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Structure and Dynamics of the Phospholamban-SERCA Complex Probed by Site-Directed EPR Spectroscopy

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We are studying the structural dynamics of phospholamban (PLB), a regulator of the SR calcium ATPase (SERCA), by combining site-directed spin-labeling and EPR spectroscopy with solid-phase peptide synthesis. PLB is a 52-residue integral membrane protein that binds and inhibits SERCA in the presence of sub-micromolar calcium concentrations. SERCA activity can be restored without dissociating the two proteins by phosphorylating PLB at Ser16 (Mueller et al., 2004). To observe the effects of phosphorylation and SERCA-binding on PLB conformation, we have synthesized PLB analogs containing the spin-labeled amino acid TOAC. Doubly-labeled mutants were studied by DEER spectroscopy, a pulsed EPR technique capable of measuring interspin distances from 2-7nm. Our results agree with published EPR dynamics data (Karim et al., 2006) showing that PLB equilibrates between an ordered, compact (T) state and an extended, dynamically disordered (R) state. Alone, PLB largely occupies the (T) state, while this equilibrium shifts moderately in favor of the (R) state upon SERCA binding or Ser16 phosphorylation. In contrast, SERCA-bound PLB becomes more ordered and compact upon phosphorylation. We have also used relaxation enhancement EPR with singly-labeled mutants to study the movement of PLB's transmembrane (TM) helix relative to the membrane plane. In these experiments, the spin-lattice relaxation rate of excited spins is enhanced by their collision with paramagnetic relaxation agents (PRAs). For TOAC residues incorporated into the TM domain, PLB motions that reposition this helix will reduce or increase the spin-label's accessibility to water-soluble PRAs, while having the reverse effect for lipid-soluble PRAs. With these experiments, we are constructing a more complete model of PLB dynamics during its interaction with SERCA.

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FRET Detected Interactions of Cardiac Membrane Proteins in Living Cells

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We have investigated the structure of phospholamban (PLB) and its regulation of the sarcoplasmic reticulum Ca-ATPase (SERCA) using fluorescence resonance energy transfer (FRET) on fluorescent fusion proteins expressed in living cells. Fusion proteins were created with either a donor fluorophore (cyan fluorescent protein, CFP), or an acceptor fluorophore (yellow fluorescent protein, YFP), attached to one terminus of the protein of interest. Both N-terminal and C-terminal fusions of CFP and YFP were made to SERCA and N-terminal fusions were made to PLB. These proteins were expressed and co-expressed in either SF21 insect cells or HEK-293 cells, preserving normal physiological function of both proteins. In fluorescence transfer recovery (FTR) experiments, FRET was calculated from the recovery of CFP fluorescence due to photobleaching of YFP. The dependence of donor fluorescence on acceptor photobleaching showed that PLB exists primarily as oligomers in cells but binds to SERCA exclusively as a monomer. Phosphorylation of PLB by PKA, after stimulation of adenylyl cyclase with forskolin, showed a change in FRET. Time-resolved fluorescence showed that the change in FRET was due primarily to a change in the structure of the SERCA-PLB complex, not to a change in protein association. The structural and functional effects of PLB mutation are also under investigation. This work was supported by NIH (GM27906, AR007612).

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Phosphomimetic Mutations Increase FXYD1 Oligomerization, but Does Not Alter its Quaternary Conformation

