Biochimica et Biophysica Acta 1827 (2013) 1332–1339

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Review

Molecular mechanisms of superoxide production by complex III: A bacterial versus human mitochondrial comparative case study ☆

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ARTICLE INFO

Article history:
Received 6 November 2012
Received in revised form 14 February 2013
Accepted 20 March 2013
Available online 28 March 2013

Keywords:
Complex III
Cytochrome bc_1
Mitochondria
Reactive oxygen species
Oxidative damages
Electron transfer

ABSTRACT

In this mini review, we briefly survey the molecular processes that lead to reactive oxygen species (ROS) production by the respiratory complex III (CIII or cytochrome bc_1). In particular, we discuss the "forward" and "reverse" electron transfer pathways that lead to superoxide generation at the quinol oxidation (Q_o) site of CIII, and the components that affect these reactions. We then describe and compare the properties of a bacterial (*Rhodobacter capsulatus*) mutant enzyme producing ROS with its mitochondrial (human cybrids) counterpart associated with a disease. The mutation under study is located at a highly conserved tyrosine residue of cytochrome *b* (Y302C in *R. capsulatus* and Y278C in human mitochondria) that is at the heart of the quinol oxidation (Q_o) site of CIII. Similarities of the major findings of bacterial and human mitochondrial cases, including decreased catalytic activity of CIII, enhanced ROS production and ensuing cellular responses and damages, are remarkable. This case illustrates the usefulness of undertaking parallel and complementary studies using biologically different yet evolutionarily related systems, such as α-proteobacteria and human mitochondria. It progresses our understanding of CIII mechanism of function and ROS production, and underlines the possible importance of supra-molecular organization of bacterial and mitochondrial respiratory chains (i.e., respirasomes) and their potential disease-associated protective roles. This article is part of a Special Issue entitled: Respiratory complex III and related bc complexes.

1. Introduction

1.1. Reactive oxygen species

Reactive oxygen species (ROS) are a group of radical or non-radical oxygen containing molecules that display high reactivity with lipids, proteins and nucleic acids. Depending on the concentration, location, and molecular context, ROS can be beneficial or harmful to cells. Increasing evidence indicates that homeostatic and physiological levels of ROS are indispensable in regulating diverse cellular processes, including ion channel/transporter function [1], Ca^{2+} spark production [2,3], protein kinase/phosphatase activation and gene expression [4]. A current view is that low levels of ROS production contribute to many essential intracellular signaling processes ranging from cell metabolism to ischemia preconditioning in eukaryotic cells [4–6]. Conversely, excessive ROS generation often leads to apoptotic and necrotic cell death [7,8] as well as to a panel of clinically distinct disorders, including neurodegeneration (e.g., Alzheimer's disease), cardiomyopathies, diabetes and cancer [9–11]. Accumulative and systemic ROS damages also underlie cell senescence and aging [6,12].

In quiescent cells, ROS are primarily produced as byproducts of mitochondrial respiration when electrons leak from the electron transport chain (ETC) (Fig. 1) [13]. Superoxide anions ($O_2^{\bullet -}$) are the primary ROS species generated by the ETC, and are converted to hydrogen peroxide (H_2O_2) either spontaneously or by the superoxide dismutase (SOD). In the presence of transition metals, $O_2^{\bullet -}$ can also be transformed into hydroxyl radicals ($^{\bullet}OH$) that are considered to be more reactive and damaging. Recent studies documented that massive increases in localized ROS production could occur during metabolic stress [6,14] and photostimulation [15]. Besides, excessive amounts of intracellular ROS ultimately contribute to necrotic or apoptotic cell death [16,17].

1.2. Oxidized and reduced quinones

Quinone molecules (oxidized Q/reduced QH_2) play a central role in $O_2^{\bullet -}$ generation because they act as direct electron donors/acceptors, or redox mediators, to reduce molecular oxygen (O_2). They are present in energy transducing membranes in large amounts as compared with other components of the respiratory chain, and serve as intermediates

Abbreviations: ROS, reactive oxygen species; ETC, electron transport chain; $O_2^{\bullet -}$, superoxide radical; $^{\bullet}OH$, hydroxyl radical; $SQ^{\bullet -}$, semiquinone radical; H_2O_2, hydrogen peroxide; CI, CII, CIII, CIV and CV, respiratory complex I, complex II, complex III (cytochrome bc_1), complex IV and complex V, respectively; SOD, superoxide dismutase

☆ This article is part of a Special Issue entitled: Respiratory complex III and related bc complexes.

