Can All Major ROS Forming Sites of the Respiratory Chain Be Activated By High FADH₂/NADH Ratios?

Ancient evolutionary constraints determine mitochondrial ROS formation

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Aspects of peroxisome evolution, uncoupling, carnitine shuttles, supercomplex formation, and missing neuronal fatty acid oxidation (FAO) are linked to reactive oxygen species (ROS) formation in respiratory chains. Oxidation of substrates with high FADH₂/NADH (F/N) ratios (e.g., FAs) initiate ROS formation in Complex I due to insufficient availability of its electron acceptor (Q) and reverse electron transport from QH₂, e.g., during FAO or glycerol-3-phosphate shuttle use. Here it is proposed that the Q-cycle of Complex III contributes to enhanced ROS formation going from low F/N ratio substrates (glucose) to high F/N substrates. This contribution is twofold: 1) Complex III uses Q as substrate, thus also competing with Complex I; 2) Complex III itself will produce more ROS under these conditions. I link this scenario to the universally observed Complex III dimerization. The Q-cycle of Complex III thus again illustrates the tension between efficient ATP generation and endogenous ROS formation. This model can explain recent findings concerning succinate and ROS-induced uncoupling.

1. Introduction

The respiratory electron transport chain (ETC) catalyses redox reactions in which an important part of the energy present in the "high energy" electrons from metabolites such as carbohydrates, proteins, and fats is stored in a proton motive force (PMF; Δp) across the mitochondrial inner membrane, because electron transfer reactions are efficiently coupled to proton "pumping" across this membrane.[1,2] An important part of the explanation for the extraordinary amounts of ATP that can be generated in this way lies in the extreme subdivision of reactions from start to finish and the use of molecular oxygen (O₂) as final electron acceptor. The last reaction is catalyzed by the ultimate ETC complex, Cytochrome c oxidase, forming water, see ref. [3] and references therein. However, this allows reverse reactions to occur. On top of that, O₂ can act as a double-edged sword by occasional premature reactions with some of the reduced centers of the ETC, giving rise to internal superoxide anions and other reactive oxygen species (ROS), which are highly damaging to the eukaryotic cell,[4,5] as they can initiate detrimental reaction cascades involving all the major groups of biological molecules. Because of the long coevolution of eukaryotes and internal ROS formation, low amounts of ROS can signal efficient antioxidant responses and thus have a beneficial effect as reflected in the concept of mitohormesis.[6–8] The superoxide anion is quickly converted to relatively stable hydrogen peroxide (by superoxide dismutase), but this ROS species can pass membrane barriers,[9] making it both a more long-range signaling molecule and potentially more detrimental.[10] Though the subject is heavily debated, the general opinion still seems to be that the most significant ETC contributions to ROS formation come from NADH:ubiquinone oxidoreductase (Complex I; on the matrix side) and ubiquinol cytochrome c oxidoreductase (Complex III; on the intermembrane side), but the physiological relevance of many studies involved has been questioned.[4,11] The PMF can be converted to ATP, containing abundant chemical potential energy, released upon hydrolysis,[2] enabling practically all eukaryotic cellular processes, but the PMF can also be dissipated in the form of heat by (pharmacological) uncoupling agents/proteins, strongly influencing ROS formation (see below).

2. FADH₂/NADH (F/N) Ratios Are Crucial For ROS Formation

The redox state of one of the central ETC electron carriers, ubiquinone, or coenzyme Q, seems to be a crucial determinant
in ROS formation by the respiratory chain. An increase in the steady-state amount of reduced Q (ubiquinol; QH$_2$) at the expense of oxidized Q, reflected in a high QH$_2$/Q ratio can most easily occur when abundant energy-rich substrate is being oxidized. Let us compare three (idealized) scenarios: low level glucose oxidation, “turbo glycolysis”, and FAO (aka beta-oxidation). The crucial electron carriers to consider are FAD/FADH$_2$ and NAD$^+$/NADH. All electrons entering the ETC in these cases either come from soluble NADH via Complex I, or via a prosthetic FADH$_2$ group attached to several ETC complexes, directly reducing Q without proton pumping (see Figure 1). I proposed that the FADH$_2$/NADH (F/N) ratio (i.e., the number of electrons passing Complex I relative to the number reducing Q directly) during catabolism is a crucial determinant of ROS formation.\cite{12} It is necessary to clarify the concept from the outset to avoid confusion. First of all, the soluble cofactor NADH and the covalently attached FADH$_2$ of ETC dehydrogenases are used here to stress the difference between reducing Q via complex I or doing so directly. It links to standard biochemical usage, as in “The TCA cycle produces 3 NADH and 1 FADH$_2$.\cite{10} Electrons from NADH enter Complex I via a covalently attached Flavin Mono Nucleotide, while an anabolic, directly Q reducing enzyme complex, Dihydroorotate dehydrogenase, involved in Pyrimidine biosynthesis, uses the same prosthetic group. Use of the catabolic F/N concept avoids these confusing details.

