Supplementary Information

Conformational Dynamics at the Inner Gate of KcsA During Activation

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Supporting Information:
Detailed experimental procedures, supporting data, supplemental Table I and supplemental figures S1-S4. This material is available free of charge via the Internet at http://pubs.acs.org.

DEER and Continuous Wave EPR

Expression and Purification of KcsA Tandem Dimer

A tandem dimer construct of an n-terminal hexahistidine tagged KcsA (KcsA TDL9), in pQE32 (Qiagen), with a 9-residue linker (ENLYFQGGT) region containing an rTEV protease site was used to determine distances between diagonal subunits as previously described 1,2. The KcsA TDL9 construct used for this study possessed a cysteine mutation at residue G116 in one of the two protomers and was used to transform XL10 Gold cells made freshly competent. Cells were grown overnight at 37° C on agar plates containing ampicillin. The next day, a colony was selected to create a starter culture in LB (Invitrogen) and ampicillin [100 mg·ml⁻¹] (Mediatech) with shaking (250 RPM) at 37° C. Subsequently, the starter culture was then used to create an overnight culture in LB and ampicillin [100 mg·ml⁻¹] with 0.2% v/v glucose. The use of glucose suppresses background expression of protein before induction 3. This overnight culture was used the next day to inoculate LB and ampicillin [100 mg·ml⁻¹] at a final concentration of 1% v/v with 0.2% v/v glucose. Cultures were grown at 37° C with shaking (250 RPM) to an O.D. of 0.6 and then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (ITPG) (Anatrace). Cells were grown for 3 hours after induction, harvested at 8900 RCF for 10 minutes at 4° C, supernatant removed, and pellets stored at -80° C.

Pellets were resuspended [10 mL/g] in Buffer A (HEPES [50 mM], KCl [200 mM], pH 7) with the addition of phenylmethylene sulfonylfluoride (PMSF) to a 1 mM concentration and 3 mg
of DNase A per liter of culture. The resuspended cells were homogenized with a French press, ultracentrifuged (30 minutes, 4°C, 158,000 RCF) to pellet membranes, supernatant discarded and then the pellet was solubilized with Buffer A supplemented with 10 mM n-dodecyl β-D maltopyranoside (DDM) (Affymetrix) at room temperature for 1 hr in the presence of β-mercaptoethanol (BME) (Sigma-Aldrich) [5 mM] and PMSF [1 mM]. Homogenate was ultracentrifuged (1 hr, 4°C, 185,000 RCF), and supernatant retained.

A semi-batch technique was used for immobilized metal affinity chromatography (IMAC). Talon Metal affinity Co²⁺ resin was pre-equilibrated with Buffer A with the addition of BME [5 mM] and DDM [1 mM] at pH 7 before loading supernatant from solubilized membranes. Resin with supernatant was incubated with continuous, gentle rotation for 1 hour at room temperature. Gravity columns were used to collect resin after incubation. Resin was washed with 15 bed volumes of Buffer A supplemented with DDM [1mM], imidazole [5 mM] and tris (2-carboxyethyl) phosphine (TCEP) (Amresco) [0.5 mM]. KcsA TDL9 was eluted from the column with 5 bed volumes of Buffer A supplemented with DDM [1mM], imidazole [500 mM], TCEP [0.1mM] at pH7 with 1 mL fractions collected. Flow, wash and fractions were analyzed using SDS-PAGE 4-20% gradient TGX gels (Bio-Rad) for purity and efficiency of IMAC. Fractions were selected and protein concentrated to a working concentration using Amicon-Ultra centrifugal filter units (30,000 MWCO)(Merck Millipore).

