The sites and topology of mitochondrial superoxide production

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Abstract

Mitochondrial superoxide production is an important source of reactive oxygen species in cells, and may cause or contribute to ageing and the diseases of ageing. Seven major sites of superoxide production in mammalian mitochondria are known and widely accepted. In descending order of maximum capacity they are the ubiquinone binding sites in complex I (site IQ) and complex III (site IIIQo), glycerol 3-phosphate dehydrogenase, the flavin in complex I (site IF), the electron transferring flavoprotein:Q oxidoreductase (ETFQOR) of fatty acid beta oxidation, and pyruvate and 2-oxoglutarate dehydrogenases. None of these sites is fully characterized and for some we only have sketchy information. The topology of the sites is important because it determines whether or not a site will produce superoxide in the mitochondrial matrix and be able to damage mitochondrial DNA. All sites produce superoxide in the matrix; site IIIQo and glycerol 3-phosphate dehydrogenase also produce superoxide to the intermembrane space. The relative contribution of each site to mitochondrial reactive oxygen species generation in the absence of electron transport inhibitors is unknown in isolated mitochondria, in cells or in vivo, and may vary considerably with species, tissue, substrate, energy demand and oxygen tension.

Keywords

Reactive oxygen species; ROS; electron transport; semiquinone; complex I; complex III; glycerol phosphate dehydrogenase; ETFQOR

1. Introduction

In the mitochondrial free radical theory of ageing (Harman, 1972), mitochondrial reactive oxygen species (ROS) generation is an inevitable consequence of oxidative ATP production, and the primary cause of macromolecular damage. Some damage is not repaired, causing progressive failure of cellular machinery, aging-related diseases, and ageing. There is considerable evidence both in favour and against the theory (Beckman and Ames, 1998; Finkel and Holbrook, 2000; Golden et al., 2002; Muller et al., 2007; Sanz et al., 2006). Most authors think that mitochondria-generated oxidative stress is important, particularly in ageing-related diseases, but is not the sole cause of ageing. According to a recent review (Muller et al., 2007), mitochondrial ROS convincingly determines lifespan in fungi (hyphal senescence in *Podospora* and chronological aging in *Saccharomyces*) and reasonably convincingly in

Caenorhabditis elegans. The case is tentative in *Drosophila* but inconclusive in mouse and human, and better tests are required.

Seven specific sites that are involved in ROS generation have been defined in isolated mammalian mitochondria using electron transport chain inhibitors (Andreyev et al., 2005; Brand et al., 2004; Jezek and Hlavata, 2005; Murphy, 2009; Raha and Robinson, 2000; Turrens, 2003), but we lack reliable measurements of their rates in the absence of such inhibitors, and there is no consensus on their relative importance. In cells our knowledge of which sites are important is even worse. It is based mostly on measurements using inhibitors of electron transport. Typically, if rotenone (a complex I inhibitor) raises ROS production in cells, endogenous ROS generation is inferred to be from complex I, but this inference is unjustifiable, and new approaches are needed.

2. The mitochondrial free radical theory of ageing

An association between mitochondrial ROS generation and age-related disease is generally accepted, although it is also agreed that the mitochondrial free radical theory's explanation of ageing is incomplete (Beckman and Ames, 1998; Finkel and Holbrook, 2000; Golden et al., 2002; Muller et al., 2007; Sanz et al., 2006). Since age is the primary risk factor for many diseases, understanding the mechanisms of ageing may allow us to significantly reduce the burden of disease and increase human healthspan. It is therefore crucial to fully understand mitochondrial ROS production to assess its role in age-related diseases and ageing, and ultimately to allow rational design of beneficial therapies.