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Phospholemman (FXYD1, or PLM) is a key regulator of Na^+-K^+ ATPase in the heart, and is principally phosphorylated by cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC). To investigate whether phosphorylation alters FXYD1 structure and oligomerization, we fused cyan or yellow fluorescent protein (CFP/YFP) to the c-terminus of FXYD1 and co-expressed the fusion proteins in AAV-293 cells. Phosphorylation of FXYD1 was mimicked by mutations S68E (PKA site) or S63E/S68E (PKC+PKA sites), and FRET from CFP-FXYD1 to YFP-FXYD1 was quantified by acceptor photobleaching. FRET increased with protein concentration up to a maximum ($FRET_{max}$), which was taken to represent the intrinsic FRET of the bound complex. We did not detect significant changes of $FRET_{max}$ with phosphomimetic mutations, suggesting the quaternary structure of FXYD1 oligomer is not grossly altered by phosphorylation. The concentration dependence of FRET also yielded the relative dissociation constant of the FXYD1 oligomer (K_d), in arbitrary units (AU). Compared to non-phosphorylatable mutant S68A, S68E showed a significant decrease in K_d (14.1 $\pm$ 2.0 and 7.3 $\pm$ 2.0 AU, respectively). The data are consistent with more avid oligomerization of pseudo-phosphorylated FXYD1. Phosphomimetic mutation of both PKC and PKA sites (S63E/S68E) resulted in a K_d of 6.6 $\pm$ 1.1 AU, suggesting there was not a significant additional increase in oligomerization *vs.* the single site mutation. Taken together, the data suggest that phosphorylation can enhance FXYD1 oligomerization without altering the architecture of the oligomeric complex. Increased FXYD1 oligomerization may have an indirect effect on the regulatory interaction of FXYD1 with Na^+-K^+ ATPase.

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Phospholemman Recruits Peroxiredoxin 6 to the Cardiac Sodium Pump

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Phospholemman (FXYD1, PLM), the principal sarcolemmal substrate for protein kinases A and C in the heart, is a regulator of the cardiac sodium pump. We investigated proteins that interact with PLM in adult rat ventricular myocytes using bifunctional crosslinking reagents and co-immunoprecipitation.

Digitonin-permeabilized ventricular myocytes were treated with the heterobifunctional crosslinking reagent sulfo-lc-smpt (distance between reactive groups 2nm, reactive towards amino and sulfhydryl amino acid side chains), and cell lysates immunoblotted for PLM. We found sulfo-lc-smpt quantitatively crosslinked PLM to a 20-25kDa protein (electrophoretic mobility of PLM 15kDa, electrophoretic mobility of crosslinked adduct 37kDa). Co-immunoprecipitation experiments indicated that the crosslinked adduct was PLM linked to the anti-oxidant protein peroxiredoxin 6 (prdx6). PLM phosphorylation at serine 68 (protein kinase A activation with 10μM forskolin) or at serines 63, 68 and threonine 69 (protein kinase C activation with 300nM PMA) had no effect on the ability of sulfo-lc-smpt to crosslink PLM to prdx6. Hydrogen peroxide treatment of ventricular myocytes (1-100μM) was also without effect on the binding of prdx6 to PLM.

In conclusion, our data suggest a new role for PLM in the heart. As well as being responsible for kinase-mediated regulation of the cardiac sodium pump, it is responsible for recruitment of prdx6 to this ion transporter. Prdx6 catalyzes the reduction of hydrogen peroxide, fatty acid hydroperoxides and phospholipid hydroperoxides using glutathione. Given the well-established sensitivity of the alpha subunit of the sodium pump to cysteine oxidation, recruitment of prdx6 to the sodium pump by PLM may be important to maintain pump activity during periods of oxidative stress.