**rTEV digestion and SDS-PAGE analysis of products**

Purified protein was then processed to cut the linker region between the two protomers using the cysteine protease Tobacco Etch Virus with the S219N mutation[^4]. KcsA TDL9 was exchanged into an rTEV buffer (HEPES [50 mM], KCl [200 mM], DDM [1mM], EDTA [0.5 mM], dithiothreitol (DTT) [1mM] at pH 8 using a PD10 column (GE Healthcare). After exchange, KcsA TDL9 was incubated at a 1:5 molar ratio of rTEV to KcsA TDL9 overnight at room temperature. An aliquot of each reaction, including controls, were analyzed with SDS-PAGE to assess efficiency of cleavage. Since KcsA is resistant to SDS[^5,6], it is important to differentiate between resistant monomers of the protein and incompletely digested constructs. Irreversible denaturation of the protein ensures that only the monomeric form of the protein are visualized on SDS-PAGE if they are successfully cut[^7,8]. Digested protein and 60% trifluorethanol (v/v)
(Sigma Aldrich) were used to ensure complete dissociation and verify digestion was completed. Reactions were processed using Size Exclusion Chromatography on a Superdex 200 10/300 GL column pre-equilibrated with Buffer A with the addition of DDM [1mM], TCEP [0.1 mM] at pH 7 to remove free DTT and isolate monodisperse, tetrameric forms of cleaved KcsA TDL9.

**Labeling KcsA TDL9**

Upon elution from the SEC, digested KcsA TDL9 G116C was reacted with a 2 rounds of spin label ([1-oxyl 2,2,5,5-tetramethylpyrrolidin-3-methyl]-methanethiosulfonate; MTS-SL) (Toronto Research Chemicals) for 30 minutes on ice. Fifteen and ten molar excess were used for the first and second incubation respectively. The reaction was then quenched with a 30x excess total probe concentration of L-cysteine. The labeled protein was then exchanged into Buffer A supplemented with DDM [1mM] at pH7 either by PD10 column for CW-EPR studies (supplementary Figure 1 for all spectra in asolectin liposomes) or SEC with a Superdex 200 10/300 GL column for DEER studies. After collection, the sample was briefly incubated at 42° C for 10 minutes and a fraction was used to verify labeling efficiency on the spectrometer. Diamagnetic spin label (Toronto Research Chemicals) was used to prepare under-labeled samples required for distance estimation using CW-EPR. Under labeling was accomplished by creating a 1:10 diamagnetic to MTS-SL ratio mixture to label the protein. Samples were then either prepared for DEER analysis by the addition of 20% glycerol to a 200 uM KcsA TDL9 G116C-MTS-SL rTEV digested sample or reconstituted into asolectin liposomes for CW analysis.

**Reconstitution of KcsA TDL9 G116 into Asolectin liposomes for CW EPR**

Asolectin (Avanti Polar Lipids) was prepared by removing chloroform using a Roto-vapor R114 rotary evaporator (Buchi) followed by further drying with an N2 stream for 10 minutes. Lipids were then reconstituted with Buffer A at pH 7 at an equivalent volume to the sample being reconstituted followed by sonication for 10 minutes. Protein was then added to the reconstituted lipid at a 1:1000 molar ratio, incubated for 30 minutes at room-temperature with continual rotation and then diluted to a final volume of 14 mL with Buffer A. Detergent was removed from the sample gradually using three rounds of incubation with Biobeads (Bio-Rad). In the first two rounds, samples were incubated with 70 mg of beads for 30 minutes with continual rotation at room temperature. In a third round, the sample was incubated overnight.
with 70 mg Biobeads. Sample was visually inspected for the presence of detergent (bubbles) before proceeding to ultracentrifugation (40,000 RPM) for 1 hour at 4° C to pellet the sample. The final pellet was resuspended in a 150 μL volume of McIlvain Buffer at the desired pH. The sample was then repelleted and resuspended in the same buffer one more time and the pH was verified with a micro pH electrode before proceeding to measurement.

**CW-EPR Spectroscopy and Data Analysis**

Spectra from fully labeled or under labeled samples in the germane pH were recorded at 145K using liquid N$_2$ in sealed glass capillary tubes with the following instrument parameters: 50 μW incident power, 100 KHz modulation frequency and 2 G amplitude modulation, 41 ms conversion time, 41 ms time constant with 1024 points of acquisition and a 300G field sweep on a Bruker EMX Spectrometer with a di-electric resonator. Broadening of dipolar spectra from pH gating were analyzed with CWDipFit to determine distances. The program may be downloaded at the URL provided at the end of this supplemental file. Error was measured as the width of the population at half-maximal intensity and normalized to pH4 to emphasize comparisons between pH increments. The average width at half-maximal intensity was 0.67 AU +/- 0.05 S.E.M. Supplementary Figure 2 presents CW data with fully labeled, under labeled and population distributions for pH7 to pH4 in 0.5 pH increments. The upper limit of CW distance determination is achieved at pH4 and further measurements are conducted in DEER (see below) with finer pH titrations.

