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Mitochondrial Q cycle–derived superoxide and chemiosmotic bioenergetics

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We examined the intrinsic relation between two interdependent and interacted processes, namely, chemiosmotic energy coupling partition and redox signaling involved in mitochondrial respiration. The following aspects of research were conducted and discussed: generation sites and release sidedness of superoxide from the Q cycle of complex III of the mitochondrial respiratory chain; the different physiological roles of PMF components, $\Delta\Psi$ and ΔpH (ΔpH^S), of the Q cycle in mitochondrial superoxide generating and partitioning; and direct feedback effects of Q cycle–derived $O_2^{\bullet-}$ on PMF energy partition through its interaction with protons in ΔpH^S to form $HO_2^{\bullet}$, leading to decreasing ΔpH^S and ATP synthesis due to its increasing effects of basic proton leak of mitochondria. The present experimental data give new evidence for our hypothesis of reactive oxygen species cycle cooperation with Q cycle and H^+ cycle in respiratory chain in keeping PMF energy partition and its equilibrium with redox signaling regulation of mitochondrial respiration.

Keywords: mitochondrial Q cycle superoxide; single electron leak; proton leak; non-Ohmic dependence; protonmotive force partition; chemiosmotic bioenergetics

Introduction

It has been established that more than 90% of oxygen consumed by aerobic eukaryotic cells is reduced to water through four steps of single electron reduction by cytochrome c oxidase of mitochondrial respiratory chain. Meanwhile, a small proportion (2–6%) of consumed oxygen *in vitro* can also be reduced partially by one or two electrons in the mid-pathway of respiratory chain to generate superoxide ($O_2^{\bullet-}$) and its derivative, hydrogen peroxide (H_2O_2) (both called as reactive oxygen species—ROS), as normal metabolic products of oxygen during respiration by isolated mitochondria, or much less ROS production *in vivo* was measured to be 0.2% of oxygen consumption in cell culture condition.[1] However, due to the largest amount of the inner mitochondrial membrane surface and of the enzymes activity of respiratory chain complexes among cellular membranes and enzyme systems, mitochondrial respiratory chain produces 95% of $O_2^{\bullet-}$ in oxidative metabolism of the living body, and the daily yield of $O_2^{\bullet-}$ reaches 3×10^7 molecules per mitochondria, and is estimated, if only 0.1% of oxygen consumption is account for $O_2^{\bullet-}$ production through 'single electron leak pathway' of oxygen reduction from mitochondrial respiratory chain, to be 400 L/per day/per adult, and about 400 mL of $O_2^{\bullet-}$ /per day/per adult, produced *in vivo*, which is much higher than the total amount of $O_2^{\bullet-}$ derived from all other enzyme system producing $O_2^{\bullet-}$ in the same *in vivo* conditions.[2,3] Therefore, mitochondrial respiratory chain-derived $O_2^{\bullet-}$ (and H_2O_2) have been investigated intensively and reviewed continuously, with emphasizing their crucial functional roles not only in a variety of harmful oxidative stress in pathogenesis, but also to be pivotal significant physiologically in redox signaling for various cellular events.[4–8] However, comparative less attention was paid to the involvement of respiratory chain-generated $O_2^{\bullet-}$ in its bioenergetic implications in mitochondrial PMF energy production and its partitioning, resulting in

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consequential downstream bioenergetic and redox signaling effects in various cell functions. To get insight into mechanisms underlying intrinsic relationship between both chemiosmotic energy coupling and redox signaling involved in mitochondrial Q cycling-derived $O_2^{\bullet-}$ generation and partitioning, we here focus particularly the following aspects of this topic, based on the research progress of our group and several relevant others, namely:

Generation sites and release sidedness of superoxide from complex III of mitochondrial respiratory chain

The Mitochondrial $O_2^{\bullet-}$ is produced mainly in mid-pathway of electron transfer chain by the one-electron leak to reduce univalent molecular O_2, instead of four electron reduction of O_2 to make water in terminal cytochrome c oxidase. So, the univalent reduction of O_2 by one-electron leak for making $O_2^{\bullet-}$ from respiratory redox enzyme complex could be considered as a "branch" of electron transfer along overall respiratory chain instead of water formation in mitochondrial respiration,[2,8] or expressed as 'bypass reaction' to form radical ubisemiquinone intermediate at Q_o site in Q cycling reactions, which has been detected and confirmed experimentally to be one-electron donor to O_2 for producing $O_2^{\bullet-}$ in mitochondria.[9–12] Therefore, mitochondrial respiration-derived $O_2^{\bullet-}$ production must be controlled by the kinetic and thermodynamic factors underlying the interaction of potential one-electron donor from components of respiratory chain with one-electron acceptor oxygen. Owing to having two unpaired electrons in antibonding orbitals with parallel spins in ground-state of O_2 that makes it to accept one electron at a time. The standard reduction potential for the transfer of an electron to O_2 to form $O_2^{\bullet-}$ is -160 mV at pH 7, for a standard state of 1 M O_2. The actual reduction potential *E*h will be determined by the relative $[O_2]$ and $[O_2^{\bullet-}]$, according to Eq. (1): [4]

$$E\mathrm{h(mV)} = -160 + 61.5\log_{10}[O_2]/\left[O_2^{\bullet-}\right] \quad (1)$$

