The NhaA Na⁺/H⁺ antiporter, new insight into structural and functional dynamics
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In 1974, Peter Mitchell and colleagues discovered sodium proton antiport activity in bacterial cells and suggested that Na⁺/H⁺ antiporter proteins have primary roles in the homeostasis of these cations. In the ensuing 40 years, sodium proton antiporter activity has been identified in the cytoplasmic and organelle membranes of almost all cells. Cloning of the NhaA gene from *Escherichia coli* opened the prokaryotic Na⁺/H⁺ antiporters’ field to molecular biology. Overexpressing and purifying the NhaA protein in a functional state, was a step that led to elucidating the bioenergetics and kinetics of the antiporter. Finally, it paved the way to structure biology and the determination of the crystal structure of NhaA at pH 3.5 (in collaboration with H. Michel, Max Planck fur Biophysics, Frankfurt, Germany). The NhaA crystal structure has provided key insight into the function and regulatory properties of antiporters, shaded new light on the general architecture of transport proteins and opened the way to structure based interdisciplinary studies of the Na⁺/H⁺ antiporters that could not otherwise have been carried out. The NhaA structural fold is unique: two inverted repeats of helices with unwound chain in each repeat cross each other. This assembly creates a very delicately electrostatic balanced area in the middle of the membrane essential for activity. Remarkably, the number of secondary transporters with the NhaA fold is steadily increasing. Several of these are even not homologues to NhaA.

Transporter is a dynamic “nano-machine” which transports the substrate from one side of the membrane to the other side. The kinetics and thermodynamics of all types of transporters can, in principle, be explained by the alternating access conceptual model (Jardetzky, 1966) in which the active site has alternating access to either side of the membrane. Therefore, to understand the NhaA functional mechanism in atomic details, we try to crystallize the active conformations of NhaA at physiological pH. In parallel, apart from identifying the importance of every residue for activity, many site directed techniques have been employed to identify the pH/Li⁺/Na⁺ induced conformational changes. Nevertheless, these results focusing on specific sites, left unknown the global movement of the protein and its coordination. We therefore employed Hydrogen-Deuterium Exchange Mass Spectroscopy (HDX-MS). This technique which has long been used for elucidating conformational changes in soluble proteins, has rarely been applied to membrane proteins because of their hydrophobicity the associated detergents. Solving the technical problems, we applied HDX-MS to NhaA and revealed a global coordinated conformational change in the architecture of NhaA upon Li⁺ binding allowing to suggest a model for cation exchange mechanism of NhaA.

Due to NhaA evolutionary conservation, we have succeeded in modeling the structure of NHE1 and NHA2, human homologues that have long been recognized as drug targets. Comprehensive evolutionary analysis of the present 6,537 representative of Na⁺/H⁺ antiporter sequences describes the full complexity of the antiporters phylogeny as a tree, and reveals a sequence motif that appears to determine the phenotypic characteristics of NhaA.
mediating the PT (the PT pore, PTP or mitochondrial megachannel, MMC) has remained a mystery. The initial suggestion that the channel proper was provided by the adenine nucleotide translocator, outer membrane VDAC and the peripheral benzodiazepine receptor (TSPO) did not stand the test of their genetic inactivation. The most recent hypothesis posits that the PTP/MMC forms following a Ca$^{2+}$-dependent conformational change of the F-ATP synthase. The hypothesis is supported by some genetic manipulations of F-ATP synthase, by electrophysiological measurements of the partially purified bovine, human, yeast and Drosophila enzymes and by site-directed mutagenesis of specific F-ATP synthase residues; while it has been questioned by studies based on genetic ablation of subunits c, b and OSCP (see [1] for a recent discussion that covers this controversy and offers an explanation for the apparent discrepancies). I will recapitulate the history of the PT, present evidence on the channel activity of highly purified F-ATP synthase and illustrate the structure-function relationship of MMC activity based on site-directed mutagenesis of critical Thr, His, Arg and Cys residues of F-ATP synthase.

References

Plenary I. - Macromolecular structure and function 1

Cryo-EM structures of complex I from mouse heart mitochondria in biochemically-defined states
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Respiratory complex I (NADH:ubiquinone oxidoreductase) is one of the largest membrane-bound enzymes in the mammalian cell. It powers ATP synthesis in mitochondria by capturing the free energy produced by electron transfer from NADH to ubiquinone and using it to drive protons across the inner membrane. Mammalian complex I contains 45 subunits. 14 core subunits house the catalytic machinery and are conserved from bacteria to humans, whereas the cohort of 31 supernumerary subunits forms an exoskeleton around the core and is specific to mammalian species. Structures of mammalian complex I, determined by single-particle electron microscopy (cryoEM) have now been determined for several species and have been steadily improving in resolution. In this talk I will discuss recent data on complex I from mouse heart mitochondria, a biomedically relevant model system. The 3.3-Å resolution structure determined in the ‘active’ state has revealed a nucleotide bound in subunit NDUFA10 (a nucleoside kinase homolog) and defined the structures of mechanistically critical elements. By comparisons with a 3.9-Å structure of the ‘deactive’ state and with known bacterial structures, differences in helical geometry in the membrane domain that occur upon activation or that alter the positions of catalytically important charged residues have also been identified. These results demonstrate the capability of cryo-EM analyses to challenge and develop mechanistic models for mammalian complex I.

Structure of the Alternative Complex III in a supercomplex with cytochrome oxidase
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Alternative Complex III (ACIII), like Complex III, catalyzes the oxidation of membrane-bound quinol and reduction of cytochrome c, but is structurally unrelated to Complex III. We solubilized the ACIII directly from membranes of Flavobacterium johnsoniae using styrene maleic acid (SMA) copolymer in the absence of traditional detergents. The ACIII was isolated as a functional 1:1 supercomplex with an aa_3-type cytochrome c oxidase (cyt aa_3) within SMA copolymer nanodiscs. We determined the structure of the ACIII component of the
supercomplex to 3.4 Å resolution by cryo-EM and constructed an atomic model for its six subunits, two of which are anchored to the lipid bilayer with N-terminal triacylated cysteine residues, resolved here for the first time. The structure also contains a [3Fe-4S] cluster, a [4Fe-4S] cluster, and six hemes c along with 11 phospholipid molecules. The ACIII is in direct contact with subunit III of the cyt aa₃ component of the supercomplex.

Regulation, functional analysis and assembly of dimeric ATP synthases in mitochondria

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Until recently, a molecular description has been lacking of how the proton motive force drives rotation in the synthetic sense, as there was no molecular structure of the interface between the membrane domain of the rotor where the trans-membrane proton pathway is thought to lie. Important contributions to the provision of this description have come recently from both X-ray crystallographic [1] and electron cryo-microscopy studies [2,3] of various intact ATP synthases. They indicate that the proton pathway is provided by polar residues in trans-membrane α-helices in the a-subunit that are tilted at 30° to the plane of the membrane. Cardiolipin molecules that bind briefly to specific sites near to the exit of the pathway are likely to participate in the release of the protons in the mitochondrial enzyme [4], but not in chloroplasts. One important question is how is this proton pathway assembled? Recent experiments have shown that the proton pathway in human mitochondria is assembled in a way that avoids the partially assembled enzyme acting in hydrolytic mode, and also avoids the proton motive force being dissipated. Dimerisation of the complex is coincident with formation of the proton channel, and dimers associate into oligomers of dimers in a subsequent step [5]. It is increasingly unlikely that the permeability transition pore is associated with the ATP synthase complex [6,7].

References

Plenary II. - Macromolecular structure and function 2

Cytochrome c oxidase – a molecular machine

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Cytochrome c oxidase is a remarkable energy transducer that seems to work almost purely by Coulombic principles without the need for significant protein conformational changes. In recent years it has become possible to follow key partial reactions of the catalytic cycle in real time, both with respect to electron and proton movements. These experiments have largely set the stage for the proton pump mechanism. The structures of the catalytic binuclear heme-copper site that is common to the huge family of heme-copper oxidases, are today well understood throughout the catalytic cycle of oxygen reduction to water based on both spectroscopic studies and quantum
chemical calculations. Here, we briefly review this progress, and add some recent details into how the proton pump mechanism is protected from failure by leakage.

Terminal oxidases of the heme-copper and bd oxidase type, a structural and functional comparison

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Nature has invented two types of membrane integrated terminal oxidases for the reduction of molecular dioxygen to water. The enormous energy inherent in this reaction is stored in the form of an electrochemical proton gradient to a large extent. The members of the superfamily of the heme-copper containing oxidases (HCOs) have invented mechanisms to pump protons across the membrane in addition to using electrons and protons from opposite sides of the membrane, whereas the evolutionary unrelated quinol oxidizing cytochrome bd oxidases rely on the release of the protons from the quinols to the external side and the uptake of protons for water formation from the internal side. We have determined the structures of members of the A and C families of the HCOs [1,2] as well as of a bd oxidase [3]. Surprisingly, and in contrast to the expectations, there are striking similarities: The oxygen binding heme is found in a central location so that the electrons needed to reduce the oxygen can be provided from two opposite sides thus reducing the danger of forming partially reduced oxygen species. In both cases a tryptophan residue is located between the first electron accepting heme and the oxygen binding heme. Nature appears to have invented the same mechanism for the prevention of the formation dangerous partially reduced oxygen species twice.


CryoEM structures of ATP synthase

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The current resolution revolution in electron cryo-microscopy (cryoEM) is having a major impact on the structure determination of large, dynamic membrane protein complexes. We have used cryoEM to investigate the structure, mechanisms and in situ arrangement of the mitochondrial and chloroplast ATP synthase. Electron cryo-tomography (cryoET) of mito–chondria from a wide range of organisms indicated that dimers of ATP synthase form long rows in the inner membrane and are a common, fundamental feature of mitochondrial morphology (Davies et al, 2012; Mühleip et al, 2016; 2017). By contrast, cryoET of chloro–plast thylakoids shows that the chloroplast ATP synthase is a monomer in the membrane (Daum et al, 2010). The single-particle cryoEM structure of the intact mitochondrial F_{1}F_{0} ATP synthase from Polytomella sp. (Allegretti et al, 2015; Klusch et al, 2017) now resolves all 14,000 sidechains in the 1.6 MDa dimer, enabling a complete chain trace including the 10 subunits of the bulky peripheral stalk. Subunit a in the membrane-embedded F_{0} motor assembly forms two aqueous channels to conduct protons to and from the protonation sites on the c-ring rotor that powers ATP generation. The structure of the complete and functional monomeric chloroplast F_{1}F_{0} ATP synthase reconstituted into lipid nanodiscs was determined at near-atomic resolution (Hahn et al, 2018). Key features of the mitochondrial and chloroplast ATP synthases, including the aqueous subunit a channels, are highly conserved. Structure-based estimates of torque generated by F_{0} agree well with published experimental data.

References

It is unclear how the mitochondrial fusion protein Optic atrophy 1 (OPA1), which inhibits cristae remodeling, protects from mitochondrial dysfunction. Here we present our recent data that identify the mitochondrial F1Fo-ATP synthase as the effector of OPA1 in mitochondrial protection. In OPA1 overexpressing cells, the loss of proton electrochemical gradient caused by respiratory chain complex III inhibition is blunted and this protection is abolished by the ATP synthase inhibitor oligomycin. Mechanistically, OPA1 and ATP synthase can interact, but recombinant OPA1 fails to promote oligomerization of purified ATP synthase reconstituted in liposomes, suggesting that OPA1 favors ATP synthase oligomerization and reversal activity by modulating cristae shape. When ATP synthase oligomers are genetically destabilized by silencing the key dimerization subunit e, OPA1 is no longer able to preserve mitochondrial function and cell viability upon complex III inhibition. Thus, OPA1 protects mitochondria from respiratory chain inhibition by stabilizing cristae shape and favoring ATP synthase oligomerization.

Mitochondrial Calcium Signaling

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Early studies of mitochondria isolated from various tissues have demonstrated that Ca\(^{2+}\) uptake is driven by the membrane potential, and is mediated by a ruthenium red-sensitive electrogenic uniport, referred to as “Ca\(^{2+}\) uniporter” (mtCU). Electrophysiological recording of mtCU documented a similar inwardly rectifying Ca\(^{2+}\) current in mitoplasts derived from different tissues but great differences appeared in the current density, which was particularly low in cardiac mitochondria. Recently, the major mtCU forming proteins have been identified, including the pore, MCU, its dominant-negative form, MCUb, a scaffold, EMRE, and Ca\(^{2+}\)-sensitive regulators, MICU1 and MICU2. To date, a MICU complex (a hetero/homo-dimer of MICU1 and MICU2) appears to determine both the threshold and cooperative activation of the mtCU by Ca\(^{2+}\), thus providing a mechanism for the sigmoidal [Ca\(^{2+}\)] dependence of the mtCU. MICU1 deletion in mouse is perinatal lethal, likely because of mitochondrial Ca\(^{2+}\) overload-induced injury. mtCU components show tissue-specific expression and MICU1 is expressed at a low level in striated muscle. The tissue specific differences in the mtCU current and molecular composition are particularly interesting in the context of the distinct calcium signaling patterns that mitochondria from different tissues have to cope with.

We have created organ-specific knockouts of MICU1 in hepatocytes, skeletal muscle fibers and neurons to determine the tissue specific physiological functions of MICU1, which studies will be shown in this presentation.
Plenary IV. - Mitochondria and cancer

Metabolic Management of Glioblastoma

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Glioblastoma multiforme (GBM) remains among the most aggressive and difficult to manage primary brain tumours in humans. Glucose and glutamine are recognized as the major fuels that drive GBM growth and invasion through glycolysis and glutaminolysis, respectively. The glutamine antagonist, 6-diazo-5-oxo-L-norleucine (DON), was administered together with a calorically restricted ketogenic diet (KD-R) to treat late-stage orthotopic growth in two syngeneic mouse models of GBM; the highly invasive mesenchymal tumour, VM-M3, and the high-grade stem cell glioma, CT-2A. DON targets glutaminolysis while the KD-R reduces glucose and, at the same time, elevates neuroprotective and non-fermentable ketone bodies. The diet/drug therapeutic strategy caused massive tumour cell death or mitotic arrest, while reversing disease symptoms and improving overall survival without toxicity. The therapeutic strategy also reduced edema, hemorrhage, and inflammation associated with rapid tumour growth. Moreover, the KD-R diet facilitated DON delivery to the brain and allowed a lower nontoxic dosage to achieve therapeutic effect. Data from human case reports will also be presented. These findings support the importance of glucose and glutamine in driving GBM growth and provide a plausible therapeutic strategy for the non-toxic metabolic management of GBM.

Michael P. Lisanti

Abstract not received

Mitochondrial dysfunction and cancer: metabolites and beyond

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Although several lines of evidence have implicated mitochondrial dysfunction in cancer aetiology, it is still unclear how and to what extent the dysregulation of mitochondrial function contributes to the behaviour of cancer cell. Today I will present some recent results obtained using a cell model with defined levels of mitochondrial DNA mutation, mTUNE, to investigate the direct consequences of mitochondrial dysfunction. We found that impaired utilization of reduced nicotinamide adenine dinucleotide (NADH) by the mitochondrial respiratory chain leads to cytosolic reductive carboxylation of glutamine as a new mechanism for cytosol-confined NADH recycling supported by malate dehydrogenase 1 (MDH1). We also observed that increased glycolysis in cells with mitochondrial dysfunction is associated with increased cell migration in an MDH1-dependent fashion. Our results elucidates a novel link between mitochondrial dysfunction and cancer metabolism, associated with changes in cell behaviour.

Displacement of Hexokinase 2 from mitochondria induces mitochondrial Ca²⁺ overload and calpain-dependent cell death in cancer cells

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Many neoplastic cell types display high levels of hexokinase isoform 2 (HK2), which enhances glucose metabolism and mainly binds to the outer mitochondrial membrane, where it contributes to cell resistance to stress stimuli and tumor growth. Therefore, HK2 constitutes a promising target for antineoplastic strategies, but HK2 inhibitors can have important side effects as they affect glucose metabolism.

We observe that in cancer cells HK2 locates at mitochondria-endoplasmic reticulum (ER) contact sites called MAMs (Mitochondria-Associated Membranes), where Ca\(^{2+}\) transfer from ER to mitochondria occurs. We have selectively detached HK2 from MAMs by using a peptide that does not perturb hexokinase enzymatic activity. Peptide treatment rapidly elicits a massive Ca\(^{2+}\) release from ER through Inositol-3-Phosphate-Receptors (IP\(_3\)-Rs). This prompts a surge in cytosolic Ca\(^{2+}\) levels, which is amplified by a rise in IP\(_3\) generation and by Ca\(^{2+}\) entry through the plasma membrane, leading to a mitochondrial Ca\(^{2+}\) overload. As a result, mitochondria stably open the permeability transition pore and undergo inner membrane depolarization in a calpain-dependent way, thus inducing cancer cell apoptosis. Administration of the HK2-targeting peptide reduces allograft growth of breast and colon cancer cells without any noxious effects on healthy tissues and causes apoptosis of chronic lymphocytic leukemia B cells freshly obtained by patients. Our findings reveal a functional connection between HK2 and Ca\(^{2+}\) fluxes in MAMs that can be exploited for novel anti-neoplastic approaches.

**Plenary V. - Transport and metabolism**

**A chloroplast-localized mitochondrial calcium uniporter homolog mediates stress-specific response in Arabidopsis plants**

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The view is emerging that chloroplasts respond to biotic and abiotic stresses with specific Ca\(^{2+}\) signals, but the relevance of this signaling has been largely underestimated, mainly due to limited information on the molecular machinery controlling chloroplast cation homeostasis. Here we unravel that a novel, chloroplast envelope-located member of the recently identified mitochondrial calcium uniporter (MCU) family, cMCU, mediates Ca\(^{2+}\) flux into this organelle in vivo. Using a toolkit of aequorin reporters targeted to chloroplast subcompartments and the cytosol in WT and knock-out lines for cMCU, we provide evidence that stress stimulus-specific Ca\(^{2+}\) dynamics in the chloroplast stroma correlate with the function of the channel. In addition, specific downstream signalling events involving kinases as well as transcription factors are influenced by cMCU activity. Plants lacking cMCU display an increased resistance to stress and recover quickly, in contrast to WT plants. Overall, a new player of a crucial aspect of the plant response system to changing environments has been identified, providing a valuable tool for the biotechnological development of new strategies to possibly alleviate the impact of climate change on agricultural productivity.

**Tryptophan rich sensory protein, TSPO: Functional insights from high resolution structures**

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The ancient conserved protein, TSPO, now known as the translocator protein 18kD, is found in all kingdoms and was originally discovered as a binding site for benzodiazepine drugs in mammalian mitochondria. An outer mitochondrial membrane protein that is highly expressed under stress conditions, it is of particular interest as a biomarker for Positron Emission Tomography imaging of inflammation in the brain, but its role in the inflammatory process is not understood. The nature of TSPO’s involvement in control of steroid hormone synthesis is also controversial, but evidence from a variety of studies and a human polymorphism suggest a connection with anxiety disorders, neurodegenerative disease and aging. Ligands that bind with high affinity include benzodiazepine drugs and analogs, cholesterol, heme, porphyrins, and retinoic acid. When purified from *E. coli*, recombinant TSPO crystallizes as an AB homodimer in a number of different crystal packings and space groups and with a porphyrin bound at the ligand binding site of monomer A. Here we report a new structure of TSPO from *Rhodobacter sphaeroides*, where a heme occupies the ligand binding site in monomer
B. The selectivity for heme binding in the B-subunit of the A-B dimer, while a partially oxidized porphyrin is only found in the A subunit, implies half-of-sites behavior and a functional role of the dimer configuration. The accelerated degradation of porphyrin by light and oxygen when bound to TSPO is not observed for heme. But porphyrin degradation still occurs in the presence of bound heme, supporting half-of-sites reactivity. The structures of several mutant forms with altered degradation activity and ligand binding behavior are reported. The physiological role of heme and porphyrin binding to TSPO is not clear, but some evidence suggests a protective function. Supported by NIH R01GM26916 and the MSU Foundation (SFM).

