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Effects of Conducting and Blocking Ions on the Structure and Stability of the Potassium Channel KcsA*

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This article reports on the interaction of conducting (K^+) and blocking (Na^+) monovalent metal ions with detergent-solubilized and lipid-reconstituted forms of the K^+ channel KcsA. Monitoring of the protein intrinsic fluorescence reveals that the two ions bind competitively to KcsA with distinct affinities (dissociation constants for the KcsA·K^+ and KcsA·Na^+ complexes of ~8 and 190 mM, respectively) and induce different conformations of the ion-bound protein. The differences in binding affinity as well as the higher K^+ concentration bathing the intracellular mouth of the channel, through which the cations gain access to the protein binding sites, should favor that only KcsA·K^+ complexes are formed under physiological-like conditions. Nevertheless, despite such prediction, it was also found that concentrations of Na^+ well below its dissociation constant and even in the presence of higher K^+ concentrations, cause a remarkable decrease in the protein thermal stability and facilitate thermal dissociation into subunits of the tetrameric KcsA, as concluded from the temperature dependence of the protein infrared spectra and from gel electrophoresis, respectively. These latter observations cannot be explained based on the occupancy of the binding sites from above and suggest that there must be additional ion binding sites, whose occupancy could not be detected by fluorescence and in which the affinity for Na^+ must be higher or at least similar to that of K^+. Moreover, cation binding as reported by means of fluorescence does not suffice to explain the large differences in free energy of stabilization involved in the formation of the KcsA·Na^+ and KcsA·K^+ complexes, which for the most part should arise from synergistic effects of the ion-mediated intersubunit interactions.

Potassium channels are complex membrane proteins widely distributed in different cell types of virtually all living organisms, where they contribute to the control of potassium flow, cell volume, release of hormones and neurotransmitters, resting potential, excitability, and behavior (1). Under normal conditions these channels are highly selective in that they allow permeation of K^+ at near diffusion-limited rates, whereas Na^+, the biologically relevant competitor, is effectively prevented from permeation (2). More recently, it has been shown that the concentration of permeating and/or blocking ions modulates the selectivity and gating properties of many of these channels (3), 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 (4, 5), whereas K^+-free media causes Na^+ permeation in some instances (6–8) or even irreversible collapse of potassium conductances (9–11).

The ground-breaking work by MacKinnon and co-workers on solving the structure of several prokaryotic potassium channels at high resolution has provided the structural basis to attempt explaining some of the above fundamental properties of these important molecules. KcsA,[4] a potassium channel from *Streptomyces lividans* (12), was the first of such structures to be solved (13) and despite the fact that it corresponds to a closed channel conformation, it has been used the most to explain 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 (Fig. 1) 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. Indeed, two of such bound K^+ ions can be seen, single file, in the KcsA crystal structure. The ion conduction pathway has also a lower and wider, water-filled region called the cavity, which opens to the cytoplasm. According to the current understanding of the process, the 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. Here, selectivity of K^+ over Na^+

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[4] The abbreviations used are: KcsA, potassium channel from *S. lividans;* DDM, dodecyl β-D-maltoside; TFE, 2, 2, 2-trifluoroethanol; CD, circular dichroism; FTIR, Fourier-transform infrared spectroscopy.

should be only slightly higher, while in the selectivity filter, it should reach a $\sim 10^3$-fold preference. The latter has been explained by the observation that 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^+ because of the higher dipole moment of the carbonyl group compared with water. 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 high energy barrier for transferring Na^+ from the water media (14). In the above hypothesis, the lack of flexibility of the selectivity filter to constrict sufficiently as to accommodate different ions plays a key role in defining the selectivity properties of the channel. Such hypothesis receives further support from the nearly identical high resolution structures seen in crystals of antibody-bound KcsA in the presence of either conducting or blocking ions (11, 15), which includes the rigid maintenance of the geometric and spatial features of the selectivity filter. This model, however, does not explain two important ion conduction features, namely: (i) the existing reports on "altered" ionic selectivity of KcsA, including Na^+ conduction, when reconstituted in different conditions (12, 16–19) or when subjected to extreme voltages in planar bilayers (7); and (ii) sodium blockade (20), which occurs at fairly low Na^+ concentrations and in the presence of a higher K^+ concentration, despite the fact that the channel exhibits a preference for K^+ over Na^+.

This article reports on direct effects of Na^+ and K^+ on KcsA in both detergent-solubilized and membrane-bound forms. Monitoring the changes in the protein intrinsic fluorescence reveals that the two ions bind competitively to KcsA with distinct affinities (K^+ having a 20–25-fold higher affinity than Na^+), leading to different conformations of the ion-bound KcsA complexes. Whereas this finding could partly explain ion selectivity by this channel, it cannot explain either functional sodium blockade, or the effects of fairly low Na^+ concentrations on the structure and stability of KcsA. The latter is observed by infrared spectroscopy but not by fluorescence, and ought to involve a second class of protein sensing sites in which Na^+ affinity must be higher or at least similar than that of K^+.

EXPERIMENTAL PROCEDURES

Protein Expression, Purification, and Reconstitution into Lipid Vesicles—Expression of the wild-type KcsA protein with an added N-terminal His_6 tag in *Escherichia coli* M15 (pRep4) cells and its purification by affinity chromatography on a Ni^{2+}-NTA-agarose column, was carried out as reported previously (21), in either 20 mM HEPES buffer, pH 7.0, 5 mM DDM, 100 mM KCl (potassium buffer), or 20 mM HEPES buffer, pH 7.0, 5 mM DDM, 100 mM NaCl (sodium buffer). Protein concentration is always given in terms of KcsA monomers, using a molar extinction coefficient of 34,950 M^{-1} cm^{-1}, as estimated from the extinction coefficients of model compounds (22).

For reconstitution of KcsA into asolectin lipid vesicles, large unilamellar vesicles of asolectin (soybean lipids, type II-S, Sigma) were first prepared (23) at 25 mg/ml in 10 mM HEPES, pH 7.5, 100 mM KCl (reconstitution buffer) and stored in liquid N_2. Purified DDM-solubilized protein was mixed with the above asolectin vesicles previously resolubilized in 5 mM DDM, at a lipid:KcsA monomer molar ratio of 2000:1, for 2 h. Reconstituted vesicles were formed by removing the detergent by gel filtration on Sephadex G-50 (fine, 15–20-ml bed volume) (20). The detergent-solubilized lipid/protein mixture (2 ml) was loaded on top of the column, and the reconstituted vesicles were eluted in the void volume at 2 ml/min. The protein-containing reconstituted fractions were pooled and centrifuged for 30 min at 300,000 × *g*. The pellet was resuspended into reconstitution buffer, divided into aliquots, and stored in liquid N_2 (24).

Fluorescence Measurements—Fluorescence measurements were taken on a SLM 8000 spectrofluorometer using 0.5-cm path-length quartz cuvettes. The samples were excited at 280 nm and the emission was recorded between 300 and 400 nm in 1-nm increments. Blank corrections were made in all spectra. A circulating Haake water bath was used to keep the temperature constant at 25 °C throughout. The results are expressed in terms of either the observed fluorescence intensity at a given wavelength or as the fluorescence spectral center of mass (intensity-weighted average emission wavelength, $\langle\lambda\rangle$), as defined by Equation 1,

$$\langle\lambda\rangle = \frac{\sum_i \lambda_i I_i}{\sum_i I_i} \qquad \text{(Eq. 1)}$$

where I_i is the fluorescence intensity measured at a wavelength λ_i (25).

