the E71A mutant in Na^+ does not exhibit sigmoidal behavior, likely because this mutant channel is already denatured (partly unfolded and dissociated into monomers) in the presence of Na^+. On the other hand, the E71A mutant in K^+ buffer exhibits a clearly sigmoidal denaturation curve, although its thermal stability (evaluated as the midpoint temperature of the cooperative transition, t_m) is greatly diminished with respect to that of wild-type KcsA under identical conditions (K^+ buffer) and in fact resembles closely that of wild-type KcsA in Na^+ medium (Figure 2A).

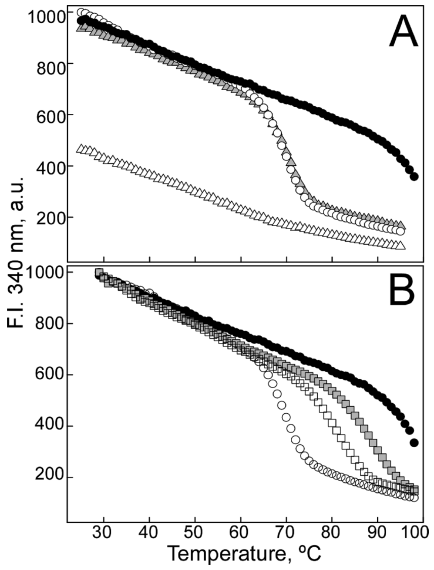

Figure 2. Fluorescence intensity monitoring of thermal denaturation of KcsA mutant channels. All experiments were conducted at a channel protein concentration of 1 μM in 20 mM HEPES (pH 7.0) and 5 mM DDM containing either KCl or NaCl at the indicated concentrations. The denaturation curves of the wild-type KcsA channel in 100 mM KCl (●) or 100 mM NaCl (○) are included in the plots for comparison. (A) E71A KcsA in 100 mM KCl (▲) shows a sigmoidal transition with a t_m of approximately 70 °C, whereas in the presence of 100 mM Na^+ (△), no sigmoidal behavior could be detected. (B) M96V KcsA shows similar sigmoidal transitions in the presence of either 100 mM KCl (■; t_m ~ 88 °C) or 100 mM NaCl (□; t_m ~ 81 °C).

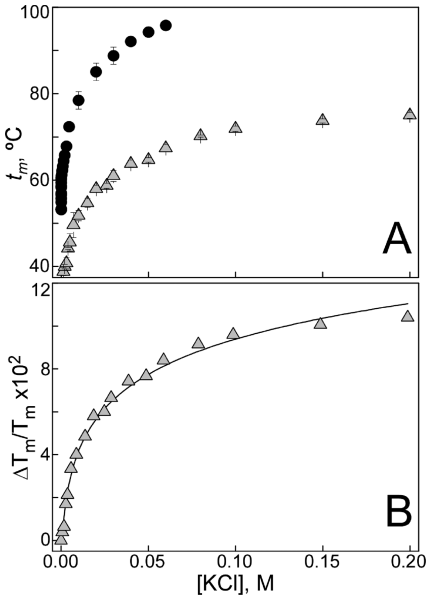

Figure 3. Dependence of the thermal stability of E71A KcsA on K^+ concentration. (A) Thermal stabilization of the mutant protein [▲; 1 μM protein in 20 mM HEPES (pH 7.0), 5 mM DDM, 5 mM NMDG, and 1.5 mM KCl] upon addition of increasing amounts of KCl. Filled circles represent the K^+-dependent stabilization of 1 μM wild-type KcsA [20 mM HEPES (pH 7.0), 5 mM DDM, 5 mM NMDG, and 1.5 mM NaCl], which have been included for comparison. Results are shown as $t_m \pm$ SD (n = 3). (B) Fitting of experimental data for binding of K^+ to E71A KcsA to eq 1. Solid lines represent the best fits of the experimental data points to the cation binding model described in Materials and Methods. The estimated dissociation constant for the single set of binding sites detected in these experiments is 3.5 ± 0.5 mM.

As for the M96V mutant channel in the presence of either cation (Figure 2B), we observed that the thermal denaturation curves fall between those seen for wild-type KcsA in K^+ and Na^+ media, showing a decrease in the thermal stability when in K^+ but an increase when in the presence of Na^+ in the buffer.

