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?**

**Stephen M. Baylor**[1], Kyle R. Gee[2], Stephen Hollingworth[1].
[1]University of Pennsylvania, Philadelphia, PA, USA, [2]Invitrogen, Carlsbad, CA, USA.

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)**

**Christoph Nowak**.
MPI for Polymer Research, Mainz, Germany.

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**

**Rachel F. Omolewu**, Teresa L. Cvetkov, Lawrence J. Prochaska.
Wright State University, Dayton, OH, USA.

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**

**Teresa L. Cvetkov**[1], Robert B. Gennis[2], Lawrence J. Prochaska[1].
[1]Wright State University, Dayton, OH, USA, [2]University of Illinois, Urbana, IL, USA.

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***

**Marco Flores**[1,2], Mark L. Paddock[3], Edward C. Abresch[3], Melvin Y. Okamura[3], Wolfgang Lubitz[2].
[1]Arizona State University, Tempe, AZ, USA, [2]Max-Planck Inst. for Bioinorganic Chemistry, Muelheim an der Ruhr, Germany, [3]Univ. of California, San Diego, La Jolla, CA, USA.