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NMR Resonance Assignments and Secondary Structure of a Mutant Form of the Human KCNE1 Channel Accessory Protein that Exhibits KCNE3-Like Function

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Abstract

KCNQ1 (Q1) is a voltage-gated potassium channel that is modulated by members of the KCNE family, the best-characterized being KCNE1 (E1) and KCNE3 (E3). The Q1/E1 complex generates a channel with delayed activation and increased conductance. This complex is expressed in cardiomyocytes where it provides the I_{Ks} current that is critical for the repolarization phase of the cardiac action potential. The Q1/E3 complex, on the other hand, is expressed in epithelial cells of the colon and stomach, where it serves as a constitutively active leak channel to help maintain water and ion homeostasis. Studies show the single transmembrane segments (TMS) present in both E1 and E3 are essential to their distinct functions. More specifically, residues FTL located near the middle of the E1 TMS are essential for the delayed activation of Q1, while the corresponding TVG sites in E3 are critical for constitutive activation of the channel. Swapping these three residues leads to the switching of the functional properties for both Q1/E1_{FTL→TVG} and Q1/E3_{TVG→FTL} complexes. This work details the backbone assignments and chemical shifts for the E1_{FTL→TVG} mutant, as determined using a suite of 3D NMR experiments along with specific and inverse amino acid isotopic labeling. The completed assignments are being used, in conjunction with other NMR experiments, to generate a 3D structure of E1_{FTL→TVG}. The results of TALOS-N analysis of the chemical shifts are reported here. The E1_{FTL→TVG} structure will be compared to the already available E1 and E3 structures to determine the roles that their TMS triplet motifs play in each protein to dictate their distinct channel-modulatory functions.

Keywords

KCNQ1; Voltage-gated potassium channel; KCNE1; KCNE3; Backbone resonance assignments; membrane protein; NMR

Biological Context:

KCNQ1 (Q1) is a 676 amino acid voltage-gated potassium channel that forms functional homotetramers in the plasma membranes of vertebrates. Mutations in either Q1 or its KCNE

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binding partners often result in human diseases such as congenital deafness and potentially fatal long QT syndrome cardiac arrhythmia (Jespersen 2005; Robbins 2001). Each Q1 subunit has six TM segments, with the first four (S1–S4) comprising the voltage sensor domain and the last two (S5, S6) contributing to the homotetrameric pore that forms the ion conduction pathway. Connecting the voltage sensor and pore domains is the S4–S5 segment. The TM S4 helix of the voltage sensory domain (S1–S4) contains four charged residues that can sense the transmembrane electrical potential of the membrane and undergoes a conformational change from its resting configuration to the activated state when the membrane electrical potential transitions from a negative to a positive value during the action potential. The activation of the voltage sensor domain is coupled via the S4–S5 connector to opening of the channel gate in the pore domain, resulting in activation of potassium ion conduction. The pore domain includes a “P-loop” between S5 and S6 helices that provides the K⁺ ion selectivity of the channel. (Jespersen 2005; Robbins 2001).

Under physiological conditions Q1 is modulated by members of the KCNE family, with E1 and E3 being the most well-studied. E1 and E3 are both contain a single TM alpha helix and bind homotetrameric Q1 to form a complex, possibly with a 4:2 KCNQ1:KCNE subunit stoichiometry (McCossan 2004). E1 has 129 residues and E3 has 103 residues. E1 and E3 are homologs, but their tissue localization and channel modulatory effects are strikingly distinct. The Q1/E1 complex is expressed in cardiomyocytes where the complex results in delayed channel activation and increased open state conductivity. The Q1/E1 complex provides the repolarizing I_{Ks} current of the cardiac action potential of cardiomyocytes (Barhanin 1996; Abbott 2017) and is essential for heart muscle contraction. On the other hand, the Q1/E3 complex is found in epithelial cells of the colon, stomach, and kidney, where its association with Q1 leads to a constitutively active “leak” channel that is critical for the secretion of potassium and chloride ions in epithelial cell fluid and salt homeostasis (Jespersen 2005; Robbins 2001; Organ-Darling 2013).

