SUPPORTING INFORMATION

Tertiary Structure of the Pore

A control residue, S362C in the middle of S5 (in FRAG), undergoes little, if any, pegylation, i.e., maximum Fpeg < 0.1. This residue is surrounded by lipid (see Fig. 5C) and is inaccessible. Though S5 and S6 are largely surrounded by lipid, the turret and loop S6 are surrounded by water and the pore helix and selectivity filter are in contact with both lipid and water. Based on the last 500 ns of the molecular dynamics simulation for the monomer, an average of 0.5, 0.7, 1.2, 1.6, 1.4 hydrogen bonds are formed to water molecules by the backbone carbonyl oxygens of selectivity filter residues TVGYG (374-378), whereas for maximally hydrated residues, e.g., turret residues DERDS (352-356), the average number is 2.9, 2.3, 2.2, 1.4, and 2.7, respectively.

Homologous tertiary folds

Using DaliLite v. 3 (http://ekhidna.biocenter.helsinki.fi/dali_server) to search a protein structure database, several examples of monomeric soluble proteins were identified that are similar (rmsd values of ≤ 3Å) to the Kv pore segment (residues 333 to 398), which includes part of S5, S6, the turret, the pore helix, the selectivity filter, and loop-S6. For example, a semi-synthetic C5a receptor antagonist, a helicase, RNA polymerases, a metal dependent phosphohydrolase, a fructose repressor, lac repressor, and syntaxin binding protein are structurally homologous to the Kv pore segment.

Intrasubunit hydrogen bonds

According to Kv1.2/2.1 numbering, a triad interaction exists between W362 (pore helix), D375 (selectivity filter), and Y373 (selectivity filter); the turn at the bottom of the pore helix is likely

facilitated by the interaction between T369 (bottom of pore helix) and V371 (bottom of selectivity filter); a pair of tight (2.7Å) hydrogen bonds between V377 (loop S6) and Q353 (turret) also secures the re-entrant loop. All of these interactions occur within the monomer.

SUPPORTING METHODS

Constructs, In vitro Translation, Pegylation

Standard methods of bacterial transformation, plasmid DNA preparation, and restriction enzyme analysis were used. The nucleotide sequence of the entire coding region was confirmed for all mutant DNA via automated cycle sequencing on an ABI 377 Sequencer using Big dye terminator chemistry (A0BI) performed by the DNA Sequencing Facility at the University of Pennsylvania School of Medicine. Capped cRNA was synthesized *in vitro* from linearized templates using Sp6 RNA polymerase (Promega™, Madison, WI). Linearized templates for Kv1.3-derived constructs were generated using BstEII or SgrAI restriction enzymes (New England Biolabs® Inc., Ipswich, MA) to produce different length DNA constructs lacking a stop codon to position the peptide at a specific location in the ribosomal exit tunnel. For extended controls tape measure constructs, a E64C mutation created a reporter cysteine. A BstEII restriction enzyme digestion site was engineered to position the reporter cysteine 32 amino acids away from the peptidyl transfrerase center (PTC). For Kv1.3 nascent peptides, a native SgrAI-cut site in S6 (cuts immediately before bp1262) was utilized. Linearized templates for T1(-) and FRAG constructs were generated using EcoRI restriction enzyme (New England Biolabs® Inc., Ipswich, MA), which cuts Kv1.3 after the stop codon. RNA was translated for 1 h at 22°C using a cell-free mammalian *in vitro* translation system (Promega™, Madison, WI) including a final concentration of 2 mM DTT,

rabbit reticulocyte lysate, amino acid mixture without methionine and [^{35}S]Cysteine/Methionine (4 µl/50 µl translation mixture; ~10 µCi/µl, Environmental Health and Radiation Safety, University of Pennsylvania, Philadelphia, PA) according to the Promega™ Protocol and Application Guide. The concentration of K+ ions in the translation reaction was 79 mM. For tertiary folding experiments, T1(-) and FRAG RNA were translated for 1 to 2 h at 30°C in the presence of canine microsomal membranes (Promega™, Madison, WI). Optimal times for translation were determined to allow peptides to integrate into membranes.

As described previously(1), 5-15 µl of the translation mixture was diluted into a final volume of 500-1000 µl 1X PBS* (Gibco™ $CaCl_2$- and $MgCl_2$-free Dulbecco's phosphate buffered saline, pH 7.4, supplemented with 4 mM $MgCl_2$, and 2 mM DTT (Invitrogen™ NuPAGE® Sample Reducing Agent) and centrifuged (Beckman Optima TLX Ultracentrifuge, Beckman TLA 100.3 rotor) through a sucrose cushion (120 µl; 0.5 M sucrose, 100 mM KCl, 5 mM $MgCl_2$, 50 mM Hepes, and 1 mM DTT; pH 7.5; to remove globin protein) for 20 min at 70,000 rpm (~245,000xg) at 4°C to isolate ribosome-bound peptides.

