9480 *Biochemistry* **2010,** *49,* 9480–9487
DOI: 10.1021/bi101235v

BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Ion Binding to KcsA: Implications in Ion Selectivity and Channel Gating[†]

M. L. Renart, I. Triano, J. A. Poveda, J. A. Encinar, A. M. Fernández, A. V. Ferrer-Montiel, J. Gómez, and J. M. González Ros*

Instituto de Biología Molecular y Celular, Universidad Miguel Hernández, Elche, 03202 Alicante, Spain

Received August 3, 2010; Revised Manuscript Received October 6, 2010

ABSTRACT: Binding of K^+ and Na^+ to the potassium channel KcsA has been characterized from the stabilization observed in the heat-induced denaturation of the protein as the ion concentration is increased. KcsA thermal denaturation is known to include (i) dissociation of the homotetrameric channel into its constituent subunits and (ii) protein unfolding. The ion concentration-dependent changes in the thermal stability of the protein, evaluated as the T_m value for thermal-induced denaturation of the protein, may suggest the existence of both high- and low-affinity K^+ binding sites of KcsA, which lend support to the tenet that channel gating may be governed by K^+ concentration-dependent transitions between different affinity states of the channel selectivity filter. We also found that Na^+ binds to KcsA with a K_D similar to that estimated electrophysiologically from channel blockade. Therefore, our findings on ion binding to KcsA partly account for K^+ over Na^+ selectivity and Na^+ blockade and argue against the strict "snug fit" hypothesis used initially to explain ion selectivity from the X-ray channel structure. Furthermore, the remarkable effects of increasing the ion concentration, K^+ in particular, on the T_m of the denaturation process evidence that synergistic effects of the metal-mediated intersubunit interactions at the channel selectivity filter are a major contributor to the stability of the tetrameric protein. This observation substantiates the notion of a role for ions as structural "effectors" of ion channels.

Ion channels recognize and selectively bind specific ions while allowing their permeation at near diffusion-limited rates. How to reconcile these two apparently opposed phenomena, ion selectivity and rapid permeation, still remains a matter of debate (*1*, *2*) The pioneering work by MacKinnon and co-workers on solving the protein structure of several prokaryotic potassium channels at high resolution provides a structural framework to attempt explaining the above fundamental properties. KcsA,[1] a potassium channel from *Streptomyces lividans*, was the first of such structures to be solved (*3*) and has been widely used to investigate the molecular basis of ion selectivity and permeation. KcsA is a homotetramer in which each subunit defines two transmembrane segments connected by a pore region that contains a tilted short α-helix (pore helix) spanning inward to about one-third of the membrane thickness and an ion selectivity filter with the sequence TVGYG unmistakably homologous to the more complex eukaryotic potassium channels, which points toward the membrane surface. The ion conduction pathway in KcsA (*3*, *4*) consists of a narrow upper region contributed by the amino acid residues within the selectivity filter, in which the backbone carbonyl oxygens point toward the pore to create a stack of multiple potassium binding sites at which K^+ may bind in a dehydrated form. The pore has also a lower and wider, water-filled region called the cavity, which opens to the cytoplasm. Selectivity for K^+ over the physiologically relevant Na^+ is exerted both at the cavity but mostly at the selectivity filter. In the cavity, the ions enter in a fully hydrated form and become partly stabilized near the inner mouth of the narrower selectivity filter by the four dipolar pore helices, which point their negatively charged C-terminal ends toward the hydrated ions. Weak selectivity for K^+ over the physiologically relevant Na^+ is found for the cavity with much stronger K^+ vs Na^+ selectivity observed at the filter ($\sim 10^3$) (*5*). The latter was initially explained based on strict geometrical constraints; i.e., the carbonyls in the selectivity filter in the X-ray structure are at precisely the right spatial locations and distances to effectively replace the hydration waters surrounding K^+. On the contrary, Na^+ ions are too small, and the selectivity filter could not be sufficiently constricted to accommodate the dehydrated ion into it, and thus, there would be a higher energy barrier for transferring Na^+ over K^+ from the water media (*6*). In this "snug-fit" hypothesis, the lack of flexibility of the selectivity filter to accommodate different ions plays a key role in defining the selectivity properties of the channel. More recently, however, an "induced-fit" hypothesis appeared in which the channel's selectivity filter is flexible enough to conform more favorably to an ion of a particular size (*1*, *7*). Finally, other hypotheses to explain selectivity are based on the way the ions become coordinated inside the selectivity filter and on the repulsion between adjacent carbonyl oxygens, with emphasis on either the chemistry of the coordinating ligands ("field strength" hypothesis (*8*)) or the number of ligands that coordinate each ion ("overcoordination" hypothesis (*9*−*12*)). Unfortunately, all of the above hypotheses can be reconciled sufficiently well with the crystallographic structures available, and therefore, additional experimental information is required if any of such alternatives is to be favored

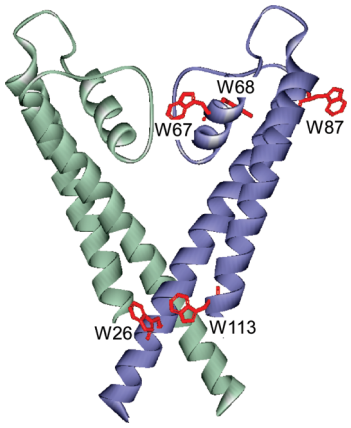
[†]Supported by grants from the Spanish DGI BFU2008-0062/BMC, BFU2009-08346, and Consolider-Ingenio 2010 CSD2-2008-00005.

*To whom correspondence should be addressed. Phone: + 34 96 6658757. Fax: + 34 96 6658758. E-mail: gonzalez.ros@umh.es.

[1]Abbreviations: KcsA, potassium channel from *Streptomyces lividans*; DDM, dodecyl β-D-maltoside; SDS−PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; CD, circular dichroism; t_m, midpoint denaturation temperature; NMDG, *N*-methyl-D-glucamine; K_D, dissociation constant; ΔG_{unf}, free energy change upon protein unfolding; ΔH_{unf}, enthalpy change upon protein unfolding.

 Published on Web 10/06/2010

Scheme 1: Structural Representation of KcsA as from PDB 1K4C[a]



[a]Only two out of the four identical subunits have been drawn for clarity. The location and numbering of the tryptophan residues have been indicated in one of the subunits, three at the extracellular mouth (W67, W68, and W87) and two at the intracellular ends of the transmembrane segments (W26 and W113).

over the others. Whatever the case might be, it is clear that the interaction of either permeating or blocking ions with ion channels goes far beyond their passive passage through the aqueous ion channel pore and approaches that of truly protein "effectors" in which the presence of a given ion at a given concentration could modulate fundamental properties in many channels, such as their selectivity or gating (*13*, *14*).