**DEER Spectroscopy and Data Analysis**

Quartz capillaries tubes (Vitrocom) were loaded with sample and introduced into a cryostat (Oxford) pre-equilibrated to 80K with Liquid N$_2$ on a Bruker Elexsys 580 EPR spectrometer with a 3 mm split-ring resonator. The cavity was tuned in continuous wave mode and over-coupled before proceeding to pulse mode. Instrument settings for a standard DEER pulse used include a 16 ns 90° pulse, a 32 ns 90° pulse with evolution times ranging from 1800-2200 ns. The time ranges were varied based on the quality of signal to noise and evolution of signal echo (i.e., distance). Pump pulses focused on the central frequency and observer pulses were placed in the low field approximately 65-70 MHz away by calculating the ELDOR frequency. 1024 points with a 1500 ns shot repetition time were used. An empirical number of scans were
determined with continuous analysis of data until sufficient signal to noise were achieved as evidenced by both the initial decay of the dipolar evolution as well as the extent and quality of the echo itself. Data were analyzed using a model-free Tikhonov regularization using the DeerAnalysis2013 program to generate a distance distribution. Supplementary Figure 3 presents data from pH3 for DEER spectroscopy. Briefly, the alpha parameter was selected which balances the effects of smoothing (ρ) (by calculating the mean square deviation of the experimental and observed form factor F) and the roughness of the fit (η) which assesses the second derivative of the population distribution. This is graphically represented as an “L-curve” (Supplementary Figure 3) with the point of inflection, or maximum curvature being selected as the alpha-value (alpha-value of 10 was selected for all three data points). The alpha-values used for all three solutions were 100. Error was measured as the width of the population at half-maximal intensity for CW data. DEER data was evaluated for the contribution of background to the signal using the validation suite in DeerAnalysis2013. Origin Pro 7.0 was used to calculate the pKa of activation by using the built-in fitting function for a Boltzmann distribution.

**Expression and Purification of rTEV**

The rTEV expression vector pRK793, in conjunction with tRNA accessory plasmid pRIL, were used with BL21 (DE3) (Agilent) competent cells to overproduce protein. Briefly, glycerol stocks of BL21 (DE3) harboring both plasmids were streaked on selective agar plates, allowed to grow overnight, and then colonies used to create starter and overnight cultures. LB media was inoculated with 0.1% v/v overnight and selective antibiotics, allowed to grow to 0.5 O.D. at 37° C and shaking (250 RPM). Temperature was reduced to 30° C, induced with IPTG [1 mM] and grown for an additional 4 hours before harvesting. Pellets were stored at -80° C.

Pellets were resuspended in 10 mL of lysis buffer (K₂HPO₄ [50 mM] pH 8, NaCl [100 mM], 10% (v/v) glycerol, and imidazole [15 mM]), homogenize x2, and polyethyleneimine (Sigma Aldrich) was added to 0.1%. Homogenate was mixed by inversion and centrifuged at 25,000 RPM RCF for 30 minutes. Supernatant was collected and processed using IMAC chromatography with Talon Metal Affinity Co²⁺ resin pre-equilibrated with lysis buffer. The column was then washed with 7x bed volume of ice cold lysis buffer. Fractions were collected by eluting the protein with elution buffer (K₂HPO₄ [50 mM] pH 8, NaCl [100 mM], 10% (v/v)
glycerol, and imidazole [200 mM]), concentrated and exchanged into a storage buffer (K$_2$HPO$_4$ [25 mM] pH 8, NaCl [200 mM], 10% (v/v) glycerol, EDTA [2mM], and DTT [10 mM] via PD10 columns. Protein was aliquoted and stored at -80°C.

**Nuclear Magnetic Resonance**

*Cloning and Site Directed Mutagenesis*

KcsA was subcloned from pQE32 (Qiagen) into pET28A (Merck Millipore) using PCR amplification with the restriction sites NheI and NdeI:

5’ GAGTCATGCTAGCTCACCGGCGGTTGTCGTCG 3’ for NheI

5’ TCCGCGGCAGCCATATGCCACCCATGC 3’ for NdeI

The PCR product was ligated into pET28A using the Quick T4 DNA Ligase (New England Biolabs). QuickChange™ Subcloning was verified by the DNA Sequencing and Genotyping Facility at The University of Chicago.