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0005-2728/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbabio.2013.03.009

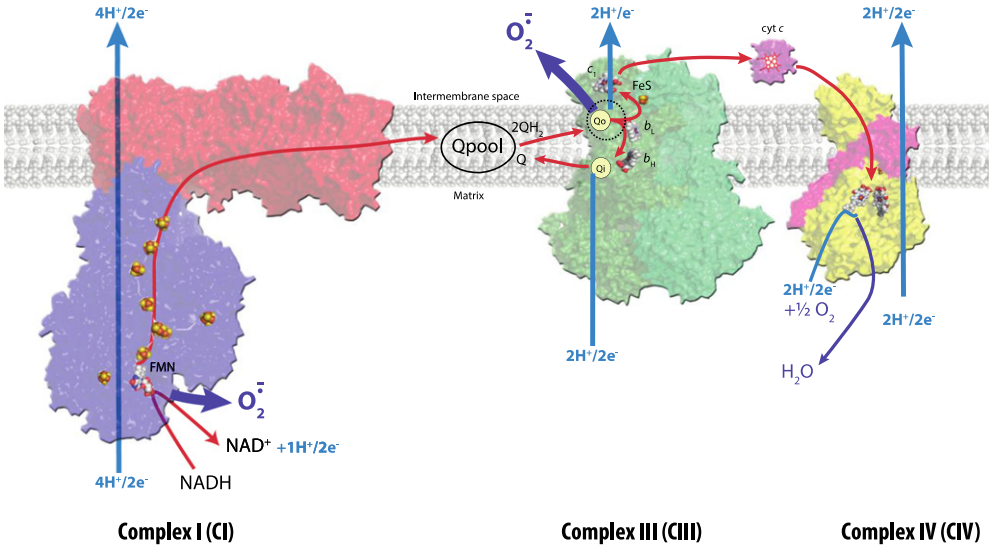

Fig. 1. Structures of CI, CIII and CIV complexes of respiratory ETC and ROS production. Overall structures of individual respiratory complexes CI (PDB 3IAM + 3M9C in red and blue for membrane integral and membrane external parts, respectively), CIII (PDB 1PP9 in green for the two monomers) and CIV (PDB 1OCC in yellow) are shown together with the Q pool and the electron carrier cytochrome *c* in purple. In CIII the iron sulfur clusters are positioned in the stigmatellin-bound form and depicted as yellow and orange balls. All heme cofactors are shown in white, red, and blue balls, except in the case of the electron carrier cytochrome *c* where heme is shown in red. The Q pool (made of oxidized Q and reduced QH_2) is indicated in black. The electron transfer and the proton uptake/release pathways are shown in red and blue arrows, respectively. The CI and CIII complexes of the mitochondrial respiratory ETC are generally considered to be the main sources of $O_2^{\bullet-}$ radicals, indicated with thick dark blue arrows. In CI, $O_2^{\bullet-}$ is thought to be produced primarily at the FMN (flavine mononucleotide) cofactor facing the matrix side, whereas in $CIII_2$ it is generated at the ubiquinol oxidation site (Q_o site) facing the inter membrane space of mitochondria.

during electron transfer reactions connecting together ETC complexes (Fig. 1). The redox chemistry of Q/QH_2 is reversible, fast, and involves two consecutive "one-electron" reduction steps via a semiquinone radical ($SQ^{\bullet-}$) intermediate. While this property of Q/QH_2 is important during energy transduction to store reducing equivalents, it also constitutes a liability for ROS generation, especially when Q/QH_2 catalysis is not well confined to specific niches in related proteins. This process is often referred to as the 'leakage of electrons' from the ETC complexes [18]. In addition to their redox functions, QH_2 molecules can also reduce O_2 via a "one-electron" reduction step leading to the formation of $O_2^{\bullet-}$, which is the main source of ROS in cells. However, direct oxidation of QH_2 by O_2 is slow and spin-forbidden, whereas the redox reactions between $SQ^{\bullet-}$ and O_2 ultimately yielding $O_2^{\bullet-}$ can be very fast [19]. These reactions strongly depend on how well $SQ^{\bullet-}$ binds to its site of generation as well as the redox potential of the $Q/SQ^{\bullet-}$ couple, thus on the stability constant and the local factors modulating its disproportionation [20]. As O_2 partitions favorably into the lipid phase, co-partitioning would allow O_2 and $SQ^{\bullet-}$ to react effectively unless the latter species is sequestered away from accessing O_2. The local concentration and stability of $SQ^{\bullet-}$ have been optimized during evolution in the case of the ETC complexes that perform Q/QH_2 redox chemistry. It is also noteworthy that the reaction leading to $O_2^{\bullet-}$ production is reversible, which allows Q to be used as a protective agent against oxidative damages by consuming $O_2^{\bullet-}$ radicals [21,22].

1.3. Production of ROS by ETC

Although still subject to controversy, it is believed that ROS are produced in the cell mainly during perturbations of respiratory chain functions [6]. Increased supply of electrons to ETC, (e.g., excess of reducing equivalents) or enhanced membrane potential generation leads to an increase of $SQ^{\bullet-}$ content in membranes, and subsequently, to higher $O_2^{\bullet-}$ production. Moreover, kinetic constraints exerted downstream of the Q pool (e.g., blocking electron flow at the level of cytochrome *c* oxidase, CIV) further enhance production of $SQ^{\bullet-}$. Conversely, moderate uncoupling of the membrane potential decreases the electron flux constraints across ETC, therefore decreasing $O_2^{\bullet-}$ production by the respiratory complexes (Fig. 1) [23]. Both of these effects can also be observed upon modification of the respiratory chain by specific chemicals [24,25]. For example, free fatty acids exert different effects on mitochondria in respect to ROS generation; they inhibit electron flux through the complex I (CI), and probably the complex III (CIII), by inducing an increase of ROS production as seen in isolated rat heart or liver mitochondria [26]. Fatty acids also induce an uncoupling effect, which seems responsible for a large decrease in ROS formation. Thus, ROS production is fine-tuned in response to changes in electron flux through ETC.

