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The aim of this study was to estimate the membrane potential of isolated mitochondria on the single-particle level. We used a fluorescence correlation spectroscopy setup with a 532-nm laser to detect fluorescence signals of single TMRE-doped mitochondria in suspension. The brightness of the fluorescent particles increased after the addition of a respiratory substrate (succinate) in the presence of rotenone and decreased after the addition of an uncoupler (dinitrophenol). Thus, the fluorescence signals of the particles correlated well with membrane potential magnitudes under our experimental conditions. Using an empirical formula of Gaussian–Lorentzian distribution of the brightness in the confocal volume, we found the fluorescence intensity of a single energized mitochondrion passing through the center of the observation volume. Given the fluorescence intensity of a single TMRE molecule, we estimated the number of TMRE molecules bound to a single mitochondrial particle. The number of mitochondrial particles per mg of protein (1.5×10^9) determined from the statistical distribution of fluorescence intensities and the magnitude of the membrane potential (190 mV) estimated by the Nernst equation were consistent with values of these parameters measured previously by other techniques.

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(S15) bc_1 complexes symposium lecture abstracts

S15/1 The Q_O site semiquinone state in isolated cytochrome bc_1 (complex III) from *Rhodobacter capsulatus*

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The Q_o site semiquinone of cytochrome bc_1 has been assigned pivotal roles in productive energy-conversion and destructive superoxide generation. After a 30 year search for direct evidence of this semiquinone state, a transient, Q_o site inhibitor sensitive semiquinone EPR radical has been revealed in a genetic heme b_H knockout, which effectively improves the energetics for semiquinone formation at the Q_o site. This first observation was performed in native membranes of the purple photosynthetic bacterium *Rhodobacter capsulatus*. To remove possibilities that the signal was a result of either an unforeseen semiquinone state in another redox protein of the native membranes, or damage resulting from knockout of heme b_H, we have examined for the Q_o site semiquinone state in isolated and purified cytochrome bc_1 equipped with a full complement of cofactors. Combined in a hybrid system with reaction centers (*Rba. sphaeroides*; thanks to Colin Wraight, Urbana-Champagne, IL), ubiquinone and cytochrome c_2 (*Rba. capsulatus* thanks to John Fitch and Michael Cusanovitch, Tucson, Az), light activation generates an EPR signal in a manner similar to that seen in native membranes and fully consistent with its identity as a key state of the cytochrome bc_1.

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S15/2 Domain conformational switch of the iron sulfur protein in cytochrome bc_1 complex is induced by the electron transfer from cytochrome b_L to b_H

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Intensive biochemical, biophysical and structural studies of the cytochrome (cyt) bc_1 complex in the past have led to the formulation of the "protonmotive Q-cycle" mechanism for electron and proton transfer in this vitally important complex. The key step of this mechanism is the separation of electrons during the oxidation of a substrate quinol at the Q_P site with both electrons transferred simultaneously to ISP and cyt b_L when the extrinsic domain of ISP (ISP-ED) is located at the *b*-position. Pre-steady state fast kinetic analysis of bc_1 demonstrates that the reduced ISP-ED moves to the c_1-position to reduce cyt c_1 only after the reduced cyt b_L is oxidized by cyt b_H. Structural analyses of Pm or Pf inhibitor loaded crystals revealed two ISP-ED binding positions on cyt *b*. However, the question of how the conformational switch of ISP-ED is initiated remains unanswered. The results obtained from analysis of inhibitory efficacy and binding affinity of Pm and Pf inhibitors, under various redox states of the bc_1 complex, suggest that the electron transfer from heme b_L to b_H is the driving force for the releasing of the reduced ISP-ED from the *b* position to c_1 position to reduce cyt c_1.

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S15/3 Regulatory interactions in the dimeric cytochrome bc_1 complex

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The dimeric cytochrome bc_1 complex catalyzes oxidation-reduction of quinol and quinone at sites located in opposite sides of the membrane in which it resides. The kinetics of electron transfer and inhibitor binding in the isolated yeast and *Pseudomonas denitrificans* bc_1 complexes reveal functional interactions between the quinol oxidation site at center P and quinone reduction site at center N in opposite monomers in conjunction with inter-monomer electron equilibration between the cytochrome *b* subunits of the dimer. The resilience of center P catalysis to inhibition caused by partial pre-reduction of the b_H hemes can be explained by inter-monomer electron transfer between the two cytochrome *b* subunits in the bc_1 dimer. A model for the mechanism of the bc_1 complex has emerged in which binding of ligands that mimic semiquinone at center N regulates half-of-the-sites reactivity at center P and binding of ligands that mimic binding of ubiquinol at center P regulates half-of-the-sites reactivity at center N. An additional feature of this model is that inhibition of quinol oxidation at the quinone reduction site is avoided by allowing catalysis in only one monomer at a time, which maximizes the number of redox acceptor centers available in cytochrome *b* for electrons coming from quinol oxidation reactions at center P and minimizes the leakage of electrons that would result in the generation of damaging oxygen radicals.

