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Modelling mitochondrial ROS production by the respiratory chain

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Abstract ROS (superoxide and oxygen peroxide in this paper) play a dual role as signalling molecules and strong oxidizing agents leading to oxidative stress. Their production mainly occurs in mitochondria although they may have other locations (such as NADPH oxidase in particular cell types). Mitochondrial ROS production depends in an interweaving way upon many factors such as the membrane potential, the cell type and the respiratory substrates. Moreover, it is experimentally difficult to quantitatively assess the contribution of each potential site in the respiratory chain. To overcome these difficulties, mathematical models have been developed with different degrees of complexity in order to analyse different physiological questions ranging from a simple reproduction/simulation of experimental results to a detailed model of the possible mechanisms leading to ROS production. Here, we analyse experimental results concerning ROS production including results still under discussion. We then critically review the three models of ROS production in the whole respiratory chain available in the literature and propose some direction for future modelling work.

Keywords (separated by '-') ROS - Superoxide - Oxygen peroxide - Respiratory chain - Modelling

Footnote Information



2 Modelling mitochondrial ROS production by the respiratory chain

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6 Abstract

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9 NADPH oxidase in particular cell types). Mitochondrial ROS production depends in an interweaving way upon many factors
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17 **Keywords** ROS · Superoxide · Oxygen peroxide · Respiratory chain · Modelling

18 Introduction

19 Accumulating evidence has suggested that reactive oxygen
20 species (ROS), such as superoxide, hydrogen peroxide and
21 other reactive forms of oxygen, play an important role in a
22 broad range of cellular signalling processes [1–4]. However,
23 at high concentrations, ROS damage proteins, lipid mem-
24 branes, DNA and triggers PTP opening [5, 6] generating
25 what is called oxidative stress. Oxidative stress is defined as
26 a perturbation in the balance between the production of reac-
27 tive oxygen species (free radicals) and antioxidant defences
28 and contributes to pathologies such as cancer, ischemic
29 cardiac injury and stroke, neurodegenerative diseases and
30 other age-related degenerative conditions [7, 8]. Given their
31 deleterious effects, ROS production is usually finely tuned
32 by ROS-scavenging systems.

33 The mitochondrial electron transport chain is one of the
34 major providers of ROS in most cells. In C2C12 myoblasts,

35 Wong et al. [9] show that 45% of ROS comes from mito-
36 chondria and 40% from NADPH oxidase. Work by many
37 investigators (see [10] for a review) has largely established
38 that complexes I and III of the mitochondrial respiratory
39 chain are the major sources of reactive oxygen species
40 (ROS), in the form of superoxide (O_2^-) and hydrogen per-
41 oxide H_2O_2 . However, despite intensive biochemical and
42 biophysical studies of electron and proton transfer in the
43 respiratory chain (for reviews, see [9, 11–15]) many ques-
44 tions about the mechanisms of O_2^- generation, particularly
45 in physiological conditions, remain unsolved.

46 The production of superoxide/hydrogen peroxide is dif-
47 ficult to assess, particularly their site of production and their
48 dependence upon the experimental conditions (respiratory
49 substrate, inhibitors). Furthermore, when working with the
50 whole respiratory chain in isolated mitochondria or in whole
51 cells, it is difficult to assess the relative contribution of each
52 separate site and to take into account contribution of the
53 scavenging systems. This is why theoretical models of ROS
54 generation can be useful to facilitate the quantitative analy-
55 sis of the features controlling mitochondrial O_2^- produc-
56 tion and help in the elucidation of experimental results and
57 eventually predict new discriminant experimental protocols.
58 Several theoretical models of ROS production by complex
59 I and III exist [16–21] and also one involving complex II

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60 [22]. However, we found only three theoretical models in
 61 the literature aiming at describing ROS production by the
 62 whole respiratory chain under different conditions. First,
 63 we will describe the main experimental results concerning
 64 ROS production for which a large consensus exists, and that
 65 theoretical models should reproduce, as well as experimental
 66 results leading to contradictory hypotheses, between which
 67 theoretical models might help to decide. We will emphasize
 68 the main points that a theoretical model must explain/simulate
 69 and finally we will proceed to the critical description of
 70 the three theoretical models.

71 Experimental data: the different sites of ROS 72 production and the role of inhibitors

73 Using specific inhibitors of different sites of ROS production
 74 (superoxide and/or hydrogen peroxide), particularly inhibitors
 75 that do not prevent electron flow and varying the respiratory
 76 substrates, the group of Martin Brand has finely dissected
 77 the different sites of mitochondrial ROS production
 78 [9, 10]. They identified eleven distinct sites associated with
 79 respiratory complexes or enzymes and they gave an estimation
 80 of the maximal ROS production flux for each site. If
 81 we limit our description to the respiratory chain complexes
 82 (Fig. 1), the main producers are: the Flavin site of complex
 83 I (I_F), the ubiquinone reducing site of complex I (I_Q), the
 84 Flavin site of complex II (II_F) and the ubiquinone oxidizing
 85 site of complex III (III_{Qo}), (see Fig. 1 in [10] and Fig. 1b in
 86 [23]). Site III_{Qo} has, at least, twice the capacity of any other
 87 site (see Fig. 2 in [10]).