Mitochondrial Uncoupling Proteins: How Many of Them?
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Mitochondria convert the energy of substrate oxidation into ATP and heat. Mitochondrial thermogenesis helps to maintain core body temperature and protects against obesity, diabetes, age-related disorders, and pathological conditions involving mitochondrial oxidative stress such as ischemia-reperfusion. Mitochondrial heat and ATP are produced from the same energy source—voltage ($\Delta V$) across the inner mitochondrial membrane generated by the electron transport chain. ATP is generated by the ATP synthase that passes $H^+$ down $\Delta V$ and uses the released energy to generate ATP from ADP and inorganic phosphate. In contrast, heat is generated by so-called uncoupling proteins (UCPs) that also pass $H^+$ down $\Delta V$ but do not generate ATP, instead letting energy dissipate as heat. A clear understanding of UCPs, and even their molecular identity in the majority of tissues, remains elusive. The primary barrier in the field has been the inability to measure the $H^+$ current mediated by UCPs (referred to as the “mitochondrial $H^+$ leak”) directly and under controlled conditions. We recently removed this major technical barrier by demonstrating that the $H^+$ leak can be measured directly from the inner mitochondrial membrane using the patch-clamp technique. This method has already helped to clarify the long-sought mechanism by which FA, the physiological activators of mitochondrial thermogenesis, activate $H^+$ leak via UCP1, the UCP of the specialized thermogenic tissue brown fat. It was also instrumental in the characterization of the molecular mechanisms of heat production in beige/brite fat, a recently identified thermogenic tissue. Here, we will discuss the molecular mechanisms of mitochondrial $H^+$ leak and thermogenesis in regular somatic tissues such as skeletal muscle, heart and liver. In particular, we will focus on the identity the UCP of regular somatic tissues and its regulatory mechanisms.

Plenary VI. - Mitochondrial physiology and signalization

Control of brain metabolism and behavior by cell-type specific mitochondrial CB$_1$ receptors

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Type-1 cannabinoid receptors (CB₁) are powerful modulators of brain physiology and behavior. We demonstrated that these receptors are present in brain mitochondrial membranes (mtCB₁) among several neuronal types. Activation of mtCB₁ downregulates intra-mitochondrial cAMP-PKA signaling to blunt cellular respiration and ATP levels. The mtCB₁ signaling affects memory performance since specific deletion or inhibition of mtCB₁ block the cannabinoid-dependent alteration of synaptic transmission and memory performance. These first results demonstrated that subcellular localization of cannabinoid receptors dictate how they impact on brain metabolism and cognition. The role of mtCB₁ among specific brain cell types is still however uncharacterized. We recently observed that mtCB₁ is also present in astrocytes where it impacts on mitochondrial activity and behavior differently than other mtCB₁ populations. Briefly, specific activation of mtCB₁ located in astroglial mitochondria impairs social behavior by altering brain glucose and lactate metabolism. By modulating the phosphorylation of specific Complex I subunits, activation of astroglial mtCB₁ receptors decreases stability and activity of complex I to attenuate reactive oxygen species levels, leading to reduced glycolytic lactate production. Conditional deletion of astroglial CB₁ receptors, genetic inhibition of cannabinoid effects on phosphorylation of Complex I subunits and brain lactate supplementation reversed cannabinoid-induced behavioral deficits in mice. Our works reveal that (mt)CB₁ can directly regulate various cellular metabolic and behavioral processes and that mitochondrial neurotransmitter receptor signaling can directly regulate brain glucose metabolism to modulate high order behavioral responses.

The Mitochondria Reticulum of Muscle Cells

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Recently both 3D high resolution and functional studies in muscle cells have revealed a tightly coupled mitochondria reticulum (MR) to rapidly distribute potential energy, in the form of the mitochondrial membrane potential (MMP), throughout skeletal muscle and heart cells. Herein the structural aspects of the MR are described using 3D Focused Ion Beam Scanning Electron Microscopy (FIB-SEM) and presented in muscle cells. A large portion of the MR conductivity is dependent on direct mitochondrial matrix continuity while in some regions of the muscle the connectivity is proposed to occur via poorly characterized electron dense regions (EDR) between adjacent mitochondria. Using a photo-activated mitochondrial uncoupler to regionally perturb the MMP, we have demonstrated that large regions of the MR are electrically coupled via a shared matrix as well as EDR structures between the mitochondria. In murine skeletal muscle cells a large fraction of the mitochondrial volume is located in regions close to capillary indentations in the cell structure. These embedded capillaries are surrounded by large pools of mitochondria near the plasma membrane that have narrow tubes which run along the I-Bands (I-Band Mitochondria Segments (IBMS)) deep into the muscle cell. It has been proposed that these IBMS serve to distribute the MMP from the large sub-sarcolemma mitochondrial pool to the more central ATP-consuming myofibril region of the muscle cell. Consistent with this notion was the observation that there is a 3-fold enhancement of MMP generating oxidative phosphorylation complex in comparison to MMP utilizing ATP synthesis enzymes in the periphery of the muscle cell when compared to central regions near the muscle ATPase activity. In cardiac cells, no IBMS exist and the coupling is exclusively through large mitochondria structures and numerous EDR connections.

These data are consistent with a mitochondria reticulum in muscle cells that couples large numbers of mitochondria together providing a rapid and uniform potential energy source throughout the cell to support ATP production.

Roles of endogenous PINK1 and Parkin in vivo – a different type of mitochondrial QC

Richard J. Youle

Abstract not received
Environmental adaptation, predisposition to common diseases, and perhaps speciation may all be linked through alterations in mitochondrial bioenergetics due to functional mitochondrial DNA (mtDNA) variants. Simple changes in the percentage of mtDNAs harboring the mt\text{\texttt{tRNALeu(UUR)} nt 3243T>G} mutant can result in a range of phenotypes from diabetes and autism, to neurodegenerative disease, to lethal pediatric disease, the phenotypic differences relating to mitochondrial signaling that alters nuclear gene expression\textsuperscript{1}.

The sequential accumulation of mtDNA mutations along radiating maternal lineages results in mtDNA haplotypes whose genetic variants remain in total linkage disequilibrium. Some functional variants are adaptive in specific environments and when they arise on a mtDNA lineage, descendent mtDNAs became regionally enriched by adaptive selection resulting in groups of related haplotypes, a haplogroup. While functional haplogroup variants may be adaptive in the original environment, they can be maladaptive in another environment. As a result, mtDNA haplogroups are now being correlated with a predisposition to a wide range of metabolic and degenerative diseases, cancer, and longevity\textsuperscript{2}.

All Eurasian mtDNA haplogroups fall into two major founding lineages, macro-haplogroup N (defined by variants m.\text{\texttt{ND3 10398A (114T)}} and m.\text{\texttt{ATP6 8701A (59T)}}) and macro-haplogroup M (variants m.\text{\texttt{ND3 10398G (114A)}} and m.\text{\texttt{ATP6 8701G (59A)}}). Additional mtDNA variants create sub-lineages, the haplogroups. Additional mutations such as the m.\text{\texttt{ND1 3394T>C (Y30H)}} variant can result in further physiological modifications. That the same mtDNA variant can be adaptive or maladaptive is demonstrated by the m.\text{\texttt{ND6 m.ND1 3394C (30H)}} variant which is maladaptive when occurring on macro-haplogroup N in association with primary Leber Hereditary Optic Neuropathy (LHON) complex I mtDNA mutations [m.\text{\texttt{ND4 nt 11778A>G (R340H)}} or m.\text{\texttt{ND6 nt 14484T>C (V64M)}}], but is adaptive for Tibetan high altitude when occurring on the macro-haplogroup M background with an odds ratio 23.7\textsuperscript{3}.

A number of mtDNA haplogroups have been associated with altered risk for neurodegenerative disease. Haplogroup Uk increases the risk of Alzheimer disease\textsuperscript{4} and the m.\text{\texttt{tRNAGln nt 4336A>G}} gene variant in association with European haplogroup H (H5a) predisposes to both Alzheimer and Parkinson disease\textsuperscript{4}. European haplogroups I, J, K, T and U are at increased risk of autism relative to lineage (H+HV), with each haplogroup has an odds ratio of ~2, with haplogroups I, J, K, T and U representing 55% of the European population\textsuperscript{6}.

mtDNA haplogroups have also been correlated with diabetes and metabolic syndrome. In an Asian study haplogroups N9a, F4, and D5 were associated with decreased risk of diabetes, while haplogroups F4 and N9a were associated with increased risk of hypertension and F4 with increased risk of obesity. Moreover, ancient adaptive haplogroup variants can modulate the clinical phenotypes of recent deleterious mutations as shown by the reduction of the phenotype neuromuscular disease to diabetes in a family with a high percentage m.\text{\texttt{tRNALeu(UUR) nt 3243G}} mutant when it occurs on the N9a haplogroup background\textsuperscript{7}.

That mtDNA haplogroup lineages are functionally important has been demonstrated by creating transmitochondrial cybrid cell lines and documenting extensive physiological differences between haplogroups\textsuperscript{2}. For example, macro-haplogroup N mtDNAs have a lower respiratory complex I activity than macro-haplogroup M mtDNAs, and different N haplogroups have different complex I activities. In all haplogroups addition of the m.\text{\texttt{ND1 3394C (30H)}} variant further reduces complex I activity\textsuperscript{2}. Mice with the same nucleus but different mtDNA haplogroups also have different phenotypes\textsuperscript{5}.

There is considerable variability in human mtDNA haplogroup association studies. However, this is likely a consequence of the extreme regional stratification of mtDNA haplogroups and the interacting effects of ancient and recent mtDNA variants graded physiological effects. What is important is the repeated demonstration that mtDNA variation modifies the relative risk of the common diseases in a wide range of populations and diseases. Thus, bioenergetic variation must be an important factor in the etiology of neurodegenerative and metabolic diseases.

Mitochondrial machineries for import and assembly of proteins

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Mitochondria have to import about 1,000 different proteins from the cytosol. The protein translocase of the outer mitochondrial membrane (TOM) functions as main protein entry gate for precursor proteins with different types of targeting signals. Upon translocation across the outer membrane through the Tom40 channel, the precursor proteins are transferred to distinct downstream machineries: the presequence translocase of the inner membrane (TIM23), the carrier translocase of the inner membrane (TIM22), the mitochondrial intermembrane space import and assembly (MIA) system, or the sorting and assembly machinery (SAM) of the outer membrane. The translocases do not function as independent units, but are connected to each other and to further mitochondrial machineries, including respiratory chain complexes, the mitochondrial contact site and cristae organizing system (MICOS) of the inner membrane, and the metabolite channel porin/VDAC of the outer membrane. Mitochondrial preprotein translocases thus participate in the formation of large protein networks that link biogenesis, bioenergetics, metabolism and membrane dynamics of the organelle.

The physiology of microbe number one

Prof. Dr. William Martin
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Life is a chemical reaction. More specifically, life is an exergonic chemical reaction. What was the chemical reaction from which the first cells arose, and what was the chemical reaction that fuelled the first free-living cells? These are questions about chemistry and physiology, but molecular evolution can contribute. The last universal common ancestor (LUCA) is the assemblage of cells from which all life evolved roughly four billion years ago. Genomes and phylogeny have yielded new avenues to understanding early evolution and LUCA. We know LUCA had the universal genetic code shared by all descendant life forms. But how did LUCA harness energy? The chemical reactions that help cells harness energy from their environments today seem almost as diverse as life itself. Which forms of energy harnessing are ancient? We looked at that question using data from sequenced microbial genomes. We found that LUCA lived from gases — H₂, CO₂, H₂S, CO, N₂ — in a setting that looked very much like a modern submarine hydrothermal vent. The classical approach to investigate LUCA using genomes is to identify genes that are present in all modern cells hence present present in LUCA. We asked which genes trace to LUCA by phylogenetic criteria. The results indicate that the first forms of life were anaerobic chemoautotrophs that evolved from preexisting geochemical processes involving exergonic reactions of H₂, metals, and CO₂.
In photosynthesis sunlight is absorbed by the photosynthetic pigments, chlorophyll and carotenoid and the resulting excited state is stored as chemical energy. This energy conversion under optimal conditions occurs with a remarkable efficiency that scientists hope to mimic in bio-inspired solar energy converting devices, based on abundant elements. Two ultrafast (femtoseconds-picoseconds, $10^{-15}$-$10^{-12}$ sec) processes are at the basis of the success of photosynthesis: excitation energy transfer in a light-harvesting antenna followed by charge separation in the photosynthetic reaction center. In plants pigments involved in light-harvesting antenna and charge separation are bound to specialized proteins that are organized in a membrane, the thylakoid membrane. Plants make do with two reaction centers that operate in series, Photosystem 1 and Photosystem 2, the former is sufficiently reducing to reduce CO$_2$, the latter has the capacity to extract the necessary electrons from water and produce molecular oxygen. Upon absorption of light collective excitations (excitons) are formed that are delocalized over a number of pigments that move extremely rapidly through the light-harvesting antenna to the reaction center in such a way that the quantum coherence is maintained even during the final charge separation. Two-dimensional (2D) electronic spectroscopy is an ultrafast laser technique that allows a visualization of how these coherences are involved in the primary processes of energy and charge transfer. Based on quantitative modeling we identify the exciton-vibrational coherences observed in 2D photon echo of the photosystem II reaction center (PSII-RC). We find that the vibrations resonant with the exciton splittings can modify the delocalization of the exciton states and produce additional states, thus promoting directed energy transfer and allowing a switch between the two charge separation pathways. We conclude that the coincidence of the frequencies of the most intense vibrations with the splittings within the manifold of exciton and charge-transfer states in the PSII-RC is not occurring by chance, but reflects a fundamental principle of how energy conversion in photosynthesis was optimized.

For a recent review of this work see:

Photosynthetic reaction centers – Robustness with increased complexity
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The ability of photosynthetic organisms to use the sun’s light as a sole source of energy sustains life on our planet. Photosystems I (PSI) and II (PSII) are large, multi-subunit, pigment–protein complexes that enable photosynthesis, but this intriguing process remains to be explained fully. Plant PSI is one of the most intricate membrane complexes in Nature. It is comprised of two complexes, a reaction center and light-harvesting LHCI. An atomic-level structural model of higher plant PSI at 2.2 Å resolution has been constructed based on new crystal form. The structure includes 16 subunits and more than 200 prosthetic groups, the majority of which are light harvesting pigments.

Recently we solved the structure of trimeric PSI from *Synechocystis* at 2.5 Å resolution. Several differences between the mesophilic and thermophilic PSI were revealed and the position of lipids between the monomers was determined. The structure of green and red algae PSI was solved at intermediary resolution. The mechanistic and evolutionary implications will be discussed.

An operon encoding PSI was identified in cyanobacterial marine viruses. We generated a PSI that mimics the salient features of the viral complex containing PsaJ-F fusion subunit. The mutant is promiscuous for its electron donors and can accept electrons from respiratory cytochromes. We solved the structure of the PsaJ-F fusion mutant as well as a monomeric PSI with subunit composition similar to the viral PSI.

The perspective of robustness versus complexity of this highly important bioenergetic complex is discussed. From our studies, and several other laboratories, it has become apparent that advancements in evolution have resulted in increased complexity, and also increased sensitivity to structural alterations, without compromising on the robustness of the system.

A novel approach for sustainable photosynthetic hydrogen production will be discussed.
Mechanism of photosynthetic water-splitting catalyzed by the Mn₄CaO₅ metal cluster in photosystem II

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Photosystem II (PSII) consists of 20 subunits with a total molecular mass of 350 kDa, and catalyzes light-induced water-splitting leading to the evolution of molecular oxygen indispensable for aerobic life on the earth. We have solved the crystal structure of the dimeric PSII from a thermophilic cyanobacterium at a resolution of 1.9 Å [1] using synchrotron radiation (SR) X-rays, which revealed a clear picture of a Mn₄CaO₅-cluster, the catalytic center for water-splitting. In order to avoid possible radiation damages and eliminate the uncertainties in the inter-atomic distances, we used femtosecond X-ray pulses from an X-ray free electron laser (XFEL) facility SACLA, Japan, to solve the structure of PSII. We used a large number of “big” PSII crystals and a “fixed-target rotational method” to collect the X-ray diffraction data, which allowed us to solve the PSII structure at 1.95 Å resolution [2]. This structure showed shortening of some Mn-Mn distances, suggesting the successful determination of the radiation damage-free structure. From this structure, we confirmed the unique position of O₅, an oxo-bridged oxygen within the Mn₄CaO₅-cluster that has a longer distances to its nearby Mn ions, suggesting its possible involvement in the O=O bond formation. We further used a pump-probe approach with a combination of small PSII crystals and serial femtosecond crystallography (SFX) by XFEL to solve the structure of the S₃ intermediate-state induced by 2-flash illumination [3]. The results obtained showed some apparent structural changes at both electron donor and accept sides induced by the 2-flash illumination. Among these changes, the most important one is the insertion of a water molecule (oxygen atom) into a position close to O₅, indicating the site of O=O bond formation. Based on these results, the mechanism of photosynthetic water-splitting will be discussed.

References

Symposium lectures

1a. Quinone reductases

Tweaking the gearbox: the mechanism and regulation of mitochondrial complex I

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At ~ 1000 kDa, mitochondrial complex I is the largest multiprotein assembly of the respiratory chain comprising one FMN, eight iron-sulfur clusters and some 40 different subunits. Defects in human complex I have been implicated in multiple pathological processes. Complex I transfers two electrons from NADH to ubiquinone to pump four protons across the inner mitochondrial membrane. High-resolution structures of mitochondrial complex I obtained by our group and others, now provide important clues on the molecular mechanism and the regulation of complex I. The key steps of energy conversion by complex I are associated with ubiquinone reduction taking place in the peripheral arm of the L-shaped complex. Thus, ubiquinone chemistry plays a pivotal role in the catalytic cycle by providing the energy for vectorial proton transport at the four putative pump sites in the membrane domain. We have proposed that ubiquinone reduction drives concerted rearrangement of three loops in the active site, thereby triggering the power stroke driving the proton pumps. We show that arresting one of the loops by an engineered disulfide bond reversibly disengages proton pumping from ubiquinone reduction [2]. This clearly demonstrates that loop-mobility is critical for energy conversion and corroborates the hypothetical two-state stabilization change mechanism proposing that complex I alternates between two structurally
defined functional states during the stepwise reduction and pronation of ubiquinone thereby exerting strokes that are transmitted to the proton pump modules. The proposed mechanism also provides a thermodynamically feasible rationale for the reverse mode of the complex I and its regulation by the active/deactive transition.


