I outline the principles that govern O$_2$•− production, and in the present review I outline the principles that govern O$_2$•− production within the matrix of mammalian mitochondria. The flux of O$_2$•− is related to the concentration of potential electron donors, the local concentration of O$_2$ and the second-order rate constants for the reactions between them. Two modes of operation by isolated mitochondria result in significant O$_2$•− production, predominantly from complex I: (i) when the mitochondria are not making ATP and consequently have a high Δp (protonmotive force) and a reduced CoQ (coenzyme Q) pool; and (ii) when there is a high NADH/NAD$^+$ ratio in the mitochondrial matrix. For mitochondria that are actively making ATP, and consequently have a lower Δp and NADH/NAD$^+$ ratio, the extent of O$_2$•− production is far lower. The generation of O$_2$•− within the mitochondrial matrix depends critically on Δp, the NADH/NAD$^+$ and CoQH$_2$/CoQ ratios and the local O$_2$ concentration, which are all highly variable and difficult to measure in vivo. Consequently, it is not possible to estimate O$_2$•− generation by mitochondria in vivo from O$_2$•−-production rates by isolated mitochondria, and such extrapolations in the literature are misleading. Even so, the description outlined here facilitates the understanding of factors that favour mitochondrial ROS production. There is a clear need to develop better methods to measure mitochondrial O$_2$•− and H$_2$O$_2$ formation in vivo, as uncertainty about these values hampers studies on the role of mitochondrial ROS in pathological oxidative damage and redox signalling.

Key words: complex I, hydrogen peroxide, mitochondrion, reactive oxygen species (ROS), respiratory chain, superoxide.

INTRODUCTION

Mitochondria are an important source of ROS (reactive oxygen species) within most mammalian cells [1–8]. This ROS production contributes to mitochondrial damage in a range of pathologies and is also important in redox signalling from the organelle to the rest of the cell [3,9]. Consequently, knowledge of how mitochondria produce ROS is vital to understand a range of currently important biomedical topics (Figure 1). The first report that the respiratory chain produced ROS came in 1966 [10], followed by the pioneering work of Chance and colleagues who showed that isolated mitochondria produce H$_2$O$_2$ [4,11,12]. Later, it was confirmed that this H$_2$O$_2$ arose from the dismutation of superoxide (O$_2$•−) generated within mitochondria [13,14]. The parallel discovery that mitochondria contain their own SOD (superoxide dismutase), MnSOD, confirmed the biological significance of mitochondrial O$_2$•− production [15]. Since then, a huge literature has developed on the sources and consequences of mitochondrial ROS production, which I will not attempt to cover systematically. Instead, I shall develop a consensus about how mitochondria produce ROS and indicate current uncertainties and future issues to be addressed. The focus of the present review is the production of the proximal mitochondrial ROS, O$_2$•−, in the mitochondrial matrix by the core metabolic machinery present in the mitochondrial inner membrane and matrix. Other potentially important ROS sources associated with the mitochondrial outer membrane or intermembrane space will not be considered [1,5]. The aim is to provide a useful resource for those working on mitochondrial ROS production, to facilitate the design and interpretation of experiments, and to stimulate new approaches.

O$_2$•−, the proximal mitochondrial ROS

Within mitochondria, O$_2$•− is produced by the one-electron reduction of O$_2$. Therefore it is the kinetic and thermodynamic factors underlying the interaction of potential one-electron donors with O$_2$ that control mitochondrial ROS production. Having two unpaired electrons in antibonding orbitals with parallel spins makes ground-state O$_2$ accept one electron at a time [16]. The standard reduction potential for the transfer of an electron to O$_2$ to form O$_2$•− is −160 mV at pH 7 [16], for a standard state of 1 M O$_2$. As the pK$_a$ of O$_2$•− is 4.7 [16], this standard reduction potential is invariant across most biological pH values. The actual reduction potential that determines the thermodynamic tendency of O$_2$ to form O$_2$•−, $E_a$, will vary with the relative concentrations of O$_2$ and O$_2$•−:

$$E_a (\text{mV}) = -160 + 61.5 \log_{10} \frac{[O_2]}{[O_2•−]} \quad (1)$$

The [O$_2$] in air-saturated aqueous buffer at 37°C is approx. 200 μM [17]; however, mitochondria in vivo are exposed to a considerably lower [O$_2$] that varies with tissue and physiological state [18]. This occurs because the mitochondrial [O$_2$] in vivo is largely set by the extracellular [O$_2$] in the tissue, which is itself determined by local O$_2$ delivery and consumption [18]. In addition, [O$_2$] decreases on going from the extracellular
ROS production by mitochondria can lead to oxidative damage to mitochondrial proteins, membranes and DNA, impairing the ability of mitochondria to synthesize ATP and to carry out their wide range of metabolic functions, including the tricarboxylic acid cycle, fatty acid oxidation, the urea cycle, amino acid metabolism, haem synthesis and FeS centre assembly that are central to the normal operation of most cells. Mitochondrial oxidative damage can also increase the tendency of mitochondria to release intermembrane space proteins such as cytochrome c (cyt c) to the cytosol by mitochondrial outer membrane permeabilization (MOMP) and thereby activate the cell’s apoptotic machinery. In addition, mitochondrial ROS production leads to induction of the mitochondrial permeability transition pore (PTP), which renders the inner membrane permeable to small molecules in situations such as ischaemia/reperfusion injury. Consequently, it is unsurprising that mitochondrial oxidative damage contributes to a wide range of pathologies. In addition, mitochondrial ROS may act as a modulatable redox signal, reversibly affecting the activity of a range of functions in the mitochondria, cytosol and nucleus.

The factors determining the rate of $O_2^{**}$ production by mitochondria are relatively straightforward. The first is the concentration of the enzyme or protein [E] containing electron carriers and a wide range of electron donors within mitochondria could potentially carry out this reaction [1]. However, only a small proportion of mitochondrial electron carriers with the thermodynamic potential to reduce $O_2$ to $O_2^{**}$ do so. Under most circumstances, small-molecule electron carriers such as NADH, NADPH, CoQH$_2$ (reduced coenzyme Q) and glutathione (GSH) do not react with $O_2$ to generate $O_2^{**}$. Instead, mitochondrial $O_2^{**}$ production takes place at redox-active prosthetic groups within proteins, or when electron carriers such as CoQH$_2$ are bound to proteins, and it is the kinetic factors that favour or prevent the one-electron reduction of $O_2$ to $O_2^{**}$ by these that determine mitochondrial $O_2^{**}$ production.