Because the observed effects of Na^+ and K^+ on the fluorescence spectra of the protein were antagonistic (see "Results"), the possibility that the two ions could bind to the protein competitively was systematically explored by monitoring the changes in the emission spectra of the protein induced by a set of seven different constant concentrations of Na^+ (ranging from 5 up to 150 mM) and increasing concentrations of K^+ from 1 to 300 mM. In all cases the protein concentration (0.2 μM) was kept constant. Assuming that both K^+ and Na^+ ions compete for the same binding site(s) on the protein, addition of K^+ to the protein solution containing a given, fixed concentration of Na^+ would partly displace them from the complex with KcsA to a degree that depends on the magnitudes of the individual dissociation constants of both complexes (KcsA·Na^+ and KcsA·K^+) and on the concentrations of both ions. Data treatment was complicated by the fact that the magnitude of the intrinsic binding constant of Na^+ to KcsA is such (see below) that the protein binding site(s) are not fully saturated under the experimental conditions used (5 mM $< [Na^+] <$ 150 mM). Therefore, both linked equilibria had to be considered simultaneously since addition of K^+ to the protein solution partially saturated with Na^+ ions would induce both, partial displacement of Na^+ from its complex with KcsA and direct binding of K^+ ions to the free available binding sites of the protein.

The exact analysis describing competitive binding of two different ligands to a protein under conditions of partial saturation

has been previously described (26, 27). Briefly, when ligands A and B (*e.g.* Na^+ and K^+), compete for the same binding site on the protein P (KcsA in this case), the two dissociation equilibria can be represented as Equations 2 and 3,

$$P{\cdot}A \overset{K_A}{\rightleftharpoons} P + A \qquad \text{(Eq. 2)}$$

$$P{\cdot}B \overset{K_B}{\rightleftharpoons} P + B \qquad \text{(Eq. 3)}$$

where K_A and K_B are dissociation constants for the binding of A and B to protein P in Equation 4.

$$K_A = \frac{[P]\cdot[A]}{[P{\cdot}A]} \quad \text{and} \quad K_B = \frac{[P]\cdot[B]}{[P{\cdot}B]} \qquad \text{(Eq. 4)}$$

The concentrations of the five species coexisting in solution (*e.g.* P, A, B, P·A, and P·B) are not lineally independent since the conservation of mass imposes the following restrictions in Equations 5–7,

$$[A]_0 = [A] + [P{\cdot}A] \qquad \text{(Eq. 5)}$$

$$[B]_0 = [B] + [P{\cdot}B] \qquad \text{(Eq. 6)}$$

$$[P]_0 = [P] + [P - A] + [P - B] \qquad \text{(Eq. 7)}$$

where $[A]_0$, $[B]_0$, and $[P]_0$ are the total concentrations of both the ligands and the target protein. Therefore, the concentrations of the two possible complexes, P·A and P·B, are given by Equation 8.

$$[P{\cdot}A] = \frac{[P]\cdot[A]_0}{K_A + [P]} \quad \text{and} \quad [P{\cdot}B] = \frac{[P]\cdot[B]_0}{K_B + [P]} \qquad \text{(Eq. 8)}$$

Substitution of Equations 5–7 into Equation 8 and rearrangement yields the cubic Equation 9,

$$[P]^3 + a\cdot[P]^2 + b\cdot[P] + c = 0 \qquad \text{(Eq. 9)}$$

where a, b, and c are given by Equations 10–12.

$$a = K_A + K_B + [A]_0 + [B]_0 - [P]_0 \qquad \text{(Eq. 10)}$$

$$b = K_B\cdot([A]_0 - [P]_0) + K_A\cdot([B]_0 - [P]_0) + K_A\cdot K_B \qquad \text{(Eq. 11)}$$

$$c = -K_A\cdot K_B\cdot[P]_0 \qquad \text{(Eq. 12)}$$

Equation 9 can be solved analytically to yield three roots, only one of them being physically meaningful (26) and given by Equation 13,

$$[P] = \frac{2\cdot\sqrt{(a^2 - 3b)}\cdot\cos(\phi/3)}{3} \qquad \text{(Eq. 13)}$$

where in Equation 14,

$$\phi = \arccos\frac{-2\cdot a^3 + 9\cdot a\cdot b - 27\cdot c}{2\cdot\sqrt{(a^2 - 3b)^3}} \qquad \text{(Eq. 14)}$$

and the concentrations of P·A and P·B are given by Equations 15 and 16.

$$[P{\cdot}A] = \frac{[A]_0\cdot\{2\cdot\sqrt{(a^2 - 3\cdot b)}\cdot\cos(\phi/3) - a\}}{3\cdot K_A + \{2\cdot\sqrt{(a^2 - 3\cdot b)}\cdot\cos(\phi/3) - a\}} \qquad \text{(Eq. 15)}$$

$$[P{\cdot}B] = \frac{[B]_0\cdot\{2\cdot\sqrt{(a^2 - 3\cdot b)}\cdot\cos(\phi/3) - a\}}{3\cdot K_B + \{2\cdot\sqrt{(a^2 - 3\cdot b)}\cdot\cos(\phi/3) - a\}} \qquad \text{(Eq. 16)}$$

At each of the constant concentrations of Na^+, increasing concentrations of K^+ induced a monotonic blue-shift in the emission spectra of the protein (see "Results" section below). Because the fluorescence spectral center of mass, $\langle\lambda\rangle$, of a fluorescence spectrum is indicative of the solvent exposure of the tryptophan residues (28), we chose this parameter as our observable in the quantification of the competition binding studies. For each set of experiments (at a given constant concentration of Na^+), the degree of saturation of the protein with K^+ (f) was obtained according to the expression in Equation 17,

$$f = \frac{\langle\lambda\rangle_0 - \langle\lambda\rangle_i}{\langle\lambda\rangle_0 - \langle\lambda\rangle_{sat}} \qquad \text{(Eq. 17)}$$

where $\langle\lambda\rangle_i$, $\langle\lambda\rangle_0$, and $\langle\lambda\rangle_{sat}$ refer to the fluorescence spectral center of mass for a fluorescence spectrum of KcsA at a given concentration of K^+, in the absence of K^+ and at a saturating concentrations of K^+ (typically above 150 mM), respectively.

The dependence of the degree of saturation of the protein with the concentration of K^+ was fitted to the competitive binding model from above by an iterative method to extract the best estimates for the true dissociation constants K_A and K_B. In brief, initial values for K_A and K_B, together with the total concentrations of protein, $[P]_0$, and both ligands, $[A]_0$ and $[B]_0$, were used to calculate initial guesses for the parameters a, b, and c (according to Equations 10–12) yielding a value for the angle ϕ (Equation 14) and, therefore, for the individual concentrations of free protein, [P], and of the two complexes, [PA] and [PB] (see Equations 13, 15, and 16). The best estimates for K_A and K_B were obtained by iterative minimization of the squared difference between the calculated degree of saturation of the protein, $f = [PB]/[P]_0$, and the experimentally determined one (Equation 17). Finally, the values reported here for the dissociation constants of the protein complexes with either Na^+ and K^+ are the average of the seven individually determined values from each set of experiments.