During complete oxidation of glucose (using glycolysis, the aspartate/malate shuttle to import NADH, plus further mitochondrial breakdown of pyruvate by oxidative decarboxylation and the TCA cycle) the respiratory chain will have to oxidize 2 FADH$_2$ and 10 NADH molecules, resulting in an F/N ratio of 0.2. In turbo glycolysis, when aspartate/malate shuttling is insufficient, the glycerol-3-phosphate dehydrogenase shuttle (GPDH) allows the very rapid entry of the electrons of cytoplasmic NADH in the form of (less energy rich) FADH$_2$. If all NADH entered in this fashion, we would end up with an F/N ratio of 0.5, as 2 cytoplasmic NADHs are exchanged for mitochondrial FADH$_2$s. During the oxidation of (saturated) FAs in the mitochondrion, coupling the product of FAO (a repeating four step process generating NADH and FADH$_2$ via the ETF/ETF:QO complex; indicated in dark green in Figure 1), acetyl-CoA, to the TCA cycle, will likewise generate high F/N ratios (approaching 0.5 with increasing FA length).\cite{8,12} Such high F/N ratios (in the presence of large amounts of reducing equivalents) would create a “problem” for Complex I. It will encounter much higher levels of ubiquinol (QH$_2$), the reduced, occupied, form of its acceptor ubiquinone (Q) during FAO than during (non-turbo)

Figure 1. ROS formation in Complex I and III due to high QH$_2$ levels during beta oxidation or use of the GPDH shuttle. A) Glucose oxidation (low F/N ratio) with adequate electron-acceptor (Q) for Complex I and III (during Q-cycle). B) High F/N ratios with insufficient electron-acceptor (Q) for Complex I and III; ROS formation at Complex I and III (via RET (“?>”). RET depends on high QH$_2$ levels and a large A$p$ (indicated). C) Decreased ROS production by either less FA oxidation (e.g., use of peroxisomes) or enhanced QH$_2$ oxidation (e.g., more uncoupling). Complex I (not to scale\cite{79}; purple, Complex II light green, ETF/ETF:QO complex dark green, GPDH pink, BC$_2$ Complex (Complex III) blue, ubiquinone (Q) red, electron flow black arrows. Quinol (QH$_2$) is oxidized at the Q$_p$ site, located on the positive (or outer/intermembrane space) side of the membrane, and produced at the Q$_n$ (“negative”) site. The specific ROS-generating site of complex I upon RET is debated: The flavin containing site (IF)\cite{79} or the Q binding site (IQ).\cite{80} IMS, intermembrane space; F, FADH$_2$ oxidizing complex; N, NADH dehydrogenase (Complex I). For details see text. Extended and adapted with permission.\cite{24} Copyright 2017, the Authors.
glucose oxidation. All other things being equal, the high F/N ratio would translate into a high QH2/Q ratio for Complex I. This, in turn, can give rise to reverse electron transport (RET; due to a combination of a larger membrane potential (Δp) and high QH2/Q ratios). Thus, we can predict more intense ROS formation in Complex I during FAO (or turbo glycolysis). This is probably the reason why the expression of mitochondrial GPDH, which is essential in turbo glycolysis, is suppressed in most tissues.[13] An important aspect to stress is the large increase in F/N ratio possible: A 2.5-fold increase can easily disturb homeostatic equilibria. It is instructive to compare this to monitoring ATP need via AMP/ATP ratios, as occurs for example in muscle by the universal eukaryotic enzyme AMP kinase (AMPK), activated by AMP (this enzyme possibly originated from a glucose-sensing mechanism that was linked to, among others, mitochondrial control[14]). Because of the reaction catalyzed by adenylate kinase (2ADP ↔ ATP + AMP), cellular AMP/ATP ratios vary as the square of the more stabilized ADP/ATP ratio.[15] Comparing muscle cells with and without exercise, this translates into a 2- to 3-fold change. Likewise, manipulating ROS formation by changing F/N ratios to signal the need for cellular adaptations is frequently observed (see below).