Characterization of Binding of Ions to the Mutant Ion Channels. The thermal denaturation assay described above has been used to study binding of K^+ and Na^+ to the E71A and M96V KcsA channel mutants under noncompetitive conditions. We chose such a procedure because the differential effects of increasing the concentration of the cations on the t_m of the protein denaturation processes are remarkably strong and strongly dependent on the type and concentration of ions present in the buffer, thus facilitating the quantitation of the ion binding phenomena. For such purposes, batches of the purified KcsA mutants were prepared in the presence of low concentrations of monovalent cations (Na^+ in the case of the M96V mutant and K^+ for the E71A mutant, at ~1.5 mM in either case); then either ≤200 mM Na^+ or K^+ was added separately to the KcsA mutant samples, and they were subjected to thermal denaturation. The low concentrations of monovalent cations used in each case were chosen as the starting point in these titrations to guarantee a low occupancy of the ion binding sites, so that most of the channel population to be titrated is initially present as the cation-free form.[33] It should be mentioned here that in the case of the E71A mutant, K^+ at <1 mM or Na^+ over the whole concentration range abolishes the characteristic sigmoidal behavior seen in the thermal denaturation curves.

Figure 3A shows the results of titrating the E71A mutant with K^+. A first observation is that K^+ increases greatly the thermal stability of the mutant channel, as the t_m is increased by more than 30 °C in response to an increase in the K^+ concentration from 1.5 to 200 mM. Second, the stabilization of the E71A mutant protein induced by K^+ can be described in terms of the occupation of a single set of fairly low affinity ion binding sites (K_D ~ 3.5 mM) (Figure 3B). Both the K_D and the increase in t_m are quite similar to those reported for the second, low-affinity K^+ binding event in the wild-type channel, ascribed to the formation of the conductive state of the selectivity filter.[24] Obviously, Na^+ binding experiments could not be conducted with the detergent-solubilized E71A mutant, as Na^+ by itself over the whole range of concentrations used in these studies causes denaturation of the protein.

For the M96V mutant channel, both K^+ and Na^+ titrations were conducted. Figure 4A shows that again, K^+ significantly increases the thermal stability of the M96V mutant channel, although the observed stabilization is weaker than in wild-type KcsA. Interestingly, the increase in t_m observed in this mutant with potassium concentration occurs in two phases, suggesting the existence of not one but two thermodynamically different sets of ion binding sites that are occupied successively as the protein becomes saturated with the ion (Figure 4B–D). The occupancy of the first set of high-affinity K^+ binding sites over the lower K^+ concentration range (<25 mM) results in an increase in the t_m of approximately 15 °C, which is followed by an additional 12 °C increase in the t_m when the second set of K^+ binding sites becomes occupied at higher concentrations (≤200 mM). The apparent dissociation constants were estimated to be approximately 57 μM for the higher-affinity

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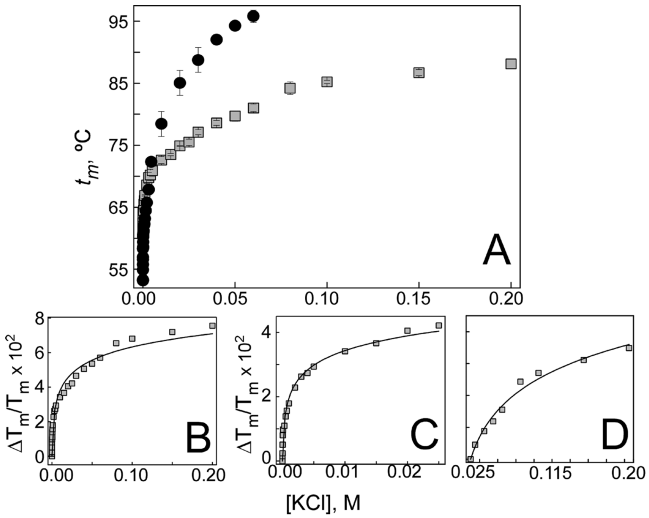

Figure 4. Dependence of the thermal stability of M96V KcsA (■) on K^+ concentration. Experimental conditions are described in the legend of Figure 3. The behavior of wild-type KcsA (●) is also included in panel A to facilitate comparison. Panel B shows that fitting to eq 1 for binding of K^+ to a single set of binding sites on the channel fails when taking into account the whole titration curve but suffices when the low K^+ (C) and high K^+ (D) concentration ranges in the titration curves are analyzed separately, suggesting that at least two different sets of K^+ binding sites are present in the KcsA protein. The estimated dissociation constants for such high- and low-affinity K^+ binding sites in M96V KcsA are 57 ± 14 μM and 41 ± 11 mM, respectively.