The respiratory complexes CI and CIII were well known for some time to be involved directly in superoxide production (Fig. 1). More recently, complex II (CII) is also implicated in this process as well [27,28]. It has been suggested that about 2–5% of O_2 consumed might lead to $O_2^{\bullet-}$ generation, of which roughly 70–80% is linked to the mechanism of function of CIII [29] based on quantitative data obtained using isolated mitochondria. However, $O_2^{\bullet-}$ production measurements depend on cell types, respiration states, materials and techniques used to quantify the chemical free radicals. More conservative predictions estimate that $O_2^{\bullet-}$ production by ETC is perhaps only 0.1% of physiological respiratory rates [30,31].

2. Complex III (CIII or cytochrome bc_1)

2.1. Structure and mechanism of function of CIII

CIII is a major multi-subunit, membrane-bound enzyme that is central to respiratory energy transduction pathways in many organisms [32]. In different species, it enzymatically converts various derivatives of QH_2 to Q, and reduces various mobile or membrane-anchored electron carriers, typically *c*-type cytochromes. CIII operates via the Q-cycle mechanism, and contributes to the formation of both the membrane potential and the proton gradient used for ATP production by ATP synthase (complex V or CV) [33–35]. Depending on the species, mitochondrial CIII contains up to eleven subunits, of which eight are not essential for enzymatic activity (referred to as the "supernumerary subunits"). These subunits are absent in most bacterial CIII, which usually contain only three subunits as the essential catalytic core of the enzyme (Fig. 2a). Due to

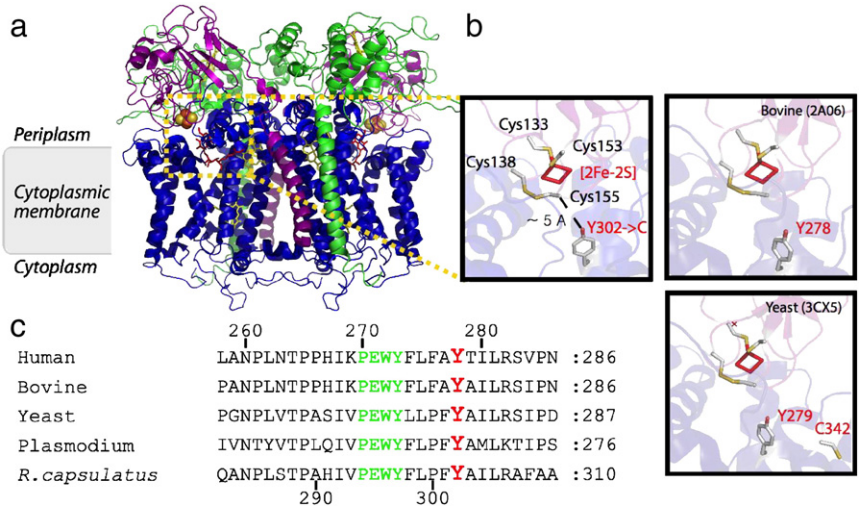

Fig. 2. Structure of CIII (cytochrome bc_1) and location of the conserved Tyr 302/278 of cytochrome *b*. **a**, The three dimensional structure of bacterial dimeric CIII bound with stigmatellin that mimics QH_2 (PDB 1ZRT) is shown. The catalytic subunits cytochrome *b*, cytochrome c_1 and the Fe/S protein are shown in blue, green and purple, respectively. Hemes are shown in yellow sticks, the [2Fe–2S] cluster in yellow and orange spheres, and the Q_o site inhibitor stigmatellin in red sticks. **b**, Close-up views of bacterial (*R. capsulatus*, PDB 1ZRT), bovine (PDB 2A06) and yeast (PDB 3CX5) mitochondrial CIII Q_o site structures. The four conserved cysteine residues of the Fe/S protein (yellow and white sticks, *R. capsulatus* numbering Cys 133 and Cys 153 acting as ligands of the [2Fe–2S] cluster and Cys 138 and Cys 155 forming a disulfide bond) are shown together with the conserved Tyr 302/278/279 of cytochrome *b* substituted with Cys (gray stick, *R. capsulatus* Y302 → C, bovine Y278C and yeast 279). In the yeast case, the Cys342 is also indicated. **c**, Sequence alignment of *R. capsulatus* cytochrome *b* residues around the conserved tyrosine 302 (278 in human and bovine, 268 in *Plasmodium* and 279 in *yeast*) and the conserved PEWY motif shown in red, and green, respectively. Human, bovine, yeast, *Plasmodium* and *R. capsulatus* refer to *Homo sapiens* (NCBI protein database accession no: P00156), *Bos taurus* (P00157), *Saccharomyces cerevisiae* (P00163), *Plasmodium yoelii* (AAC25924), and *Rhodobacter capsulatus* (P08502) cytochrome bc_1, respectively.