*Expression and labeling*

Freshly made BL21 (DE3) pLysS competent cells (Merck Millipore) were transformed with pET28A KcsA WT and grown overnight at 37°C on agar plates with selective antibiotic. The next day a single colony was grown in LB and kanamycin [30 ug·ml$^{-1}$] at 37°C with shaking (250 RPM) for 8 hours before starting overnight cultures in the same growing conditions. Overnight cultures were used to inoculate LB and kanamycin [30 ug·ml$^{-1}$] at a final concentration of 1% v/v. Cultures were grown until O.D. of 0.6 at which point the biomass was increased 2 fold$^{15,16}$ by combination of pellets. Cultures were spun at 4000 RCF for 10 minutes at 4°C. The pellet was resuspended with 10 mL of sterile defined M9 media, returned to 990 mL of sterile defined media and allow to acclimate for 1 hour at 37°C with shaking (250 RPM) before induction with IPTG [1mM] (Anatrace). Pellets were harvested 4 hours later at 9,000 RCF for 10 minutes.

Defined media consisted of the following reagents: 1g $^{15}$NH$_4$Cl, 3g KH$_2$PO$_4$, 0.5 g NaCl, 12.8g Na$_2$HPO$_4$·7H$_2$O, 2g glucose, 492 mg MgSO$_4$·7H$_2$O, 14.7 mg CaCl$_2$·2H$_2$O, 100 mg thiamine hydrochloride, 30 mg of kanamycin sulfate and 1 g of $^3$H/$^{15}$N Isogro in 1L of 100% deuterium oxide and 1 generic tablet of multivitamin (Walgreens). Unless noted, all other reagents were obtained from Sigma Aldrich.
**Purification**

Frozen pellets were resuspended 10 mg/g in Buffer A with 1 mM PMSF (Sigma Aldrich) and 3 mg of DNase A per liter of culture. The suspension was homogenized and ultracentrifuged for 30 minutes at 4°C at 158,000 RCF to pellet membranes and remove soluble proteins. The pellet was then resuspended in Buffer A with the addition of 10 mM DDM (Affymetrix) and rotated for 1 hour at room temperature to solubilize membrane proteins. The resulting homogenate was then re-centrifuged at 185,000 RCF for 1 hour at 4°C. Supernatant was then incubated with pre-equilibrated Talon Metal Affinity Co²⁺ resin (Clontech) (Buffer A + DDM [1 mM]) for 1 hour with gentle rotation at room temperature. Resin was collected by gravity columns, washed with Buffer A with DDM [1mM] and imidazole [5 mM] for 15 bed volumes and then eluted with Buffer A with DDM [1mM] and imidazole [500 mM] for 5 bed volumes. Fractions were collected in 1 mL increments with wash, flow and fractions analyzed by SDS-PAGE. Fractions were selected, protein concentrated (Amicon Ultra-4 centrifugal filter units at 30,000 MWCO) (Merck Millipore). The resulting protein was digested at a 1:15 mass/mass ratio of α-chymotrypsin (Sigma Aldrich) to KcsA overnight at 4°C with gentle rotation. The working volume was maintained at a level that would permit efficient digestion with α-chymotrypsin (Sigma Aldrich) (i.e., α-chymotrypsin < 10 µg/mL). The resulting cleaved protein KcsA WT (Δ125) was purified via Size Exclusion Chromatography using a Superdex 200 10/300 GL (GE Healthcare Life Sciences) column pre-equilibrated with Buffer A plus 1 mM DDM. Fractions were collected and analyzed with SDS-PAGE to assess cleavage efficiency, SEC by comparison to elution volume of non-cleaved protein, and multiple angle light scattering (Wyatt) to confirm cleavage. The final KcsA Δ125 preparation was then exchanged into an NMR buffer (MOPS [10 mM], KCl [100 mM] Fos-Choline-12 ([5 mM], germine pH) via PD10 desalting column (GE Healthcare Life Sciences) and concentrated to working range of [250-300 µM tetramer] for analysis using centrifugal filter devices with a MWCO of 30,000 Da. A 10% v/v D₂O/TSS (Sigma Aldrich) standard was added to the sample and placed within a Shigemi tube and sealed before introduction to the spectrometer. The samples concentration was determined by A₂₈₀ absorbance and the pH verified with a Ross Ultra semi-micro pH probe and Accumet AP110 portable pH meter (Thermo Fisher).
NMR Experiments and data analysis