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 2-nm resolution. The samples were thermostatted with a Jasco Peltier system and contained into 0.1-cm path-length quartz cuvettes. The molar ellipticity per residue, $[\Theta]$, was calculated as in Equation 18,

$$[\Theta] = \frac{\Theta}{10\times l\times c\times(N-1)} \qquad \text{(Eq. 18)}$$

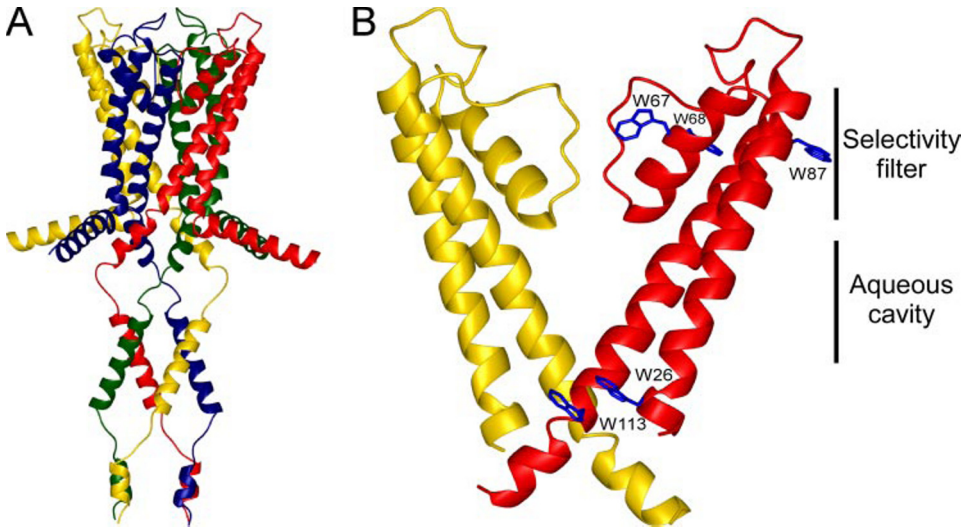


FIGURE 1. *Panel A* shows a structural model for the full-length 1–160, tetrameric KcsA obtained by optimization from the α-carbon trace (1F6G) from Cortes *et al.* (37) and the crystallographic structure determined for the KcsA-(23–119) (1BL8) by Doyle *et al.* (13) (see Ref. 24 for details). *Panel B* zooms in the transmembrane portion of the channel (only two of the four identical subunits have been drawn for clarity) to indicate the main features of the selectivity filter and the aqueous cavity comprising the ion conduction pathway. The location and numbering of the tryptophan residues have been indicated in one of the subunits.

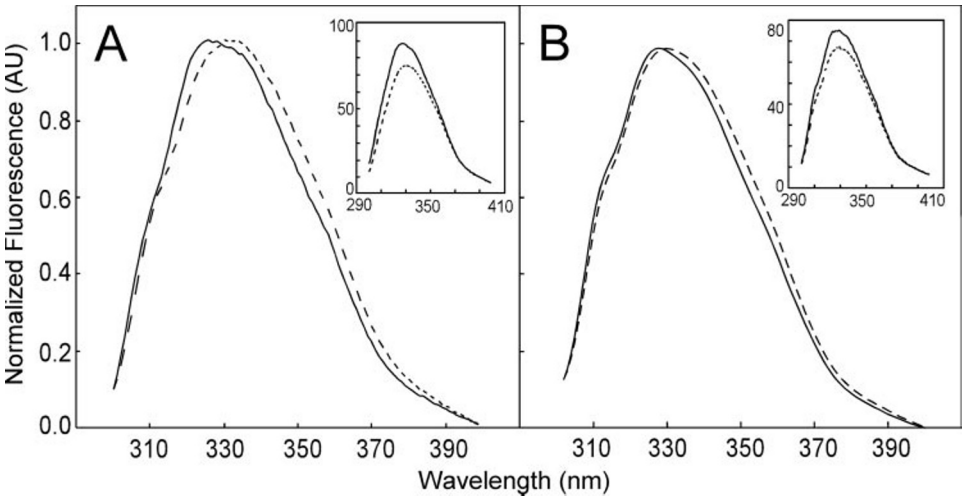


FIGURE 2. **Cation-induced changes in the intrinsic fluorescence of wild-type KcsA.** *Panel A* shows the normalized fluorescence emission spectra exhibited by detergent-solubilized KcsA in buffers containing either 100 mM KCl (*continuous line*) or 100 mM NaCl (*discontinuous line*) under otherwise identical conditions. *Panel B* illustrates comparable cation-induced phenomena, but using KcsA reconstituted in asolectin lipid vesicles. Again, the continuous line corresponds to the sample in the KCl buffer, while the discontinuous line corresponds to the NaCl sample (see "Experimental Procedures"). The *inset* shows the spectra without normalization to appreciate the differences in the intensity of the fluorescence emitted by the two samples.

where Θ is the measured ellipticity; *l*, the path length; *c*, the KcsA molar concentration in terms of KcsA monomers, and *N*, the number of amino acid residues.

Fourier Transform Infrared Spectroscopy—For infrared amide I′ band recordings, aliquots containing 400 μg (1.1 mM) of KcsA reconstituted into asolectine lipids were washed twice with 10 mM HEPES buffer, pH 7.0, containing one of the following ionic concentrations: (*a*) 100 mM KCl, (*b*) 98 mM KCl, 2 mM NaCl, (*c*) 90 mM KCl, 10 mM NaCl; (*d*) 50 mM KCl, 50 mM NaCl, and (*e*) 100 mM NaCl. The first wash was done by ultracentrifugation during 30 min at 55,000 rpm in a TFT-70 Beckman rotor. The pellet was resuspended in 3 ml of the corresponding buffer and kept at 4 °C during 14 h. Then, samples were washed again by centrifugation in identical conditions and the second pellet was resuspended in 50 μl of buffer, dehydrated in a SpeedVac Savant rotary evaporator and finally resuspended in 20 μl of plain D_2O to avoid the interference of H_2O infrared absorbance (1645 cm^{-1}).

Routinely, infrared spectra were taken in triplicate from different KcsA samples in a Bruker IF66s instrument equipped with a DTGS detector. Buffer contribution was subtracted from the individual spectra and spectral noise was reduced as described previously (29). The protein secondary structure was estimated from 600 scans of IR spectra by decomposition of the amide I′ band into its spectral components (29). For temperature-dependent studies, the samples were submitted to heating cycles at each at the indicated temperatures. Each step in such heating cycles included (i) a step-like increase in temperature, (ii) a stabilization period of the sample (or plain buffer) in the IR cell at each selected temperature and (iii) a period of spectral acquisition (200 spectral scans). The duration of a complete heating cycle was of ~3 h.

SDS-PAGE—Solubilized KcsA in 20 mM HEPES buffer, pH 7.0, 5 mM DDM containing 100 mM NaCl, or 100 mM KCl were incubated with different concentrations of TFE at room temperature for 30 min (21). Then, sample aliquots containing the same amounts of protein were mixed at a 4:1 volume ratio with electrophoresis sample buffer (20 mM Tris, pH 6.8, 20% glycerol, 0.1% bromphenol blue and 4% SDS) and run in 13.5% PAGE in the presence of 0.1% SDS (30). After Coomassie Brilliant Blue staining, the intensity of the bands was measured by densitometry.