88 ROS production by complex I

89 Site I_F has long been proposed as a site of superoxide pro-
 90 duction [24, 25]. However, Brand et al. [10, 26] show that
 91 much of the ROS production previously ascribed to site
 92 I_F truly arises from other dehydrogenases, particularly

93 sites O_F (2-oxoglutarate dehydrogenase) and P_F (pyruvate
 94 dehydrogenase).

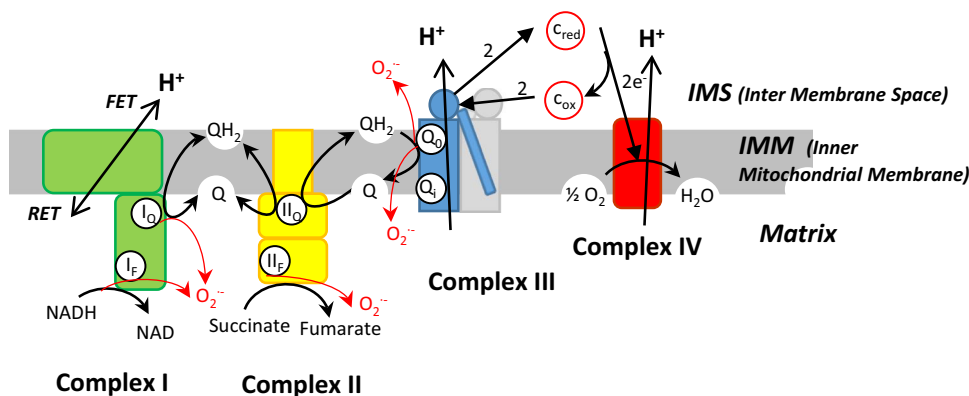
95 Site I_Q during reverse electron transport (RET), the
 96 majority of ROS arise from site I_Q . In RET (Fig. 2a), elec-
 97 trons are forced back into complex I by the high QH_2/Q
 98 ratio and the high proton motive force generated by electron
 99 flow through complexes III and IV. In this process, the elec-
 100 tron flow is driven backwards by the consumption of proton
 101 motive force. Thus, production of ROS at site I_Q during
 102 reverse electron transport has a strong dependence on proton
 103 motive force [27–32]. However, several authors showed that
 104 it is much more sensitive to the magnitude of the pH gradi-
 105 ent than of the membrane potential, even at constant proton
 106 motive force [30, 31, 33]. These results suggest that site I_Q
 107 is linked to an electroneutral proton-translocating step in
 108 the proton-pumping mechanism of complex I [34, 35]. The
 109 localization at site I_Q is confirmed by the inhibition of ROS
 110 production at this site by rotenone. Then addition of anti-
 111 mycin stimulates ROS production at site III_{Qo} (Figs. 2b, 4).

ROS production by complex II

113 The Flavin site II_F (Fig. 3) is supposed to be the site of ROS
 114 production in complex II and displays a similar maximal
 115 capacity of ROS production as site I_Q [10]. However, Grive-
 116 nnikova et al. [22] claim that their data are indicative of the
 117 [3Fe–4S] centre, close to the ubiquinone reduction site, as
 118 the site of superoxide generation in this complex. Wild-type
 119 complex II makes little contribution to ROS production in
 120 isolated mammalian mitochondria under normal conditions
 121 [36]. However, mutations in this complex can lead to abun-
 122 dant ROS production and cause pathologies [37]. ROS pro-
 123 duction by the Flavin site of complex II, site II_F , in isolated
 124 mitochondria [23] requires two conditions: there must be a
 125 source of electrons to reduce the Flavin (succinate), and the
 126 site must be open, probably to allow access of oxygen [10].

127 This behaviour results in a bell-shaped response of
 128 ROS production to succinate concentration, with a maxi-
 129 mum in the region of the K_M of complex II for succinate

Fig. 1 Possible sites of ROS production (red arrows) in respiratory chain. In the text, the site Q_o and Q_i of complex III are called III_{Qo} and III_{Qi} . All ROS are generated in the matrix except for ROS in III_{Qo} which are partly extruded in the IMS. *FET* forward electron transport (with NADH), *RET* reverse electron transport (with succinate)



