Generation of Reactive Oxygen Species by Mitochondria

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Reactive oxygen species (ROS) are series of chemical products originated from one or several electron reductions of oxygen. ROS are involved in physiology and disease and can also be both cause and consequence of many biological scenarios. Mitochondria are the main source of ROS in the cell and, particularly, the enzymes in the electron transport chain are the major contributors to this phenomenon.

mitochondria ROS mechanism signalling disease supercomplexes

1. Introduction

All metazoans require oxygen to survive as it is used by the mitochondria to obtain operational energy. Mitochondria are composed by a double membrane, the inner mitochondrial membrane (IMM) and the outer mitochondrial membrane (OMM), which are separated by the intermembrane space (IMS) and hold the interior of the organelle, the mitochondrial matrix, isolated form the cytoplasm. These organelles use a panoply of carbon sources, from catabolic to anabolic pathways, which generate reducing equivalents useful for the formation of adenosine 5′-triphosphate (ATP). Reducing equivalents, such as nicotinamide adenine dinucleotide hydrogen (NADH) or flavin adenine dinucleotide dihydrogen ($FADH_2$), are co-factors in multiple reactions that store electrons derived from metabolic oxidations [1]. NADH flux independently and act as substrate of mitochondrial complex I (CI), which, in turn, reduces ubiquinone to ubiquinol. On the other hand, $FADH_2$ remains linked to the enzymes that are in contact with the IMM, where they interact with CoQ to recycle their FAD cofactors by reducing ubiquinone to ubiquinol. The most prominent example of FAD-dependent enzymes in mitochondria is complex II (CII). Complex III (CIII) oxidizes ubiquinol to reduce cytochrome c, which, subsequently, donates its electron to complex IV (CIV). Finally, CIV reduces oxygen to water. CI, CIII, and CIV couple the electron flux to the ejection of H^+ across the IMM, creating a H^+ electrochemical gradient (negative and alkaline inside), the proton motive force (Δp). Δp is used by a fifth complex, the ATP synthase (CV), to transfer H^+ back to the mitochondrial matrix in an energy-releasing process, which is used to phosphorylate adenosine 5′-diphosphate (ADP) into ATP. As long as the oxygen consumption matches the phosphorylation of ADP, the respiration is coupled to ATP synthesis. Complexes from CI to CIV comprise the mitochondrial electron transport chain (mETC), which, together with CV, form the oxidative phosphorylation system (OXPHOS; **Figure 1**) [1].

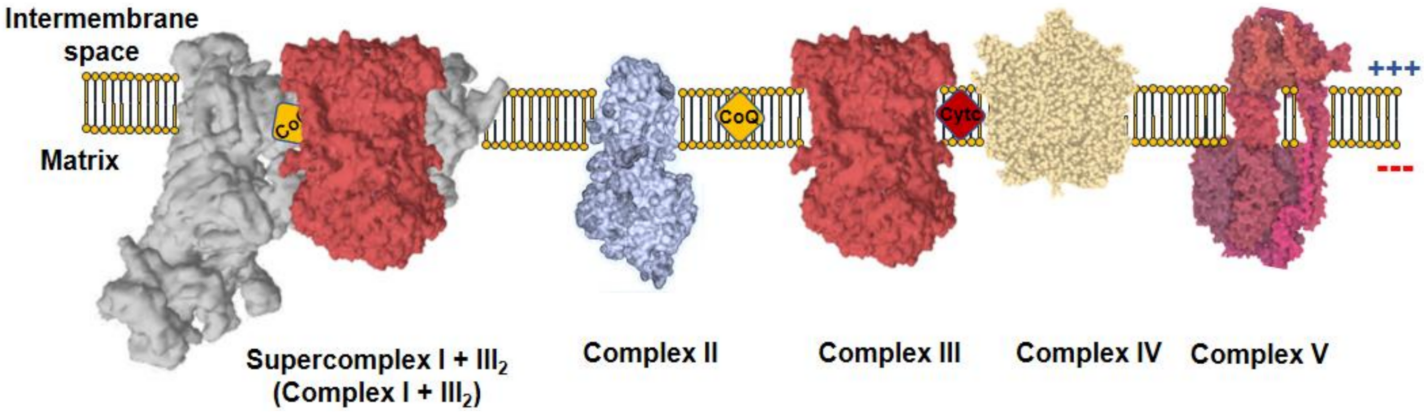

Figure 1. Mitochondrial oxidative phosphorylation system (OXPHOS). The inner mitochondrial membrane (IMM) comprises five protein complexes, which couple the transfer of electrons to H^+ pumping. Charge distribution across the IMM produces a $\Delta\Psi m$, negative inside. Complex I (CI) is normally found inside supercomplexes with complex III (CIII) (supercomplex I + III_2) or CIII+complex IV (CIV) (N-respirasome), a particularly relevant feature for mitochondrial reactive oxygen species (ROS) production.