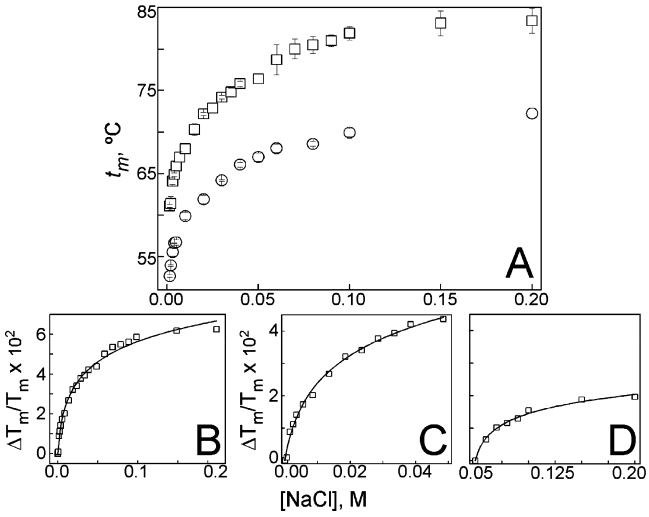

Figure 5. Dependence of the thermal stability of M96V KcsA (□) on Na^+ concentration. Experimental conditions are as described in the legend of Figure 3. The behavior of wild-type KcsA (○), in which Na^+ is less stabilizing than in the M96V mutant, is also included in panel A to facilitate comparison. Panel B shows that as in the K^+ titration, fitting to eq 1 fails when taking into account the whole Na^+ titration curve but suffices when the low Na^+ (C) and high Na^+ (D) concentration ranges are analyzed separately, suggesting that, again, at least two different sets of Na^+ binding sites are present in the M96V KcsA mutant channel. The estimated dissociation constants for such high- and low-affinity Na^+ binding sites in M96V KcsA are 3.8 ± 0.5 and 54 ± 12 mM, respectively.

sites and 41 mM for the lower-affinity sites. These indicate an approximately 20-fold decrease in the affinities for K^+ in this mutant channel with respect to those exhibited by the corresponding high- and low-affinity sites in wild-type KcsA (1.9 μM and 2.5 mM, respectively).[24]

Figure 5 shows the results obtained from titration of the M96V mutant channel with increasing concentrations of Na^+. First, we observed that Na^+ stabilizes the M96V mutant channel against thermal denaturation (i.e., it increases the t_m) more markedly than in the wild-type KcsA protein (Figure 5A). Second, unexpectedly, we found that the increase in t_m observed in this mutant with Na^+ concentration takes place in two phases, instead of being monophasic as in the wild-type protein. This suggests that, like that seen in the binding of K^+, there are two thermodynamically different sets of Na^+ binding sites in this mutant protein that are occupied successively as the protein becomes saturated with the ion (Figure 5B–D). The occupancy of the first set of higher-affinity Na^+ binding sites increases the t_m by nearly 16 °C (when the Na^+ concentration reaches 50 mM) and has a K_D value of approximately 3.8 mM, very similar to the single event reported in the wild-type channel (3.3 mM). A further increase in the Na^+ concentration to 200 mM leads to an additional increase in the t_m of 6 °C when the second set of Na^+ sites are occupied, with an apparent K_D for the ion binding process of approximately 54 mM.

Ion Permeation through the E71A and M96V KcsA Mutant Channels. Wild-type KcsA reconstituted into giant liposomes exhibits different patterns of channel activity, which have been correlated with different supramolecular assemblies and interactions among individual channels.[44] The more frequent pattern has a characteristic low opening probability (LOP pattern) and most other features reported for KcsA reconstituted into planar lipid bilayers.[45−47] The predominant events in this LOP pattern correspond to the gating of individual single channels or the positive coupling of two KcsA channels. However, other activity patterns are also detected almost as frequently, which are characterized by a high channel opening probability (HOP patterns) and by the positive coupling of several (most often five or multiples of five) concerted channels.[44]