3. Internal ROS Formation Explains a Host of Eukaryotic Features

I speculated that the resulting ROS formation by Complex I could explain many eukaryotic features, at both cellular and higher order levels. At the cellular level, the increase in severe, indiscriminately reacting, ROS produced by the new endosymbiont (the mitochondrion to be) inside the merged pre-eukaryote, would constitute a driving force to have FAO occur in new cellular organelles, peroxisomes, generating NADH in the “absence” of FADH2 for the ETC.[12,16] Peroxisomes most likely evolved to decrease the total amount of FAO in mitochondria, lowering the overall F/N ratios.[8,12,17] Recent findings that peroxisomal FAO is almost completely derived from the endosymbiont (i.e., most genes involved are of alpha-proteobacterial origin), copying the process except for the step involving FAD/FADH2[12] and that new peroxisomes can be formed when mitochondrial and ER-derived pre-peroxisomes come together are in line with predictions of the model.[19,20] To avoid confusion, I should mention that the oldest, most conserved, peroxisomal pathway is beta-oxidation. The relative distribution of this pathway over mitochondria and peroxisomes can differ profoundly between eukaryotes, due to the trade-off between ATP and ROS formation (see below). High energy consumers such as animals use more ATP, so they only oxidize very long chain FAs in peroxisomes, which though a minor component, can signal increased FAO and are very effective in lowering F/N ratios.[8,12] Besides beta-oxidation, peroxisomes have acquired a diversity of other functions in different eukaryotes, which explains their importance for normal neuronal function in the absence of FA catabolism.

Some of the other eukaryotic cellular innovations that make sense in this light are the evolution of carnitine-dependent mitochondrial import of FAs, supercomplex formation, and mitochondrial uncoupling proteins,[8,17,21] which will be dealt with below. At higher order levels, the surprising fact just indicated, that cells consuming large amounts of energy such as neurons do not use the most abundantly stored, energy-rich compound (fat, i.e., esterified FAs), can be understood invoking the model. Use of “high-ROS” substrates such as FAs causes extensive oxidative damage because of the need for mitochondrial FAO. Here, I should mention that circumstantial evidence for the characterization of FA as a “high-ROS” substrate is very strong, but direct evidence using isolated mitochondria is still limited (see below). One might ask why other intensive ATP consumers such as heart or skeletal muscle cells do (constitutively) use FAO. Individual neurons contain irrereplaceable information (encoding memory by their highly specific interconnections build up in a lifetime). This does not hold true for the other major energy-consuming cells just mentioned, which allow higher numbers to be lost and/or replaced.

4. The Conflict Between Efficient ATP Generation and ROS Formation Has Highly Variable Outcomes

That a sudden large membrane potential (Δp) shift, especially in conjunction with high F/N ratio substrates, would lead to intense ROS formation is reflected in further adaptations to the ETC in high ATP consuming tissues, such as liver, kidney, and heart, which use varying catabolic substrates. In these organs, different residues of cytochrome c can be phosphorylated at high levels, leading to reductions in Complex IV activity and, thus, Δp, which would lower ROS formation.[22] There are indications that AMPK, mentioned above, might be involved.[22] This might make sense, because a strong induction of catabolism to heighten ATP production would give rise to the danger of ROS formation. AMPK is even suspected to be regulated by ROS directly via redox sensitive cysteine residues. However, recent experimental probing could find no evidence for such direct ROS monitoring.[23]

At the other end of the spectrum, we have eukaryotic adaptations that reflect less dominance of efficient ATP generation, leading to lower intrinsic ROS formation. In yeasts (characterized by dormancy until plenty of catabolic substrate is available) we find many alterations that can lessen ROS formation. At its most extreme, we find the disappearance of Complex I in Saccharomyces cerevisiae. Instead yeast has alternative, non-proton pumping (!), NADH dehydrogenase(s) feeding into the Q-pool and an alternative oxidase using QH2 as substrate which completely bypasses Complex III and IV. This highly inefficient system should lower endogenous ROS formation very effectively. I have speculated about the selective forces giving rise to this phenotype in ref.[24]. Not all yeasts differ so extremely from animals in their mitochondrial pathways. Ustilago maydis has a normal mitochondrial ETC, GPDH, and FAO as well as inducible peroxisomal FAO. However, it also encodes an alternative to Complex I, located at the intermembrane side, and an alternative oxidase (responsible for 20% of the O2 consumption, but likely activated upon incubation with ROS, as occurs in plants; see below).[25,26] Of course, the eukaryotic uptake of the cyanobacterium that would become the chloroplast, heavily influenced the mitochondrial ETC, as energy efficiency could thus become much less important for plants. Indeed, plant mitochondria contain multiple FAD-containing NAD(P)H dehydrogenases and an alternative oxidase contacting the Q pool.[27] All these energy inefficient alternatives make the (effect of) F/N ratios of certain
catabolic pathways much harder to predict in plant (tissues) as well as yeasts. Even so, though these alternatives would lower the use of Complexes I, III and IV (and, thus, Ap), the F/N ratio of catabolic substrates (presuming the presence of Complex I) would still vary considerably, influencing mitochondrial ROS formation.

