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Supporting Online Material for

Ion Selectivity in a Semisynthetic K^+ Channel Locked in the Conductive Conformation

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Supplementary Information

METHODS

Semi-synthesis of KcsA$^{D\text{-}Ala77}$. The KcsA polypeptides with the Gly^{77}→D-Ala substitution were generated by an expressed protein ligation (EPL) of a synthetic C-peptide corresponding to residues 70-123 and a recombinantly expressed N-peptide thioester corresponding to residues 1-69. Protocols for the synthesis/purification of the C-peptide and recombinant expression/purification of the N-peptide thioester have been previously described and were used without modification (*S1, S2, S3*). Purified peptides were then ligated using established procedures and the resulting KcsA analogues folded and purified as previously described. In addition to the Gly^{77}→D-Ala substitution, the semi-synthetic proteins used for the structural studies contained the following amino acid substitutions; Ser^{69}→Ala, Val^{70}→Cys (at the ligation site). The semi-synthetic protein used for electrophysiological studies contained, in addition to the above, the following amino acid substitutions; Gln^{58}→Ala, Thr^{61}→Ser, Arg^{64}→Asp (mutations that render KcsA sensitive to inhibition by $AgTx_2$) and Ala^{98}→Gly (a mutation that increases the open probability of KcsA) (*S2*). The KcsA protein used as the wild type control in the electrophysiological analysis contained the following amino acid substitutions Gln^{58}→Ala, Thr^{61}→Ser, Arg^{64}→Asp and Ala^{98}→Gly. This recombinant protein was expressed and purified as previously described (*S4*).

Crystallography. KcsA$^{D\text{-}Ala77}$ was crystallized as complex with Fab in the presence of 150 mM KCl at 20 ^{o}C using the sitting drop method (*S5*). The crystallization

solution consisted of 20-25% PEG400, 50 mM magnesium acetate, pH 5.5-6.5. Cryo-protection of the crystals was achieved by increasing the PEG concentration in the reservoir to 40%. All crystals were flash frozen in liquid propane cooled in liquid nitrogen. Crystals of the complex in the presence of 1 mM KCl were obtained using two different methods. In the first method, the KcsA-Fab complex was dialysed extensively against a buffer containing 1 mM KCl + 149 mM NaCl prior to crystallization. Alternatively, crystals of the KcsA-Fab complex obtained in the presence of 150 mM KCl were washed twice in a wash solution that was identical to the crystallization solution except that it contained 1 mM KCl + 149 mM NaCl. The crystals were incubated overnight in the low K^+ solution and then cryoprotected and frozen for data collection. Of the data sets collected, the higher resolution data-set obtained from a crystal prepared using the second method is reported in this paper.

Data were collected at beam-line X25 of the National Synchrotron Light Source, Brookhaven National Laboratory. The data were processed and scaled using Denzo and Scalepack form the HKL program suite (*S6*). The structures were solved by molecular replacement using the published KcsA-Fab structure (PDB code: 1K4C) as the search model. The initial model was modified to incorporate the amino acid changes present in the semi-synthetic KcsA molecule and then refined by cycles of manual rebuilding using O (*S7*) and refinement using CNS (*S8*). The final model of the D-Ala mutant at high K^+ contains 4035 protein atoms, 7 K^+ ions, 1 lipid molecule and 266 H_2O molecules and is refined to R_{work} and R_{free} of 23.3% and 24.2%, respectively. The final model of the D-Ala mutant at low K^+ contains 4061 protein atoms, 5 K^+ ions, 1 lipid molecule and 49 H_2O molecules and is refined to R_{work} and R_{free} of 23.3% and 25.1%,

respectively. Data collection and refinement statistics are given in Table S1, which is provided in the Supplementary information. Coordinates have been deposited in the Protein Data Bank under accession code 2IH3 for the high K^+ structure and 2IH1 for the low K^+ structure.

Electrophysiology. The semi-synthetic or recombinant KcsA molecules were reconstituted into lipid vesicles composed of 1-Palmitoyl-2-Oleoyl-Glycero-3-Phosphoethanolamine (POPE) (7.5 mg/ml) and 1-Palmitoyl-2-Oleoyl-Glycero-3-Phosphatidylglycerol (POPG) (2.5 mg/ml) at a protein to lipid ratio of 0.5-1:10 (*S9*). For measurements of channel activity, the lipid vesicles were fused with planar lipid bilayers composed of POPE (15 mg/ml) and POPG (5 mg/ml) painted over a 300μ hole in a polystyrene partition separating the internal (pH 4.0) and external solutions (pH 7.0) as previously described. Internal pH 4.0 is required to open the gate of the KcsA K^+ channel (*S10, S11*). Leak currents were determined by blocking the current through the channel with 5μM $AgTx_2$, which was added to the external side.

The focus of this study is on the properties of ion conduction through the pore rather than on channel gating. However, we studied gating by analyzing open time histograms from bilayers containing single channels or a sufficiently small number of channels so that double open events were not observed. For wild-type KcsA (containing mutation A98G and 35 c-terminal amino acids truncated) the predominant open state had a mean lifetime of 11 msec. For $KcsA^{D\text{-}Ala77}$ the predominant open state had a mean lifetime of 7 msec. The gating properties of these channels are not dramatically different.

Table S1. Crystallographic data collection and refinement statistics

	High K^+	Low K^+
Data Collection		
Resolution (Å)	1.7	2.4
R_{sym} [a]	0.064(0.38)	0.063(0.496)
Completeness(%)	98.1(87.6)	95.1(90.7)
I/σ	20.7(2.0)	21.6(2.7)
Redundancy	2.9(1.9)	4.1(3.9)
Refinement statistics		
R_{free}/R_{work}(%) [b]	24.2/23.3	25.1/23.3
Mean *B*-factor ($Å^2$)	58.9	52.2
Root mean square difference		
Bond lengths (Å)	0.013	0.014
Bond angles (^{o})	1.6	1.7

[a] $R_{sym} = \sum |I_i - \langle I_i \rangle| / \sum I_i$ where $\langle I_i \rangle$ is the average intensity of symmetry-equivalent reflections.
[b] $R = \sum |F_o - F| / \sum F_o$, 10% of the reflections that were excluded from the refinement were used in the R_{free} calculation for the high K^+ structure while 5% were excluded for R_{free} calculation for the low K^+ structure.
Numbers in brackets are statistics for the last resolution shell.

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