RESULTS

Cation-induced Fluorescence Changes—The tetrameric potassium channel KcsA contains five tryptophan residues per subunit (Fig. 1), which are located either at the ends of the two transmembrane segments (Trp^{26}, Trp^{87}, and Trp^{113}) or at the pore region that connects them (Trp^{67} and Trp^{68}) and define two rings of such aromatic residues buried in the bilayer, nearly parallel to the intracellular and extracellular

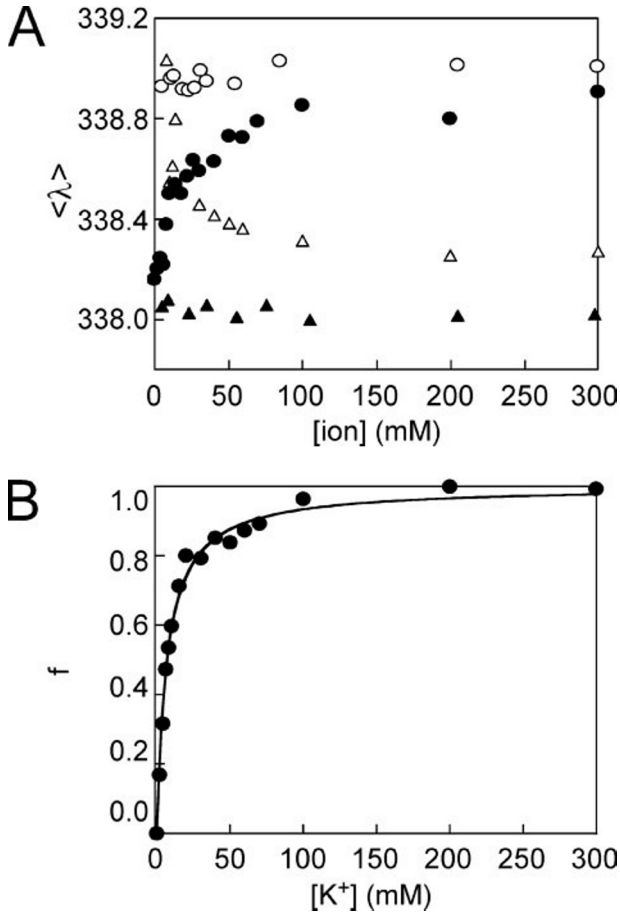


FIGURE 3. *Panel A* illustrates the antagonizing effects of Na^+ and K^+ on the fluorescence spectral center of mass of detergent-solubilized KcsA (0. 2 μM). *Closed symbols* correspond to a protein sample in 20 mM HEPES buffer, pH 7.0, containing 5 mM DDM and 5 mM KCl titrated at the indicated concentrations by either KCl (*closed triangles*) or NaCl (*closed circles*). *Open symbols* correspond to a similar protein sample in 10 mM HEPES buffer, pH 7.0, containing 5 mM DDM and 100 mM NaCl titrated at the indicated concentrations by either KCl (*open triangles*) or NaCl (*open circles*). *Panel B* illustrates the change in the experimental degree of KcsA saturation as a function of K^+ concentration (*closed circles*), in 20 mM HEPES buffer, pH 7.0, containing 5 mM DDM and a constant concentration of NaCl of 100 mM for the particular experiment shown in the figure. The *continuous line* represent the best-fit of the experimental data points to the competitive Na^+ and K^+ binding model described under "Experimental Procedures." Similar titrations were performed at 7 different concentrations of Na^+ in the range 4–150 mM. The mean intrinsic dissociation constants of the KcsA·K^+ and KcsA·Na^+ complexes were estimated as 8 $\pm$ 2 mM and 190 $\pm$ 80 mM, respectively, where the errors represent the S.D. from the mean.

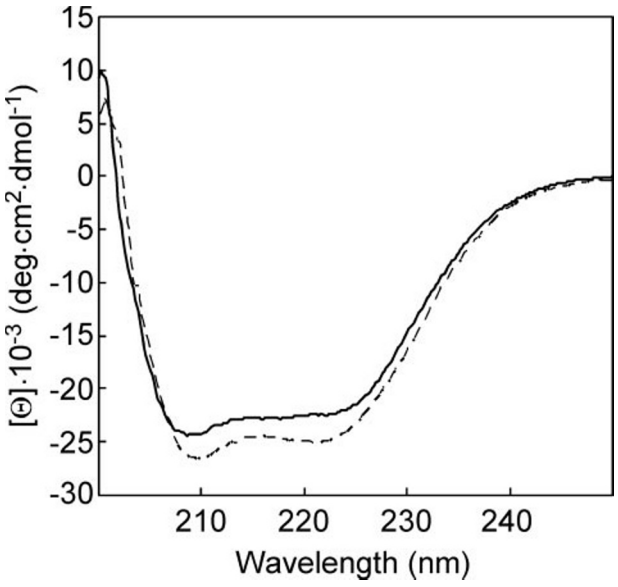


FIGURE 4. **Far UV circular dichroism spectra of identical aliquots of detergent-solubilized wild-type KcsA (8.5 μM) in buffers containing either 100 mM KCl (*continuous line*) or 100 mM NaCl (*discontinuous line*) under otherwise identical conditions.** Estimation of the secondary structure of the protein from these data were carried out using the CDNN (neural network circular dichroism deconvolution) program (48), which indicates that the sample in Na^+ exhibits a slight increase of ~5–10% in the α-helical motif compared with that seen in K^+.

membrane-water interphases, respectively. Fig. 2*A* shows the intrinsic fluorescence spectra of DDM-solubilized KcsA purified in buffers containing either 100 mM KCl or 100 mM NaCl, respectively. In the K^+-containing buffer the emission maximum is blue-shifted and the intensity increased with respect to those seen in the Na^+-containing buffer. Such differences in the emission maxima and intensities are small but highly reproducible and clearly suggest a different exposure of the tryptophan residues to the solvent when in the presence of the different monovalent cations.

KcsA was also reconstituted into asolectin lipid vesicles in an attempt to check whether the differential effects of Na^+ and K^+ on the environment of the tryptophan residues observed in detergent solution, could also be detected in a more physiological environment such as that provided by a lipid bilayer. Fig. 2*B* shows the fluorescence emission spectra of KcsA reconstituted into lipid vesicles and in the presence of 100 mM of either NaCl or KCl. Similarly to the results shown in the detergent-solubilized KcsA preparations, the sample in K^+-containing buffer exhibited a blue shift and an increase in the fluorescence intensity relative to the sample in Na^+-containing buffer. Such a similarity between detergent-solubilized and reconstituted preparations reinforces the notion of a structural change caused by the different monovalent cations and suggests that the site(s) on the KcsA protein sensing their presence are as accessible in the reconstituted lipid vesicles as they are in the detergent-solubilized KcsA.

When reconstituted into asolectin vesicles, KcsA has been reported to insert into the bilayer asymmetrically (nonrandomly), exposing its intracellular side to the extravesicular media, *i.e.* adopting an "inside-out" orientation (31). We confirmed such finding in our reconstituted vesicles by using agarose-immobilized chymotrypsin added to the media, which indeed resulted in the cleavage of the 126–160 cytoplasmic domain from the whole KcsA population (not shown). Then, we took advantage of this fully asymmetric orientation of the reconstituted KcsA to design experiments in which the different monovalent cations were added in and out of the vesicles in an attempt to see whether there was some specificity on their action when there were contained either in the intravesicular or the extravesicular compartments. To this end, a sufficiently concentrated preparation of reconstituted KcsA vesicles in, for instance, 100 mM KCl, was diluted 150-fold in 100 mM NaCl buffer and the fluorecescence emission spectra taken immediately afterward. Assuming that the vesicles are sufficiently impermeable to the monovalent cations during the time course