On account of the standard reduction potentials of electron carriers in the four mitochondrial complexes span the range of redox potentials from –0.32 V (NADH at complex I) to +0.82 V (O_2 at complex IV), so, theoretically, many components of the respiratory complexes in respiratory chain could thermodynamically transfer an electron to O_2. However, in fact, only a small proportion of mitochondrial electron carriers with the thermodynamic potential of reducing O_2 to $O_2^{\bullet-}$ was found to do so. NADH, NADPH, reduced coenzyme Q ($CoQH_2$) and glutathione (GSH) do not react with O_2 to generate $O_2^{\bullet-}$. Mitochondrial $O_2^{\bullet-}$ production mainly takes place at redox-active prosthetic groups within proteins, or when electron carriers such as reduced ubiquinol ($CoQH_2$) are bound to proteins, and the kinetic factors that favor or prevent the one-electron reduction of O_2 to $O_2^{\bullet-}$ can determine mitochondrial $O_2^{\bullet-}$ production.[10,11] Therefore, the single electron transfers to oxygen in respiratory chain are tightly controlled and almost simultaneously coupled with the second electron transfer of redox complex of respiratory chain in coupling with proton translocation across mitochondrial membrane for setup PMF, common energy intermediate in chemiosmotic bioenergetics. So, according to Mitchell chemiosmotic theory, the redox energy (ΔE_h) released from electron transfer along the respiratory chain is conserved into PMF (ΔP), consisting both components of $\Delta\Psi$ and ΔpH (ΔpH^S, as in our recent paper of Xiong *et al.*[21]), and both energy sources can be used to synthesize ATP (ΔE_{ATP}) from ADP and Pi by H^+ re-entering the matrix at ATP synthase, or to coupling cation/anion translocation across mitochondrial membranes for cellular ions homeostasis, or to produce heat (ΔE_H) by proton leak through H^+ membrane diffusion process, and these processes described are thermodynamically reversible, as expressed in

$$\Delta E_h = \Delta E_P = \Delta E_{ATP}\,(\mathrm{and/or}\,\Delta E_H) \quad (2)$$

Therefore, mitochondrial respiration-derived $O_2^{\bullet-}$ generation can be considered as a "branch" of electron transfer chain of mitochondria and should be interconnected within energy transduction reactions in chemiosmotic bioenergetics. This also raises an interesting question whether and how both basically essential processes of mitochondria, mentioned above, may interplay with one another, and does mitochondrial $O_2^{\bullet-}$ generated from respiration affect energy coupling functions *per se* in mitochondrial oxidative phosphorylation or vice versa?

To better understanding and deeper discovering the underlying mechanisms of raised questions above, it is logical to determine which component(s) in mitochondrial respiratory chain has been established and/or commonly recognized as single

electron donor to oxygen for making $O_2^{\bullet-}$, or to serve as generating sites of superoxide in redox enzyme complexes of mitochondrial respiratory chain *in vivo* and *in vitro*? As discussed in recent reviews mentioned, the precise site(s) in complex I for leakage of single electron to oxygen for making $O_2^{\bullet-}$ has not been established yet, and its $O_2^{\bullet-}$ release sidedness is mainly into mitochondrial matrix and normally can not release out off to cytosol due to the presence of much higher amounts of its scavenging enzyme systems in matrix, as experimentally demonstrated in our recent paper[1] and discussed by Murphy in 2009.[4] Besides, the cytochrome c-p66shc system in respiratory chain has been also established to be the electron sources from respiratory chain to reduce dioxygen molecule for making H_2O_2 directly with no formation of $O_2^{\bullet-}$ as intermediate step.[9,11,12] However, ubisemiquinone at Qo site of Q cycle in complex III has shown to be most suitable candidate to meet the requirement for exploring such complicated problem mentioned above. This is because that the electron transfer sequences of Q cycle in coupling with proton translocation by complex III (cyt bc_1) for setting up PMF that occupy and constitute the central part of respiratory chain in mitochondria and in other energy transducing membrane system, and have been studied well both in enzyme-complex structure and in the molecular basis for elucidating mechanisms of Q cycle theory, proposed originally by P. Mitchell 30 years ago.[9–12] Recently, by using continuous wave and pulsed EPR spectroscopy, Cage *et al.*[11] provided first direct evidence for detection of a ubisemiquinone radical generated at Q_o site of complex III, which was shown to be sensitive to both specific inhibitors and mutations within the Q_o site as well as O_2, suggesting that it is the elusive intermediate responsible for superoxide production. Paramagnetic interactions also show that the new semiquinone species is buried in the protein in or near the Q_o site but not strongly interacting with the 2Fe2S cluster.[11] Under optimal conditions, however, normal electron flow in the Q-cycle is able to out-compete these bypass reactions kinetically through some unknown mechanism(s) to control the reactive intermediates of the Q_o site. The rates and yield of the bypass reactions are greatly increased upon slowing down the 'normal' Q-cycle reactions by exposure to certain inhibitors, by mutations in the bc complex, or by buildup of a large PMF. Some of these conditions, particularly high PMF or the accumulation of mitochondrial mutations with cellular ageing expected *in vivo*, suggesting that Q-cycle bypass reactions for generating $O_2^{\bullet-}$ are physiologically relevant.[10,11] Most importantly, it has been also established that Q cycle–derived $O_2^{\bullet-}$ anions mostly release outside into mitochondrial intermembranes space and some sort of the anion radicals can be also leak back into matrix across inner mitochondrial membranes, but the mechanism(s) for this are still unknown, although various hypothetical models have been proposed.[5,13,14,19,24,27] Therefore, the release sidedness for Q cycle–derived $O_2^{\bullet-}$ and its regulation have significant importance not only in the basic aspect of chemiosmotic bioenergetics, but also related to its downstream redox signaling pathway in different cellular events in physiopathology.[1,2,5–8] Thus, in the following part of this paper we will present our research data and discuss the interrelationship between the Q cycle derived-$O_2^{\bullet-}$ and its interaction with energy transduction functions of complex III of mitochondrial respiratory chain with respect to chemiosmotic bioenergetics.

