mechanisms by which mitochondrial enzymes generate toxic free radicals. Due to its low potential cofactors, mitochondrial complex I is a prime candidate for significant radical production. Indeed, studies on both isolated complex I [1] and intact mitochondria [2] have shown that complex I can generate significant levels of the reactive oxygen species (ROS) superoxide and hydrogen peroxide. During NADH oxidation, studies of the isolated enzyme have described a single site of ROS production (the flavin mononucleotide). Conversely work on intact mitochondria has suggested that a second site contributes during NADH oxidation, and the locus of ROS production during reverse catalysis is unclear. Reverse catalysis is not possible with the isolated enzyme. To resolve the mechanisms of ROS production in both directions of catalysis we have prepared tightly coupled submitochondrial particles (SMPs) from bovine heart mitochondria. Because they are inside out with respect to mitochondria we have direct access to the catalytic sites of the respiratory complexes, and the ability to detect the ROS produced directly (without interference from any antioxidant protection systems). Here, we describe how ROS production by complex I responds to the NAD^+/NADH ratio, to the presence of inhibitors, and to the proton motive force. The influence of semiquinone intermediates is explored during catalytic turnover. Consequently, we provide a unified molecular mechanism for ROS production by complex I.

References

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5P.16 Growing fast and dying young: A mitochondrial coupling problem?

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Organism body size is known to be positively correlated with longevity at least at inter-specific level. However, the interplay between growth and senescence is still poorly documented at the intra-specific level, especially in ectotherms. Here, we present an intra-species comparison of two neighbouring populations of frogs (*Rana temporaria*) that present large differences in both body mass (2–3 fold, at same age) and lifespan (being shorter in large morph than in small one). In the light of the mitochondrial free radical theory of aging, we hypothesised that an alteration in the mitochondrial functioning would play a part in differential growth rates and survival. Thus, we assessed key parameters of frog's liver mitochondria from both populations enabling a comparison between fast and low growth rate phenotype (hereafter called fast GR and low GR).Our data shows that the efficiency of oxidative phosphorylation process (ATP/O ratio), in liver mitochondria, was three-fold higher in fast GR frogs than in low GR ones ($P<0.05$). However, no age effect (with 3, 4 and 5 years-old individuals) was demonstrated on ATP/O ratio, neither in low nor in fast GR. Interestingly, phosphorylating (State 3) and non phosphorylating (State 4) respiration rates were identical in both populations ($P=0.87$ and $P=0.30$, respectively) while the maximal rate of ATP synthesis was 2.4-fold higher in liver mitochondria of fast GR than in low GR phenotype (2.15 ± 0.16 *vs.* 0.91 ± 0.24 nmol ATP/min mg protein, respectively; $P<0.05$).As Cytochrome Oxidase activity remained unchanged in liver mitochondria from both frog populations it could not explain these original results. Nevertheless, the higher rate of ATP hydrolysis by the ATP synthase complex observed in fast GR phenotype (62.82 ± 10.75 *vs.* 33.34 ± 7.24 nmol ATP/min mg protein, fast GR and low GR phenotype, respectively; $P<0.05$) could partly explain our results. For the first time, we describe an important age-independent association between mitochondrial plasticity (affecting the ATP production) and growth rate. It is now important to describe how such plasticity, which affects the efficiency of oxidative phosphorylation process, impact on ROS production and antioxidant defenses.

Keywords: Amphibian, lifespan, growth rate, mitochondria, ATP/O ratio.

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5P.17 Design and engineering of superoxide oxidoreductase activity in new artificial proteins

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Superoxide(SO) is a ubiquitous product or by product among cellular metabolic reactions, though many sources remain to be identified. Its production may be purposeful as a cell-signaling molecule, through conversion to hydrogen peroxide, or as a bactericidal agent used by neutrophils. However, SO can also have deleterious effects, especially after converting to more potent radicals *via* peroxide or nitrous oxide. These ROS are implicated in oxidative stress and age-related cellular malfunctions. Resolution of ROS production at specific cofactor sites within implicated proteins, such as Complexes I and III, has been elusive. This is most likely due to the presence of multiple sites of generation. To address this challenge we use step wise engineering approaches to design artificial enzymes that can resolve the mechanisms of ROS production at individual sites. Artificial 4-helixbundle proteins have been synthesized that ligate hemes, flavins and quinones, allowing for 1 or 2 electron transfer (ET) to O_2. So far, results have been obtained for heme proteins. Flavin- and quinone-containing proteins will soon be studied for their SO-generating activities. Two heme-ligating variants were derived from an artificial oxygen transport protein [1] to examine two means of SO generation: inner-and outer-sphere ET. The parent protein bound O_2 stably and SO generation was undetectable. One variant was redesigned to render the heme water-accessible. This destabilized the oxyferrous state and yields SO by inner-sphere ET as in globins. The other variant was redesigned to lack strain essential for O_2-binding but to retain water-inaccessibility. As designed, it failed to bind O_2 and yielded SO by outer-sphere ET. Both variants produce SO at rate that matches SO-generating enzymes such as NADPH oxidases. SO was detected with the SO-specific chemiluminescent probe methyl-cypridina-luciferin analogue (MCLA). SO generation was monitored by stopped-flow while heme oxidation was monitored independently by UV–Visible spectroscopy. This work demonstrates the design and engineering of multiple mechanisms of ROS production in artificial proteins that display catalytic activity approaching that of natural enzymes.