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Mitochondrial Superoxide Radical Formation is Controlled by Electron Bifurcation to the High and Low Potential Pathways

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The generation of oxygen radicals in biological systems and their sites of intracellular release have been subject of numerous studies in the last decades. Based on these studies mitochondria are considered to be the major source of intracellular oxygen radicals. Although this finding is more or less accepted, the mechanism of univalent oxygen reduction in mitochondria is still obscure. One of the most critical electron transfer steps in the respiratory chain is the electron bifurcation at the cytochrome bc_1 complex. Recent studies with genetically mutated mitochondria have made it clear that electron bifurcation from ubiquinol to the cytochrome bc_1 complex requires the free mobility of the head domain of the Rieske iron–sulfur protein. On the other hand, it has been long known that inhibition of electron bifurcation by antimycin A causes leakage of single electrons to dioxygen, which results in the release of superoxide radicals. These findings lead us to study whether hindrance of the interaction of ubiquinol with the cytochrome bc_1 complex is the regulator of single electron diversion to oxygen. Hindrance of electron bifurcation was observed following alterations of the physical state of membrane phospholipids in which the cytochrome bc_1 complex is inserted. Irrespective of whether the fluidity of the membrane lipids was elevated or decreased, electron flow rates to the Rieske iron–sulfur protein were drastically reduced. Concomitantly superoxide radicals were released from these mitochondria, strongly suggesting an effect on the mobility of the head domain of the Rieske iron–sulfur protein. This revealed the involvement of the ubiquinol cytochrome bc_1 redox couple in mitochondrial superoxide formation. The regulator, which controls leakage of electrons to oxygen, appears to be the electron-branching activity of the cytochrome bc_1 complex.

Keywords: Electron bifurcation; Low potential pathways; Mitochondrial superoxide formation, Cytochrome bc_1 complex

Abbreviations: DTPA, diethylenetriaminepentaacetic acid; ESR, electron spin resonance; HRP, horseradish peroxidase; HVA, homovanillic acid; $O_2^{\cdot-}$, superoxide radical; RHM, rat heart mitochondria; SMP, submitochondrial particles; SOD, superoxide dismutase; TRIS, tris(hydroxymethyl)-aminomethane; UQ, ubiquinone; $UQ^{\cdot-}$, ubisemiquinone; UQH_2, ubiquinol

INTRODUCTION

Dioxygen is the terminal electron acceptor of cell respiration in aerobic systems. The electron configuration of dioxygen in the outer orbital forbids the transfer of paired electrons. Hence, reduction of O_2 to water requires four consecutive single electron transfer steps. This necessarily involves two reduction intermediates with paramagnetic properties which, however, are not set free at the site where oxygen is reduced to water.[1] Nevertheless, although controversial, mitochondria have been considered for many years to play a major role in cellular ROS formation.[2–5] All reports dealing with this question have been based on studies with isolated mitochondria. Since bioenergetic activities of mitochondria may be readily affected during isolation from the tissue, it is still not clear whether or not mitochondria, when operating in the cell, can be considered as

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a potential O_2-radical source. There is, however, indirect evidence that biological systems in contact with oxygen generate O_2-radicals.

i) Aerobic but not anaerobic biological organisms have developed enzymatic and non-enzymatic strategies to cope with prooxidants and their reaction products.[6]
ii) Aerobic systems accumulate oxidatively modified biomolecules indicative of the existence of reactive oxygen species.[7]

Although other sources of oxygen radicals have been proven to exist in tissues, mitochondria are generally considered to play a key role in cellular oxygen radical generation.[8,9]

In fact, mitochondria exhibit bioenergetic properties which favor these organelles over cytochrome oxidase as a permanent radical source. Direct electron leakage from the respiratory chain to dioxygen has been considered for many years to result from redox-cycling ubiquinones in contact with the bc_1 complex.[10] Superoxide radical formation is reported to occur in the resting state whilst mitochondrial respiration is not linked to ATP production. Complex I has also recently been suggested to contribute to cellular superoxide radical generation by the group of Barja[11,12] independently of the respiratory state. However, this finding has not been repeated so far and seems to be controversial. We have earlier reported that contradictory results may be due to the use of inadequate detection systems and/or non-uniform preparative isolation procedures.[13]

In this study, we will present experimental evidence that regular mitochondrial respiration is not associated with superoxide generation. On the other hand experiments will reveal where and under which conditions single electrons may leak out from the respiratory chain to external oxygen.