Coupling mechanism of complex I
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NADH-ubiquinone oxidoreductase (complex I) is the first and largest enzyme in the respiratory chain of mitochondria and many bacteria. It couples electron transfer between NADH and ubiquinone to the translocation of four protons across the membrane. It is an L-shaped assembly formed by the membrane and hydrophilic arms. Mammalian mitochondrial complex I consists of 45 subunits of ~1 MDa, whilst the prokaryotic enzyme is simpler and consists of 14 conserved “core” subunits. We have determined first atomic structures of complex I using bacterial enzyme, starting with the hydrophilic domain, followed by the membrane domain and the structure of the entire *Thermus thermophilus* complex. Recently we solved the first nearly complete structure of the entire mammalian complex I by the latest cryo-EM methods at 3.9 Å resolution. We have also determined the architecture of the mammalian “respirasome”, a 1.7 MDa supercomplex containing complexes I, III, and IV. Our structures suggest a unique mechanism of coupling between electron transfer in the hydrophilic domain and proton translocation in the membrane domain of complex I via long-range (up to ~200 Å) conformational changes.

I will discuss our current work, which is aimed at elucidating the molecular details of the coupling mechanism through determination of structures of the complex in different redox states with various bound substrates/inhibitors.

Molecular mechanism of long-range proton-electron coupling in respiratory complex I
Prof. Dr. Ville R. I. Kaila
Department of Chemistry, Technical University Munich (TUM), Germany

Biological energy conversion is driven by remarkable proteins that capture and convert chemical and light energy into other energy forms. In this talk, I will describe our recent work on the redox-driven proton pump, the respiratory complex I, that reduces quinone to quinol and couples the free energy released to pumping protons across a biological membrane, up to ca. 200 Å away from the active site. We find that coupled electrostatics-, conformational-, and hydration changes are essential for the function of this remarkable enzyme [1-11], with distinct similarities to other biological energy-transducing proteins [5,10]. I will also describe how the dynamics and catalysis are modulated in complex I by the protein structure and surrounding membrane, and present a new putative molecular mechanism for long-range proton-electron coupling process.

References
Large scale atomistic simulations unveil key role of ubiquinone dynamics in proton pumping by mitochondrial complex I
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In mitochondria, ATP is synthesized by utilizing the established proton gradient across the inner mitochondrial membrane. Respiratory complex I, the first enzyme in the mitochondrial electron transport chain, contributes to proton electrochemical gradient by pumping protons across the membrane in tight coupling to the redox reactions of ubiquinone that take place at a maximal distance of ~200 Å. How redox energy of ubiquinone drives long-ranged proton pumping in complex I remains a major question in the field of bioenergetics. Earlier, by performing atomistic simulations of a large model of bacterial complex I (ca. 850000 atoms), we identified the role of protein and water dynamics in proton translocation, combined with local redox-coupled conformational transitions [1,2]. Our extended microseconds long molecular dynamics simulations of small-to-large model systems reveal ubiquinone dynamics as a central element in proton pumping by respiratory complex I. The new simulation results, combined with earlier data [1], are found to be in agreement with our recent mechanistic proposals [3].

References:

Superoxide: quinone oxidoreductase – a new player in the respiratory chain?
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Superoxide is a reactive oxygen species produced during aerobic metabolism in mitochondria and prokaryotes. It causes damage to lipids, proteins and DNA and is implicated in cancer, cardiovascular disease, neurodegenerative disorders and ageing. Soluble superoxide dismutases, that disproportionate superoxide to molecular oxygen and hydrogen peroxide, make up the universal biochemical defense against superoxide. In some anaerobic organisms an enzyme reducing superoxide to hydrogen peroxide only has also been identified. Strikingly, these ROS-generating mechanisms remain the only identified protection against superoxide in biology. Here, we define a previously undescribed biochemical reaction, catalyzed by the integral membrane protein CybB of Escherichia coli, in which superoxide is directly oxidized to molecular oxygen and the sequestered electron is funneled to ubiquinone in a diffusion-limited reaction, the protein thus functions as a superoxide:quinone oxidoreductase. Using in vitro experiments, we unravel the electron transport processes from superoxide to ubiquinone. Furthermore, we show that liposome embedded CybB efficiently quenches
superoxide generated at the membrane and reduces the amount of superoxide released from respiring membranes. We also present the 2.0-Å crystal structure of CybB, showing an integral membrane di-heme cytochrome poised for electron transfer from the periplasmic side and proton uptake from the cytoplasm. The electrogenic configuration of CybB suggests a mechanism, in which energy loss during superoxide detoxification is minimized by contributing to the electrochemical potential and reduction of the quinone pool, while avoiding generation of other reactive oxygen species like hydrogen peroxide.

Reference:

1b. Oxidative stress

Human 2-oxo acid dehydrogenase multienzyme complexes: experimental observation of the thiamin diphosphate-enamine radical species and its contribution to generation of superoxide and hydrogen peroxide *in vitro*

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We here review our recent findings on the human 2-oxoacid dehydrogenase complexes (hOADH) that have roles at key junctions in human metabolism and could contribute to oxidative stress in mitochondria. We have reported that the thiamin diphosphate (ThDP)-dependent E1 components of 2-oxoglutarate dehydrogenase complex (OGDHc) in the TCA cycle and 2-oxoadipate dehydrogenase complex (OADHc) on the L-lysine degradation pathway could provide ThDP-enamine radical intermediates leading to the concomitant formation of the reactive oxygen species superoxide and $H_2O_2$ in the forward physiological direction (direct electron flow). This pathway of reactive oxygen species formation joins the well-documented dihydrolipoyl dehydrogenase pathway (hE3 component with FAD bound), in the reverse direction not requiring substrate in mitochondria. With individually expressed components of the hOGDHc, 2-oxoglutarate dehydrogenase (hE1o), dihydrolipoyl succinyltransferase (hE2o), hE3 and 2-oxoadipate dehydrogenase (hE1a) we had an opportunity to investigate O$_2$-induced oxidation-reduction chemistry. The following could be concluded: (i) A stable ThDP-derived radical consistent with the C2(α-hydroxy)-α-carboxylpropylidene ThDP cation radical could be detected by electron paramagnetic resonance (EPR) spectroscopy on aerobic oxidation of 2-oxoglutarate (OG) by hE1o. (ii) Less than 1% of hE1o active centers were occupied by radical species on oxidation of OG with a half-life of approximately 1 min; ca. 0.2% of active center occupation was estimated for hE1a on oxidation of 2-oxoadipate (OA). No radical species were identified on human pyruvate dehydrogenase component from pyruvate, suggesting substrate specificity. (iii) Concurrently, with the radical formation, the OGDHc could produce $H_2O_2$ from both OG and OA. (iv) The hE1a was found to recruit the hE2o and hE3 components of the OGDHc for glutaryl-CoA, superoxide and $H_2O_2$ production from decarboxylation of OA. (v) The hE1o and hE1a experience dramatically different regulation: both succinyl-CoA and glutaryl-CoA significantly reduced the hE1o, but not the hE1a activity while assembled with hE2o and hE3 components. The studies led to the following mechanistic conclusions. (i) The stability of the ThDP-enamine radical derived from OG on hE1o and from OA on hE1a, compared with that derived from pyruvate (hE1p), is likely due to some stabilization afforded by the side chain C5 or α-carboxyl group (OG), or the C6 or α-carboxyl group (OA). (ii) The low occupancy of hE1o and hE1a active centers by the ThDP-enamine radical suggests that the "on-pathway" mechanism produces succinyl-CoA and glutaryl-CoA via 2-electron enamine oxidation (by an ionic mechanism) proposed earlier in the model reaction. (iii) The results suggest that both hOGDHc and hOADHc could produce reactive oxygen species perhaps of importance in human physiology and disease.

Site-specific ROS signalling during ageing

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Reactive Oxygen Species (ROS) have been extensively studied but still much is unknown about how they operate in vivo. ROS can cause oxidative damage, but they are also essential messengers that maintain cellular homeostasis. Accordingly, boosting ROS levels has been shown as an effective strategy to extend lifespan in few animal species. In the past, we have shown that expression of the alternative NADH dehydrogenase from *Saccharomyces cerevisiae* in *Drosophila melanogaster* stimulates generation of mitochondrial ROS via Reverse Electron Transport (ROS-RET) and extends *Drosophila’s* lifespan[1]. Here, I will show how CI can produces ROS both in forward and reverse direction in the fly brain. I will show the main metabolic changes that trigger the production of ROS-RET, and which proteomics changes are produced in response to changes in the way CI generates ROS. Finally, I will show that suppressing ROS-RET under stress conditions inhibits mitochondrial fission and decreases survival.

References

UCP3 regulation in response to oxidative stress and its role in cardioprotection
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UCP3 is a member of the mitochondrial uncoupling protein (UCP) family, which is expressed predominantly in skeletal muscle, but also in brown adipose tissue and heart. Its precise function is not clearly established. Physiological increases in UCP3 expression facilitate fatty acid oxidation, suggesting a role in lipid metabolism. Growing experimental evidence indicates that UCP3 protect against excessive mitochondrial superoxide generation and oxidative stress. Superoxide and the lipid peroxidation product 4-hydroxynonenal (HNE) activate UCp3s, leading to lowered protonmotive force and decreased superoxide production. In addition, hydrogen peroxide and HNE induce UCP3 expression [1]. Furthermore, UCP3 activity is regulated by glutathionylation. Cardiac ischemia-reperfusion (IR) injury, a condition known to increase reactive oxygen species production, occurs when the blood supply to the heart is blocked and then restored. Results from other groups and our own group indicate that UCP3 protects against cardiac IR injury and is involved in the protective phenomenon of ischemic preconditioning (IPC) [2]. We have found that isolated perfused hearts from UCP3 knockout mice have larger infarct size after IR than wild-type littermates, and that protection by IPC is lost in these mice. Similarly, UCP3 knockout mice have increased infarct area following left anterior descending artery occlusion compared with wild-type mice. How UCP3 protects the heart, however, is presently unclear. We are currently working to better understand the nature of this protection.


E3-deficiency by the pathogenic mutations of the human dihydrolipoamide dehydrogenase: elucidation of the molecular pathomechanism by a multifaceted structural approach
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The α-ketoglutarate dehydrogenase complex (KGDHc) represents a rate-limiting step in the Krebs cycle catalyzing the oxidative decarboxylation of α-ketoglutarate while generating succinyl-CoA and NADH. The KGDHc is one of the major generators of oxidative stress in the mitochondrion under pathological conditions; malfunctioning of and reactive oxygen species (ROS) generation by KGDHc are implicated in the progression of senescence/aging, neurodegenerative diseases, ischemia-reperfusion, hypoxia- and glutamate-induced cerebral damage, E3-deficiency, among others. ROS generation by KGDHc is attributed to the homodimeric flavoenzyme E3 component, which is also part of the pyruvate dehydrogenase complex (PDHc) and a few other dehydrogenase complexes. Pathogenic mutations of hE3 (h for human) lead to an inherited, often lethal disease known as E3-deficiency, the clinical course of E3-deficiency is greatly diversified and often involves cardiological and/or neurological symptoms. Selected pathogenic mutations of hE3 stimulate the ROS generation by hE3 and hKGDHc or impair the recruitment of hE3 to the harboring complexes, which are likely to be important
factors in the respective molecular pathogenesis. Structural alterations of the 14 pathogenic variants of hE3 and their role in the respective molecular pathogenesis have been investigated in our laboratory by a multifaceted structural approach, which involves CD spectroscopy, MD simulation, H/D-exchange mass spectrometry, electron microscopy, NMR spectroscopy, and X-ray crystallography. The biophysical approaches are accompanied by biochemical assays for assessing i., FAD-content, ii., ROS-generating capacities and enzymatic activities in the forward or the reverse catalytic directions as a function of pH, in the presence of affectors, and using E3 reconstructed with other recombinant hPDHc or hKGDHc components (E1, E2), and iii., monomerization via calibrated size-exclusion chromatography coupled to nano-LC MS.

Mitochondrial Reactive Oxygen Species Production and Oxygen Level: Linear Dependence or Not?
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Reactive oxygen species (ROS) are by-products of aerobic mitochondrial metabolism that are involved in both physiological cellular signaling pathways and pathophysiological processes of tissue damage including brain ischemia-reperfusion injury. The mitochondrial respiratory chain is considered a major source of ROS; however, there is little agreement on how ROS production depends on oxygen concentration ([O2]). We measured H2O2 release by intact mouse brain mitochondria during oxidation of different substrates at various oxygen concentrations. We found the highest rate of H2O2 release occurs under conditions of reverse electron transfer, when mitochondria oxidize succinate or glycerol 3-phosphate. For the first time, we determined that H2O2 release by respiring mitochondria depends linearly on oxygen concentration in physiological range (>200µM [O2]) during both reverse and forward electron transfer and with any substrate used. We found that Complex III is not the major contributor to ROS generation under physiological conditions. H2O2 production by complex III is significant only in the presence of antimycin A, and in this case the oxygen dependence manifested mixed (linear and hyperbolic) kinetics. We also demonstrated that when the quinone pool is reduced, Complex II can significantly contribute to H2O2 production even in the absence of its substrate succinate.

Our results underscore the critical importance of reverse electron transfer in the brain, where a significant amount of succinate can be accumulated during ischemia and provide backflow of electrons upstream to complex I at the early stages of reperfusion. Our study demonstrates a linear dependence of mitochondrial H2O2 release on oxygen concentration in the absence of inhibitors, indicating that ROS production in brain mitochondria under hypoxia is lower than in normoxia.

2a. Regulation of respiratory chain, from subunits to supercomplexes

The protonmotive force under pressure: an isomorphic analysis
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‘... the sum of the electrical pressure difference and the osmotic pressure difference (i.e. the electrochemical potential difference) of protons’
[1] links to non-ohmic flux-force relationships between proton leak and protonmotive force (pmf). This is experimentally established, has direct consequences on mitochondrial physiology, but is theoretically little understood. Here I distinguish pressure from potential differences (diffusion: ∆µH+ or ∆dFpH+; electric: ∆Ψ or ∆elFpH+), to explain non-ohmic flux-force relationships on the basis of four thermodynamic theorems. (1) Einstein’s diffusion equation explains the concentration gradient (dc/dz) in Fick’s law as the product of chemical potential gradient (the vector force and resistance determine the velocity, v, of a particle) and local concentration, c. This yields the chemical pressure gradient (van’t Hoff equation): dc/dz = RT dc/dz. Flux is the product of ν and c; c varies with force. Therefore, flux-force relationships are non-linear. (2) The pmf is not a vector force; the gradient is replaced by a pressure difference, and local concentration by a distribution function or free activity, α. Flux is a function of α and force, Jα = -b·α·ΔFα = -b·ΔαFα. (3) At ∆elFpH = -ΔdFpH, the diffusion pressure of protons, ∆dFpH = RT·ΔµH+ [Pa J m-2] is balanced by electric pressure, maintained by counterions of H+.
Diffusional and electric pressures are isomorphic, additive, and yield protonmotive pressure (pmp). (4) The dependence of proton leak on pmf varies with $\Delta F$ versus $\Delta F_H^+$, in agreement with experimental evidence. The flux-force relationship is concave at high mitochondrial volume fractions, but near-exponential at small mt-matrix volume ratios. Linear flux-pmp relationships imply a near-exponential dependence of the proton leak on the pmf.

Reference:

Regulatory Interactions within the Respiratory Chain
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The respiratory supercomplex factor 1 (Rcf 1) in S. cerevisiae is an assembly factor of cytochrome c oxidase (CytO) [1, 2], but it also binds to and regulates the activity of the enzyme [3]. In addition, Rcf1 shifts the equilibrium of membrane-bound cyt. bc, and CytO toward formation of cyt. bc-CytO supercomplexes. Furthermore, our data show that Rcf1 binds cyt. c [4, 5], in agreement with data showing stoichiometric binding of cyt. c to CytO in the mitochondrial membrane [6].

In the absence of Rcf1 there are two main CytO populations: (i) one that is incorrectly assembled and harbors hemes with altered absorbance spectra and, (ii) one that is presumably correctly assembled and that could be purified using affinity chromatography, but displayed a lower O2-reduction activity due to structural changes at the heme-copper catalytic site (Schäfer et al. Sci. Rep. in press).

The O2-reduction activity of CytO purified from the strain with genetically removed Rcf1 could be increased to that of the wild-type CytO upon liposome co-reconstitution of the enzyme with Rcf1 protein expressed in E. coli (Nilsson, Schäfer, Zhou et al., in preparation).

Collectively, the data indicate that in the cyt. bc-CytO supercomplex, binding of cyt. c to Rcf1 may establish an electron-transfer pathway between the two complexes, as is observed in supercomplexes from bacteria that lack a water-soluble cyt. c [7]. Furthermore, the reversible binding of Rcf1 to CytO may reflect a mechanism to modulate the CytO activity thereby controlling energy conservation of the respiratory chain.


3D super-resolution microscopy reflects mitochondrial cristae alternations and mtDNA nucleoid size and distribution
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The 3D immunocytochemistry imaging by 3D direct stochastic optical reconstruction microscopy (dSTORM) possesses an advantage in the ability to resolve structures and protein proximities much smaller than the diameter of the mitochondrial network tubules (300–400 nm). We have elaborated novel 3D data analysis, using the Ripley’s K-function modeling spatial patterns and transferring them into histograms of distance distribution function. The histograms provide most frequent distances (MFD) between the localized single antibody molecules and may reveal some repeating features, such as yet unexplored entities of mitochondrion, mitochondrial cristae and nucleoids (the confined sites of mitochondrial DNA – mtDNA - in complex with proteins [2]). Using transmission electron microscopy, we have found that upon glucose-stimulated insulin secretion (GSIS) in model pancreatic βcells, INS-1E cells, cristae are narrowed. The average 20 nm and 15 nm cristae width at “fasting” 0 and 3 mM glucose decreased to 9 nm or 8 nm at 11 or 20 mM
As we know, the mitochondrial OXPHOS systems is compartmented in cristae. Cristae apparently are further divided into the rim compartment, occupied by strings of ATP synthase dimers, and sheets, where the respiratory complexes are found. The respiratory complexes can further assemble into supercomplexes, which is likely a plastic and reversible process. What we know little is how defined this distribution of OXPHOS complexes is, or, rephrased, how mobile single OXPHOS complexes are in the membrane. We addressed this important question by super-resolution microscopy using a modified TIRF illumination and self-labeling tags at diverse OXPHOS proteins. By localizing and tracking single OXPHOS proteins, we dissect mobility patterns and reveal the subcompartmental localization. First, we found that OXPHOS complexes are mobile, in particular, and most unexpectedly, the F1FO ATP synthase [1–4]. Second, the movement is mostly but not exclusively confined to cristae compartments. Third, the spatio-temporal organization alters in dependence on metabolic supply. These observations shed new light on the apparent sub-compartmentation of mitochondrial OXPHOS complexes.


