BJ www.biochemj.org

BJ Energy

Biochem. J. (2009) **417**, 1–13 (Printed in Great Britain) doi:10.1042/BJ20081386

multimedia

REVIEW ARTICLE

How mitochondria produce reactive oxygen species

Michael P. MURPHY[1]

MRC Dunn Human Nutrition Unit, Hills Road, Cambridge CB2 0XY, U.K.

The production of ROS (reactive oxygen species) by mammalian mitochondria is important because it underlies oxidative damage in many pathologies and contributes to retrograde redox signalling from the organelle to the cytosol and nucleus. Superoxide ($O_2^{\bullet-}$) is the proximal mitochondrial ROS, and in the present review I outline the principles that govern $O_2^{\bullet-}$ production within the matrix of mammalian mitochondria. The flux of $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ production is far lower. The generation of $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ generation by mitochondria *in vivo* from $O_2^{\bullet-}$-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 $O_2^{\bullet-}$ and H_2O_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_2O_2 [4,11,12]. Later, it was confirmed that this H_2O_2 arose from the dismutation of superoxide ($O_2^{\bullet-}$) generated within mitochondria [13,14]. The parallel discovery that mitochondria contain their own SOD (superoxide dismutase), MnSOD, confirmed the biological significance of mitochondrial $O_2^{\bullet-}$ 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, $O_2^{\bullet-}$, 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.

$O_2^{\bullet-}$, THE PROXIMAL MITOCHONDRIAL ROS

Within mitochondria, $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ is −160 mV at pH 7 [16], for a standard state of 1 M O_2. As the pK_a of $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$, E_h, will vary with the relative concentrations of O_2 and $O_2^{\bullet-}$:

$$E_h(\mathrm{mV}) = -160 + 61.5 \log_{10} \frac{[O_2]}{[O_2^{\bullet-}]} \quad (1)$$

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

Abbreviations used: CoQ, coenzyme Q; $CoQH_2$, reduced CoQ; DPI, diphenyleneiodonium; ETF, electron transfer flavoprotein; α-GPDH, α-glycerophosphate dehydrogenase; HIF-1, hypoxia-inducible factor-1; αKGDH, 2-oxoglutarate dehydrogenase (α-ketoglutarate dehydrogenase); PHD, prolyl hydroxylase; RET, reverse electron transport; ROS, reactive oxygen species; SMP, submitochondrial particle; SOD, superoxide dismutase.

[1] email mpm@mrc-dunn.cam.ac.uk

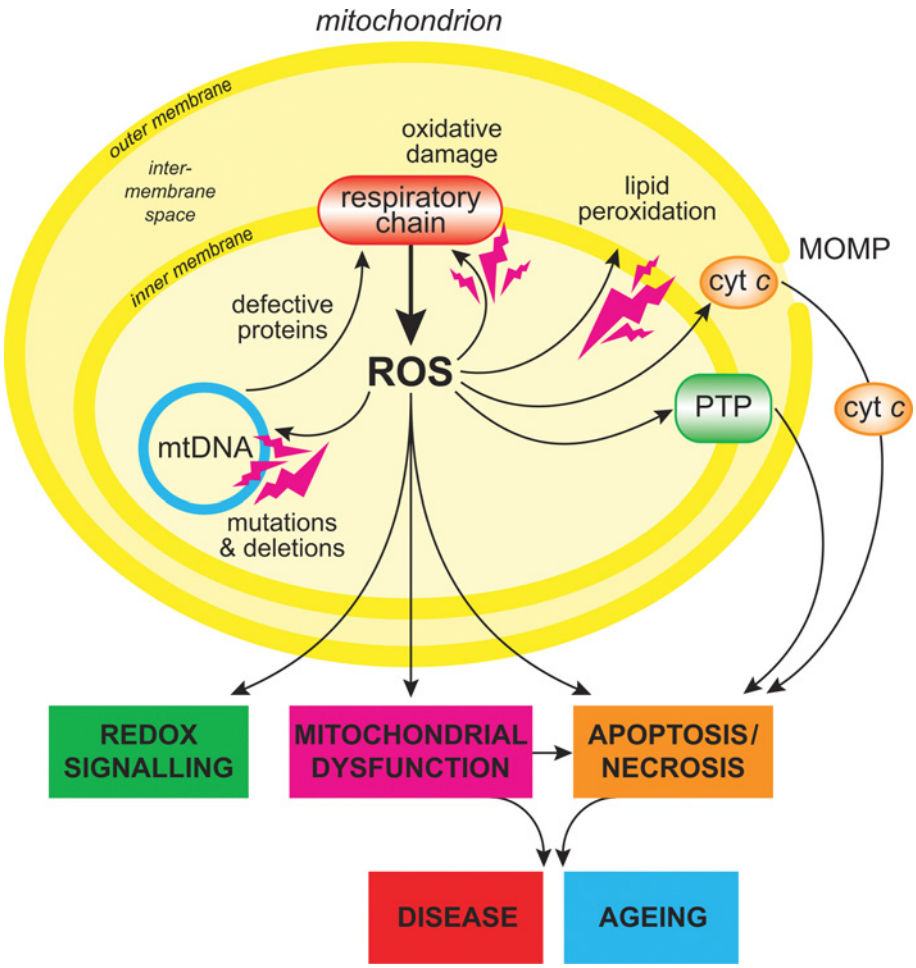

Figure 1 Overview of mitochondrial ROS production

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.

environment to the mitochondrion, owing to O_2 consumption by cytochrome oxidase [18,19]. Plausible estimates for the mitochondrial $[O_2]$ *in vivo* are in the range 3–30 μM [2]. The steady-state intramitochondrial $[O_2^{\bullet-}]$ is difficult to measure accurately, but it is likely to be very low because of micromolar concentrations of MnSOD in the mitochondrial matrix (e.g. 11 μM MnSOD in rat liver mitochondria [20]). This MnSOD will very rapidly ($k \approx 2.3 \times 10^9$ $M^{-1} \cdot s^{-1}$) catalyse dismutation of $O_2^{\bullet-}$ to H_2O_2 by a process that is first-order with respect to $[O_2^{\bullet-}]$ [2,4,5]. Furthermore, by acting as a very effective $O_2^{\bullet-}$ sink, MnSOD will favour the reaction of O_2 with electron donors to form $O_2^{\bullet-}$, in effect increasing the flux of electrons from electron donors to H_2O_2 [21,22]. Tentative estimates for $[O_2^{\bullet-}]$ within the mitochondrial matrix are in the range 10–200 pM [4,5]. By combining these with a plausible intramitochondrial $[O_2]$ of 25 μM [2], eqn (1) gives E_h in the range 150–230 mV for the reduction of O_2 to $O_2^{\bullet-}$. Even for a low $[O_2]$ of 1 μM and a high $[O_2^{\bullet-}]$ of 200 pM, the E_h value obtained is 68 mV.

Therefore, *in vivo*, the one-electron reduction of O_2 to $O_2^{\bullet-}$ is thermodynamically favoured, even by relatively oxidizing redox couples, 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$. Instead, mitochondrial $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ by these that determine mitochondrial $O_2^{\bullet-}$ production.

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

The factors determining the rate of $O_2^{\bullet-}$ 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 O_2 to form $O_2^{\bullet-}$. The second is the proportion (P_R) of this enzyme's electron carrier present in a redox form that can react with O_2. As many redox-active groups exist only transiently in a state that can react with O_2, P_R is a time-average. The remaining factors are the local $[O_2]$ and the second-order rate constant (k_E) for the reaction of that electron carrier with O_2 to form $O_2^{\bullet-}$. For a given enzyme or protein (E), the rate of $O_2^{\bullet-}$ production is:

$$\left(\frac{d[O_2^{\bullet-}]}{dt}\right)_E = k_E[O_2]P_R[E] \quad (2)$$

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:

$$\left(\frac{d[O_2^{\bullet-}]}{dt}\right)_{Total} = [O_2]\sum_i (k_E P_R[E])_i \quad (3)$$

The concentration of the enzyme responsible for $O_2^{\bullet-}$ 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 K_m of cytochrome oxidase for O_2 is very low ($<1\ \mu$M [25]), changes in $[O_2]$ should have little direct effect on mitochondrial function and instead are most likely to interact by affecting $O_2^{\bullet-}$ production. The generation of $O_2^{\bullet-}$ or H_2O_2 by isolated respiratory complexes, SMPs (submitochondrial particles) or mitochondria increases when $[O_2]$ is raised above the normal atmospheric level of 21 % O_2, and this increase is roughly proportional to $[O_2]$, at least over the lower range of supraphysiological $[O_2]$ [4,12,26–28]. Fewer studies have looked at the effects of decreasing $[O_2]$, but $O_2^{\bullet-}$ production by isolated complex I decreases linearly with lowered $[O_2]$ [28], and there is a decrease in H_2O_2 production by isolated mitochondria when $[O_2]$ is lowered below that of air-saturated medium [27,29,30]. However, in one detailed study, the rate of H_2O_2 production did not change as $[O_2]$ was lowered from $\sim 200\ \mu$M to $\sim 5\ \mu$M and only decreased when $[O_2]$ 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 H_2O_2 production or whether it reflects a genuine phenomenon.

