"Quantifying the Diffusion of Membrane Proteins and Peptides in Black Lipid Membranes with Dual Focus Fluorescence Correlation Spectroscopy"

Supporting Information

The applicability of the Saffman-Delbrück model considering the influence of membrane protein interactions such as hydrophobic mismatch was studied by measuring the diffusion of membrane proteins of different sizes.

The membrane proteins Cytochrome-B5, KcsA, EcClC and AcrB were reconstituted with two different approaches: direct addition of the protein solution to the buffer surrounding the bilayer and SNARE-mediated vesicle fusion. In the following Supplementary Information, different control experiments to characterize the respective structure of KcsA inside the membrane are presented to support our theory that it can incorporate as a monomer or tetramer depending on the reconstitution method used as shown in ref. (1).

KcsA was chosen as an example because it has the highest stability compared to EcClC and AcrB. Moreover, the protein's longer opening times and larger currents as compared to EcClC allow for an easier electrophysiology measurement even in the presence of noise. While the KcsA tetramer forms one transmembrane pore, the EcClC dimer consists of two separate pores. It has been suggested that these pores can function independently, even in monomeric EcClC (2,3). Therefore, distinguishing EcClC monomers and dimers by means of electrophysiology is much more difficult than distinguishing KcsA monomers and tetramers. AcrB was not chosen because it is a multi-drug efflux pump which works in complex with two other proteins, AcrA and TolC (4). Therefore, its functionality could not be investigated by means of electrophysiology with our experimental setup.

Direct addition of KcsA to the BLM

In order to check which KcsA species is present upon direct addition of the protein to the BLM system, the experimental conditions of the direct addition were recreated. KcsA was measured in solution in the presence of surfactant and after surfactant dilution. In the presence of surfactant micelles, the correlation curves cannot be fitted in a meaningful way (figure S1). Their shape indicates strong polydispersity which can be attributed to differently sized micelles stabilizing KcsA.

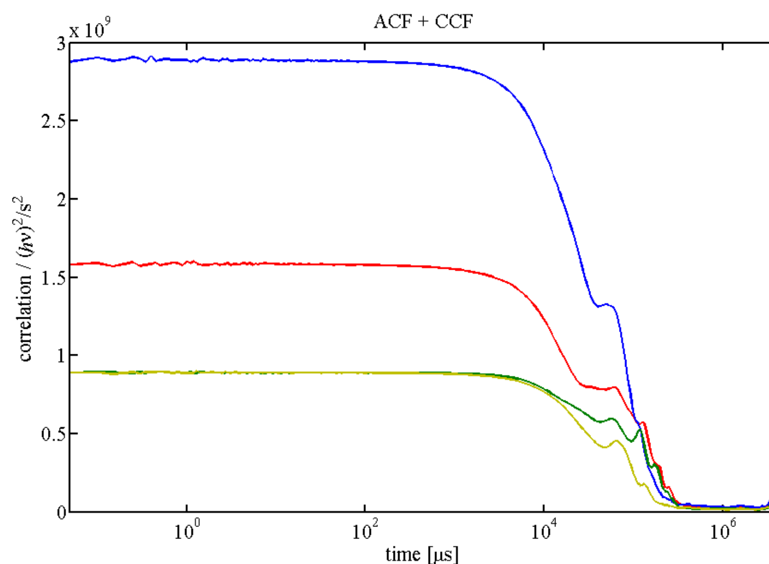

Figure S1: Autocorrelation (blue and red) and crosscorrelation (green and yellow) curves of KcsA^{Alexa647} in PBS with 5 mM DM. The sample is highly polydisperse.

Upon dilution, the correlation curves become smooth and can be fitted (figure S2). The resulting diffusion coefficients correspond to the ones expected for KcsA monomers.