The different physiological role of PMF components, $\Delta\Psi$ and ΔpH (ΔpH^S), of Q cycle in mitochondrial superoxide generating and partitioning

As it has been discussed above that one single-electron donor sources from respiratory chain in mitochondria for oxygen to making $O_2^{\bullet-}$ mostly comes from ubisemiquinone radicals, suggested previously and determined very recently to be formed at Qo site in complex III, as "bypass reaction" intermediate radicals in Q cycle, although some sort of electron donor from other component of Q cycle, such as cyt b, is not excluded.[9,13,14,25] However, the precise mechanisms of how single electron donors in components within Q cycle and the electron acceptor-molecule di-oxygen interact with each other to form $O_2^{\bullet-}$, and which kind of thermodynamic energetic factors in association with Q cycling reactions may control and regulate 'bypass reaction" for $O_2^{\bullet-}$ formation, are still remain to be far from being clear and needed to be elucidated.[5,9,10,12]

Cadenas and Boveris observed that the formation rate of mitochondrial H_2O_2, being derived from of $O_2^{\bullet-}$, was significantly affected by ions protonophore.[22] The authors also showed that

much higher increase in ROS formation was associated with inhibition of electron transfer of Q cycle, than that of control with no inhibition of Q cycling activity. However, the problem concerning the relationship between ROS production and mitochondrial membrane protons (PMF) potentials of remains not elucidated at that time.[22] The situation was also indicated as in the review paper by Garland in 1991,[35] emphasizing that if a component of mitochondrial respiratory chain like cytochrome b is the $O_2^{\bullet}$ generating autoxidisable component, is its reactivity with oxygen influenced by chemiosmotic effects such as the membrane potential?[35] It appears that from 1995 to 1999, our group presented for first time a series experimental evidence to indicate that both $O_2^{\bullet -}$ and H_2O_2 generation in rat heart mitochondria respiring succinate with rotenone were found to be controlled and dependent on protonic membrane potentials (energized $\Delta\psi_E$) of mitochondria.[1,2,13,14,20]

In our original observations expressed in Figure 1, four sets of experimental parameters for state 4 respiration rate, $O_2^{\bullet -}$ and H_2O_2 generation rates and the rate of cyt b reduction in rat heart

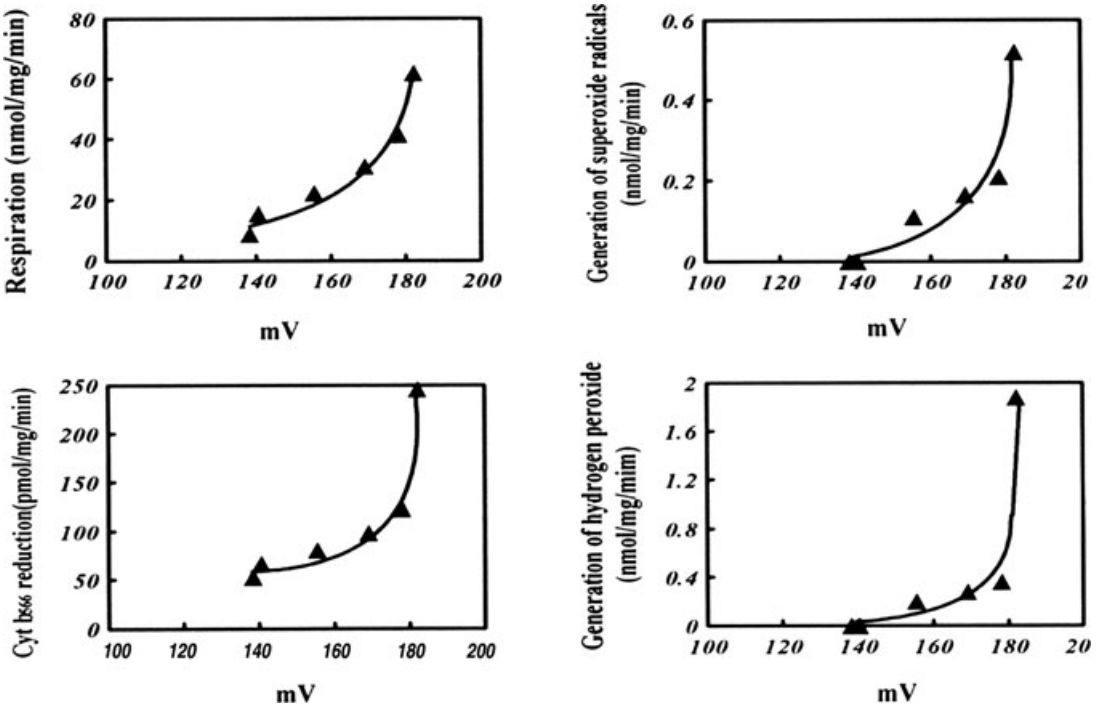

Figure 1. Correlation between the rates of state 4 respiration, superoxide, and hydrogen peroxide generation, cytochrome *b*566 reduction, and the transmembrane potential in rat heart mitochondria (from Liu and Huang,[13] with permission). (1) The respiration rate of mitochondria was measured using a Clark oxygen electrode on YSI Model 53 Oxygen Monitor. The reaction medium contains in 2 mL (mM): sucrose, 225; KCl, 20; Tris-HCl, 15, pH 7.2; KH_2PO_4, 17; $MgCl_2$ 7; BSA, 1 mg/mL; mitochondria protein, 1 mg/mL. Temperature, 258C. Succinate at 2.5 mM was added to initiate the reaction. Malonate was 0–5 mM. (2) The transmembrane potential $\Delta\psi$E of mitochondria was assayed fluorospectrometrically in a dialysis flow cell using rhodamine-123 as a probe. The reaction medium was 0.5 mL containing (mM):sucrose, 225; KCl, 10; KH_2PO_4, 10; MgCl2, 5; Tris-Cl, 2, pH, 7.4; rotenone, 1 mM; mitochondrial protein,1 mg/mL; succinate, 2.5 mM. Rhodamine-123 (0.4–0.8 mM) was added as standard to calibrate the fluorescence intensity and the transmembrane potential, $\Delta\psi$E, was calculated according to Nernst equation. (3) Cytochrome *b*566 reduction was measured on Hitachi 557 double-wavelength spectrophotometer following the absorbance changes at 566–575 nm according to Boveris and Chance (1973). (4) Superoxide was assayed on luminometer using 2-methyl-6-(*p*-methyoxyl)-3,7-dihydroimidozol [1,2-A′]pyrazin-3-one (MCLA) as probe according to Nakano.[36] The reaction medium (1.0 mL) contains (mM): sucrose,300; Tris-HCl, 10, pH 7.5; KPO_4, 10; KCl, 10 $MgCl_2$, 5; MCLA, 4 mM; mitochondria was 0.1 mg/mL. (5) Hydrogen peroxide was determined according to Boveris and Chance (1973). The reaction medium (2 mL) contains (mM): sucrose, 75; mannitol, 225; Tris-HCl, pH 7.3, 20; mitochondria, 1 mg/mL; horse radish peroxidase, 1.7 mM; mitochondria was 0.1 mg/mL. Succinate (5 mM) was added to initiate the reaction. Temperature, 25 °C. The activity of the enzyme was monitored by following the absorbance changes at 417–407 nm (ΔI mM = 50 mM–1/cm).