The dependence of mitochondrial H_2O_2 production on $[O_2]$ is likely to be an important factor in the variation of ROS production *in vivo* because extracellular $[O_2]$ varies with physiological state, and there are $[O_2]$ gradients from the circulation to the mitochondria where O_2 consumption by cytochrome oxidase decreases $[O_2]$ locally [18,19]. Thus increasing or decreasing the rate of O_2 consumption by mitochondria may be an important aspect for modifying $O_2^{\bullet-}$ production *in vivo* by altering the local $[O_2]$ [19]. Furthermore, as O_2 is approx. 3-fold more soluble within membranes than in water [31], O_2 may be concentrated close to electron carriers within the membrane. Physiological levels of nitric oxide ($NO^{\bullet}$) compete with O_2 for cytochrome oxidase when $[O_2]$ is low, effectively raising the apparent K_m of this enzyme [32–35], and may thus alter the local $[O_2]$ around mitochondria, leading to changes in $O_2^{\bullet-}$ production [36–38].

Probably the most important factor that determines $O_2^{\bullet-}$ production by mitochondria is the proportion, P_R, of a given electron carrier that is reactive with O_2 to form $O_2^{\bullet-}$, as P_R responds rapidly to a range of biological situations. The relationship between P_R 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 O_2 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, P_R will be affected by changes in the carrier's E_m 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 P_R by affecting these parameters, with changes in Δp (protonmotive force) likely to be particularly important as it significantly affects the P_R of electron carriers and it varies rapidly in response to changes in mitochondrial ATP synthesis.

The final factor affecting the rate of $O_2^{\bullet-}$ production by electron carriers within proteins is the second-order rate constant (k_E) of their reaction with O_2. The reaction between protein-bound electron carriers and O_2 to form $O_2^{\bullet-}$ 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 O_2, and the rate is very dependent on the distance between O_2 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 O_2 to form $O_2^{\bullet-}$, with the bulk of the protein acting as an insulator to keep O_2 at a safe distance from the carriers and thereby minimize $O_2^{\bullet-}$ production [41]. Consequently, $O_2^{\bullet-}$ production will probably occur at sites where O_2 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 O_2 has been investigated for flavoenzymes that activate O_2, where the reduction of O_2 to $O_2^{\bullet-}$ is often a precursor to further reactions [42]. For the flavoenzyme glucose oxidase, the rate of O_2 reduction to H_2O_2 is $\sim 10^6\ M^{-1} \cdot s^{-1}$ [39], and, as the rate-limiting step is O_2 reduction to $O_2^{\bullet-}$ [39], this indicates that $O_2^{\bullet-}$ production by protein-bound electron carriers can be rapid. Of course, the production of H_2O_2 is a consequence of the normal physiological function of glucose oxidase, and for other flavoproteins, where electron transfer to O_2 to form $O_2^{\bullet-}$ may be a side reaction, the rate varies over five orders of magnitude [43]. Even so, the rate of reduction of O_2 by the reduced FMN of complex I to form $O_2^{\bullet-}$ is $\sim 40\ O_2^{\bullet-} \cdot min^{-1}$ [28], corresponding to a second-order rate constant of $\sim 10^3\ M^{-1} \cdot s^{-1}$. Thus the second-order rate constants (k_E) for $O_2^{\bullet-}$ 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 O_2. Therefore alterations to a protein that enabled O_2 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 $O_2^{\bullet-}$ production, and potentially play a regulatory function. Such factors determining the second-order rate constant for the reaction of O_2 with protein electron carriers could determine mitochondrial $O_2^{\bullet-}$ production, but little is known about how k_E may be varied by different enzymes within mitochondria.

MEASUREMENT OF $O_2^{\bullet-}$ PRODUCTION BY ISOLATED MITOCHONDRIA

Although many purified mitochondrial proteins can be manipulated so as to produce $O_2^{\bullet-}$, the physiological relevance of this is limited. Therefore it is important to understand $O_2^{\bullet-}$ production

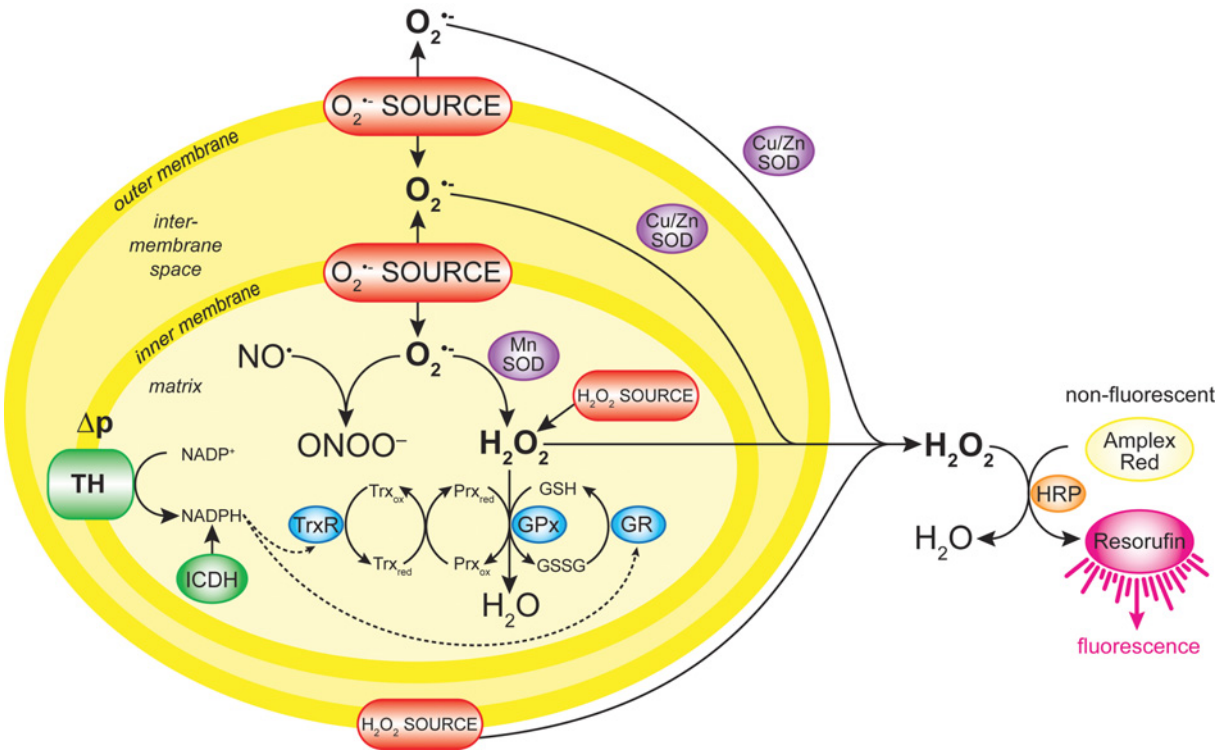

Figure 2 Measurement of H_2O_2 production by isolated mitochondria

The production of $O_2^{\bullet-}$ within the mitochondrial matrix, intermembrane space and outer membrane leads to the formation of H_2O_2 from SOD-catalysed dismutation. Some $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ within mitochondria is challenging due to its rapid dismutation in the presence of $\sim 10\ \mu$M MnSOD ($k \approx 2 \times 10^9\ M^{-1} \cdot s^{-1}$ [2,5]), which leads to very low steady-state matrix [$O_2^{\bullet-}$] and competes with $O_2^{\bullet-}$ detection systems [44]. {The spontaneous dismutation of $O_2^{\bullet-}$ ($k \approx 10^6$ [16]) is less important because it is second-order with respect to [$O_2^{\bullet-}$], which is very low in the presence of MnSOD.} Studies have been carried out on $O_2^{\bullet-}$ production by mitochondria lacking MnSOD [44], but there is always the concern that the absence of MnSOD may decrease the flux of $O_2^{\bullet-}$ by allowing the back reaction between $O_2^{\bullet-}$ and the electron donor [21,22], as well as having the potential to damage the system [45,46]. The use of $O_2^{\bullet-}$-sensitive dyes such as hydroethidine and MitoSOX™ [47–50], measurement of the reaction of $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ production. Even so, measurement is challenging, and the quantification of $O_2^{\bullet-}$ production by isolated mitochondria is not done routinely.