Age-related disease can be caused by overproduction of superoxide, resulting in molecular damage. Mitochondrial superoxide is detoxified to H_2O_2 by superoxide dismutases (matrix Mn-SOD, cytosolic Cu/Zn-SOD), then to O_2 and H_2O by antioxidant defences, including catalase or glutathione peroxidase. However, these antioxidants are imperfect, and superoxide that evades them damages proteins, lipids and DNA. H_2O_2 is relatively unreactive, but in the presence of Fe(III) it forms reactive hydroxyl radicals, initiating membrane lipid peroxidation (Halliwell and Gutteridge, 1999). The products of sugar, protein and lipid oxidation cause secondary damage to proteins (Shigenaga et al., 1994; Sohal and Weindruch, 1996). Thus, mitochondrial superoxide causes 'oxidative stress'. The toxicity of matrix superoxide is shown by Mn-SOD knockout mice, which live only 10–20 days even in the presence of antioxidants (Lebovitz et al., 1996; Li et al., 1995). In contrast, cytosolic superoxide is not lethal in Cu/ZnSOD knockout mice (although they are sensitive to oxidative stress (Ho et al., 1998)) showing that extramitochondrial superoxide is less toxic. Many pathologies are related to oxidative stress, including atherosclerosis, hypertension, ischemia-reperfusion, inflammation, cancer, diabetes, Parkinson's and Alzheimer's disease (Halliwell and Gutteridge, 1999).

In ageing, there is good evidence for the importance of mitochondrial ROS generation (Cadenas and Davies, 2000). Beckman and Ames (1998) discuss 14 lines of evidence for the free radical theory, most implicating mitochondrial ROS. However, many are correlative and do not prove causality. One intriguing inverse correlation, between maximum lifespan and mitochondrial ROS production during reverse electron transport through complex I in different species (Barja et al., 1994; Ku et al., 1993; Lambert et al., 2007), is hard to explain if complex I ROS production does not contribute to ageing. More direct tests involve altering antioxidant defences, but give ambiguous results. Overexpression of Cu/Zn-SOD in *Drosophila* either has no effect (Seto et al., 1990), or increases lifespan (Sun et al., 2002). SOD/catalase mimetics increase lifespan in *C.elegans* (Melov et al., 2000), but only under specific conditions (Keaney and Gems, 2003). Overexpression of antioxidant defences in mice does not generally increase lifespan (Perez et al., 2009). In principle, the best test is to show that decreasing mitochondrial ROS production slows ageing. A striking result from RNAi screens is that knockdown of most

mitochondrial electron transport proteins extends life in *C. elegans* (Dillin et al., 2002; Lee et al., 2003). However, because we do not know which sites in the electron transport chain produce the most ROS, it is hard to know how to alter ROS production without also altering ATP synthesis.

3. Mitochondria as a source of ROS

The respiratory chain produces superoxide when single electrons leak to O_2 as electron pairs flow down the chain (Chance et al., 1979). Interest in mitochondrial ROS began more than 40 years ago (Boveris and Chance, 1973; Boveris et al., 1972; Hinkle et al., 1967; Jensen, 1966) and has been well-reviewed (Andreyev et al., 2005; Brand et al., 2004; Jezek and Hlavata, 2005; Murphy, 2009; Raha and Robinson, 2000; Turrens, 2003). There are currently seven separate sites of mammalian mitochondrial ROS production that have been identified and widely accepted (Fig. 1) and these are discussed in more depth below. The sites with the greatest maximum capacities to produce ROS are at complex I (site IQ) and complex III (site IIIQo) (Barja, 1999; Chen et al., 2003; Kudin et al., 2004; Liu et al., 2002; Raha and Robinson, 2000; St-Pierre et al., 2002; Votyakova and Reynolds, 2001), although the other five sites also have significant maximum rates (Andreyev et al., 2005) (Fig. 1). The topology may be vital, given the location of mitochondrial DNA in the matrix and its susceptibility to oxidative damage. Complex I produces superoxide to the matrix, whereas complex III produces it to the matrix and intermembrane space at about equal rates under de-energized conditions (Fig. 1) (Han et al., 2003; Han et al., 2001; Miwa and Brand, 2005; Miwa et al., 2003; Muller et al., 2004; St-Pierre et al., 2002).

In isolated cells, or in vivo, it is thought that mitochondria produce significant amounts of ROS (Skulachev, 1996), although the evidence is not fully compelling. The main indirect evidence comes from the lifespan-shortening effect of Mn-SOD knockout in mice (Lebovitz et al., 1996; Li et al., 1995). Fluorescent probes such as dichlorodihydrofluorescein or dihydrorhodamine have been used in hundreds of studies to infer mitochondrial ROS production in cells, but suffer from interpretational problems (Wardman, 2007). Hydroethidine and its mitochondria-targeted form, mitoSOX, may be more reliable (Jekabsons and Nicholls, 2006; Johnson-Cadwell et al., 2007; Mukhopadhyay et al., 2007; Song et al., 2007).