5. How Does the Q-Cycle Fit in?

Ubiquinol cytochrome c oxidoreductase (the bc1 complex; Complex III) is an almost universally present and crucial part of the ETC in mitochondria of eukaryotic cells. It catalyses a redox reaction that allows an important part of the energy present in the “high energy” electrons to end up in the proton motive force (PMF) across the mitochondrial inner membrane, because its electron transfer reactions are efficiently coupled to proton transfer.[28] The biochemical reaction in the absence of ROS formation and/or RET is given as:

\[
QH_2 + 2 \text{Cytochrome C}^{1+} + 2H^+ \text{matrix} \rightarrow Q + 2 \text{Cytochrome C}^{3+} + 4H^+ \text{intermembrane space}
\]

The enzyme constitutes the only available route of further electron flow from several possible upstream catabolic pathways (e.g., the TCA cycle enzyme succinate dehydrogenase, also referred to as Complex II[29]; the ETF/ETF:QO complex involved in FAO[30]; the GPDH shuttle,[13] and the oxidation of NADH by Complex I), all reducing the Q pool (Figure 1).[30] Quinones, such as Q, are lipophilic mobile electron carriers endowing electrons to end up in the proton transfer (to the hydrophilic NADH (mostly matrix) molecule and (intermembrane) cytochrome c protein. The complex catalyses the exergonic two-electron oxidation of QH2 to two one-electron reductions of cytochrome c while transferring protons across the mitochondrial inner membrane (by releasing four protons while consuming two matrix protons). This intricate process is accomplished using a two-step reaction, known as the Q-cycle, during which electron flow is bifurcated. Step 1: QH2 is oxidized at the Qp (Qo) site, found on the positive (intermembrane space; IMS) side or “outside” of a bacterium. One electron passes down a high potential chain, via the Rieske iron sulphur group and cytochrome c1, in the end reducing cytochrome c at this side. The other electron passes the low potential chain via b-type haem groups to partially reduce Q, likely forming a (stable) semi-quinone at the Qn (Qi) site, located at the matrix (negative) side of the membrane or “inside” of a bacterium. Step 2: A second QH2 is oxidized at the Qp site, again using one electron to reduce a cytochrome c molecule, while the other electron follows the low potential chain and allows a QH2 to be released from the Qn site (using two matrix protons). This means that normally operating Complex III both produces and consumes Q (l), in order to efficiently contribute to the PMF.

We can predict that Complex III shall, both directly and indirectly, contribute to elevated ROS formation whenever we have both a high Ap and high QH2/Q ratios. Why? One might say that because Complex III also uses Q as substrate, Complex I must compete with it, as well as with the FADH2 containing complexes. Apart from that, Complex III will start to produce more ROS itself when electrons cannot easily be given back to Q.

In both cases electron flow will be somewhat halted, RET can occur and vulnerable reduced sites in both complexes can react with O2 to create ROS. How does this model stand up to scrutiny?

6. Looking at Complex III in the Light of ROS Formation

6.1. What Role Do Semiquinones Play?

Complex III is known to have a central role in (controlling) both energy transduction and ROS production. However, many of the specific molecular details and mechanisms are unknown and still debated. Much of what is known and agreed upon in this respect could be deduced from X-ray data obtained with crystals of dimers of the mammalian complex. Reconstructed structures allowed the location of major catalytic components in the complex.[31–34] This structural information helped to get the Q-cycle mechanism broadly accepted,[35] though differences with regard to the precise sequence of events involving the partial redox reactions that occur remain. An “activated” Q-cycle mechanism in which QH2 oxidation at the Qn site primes the complex for catalysis at the Qp site first is championed by Mulkidjianian.[36] Whether this is a viable alternative does not seem to be highly relevant in the context of our present hypothesis. In the following, a “general” Q-cycle model is presupposed. More about complex III and details of the Q-cycle mechanism, such as the role of iron sulphur protein head domain mobility in catalytic function,[34] can be found in ref. [37,38].