MATERIALS AND METHODS

Detection of Mitochondrial H_2O_2 Release by Means of Fluorescence Spectroscopy

Rat heart mitochondria (RHM) were prepared from male Sprague–Dawley rats as described in Ref. [13] RHM preparations used in this study were checked for their bioenergetic integrity (proton leakage, membrane potential, P/O ratio) and their purity with respect to adhering mitochondrial fragments (less than 3%; see Ref. [14]) RHM (0.5 mg protein/ml) were suspended in 0.3 M sucrose, 20 mM triethanolamine, 1 mM diethylenetriaminepentaacetic acid (DTPA), 0.5 mg/ml bovine serum albumin (pH 7.4, 25°C) and supplemented with 4 mM inorganic phosphate and 10 mM succinate or 5 mM glutamate/5 mM malate as respiratory substrates, respectively. H_2O_2 was determined using the horseradish peroxidase (HRP)-catalyzed oxidation of hydrogen donors such as scopoletin[15,16] or homovanillic acid (HVA),[11,12] respectively. Mitochondrial H_2O_2 liberation was detected either invasively, e.g. when the detection system was in direct contact with H_2O_2-releasing mitochondria or noninvasively, e.g. when mitochondria were made to produce H_2O_2 in the absence of the detection system as described in Ref. [13]. In the latter case RHM were allowed to equilibrate for 3 min at 25°C and sedimented by centrifugation (5 min, 9000*g*). The resulting supernatants containing the accumulated H_2O_2 were placed in a fluorescence cuvette and supplemented with 5 μM scopoletin or 100 μM HVA, respectively. Following HRP-catalyzed reaction (10 U/ml) the concentration of released H_2O_2 was quantified from fluorescence changes of scopoletin (excitation: 366 nm, emission: 460 nm) or HVA (excitation: 312 nm, emission: 420 nm) using calibration curves with known amounts of H_2O_2. 2 μg/ml antimycin A was added to stimulate $O_2^{\cdot-}$-derived H_2O_2 release. Catalase (725 U/ml) was added to prove the specificity of the detection system.

Preparation of Submitochondrial Particles (SMP) Enriched With Cholesterol and Erucic Acid

SMP were prepared from beef heart according to Nohl and Hegner.[17] 400 μl of SMP were lyophilized yielding about 60 mg of dried lyophilisate. Each 20 mg of lyophilisate were mixed with 500 μl of pentane, 1.5 μl of ethanol, and 2.44 μmol of cholesterol or 2.79 μmol of erucic acid. After removal of organic solvents SMP were resuspended in 200 μl of water.

Spin Labeling of Liposomes and Modified SMP

The spin label (5-doxyl-stearic acid) concentration was adjusted to 30 nmol/mg of lipid. Electron spin resonance (ESR) spectra of spin-labeled liposomes and SMP were measured in a quartz flat cell using a Bruker EMX spectrometer. Order parameters were calculated as described previously.[18]

Detection of $O_2^{\cdot-}$ Formation by SMP

Generation of superoxide radicals was followed by the superoxide dismutase (SOD)-sensitive reduction of acetylated cytochrome c.[19] SMP (0.2 mg of protein/ml), acetylated cytochrome c (10 μM), and succinate (6 mM) were dissolved in 2 ml of oxygen-saturated buffer (150 mM KCl, 20 mM triethanolamine, 1 mM DTPA, pH 7.5), and SOD (20 μg/ml) if required. The reduction of acetylated cytochrome c

was measured by a UV–vis spectrometer (Aminco DW2000), which was set to 550 nm in the double beam mode. Molar amounts were calculated using an extinction coefficient of 21 mM^{-1}/cm.