NMR spectra were completed on a deuterated -uniform $^{15}\text{N}$ labeled KcsA WT (Δ125) using Tranverse Relaxation-Optimized Spectroscopy (TROSY)\textsuperscript{17}. Measurements were made on a Bruker Avance III HD 600 MHz spectrometer equipped with an inverse triple resonance room temperature probe with z-gradient operating at a proton frequency of 600.13 MHz. Spectra were recorded at 323K using a spectral width of 9615.385 Hz for proton and 1935.734 Hz nitrogen with 2048 complex points for proton and 256 complex points for nitrogen and a total of 64 scans. A recovery delay of 1s was used. The optimized 90° pulse lengths for both proton and $^{15}\text{N}$ were used and had values typically around 9.75 μs to 10.07 μs and 35-37 μs respectively.

$^{1}T_{1}$-TROSY and $^{2}T_{2}$-TROSY measurements were made of the backbone of peak G116 during pH gating using the same deuterated -uniform $^{15}\text{N}$ labeled KcsA WT (Δ125) protein\textsuperscript{18}. These techniques were selected based on the size of our protein and its deuterated state\textsuperscript{18}. The $T_{1}$ values were determined from multiple different relaxation delays (0.01s, 0.02s, 0.05s, 0.1s, 0.50s, 0.750s, 1s and 2s). The $T_{2}$ values were determined from nine different relaxation delays (0.02s, 0.04s, 0.06s, 0.08s, 0.1s, 0.12s, 0.140s, 0.160s, 0.180s, 0.200s, and 0.240s). In both protocols, spectra were recorded with 2048 complex points for proton and 128 complex points for nitrogen with a total of 24 scans. NMRViewJ was used to analyze data. The following simple exponential equation was used to fit data points and determine $T_{1}$ or $T_{2}$ values with associated error:

$$y = a * e^{(-b*x)}$$

The $T_{1}$ times measured were pH7 (466ms +/- 183ms), pH4 (606 ms +/- 347 ms) and pH3 (902 ms +/- 125ms) (see Figure 2C and supplementary Figure 4). The residue G116 (located near the inner gate) possesses a faster $T_{2}$ time at pH7 than pH3 (20 ms +/- 9ms (green) vs. 82ms +/- 2ms (red) respectively. The product of $T_{1}$*$T_{2}$ suggests that the exchange we are seeing is not due to fast internal motions but rather slow ones since they differ between our two conditions: pH7 (9320) versus pH3 (73694)\textsuperscript{19}.

For titrations and $T_{1}$/T2 measurements spectra were processed using NMRPipe\textsuperscript{20} and visualized and analyzed with NMRViewJ (OneMoon Scientific). Chemical shift measurements were performed using the techniques of Imai et al., 2010\textsuperscript{21}. The correction factor used for this study was calculated from the ratio of the variance of the chemical shifts for $^{1}\text{H}$ and $^{15}\text{N}$ for
phenylalanine from the Biological Magnetic Resonance Data Bank (BMRB) 32 which was 6.4. The follow equation was used:

$$\Delta \delta = \sqrt{(\Delta \delta_{1H}^2 + \frac{(\Delta \delta_{15N})^2}{6.4})}$$

**Molecular Dynamics Simulations**

**System Construction**

Tetrameric coordinates of C-terminal truncated KcsA in closed (PDB: 1K4C 23) and open (PDB: 3F5W, 3F7V, and 3F7Y 24) conformations were retrieved from the Orientations of Proteins in Membranes database 25. The unresolved residues and/or unwound helical segments at the N- and C-termini of the open structures (3F5W, 3F7V and 3F7Y) were modeled by extending the respective helices based on the closed structure (1K4C). Specifically, the regions S22–G30 and R117–H124 were modeled for structures 3F5W and 3F7V, by adopting coordinates from 1K4C after superimposing backbone atoms of G30–I38 and A109–R117, respectively; the regions S22–A29 and L110–H124 in 3F7Y were modeled in the same manner by superimposing G30–I38 and S102–A109, respectively. The structures were first minimized for 3000 steps *in vacuo* before membrane placement, during which the backbone atoms of well-resolved residues (T33–G116 in 3F5W and 3F7V; G30–A109 in 3F7Y) were fixed.