Subunit composition of the membrane energy-transducing complexes in diverse bacteria and archaea
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The latest update of the Clusters of Orthologous Genes (COG) database (https://www.ncbi.nlm.nih.gov/COG/) covers complete genomes of 628 bacteria and 83 archaea, representing 693 distinct prokaryotic genera. COG phylogenetic profiles graphically display the presence or absence of members of that COG in every covered genome. Grouping these phylogenetic profiles by phylogeny provides an easy way to judge which microbial groups encode – or do not encode – the given protein. Individual components of a multi-subunit enzyme complex can be expected to have the same phylogenetic profiles. Thus, non-uniform phylogenetic patterns for components of the same multi-subunit complex may be indicative of gene acquisition and loss in the course of evolution. Comparison of the phylogenetic patterns and operon organization for individual subunits of F0F1-ATPase, A/V-type ATPases, H+- and Na+-translocating NADH oxidoreductases, and other membrane energy-transducing complexes uncovered an unexpected diversity of these enzymes in bacteria and archaea. These findings include, among others, (i) the absence of membrane rotary ATPases in several intracellular symbionts; (ii) widespread distribution of A/V-type ATPases among diverse bacterial phyla; (iii) poor conservation of the C, F and G/H subunits in crenarchaeal and many bacterial A/V-type ATPase operons; (iv) frequent loss of the ndh operons in bacteria; and (v) widespread presence of Na+-pumps and A/V-type ATPases among various bacterial pathogens. These findings reveal rampant lateral
gene transfer of the ATPase operons and question the existing paradigm on the origin and evolution of membrane energy-transducing complexes. The exclusive presence of the Na⁺-translocating ion pumps and A+V-type ATPases in some important bacterial pathogens, including chlamydia, spirochetes and streptococci, suggests that these enzymes could serve as targets for a new generation of antibacterial compounds.

The atypical subunit composition of oxidative phosphorylation complexes is associated with original extra structural domains in *Euglena gracilis*

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In mitochondrial oxidative phosphorylation, electron transfer from NADH or succinate to oxygen by a series of large protein complexes in the inner mitochondrial membrane (complexes I - IV) is coupled to the generation of an electrochemical proton gradient, the energy of which is utilized by FₐFₒ ATP synthase (complex V) to generate ATP. In *Euglena gracilis*, a non-parasitic secondary green alga related to trypanosomes, these respiratory complexes totalize more than 40 Euglenozoa-specific subunits along with about 50 classical subunits described in other eukaryotes [1]. Here mitochondrial complexes I, III, IV and V were purified by a two-step chromatographic procedure and their subunit compositions were resolved by a three-dimensional gel electrophoresis (BN/SDS/SDS). The purified complexes were also studied by electron microscopy followed by single-particle analysis. Even if the overall structures of the four complexes are similar to the structure of canonical enzymes (e.g. from mammals), an extra domain located at the tip of the peripheral arm of complex I and a "helmet-like" domain on the top of the cytochrome c binding region in complex IV were observed. In the case of complex V, new features include a large membrane-spanning region joining the monomers, an external peripheral stalk and a structure that goes through the membrane and reaches the inter membrane space below the c-ring, the latter having not been reported for any mitochondrial F-ATPase [2, 3].

References:

Control of UCP1 activity in brown-fat mitochondria
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Brown adipose tissue (BAT) mitochondria contain uncoupling protein 1 (UCP1), the activity of which is rate-limiting for nonshivering thermogenesis. UCP1 is inhibited by GDP experimentally and by cytosolic ATP within brown-fat cells. Fatty acids are able to overcome this inhibition and re-activate UCP1. A kinetic analysis of UCP1-dependent thermogenesis indicates that the interaction between fatty acids and GDP can be fully described as being competitive. We have found that a wide range of different structures, only vaguely resembling fatty acids, are able to activate UCP1. The limitation for fatty acid chain length is 8-10 carbonyl groups. Increased unsaturation does not influence activation. Fatty aldehydes (4-hydroxy-2-nonenal and trans-2-nonenal) are not active; therefore, the
carboxyl group of the activators is absolutely required for their function. Introduction of several carboxyl groups, as in \( \beta,\beta' \)-methyl-substituted \( \alpha,\omega \)-dioic fatty acids (MEDICA 16), does not significantly diminish their activity. Noticeably, even flip-flop-incapable fatty acids (12-hydroxylation, dodecanedicarboxylic, benzenoheptanoic acid) demonstrate UCP1-dependent function as thermogenic (re)activators. Substitution of hydrogen by fluorine in perfluorooctanoic acid improves the UCP1 (re)activation ability as compared with octanoic acid. Also, other structures, not displaying much similarity to aliphatic fatty acids, can activate UCP1. Until recently, the widely accepted view was that UCP1 activity is strongly correlated with UCP1 content. However, overexpression of CIDEA led to inhibition of UCP1 activity, despite preserved content. We observed as expected a similar phenomenon in respiratory chain-deficient BAT mitochondria from mtDNA mutator mice. It is currently unclear whether the activity of UCP1 is decreased due to modifications of the UCP1 molecule itself, or to alterations in the environment of the UCP1 molecule.

2b. Mitochondrial physiology, pathology

Coenzyme Q10 depletion induces endogenous hypoxia in cultured cells

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Coenzyme Q biosynthesis is a complex process occurring in both the cytosol and the mitochondrial matrix. The cytosolic pathway is shared with cholesterol biosynthesis through the mevalonate pathway, while the biosynthesis of the benzoquinone ring starts from \( p \)-hydroxybenzoic acid (4-HB), derived from tyrosine. A crucial step in the CoQ assembly is the insertion of the isoprenyl chain in the aromatic ring of 4-HB catalysed by 4-para-hydroxybenzoate: polyprenyl transferase (Coq2). We used 4-nitrobenzoic acid (4-NB) as a competitive inhibitor of Coq2 to induce CoQ depletion [1]. We found that CoQ depleted cells showed increased cholesterol levels, increased HIF-1\( \alpha \) levels and a reduced intracellular oxygen tension in addition to the well-known effects associated with CoQ depletion (low respiratory capacity, low ATP content and high ROS production). The analysis of the chemical-physical state of the cellular membranes showed an increased membrane rigidity that, in our opinion, is responsible for the reduced oxygen uptake. Moreover, we found increased cytosolic NADH levels that contribute to the stabilization of the hypoxic factor HIF-1\( \alpha \), which is responsible for the rearrangement of cellular metabolic status that cannot be completely restored by CoQ10 supplementation. These findings could be relevant for clinical interest suggesting an explanation for the lack of effectiveness of CoQ10 supplementation therapy observed in a number of patients affected by primary CoQ10 deficiency syndrome. Moreover, our data provide new insights on the effect of CoQ10 depletion in cells and sheds light on the mechanisms that underlie CoQ10 deficiency syndrome pathogenesis.


Knockout of DAPIT protein disrupts ATP synthase oligomerisation and has a profound role in regulation of glucose homeostasis

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FoF1-ATP synthase is the key enzyme of mitochondrial energy provision, responsible for production of most of the cellular ATP. Recently, DAPIT protein (Diabetes Associated Protein in Insulin sensitive Tissues), has been found associated with the enzyme, but its
biological role is largely enigmatic. To elucidate the importance of this novel protein we produced rat knockout of DAPIT on unique SHR background. DAPIT-/- animals were viable and possessed normal levels of fully assembled ATP synthase. However, this was predominantly present in the monomeric form. Contrary to proposed role of ATP synthase dimers in mitochondrial cristae formation, we observed only minor changes in cristae morphology in heart of DAPIT-/- animals as well as in HEK293 DAPIT knockdown model. We observed mild isolated ATP synthase deficiency in DAPIT-/- animals, with both ADP phosphorylating and ATP hydrolysing activities reduced by ≈10% in studied tissues, i.e. liver and heart. DAPIT-/- animals had 20-30% lower body weight and pronounced decrease in total adiposity (by 40%). Based on indirect calorimetry, DAPIT-/- animals preferred utilisation of glucose to other substrates. This was replicated at the tissue level, with higher glucose oxidation in DAPIT-/- skeletal muscle. Serum levels of glucose were unchanged, but DAPIT-/- animals were significantly more insulin sensitive with decreased levels of serum insulin as well as area under curve in OGTT test. This is due to the improved peripheral insulin sensitivity, as glucose-stimulated insulin secretion from pancreatic islets was normal in DAPIT-/- animals. High fat diet led to further dissociation of phenotype between control and knockout animals.

In conclusion, absence of DAPIT protein leads towards preferential oxidation of glucose, increases insulin sensitivity and decreases total adiposity in rat. It implicates that mitochondrial ATP synthase can be directly involved in regulation of glucose homeostasis.

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Mitochondrial uncoupling protein 3 is a biomarker of heart development and fatty acid β-oxidation metabolism

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The importance of mitochondria in the pathogenesis of cardiovascular diseases is widely acknowledged, however, particular mechanisms are still far from being understood. In this study we, for the first time, reveal the connection between mitochondrial uncoupling proteins 2 and 3 [1] as well as the predominant type of metabolism in cardiomyocytes at different stages of heart development and stem cell differentiation. The results are important for stem cell-based treatment of heart diseases, because they demonstrate that the electrical activity of cardiomyocytes derived from embryonic stem cells may not be sufficient for their successful employment for damaged tissue substitution. We suggest that the expression ratio between UCP2 and UCP3 indicates the metabolism type in the heart and may be an important diagnostic criterion for the characterisation of the degree of cardiomyocyte differentiation and/or severity of heart failure.

REFERENCES

Cyclophilin D isomerase activity controls FoF1-ATP synthase oligomerization and mitochondrial ultrastructure

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Permeability transition (PT) is a key event leading to mitochondrial swelling, bioenergetic collapse and the release of low molecular weight molecules [2]. Despite its relevance for cell death, its full molecular identity remains unknown [2]. Departing from the current view...
of F₀F₁-ATP synthase as core component of PT [5], we characterized the role of its only accepted activator cyclophylin D (CyPD), a matrix isomerase [3] known to interact with F₀F₁-ATP synthase [1], on mitochondrial ultrastructure. We show that CyPD isomerase activity defines F₀F₁-ATP synthase oligomerization, mitochondrial bioenergetics and PT occurrence upon Ca²⁺ overload. Notably, CyPD impinges on F₀F₁-ATP synthase conformation to shape the cristae, in line with the occurrence of PT as an inner mitochondrial membrane event. Our results place CyPD as a physiological regulator of F₀F₁-ATP synthase conformation and bioenergetics and present its cristae remodeling activity as a prerequisite for PT [4].

References

Pore formation by yeast mitochondrial ATP synthase involves subunits e, g and b

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The permeability transition pore (PTP) is an unselective, Ca²⁺-dependent high conductance channel of the inner mitochondrial membrane whose molecular identity has long remained a mystery. A recent hypothesis is that F-ATP synthase forms the pore, since it consistently generates Ca²⁺-activated channels in mitochondria from bovine hearts, human cells, yeast and drosophila. However, how it can create a channel and which are core proteins is still a matter of debate. In yeast, null mutant for the F-ATP synthase e and g subunits show desensitization of pore opening to Ca²⁺ [1]. Here, we show that genetic ablation of these subunits decreases channel conductance about tenfold and nearly abolishes PTP-dependent swelling. As reported in the recent Fo structure, subunits e and g create a distinct lateral domain together with the first transmembrane (TM) α-helix of subunit b, a key component of the lateral stalk [2]. Interestingly, ablation of the TM α-helix in the e/g null background further affected channel activity, indicating that the lateral domain is a prime candidate for pore formation. Subunits e and g are only present in eukaryotes and may have evolved to confer this novel channel function to F-ATP synthase.

References:

A Role for Mitochondrial Uncoupling Protein 3 in CD4+ T Cell Function

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Uncoupling proteins (UCPs) are members of the mitochondrial anion carrier superfamily that can mediate the transfer of protons into the mitochondrial matrix from the intermembrane space. Our laboratory has previously reported evidence of UCP3 expression in thymocytes. Thymocytes develop into mature T cells before exiting the thymus and travelling to the periphery where they wait to carry out an immune response. Here, we demonstrate that Ucp3 is expressed in peripheral naïve CD4⁺ T cells at the mRNA level before being markedly downregulated following cell activation. Ucp3⁻⁻ non-polarized, activated T cells (T naïve cells) produce significantly more IL-2, which is reflected by an increase in CD25 and CD69 expression as well as an increase in cell proliferation. However, it is also followed by a decrease in cell viability and IFN-γ production 72 h post-stimulation. It is thought that the increased IL-2 levels observed in Ucp3⁻⁻ T naïve cells are promoting early T cell activation but also subsequently inducing activation-induced cell death (AICD). The altered IL-2 expression observed between Ucp3⁺⁺ and Ucp3⁻⁻ T naïve cells also appears to have a crucial impact on the generation of T r17 and regulatory T (T naïve) cells in vitro. T r17 cells are a subset of T cells that are highly pro-inflammatory, while T naïve cells act to suppress the immune response. An increased frequency of FoxP3⁺ T naïve cells is generated in vitro from Ucp3⁻⁻ mice compared to Ucp3⁺⁺ mice, while Ucp3⁻⁻ T r17 cells generated in vitro display lower cell survival/viability and decreased IL-17A production compared to their Ucp3⁺⁺ counterparts. We postulate that UCP3 is acting to restrict the activation of naïve T cells. UCP3 may be acting as a rheostat to dampen signals following T cell receptor and CD28 co-receptor ligation, thereby preventing early activation and AICD. That Ucp3 ablation alters the T r17:T naïve cell balance implicates UCP3 as a potential target for the treatment of some forms of autoimmunity.

Tissue- and substrate-specific patterns in the oxygen kinetics of mitochondrial respiration

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In most tissues mitochondria (mt) respire in a low oxygen (O₂) environment, where intracellular partial O₂ pressures (pO₂) may exert control over OXPHOS. It is well established that the affinity of cytochrome c oxidase (CIV) for O₂ decreases with increasing enzyme turnover [1] and that mt pO₂ (pO₂ at half-maximum O₂ flux, J(O₂)) is a function of coupling and J(O₂) [2]. In our study of tissue-specific O₂ kinetics, we investigated the influence of pathway and coupling control on mt pO₂ with various fuel substrates in OXPHOS-, LEAK- and ET-states in mt isolated from mouse brain, heart and liver. Isolated mt were incubated in Oroboros O2k High-Resolution FluORespirometers. Kinetic data was obtained during aerobic-anaerobic transitions with high time-resolution. pO₂ values were calculated using the O2kinetics software for automatic calibration and correction of O₂ signals, data processing and curve fitting. pO₂ ranged from 0.006 to 0.07 kPa for NADH-linked LEAK respiration with glutamate&malate (GM), and NADH-&succinate-linked OXPHOS capacity with GM and pyruvate, in agreement with and extending the literature. pO₂ increased with an increase from 25 °C to 37 °C. In heart and liver, pO₂ was higher in OXPHOS- than in LEAK-states, increasing proportionally with CIV turnover. Surprisingly, however, brain mt did not follow this kinetic pattern in S-linked coupling control states, irrespective of rotenone addition, with pO₂ values in LEAK up to 2-times higher than in OXPHOS, despite a 3-4 fold decline of J(O₂). Further studies are underway to elucidate the underlying mechanisms, and to address the question if mouse brain is an exception or representative of a general pattern.

We have developed a new mitochondrial function assay technology that measures the rates of metabolism of mitochondrial substrates and the sensitivity of metabolism of these substrates to mitochondrial inhibitors. The technology employs saponin permeabilized cells and a redox dye added to 96-well microplates that contain mitochondrial substrates or inhibitors precoated and dried into the wells. The MitoPlate S-1™ has a triplicate repeat of a set of 31 substrates. Mitochondrial function is assayed by measuring the rates of dye reduction from electrons flowing into and through the electron transport chain from substrates whose oxidation produces NADH (e.g., L-malate) or FADH₂ (e.g., succinate). The electrons donated to complex 1 or complex 2 travel to the distal portion of the electron transport chain where a tetrazolium redox dye (MC) acts as a terminal electron acceptor and changes from colorless to a purple formazan upon reduction. All 96 assays in the MitoPlate are run concurrently, and each assay provides different information because each substrate follows a different metabolic route using different transporters to enter the mitochondria, and then different dehydrogenases to produce NADH or FADH₂. The MitoPlate S-1™ can also be used to assess the activity and specificity of substrate transport inhibitors, dehydrogenase inhibitors, or electron transport chain inhibitors. A second assay plate, the MitoPlate I-1™, provides another assessment of mitochondrial function by measuring the sensitivity of mitochondrial electron flow to a set of 22 diverse inhibitors titrated at 4 dilutions. The I-1 plates can be run using any of the NADH or FADH₂ producing substrates, each providing additional information. Using these new assays we show that the mitochondria from different cell types exhibit different functional properties. This new technology will assist efforts to understand how mitochondria change in cell models of human disorders that have a mitochondrial basis.

3a. Quinol oxidases and terminal oxidases

Two-electron and two-proton quinone redox reactions are integral part of operation of complexes I-III of mitochondrial electron transport chain. Typically, these reactions are step-wise and involve semiquinone radical intermediates, thus analysis of spectroscopic properties of these radicals is expected to provide insights into the mechanism of catalytic reactions. Here, we reflect on complex III (cytochrome bc₁) and new spectroscopic analyses of semiquinones in both the quinol oxidation and quione reduction sites (Qo and Qi sites, respectively).

In the Qo site, a metastable state nonreactive with oxygen is formed during enzymatic turnover [1]. In this state, a semiquinone intermediate interacts with a reduced Rieske cluster via spin-spin exchange. This state was also detected in photosynthetic cytochrome bc₁/b₆f complexes [2]. We propose that this state safely holds electrons at a local energetic minimum becoming possible element of regulation of ROS production by cytochrome bc₁/b₆f complexes [2].

In the Qi site, two populations of semiquinone differing in spin lattice relaxation properties are present [3]. The fast-relaxing semiquinone corresponds to the form that is magnetically coupled with oxidized heme b₅, while the slow-relaxing semiquinone reflects the form present along with reduced (and diamagnetic) heme b₅. The two populations of SQ reflect possible electron equilibration within the four hemes b and the two Q sites of the dimer [4] and also call for reinvestigation of thermodynamic properties of this semiquinone. Properties of newly reported semiquinones at the Qo and the Qi sites offer new perspective for understanding mechanism of regulation of electron flow through cofactor chains of cytochrome bc₁.

The mitochondrial cytochrome bc₁ complex (cyt bc₁) is essential for oxidative phosphorylation, the most efficient energy conversion machinery of eukaryotic organisms. The development of cyt bc₁ inhibitors is of interest for clinical and agricultural applications. Cyt bc₁ catalyzes ubiquinol-dependent cytochrome c reduction via the Q cycle mechanism. It has two catalytic centers, the Qo site at which ubiquinol is oxidized, and the Qi site at which ubiquinone is reduced. During the enzymatic forward reaction, electrons are transferred from the Qo to the Qi site. Blocking either Qo or Qi site inhibits the enzyme activity efficiently and cyt bc₁ loses its function in energy conversion. Qo site inhibitors are widely applied as therapeutics and agrochemicals and are well characterized. For instance, our structural analysis of atovaquone-inhibited cyt bc₁ revealed the molecular basis of antimalarial drug action of atovaquone, which is in clinical use [1]. In contrast, Qi site inhibitors are less well-known, but are currently attracting the attention of developers as there is an urgent need in combating resistance against Qo site inhibitors. We determined the X-ray structure of cyt bc₁ from Saccharomyces cerevisiae with the bound Qi site inhibitor ilicicolin H at high resolution. The structural analysis provided a detailed characterization of the binding mode of the inhibitor. Comparison with a high-resolution structure of the same complex with the Qi site inhibitor antimycin A highlighted the unique features of the ilicicolin binding mode. A comprehensive sequence analysis of cytochrome b, the subunit harboring the catalytic sites of cyt bc₁, suggests that Q-site inhibitors have a high tendency for species-specific inhibitory action.