mitochondria, all exhibited close parallel correlative curves with same nonlinear dependence (non-Ohmic relationship) on $\Delta\psi_E$ in the range from 120 to 180 mv, a range of energized mitochondrial membrane potentials of protons derived from coupling respiration. The maximal sharp increases in $O_2^{\bullet-}$ and H_2O_2 generation were observed at above $\Delta\psi_E$ of 180 mv of mitochondria, while a small decrease in 5-0 mv of $\Delta\psi_E$ from 180 mv to about 170 mv was found to decrease ROS production rapidly and largely in an amount corresponding to 70–90% of original values of both ROS species. That the decrease extent in state 4 respiration rates and cyt b reduction versus $\Delta\psi_E$ within the same range between 120 and 180 mv, was found to be the same tendency as both ROS decrease versus $\Delta\psi_E$. From Figure 1, one can make conclusion that the rate of $O_2^{\bullet-}$ generation (one electron reduction of O_2 by complex III), the rate of H_2O_2 formation (two electron reduction of O_2 by SOD dismutase and/or auto-dismutation of $O_2^{\bullet-}$) and the rates of state 4 respiration (four electron reduction rate of O_2 by complex IV) as well as cyt b_{566} reduction rate (one electron reduction rate by ubisemiquinone at Qo site of complex III during Q cyling reaction) are the functions of $\Delta\psi_E$, or just PMF, and not related to the rest membrane potentials, $\Delta\psi_R$ of mitochondria, the upper level of which reaches to about 120–130 mv, derived from intrinsic fixed charge differences between both surfaces of inner mitochondrial membrane.[6,13,20,21]

Figure 2 shows that both CCCP and nigericin, known uncouplers of $\Delta\psi_E$ and ΔpH (ΔpH^S), were found to be inhibitory for $O_2^{\bullet-}$ and H_2O_2 generation in rat heart mitochondria as well as also in rat liver mitochondria from euthyroid and hyperthyroid rats.[1,2,13,14,20,21] Antimycin A was found to stimulate an additional increases in $O_2^{\bullet-}$ and H_2O_2 production after that derived from state 4 respiration of Q cycle operation in the absence of antimycin A, which was demonstrated clearly in the insert 1 of Figure 2. This also indicates that the generation of $O_2^{\bullet-}$ and H_2O_2 are controlled separately or independently by two components, $\Delta\psi$ and ΔpH (ΔpH^S) of PMF involved in Q cycle of mitochondrial respiration. It should be noted that a clear nonlinear relationship (disproportionality) between state 4 reparation and $\Delta\psi_E$ was first discovered by Nicholls and subsequently confirmed by different groups[1,2,13,14] and the results of the first set of our experiment in Figure 1, was also shown to be consistent well with Nicholls findings, although the underlying mechanisms of this phenomenon are still not fully understood. However, our new findings concerning the nonlinear relationship between ROS production and $\Delta\psi_E$ and ΔpH (ΔpH^S) as expressed in Figures 1 and 2, may have general pathophysiological significance in better understanding these events involved in mitochondrial respiration-derived ROS generation and the mechanisms controlled by $\Delta\psi_E$ and ΔpH(ΔpH^S) of PMF, that become to be commonly recognized in mitochondrial research field at present. Data of Insert 1 in Figure 2, indicated that in condition of our experiments, $O_2^{\bullet-}$ generation in heart mitochondria was shown to be taken place by ubiquinol oxidation at Qo site, and was sensitive to myxothiazol and antimycin A, and could be abolished by exogenous added SOD. These findings in Figure 1 were firstly cited by Skulachev[15] and later experimentally reconfirmed by his group,[16–18] as well as by Brand's laboratory,[19] and since then have been reviewed in literature positively and continuously.[4,5,18,19,25–30,31]

However, as discussed in the first section of this article, there are many questions still remain unclear and needed to be resolved,[5] such as, for example, what are the precise mechanisms for the effects of both components of PMF on Q cycle linked $O_2^{\bullet-}$ generation? Or, do both components of PMF have the same effects, or do they have different action mechanisms on it?