their structural simplicity and evolutionarily conserved sequences and structures, the facultative phototrophic bacteria (*Rhodobacter capsulatus* and *Rhodobacter sphaeroides*) and *Paracoccus denitrificans* are widely used organisms as CIII models for their mitochondrial counterparts [36–38]. The three universally conserved catalytic subunits of CIII are the cytochrome *b*, the Fe/S (also called the Rieske) protein and the cytochrome c_1. Cytochrome *b* is an integral membrane protein, whereas the Fe/S protein and cytochrome c_1 are membrane-anchored by their amino- and carboxyl-terminal helices, respectively. These three subunits carry specific cofactors that are required for the catalytic activity of the enzyme. These cofactors are two *b*-type hemes (axially coordinated protophorphyrin IX-iron) with one low (b_L) and one high (b_H) E_m of cytochrome *b*, the [2Fe–2S] cluster with a high redox midpoint potential (E_m) of the Fe/S protein, and a high E_m *c*-type heme (covalently bound protophorphyrin IX-iron) of cytochrome c_1 (Fig. 2a). Most bacterial and mitochondrial purified CIII form dimers, and their three-dimensional structures depict these proteins as symmetrical homodimers [36–42]. In the case of *R. capsulatus* CIII, monomeric forms of the enzyme are neither active nor stable. However, tetrameric forms of bacterial CIII have been reported in at least two instances. In *R. capsulatus*, upon fusion of cytochrome c_1 with its physiological electron carrier cytochrome c_y, formation of active CIII tetramers was observed [43]. The *P. denitrificans* native enzyme forms tetramers, but elimination of a naturally present amino-terminal extension of cytochrome c_1 was reported to yield dimeric CIII [36].

Each monomer of the bacterial enzyme contains the three catalytic subunits in an unusual organization. Cytochrome *b* with its eight transmembrane helices forms the membrane-embedded core to which the other two subunits are bound. Facing the lipid layer on cytochrome *b*, two Q/QH_2 binding (QH_2 oxidation (Q_o) and Q reduction (Q_i)) sites are located on the positive (*p*) and negative (*n*) sides of the membrane. The carboxyl-terminal helix of cytochrome c_1 interacts closely with the fifth helix of cytochrome *b* to form a cytochrome *b*-c_1 core, which interacts with the mobile head domain of the Fe/S protein, leaving its amino terminal membrane helix (i.e., tail) associated with cytochrome *b* of the other monomer. In agreement with this organization, a stable dimeric cytochrome *b*-c_1 subcomplex has been purified from *R. capsulatus*. Reconstitution of this subcomplex into an active enzyme was achieved when a full-length Fe/S protein was used, but not with a truncated Fe/S protein lacking its amino-terminal tail domain [44]. The mobility of the Fe/S protein head domain between cytochrome *b* and cytochrome c_1 and its [2Fe–2S] cluster is essential for CIII activity [45,46].

According to the proton-motive Q-cycle mechanism, upon the diffusion of a QH_2 molecule from the Q-pool to the Q_o site of CIII, the oxidized [2Fe–2S] cluster of the Fe/S protein oxidizes this QH_2 and conveys a single electron via its mobile head domain to the oxidized cytochrome c_1. This electron is then transferred down the ETC to a terminal oxidase (e.g., CIV). The highly unstable $SQ^{\bullet-}$ radical thus produced at the Q_o site gives an electron to heme b_L of cytochrome *b*, which rapidly transfers it to heme b_H across the lipid bilayer, to generate membrane potential and a stable $SQ^{\bullet-}$ at the Q_i site. Completion of the catalytic turnover of CIII involves a second QH_2 oxidation at the Q_o site of the dimeric CIII, via the same sequence of events described above, converting $SQ^{\bullet-}$ at the Q_i site to a QH_2 to be released from the enzyme. The Q_o and Q_i sites of CIII are not identical with respect to their ability to interact with the $SQ^{\bullet-}$ species. While the $SQ^{\bullet-}$ at the Q_i site is well characterized by EPR spectroscopy [47–49], $SQ^{\bullet-}$ at the Q_o site is a subject of controversy. It is difficult to detect the latter species experimentally, and it was only seen under specific conditions at extremely low amounts [50,51]. Moreover, no structural information is yet available about the exact position of Q/QH_2 at the Q_o site. Thus, detailed descriptions of the events that follow QH_2 oxidation by the [2Fe–2S] cluster of the Fe/S protein until the transfer of the second electron to heme b_L of cytochrome *b* remain unknown.