Δp is actively generated by the OXPHOS system; therefore, it reflects an active process of charge separation across the IMM, unlike the plasma membrane in which the charge separation is carried out by diffusion potential. Δp can be further decomposed in mitochondrial membrane potential ($\Delta\Psi m$) and pH gradient (ΔpHm). $\Delta\Psi m$ is created by the difference in charge distribution across the IMM, being positive in the IMS and negative in the matrix. Similarly, the distribution of H^+ across the IMM makes the IMS acidic and the matrix alkaline. An often-forgotten integral inner membrane protein that contribute to the H^+ gradient is the NAD(P) transhydrogenase or NNT. NNT couples hydride transfer of reducing the equivalent between NADH and NADP to proton translocation across the inner mitochondrial membrane at the expenses of Δp, generating NADPH of the proton gradient, but can work in reverse to generate Δp and NADH from NADPH [2]. In addition to its role in ATP synthesis, the Δp is utilized by the mitochondria as the driving force to import proteins, metabolites and to balance the ion fluxes across the IMM. Interestingly, the movement of ions and charged molecules across the IMM impacts the Δp.

One of the more relevant ions that cross the IMM is Ca^{2+}. Mitochondrial Ca^{2+} uptake is carried out by the inwardly rectifying mitochondrial Ca^{2+} uniporter (MCU), a highly specific channel for Ca^{2+} [3]. Thus, the MCU passes Ca^{2+} down the electrochemical gradient without coupling ATP hydrolysis or transport other ions. Mitochondrion Ca^{2+} extrusion is primarily carried out by the mitochondrial Na^+/Ca^{2+} exchanger (NCLX) [4][5] in an electrophoretic process that mediates the extrusion of $3Na^+$ per $1Ca^{2+}$. The activity of NCLX depends on the Na^+ gradient created by the fast acting mitochondrial Na^+/H^+ exchanger (mNHE), which parallels Na^+ gradient to ΔpHm [6]. In this way, Ca^{2+} and Na^+ homeostasis becomes engaged to the activity of OXPHOS. Notably, despite the importance of these exchangers for mitochondrial homeostasis, their molecular identities have remained unknown until recently [5][7][8], with the exception of the mNHE whose molecular identification remains to be confirmed.

All organisms are subjected to acute changes in oxygen availability (hyperoxia and hypoxia, respectively) and both result in the production of reactive oxygen species (ROS). ROS are most commonly the product of subsequent

one-electron reduction of oxygen. Thus, one electron reduction of oxygen produces superoxide anion ($O_2^{\bullet-}$), which is the most common first step in all ROS-producing enzymes and a very toxic species. One-electron reduction of $O_2^{\bullet-}$ produces hydrogen peroxide (H_2O_2), which is the best-known ROS acting as second messenger, normally due to its ability of reversibly oxidizing thiols groups on proteins. Subsequent one-electron reduction of H_2O_2 yields hydroxyl radical ($^{\bullet}OH$), which is an extremely harmful ROS that is notoriously involved in toxic reactions, such as the Fenton reaction. Mitochondrial ROS are involved in numerous physiological processes [9][10][11][12][13][14][15] and also link the progression from tissue homeostasis to disease [16][17][18][19]. Very surprisingly, the mechanisms of ROS production in live cells and tissues are poorly understood. Yet, thanks to the work on isolated mitochondria, researchers have a progressively deeper knowledge of how these organelles produce ROS [20][21][22][23][24].

2. Modes of ROS Production by Mitochondria

Classical experiments with mETC inhibitors pointed to CI and CIII as the major sources of ROS within the mitochondria and the cell. From a general point of view, potential sites of ROS production are triggered depending on the respiration substrate, membrane potential and, if present, the inhibitor used.