Broad consensus regarding the Q-cycle mechanism does not preclude debate about the details of several of the biochemical reactions occurring in Complex III. I will name but a few. Existence of semiquinones upon QH2 oxidation at the Qp site has been vigorously debated, a direct two-electron oxidation also being proposed.[39,40] The consensus seems to be moving towards real (though very short-lived) semiquinone intermediates at the Qp site, but this debate is not over yet (see e.g., Pietras et al.[41,42]). A stable intermediate semiquinone at the Qn site has been studied, see for example, ref. [43] and references therein. As semiquinones have been implicated in ROS production,[44,45] these discussions are not purely academic. However, whether ROS is coming from such a source directly at the Qp side in Complex III is still very difficult to answer.[6,11,41,45]

6.2. Why Do We Always Find Complex III Dimers?

Complex III (in prokaryotic context: the bc1 complex, in cyanobacterial/chloroplast context: the b6f complex) is known to be present as a dimer: for example, the bovine mitochondrial complex,[32] the evolutionarily related a-proteobacterial Paracoccus denitrificans complex, which can be studied as a dimer upon N-terminal truncation of the cytochrome c3 subunit,[46] the yeast mitochondrial complex,[39,47] the purple bacterium, Rhodobacter capsulatus, complex,[48] and the spinach chloroplast complex.[49] Dynamic, large-scale, supercomplex formation involving different parts of the ETC (for an example of their dynamic adjustment in
response to ROS production see below) is mostly a eukaryotic, mitochondrial, affair (but some ETC higher order complex formation can occur in bacteria\[50\]). However, the dimeric state of “complex III” is abundantly found in prokaryotes. Such a constant finding in organisms over wide evolutionary timescales implies functional advantages. Surprisingly, the ability of the complex to function as a real dimer (that engages in inter-electron exchange, as opposed to having merely a hypothetical role in overall stability) has been debated. However, there seems to be enough evidence of sufficiently fast inter-monomer electron transfer in vivo to claim that it is part of the explanation for the formation and function of dimers (refs. \[51,52\] and references therein). Thus, a highly interesting proposal (involving ROS production) to explain dimer formation was put forward by Covian and Trumpower. They shrewdly stressed that: “Having two sites catalysing the same net reaction in opposite directions (oxidoreduction of quinol/quinone) introduces the potential for significant inhibition of quinol oxidation at center P by the reversibility of reactions at center N.\[52\] Such an inhibition would not only hamper overall flow, but also lead to ROS formation on, or in the neighborhood of, site Qp. Using kinetic modelling these authors showed that Qp catalysis could be protected from inhibition by partial pre-reduction of the b-type haem groups, and thus possible superoxide formation due to RET, by inter-electronic equilibration between monomers of the dimeric Complex III structure.\[38\] One might say the dimer strongly increases chances for the low potential electrons to find a way out, especially when pre-reduction at a single site is already rare (QH₂ < Q). Covian and Trumpower also compared the effects of dimerization for overall electron densities in the complex under varying conditions with respect to changes in QH₂/Q and Δp.

6.3. High QH₂/Q and Δp Lead to ROS Formation in the ETC

Surprisingly, information regarding the QH₂/Q ratios during “normal” mitochondrial respiration is scant. In pioneering studies, it was found to be (somewhat) below 1 in uncoupled submitochondrial particles (i.e., the majority of the co-enzyme is oxidized\[53\]). Other studies have QH₂/Q ratios clearly above 1; see ref. \[54\] and references therein. However, uncoupling is anything but physiological and a single undifferentiated Q-pool was envisaged, while the supercomplex formation found in mitochondria makes this highly unlikely.\[55\] I think it is fair to state that “The redox state of the Q pool during rest, work, and pathophysiological conditions has been debated for decades.\[54\] Be that as it may, a lot of (very recent) studies have shown that mammalian cells use influx of substrates with high F/N ratios, such as FAs and succinate, leading to QH₂ increase and subsequent ROS signaling, to adjust to changing metabolic conditions and/or differentiate.\[8,21,56,57\]