2.2. Superoxide production at the Q_o site

Importance of the structural integrity of bacterial CIII for maximal rate of catalysis and minimal rate of electron leakage to O_2 is known. Heat-inactivated or proteinase K-digested CIII [52], and catalytically impaired mutants producing higher amounts of $O_2^{\bullet-}$ [53,54] have been reported. Bifurcated electron transfer from QH_2 to the [2Fe–2S] cluster of the Fe/S protein and to the heme b_L of cytochrome *b* at the Q_o site infers that either preventing the formation of a $SQ^{\bullet-}$, or entrapping it within CIII to avoid its interaction with O_2, should

prevent $O_2^{\bullet-}$ production. Conditions favoring the generation of $SQ^{\bullet-}$ should enhance $O_2^{\bullet-}$ production as analyzed in detail by Oscyzka et al., [18,53,54]. Two different situations lead to $SQ^{\bullet-}$ generation: a *semiforward* electron pathway that produces a $SQ^{\bullet-}$ following oxidation of QH_2 by the Fe/S protein [20,50,51,53–57], and a *semireverse* electron transfer pathway that involves electron transfer from reduced heme b_L of cytochrome *b* to a Q bound at the Q_o site to yield a $SQ^{\bullet-}$ (referred to as "forward" and "reverse" for simplicity) (Fig. 1) [53,54,58,59].

2.2.1. Forward electron transfer for $SQ^{\bullet-}$ generation at the Q_o site of CIII

Earlier studies focused mainly on the production of $O_2^{\bullet-}$ at the Q_o site via the forward electron transfer pathway [20,50,51,55–57]. Historically, the pioneering work of Chance proposed that the residual cytochrome *c* reduction activity seen when CIII is inhibited with antimycin A was closely associated with $O_2^{\bullet-}$ production [60,61]. Accordingly, both electrons from QH_2 oxidation would be transferred to the electron carrier cytochrome *c*, but via two disparate pathways. One electron would be delivered to cytochrome *c* via the high-potential chain (i.e., the Fe/S protein and cytochrome c_1), while the other electron would be conveyed to O_2 to yield $O_2^{\bullet-}$ which would rapidly oxidize cytochrome *c*. Later on, occurrence of this process was supported by the fact that chemical destruction of the [2Fe–2S] cluster of the Fe/S protein [62] or maintenance of this cluster in a reduced state [20] inhibited $O_2^{\bullet-}$ formation. Similarly, inhibiting reduction of Q at the Q_i site (e.g., using antimycin A) significantly increased Q_o site mediated $O_2^{\bullet-}$ production. Studies of the effects of specific Q_o and Q_i site inhibitors on $O_2^{\bullet-}$ production described the complementary bypass reactions in details [20]. For instance, decreasing the rate of electron transfer between the hemes b_L and b_H of cytochrome *b*, or abolishing the subsequent oxidation of these hemes via the Q_i site inhibitor antimycin A, resulted in the accumulation of electrons on cytochrome *b* [18]. This led to the accumulation of $SQ^{\bullet-}$ at the Q_o site and to the leak of electrons to O_2 to generate $O_2^{\bullet-}$ [20]. In general, if a $SQ^{\bullet-}$ is formed at the Q_o site (i.e., via a non concerted electron bifurcation) during the normal turnover of a native CIII, $O_2^{\bullet-}$ production is expected to be quite low to minimize electron leakage and energy waste. However, $O_2^{\bullet-}$ production at the Q_o site might become significant under compromising conditions, such as a highly reduced Q pool, presence of antimycin A-like molecules inhibiting oxidation of reduced *b* hemes of cytochrome *b*, extremely high membrane potential, or specific Q_o site mutations (see Section 3). Such conditions may occur in damaged CIII enzymes, or under extreme physiological situations (e.g., ischemia and reperfusion) [63,64].

Recently, a variant of the forward electron transfer pathway was proposed for *R. sphaeroides* CIII. In contrast to the earlier studies, this model postulated that under physiological conditions, $O_2^{\bullet-}$ production is not the result of a bypass reaction during the Q-cycle, but is a regulatory step for enhancing Q_o site catalysis [52,65]. The authors entertained the idea that O_2 might act as a redox mediator during oxidation of QH_2 and reduction of heme b_L of cytochrome *b*. Accordingly, $O_2^{\bullet-}$ formation and CIII activity would increase together as a function of O_2 concentration available during the assay conditions. However, the relevance and validity of the relatively mild effects (<2 fold) observed on enzyme activity require additional investigations [65].

2.2.2. Reverse electron transfer for $SQ^{\bullet-}$ generation at the Q_o site of CIII

In recent years, several studies focused on the bypass reactions of the Q-cycle yielding $O_2^{\bullet-}$ production via a reverse electron transfer pathway. It appears that partial oxidation of the Q pool in a physiologically relevant scenario significantly increases the rates of $O_2^{\bullet-}$ production by antimycin A inhibited CIII [58]. Using submitochondrial particles, Dröse and Brandt observed that CIII mediated ROS production was higher when CII activity was partially inhibited by malonate (or oxaloacetate), linking the Q pool redox state to $O_2^{\bullet-}$ production via the Q_o site of CIII. They proposed that the $O_2^{\bullet-}$ thus generated was produced at the Q_o site by reverse electron transfer from reduced heme b_L of cytochrome *b* to O_2 via a $SQ^{\bullet-}$ intermediate acting as a redox mediator. Quinlan et al. further supported this proposal showing that this effect might be directly driven by the redox state of hemes b_L and b_H of cytochrome *b* that are sensitive to the Q pool redox state and membrane potential [59]. Additional studies using bacterial CIII mutants indicated that $O_2^{\bullet-}$ production at the Q_o site also involved reverse electron transfer from reduced heme b_L of cytochrome *b*, and Osyczka's group proposed a "kinetic" mechanism to account for its occurrence [54]. Accordingly, the movement of the Fe/S protein [2Fe–2S] cluster from the Q_o site increased $O_2^{\bullet-}$ generation, whereas its stagnation at the Q_o site decreased it. This observation correlated the production of ROS with the position of the Fe/S protein head domain on cytochrome *b*. Assuming that the movement of the reduced Fe/S protein is not obligatorily "concerted" with electron transfer from $SQ^{\bullet-}$ to cytochrome b_L heme, ROS generation could be rationalized as the result of a kinetic competition between the internal reactions involving the cofactors of CIII, the Q residing at the Q_o site, and the reaction of $SQ^{\bullet-}$ with O_2 [54].

