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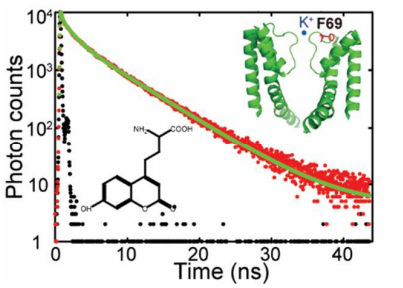

Fluorescence lifetime and time-resolved anisotropy measurements were applied for characterizing ion preference of the NaK channel.

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K^+ preference at the NaK channel entrance revealed by fluorescence lifetime and anisotropy analysis of site-specifically incorporated (7-hydroxycoumarin-4-yl)ethylglycine†

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The fluorescent unnatural amino acid, (7-hydroxycoumarin-4-yl)ethylglycine (HC), was site-specifically incorporated at the Phe69 site, close to the entrance of the selectivity filter of the NaK channel. Decreased fluorescence lifetime and elevated time-resolved anisotropy of NaK-F69HC in buffers with high K^+/Na^+ molar ratios indicated the K^+ preference at the entrance of NaK channel, consistent with previous crystal structure results of the NaK channel.

Ion selectivity is essential for the physiological functions of ion channels, allowing the passage of specific ions through their conductance pore.[1] Physiological and structural studies of ion channels in the past have identified different levels of selectivity for various ion channels.[2] The NaK channel from *B. cereus* shares overall structural similarities with the K^+ channel, KcsA, except for its selectivity filter. The NaK channel can conduct both Na^+ and K^+ ions, making it a unique model for ion selectivity studies.[3] Conventionally, the ion selectivity or preference analysis of different ions to a specific ion channel is conducted through electrophysiology studies.[4] Recently, crystallographic electron density analysis and isothermal titration calorimetry measurements of wild type and mutant NaK channels in different Na^+ or K^+ concentrations illustrated the K^+ preference of the NaK channel.[5] To cross-validate or verify the ion preference of an ion channel, the development of other biophysical methods is necessary and helpful for the further exploration of the mechanism of ion selectivity.

Fluorescence spectroscopy has frequently been used to study folding or conformational change of proteins in different functional states.[6] The signal intensity and maximum emission wavelength (λ_{max}^{em}) are very sensitive to environmental polarity changes around a specific fluorophore. The fluorescence quenching or intensity attenuation can be observed for specific interactions between the fluorophore group and charged compounds.[7] Normally, the fluorescence intensity can be affected not only by the environmental polarity change or quench interactions, but also by continuous light irradiation bleaching or scattering.[8] Recently, the picosecond pulsed laser has enabled fluorescence lifetime measurements. The fluorescence lifetime is the average amount of time that a fluorophore remains in the excited state following pulse excitation, avoiding the effects of light bleaching or scattering.[8b, 9] Therefore, the fluorescence lifetime can provide more reliable information on environmental changes or quenching interactions of the fluorophore.[10] Consequently, protein conformation and dynamics studies using fluorescence lifetime measurement will be superior to fluorescence intensity or λ_{max}^{em} analysis.[11] Recently, fluorescence lifetime measurements of tetramethylrhodamine (TMR)-labelled KcsA illustrated the strong correlations between the channel open (or closed) state and the fast fluorescence decay (or slow decay), despite of the requirements for fluorescent probe labelling after protein purification.[12]

Coumarin and its derivatives are known to be an important class of fluorescent probes, particularly because of their good photochemical properties, chemical stability and ease of synthesis.[13] Recently, the site-specific incorporation of (7-hydroxycoumarin-4-yl)ethylglycine (HC) into a protein attracted many attentions in protein fluorescence studies, due to its much smaller size than the green fluorescent protein GFP (263 Da versus 27 kDa) and amenability to site-specific protein incorporation using the unnatural amino acid-based orthogonal translational scheme.[13a, 14]

To develop the method for ion preference studies of ion channels, HC was site-specifically incorporated at the Phe69 site of the NaK channel (detailed experimental procedures can be found in the ESI†). The Phe69 site is located close to the selectivity filter of the channel (Fig. 1A) and hopefully, the fluorophore at this site can sensitively reflect the passage of Na^+ or K^+ through the channel pore. To verify the function of

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NaK-F69HC, the purified NaK-F69HC protein (Fig. 1B) was reconstituted into liposomes containing 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) for single-channel electrophysiology measurements (see ESI†). Single-channel traces of the sample demonstrated about 1 pA conductance with a holding potential of either +30 mV (upper panel) or -30 mV (lower panel, Fig. 1C). The single-channel conductance amplitude and open probability of NaK-F69HC (Fig. 1C) were similar to the wild-type NaK channel reconstituted in POPC/POPG liposomes (Fig. S1, ESI†), although some minor deviations were observed between NaK-F69HC and wild-type NaK channel in liposomes, probably due to complicate procedures in the proteo-liposome preparation. The observed similar channel conductance strongly suggested that there was no obvious negative functional or conformational interference on NaK channel from the incorporation of HC.