Pre-steady State Reduction Kinetics of Cytochrome b and c_1 in SMP

Fast reduction kinetics of cytochromes in SMP were followed with an Aminco DW2000 UV–vis spectrometer equipped with a stopped flow reactor (MillieFlow SLM). The two syringes of the reactor contained 3 ml of preparation buffer (0.25 M sucrose, 10 mM TRIS, 1 mM EDTA), succinate (20 mM) and SMP (0.9 mg of protein/ml), KCN (3.34 mM), deoxycholate (0.05%), respectively. From kinetic traces of both cytochromes prior to reaching a steady state absorption the cytochrome reduction rates were calculated ($\varepsilon_{cyt\,b,563-577\,nm} = 14.3\,mM^{-1}$/cm, $\varepsilon_{cyt\,c1,554-540\,nm} = 18.8\,mM^{-1}$/cm).[20]

Detection of Autoxidizing Ubisemiquinones in Heart Mitochondria

RHM (16.85 mg of protein/ml) were supplemented with succinate (20 mM)/fumarate (4 mM) after preincubation with NADH (10 mM, 25 min at 4°C). During ESR power saturation experiments Cr^{3+} (50 mM) was added if required. ESR measurements were carried out at 200 K using a flow dewar and a temperature control unit.

Sensitivity of Ubiquinone Reduction States Towards Oxygen

Ubiquinone (UQ) was reduced to ubiquinol (UQH_2) according to Maguire *et al.*.[21] For testing the effect of oxygen UQH_2 solutions were mixed with buffered UQ solutions, which were adjusted to the desired pH-value under anaerobic conditions. Later the mixture was exposed to air oxygen. Anaerobic conditions were established by bubbling nitrogen for 15 min. ESR spectra were recorded with a Bruker ER 200 D-SRC in a quartz flat cell. General ESR settings were: microwave frequency, 9.6–9.7 GHz; modulation frequency, 100 kHz; microwave power, 10 mW; modulation amplitude, 0.28 G; sweep, 30 G.

RESULTS

Critical Evaluation of Mitochondrial H_2O_2 Detection

The quantitative registration of $O_2^{\cdot-}$ radicals derived from intact mitochondria is impeded by their inaccessibility to most compounds normally used in conventional detection systems and by the instability of the radical itself. Thus the assessment of $O_2^{\cdot-}$ generation in suspensions of intact mitochondria is derived from the detection of the stable dismutation product H_2O_2 which equilibrates with the hydrophilic phase. The dye system indicating H_2O_2 formation is normally in direct contact with mitochondria. Figure 1 shows that mitochondria drastically affect the fluorescence increase of HVA following HRP-catalyzed oxidation through H_2O_2. In addition, when comparing HVA as the H_2O_2 indicator with scopoletin, the two most frequently

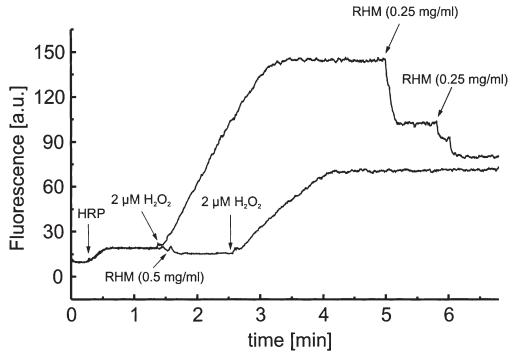

FIGURE 1 Influence of succinate-respiring rat heart mitochondria (RHM) on time scans of peroxidase-catalyzed (HRP) H_2O_2-mediated fluorescence increase of homovanillic acid (HVA).

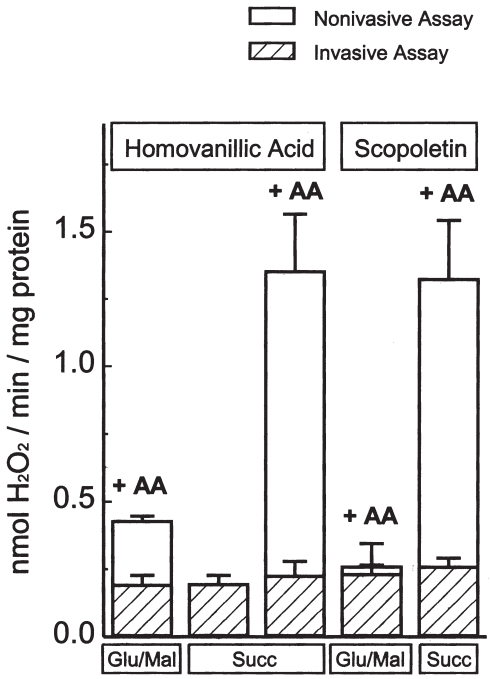

FIGURE 2 H_2O_2 release from RHM respiring glutamate/malate (Glu/Mal) or succinate (Succ). When indicated antimycin A (AA) was added to start the H_2O_2 production. H_2O_2 liberation was detected either invasively, e.g. when the detection system was in direct contact with H_2O_2-releasing RHM or noninvasively, e.g. when RHM were made to produce H_2O_2 in the absence of the detection system (HRP and HVA or scopoletin) and H_2O_2 was determined afterwards in the mitochondrial supernatant. Data represent means ± SEM of 4–9 independent mitochondrial preparations.