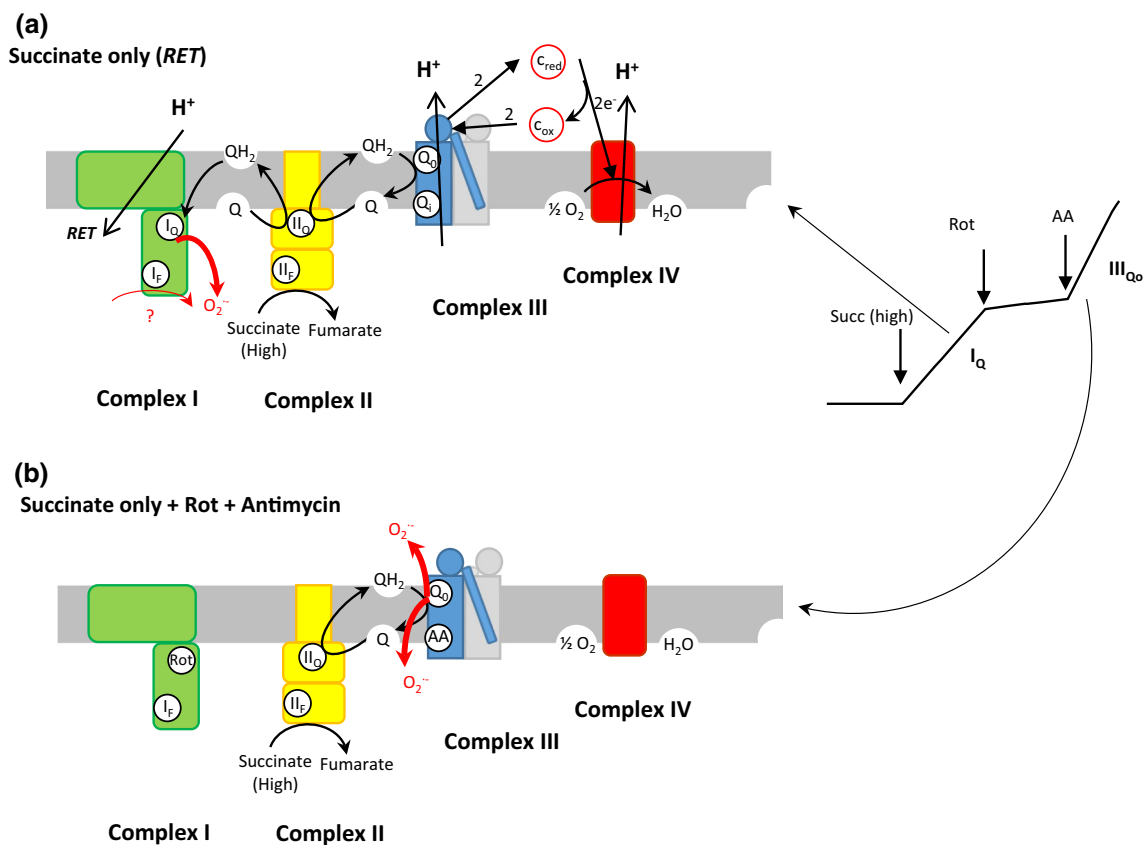


Fig. 2 ROS production in the respiratory chain by complex I and III with succinate. **a** RET results from the addition of succinate alone at high concentration. The thickness of the red arrows corresponds to the first addition of succinate without any inhibitor. Rotenone inhibits

this ROS production. **b** High ROS production by complex III in the presence of antimycin A (AA) [a similar ROS production occurs at complex III with antimycin when electrons are supplied by NADH in the absence of succinate and rotenone (Rot)]

130 (100–500 μM) [22, 23, 38]. The capacity for ROS production
 131 at site II_F can be measured in two particular conditions
 132 illustrated in Fig. 3 in the presence of inhibitors of complexes
 133 I and III [10]. In rat skeletal muscle mitochondria,
 134 the maximum capacity for ROS production of site II_F is very
 135 high, of the same order as site I_Q (Fig. 2 in [10]).

136 ROS production by complex III (bc₁ complex) at site 137 III_{Q_0}

138 Complex III (Fig. 4) at site Q_0 (III_{Q_0} site) has the highest
 139 capacity of ROS production (Fig. 2 in [10]). It is commonly
 140 accepted that the complex III ROS production is due to the
 141 formation during the catalytic process of an instable semiquinone
 142 SQ in Q_0 . However, the mechanism of a semiquinone formation
 143 in Q_0 is still the matter of controversy, which has to be taken
 144 into account for modelling. It is widely accepted that the
 145 modified Q-cycle mechanism proposed by Mitchell [39, 40] and
 146 subsequently refined by Crofts [41, 42] correctly describes the
 147 bc₁ complex operation. It is based on a bifurcation of the two
 148 electrons

coming from the QH_2 molecule bound at the Q_0 site. 149
 The first electron is transferred to the iron sulphur protein 150
 (ISP) and the second to the lower potential heme b_L . 151
 The electron on heme b_L moves within the cytochrome 152
 b to reduce the higher potential heme b_H , which in turn 153
 reduces ubiquinone (Q) at a second ubiquinone binding 154
 site Q_i (Fig. 4). The transfer of an electron from quinol 155
 to cytochrome c is a complex process involving: (i) a 156
 first electron transfer from quinol bound at the catalytic 157
 Q_0 site to a [2Fe–2S] cluster situated in the head of the 158
 Rieske iron sulphur protein (ISP) anchored in the inner 159
 mitochondrial membrane (ii) a large-scale movement of 160
 the head of reduced ISP towards cytochrome c_1 , (iii) the 161
 reduction of cytochrome c_1 and eventually, (iv) the 162
 reduction of cytochrome c by cytochrome c_1 and the return 163
 of ISP head to site Q_0 . How the steps following the transfer 164
 of the first electron on ISP interweave with the transfer 165
 of the second electron on b_L then b_H , is still debated. 166
 Three main scenarios (Fig. 5) have been proposed which 167
 may have implications for the semiquinone formation/lifetime 168
 and its reaction with oxygen: 169