Photosynthesis & Photoreceptors

887-Pos

Identifying the Quencher of Excited State Energy in Photosynthetic Antennae

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Excess energy dissipation in plants in high-light conditions requires the formation of a quenching site. Although several different quenching mechanisms have been proposed, all of them involve pigment-pigment interactions between chromophores coordinated to the antenna complexes of Photosystem II. The best quencher-candidates are Chlorophyll-Chlorophyll and Chlorophyll-carotenoid pairs, likely belonging to the same Lhcb complex, which switches between a light-harvesting and a dissipative state, in this way changing the strength of the interaction. In principle all the antenna complexes can contain a quencher, as suggested by the analysis of *Arabidopsis* Lhcb-depleted lines, which have shown that none of the Lhcb is *per se* necessary for NPQ, although the absence of all of them leaves the system unprotected. This suggests that more than one antenna complex can act as a quencher and thus should contain a quenching site. Previous proposals have suggested a role for Chl 612 interacting with site L1 and Chl 603 interacting with site L2, but also other Chls located in the proximity of the carotenoids can be a putative quenching site. The spectroscopic properties of most of the Chls coordinated to several Lhcb complexes, their interactions with neighbouring carotenoids and their effect on the excited state lifetimes of the complexes have been investigated by combining mutation analysis with time-resolved spectroscopy. The experiments have been performed both in solution, where the light-harvesting conformation dominates,

and in aggregates, which are widely used to mimic the quenching state *in vivo*. The experiments indentify several quenching sites in the aggregates.

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Effect of Antenna-Depletion in Photosystem II on Excitation Energy Transfer in *Arabidopsis thaliana*

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The role of individual photosynthetic antenna complexes of Photosystem II (PSII) both in membrane organization and excitation energy transfer have been investigated. Thylakoid membranes from wild-type (WT) *Arabidopsis thaliana*, and three mutants lacking light-harvesting complexes CP24, CP26 or CP29, respectively, were studied by picosecond-fluorescence spectroscopy. By using different excitation/detection wavelength combinations it was possible for the first time to separate PSI and PSII fluorescence kinetics. The sub-100 ps component, previously ascribed entirely to PSI, turns out to be partly due to PSII. Moreover, the migration time of excitations from antenna to PSII reaction center (RC) was determined for the first time for thylakoid membranes. It is four times longer than for PSII-only membranes, due to additional antenna complexes, which are less well connected to the RC. The results in the absence of CP26 are very similar to those of WT, demonstrating that the PSII organization is not disturbed. However, the kinetics in the absence of CP29 and, especially, of CP24 show that a large fraction of the light-harvesting complexes becomes badly connected to the RCs. Interestingly, the excited-state lifetimes of the ''disconnected'' light-harvesting complexes appear to be substantially quenched.

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Spectroscopic Determination of HOMO and LUMO Energies of Retinal in Bacteriorhodopsin for Solar Cell Applications

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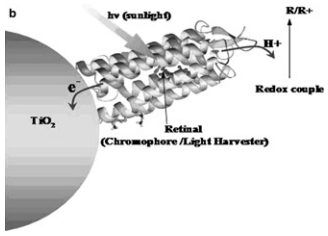
Bacteriorhodopsin (bR) is a potential sensitizer for bio-sensitized solar cells (Fig. 1). In this study, the energies of the Highest Occupied Molecular Orbital (HOMO) and the Lowest Unoccupied Molecular Orbital (LUMO) of retinal in bR are investigated using X-ray Photoemission Spectroscopy (XPS), X-ray Absorption Spectroscopy (XAS), and Ultraviolet Photoemission Spectroscopy (UPS). With the combination of XPS, XAS and UPS methods, the absolute energies of the HOMO and LUMO can be determined for comparison to the valence and conduction band energies of the biosensitized semiconductor. The HOMO-LUMO gap of retinal was spectroscopically determined to be 2.49 eV. For comparison, we also test the feasibility of DFT calculations in determining the HOMO-LUMO gap of free retinal. Using the G-311G basis set, the calculated HOMO-LUMO gap was 2.69 eV. The results shows that the DFT method overestimates the experimentally found band gap; consequently, higher level calculations are required.