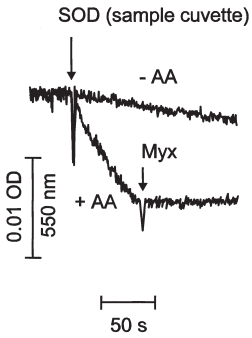

FIGURE 3 Univalent reduction of oxygen by antimycin A-inhibited submitochondrial particles and its sensitivity to myxothiazol. Generation of superoxide radicals was assayed by the SOD-sensitive reduction of acetylated cytochrome c, which was followed photometrically at 550 nm.

applied fluorescence methods for H_2O_2 in mitochondria, the results obtained were not identical. While in the case of HVA, state IV respiration driven by succinate affects fluorescence according to the existence of H_2O_2 (Fig. 2) in a catalase-insensitive manner, no H_2O_2 was found under otherwise identical conditions using scopoletin (data not shown). This observation leads us to separate the detection system from the mitochondria. As compared with conventional invasive methods, H_2O_2 formation rates when assessed in the supernatant after separation from the mitochondrial pellet were found to be higher, especially when succinate was used as substrate. This was valid both for scopoletin and HVA. Irrespective of the dye or method applied, antimycin A was required to obtain H_2O_2-related fluorescence changes.

Leakage Site of Single Electrons to Dioxygen

Figure 3 shows that antimycin A-induced $O_2^{\cdot-}$ formation in SMP is totally suppressed when myxothiazol is present. Since myxothiazol interferes with the electron transfer from ubisemiquinone to low potential cytochrome b of the bc_1 complex, autoxidation of the semiquinone pool associated with the bc_1 complex is assumed to be responsible for antimycin-induced $O_2^{\cdot-}$ generation. Some myxothiazol-sensitive $O_2^{\cdot-}$ generation was also observed in the absence of antimycin A. Spontaneous $O_2^{\cdot-}$ release after supplementation with succinate was higher when the intensity of the sonic treatment of RHM to disrupt mitochondrial membranes during SMP preparation was increased (not shown).

Figure 4 demonstrates that deprotonated (but not protonated—not shown) ubisemiquinones are sensitive to oxygen. The expected reaction product is the superoxide radical. Charged ubisemiquinones were obtained from a comproportionation reaction of deprotonated divalently reduced ubiquinone in the presence of equal amounts of totally oxidized ubiquinone. Deprotonation of divalently reduced ubiquinone requires a strong alkaline pH-value (pH = 13). On the contrary, ubisemiquinones of the mitochondrial respiratory chain are permanently formed by one-electron redox-cycling in direct contact with the respective redox partners. The physical interaction with the respective redox partners stabilizes the otherwise instable intermediate reduction state of ubiquinone preventing both disproportionation and autoxidation. The