The frequent assertion that 1–4% of mitochondrial O_2 consumption is diverted to ROS in vivo is wrong. It is based on the maximum rate of superoxide production from complex III in mitochondria in the presence of antimycin, at saturating substrate and O_2 (Chance et al., 1979). Less unrealistic conditions predict lower values, only 0.15% (Hansford et al., 1997; St-Pierre et al., 2002). Which of the specific sites of mitochondrial ROS production is most active in cells in the absence of inhibitors of the electron transport chain is unclear.

4. Measurement of ROS production in isolated mitochondria

Superoxide does not cross the mitochondrial inner membrane, so bulk phase assays using isolated mitochondria do not register matrix superoxide. However, matrix Mn-SOD converts most of the matrix superoxide to H_2O_2, which diffuses to the medium for assay. The standard assay of extramitochondrial H_2O_2 is to add horseradish peroxidase (to reduce H_2O_2 to H_2O) and a substrate (e.g. amplex red) that is oxidized to a fluorescent product (resorufin). The method underestimates matrix superoxide production if significant amounts evade Mn- SOD in the matrix, or if significant amounts of H_2O_2 are scavenged by peroxidases such as glutathione peroxidase or catalase. Early work used homovanillic acid for detection (Barja, 2002). However, this suffers from high background rates and low accuracy, and the native rates of superoxide production during oxidation of pyruvate and malate in skeletal muscle or heart mitochondria are at or below the detection limit (St-Pierre et al., 2002). Amplex red or amplex ultra red are better probes, giving a greater signal and low backgrounds, although it is still

necessary to control for fluorescence quenching and to calibrate carefully (Lambert et al., 2007; Lambert et al., 2008a; Lambert et al., 2008b). With amplex red, native rates of superoxide production of less than 0.1 $nmol.min^{-1}.(mg\ protein)^{-1}$ can be measured (Lambert et al., 2007). This compares to 3 to 6 $nmol.min^{-1}.(mg\ protein)^{-1}$ during reverse electron transport, or forward electron transport plus antimycin in mammalian heart or skeletal muscle mitochondria or Drosophila mitochondria (Lambert and Brand, 2004b; Lambert et al., 2008b; Miwa et al., 2003). Matrix aconitase can also be used to assay matrix superoxide under different conditions (Talbot et al., 2004). Aconitase has a labile active site Fe-S centre, and is sensitive to superoxide. If calibrated using known rates of superoxide production, e.g. from site IF, aconitase inactivation kinetics can report matrix ROS production in absolute units (Miwa and Brand, 2005).

5. Sites of mitochondrial ROS production

The relative importance of each site to total superoxide production in isolated mitochondria is contentious, partly because of different assays, different substrates and different sources of mitochondria. Most assays of superoxide production from defined sites measure maximal capacities for superoxide production, and the actual rate from each site in the absence of inhibitors is not known. During reverse electron transport from succinate to $NAD^{\pm}$, complex I can produce superoxide at high rates (Han et al., 2003; Hansford et al., 1997; Kushnareva et al., 2002; Lambert and Brand, 2004a, b; Liu et al., 2002; Turrens and Boveris, 1980; Votyakova and Reynolds, 2001), although the physiological relevance is unclear (Muller et al., 2008). During forward electron transport from NAD-linked substrates (which may be more physiological), mostmitochondria produce superoxide at high rates after addition of inhibitors such as rotenone (for complex I) (Han et al., 2003; Hansford et al., 1997; Lambert and Brand, 2004a, b; Liu et al., 2002; St-Pierre et al., 2002; Votyakova and Reynolds, 2001) or antimycin A (for complex III) (Chen et al., 2003; Gyulkhandanyan and Pennefather, 2004; Herrero and Barja, 1997; Kwong and Sohal, 1998; Liu et al., 2002; McLennan and Degli Esposti, 2000; Raha et al., 2000; St-Pierre et al., 2002; Turrens et al., 1985). Other physiologically-relevant substrates, such as fatty acids and glycerol 3-phosphate, may cause superoxide production from sites that are less active during pyruvate oxidation, such as ETF–Q oxidoreductase and glycerol 3-phosphate dehydrogenase.