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Photosynthetic Antenna Systems: The Place Where Light Interfaces with Biology

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All photosynthetic organisms contain a light-gathering antenna system, which functions to collect light and transfer energy to the reaction center complex where electron transfer reactions take place. Our work centers on the antenna complexes found in green photosynthetic bacteria, which include chlorosomes, the Fenna-Matthews-Olson (FMO) antenna protein and integral-membrane antenna and reaction center complexes. All of these complexes are involved in the light-energy collection process in these organisms, which are adapted for life in very low light intensities. Chlorosomes are ellipsoidal structures attached to the cytoplasmic side of the inner cell membrane. These antenna complexes provide a very large absorption cross section for light capture. Evidence is overwhelming that the chlorosome represents a very different type of antenna from that found in any other photosynthetic system yet studied. Chlorosomes do not contain traditional pigment-proteins, in which the pigments bind to specific sites on proteins. These systems are of interest from both a basic science perspective of what is the structure of this unique class of photosynthetic antennas and how they work so efficiently, as well as more applied aspects in which the principles of self organization and extraordinary pigment properties that characterize these systems are used in a bio-mimetic approach to devise artificial light-energy capture systems. Recent work involves studies on the structure of the FMO antenna complex and the architecture of the membrane that includes the chlorosome, FMO protein and reaction center. Additional work involves using chlorosomes as part of bio-hybrid systems in which the biological complex feeds energy to an inorganic semiconductor substrate such as titanium dioxide.

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Investigating The CP29 Photosynthetic Light Harvesting Complex with 2D Electronic Spectroscopy

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Though chlorophyll-binding protein CP29, a light harvesting complex in photosystem II of green plants, is proposed to play a role in the regulation of potentially-damaging excess chlorophyll excitations in the supercomplex, little is known about its spatial structure and its relation to excitation energy transfer dynamics and photoprotective action. In vivo, the presence of carotenoid pigment zeaxanthin is correlated with the quenching of excess chlorophyll excitations. Although the mechanism of quenching is still unknown, it is evident that CP29 exchanges carotenoid pigments depending on illumination conditions–in low light, the complex binds violaxanthin, while in high light zeaxanthin is bound.

We probe the chlorophyll Qy band of isolated CP29, binding either violaxanthin or zeaxanthin, using conventional and polarization-sensitive two-dimensional electronic spectroscopy (2DES) in order to better characterize electronic and spatial structure. 2DES resolves both excitation and emission energy of the molecular complexes being studied, providing a picture of the correlations between multiple excited states and revealing the presence of states that may go undetected by other spectroscopies. It also provides a direct map of excitation energy transfer processes within the complex by identifying signals from correlated donor and acceptor energies. Polarization-dependent studies provide clues in particular about chromophore configuration. We furthermore investigate whether the binding of zeaxanthin alters the excitation energy landscape and the resulting dynamics of CP29 to potentially modulate the quenching of excess excitations.

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The Ligand Environment of the S_2 State of Photosystem Ii: A Study of the Hyperfine Interactions of the Tetranuclear Manganese Cluster by 2D Hyscore Spectroscopy.‡

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The solar water-splitting protein complex, photosystem II (PSII), catalyzes the light-driven oxidation of water to dioxygen in Nature. The four-electron water oxidation reaction occurs at the tetranuclear manganese-calcium-oxo (Mn_4Ca-oxo) cluster that is present in the oxygen-evolving complex (OEC) of PSII. The mechanism of light-driven water oxidation has been a subject of intense interest and the OEC of PSII has been studied extensively by structural methods. While the recent X-ray crystal structures, single crystal EXAFS and EPR spectroscopy investigations provide a model for the geometry of the catalytic Mn_4Ca-oxo cluster, there is limited knowledge of the protein environment that surrounds the catalytic site. It is suggested that the binding and activation of the substrate water molecules at the Mn_4Ca-oxo cluster in the OEC of PSII are facilitated by key amino acid residues that could be ligated to the catalytic cluster. In this study, we demonstrate the application of two-dimensional (2D) hyperfine sublevel correlation spectroscopy to determine the magnetic couplings of the S_2 state of PSII. We utilize 2D difference spectroscopy to facilitate unambiguous assignments of the spectral features arising from the substrate molecules and surrounding amino acid residues in the S_2 state of PSII. The results presented here, for the first time, identify previously unknown ligands to the catalytic