EPR Spectroscopic Distance Measurements of the KCNE1 Membrane Protein in Micelles and Lipid Bilayers

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Together, these components comprise a complete pressure-jump EPR system for quantifying millisecond or slower conformational exchange rates in proteins. Preliminary results on conformational exchange rates in apo-myoglobin and T4 Lysozyme cavity mutants will be presented.

2062-Pos Board B832

Probing the Structural and Dynamic Properties of KCNE1 using Site-Directed Spin Labeling EPR Spectroscopy

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KCNE1 (minK) is a membrane protein known to modulate the function of the KCNQ1 voltage-gated potassium ion channel in the human heart. KCNE1 has been over expressed and purified into SDS detergent and reconstituted into POPC/POPG lipid vesicles. We show that that KCNE1 maintains its ability to associate with and modulate KCNQ1 K+ channel activity when placed into POPC/POPG liposomes. Functional measurements of KCNE1 proteoliposomes were performed in Xenopus oocytes expressing KCNQ1 (KV7.1). Our CD measurements show that KCNE1 undergoes a global change in secondary structure when removed from a micelle environment and placed in a lipid bilayer. CW-EPR and Power Saturation EPR experiments have been used to examine the structural and dynamic properties of KCNE1 in micelles and lipid bilayer vesicles. The results indicate that KCNE1 inserts into the POPC/POPG vesicles, S28C and R32C are solvent-exposed and located outside the membrane, and S64C and F54C are buried inside the POPC/POPG lipid bilayer. The differences of KCNE1 placed into either micelles or lipid bilayers will be discussed.

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Structural and Dynamic Response to Core Repacking Substitutions in T4 Lysozyme Detected by Site-Directed Spin Labeling

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Proteins can evolve to gain new functionality *via* mutations that decrease stability, and presumably, increase flexibility. Particularly interesting examples are mutations that modify the hydrophobic core packing and create new functions, *e.g.* novel ligand binding sites created by cavity-forming mutants. Many core repacking mutants of T4 Lysozyme (T4L) have been characterized, some of which bind non-polar ligands. Overall, the crystal structures of these mutants are very similar to the WT and offer little insight into the mechanism whereby the ligand reaches the cavity. Site-Directed Spin Labeling (SDSL) has been applied to investigate the structural and dynamical response of T4L in solution to 28 core repacking mutations, many of which have known crystal structures. Remarkably, the most common response in the cavity-creating susbtitutions is the appearance of a second conformational substate, with populations as high as 70 %, showing that the cavities allows for an alternative protein fold, at least locally. Osmotic perturbation shows that the conformations are in slow exchange on the EPR time scale. Thus, the cavity mutations excite molecular flexibility, with a characteristic time scale $>$ 100 ns, probably in the μs-ms range. Interspin distance measurements carried out for two of the cavity mutants to study the magnitude and nature of the conformational rearrangement suggest the interesting possibility that the alternative states are also present in the WT protein, but at a different ratio.

In some cases, ns backbone modes were also excited by the core mutation, but the effects were subtle. Remarkably, the type and magnitude of the changes in the protein flexibility were not necessarily correlated with the extent of destabilization by the substitution. The relationship of the cavity-excited states to the mechanism of ligand entry is under study.

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EPR Method for ex vivo Detection of Reactive Oxygen Species in Tissue

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Measurement of free radicals, *ex vivo,* in biological tissue with continuous wave electron paramagnetic resonance (EPR) has been problematic. Here, a novel EPR spin trapping technique has been developed that places one or two large segments of porcine arterial tissue along with supernatant directly into a standard EPR spectrometer cavity at liquid nitrogen temperature. This approach is validated in porcine arterial tissues treated as controls *in vivo*. To investigate that this method has lower extraneous ROS generation than methods which slice tissue into thin slivers before assaying for reactive oxygen species (ROS), we take two sets of coronary arterial tissue from the same animal. In the first set, we excise one or two large segments of tissue, and in the second set we section the tissue into ten pieces. In both cases, tissue was harvested, placed in physiological saline solution with 190-mM PBN (N-*tert*-butyl-α-phenylnitrone) and incubated in the dark for 2 hours at physiological temperature while gently being stirred. Tissue and supernatant were then loaded into a syringe and frozen at minus eighty degrees Celsius until EPR analysis. Although absorption of microwaves by an aqueous sample typically at room temperature limits the size of the sample; this method can accommodate as large as 20-mm^3 tissue volume into the EPR cavity at liquid nitrogen temperature. In our experiments the tissue volumes were in a range of values between 2 mm^3 and 5 mm^3,and the EPR spectra were normalized with respect to tissue volume.