The fluorescence lifetimes of purified NaK-F69HC in buffers containing decylmaltoside (DM) were measured using a time-correlated single-photon counting fluorimeter (DeltaFlex, Horiba Scientific) equipped with a 374 nm diode-pulsed laser and a picosecond photon detector. To determine the ion preference of NaK-F69HC for Na^+ or K^+, six samples of the protein were prepared in buffers containing a total of 200 mM chloride salts at six different K^+/Na^+ molar ratios (0/100; 20/80; 40/60; 60/40; 80/20; 100/0). The NaK-F69HC fluorescence lifetime measurements were conducted using these samples. For each measurement, a total of 4096 channels with a time-to-amplitude conversion range of 27 ps/channel were applied for data acquisition. The instrument response function was obtained at the excitation wavelength of 374 nm using 0.01% dilution of Ludox AS40 colloidal silica (Sigma-Aldrich) in deionized H_2O. Fluorescence intensity decay was measured with a vertically orientated polarizer on the excitation and the emission polarizer at the magic angle (54.7° to the vertical) to remove polarization effects. The sample was prepared with absorbance at 374 nm below 0.1 to avoid inner filter effects. All decays were collected with a peak pre-set of 10,000 counts.

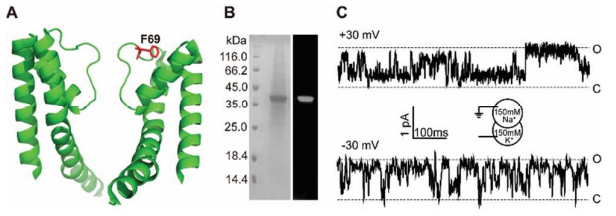

Fig. 1 The reconstituted NaK-F69HC in POPC/POPG liposomes was shown to retain normal channel function. (A) Ribbon representation of NaK (PDB number 3E8H) viewed from the membrane with the front and rear subunits removed. The fluorescent unnatural amino acid, HC, was incorporated at the Phe69 site (represented in red line). (B) Coomassie-stained SDS-PAGE gel (left) and the fluorescence image (right) of the purified NaK-F69HC protein. (C) Single-channel traces of NaK-F69HC reconstituted into lipid bilayers (POPC : POPG = 3:1) at ±30 mV.

Different components of fluorescence lifetime were derived through fitting the fluorescence intensity decay using a sum of multiple exponential components:

$$F(t) = A + \sum_{i=1}^{n} \alpha_i \exp\left(-\frac{t}{\tau_i}\right). \quad (1)$$

τ_i is the lifetime, α_i is a pre-exponential factor or weighting factor representing the amplitude of the component at t = 0, and n is the number of lifetimes. The best acceptable fluorescence lifetime components were obtained with a data-fitting chi-squared value (χ^2) below 1.2, and there were no further meaningfully improvements with the addition of an extra decay component. The overall average lifetime was calculated as the sum of normalized pre-exponential multiplied by the lifetime:

$$\langle\tau\rangle = \sum_{i=1}^{n} \alpha_i \tau_i. \quad (2)$$

Fig. 2 shows the fluorescence intensity decays analyses of NaK-F69HC in buffers containing K^+/Na^+ at molar ratios of 0/100 (Fig. 2A), 20/80 (Fig. 2B), 60/40 (Fig. 2C) and 100/0 (Fig. 2D). A total of three components of the fluorescence lifetime of HC in the NaK-F69HC channel were derived from the fitting of fluorescence intensity decay. Since the lifetime in a protein is very sensitive to the local environment around the fluorophore probe, the three components of the fluorescence lifetime indicated three different conformations of the fluorophore in NaK-F69HC. The three lifetime values (τ, a high τ value indicates stable fluorophore environment or slow decay, while a low τ value indicates a fast decay or high probability of quenching) and corresponding weighting factors (α, relative population of the fluorophore with the lifetime value) were derived for NaK-F69HC in six buffers at different K^+/Na^+ molar ratios (Table S1, ESI†). The three fluorescence lifetime values were approximately 5.70 ns, 1.65 ns and 0.16 ns. The weighting factors (α_1, α_2 and α_3) for the three lifetimes were similar when the K^+/Na^+ molar ratios were no more than 60/40 (Fig. 2E). With increasing K^+ concentration (with complementary decreased of Na^+ concentration), α_1 for the highest fluorescence lifetime value (5.70 ns) decreased from 0.43 to 0.28, α_2 for the medium level fluorescence lifetime