Under normal conditions, coupled respiration on glutamate/malate (GM) or pyruvate/malate (PM) activates the Krebs cycle enzymes 2-oxoglutarate dehydrogenase (OGDH), malate dehydrogenase (MDH), and pyruvate dehydrogenase (PDH), and maintains a low membrane potential as CV is producing ATP. OGDH, PDH, and MDH reduce NAD^+ to NADH, which is, in turn, a substrate of CI. As electrons flow down the mETC they eventually reach CIII and CIV. In this situation, the production of ROS is low, but measurable and is commonly assigned to CI, the outer ubiquinone-binding site of CIII ($CIII_o$) and OGDH [22][23][24] in the so-called forward electron transfer (FET) (**Figure 2**A). This mode of ROS production can be exacerbated by ubiquinone-binding site inhibitors of CI, such as rotenone or piericidin A [25][26][27][28], or by inhibitors of the inner ubiquinone-binding site of CIII ($CIII_i$), such as antimicyn A [29][30]. On the other hand, to decrease this type of ROS production, especially after the application of the mentioned compounds, inhibitors of the flavin site of CI, such as diphenyleneiodonium (DPI; [31][32]), $CIII_o$ blockers, such as myxothiazol, stigmatellin, or mucidin [33], and OGDH inhibitors, such as succinyl phosphonate or 3-methyl-2-oxopentanoate [24][34], can be applied.

