average SR Ca^{2+} depletion is 13% in response to 4-CmC and 4.5% in response to prolonged and maximal sarcolemmal depolarization; and (5) the time-to-peak of intra-SR Ca^{2+} release and cytoplasmic Ca^{2+} transient at maximal sarcolemmal depolarization do not differ significantly.

1220-Pos Board B64

Which Low-affinity Fluorescent Calcium Indicators Accurately Track The Change In Myoplasmic Free Calcium Concentration (Δ[Ca]) In Skeletal Muscle?

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In vertebrate twitch fibers, spatially-averaged Δ[Ca] elicited by an action potential (AP) is large and brief. Consequently, Δ[Ca] is more accurately measured with low-affinity than with high-affinity Ca indicators (Hirota et al., 1989; Baylor and Hollingworth, 1998). Previous studies with low-affinity fluorescent indicators found that the time course of Δ[Ca] is quite accurately monitored with furaptra (= mag-fura-2), mag-fura-5, and mag-indo-1 (Konishi et al., 1991; Zhao et al, 1996). Because these tri-carboxylate Ca indicators have some sensitivity to free magnesium ([Mg]), we have evaluated three low-affinity tetra-carboxylate indicators, fura-5N, OGB-5N, and fluo-5N, which have negligible [Mg] sensitivity. To do so, resting fluorescence (F_R) and fluorescence changes elicited by an AP (ΔF) were measured at 16 °C in frog single fibers micro-injected with both furaptra and one of the other three indicators. Disappointingly, with the other three indicators, the full-duration at half maximum of ΔF was larger than that with furaptra, on average, by 37, 51, and 53%, respectively, increases that do not appear to arise from increased saturation of indicator with Ca. We also evaluated mag-fluo-4 (cf. Caputo et al., 2004), another tri-carboxylate indicator. Encouragingly, mag-fluo-4's ΔF time course was essentially identical to furaptra's while its signal-noise ratio with visible excitation wavelengths was an order of magnitude larger (for similar concentrations of indicator). However, because F_R of mag-fluo-4 probably arises largely from indicator molecules bound with Mg, calibration of mag-fluo-4's $\Delta F/F_R$ in terms of Δ[Ca] is likely to be more sensitive to variations in [Mg]. Also, with mag-fluo-4, unlike furaptra, fluorescence anisotropy values varied with the plane of polarization of the exciting light, thus revealing a population of mag-fluo-4 molecules that are oriented and presumably bound to structural components within the fiber.

Electron & Proton Transfer

1221-Pos Board B65

Redox Kinetics Of Cytochrome C Oxidase By Electrochemically-induced Time-resolved Surface-enhanced Infrared Absorption Spectroscopy (tr-Seiras)

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For use with surface-enhanced infrared absorption spectroscopy, SEIRAS, we designed a two-layer gold surface for use with electrochemistry which consists of a conducting underlayer onto which Au nanoparticles (AuNPs) are grown by self-catalyzed electroless deposition. This enabled us to apply time-resolved (tr)-SEIRAS to cytochrome c oxidase (CcO) from *R. sphaeroides* immobilized in a strictly oriented fashion on the two-layer gold surface. The enzyme was excited by direct electron transfer (electronic wiring) to the Cu_A redox center. Electrons then travelled through the rest of the electron transfer chain to heme a, heme a_3 and Cu_B without using any mediators. Kinetic constants were obtained by applying periodic potential pulses and recording spectral changes as a function of time. Excitation frequencies were varied in a wide range between 0.7 Hz and 2 kHz. Time resolved spectra were analyzed by phase-sensitive detection. A wide range of kinetic constants was obtained thereby separating these parameters from the contribution due to charging currents.

Tr-SEIRA spectra of the cytochrome c oxidase at different excitation frequencies
10 Hz 250 Hz

Ch. Nowak, Ch. Luening, D. Schach, D. Baurecht, W. Knoll, R. L. C. Naumann, electron transfer kinetics of cytochrome c in the sub-ms time regime using time-resolved SEIRAS, JPC(C) under review.