In contrast with the difficulties of assessing $O_2^{\bullet-}$ directly, intramitochondrial $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ is necessarily converted into H_2O_2 *in vivo*, as some $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ ($k \approx 10^{10}\ M^{-1} \cdot s^{-1}$) 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 $O_2^{\bullet-}$ formation is the sum of the H_2O_2 measured effluxing from the mitochondria, the $O_2^{\bullet-}$ sinks, the rate of H_2O_2 degradation to water, minus H_2O_2 production from outside mitochondria (eqn 4) (Figure 2):

$$\frac{1}{2}\left(\frac{d[O_2^{\bullet-}]}{dt}\right)_{Mito} = \left(\frac{d[H_2O_2]}{dt}\right)_{Measured} + \frac{1}{2}\left(\frac{d[O_2^{\bullet-}]}{dt}\right)_{Sink} + \left(\frac{d[H_2O_2]}{dt}\right)_{Degr} - \left(\frac{d[H_2O_2]}{dt}\right)_{Ext} \quad (4)$$

Consequently, there is a threshold of intramitochondrial $O_2^{\bullet-}$ formation before it exceeds scavenging by $O_2^{\bullet-}$ sinks and peroxidases, and generates sufficient H_2O_2 to be measured externally [1,61]. A corollary is that there may be significant $O_2^{\bullet-}$ formation within mitochondria that goes undetected, which is supported by the finding that H_2O_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_2O_2 [69–71], and thus the mitochondrial H_2O_2-degradation rate may vary considerably with condition and history of the organelle. Consistent with this, exposure of mitochondria to H_2O_2 leads to greater H_2O_2 efflux [27], and may be one way in which H_2O_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_2O_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 $O_2^{\bullet-}$ production and matrix H_2O_2 degradation. Finally, it is generally assumed that H_2O_2 permeates directly through the mitochondrial membrane; however, H_2O_2 diffusion across the plasma membrane is facilitated by aquaporins [76,77], and the possibility remains that H_2O_2 from mitochondria may actually be protein-mediated, perhaps leading to another level of control over H_2O_2 efflux. Therefore, although inferring mitochondrial $O_2^{\bullet-}$ production by measuring H_2O_2 efflux is robust and reliable, a number of points must be borne in mind when interpreting these data.

$O_2^{\bullet-}$ PRODUCTION WITHIN ISOLATED MITOCHONDRIA

Sufficient studies have been carried out on the efflux of H_2O_2 from isolated mitochondria to enable generalizations about the conditions that favour $O_2^{\bullet-}$ production and the mitochondrial sources of $O_2^{\bullet-}$. There are two main modes of operation by isolated mitochondria that lead to extensive H_2O_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_2O_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 $O_2^{\bullet-}$ production under these three modes of mitochondrial operation and thereby infer where and how $O_2^{\bullet-}$ is produced *in vivo*.

Sites of $O_2^{\bullet-}$ 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

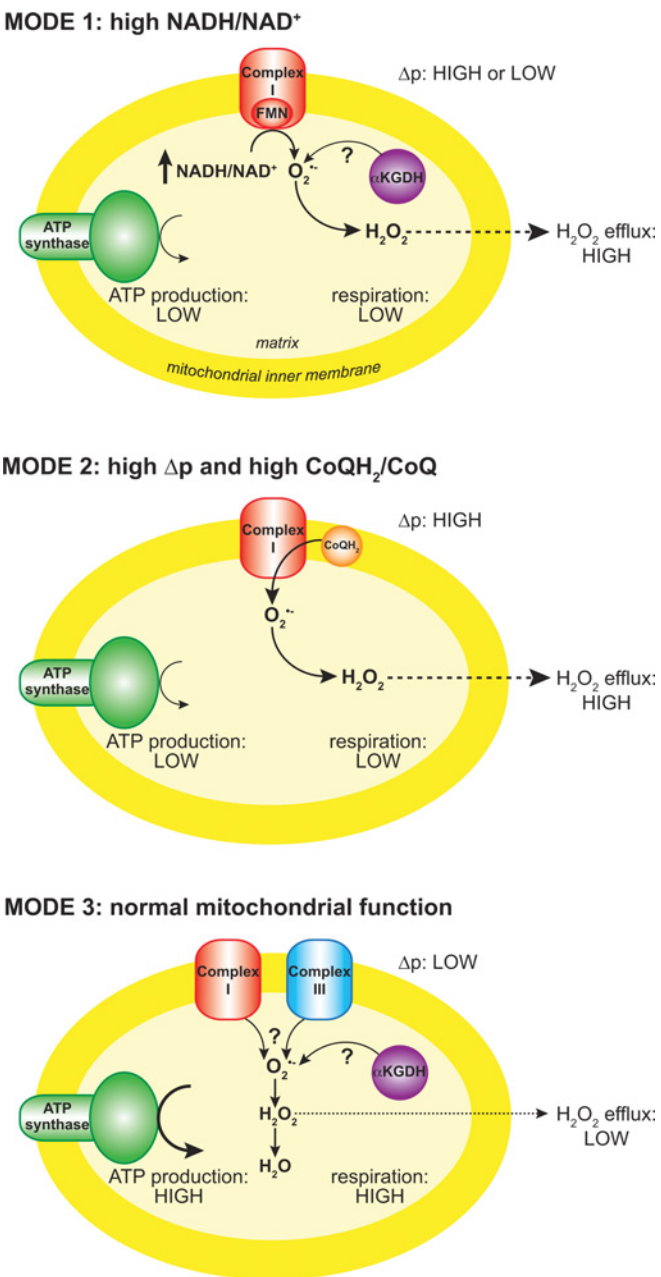

Figure 3 Modes of mitochondrial operation that lead to $O_2^{\bullet-}$ production

There are three modes of mitochondrial operation that are associated with $O_2^{\bullet-}$ production. In mode 1, the NADH pool is reduced, for example by damage to the respiratory chain, loss of cytochrome *c* during apoptosis or low ATP demand. This leads to a rate of $O_2^{\bullet-}$ formation at the FMN of complex I that is determined by the extent of FMN reduction which is in turn set by the NADH/NAD^+ ratio. Other sites such as αKGDH may also contribute. In mode 2, there is no ATP production and there is a high Δp and a reduced CoQ pool which leads to RET through complex I, producing large amounts of $O_2^{\bullet-}$. In mode 3, mitochondria are actively making ATP and consequently have a lower Δp than in mode 2 and a more oxidized NADH pool than in mode 1. Under these conditions, the flux of $O_2^{\bullet-}$ within mitochondria is far lower than in modes 1 and 2, and the $O_2^{\bullet-}$ sources are unclear.

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 O_2, so that O_2 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 O_2 [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 H_2O_2 production [83]. Subsequently, it was shown that isolated complex I in the presence of NADH produces $O_2^{\bullet-}$ and that this generation is enhanced by the inhibitor rotenone which binds to the CoQ-binding site [84]. The mechanism of $O_2^{\bullet-}$ production by isolated complex I is now reasonably well understood [28,85]. The isolated complex produces $O_2^{\bullet-}$ from the reaction of O_2 with the fully reduced FMN, and the proportion of the FMN that is fully reduced (P_R) is set by the NADH/NAD^+ ratio [28,85]. This model explains why inhibition of complex I with rotenone increases $O_2^{\bullet-}$ production, as it will lead to a back up of electrons onto FMN which will produce $O_2^{\bullet-}$ [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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ are produced from complex I [87]. An elegant demonstration of the importance of an elevated NADH/NAD^+ ratio for $O_2^{\bullet-}$ production from complex I is that oxidation of the NADH pool in mammalian mitochondria by expression of a yeast NADH dehydrogenase decreases $O_2^{\bullet-}$ production [91].

The other mechanism by which complex I produces large amounts of $O_2^{\bullet-}$ 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 $CoQH_2$ into complex I, and can reduce NAD^+ to NADH at the FMN site [92]. RET was first associated with H_2O_2 production in SMPs respiring on succinate [83,93]. Later, it was shown that isolated mitochondria respiring on succinate, also generate large amounts of $O_2^{\bullet-}$ from complex I by RET [94]. Since then it has become clear that there is extensive $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$-producing site(s) within complex I. Intriguingly, $O_2^{\bullet-}$ production by complex I is more sensitive to changes in the ΔpH (pH gradient) component of the Δp, than to the $\Delta\psi$ (membrane potential) component [79,96].