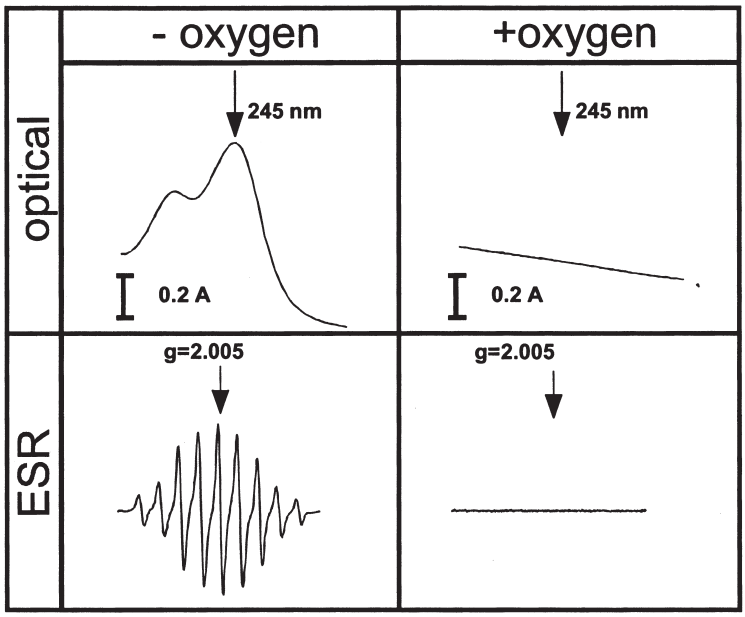

FIGURE 4 Effect of oxygen on optical and ESR spectra of semiquinone radicals. Deprotonated ubisemiquinone was obtained from a comproportionation reaction of deprotonated divalently reduced ubiquinone (UQ^{2-}) in the presence of equal amounts of ubiquinone. The pK-value of UQ^{2-} requires a TRIS buffered solution around pH 13. Final concentrations: UQH_2, 0.15 mM; UQ, 0.15 mM; water, 2.5%; ethanol, 97.5%; TRIS, 2.5 mM.

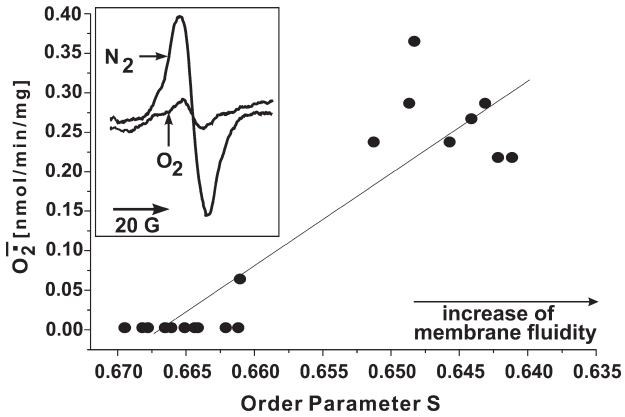

FIGURE 5 Superoxide radical formation correlated with membrane fluidity changes (order parameter S) in heart mitochondria following toluene accumulation in the inner membrane. Insert: Sensitivity of mitochondrial ubisemiquinone towards oxygen after toluene treatment. Final toluene concentrations were 1.77 mM (84.4 nmol/mg protein), 3.08 mM (200 nmol/mg protein) and 3.66 mM (223.4 nmol/mg protein), respectively. Preincubation time was 25 min.

pK-value (5.9) of ubisemiquinones suggests that in mitochondria the majority of this redox-cycling electron carrier is in the deprotonated state. Does this contradict the above finding, that intact mitochondria do not leak single electrons from the respiratory chain because $O_2^{\cdot-}$ formation was excluded?

Conditions for Ubisemiquinone-mediated Electron Leakage

Deprotonated ubisemiquinone was shown in Fig. 4 to destabilize in aqueous TRIS buffer as soon as oxygen was allowed to equilibrate with the solution. Although most of mitochondrial ubisemiquinone is in the deprotonated state (pK = 5.9) the respective ESR-signal was repeatedly shown to remain unchanged in the presence of oxygen.[3,22]

About 1–2% of total mitochondrial ubiquinone is reported to exist in the semiquinone form. Demonstration of this small paramagnetic fraction by means of ESR would not be possible if ubisemiquinones decompose in the respiratory chain by autoxidation or disproportionation. The relatively high microwave power saturation value required to obtain the semiquinone-related ESR-signal reveals a strong dipolar interaction with the catalytic metal center of the respective redox partner.[22] This interaction stabilizes the deprotonated ubisemiquinones such that autoxidation cannot occur. We have earlier described a novel ubisemiquinone species associated with complex I of the respiratory chain which requires unusually low microwave power to achieve saturation conditions compared to regular ubisemiquinones.[18]

This particular semiquinone species is only present when the external NADH dehydrogenase is activated by cytosolic NADH levels exceeding physiological values. The ESR spectrum of this semiquinone compound is sensitive to paramagnetic chromium salts, which, although not soluble in the mitochondrial membrane, affect the signal by spin–spin interaction. As a result the microwave power required for saturation of the ubisemiquinone derived ESR amplitude decreases giving rise to an increase of the amplitude to power ratio. This finding reveals that this ubisemiquinone species operates close to the bordering aqueous phase of the membrane. As earlier reported, heart mitochondria, when supplemented with external NADH, release $O_2^{\cdot-}$ radicals which most likely stem from autoxidation of this particular semiquinone species.[23]