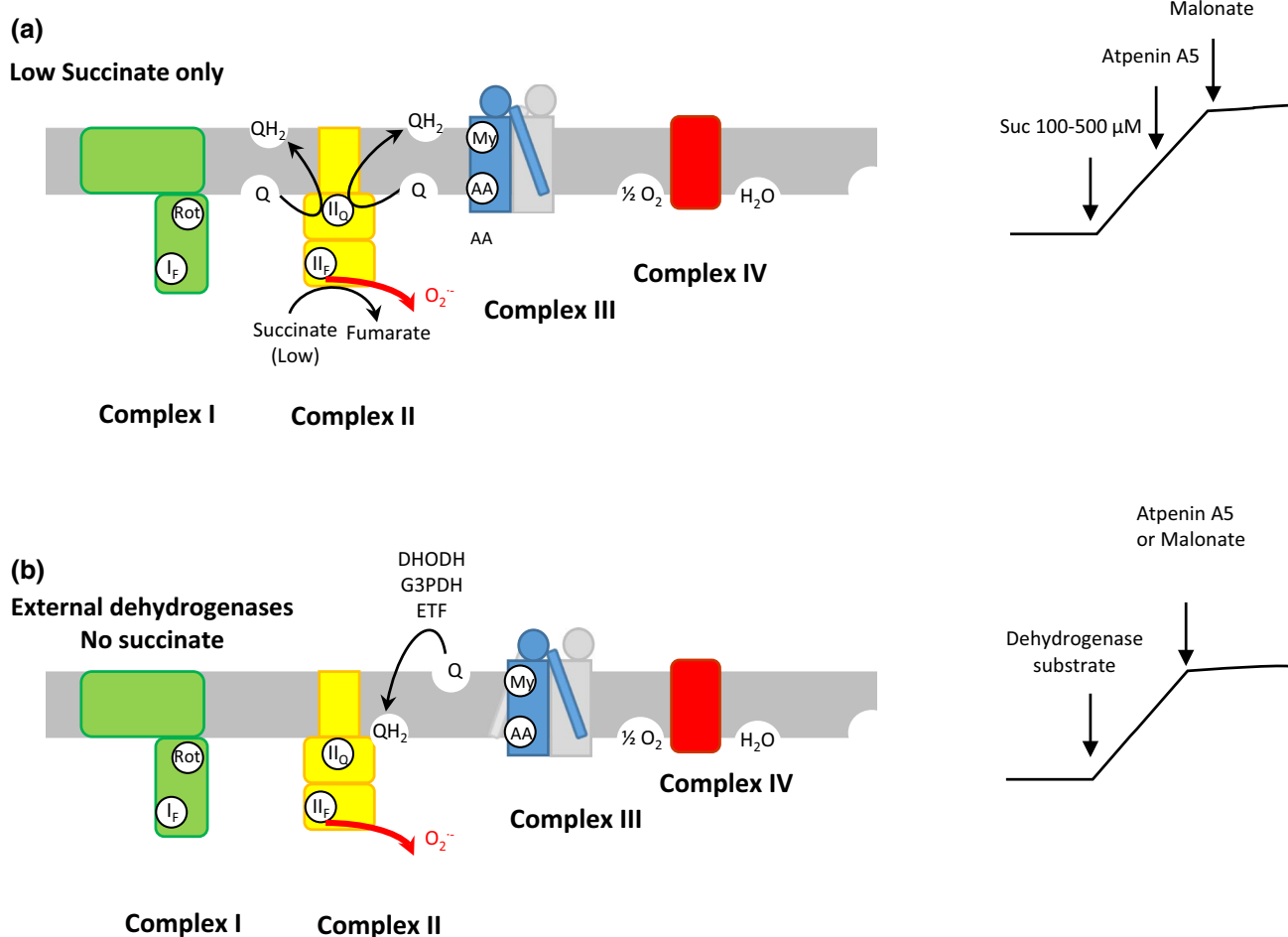


Fig. 3 ROS production at site II_F (a), complex II in the presence of rotenone, myxothiazol and antimycin generates ROS in the forward reaction: succinate around its K_M (100–500 μM) supplies electrons to reduce Q to QH_2 which accumulates and blocks electron pathway generating ROS in II_F . Malonate or high succinate concentrations cancel ROS formations but not Atpenin A5 binding in II_Q . **b**

ROS production by complex II in the reverse reaction with electrons supplied by other dehydrogenases to accumulate QH_2 and provide electrons in II_F to form ROS. In these conditions, ROS formation is inhibited by Atpenin A5 or Malonate or high succinate concentrations (after [10, 23])

170 a. The ISP leaves the Qo site to reduce c_1 before the
171 second electron jumps on b_L and then b_H [43, 44] (Fig. 5a).

172 b. The ISP leaves the Qo site to reduce c_1 after the sec-
173 ond electron transfers from b_L to b_H [45, 46] (Fig. 5b).