The complex I site producing $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ production is the same during RET as it is for $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ production than reduction of FMN alone, suggesting a role for the CoQ-binding site in RET $O_2^{\bullet-}$ production [95]. In addition, there is not a unique relationship between the mitochondrial NADH/NAD^+ ratio and $O_2^{\bullet-}$ production by complex I under different conditions [98]. This suggests that $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ generated during RET is not produced at the FMN. If $O_2^{\bullet-}$ 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 $CoQH_2$, while pumping four H^+ across the mitochondrial inner membrane by an unknown mechanism. The intriguing pH- and ΔpH-dependence of $O_2^{\bullet-}$ production during RET [79] may suggest that critical semiquinones and semiquinolates are formed during proton pumping that react directly with O_2 to form $O_2^{\bullet-}$ [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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ production during RET is not known, the rate of $O_2^{\bullet-}$ 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 Q_i and Q_o sites [99]. Complex III has for a long time been regarded as a source of $O_2^{\bullet-}$ within mitochondria [84,100]. When supplied with $CoQH_2$ and when the Q_i site is inhibited by antimycin, complex III produces large amounts of $O_2^{\bullet-}$ from the reaction of O_2 with a ubisemiquinone bound to the Q_o site [84,89,100–103]. This $O_2^{\bullet-}$ is released from complex III to both sides of the inner membrane [24,27,52,104]. However, in the absence of antimycin, the Q_o site ubisemiquinone is not stabilized and $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ production by complex III [100], therefore reduction of the CoQ pool is not sufficient to generate $O_2^{\bullet-}$ at complex III. It may be that high Δp stabilizes the Q_o site ubisemiquinone; however, the very high production of $O_2^{\bullet-}$ by complex I during RET in the presence of succinate is abolished completely by rotenone [87,95]. Under these conditions, there is a large Δp and a reduced CoQ pool, suggesting that the maximal $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ production by complex I is low, the contribution of complex III to the overall $O_2^{\bullet-}$ flux may then become relatively significant.

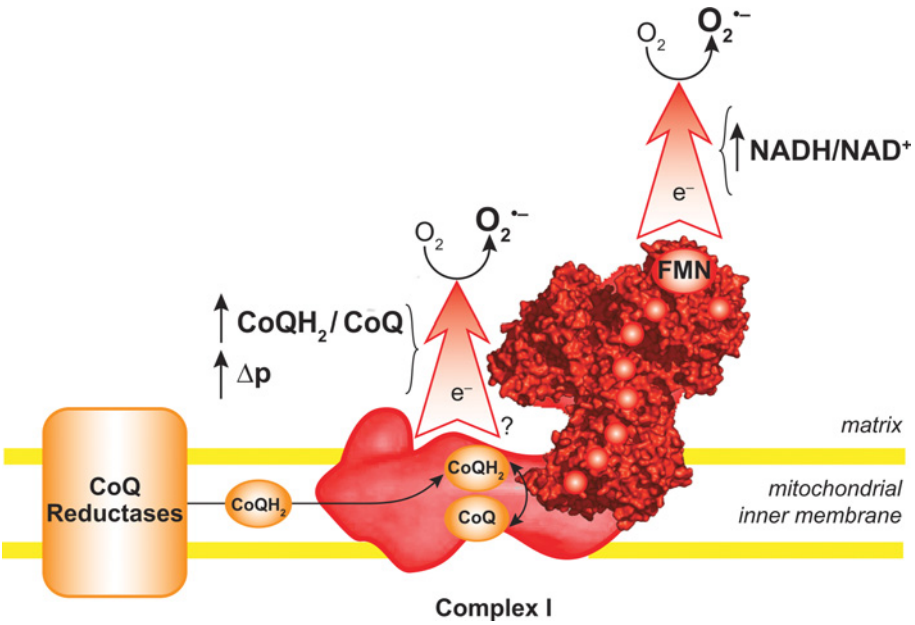

Figure 4 Production of $O_2^{\bullet-}$ by complex I

The cartoon of complex I is a chimaera modelled on the hydrophobic arm of *Yarrowia lipolytica* obtained by electron microscopy [132] and the crystal structure of the hydrophilic arm from *Thermus thermophilus* [82]. The location of the FMN and the FeS centres in the water-soluble arm are indicated, along with the putative CoQ-binding site. In mode 1, there is extensive $O_2^{\bullet-}$ production from the FMN in response to a reduced NADH pool. In mode 2, a high Δp and a reduced CoQ pool lead to RET and a high flux of $O_2^{\bullet-}$ from the complex. The site of this $O_2^{\bullet-}$ production is uncertain, hence the question mark, but may be associated with the CoQ-binding site(s).

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 $O_2^{\bullet-}$ production. Therefore, although complex III can be induced to produce $O_2^{\bullet-}$ with the inhibitor antimycin, its production in mitochondria under physiological conditions is far lower and is negligible compared with the maximum rates of $O_2^{\bullet-}$ production from complex I.

Other sites of $O_2^{\bullet-}$ production within mitochondria

Many other sites within mitochondria may also produce $O_2^{\bullet-}$ or H_2O_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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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, $O_2^{\bullet-}$ production from complex I is negligible and the contribution from sites such as αKGDH is proportionally more significant [106].

Many potential sites of $O_2^{\bullet-}$ 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_2O_2 production [12,90], primarily from RET at complex I; however, ETF:CoQ reductase may itself produce ROS [24]. Dihydro-orotate 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 $O_2^{\bullet-}$ production [110]; however, it is uncertain whether $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ or H_2O_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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ production by isolated mitochondria

It is possible to draw some tentative conclusions about $O_2^{\bullet-}$ production by isolated mitochondria. Two modes of operation lead mitochondria to produce large amounts of $O_2^{\bullet-}$: 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 $O_2^{\bullet-}$ production is complex I, in keeping with a growing view that complex I is the major source of $O_2^{\bullet-}$ within mitochondria *in vivo* [1,7,85,118]. Even so, the accumulation of NADH that occurs in modes 1 and 2 may lead to $O_2^{\bullet-}$ production from other sites such as αKGDH. The mechanisms of $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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 $NO^{\bullet}$. Treatments that oxidize the NADH pool, such as overexpressing NADH oxidases, should decrease the production of $O_2^{\bullet-}$ in mode 1, while rotenone and other respiratory inhibitors should increase it (antimycin would not be diagnostic here as it will increase $O_2^{\bullet-}$ production at complex III). Whether mode 2 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ production by RET and greatly decrease $O_2^{\bullet-}$ production at the FMN of complex I, making H_2O_2 efflux from mitochondria negligible compared with modes 1 or 2 (e.g. [4,78,89,119]). However, there may still be $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ production may account for most of the overall exposure of mitochondria to $O_2^{\bullet-}$. Thus, even though the rate of $O_2^{\bullet-}$ 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_2O_2 PRODUCTION BY MITOCHONDRIA

Figure 3 gives a reasonable description of mitochondrial $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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_2O_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_2O_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_2O_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_o site [126]. Although ongoing work may refine details of this model, the question remains of how lowering $[O_2]$ can increase H_2O_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 $O_2^{\bullet-}$ production directly, for example by altering the stability of the ubisemiquinone radical at complex III, the rate of degradation or release of H_2O_2 from mitochondria or the sidedness of $O_2^{\bullet-}$ 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 $[NO^{\bullet}]$ might modulate the response of cytochrome oxidase to low $[O_2]$ [127], thereby altering the P_R 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

mitochondrial H_2O_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 $O_2^{\bullet-}$ DO MAMMALIAN MITOCHONDRIA PRODUCE *IN VIVO*?

To investigate the significance of mitochondrial $O_2^{\bullet-}$ production in oxidative damage and redox signalling, it is necessary to know how much $O_2^{\bullet-}$ is generated by mitochondria *in vivo*. The greatest rate of H_2O_2 production by isolated mitochondria occurs during mode 2 when mitochondria have a high Δp, 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 $O_2^{\bullet-}$ [1,4,27]. Since it was first published by Chance and colleagues [4,12], this value of 1–2 % of respiration going to $O_2^{\bullet-}$ has propagated through the literature and has been used erroneously to estimate mitochondrial $O_2^{\bullet-}$ 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_2O_2 production by isolated mitochondria to the *in vivo* situation invalid. First, maximal $O_2^{\bullet-}$ 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_2O_2 [57], while use of the physiological substrate palmitoyl-CoA decreases H_2O_2 production to approx. 0.15 % of respiration [24], and, when glutamate/malate are used as substrates, H_2O_2 production accounts for approx. 0.12 % of respiration [27]. Secondly, measurements on isolated mitochondria are generally made using air-saturated medium containing $\sim$200 μM O_2. As mitochondrial $O_2^{\bullet-}$ production is probably proportional to $[O_2]$ and the physiological $[O_2]$ around mitochondria is approx. 10–50 μM, $O_2^{\bullet-}$ 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* $O_2^{\bullet-}$ 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 Δp and relatively oxidized NADH and CoQ pools. Consequently, their rates of H_2O_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 $O_2^{\bullet-}$ *in vitro*, these values cannot be extrapolated to the *in vivo* situation where mitochondrial $O_2^{\bullet-}$ production will be far, far lower.