1222-Pos Board B66

Isolation and Characterization of Site-directed Mutants in the Highly Conserved Dicyclohexylcarbodiimide Binding Site in Subunit III of *Rhodobacter sphaeroides* Cytochrome c Oxidase

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Cytochrome *c* oxidase (COX) is the final electron acceptor in mitochondrial respiratory chain and in many bacterial species including *Rhodobacter sphaeroides.* Electron transfer is coupled with the pumping of protons across the membrane. Previous work has shown that reaction of beef COX with dicyclohexylcarbodiimide (DCCD) resulted in an inhibition of proton translocation by covalently binding to the conserved amino acid residue E90 located in a nonpolar region of subunit III (SIII). E90 is involved in a bonding pair with another conserved residue H212, possibly connected by a salt bridge or a hydrogen bond pair in the three dimensional structure of SIII. Our goal was to test whether the retention of the E90-H212 linkage and the spatial arrangements of these amino acid residues were critical for electron transfer and proton pumping activities of the enzyme. In the current work, we investigated the functional role of these amino acids through the creation of three mutants in SIII_H212E, E90H, and E90H/H212E. Each of the visible absorbance spectra of the three mutant proteins in the bacterial membranes exhibited similar properties as wild type COX. Conversely, the spectrum of isolated and purified COX mutant SIII E90H displayed a blue shift of 3 nm. SDS-PAGE verified that subunit III was present. Electron transfer activity assays of E90H showed an approximate 40% decrease in activity when compared to wild type enzyme and that the mutant did not undergo suicide inactivation during steady-state turnover. Proton pumping activity of the mutants reconstituted into liposomes will be discussed.

1223-Pos Board B67

Functionality of Single-Cysteine Mutants in Subunit III of *Rhodobacter sphaeroides* Cytochrome *c* Oxidase

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Cytochrome *c* oxidase (COX) catalyzes the reduction of oxygen to water using ferrocytochrome *c* and conserves the energy of this reaction by translocating protons across the bacterial or inner-mitochrondrial membrane. COX from *Rhodobacter sphaeroides* is a four subunit transmembrane protein that serves as a model for the mitochondrial enzyme. Subunit I and II contain the redox centers and proton pathways necessary for redox chemistry and proton translocation. The indispensable role of subunit III is an area still being investigated. This work examines the functionality of three mutant forms of COX - one in which all cysteines have been removed from the enzyme (CA1CS3), and two in which single cysteines are reintroduced into CA1CS3 at specific locals in subunit III (A4C, S187C). The single cysteine mutants provide a means to specifically target thiol-reactive probes to areas of interest in COX subunit III. The A4C mutant allows for a probe to be placed at the mouth of the D-channel - an important proton-conducting pathway necessary for the pumping and redox activities of COX. Bioconjugation of S187C would place a probe on an exterior loop which is thought to undergo redox-linked transient conformational changes. All three mutants were expressed and purified, and their absorbance spectra are identical to wildtype, indicating that the heme active centers are unperturbed. SDS-PAGE gels show that all three mutants retain wildtype subunit composition. The oxygen reduction activity of the mutants are also comparable to wildtype, with values between 1200-1600 $e^-/s*mol$ at pH 7.4. In conclusion, these results indicate that the cysteine-free mutant and two mutants in which single cysteines are reintroduced at non-conserved locations retain wildtype functionality, indicating that cytochrome *c* oxidase subunit III is a candidate for cysteine scanning-mutagenesis studies utilizing thiol-reactive probes.

1224-Pos Board B68

ENDOR Spectroscopy Shows that Q_A Remains in the Same Orientation Upon Reduction in Reaction Centers from *Rhodobacter Sphaeroides*

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It has been, recently, proposed that Q_A moves and rotates ~60° upon its reduction.[1] Here we investigated possible changes using ENDOR spectroscopy that provides a very sensitive (to ~0.01 Å) probe for the binding site of $Q_A^{\bullet-}$.[2] Identical RC samples were made - (*a*) one frozen in the dark (ground state) and then illuminated generating $D^{\bullet+}Q_A^{\bullet-}$ and (*b*) one frozen under illumination (excited state) which trapped $D^{\bullet+}Q_A^{\bullet-}$ in ~70% of the RCs at 80 K. Figure 1 shows the resultant ^{1}H ENDOR spectra of $Q_A^{\bullet-}$. The peaks labeled L_1, L_2 and L_3 correspond to the two H-bonds to $Q_A^{\bullet-}$.[2,3] Essentially no differences in the ENDOR spectra were observed indicating that the interactions of $Q_A^{\bullet-}$ with the protein are the same in the ground state as in the excited state. These results are irreconcilable with the proposed rotation.[1] Thus, Q_A is preset in an environment that favors its reduction.