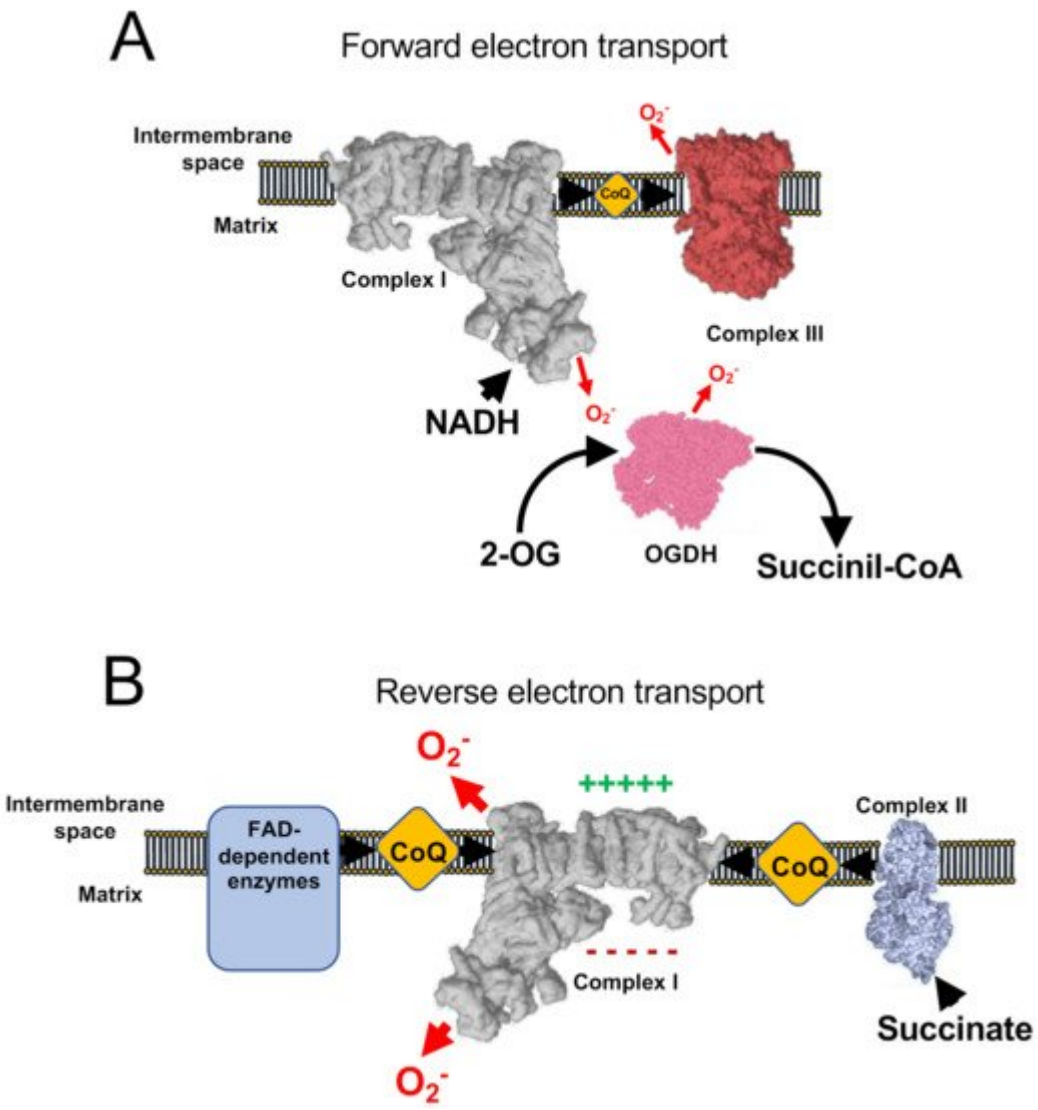

Figure 2. ROS production involving respiratory Complex I. (**A**) Under normal conditions, minimal amount of mitochondrial ROS are produced, nicotinamide adenine dinucleotide hydrogen (NADH) is oxidized at a high rate, as well as ubiquinol and 2-oxoglutarate. (**B**) Under conditions of normal-to-high $\Delta\Psi m$ and accumulation of reduced CoQ (or succinate) complex I works in the reverse mode, producing high rates of ROS. In addition, a few flavin adenine dinucleotide (FAD)-dependent enzymes have also been shown to contribute to reverse electron transfer (RET).

Succinate is the substrate of the Krebs cycle enzyme succinate dehydrogenase (SDH), also known as CII. SDH is representative of a variety of FAD dependent enzymes in the IMM that reduce ubiquinone, such as glyceraldehyde-3-phosphate dehydrogenase (G3PDH), dihydroorotate dehydrogenase (DHODH), and electron-transferring flavoprotein (ETF) [35]. When reducing potential is provided by CII, or to a lesser extent by other FAD-dependent enzymes, mitochondria over reduce ubiquinone and, under conditions of mitochondrial hyperpolarization (i.e., inactive CV), electrons are able to flow back through CI, reducing NAD^+ into NADH and producing superoxide. This process is known as reverse electron transfer (RET) (**Figure 2**B). RET is the mode that produces the largest levels of ROS [36] and has been observed in both physiological [12][26] and pathophysiological situations [16][37][38]. Notably, the exact site of ROS production by RET is not yet clear as some authors propose that it occurs at the flavin site of

CI (CI_N; [36][39][40]), whereas others suggest the ubiquinone-binding site of CI (CI_Q) plus CI_N [41][42] or the iron-sulphur cluster N2 [43] as the main actors. The investigations of ROS production by RET have used a variety of commonly used mitochondrial drugs to halt this mechanism: (i) CI inhibitors such as rotenone, DPI [20][39] or piericidin A [42]; (ii) OXPHOS uncouplers, which are $\Delta\Psi m$-disrupting molecules, such as FCCP [12][20][22][44]. As CI inhibitors can impede other modes of ROS production (see below), it is recommended to not only defining RET by its sensitivity to CI blockers (e.g., rotenone sensitivity), but also by using OXPHOS uncouplers. RET can be exacerbated by the incorporation of molecules to increase Δp [20], such as the CV inhibitor oligomycin or ATP, and it has been proposed that RET can be modulated by the activity of NNT as regulator of the NADH concentration [45][46]. Recently, it has been shown that mice harbouring ND6-P25L mutation in their mitochondrial DNA [47] are unable to produce rotenone-sensitive RET, though they still display some residual FCCP sensitivity [48]. Such inability is due to the capacity of CI to enter into its deactive form in every catalytic cycle, possibly allowing the enzyme to undergo active/deactive (A/D) transition very rapidly under hypoxia [48]. In addition, residual ROS production under succinate oxidation may be caused by the $CIII_o$ [23][49].

As commented above, independently of the electron source, CIII is able to produce ROS upon inhibition with specific molecules. $CIII_i$ inhibitors, such as Antimycin A promote the accumulation of electrons inside CIII, which reach the $CIII_o$ site, producing superoxide anion. It is important to note that $CIII_o$ blockers, such as myxothiazol or stigmatellin do not induce the production of ROS as they render the complex completely oxidized. Indeed, $CIII_o$ inhibitors block the production of ROS by Antimycin A.

Under specific experimental conditions, when CI and CIII are inhibited and the concentration of succinate is low, CII can produce ROS at a significant rate (**Figure 3**). CII can also operate in forward and reverse modes as it can accept electrons both from succinate (forward) and ubiquinol (reverse). When working in the reverse mode (**Figure 3**A), CII can produce ROS sensitive to the ubiquinone-binding site inhibitor atpenin A5, and to the flavin-binding inhibitor malonate. However, when working on the forward mode (**Figure 3**B), CII can produce ROS that are only sensitive to monate, indicating that the site of ROS production under both modes is the flavin [49].