Reference:

Cytochrome bd in mycobacteria
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Targeting respiration and ATP synthesis has received strong interest as a new strategy for combatting drug-resistant Mycobacterium tuberculosis [1]. Previously we have shown that that genetic inactivation of mycobacterial cytochrome bd enhances the susceptibility to hydrogen peroxide and antibiotic-induced stress [2]. Using an mCherry-based fluorescent reporter we here detect stress-induced expression of mycobacterial cytochrome bd in vitro and in vivo infection models [3]. We also show that inhibition of cytochrome bd by aurachin D invoked bactericidal activity of the cytochrome bcc inhibitor Q203, a drug which otherwise is bacteriostatic [4]. In biochemical assays using inverted membrane vesicles from Mycobacterium tuberculosis and Mycobacterium smegmatis we found that inhibition of respiratory chain activity by Q203 was incomplete, but could be enhanced by aurachin D. These results indicate that simultaneously targeting the cytochrome bcc and the cytochrome bd branch of the respiratory chain may turn out as effective strategy for combating M. tuberculosis.

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SuperComplexes Assembly Factor 1 (SCAF1) shapes metabolism
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Respiratory chain complexes can superassemble into quaternary structures called supercomplexes that optimize cellular metabolism. The interaction between complexes III (CIII) and IV (CIV) is modulated by supercomplex assembly factor 1 (SCAF1, also known as COX7A2L). This protein is mutated in C57BL/6 mouse strains (with the loss of two amino acids) rendering it inactive. We demonstrated by combining deep proteomics and immunodetection analysis, that SCAF1 is always required for the interaction between CIII and CIV. In order to study the physiological role of SCAF1 we created: (i) purebred C57BL/6J mice harbouring SCAF1+/−, (ii) C57BL/6J mice SCAF1−/− and (iii) zebrafish SCAF1−/−.

The lack of SCAF1−/− modifies the efficiency of the OXPHOS system promoting conspicuous physiological differences both in mice and zebrafish models.

3b. Frontiers in mitochondrial research

Selective segregation of mitochondria by stem cells
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Cell fate changes in adult stem cells coincide with profound metabolic rewiring. However, how metabolism influences fate determination remains unclear. We have studied the selective inheritance of organelles in the specific case of asymmetric cell division, and previously found that the daughter cell becoming the new stem cell inherits chronologically newer mitochondria than the daughter cell that differentiates. In ongoing studies, we have discovered that mitochondrial age-classes are proteomically and functionally distinct, and guide daughter cell fate immediately upon asymmetric divisions. While chronologically old mitochondria support oxidative respiration, new organelles are immature and metabolically less active. Upon cell division, selectively segregated mitochondrial age-classes elicit a metabolic bias in progeny cells, with old mitochondria imposing oxidative energy metabolism inducing differentiation. High pentose phosphate pathway flux, promoting redox maintenance, is favored in cells receiving newly synthesized mitochondria, and is required to maintain stemness during early fate determination after division. Our results demonstrate that fate decisions are susceptible to intrinsic metabolic bias imposed by selectively inherited mitochondria.

Critical role of mitophagy in inflammasome activation and hepatocarcinogenesis

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Accumulating evidence has proved that mitochondrial metabolism and functions are closely linked with tumor initiation and progression. Mitophagy, a selective process that removes damaged or unwanted mitochondria, was suggested to play a role in mitochondrial quality control and metabolic reprogramming. Previously, we have revealed that FUNDC1, a mitochondrial outer-membrane protein, functions as a mitophagy receptor to mediate hypoxia-induced mitophagy. FUNDC1 harbors an LC-3–interacting region (LIR) and interacts with LC-3 to mediate mitophagy both in cultured cell systems and in (patho-)physiological settings. Here we discover that enhanced expression of FUNDC1 protects against diethylnitrosamine (DEN)-induced HCC, whereas specific knockout of FUNDC1 in hepatocytes promotes HCC initiation and progression. Hepatocyte-specific FUNDC1 ablation results in the accumulation of damaged mitochondria and elicits a cascade of events involving inflammasome activation, leading to hyperproliferation of hepatocytes and promotion of hepatocellular carcinoma. Our results uncover the critical role of FUNDC1-mediated mitophagy in inflammasome activation and hepatocarcinogenesis.

Co-regulation of mitochondrial and cytosolic translation programs

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Oxidative phosphorylation complexes pose a unique challenge for the cell, because their subunits are encoded on the nuclear genome and on the mitochondrial genome. Recently we showed that the mitochondrial and nuclear transcription programs are not coordinated during mitochondrial biogenesis in S. cerevisiae. Rather there are synchronized translation programs across compartments. In human cells, it remains unclear whether translation programs will be coordinated due to the vast differences between yeast and human mitochondrial gene expression, especially with respect to translation regulation. To investigate mitochondrial translation in human cells, we optimized ribosome profiling to robustly capture the unique human mitoribosome. Our data reveal the dynamics of human mitochondrial translation, shedding light on mitochondrial translation initiation and the precision of mitochondrial translation termination. Experiments are ongoing to determine whether mitochondrial and cytosolic translation programs are co-regulated in human cells.

Mitochondria and Adaptations in Hibernation

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Hibernating mammals survive profound hypothermia and metabolic suppression without cellular injury, a remarkable biological phenomenon with potential medical applications. We study adaptive mechanisms, such as mitochondrial modulations, that promote cell/tissue survival and function during hibernation. Comparison between human and ground squirrel iPSC-derived neurons revealed
differential mitochondrial responses to cold. In human iPSC-neurons cold triggered mitochondrial stress, resulting in reactive oxygen species overproduction and lysosomal membrane permeabilization, contributing to microtubule destruction. Manipulations of these pathways endowed microtubule stability upon human iPSC-neurons and retina from rat (a non-hibernator). Furthermore, these treatments significantly improved microtubule integrity in cold stored kidneys, demonstrating the potential for prolonging shelf-life of organ transplants. Thus, cold-adaptive strategies from hibernators may be adopted for vital clinical applications.

**Wnt signaling mediates intercellular mitochondrial unfolded protein response**

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In a number of neurodegenerative diseases, the accumulation of misfolded proteins and toxic aggregates is often accompanied with widespread peripheral metabolic changes. The mechanism by which the nervous system elicits distal proteotoxic stresses remains unknown. Studies in *C. elegans* have established that the expression of the HD-causing polyQ40 protein in neurons initiates the mitochondrial unfolded protein response (UPR<sub>mt</sub>) in the intestine, a process that induces global alteration of transcription networks to maintain a functional mitochondrial proteome. These studies have led to the concept of a "mitokine" - a signal generated in cells experiencing mitochondrial stress that is secreted, propagated, and subsequently perceived by peripheral tissues to regulate mitochondrial proteostasis. We found that animals use retromer-dependent Wnt signaling to propagate mitochondrial stress signals from the nervous system to peripheral tissues. Neuronal expression of the Wnt ligand is sufficient to induce cell-non-autonomous UPR<sub>mt</sub> in the intestine in a retromer complex-, canonical Wnt signaling-, and serotonin-dependent manner, clearly implicating Wnt signaling as a strong candidate for the 'mitokine' signal.

**4a. Terminal oxidases**

**Subunit Structure and Regulation of Cytochrome c Oxidase**

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Cytochrome c oxidase (COX or complex IV) is the terminal enzyme complex of the mitochondrial respiratory chain. It catalyzes the reduction of oxygen to water coupled to proton translocation across the inner mitochondrial membrane. Together with complexes I and III this generates the proton motive force driving ATP synthesis by complex V.

The recently developed complexome profiling approach, which exploits the powerful combination of blue-native electrophoresis and state-of-the-art mass spectrometry, is ideally suited to characterize subunit composition and multiple assembly intermediates comprehensively in a single experiment and allowed us to decipher the assembly pathway of mitochondrial complex I in great detail [1]. Here, we have applied complexome profiling to study the subunit assembly and composition of human COX. We present evidence that its assembly from three mitochondrial and more than 10 nuclear encoded subunits follows a precisely coordinated, module-based pathway. Fully assembled COX then associates with complexes I and III into supercomplexes.

We have identified new proteins that comply with the characteristics of subunits or assembly factors. This not only deepens our understanding of the structure and function of this respiratory chain complex, but will also shed further light on its involvement in regulatory and disease mechanisms, as already reported recently for MR-1S, a newly identified assembly factor of complex IV [2].


Cytochrome c oxidase subunit 4 isoform switch results in modulation of oxygen affinity

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Regulation of cytochrome c oxidase (COX), the terminal enzyme of respiratory chain, is realized through tissue-, development- or environment-controlled expression of subunits’ isoforms. COX4 subunit is thought to optimize respiratory chain function according to oxygen-controlled expression of its isoforms COX4i1 and COX4i2. However, details of functional alterations between the two variants have not yet been described. Employing CRISPR CAS9-10A technology, we created HEK293-based cellular model with complete absence of both COX4 isoforms (COX4i1/4i2 KO), resulting in full reliance on OXPHOS-independent ATP production. COX4i1/4i2 KO were subsequently utilized as a platform for knock-in of COX4i1 or COX4i2 using stable overexpression from pcDNA 3.1 vector. Expression of both isoforms complemented the respiratory defect of COX4i1/4i2 KO. The content of COX as well as its ability to incorporate into supercomplexes were comparable in COX4i1 and COX4i2 expressing cells. Respiratory rates of permeabilized cells in OXPHOS (coupled, state 3) and ETC (uncoupled, state 3u) states, as well as COX capacity were not distinguishable between cells expressing either isoform of COX4. However, significant changes were uncovered in COX oxygen kinetics. The p50 parameter (partial pressure of oxygen at half-maximal respiration) was approximately 2-fold increased in COX4i2 versus COX4i1 cells. These findings indicate decreased oxygen affinity of COX4i2-containing enzyme. The modulation of oxygen kinetics was not due to putative modification of cysteine residues unique to isoform 2, as cells expressing mutant variants of COX4i2 (C40S, C54S, C108S) displayed similar respiratory rates and p50 values as wild-type COX4i2. Using this model, we further plan to investigate the ability of COX4 isoforms to serve as mitochondrial energy and redox sensors for regulation of ATP production and oxidative stress response during normoxia and hypoxia.

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A Common Core Coupling Mechanism for Mitochondrial and Bacterial A-type Cytochrome c Oxidases?

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The core structures of bovine and yeast mitochondrial cytochrome c oxidases (CcO) and ‘A1-type’ homologues of bacterial CcO are similar. All contain two hydrophilic networks of amino acids and waters termed the D and K channels. A third hydrophilic network, the H channel, is prominent in bovine mitochondrial CcOs and is also evident in yeast and bacterial A1-type CcOs, though with some notable differences. In bacterial systems, there is substantial evidence that the D channel provides the route for pumped protons to a proton trap site, though the exit route from this trap site remains uncertain. In contrast, in mammalian, but not in yeast, mitochondrial CcO structural and functional data have suggested that the ‘lower’ part of the H channel instead provides the route for pumped protons to the trap site, and that its ‘top’ half provides the exit route into the P phase. Possible function(s) of the mitochondrial H channel were further explored both with MD simulations and with yeast CcO mutants. Simulations of the properties of the H channel of bovine and yeast mitochondrial CcOs support the notion that the ‘top’ part of the H channel structure could form a route for proton exit from the trap. However, simulations were unable to support the notion that the ‘lower’ region around H413 (bovine numbering) provides a proton transfer route into the trap site unless H413 can be protonated. The pK of H413 was estimated to be very low and redox titration of mutants of the equivalent position in yeast CcO (Q413) indicate only a weak electrostatic interaction of this position with heme a. H channel residues around haem a support a role in dielectric modulation of haem A properties. Hence, overall, some degree of convergence of core mechanism of bacterial, yeast and bovine CcOs may be proposed. Evidence for allosteric regulation of core activity that could be mediated by modulation of the H channel in bovine and yeast CcOs will be reviewed.

Heme-Copper Oxidases – Mechanisms for Chemistry and Energy Conservation, Including Proton Pumping

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Heme-copper oxidases (HcUs) are membrane-bound enzymes involved in aerobic and anaerobic respiration. Cytochrome c oxidase (CcO) belongs to this family, and reduces molecular oxygen to water as the final step in aerobic respiration. Nitric oxide reductase
(NOR) also belongs to the HCuO superfamily, and reduces nitric oxide to nitrous oxide and water as a key step of denitrification. Both reactions are quite exergonic, and an interesting observation is that while oxygen reduction in CcO is coupled to conservation of a significant part of the released free energy by the formation of a proton electrochemical gradient across the membrane, NO reduction in NOR is not. Another interesting observation is that for certain subfamilies of heme-copper oxidases, the enzyme can use both O₂ and NO as substrate in the reduction reaction, which provides an excellent opportunity for deeper mechanistic insights. Quantum mechanical calculations (density functional theory) have been used to study both O₂ and NO reduction in different types of CcOs, and also in cytochrome c dependent NOR (cNOR). Based on a combination of the results from the calculations and experimental data, a number of interesting general conclusions can be drawn: The efficient energy conservation in CcO can be explained, in terms of both energetics and mechanisms. An active site tyrosine, found to be redox-active, is shown to play a role both in the smooth distribution of the released energy over the reduction steps, and in making it possible to transfer two protons per electron, i.e. for proton pumping. The intrinsic reduction potential of the active site copper is shown to be significantly higher than expected from experimental data. The lack of energy conservation in cNOR is shown to be due to the low reduction potentials of the active site cofactors, which in turn leads to fast reduction of the toxic NO molecule. The difference in NO reduction ability between different types of CcOs is also explained.

Snapshot of an Oxygen Intermediate in the Catalytic Reaction of Cytochrome c Oxidase

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Cytochrome c oxidase (CcO) reduces dioxygen to water and harnesses the chemical energy to drive proton translocation across the inner mitochondrial membrane by an unresolved mechanism. By using time-resolved serial femtosecond crystallography with an X-ray free electron laser, we identified a transient intermediate in the oxygen reduction reaction of bovine CcO microcrystals [1]. It is assigned as the F-intermediate and is characterized by distinct metal redox states and protein conformations. The heme a₃ iron atom is in a ferryl (Fe₄=O²⁻) configuration and heme a is oxidized but Cu A is reduced. A helix-X segment is poised in an open conformation [2]; the farnesyl sidechain of heme a is H-bonded to S382; and Loop-I-II adopts a unique conformation. These data offer new insights into the mechanism by which the oxygen chemistry drives unidirectional proton translocation [1].

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4b. Bioenergetics of CNS diseases

OXPHOS organization in neurons and astrocytes regulates brain metabolism

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Neurons tightly depend on mitochondrial oxidative (OXPHOS) metabolism for function and survival, whereas astrocytes mostly rely on glycolysis (1). A key factor that accounts for these cell-specific metabolic programs is PFKFB3 (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3), i.e. the pro-glycolytic enzyme that activates PFK1 (6-phosphofructo-1-kinase). Being a substrate of the E3 ubiquitin ligase, anaphase-promoting complex/cyclosome (APC/C)-Cdh1 (1,2), PFKFB3 entirely controls the different glycolytic shapes of neurons and astrocytes (2). In astrocytes, APC/C-Cdh1 activity is very low, which allows PFKFB3 to be stable to keep glycolysis high (2). However, in neurons APC/C-Cdh1 activity is very high, which promotes continuous degradation of PFKFB3 to keep glycolysis low (2). Such glycolytic control by this APC/C-Cdh1–PFKFB3 axis supports a mechanistic ground for the astrocyte-neuronal lactate shuttle. Furthermore, genetically engineered mice to force PFKFB3 expression specifically in neurons in vivo exhibit cognitive decline and motor imbalance, which are rescued by selectively scavenging mitochondrial ROS in these neurons. Thus, neuronal glycolysis regulates mitochondrial redox metabolism and behaviour.
Likewise, in the highly glycolytic astrocytes the OXPHOS energy efficiency is low and dictates high mitochondrial ROS under physiological conditions (3). Deciphering the physiological roles of mitochondrial ROS in the brain are currently being investigated in our laboratory using genetically engineered mice with attenuated mitochondrial ROS in astrocytes in vivo.

References

Thiamine preserves mitochondrial function in a rat model of traumatic brain injury, preventing inactivation of the 2-oxoglutarate dehydrogenase complex
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Based on the fact that traumatic brain injury is associated with mitochondrial dysfunction we aimed at localization of mitochondrial defect and attempted to correct it by thiamine. Adult male Sprague-Dawley rats were subjected to lateral fluid percussion traumatic brain injury. Thiamine was administered 1 h prior to trauma; cortex was extracted for analysis 4h and 3d after trauma. Increased expression of inducible nitric oxide synthase (iNOS) and tumor necrosis factor receptor 1 (TNF-R1) by 4h was accompanied by a decrease in mitochondrial respiration with glutamate but neither with pyruvate nor succinate. Assays of TCA cycle flux-limiting 2-oxoglutarate dehydrogenase complex (OGDHC) and functionally linked enzymes (glutamate dehydrogenase, glutamine synthetase, pyruvate dehydrogenase, malate dehydrogenase and malic enzyme) indicated that only OGDHC activity was decreased. Application of the OGDHC coenzyme precursor thiamine rescued the activity of OGDHC and restored mitochondrial respiration. These effects were not mediated by changes in the expression of the OGDHC sub-units (E1k and E3), suggesting post-translational mechanism of thiamine effects. By the third day after TBI, thiamine treatment also decreased expression of TNF-R1. Specific markers of unfolded protein response did not change in response to thiamine. Our data point to OGDHC as a major site of damage in mitochondria upon traumatic brain injury, which is associated with neuroinflammation and can be corrected by thiamine. Further studies are required to evaluate the pathological impact of these findings in clinical settings.

Inosine reverses motor neuron toxicity observed in amyotrophic lateral sclerosis patient astrocytes with an adenosine deaminase deficiency
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As clinical evidence supports a negative impact of dysfunctional energy metabolism on disease progression in amyotrophic lateral sclerosis (ALS) [1], it is vital to understand how the metabolic pathways are altered and whether they can be restored to slow disease progression. To test the hypothesis that supplementation of the energy substrates involved in dysfunctional pathways back into the cell would restore the metabolic defect, we employed a phenotypic metabolic assay [2] to analyse the basis of the catabolic defect in ALS. We profiled fibroblasts and induced neuronal progenitor (iNPC) derived iAstrocytes from familial and sporadic ALS cases and from controls. This was followed up by western blot and PCR analysis of components involved in the dysfunctional pathways and
supplementation assays measuring ATP and metabolic flux levels. Finally, we assessed motor neuron survival in co-culture with the supplemented iAstrocytes. ALS patient iAstrocytes and fibroblasts displayed reduced NAD(P)H production in the presence of adenosine due to a reduction of adenosine deaminase (ADA) at both the RNA and protein level. Patient iAstrocytes were more susceptible to adenosine-induced toxicity, which could be mimicked by inhibiting ADA in controls. ADA inhibition in control iAstrocytes led to increased motor neuron toxicity in co-cultures similar to the levels observed with patient derived iAstrocytes. Inosine supplementation in iAstrocytes increased glycolytic energy output, leading to an increase in motor neuron survival in co-culture. We have identified a novel dysfunctional pathway in ALS which may be caused by a common mechanism of dysfunction in both familial and sporadic patient groups. Inosine supplementation in combination with ADA level modulation may be beneficial in ALS and may slow down disease progression.