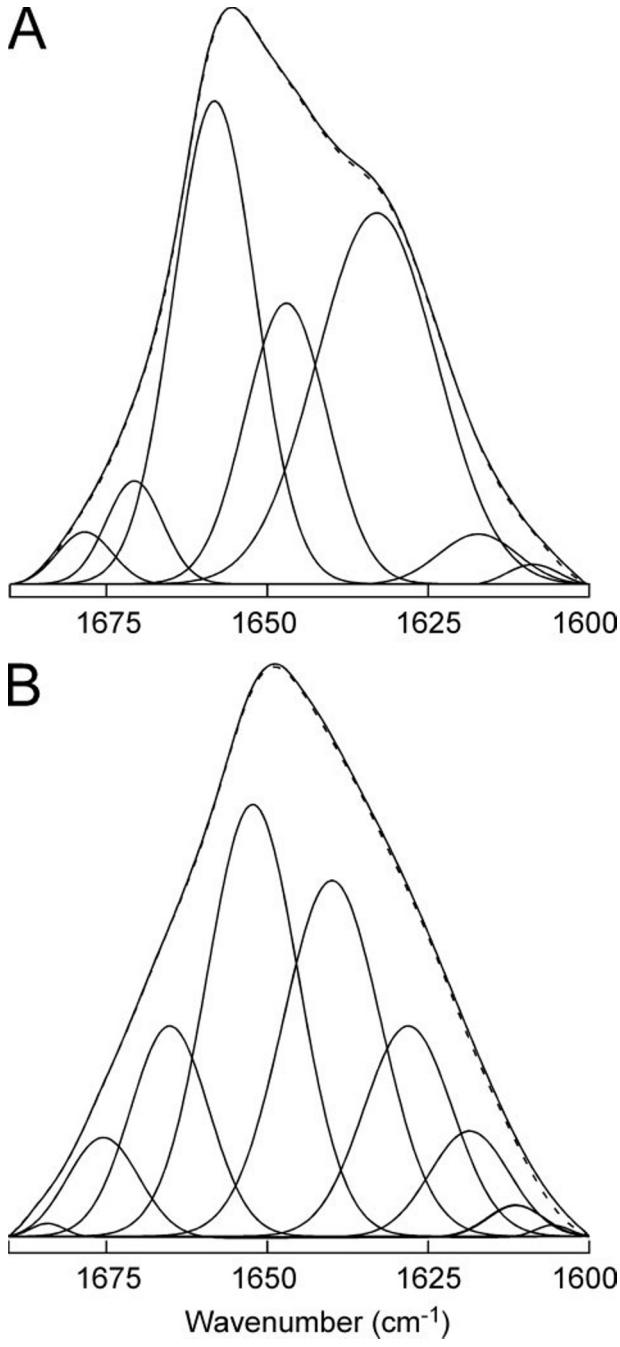
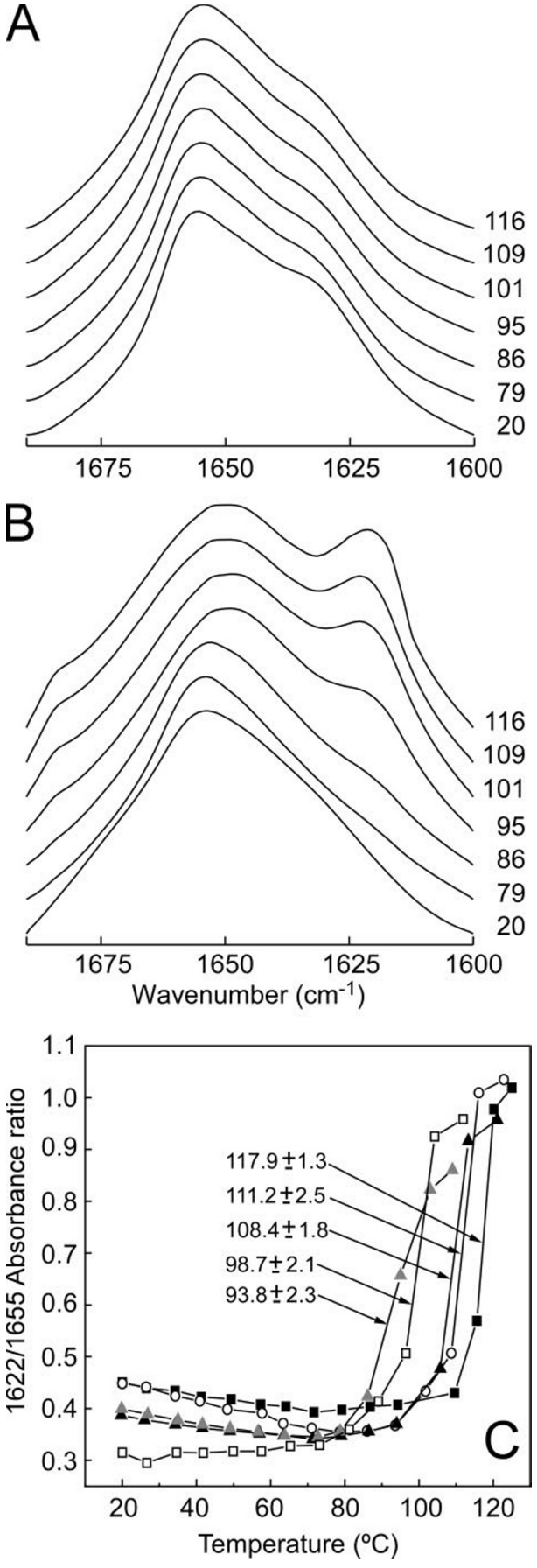


FIGURE 5. **Representative amide I′ band profiles in the infrared spectra of wild-type KcsA reconstituted in asolectin lipid vesicles in D_2O buffers containing either 100 mM KCl (*panel A*) or 100 mM NaCl (*panel B*) under otherwise identical conditions.** Both panels include: (i) the recorded amide I′ band spectral envelope (*continuous line*), (ii) the component bands obtained by decomposition of the amide I′ band (29), and (iii) the reconstruction of the amide I′ band from the observed spectral components (*dashed line*). Band assignments are: 1657 cm^{-1} to α-helix; 1670 and 1680 cm^{-1} to β-turns; 1631 and 1617 cm^{-1} to intramolecular and intermolecular vibrations of β-sheets, respectively; 1644 cm^{-1} to non-ordered conformations (35).

of the experiment, this dilution should essentially lead to have intravesicular K^+ and extravesicular Na^+. Indeed, the practical impermeability of the vesicles to monovalent cations was checked using a vesicle-entrapped fluorophore (PTSA, 1,3,6,8-pyrene tetrasulfonic acid) and externally added Tl^+ (32). The latter cation, in addition to being highly permeable through potassium channels, is an efficient collisional quencher of fluorescence and therefore its entrance into the vesicle can be easily monitored by measuring the quenching of the fluorescent probe, which was found to respond to a slow time-dependant process (*i.e.* the vesicles seem impermeable in practical terms). All possible four combinations were attempted in the dilution experiments, *i.e.* KcsA preparations in NaCl diluted into Na^+ or K^+ buffers, or KcsA preparations in KCl diluted into Na^+ or K^+ buffers. The conclusions from these experiments were that, regardless of the intravesicular cation, the samples containing extravesicular Na^+ show the spectrum of KcsA in sodium, while those having extravesicular K^+, have always the spectrum of KcsA in potassium, such as those shown in Fig. 2*B*. Although these experiments might be affected by a small extent of passive leakage of cations occurring during spectral adquisition, they suggest that the protein site(s) sensing the presence of the different monovalent cations is (are) accessible from the extravesicular side of the reconstituted vesicles to which the intracellular mouth of KcsA is exposed. Such a conclusion also seems consistent with the asymmetry found in reconstituted systems when measuring Na^+ blockade of KcsA, in which Na^+ gains access to the blocking site in the channel only when added to the intracellular solution (7, 18).