We previously reported on binding of Na^+ and K^+ to KcsA. The channel contains five tryptophan residues per subunit located at both ends of the transmembrane helical segments of the protein. Two of these residues are positioned at the intracellular membrane interface (W26 and W113) and three at the opposite side of the channel (W67, W68, and W87) (Scheme 1) (*3*). Monitoring the spectral shift of the protein intrinsic fluorescence in the presence of increasing concentration of the monovalent cations reveals that the two ions bind competitively to KcsA with distinct affinities, leading to different conformations of the ion-bound KcsA complexes (*15*). Here we used the intrinsic fluorescence to monitor thermal denaturation of KcsA as it provides a much better experimental observable than previous spectral shift-based measurements. This allows for the observation of different sets of ion binding sites which we were unable to observe before. Our findings on the existence of high- and low-affinity potassium binding sites on KcsA lend support to the idea that gating may be governed by K^+ concentration-dependent transitions between different affinity states of the selectivity filter (*16*). We also found that Na^+ binds to KcsA with a K_D similar to that estimated electrophysiologically from channel blockade (*17*). Therefore, it seems that our findings on the ion binding properties of KcsA partly account for K^+ over Na^+ selectivity and Na^+ blockade and also argue against the strict "snug fit" hypothesis.

MATERIALS AND METHODS

Mutagenesis and Purification of KcsA. KcsA tryptophan mutants were obtained through site-directed mutagenesis using wild-type gen inserted in pQE30 plasmid (Qiagen) as template. In all cases Trp residues were mutated to Phe to obtain the more conservative change at each position without introducing any other fluorescent amino acid (such as tyrosine). Development of single, double, and triple mutants was carried out in several steps using the following synthetic oligonucleotides (sense and antisense, respectively) (Invitrogen): W26F, 5′-AGTCGCCTGCACGCGAGGGCCGCG-3′ and 5′-CGCGGCCCTCGCGTGCAGCGCACT-3′/W87F, 5′-CCCGTGACTCTGTTCGCCGGCTC-3′ and 5′-GAGCCGGCCGAACAGAGTCACGGG-3′/W113F, 5′ GCGCTGGCCACCTTCTTCGTCGGC-3′ and 5′-GCCGACGAAGAAGGTGGCCAGCGC-3′/W67,68F, 5′-CGGGCGCTGTTCTTCTCCGTGGAG-3′ and 5′-CTCCACGAAGAACAGCGCCCG-3′. After Pfu Turbo polymerase (Stratagene) chain reaction mutagenesis, the native methylated DNA templates were digested with *Dpn*I (Fermentas) for 2 h at 37 °C. The mutations were confirmed by dideoxynucleotide sequencing. In all, we were able to obtain the following mutants: W26F, W113F, W26,113F, W87F, W26,87,113F, and W67,68F.

Expression of the wild-type KcsA protein and Trp mutants, all with an added N-terminal hexahistidine tag in *Escherichia coli* M15 (pRep4) cells, and its purification by affinity chromatography on a Ni^{2+}-Sepharose (GE Healthcare) column were carried out as reported previously (*18*). The final buffer used with the stock of purified protein was 20 mM HEPES (Sigma-Aldrich), pH 7.0, containing 5 mM DDM (Calbiochem), and 100 mM NaCl or 100 mM KCl (Merck). Protein concentration was routinely determined from the absorbance at 280 nm, using the following molar extinction coefficients for the KcsA monomer, estimated from the extinction coefficients of model compounds (*19*): 34950 (WT), 29450 (single mutants), 23950 (double mutants), and 18450 $M^{-1}\cdot cm^{-1}$ (triple mutants). KcsA concentration values in this report are always given in terms of KcsA monomers.

SDS−PAGE. Aliquots of WT and mutant channels were mixed with electrophoresis sample buffer (*20*) and loaded into a 13.5% polyacrilamide gel to check the tetramer integrity in each case. Protein bands were visualized after Coomassie Brilliant Blue staining.

Fluorescence Measurements. Fluorescence spectra were taken on a SLM 8000 spectrofluorometer using 0.5 cm pathlength quartz cuvettes, as described in ref *15*. The samples were excited at 280 nm, and the emission was recorded between 300 and 400 nm in 1 nm increments. Changes of intensity-weighted average emission wavelength $\langle\lambda\rangle$ (*21*) according to cation concentration were carried out by adding increasing amounts of a stock of KCl to a solution of 1 μM KcsA in 20 mM HEPES, pH 7.0, 5 mM DDM, 5 mM *N*-methyl-D-glucamine (NMDG), and 3 mM NaCl.

Fluorescence Monitoring of Thermal Denaturation. Thermal denaturation of KcsA was monitored in a Varian Cary Eclipse 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 (*22*). The thermal-induced denaturation of the protein has been shown to be irreversible and to involve both the dissociation of its tetrameric structure and the (partial) unfolding of the monomers (*22*). Therefore, the heat-induced denaturation process is under kinetic control, and the characteristic parameters of the transition (specifically the enthalpy change upon denaturation, ΔH, and the denaturation temperature, T_m) are both concentration and scan rate dependent. In order to allow for the investigation of the effect of the concentration of either K^+ or Na^+ on the thermal stability of the protein (see below), all

experiments were performed at a fixed concentration of the protein (1 μM) and identical heating rate (0.6 °C/min).

Two types of experiments were performed in order to characterize the binding of K^+ and Na^+ to KcsA: (a) Competitive conditions, in which the thermal stability of 1 μM KcsA in 20 mM HEPES, pH 7.0, and 5 mM DDM was measured in the presence of increasing amounts of KCl (Sigma-Ultra), while NaCl (Sigma-Ultra) concentration was decreased accordingly to keep the final concentration of both chloride salts constant at 100 mM. Since the presence of high concentrations of potassium makes KcsA thermal stability too high to allow the complete monitorization of the transition, 12% 2,2,2-trifluoethanol (TFE) was added to all of the samples as a protein destabilizer to facilitate the measurements but allowing KcsA to remain in its native tetrameric structure (*18*, *23*). (b) Noncompetitive conditions, in which the experiments were performed 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. Initial Na^+ concentration, coming from the protein stock, was 1.5 mM. Increasing amounts of either NaCl or KCl 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 increase of temperature) to a two-state unfolding model, assuming a linear dependence of the pre- and posttransition baselines on temperature (*24*).