Allowing for these caveats, can we estimate roughly how much $O_2^{\bullet-}$ 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 Δp 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ that leads to this damage.

Although estimates of mitochondrial $O_2^{\bullet-}$ production as a proportion of respiration rate are not possible, can we infer from studies of mitochondria *ex vivo* the extent of $O_2^{\bullet-}$ production *in vivo*? The maximum $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ production rate depends so closely on factors such as Δp 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ production *in vivo*. A classic estimate of H_2O_2 production was made in perfused liver where H_2O_2 production by the whole organ was measured from changes in catalase compound I [129]. Approx. 80 nmol of H_2O_2/min per g of wet weight was produced in the tissue [127], with about 12 nmol of H_2O_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_2O_2 production *in situ* was the same as for isolated mitochondria respiring on succinate, when they would have been producing H_2O_2 by RET [90]. Therefore we know little about the actual flux of $O_2^{\bullet-}$ within mitochondria *in vivo*, about how it changes under different physiological circumstance, or about its quantititive 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 $O_2^{\bullet-}$ production within isolated mitochondria and have shown that there are two modes of high $O_2^{\bullet-}$ production, predominantly, but not exclusively, from complex I: by RET when the Δp is high and the CoQ pool is reduced, and from the FMN when the NADH/NAD^+ ratio is high (Figure 3). When mitochondria are actively making ATP, the rate of $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ production *in vivo*. Even so, the description given here of the factors underlying mitochondrial $O_2^{\bullet-}$ 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_2O_2 that 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_2O_2 efflux could act as a retrograde signal to the cell, reporting on mitochondrial Δp or the redox state of the NADH pool, and thus enable

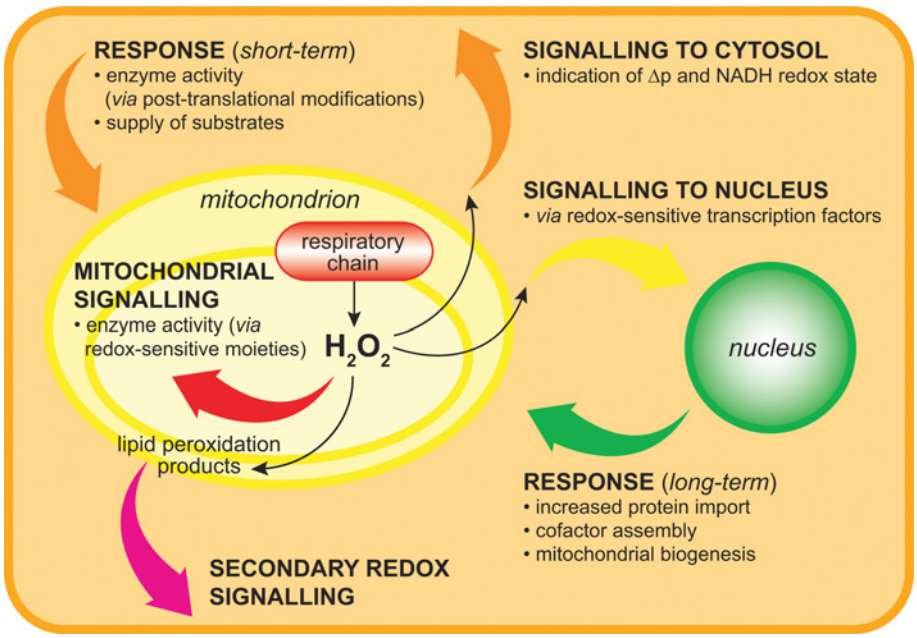

Figure 5 Possible mechanisms of mitochondrial redox signalling

The production of H_2O_2 from mitochondria is a potential redox signal. H_2O_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_2O_2 production from mitochondria will depend on factors such as Δp 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 $O_2^{\bullet-}$ production by the respiratory chain by post-translational modification. Alteration of the activity of mitochondrial peroxidases could also modulate H_2O_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_2O_2, could act as secondary redox signals.

the activity, transcription, translation, import or degradation of mitochondrial components to be adjusted accordingly. It is also likely that external signals can alter $O_2^{\bullet-}$ production through the post-translational modification of the respiratory chain, or by altering H_2O_2 efflux from mitochondria by modulating the activity of matrix peroxidases such as peroxiredoxins. However, it is clear that to address these vital issues, better methods are required to assess mitochondrial Δp and the reduction potential of critical redox couples within mitochondria *in vivo*, and thus enable the tendency of mitochondria to produce $O_2^{\bullet-}$ to be inferred. Furthermore, techniques to measure the production of mitochondrial $O_2^{\bullet-}$ and H_2O_2 directly *in vivo* also need to be developed. Such innovations are essential to understand the role of mitochondrial $O_2^{\bullet-}$ production in redox signalling and in pathological oxidative damage.

ACKNOWLEDGEMENTS

I thank Martin Brand, Paul Brookes, Helena Cochemé, Judy Hirst, Thomas Hurd, Andrew James, Adrian Lambert, Paul Schumacker and Jan Trnka for helpful comments and discussions, and Helena Cochemé for producing the figures.

FUNDING

Work is supported by the Medical Research Council (U.K.).

REFERENCES

1 Andreyev, A. Y., Kushnareva, Y. E. and Starkov, A. A. (2005) Mitochondrial metabolism of reactive oxygen species. Biochemistry (Moscow) **70**, 200–214
2 Turrens, J. F. (2003) Mitochondrial formation of reactive oxygen species. J. Physiol. **552**, 335–344
3 Balaban, R. S., Nemoto, S. and Finkel, T. (2005) Mitochondria, oxidants, and aging. Cell **120**, 483–495
4 Chance, B., Sies, H. and Boveris, A. (1979) Hydroperoxide metabolism in mammalian organs. Physiol. Rev. **59**, 527–605
5 Cadenas, E. and Davies, K. J. (2000) Mitochondrial free radical generation, oxidative stress, and aging. Free Radical Biol. Med. **29**, 222–230
6 Raha, S. and Robinson, B. H. (2000) Mitochondria, oxygen free radicals, disease and ageing. Trends Biochem. **25**, 502–508
7 Adam-Vizi, V. and Chinopoulos, C. (2006) Bioenergetics and the formation of mitochondrial reactive oxygen species. Trends Pharmacol. Sci. **27**, 639–645
8 Muller, F. (2000) The nature and mechanism of superoxide production by the electron transport chain. J. Am. Aging Assoc. **23**, 227–253
9 Droge, W. (2002) Free radicals in the physiological control of cell function. Physiol. Rev. **82**, 47–95
10 Jensen, P. K. (1966) Antimycin-insensitive oxidation of succinate and reduced nicotinamide-adenine dinucleotide in electron-transport particles. I. pH dependency and hydrogen peroxide formation. Biochim. Biophys. Acta **122**, 157–166
11 Loschen, G., Flohe, L. and Chance, B. (1971) Respiratory chain linked H_2O_2 production in pigeon heart mitochondria. FEBS Lett. **18**, 261–264
12 Boveris, A. and Chance, B. (1973) The mitochondrial generation of hydrogen peroxide: general properties and effect of hyperbaric oxygen. Biochem. J. **134**, 707–716
13 Loschen, G., Azzi, A., Richter, C. and Flohe, L. (1974) Superoxide radicals as precursors of mitochondrial hydrogen peroxide. FEBS Lett. **42**, 68–72
14 Forman, H. J. and Kennedy, J. A. (1974) Role of superoxide radical in mitochondrial dehydrogenase reactions. Biochem. Biophys. Res. Commun. **60**, 1044–1050
15 Weisiger, R. A. and Fridovich, I. (1973) Superoxide dismutase: organelle specificity. J. Biol. Chem. **248**, 3582–3592
16 Sawyer, D. T. and Valentine, J. S. (1981) How super is superoxide? Acc. Chem. Res. **14**, 393–400
17 Reynafarje, B., Costa, L. E. and Lehninger, A. L. (1985) O_2 solubility in aqueous media determined by a kinetic method. Anal. Biochem. **145**, 406–418
18 Erecinska, M. and Silver, I. A. (2001) Tissue oxygen tension and brain sensitivity to hypoxia. Respir. Physiol. **128**, 263–276