**THE CONTROL OF $O_2^{**}$ PRODUCTION BY PROTEIN-BOUND REDOX GROUPS**

The factors determining the rate of $O_2^{**}$ production by mitochondria are relatively straightforward. The first is the concentration of the enzyme or protein [E] containing electron carriers
that can exist in a redox form able to react with O₂ to form O₂•−. The second is the proportion (Pₚ) of this enzyme’s electron carrier present in a redox form that can react with O₂. As many redox-active groups exist only transiently in a state that can react with O₂, Pₚ is a time-average. The remaining factors are the local [O₂] and the second-order rate constant (kₑ) for the reaction of that electron carrier with O₂ to form O₂•−. For a given enzyme or protein (E), the rate of O₂•− production is:

$$\frac{d[O_2^{•−}]}{dt} = kₑ[O_2]Pₚ[E]$$  \hspace{1cm} (2)

This can be extended to consider several potential electron-donor sites within mitochondria, and also to take into account multiple electron donor sites within a single protein:

$$\frac{d[O_2^{•−}]}{dt} = [O_2] \sum (kₑPₚ[E])$$  \hspace{1cm} (3)

The concentration of the enzyme responsible for O₂•− production, [E], will vary with organism, tissue, state, age or hormonal status, and may underlie many of the changes in maximum ROS production capacity between tissues [23]; for example, complex I content may explain the different maximum capacities of pigeon and rat heart mitochondria [24].

As the apparent Kₐ of cytochrome oxidase for O₂ is very low (<1 µM [25]), changes in [O₂] should have little direct effect on mitochondrial function and instead are mostly likely to interact by affecting O₂•− production. The generation of O₂•− or H₂O₂ by isolated respiratory complexes, SMPs (submitochondrial particles) or mitochondria increases when [O₂] is raised above the normal atmospheric level of 21 % O₂, and this increase is roughly proportional to [O₂]; at least over the lower range of supraphysiological [O₂] [4,12,26–28]. Fewer studies have looked at the effects of decreasing [O₂], but O₂•− production by isolated complex I decreases linearly with lowered [O₂] [28], and there is a decrease in H₂O₂ production by isolated mitochondria when [O₂] is lowered below that of air-saturated medium [27,29,30]. However, in one detailed study, the rate of H₂O₂ production did not change as [O₂] was lowered from ~200 µM to ~5 µM and only decreased when [O₂] was lowered below 5 µM [29]; further investigation of this important issue is required to see whether it arises due to the technical difficulties of measuring low levels of H₂O₂ production or whether it reflects a genuine phenomenon.

The dependence of mitochondrial H₂O₂ production on [O₂] is likely to be an important factor in the variation of ROS production in vivo because extracellular [O₂] varies with physiological state, and there are [O₂] gradients from the circulation to the mitochondria where O₂ consumption by cytochrome oxidase decreases [O₂] locally [18,19]. Thus increasing or decreasing the rate of O₂ consumption by mitochondria may be an important aspect for modifying O₂•− production in vivo by altering the local [O₂] [19]. Furthermore, as O₂ is approx. 3-fold more soluble within membranes than in water [31], O₂ may be concentrated close to electron carriers within the membrane. Physiological levels of nitric oxide (NO•) compete with O₂ for cytochrome oxidase when [O₂] is low, effectively raising the apparent Kₐ of this enzyme [32–35], and may thus alter the local [O₂] around mitochondria, leading to changes in O₂•− production [36–38].

Probably the most important factor that determines O₂•− production by mitochondria is the proportion, Pₚ, of a given electron carrier that is reactive with O₂ to form O₂•−, as Pₚ responds rapidly to a range of biological situations. The relationship between Pₚ and the overall reduction state of an electron carrier group in a protein may be complicated, as the redox form that donates an electron to O₂ is not necessarily fully reduced. Instead, a partially reduced form, such as a semiquinone, that can respond to the cumulative reduction of an electron carrier system may be the critical electron donor. For any electron carrier, Pₚ will be affected by changes in the carrier’s Eₚ and in its rate of electron supply and release, all of which can be altered by inhibition, damage, mutation or post-translational modification to protein complexes distal or proximal to the site, or to the protein itself. Many other factors can have an impact on Pₚ by affecting these parameters, with changes in Δp (protonmotive force) likely to be particularly important as it significantly affects the Pₚ of electron carriers and it varies rapidly in response to changes in mitochondrial ATP synthesis.

The final factor affecting the rate of O₂•− production by electron carriers within proteins is the second-order rate constant (kₑ) of their reaction with O₂. The reaction between protein-bound electron carriers and O₂ to form O₂•− is generally thought to occur through an outer-sphere mechanism described by the Marcus theory [39,40]. In this mechanism, an electron tunnels from the electron donor to O₂, and the rate is very dependent on the distance between O₂ and the electron donor [39,40]. This is similar to electron movement down the respiratory chain which occurs by electron tunnelling from carrier to carrier, with a maximum distance of approx. 14 Å (1 Å = 0.1 nm) between each carrier for effective tunnelling to occur [41]. Similar distance constraints probably apply to the reaction of protein-bound electron carriers in the respiratory chain with O₂ to form O₂•−, with the bulk of the protein acting as an insulator to keep O₂ at a safe distance from the carriers and thereby minimize O₂•− production [41]. Consequently, O₂•− production will probably occur at sites where O₂ can approach closely to electron carriers, such as at active sites exposed to the aqueous phase or to the membrane core.

The rate of electron transfer from protein electron carriers to O₂ has been investigated for flavoenzymes that activate O₂, where the reduction of O₂ to O₂•− is often a precursor to further reactions [42]. For the flavoenzyme glucose oxidase, the rate of O₂ reduction to H₂O₂ in H₂O₂ solution is ~10¹⁰ M⁻¹ s⁻¹ [39], and, as the rate-limiting step is O₂ reduction to O₂•− [39], this indicates that O₂•− production by protein-bound electron carriers can be rapid. Of course, the production of H₂O₂ is a consequence of the normal physiological function of glucose oxidase, and for other flavoproteins, where electron transfer to O₂ to form O₂•− may be a side reaction, the rate varies over five orders of magnitude [43]. Even so, the rate of reduction of O₂ by the reduced FMD of complex I to form O₂•− is ~40 O₂•− · min⁻¹ · kg⁻¹ [28], corresponding to a second-order rate constant of ~10¹⁰ M⁻¹ · s⁻¹. Thus the second-order rate constants (kₑ) for O₂•− production as a side reaction by protein-bound electron carriers can be rapid, although it is likely to vary markedly with the environment of the electron donor and its accessibility to O₂. Therefore alterations to a protein that enabled O₂ to approach more closely to the electron carrier, such as by damage, mutation, post-translational modification, conformational change or quaternary interactions, could lead to dramatic changes in the rate of O₂•− production, and potentially play a regulatory function. Such factors determining the second-order rate constant for the reaction of O₂ with protein electron carriers could determine mitochondrial O₂•− production, but little is known about how kₑ may be varied by different enzymes within mitochondria.