174 c. A bypass/short-circuit mechanism can occur when
175 reduced b_L transfers its electron not on b_H but in the reverse
176 direction on a quinone in Qo , which can be either the product
177 of the reaction or of a newly bound quinone [47–49] (Fig. 5c).

178 Summary of the main points to consider 179 in a mathematical model of mitochondrial ROS 180 generation

181 • The main site of ROS production by the respiratory
182 chain are III_{QO} , I_Q and I_F with a decreased maximum capac-
183 ity in this order.

184 • In III_{QO} and I_Q , ROS are produced by the semi-
185 quinone Q^- . The ROS production in I_F is through the fully
186 reduced $FMNH^-$ species.

187 • All ROS species by respiratory chain are produced
188 in the matrix except for III_{QO} site, which produces
189 ROS both in the matrix and in the intermembrane space.

190 • ROS production can also occur in the matrix at
191 the FMN site of complex II (II_F) at low succinate con-
192 centration around 100–500 μM , with a maximal capacity
193 analogous to the one of I_Q .

194 • The relative contributions of distinct mitochon-
195 drial sites depend on many factors: the substrates being
196 oxidized, the energetic demands of the cell, the transmem-
197 brane potential, the amount of QH_2 pool and ultimately the
198 cell type.

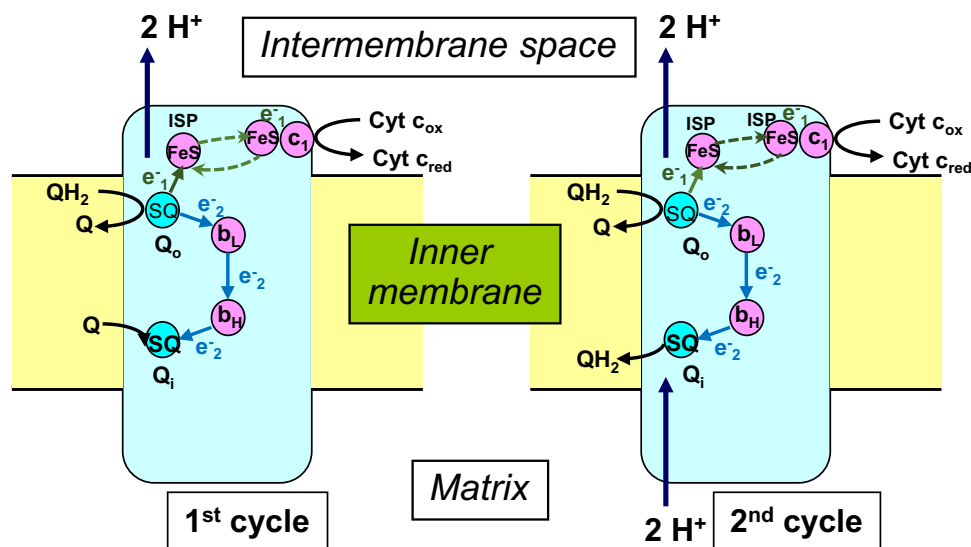


Fig. 4 Modified Q-cycle and ROS generation in complex III. The mechanism of modified Q-cycle [41, 42] involves the bifurcation of electrons in Q_o . The first electron (green pathway) reduces a FeS centre in the head of the Rieske protein which moves towards c_1 (dotted green arrows). An unstable semiquinone (SQ) can be formed before

the second electron (blue pathway) is transferred to a Q molecule in Q_i to form a stable semiquinone (bold SQ). A second cycle of QH_2 oxidation in Q_o is necessary to completely reduce the SQ in Q_i in a new QH_2 molecule. After the two cycles, two Cyt_{ox} have been reduced in two Cyt_{red} and a net QH_2 has been oxidized to Q