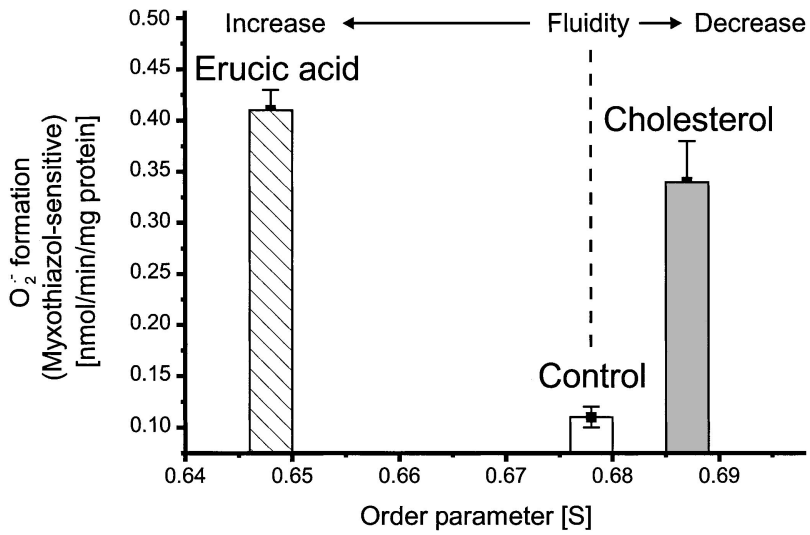

FIGURE 6 Submitochondrial particles enriched with cholesterol or erucic acid exhibit order parameter changes in opposite directions. Order parameter changes in any direction are linked to the increased release of superoxide radicals. Control samples were subjected to an analogous treatment but without cholesterol and erucic acid.

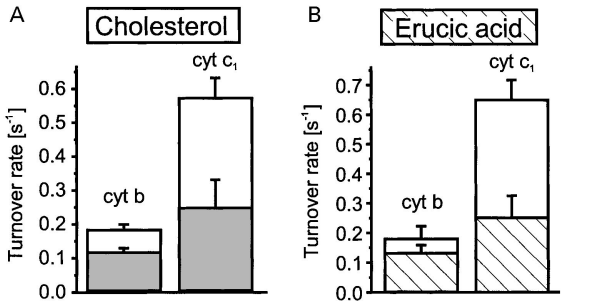

FIGURE 7 Pre-steady state reduction rates of cytochromes b and c_1 in cyanide-inhibited SMP, which were treated as described in Fig. 6. Both cholesterol (A) and erucic acid (B) incorporation decreased cytochrome reduction rates in SMP. Initial reduction rates were determined from kinetic traces during the first second after supplementation with succinate as a substrate. The ratio was calculated from the cytochrome b reduction rate divided by the reduction rate of cytochrome c_1.

We conclude from these findings, that autoxidation of mitochondrial ubisemiquinones, involved in redox-cycling of the respiratory chain, can be expected: (i) when the interaction with the respective redox partner is affected and (ii) when the semiquinone is within reach of the aqueous phase.

Incorporation of toluene into the lipid phase of the inner membrane of RHM was found to change conditions for redox-cycling ubisemiquinones in a way which favors autoxidation and the subsequent release of $O_2^{\cdot-}$ radicals (Fig. 5). Toluene incorporation changes the natural basic order of membrane lipids. Order parameters determined by means of the spin–labeling–ESR technique reveal a correlation between the amount of toluene incorporated and the decrease of order parameters. The latter indicates increasing membrane fluidity which was found to correlate linearly with the amount of $O_2^{\cdot-}$ released from RHM. This finding, however, is in contrast to the stimulation of $O_2^{\cdot-}$ generation in senescent mitochondria which were earlier shown to have a more rigid membrane.[24] We therefore focused our interest on the underlying mechanism of physical state alterations as the potential trigger of mitochondrial $O_2^{\cdot-}$ radical generation. The model systems used to affect the basic structure of the phospholipid order were selected on the basis of relevant physiological as well as toxicological parameters. Cholesterol incorporation into the membrane of heart SMP is in line with reports that the membrane content of cholesterol increases with aging.[25,26] Erucic acid, which is a component of rape oil, has been suggested to trigger cardiotoxicity.[27]