**MEASUREMENT OF O₂•− PRODUCTION BY ISOLATED MITOCHONDRIA**

Although many purified mitochondrial proteins can be manipulated so as to produce O₂•−, the physiological relevance of this is limited. Therefore it is important to understand O₂•− production
The production of $O_2^{•−}$ within the mitochondrial matrix, intermembrane space and outer membrane leads to the formation of $H_2O_2$ from SOD-catalysed dismutation. Some $O_2^{•−}$ can react directly with nitric oxide (NO•) to form peroxynitrite (ONOO•). There are also sources outside mitochondria that produce $H_2O_2$ directly. The $H_2O_2$ efflux from mitochondria can be measured following reaction with a non-fluorescent substrate such as Amplex Red in conjunction with horseradish peroxidase (HRP) to form a fluorescent product, resorufin. Within mitochondria $H_2O_2$ is degraded by glutathione peroxidases (GPx) or peroxiredoxins (Prx) which depend on glutathione (GSH) and thioredoxin-2 (Trx) for their reduction respectively. Glutathione disulfide (GSSG) is reduced back to GSH by glutathione reductase (GR). Trx is reduced by thioredoxin reductase-2 (TrxR). Both enzymes receive reducing equivalents from the NADPH pool, which is kept reduced by the Δp-dependent transhydrogenase (TH), and by isocitrate dehydrogenase (ICDH). Note that many mitochondrial preparations, particularly those from the liver, contain large amounts of catalase contamination. The effects of such extramitochondrial $H_2O_2$ sinks are not indicated here as they are usually accounted for by appropriate $H_2O_2$ calibration curves in the presence of mitochondria. ox, oxidized; red, reduced. An animated version of this Figure can be seen at http://www.BiochemJ.org/bj/417/0001/bj4170001add.htm.

within isolated mitochondria under conditions that mimic those that may arise in vivo under physiological or pathological conditions. A constraint of using isolated mitochondria is that the system is complicated and difficult to manipulate. In particular, the direct measurement of $O_2^{•−}$ within mitochondria is challenging due to its rapid dismutation in the presence of $\sim 10 \mu M$ MnSOD ($k \approx 2 \times 10^9 M^{-1} s^{-1}$ [2,5]), which leads to very low steady-state matrix $[O_2^{•−}]$ and competes with $O_2^{•−}$ detection systems [44]. The spontaneous dismutation of $O_2^{•−}$ ($k \approx 10^9$ [16]) is less important because it is second-order with respect to $[O_2^{•−}]$, which is very low in the presence of MnSOD.) Studies have been carried out on $O_2^{•−}$ production by mitochondria lacking MnSOD [44], but there is always the concern that the absence of MnSOD may decrease the flux of $O_2^{•−}$ by allowing the back reaction between $O_2^{•−}$ and the electron donor [21,22], as well as having the potential to damage the system [45,46]. The use of $O_2^{•−}$-sensitive dyes such as hydroethidine and MitoSOX™ [47–50], measurement of the reaction of $O_2^{•−}$ with compounds to form chemiluminescent products [44,51], spin trapping [52] or measurement of the inactivation rate of aconitase [53] do provide useful information on mitochondrial $O_2^{•−}$ production. Even so, measurement is challenging, and the quantification of $O_2^{•−}$ production by isolated mitochondria is not done routinely.

In contrast with the difficulties of assessing $O_2^{•−}$ directly, intramitochondrial $O_2^{•−}$ flux can be readily measured in isolated mitochondria following its dismutation to $H_2O_2$ by MnSOD and subsequent diffusion from the mitochondria [11,54,55]. There are a number of ways of assaying this $H_2O_2$, but typically this is now done by measuring the oxidation of a non-fluorescent dye by $H_2O_2$ in conjunction with a peroxidase to form a fluorescent product (Figure 2) [11,55–57]. Although such assays are reliable, sensitive and robust, there are a number of issues to be considered in interpreting these data and in extrapolating from isolated mitochondria to the in vivo situation. First, not all $O_2^{•−}$ is necessarily converted into $H_2O_2$ in vivo, as some $O_2^{•−}$ could react with other electron acceptors or with NO• within mitochondria [58,59]. Although the extent of these reactions is not known, situations may arise where they are significant, particularly as physiological levels of NO• can react very rapidly with $O_2^{•−}$ ($k \approx 10^{10} M^{-1} s^{-1}$) to form peroxynitrite (ONOO•) [58,59]. Secondly, measurement of $H_2O_2$ efflux from mitochondria will also be affected by $H_2O_2$ produced in the intermembrane space or outer membrane, which can be significant under some conditions [1,52,60]. Thirdly, and most importantly, not all $H_2O_2$ produced within the mitochondrial matrix will survive to efflux from the mitochondria, owing to matrix peroxidases that consume $H_2O_2$ [1,61–63]. These include peroxiredoxins 3 and 5 [64], catalase [65,66] and glutathione peroxidases 1 and 4 [67], with the peroxiredoxins probably of the greatest significance [64]. Thus the rate of matrix $O_2^{•−}$ formation is the sum of the $H_2O_2$ measured effluxing from the mitochondria, the $O_2^{•−}$ sinks, the rate of $H_2O_2$ degradation to water, minus $H_2O_2$ production from outside mitochondria (eqn 4) (Figure 2):