In 2010, we have shown that, by inserting pH sensitive fluorescein-phosphatidyl-ethanolamine (F-PE) into mitoplast surface and measuring formation of ΔpH^S supported by succinate oxidation in the presence of rotenone and Ap5A, the interfacial proton current of PMF (ΔpH^S component of ΔpH in PMF) of mitoplasts isolated from rat liver mitochondria, can serve as the primary energy sources for ATP synthesis.[21] The functional role of $\Delta\psi_E$ in ATP synthesis was also shown to be its balancing effects for compensation to support ΔpH^S, an essential component of ΔpH in PMF. Antimycin A decreased mitoplast ΔpH^S and also ATP synthesis in a similar or identical pattern of inhibition on both processes in mitoplast and mitochondria, but not similar with that of $\Delta\Psi_E$. Antimycin A could prevent the interfacial pH decrease and the preventing effects exhibited in a concentration-dependent manner.[21] Meanwhile,

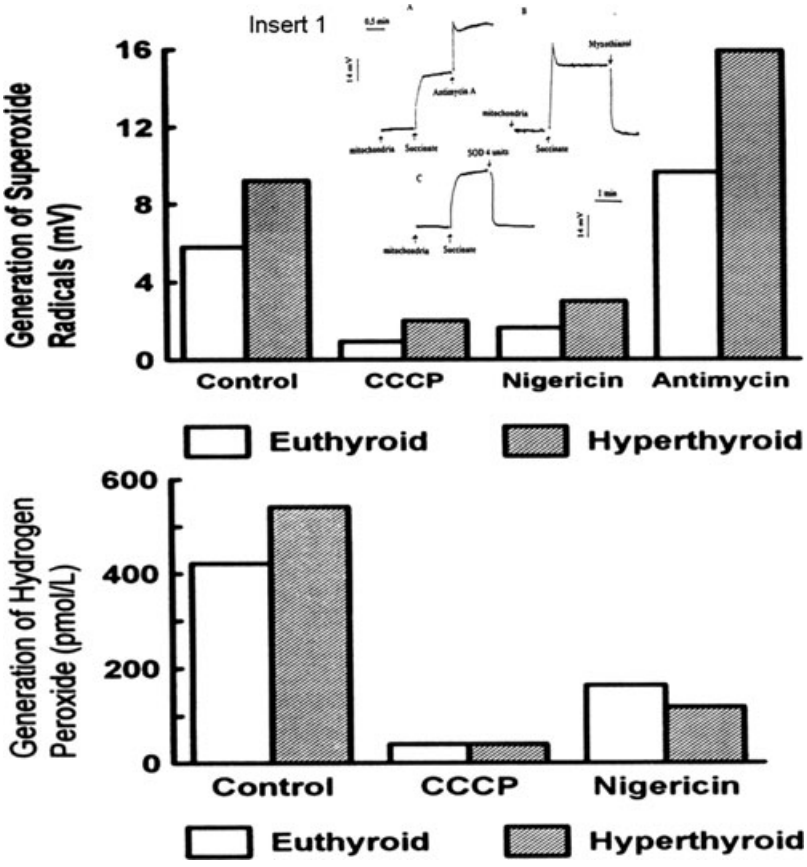

Figure 2. Comparison of superoxide radicals generation in euthyuroid and hyperthyrouid rat liver mitochondria. Reproduced from Refs. 2, 13, and 14, with permission from AOCS Press, USA. Superoxide radicals ($O_2^{\bullet-}$) were assayed on Luminometer 1250 type (LKB) using (MCLA) as a probe according to Nakano[36] and described as in the below condition. 1 mL of the reaction medium contains (mM): Sucrose, 300; Tris-HCl pH 7.5, 10; Potassium phosphate, 10; KCl, 10; $MgCl_2$, 0.05; Rotenone, 3 μM; MCLA, 4 μM; and 0.1 mg/mL mitochondria. Five mM succinate was added to initiate the reaction. Temperature, 25 °C. In the case of CCCP, nigericin, and antimycin A, the final concentrations of these reagents were 4, 12, and 9 μM, respectively. Hydrogen peroxide was determined was determined as in Figure 1 in Refs. 2 and 13. Insert 1. Effects of myxothiazol, antimycin A, and SOD on the chemiluminescence intensity of MCLA excited by superoxide generated from mitochondria respiring succinate in the presence of rotenone (myxothiazol or antimycin A: 5 mg/mL, SOD: 4 units/mL of reaction medium).

because antimycin A is well known to inhibit electron transfer from cytochrome b_{562} to oxidized ubisemiquinone for re-reduction of Q to form full reduced QH_2 at Qi binding site in complex III, leading to partial inhibition of succinate oxidation and increasing effects on $O_2^{\bullet-}$ generation through Q cycling activity, as also shown in insert I of Figure 2. This could be one of usual reasonable explanations for the inhibitory effects of antimycin A on electron transfer reaction of Q cycle. However, that the prevent effects of antimycin A on both interfacial pH decreases of mitoplasts energized by succinate and on the resultant ATP synthesis in mitochondrial energy transducing membranes exhibited a very close parallel correlation, as shown also in Figure 3, has been considered as a new finding concerning the action mechanism of antimycin A in Q cycle.[21] Since this novel discovery is very interesting for being a new evidence to prove existence of an intrinsic interaction between $O_2^{\bullet-}$ and energized H^+ in ΔpH^S component of PMF involved in Q cycle linked chemiosmotic bioenergetics, particularly, in $O_2^{\bullet-}$ inducing basic proton leak mechanisms in PMF energy partition of mitochondria.[2,13,14,20] The typical experiments are presented here in Figure 3.