SMP from heart mitochondria were prepared by sonic treatment. As can be seen from Fig. 6, the untreated particles have a small basic release rate of $O_2^{\cdot-}$ radicals which, however, was drastically stimulated when SMP were preloaded with cholesterol or erucic acid. Order parameters of the latter clearly deviated from the controls. Although cholesterol and erucic acid had an opposite effect, cholesterol decreased, and erucic acid strongly increased, membrane fluidity, the effect on $O_2^{\cdot-}$ generation was the same. It appears that any impact on the lipid structure of the mitochondrial membrane results in the leakage of single electrons from the respiratory chain to O_2 out of sequence. This may also explain the apparent contradiction between intact mitochondria, which were shown to respire without $O_2^{\cdot-}$ release, and mitochondrial membranes, which release small amounts of $O_2^{\cdot-}$ following sonic treatment to obtain SMP.

Electron Bifurcation from Ubiquinol to the bc_1 Complex Regulates Ubisemiquinone Autoxidation

Figure 7 reveals that electron flow rates from ubiquinol to the biological oxidants of the bc_1 complex were clearly affected in SMP preloaded with cholesterol or erucic acid. The inhibition of electron flow rates to cytochrome c_1 via the Rieske iron–sulfur protein was much more marked than the transfer of the second electron to low potential cytochrome b. Myxothiazol, which intercepts the electron bifurcation from ubiquinol to the bc_1 complex, was found to totally suppress $O_2^{\cdot-}$ release from the pretreated SMP.

DISCUSSION

The present study confirms earlier reports on the potential role of redox-cycling UQ in mitochondrial $O_2^{\cdot-}$ radical formation.[8,9,15] Here we have shown that only deprotonated $UQ^{\cdot-}$ serve as univalent reductants of dioxygen. Although the pK-value keeps mitochondrial ubisemiquinone mainly in the deprotonated state, autoxidation normally does not occur. This was clearly shown by the recently developed non-invasive H_2O_2-detection method[4] which excludes any alterations of the regular mitochondrial electron flow from interfering with reactants of the dye system. In agreement with all studies in this field, $O_2^{\cdot-}$-derived H_2O_2 formation was, however, found in antimycin A inhibited mitochondria. Both antimycin A induced and spontaneous ROS formation of SMP were sensitive to myxothiazol. From these observations we concluded that sonic treatment of mitochondria, which is required to obtain SMP, or the presence of antimycin A, exert similar effects on the ubiquinol/bc_1-redox couple of the respiratory chain.

Antimycin A primarily affects the bifurcating electron transfer from ubiquinol via ubisemiquinone to the low potential pathway. In contrast, in mitochondrial membranes in which the phospholipid ground structure was changed, the electron flux to the high potential pathway was significantly

impaired, most likely by an impediment to the regular conformational arrangement of the bc_1 complex. Also, sonic pretreatment for SMP preparation may change the order of phospholipids such that electron bifurcation from ubiquinol to the bc_1 complex is affected. We conclude from these observation that any impediment to regular electron bifurcation to the high and low potential pathways results in the leakage of single electrons from autoxidizing $UQ^{\cdot-}$. The underlying mechanism causing autoxidation of otherwise stable mitochondrial $UQ^{\cdot-}$ at the bc_1 complex may be multifactorial. Inhibition of proton-gated ubiquinol oxidation leads to non-physiological accumulation of ubiquinol. As a consequence, ubiquinol concentrations at the Rieske iron–sulfur protein/cyt b_L binding domain exceed ubiquinone levels in this pocket. In a chemical reaction system we have observed that such a situation stimulates the comproportionation reaction to ubisemiquinones (see Eq. (1)).

$$UQH_2 + UQ \rightleftharpoons 2UQ^{\cdot-} + 2H^+ \quad (1)$$

The reaction is driven by the amount of ubiquinol. Ubisemiquinones emerging from this reaction pathway are likely to be destabilized by autoxidation when the natural stabilizing binding capacity is exhausted. Other effects favoring autoxidation of electron-carrying ubisemiquinones may result from changes of redox potential, physical binding properties, or altered activation energy barriers in the first deprotonation step. We believe that we have described here a general mechanism by which the respiratory chain switches from well controlled electron transfer to a $O_2^{\cdot-}$ radical source. We have work in progress to examine the effect of the membrane potential and/or proton motive force on the electron leakage from the bifurcation pathway.

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