A novel model of Leber’s hereditary optic neuropathy: respiratory insights and effects of idebenone

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There are few treatment options for Leber’s hereditary optic neuropathy (LHON) patients, due in part to the difficulty in creating experimental LHON models. Targeted disruption of proteins encoded by mitochondrial DNA has been bolstered in recent years by several promising approaches, yet each of these has their limitations. In this study, we utilized Artificial Site-specific RNA Endonucleases (ASREs), a novel mitochondrial RNA engineering platform, to create a cell model of LHON. By combining ASRE ablation of a target gene with the expression of corresponding human disease allele integrated into the nuclear genome as a transgene, unique cell culture models of human mitochondrial disease can be generated. Human embryonic kidney (HEK293) cells and C2C12 cells were transfected with a drug-inducible ASRE designed to decrease the expression of complex I subunit ND1 (ASRE-ND1). In a separate cohort of cells, the ASRE-engineered cells were transfected with piggyBac (PB) transposon vectors that carried a cumate-inducible wild type ND1 (PB-ND1) or ND1 gene associated with some forms of LHON [PB-ND1(G3640A)]. ASRE-ND1 cells displayed a heightened susceptibility to mitochondrial toxicity. High-resolution respirometry in permeabilized cells revealed lower complex I-dependent respiration after ASRE-ND1 activation (respiration decreased from 13.2±0.7 pmol/sec*million cells to 11.0±0.4 pmol/sec*million cells). Expressing wild type PB-ND1 rescued complex I-dependent respiration to 14.5±0.4 pmol/sec*million cells. Interestingly, expression of the PB-ND1(G3649) LHON mutant in ASRE-ND1 cells did not restore complex I-dependent respiration. Respiration in ASRE-ND1 cells expressing PB-ND1(G3649) was acutely improved with idebenone. These data highlight the feasibility of creating cell models of mitochondrial disease using the ASRE platform. We anticipate this technology will lead to the development of new screening platforms for emerging therapeutic interventions.

Alterations in the Circadian Clock-Dependent Mitochondrial Functions in Parkinson Patient Fibroblasts Carrying Mutated PARK2

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Increasing evidences highlighted a tight connection between circadian rhythms and mitochondria function. In particular, the mitochondrial quality control proved to undergo circadian oscillations under the control of master clock genes which also affects the mitochondria bioenergetics circadian rhythm. Parkinson’s disease (PD) is a chronic neurodegenerative disease characterized by a selective loss of dopaminergic neurons. Almost half of the autosomal recessive forms of juvenile parkinsonism has been associated with
mutations in the PARK2 gene coding for parkin, shown to be involved in the mitophagy-mediated mitochondria quality control. The aim of this study was to investigate in fibroblast from PD patients with parkin mutations the interplay between mitochondria bioenergetics and the cell-autonomous circadian clocks. Using three different in-vitro-synchronized protocols, we demonstrated that normal/control fibroblasts displayed a rhythmic oscillation of both their mitochondrial respiration and glycolytic activity. Conversely, in the fibroblasts from PD patients a robust dampening of the oscillatory bioenergetics functions was observed. Analysis of the major clockwork-related genes resulted in deregulation of their expression profile. To verify if impaired mitochondrial functions, such those hallmarking parkin-mutated cells, were by their own responsible of the altered clock genes expression we tested the impact of OxPhos inhibitors, under non-cytotoxic conditions. The results obtained clearly show that functional mitochondrial OxPhos is required to preserve the autonomous circadian oscillation of the clock gene, confirmed by similar analysis carried out with Rho0 fibroblasts. The presented results indicate a reciprocal interplay between the circadian clock genes machinery and the mitochondrial energy metabolism and point to a parkin-dependent mechanism of regulation unveiling a hitherto unappreciated level of complexity in neurodegenerative diseases.

5a. ATP synthases and ATPases

Monitoring single \(F_{\text{Fo}}F_{\text{F1}}\)-ATP synthases at work in the ABELtrap

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Since 20 years we focus on the mechanochemistry of \(F_{\text{Fo}}F_{\text{F1}}\)-ATP synthase from \(E. coli\). The enzyme operates as a bidirectional motor protein. We developed a single-molecule Förster resonance energy transfer (smFRET) approach to monitor internal subunit rotation in solution. Our previous confocal smFRET studies revealed distinct step sizes and elastic deformations of the two rotary motors of \(F_{\text{Fo}}F_{\text{F1}}\)-ATP synthase [1-3] and its regulatory mechanism [4]. Yet, the drawbacks of these measurements were short observation times and large intensity fluctuations inherent to free diffusion through a confocal detection volume. Therefore we implemented an anti-Brownian electrokinetic trap (ABELtrap, invented by A. E. Cohen and W. E. Moerner) to hold single enzymes in place during the smFRET measurements [5]. Extended observation times up to seconds allowed for analysis of ATP concentration-dependent fast rotation of the \(F_{\text{1}}\) and \(F_{\text{r}}\) motor.

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The uniqueness of subunit \(\alpha\), \(\gamma\) and \(\epsilon\) of mycobacterial \(F\text{-ATP synthases: Evolutionary variants for niche adaptation}

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The \(F_{\text{o}}F_{\text{r}}\) ATP synthase of the pathogenic bacterium Mycobacterium tuberculosis (Mtbo) is essential for the viability of growing and non-growing persister cells of the pathogen [1]. This enzyme complex is composed of nine subunits in the stoichiometry of \(\alpha_{3}\beta_{3}\gamma\delta\varepsilon\cdot\alpha_{2}b_{2}b'_{2}c_{9}\) and organized in a membrane-embedded \(F_{\text{o}}\) domain \((a:b:b':c_{9-12})\) and a water soluble \(F_{\text{r}}\) part \((\alpha_{3}\beta_{2}\gamma\delta\varepsilon\cdot\varepsilon)\). A characteristic feature of the mycobacterial \(F\text{-ATP synthase is its inability to establish a significant proton gradient during ATP hydrolysis, and its low or latent ATPase activity in the fast- or slow-growing form [2-3].}
The presentation reveals that at least the three subunits α, γ and ε contribute to the important enzymatic differences of mycobacterial F-ATP synthases in suppression of proton pumping and proton motive force (PMF) formation [3-5], which is essential because dissipating the PMF is lethal to mycobacteria, as well as in ATP formation, employing them as potential drug targets. Our novel atomic structure of the rotating subunit ε in solution provides insights into a new mechanism of coupling proton-conduction with ATP synthesis and identifies a second binding site of the TB drug bedaquiline (BDQ, Sirturo®) [5].

Using a combination of recombineering, single molecule, solution X-ray scattering, NMR spectroscopy and electron microscopy it will be shown that unique mycobacterial stretches inside the nucleotide-binding subunit α, γ and ε influence cell growth, ATPase activity and ATP synthesis of the pathogen [3-5, unpublished data].

These data formed a platform to identify and synthesize new compounds, which effectively inhibit ATPase and ATP synthesis of mycobacterial F-ATP synthase and inhibit growth of *Mycobacterium smegmatis*, *M. bovis* as well as *M. tuberculosis* [unpublished data].

**References**


Unidirectional control of the F<sub>1</sub>F<sub>0</sub>-ATPase/synthase nanomotor by the ζ pawl-ratchet inhibitor protein of *Paracoccus denitrificans*

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The ATP synthase is a reversible nanomotor that gyrates its central rotor clockwise (CW) to synthesize ATP and counter clockwise (CCW) to hydrolyse it. In bacteria and mitochondria, two canonical natural inhibitor proteins, namely the ε and IF<sub>1</sub> subunits, prevent the wasteful CCW F<sub>1</sub>F<sub>0</sub>-ATPase activity by blocking γ rotation at the αDP<sub>1</sub>βDP<sub>1</sub>γ interface of the F<sub>1</sub> portion. In *Paracoccus denitrificans* and related α-proteobacteria, we discovered a different natural F<sub>1</sub>-ATPase inhibitor named ζ, which also binds to the same F<sub>1</sub> catalytic interface [1], shifting its inhibitory N-terminus from an intrinsically disordered region (IDPr) to an α-helix. We showed for the first time the key role of a natural ATP synthase inhibitor by the distinctive phenotype of a Δζ knockout mutant in *P. denitrificans* [2].

ζ blocks exclusively the CCW F<sub>1</sub>F<sub>0</sub>-ATPase rotation without affecting the CW-F<sub>1</sub>F<sub>0</sub>-ATP synthase turnover, preventing wasteful ATP hydrolysis by working as an unidirectional pawl-ratchet inhibitor. Funding: DGAPA-PAPIIT IN-221216; CONACyT CB-167622.

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A molecular transfer theory that combines chemical reaction theory and structural mechanical elasticity is used to treat single molecule imaging and magnetic tweezer manipulation experiments in the F1-ATPase. The theory provides a method to calculate the rate and equilibrium constants for individual power-stroke substeps involving ATP binding, catalysis and release of products as a function of the rotor angle. It also serves as the basis for correcting the estimated rate constants due to statistical noise in the photon counting trajectories from the single-molecule fluorescence experiments. It captures key features of the single molecule data, including the exponential dependence of the forward and reverse rate constants over the limited range of the stalling experiments, or the turn-over of these trends in the extended range of the controlled rotation experiments. It establishes the relationship between the discrete dwell angles in imaging experiments and the continuous angular dependence of the rate constants in the manipulation experiments. Despite its formal simplicity the model leads to the correct quantitative prediction of the controlled rotation rate constants on fluorescent ATP without any adjustable parameters, using independent experimental data from ensemble biochemical, FRET, crystallography and single-molecule imaging experiments. The model is currently extended to force spectroscopy experiments in myosin V and to reveal the dynamics of transitions between dwells “hidden” in fast microsecond imaging.


F1-ATPase Dwell and Power Stroke Relationships
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To complete a full revolution, the F1-ATPase $\alpha$-subunit undergoes 3 successive power strokes separated by catalytic dwells when ATP hydrolysis occurs. The power stroke is interrupted by a second ATP-binding dwell at limiting ATP concentrations. Årrhenius analysis of the power stroke angular velocity versus rotational position revealed negative activation energy values of the power stroke during the first 60° after the catalytic dwell (Phase-1) that varied parabolically with a minimum at 34°, the optimum ATP-binding dwell position [1]. These results indicate that the energy that powers Phase-1 rotation is derived from elastic energy from a torsion spring with a spring constant of $k = 50 k_BT\cdot\text{rad}^{-2}$. Single-molecule experiments examined the rotary oscillations of subunit-$\alpha$ that occur during the catalytic dwell, and the effects of the ATP-binding dwell on the angular velocity profile of the power stroke. The effects on both of these parameters that result from mutations that weaken the anchor between the $\alpha$-subunit coiled-coil and the $\alpha_3$-ring of F1 that serves as a stator for the motor were also investigated. The results provide new insight into the means by which elastic energy stored in the $\alpha$ subunit coiled-coil is used to power rotation in the F1-ATPase molecular motor.


5b. Targeting of bioenergetic organelles for mitochondrial diseases

Targeting the mitochondrial electron transport chain of Plasmodium falciparum: Opportunities and challenges towards the development of improved antimalarials for the elimination era
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Despite intense efforts, there has not been a truly new antimalarial, possessing a novel mechanism of action, registered for some 20 years. By virtue of a novel mode of action, it is hoped that the global challenge of multidrug-resistant parasites can be overcome, as well as developing drugs that possess prophylaxis and/or transmission-blocking properties, towards an elimination agenda. Cytochrome bc₁ is a proven drug target in the prevention and treatment of malaria. Medicinal chemistry activity has largely focused on three classes of bc₁ inhibitors including acridinediones, pyridones and quinolone aryl esters, as well as inhibitors of dihydroorotate dehydrogenase that includes triazolopyrimidines and benzimidazoles. Parasite resistance mechanisms to atovaquone can be overcome by targeting the Qi-site of the bc₁ complex, however this can lead to acute toxicity issues. Common barriers to progress and opportunities for novel chemistry and potential additional electron transport chain targets are discussed in the context of the target candidate profiles for uncomplicated malaria.

Targeting the alternative oxidase for antitrypanosomal drug development
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The alternative oxidase (AOX) is a monotopic diiron carboxylate protein which catalyzes the four-electron reduction of dioxygen to water by ubiquinol. The blood stream forms of Trypanosoma brucei, a protozoan parasite causing Human African Trypanosomiasis, heavily depend on AOX for survival. The absence of AOX in the mammalian genome makes TAO a promising target for the development of new antitrypanosomal drugs. Although we have recently determined the crystal structure of Trypanosoma brucei AOX (TAO) in the presence and absence of ascofuranone (AF) derivatives (which are potent mixed type inhibitors) the mechanism by which ubiquinol and dioxygen binds to TAO remain inconclusive. We have recently identified ferulenol as the first competitive inhibitor of AOX which has been used to probe the binding site of ubiquinol. AF and ferulenol have similar potency as judged by IC₅₀s against recombinant TAO, however, differing over 1000-fold in their trypanocidal activity. Inhibitor binding kinetics revealed by surface plasmon resonance show that ferulenol have over 1000-fold higher $K_{off}$ compared to AF, which correlated well with their Trypanocidal activity. Moreover, $K_{m}/K_c$ ratio suggests that AF and ferulenol possess the potential to be a transition-state analog and substrate/product analogues of TAO, respectively. The structure of the TAO-ferulenol complex, determined at 2.7 Å, provided insights into ubiquinol binding and has also identified a potential dioxygen molecule bound in a side-on conformation to the diiron center for the first time.

Bioenergetic consequences of xenotopic expression of Ciona intestinalis alternative oxidase (AOX) in the mouse
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Mitochondrial respiratory inhibition induces oxidative stress, redox imbalance and metabolic stalling and is the underlying cause for a number of human disorders. Conversely, “primitive” organisms and plants, but not mammals, express alternative oxidases (AOX) that can bypass the cytochrome part of the respiratory chain passing electrons directly from ubiquinol to oxygen thus restoring mitochondrial electron transport and relieving cell stress.
We established a mouse model xenotopically expressing *Ciona intestinalis* AOX. Phenotypically, the AOX mouse shows only subtle alterations [1]. It remained unclear, however, how AOX affects the endogenous mitochondrial respiration. Here we show that AOX does not directly interact with any of the classical respiratory complexes, it allows complex I-driven oxidative phosphorylation and retains the mitochondrial membrane potential when the cytochrome part of the respiratory chain is inhibited. Furthermore, AOX decreases the level of succinate-driven reactive oxygen species produced by reverse electron transport.

Taken together, our data support the concept of respiratory restoration and stress relieve by AOX and make the AOX mouse model a valuable tool to decipher respiratory control and disease mechanisms.

or NTOs). Lately, another group of cyanobacterial anion pumps with a very distinct primary structure was reported [1]. Here, we studied the chloride-transporting photocycle of a representative of this new group, *Mastigocladus repens* rhodopsin (MastR), using time-resolved spectroscopy in the infrared and visible ranges and site-directed mutagenesis [2]. We found that, in accordance with its unique amino acid sequence containing many polar residues in the transmembrane region of the protein, its photocycle features a number of unusual molecular events not known for other anion-pumping rhodopsins. It appears that light-driven chloride ion transfers by MastR are coupled with translocation of protons and water molecules as well as perturbation of several polar sidechains. Of a particular interest is transient deprotonation of Asp-85, homologous to the cytoplasmic proton donor of light-driven proton pumps (such as Asp-96 of bacteriorhodopsin and Glu-108 of proteorhodopsin), which may serve as a regulatory mechanism.


**Molecular mechanism of channelrhodopsin**

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The discovery of channelrhodopsins introduced a new class of ion channels whose conductance can be remotely controlled by light, the feature that founded optogenetics. To explore the connection between the gating mechanism and the influx and efflux of water molecules in channelrhodopsin-2 (ChR2), we have integrated light-induced time-resolved infrared spectroscopy and electrophysiology. Cross-correlation analysis revealed that ion conductance tallies with peptide backbone amide I vibrational changes that report on the hydration of transmembrane α-helices. We show that D253 accepts the proton released by the Schiff base (τ₁/₂ = 10 μs), the latter being reprotonated by D156 (τ₁/₂ = 2 ms) which is part of the DC gate. Previous conclusions on the involvement of E90 in channel opening are ruled out by demonstrating that E90 deprotonates exclusively in the non-conductive P₄₄₈₀ state. Our results merge into a mechanistic proposal that relates the observed proton transfer reactions, protein conformational changes and backbone hydration to the gating of the cation channel. Our results will not only contribute to improve the properties of this optogenetic tool but will also help in elucidating the temporal sequences of ion channeling across the cellular membrane.

**References**


**Light-driven sodium-pumping rhodopsin: A new concept of active transport**

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Ion pumps perform active transport of ions by using energy. The active transport mechanism can be illustrated by the Panama Canal model, which considers two gates and a gain in energy. The Panama Canal model is consistent with the alternating access model that is used to describe active transport, in which the substrate ion is bound, energized, and released. It was generally accepted that energization occurs only for an ion-bound protein, but not for an ion-unbound protein. Light-driven proton and chloride pumps, two of the best studied pumps, are represented by the Panama Canal model. In this case, light absorption takes place for the bound state of ions (proton and chloride ions) in the active center (protonated Schiff base of the retinal chromophore) [1]. In contrast, a recently discovered
light-driven sodium pump, *Krokinobacter eikastus* rhodopsin 2 (KR2), is a unique active transporter that does not bind the transport substrate, the sodium ion, in its resting state [2,3]. The molecular architecture and photoreaction cycle of the light-driven sodium pump are very similar to those of proton and chloride pumps, although sodium ions are actively transported without initial binding. Sodium uptake is a diffusive process, but the presence of two gates allows the unidirectional transport of sodium ions [4]. In this sense, the light-driven sodium pump is also represented by a modified Panama Canal model. Molecular mechanism of light-driven sodium pump will be discussed in comparison with other active transporters.

References:

**Electrophysiology of the engineered light-driven sodium pump eKR2 and its conversion into a channel**

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Light-driven ion pumps are microbial rhodopsins, which actively transport ions across the plasma membrane. These proteins fascinate with their outstanding feature that these convert light energy directly into an electrochemical gradient. In 2013, the discovery of outward directed light-driven sodium pumps (NaRs) expanded the family of microbial rhodopsins [1]. The first described and best-characterized NaR is KR2 from the marine bacterium *Dokdonia eikasta*.

Poor membrane targeting hindered the electrophysiological characterization and optogenetic application of KR2 in mammalian cells so far. To overcome this drawback, we designed the enhanced KR2 (eKR2) with excellent membrane targeting and higher photocurrent amplitudes [2]. Successful expression of eKR2 in cultured hippocampal mouse neurons allowed reversible inhibition of action potential firing. Ion selectivity measurements in ND7/23-cells revealed that stationary photocurrents are primarily carried by sodium ions whereas the intracellular proton concentration had no significant influence on the photocurrent size.

Furthermore, we transferred our knowledge about the conversion of light-driven proton pumps into channel-like ion transporters to NaRs [3]. We found that introduction of ion leakiness into eKR2 by site-directed mutagenesis is possible and generates channel-like variants with different ion selectivity for sodium, potassium and lithium - but without detectable proton conductance.

concentration gradient involves protein structural changes. Collaborative studies performed at SACLA (an XFEL in Japan) and the LCLS (an XFEL in Stanford, USA) have probed structural changes in microcrystals on a time-scale from femtoseconds to milliseconds. Structural results from these studies enabled a complete picture of structural changes occurring during proton pumping by bacteriorhodopsin to be recovered [1,2].