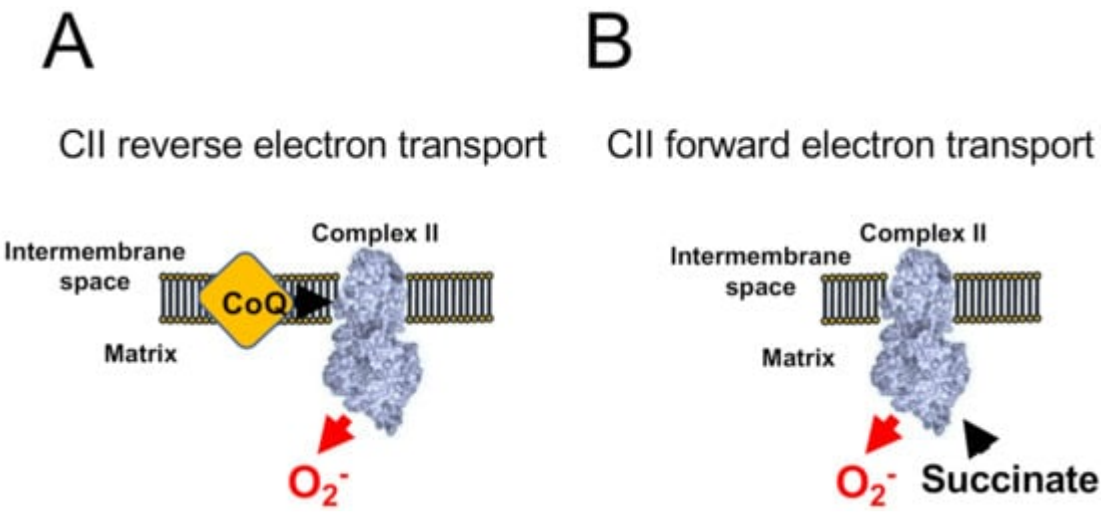

Figure 3. ROS production by Complex II. CII-dependent ROS production has been shown to occur only when CI and CIII are blocked. (**A**) When CoQ is highly reduced CII generates atpenin A5-sensitive ROS in its reverse reaction. (**B**) The accumulation of succinate promotes malonate-sensitive ROS in its forward reaction.

To note, ROS production triggered by oxidation of G3P can also be promoted by other sources, such as CI (RET) and, more scarcely, $CIII_O$ [23][49]. In very specific experimental conditions, in which CI and CIII are inhibited, DHODH has been shown to produce ROS through CII and, to a lesser extent, by itself [50]. Moreover, OGDH, branched-chain 2-oxoacid dehydrogenase (BCODH) and pyruvate dehydrogenase (PDH) are potential sources of ROS under precise experimental conditions (**Table 1**) [34].

Table 1. Summary of the main ROS sources in mitochondria. Respiratory enzymes are able to produce ROS in forward and reverse reactions, as well as under specific physiological conditions, such during acute hypoxia. Biological material is underlined.

Name and Source	*System*	*Substrate(s)/Conditions*	*Potentiator(s)*	*Inhibitor(s)*	*References*
FET (CI and $CIII_O$)	Tissues, cells, and isolated mitochondria	Cells and Tissues: standard culture media Isolated Mitochondria: pyruvate, malate and glutamate	Rotenone, Piericidin A or Antimycin A	DPI, myxothiazol, stigmatellin or mucidin	[25][26][28][29] [30][31][32][33] [34]
RET (CI_N)	Cells and isolated mitochondria	Succinate or G3P	CV inhibitors or ATP	CI and CII inhibitors and OXPHOS uncouplers (FCCP)	[13][16][20][22] [36][37][38][39] [40][41][42][44]
$CIII_O$	Cells and isolated mitochondria	NADH or succinate	Antimycin A	Myxothiazol or stigmatellin	[29][30][51]
CII-derived forward ROS production	Isolated mitochondria	Low succinate concentration, CI and CIII inhibited	-	Malonate	[49]
CII-derived reverse ROS production	Isolated mitochondria	Ubiquinol concentration, CI and CIII inhibited	-	Atpenin A5 and malonate	[49]
Hypoxic ROS	Tissues, cells and isolated mitochondria	Cells and Tissues: normal culture media Isolated Mitochondria: Malate, glutamate, $CaCl_2$ and NaCl	Monensin, Nigericin, FCCP (in normoxic cells)	Rotenone, piericidin A, myxothiazol, malonate,	[52]

Name and Source	System	Substrate(s)/Conditions	Potentiator(s)	Inhibitor(s)	References
				NCLX inhibitors (preincubated)	[53]

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Nowadays, though the relationship between chronic adaptation to hypoxia and the production of ROS is still unclear, acute responses to hypoxia is undoubtedly associated to hypoxic ROS generation [52][56][57][58][59][60].