Cation Binding Studies—The spectral shifts induced by Na^+ (red shift) and K^+ (blue shift) in the protein intrinsic fluorescence emission (see above) were used as a tool to explore whether the two monovalent cations antagonize each other in their interaction with the detergent-solubilized protein. Fig. 3*A* shows that indeed, the spectral red shift induced by a fixed concentration of Na^+ can be reversed by adding increasing concentrations of K^+ and *vice versa*. These observations suggest that Na^+, the physiological competitor of K^+ in accessing the ion conduction pathway of K^+ channels, competes with K^+ for binding to the KcsA protein in the detergent solution. It should be noticed, however, that K^+ is much more efficient (lower concentrations are needed) in reversing the spectral effects induced by Na^+, than it is Na^+ in reversing those of K^+.

In an attempt to quantitate the presumably competitive binding process, experiments were conducted in which different Na^+ concentrations were kept constant throughout, while the K^+ concentration was increased from 5 up to 300 mM in each of the samples (see "Experimental Procedures"). Fig. 3*B* illustrates the changes in the degree of saturation of KcsA by K^+ at a given constant concentration of Na^+ (100 mM in the experiments presented in the figure). In these experiments, the emission spectrum of the protein is progressively blue-shifted as the concentration of K^+ increases, finally reaching a plateau which, for the experiment shown in the figure, occurs at concentrations of K^+ above 200 mM. Similar titrations of KcsA with increasing concentrations of K^+ were performed at different (constant) concentrations of Na^+ ions (different degrees of saturation of the protein binding sites). Direct fitting of each set of data (performed at a constant concentration of Na^+ ions) to a single binding equilibrium (dissociation of KcsA·K^+ complex) yielded dissociation constants, K_d, in the millimolar range that monotonically increased (from 6 mM up to 16 mM) with the concentration of Na^+ (maintained constant for each set of data



from 4 mM up to 150 mM, respectively). Again, the most plausible explanation for this behavior is that both K^+ and Na^+ ions indeed compete for the same binding site(s) on the protein. Therefore the experimental data were fitted to the competitive binding model for Na^+ and K^+ binding to KcsA under conditions of partial saturation (see "Experimental Procedures"). The mean intrinsic dissociation constants of the complexes between KcsA and both K^+ and Na^+ were estimated to be 8 ± 2 mM and 190 ± 80 mM, respectively, where the errors represent the S.D. from the mean. It is interesting to note that these values for the dissociation constants obtained for both KcsA·K^+ and KcsA·Na^+ complexes are lower than those observed for the dissociation of complexes of these two cations and a monodentate chelator such as acetate (*e.g.* 2700 and 540 mM for K^+ and Na^+, respectively); a bidentate chelator such as oxalate (500 and 320 mM, respectively) or an hexadentate chelator such as EDTA (160 and 14 mM, respectively) (33). The above comparisons rule out that the binding of both Na^+ and K^+ to KcsA could be ascribed to the recognition of the cations by single carboxylate groups at the surface of the protein and suggest that the putative cation binding site(s) in the protein is(are) contributed by several functional groups, probably belonging to amino acid residues from different subunits of the protein (see below when discussing the thermal stability of the protein).

Changes in the Secondary Structure of KcsA—To study whether the above changes in tryptophan exposure upon binding of the cations were accompanied by detectable changes in the secondary structure of KcsA, we carried out circular dichroism (CD) and Fourier-transform infrared (FTIR) studies in DDM-solubilized KcsA and in KcsA reconstituted into asolectin vesicles, respectively.

Fig. 4 shows the CD spectra of detergent-solubilized KcsA recorded in buffers containing either 100 mM KCl or 100 mM NaCl. The differences seen between the two spectra, particularly in the ellipticity at 222 nm characteristic of α-helices, indicate slightly different contents in that secondary structural motif in the solubilized protein for the two ionic conditions used in these studies.

To study the secondary structure of KcsA reconstituted into asolectin vesicles we used FTIR instead of CD spectroscopy because of the great scattering interference when using the latter technique with reconstituted vesicles. Fig. 5 shows the conformationally sensitive amide I' band from the infrared spectra of reconstituted KcsA in D_2O buffers containing either 100 mM KCl (*panel A*) or 100 mM NaCl (*panel B*). The strong amide I′ band, comprising the 1600–1700 cm^{-1} infrared spectral region results primarily from stretching vibrations of C=O groups in

FIGURE 6. **Representative temperature dependence of the amide I′ band of KcsA reconstituted in asolectin lipid vesicles in D_2O buffers containing either 100 mM KCl (*panel A*) or 100 mM NaCl (*panel B*) under otherwise identical conditions.** Temperatures of spectral acquisition are indicated to the *right* of each spectrum. *Panel C* shows the temperature dependence of the spectral changes in terms of the normalized 1622 to 1655 cm^{-1} absorbance ratio for a series of samples prepared in either 100 mM KCl (■), 2 mM NaCl and 98 mM KCl (○), 10 mM NaCl and 90 mM KCl (▲), 50 mM NaCl and 50 mM KCl (□) or 100 mM NaCl (*gray triangle*), under otherwise identical conditions. Numbers within the panel indicate the estimated midpoint temperatures (mean ± S.D. from three different samples) for thermal denaturation of the proteins in the different experimental conditions.