Figure 6 shows recordings from excised giant liposome patches containing the reconstituted E71A (Figure 6A,B) or M96V (Figure 6C–F) KcsA mutant. The E71A mutant exhibits three major relevant features: (i) a greatly diminished ability to undergo coupled gating (i.e., uncoupled single-channel currents are detected as the predominant gating event in most of the cases), (ii) a very high channel opening probability, and (iii) a low rate of inactivation on the experimental time scale. These features essentially coincide with reports by others[26,27,48,49] and cause the predominant events seen in the E71A recordings that correspond to a single channel "frozen" in a conductive conformation for K^+. Interestingly, the E71A mutant channel maintains such K^+ permeation features even at low concentrations of the cation (down to 5 mM on both sides of the liposome patch), suggesting that the frozen conductive conformation mentioned previously is indeed constitutive in the E71A mutant and does not collapse in response to low K^+ concentrations. This seems also fully consistent with recently published crystallographic evidence of the lack of collapse of the E71A mutant in the absence of K^+.[48]

As stated above, substituting K^+ with Na^+ causes the E71A channel in detergent solution to partly unfold and dissociate into its constituent subunits, thus preventing Na^+ binding experiments from being conducted. Interestingly, when reconstituted into asolectin liposomes, the E71A mutant channel remains as a tetramer as determined by SDS–PAGE upon extensive exposure to Na^+ buffers (data not shown). This