During the first minutes of hypoxia, CI undergoes the active/deactive (A/D) transition, which consists of a conformational change involving ND3 and other CI subunits. ND3 rearranges to expose its Cys39 [61][62][63][64]. A/D transition is a characteristic dormant state of CI that is not able to perform its enzymatic activity and, therefore, is not able to pump H^+ [27][65]. As CI becomes deactive in acute hypoxia, the mitochondrial matrix acidifies and the calcium phosphate precipitates in the matrix partially dissolve, liberating free Ca^{2+}. The rise in matrix [Ca^{2+}] activates the mitochondrial Ca^{2+}/Na^+ antiporter (NCLX), which promotes the entry of Na^+ into the mitochondria. Na^+ accumulates in the matrix and interacts with the carbonyl group in the phospholipids of the inner leaflet of the IMM. The interaction Na^+:phospholipid promotes the formation of phospholipid aggregates, which, in turn, diminish the fluidity of IMM. The decrease in IMM fluidity lowers ubiquinol transfer between CII and CIII, while the transfer between CI and CIII is preserved as they are arranged into supercomplexes [66][67]. The decrease in ubiquinol transfer between CII and CIII promotes the generation of superoxide at the level of $CIII_o$ due to the uncoupling of the Q cycle. This mechanism highlights the role of Na^+ as second messenger, controlling OXPHOS and hypoxic redox signalling [52].

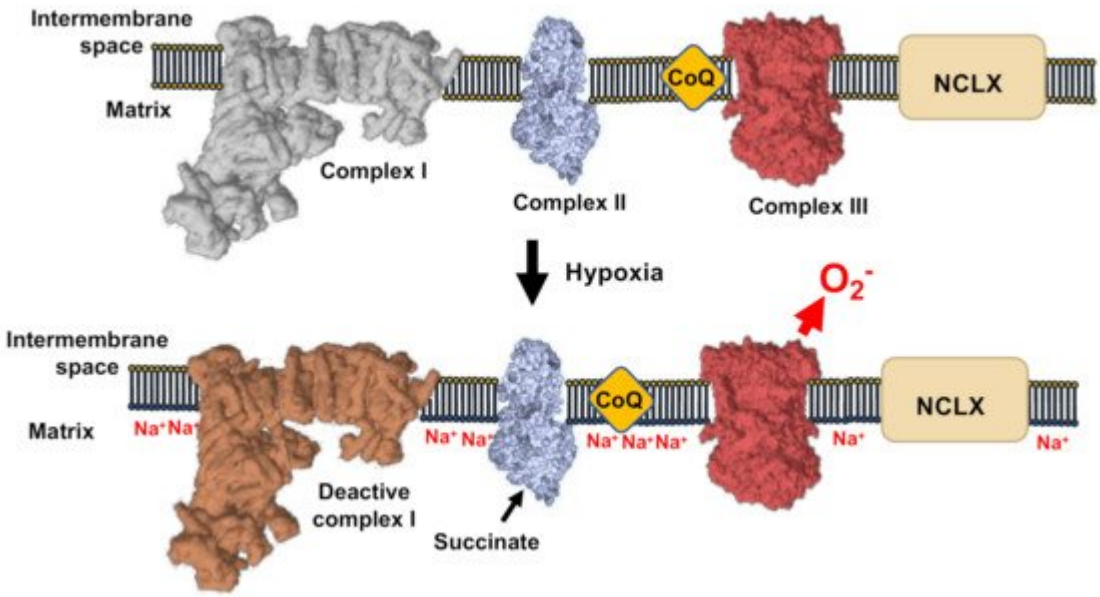

Figure 4. Mechanism of ROS production in hypoxia. CI-deactivation upon oxygen reduction induce de acidification of the mitochondrial matrix, the subsequent solubilization of matrix Ca^{2+} precipitates. The concomitant elevation of Ca^{2+} concentration activate the Ca^{2+}/Na^+ antiporter (NCLX). This causes the elevation of Na^+ that decrease the inner membrane fluidity affecting the free CoQ diffusion and increase ROS production by CIII.

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