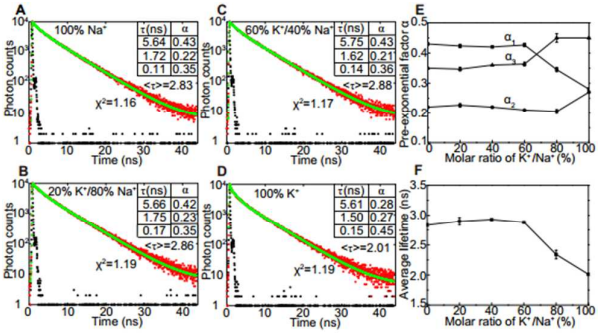

Fig. 2 Discrete analysis of fluorescence intensity decays of NaK-F69HC in buffers containing different K^+/Na^+ molar ratios. Decays were fitted using Eq. 1. (A) 0% K^+/100% Na^+. (B) 20% K^+/80% Na^+. (C) 60% K^+/40% Na^+. (D) 100% K^+/0% Na^+. (E) Weighting factor represented the amplitude of each lifetime component. (F) Average fluorescence lifetime of NaK-F69HC at different K^+/Na^+ molar ratios, calculated using Eq. 2.

value (1.65 ns) increased from 0.22 to 0.27 (only at 100% K^+), and α_3 for the smallest fluorescence lifetime value (0.16 ns) increased from around 0.35 to 0.45. Moreover, as shown in Fig. 2F, the overall average lifetime values of NaK-F69HC decreased from 2.93 ns (K^+/Na^+ = 40/60) to 2.01 ns (K^+/Na^+ = 100/0). The observed decrease of stable fluorophore population (τ_1 = 5.70 ns), increased population of labile fluorophore (easily quenched, τ_3 = 0.16 ns) and decreased overall average lifetime values at increased K^+ concentrations (in buffers containing constant cation concentrations or ionic strengths) are shown in Fig. 2E and 2F. In parallel, the fluorescence lifetime for the fluorophore compound, HC, was measured under the same buffer conditions as NaK-F69HC, with either 200 mM NaCl or 200 mM KCl. The measured fluorescence lifetime values were almost the same (Fig. S2, ESI†). These results immediately excluded different influences of Na^+ or K^+ on the fluorescence lifetime of HC, while the fluorescence intensity attenuation of NaK-F69HC in buffers containing elevated K^+/Na^+ molar ratio (but constant ionic strength) strongly suggested that the fluorophore of NaK-F69HC was easily be quenched by elevated K^+ concentrations, and there is less interaction between the fluorophore and Na^+.

Therefore, the observed decreased overall average lifetime of NaK-F69HC at elevated K^+ condition represented the K^+ preference state of NaK channel.[15] As shown in Fig. 2E and 2F, the decreased overall average lifetime of NaK-F69HC was due to simultaneous decrease of α_1 (τ_1 = 5.70 ns) and complementary increase of α_3 (τ_3 = 0.16 ns) , together with minor increase of α_2 (τ_2 = 1.65 ns) with elevated K^+ concentration. Then, the conformation 3 (τ_3 = 0.16 ns) might represent the K^+ preference state of NaK-F69HC, while conformation 1 (τ_1 = 5.70 ns) represents the K^+ non-preference state of NaK-F69HC (especially at low K^+ condition). Recently, strong correlation was observed between the channel open state and the decreased lifetime of attached TMR to the pore-lining residues of KcsA.[12] This was consistent with our observations that the decreased HC lifetime of NaK-F69HC site at the pore entrance was due to the stronger interaction between K^+ and NaK pore region[15] and consequent cationic quenching effect on the fluorophore during the K^+ flow through the NaK channel.

The previously reported crystal structures of NaK channel with different cations illustrated ion binding properties of the NaK selectivity filter.[15] Although the NaK channel can conduct Na^+, K^+ and other cations, the external site (around Gly67) was reported to have higher selectivity for K^+ compared with Na^+, while the internal sites (Val64 and Thr63) in the selectivity filter demonstrated similar selectivity for Na^+ and K^+ (Fig. S3, ESI†).[15] The observed specific binding of K^+ close to Gly67 consequently increased local K^+ concentrations. Under these circumstances, the relative high concentration of K^+ could have a greater charge-induced quenching effect on the proximate fluorophore at the Phe69 site of NaK-F69HC. Although the F69 site was surrounded by several acidic residues, the non-specific binding of K^+ and Na^+ should have similar influences on the NaK channel. With the coordinating preference of K^+ over Na^+ in the NaK channel, especially at the Gly67 site[15] and Gly65 site (unpublished solid state NMR data), the elevated K^+ concentration or K^+/Na^+ molar ratio might result in more K^+ around the pore entrance (F69 site) of the NaK channel, leading to consequent cationic quenching effect on NaK-F69HC and decreased fluorescent lifetime values of HC.