5.1. ROS production at Complex I: centre IF

Several pathologies are associated with elevated production of superoxide by defective complex I (Raha and Robinson, 2000), and it is important to identify the sites of superoxide production within this large multi-subunit complex. The redox centres are flavin mononucleotide (FMN), eight Fe-S clusters (N1a, N1b, N2, N3, N4, N5, N6a and N6b), and Q. The complex has a hydrophobic arm and a matrix-protruding hydrophilic arm (containing FMN and the Fe-S clusters). The Q binding site is at the junction between the arms. How electron transfer is coupled to proton pumping is a major unresolved question (Sharma et al., 2009).

In isolated complex I, the fully reduced flavin (site IF) dominates superoxide production (Kussmaul and Hirst, 2006). In isolated mitochondria, substrates for forward electron transport that feed into complex I (e.g. pyruvate plus malate) give low rates of superoxide production (Gyulkhandanyan and Pennefather, 2004; Hansford et al., 1997; Kudin et al., 2004; Kushnareva et al., 2002; Kwong and Sohal, 1998; Lambert and Brand, 2004b; Liu et al., 2002; Votyakova and Reynolds, 2001). Under these 'physiological' conditions, site IF is relatively oxidized, and the balance between superoxide from this site and the remaining sites in the electron transport chain is currently unknown. Rotenone blocks the quinine-binding site of complex I, causing electrons to back up and fully reduce the upstream redox centres. This increases the low rate several-fold, suggesting that complex I has the capacity to make superoxide at significant rates

during forward electron transport from a site upstream of the rotenone block. This site is usually identified as site IF. However, it may also include matrix dehydrogenases (pyruvate dehydrogenase and oxoglutarate dehydrogenase) (Chinta et al., 2009; Gazaryan et al., 2002; Starkov et al., 2004; Tretter and Adam-Vizi, 2004). Site IF has been championed from inhibitor studies (Johnson et al., 2003; Liu et al., 2002; Young et al., 2002) and on thermodynamic grounds (Kudin et al., 2004). Fe-S clusters (Herrero and Barja, 2000), specifically N1a (Kushnareva et al., 2002) or N2 (Genova et al., 2001), have also been proposed. However, there is now general agreement that superoxide production from rotenone-inhibited mitochondria in the presence of substrates that reduce matrix NAD is primarily from site IF (and from upstream dehydrogenases).

5.2. ROS production at Complex I: centre IQ

The maximum rate of superoxide production by complex I in mitochondria varies considerably. Succinate or glycerol 3-phosphate give the highest rates (Gyulkhandanyan and Pennefather, 2004; Hansford et al., 1997; Kushnareva et al., 2002; Lambert and Brand, 2004b; Liu et al., 2002; Miwa et al., 2003; Ohnishi et al., 2005; Votyakova and Reynolds, 2001). These substrates reduce ubiquinone (Q) to QH_2 and generate a protonmotive force, driving electrons thermodynamically uphill through complex I to reduce $NAD^{\pm}$ to NADH. Rotenone (the complex I Q site inhibitor) markedly diminishes superoxide production (Gyulkhandanyan and Pennefather, 2004; Hansford et al., 1997; Kushnareva et al., 2002; Lambert and Brand, 2004b; Liu et al., 2002; Ohnishi et al., 2005; Votyakova and Reynolds, 2001), showing that large amounts of superoxide are generated by complex I during reverse electron transport from QH_2 to $NAD^{\pm}$. This superoxide appears to come from the quinine-binding site in complex I, site IQ. Maximum superoxide production from complex I during reverse electron transport is much faster than the maximum rate from the flavin during forward electron transport, so site IF has a lower maximum capacity and contributes little to the high rate during reverse electron transport (Fig. 1).