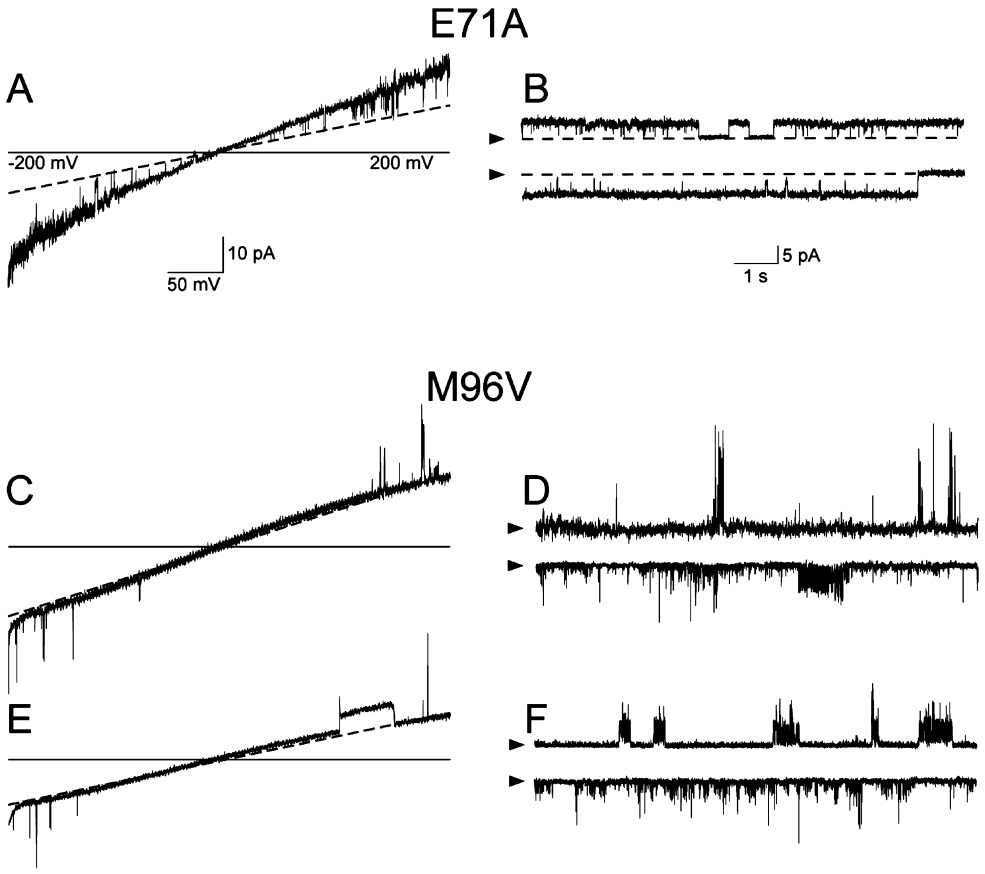

Figure 6. (A, C, and E) Typical voltage ramps (−200 to 200 mV, 133 mV/s) obtained by patch clamping excised inside-out patches from reconstituted giant liposomes containing the E71A (A) or M96V (C and E) KcsA mutant. Symmetrical solutions of K^+ (100 mM KCl on both extra- and intracellular sides for panels A and C) or symmetrical Na^+ solutions (100 mM NaCl on both sides for panel E) were routinely used in the experiments. (B, D, and F) Recordings from the mutant channels taken at 150 and −150 mV (top and bottom traces, respectively, in each of the panels) under otherwise identical experimental conditions. Notice the occurrence of single-channel as well as coupled gating events in the M96V channel in either a K^+ (D) or a Na^+ (F) solution. In this figure and Figure 7, the dashed line and the arrowheads indicate the closed channel state. Channel openings appear as upward (at positive voltages) or downward (at negative voltages) deflections over the closed state line.

is evidence that the insertion of the protein into the lipid bilayer provides protection to the mutant channel against ion-induced dissociation and unfolding and, more relevant to this work, allows channel recordings to be taken for this mutant channel in the presence of Na^+. We found that the mutant E71A channel exhibits blockade by Na^+ when added intracellularly, while no Na^+ currents were detected under bi-ionic conditions, with the Na^+ solution bathing the extracellular side of the channel (Figure 7A). These latter observations, along with reports by other authors on the selectivity of the E71A mutant[26,48,50] and a similar E71V mutant,[51] suggested that the mutation retains qualitatively the selectivity properties of KcsA,[47] which clearly prefers K^+ over Na^+ (for instance, Na^+/K^+ permeability ratios ranging 0.03 to 0.14 are reported in ref 48). This is further confirmed by the observation of a shift to negative values in the reversal potential under bi-ionic conditions (Figure 7B), which indicates a marked preference of the E71A mutant for K^+ versus Na^+. Nonetheless, ion selectivity of the E71A mutant in strict terms seems to be a controversial issue, and while some authors report selectivity properties comparable to that of the wild-type channel,[26,50] others have found a decreased selectivity for K^+ versus other monovalent metal ions.[48]

The M96V mutant channel was assayed in both K^+ and Na^+ media. Because of the similarity between the crystal structure of this mutant channel and that of wild-type KcsA in presence of Na^+ (see Scheme 1), M96V was identified as a collapsed channel, although no electrophysiological evidence was provided to demonstrate that fact.[21] We found that although the M96V mutant is inactivated ∼4-fold faster than wild-type KcsA, this mutant channel cannot be identified as a collapsed channel. Indeed, the M96V channel when bathed in symmetrical K^+ solutions shows an electrophysiological behavior reminiscent of that seen in wild-type KcsA.[44] This includes the single-channel conductance, the flickering behavior, or the presence of LOP and HOP patterns of electrical activity comprising gating events ranging from single channels to the coupling of large assemblies of channels (Figure 6D). Additionally, opposite to the case for the wild-type channel, Na^+ does not block the mutant M96V channel, which indeed conducts Na^+ almost as efficiently as K^+ when using either symmetrical solutions [mean slope single-channel conductances of 31 ± 1 and 29 ± 1 pS have been determined in 100 mM KCl and 100 mM NaCl, respectively (Figure 6E,F)] or bi-ionic conditions (Figure 7C). Therefore, the most salient features of the M96V mutant channel are its rapid inactivation and its ability to accept both Na^+ and K^+ as conducting ionic species.