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EPR Spectroscopic Distance Measurements of the KCNE1 Membrane Protein in Micelles and Lipid Bilayers

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Distance measurements between two strategically placed nitroxide spin labels can provide pertinent structural information on complicated biological systems such as membrane proteins. Distances of approximately 8 Å - 25 Å can be determined by measuring line broadening due to dipolar coupling using continuous wave (CW)-EPR spectroscopy. The short range distances can be measured between isotopically coupled nuclear spins and nitroxide electronic spin labels up to a distance of about 8 Å using Electron Spin Echo Envelope Modulation (ESEEM) and long range distances of 20 Å - 70Å between two nitroxide electronic spin labels using Double Electron Electron Resonance (DEER). The transmembrane domain (TMD) of KCNE1 binds to the pore domain of the KCNQ1 channel modulating the channel's gating. In order to characterize the structural properties of the TMD of KCNE1, double cysteine mutants were prepared for TMD of KCNE1 and further modified by the addition of a MTSL nitroxide spin label. The purified proteins were reconstituted into LMPG detergent micelles and POPC/POPG bilayer vesicles. Dipolar broadening CW-EPR experiments were performed on the double mutants F53C-L63C, F56C-S64C, L59C-Y65C, and L59C-S64C at 225K; and DEER experiments on double mutants V50C-S68C, V47C-I66C at 80K. Molecular Dynamics Modeling study was performed on these mutants using nanoscale molecular dynamics (NAMD) with the molecular graphics software VMD. A 1 ns molecular dynamics simulation was ran for each double mutants and distance distribution was obtained by analyzing the trajectory data file using VMD. The distance distributions obtained from dipolar broadening CW EPR and DEER are in good agreement with NAMD/VMD and MMM modeling results.

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Structural Transition of N-Terminal Extension of Cardiac Troponin-I Complexed with Troponin-C caused by PKC-Mediated Phosphorylation, as Revealed by Spin Labeling EPR

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Cardiac troponin (cTn) complex consisting of cTnI, cTnC and cTnT is responsible for cardiac mechanotransduction. Aberrent phosophorylation of cTnI by PKC on S42/44 or its phosphorylation mimic mutant S42/44D decreases Ca^{2+} sensitivity and tension force, consequently generating heart failure. Until now, consequence of cTnI by PKC phosphorylation on molecular level and associated structural mechanisms involved in it are still elusive. Combined with previously published NMR spectra and our bioinformatics analysis, this distinct N-extension of cTnI is strongly shown to be intrinsically disordered. To simulate the possible conformation of this intrinsically disordered region and potential conformational transition caused by PKC when binding with cTnC, Monte Carlo simulation was implemented to explore potential docking sites on cTnC, of which all the predicted structures were evaluated by total and binding energy with the criteria under RosettaCommons software suite. We screened around 10,000 structures for cTnC-I and cTnC-I (S42/44D) complexes, respectively. For cTnC-I complex, flexible N-extension of cTnI docked on N-domain of cTnC, putatively assisting to stabilize the full-open state of cTnC which binds strongly the regulatory region of cTnI to initiate muscle contraction. In contrast, as for cTnC-I (S42/44D), it localized on C-domain of cTnC, thus deficiently to maintain full-open state of cTnC, causing a weak interaction between the regulatory region of cTnI (S42/44D) and cTnC to generate heart failure. To verify our prediction, we are measuring residual mobility and inter-residual distance from spin labels placed on cTnI or cTnI (S42/44D) and cTnC. Preliminary mobility measurement of MSL-labeled cTnC(C84) indicated loose interaction between the regulatory region of cTnI (S42/44D) and cTnC. Measurements on the other sites are in progress.