6b. Bioenergetics: Disease and health

The pathomechanism of COX deficiency includes nuclear DNA damage and replicative stress
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Mitochondrial cytochrome c oxidase (COX, respiratory chain complex IV), contributes to ATP production via oxidative phosphorylation (OXPHOS). The mammalian COX is a dimeric multi-protein complex composed of 13 subunits. The three mitochondrial-encoded subunits comprise the catalytic core, while the remaining subunits, encoded by the nuclear DNA perform regulatory functions.
Isolated COX deficiency is a mitochondrial disease, presenting with a marked clinical heterogeneity ranging from fatal neonatal, lactic acidosis to adult myopathy affecting numerous organs including brain, heart, muscle, liver, hematopoiesis and pancreas. Mutations in more than 30 genes, in both mitochondrial and nuclear DNA, affecting either structural subunits of the enzyme or proteins involved in its biogenesis are associated with human disease.
Among these, is the K101N mutation in the common isoform of COX4 (COX4-1), we recently reported. The relatively mild presentation could be explained by upregulation of the COX4-2 isoform, while the Fanconi anemia-like features, suggested genomic instability. This was confirmed in the patient’s fibroblasts by detecting increased frequency of double stranded DNA breaks (DSB) in the nuclear DNA, by phospho histone H2AX Ser139 staining and neutral comet assay. DSB was also present in COX6B1 deficient fibroblasts and KCN treated control fibroblasts grown in high glucose medium where ATP was normal and without evidence of oxidative stress. In contrast, DSB was not detected when cell growth was impaired (glucose free or serum free medium). Interestingly, nicotinamide riboside (NR) ameliorated DSB in the COX mutated fibroblasts while polymerase (PARP) inhibitor had a marked negative effect and antioxidants did not show any significant effect.
Although additional investigation is needed, our findings raise the possibility that the pathomechanism of COX deficiency and maybe also in some other OXPHOS defects, include nuclear DNA damage resulting from nicotinamide adenine dinucleotide (NAD+) deficit combined replicative stress, rather than oxidative stress and energy depletion.

Exogenous NAD+ prevents galactose-induced death of Leigh Syndrome patient fibroblasts with isolated complex I deficiency
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The pathomechanism of Leigh syndrome (LS) still remains poorly understood. Primary fibroblasts from LS patients with isolated complex I (CI) deficiency display increased mitochondrial NAD(P)H autofluorescence, suggesting that NAD+ to NADH recycling is impaired. Here we investigated the hypothesis that LS fibroblasts display “reductive stress” impacting on cell viability and bioenergetics.
To this end we optimized a cell culture strategy in which glucose in the medium was replaced by galactose. At 96 h, this manoeuvre specifically induced cell death in LS fibroblasts but not in control (CT) cells. High-content microscopy revealed that certain (cell
permeable) metabolites inhibited galactose-induced LS cell death, whereas others were ineffective. Similarly, supplementing the galactose medium with extracellular NAD⁺ (eNAD) increased intracellular NAD⁺ levels (iNAD) and inhibited LS cell death. Analysis of mitochondrial morphofunction at 24 h revealed that eNAD differentially affected CT and LS cells. Galactose treatment increased reactive oxygen species (ROS) levels and reduced cellular ATP content in CT and LS cells. These aberrations were fully normalized by eNAD. Our results suggest that LS patient cells suffer from reductive stress when placed in galactose medium and that NAD⁺ prevents this stress thereby maintaining cell viability.

Targeting the mitochondrial trifunctional protein in oxidative lung carcinomas

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Metabolic reprogramming is a common hallmark of cancer but a large variability in tumor bioenergetics exists between patients. Using high-resolution respirometry, we identified two human lung adenocarcinoma subgroups: high (OX+) mitochondrial respiration and low (OX-) mitochondrial respiration. The OX+ tumors poorly incorporated [18F] fluorodeoxy-glucose and showed increased expression of the mitochondrial trifunctional fatty oxidation enzyme (MTP) compared to the paired adjacent tissue. Genetic inhibition of MTP altered OX+ tumor growth in vivo. Trimetazidine, an approved drug inhibitor of MTP used in cardiology, also reduced tumor growth and induced destabilization of respiratory chain complex I assembly, leading to a cellular redox and energy crisis. MTP expression in tumors varied in negative correlation with [18F] fluorodeoxy-glucose incorporation, and patients with high MTP-expressing tumors had lower survival rates. These findings provide proof-of-concept data for preclinical precision bioenergetic medicine in lung carcinomas.

Exercise-induced mitochondrial biogenesis: is it a key for diseases prevention?

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Mitochondrial content, dynamics and function, influence the generation of ROS and the capacity of the antioxidant system. Exercise training increases mitochondrial levels in brain, heart, liver, kidney and testis, and it is known that regular exercise attenuates the diseases in these organs. During exercise coactivator peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) can be activated by AMP-activated protein kinase (AMPK), silent mating type information regulation 2 homolog 1 (SIRT1), Ca²⁺/calmodulin-dependent protein kinase IV, calcineurin A, and ROS. PGC-1α is not just activated by ROS, it can control the level of ROS partly through the activation of NRF-2 that binds to antioxidant response element (ARE). Besides the activation of ARE, PGC-1α might be important to exercise- induced increases in the expression of SOD2. Therefore, the exercise-induced PGC-1α levels can be one of the reason why exercise results better metabolism to brain, which is important to prevent Alzheimer Diseases, attenuation of the loss of
mitochondrial mass in the kidney is important to maintain normal function. Exercise-mediated redox signaling through PGC-1α and SIRT1 pathways might be important against chronic inflammation, and all kinds of age-associated diseases.

**The Control of Oxidative Phosphorylation in Healthy and Type 2 Diabetic Human Pancreatic β-Cells**

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Mitochondrial metabolism plays a central role in insulin secretion in pancreatic β-cells by ATP synthesis and by pathways that amplify insulin secretion with possibly distinct mechanisms. The operation and relative importance of these two pathways are controversial. Type 2 diabetes (T2D) is hallmarked by relative insulin insufficiency. Secretory dysfunction and compromised mitochondrial metabolism have been demonstrated in T2D human pancreatic islets and β-cells, and also in rodent models of T2D. Causes of this secretory dysfunction are unclear. Novel mitochondrial assay technology developed by us now allows mechanistic studies of how cellular energy metabolism controls insulin secretion in micro-scale primary cell cultures. Mitochondrial membrane potential (ΔψM), cell respiration and insulin secretion were followed in dispersed islet cell cultures. In healthy β-cells, when glucose concentration is elevated, increased metabolism results in a substantial (30-40 mV) ΔψM hyperpolarization, as well as in increased rates of ATP synthesis and turnover marked by faster (doubled) cell respiration. I find that the hyperpolarization of the ΔψM induced by glucose, which drives high ATP/ADP, is necessary for and predicts insulin secretion. Using modular kinetic analysis, properties of cellular energy metabolism that enable a large glucose-induced change in ΔψM in human β-cells were explored. I found that an ATP-synthesis-dependent pathway activates glucose or substrate oxidation, acting as a positive feedback in energy metabolism. This novel activation mechanism is essential for a high magnitude glucose-induced ΔψM hyperpolarization and therefore for insulin secretion. Data from human T2D β-cells suggest the impairment of this activation pathway. An altered kinetic response of glucose oxidation to ΔψM may explain bioenergetic dysfunction in T2D β-cells.

**PTP round table**

*John E. Walker*

Abstract not received

*Paolo Bernardi*

Abstract not received

**ATP synthase in neuronal development, neurodegeneration and plasticity**

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We have recently found that neuronal mitochondrial function is highly developmentally regulated; that developing synaptic mitochondria have an inner membrane leak that reduces mitochondrial membrane potential. This is due to a programmed inefficiency of ATP synthesis. We find that the anti-apoptotic protein Bcl-xL, the autism gene FMRP and the familial Parkinson's gene DJ1 are developmentally regulated to alter the balance between glycolytic and oxidative metabolism. The switch brings about developmental maturation and enhances synaptic plasticity underlying learning and memory formation. We suggest that synaptic activity causes translation of genes that close the inner membrane leak, switching the behavior of the ATP synthase from ATP hydrolyzing to ATP synthesizing. During synaptic stimulation, movement of proteins into the inner membrane closes the leak to bring about the metabolic switch. These proteins include DJ1 and Bcl-xL but also the F1 components themselves, that are made in response to synaptic...
stimulation and change the stoichiometry between F1 and FO. ATP production now phosphorylates downstream targets. The first of many targets has been identified and it is EF2, a known regulator of protein translation, particularly of synaptic proteins required for learning and memory formation. Given that the inner membrane leak regulates protein translation, we tested if change in level of the c-subunit leak channel in the inner membrane regulates protein translation. We find that we reduce the abnormally high levels of protein translation in the Fragile X mouse by depletion of c-subunit while overexpression increases protein translation. Pharmacological modulators of ATP synthase, CsA and Dexpramipexole, also decrease protein translation in the mutant mouse and in human cells. The c-subunit leak is regulated during development to promote the onset of oxidative phosphorylation and regulate the translation machinery to produce proteins needed for synaptic maturation.

ATP synthase in neuronal development, neurodegeneration and plasticity

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In vitro assays ranging from pmf-driven ATP synthesis in proteoliposomes to the X-ray crystal structures of the F1 subcomplex and direct observation of mechanical rotation at the single molecule level have been the driving force of the F-ATPase field [1-3]. Matching this tradition, six years ago based on in vitro measurements of planar bilayer reconstituted bovine F-ATP synthase Bernardi and colleagues proposed that the molecular identity of the mitochondrial permeability transition pore (ptp) is the mitochondrial F-ATP synthase dimer [4, 5]. However, limits in the experimental set-up, such as low purity of the protein sample and limited biochemical characterization after bilayer reconstitution, left room for doubt on the interpretation of the experimental results [6]. To address these open issues, we have employed highly purified mammalian F-ATP synthase from bovine heart. The preparation displays H+ pumping activity in proteoliposomes, which were used for the electrophysiological characterization in planar lipid bilayers. The observed channel properties match those of the ptp, including sensitivity to Ca2+ alone. Thus, a molecular identity of the ptp other than the mitochondrial F-ATP synthase is very unlikely.

References

7a. Channels and transporters

Luigi Palmieri

Abstract not received
The DOs and DON'Ts of secondary-active transporters: lessons from the Na⁺/Ca²⁺ exchanger
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Secondary-active transporters catalyze the uptake and efflux of substances across biological membranes, driven by the electrochemical potential gradient of one of the transported species, typically Na⁺ or H⁺. It is increasingly evident that the mechanism of these proteins involves a conformational cycle whereby one or more ion/substrate binding sites within the transporter become alternatively exposed to the intracellular or extracellular space, but not both simultaneously. It is underappreciated, however, that for this mechanism to result in active transport, the interconversion between conformational states open to one or the other side of the membrane must be strictly self-inhibited unless the appropriate type and number of substrates are concurrently recognized. Indeed, the physiological character of a given transporter – whether it is a symporter or antiporter, for example, or its precise substrate stoichiometry – is ultimately determined by precisely which substrate occupancy states permit this conformational interconversion and which do not. In this presentation, I will discuss the physical basis of this fundamental mechanism of allosteric control, using a prokaryotic homolog of the cardiac Na⁺/Ca²⁺ exchanger as a model system.

VDAC isoforms in S. cerevisiae
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The permeability of Mitochondrial Outer Membrane in eukaryotes is possible by the presence of Voltage-Dependent Anion Channel (VDAC) proteins, a conserved group of β-barrel porins allowing mainly ATP/ADP and ions exchange between cytosol and mitochondria. The budding yeast Saccharomyces cerevisiae has two genes encoding two distinct VDAC proteins (yVDAC1 and 2). yVDAC1 is the main porin and is necessary during respiration: cells lacking VDAC1 (por1⁺), indeed, show a strong impairment of yeast growth in non-fermentable condition. In cell, yVDAC2 does not complement the lack of yVDAC1. We found that this is due to a very reduced expression of POR2 gene [1], since the addition of stimulatory factors, as induction of hSOD1, raises yVDAC2 and restore a respiratory function [1]. Genetic enrichment of yeast cells allowed us to purify yVDAC2, whose functional features showed to be the same of classical porins [2]. In this work we investigated the transcriptomic set up of por1⁺/mutant and of the same strain carrying hSOD1. In agreement with microarray analysis, the oxygraphic and enzymatic determinations show that the por1⁺/mutant has a profound modification of the bioenergetic metabolism, which is restored, but in a peculiar way, by the addition of hSOD1. The results and a possible interpretation of a different role of the two yVDAC isoforms will be presented.


Mitochondrial regulation of mitochondrial potassium channels
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Lecture will present the most interesting issues about new regulation and pharmacology of the mitochondrial potassium channels. There few mitochondrial potassium channels contributing to the potassium permeability of the inner mitochondrial membrane: ATP-regulated channel, calcium-regulated channels (large, intermediate and small conductance), voltage-regulated Kv1.3 and Kv7.4 channels, two-pore-domain TASK-3 channel and SLO2 channel. The primary function of the mitochondrial potassium channels is regulation of the mitochondrial membrane potential. Additionally, mitochondrial potassium channels alter cellular respiration, regulation of the mitochondrial volume and ROS synthesis. These channels are targets for various drugs including potassium channels openers such as diazoxide. The focus of the presentation will be on mitochondrial specific regulation of mitochondrial potassium channels such as...
interaction with respiratory chain proteins and gas molecules (CO, NO and H₂S). Additionally, stretch regulation of mitochondrial calcium-regulated potassium channels.

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The structural mechanism of transport by the mitochondrial ADP/ATP carrier

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Mitochondrial ADP/ATP carriers in the inner membrane belong to the mitochondrial carrier family and they transport ADP into the mitochondrial matrix for ATP synthesis, and ATP out to the cytosol to fuel the cell. They cycle between cytoplasmic-open and matrix-open states, in which the substrate-binding site is alternately accessible from either compartment for ADP or ATP binding. We will present data that will resolve their mechanism in atomic detail. In agreement with earlier published data, the structural evidence demonstrates that the carrier functions as a monomer [1] and has a single substrate binding site [2,3] and two salt bridge networks that regulate access to this site in a conformation-dependent way [3-5]. The structural analysis also explains the role of all conserved sequence features of mitochondrial carriers, showing that the mechanism is universal for this class of transport proteins.

References


The human SLC1A5 amino acid transporter: structure/function relationships, regulatory aspects and involvement in energy metabolism

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The human ASCT2 transporter mediates Na-dependent antiport of neutral amino acids across plasma membrane with the function of balancing intracellular amino acid pool. Despite the acronym standing for Ala, Ser and Cys Transporter, Gln revealed to be the preferred substrate. Gln taken up by ASCT2 is used for energy purposes under proliferative conditions. Indeed, the transporter is over-expressed in most human cancers, which use Gln as the major energy source. To characterize the human transporter, the protein was produced in P.pastoris, purified and assayed in proteoliposomes for transport function. A functional and kinetic asymmetry has been demonstrated in most human cancers, which use Gln as the major energy source. To characterize the human transporter, the protein was produced in P.pastoris, purified and assayed in proteoliposomes for transport function. A functional and kinetic asymmetry has been demonstrated in most human cancers, which use Gln as the major energy source. To characterize the human transporter, the protein was produced in P.pastoris, purified and assayed in proteoliposomes for transport function. A functional and kinetic asymmetry has been demonstrated in most human cancers, which use Gln as the major energy source. To characterize the human transporter, the protein was produced in P.pastoris, purified and assayed in proteoliposomes for transport function. A functional and kinetic asymmetry has been demonstrated in most human cancers, which use Gln as the major energy source.
reducing and oxidizing agents was investigated: transport activity increased upon treatment with reducing agent DTE, i.e., when Cys residues are reduced. Methyl-Hg, which binds to SH groups, was able to inhibit WT and seven out of eight mutants. C467A loses the sensitivity to both DTE activation and Methyl-Hg inhibition. The C467A showed a Km for Gln one order of magnitude higher than that of WT. Moreover, the C467 residue is localized in the substrate binding region of the protein, as suggested by bioinformatics on the basis of the recent 3D structure of hASCT2. Thus, C467 residue is crucial for both substrate binding and modulation of hASCT2. Indeed, physiological signaling molecules such as nitric oxide and GSH interact with the transporter through C467, modulating its transport function.

7b.

Bioenergetics of cancer

How do cancer cells harness energy?
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Cancer cells in vivo often exhibit ultrastructural and biochemical abnormalities of their mitochondria. This disorderliness is incompatible with the structural and functional ‘discipline’ required for oxidative phosphorylation (OXPHOS). Furthermore, the increase in glycolysis underlying Warburg effect serves the purpose of yielding metabolic intermediates shuttled towards the anabolic needs of the cancer, and less so for producing ATP. Under these conditions, the obvious question is where do cancer cells get their energy for growth and invasion? Here, evidence will be shown suggesting that mitochondrial substrate-level phosphorylation (mSLP) substantiated by succinate-CoA ligase can compensate for the impairment in OXPHOS in cancer cells. mSLP is fueled by catabolism of glutamine, a hallmark of many tumor types. The identification of mSLP and upstream pathways encompassing glutamine catabolism could be breakthrough targets for cancer management.

Reversing Wrinkled Skin and Lost Hair in Mice by Restoring Mitochondrial Function
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We have created an inducible (mtDNA-depleter mouse expressing, in the polymerase domain of POLG1, a dominant-negative mutation to induce depletion of mtDNA in different tissues. These mice showed reduced mtDNA content, changes in mitochondrial protein expression and reduced stability of mitochondrial oxidative phosphorylation complexes. We demonstrate that ubiquitous depletion of mtDNA in mice has profound and predominant effects on the skin resulting in wrinkles and hair loss. Development of skin wrinkles was associated with the hyperproliferation of epidermis, increased expression of MMPs and decreased expression of TIMP1. We also found increased inflammation that may be an underlying contributing factor in phenotypic changes in the skin. Histopathologic analyses revealed dysfunctional hair follicles. The mice also showed changes in expression of aging-associated markers including IGF1R, KLOTHO, VEGF, and MRPS5. The rescue experiment revealed that, by turning off the mutant POLG1 transgene expression and restoring the mtDNA content to the wild-type level in the whole animal (mtDNA-repleter) the skin and hair phenotypes revert to normal. These studies present first in vivo evidence that the skin wrinkles and loss of hair can be reversed by restoring mitochondrial function. Together, we have developed a mtDNA-depleter and repleter mouse model. This mouse will provide an unprecedented opportunity to achieve tissue-specific modulation of mitochondrial functions to determine, for various tissues and organs, the role of mitochondria in vivo and expand our knowledge of how mitochondria contribute to the pathogenesis of human diseases beyond their well-established roles in metabolism and cell death. The development of mtDNA-depleter and repleter mouse would also provide an impetus to the research about the development of preventative and therapeutic strategies to augment the mitochondrial functions for the treatment of mitochondrial and mitochondria related diseases.
Abstract not received

Metabolic vulnerabilities in solid tumors predicted by rapid ex vivo functional analysis
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Precision medicine has been equalized to genomic methods, but even if the definition is expanded with proteomic or other static methods, significant limits apply. In case of drugs targeting tumor metabolism, standard static markers fail to describe functional behavior of the targeted intra-tumoral processes and, hence, fail to preselect patients for such treatments. Therefore, without questioning the role of genomic methods, it is evident that additional tools are necessary to make further segregation among cancer patients. Here, using human breast cancer samples and respective cell lines, we show that our rapid ex vivo functional analysis reveals metabolic dependencies that cannot be predicted based on static properties like abundance of certain central enzymes or even number of mitochondria. Specifically, mitochondria in permeabilized samples turn selected substrates into wide variety of metabolites in varying amounts. Subsequent introduction of drugs cause disturbances in the metabolite profiles that in turn predict susceptibility to tested inhibitors or presence of pathways driving resistance. Analysis is conducted on viable tissue that eliminates artefacts arising from culturing the tumors. Taken together, functional predictive biomarkers can have critical role in detecting therapeutic opportunities in breast cancer patients that currently cannot be correctly assigned to available metabolic treatments.