19 Skulachev, V. P. (1996) Role of uncoupled and non-coupled oxidations in maintenance of safely low levels of oxygen and its one-electron reductants. Q. Rev. Biophys. **29**, 169–202

20 Tyler, D. D. (1975) Polarographic assay and intracellular distribution of superoxide dismutase in rat liver. Biochem. J. **147**, 493–504

21 Winterbourn, C. C., French, J. K. and Claridge, R. F. (1978) Superoxide dismutase as an inhibitor of reactions of semiquinone radicals. FEBS Lett. **94**, 269–272

22 Schafer, F. Q. and Buettner, G. R. (2001) Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radical Biol. Med. **30**, 1191–1212

23 Barja, G. (1999) Mitochondrial oxygen radical generation and leak: sites of production in states 4 and 3, organ specificity, and relation to aging and longevity. J. Bioenerg. Biomembr. **31**, 347–366

24 St-Pierre, J., Buckingham, J. A., Roebuck, S. J. and Brand, M. D. (2002) Topology of superoxide production from different sites in the mitochondrial electron transport chain. J. Biol. Chem. **277**, 44784–44790

25 Wikstrom, M., Krab, K. and Saraste, M. (1981) Cytochrome Oxidase: a Synthesis, Academic Press, London

26 Turrens, J. F., Freeman, B. A., Levitt, J. G. and Crapo, J. D. (1982) The effect of hyperoxia on superoxide production by lung submitochondrial particles. Arch. Biochem. Biophys. **217**, 401–410

27 Kudin, A. P., Bimpong-Buta, N. Y., Vielhaber, S., Elger, C. E. and Kunz, W. S. (2004) Characterization of superoxide-producing sites in isolated brain mitochondria. J. Biol. Chem. **279**, 4127–4135

28 Kussmaul, L. and Hirst, J. (2006) The mechanism of superoxide production by NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria. Proc. Natl. Acad. Sci. U.S.A. **103**, 7607–7612

29 Hoffman, D. L., Salter, J. D. and Brookes, P. S. (2007) Response of mitochondrial reactive oxygen species generation to steady-state oxygen tension: implications for hypoxic cell signaling. Am. J. Physiol. Heart Circ. Physiol. **292**, H101–H108

30 Alvarez, S., Valdez, L. B., Zaobornyj, T. and Boveris, A. (2003) Oxygen dependence of mitochondrial nitric oxide synthase activity. Biochem. Biophys. Res. Commun. **305**, 771–775

31 Shiva, S., Brookes, P. S., Patel, R. P., Anderson, P. G. and Darley-Usmar, V. M. (2001) Nitric oxide partitioning into mitochondrial membranes and the control of respiration at cytochrome *c* oxidase. Proc. Natl. Acad. Sci. U.S.A. **98**, 7212–7217

32 Brown, G. C. and Cooper, C. E. (1994) Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. FEBS Lett. **356**, 295–298

33 Brown, G. C. (1995) Nitric oxide regulates mitochondrial respiration and cell functions by inhibiting cytochrome oxidase. FEBS Lett. **369**, 136–139

34 Cleeter, M. J. W., Cooper, J. M., Darley-Usmar, V. M., Moncada, S. and Schapira, A. H. V. (1994) Reversible inhibition of cytochrome *c* oxidase, the terminal enzyme of mitochondrial respiratory chain, by nitric oxide. FEBS Lett. **345**, 50–54

35 Brown, G. C. (1999) Nitric oxide and mitochondrial respiration. Biochim. Biophys. Acta **1411**, 351–369

36 Mateo, J., García-Lecea, M., Cadenas, S., Hernández, C. and Moncada, S. (2003) Regulation of hypoxia-inducible factor-1α by nitric oxide through mitochondria-dependent and -independent pathways. Biochem J. **376**, 537–544

37 Hagen, T., Taylor, C. T., Lam, F. and Moncada, S. (2003) Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF1α. Science **302**, 1975–1978

38 Moncada, S. and Erusalimsky, J. D. (2002) Does nitric oxide modulate mitochondrial energy generation and apoptosis? Nat. Rev. Mol. Cell Biol. **3**, 214–220

39 Klinman, J. P. (2007) How do enzymes activate oxygen without inactivating themselves? Acc. Chem. Res. **40**, 325–333

40 Marcus, R. A. and Sutin, N. (1985) Electron transfers in chemistry and biology. Biochim. Biophys. Acta **811**, 265–322

41 Moser, C. C., Farid, T. A., Chobot, S. E. and Dutton, P. L. (2006) Electron tunneling chains of mitochondria. Biochim. Biophys. Acta **1757**, 1096–1109

42 Massey, V. (1994) Activation of molecular oxygen by flavins and flavoproteins. J. Biol. Chem. **269**, 22459–22462

43 Imlay, J. A. (1995) A metabolic enzyme that rapidly produces superoxide, fumarate reductase of *Escherichia coli*. J. Biol. Chem. **270**, 19767–19777

44 Cochemé, H. M. and Murphy, M. P. (2008) Complex I is the major site of mitochondrial superoxide production by paraquat. J. Biol. Chem. **283**, 1786–1798

45 Li, Y., Huang, T.-T., Carlson, E. J., Melov, S., Ursell, P. C., Olson, J. L., Noble, L. J., Yoshimura, M. P., Berger, C., Chan, P. H. et al. (1995) Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. Nat. Genet. **11**, 376–381

46 Lebovitz, R. M., Zhang, H., Vogel, H., Cartwright, J., Dionne, L., Lu, N., Huang, S. and Matzuk, M. M. (1996) Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. Proc. Natl. Acad. Sci. U.S.A. **93**, 9782–9787

47 Zhao, H., Joseph, J., Fales, H. M., Sokoloski, E. A., Levine, R. L., Vasquez-Vivar, J. and Kalyanaraman, B. (2005) Detection and characterization of the product of hydroethidine and intracellular superoxide by HPLC and limitations of fluorescence. Proc. Natl. Acad. Sci. U.S.A. **102**, 5727–5732

48 Robinson, K. M., Janes, M. S., Pehar, M., Monette, J. S., Ross, M. F., Hagen, T. M., Murphy, M. P. and Beckman, J. S. (2006) Selective fluorescent imaging of superoxide *in vivo* using ethidium-based probes. Proc. Natl. Acad. Sci. U.S.A. **103**, 15038–15043

49 Zielonka, J., Vasquez-Vivar, J. and Kalyanaraman, B. (2008) Detection of 2-hydroxyethidium in cellular systems: a unique marker product of superoxide and hydroethidine. Nat. Protoc. **3**, 8–21

50 Zielonka, J., Srinivasan, S., Hardy, M., Ouari, O., Lopez, M., Vasquez-Vivar, J., Avadhani, N. G. and Kalyanaraman, B. (2008) Cytochrome *c*-mediated oxidation of hydroethidine and mito-hydroethidine in mitochondria: identification of homo- and heterodimers. Free Radical Biol. Med. **44**, 835–846

51 Lucas, M. and Solano, F. (1992) Coelenterazine is a superoxide anion-sensitive chemiluminescent probe: its usefulness in the assay of respiratory burst in neutrophils. Anal. Biochem. **206**, 273–277

52 Han, D., Antunes, F., Canali, R., Rettori, D. and Cadenas, E. (2003) Voltage-dependent anion channels control the release of the superoxide anion from mitochondria to cytosol. J. Biol. Chem. **278**, 5557–5563

53 Gardner, P. R. (2002) Aconitase: sensitive target and measure of superoxide. Methods Enzmol. **349**, 9–23

54 Boveris, A. (1984) Determination of the production of superoxide radicals and hydrogen peroxide in mitochondria. Methods Enzymol. **105**, 429–435

55 Barja, G. (1999) Kinetic measurement of mitochondrial oxygen radical production. In Methods in Aging Research, pp. 533–548, CRC Press, Boca Raton

56 Sohal, R. S. (1991) Hydrogen peroxide production by mitochondria may be a biomarker of aging. Mech. Ageing Dev. **60**, 189–198

57 Hansford, R. G., Hogue, B. A. and Mildaziene, V. (1997) Dependence of H_2O_2 formation by rat heart mitochondria on substrate availability and donor age. J. Bioenerg. Biomembr. **29**, 89–95

58 Szabo, C., Ischiropoulos, H. and Radi, R. (2007) Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. Nat. Rev. **6**, 662–680

59 Packer, M. A., Porteous, C. M. and Murphy, M. P. (1996) Mitochondrial superoxide production in the presence of nitric oxide leads to the formation of peroxynitrite. Biochem. Mol. Biol. Int. **40**, 527–534