A comparable state of confusion exists with regard to the “normal” mitochondrial membrane potential (Δp). It is clear that conditions of larger Δp can occur more easily than originally presumed; see ref. \[8\] and references therein. Looking specifically at ROS formation by Complex III, Brand and co-workers found a decrease in the presence of a membrane potential.\[58\] However, others found a strong increase in the rate of ROS production with increasing membrane potential.\[59\] Considering that the first result was obtained in the presence of the specific Complex III inhibitor, antimycin, it is probably safe to say that larger Δps should normally correlate with higher ROS production, because it reflects the necessary, unavoidable, by-product of respiratory control. Indeed, another worthwhile way of envisaging ROS formation by the Q-cycle and other parts of the ETC comes from an evolutionary perspective. Also in prokaryotic systems, evolutionary constraints must give rise to side reactions of ETCs. The mechanism of respiratory control (efficiently halting food oxidization in the absence of ADP, signaling falling energy demands) works by the build-up of Δp as soon as ATP-synthase cannot use the PMF to a sufficient extent to prevent this from happening. The ETC cannot transfer additional protons from the inside (matrix) to the outside (IMS) under such conditions and electrons “stuck” in the ETC are available for side reactions. Thus, selection for bacterial efficiency would end up as a tendency toward detrimental internal ROS formation in eukaryotes.\[60\] In conclusion, most models indeed show ROS production by Complex III to positively correlate with QH₂/Q and Δp.\[18,54\]

6.4. Understanding ETC ROS Formation in the Light of F/N and QH₂/Q Ratios Illuminates Seemingly Disparate Biological Phenomena

The central proton transferring complex of the ETC uses a unique mechanism to couple H⁺ transport to electron transport. Though the Q-cycle at first sight seems to be a very complex mechanism (oxido-reduction of QH₂/Q; in which two sites perform the same reaction but in opposite directions), it actually is the simplest way of transferring H⁺ over membranes in the absence of channels! In essence, protons are taken up inside by Q and given off outside by QH₂. The mechanism does not involve a complex cascade of proton travel via amino acid side chains, and thus it might represent one of the oldest mechanisms to couple electron shuttling to ion transport over membranes. The central role of Q as a “universal” acceptor allowing diverse catabolic routes to converge on the complex also supports such a notion. However, some researchers might not subscribe to the idea, because of the possible evolutionary primacy of a sodium-motive force energy intermediate, see for example, ref. \[61\]. Whatever the specific age of this ancient enzyme complex, having two sites catalyzing opposite reactions automatically gives rise to all kinds of possible electron flow limitations and easily occurring reverse reactions, with ROS formation as a result. High QH₂ levels would directly limit availability of the necessary substrate Q at the Qn site, confronting Complex I with an added competitor for this substrate, and negatively influencing the overall speed in the ETC under such conditions. All this would lead to extra ROS formation in both Complex I (as a result of RET) and Complex III. This nicely explains the universal dimerization of Complex III observed. However, starting with the merger of an archaeon with the “alpha-proteobacterial” mitochondrion precursor, these problems become more acute. Much of the internal space of eukaryotes is taken up by mitochondria (individually or as
networks), that oxidize multiple substrates. One can imagine that especially sudden shifts in metabolism would lead to strongly increased ROS formation (as reflected in the recent rash of discoveries of mitochondrial ROS as a mammalian cellular differentiation mechanism alluded to above).\[8\] To give just a few examples: when switched to FAO, the Q oxidation of mice fibroblasts is insufficient, and ensuing increased ROS formation in complex I will damage some of its subunits, which eventually results in reconfiguration of supercomplexes. Thus, the QH2/Q ratio adjusts the ETC to the high F/N ratio associated with FAO.\[21\] Mills et al.\[56\] describe how LPS stimulates macrophages to shift to glycolysis but also upregulate succinate levels. The