In all simulation systems, mutations created during the crystallization studies were all restored to the WT sequence, except for the closed KcsA system (1K4C), in which the L90C mutation was preserved. The N-termini at S22 of all four monomers were capped with acetamide groups in all systems, whereas all the C-termini of H124 were modeled as standard charged carboxylate groups. E71 was modeled in its protonated form in all the systems. To simulate the pH condition resulting in the inner gate opening, residues H25, E118 and E120 in the three open KcsA systems (3F5W, 3F7V and 3F7Y) were protonated in the simulations, and in their deprotonated (default) form in the closed KcsA simulation. The resulting four tetrameric KcsA structural models were all placed in POPE bilayers of 100 × 100 Å² dimension. The membrane-embedded channels were then solvated in water boxes of 100 Å thickness, and ionized with 200 mM KCl while preserving the K⁺ ions present in the crystal structures.
Simulation Protocols

MD simulations were conducted using NAMD 2.9 with CHARMM27 force field for protein, CHARMM36 force field for lipids, and the TIP3P model for water. All simulations were carried out as NPT ensembles unless otherwise stated. The temperature was maintained using Langevin dynamics with a damping coefficient of $\gamma = 0.5$ ps$^{-1}$, and the pressure was maintained using the Nosé-Hoover Langevin piston method. Particle mesh Ewald method was used to calculate long-range electrostatic interactions beyond the 12 Å cutoff distance under periodic boundary conditions. Simulations were integrated in 2 fs steps, except for the initial equilibration period during which 1 fs steps were used.

The equilibration of the four systems included two phases: i) a 0.5 ns simulation in NVT ensemble to “melt” the lipid bilayer, during which only the acyl chains of the lipid molecules were allowed to move while all other atoms fixed; ii) a 5 ns simulation where the backbone atoms present in the original crystal structures (1K4C: all residues, 3F5W and 3F7V: T33–G116, 3F7Y: G30–A109) were harmonically restrained with a force constant of $k = 5$ kcal/mol·Å$^2$. The production runs were carried out for 100 ns and coordinates were recorded every 5 ps. The program VMD was used for data analysis and generation of molecular images.

PROGRAMS

CWDipfit: http://www.sb.fsu.edu/~fajer/Programs/CWdipFit/cwdipfit.html

| pH  | Distance (Å) | Error (Standard Deviation) (Å) |
|-----|--------------|---------------------------------|
| 3.5 | 23           | 5.2                             |
| 3.3 | 23.4         | 4.5                             |
| 3.0 | 23.7         | 4.5                             |

Table I: Distance and error estimation of DEER Analysis 2013 on Tandem Dimer labeled KcsA.
Figure 1 Data analysis of CW Spectra for KcsA Tandem Dimer spin labeled at G116 using CWDipFit. (Left). Fully labeled spectra (magenta) and under labeled spectra (blue) for KcsA TDL9 from pH7 to pH4.
Figure 2 – DEER Analysis 2011 – Analysis of KcsA TDL9 at three different pH values. In each section, the L-curve, Background, Dipolar Evolution and subsequent population distribution are given. The error in background is displayed at gray bars on the distance distributions.
Figure 3 – T1 relaxation of KcsAWT Δ125 G116. (A) T1-TROSY measurements of KcsA residue G116 at pH3 (red), pH4 (yellow) and pH7 (green) at differing delays (filled square) and the fitted exponential decay (colored line). (B) Histogram of mean T1 values for G116 +/- standard deviation.
**Figure 5** $^{15}$N – $^1$H TROSY spectra of KcsA Δ125. pH titration of uniformly $^{15}$N labeled, deuterated KcsA Δ125. The Δ125 construct was used to ease spectral congestion, making spectra easier to interpret.
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