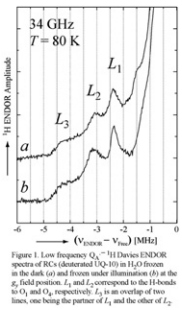

Figure 1. Low frequency $Q_A^{\bullet-}$ ^{1}H Davies ENDOR spectra of RCs (deuterated UQ-10) in H_2O frozen in the dark (*a*) and frozen under illumination (*b*) at the g_z field position. L_1 and L_2 correspond to the H-bonds to O_1 and O_4, respectively. L_3 is an overlap of two lines, one being the partner of L_1 and the other of L_2.

[1] Heinent et al. (2007), *J. Am. Chem. Soc*. **129**, 15935. [2] Flores et al. (2007), *Biophys. J*. **92**, 671. [3] Sinnecker et al. (2006), *Phys. Chem. Chem. Phys.* **8**, 5659.

1225-Pos Board B69

Demethyl Ubiquinone Inhibits Catalytic QB Activity In Reaction Centers From Rhodobacter sphaeroides*

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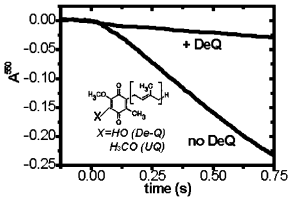
Catalytic light induced electron transfer in the photosynthetic reaction center (RC) involves reduction of the loosely bound secondary quinone Q_B. In this study we investigated the activity of demethyl ubiquionone (De-Q), the immediate precursor for the synthesis of ubiquinone (UQ), a critical component of the electron transport pathways in both prokaryotes and eukaryotes (1). Upon addition of 2 microM De-Q (K_D ~ 0.2 microM), catalytic Q_B activity was inhibited > 10-fold at pH 8 (Figure). These results show that De-Q binds more tightly than UQ thereby inhibiting its reduction. In addition, optical shifts of bacteriocholorophyll were observed consistent with an anionic De-Q; the solution pKa of De-Q was measured to be ~ 6 (not shown). We propose that De-Q near neutral pH is anionic which facilitates binding acting as a non-reducible analog of ubisemiquinone in the RC. (1) Poon *et al.* (1999) *J Biol Chem.* **274**, 21665-21672. *Supported by NIH (GM 41637).



Light induced electron turnover in the presence and absence of 2 microM DeQ (shown in inset); DeQ differs from UQ in that a titratable hydroxy group replaces a methoxy group.

1226-Pos Board B70

Kinetics and Energetics of Electron Transfer Reactions in a Photosynthetic Bacterial Reaction Center Assembled with Zinc Bacteriochlorophylls

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Electron transfer processes were studied in the reaction center (RC) of a *Rhodobacter sphaeroides* magnesium chelatase (*bchD*) mutant that assembles with six chemically identical chlorin molecules. A previous study [Jaschke & Beatty, Biochemistry, 2007] and this work show the complete absence of bacteriochlorophyll (containing Mg as the metal) and bacteriopheophytin from the *bchD* mutant RC. Instead, bacteriochlorophylls containing a Zn atom as the metal (Zn-BChl) occupy the binding sites of the special pair (P), accessory bacteriochlorophyll (B), and primary electron acceptor (H). In spite of significant differences in cofactor composition, electron transfer from excited P through B to H proceeds with high efficiency and with rates nearly identical to the wild type RC. The rate of electron transfer from H to Q_A is also the same as that observed in the wild type RC. Thus, the protein-cofactor interactions, mainly through electron sharing between the metal of the BChl and the protein, play an important role in adjusting the energies of the cofactors to form an efficient electron transfer system. The study also suggests that the overall electron transfer from P to H is more sensitive to the energy change between P and B than B and H, and can tolerate a large variation in the redox energy of H.