Mathematical models of ROS production through the whole respiratory chain

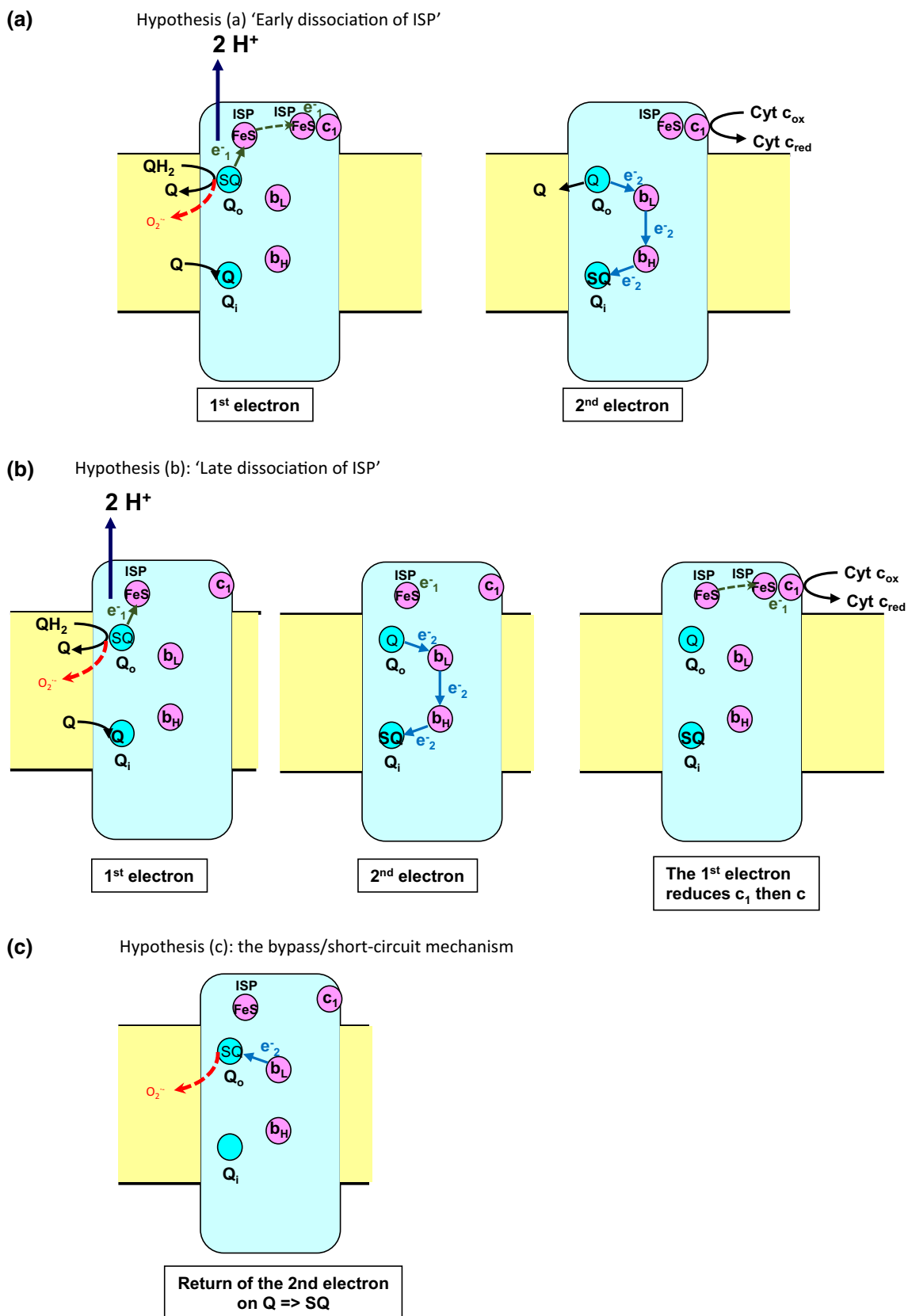
Models of the group of Aon and Cortassa

In [50], Kembro et al. extended previous mathematical models of the mitochondrial respiratory chain [51, 52] to account for ROS production. Their ROS production model is purely phenomenological, with a function called ‘shunt’ which is a small percentage of the rate of respiration (VO_2) and depends on the state 3 or 4 of the respiration rate (Table 2 in [50]). In addition, the model involves the important contribution of ROS scavenging systems to study the balance between ROS production and scavenging in different redox environments. Model simulations were compared with experiments from isolated heart mitochondria reported in the same paper. However, in their conclusions the authors note that their model “is unable to simulate the increase in ROS levels when mitochondria evolve into state 4 respiration” [50].

Shortly after, the same authors published a new model [53] which is, above all, a detailed respiratory chain model including variables describing the concentrations of ubiquinone, ubiquinol, and ubiquinol, along with the oxidation states of cytochrome c and the redox centres in complex III, i.e., the high- and low-potential b-hemes (b_H and b_L) and cytochrome c_1 . They use the forward and reverse rate constants for electron transfer of complexes II–IV from the model of Demin et al. [54, 55]. A model of complex I was based on the non-equilibrium thermodynamic description

from Magnus and Keizer’s mitochondrial model [56]. First, the authors fit the experimental results in which the variables such as b_L and b_H reduction states [57, 58] are measured because these variables play an important role in semiquinone concentration at the Q_o site of complex III. The production of ROS in complex I is supposed to occur from $FMNH^-$ according to Pryde and Hirst [59], although the authors test the possibility of ROS production in site I_Q (but in the absence of ROS production by $FMNH^-$); they claim that they obtain similar results (not shown) and conclude that “Given that the modelling results for the two different hypotheses were identical, this modelling experiment was unable to distinguish between the two mechanisms” [53]. They also reproduce the pH dependence of ROS production by complex I observed in [33] (Fig. 5 in [53]). The ROS production from complex III is modelled from Demin and involves the semiquinone in Q_o , a highly reduced quinone pool and a high proton motive force [54, 55]. The authors were aware of other hypotheses [47, 60] but did not take them into account in their model.