Studies have shown the TM region of both E1 and E3 is necessary for their distinct functions when bound to Q1. More specifically, amino acids F57, T58, and L59 of E1 and T71, V72, and G73 of E3, both located near the center of their respective TM helices, are critical to establishing their functional differences. Studies indicate that when these sites in E1 are swapped with the corresponding wild type sites of E3 to generate the E3_{TVG}→_{FTL} mutant, it modulates Q1 in a manner that closely resembles modulation by wild type E1. In particular, delayed Q1 channel activation is induced by binding of the E3_{TVG}→_{FTL} mutant to the channel. Conversely, swapping in the E3 triplet motif into E1 to generate E1_{FTL}→_{TVG} leads to E3-like constitutive activation of the Q1 channel (Melman 2002; Melman 2000; Barro-Soria 2017). It is clear from these results that the triplet motifs located near the middle of the E1 and E3 TM segments are critical. However, we have little information about how these motifs alter the structure and/or dynamics of each KCNE subunit or how these changes result in altered channel interaction and functional modulation. In this study, we report the backbone NMR assignments and secondary structure of the E1_{FTL}→_{TVG} mutant that exhibits E3-like channel modulatory behavior. The results of this work provide a first step in comparing results for this mutant with previously NMR-determined structures of wild type E1 (Tian 2007, Kang 2008) and wild type E3 (Kang 2010, Kronke 2016). Our TALOS-N analysis of the chemical shifts shows that the secondary structure of E1_{FTL}→_{TVG} at the

mutation site exhibited an increase in helical structure relative to what is seen in wild type E1, becoming more like E3. The completed backbone assignments for the E1_{FTL→TVG} mutant also provide the basis for future studies of the 3D structure and dynamics of this membrane protein.

Methods and Materials

Cloning of KCNE1_{FTL→TVG}

cDNA encoding full length E1_{FTL→TVG} was created from the wild type E1 template using the QuickChange mutagenesis kit from Qiagen consisting of Pfu polymerase and Dpn1, and cloned into a pET16b expression vector according to the protocol in (Tian 2007). The E1_{FTL→TVG} mutant construct employed in this study includes not only the wild type E1 residues 71–73 (FTL) replaced with the corresponding residues of E3 (TVG), but also includes mutation of the single wild type cysteine (residue 106) to serine and an N-terminal MGHHHHHHG- purification tag (139 residues, with tag). The DNA was then transformed into XL1-Blue competent cells and plated on an agar gel plate with appropriate antibiotics and incubated overnight at 37C. Single colonies were picked, used to inoculate LB medium, and mini-prepped using a Qiagen QIAprep Spin kit. The mini-prepped samples were submitted for sequencing to confirm that the mutations were correctly inserted.

Overexpression of KCNE1_{FTL→TVG}

The vector encoding His₆-E1_{FTL→TVG} was transformed into BL21(DE3) CodonPlus RP competent cells using a heat-shock technique, and the cells were plated on agar with the appropriate antibiotics overnight at 37C. Single colonies were picked and added to LB medium with appropriate antibiotics then agitated at 37C for 6–8 hours or until solutions were turbid. 2–3 mL of this starter culture was added to 1L of ¹⁵N-M9 media (0.1 mM CaCl₂, 1 mM MgSO₄, 0.4% glucose, 10 mL 1X MEM vitamins from Corning Cellgro, 100ug/ml of ampicillin, and 34 ug/ml of chloramphenicol). This was followed by rotary shaking at 25C for 16–18 hours until the optical density (OD₆₀₀) was between 0.7 and 1.0. Cell expression was induced using 1 mM IPTG, followed by continued rotary shaking at 37C for 6 hours. The cells expressing recombinant E1_{FTL→TVG} were harvested at 4C at 6,000 X g, flash frozen, and stored at –80C until ready for purification.