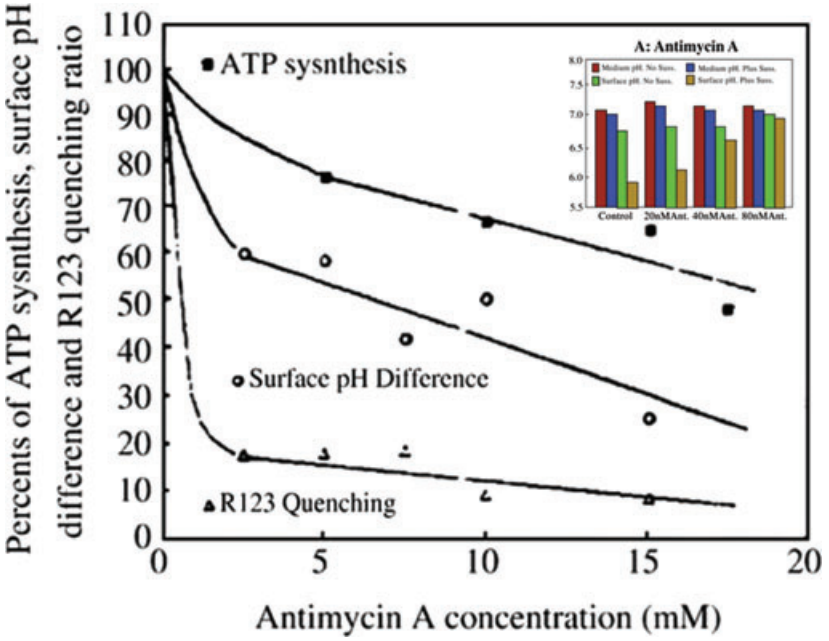

Figure 3. Correlation of the rates of ATP synthesis, the interfacial pH difference, and Rhodamine-123 fluorescence quenching ratios in mitoplasts: The effects of increasing concentrations of antimycin A. The reaction medium (2 mL, at 25 °C) contains 2 mM Hepes-KOH (pH 7.0), 1 mM H_2PO_4-K_2HPO_4, 70 mM Sucrose, 220 mM Mannitol, 4 mM $MgCl_2$, 2 μm Rotenone, 2.5 μ M Ap5A, 0.5 mg protein/mL of mitoplasts labeled with 80 ng FPE/mg protein. 0.1 mM ADP and Antimycin A were added as indicated in the figure. 5 mM succinate was added to initiate the reaction. The results of control group (no Antimycin A) were assumed to be 100%, which were rates of ATP synthesis = 35 mmol/mg/min; the interfacial pH difference = 0.74 pH units; and Rhodamine-23 quenching ratios = 44.9%, respectively. Insert 2. The changes in medium pH and in the interfacial pH of mitoplast membranes oxidizing succinate with different inhibitors of antimycin A. The reaction medium in Insert 2 contains (2 mL, at 25 °C) 2 mM Hepes–KOH (pH 7.0), 70 mM sucrose, 220 mM mannitol, 4 mM $MgCl_2$, 2 mM rotenone, mitoplasts (1 mg protein/mL) labeled with 80 ng F-PE/mg protein of mitoplasts. Experimental groups include control (5 mM succinate only), 20 nM antimycin A (5 mM succinate and 20 nM antimycin A), 40 nM antimycin A (5 mM succinate, and 40 nM antimycin A), and 80 nm antimycin A (5 mM succinate and 80 nM antimycin A). Succinate (5 mM) was added to initiate oxidation reaction for energization of mitoplasts.

Based on analyzing our new data in Figures 1 and 2, and the experiments discussed in Figure 3,[21] one may tentatively conclude that, ΔpH^S, in addition to its main role to serve as primary energy sources in ATP synthesis, and also is able partially to prevent $O_2^{\bullet-}$ generation in Q cycle due to its surfacial proton current on mitoplast membrane for setup ΔpH^S in PMF across mitochondrial membrane, while ΔpH^S in PMF is not used for ATP synthesis (during state 4 respiration). By contract, antimycin A decreases surfacial proton current and ΔpH^S, resulting in suppressing ATP synthesis, probably due to its increasing effect on an additional generation of $O_2^{\bullet-}$, derived from ubisemiquinone radicals at Q_o site in Q cycle. This could be done by its inhibiting on electron transfer from b_{562} to oxidized ubiquinone at Q_i site in Q cycle, making ubisemiquinone at Q_o site to donor single-electron leak to oxygen to form $O_2^{\bullet-}$ (Fig. 2, insert 1), the later could induce proton leak leading decreases in ΔpH^S and ATP synthesis in mitochondria, as shown in Figure 3, and discussed later in Figure 4. Thus, when ΔpH^S was used in ATP synthesis, or the surfacial proton current was prevented by PMF or by antimycin A inhibiting effects on electron transfer in Q cycle, the redox energy of respiratory chain could also switch in a "bypass reaction" from Q cycle to drive one electron leak to make oxygen forming $O_2^{\bullet-}$, that constitutes a small part of the product of oxygen consumption in mitochondria.

Therefore, in order to deeper and better understand the action mechanism of antimycin A on bioenergetic functioning of Q cycle, many questions could be addressed: What is the interplay mechanism or relationship between the decrease in ATP synthesis by the prevention of protonation on

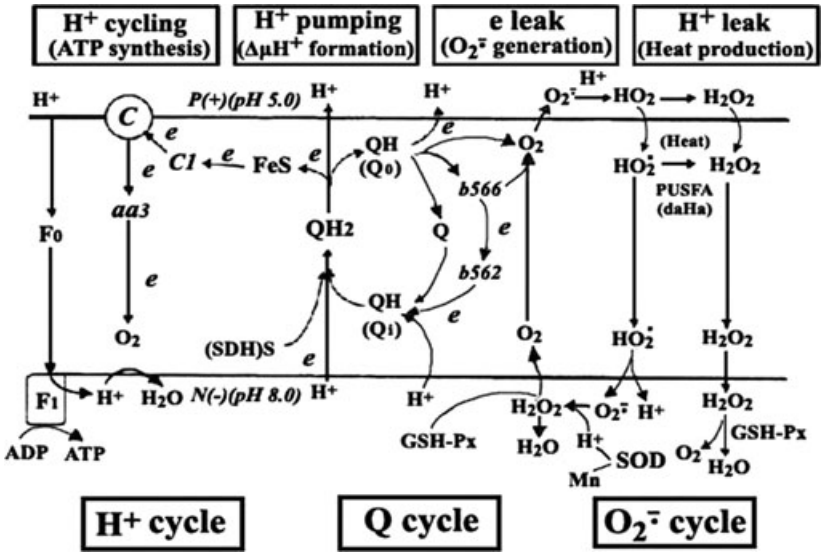

Figure 4. **A hypothetical model of cooperation of "reactive oxygen cycle" with Q cycle and the proton cycle in the respiratory chain of mitochondria (From Refs. 2, 13, and 14, with permission).**

mitoplast membrane surface leading to decrease of ΔpH^S and the increase in Q cycle-derived $O_2^{\bullet-}$ generation in Q cycle of complex III in mitochondria? If the answer was like this: $O_2^{\bullet-}$ generated in Q cycle is able to decrease ΔpH^S by its preventing or inhibiting effect on surface protonation of mitoplast during Q cycling, and the next questions will be: How $O_2^{\bullet-}$ can do this? Does and how Q cycling-derived $O_2^{\bullet-}$ affect back on energy partition functions of PMF-Q cycle reactions? These interesting questions will be discussed in next section of this paper.