The stabilization of the native state of the protein relative to its denaturated one as a consequence of the binding of a certain ligand, L, will depend on both the concentration of the ligand, [L], and the dissociation constant of the protein−ligand complex, K_D (*25*, *26*)

$$\Delta G_{unf} = \Delta G_0 + RT \ln\left(1 + \frac{[L]}{K_D}\right) \quad (1)$$

where ΔG_{unf} and ΔG_0 are the free energy changes upon unfolding in the presence and absence of ligand, respectively. Therefore, we can visualize the stabilizing effect of bound ligand as arising from the additional free energy required to remove the ligand from its binding site in the protein native conformation. This energetic penalty for the unfolding of the protein−ligand complex relative to the native protein would be higher as the free concentration of ligand increases, and for a given concentration of ligand, the stabilization will be higher as the affinity of the ligand for the protein becomes larger (lower dissociation constant).

The raising of the midpoint transition temperature, T_m, for the heat-induced denaturation of the protein in the presence of increasing concentrations of ligand can be used to estimate the dissociation constant of the protein−ligand complex (*25*−*27*)

$$\frac{\Delta T_m}{T_m} = \frac{T_m - (T_m)_0}{T_m} = \frac{R(T_m)_0}{\Delta H_0} \ln\left(1 + \frac{[L]}{K_D}\right) \quad (2)$$

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 the enthalpy change upon protein denaturation in the absence of ligand.

Equations 1 and 2 are derived for the simple case of a protein with a single binding site whose unfolding can be represented by a reversible two-state mechanism. The heat-induced denaturation of KcsA is an irreversible process involving the dissociation of the tetramer and the partial unfolding of its monomers (*22*) and may involve changes in the population of multiple monovalent binding sites. Therefore, the apparent K_D values extracted from the K^+ and Na^+ concentration dependence of the protein thermal stability, T_m, should be considered as estimations of the true thermodynamical parameters that could be only obtained from equilibrium-based approaches. Nevertheless, the limitations imposed by the nonequilibrium nature of the physical observable from which the estimations for the dissociation constants are extracted do not compromise the main conclusions derived from the very different thermal stability of the protein in the presence of either K^+ or Na^+ (see Results and Discussion sections below).

Circular Dichroism. Far-UV circular dichroism (CD) spectra were taken on a Jasco J810 spectropolarimeter at a 100 nm/min scan rate and using a 0.5 nm resolution. The mean residue ellipticity, θ_{MRW}, was calculated as in ref *22*. Aliquots of KcsA were diluted with 20 mM Tris, pH 7.0, 5 mM DDM, and 50 mM Na_2SO_4 to a final concentration of 5 μM in order to get the average of six spectra. In these experiments the HEPES buffer was changed to Tris, and the chloride salts were substituted by sulfates to reduce the absorption below 200 nm.

RESULTS

Monitoring Ion Binding to the KcsA Channel by Using a Thermal Denaturation Assay. We have previously shown that KcsA in detergent solution undergoes an irreversible, cooperative process of thermal denaturation as the temperature is raised, which includes the dissociation of the tetrameric protein into individual monomers, concomitantly to its partial unfolding (*22*). Figure 1A shows examples of intrinsic fluorescence monitoring of such thermal denaturation process under conditions in which different concentrations of either sodium or potassium ions are present in the detergent solution containing the purified KcsA. In all the cases tested, it is observed that, in the lower temperature range (the native state baseline), the protein intrinsic fluorescence decreases linearly with temperature in accordance with the general increase in quenching of fluorescence with temperature (*28*), then goes into a sigmoidal-like region with a steeper slope and a characteristic midpoint (t_m) temperature, and finally, in the higher temperature range (the denatured state baseline), returns to a linear decrease with a slope different from that seen at lower temperatures reflecting the change of the local environment of the Trp residues as a consequence of the thermally induced conformational change of the protein. Figure 1A also shows that the characteristic t_m value for the heat-induced dissociation and unfolding process is strongly dependent on the type and concentration of ions present in the buffer. This is due to the role of certain ions on stabilizing the native state of the KcsA protein (*14*, *15*). In particular, potassium ions at sufficiently high concentrations increase the t_m to values near the boiling point of water, which prevents the acquisition of a good baseline for the denatured state in our aqueous samples. Lowering the potassium concentration to 2.5 mM decreases the t_m by more than 30 °C, thus providing an experimental observable much more sensitive than the previously used spectral shift of the fluorescence emission to probe the effects of potassium ions on the protein structure and stability (*15*). Likewise, the t_m obtained from samples containing high or low concentrations of sodium differ by nearly 20 °C.

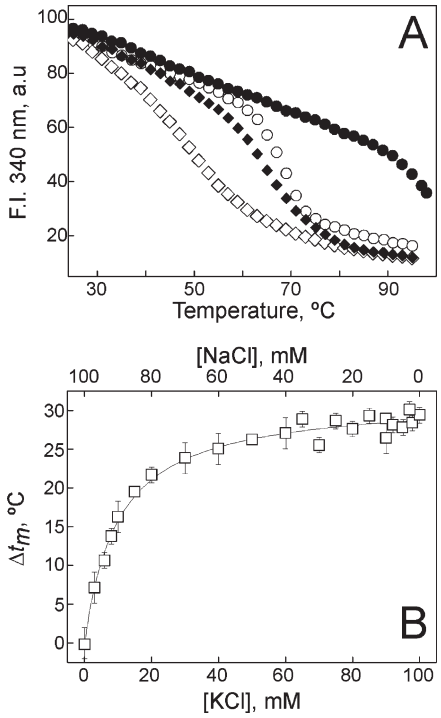


FIGURE 1: Fluorescence monitoring of thermal denaturation of KcsA in DDM solution. Panel A: The selected experimental traces to illustrate these experiments show the changes in the KcsA intrinsic fluorescence emission at 340 nm upon excitation at 280 nm, as the temperature is increased. KcsA samples (1 μM) were in 20 mM HEPES buffer, pH 7.0, containing 5 mM DDM, 5 mM NMDG, and 2 mM NaCl (◇), 2 mM KCl (◆), 100 mM NaCl (○), or 100 mM KCl (●). The t_m estimated from each experiment was 52, 67, and 70 °C, respectively. T_m cannot be determined in the presence of 100 mM KCl due to the high thermal stability of the channel in this condition. Panel B: Dependence of the t_m values on the concentration of Na^+ and K^+ chloride salts (using "competitive" conditions; see Materials and Methods). This experiment illustrates the antagonizing effects of Na^+ and K^+ on the thermal stability of 1 μM KcsA in 20 mM HEPES, pH 7.0, 5 mM DDM, and 12% TFE. Results are shown as the increase in the $t_m \pm$ standard deviation of the media (SD) ($n = 3$).