There is some controversy about the presence of two separate sites of superoxide production in complex I (sites IF and IQ) rather than a single site (Hirst et al., 2008; Lambert et al., 2008a; Lambert et al., 2008b), and more work needs to be done to fully resolve this issue. Complicating the analysis, some of the inhibitors used to identify sites in complex I are poorly characterized. They include 'FMN-specific' diphenyleneiodonium, Fe-S centre inhibitors such as *p*-chloromercuribenzoate and ethoxyformic anhydride, and a variety of Q site inhibitors (Degli Esposti, 1998). In our hands, (except for rotenone and piericidin) these inhibitors also uncouple and lack site-specificity, making them unreliable for identifying ROS sites. In our current model of superoxide production during reverse electron transport, the majority originates from site IQ. Site IF produces superoxide at modest rates that only dominate when site IQ is inactive and the flavin is fully reduced, such as during forward electron transport in the presence of rotenone and lack of a pH gradient (Lambert and Brand, 2004a). Site IQ is not normally very active during forward electron transport. It only generates ROS during forward electron transport when very specific conditions are met: the presence of both a large pH gradient and a specific inhibitor of the Q-binding site, such as myxothiazol, which may distort the site to allow ROS generation and mimic the state that occurs during reverse electron transport (Lambert and Brand, 2004a). Rotenone has a similar, but less marked, effect in the presence of a pH gradient. In the absence of a pH gradient it gives the same ROS production as stigmatellin, a complex III inhibitor, so ROS production during forward electron transport in the presence of rotenone or stigmatellin comes from site IF (plus upstream sites).

We have tested these assignments of IQ and IF in mitochondria. Previous evidence for the involvement of site IF during reverse electron transport, based on the use of a 'flavin-site specific' inhibitor, diphenyleneiodonium, is flawed because the inhibitor works not at site IF

but at site IQ in mitochondria in short-term incubations (Lambert et al., 2008a). We have also disproved suggestions that ROS production from complex I during reverse electron transport is from site IF (or upstream) and can be explained by excessive reduction of the flavin under these conditions – we find that the NAD(P)H pool (which reports the flavin redox state) during reverse electron transport is not more, but less reduced than during forward electron transport with rotenone (Lambert et al., 2008b).

These issues have raised the question of whether the major complex I ROS-producing site (IQ) is active in vivo. The answer is unknown, but is crucial to testing and fully formulating the mitochondrial free radical theory of ageing. Some in vitro evidence suggests that it may be active (Muller et al., 2008; St-Pierre et al., 2002). The strongest indirect evidence that the site is physiologically active is that the activity of site IQ is inversely correlated with longevity in mitochondria from different species (Barja et al., 1994; Ku et al., 1993) even when confounding effects of body mass are eliminated (Lambert et al., 2007). However, better and more direct evidence of the importance (or not) of site IQ, and indeed of all the mitochondrial sites, is required, firstly in mitochondria oxidizing 'physiological' substrates and secondly in cells.

The components of protonmotive force (particularly the pH gradient (Lambert and Brand, 2004b)) dramatically affect superoxide production by site IQ (Gyulkhandanyan and Pennefather, 2004; Hansford et al., 1997; Korshunov et al., 1997; Lambert and Brand, 2004a, b; Liu, 1997; Liu et al., 2002; Votyakova and Reynolds, 2001). Therefore mild uncoupling, which slightly lowers both components, greatly decreases the high superoxide production during reverse electron transport. Mild uncoupling also tends to oxidize other electron carriers and decrease the local oxygen tension, decreasing superoxide production from the other sites as well. This has led to concept of "uncoupling to survive", which proposes that lowering protonmotive force by mild uncoupling lowers superoxide production whilst allowing sufficient ATP production. This might lower oxidative damage and increase lifespan (Brand, 2000; Skulachev, 1996).

5.3. ROS production at Complex III: centre IIIQo

Superoxide is produced by the single electron reduction of O_2 by electron carriers. In complex III these are cytochromes b_{566}, b_{562} and c_1, the Rieske Fe-S centre, and the quinones at centres 'i' and 'o' (Zhang et al., 2000). The main reductant is the semiquinone at centre o (IIIQo) (Boveris and Chance, 1973; Cadenas et al., 1977; Turrens et al., 1985), consistent with a Q cycle mechanism (Turrens, 2003). In mitochondria, complex III produces superoxide at high rates only in the presence of antimycin (Chen et al., 2003; Gyulkhandanyan and Pennefather, 2004; Herrero and Barja, 1997; Kwong and Sohal, 1998; Liu et al., 2002; McLennan and Degli Esposti, 2000; Raha et al., 2000; St-Pierre et al., 2002; Turrens et al., 1985). This inhibitor blocks electron transfer from b_{566} to the quinone at centre i, causing a small build-up of semiquinone at centre o, which readily reduces O_2 to superoxide (Cape et al., 2007). Centre o inhibitors of complex III (e.g. myxothiazol or stigmatellin), stop electrons accessing centre o, and prevent these high rates (Ksenzenko et al., 1983; Raha et al., 2000; Turrens et al., 1985), suggesting that the main superoxide site at complex III is the semiquinone at centre o, with little from neighbouring sites, such as centre i, pool ubiquinone (QH_2/Q) or complex II (Miwa et al., 2003; St-Pierre et al., 2002). Over-reduction of site IIIQo decreases the concentration of the reactive semiquinone at centre o, and lowers superoxide production from this site below the maximum rate obtainable under slightly more oxidizing conditions (Drose and Brandt, 2008; Erecinska and Wilson, 1976).