Direct feedback effects of Q cycle–derived $O_2^{\bullet-}$ on PMF energy partition

Originally, P. Mitchell in 1966 postulated that the energy in the form of PMF not only drives transport of H^+ back into matrix through ATP synthase to make ATP (chemical energy), but also drives exchanges of H^+ with anions or cations of opposing sides of mitochondrial inner membrane for keeping constant electrical charge difference (osmotic energy), as well as drives H^+ diffusion directly across membrane lipid bilayer (proton leak) to convert chemiosmotic energy of protons for heat production (heat energy). According to Mitchell, the total amount of H^+ ejected to the outside of mitochondrial inner membrane (*P* side, as indicated in Fig. 4) in coupling with electron transport activity must equal to the amount of H^+ return back into inner side (*N* side) of mitochondrial inner membrane.[10,11] Mitchell's theory explains how cells can generate different proton concentrations on a membrane's opposite sides and how this membrane potential of protons can be used to provide energy for cellular processes.

Nicholls was the first to demonstrate experimentally that proton leak rate in mammalian mitochondria increase disproportionally with increasing mitochondrial $\Delta\psi$ [31]. Brand's group showed that the proton leak account for about 26% of the oxygen consumption in rest liver cell and about 52% in resting rat skeletal muscle. Besides, Challomer provided an upper limit estimate of 12% for the contribution of proton leak to the respiration rate of intact heart.[31,32] These authors further emphasized that the proton leak is an important component of cellular metabolism and suggested several different functions, including heat production and reduction of harmful free radical formation. At present, there are two types or pathways of proton leak in mitochondria: basic proton leak and inducible proton leak. Basic proton link is commonly known to be present in all kinds of mitochondria. In the case of the 'inducible' proton leak, some mitochondrial membrane proteins, for example, uncoupling protein I, exist only in the mitochondria of brown adipocyte tissues and function mainly in

thermogenesis, and its anion carrier family members (UCPs and/or ANT), which are suggested to be involved or mediated in the proton leak processes, when the protein(s) are induced to express in the inner mitochondrial membranes. In following, we will present our data to discuss only the basic proton leak mechanisms in rat heart and liver mitochondria (in which UCPs are not found to be expressed or activated), but not involved in the 'inducible' one, although both pathway of 'proton leaks' mechanisms are still far from being clear.[5,27,31,32]

We were first in 1991 to postulate mitochondrial respiration-derived $O_2^{\bullet-}$, as endogenous protonophore to induce basic proton leak in mitochondria, and then published some our original experiments to support this idea in 1995 in Chinese and in English version in 1996.[13,20] Three sets of experiment were conducted in our laboratory to show:

(1) By incubating the isolated rat heart mitochondria with xanthine-xanthine oxidase as an exogenous source of $O_2^{\bullet-}$, and by ischemia-reperfusion procedure for isolate rat heart as an endogenous $O_2^{\bullet-}$ generating source, we were able to find that both sources of $O_2^{\bullet-}$ could induce the same injurious effects on mitochondria resulting in decreasing energetic $\Delta\psi_E$, $H^+/2e$ ratio of respiratory chain activity and increasing proton leak of mitochondria respiring succinate with rotenone. The injurious effects of these $O_2^{\bullet-}$ sources on these parameters exhibited a dose or reaction time dependent manner. However, both sources of $O_2^{\bullet-}$ had no effects on respiratory chain enzyme activity *per se*, and on nonenergized, or rest membrane potential of mitochondria, $\Delta\psi_R$. Superoxide scavengers, 3,4-hydroxylphenol lactate, had an obvious protective effect on both $O_2^{\bullet-}$ sources of exogenous xanthine/xanthine oxidase system and endogenous $O_2^{\bullet-}$ from ischemia-reperfusion procedure derived $O_2^{\bullet-}$ from rat heart mitochondria, which were shown to decrease proton pump activity, $H^+/2e$ ratio and energized transmembrane potentials of mitochondria, $\Delta\psi_E$. These sets of experiments not only showed that $O_2^{\bullet-}$ induce an increase in basic proton leak of mitochondria directly related to molecular mechanism of early stage damage of ischemia-reperfusion procedure on heart, but also provide evidence to support our hypothesis that respiratory chain electron leak–derived $O_2^{\bullet-}$ may be one of the mechanistic causes of basic proton leakage of mitochondria.[1,2,13]