Tertiary Structure Experiments

Each sample of the translation mixture (5-15 µl) was diluted into a final volume of 500-1000 µl 1X PBS* containing 2 mM DTT (Gibco™ $CaCl_2$- and $MgCl_2$-free Dulbecco's phosphate buffered saline, pH 7.4, supplemented with 4 mM $MgCl_2$) and centrifuged (Beckman Optima TLX Ultracentrifuge, Beckman TLA 100.3 rotor) through a sucrose cushion (120 µl; 0.5 M sucrose, 100 mM KCl, 5 mM $MgCl_2$, 50 mM Hepes, and 1 mM DTT; pH 7.5; to remove globin protein) for 5 min at 55,000 rpm. Pellets containing membrane-embedded peptides were

resuspended on ice with 50 to 100 µl of 0.1% Anatrace™ n-dodecyl-β-D-maltopyranoside, Anagrade® (C_{12}M, prepared in PBS* supplemented with 50-500 µM DTT) and incubated 1 h on ice to dissolve membranes. C_{12}M was used to solubilize the microsomal membranes following translation because it is typically non-denaturing and has been used for solubilization of K+ channels for assembly assays and ion flux assays, as well as for structure determination of membrane proteins (2-4). Effective resuspension required careful (avoiding bubble formation) and repetitive pipetting (>100 times). C_{12}M allows the membrane to be removed while preserving the intact structure of the protein. Samples were centrifuged to pellet undissolved membrane and the supernatant containing solubilized peptides was transferred to a fresh tube and an equal volume of PBS* solution containing PEG-MAL (final PEG-MAL concentration was 1-2 mM). Samples were incubated on ice for times indicated from 0 to 3 h, and. The pegylation reaction was quenched with 100-fold excess DTT at ambient temperature for 10 min and then 0°C for 10 min.

Gel Electrophoresis and Fluorography.

For secondary folding experiments, the final sample was centrifuged (Beckmann Optima TLX Ultracentrifuge, Beckman TLA 100.3 rotor) for 20 min at 70,000 rpm (~245,000xg) at 4°C to collect ribosome-bound peptides. For tertiary folding experiments, the final sample was precipitated in 1 ml cold acid acetone (from stock prepared by adding 20 µl concentrated HCl to 50 uL of acetone) over night at -20°C and centrifuged (Eppendorf Centrifuge 5415C) for 30 min at 14,000 rpm at 4°C to collect precipitated peptide. The samples were then resuspended in 6 µl loading buffer (Invitrogen™ NuPAGE® 4X LDS), 2 µl reducing solution (500 mM DTT; 10X Invitrogen™ NuPAGE® Sample Reducing Agent), and 16 µl nuclease-free water. For secondary

folding experiments, 1 to 2 µl of 500 µg/ml RNase (Roche Diagnostics, Indianapolis, IN) was added and incubated for 15 min at ambient temperature to detach peptide from the ribosome by digesting the tRNA. Samples were denatured at 70°C for 10 min and fractionated on NuPAGE® Novex® precast 10%, 12%, or 4-12% gradient Bis-Tris Mini gels (Invitrogen™). Typically, MOPS or MES SDS running buffer and load buffer containing Invitrogen™ NuPAGE® Antioxidant to maintain reduced conditions. Gels were run at 200 V for 45 min or 1 h for secondary and tertiary folding experiments, respectively. Gels were soaked for 30 min in a standard methanol/acetic acid fixation solution and treated with Amplify™ (Amersham Biosciences, GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK) fluorographic reagent to enhance ^{35}[S] fluorography and dried under vacuum at 70°C (BioRad 583 Gel Dryer). ^{14}[C]-labeled protein molecular weight markers were used as standards. Bands appear at approximately 8 kD (FRAG/SgrAI), 15 kD (tape measure extended control, TM), 20 kD (FRAG), or 35 kD (T1(-)). Gels were then exposed to Kodak™ X-AR high-resolution film at -70°C for 16-18 h and developed for qualitative analysis. Gels were also exposed to a Storage Phosphor Screen (Amersham Biosciences, GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK) for 16 to 48 h according to ^{35}[S] signal strength and were imaged using a Typhoon™ scanner (GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK). The ^{35}S signal captured in these images was quantified using ImageQuant™ computer software (GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK). Fpeg, the fraction of peptide labeled by PEG-MAL, was determined by calculating the ratio of pegylated to total peptide. Time course data were fit to a single exponential function to calculate rate constants.

Molecular Dynamics.

The atomic model (29425 atoms) consists of the Kv1.2 pore domain of 99 amino acids (1528 atoms; corresponding to residues 341-440 in Kv1.3), 90 POPC molecules (45 in the bottom and 45 in the top layer), 5269 water molecules. The system was made electrically neutral by the addition of 17 K^+ and 13 Cl^- ions giving a concentration of 150 mM KCl. The CHARMM-GUI Membrane Builder (www.charmm-gui.org) was used to assemble the initial configuration of the system. After 50 ns equilibration, Kv1.2 was generated and then simulated for another 650 ns. All-atom molecular dynamics simulation was performed using the program NAMD (5). The PARAM27 all-atom potential energy function for protein and phospholipids from CHARMM was used (6). Periodic boundary conditions were applied in three dimensions. Electrostatic interactions were calculated based on the Particle Mesh Ewald algorithm (7). The simulation was carried out at constant pressure (1 bar) and temperature (300 K) (NPT) using Langevin dynamics. Analysis of the 650 ns trajectory was performed using the Tcl command interface of the molecular viewing program VMD (8).

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