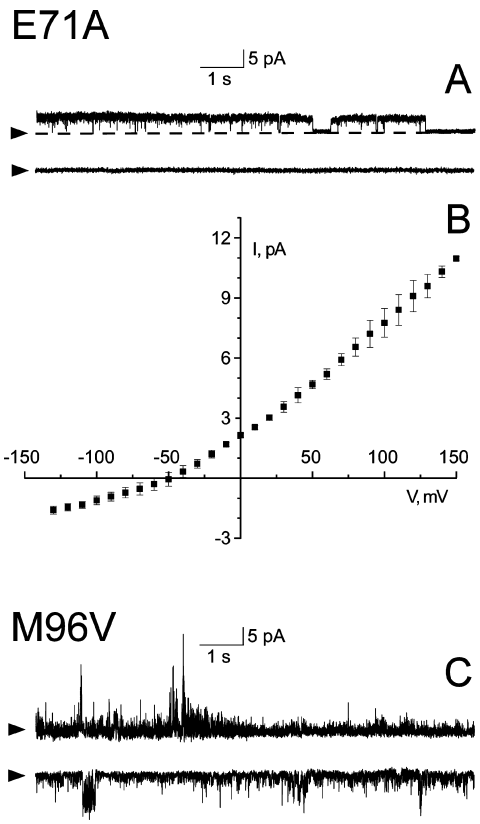

Figure 7. (A and C) Representative recordings taken at 150 and −150 mV (top and bottom traces, respectively) obtained under bi-ionic conditions [10 mM HEPES buffer (pH 7) and 100 mM NaCl in the pipet solution and 10 mM MES buffer (pH 4) and 100 mM KCl in the bath] by patch clamping excised, inside-out patches bearing E71A (A) and M96V (C) KcsA mutant channels. Notice that Na^+ currents (appearing as inward currents at negative voltages) were observed only in the M96V mutant. (B) *I*−*V* plot for E71A mutant-mediated currents measured also under bi-ionic conditions but at higher salt concentrations to allow more precise current measurements (400 mM KCl on the intracellular side; 390 mM NaCl and 10 mM KCl on the extracellular side, respectively, instead of 100 mM KCl and 100 mM NaCl, which were used for the measurements in panels A and C). Symbols represent the average value of the current (±SD) at the different holding potentials taken from five separate experiments.

DISCUSSION

Here we report on experiments that examined binding of ions to mutant KcsA channels presumed to be frozen in defined conformations of their selectivity filters. The rationale behind these experiments is that such defined conformations could perhaps be correlated with specific ion binding properties. For this purpose, we chose the E71A and M96V mutants of KcsA (see Scheme 1 for details about their structure), which were reported to be fixed in a mostly open, conductive conformation[26,27] and in a collapsed, nonconductive state,[21] respectively. As for the E71A mutant, our patch-clamp measurements on reconstituted giant liposomes essentially coincide with the observations by others[26,48] and led to the conclusion that the E71A mutant has a very low rate of inactivation and a very high opening probability. Therefore, the E71A mutant can indeed be considered in practical terms as a channel locked in a conductive conformation, which remains without collapsing even at low potassium concentrations. Interestingly, K^+ binding experiments with this mutant channel reveal the existence of just a single set of low-affinity K^+ binding sites, which by analogy with the low-affinity, wild-type KcsA·2K^+ conductive complex mentioned in the introductory section seems consistent with the behavior expected for a conductive conformation. A low-affinity, conductive complex would certainly favor the occurrence of ion permeation, but it does not guarantee selectivity in the E71A mutant channel. In fact, exclusively on the basis of the binding affinity features, it should be predicted that this mutant channel would not be selective for K^+ and, thus, should be expected to conduct other ionic species as well. Such a prediction, however, is in contrast with reports by other authors,[26,48,50,51] as well as with our own experimental findings on the selectivity for K^+ of the E71A channel, which seems comparable to that of wild-type KcsA. A possible explanation of this apparent discrepancy is that the set of high-affinity K^+ binding sites required to conform to our hypothesis is not thermodynamically stable in detergent solution and, therefore, cannot be detected in our ion binding experiments. The observed dissociation of the tetrameric E71A mutant into subunits in detergent solution either at a very low potassium concentration or in the presence of sodium (i.e., with a low occupancy of binding sites in either case) seemingly supports this interpretation. Also, given the increased stability observed for this protein when it is reconstituted into a lipid bilayer, in which the tetrameric structure is preserved regardless of ion type and concentration, it should be expected that the putative set of high-affinity K^+ binding sites would also be protected by the membrane lipids to provide the observed selectivity.

The M96V mutant was originally predicted to be a collapsed form of the KcsA channel on the basis of its crystallographic structure, although no electrophysiological measurements were provided to confirm such a claim.[21] Here we found that the M96V mutant is inactivated quite rapidly, but prior to losing its activity, it behaves somewhat like the wild-type channel in that it displays LOP and HOP patterns of electrical activity. Nonetheless, the M96V mutant differs greatly from wild-type KcsA in two main aspects: (1) the much higher rate of inactivation and (2) the conduction of Na^+, which take place here as with K^+. This latter functional peculiarity of the M96V mutant channel is somewhat expected from the alteration seen in the S2 site, which is thought to be mostly responsible for ion selectivity in KcsA[52] and is of particular relevance to the ion binding measurements, because two sets of high- and low-affinity binding sites have been detected in this study for Na^+ and K^+. This is reminiscent of that seen in the binding of the conducting species, K^+, to wild-type KcsA,[24] and therefore, it seemingly predicts that both Na^+ and K^+ should be conducted by the M96V mutant, which is indeed observed experimentally. This is consistent with the marked similarity of the fluorescence spectra of the M96V mutant in sodium and potassium, which suggests that both ions induce a similar conformation of the selectivity filter. Also, the comparison with the behavior of wild-type KcsA in K^+ predicts that the occurrence of low- and high-affinity binding sites for both cations in the M96V mutant should result in somewhat similar ion concentration-dependent affinity state transitions, and thus, gating features qualitatively similar to that of wild-type KcsA should be expected. This seems to correlate with the findings of LOP and HOP patterns of activity for the M96V mutant channel in the presence of either Na^+ or K^+. Moreover, even the rapid disappearance of activity in the M96V patches is plausibly explained by the ion binding results: For the observed affinities and at the cation