Zebrafish (Danio rerio) as a model to study the pathophysiological role of the mitochondrial chaperone TRAP1
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Expression of the Hsp90-family chaperone TRAP1 is restricted to mitochondria and increased in most tumor types. We have previously shown that TRAP1 elicits the stabilization of the transcriptional factors HIF1α, thus generating a pseudohypoxic state that supports neoplastic growth. Following oncogenic activation of the Ras/ERK pathway TRAP1 is phosphorylated by ERK1/2, leading to inhibition of succinate dehydrogenase (SDH), the complex II of respiratory chain, and to the ensuing accumulation of the oncometabolite succinate, which eventually stabilizes HIF1α. However, little is known about the timing and importance of TRAP1 induction during the process of neoplastic onset and growth, as well as on the role of TRAP1 in the physiology of non-transformed cells.

To answer these questions, we decided to exploit the Zebrafish (Danio rerio) model, whose bioenergetic features are still poorly investigated. We found that TRAP1 is highly expressed during the early stages of Zebrafish embryo development, but its levels are dramatically decreased at 96 hpf. Notably, we detected an increase in TRAP1 mRNA and protein levels after HIF1α stabilization that caused a down-regulation of SDH activity. An in silico analysis of Zebrafish TRAP1 promoter unveiled the presence of hypoxa-responsive elements motifs that are the target of HIF1 transcriptional activity. Moreover, we found that TRAP1 is absent in Zebrafish pancreas, but it is highly expressed in a model of pancreatic adenocarcinoma induced by KRasG12D expression in Ptf1a positive cells, where it causes a strong decrease of SDH activity.

Altogether, these data suggest the existence of a positive feedback loop between HIF1 α and TRAP1, in which HIF1α stabilization induces TRAP1 expression acting at the transcriptional level, and in turn TRAP1, through SDH inhibition, leads to HIF1 α stabilization. This regulatory mechanism could play an important role for the adaptation of cells to fluctuating level of oxygen both in embryogenesis and during the process of neoplastic transformation.

The ATP synthase inhibitor protein IF1 plays a significant role in cancer metabolic flexibility
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In eukaryotes the ATP synthase complex is regulated by the endogenous inhibitor protein, IF1, an 81-residue protein in human mitochondria. It binds the enzyme, inhibiting its hydrolytic activity when the mitochondrial membrane potential ($\Delta \psi_m$) falls, as it occurs in ischemic tissues [1-2]. In the last decades it has been reported that cancer cells overexpress IF1, suggesting that it can play other critical roles in metabolic reprogramming [3-5]. The molecular mechanisms at the basis of these roles are still under debate and warrant in-depth investigations. We examined the role exerted by IF1 in human 143B osteosarcoma cells. In normoxia, metabolic and bioenergetic analysis of osteosarcoma cells showed reduced OXPHOS rate when the IF1 expression was abolished. Exposure of cancer cells to different hypoxia levels down to 0.1% O2 (about 1 $\alpha_M$), unexpectedly showed that $\Delta \psi_m$ was still preserved and it was independent of the presence of IF1. However, when we induced an anoxia-mimicking condition as it occurs in vivo in solid tumors, by collapsing $\Delta \mu_H^+$ with the uncoupler FCCP, the IF1-silenced clones reversed the ATP synthase activity and hydrolyzing ATP the electrochemical proton gradient was partially reconstituted. At variance, IF1 expressing cells did not hydrolyze ATP, indicating that IF1 completely inhibited the ATP synthase, preserving the cells energy charge. Therefore, IF1 overexpression in cancer contributes the metabolic flexibility of the cells in both normoxia and anoxia (or near-anoxia).


8a. Antennae and photosystems

Cryo-EM structure of maize PSI-LHCI-LHCII supercomplex
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Photosynthesis is one of the most amazing chemical reactions in the planet. The light-driven electron transport of photosynthesis is accomplished by photosystems I and II (PSI and PSII). In the natural environment, the fluctuating illumination can cause unequal excitation of the two photosystems due to the different light absorption properties of their antenna systems. Balanced light harvesting is crucial for efficient photosynthesis, and plants have evolved sophisticated regulatory mechanisms in order to optimize the photosynthetic efficiency and to avoid photo-damage. State transitions are one of the short-term adaptation mechanisms[1, 2]. During state transitions, the trimeric LHCII is reversibly phosphorylated and de-phosphorylated, and migrates between the two photosystems. Under light conditions favoring PSII excitation, over-excitation of PSII leads to the activation of LHCII kinase and subsequent phosphorylation of the N-terminal region of LHCII. A portion of the phosphorylated LHCIIIs move laterally within the thylakoid membrane from PSII to PSI, forming the PSI-LHCI-LHCII supercomplex, increasing energy transfer towards PSI core[3, 4]. We solved the cryo-electron microscopy (cryo-EM) structure of maize PSI-LHCI-LHCII supercomplex at 3.3 Å resolution. Total of 21 protein subunits, 202 chlorophylls, 47 carotenoids and numerous other cofactors were identified in the final structure. Two PSI core subunits (PsaN and PsaO) absent in the previously reported crystal structures were identified. In addition, the phosphorylation site in LHCII was solved and the detailed interactions between LHCII and PSI were revealed. The structure showed that PsaN and PsaO are at the PSI-LHCI interface and the PSI-LHCII interface, respectively. Each subunit relays excitation to PSI core through a pair of chlorophyll molecules, thus revealing previously-unseen paths for energy transfer between the antennas and the PSI core.

References:
Structure and Function of Photosystem I Complexes and Potential Implications on Photosynthetic Electron Transport Regulation in Microalgae

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The PSI-LHCI complex is at the crossroad of controlling linear (LEF) and cyclic electron flow (CEF). In *Chlamydomonas reinhardtii*, PSI-LHCI is involved in the formation of a potential CEF supercomplex comprising in addition Cyt b6f, FNR, LHCI, LHCII, PGRL1, PETO, ANR1 and CAS. There is further evidence that the dynamic association of FNR with PSI-LHCI might channel electrons towards distinct routes [1]. To mechanistically understand the role of PSI-LHCI in partitioning of LEF and CEF, structure and function was analyzed in detail. Cross-linking experiments followed by immunodetection and mass spectrometry allowed the determination of the spatial configuration of LHCI at *C. reinhardtii* PSI. Eight LHCI subunits are bound to the PSI core at the side of PSAF in two layers: LHCA1-LHCA8-LHCA7-LHCA3 from PSAG to PSAK in the inner layer and LHCA1-LHCA4-LHCA6-LHCA5 in the outer layer. LHCA2 and LHCA9 are bound to PSAB between PSAG and PSAH of the PSI core: PSAG-LHCA9-LHCA2-PSAH. LHCA1 was identified as a potential interaction partner of FNR at PSI in *C. reinhardtii*. Further, a PSI-LHCI-FNR-cyt b6f supercomplex was isolated from *C. reinhardtii* under anaerobic conditions. Single particle analysis after electron microscopy identified top-view projections of the supercomplex. Based on molecular modeling and mass spectrometric analysis we propose a model in which dissociation of LHCA2 and LHCA9 from PSI supports formation of this CEF supercomplex, as a Δlhca2 knockout mutant possesses constitutively enhanced CEF. Thus, we propose, that partitioning of LEF and CEF requires a remodeling of the PSI-LHCI containing protein complexes in *C. reinhardtii*.

References

X-ray and NMR studies of the complex between Ferredoxin and Photosystem I

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Photosystem I (PSI) mediates the final step in light-driven electron transfer to the stromal electron carrier protein ferredoxin (Fd). Although structures of electron transfer complexes (ETCs) comprising Fd and Fd-dependent enzymes are available, that of the pivotal PSI-Fd complex remains unknown. To understand the structural basis for the dynamics and efficiency of the electron transfer reaction mediated by Fd, we studied the PSI-Fd ETC by X-ray crystallography combined with biochemical and NMR spectroscopic analyses. Two types of recombinant Fd from *T. elongatus* were prepared for complex formation: the wild-type Fd and a redox-inactive Gallium-substituted Fd with a [2Ga-2S] cluster to mimic oxidized Fd [1]. X-ray structures of both complexes were solved at 4.3 and 4.2 Å, respectively [2]. The trimeric PSI complex binds three Fds in a non-equivalent manner. While each is recognized by a PSI protomer in a similar orientation, the distances between Fds and the PSI redox centres differ (ranging from 8.3 to 9.6 Å). Fd binding to the PSI trimer thus entails loss of the exact three-fold symmetry of the latter’s soluble subunits, inducing structural perturbations which are transferred to the lumen through Psaf. The asymmetric arrangement of Fd and concerted structural changes in PSI may result in sequential ETC
formation. We propose a dynamic structural basis for productive complex formation, which supports fast electron transfer between PSI
and Fd.


Energy transfer in LHCII resolved by 2D electronic spectroscopy at ambient and low temperatures

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Efficient photosynthetic light energy conversion depends on the ability of the photosynthetic apparatus to transfer the captured solar
energy to the photochemical reaction centres without losses. Electronic dipole-dipole (exciton) couplings between chlorophylls in LHCII,
the main light-harvesting antenna of Photosystem II, result in excitation energy transfer (EET) times from hundreds of femtoseconds to
picoseconds. Despite the abundance of data, the presently existing models report different kinetics of EET in LHCII. Two-dimensional
electronic spectroscopy (2DES) is a powerful technique for mapping EET pathways. We have measured the excited-state dynamics in
isolated plant LHCII by 2DES at ambient and cryogenic temperatures. Using broadband excitation pulses tuned to the lowest-lying
electronic excited state of chlorophyll $a$ in LHCII, we resolved simultaneous downhill and uphill EET. The 2DES data unequivocally
confirm that spectral equilibration in the Chl $a$ exciton manifold of LHCII is complete within ca. 10 ps at room temperature (Akhtar et al.,
J. Phys. Chem. Lett. 8, 2017). It can be expected that as the temperature decreases, the population of higher-energy states by uphill
EET will diminish, according to the Boltzmann distribution. This was experimentally confirmed by comparing the uphill/downhill cross-
peaks in the 2D spectra at different temperatures. Furthermore, we found strong non-trivial temperature dependence of the EET kinetics
in the complex. A temperature-dependent model of EET in LHCII can be proposed based on these results.

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Global Spectroscopic Analysis to Study The Regulation Of The Proton Motive Force In Photosynthetic Organisms

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In natural variable environments, photosynthetic organisms rapidly adjust photosynthesis for optimum balance between photochemistry
and photoprotection. This mainly occurs via the generation of the proton motive force (pmf) thanks to the coupling between electron
transfer in thylakoid membranes and proton release in the inner lumen compartment. The pmf not only comprises a $H^+$ gradient ($\Delta pH$),
but also an electric field ($\Delta V$), negative on the stromal side. The latter is generated by electron flow, due to the asymmetric location of
the electron donors and acceptors of the different photosynthetic complexes. The generation of a $\Delta V$ also affects the absorption
characteristics of some photosynthetic pigments, leading to a shift of their absorption spectrum. This phenomenon, known as the
electrochromic shift (ECS), can be used to monitor the pmf in vivo. Recent studies in the model plant Arabidopsis thaliana based on
time resolved analysis of the ECS signal have suggested an active role of ion fluxes across the thylakoid membranes in the regulation of
the pmf mainly via the activity of the TPK3 potassium channel and the K+/H+ antiporter KEA3. In this talk, I will discuss the pmf
features of *Chlamydomonas reinhardtii*, *Arabidopsis thaliana* and the diatom *Phaeodactylum tricornutum* using *tpk3* and *kea3* mutants and/or global fit analysis of the ECS signal. Differences and common features will be highlighted and discussed in terms of the different responses of photosynthesis to the environment.


8b. Variability of mitochondrial functions and pathologies

Sirtuins, NAD+ and Mitochondrial Bioenergetics: a Critique
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There is intense interest among cellular physiologists in the possible roles that protein α-lysine acylation may play in the control of mitochondrial metabolism, bioenergetics and transcriptional control. Multiple acyl-CoA species, particularly in the matrix, are able to acylate mitochondrial proteins by an apparently non-enzymatic mechanism, and a steady-state acylation status is maintained by sirtuin deacylases, with the stoichiometric utilization of NAD+. Protein acylation is usually, but not universally, considered to inhibit function, and the corollary is that enhancing sirtuin activity, for example by increasing local free NAD+, will decrease steady-state acylation and have a beneficial effect. This postulate is the basis of a vast literature dealing with normal physiology, aging, and pathological conditions from Alzheimer’s to hearing loss. The bioenergetic community has the ability to critically evaluate this literature, and in particular to establish the extent to which claims are consistent with our understanding of mitochondrial function, and, conversely, to determine whether accepted metabolic and bioenergetic parameters may need to be reassessed to take account of acylation.

**NAD+ homeostasis plays role in mitochondrial biogenesis during beige adipocyte differentiation**

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Classically, adipose tissue or adipocytes are classified either as white (unilocular) adipocytes, responsible for fatty acid storage, or as brown (multilocular) adipocytes, responsible for fatty acid oxidation and heat generation. This dichotomy was revised recently by the discovery of beige adipocytes that morphologically look like white adipocytes, while are functionally similar to brown adipocytes. Apparently, beige adipocytes are crucial in energy homeostasis and in metabolic diseases such as type II diabetes. Beige adipocytes differentiate from a myoblastoid precursor and yet little is known about the differentiation process. Apparently, a major determining factor towards beige differentiation is the deacetylation of peroxisome proliferator-activated receptor γ. We wanted to enlarge this picture and assess whether the modulation of the NAD+ pool or cellular energy sensors have role in beige determination or differentiation.

We used a PARP inhibitor, olaparib, an NAD+ precursor, nicotinamide-riboside to boost cellular NAD+ pools and AICAR for the activation of AMPK. Human adipose tissue-derived stem cells were differentiated to white and beige adipocytes, in addition, we treated white adipocytes with the drug indicated beforehand. We assessed markers of beige differentiation, mitochondrial oxidation,
mitochondrial morphology and members of the cellular energy sensor web. Our data suggest that the reprogramming of the energy sensor web plays role in the (trans)differentiation process towards beige adipocytes.

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Interaction of Alzheimer’s disease triggering amyloid beta peptides with membranes and organelles: bioenergetical consequences
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Alzheimer’s disease affects 47 million people worldwide. No significant progress has been made in research for early diagnosis and efficient therapy. One of the characteristic histopathological markers of AD is the presence of extracellular plaques consisting predominantly of amyloid-β peptides (Aβ) with 40 or 42 amino acids. Recent research suggests that monomers and/or soluble oligomers of Aβ are responsible for AD symptoms but not the extracellular plaques. To study cellular and organelle trafficking of Aβ, to identify its target(s), Aβ42 peptide monomers/small oligomers were externally applied to mammalian cells (human neuroblastoma and rat oligodendroglia). Monomeric/oligomeric peptides entered cells, as proven by confocal fluorescence microscopy by employing fluorescently labeled Aβ42 peptides. In time and space the pathway of Aβ peptides from the outside of the cell across the plasma membrane to internal target membranes of specific organelles was tracked. Aβ peptides initially co-localized with the plasma membrane and thereafter entered the cells and trafficked to organelles, e.g., predominantly to lysosomes. The deep insertion of Aβ in lipid bilayers that subsequently induced membrane perturbations and alterations in lipid dynamics was verified by neutron scattering and fluorescence polarization. Aβ affected numerous physiological cell parameters, such as ROS and ATP concentration, viability (necrosis/apoptosis), mitochondrial membrane potential and viscosity. The Aβ-induced alterations might cause neuronal cell death and AD pathology.

Mitochondrial cAMP augments Ca2+ uptake into the organelle to support steroidogenesis
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Cytoplasmic Ca2+ signals are followed by mitochondrial Ca2+ uptake. The ensuing mitochondrial Ca2+ signal regulates pivotal processes (e.g. ATP synthesis, apoptosis) and modulates specialised cell functions such as hormone secretion. Ca2+ elevations also activate the matrix soluble adenylyl cyclase (sAC) resulting in an intramitochondrial cAMP (mt-cAMP) signal which was recently shown to augments aldosterone production in human adrenocortical H295R cells [1]. Since steroidogenesis positively correlates with intramitochondrial [Ca2+] in various experimental paradigms, we hypothesised that mt-cAMP supports steroidogenesis via the intensification of mitochondrial Ca2+ accumulation.

Silencing of sAC decelerated mitochondrial Ca2+ accumulation whereas membrane permeable cAMP analogues and the inhibition of the matrix phosphodiesterase (PDE2A) both accelerated mitochondrial Ca2+ uptake. The augmenting effect of mt-cAMP on mitochondrial Ca2+ accumulation was not dependent on mitochondrial membrane potential and was insensitive to the blockade of mitochondrial Ca2+ efflux. Finally, mitochondrial overexpression of wild-type sAC increased mitochondrial cAMP formation, accelerated mitochondrial Ca2+ uptake and potentiated aldosterone production as compared to the overexpression of the enzymatically inactive mitochondrial sAC variant.

Overall, Ca2+-dependent formation of mt-cAMP boosts additional Ca2+ uptake into the organelle and this positive feed-back loop may accelerate the hormonal response when rapid hypersecretion of mineralocorticoids is essential (e.g. blood loss).

References
Cold and other environmental factors induce “browning” of white fat depots—development of beige adipocytes with morphological and functional resemblance to brown fat. Similar to brown fat, beige adipocytes are assumed to express mitochondrial uncoupling protein 1 (UCP1) and to be thermogenic due to the UCP1-mediated H⁺ leak across the inner mitochondrial membrane. However, this assumption has never been tested directly. We used mitochondrial patch-clamping to compare H⁺ leak properties in brown fat and beige fat of two distinct white fat depots: inguinal and epididymal [1]. We demonstrated that β3-adrenergic receptor stimulation leads to development of beige adipocytes in both inguinal and epididymal fat (lipid droplet fragmentation, active mitochondrial biogenesis). However, while all inguinal beige mitochondria exhibit UCP1-dependent H⁺ current similar to that of brown fat, the large majority of epididymal beige mitochondria have no UCP1-dependent H⁺ leak. In contrast to the current belief that all beige adipocytes are capable of UCP1-dependent thermogenesis, our data clearly demonstrate the existence of UCP1-positive and UCP1-negative beige fat cells. Although UCP1-negative beige adipocyte mitochondria are devoid of UCP1-dependent thermogenesis, these cells are thermogenic due to robust mitochondrial creatine-dependent substrate cycling. Thus, we demonstrate the existence of a UCP1-negative thermogenic adipocyte within white fat depots. A better understanding of the thermogenic pathways employed by the UCP1-negative beige fat cells will help develop new approaches to the treatment of the metabolic syndrome.


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