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Protein Extraction Reagent According to Manufacturer's Instruction

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Description

The discovery of bacterial Navs has been important in addressing our lack of structural insight into eukaryotic Navs. Bacterial Navs are simplified homologues of eukaryotic Navs; their sequences are analogous to one domain (i.e. six TMS segments with a voltage sensor and a pore forming region) of a eukaryotic Nav and they form functional channels from homotetrameric assembly. Their minimalist structure has enabled their atomic-level structures to be resolved, which together with their electrophysiological characterization and atomic simulations based on the resolved structures, have been pivotal in gaining detailed understanding of ion permeation and gating for Navs.

Limitations of Bacterial Models

However, there are several limitations of these bacterial models for the understanding eucaryotic Nav channels. Foremost is that (unlike eukaryotic Nav channels) bacterial channels display radial symmetry (a consequence of their homotetrameric structure). Consequently, the bacterial counterparts cannot be used to investigate experimentally the distinct role of the four individual domains of eukaryotic Nav channels and as homotetramers, it is not possible to generate asymmetry in a bacterial Nav channel. For example, Xia et al. constructed a model of NavRh with the Selectivity Filter (SF) mutated from the radially-symmetrical glutamate ring to the asymmetric ring of DEKA (to mimic the SF in eukaryotic Nav channels) and through MD simulations of Na+ permeation proposed a model to explain Na+/K+ selectivity in mammalian Nav channels. Currently the predictions of the simulation study cannot be experimentally tested.

Whole-cell patch clamp recordings were acquired with Axopatch 200 series amplifiers (Molecular Devices, Sunnyvale, USA). Signals were digitized using Digidata1322 (Molecular Devices, Sunnyvale, USA). Data were filtered at 1 or 2 kHz. All the experiments were performed at 20 °C. Patch pipettes were produced by a pipette puller (model 730, KOPF instrument, USA) from KIMAX melting point capillary tubes (34500–99; Kimble Company, USA). Pipettes had resistances between 2 and 4 MΩ after filling with intracellular solution. Shanks of the pipettes tip were coated with bee's wax to reduce pipette capacitance.

For each dodecamer, a dilute system without crowders (0% crowder fraction) and three systems with different protein crowder volume fractions (20, 30, and 40%) were prepared. Protein G (PDB: 1PGB) was selected as the crowder protein due to its small size and stability in computer simulations. We used neutral protein G models molecules introduced in previous work, where D36, D40, E19 and E42 are protonated. In the previous study, both, the charged and neutralized variants of protein G were studied under crowded conditions similar to the systems studied here but without DNA and both were found to be stable in simulations.

Extraction of Protein

Protein extracted from overnight cultures of S. cerevisiae (SCM-ura but with glucose replaced with 2% galactose and 2% raffinose to induce protein expression) was conducted by treating yeasts with 2 M of lithium acetate (LiAc) for 5 min and then 0.4 M of NaOH for 10 min at room temperature. Supernatant was tested after centrifugation at 13,000 g for 15 min at 4 °C. Protein expression was induced in E. coli by culturing in LB containing 0.4 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) for 1 h at 37 °C with shaking at 150 rpm. After washing, bacteria were lysed with Y-PER™ Yeast Protein Extraction Reagent according to manufacturer's instruction (Thermo Scientific) with addition of proteinase inhibitor for 20 min at room temperature. Supernatant after centrifugation at 13,000 g for 15 min was retained for analysis.

Protein G is not known to specifically interact with DNA and we chose the net-neutral form to reduce electrostatic interactions with the highly charged DNA to focus on more general crowding effects while still maintaining protein-like crowders. The crowded systems (20, 30, and 40%) consisted of one dodecamer and 8 protein G molecules, whereas the dilute systems only contained one dodecamer. Simulation box sizes were varied between 53.2–61.3 Å to obtain the abovementioned crowder volume fractions. The box sizes were varied instead of the number of protein copies to achieve exactly the target crowder fractions and minimize computational costs at the higher concentrations as in previous work. There is no experimental evidence for a specific DNA-protein G complex that is stable over long time and consequently the system is assumed to be fully dynamic in the liquid state with molecular interactions varying transiently. To avoid biasing towards any specific initial protein G-DNA interaction, the initial crowded systems were set up by randomly rotating and placing the DNA dodecamer and the crowder proteins in the simulation box using a protocol developed previously. Different replicates of each system had different initial orientations and placements of the DNA and the surrounding crowders. All systems were solvated with explicit TIP3P (three-site transferable intermolecular potential) water molecules. To neutralize the DNA dodecamer, 22 sodium ions were added to the systems. In order to keep the ion molality of all systems the same, 6 and 12 additional pairs of sodium and chloride ions were added to 30% and 0/20% systems, respectively. Therefore, all systems had 0.45 mol/kg ion molality In an attempt to address this problem and to gain further insights in to the molecular mechanisms of ion permeation in eukaryotic Nav channels using their bacterial counterparts, we attempted to generate a concatenated bacterial Nav channel in which four monomer subunits are covalently linked to form a single polypeptide (and thus mirroring the structure of their eukaryotic counterparts).

It was envisaged that such a structure would enable targeted mutation of individual domains of the concatemer and thus permit experimental testing of bacterial channels exhibiting asymmetry in the pore of the bacterial channels (e.g. Xia et al. study). We report intact expression of NaChBac and NavMs and NavAb concatemers but that stable expression was dependent on the expression system employed. Surprisingly, NaChBac concatemer was inherently unstable. However, NavMs concatemers could be expressed intact in mammalian cells and were amenable to electrophysiological investigation using the patch clamp technique.