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Chapter 11

Reactive Oxygen Species Production by Mitochondria

Adrian J. Lambert and Martin D. Brand

Abstract

Oxidative damage to cellular macromolecules is believed to underlie the development of many pathological states and aging. The agents responsible for this damage are generally thought to be reactive oxygen species, such as superoxide, hydrogen peroxide, and hydroxyl radical. The main source of reactive species production within most cells is the mitochondria. Within the mitochondria the primary reactive oxygen species produced is superoxide, most of which is converted to hydrogen peroxide by the action of superoxide dismutase. The production of superoxide by mitochondria has been localized to several enzymes of the electron transport chain, including Complexes I and III and glycerol-3-phosphate dehydrogenase. In this chapter the current consensus view of sites, rates, mechanisms, and topology of superoxide production by mitochondria is described. A brief overview of the methods for measuring reactive oxygen species production in isolated mitochondria and cells is also presented.

Key words: Superoxide, hydrogen peroxide, Complex I, Complex III, succinate, rotenone, antimycin, myxothiazol.

1. Introduction

The term "free radical" is used to denote any atom or molecule with an unpaired electron in its outermost shell. In biology, the term tends to have negative connotations, since free radicals are associated with high, indiscriminate reactivity that can lead to molecular damage. The free radical theory of aging is based on this premise *(1)*. The term reactive oxygen species (ROS) is usually used to signify any oxygen-containing molecule (radical or non-radical) capable of initiating some kind of deleterious reaction. These include superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), peroxyl radical ($RO_2^{\bullet}$), alkoxyl radical ($RO^{\bullet}$), hydroperoxyl radical ($HO_2^{\bullet}$), hypochlorous acid (HOCl),

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and singlet oxygen ($^1O_2$). Research has tended to focus on the negative aspects of ROS, however, they do participate in essential functions, such as redox signaling and the killing of bacteria by neutrophils and macrophages *(2)*.

Most work on ROS has centered on superoxide, hydrogen peroxide, and the hydroxyl radical, which are formed as follows. The one-electron reduction of oxygen results in the formation of superoxide (reaction 11.1), dismutation of two superoxide molecules yields hydrogen peroxide and oxygen (reaction 11.2), and the oxidation of ferric iron by hydrogen peroxide yields the hydroxyl radical and the hydroxide anion (reaction 11.3):

$$O_2 + e^- \rightarrow O_2^{\bullet -} \quad (11.1)$$

$$2O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2 \quad (11.2)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + OH^- \quad (11.3)$$

The discovery of superoxide dismutase (SOD), a naturally occurring enzyme that catalyzes reaction (11.2), was a key event in demonstrating the importance of ROS in vivo *(3)*. This importance was confirmed more recently by the demonstration that mice null for the mitochondrial form of SOD (MnSOD or SOD2) exhibit severe pathologies and have a markedly curtailed lifespan *(4, 5)*. In addition to SOD, cells contain many other systems dedicated to the control of ROS levels within the cell. These include small metabolites (e.g., ascorbic acid, α-tocopherol) that scavenge ROS and enzymes such as catalase and glutathione peroxidase that convert ROS to less reactive products. Despite the elaborate defense systems, it appears that control over ROS levels in the cell is not perfect. Overproduction or imperfect removal of ROS results in oxidative stress, which is associated with oxidative damage to DNA, proteins, and lipids. Excessive (or limited) ROS may also perturb redox signaling pathways, which may lead to aberrant gene expression and cellular dysfunction. There is now a wealth of evidence indicating that ROS are intimately involved in the development of major diseases, including cancer, diabetes, Parkinson's disease, and Alzheimer's disease *(6)*.

Mitochondria are responsible for the majority of ATP production in the cell. The energy for ATP synthesis comes from the electrochemical proton gradient across the mitochondrial inner membrane. This gradient (called proton motive force) is composed of a pH component (ΔpH) and an electrical component ($\Delta\psi$), and is formed by the enzymes of the electron transport chain as they pump protons from the mitochondrial matrix to the intermembrane space. The energy required for the proton pumping is obtained as the electrons are passed from carriers in Complex I with high potential energy through Complex III to carriers in Complex IV with lower potential energy. Pairs of electrons enter the chain from reduced substrates at Complex I, Complex II, and

other dehydrogenases, and reduce oxygen to water at Complex IV. However, at certain sites within the chain, electrons are passed singly to carriers such as FeS centers whose chemistry demands that they take electrons one at a time or to flavins or quinones that have stable one-electron reduced intermediates. It is these single electrons that may occasionally react directly with oxygen instead of passing on down the respiratory chain to Complex IV, resulting in superoxide formation (reaction 11.1). Understanding the sites, mechanisms, topology, and regulation of superoxide production by mitochondria is of great importance to understanding the damage ROS cause and the removal and repair systems that need to be in place, and the majority of this review is focused on these areas. First, we provide an overview of the methods commonly deployed to detect and measure ROS.