60 Giorgio, M., Migliaccio, E., Orsini, F., Paolucci, D., Moroni, M., Contursi, C., Pelliccia, G., Luzi, L., Minucci, S., Marcaccio, M. et al. (2005) Electron transfer between cytochrome *c* and $p66^{Shc}$ generates reactive oxygen species that trigger mitochondrial apoptosis. Cell **122**, 221–233

61 Zoccarato, F., Cavallini, L. and Alexandre, A. (2004) Respiration-dependent removal of exogenous H_2O_2 in brain mitochondria: inhibition by Ca^{2+}. J. Biol. Chem. **279**, 4166–4174

62 Rhee, S. G., Yang, K. S., Kang, S. W., Woo, H. A. and Chang, T. S. (2005) Controlled elimination of intracellular H_2O_2: regulation of peroxiredoxin, catalase, and glutathione peroxidase via post-translational modification. Antioxid. Redox Signaling **7**, 619–626

63 Hurd, T. R., Costa, N. J., Dahm, C. C., Beer, S. M., Brown, S. E., Filipovska, A. and Murphy, M. P. (2005) Glutathionylation of mitochondrial proteins. Antioxid. Redox Signaling **7**, 999–1010

64 Rhee, S. G., Kang, S. W., Chang, T. S., Jeong, W. and Kim, K. (2001) Peroxiredoxin, a novel family of peroxidases. IUBMB Life **52**, 35–41

65 Salvi, M., Battaglia, V., Brunati, A. M., La Rocca, N., Tibaldi, E., Pietrangeli, P., Marcocci, L., Mondovi, B., Rossi, C. A. and Toninello, A. (2007) Catalase takes part in rat liver mitochondria oxidative stress defense. J. Biol. Chem. **282**, 24407–24415

66 Radi, R., Turrens, J. F., Chang, L. Y., Bush, K. M., Crapo, J. D. and Freeman, B. A. (1991) Detection of catalase in rat heart mitochondria. J. Biol. Chem. **266**, 22028–22034

67 Imai, H. and Nakagawa, Y. (2003) Biological significance of phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) in mammalian cells. Free Radical Biol. Med. **34**, 145–169

68 Rigobello, M. P., Folda, A., Scutari, G. and Bindoli, A. (2005) The modulation of thiol redox state affects the production and metabolism of hydrogen peroxide by heart mitochondria. Arch. Biochem. Biophys. **441**, 112–122

69 Rhee, S. G., Kang, S. W., Jeong, W., Chang, T. S., Yang, K. S. and Woo, H. A. (2005) Intracellular messenger function of hydrogen peroxide and its regulation by peroxiredoxins. Curr. Opin. Cell Biol. **17**, 183–189

70 Cox, A. G., Pullar, J. M., Hughes, G., Ledgerwood, E. C. and Hampton, M. B. (2008) Oxidation of mitochondrial peroxiredoxin 3 during the initiation of receptor-mediated apoptosis. Free Radical Biol. Med. **44**, 1001–1009

71 Hurd, T. R., Prime, T. A., Harbour, M. E., Lilley, K. S. and Murphy, M. P. (2007) Detection of reactive oxygen species-sensitive thiol proteins by redox difference gel electrophoresis: implications for mitochondrial redox signaling. J. Biol. Chem. **282**, 22040–22051