Figure 2. Making a virtue of necessity? All major ETC components using FADH2 as a prosthetic group induce uncoupling protein (UCP) activity directly or indirectly when used disproportionately as a point of entry for their electrons (i.e., under conditions with high F/N ratios). ROS formation due to high QH2 levels and increased Δp during extra succinate/FA oxidation or use of the GPDH shuttle. A) Glucose oxidation (low F/N ratio) only moderately using Succinate dehydrogenase (Complex II). B) High F/N ratios with high QH2 levels and a large Δp (indicated). C) ROS reduction by enhanced QH2 oxidation (more uncoupling). Complex II light green, ETF/ETF:QO complex (FA oxidation) dark green, GPDH (“turbo glycolysis”) pink, ubiquinone (Q) red, uncoupling protein blue/purple, electron flow black arrows. F) FADH2 oxidizing complex; IMS, intermembrane space. For details see text.
resulting \( \text{QH}_2/\text{Q} \) increase, together with elevated \( \Delta \nu \), leads to ROS production and a pro-inflammatory phenotype. Chouchani et al. showed that accumulation of succinate also controls activation of thermogenesis in brown fat, again by ROS production in the ETC. A mitochondrial dicarboxylate carrier allows access to cytosolic succinate, so that brown adipocyte mitochondria can take up the necessary succinate. The specific ROS formation site(s) is still unclear. Thermogenesis results from the stimulation of the uncoupling protein 1 (UCP1)\[^{[57]}\]; see below.

7. ROS As a Mammalian Cell Signal: The Example of UCPs

7.1. UCP Induction Is Linked to High F/N Ratio Substrates

Upon speculating about the problems associated with substrates that represent high F/N ratios, I described how the oxidation of FA's induced uncoupling proteins (UCPs) in both number and activity. UCPs lower \( \Delta \nu \), which reduces RET and subsequent ROS formation by complex I and III (see Figures 1 and 2). UCP2 is regulated at the transcriptional and translational levels, as well as via direct activation. It is upregulated in response to FAs (e.g., by PPAR transcription factors, signalling FA presence), high \( \text{QH}_2/\text{Q} \) and ROS, see ref.\[^{[8]}\] and references therein. In cells that can use turbo glycolysis, UCP2 and UCP3 transcription is activated by the thyroid T3 hormone,\[^{[62]}\] which also activates the GPDH shuttle,\[^{[63]}\] and see ref.\[^{[124]}\]. The latest example of succinate inducing UCP1 in brown, that is, mitochondria rich, adipose tissue\[^{[67]}\] demonstrates that specific increases in the activity of every single major ETC component using \( \text{FADH}_2 \) as a prosthetic group can enhance uncoupling protein (UCP) activity. The presumed old evolutionary link between uncoupling and high F/N (\( \text{QH}_2/\text{Q} \)) ratios reappears independent of the specific \( \text{FADH}_2 \)-utilizing complex used (see Figure 2). Of note, FAs and high concentrations of succinate both induce UCP activity for FAO, but they do so in completely different metabolic contexts (sustained high ATP generation vs. thermogenesis) and using different UCPs. The observation that succinate is used in brown adipose tissue might reflect the fact that in the adipocyte lineage (with much lower levels of FAO, which can be further suppressed by insulin) other ROS-generating mechanisms are needed, though FAO-related ROS damage can occur in white adipose tissue in rodents which have been fed high-fat diets.\[^{[64–66]}\]

7.2. Does UCP Induction Lower ROS Formation?

One might expect (in light of all the circumstantial evidence given above) that the proposed ROS-lowering effects of UCPs have been easy to prove experimentally. However, this is not the case. As so frequently observed, results are mixed when studying mitochondrial ROS formation, because in all studies regarding its production, approaches used to measure ROS are different. Thus, apart from the multi-layered adaptations found in “higher” eukaryotes, another level of complexity is added when interpreting data. Indirect indications are easier to obtain, but more difficult to interpret with regard to the molecular mechanisms involved.

Using mitochondria from brown adipose tissue of mice, Dlasková et al. show that inhibition or ablation of UCP1 indeed increases both \( \Delta \nu \) and ROS formation, while Kazak et al. demonstrate that such ablated mitochondria become dysfunctional by induction of ROS production via RET to Complex I.\[^{[67–69]}\] For UCP2, a well-controlled study revealed induction of host UCP2 expression during Leishmania infection, lowering ROS generation in order to suppress macrophage defence mechanisms,\[^{[70]}\] and UCP2 also inhibited ROS-mediated apoptosis in A549 cancer cells (during hypoxia).\[^{[71]}\] UCP3 has been shown to lower ROS formation in isolated mouse mitochondria as well, by so-called “mild uncoupling,”\[^{[72]}\] especially when UCP1 is considered, this concept and the related link between UCP function and ROS suppression is not supported by experiments performed by Cannon, Nedergaard et al., who found no indications of UCP1 diminishing oxidative damage, see for example, refs.\[^{[63,73–75]}\]

8. Conclusions and Outlook

Since I proposed that peroxisomes are a way by which eukaryotes reduce mitochondrial F/N ratios,\[^{[12]}\] many findings have supported the model and its relevance for the evolution of peroxisomes. Thus, the advent of peroxisomes nicely fits in with symbiogenesis, the theoretical stance stating that eukaryogenesis can only be understood in the light of mutual adaptations of archaean “host” and bacterial endosymbiont.\[^{[18,19,24,75]}\] Are there specific aspects linked to the role of Complex III in this respect? Only that it might be argued that direct contributions of Complex III to “external” (IMS) superoxide formation during FAO, such as considered here, might have made the challenge of a ROS forming cell/organelle inside the new conglomerate cell during early stages of eukaryogenesis even more acute.