Purification of KCNE1_{FTL→TVG} into LMPG detergent micelles:

Frozen cell pellets containing overexpressed E1 were thawed and resuspended in lysis buffer (75 mM Tris-base, 300 mM NaCl, 0.2 mM EDTA, pH 7.8) at 20 mL per gram of cell pellet. Added to dissolved cell pellets were 2 uL of LDR solution (100mg/mL lysozyme, 10 mg/ml DNase, 10 mg/mL RNase, 10% glycerol) per mL of lysis buffer. To complete cell lysis conditions, we added the protease inhibitor PMSF to a final concentration of 1 mM and magnesium acetate to a final concentration of 5 mM. This solution was tumbled for 30 minutes at 4C. This solution was then probe-sonicated on ice for five minutes with alternating 5-second pulse and 5-second rest cycles. The lysed cells were centrifuged for 20 minutes at 20,000 × g at 4C. The supernatant was discarded, and the inclusion body pellet was homogenized in lysis buffer. The homogenized pellet was sonicated on ice and centrifuged using the same conditions as listed above, before being resuspended in 10mL of

an 8M urea and 0.2% lauroyl sarcosine solution per gram of wet cells. This solution was tumbled overnight at 4C.

The dissolved inclusion bodies were centrifuged at $30,000 \times g$ for 45 minutes at 4C to remove debris. This supernatant was mixed with 1.2 mL of Ni-NTA resin per gram of wet cell pellet and tumbled at 4C for 1 hour. The solution was transferred to a gravity-flow column, allowing the supernatant to pass through, leaving the resin with bound protein. The resin was washed with 10 column volumes of a wash buffer (20 mM tris, pH 7.8, 150 mM NaCl, 0.2% lauroyl sarcosine) to remove urea. The resin was then washed with 10 column volumes of a buffer exchange solution (20 mM tris, pH 7.8, 150mM NaCl, 30 mM imidazole, 0.2% LMPG) to exchange in the non-denaturing detergent LMPG and to elute loosely bound impurities. The protein was eluted with 10 ml of 300 mM analytical grade imidazole at pH 7.8 with 0.2% LMPG. The protein concentration was determined by measuring light absorption at 280nm using an extinction coefficient of 1.29 OD per mg/mL per cm. The purification gel indicated that the protein had minimal impurities and exhibited an apparent molecular weight of 15.4 kDa. Our purification technique yielded approximately 2.5 mg of pure protein per liter of medium.

NMR Sample Preparation and NMR Spectroscopy

Uniformly ^{15}N -labeled His6-E1_{FTL}→TVG was exchanged from the elution buffer listed above into an NMR buffer (10% D₂O, 50 mM imidazole, 50 mM NaCl, 2 mM EDTA, 0.05% LMPG, pH 6.5) using a 10 kDa molecular weight cut-off Amicon Ultra-15 centrifugal filter. Using the same centrifugal filter method, E1_{FTL}→TVG was concentrated to 1 mM, and 200 uL was transferred to a 5 mm NMR tube. Using a Bruker 800 MHz spectrometer, a 2-dimensional ^1H , ^{15}N -TROSY spectrum of U- ^{15}N - E1_{FTL}→TVG was collected at 45C. 256×512 points were collected in the ^{15}N dimension and ^1H dimension, respectively, and the spectra were processed in TopSpin.

3D Experiments for Assignments, Single Amino Acid Labeling and Inverse Labeling

Uniformly ^{13}C , ^{15}N , isotopically-labeled E1_{FTL}→TVG was prepared and purified using the methods listed above, with the addition of 4 g of $^{13}\text{C}_6$ -glucose instead of ^{12}C -glucose to the minimal medium. To make resonance assignments, the following experiments were used: 3D TROSY-based pulse sequences (HNCACB, HNCO, HNCA, HNCACO, HNCB), inverse labeling (unlabeled Thr or Ser in otherwise ^{15}N -labeled protein), and specific amino acid labeling (^{15}N -labeled Ala, Val, Phe, Leu, Met, Ile, or Thr in otherwise unlabeled protein). Inverse-labeled E1_{FTL}→TVG was expressed and purified using the standard method except that 1g of the unlabeled amino acid of choice was included in the ^{15}N -M9 media.