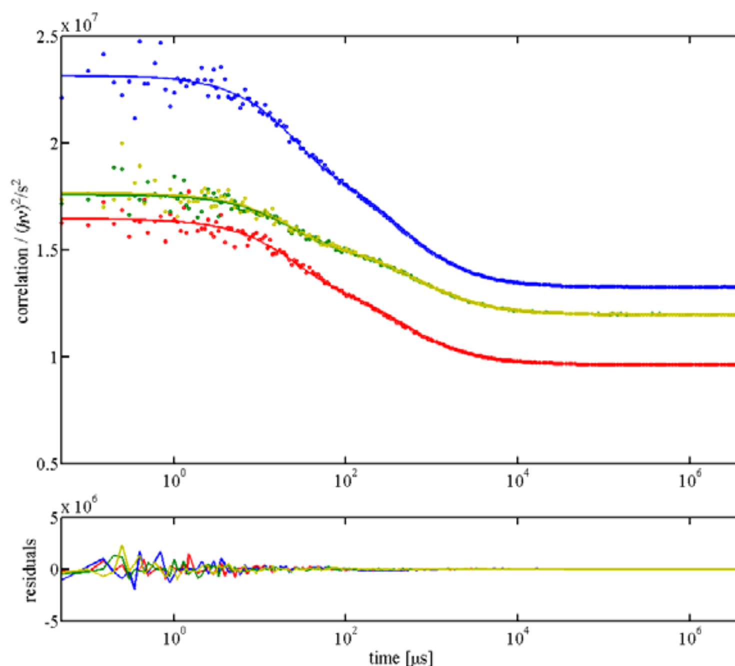

Figure S2: Autocorrelation (blue and red) and crosscorrelation curves (green and yellow) for KcsA without surfactant. The obtained diffusion coefficient D = 280 (± 7) $\mu m^2 s^{-1}$ corresponds to a hydrodynamic radius of 0.8 nm matching the size of a KcsA monomer. The correlation decay component at short times is caused by triplet-state photophysics of the dye (Alexa647) and was fitted with an additional exponential term.

A dilution factor of 1:100 was chosen because it corresponds to the dilution used during the BLM preparation and measurements. In BLM experiments, the chip to which the protein solution is added has a volume of 300 µL. The protein is therefore diluted 1:60 upon addition to the chip. During BLM formation the chip is flushed with additional solvent (usually about 1 mL) which leads to an overall dilution of at least 1:100, i.e. well below the surfactant's cmc, before the protein is incorporated into the lipid membrane. Diluting the sample 1:1000 and performing 2fFCS measurements in solution yields similarly smooth correlation curves and the same diffusion coefficient.

In a second control experiment, the protein was heated to 95 °C for 10 minutes after purification and labeling (i.e. in presence of surfactant) to generate monomers. The resulting sample was analyzed by SDS PAGE (figures S3 and S4 depict the SDS PAGEs before and after heating, respectively), which clearly shows that after heating only monomers are present in the sample.

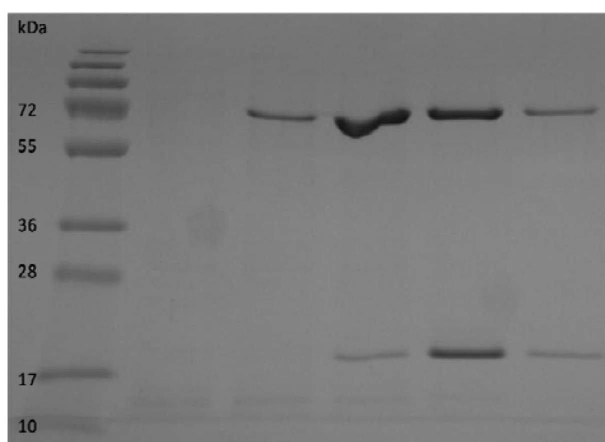

Figure S3: SDS-PAGE of KcsA after purification without heating. The left lane shows the marker, the other lanes represent elution fractions from Ni-NTA purification via His-Tag. KcsA monomers (≈ 18 kDa) and tetramers (≈ 67 kDa) are present after purification.