concentration of 100 mM (either K^+ or Na^+) used in the recordings, the conductive channel states in the M96V mutant could not be fully populated, at equilibrium nor kinetically. This should favor the nonconductive, higher-affinity states, which in turn increases the possibility of channel collapse, a phenomenon favored by the low occupancy of the selectivity filter by the permeating ion. It should be noted here that a previous isothermal scanning calorimetry study[21] did not show binding of K^+ to the M96V mutant. Such an apparent discrepancy could be explained on the basis of the experimental conditions used by those authors to study K^+ binding (in the continuous presence of 100 mM Na^+), along with the dissociation constants for the two cations determined here, which clearly preclude the observation of K^+ binding in those experiments.

Our attempts to explain ion permeation features of model ion channels on the basis of ion binding parameters encounter a major conceptual difficulty in that the two major types of experimental results used here, i.e., patch-clamp recordings and ion binding measurements (or reported X-ray crystallography data), are obtained under markedly different experimental conditions. Briefly, the patch-clamp measurements (i) are probably conducted under far-from-equilibrium conditions (and, thus, are kinetically limited by potential energy barriers to ions and other factors), (ii) use the ion channel proteins reconstituted into giant liposomes, and (iii) record ionic currents that imply that the outer and inner gates in the KcsA channel (at the selectivity filter and at the intracellular ends of transmembrane helical segments, respectively) must both be opened for ions to flow. In contrast, ion binding experiments (i) use a much longer experimental time scale (equilibrium or near-equilibrium conditions) and (ii) are conducted with detergent-solubilized channel proteins and at neutral pH in an attempt to avoid gating by the inner gate and, thus, to underline inasmuch as possible the role of the selectivity filter in the process. In spite of these experimental differences, it seems remarkable that certain channel gating, ion selectivity, and permeation features can be grossly predicted on the basis of fairly simple and straightforward ion binding experiments. We propose the term "ion affinity hypothesis" to refer to these attempts to partly explain ion channel function on the basis of ion binding by the channel protein. In essence, we found that ion binding is a major source of stability for the channel protein. This is caused by ion-mediated intersubunit interactions at the channel's selectivity filter, bridging adjacent subunits and holding together the characteristic tetrameric assembly.[33,35] Indeed, weakening such interactions by modifying the selectivity filter and by using an inappropriate ion, such as the E71A mutant in detergent solution and in a Na^+ medium, results in partial unfolding and complete dissociation of the channel into its constituent subunits. Second, depending upon ion type and concentration, binding of ions leads to different conformations of the channel protein that exhibit different ion binding affinities. High-affinity states guarantee binding of specific ions and thus, via ion selectivity, have a lower occupation of the available binding sites and are nonconductive. On the other hand, low-affinity states would not be expected to discriminate well among different ions present at moderate or high concentrations, have a higher ion occupancy of the available binding sites, and most importantly allow ion permeation to occur. Finally, as similarly proposed previously by others,[33,53] it seems likely that the conformational transitions between high-affinity (nonconducting) and low-affinity (conducting) states constitute the basis of channel gating.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

CD, circular dichroism; DDM, dodecyl β-D-maltoside; ΔH_o, enthalpy change upon protein unfolding in the absence of ligand; KcsA, potassium channel from *Streptomyces lividans*; K_D, dissociation constant; LOP, low-opening probability pattern; HOP, high-opening probability pattern; NMDG, *N*-methyl-D-glucamine; SD, standard deviation; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; t_m, midpoint denaturation temperature (in degrees Celsius).

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