![Figure 2 Measurement of $H_2O_2$ production by isolated mitochondria](https://www.BiochemJ.org/bj/417/0001/bj4170001add.htm)
Consistent with this, exposure of mitochondria to H$_2$O$_2$ leads to greater H$_2$O$_2$ efflux [27], and may be one way in which H$_2$O$_2$ efflux from mitochondria is regulated by a feed-forward cycle of ROS induced ROS efflux. A related factor is that many mitochondrial manipulations affect the activity of H$_2$O$_2$-degradation systems, which are linked to the GSH/GSSG ratio through glutathione peroxidases [67] and to the thioredoxin-2 oxidized/reduced ratio through peroxiredoxins [62,63,68,72,73]. These in turn are affected by the NADPH/NADP$^+$ ratio which is set by the activity of the transhydrogenase and of NADP$^+$-dependent isocitrate dehydrogenase [74,75]. Thus interventions that alter mitochondrial substrate supply or Δp could affect both O$_2$•− production and matrix H$_2$O$_2$ degradation. Finally, it is generally assumed that H$_2$O$_2$ permecates directly through the mitochondrial membrane; however, H$_2$O$_2$ diffusion across the plasma membrane is facilitated by aquaporins [76,77], and the possibility remains that H$_2$O$_2$ from mitochondria may actually be protein-mediated, perhaps leading to another level of control over H$_2$O$_2$ efflux. Therefore, although inferring mitochondrial O$_2$•− production by measuring H$_2$O$_2$ efflux is robust and reliable, a number of points must be borne in mind when interpreting these data.

**O$_2$•− PRODUCTION WITHIN ISOLATED MITOCHONDRIA**

Sufficient studies have been carried out on the efflux of H$_2$O$_2$ from isolated mitochondria to enable generalizations about the conditions that favour O$_2$•− production and the mitochondrial sources of O$_2$•−. There are two main modes of operation by isolated mitochondria that lead to extensive H$_2$O$_2$ efflux (Figure 3). The first mode occurs when there is a high NADH/NAD$^+$ ratio in the matrix [27,28]. The second mode is when there is a highly reduced CoQ (coenzyme Q) pool, in conjunction with a maximal Δp and no ATP synthesis [57,78,79]. The third mode of mitochondrial operation is when the mitochondria are working normally making ATP (i.e. they are in, or close to, state 3), or using the Δp for other functions such as thermogenesis. In this third mode of operation, H$_2$O$_2$ efflux from mitochondria is negligible compared with modes 1 or 2. Our next focus is to understand in detail the sites and mechanisms of O$_2$•− production under these three modes of mitochondrial operation and thereby infer where and how O$_2$•− is produced in vivo.

**Sites of O$_2$•− production within isolated mitochondria**

Complex I

Mammalian complex I is the entry point for electrons from NADH into the respiratory chain and is a ~1 MDa complex comprising 45 polypeptides [80,81]. An FMN cofactor accepts electrons from NADH and passes them through a chain of seven FeS (iron–sulfur) centres to the CoQ reduction site, with another FeS centre (N1a) close to the FMN, but not thought to be involved in electron transfer to CoQ [80,81]. The structure of the water-soluble arm

\[
\frac{1}{2} \left( \frac{d[O_2^{•−}]}{dr} \right)_{\text{Min}} = \left( \frac{d[H_2O_2]}{dr} \right)_{\text{Meas}} + \frac{1}{2} \left( \frac{d[O_2^{•−}]}{dr} \right)_{\text{Sink}} + \left( \frac{d[H_2O_2]}{dr} \right)_{\text{Degr}} - \left( \frac{d[H_2O_2]}{dr} \right)_{\text{Ext}}
\]

Consequently, there is a threshold of intramitochondrial O$_2$•− formation before it exceeds scavenging by O$_2$•− sinks and peroxidases, and generates sufficient H$_2$O$_2$ to be measured externally [1,61]. A corollary is that there may be significant O$_2$•− formation within mitochondria that goes undetected, which is supported by the finding that H$_2$O$_2$ efflux from mitochondria increases on inhibition of peroxidases [1,68]. An interesting extension is that the activity of intramitochondrial peroxidases such as peroxiredoxins is decreased on exposure to H$_2$O$_2$ [69–71], and thus the mitochondrial H$_2$O$_2$-degradation rate may vary considerably with condition and history of the organelle. Consistent with this, exposure of mitochondria to H$_2$O$_2$ leads to greater H$_2$O$_2$ efflux [27], and may be one way in which H$_2$O$_2$ efflux from mitochondria is regulated by a feed-forward cycle of ROS-induced ROS efflux. A related factor is that many mitochondrial manipulations affect the activity of H$_2$O$_2$-degradation systems, which are linked to the GSH/GSSG ratio through glutathione peroxidases [67] and to the thioredoxin-2 oxidized/reduced ratio through peroxiredoxins [62,63,68,72,73]. These in turn are affected by the NADPH/NADP$^+$ ratio which is set by the activity of the transhydrogenase and of NADP$^+$-dependent isocitrate dehydrogenase [74,75]. Thus interventions that alter mitochondrial substrate supply or Δp could affect both O$_2$•− production and matrix H$_2$O$_2$ degradation. Finally, it is generally assumed that H$_2$O$_2$ permeates directly through the mitochondrial membrane; however, H$_2$O$_2$ diffusion across the plasma membrane is facilitated by aquaporins [76,77], and the possibility remains that H$_2$O$_2$ from mitochondria may actually be protein-mediated, perhaps leading to another level of control over H$_2$O$_2$ efflux. Therefore, although inferring mitochondrial O$_2$•− production by measuring H$_2$O$_2$ efflux is robust and reliable, a number of points must be borne in mind when interpreting these data.

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\]
of complex I from Thermus thermophilus, which contains the FMN and the FeS centres, is known and is likely to be very similar to that in mammals [80–82]. However, the structure of the CoQ reduction site in the hydrophobic arm is not known and the mechanism of proton pumping by complex I is uncertain [80–82]. The Thermus structure indicates that the seven FeS centres in the hydrophilic arm involved in passing electrons from FMN to CoQ are reasonably well shielded from O₂, so that O₂ is most likely to access electron carriers at the FMN and CoQ sites, although there are FeS centres at both termini which could also donate electrons to O₂ [81].