Further time-resolved fluorescence anisotropy measurements were conducted using DeltaFlex. The vertically ($I_{VV}(t)$) and orthogonally ($I_{VH}(t)$) polarized emission decays were collected while samples were excited with vertically polarized light. The peak difference was set to 10,000 counts between $I_{VV}(t)$ and $I_{VH}(t)$. The polarization bias (G factor) of the fluorescence detection system was determined by measuring two additional decays, $I_{HV}(t)$ and $I_{HH}(t)$, while the samples were excited with orthogonally polarized light and the emission was monitored with a polarizer oriented in the vertical and orthogonal directions. The fluorescence lifetime based anisotropy measurements retained the advantage, due to the lack of bleaching or scattering interference and providing accurate motion information of the fluorophore.[8b, 16] The time-resolved anisotropy, r(t), was calculated according to the following equation:

$$r(t) = \frac{I_{VV}(t) - GI_{VH}(t)}{I_{VV}(t) + 2GI_{VH}(t)}, \quad (3)$$

where $G = \int I_{HV}(t)\,dt / \int I_{HH}(t)\,dt$.

Then, the fluorescence anisotropy decay could be fitted using the following function:

$$r(t) = r_\infty + (r_0 - r_\infty)\exp(-t/\tau_r) \quad (4)$$

where r_∞ is the limiting anisotropy at infinite time, r_0 is the initial anisotropy at time zero, and τ_r is the rotational correlation time.

In Fig. 3A–3D, the fluorescence anisotropy measurements of NaK-F69HC in buffers at different K^+/Na^+ molar ratios are shown. Consequently, the time-resolved fluorescence anisotropy could provide an accurate estimation of the side chain motion (τ_r) of the fluorophore in NaK-F69HC (Fig. 3E). Interestingly, much slower motion (large τ_r) was observed for

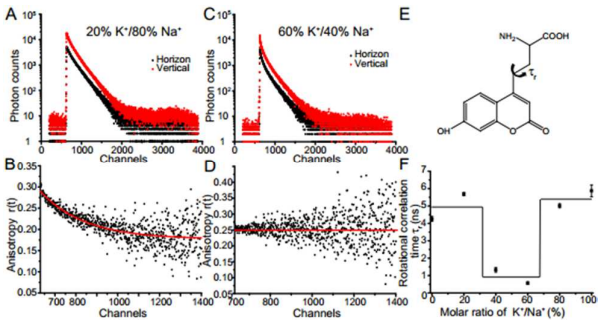

Fig. 3 Fluorescence anisotropy analysis of NaK-F69HC in buffers containing different K^+/Na^+ molar ratios. (A) The fluorescence intensity decays with polarized detection and (B) the anisotropy analysis of sample in buffer containing 20% K^+/80% Na^+. (C) The fluorescence intensity decay with polarized detection and (D) the anisotropy analysis of samples in buffer containing 60% K^+/40% Na^+. (E) Side chain motion representation of HC. (F) Rotational correlation time τ_r of NaK-F69HC at different K^+/Na^+ molar ratios.

the NaK-F69HC in buffer with 80% or 100% Na^+ or K^+, while faster motion (small τ_r) was observed for the sample in buffer with 40% or 60% Na^+ or K^+ (Fig. 3F). The observed slow motion, or large τ_r, indicated that the locally concentrated Na^+ or K^+ induced relatively high viscosity around the fluorophore or that the different channel conformations in unbalanced K^+/Na^+ molar ratios restricted motion of the fluorophore. The faster motion, or small τ_r, indicated that balanced Na^+ and K^+ prevented any ion preference or specific cation-fluorophore interaction in the NaK-F69HC channel.

In summary, the decreased fluorescence lifetime of the fluorophore in NaK-F69HC at high molar ratios of K^+/Na^+ implies that the NaK channel has a higher K^+ preference in the entrance region of the channel, consistent with previous reports on the specific binding of K^+ (not Na^+) around the Gly67 site.[15] The time-resolved fluorescence anisotropy and consequent estimation of rotational correlation time revealed reduced motion of the fluorophore in NaK-F69HC when the K^+/Na^+ molar ratio was unbalanced and fast motion of the fluorophore when the K^+/Na^+ molar ratio was balanced. This strongly suggests that unbalanced cation molar ratios might be the basis for the ion preference of an ion channel.

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