In addition, they added to their model of ROS production a previous minimal model of ROS scavenging [61], which allowed them to draw a U-shaped dependence of the ROS balance between production and scavenging as a function of mitochondrial redox environment (sum of redox potentials for NADH, NADPH and GSH weighted by their respective concentrations [62]): the measured ROS production is high in reducing environment which favours ROS production which cannot be destroyed at limited scavenging mechanisms, but are also high in oxidizing environment,



Author Proof

◀**Fig. 5** ISP movement, transfer of the second electron and ROS formation. The dotted red arrows indicate the possible formation of O_2^- from the semiquinone SQ in Qo **a** ‘early dissociation hypothesis’: O_2^- formation can occur in the same time as reduced ISP leaves Qo site, i.e. before the second electron is transferred to heme b_L ; **b** ‘late dissociation hypothesis’: reduced ISP leaves Qo site after the second electron is transferred from b_L to b_H and **c** return of the electron of reduced b_L on a Q molecule in Qo forming the semiquinone which can react with oxygen

257 as, despite a lower ROS production, the scavenging mecha-
258 nisms are also less efficient (less regenerated in oxidizing
259 environment). The intermediate environment corresponds
260 to a minimum in the ROS production.

261 More recently, the same authors proposed an experi-
262 mental and theoretical approach to assess the effects of
263 β -oxidation in the heart on redox and energy metabolism
264 [63]. They described the antagonist effects of fatty acids as
265 respiratory substrates but also as uncouplers on the respira-
266 tion rate (VO_2) and on the ROS production rate in relation-
267 ship with the level of the antioxidant system.

268 **Markevich’s model [64]**

269 In order to study the mitochondrial production of ROS in
270 different conditions of respiratory substrates and membrane
271 potential ($\Delta\psi$), Markevich and Hoek developed an elabo-
272 rate computational model of the whole respiratory chain.
273 Complex II, modelled by only one simple rate equation, is
274 not taken into account in their work for ROS production.
275 Electron transfers inside complex I and III are detailed with
276 several sites of ROS production. In accordance with Kuss-
277 maul and Hirst [24], they proposed that O_2^- is formed by
278 the transfer of one electron from the fully reduced Flavin
279 FMNH⁻ to O_2 . In addition, they assumed that the semiqui-
280 none Q^- in site I_Q is a second site of O_2^- formation in Com-
281 plex I. Regarding complex III, they considered, as generally
282 accepted, that the unstable semiquinone Q^- in site Qo is
283 the site of O_2^- formation. According to the various mecha-
284 nisms of electron transfer that have been proposed in Qo
285 (see above), Markevich and Hoek compare three variations
286 of their model. The first one considers that O_2^- formation
287 occurs at the same time as the reduced Rieske protein (Iron
288 Sulphur Protein or ISP) leaves the site Q_o to transfer its elec-
289 tron to c_1 (‘early dissociation of ISP’, Fig. 5a) i.e. before the
290 second electron is transferred to the heme b_L [43, 65]. The
291 second variation suggests that the Rieske protein leaves the
292 Qo site after the transfer of the second electron to b_L and
293 then to b_H (‘late dissociation of ISP’, Fig. 5b) [46]. In a third
294 variation proposed by Dröse and Brandt [47] they considered
295 that the oxidized quinone can leave the Qo site before ISP
296 and before b_L transfers its electron to b_H . This may allow
297 the return of the reduced b_L electron on an oxidized Q mol-
298 ecule (bypass/short-circuit mechanism also proposed in [48,

49]) by the reversion of the reactions to form a semiquinone
which can react with O_2 and explain the activating role of the
oxidized quinone in O_2^- formation by complex III (Fig. 5c).

The values of the kinetic parameters of superoxide pro-
duction were chosen such that the computer-simulated rates
of ROS generation were close to those observed in liver
mitochondria [66, 67].

Their model is detailed enough to dissect ROS production
at each site in different experimental conditions, particularly
as a function of the transmembrane potential $\Delta\psi$ with dif-
ferent respiratory substrates (NADH alone, succinate alone,
NADH⁺ succinate and NADH⁺ Rotenone (ROT), Fig. 3 in
[64]). The difference in ROS production when pH or $\Delta\psi$
were changed is noteworthy (Fig. 4 in [64]). Furthermore,
comparing the different variations of electron transfer in site
Qo of complex III, they proposed that the scenario with ‘late
dissociation of ISP’ is more likely. Finally, their third vari-
ation of the model (with ‘late dissociation of ISP’ and with
binding of Q when $cytb_L$ is reduced) qualitatively repro-
duced the results of Dröse and Brand [47] and of Quinlan
et al. [19] (see also Fig. 12 in [64]). They dissected this
behaviour by analysing the amount of individual species,
oxidized Q, reduced b_L and the b_L^- .Q.ISP complex (Fig. 12B
in [64]). Their results stressed the necessity of a detailed
modelling in the multifaceted field of ROS production by
the respiratory chain. Furthermore Markevich and Hoek
showed another advantage of modelling, i.e. generating
testable hypotheses, some of them were confirmed later on,
such as the ROS production by RET in I_Q [10]. Incidentally,
they offer a careful model of the calculation of the different
volumes and concentrations of the different mitochondrial
compartments (membrane, matrix, etc.). In fact, this very
detailed model is not far from a complete model of the res-
piratory chain, i.e. the expression of VO_2 as a function of
different substrates (Fig. 3E in their paper) and can certainly
be used to test other hypotheses.