3. Defective CIII catalysis and enhanced ROS production due to specific mutations

3.1. Bacterial CIII mutations and ROS production

Studies using bacterial CIII also highlighted some specific amino acid residues as key contributors for affecting ROS production. For instance, the M183K or M183L substitutions in *R. capsulatus* cytochrome c_1 drastically decreased the E_m of heme c_1, severely impeding electron flow kinetics through the high potential chain of CIII, and enhancing $O_2^{\bullet-}$ production during Q_o site catalysis [54]. In a recent study, Lee et al. described a different role played by some amino acid residues of cytochrome *b* in controlling $O_2^{\bullet-}$ production via the Q_o site of the bacterial CIII [66] (Fig. 2b). In *R. capsulatus*, substitution of the conserved Y302 of cytochrome *b* with any other amino acid residue decreased CIII activity. Concomitantly, it increased $O_2^{\bullet-}$ production independently of antimycin A inhibition or other treatments known to enhance this process [66]. These findings indicated that some cytochrome *b* residues are critical for suppressing ROS production at the Q_o site of the enzyme. Various structures have depicted this tyrosine side chain in slightly different H-bonding patterns, depending on the position of the Fe/S protein head domain and the occupant of the Q_o site [67]. Moreover, the hydroxyl group of this residue is within H-bonding distance from a cluster of H_2O molecules in a high resolution structure [68]. Although it is unclear how Y302X (X being any amino acid) mutations enhance mechanistically $O_2^{\bullet-}$ production, the finding that even the Y302F substitution increases $O_2^{\bullet-}$ production suggests that it may be linked to the loss of the fixed H_2O cluster coordinated in the native enzyme by the hydroxyl group of Y302 [66]. Accordingly, any mutant losing the hydroxyl group would exhibit decreased CIII activity due to the incorrect positioning of the Fe/S protein head domain. Concomitantly, it would also produce increased $O_2^{\bullet-}$ due to the uncoordinated mobility of the Fe/S protein head domain vis-a-vis the electron transfer from $SQ^{\bullet-}$ to cytochrome b_L, and the ensuing undesirable electron leakage to O_2 during Q_o site catalysis.

The counterparts of *R. capsulatus* Y302 in other species, in particular the malarial (Y268) [69,70], yeast (Y279) [71–73] and human (Y278C) mitochondrial mutants [74,75] were also studied with respect to decreased CIII catalysis and enhanced ROS production. Decreased CIII activities were reported for all mutants, and enhanced $O_2^{\bullet-}$ production was described for several yeast and human mutants (see below). The overall findings indicate that a number of amino acid residues of cytochrome *b* at the Q_o site affect both CIII catalysis and ROS production. Whether or not all catalytically defective Q_o

site mutants always produce enhanced ROS, as a general property of the CIII enzyme, is unknown.

3.2. Bacterial cytochrome b Y302C mutation forms an inter subunit disulfide bond

The bacterial mutant carrying the cytochrome *b* Y302C mutation was studied in detail [66]. This mutant supported CIII-dependent anoxygenic photosynthetic growth of *R. capsulatus*. However, it progressively lost its CIII activity upon exposure to air due to slow oxidative disintegration of the [2Fe–2S] cluster in its Fe/S protein both in chromatophore membranes and in purified samples [66]. On the other hand, although the homologous yeast cytochrome *b* Y279C mutant also produced ROS [73], its Fe/S protein [2Fe–2S] cluster did not exhibit oxidative damage [71,73]. In the case of *R. capsulatus*, oxidative disintegration of the [2Fe–2S] cluster required not only the presence of O_2, but also the catalytic activity of the Q_o site and the presence of a free thiol group at position 302. Strict anaerobiosis, highly reducing conditions, as well as use of Q_o site inhibitor stigmatellin or thiol-alkylating reagents (e.g., iodoacetamide or N-ethyl-maleimide) abolished the oxidative damages in the Y302C mutant [66]. Using the bacterial mutant CIII, mass spectrometry analyses revealed for the first time that the mutant cytochrome *b* and the Fe/S protein subunits of CIII were covalently cross-linked to each other by an inter subunit disulfide bond formed between the thiol groups of cytochrome *b* Y302C and the Fe/S protein C155. It was therefore proposed that the ROS-induced cysteine redox chemistry reduced the intra molecular disulfide bridge, which is naturally present in the Fe/S protein and stabilizes its [2Fe–2S], to render this cluster oxygen labile and the mutant CIII air-sensitive (Fig. 2b) [66].