The superoxide production rate from site IIIQo in coupled mitochondria oxidizing NAD-linked substrates in the absence of antimycin is unknown, because addition of myxothiazol or stigmatellin to block this site also indirectly perturbs the complex I sites by collapsing the protonmotive force and altering the redox state of the Q and NAD pools. Some authors propose

that site IIIQo is the main site under physiological conditions (Chen et al., 2003); others argue that its role is minor (Kushnareva et al., 2002; Lambert and Brand, 2004a; Liu et al., 2002; Votyakova and Reynolds, 2001). Definitive experiments are needed.

5.4. ROS production at other mitochondrial sites

Complex II mutations and consequent overproduction of ROS causes disease in humans (Rustin et al., 2002) and *C. elegans* (Senoo-Matsuda et al., 2001). Compared to the maximum rates of superoxide production at complexes I or III, rates at complex II (succinate dehydrogenase) are normally negligible (Chen et al., 2003; Gyulkhandanyan and Pennefather, 2004; Hansford et al., 1997; McLennan and Degli Esposti, 2000; Miwa et al., 2003; Turrens et al., 1985) (Fig. 1) because the enzyme suppresses the formation of a flavin radical in the active site. The rates during succinate oxidation by fumarate reductase, which catalyses the reverse reaction in *Escherichia coli* and lacks this mechanism, are much greater (Messner and Imlay, 2002).

Glycerol 3-phosphate dehydrogenase generates ROS in mitochondria (Drahota et al., 2002; Gyulkhandanyan and Pennefather, 2004; Kwong and Sohal, 1998; Miwa et al., 2003; Sekhar et al., 1987; Tretter et al., 2007). Topological measurements show that superoxide is produced to both sides of the membrane (Miwa et al., 2003). Since the crystal structure shows that the flavin is well outside the plane of the membrane (in the intermembrane space) (Yeh et al., 2008), the membrane-buried quinone-binding site of this dehydrogenase may be the main site of superoxide production. Further study on this site is needed.

During oxidation of fatty acids the electron transferring flavoprotein Q oxidoreductase (ETFQOR) may produce superoxide at significant rates to the matrix side of the membrane (St-Pierre et al., 2002), but this site remains poorly characterized.

ROS production from sites downstream of complex III (cytochrome *c*, complex IV) can be measured by difference when these sites are fully reduced by addition of cyanide (subtracting off measured rates from upstream); they make little contribution (Miwa et al., 2003).

Other mitochondrial sites can generate ROS (Andreyev et al., 2005), including 2- oxoglutarate and pyruvate dehydrogenases (Chinta et al., 2009; Gazaryan et al., 2002; Starkov et al., 2004; Tretter and Adam-Vizi, 2004) and non-mammalian NADH-Q oxidoreductases (Fang and Beattie, 2003). Their ROS production is poorly characterized and deserves further work.

6. Topology of mitochondrial superoxide production

The topology of ROS production is crucial for which sites might most damage mtDNA. In intact mitochondria superoxide that is produced to the matrix is converted by matrix Mn-SOD to H_2O_2, which diffuses out and is assayed in the extramitochondrial medium. However, superoxide that is produced to the intermembrane space requires added SOD to be fully converted to H_2O_2 for assay, so H_2O_2 production that is dependent on added SOD is produced to the intermembrane space not to the matrix. This assay accurately identifies external superoxide production from particular sites, and enables estimation of the topology of superoxide production, although the quantitative estimate of sidedness is crude because of effects of endogenous external dismutases and internal peroxidases (Miwa and Brand, 2005; Miwa et al., 2003; St-Pierre et al., 2002; Muller et al., 2004). Using this assay, the topology of superoxide production from each of the electron transport chain sites has been determined (Fig. 1). Because of their matrix localization, pyruvate dehydrogenase and oxoglutarate dehydrogenase are presumed to make superoxide exclusively in the matrix. All of the electron transport chain sites also produce superoxide exclusively to the matrix, except for complex III

and glycerol 3-phosphate dehydrogenase (Miwa and Brand, 2005; Miwa et al., 2003; St-Pierre et al., 2002).