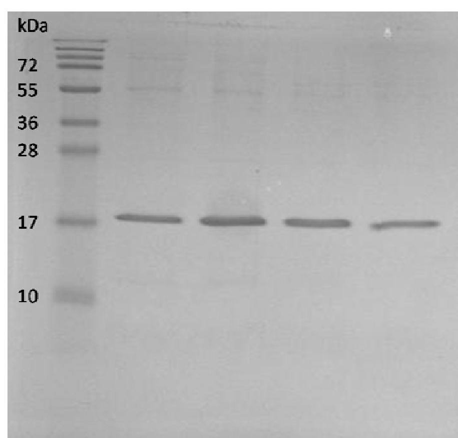

Figure S4: SDS-PAGE of KcsA after purification and heating to 95 °C for 10 minutes. Only the monomeric form (≈ 18 kDa) of KcsA is present in all fractions. The left lane shows the marker, the other lanes show heated elution fractions from Ni-NTA purification via His-Tag.

The heated fluorescently labeled KcsA sample was then added to the BLM system. The protein incorporated into the bilayer, which was shown by fluorescence imaging. The measured diffusion coefficient was 9.1 (± 0.2) $\mu m^2 s^{-1}$. Without heating, a diffusion coefficient of 9.3 (± 0.5) $\mu m^2 s^{-1}$ for KcsA was obtained. The mean values of the diffusion coefficients differ by only 1.4 %, which suggests that in both experiments the same species was measured. An exemplary correlation curve is shown in figure S5.

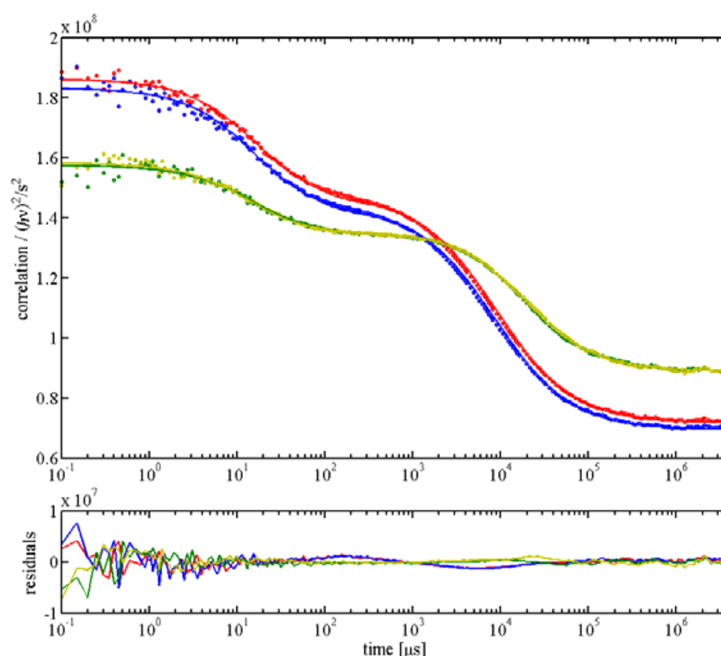

Figure S5: Autocorrelation (blue and red) and crosscorrelation (green and yellow) curves of KcsA^{Alexa647} in POPC/POPE Black Lipid Membrane. The correlation decay component at short times is caused by triplet-state photophysics of the dye (Alexa647) and was fitted with an additional exponential term.

SNARE-mediated vesicle fusion

The second approach chosen to reconstitute proteins into BLMs was SNARE-mediated vesicle fusion. The membrane proteins were first incorporated into large unilamellar vesicles (LUVs) and then fused to the BLM using SNARE proteins.

The structure of KcsA in the BLM after SNARE-mediated vesicle fusion was investigated by electrophysiological measurements. For BLM formation, DOPG was added to the POPC/POPE bilayer mixture to a final concentration of 1 mg/mL, since KcsA gating requires the presence of negatively charged lipids (1). The KcsA diffusion coefficient in both lipid mixtures POPC/POPE and POPC/POPE/DOPG was determined with 2fFCS and found to be identical.