Theoretical considerations seem to dictate that ROS formation by Complex III should increase when mitochondria use FAs as the dominant catabolic substrate (all else being equal). Do experiments show this to be true? As always (compare the ongoing discussions regarding specific sites of ROS formation in the ETC), results are mixed. But an extensive study using isolated skeletal muscle mitochondria, catabolizing long-chain FAs indeed showed ROS formation exceeding that of normal NADH-linked (glutamate/malate) substrates.\[^{[76]}\] The authors state that in their setup: “Observations support complex III, not complex I, as an important site of ROS formation during FAO.” This is mostly based on a topological argument (substantial superoxide formation occurred at the intermembrane site, only with the FA substrate). The contribution of Complex III seems clear, but the lack of significant ROS contributions from Complex I might make sense when considering that the authors only find an increase in \( \Delta \nu \) using up to 5 \( \mu \)M of their FA substrate, after which no further increase could be obtained.
Tissues normally using FAO express UCPs, which are directly activated by higher FA concentrations, lowering Δψ and thus blunting ROS formation by Complex I via RET. This constitutes a nice example of a highly evolved eukaryotic ROS defense mechanism.

Seifert et al. also used their preparations to look at the effect of carnitine. The carnitine shuttle is a specific eukaryotic invention: Longer FAs can only be imported, and thus used as substrates for mitochondrial FAO, in the form of carnitine (a small zwitterionic molecule with a trimethylammonium and a carboxy group) esters. I previously speculated that the extreme mitochondrial matrix concentrations of carnitine during FAO might reflect (in)direct anti-oxidative properties. The results of Seifert et al. are complex, but indeed support important roles for carnitine in limiting ROS formation during FAO. They compared muscle and liver mitochondria and found that carnitine limited the amount of long chain FA catabolic intermediates in both preparations, though much less in the liver organelle. Muscle mitochondria, but not liver mitochondria, had a FA-specific increase in Δψ and oxygen consumption, without an increase in ROS formation. In liver mitochondria the presence of carnitine suppressed ROS formation. The authors speculated that carnitine exerts (part of) its protective effect by limiting the intermediates mentioned. But this cannot fully explain the effect observed in liver preparations, where oxygen consumption went down (not significantly), while ROS formation was significantly inhibited. It is clear that carnitine has many ROS-protective properties, but its own scavenging potential, when looking at the molecular structure, looks weak. Inhibiting ROS-generating Fenton reactions by chelating metal ions using coordinated multiples of their carboxy groups seems a better explanation. One might speculate that in that case the positively charged trimethylammonium part might position it better explanation. One might speculate that in that case the positively charged trimethylammonium part might position it near the matrix side of the inner membrane, close to, for example, the mitochondrial DNA.

As can be deduced from the experimental results I discussed above, some of the questions we will have to answer are obvious. How does carnitine limit ROS formation during FAO? What are the relative contributions of Complex I and III to ROS formation when burning high F/N ratio substrates and how are they influenced by specific eukaryotic (tissue) adaptations? Will we see more instances where these old pathways are put to new use, making a virtue of necessity? The multilayered and elusive nature of mitochondrial ROS formation still poses a unique scientific challenge.

**Abbreviations**

AMPK, AMP kinase; ETC, electron transport chain; FA, Fatty acid; FAO, Fatty acid oxidation; F/N ratio, FADH2/NADH ratio; GPDH, glycerol-3-phosphate dehydrogenase shuttle; IMS, intermembrane space; PMF, proton-motive force; RET, reverse electron transport; ROS, reactive oxygen species; UCP, uncoupling protein.

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**Conflict of Interest**

The author declares no conflict of interest.

**Keywords**

β-oxidation, carnitine, FADH2/NADH ratio, peroxisomes, Q-cycle, reverse electron transport (RET), symbiogenesis

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