Figure 1B shows the KcsA t_m variation upon binding of sodium and potassium ions to the native state of KcsA. In this experiment we varied the concentration of sodium and potassium in order to maintain the total chloride salt concentration at 100 mM. These conditions result in a competitive ion binding to KcsA, which is in accordance with the report from fluorescence spectral shift data (*15*). As stated above, the protein t_m values in the presence of potassium may be too high, and then, we added 12% TFE to all of the samples in these experiments to facilitate the measurements. TFE is an efficient destabilizer of the KcsA protein, and at the low concentration used here it just lowers the protein's t_m to a more amenable range for the study, while the protein remains in its characteristic tetrameric form (*18*, *23*). Figure 1B also shows that potassium remarkably stabilizes the protein relative to sodium, as indicated by an increase in the t_m of nearly 30 °C. Finally, it is also shown that potassium antagonizes the effects of sodium much more effectively than sodium antagonizes those of potassium, which reflects the higher free energy penalty derived from the removal of potassium versus sodium from the native state of the KcsA protein upon its denaturation (see eq 1). This observation is in agreement with the previously reported higher affinity of potassium versus sodium ions to KcsA (*15*).

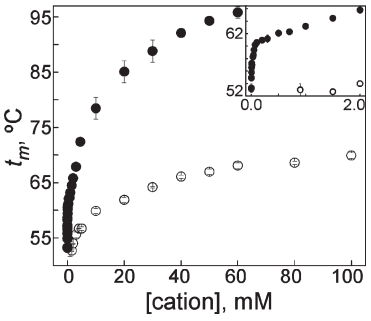


FIGURE 2: KcsA thermal stability dependence on cation concentration (under "noncompetitive" conditions; see Materials and Methods). Closed circles represent the stabilization of KcsA (1 μM, 20 mM HEPES, pH 7.0, 5 mM DDM, 5 mM NMDG, and 1.5 mM NaCl) upon addition of increasing amounts of KCl. Inset zooms on the first stabilization event, which is induced by K^+ in the μM range. Open circles represent the thermal stabilization of KcsA induced by Na^+ binding to the channel. Results are shown as $t_m \pm$ SD ($n = 3$).

Because of the wide range of variation in the protein t_m as an experimental observable, we use the thermal denaturation assay to study in more detail ion binding to KcsA under noncompetitive conditions and in the absence of TFE. For such purposes, batches of the purified KcsA protein were prepared in as low as possible concentrations of sodium (namely, 1.5 mM), and then either sodium (up to 100 mM) or potassium (up to 60 mM) was added separately to KcsA samples and submitted to thermal denaturation. Such a low concentration of sodium was chosen as the starting point for these titrations to guarantee an initial very low ion occupancy of the binding sites, as KcsA has a reported K_D for sodium near 190 mM (*15*) and thus most of the channel population should be present as the cation-free form.

Figure 2 illustrates that both monovalent cations stabilize the protein against thermal denaturation in an ion concentration-dependent manner. Nonetheless, potassium is a much better stabilizer than sodium (as could be deduced from Figure 1), and that explains why under competition conditions such as that in Figure 1B it gave the wrong impression that potassium stabilizes the protein while sodium destabilizes it. An additional observation from the experiments in Figure 2 is that the increase in t_m with potassium concentration occurs in two phases, while the effect of sodium seems to follow a simpler process. This observation receives further support from the data fitting shown in Figure 3. Indeed, the effect of sodium on the thermal denaturation of KcsA seemingly responds to a single binding event where occupation of a single set of ion binding sites with an apparent K_D of 3.5 mM suffices to fit the experimental data (Figure 3A). This is clearly in contrast with the case of potassium (Figure 3B and inset to Figure 2), where the biphasic behavior in the K^+ concentration-dependent stabilization of the protein strongly suggests the existence of two thermodynamically different sets of ion binding sites that seem to be occupied successively as the protein becomes saturated with the ion (Figure 3C,D). In this latter case, the occupancy of the first set of high-affinity potassium binding sites at low potassium concentrations (0 − 0.1 mM) results in an increase of approximately 8 °C in the t_m, which is followed by an additional 35 °C increase in the t_m when potassium concentration is raised from 0.1 to 60 mM. Figure 3C shows the fit of the stabilization of the protein against its thermal denaturation as a function of ligand concentration to eq 2 in the low potassium concentration interval ($0 < [K^+] < 0.1$ mM). The observed stabilization of the protein is consistent with a single

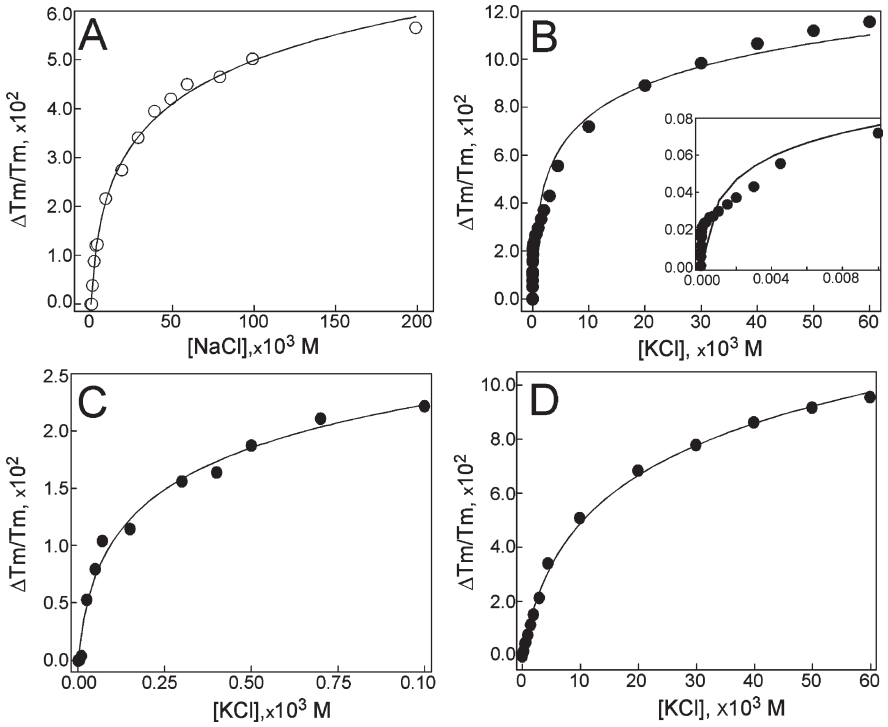


FIGURE 3: Data fitting from experimental data from Figure 2 to eq 2. Continuous lines represent the best fit of the experimental data points to the cation binding model described under Materials and Methods. Panel A illustrates the fitting for binding of Na^+ to a single set of binding sites on the channel ($K_d = 3.3 \pm 0.5$ mM). In the case of K^+, such fitting fails when taking into account the whole titration curve (panel B and its inset), but it suffices when the low K^+ (panel C) and the high K^+ concentration ranges (panel D) 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 KcsA are 1.9 ± 0.5 μM and 2.5 ± 0.2 mM, respectively.