Bazil and Vinnakota’s model [68]

Bazil et al. [68] integrated their previous models of super-
oxide and hydrogen peroxide production by complexes I
[20] and III [69] into an updated Beard’s model of oxidative
phosphorylation [70] that can simulate both the respiratory
dynamics associated with ATP production and the kinetics
of ROS production in a single integrated system. The authors
distinguished hydrogen peroxide and superoxide generation.

The authors showed that the kinetic control is distributed
and depends upon the experimental conditions as experi-
mentally reported by many authors [71–73] and they fitted
experimental results obtained using isolated rat heart mito-
chondria at low (1 mM) and high (5 mM) Pi concentrations.

They showed that ROS production depends indirectly on
Pi concentration through changes in pH and $\Delta\psi$.

Model simulations predicted that complex III is responsible for more ROS production during physiological working conditions relative to complex I in the condition of forward electron transport, where electrons are transferred along the Electron Transfer Chain (ETC) from NADH and succinate to O_2 . However, simulating ischemia/reperfusion mechanism, they showed that an accumulation of succinate leading to a highly reduced quinone pool can explain a burst of ROS generated in complex I corroborating the experimental results of Chouchani et al. [8] (who also used a mathematical model of ROS production in complex I [74]). Of note the authors explored the bistability behaviour reported in [16–18] characterized by different rates of ROS production in the same conditions. This is mainly due to their model of ROS production by complex III [69] as emphasized in several complex III models aimed to simulate this phenomenon [16–18, 21].

Conclusion and future prospective

We have analysed in this review the three models currently available for ROS production by the whole respiratory chain. Two of them focused on ROS production in heart [50, 53, 68] in which ROS production during ischemia–reperfusion is a medical concern. The third one by Markevich and Hoek [64] estimated parameters from liver mitochondria but is in fact rather general. It is difficult to compare these models because their aims were different. The models of Aon and Cortassa and of Bazil and Vinnakota tried to simulate in the simplest way possible the experimental results in the heart. Furthermore, Aon and Cortassa studied the physiological interplay of the scavenging mechanisms with ROS production. On the contrary, the Markevich and Hoek model [64] aimed at understanding the intimate mechanisms of ROS production in different conditions. Their model is more detailed than the two others and analysing the consequences of different scenarios for the complex reaction of complex III, proposed a mechanism of the controversial mode of bifurcation of electrons in the Q_o site of bc_1 complex. This illustrates two purposes of modelling, either to derive a phenomenological simple model which can be used to study physiological question such as the balance between ROS production and scavenging in different redox situations or to test different mechanistic hypotheses of ROS production as done by Markevich and Hoek [64]. However, even in this latter case some simplifications were made. For instance, they bring together almost all FeS centres redox reactions of complex I in one reaction and concerning complex III, they assume the release of reduced ISP from Q_o in the same time as the second electron transfer on b_L , which is far from acknowledged.

Another hypothesis, made in all models, is the localization of ROS production. A general consensus is emerging that I_F , I_Q , II_F and III_{Q_o} are the main sites of ROS production in the respiratory chain and there are good evaluations of their maximal capacities [10]. All models take these sites as ROS production sites, excluding site II_F . In addition, some indications exist of the possibility of ROS production at other sites. In principle, any reduced redox centre with midpoint potential close to that of the O_2/O_2^- couple (-160 mV), should be able to produce O_2^- when O_2 is (spatially) close enough to accept an electron. The respiratory chain complexes contain iron–sulphur centres and hemes which are, in principle, able to transfer their electrons to O_2 . The limiting factor will be the distance, the O_2 molecule being at the closest at the surface of the protein. This is perhaps the reason why the binding sites, opened on the external medium and allowing O_2 to diffuse near the redox active site, are favoured for ROS generation. Although left aside by a number of experimenters, there have been published experiments proposing such redox centres as producing superoxide. For complex I, the iron–sulphur centre N_2 which is close to the quinone binding site has been proposed in [75], as well as the iron–sulphur centre $N1a$ which is not very far from the FMN in [76]. The Fe_3S_4 iron–sulphur centre of complex II which is close to the quinone binding site was proposed as a superoxide producing site [22]. Similarly, it has been proposed that reduced heme b_L is able to generate ROS in complex III [77]. One way of approaching this problem without any a priori would be to calculate the probability of reacting with the oxygen of all the redox centres of the respiratory complexes by using the equations developed by Moser and Dutton [78], that considers the distance and the difference in redox potential between the redox centres. Such a stochastic treatment takes into account all possible reactions (i.e. the reactions with a reasonable probability) and all the different oxidized/reduced species, but only when they are produced contrary to what occurs using differential equations (see for instance [79, 80]).

Of note, all models listed above are studied at steady-state. Let us also emphasize that experimental studies of transient phases of ROS production might be more informative on the intimate mechanisms than the simple consideration of steady-states.

To sum up, it appears that a complete model of ROS production by the respiratory chain still remains to be developed by incorporating at least the generation of ROS by the dehydrogenases of the Krebs cycle (including the II_F site of the succinate dehydrogenase) in a deterministic (differential equations) or/and a stochastic approach.

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