The original view was that ROS production by complex III was directed towards the matrix, but the crystal structure (Iwata et al., 1998) showed that site IIIQo faces the cytoplasmic side of the membrane, making it unclear how this site could generate matrix superoxide. We found that ROS from IIIQo was generated partly outside and partly inside the mitochondria (St-Pierre et al., 2002). The same held in Drosophila mitochondria, where superoxide from both site IIIQo and glycerol 3-phosphate dehydrogenase was directed about equally to each side of the membrane (Miwa and Brand, 2005; Miwa et al., 2003). Approximately equal production of site IIIQo superoxide to each side of the membrane was also shown in an elegant study in mammalian mitochondria (Muller et al., 2004). The mechanism by which these sites generate superoxide to both sides of the membrane is not fully resolved, but it probably involves release of semiquinone or hydroperoxyl radicals within the membrane bilayer, followed by diffusion and reaction (with oxygen or by deprotonation, respectively) to form superoxide which spills out to both sides of the membrane (Muller et al., 2004).

7. ROS production by mitochondria in cells

Mitochondria are frequently asserted to be the main producers of ROS in cells (Skulachev, 1996), but this conclusion is not thoroughly established. In some cells, other sources, such as cytochrome P450 (Caro and Cederbaum, 2004) or plasma membrane NADPH oxidases (Babior et al., 2002), produce large amounts of ROS (Jezek and Hlavata, 2005), and it remains to be established what proportion of cellular ROS production is mitochondrial in different cells. Since mitochondrial ROS production may depend hyperbolically on oxygen tension, with different oxygen affinities at different sites (Hoffman and Brookes, 2009), the contribution of different sites may vary as oxygen tension in a cell rises and falls.

Two problems hinder identification of the specific mitochondrial sites of ROS production in cells. The first is the methods to quantify ROS production. Fluorescent probes such as dichlorodihydrofluorescein or dihydrorhodamine have interpretational problems – their specificity is often unclear and they may themselves cause ROS production (Wardman, 2007). Hydroethidine and mitoSOX may be more reliable (Jekabsons and Nicholls, 2006; Johnson-Cadwell et al., 2007; Mukhopadhyay et al., 2007; Song et al., 2007). The second is the use of electron transport inhibitors to identify sites of ROS production, which poses the same problems as with isolated mitochondria. It is unknown whether reverse electron transport and the associated high superoxide production occur in cells. Some studies (Aon et al., 2003; Chandel et al., 1998; Li and Trush, 1998; Parthasarathi et al., 2002; Quillet-Mary et al., 1997; Schuchmann and Heinemann, 2000; Vrablic et al., 2001; Zamzami et al., 1995) find that rotenone decreases cellular ROS formation, suggesting that site IQ (or IIIQo) is active in cells. Conversely, many studies report that rotenone increases ROS production in cells (Barrientos and Moraes, 1999; Li et al., 2003; Nakamura et al., 2001; Siraki et al., 2002). The latter result is often taken to imply the involvement of site IF in the cells, however, this conclusion is erroneous, because rotenone addition reduces site IF and increases ROS generation, regardless of whether it was sufficiently reduced to produce significant ROS before inhibition. Therefore, the observation of increased ROS production in cells following addition of rotenone cannot be used to infer the identity of the native mitochondrial site of ROS production, and other methods need to be developed.

8. Conclusion

Mitochondrial ROS production is clearly involved in ageing and the diseases of ageing, but its exact roles and importance remain ambiguous. We now know the identity of seven sites within

mitochondria that generate superoxide, and have a good idea of their topology and their relative maximum capacities (Fig. 1). What we do not know is the rate at which these sites run under different physiological conditions of substrate supply and energy demand in isolated mitochondria, in intact cells, and in vivo. Working out ways to make such measurements is an important future challenge, but very necessary if we are to fully understand mitochondrial ROS production and its role in ageing and disease.