2. Detection of Reactive Oxygen Species in Mitochondria and Cells

2.1. Measurement of Superoxide in Isolated Mitochondria

Most of our understanding of the mechanics of ROS production has been obtained using isolated mitochondria, because of their experimental accessibility. Most superoxide detection systems rely on the reaction of some compound with superoxide that results in a change in the characteristics of that compound, such as a change in absorbance, fluorescence, luminescence, or paramagnetism. Detector compounds include acetylated cytochrome *c*, epinephrine, dihydroethidium, lucigenin, coelenterazine, and DMPO *(7)*. However, in intact mitochondria, direct detection of superoxide that is produced in the matrix using these probes is problematic for various reasons. Firstly, since superoxide does not cross the mitochondrial inner membrane, it cannot diffuse out of the mitochondria, thus the probe must be delivered into the matrix where it may undergo a variety of non-specific reactions. Of particular concern is the ability of some probes to undergo redox cycling which results in the production of superoxide by the probe itself. Secondly, if the probe is charged or becomes charged after reacting with superoxide, then it may distribute across the mitochondrial inner membrane according to the membrane potential, which may change its physico-chemical properties. Direct quantification of the rate of superoxide production is difficult as "standard" superoxide cannot be added to the matrix to generate a standard curve.

There are two alternative methods to measure mitochondrial superoxide production, both of them are indirect. The first is by the inactivation of aconitase, which is a mitochondrial matrix enzyme containing a labile iron in its iron–sulfur cluster that is superoxide sensitive. At any given moment, mitochondrial aconitase activity

depends on its rate of inactivation by superoxide (and possibly other ROS) and its rate of reactivation by reduction and replacement of iron into the cluster. The aconitase assay is based on the following scheme where (11.4) is catalyzed by aconitase, (11.5) is catalyzed by isocitrate dehydrogenase, and the reaction is monitored by following the rate of increase in NADPH absorbance or fluorescence:

$$\text{Citrate} \rightarrow \text{Isocitrate} \tag{11.4}$$

$$\text{Isocitrate} + \text{NADP}^+ \rightarrow \alpha - \text{Ketoglutarate} + \text{NADPH} + \text{H}^+ \tag{11.5}$$

Typically, mitochondria are incubated under the conditions of interest for a specified amount of time. The mitochondria are then lysed and the aconitase activity determined. A relative drop in activity compared to controls is an index of superoxide produced during the incubation period *(8–10)*. One drawback with this method is that quantification of absolute rates of superoxide production is not possible.

The second (and most commonly used) way to assess superoxide production indirectly is by measuring hydrogen peroxide, which readily diffuses out of the mitochondria and is amenable to simple detection protocols. The method relies on the fact that most of the superoxide produced by mitochondria is converted to hydrogen peroxide by endogenous (and if desired, externally added) superoxide dismutase. The method assumes that any removal processes of hydrogen peroxide in the matrix do not change under different experimental conditions. Common assays used to assess mitochondrial hydrogen peroxide production are based on the oxidation of a reduced detector compound coupled to the enzymatic reduction of hydrogen peroxide by horseradish peroxidase (HRP) (11.6 to 11.8)

$$\text{HRP} + \text{H}_2\text{O}_2 \rightarrow \text{HRP} - \text{H}_2\text{O}_2 \tag{11.6}$$

$$\text{HRP} - \text{H}_2\text{O}_2 + \text{AH}_2 \rightarrow \text{HRP} + 2\text{H}_2\text{O} + \text{A} \tag{11.7}$$

or

$$\text{HRP} - \text{H}_2\text{O}_2 + 2\text{AH} \rightarrow \text{HRP} + 2\text{H}_2\text{O} + \text{A}_2 \tag{11.8}$$

where AH_2 is typically amplex red or dihydrochlorofluorescein, and AH is *p*-hydroxyphenylacetic acid or homovanillic acid *(7)*. The increase in A or A_2 is followed fluorometrically. The advantage of this method is that quantification is easily achievable by means of a standard curve obtained by the addition of known amounts of hydrogen peroxide to the system. This allows for direct comparison of rates from different laboratories, a selection of which are presented in **Table 11.1**. However, as with all methods, consideration should be given to appropriate controls. For example,