The first demonstration of ROS production by complex I was in SMPs where reduction of the CoQ pool and generation of a large Δp by succinate led to uncoupler-sensitive H₂O₂ production [83]. Subsequently, it was shown that isolated complex I in the presence of NADH produces O₂•− and that this generation is enhanced by the inhibitor rotenone which binds to the CoQ-binding site [84]. The mechanism of O₂•− production by isolated complex I is now reasonably well understood [28,85]. The isolated complex produces O₂•− from the reaction of O₂ with the fully reduced FMN, and the proportion of the FMN that is fully reduced (Pₐ) is set by the NADH/NAD⁺ ratio [28,85]. This model explains why inhibition of complex I with rotenone increases O₂•− production, as it will lead to a back up of electrons onto FMN which will produce O₂•− [86,87]. For complex I within intact mitochondria, the proportion of fully reduced FMN is thought to be set by the NADH/NAD⁺ ratio, so inhibition of the respiratory chain by damage, mutation, ischaemia, loss of cytochrome c or by the build up of NADH due to low ATP demand and consequent low respiration rate will increase the NADH/NAD⁺ ratio and lead to O₂•− formation [27,28,57,88–90]. In contrast, for most situations where mitochondria are respiring normally on NADH-linked substrates and the NADH/NAD⁺ ratio is relatively low, only small amounts of O₂•− are produced from complex I [87]. An elegant demonstration of the importance of an elevated NADH/NAD⁺ ratio for O₂•− production from complex I is that oxidation of the NADH pool in mammalian mitochondria by expression of a yeast NADH dehydrogenase decreases O₂•− production [91].

The other mechanism by which complex I produces large amounts of O₂•− is during RET (reverse electron transport) [7,27,87,89]. RET occurs for mitochondria operating in mode 2 when electron supply reduces the CoQ pool, which in the presence of a significant Δp forces electrons back from CoQH₂ into complex I, and can reduce NAD⁺ to NADH at the FMN site [92]. RET was first associated with H₂O₂ production in SMPs respiring on succinate [83,93]. Later, it was shown that isolated mitochondria respiring on succinate, also generate large amounts of O₂•− from complex I by RET [94]. Since then it has become clear that there is extensive O₂•− production by RET at complex I in isolated brain, heart, muscle and liver mitochondria under conditions of high Δp with electron supply to the CoQ pool from succinate, α-glycerophosphate or fatty acid oxidation [7,24,87,89]. This O₂•− production by RET is abolished by rotenone, confirming that it is due to electrons entering into complex I through the CoQ-binding site(s) [95]. Of particular note, the dependence of RET-associated O₂•− production on Δp is very steep and is abolished completely by a small decrease in Δp [79,87], presumably by decreasing the thermodynamic driving force pushing electrons to the O₂•−-producing site(s) within complex I. Intriguingly, O₂•− production by complex I is more sensitive to changes in the ΔpH (pH gradient) component of the Δp, than to the Δψ (membrane potential) component [79,96].

The complex I site producing O₂•− during RET is unclear [85,97]. The simplest possibility is that RET forces electrons right back through complex I to the FMN, and that the site of O₂•− production is the same during RET as it is for O₂•− production from the reduced FMN in response to an elevated NADH/NAD⁺ ratio [85,89]. Consistent with this, the flavin inhibitor DPI (diphenyleneiodonium) blocks RET-associated O₂•− production by complex I [89]; however, this does not confirm the involvement of the FMN site, as DPI has other interactions with mitochondria [97]. Furthermore, disruption of the CoQ-binding site in complex I under conditions where there was a high ΔpH led to far more extensive O₂•− production than reduction of FMN alone, suggesting a role for the CoQ-binding site in RET O₂•− production [95]. In addition, there is not a unique relationship between the mitochondrial NADH/NAD⁺ ratio and O₂•− production by complex I under different conditions [98]. This suggests that O₂•− production from complex I may occur both by RET and from the reduction of FMN by the NADH pool simultaneously, and that most of the O₂•− generated during RET is not produced at the FMN. If O₂•− is not produced at FMN during RET, then the focus shifts to the CoQ-binding site, where two electrons are passed from the N2 FeS centre to CoQ, reducing it to CoQH₂, while pumping four H⁺ across the mitochondrial inner membrane by an unknown mechanism. The intriguing pH- and ΔpH-dependence of O₂•− production during RET [79] may suggest that critical semiquinones and semiquinolates are formed during proton pumping that react directly with O₂ to form O₂•− [57,88,95]. However, our lack of knowledge of the full structure of complex I and its exact mechanism of proton pumping hampers progress. More positively, it may be that investigating the mechanism of O₂•− production [95], and the unusual interaction of complex I with electron acceptors during RET [44], may shed light on the mechanism of proton pumping by complex I.

To summarize, complex I produces large amounts of O₂•− by two mechanisms: when the matrix NADH/NAD⁺ ratio is high, leading to a reduced FMN site on complex I, and when electron donation to the CoQ pool is coupled with a high Δp leading to RET (Figure 4). Although the site of O₂•− production during RET is not known, the rate of O₂•− production under RET seems to be the highest that can occur in mitochondria [71,79,95].

Complex III

Complex III funnels electrons from the CoQ pool to cytochrome c. The monomer is ~240 kDa and comprises 11 polypeptides, three haems and an FeS centre, and it interacts transiently with CoQ during the Q-cycle at the Qₐ and Qₜ sites [99]. Complex III has for a long time been regarded as a source of O₂•− within mitochondria [84,100]. When supplied with CoQ₉ and when the Q site is inhibited by antimycin, complex III produces large amounts of O₂•− from the reaction of O₂ with a ubisemiquinone bound to the Q site [84,89,100–103]. This O₂•− is released from complex III to both sides of the inner membrane [24,27,52,104]. However, in the absence of antimycin, the Q site ubisemiquinone is not stabilized and O₂•− production by complex III is low [105]. Inhibition of respiration at points distal to complex III with cyanide or by loss of cytochrome c does not increase O₂•− production by complex III [100], therefore reduction of the CoQ pool is not sufficient to generate O₂•− at complex III. It may be that high Δp stabilizes the Q site ubisemiquinone; however, the very high production of O₂•− by complex I during RET in the presence of succinate is abolished completely by rotenone [85,95]. Under these conditions, there is a large Δp and a reduced CoQ pool, suggesting that the maximal O₂•− production by uninhibited complex III is negligible compared with that by complex I during RET. However, it is possible that, for mitochondria operating in mode 3, when O₂•− production by complex I is low, the contribution of complex III to the overall O₂•− flux may then become relatively significant.
Under these conditions, factors that affect the stability of the ubisemiquinone radical in the Q_{o} site, such as loss of cytochrome c or changes in Δp or in the redox state of the CoQ and cytochrome c pools, may modulate O_{2}^{•−} production. Therefore, although complex III can be induced to produce O_{2}^{•−} with the inhibitor antimycin, its production in mitochondria under physiological conditions is far lower and is negligible compared with the maximum rates of O_{2}^{•−} production from complex I.

Other sites of O_{2}^{•−} production within mitochondria

Many other sites within mitochondria may also produce O_{2}^{•−} or H_{2}O_{2}. It is convenient to divide most of these into sites that interact with the matrix NADH pool and those that are connected to the CoQ pool within the inner membrane.