1227-Pos Board B71

Eseem And Hyscore Analysis Of Q_A- In Native And 15N Labeled Reaction Centers From *Rhodobacter sphaeroides*

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Reduction of the primary acceptor quinone (Q_A) in the photosynthetic reaction center *Rhodobacter sphaeroides* generates a semiquinone anion radical. This radical species has been studied using electron spin echo envelope modulation (ESEEM). Evidence has supported hydrogen bonding between the carbonyl groups of Q_A and nitrogen from His M219 and peptide nitrogen from Ala M260. In this study 3-pulse ESEEM and 2D-HYSCORE measurements on native and ^{15}N labeled reaction centers were used to directly measure the hyperfine interactions (hfi) between the semiquinone and surrounding nitrogen nuclei. 3-pulse ESEEM spectra of native reaction centers looked similar to previously reported results (Spoyalov et. al. 1996). Nuclear quadrupole coupling (nqc) produced a very complicated HYSCORE spectrum, but ^{15}N labeling eliminated the nqc and allowed for hfi to be measured. HYSCORE measurements showed the semiquinone coupled to 2 unique nitrogen nuclei. One set of cross peaks appeared far more intense in the spectrum. The difference in intensity suggests contributions to nitrogen coupling in addition to geometry.

1228-Pos Board B72

A Neutral Mutation Changes the Ionic Strength Dependence of the Rate of Electron Transfer between Cyt c2 and RCs from Rb. sphaeroides

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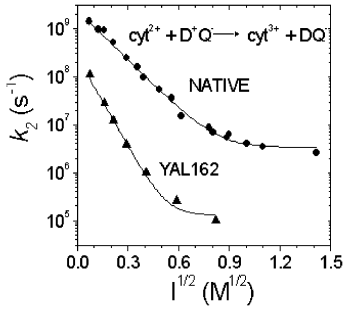
The contact between the electron transfer proteins cytochrome c_2 (cyt) and Reaction Center (RC) is centered on the hydrophobic residue Tyr L162. In the YAL162 mutant a greater ionic strength dependence of the second order electron transfer rate constant k_2 was found even though no charge changes were made. We explain this result by a transition state model (figure). For Native RC, k_2 is diffusion limited (electron transfer occurs before dissociation) and decreases with ionic strength due to increasing energy of the transition state for association. For YAL162 RCs, mutation of Tyr L162 increases the dissociation rate and decreases the electron transfer rate so that k_2 is in the fast exchange (pre-equilibration) regime (dissociation occurs before electron transfer). Here ionic strength effects are due to changes in binding energy, which are greater than changes in transition state energy (by ~2-fold) accounting for the steeper slope for the mutant. The decreased electron transfer rate due to this mutation demonstrates the importance of hydrophobic interactions in binding and electron transfer.



(1)Gong *et al.* (2003) *Biochem.* **42**, 14492.
*Supported by NIH(GM41637).

1229-Pos Board B73

Generation, Characterization And Crystallization Of A Subunit Iv Fused Mutant Cytochrome bc_1 Complex From *Rhodobacter Sphaeroides*

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The cytochrome bc_1 complex from *Rhodobacter sphaeroides* contains a three-subunit core complex and a supernumerary subunit (subunit IV). Although a 2.1 Å resolution x-ray crystallographic study of the wild-type complex has been achieved recently, the dissociation of subunit IV during crystallization has undermined structural information of subunit IV. To overcome this difficulty, we have constructed and characterized mutants with the N-terminus of subunit IV fused to the C-terminus of cyt. c_1 (c_1-IV fusion). A polyglycine (6 or 14 residues) linker was placed between the two involved proteins to ease the constraint that might result from the fusion of two subunits in the assembling process. A 6-histidine tag was placed at the C-terminus of IV (c_1-6G-IV_{His} and c_1-14G-IV_{His}) for the ease of purification. Both mutant cells grew photosynthetically