(2) In next series of experiments, we also demonstrated that an exogenous $O_2^{\bullet-}$ sources from xanthibe/xanthine oxidase system and an endogenous $O_2^{\bullet-}$ sources from mitochondrial Q cycle-linked $O_2^{\bullet-}$ derived system by antimycin A stimulation, were found to be able to remove energized protons on membrane surface of mitochondria or mitoplast with succcinate oxidation in the presence of rotenone, leading to membrane surface pH increase, or decrease in ΔpH (ΔpH^S) of PMF across mitoplast membrane. The proton concentration changes on mitoplast membrane surface were detected by fluorescence changes of inserted pH sensitive fluorescien-phosphatidylethanolamine probes. Figure 3 and the insert 2, present the positive results from experiments with antimycin A, which shows that mitoplast isolated from rat liver mitochondria energized by succinate oxidation (with rotenone) can induce protonation on mitoplast surface with a pH decrease for about one pH unit (from pH about 6.8 to pH 5.8, as shown in Insert 2). This pH decrease on membrane surface of mitoplasts is able to establish pH gradient between both sides of mitochondrial inner membrane and constitutes ΔpH (ΔpH^S) component of PMF in Q cycle operation. The addition of antimycin A showed preventing effects on surface protonation of mitoplast in a dose dependent manner. The similar separate experiments (Fig. 3) demonstrated that antimycin A also give a close parallel curves of inhibiting effects on both ATP synthesis and mitoplast surface protonation (or ΔpH^S decrease). In considering the additionally stimulating effects of antimycin A on mitochondrial Q cycle–derived $O_2^{\bullet-}$ production (as shown in insert 1 of Fig. 2), it is evident that $O_2^{\bullet-}$ is the causative factor decreasing in mitoplast surface protonation and its ΔpH^S, resulting in decreasing ATP synthesis, mainly due to the proton leak of protonated $HO_2^{\bullet}$ formed at membrane surface of

mitoplasts, derived from the interaction between H^+ and $O_2^{\bullet-}$ to form $HO_2^\bullet$ in an equilibrium of the following reaction with pKa of 4.8 (see Ref. 2)

$$O_2^{\bullet-} + H^+ = HO_2^\bullet (pK_a = 4.8) \quad (3)$$

This is because $O_2^{\bullet-}$ is a kind of oxygen free radical, capable of donating electron (reduction activity) and also to accept electron (oxidation activity). $O_2^{\bullet-}$ can form H_2O_2 by self-dismutation and possesses an alkaline property to accept H^+ to form $HO_2^\bullet$, perhydroxyl. So, the pH value on membrane surface of mitoplasts oxidizing succinate with rotenone, was detected to be about 6.0 unit, in comparison with medium pH of 7.2, shown as in Figure 3, and the direction of equilibrium of Eq. (3), $H^+ + O_2^{\bullet-} = HO_2^\bullet$ will move toward to right-hand side to form more of about 10% of $HO_2^\bullet$. After being permeable through proton leakage from out side of mitoplast into matrix, where HO_2 dissociates to leak H^+ and $O_2^{\bullet-}$ for the direction of equilibrium of Eq. (3) will move toward to left-hand side under alkaline condition in matrix of mitoplast(pH = 7.5–8.0). Moreover, in contract to $O_2^{\bullet-}$, $HO_2^\bullet$ has a much stronger reductive and oxidative ability than that of $O_2^{\bullet-}$ and as being a neutral charge compound and much stronger membrane permeable prosperity than that of $O_2^{\bullet-}$. Thus, it is probable that this is the mechanism of proton leak by $O_2^{\bullet-}$ derived from respiratory chain through its pK-dependent formation of $HO_2^\bullet$, leading to its subsequential decrease in ΔpH^S of PMF and its feedback effects of decreasing $O_2^{\bullet-}$ generation in mitochondrial respiration.

(3) The pH changes in matrix side of mitochocndria or mitoplasts in association with proton pumping induced by succinate oxidation with rotenone, or with proton leak induced by exogeneous and endogeneous sources of superoxide could be detected by monitoring fluorescence intensity changes of BCECF/AM preloaded in mitochondria matrix. Both experiments with BCECF/AM, pH sensitive fluorescence probe labeling have given positive results to confirm that superoxide induces basic proton leak through its interaction with energized H^+ (or ΔpH^S) to form permeable $HO_2^\bullet$ on mitochondrial or mitoplast surface.[2,13,14]

Because proton leak in mitochondrial respiratory chain is particularly significant in PMF-energy transformation and partition, as well as in regulation of Q cycle–derived $O_2^{\bullet-}$ generation and its release sidedness involved in the downstream redox signaling effects in cellular physiopathology, but its molecular mechanism is still poorly understood. Therefore, the experimental results presented here may have significant implication in supporting the hypothesis, that mitochondrial Q cycle–derived $O_2^{\bullet-}$ may play a role as endogenous protonophore, and that could constitute a solid basis for our theoretical model of "cooperation of a reactive oxygen cycle with Q cycle and proton cycle in the respiratory chain of mitochondria.[1,2,13,14,20,21,33] This model emphasizes that during state 4 respiration, an interaction between an electron leak (a branch of electron transfer directly from the respiratory chain to form $O_2^{\bullet-}$, but not H_2O) and a proton leak (a branch pathway which uses PMF to produce heat, but not ATP) may take place in cooperation with the Q and proton cycles in mitochondria through the consumption of H^+ by $O_2^{\bullet-}$ anions to form a protonated $HO_2^\bullet$, perhydroxyl, which is directly permeable across the inner mitochondrial membrane and induces proton leakage and a decrease of PMF. $O_2^{\bullet-}$ generation in the mitochondrial respiratory chain and its cycling across the inner membrane may have the role of an endogenous protonophore in regulating and partitioning energy transduction and heat production,[13,14] as well as in pathogenesis of mitochondrial diseases, aging, and apoptosis.[5,6,18,26–32,34] Based on the presentation of experimental evidence as well as their theoritical ananlysis and discussion on the findings of PMF ($\Delta\psi$ and ΔpH^S) for controlling and regulating mitochondrial ROS generation in non-Ohmic manner through affecting redox state and lifetime of ubisemiquinone of Q cycle has been commonly recognized as novel function of PMF in addition to its traditional role in energy transduction for ATP synthesis and in energy partition, and the theoretical basis of Mitchell's chemiosmotic theory could be involved not only in energy transduction and ions translocation of mitochondria, but also spread to affect cellular redox signaling through its controlling mechanism for mitochondrial ROS production.[33]

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Conflicts of interest

The author declares no conflicts of interest.

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