binding site becoming increasingly occupied with an apparent dissociation constant, K_D, of 0.002 mM. As the concentration of ligand increases from 0.1 to 60 mM, the protein is further stabilized. This stabilization arises not only from the occupancy of a second binding site of the protein (with lower affinity than the one detected at very low potassium concentrations) but also from the stabilization of the protein exerted by the high-affinity binding site at concentrations of ligand well above those necessary for its complete saturation (see eq 2). Figure 3D shows the fit of the stabilization of the protein ($\Delta T_m/T_m$) as a function of potassium concentration in the interval $0.1 < [K^+] < 60$ mM according to eq 2. The estimated apparent dissociation constant, K_D, for this binding site, 2.5 mM, must be considered a lower limit of the real K_D due to the contribution of the high-affinity binding site to the observed stabilization of the protein.

Use of Tryptophan Mutants To Ascribe the Structural Effects of Ion Binding to KcsA to Rearrangements of Local Protein Domains. The intrinsic fluorescence spectrum of KcsA complexed with sodium is red shifted and decreased in intensity compared to that of the potassium complex (*15*). The observation that the protein intrinsic fluorescence allows monitoring the effects of ions on the protein structure prompted us to use tryptophan mutants in an attempt to determine experimentally which regions of the protein respond to ion binding. KcsA has five tryptophan residues per subunit, which are exclusively located at both ends of the transmembrane helical segments defining the extracellular (W67, W68, and W87) and the intracellular (W26 and W113) lipid−water interfaces, respectively (*3*). For the following experiments we substituted tryptophan by phenylalanine residues, a conservative mutation, so that the fluorescence from each of the tryptophan residues could specifically be turned off, while minimally altering the protein structure in the resulting mutants. Figure 4 illustrates the most relevant features of some of such tryptophan mutants. We were aware of the difficulties encountered by Perozo and co-workers in their attempts to prepare W67A and W68A mutants (*29*), and indeed, we found that the double W67,68F mutant was always expressed at very low yields and the expression products did not tetramerize in a stable manner (Figure 4A). Therefore, we decided to maintain W67 and W68 as such in all of our mutants. On the contrary, W26F, W87F, and W113F mutations, either as single, double, or triple point mutants, exhibited expression yields similar to that of the wild-type channel and produced tetrameric proteins in which the secondary structure and the stability were also very similar to that of the wild-type channel (Figure 4). Moreover, functional patch-clamp measurements of these mutant channels reconstituted into asolectin giant liposomes were also very similar to that of the wild-type channel (data not shown).

Once it was shown that the structure, stability, and function of the KcsA mutants lacking W26, W87, and W113 residues were comparable to that of the wild-type protein, we tested their responses to the presence of different concentrations of sodium or potassium ions, which in the wild-type KcsA results in characteristic spectral red and blue shifts of the fluorescence emission, respectively (*15*). Figure 5 shows that the concentration-dependent responses to the presence of sodium or potassium in the wild-type protein were emulated very closely by either the double W26,113F or the triple W26,87,113F mutants, both in terms of the changes in spectral shape and in the extent of spectral shift (obviously the fluorescence intensity decreases as the number of tryptophan residues in the mutant protein are reduced). These observations suggest that W67 and W68, which are the only tryptophans present in the triple mutant, are "sensitive" to the conformational changes associated to ion. As a corollary from

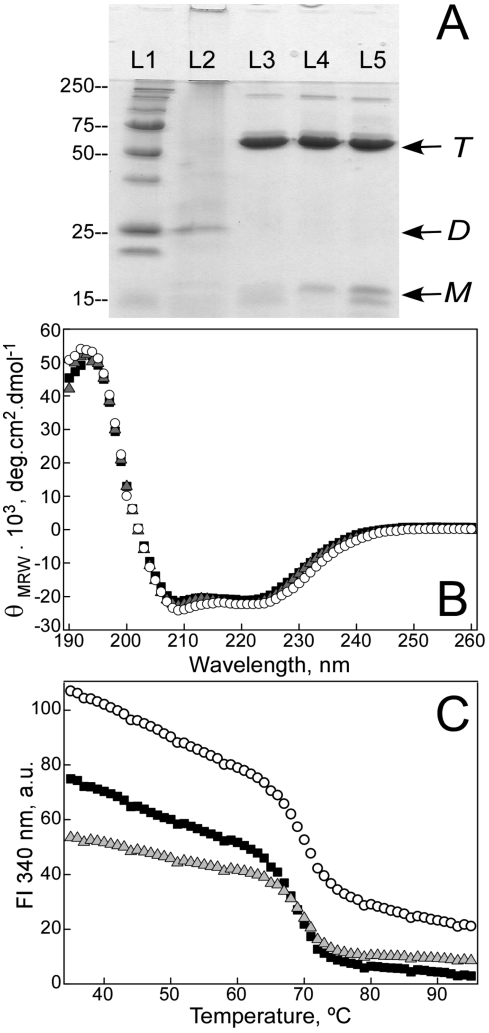


FIGURE 4: Biophysical characterization of tryptophan mutants of KcsA. Panel A: SDS−PAGE (13.5%) of 10 μg of KcsA W67,68F (lane 2), W26,113F (lane 3), W26,87,113F (lane 4), and WT (lane 5). The position corresponding to different oligomeric states of the KcsA channel is indicated as T (tetramer), D (dimer), and M (single monomer). Molecular weight markers are shown as a reference (lane 1). Panel B: Far-UV CD spectra of W26,113F (■), W26,87,113F (△), and WT (○) KcsA. Samples (5 μM) were in 20 mM Tris buffer, pH 7.0, 5 mM DDM, and 100 mM Na_2SO_4. Panel C: Fluorescence monitoring of thermal stability of 1 μM KcsA in 20 mM HEPES, pH 7.0, 5 mM DDM, and 100 mM NaCl. Symbols are identical to those used in panel B. Data fitting resulted in t_m of approximately 70 °C in all cases.