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S15/4 The loneliness of the electrons in the bc_1 complex

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A stochastic approach based on Gillespie algorithm is particularly well adapted to describe the time course of the redox reactions that occur inside the respiratory chain complexes because they involve the motion of single electrons between individual unique redox centers of a given complex and not populations of electrons and redox centers as usually considered in ordinary differential equations. In this way we approach the molecular functioning of the bc_1 complex based on its known crystallographic structure and the rate constants of electron tunnelling derived from the Moser and Dutton phenomenological equation. The main features of our simulations are the dominant and robust emergence of a Q-cycle mechanism and the near absence of short-circuits in the normal functioning of the bc_1 complex. Thus, in our paper, the Mitchell Q-cycle no longer appears as an *a priori* hypothesis but arises out of the bc_1 complex structure and of the kinetic laws of redox reactions.

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(S15) bc_1 complexes symposium abstracts (poster and raised abstracts)

S15.5 Construction of a bacterial bc_1 complex hetero dimer

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The bc_1 complex is a homodimer. When the first structures were published it was an unexpected result: both monomers are intertwined by their ISP subunits: The head domain of the ISP anchored in monomer A belongs to the Qo center of monomer B, and *vice versa*. These findings raise the question whether the two monomers are also functionally interacting, as indicated by recent results. To answer this experimentally two operons were constructed to express two different monomers: one is wild type, the other carries an inactivating mutation in the cytochrome *b*. Statistically, assembly will result in 50% heterodimeric complexes. These are detected and purified by different tags. We assume that the activity of such a complex is 50% of wild type if there is no cooperativity between monomers. In case of an interaction, a lower activity should result from kinetics. We chose two different tags, Strep-tag II and His-tag, which were cloned and tested in different positions in the *b* subunit of the complex. These were checked for activity, spectral properties, resin binding and assembly. We were able to introduce an inactivating mutation in the cytochrome *b* that does not disturb subunit assembly or spectral properties. Both operons were stably introduced in a *P. denitrificans* bc_1 complex deletion strain and co-expression of both complexes were verified by their respective tags.

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S15.6 The unprecedented peroxidase-like activity by nitrophorin-2, the no carrying heme protein from *Rhodnius prolixus*

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We have characterized the NO carrier protein Nitrophorin-2 (NP2) and its variants from *Rhodnius prolixus* for their reaction with H_2O_2 and peroxyacetic acid (PAA). The enzyme demonstrated substantial peroxidase activity with a pH optimum of 6.8 using ABTS and o-dianisidine. The K_m for ABTS (500 μM) is comparable to that reported for some of the catalase-peroxidases (KatGs). The K_m for H_2O_2 (1.1 mM) was much higher than K_m for PAA (32 μM) but comparable to the values reported for some KatGs. Tyr38Ala variant showed lower peroxidase activity but with very high K_m values for both H_2O_2 and PAA. The stopped flow analysis of the wild type and the variants was consistent with the formation of Compound I ([Fe(IV)=O Por.$^+$]) but with different rates. The 9 GHz-EPR spectra showed the formation of two different [Fe(IV)=O Por.$^+$] species, one weakly ferromagnetically coupled signal (typical of peroxidases) at basic pH, and a novel strongly ferromagnetically coupled signal at neutral pH, exclusively observed in model heme complexes. Moreover, we also identified an [FeIV= O Tyr.] species formed by intra-molecular electron transfer. Characterization of NP2 variants indicated Tyr85 being the site for the protein radical. Our results suggest that NP2 can perform not only the "heme-edge" oxidation but can also use alternative protein-based radical intermediates as shown in the case of KatGs.

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S15.7 Estimation of the lifetime of the complex between cytochrome *c* and cytochrome bc_1 using electron paramagnetic resonance

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Electron transfer (ET) between cytochrome *c* and cytochrome bc_1 is an integral part of several energy-conserving systems, including mitochondrial respiration. We investigate the molecular association of those two proteins using ET-independent electron paramagnetic resonance techniques (EPR). We employed site-directed spin labeling to modify bacterial and mitochondrial cytochromes *c* at several surface-exposed positions. Continuous wave EPR spectra and saturation recoveries of those forms recorded in the absence and presence of cytochrome bc_1 demonstrated that EPR detects a binding of cytochrome *c* to cytochrome bc_1. The bound cytochrome *c* fraction successively decreases as the ionic strength increases with a limit of approximately 120 mM NaCl above which essentially no bound cytochrome *c* can be detected by EPR. This dynamic equilibrium between bound and free cytochrome *c* exposed by EPR allowed us to estimate that the average lifetime of the tightly-bound complex decreases from over 100 μs at low ionic strength to less than 400 ns at the physiological ionic strength. This strongly supports an early idea of diffusion-coupled reactions that link the soluble electron carriers with the membranous complexes, which, we believe, provide robust means to regulate electron flow through these complexes. *AO is The Wellcome Trust International Senior Research Fellow.

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