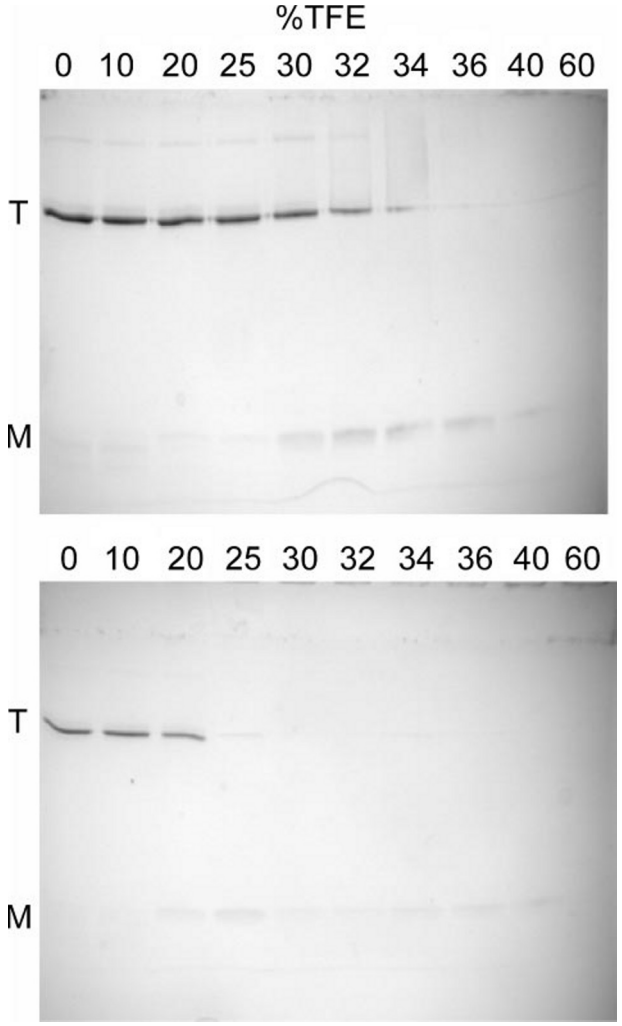


FIGURE 7. **Representative SDS-PAGE analysis of the dissociation of detergent-solubilized KcsA into its constituent subunits by increasing concentrations of TFE.** The solubilized protein samples were prepared in buffers containing either 100 mM KCl (*upper panel*) or 100 mM NaCl (*lower panel*) under otherwise identical conditions. Sample aliquots at a protein concentration of 2.5 μM, were treated with TFE for 30 min to the final TFE concentrations (v/v) given in the figure, then mixed 4:1 (by volume) with electrophoresis sample buffer and deposited into the wells of an 13.5% acrylamide gel. The figure shows representative Coomassie Blue-stained gels in which the tetrameric (*T*) and monomeric (*M*) forms of KcsA are indicated. It should be noticed that, as reported by others (39), the presence of TFE in the samples causes that the lower molecular weight components appear as faint bands in the gel or do not appear at all and under these conditions, only the KcsA tetramer could be reliably measured by densitometry. Such densitometric scans estimated that the TFE concentrations needed to dissociate 50% of the tetrameric KcsA were 27.8 and 20% (v/v) in the sample containing 100 mM KCl or 100 mM NaCl, respectively.

peptide bonds (34), the exact frequencies of which are determined by the particular secondary structure adopted by the protein. In the presence of 100 mM KCl, the amide I′ band of the reconstituted KcsA exhibits a fairly asymmetrical shape with a maximum centered at 1655 cm^{-1} and a prominent shoulder at 1631 cm^{-1}, which are assigned to α-helical and β-structure spectral components, respectively. The latter shoulder practically disappears from the amide I′ band in the spectrum taken in 100 mM NaCl, which also has a more symmetrical shape and exhibits a maximum at 1653 cm^{-1}, thus, suggesting the occurrence of significant changes in the secondary structure of the reconstituted KcsA as a consequence of the exposure to the different monovalent cations. Indeed, curve-fitting the amide I′ band to a mathematical composition of its overlapping spectral components (29) (not shown) confirms that the protein in Na^+ loses much of a β-structure component at 1631 cm^{-1} compared with the K^+ sample, while there is an increase in non-ordered structures at 1644 cm^{-1}. On the contrary, the α-helical component seen at 1657 cm^{-1} seems fairly constant in both samples.

Changes in the Stability of KcsA—FTIR monitoring of the amide I′ band at increasing temperatures has been used to assess thermal stability in the asolectin-reconstituted KcsA samples (35). Fig. 6 illustrates the temperature-induced changes on the spectral shape of the amide I′ band of samples in either K^+ or Na^+ buffers. The reconstituted KcsA sample in 100 mM KCl buffer (*panel A*) is remarkably heat-resistant and although there are band widening and other spectral changes as the temperature increases, these are much less noticeable than in the spectra taken in the sample in NaCl buffer (*panel B*) in which amide I′ band widening as well as the appearance of bands at 1685 and 1622 cm^{-1}, characteristic of aggregation of thermally denatured proteins (36) are clearly detected at much lower temperatures, suggesting a remarkably lower protein thermal stability under these conditions. Moreover, Fig. 6*C* shows that the destabilizing effects of Na^+ on the KcsA spectra could already be noticed in buffers containing as low as 2 mM NaCl (plus the accompanying 98 mM KCl), that is, at Na^+ concentrations much lower than the K_d for Na^+ binding to KcsA determined from the fluorescence measurements from above.

In an attempt to further explore the nature and the extent of the protein stability changes shown above, DDM-solubilized KcsA samples in either 100 mM KCl or 100 mM NaCl buffers were submitted to treatment with 2,2,2-trifluoroethanol (TFE), a protein-destabilizing agent, which mixed at defined percentages in the aqueous media, is known to efficiently unfold and dissociate KcsA (21). In these experiments, because the homotetrameric KcsA is very stable in SDS (24, 37, 38), analysis by SDS-PAGE provides a simple means to study the effects of destabilizing agents such as TFE on the oligomerization state of KcsA (39). The gels in Fig. 7 illustrate such experiments and clearly show that dissociation of KcsA tetramers in 100 mM NaCl requires a lower concentration of TFE than those KcsA samples in the presence of 100 mM KCl, strongly suggesting once again that the presence of the different monovalent cations in the KcsA samples are relevant determinants of the protein structure and stability. Moreover, as in our previous report on the TFE-induced unfolding of KcsA (21) additional experiments were carried out using either fluorescence or CD monitoring of the effects of TFE on the KcsA protein, both in 100 mM KCl and 100 mM NaCl buffers (data not shown). These experiments further confirmed a clear destabilization of the protein in NaCl compared with that in KCl, affecting mainly the midpoint concentration of TFE needed to induce the cooperative and reversible unfolding of the protein.

DISCUSSION

This article reports on direct spectroscopic measurements on the interaction of conducting (K^+) and blocking (Na^+)

monovalent cations with the K^+ channel KcsA. The differential effects of the cations on the intrinsic fluorescence of the protein, a blue shift and an increase in the emission intensity for K^+ and the opposite effects for Na^+, are seen both in the detergent-solubilized protein and in reconstituted lipid vesicles, suggesting that the cation-interacting sites on the protein structure are similarly accessible to the cations in the two different experimental systems. Based on previous studies (7, 15, 40, 41), such cation-interacting sites are located at the channel internal aqueous cavity and selectivity filter and thus, it follows that the cations must enter into the cavity though the channel cytoplasmic mouth, prior to binding to the specific sites. This is consistent with our observations that in reconstituted membranes, in which the protein inserts into the lipid bilayer in a defined "inside-out" orientation, the cation effects can be observed only when added to the cytoplasmic (extravesicular) side of the protein.

Experiments made in the presence of a constant concentration of one of the cations and varying concentrations of the other indicate that the two cations antagonize each other's effects on the protein intrinsic fluorescence, K^+ being more efficient in displacing Na^+, than it is Na^+ in antagonizing K^+. Because the above observations suggested that the interaction of the two cations with the protein might occur on a competitive basis, systematic binding experiments were carried out to see whether the experimental data would conform to a competitive binding model under conditions of partial saturation of a single class of cation binding sites. The fitting of the experimental data to such a model was excellent and provided true dissociation constants of the complexes between KcsA and either K^+ or Na^+ of ~8 and 190 mM, respectively, indicating that the affinity of K^+ for binding to the KcsA protein is ~20–25-fold higher than that exhibited by Na^+. These differences in binding affinity seem larger than the 5–7-fold differences reported previously from electrophysiological measurements of channel function (7) or from crystallographic data (15), respectively. Also, such affinities, particularly that found for K^+, are higher than those exhibited against mono, bi, and hexadentate chelators such as acetate, oxalate, and EDTA, respectively, suggesting that the protein binding site is highly structured and must comprise several amino acid side-chains, which are likely contributed from different subunits, in agreement with the proposal by MacKinnon and co-workers (42, 43) on the nature and diversity of contributing elements to the cation binding sites on the channel internal cavity and selectivity filter.