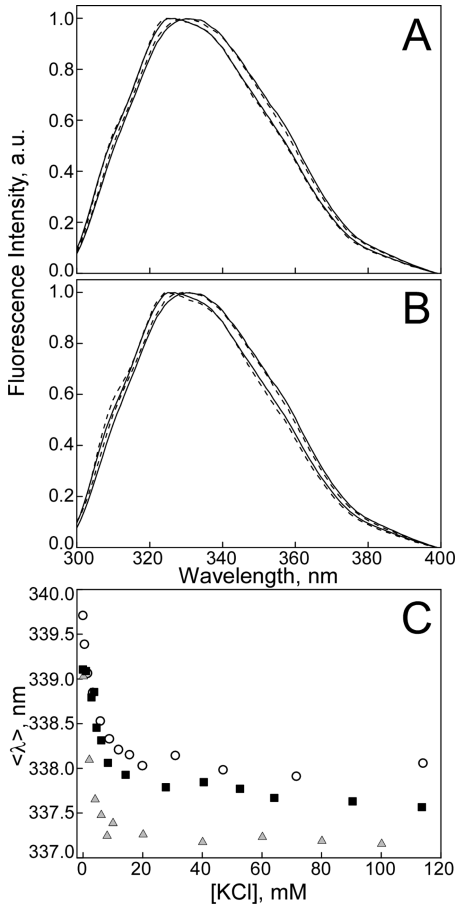


FIGURE 5: Normalized emission spectra of the intrinsic fluorescence exhibited by W26,113F (panel A) and W26,87,113F (panel B) (both in dashed lines) and WT KcsA (continuous line, shown as a control in both panels). Two micromolar KcsA samples were diluted in 20 mM HEPES, pH 7.0, 5 mM DDM, and 100 mM KCl (blue shifted) or 100 mM NaCl (red shifted). Panel C illustrates the antagonizing effect of K^+ over the intensity-weighted average emission wavelength $\langle\lambda\rangle$ of a 1 μM KcsA sample in 20 mM HEPES, pH 7.0, 5 mM DDM, and 3 mM NaCl. WT (○), W26,113F (■), and W26,87,113F (△) samples were titrated with increasing amounts of KCl, and fluorescence spectra were recorded in order to calculate $\langle\lambda\rangle$ in each case.

the above, it should be concluded that the effects of both monovalent cations on rearranging the protein structure are produced mainly at protein domains near residues W67 and W68. These two residues are located at the short helix, nearest to the channel selectivity filter, with their indole side chains practically in contact with the polypeptide backbone of the selectivity filter (*3*, *29*, *30*). Therefore, the channel selectivity filter and its immediate surroundings appear as a relevant site involved both in ion binding and in the ion binding-induced protein conformational change.

DISCUSSION

This paper reports on the fluorescence monitoring of thermal denaturation of the potassium channel KcsA as a tool to study its interaction with permeating (potassium) and blocking (sodium) monovalent cations. The differential effects of the cations on the experimental observable, the t_m of the protein denaturation process, are easily detectable and remarkably large in magnitude. Our results strongly argue toward the existence of two sets (high and low affinity) of potassium binding sites and one single set of sodium binding sites in KcsA. The stabilization of the native state of KcsA by both sodium and potassium ions, as shown by the concentration-dependent increase in t_m, would simply reflect the overall free energy change upon binding, ΔG, of those ions to either one (in the case of sodium) or two sets of binding sites in the case of potassium. Binding of the cations to the protein clearly antagonizes each other according to their relative affinity, potassium being more effective in antagonizing sodium than it is sodium in antagonizing potassium and, thus, contributing to the expected ion selectivity properties of KcsA as a potassium-specific channel.

The X-ray structure of KcsA shows the cation binding sites located at the channel internal aqueous cavity and at the selectivity filter (*4*, *5*, *31*), from which it follows that ions must first enter into the cavity through the intracellular channel mouth before ion binding to the specific sites may occur. Crystal structures of KcsA under different ionic conditions in the crystallization media have shown that the channel selectivity filter may

exist in two distinct conformations associated to the presence of low and high concentrations of potassium ions (*4*, *32*, *33*). At 5 mM potassium, the filter adopts 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 one potassium ion distributed between those two sites. As the potassium concentration is increased up to 20 mM, a second ion enters the middle of the filter (sites S2 or S3), and a conformational change occurs to the conductive state, which has an average occupation of two potassium ions per channel (*32*, *34*). Simulation studies predict binding of potassium to the S2 site is required for the conformational change to occur (*35*), while both S2 and S3 sites seem to undergo substantial changes in their architecture when in the absence or in the presence of low potassium concentrations (*36*). In fact, we carried out the experiments reported here at pH 7.0 to avoid the acid-pH-dependent opening of the intracellular channel "inner gate" (*37*) and, thus, to emphasize the events at the selectivity filter.

On the basis of the above observations, we have ascribed the high-affinity binding site for potassium suggested by our results to the formation of the nonconductive KcsA*·K^+. Likewise, as the concentration of potassium is increased, the lower affinity binding of an additional ion would take place to produce the conductive form of the KcsA·K^+ complex:

$$\text{KcsA} + K^+ \leftrightarrow \text{KcsA}^* \cdot K^+ + K^+ \leftrightarrow \text{KcsA} \cdot 2K^+$$

These or similar affinity state transitions in the conformation of the selectivity filter have been invoked to explain channel gating (*16*). Thus, according to this proposal, a nonconductive channel would have a high affinity for potassium and, thus, be highly selective for potassium versus sodium. On the contrary, an open channel would have a decreased affinity for potassium to facilitate potassium flow at a high rate.

Similarly, the single binding site for sodium suggested by the monophasic protein stabilization upon increase in sodium concentration has been ascribed to the formation of a nonconductive KcsA**·Na^+ complex:

$$\text{KcsA} + Na^+ \leftrightarrow \text{KcsA}^{**} \cdot Na^+$$

Therefore, under physiological-like conditions, it would be the relation between the concentration of sodium and potassium and their relative dissociation constants that determine the degree of occupancy and the type of ion bound to the channel, regulating in this way its permeation state. For instance, an open channel would result from a high degree of occupancy by potassium.

As to the nature of the putative sodium sites, it was first anticipated that sodium ions cannot bind to S2 or S3 sites (*34*), while more recent crystallographic data proposed a "B" site for sodium binding between S4 and S3, in the plane of the backbone carbonyls of T75 (*38*). Interestingly, binding of sodium to the "B" site requires that no potassium is present at S4 and S3 sites (*38*). This might explain why the apparent K_D value estimated here for sodium binding (~3.5 mM) in the absence of potassium is significantly lower than that previously estimated under conditions of competition between the two cations (~190 mM (*15*)), which closely resembles electrophysiological estimates extrapolated at 0 mV using physiological-like solutions (*17*). Moreover, "*in silico*" studies (*39*) predict that while the selectivity filter is flexible enough to accommodate a dehydrated sodium forming an appropriately sized 5-coordinate cavity, the total energy of the complex with sodium is significantly less favorable than that with potassium, so that ion selectivity would partly arise from the selectivity filter inability to completely dehydrate sodium and minimize the energetic cost of complex formation. This hypothesis seems to be consistent with the large differences in thermal stability detected here for the complexes between the protein and the two monovalent cations.