72 Holmgren, A. (1985) Thioredoxin. Annu. Rev. Biochem. **54**, 237–271
73 Hurd, T. R., Filipovska, A., Costa, N. J., Dahm, C. C. and Murphy, M. P. (2005) Disulphide formation on mitochondrial protein thiols. Biochem. Soc. Trans. **33**, 1390–1393
74 Rydström, J. (2006) Mitochondrial NADPH, transhydrogenase and disease. Biochim. Biophys. Acta **1757**, 721–726
75 Sazanov, L. A. and Jackson, J. B. (1994) Proton-translocating transhydrogenase and NAD- and NADP-linked isocitrate dehydrogenases operate in a substrate cycle which contributes to fine regulation of the tricarboxylic acid cycle activity in mitochondria. FEBS Lett. **344**, 109–116
76 Bienert, G. P., Moller, A. L., Kristiansen, K. A., Schulz, A., Moller, I. M., Schjoerring, J. K. and Jahn, T. P. (2007) Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. J. Biol. Chem. **282**, 1183–1192
77 Bienert, G. P., Schjoerring, J. K. and Jahn, T. P. (2006) Membrane transport of hydrogen peroxide. Biochim. Biophys. Acta **1758**, 994–1003
78 Korshunov, S. S., Skulachev, V. P. and Starkov, A. A. (1997) High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. FEBS Lett. **416**, 15–18
79 Lambert, A. J. and Brand, M. D. (2004) Superoxide production by NADH:ubiquinone oxidoreductase (complex I) depends on the pH gradient across the mitochondrial inner membrane. Biochem. J. **382**, 511–517
80 Hirst, J., Carroll, J., Fearnley, I. M., Shannon, R. J. and Walker, J. E. (2003) The nuclear encoded subunits of complex I from bovine heart mitochondria. Biochim. Biophys. Acta **1604**, 135–150
81 Sazanov, L. A. (2007) Respiratory complex I: mechanistic and structural insights provided by the crystal structure of the hydrophilic domain. Biochemistry **46**, 2275–2288
82 Sazanov, L. A. and Hinchliffe, P. (2006) Structure of the hydrophilic domain of respiratory complex I from *Thermus thermophilus*. Science **311**, 1430–1436
83 Hinkle, P. C., Butow, R. A., Racker, E. and Chance, B. (1967) Partial resolution of the enzymes catalyzing oxidative phosphorylation. XV. Reverse electron transfer in the flavin-cytochrome β region of the respiratory chain of beef heart submitochondrial particles. J. Biol. Chem. **242**, 5169–5173
84 Cadenas, E., Boveris, A., Ragan, C. I. and Stoppani, A. O. (1977) Production of superoxide radicals and hydrogen peroxide by NADH–ubiquinone reductase and ubiquinol–cytochrome *c* reductase from beef-heart mitochondria. Arch. Biochem. Biophys. **180**, 248–257
85 Hirst, J., King, M. S. and Pryde, K. R. (2008) The production of reactive oxygen species by complex I. Biochem. Soc. Trans. **36**, 976–980
86 Takeshige, K. and Minakami, S. (1979) NADH- and NADPH-dependent formation of superoxide anions by bovine heart submitochondrial particles and NADH–ubiquinone reductase preparation. Biochem. J. **180**, 129–135
87 Votyakova, T. V. and Reynolds, I. J. (2001) $\Delta\psi_m$-Dependent and -independent production of reactive oxygen species by rat brain mitochondria. J. Neurochem. **79**, 266–277
88 Kushnareva, Y., Murphy, A. N. and Andreyev, A. (2002) Complex I-mediated reactive oxygen species generation: modulation by cytochrome *c* and NAD(P)$^+$ oxidation–reduction state. Biochem. J. **368**, 545–553
89 Liu, Y., Fiskum, G. and Schubert, D. (2002) Generation of reactive oxygen species by the mitochondrial electron transport chain. J. Neurochem. **80**, 780–787
90 Boveris, A., Oshino, N. and Chance, B. (1972) The cellular production of hydrogen peroxide. Biochem. J. **128**, 617–630
91 Seo, B. B., Marella, M., Yagi, T. and Matsuno-Yagi, A. (2006) The single subunit NADH dehydrogenase reduces generation of reactive oxygen species from complex I. FEBS Lett. **580**, 6105–6108
92 Chance, B. and Hollunger, G. (1961) The interaction of energy and electron transfer reactions in mitochondria. I. General properties and nature of the products of succinate-linked reduction of pyridine nucleotide. J Biol. Chem. **236**, 1534–1543
93 Krishnamoorthy, G. and Hinkle, P. C. (1988) Studies on the electron transfer pathway, topography of iron–sulfur centers, and site of coupling in NADH-Q oxidoreductase. J. Biol. Chem. **263**, 17566–17575
94 Cino, M. and Del Maestro, R. F. (1989) Generation of hydrogen peroxide by brain mitochondria: the effect of reoxygenation following postdecapitative ischemia. Arch. Biochem. Biophys. **269**, 623–638
95 Lambert, A. J. and Brand, M. D. (2004) Inhibitors of the quinone-binding site allow rapid superoxide production from mitochondrial NADH:ubiquinone oxidoreductase (complex I). J. Biol. Chem. **279**, 39414–39420
96 Liu, S. S. (1997) Generating, partitioning, targeting and functioning of superoxide in mitochondria. Biosci. Rep. **17**, 259–272
97 Lambert, A. J., Buckingham, J. A., Boysen, H. M. and Brand, M. D. (2008) Diphenyleneiodonium acutely inhibits reactive oxygen species production by mitochondrial complex I during reverse, but not forward electron transport. Biochim. Biophys. Acta **1777**, 397–403
98 Lambert, A. J., Buckingham, J. A. and Brand, M. D. (2008) Dissociation of superoxide production by mitochondrial complex I from NAD(P)H redox state. FEBS Lett. **582**, 1711–1714
99 Iwata, S., Lee, J. W., Okada, K., Lee, J. K., Iwata, M., Rasmussen, B., Link, T. A., Ramaswamy, S. and Jap, B. K. (1998) Complete structure of the 11-subunit bovine mitochondrial cytochrome bc_1 complex. Science **281**, 64–71
100 Turrens, J. F., Alexandre, A. and Lehninger, A. L. (1985) Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. Arch. Biochem. Biophys. **237**, 408–414
101 Zhang, L., Yu, L. and Yu, C. A. (1998) Generation of superoxide anion by succinate–cytochrome *c* reductase from bovine heart mitochondria. J. Biol. Chem. **273**, 33972–33976
102 Rich, P. R. and Bonner, W. D. (1978) The sites of superoxide anion generation in higher plant mitochondria. Arch. Biochem. Biophys. **188**, 206–213
103 Grigolava, I. V., Ksenzenko, M., Konstantinob, A. A., Tikhonov, A. N. and Kerimov, T. M. (1980) Tiron as a spin-trap for superoxide radicals produced by the respiratory chain of submitochondrial particles. Biochemisty (Moscow) **45**, 75–82
104 Muller, F. L., Liu, Y. and Van Remmen, H. (2004) Complex III releases superoxide to both sides of the inner mitochondrial membrane. J. Biol. Chem. **279**, 49064–49073
105 Forman, H. J. and Azzi, A. (1997) On the virtual existence of superoxide anions in mitochondria: thoughts regarding its role in pathophysiology. FASEB J. **11**, 374–375
106 Starkov, A. A., Fiskum, G., Chinopoulos, C., Lorenzo, B. J., Browne, S. E., Patel, M. S. and Beal, M. F. (2004) Mitochondrial α-ketoglutarate dehydrogenase complex generates reactive oxygen species. J. Neurosci. **24**, 7779–7788
107 Tretter, L. and Adam-Vizi, V. (2004) Generation of reactive oxygen species in the reaction catalyzed by α-ketoglutarate dehydrogenase. J. Neurosci. **24**, 7771–7778
108 Bunik, V. I. and Sievers, C. (2002) Inactivation of the 2-oxo acid dehydrogenase complexes upon generation of intrinsic radical species. Eur. J. Biochem. **269**, 5004–5015
109 Eaton, S. (2002) Control of mitochondrial β-oxidation flux. Prog. Lipid Res. **41**, 197–239
110 Forman, H. J. and Kennedy, J. (1976) Dihydroorotate-dependent superoxide production in rat brain and liver: a function of the primary dehydrogenase. Arch. Biochem. Biophys. **173**, 219–224
111 Koza, R. A., Kozak, U. C., Brown, L. J., Leiter, E. H., MacDonald, M. J. and Kozak, L. P. (1996) Sequence and tissue-dependent RNA expression of mouse FAD-linked glycerol-3-phosphate dehydrogenase. Arch. Biochem. Biophys. **336**, 97–104
112 Drahota, Z., Chowdhury, S. K., Floryk, D., Mracek, T., Wilhelm, J., Rauchova, H., Lenaz, G. and Houstek, J. (2002) Glycerophosphate-dependent hydrogen peroxide production by brown adipose tissue mitochondria and its activation by ferricyanide. J. Bioenerg. Biomembr. **34**, 105–113
113 Tretter, L., Takacs, K., Hegedus, V. and Adam-Vizi, V. (2007) Characteristics of α-glycerophosphate-evoked H_2O_2 generation in brain mitochondria. J. Neurochem. **100**, 650–663
114 Miwa, S., St-Pierre, J., Partridge, L. and Brand, M. D. (2003) Superoxide and hydrogen peroxide production by Drosophila mitochondria. Free Radical Biol. Med. **35**, 938–948
115 Guzy, R. D., Sharma, B., Bell, E., Chandel, N. S. and Schumacker, P. T. (2008) Loss of the SdhB, but not the SdhA, subunit of complex II triggers reactive oxygen species-dependent hypoxia-inducible factor activation and tumorigenesis. Mol. Cell. Biol. **28**, 718–731
116 Hanukoglu, I. (2006) Antioxidant protective mechanisms against reactive oxygen species (ROS) generated by mitochondrial P450 systems in steroidogenic cells. Drug Metab. Rev. **38**, 171–196
117 Hanukoglu, I., Rapoport, R., Weiner, L. and Sklan, D. (1993) Electron leakage from the mitochondrial NADPH–adrenodoxin reductase–adrenodoxin–P450scc (cholesterol side chain cleavage) system. Arch. Biochem. Biophys. **305**, 489–498
118 Brand, M. D., Affourtit, C., Esteves, T. C., Green, K., Lambert, A. J., Miwa, S., Pakay, J. L. and Parker, N. (2004) Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. Free Radical Biol. Med. **37**, 755–767
119 Starkov, A. A. and Fiskum, G. (2003) Regulation of brain mitochondrial H_2O_2 production by membrane potential and NAD(P)H redox state. J. Neurochem. **86**, 1101–1107
120 Chandel, N. S., Maltepe, E., Goldwasser, E., Mathieu, C. E., Simon, M. C. and Schumacker, P. T. (1998) Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. Proc. Natl. Acad. Sci. U.S.A. **95**, 11715–11720

121 Guzy, R. D. and Schumacker, P. T. (2006) Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia. Exp. Physiol. **91**, 807–819
122 Schofield, C. J. and Ratcliffe, P. J. (2004) Oxygen sensing by HIF hydroxylases. Nat. Rev. Mol. Cell Biol. **5**, 343–354
123 Semenza, G. L. (2004) O_2-regulated gene expression: transcriptional control of cardiorespiratory physiology by HIF-1. J. Appl. Physiol. **96**, 1173–1177
124 Sanjuan-Pla, A., Cervera, A. M., Apostolova, N., Garcia-Bou, R., Victor, V. M., Murphy, M. P. and McCreath, K. J. (2005) A targeted antioxidant reveals the importance of mitochondrial reactive oxygen species in the hypoxic signaling of HIF-1α. FEBS Lett. **579**, 2669–2674
125 Guzy, R. D., Hoyos, B., Robin, E., Chen, H., Liu, L., Mansfield, K. D., Simon, M. C., Hammerling, U. and Schumacker, P. T. (2005) Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing. Cell Metab. **1**, 401–408
126 Bell, E. L., Klimova, T. A., Eisenbart, J., Moraes, C. T., Murphy, M. P., Budinger, G. R. and Chandel, N. S. (2007) The Q_o site of the mitochondrial complex III is required for the transduction of hypoxic signaling via reactive oxygen species production. J. Cell Biol. **177**, 1029–1036
127 Palacios-Callender, M., Quintero, M., Hollis, V. S., Springett, R. J. and Moncada, S. (2004) Endogenous NO regulates superoxide production at low oxygen concentrations by modifying the redox state of cytochrome *c* oxidase. Proc. Natl. Acad. Sci. U.S.A. **101**, 7630–7635
128 Beckman, K. B. and Ames, B. N. (1998) The free radical theory of aging matures. Physiol. Rev. **78**, 547–581
129 Oshino, N., Jamieson, D. and Chance, B. (1975) The properties of hydrogen peroxide production under hyperoxic and hypoxic conditions of perfused rat liver. Biochem. J. **146**, 53–65
130 Murphy, M. P. and Smith, R. A. (2007) Targeting antioxidants to mitochondria by conjugation to lipophilic cations. Annu. Rev. Pharmacol. Toxicol. **47**, 629–656
131 Hurd, T. R., Prime, T. A., Harbour, M. E., Lilley, K. S. and Murphy, M. P. (2007) Detection of reactive oxygen species-sensitive thiol proteins by redox difference gel electrophoresis: implications for mitochondrial redox signaling. J. Biol. Chem. **282**, 22040–22051
132 Radermacher, M., Ruiz, T., Clason, T., Benjamin, S., Brandt, U. and Zickermann, V. (2006) The three-dimensional structure of complex I from *Yarrowia lipolytica*: a highly dynamic enzyme. J. Struct. Biol. **154**, 269–279

Received 8 July 2008/13 August 2008; accepted 14 August 2008
Published on the Internet 12 December 2008, doi:10.1042/BJ20081386