When respiratory chain activity is low, or when there is RET, the NADH/NAD\(^{+}\) ratio will increase and this may lead to O_{2}^{•−} production at other sites connected to the NADH pool, in addition to complex I. When complex I is inhibited with rotenone, there is a reduced NADH pool and ROS production from complex I, but addition of 2-oxoglutarate (α-ketoglutarate) as a substrate increases ROS production further, suggesting that the combination of 2-oxoglutarate with a reduced NADH pool may lead to significant ROS production from αKGDH [2-oxoglutarate dehydrogenase (α-ketoglutarate dehydrogenase)] [7,106–108]. One component of αKGDH is dihydrolipoamide dehydrogenase, which contains a flavin that can produce ROS when its electron acceptor NAD\(^{+}\) is limiting [7,106–108]. Decreasing the expression of dihydrolipoamide dehydrogenase in mouse fibroblasts decreased O_{2}^{•−} production in mitochondria isolated from these cells [7,106–108]. A similar situation may exist for other enzymes that contain dihydrolipoamide dehydrogenase, such as pyruvate dehydrogenase, but their capacity for O_{2}^{•−} production seems to be less than that of αKGDH [106]. Therefore, under conditions of high NADH/NAD\(^{+}\) ratio, not only complex I, but also αKGDH, and perhaps other enzymes linked to the NADH pool, may contribute to O_{2}^{•−} production in a manner that depends on the mitochondrial substrates. It may be that when mitochondria are operating in mode 3 and are actively making ATP, O_{2}^{•−} production from complex I is negligible and the contribution from sites such as αKGDH is proportionally more significant [106].

Many potential sites of O_{2}^{•−} production interact with the CoQ pool. Fatty acid oxidation in the matrix reduces ETF (electron transfer flavoprotein) which passes its electrons to CoQ via ETF:CoQ oxidoreductase on the matrix surface of the inner membrane [109]. Oxidation of palmitoyl-CoA by mitochondria leads to H_{2}O_{2} production [12,90], primarily from RET at complex I; however, ETF:CoQ reductase may itself produce ROS [24]. Dihydroorotase dehydrogenase on the outer surface of the inner membrane catalyses oxidation of dihydro- orotate to orotate with reduction of CoQ, and dihydro- orotate oxidation is associated with mitochondrial O_{2}^{•−} production [110]; however, it is uncertain whether O_{2}^{•−} is produced by dihydro- orotate dehydrogenase itself. On the outer surface of the inner membrane, αGPDH (α-glycerophosphate dehydrogenase) takes electrons from α-glycerophosphate to CoQ [111–113]. In brown adipose tissue mitochondria [112], and in Drosophila mitochondria [114], oxidation of α-glycerophosphate is associated with ROS production, much of this due to RET from complex I, although some is produced from αGPDH itself on the outer surface of the mitochondrial inner membrane [113]. However, the physiological significance of this is unclear, as αGPDH is expressed at relatively low levels in most mammalian tissues [111,112], although it may be important in the brain [113]. Complex II oxidizes succinate passing electrons to CoQ, but, although the damaged or mutated complex can produce ROS [101,115], it seems that all O_{2}^{•−} production during succinate oxidation arises from complex I by RET. Therefore the activity of a number of CoQ-linked enzymes is associated with increased
ROS production, primarily by favouring RET at complex I, but, in some cases, they may produce ROS directly themselves. In addition to the sites discussed above, there are many other mitochondrial enzymes that can be induced to produce O$_2^•-$ or H$_2$O$_2$. Some of these are not connected to the NADH or CoQ pools, such as the adrenodoxin reductase/adrenodoxin/cytochrome P$_{450}$ system in the mitochondrial matrix that receives electrons from the NADPH pool [116,117]. Therefore it is probable that many other sites of mitochondrial O$_2^•-$ production remain to be discovered, but whether they make a quantitatively significant contribution to mitochondrial ROS production under physiological conditions is unclear [1].

Overview of O$_2^•-$ production by isolated mitochondria

It is possible to draw some tentative conclusions about O$_2^•-$ production by isolated mitochondria. Two modes of operation lead mitochondria to produce large amounts of O$_2^•-$: mode 1, when there is a build up of NADH, and mode 2, when there is a large Δp and a reduced CoQ pool (Figure 3). In both modes, the predominant site of O$_2^•-$ production is complex I, in keeping with a growing view that complex I is the major source of O$_2^•-$ within mitochondria in vivo [1,7,85,118]. Even so, the accumulation of NADH that occurs in modes 1 and 2 may lead to O$_2^•-$ production from other sites such as αKGDH. The mechanisms of O$_2^•-$ production from complex I in the two modes are quite distinct, being by reduction of FMN by NADH in mode 1 and by RET in mode 2. Of course, in some situations, both mode 1 and mode 2 may operate simultaneously when a build up of NADH and CoQ coincides with a high Δp.

The mode 1 production of O$_2^•-$ probably occurs in vivo under conditions where damage to the respiratory chain, slow respiration or ischaemia leads to a build up of NADH. This may occur during cytochrome c release in apoptosis and following inhibition of respiration at cytochrome oxidase by NO•. Treatments that oxidize the NADH pool, such as overexpressing NADH oxidases, should decrease the production of O$_2^•-$ in mode 1, while rotenone and other respiratory inhibitors should increase it (antimycin would not be diagnostic here as it will increase O$_2^•-$ production at complex III). Whether mode 2 O$_2^•-$ production occurs in cultured cells or in vivo is not known. If it does, it would be associated with a high Δp and a reduced CoQ pool, and will be very sensitive to mild uncoupling and to inhibition by rotenone. The generation of O$_2^•-$ by both modes 1 and 2 will be decreased by mild uncoupling, perhaps suggesting that the physiological function of uncoupling proteins 2 and -3 is to modulate mitochondrial O$_2^•-$ production [118]. Mode 3 occurs when mitochondria are synthesizing ATP or utilizing Δp for other functions. Under these conditions, the lowered Δp and the oxidized NADH pool prevent O$_2^•-$ production by RET and greatly decrease O$_2^•-$ production at the FMN of complex I, making H$_2$O$_2$ efflux from mitochondria negligible compared with modes 1 or 2 (e.g. [4,78,89,119]). However, there may still be O$_2^•-$ production within mitochondria operating in mode 3 that is below the threshold consumed by mitochondrial peroxidases (eqn 4) [68]. Although the sites of this putative O$_2^•-$ production in mode 3 are unclear, complex I, complex III and matrix enzymes such as αKGDH could all contribute. A further complication is that in vivo substrate supply to the respiratory chain is stringently regulated by factors such as dietary and hormonal status, leading to alterations in the steady-state reduction potential of mitochondrial electron carriers. As mitochondria in vivo probably spend much of their time synthesizing ATP, this mode of O$_2^•-$ production may account for most of the overall exposure of mitochondria to O$_2^•-$: Thus, even though the rate of O$_2^•-$ production in mode 3 is relatively low and the sources are poorly understood, it may turn out to be the mode of greatest biological importance and be responsible for the long-term accumulation of mitochondrial oxidative damage and for the extensive tissue damage seen in the absence of MnSOD [45,46].