PBS buffer containing 400 mM KCl (pH 4) was used on both sides of the membrane to allow for KcsA gating. KcsA opening could be detected using +200 mV and -200 mV pulses (figure S6). On average, 2.6 pA and 2.3 pA currents were detected per burst at -200 mV and 200 mV, respectively. There are no values published for the exact conditions used in our experiment. The published data (5,6) suggest larger values but it has also been shown that sodium ions can partially block KcsA channels and decrease the measured conductance (6). Since we are working in PBS buffer which contains large amounts of Na^+ ions, it is likely that the channels are also partially blocked in our experiment. Thus, we conclude our data is consistent with the studies previously published.

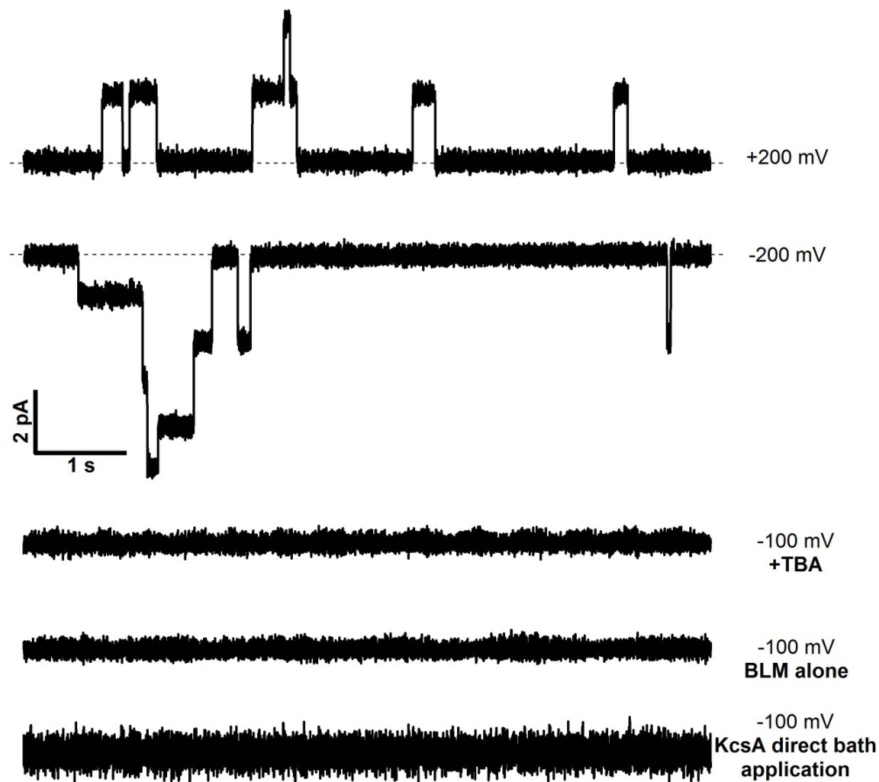

Figure S6: Electrophysiology data of KcsA. Top: KcsA (tetramer) incorporated via SNARE-mediated vesicle fusion into a POPC/POPE/DOPG BLM. As buffer PBS (pH 4) with 400 mM KCl was used. Bottom: control experiments – KcsA was blocked with tert-butyl ammonium (TBA) and the BLM was measured without protein.

We were also able to measure a sub-conductance state which is known to exist for KcsA (7) and which was about half of the conductance.

Electrophysiology measurements of the BLM without protein and the BLM with KcsA channels in presence of the potassium channel blocker tetra-butyl ammonium (TBA) (5) were performed as additional controls. Conductance steps were only observed when KcsA was incorporated into the BLM via SNARE-mediated vesicle fusion. The conductance steps were completely abolished in the presence of TBA. TBA was added at a concentration of 1 mM to the buffer surrounding the BLM. The final concentration is estimated to be 0.08 mM. The BLM by itself did not show conductance steps. Therefore, the observed steps in the first experiment can be attributed to KcsA channels.

Electrophysiology was also performed with KcsA directly added to the BLM. In this case, no conductance steps were observed, which confirms that only monomers are incorporated into the BLM.

Supporting References

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