The striking difference seen in respect to the stability of their Fe/S protein [2Fe–2S] clusters between the bacterial Y302C and its yeast counterpart Y279C is intriguing. Comparison of *R. capsulatus* and *S. cerevisiae* cytochrome *b* amino acid sequences show that while the yeast protein has several cysteine residues, the bacterial counterpart has none. In the former species, one of these cysteine residues (C342, yeast numbering) is structurally located nearby the Y279 (Fig. 2b). Whether the presence of additional cysteine residues counteracts the effect of Y279C mutation (for example by promoting an intra molecular disulfide bond within cytochrome *b)* is unknown. Mass spectrometry analyses of purified native, Y279F and Y279C yeast CIII enzymes were conducted in our group. In the case of Y279C mutant, the data indicated that the trypsin-gluC fragment encompassing Y279 (W^{273}YLLPFX^{279}AILR^{283}, where X^{279} is Y, F or C in native, Y279F and Y279C mutants, respectively) is only detectable after dithiothreitol (DTT) reduction and iodoacetamide alkylation (unpublished data). This finding suggests that in the yeast mutant the cysteine residue at position 279 might also be modified by a DTT-cleavable chemical group of unknown identity. Inspection of the bovine (Fig. 2b) and also human cytochrome *b* sequence indicates that, although they also contain several cysteine residues, none of them is structurally located in the vicinity of Y278 (homologue of *R. capsulatus* Y302). Whether the oxidative disintegration of the Fe/S protein [2Fe–2S] observed with the bacterial CIII also occurs in mammalian CIII remains to be seen.

3.3. Human mitochondrial cytochrome b Y278C mutation and ROS production

Very recently, a human mitochondrial CIII produced by a homoplasmic cybrid line generated using fibroblasts of a patient bearing the m.15579A > G (p.Y278C, i.e., the human homologue of *R. capsulatus* cytochrome *b* Y302C mutation) (Fig. 2c) heteroplasmic mutation became available [74,75]. This mutation was identified in a patient with severe exercise intolerance and multisystem disorders [76], and provided a unique opportunity to extend the significance of the findings emanating from the bacterial case. Comparison of appropriate homoplasmic cybrids carrying either the wild type or the cytochrome *b* Y278C mutation showed increased intolerance to galactose (hallmark of defective oxidative phosphorylation), drastic loss of CIII activity (over ~90%) and highly decreased CIII-dependent oxidative phosphorylation in the mutant [75]. However, the enzymatic activities of the individual CI and CII complexes, as well as the coupled activities of the CI + III and CII + III supercomplexes were little affected in the mutant. Moreover, no complete loss of the ETC driven membrane potential, or ATP synthesis was observed, suggesting that CI and CII were able to sustain some ATP production despite the low CIII activity. Excitingly, as observed with the bacterial CIII, enhanced $O_2^{\bullet-}$ production was seen in mitochondria isolated from mutant cybrids, compared to wild type cells. Moreover, an imbalance in homeostasis of the major intracellular antioxidant (i.e., an increase of the ratio of oxidized (GSSG) versus reduced (GSH) glutathione) was observed, in agreement with increased oxidative stress in mutant cybrids [75]. Indeed, the CI, CIII and CI + III activities increased significantly when mitochondrial preparations were carried out in the presence of DTT. Due to material limitations, reliable detection of the Fe/S protein [2Fe–2S] cluster by EPR spectroscopy has not yet been achieved even with mitochondria from wild type human cybrids, leaving open the question of oxidative disintegration of the Fe/S protein [2Fe–2S] cluster via ROS production.

Similar to the bacterial case, no subunit assembly defect of CIII was seen with the human cytochrome *b* Y278C mutation as compared to wild type cybrids, based on SDS-PAGE/immunoblots. Wild type and mutant mitochondria contained similar amounts of CIII as revealed by BN-PAGE of dodecylmaltoside dispersed mitoplasts. However, BN-PAGE analyses of digitonin dispersed mitochondrial respirasomes showed decreased amounts of CIII dimers and CIII + IV supercomplexes, but slightly compensatory increased levels of $CI_1III_2IV_n$ (n = number of monomers) supercomplexes [75]. The overall data suggest that supra-molecular interactions between the respiratory complexes are important for maintaining basal respiratory ETC function in the Y278C mutant. An emerging hypothesis from this ongoing work is that CIII activity might be better protected against oxidative damages when the mutant CIII is part of the $CI_1III_2IV_n$ supercomplexes (Fig. 3) [77]. Thus, comparative studies conducted for the first time with bacterial and human mitochondrial CIII bearing the same homologous mutation (Y302C and Y278C, respectively) suggest a new protective role for supra-molecular organization of respiratory complexes in membranes.