THE PARADOX OF HYPOXIC H$_2$O$_2$ PRODUCTION BY MITOCHONDRIA

Figure 3 gives a reasonable description of mitochondrial O$_2^•-$ production. However, mitochondrial ROS production is also reported to increase under conditions of very low [O$_2$], which is paradoxical and seems to contradict the dependence of mitochondrial O$_2^•-$ production on [O$_2$] given in eqn (2). These hypoxic effects are seen in cultured cells when ambient O$_2$ is decreased from 21 % O$_2$ to 1–3 % O$_2$ [120,121]. This corresponds to an equilibrium [O$_2$] of 10–20 μM, although the local [O$_2$] around mitochondria will be lower.

The discovery of increased mitochondrial ROS production during hypoxia arose from investigations of HIF-1 (hypoxia-inducible factor-1), which plays a central role in the response of cells to hypoxia [122,123]. HIF-1 is a heterodimer comprising HIF-1α and HIF-1β that translocates to the nucleus and there, in association with other proteins, initiates transcription of a number of genes in response to hypoxia [122,123]. HIF-1α is constitutively expressed, but, under normoxia, it is rapidly hydroxylated on proline residues by PHD (prolyl hydroxylase), which uses 2-oxoglutarate and O$_2$ as substrates, marking HIF-1α for rapid degradation by the ubiquitin–proteasome system [122,123]. When the [O$_2$] falls, HIF-1α is no longer degraded, allowing the HIF-1 heterodimer to form and induce the transcription of a series of hypoxia-sensitive genes. Further regulation of HIF-1 occurs because PHD activity is sensitive to H$_2$O$_2$, probably through reaction with non-haem iron in its active site [120,121]. Thus the mitochondrial respiratory chain may act as an O$_2$ sensor, releasing H$_2$O$_2$ under hypoxic conditions to decreases the activity of PHD, thereby stabilizing HIF-1α and modulating its response to hypoxia [120,121].

The evidence that hypoxia increases mitochondrial ROS production in cultured cells comes from measurements of cytosolic ROS using several different probes, and from showing that the effects of hypoxia-induced mitochondrial ROS on HIF-1 can be blocked by mitochondria-targeted antioxidants [120,121,124]. Further studies have shown that a functional respiratory chain is required, that loss of cytochrome c, or the Rieske FeS centre of complex III, abolishes this ROS signal [121,125], and that the direct addition of H$_2$O$_2$ overcomes this blockade [126]. These studies have led to the proposal that the source of the ROS is complex III [121], possibly a ubisemiquinone at the Q$_{1}$ site [126]. Although ongoing work may refine details of this model, the question remains of how lowering [O$_2$] can increase H$_2$O$_2$ efflux from mitochondria. When isolated mitochondria were maintained at low [O$_2$], ROS production decreased as the [O$_2$] was lowered from approx. 5 μM O$_2$ to anoxia [29]. This finding makes it unlikely that the decrease in [O$_2$] itself affects O$_2^•-$ production directly, for example by altering the stability of the ubiquisemiquinone radical at complex III, the rate of degradation or release of H$_2$O$_2$ from mitochondria or the sidedness of O$_2^•-$ release from complex III [104,121]. Instead it suggests that the low [O$_2$] environment requires additional factors that occur in the hypoxic cell environment to increase mitochondrial ROS efflux. For example, changes in [NO•] might modulate the response of cytochrome oxidase to low [O$_2$] [127], thereby altering the Ps of protein redox groups. Alternatively, hypoxia could act through cell signalling pathways to decrease the activity of mitochondrial matrix or intermembrane space peroxidases, thereby increasing
The system of mitochondrial O$_2^*$ efflux. Clearly, more work is required to unravel the mechanism of increased mitochondrial ROS production during hypoxia, but it remains an intriguing puzzle, and our understanding of mitochondrial ROS production will be incomplete until there is a satisfactory explanation for this phenomenon.

**HOW MUCH O$_2^*$** **DO MAMMALIAN MITOCHONDRIA PRODUCE IN VIVO?**

To investigate the significance of mitochondrial O$_2^*$ production in oxidative damage and redox signalling, it is necessary to know how much O$_2^*$ is generated by mitochondria *in vivo*. The greatest rate of H$_2$O$_2$ production by isolated mitochondria occurs during mode 2 when mitochondria have a high Δψ, a reduced CoQ pool and are not making ATP. Under these conditions, ROS production is primarily by RET at complex I, and approx. 1–2% of the O$_2$ consumed by isolated mitochondria under these conditions forms O$_2^*$ [1,4,27]. Since it was first published by Chance and colleagues [4,12], this value of 1–2% of respiration going to O$_2^*$ has propagated through the literature and has been used erroneously to estimate mitochondrial O$_2^*$ production *in vivo*, even though the original authors made it clear that it only applied to particular experimental conditions [12].

Several factors make extrapolation of H$_2$O$_2$ production by isolated mitochondria to the *in vivo* situation invalid. First, maximal O$_2^*$ production by isolated mitochondria occurs during RET using saturating levels of substrates such as succinate, which are at lower concentrations *in vivo*. When lower concentrations of succinate are used, approx. 0.4–0.8% of respiration produces H$_2$O$_2$ [57], while use of the physiological substrate palmityl-CoA decreases H$_2$O$_2$ production to approx. 0.15% of respiration [24], and, when glutamate/malate are used as substrates, H$_2$O$_2$ production accounts for approx. 0.12% of respiration [27]. Secondly, measurements on isolated mitochondria are generally made using air-saturated medium containing ~200 μM O$_2$. As mitochondrial O$_2^*$ production is probably proportional to [O$_2$] and the physiological [O$_2$] around mitochondria is approx. 10–50 μM, O$_2^*$ production may be 5–10-fold lower than for isolated mitochondria in the same state. The third and most important factor limiting extrapolation of *in vitro* O$_2^*$ production to the situation *in vivo* is that mitochondria *in vivo* are likely to be making ATP and will thus be operating in mode 3 with a lowered Δψ and relatively oxidized NADH and CoQ pools. Consequently, their rates of H$_2$O$_2$ efflux are negligible compared with modes 1 or 2. Therefore, although it is valid to say that 0.12–2% of respiration goes to O$_2^*$ *in vitro*, these values cannot be extrapolated to the *in vivo* situation where mitochondrial O$_2^*$ production will be far, far lower.