It should be noted that the notation KcsA* and KcsA** for the protein moiety in the above nonconductive complexes with potassium and sodium, respectively, has been used here merely to indicate that the conformations adopted by KcsA within those complexes are likely to be different (*15*).

As discussed above, the X-ray data point out to the selectivity filter and the channel internal aqueous cavity as the sites involved in ion binding by KcsA. NMR data also support that specific residues within the selectivity filter are indeed involved in potassium binding (*40*). Here we used tryptophan mutants to figure out which protein domains within the KcsA structure become affected by ion binding. In an attempt to maintain most of the structural features of the wild-type channel in the mutant proteins, we use a very conservative mutation, substituting tryptophans by phenylalanines. Still, W67 and W68 could not be mutated without seriously risking the integrity of the channel. Such residues are presumably involved in stabilizing the selectivity filter by hydrogen bonding to residues such as Y78 and E71 (*30*, *36*, *41*), and perhaps the disruption of such interactions in the W67 and W68 mutants explains why they are deleterious for the maintenance of the oligomeric structure of KcsA. In contrast with these observations, we found that we could eliminate all three W26, W87, and W113 residues to produce a triple mutant very similar in structure and stability to the wild-type channel. Such triple mutant in which all of the fluorescence comes exclusively from W67 and W68 responds to the presence of ions in a manner similar to that seen in the wild-type protein, therefore suggesting that the protein domain neighboring residues W67 and W68, i.e., the short helix and the selectivity filter itself, are those mainly involved in the protein structural rearrangements that follow ion binding and in the gating transitions between different affinity states of the protein.

The local nature and the limited extent of the conformational changes in KcsA associated to ion binding, particularly potassium, contrast greatly with the remarkable consequences of ion binding on the protein thermal stability. To explain this, it should be considered that thermal denaturation of KcsA includes both the unfolding and the dissociation of the tetrameric protein into its constituent subunits (*22*, *42*, *43*). Protein unfolding is limited in KcsA denaturation, and indeed the thermally denatured state presents a considerable amount of residual structure (*22*). Thus, most of the free energy of stabilization of the native state of the potassium-bound KcsA complex should come from the synergistic effect of the metal-mediated intersubunit interactions. According to this, the binding of potassium by the native state of the protein would lead to a conformation in which the interactions among the different subunits making up the cation binding sites are much strengthened over the ones taking place in the sodium-bound conformation. Therefore, the differences in the thermal stabilities of the two complexes would mainly reflect the (large) differences in the free energy necessary for the disruption of the intersubunit interactions established within the potassium-bound or sodium-bound homotetramer and also the (small) differences in the free energy change upon potassium or sodium ion binding to the protein. These results extend the previous proposal on a role for ions as structural "effectors" of

KcsA (*15*, *44*), as it seems to be also the case in many other monovalent metal ion-binding proteins (*45*). Such an "effector" role includes favoring or disfavoring a given protein conformation by the bound ions in a cofactor-like or allosteric manner, as well as bridging together the channel protein subunits with variable strength through multiple contacts within the subunit interphases likely at both the channel internal cavity and the selectivity filter. Bridging together adjacent subunits within the tetrameric KcsA seems to be also the main role of anionic phospholipids binding to intersubunit "nonannular" lipid binding sites (*22*). In fact, based on the magnitude of the observed protein stabilization due to either binding of potassium or anionic phospholipids to their corresponding intersubunit binding sites on the protein, it is likely that these two processes are major contributors to the remarkable stability and tetrameric nature of the KcsA channel.