4. Perspectives

Based on structural, biochemical and clinical studies, an integrated model for ROS production in ETC, involving CI, CII and CIII complexes with their higher order of organizations and physiological regulations is emerging [54,78]. In the case of the Q_o site of CIII, factors such as the membrane potential, availability of oxidized and reduced equivalents (Q and cytochrome *c*), redox state of the enzyme, and the presence of critical amino acid residues located at specific locations, together control the rate and the amount of ROS production. As described here, recently initiated comparative studies of bacterial and human mitochondrial CIII suggest a new role for supra-molecular organizations of the ETC complexes in membranes. Formation of respirasomes appears to improve not only the substrate/product channeling between the related enzymes [79], but might also endow them with higher degrees of stability and protection against oxidative damages by restricting ROS generation (Fig. 3) [80].

Current challenges lay in the development of new tools to improve specific detection and differentiation of various types of ROS free radicals (e.g., $O_2^{\bullet-}$, $^{\bullet}OH$, $^{\bullet}H$) at very low concentrations, especially when using integrated systems like respirasomes, mitochondria or whole cells, to better define their role(s) in both signal transduction and oxidative stress. Undoubtedly, future studies will better address whether the organization into larger macromolecular entities protects the structural

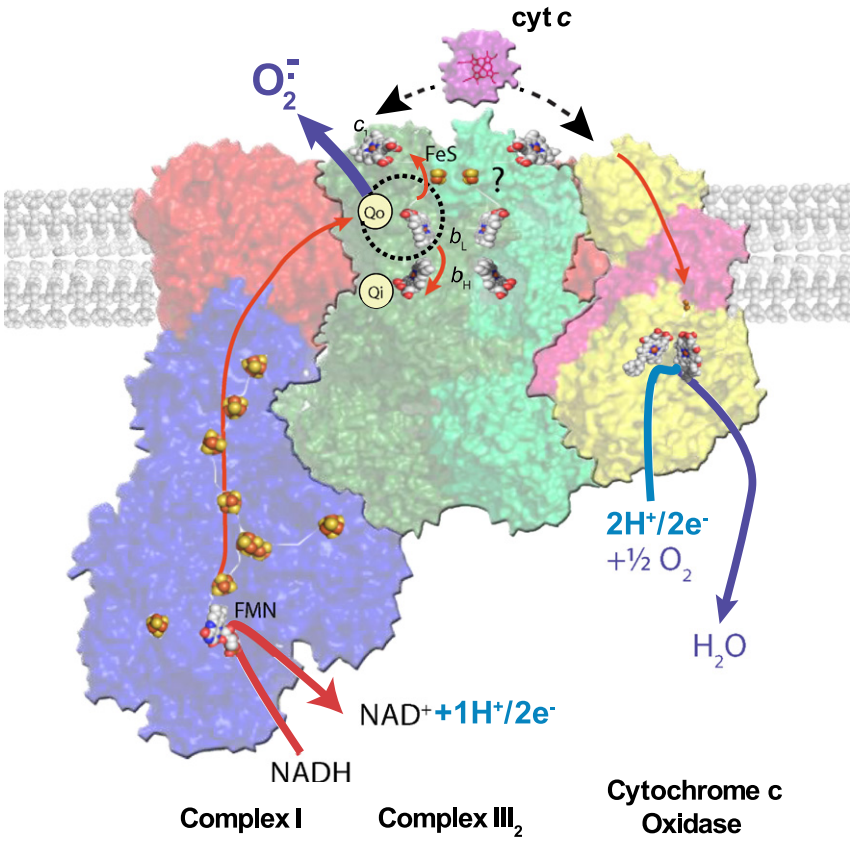

Fig. 3. Organization of ETC complexes into supercomplexes. A hypothetical higher level of organization of the respiratory complexes CI, CIII and CIV, modified from the recent single-particle electron cryo-microscopy mediated structure (PDB 2YBB) of the supercomplex $CI_1CIII_2CIV_1$ from bovine CIII and CIV and bacterial CI [77], is shown. The higher-level structural organization into larger macromolecular entities is assumed to provide structural integrity to the individual ETC complexes by maximizing inter-complex electron flux, and minimizing possible oxidative damages associated with electron leakages. A plausible protective effect exerted at the Q_o site of CIII via its close association with CI is depicted for illustrative purposes. All of the other features shown are as described in the legend of Fig. 1.

integrity of native and mutant ETC complexes, and minimizes oxidative damages associated with electron leakages. Emerging investigations of novel proteins promoting formation of supercomplexes [81–83], and determination of ROS-mediated damages on surrounding phospholipids promise to be very informative. The lipochaperone cardiolipin [84] (and also ornithine lipid in some species [85]) is already implicated in affecting the catalytic activity, formation, stability and reconstitution of supra molecular organization of the ETC complexes [86]. Lipidomic analyses using mass spectrometry will allow accurate evaluation of subtle changes in lipid profiles of intact mitochondria, isolated complexes and supercomplexes [84]. Hopefully, these studies will merge together to pave the way towards the development of novel and specific therapeutic interventions for patients with mitochondrial CIII related diseases.

Acknowledgements

This work was supported by NIH grant GM 38237 to FD and MR.

The authors thank Dr. B. Meunier for kindly providing the *Saccharomyces cerevisiae* cytochrome *b* Y279C mutant, and Drs. H. De Bari and E. Berry for providing the purified samples of *S. cerevisiae* native CIII and its cytochrome *b* Y278F and Y278C mutant derivatives.

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