Allowing for these caveats, we can estimate roughly how much O$_2^*$ is produced by mitochondria *in vivo*. Unfortunately the answer at the moment is no, because we know little about basic mitochondrial function *in vivo*. For example, the proportion of time that mitochondria spend actively making ATP (i.e. in state 3) or with a high Δψ and a low rate of ATP synthesis (i.e. in state 4) *in vivo* is not known. The proportion of mitochondria with a reduced NADH pool and whether RET occurs *in vivo* are also unclear. If, as is probable, mitochondria *in vivo* spend most of their time close to state 3, then we have very limited knowledge of how much O$_2^*$ production occurs in the matrix. Therefore, although there is considerable evidence for the accumulation of oxidative damage within mitochondria *in vivo* [3,128], from which we can infer that mitochondrial ROS production does occur, it is difficult to estimate the flux of mitochondrial O$_2^*$ that leads to this damage.

Although estimates of mitochondrial O$_2^*$ production as a proportion of respiration rate are not possible, we can infer from studies of mitochondria *ex vivo* the extent of O$_2^*$ production *in vivo*? The maximum O$_2^*$ production rate *in vivo* is proportional to the content of respiratory complexes such as complex I, and thus correlates with maximum respiration rate. However, as the actual O$_2^*$ production rate depends so closely on factors such as Δψ and NADH/NAD$^+$ ratio, which vary markedly *in vivo*, it is not possible to say that changes in ROS production by mitochondria isolated from animals of different ages or hormonal status bear any relation to differences in mitochondrial ROS production *in vivo*. Is it at least possible to infer *in vivo* rates of mitochondrial O$_2^*$ production from direct measurements of ROS within living tissue? At the moment, the answer is also no, because quantification is challenging and currently there are no reliable estimates of mitochondrial O$_2^*$ production *in vivo*. A classic estimate of H$_2$O$_2$ production was made in perfused liver where H$_2$O$_2$ production by the whole organ was measured from changes in catalase compound I [129]. Approx. 80 nmol of H$_2$O$_2$/min per g of wet weight was produced in the tissue [127], with about 12 nmol of H$_2$O$_2$/min per g of wet weight estimated to come from mitochondria [90]. However, one of the many assumptions made in this work was that mitochondrial H$_2$O$_2$ production *in situ* was the same as for isolated mitochondria respiring on succinate, when they would have been producing H$_2$O$_2$ by RET [90]. Therefore we know little about the actual flux of O$_2^*$ within mitochondria *in vivo*, about how it changes under different physiological circumstance, or about its quantitative importance relative to other sources of ROS. More work is required to develop methods to measure mitochondrial ROS production *in vivo*.

**CONCLUSIONS**

I have outlined the factors that lead to O$_2^*$ production within isolated mitochondria and have shown that there are two modes of high O$_2^*$ production, predominantly, but not exclusively, from complex I: by RET when the Δψ is high and the CoQ pool is reduced, and from the FMO when the NADH/NAD$^+$ ratio is high (Figure 3). When mitochondria are actively making ATP, the rate of O$_2^*$ production is far lower and the sites of production are uncertain. This suggests that *in vivo* conditions leading to RET or an accumulation of NADH will favour O$_2^*$ production. However, the extent to which these situations arise *in vivo* is not known, and, at the moment, it is not possible to estimate the rate of mitochondrial O$_2^*$ production *in vivo*. Even so, the description given here of the factors underlying mitochondrial O$_2^*$ production will enable the design and interpretation of experiments to assess the physiological and pathological significance of mitochondrial ROS production *in vivo*. One area where such knowledge is vital is in the large number of pathologies where mitochondrial disruption leads to oxidative damage [3,5]. This raises the possibility that better understanding of how mitochondria produce ROS will lead to the rational design of therapies to minimize mitochondrial oxidative damage [130]. Also important is the likelihood that ROS production by mitochondria is a redox signal integrating mitochondrial function with that of the rest of the cell [3,9].

Redox signalling can occur by mitochondria releasing H$_2$O$_2$, which modulates the activity of target proteins through the reversible oxidation of critical protein thiols [9,131], thus altering the activity of enzymes, kinases, phosphatases and transcription factors in mitochondria, the cytosol or the nucleus (Figure 5). Although little is known currently about mitochondrial redox signalling, the description of ROS production developed here shows how it can occur and will prove useful in investigating this rapidly developing area. For example, mitochondrial H$_2$O$_2$ efflux could act as a retrograde signal to the cell, reporting on mitochondrial Δψ or the redox state of the NADH pool, and thus enable
The production of H$_2$O$_2$ from mitochondria is a potential redox signal. H$_2$O$_2$ generated by mitochondria can reversibly alter the activity of proteins with critical protein thiols by modifying them to intra- or inter-protein disulfides, or to mixed disulfides with GSH. These modifications can occur on mitochondrial, cytosolic or nuclear enzymes, carriers or transcription factors, transiently altering their activities. The change in activity can be reversed by reducing the modified protein thiol by endogenous thiol reductants such as GSH or thioredoxin. As the extent of H$_2$O$_2$ production from mitochondria will depend on factors such as ΔΨ or the redox state of the NADH pool, it can act as a retrograde signal to the rest of the cell, reporting on mitochondrial status. This signal can then lead to the short-term modification of, for example, pathways supplying substrates to the mitochondria. Alternatively, longer-term modifications can occur through modifying redox-sensitive transcription factors that adjust the production of mitochondrial components. In addition, external signals may modify O$_2$•− production by the respiratory chain by post-translational modification. Alteration of the activity of mitochondrial peroxidases could also modulate H$_2$O$_2$ efflux from mitochondria to the rest of the cell. It is also possible that secondary redox signals, such as lipid peroxidation products derived from H$_2$O$_2$, could act as secondary redox signals.

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