The differences in the protein intrinsic fluorescence presented above are accompanied by cation-induced changes in the protein secondary structure. In detergent-solubilized KcsA such changes have been followed by far UV CD spectroscopy, which reveals that presence of Na^+ slightly increases the α-helical contents in the protein structure with respect to that seen in the presence of K^+. In KcsA reconstituted into asolectin lipid vesicles, however, the major changes in secondary structure involve a significant alteration in β-sheet and non-ordered structural elements, as indicated by the differences observed in the shape and spectral components of the infrared protein amide I′ band in the presence of each of the two monovalent cations. The differences observed in the CD and FTIR measurements seem consistent with the different structure adopted by the protein when in the detergent-solubilized or vesicle-reconstituted forms (35) but most importantly, regardless of the structural motifs involved, such structural alterations along with the differences in the tryptophan exposure seen in the fluorescence experiments from the previous paragraph, seem clearly in contrast with the almost absolute maintenance of the protein structure reported in crystals of antibody-bound KcsA in the presence of conducting and blocking cations (11). This apparent discrepancy with the existing x-ray data might be important, as our current understanding of ion permeation and blockade is based on MacKinnon's proposal on a fixed protein structure, in which selective ion conduction is permitted as long as the size of the dehydrated ion species conforms precisely to the geometry of the channel's selectivity filter, which remains constant throughout (11, 43). Conversely, our data seemingly indicate that there are significantly different protein conformations associated to the presence of either K^+ or Na^+. These experimental results receive apparent support from molecular dynamics simulations for the KcsA channel in a phospholipid bilayer (44), which predicted significant protein conformational changes affecting the selectivity filter when challenged by K^+ or Na^+. We do not fully understand the reasons for the apparent discrepancy with the crystallographic data, but it is possible that the conformation of KcsA in the crystals might not be as flexible to respond to the presence of the different cations as in the reconstituted vesicles or in the detergent solutions used here. Another possibility is that the presence of bound antibodies in the x-ray measurements could partly prevent the protein from undergoing the conformational changes detected in our studies. Whatever the explanation might be, the main conclusions from these studies are that (i) the KcsA protein exhibits a reasonable specificity to distinguish between the two competing cations, being K^+ preferred over Na^+ by a factor of 20–25-fold, and (ii) binding of the cations leads to the formation of structurally different KcsA·K^+ and KcsA·Na^+ complexes, whose individual population would be dictated by the available concentrations of both Na^+ and K^+, mainly at the intracellular side of the channel. This latter statement implies that under physiological-like conditions, *i.e.* higher intracellular concentration of K^+ over Na^+, the conducting, K^+-bound conformation of KcsA prevails.

The above conclusions seem consistent with the ionic selectivity exhibited by KcsA, being a potassium channel, and could also be related to the observed collapse of potassium channels seen in the presence of low K^+ (11) or to the permeation of Na^+ observed under conditions favorable to Na^+ (7), as these phenomena could partly be explained based on the occurrence of different protein conformations induced by the two cations. However, our model cannot explain other important features such as sodium blockade of potassium channels, which occurs at concentrations of sodium lower than that of potassium. Thus, we have to conclude that the intrinsic fluorescence monitoring does not provide a complete picture of the interaction of the cations with the KcsA protein and indeed, our results from FTIR monitoring of protein thermal stability come to confirm such perception. KcsA is known to be a remarkably stable protein (35, 37, 45) and indeed, the midpoint temperature for its

thermal denaturation when in the presence of 100 mM KCl is as high as 117 °C, but it drops to about 93 °C in the presence of 100 mM NaCl, *i.e.* a remarkable decrease of ~24 °C. Moreover, it is observed that addition of Na^+, even at concentrations as low as a few millimolar and in the presence of a higher potassium concentration causes already a noticeable decrease in such midpoint temperatures, an effect on the protein that could not be anticipated based on the differences in affinity seen in the fluorescence measurements from above. This unexpected discrepancy points toward the existence of another set of binding sites for both K^+ and Na^+ different from that thermodynamically characterized from the changes in the protein intrinsic fluorescence. According to this hypothesis, the intrinsic fluorescence of the protein would be mostly insensitive to the binding of either Na^+ or K^+ to this new set of interacting sites which, in turn, would have a profound effect on the conformational stability of the tetrameric protein. Moreover, since the destabilizing effect of Na^+ on the thermal stability of the protein is significant at concentrations as low as 2 mM (and in the presence of K^+ at a concentration of 98 mM), we can anticipate that the affinity of these latter sites for Na^+ should be higher than that observed for the site(s) thermodynamically characterized (K_d = 190 mM), the dissociation constant being of the same order or even lower than the one for K^+ (probably in the low millimolar range). Most interestingly, the range of sodium concentrations at which its effects on the protein stability can be addressed, are essentially identical to those causing sodium blockade of channel activity (18), which further attests to a possible physiological relevance for the process.

The thermal stability observations are also nicely complemented by studies using detergent-solubilized KcsA and TFE, instead of heat, as a protein destabilizing agent. At moderate concentrations and in K^+-containing buffers, TFE has been shown to reversibly unfold and dissociate the tetrameric KcsA, while at higher concentrations it causes the irreversible denaturation of the protein (21). Here we show that substitution of K^+ by Na^+ in the detergent-solubilized KcsA preparations causes that the above unfolding and dissociation processes occur at much lower TFE concentrations, as reported already by others who used only Na^+-containing buffers (21, 39), confirming once again that presence of Na^+ and lack of K^+ destabilizes greatly the protein structure. Thus, the concomitant occurrence of both, protein unfolding and dissociation into subunits could explain the large differences seen in the midpoint temperatures in the thermal stability experiments in the presence of K^+ or Na^+, as discussed in the previous paragraph. Such large differences cannot be ascribed solely to the difference in free energy change upon K^+ and Na^+ binding, which are expected to be small for binding events characterized by dissociation constants in the order of millimolar. Therefore, most of the free energy of stabilization of the native state of the K^+-bound KcsA complex over the Na^+-bound one should come from the synergistic effect of the metal-mediated intersubunit interactions. According to this picture, the binding of K^+ by the native state of the protein would lead to a conformation in which the interactions among the different subunits involved in the cation binding site are much enhanced over the ones taking place in the Na^+-bound conformation. Because the unfolding of the protein (either induced by heat or by increasing concentrations of TFE) brings about the dissociation of the homotetramer (together with the disappearance of the metal binding site) the differences in the thermal stabilities of the two complexes (KcsA·K^+ and KcsA·Na^+) would mainly reflect the (large) differences in the free energy necessary for the disruption of the intersubunit interactions established within the K^+-bound or Na^+-bound homotetramer and also the (small) differences in the free energy change upon K^+ or Na^+ binding to the protein.

These results seem also consistent with those reported recently by monitoring thermal dissociation of KcsA by SDS-PAGE in the presence of different inorganic cations (46) and extend the previous proposal on a role for the ions as structural "effectors" of the channel protein (3). According to the evidence provided here, such an "effector" role includes favoring or disfavoring a given protein conformation by the bound ions, 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. This seems reminiscent of the "induced-fit" phenomena caused by ligand binding on the structure of many enzymes during the formation of enzyme-substrate complexes, and indeed, a recent report has reviewed a fairly high number of monovalent metal ion-binding enzymes with remarkable structural similarities to ion channels and in which the cations play either a role as cofactor-like or allosteric (47).

Thus, as a conclusion from the latter protein stability studies, we believe that there must be a second set of cation interacting sites on the KcsA protein, whose occupancy does not alter the protein intrinsic fluorescence, but affects quite dramatically protein stability and oligomerization. The affinity of such sites for Na^+ and K^+ must be similar or even higher for Na^+. Particularly in regard to this latter ion, Na^+ concentrations found to cause noticeable protein destabilization and structural rearrangement are in fact very similar to those reportedly causing functional blockade of the channel and thus, it seems likely that in addition to physical occlusion of the channel by Na^+ (11, 43), there could be that Na^+ blockade involves also the adoption of a different, Na^+-induced conformation by the channel protein.

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