REFERENCES

1. Fowler, P. W., Tai, K., and Sansom, M. S. (2008) The selectivity of K^+ ion channels: testing the hypotheses. *Biophys. J. 95*, 5062–5072.
2. Grabe, M., Bichet, D., Qian, X., Jan, Y. N., and Jan, L. Y. (2006) K^+ channel selectivity depends on kinetic as well as thermodynamic factors. *Proc. Natl. Acad. Sci. U.S.A. 103*, 14361–14366.
3. Doyle, D. A., Morais, C. J., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. *Science 280*, 69–77.
4. Zhou, Y., Morais-Cabral, J. H., Kaufman, A., and MacKinnon, R. (2001) Chemistry of ion coordination and hydration revealed by a K^+ channel-Fab complex at 2.0 Å resolution. *Nature 414*, 43–48.
5. Zhou, Y., and MacKinnon, R. (2004) Ion binding affinity in the cavity of the KcsA potassium channel. *Biochemistry 43*, 4978–4982.
6. MacKinnon, R. (2003) Potassium channels. *FEBS Lett. 555*, 62–65.
7. Gouaux, E., and MacKinnon, R. (2005) Principles of selective ion transport in channels and pumps. *Science 310*, 1461–1465.
8. Noskov, S. Y., Berneche, S., and Roux, B. (2004) Control of ion selectivity in potassium channels by electrostatic and dynamic properties of carbonyl ligands. *Nature 431*, 830–834.
9. Bostick, D. L., and Brooks, C. L., III (2007) Selectivity in K^+ channels is due to topological control of the permeant ion's coordinated state. *Proc. Natl. Acad. Sci. U.S.A. 104*, 9260–9265.
10. Thomas, M., Jayatilaka, D., and Corry, B. (2007) The predominant role of coordination number in potassium channel selectivity. *Biophys. J. 93*, 2635–2643.
11. Varma, S., and Rempe, S. B. (2007) Tuning ion coordination architectures to enable selective partitioning. *Biophys. J. 93*, 1093–1099.
12. Varma, S., Sabo, D., and Rempe, S. B. (2008) K^+/Na^+ selectivity in K channels and valinomycin: over-coordination versus cavity-size constraints. *J. Mol. Biol. 376*, 13–22.
13. Yellen, G. (1997) Single channel seeks permeant ion for brief but intimate relationship. *J. Gen. Physiol. 110*, 83–85.
14. Krishnan, M. N., Bingham, J. P., Lee, S. H., Trombley, P., and Moczydlowski, E. (2005) Functional role and affinity of inorganic cations in stabilizing the tetrameric structure of the KcsA K^+ channel. *J. Gen. Physiol. 126*, 271–283.
15. Renart, M. L., Barrera, F. N., Molina, M. L., Encinar, J. A., Poveda, J. A., Fernandez, A. M., Gomez, J., and Gonzalez-Ros, J. M. (2006) Effects of conducting and blocking ions on the structure and stability of the potassium channel KcsA. *J. Biol. Chem. 281*, 29905–29915.
16. VanDongen, A. M. (2004) K channel gating by an affinity-switching selectivity filter. *Proc. Natl. Acad. Sci. U.S.A. 101*, 3248–3252.
17. Nimigean, C. M., and Miller, C. (2002) Na^+ block and permeation in a K^+ channel of known structure. *J. Gen. Physiol. 120*, 323–335.
18. Barrera, F. N., Renart, M. L., Molina, M. L., Poveda, J. A., Encinar, J. A., Fernandez, A. M., Neira, J. L., and Gonzalez-Ros, J. M. (2005) Unfolding and refolding in vitro of a tetrameric, alpha-helical membrane protein: the prokaryotic potassium channel KcsA. *Biochemistry 44*, 14344–14352.
19. Pace, C. N., and Scholtz, J. M. (1997) Protein Structure: A Practical Approach, IRL Press at Oxford University Press, Oxford, U.K.
20. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*, 680–685.
21. Lopes, D. H., Chapeaurouge, A., Manderson, G. A., Johansson, J. S., and Ferreira, S. T. (2004) Redesigning the folding energetics of a model three-helix bundle protein by site-directed mutagenesis. *J. Biol. Chem. 279*, 10991–10996.
22. Triano, I., Barrera, F. N., Renart, M. L., Molina, M. L., Fernandez-Ballester, G., Poveda, J. A., Fernandez, A. M., Encinar, J. A., Ferrer-Montiel, A. V., Otzen, D., and Gonzalez-Ros, J. M. (2010) Occupancy of nonannular lipid binding sites on KcsA greatly increases the stability of the tetrameric protein, *Biochemistry* (in press).
23. Barrera, F. N., Renart, M. L., Poveda, J. A., de, K. B., Killian, J. A., and Gonzalez-Ros, J. M. (2008) Protein self-assembly and lipid binding in the folding of the potassium channel KcsA. *Biochemistry 47*, 2123–2133.
24. Sehgal, P., and Otzen, D. E. (2006) Thermodynamics of unfolding of an integral membrane protein in mixed micelles. *Protein Sci. 15*, 890–899.
25. Cooper, A., Nutley, M. A., and Wadood, A. (2000) Differential scanning microcalorimetry, in Protein-Ligand Interactions: hydrodynamics and calorimetry (Harding, S. E., and Chowdhry, B. Z., Eds.) pp 287−318, Oxford University Press, Oxford, U.K.
26. Sturtevant, J. M. (1987) Biochemical applications of differential scanning calorimetry. *Annu. Rev. Phys. Chem. 38*, 463–488.
27. Sanchez-Ruiz, J. M. (2007) Ligand effects on protein thermodynamic stability. *Biophys. Chem. 126*, 43–49.
28. Schmid, F. X. (1997) Optical spectroscopy to characterize protein conformation, in Protein Structure: A Practical Approach (Creighton, T. E., Ed.) 2nd ed., pp 261−298, IRL Press, Oxford, U.K.
29. Cordero-Morales, J. F., Cuello, L. G., Zhao, Y., Jogini, V., Cortes, D. M., Roux, B., and Perozo, E. (2006) Molecular determinants of gating at the potassium-channel selectivity filter. *Nat. Struct. Mol. Biol. 13*, 311–318.
30. Shealy, R. T., Murphy, A. D., Ramarathnam, R., Jakobsson, E., and Subramaniam, S. (2003) Sequence-function analysis of the K^+-selective family of ion channels using a comprehensive alignment and the KcsA channel structure. *Biophys. J. 84*, 2929–2942.
31. Jiang, Y., and MacKinnon, R. (2000) The barium site in a potassium channel by x-ray crystallography. *J. Gen. Physiol. 115*, 269–272.
32. Morais-Cabral, J. H., Zhou, Y., and MacKinnon, R. (2001) Energetic optimization of ion conduction rate by the K^+ selectivity filter. *Nature 414*, 37–42.
33. Zhou, Y., and MacKinnon, R. (2003) The occupancy of ions in the K^+ selectivity filter: charge balance and coupling of ion binding to a protein conformational change underlie high conduction rates. *J. Mol. Biol. 333*, 965–975.
34. Lockless, S. W., Zhou, M., and MacKinnon, R. (2007) Structural and thermodynamic properties of selective ion binding in a K^+ channel. *PLoS Biol. 5*, e121.
35. Berneche, S., and Roux, B. (2005) A gate in the selectivity filter of potassium channels. *Structure 13*, 591–600.
36. Domene, C., and Furini, S. (2009) Dynamics, energetics, and selectivity of the low-K^+ KcsA channel structure. *J. Mol. Biol. 389*, 637–645.
37. Blunck, R., Cordero-Morales, J. F., Cuello, L. G., Perozo, E., and Bezanilla, F. (2006) Detection of the opening of the bundle crossing in KcsA with fluorescence lifetime spectroscopy reveals the existence of two gates for ion conduction. *J. Gen. Physiol. 128*, 569–581.
38. Thompson, A. N., Kim, I., Panosian, T. D., Iverson, T. M., Allen, T. W., and Nimigean, C. M. (2009) Mechanism of potassium-channel selectivity revealed by Na(+) and Li(+) binding sites within the KcsA pore. *Nat. Struct. Mol. Biol. 16*, 1317–1324.
39. Miloshevsky, G. V., and Jordan, P. C. (2008) Conformational changes in the selectivity filter of the open-state KcsA channel: an energy minimization study. *Biophys. J. 95*, 3239–3251.
40. Imai, S., Osawa, M., Takeuchi, K., and Shimada, I. (2010) Structural basis underlying the dual gate properties of KcsA. *Proc. Natl. Acad. Sci. U.S.A. 107*, 6216–6221.
41. Shrivastava, I. H., Tieleman, D. P., Biggin, P. C., and Sansom, M. S. (2002) K(+) versus Na(+) ions in a K channel selectivity filter: a simulation study. *Biophys. J. 83*, 633–645.
42. Heginbotham, L., Odessey, E., and Miller, C. (1997) Tetrameric stoichiometry of a prokaryotic K^+ channel. *Biochemistry 36*, 10335–10342.
43. Molina, M. L., Encinar, J. A., Barrera, F. N., Fernandez-Ballester, G., Riquelme, G., and Gonzalez-Ros, J. M. (2004) Influence of C-terminal protein domains and protein-lipid interactions on tetramerization and stability of the potassium channel KcsA. *Biochemistry 43*, 14924–14931.
44. Yellen, G. (1997) Single channel seeks permeant ion for brief but intimate relationship. *J. Gen. Physiol. 110*, 83–85.
45. DiCera, E. (2006) A structural perspective on enzymes activated by monovalent cations. *J. Biol. Chem. 281*, 1305–1308.