To express protein with labeling of a specific ^{15}N amino acid, CT19 cells containing unlabeled-E1_{FTL}→TVG were grown in LB medium until OD600 reached 0.5, then gently spun down and washed. Washed cell pellets were resuspended in unlabeled-M9 medium with specific antibiotics, 0.5g of each of the 7 unlabeled amino acids (Val, Ala, Phe, Leu, Ile, Tyr, Asp), 0.1g of unlabeled Trp and 0.5g of the specific ^{15}N -labeled amino acid of choice. These cells were induced, harvested, and the specifically ^{15}N -labeled E1_{FTL}→TVG protein was purified using the methods listed above. All experiments were conducted using either an

800MHz or 900MHz Bruker Avance III NMR spectrometer using a TROSY pulse sequence, with the data being processed using TopSpin.

Assignments and data deposition

The dispersion of peaks seen in the TROSY spectrum of the E1_{FTL→TVG} is typical for a reasonably well-behaved single-span membrane protein in detergent micelles (Fig.1). Since the previously published assignments of WT E1 were determined under the same conditions in LMPG micelles as E1_{FTL→TVG}, several of the assignments could be transferred to E1_{FTL→TVG} (Tian 2007). 3D-TROSY based experiments were used to confirm the assignments that were transferred, to make assignments that could not be transferred, and to provide chemical shift data for CO, C_{alpha}, C_{beta}, H_{alpha}, HN, and N. Inverse labeling and specific amino acids labeling (Sup. Fig 1) were used to help fill in missing assignments not obtained by transferring or sequential backbone walking using 3D data (Sup. Fig. 2). A total of 86 percent of the backbone resonances were assigned using these methods, with 98 percent of the TM region and juxta-TM region being assigned. G0, the residue that connects the MGHHHHHH- tag to the E1_{FTL→TVG}, yields a peak in the glycine region, along with the protein's 9 other glycine residues. While some of the peaks from E1_{FTL→TVG} aligned well with those from wild type E1, many others exhibited significant shifts in both resonance positions. The majority of these shifted peaks lie within the transmembrane regions, as might be expected as this was the site of the triplet substitution. Residues with the most highly shifted peaks (G52, F54, G55, F56, G60, L63) are each within 5 residues of the TVG substitution hinting that there might be structural changes from E1 at and around the TVG mutation site. Our assignments were analyzed by the Assignment Validation Software (AVS) and the Probabilistic Approach to NMR Assignment and Validation (PANAV) programs and not indicate and anomalous chemical shifts or inconsistent assignments (Moseley 2004, Wang 2010).

The chemical shift measurements were input into the TALOS-N web server for secondary structure analysis (Shen 2013). This data indicates that E1_{FTL→TVG} contains three alpha helices separated mainly by random coils, perhaps with a couple of additional short and unstable helices (Fig. 2). When the TALOS-N results for E1_{FTL→TVG} were compared to previously published results for E1 (Tian 2007), we notice a slight change within the transmembrane (TM) helix. The TMS of WT E1 exhibited a modest drop in helicity at and immediately after the wildtype FTL triplet that is critical for its unique function. E1_{FTL→TVG} exhibited increased helicity at this region. The E1_{FTL→TVG} secondary structure also was compared to the published results for wild type E3, which exhibits a continuous helix throughout the full length of its TM segment (Kronke 2016). This data supports the idea that TM segment in E1_{FTL→TVG} is structurally more similar to E3 than it is to wild type E1, possibly helping to explain why the triplet mutation converts E1_{FTL→TVG} into a protein with similar functionality to E3. Assignments for E1_{FTL→TVG} were deposited into the BMRB under entry code 27640.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Assigned ^1H - ^{15}N TROSY spectrum of E1_{FTL→TVG}. The sample was in 8% LMPG micelles at a concentration of 1 mM protein. It was collected at pH 6.5 at 45°C using an 800 MHz Bruker spectrophotometer. All NMR data were processed using TopSpin

Figure. 2.

Prediction of the location of alpha-helices in E1_{TVG} based on TALOS-N analysis of the experimental chemical shifts. The Y axis indicates the alpha-helical probability determined for each residue. This plot indicates three major helical segments, including the TM segment, which extends from residues 42 to 72