Acknowledgments

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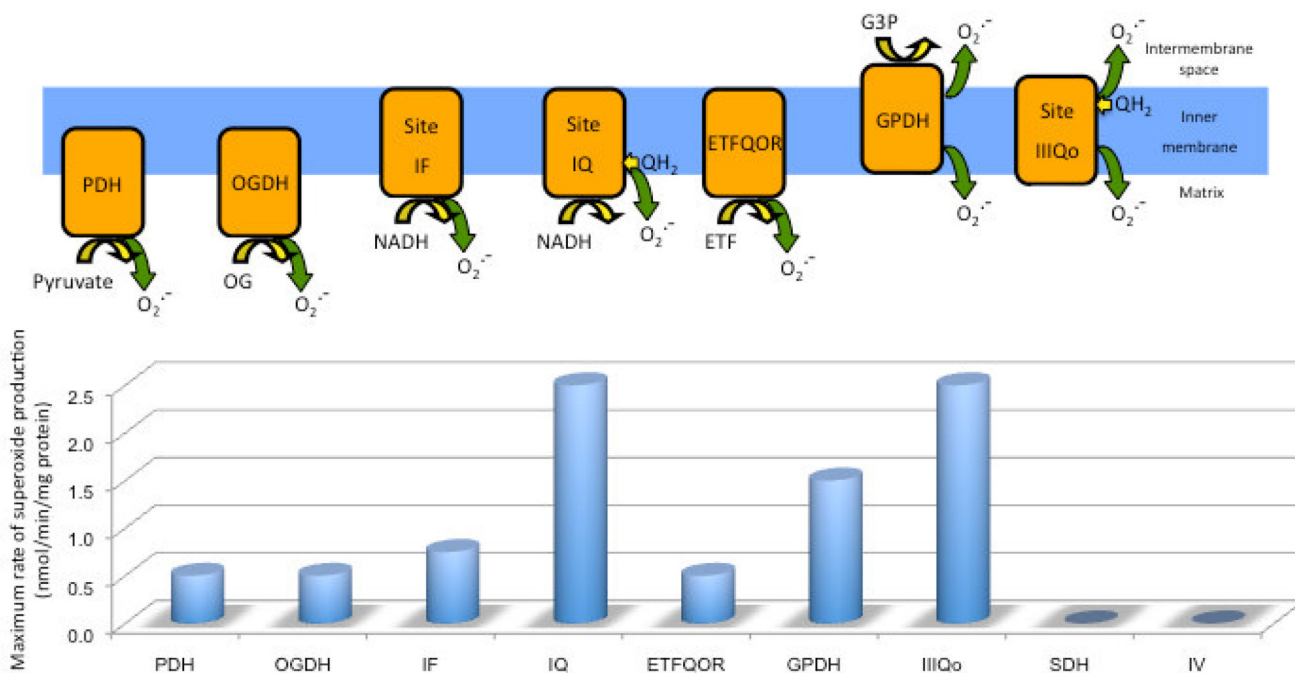
Figure 1. The sites and topology of mitochondrial superoxide production
The seven identified sites of superoxide production in mitochondria are shown in the upper diagram, with an indication of the location of their substrate-binding sites and the topology of their superoxide production. PDH, pyruvate dehydrogenase; OGDH, 2-oxoglutarate dehydrogenase; site IF, the FMN-containing NADH binding site of complex I; site IQ, ubiquinone reduction site of complex I; ETFQOR, electron transferring flavoprotein ubiquinone oxidoreductase – the entry point of electrons from flavin-linked beta-oxidation of fatty acids via ETF (electron transferring flavoprotein) to ubiquinone in the electron transport chain; GPDH, glycerol 3-phosphate dehydrogenase; site IIIQo, the outer quinone-binding site of the Q-cycle in complex III. PDH and OGDH may also produce hydrogen peroxide directly. The lower panel shows representative values of the maximum superoxide production rate from each site, and from SDH (succinate dehydrogenase; complex II) and IV (complex IV). Rates are for rat skeletal muscle mitochondria, from Lambert et al., 2007; Lambert and Brand, 2004a, b; Lambert et al., 2008a; Lambert et al., 2008b; St-Pierre et al., 2002 and Jason Treberg, Casey Quinlan and Martin D Brand (unpublished observations), and are greatest from sites IQ and IIIQo, followed by GPDH, then sites IF, EFTQOR, PDH and OGDH. There is insignificant superoxide production from complex II and complex IV (or from pool Q and cytochrome c, not shown).