Table 11.1
Rates of hydrogen peroxide production in mitochondria isolated from rat

Tissue	Substrates	Rate	Reference
Skeletal muscle	Succinate	2.54	*(30, 47)*
	Succinate + rotenone	0.05	
	Pyruvate and malate	0.02	
	Pyruvate and malate + rotenone	0.47	
Heart	Succinate	0.63	*(28)*
	Succinate + rotenone	0.06	
	Glutamate and malate	0.004	
	Glutamate and malate + rotenone	0.22	
Heart	Succinate	2.8	*(37)*
	Succinate + rotenone	0.1	
	Pyruvate and malate	0.05	
	Pyruvate and malate + rotenone	0.25	
Heart	Succinate	1.03	*(49)*
	Succinate + rotenone	0.52	
	Pyruvate and malate	1.83	
	Pyruvate and malate + rotenone	3.62	
Brain	Succinate	1.39	*(32)*
	Succinate + rotenone	0.17	
	Glutamate and malate	0	
	Glutamate and malate + rotenone	0.43	
Brain	Succinate	0.39	*(31)*
	Succinate + rotenone	0	
	Glutamate and malate	0	
	Glutamate and malate + rotenone	0.25	

Rates are in nmol H_2O_2/min/mg mitochondrial protein.

considerable background rates of fluorescence increase were obtained with homovanillic acid as a probe, even in the absence of mitochondria *(11)*.

2.2. Measurement of Reactive Oxygen Species in Intact Cells

To determine the physiological or pathological importance of ROS production by mitochondria, measurements need to be made in intact cells, but such measurements are much less reliable than those on isolated mitochondria. Most methods for the detection of ROS in cells rely on changes in fluorescence of a detector compound. Typically, the cells are incubated with the probe for a given time, then the cells are examined by either confocal microscopy, flow cytometry, or conventional fluorescence. In general the techniques are fraught with potential hazards, such as autooxidation of the probes when illuminated, non-selectivity for particular kinds of ROS, redox cycling, toxicity, and loss of the fluorophore from the

cell. Nonetheless, many studies have employed various probes as indicators of ROS production in cells *(7)*. The common probes in use are briefly described below.

Dihydroethidium (DHE, also called hydroethidine), undergoes a two-electron oxidation to ethidium, the fluorescence of which increases when it binds to DNA. The oxidation appears to be relatively specific for superoxide *(12)*. However, since ethidium is a cation it will tend to accumulate inside the mitochondria according to the magnitude of the membrane potential, $\Delta\psi$. This will alter its availability to bind nuclear and mitochondrial DNA, thus the fluorescent yield obtained does not necessarily reflect the rates of superoxide formation *(13)*. Furthermore, ethidium is not the only fluorescent product of DHE oxidation, as 2-hydroxyethidium is also formed, and there is evidence that DHE can catalyze the dismutation of superoxide *(12, 14)*. A recent study showed that more reliable results are obtainable by HPLC with electrochemical detection, rather than detection by fluorescence *(14)*. The attachment of DHE to the lipophilic cation triphenylphosphonium (TPP^+) via a hexyl carbon chain results in a compound termed MitoSOX red, which is targeted to mitochondria by the TPP^+ moiety. There is evidence to suggest that this probe offers improved selectivity for mitochondrial superoxide produced in the matrix *(15)*.

Dihydroflourescein (H_2F) and related compounds such as dihydrochlorofluorescein (H_2DCF), carboxydihydrochlorofluorescein (carboxy-H_2DCF), and dihydrochlorofluorescein diacetate (H_2DCF-DA) are commonly used indicators of cytoplasmic hydrogen peroxide production *(16)*. These probes are oxidized from the poorly fluorescent dihydro-compounds to the highly fluorescent parent molecules. However, they appear to have poor and undefined specificity, as they can apparently be oxidized by reactive nitrogen species and cellular peroxidases even in the absence of hydrogen peroxide *(7, 17, 18)*. Dihydrorhodamine 123 is another compound that is oxidized to a fluorescent parent compound, rhodamine 123. Like ethidium, this hydrophilic cation will accumulate into the mitochondrial matrix depending on the membrane potential, and like H_2DCF and related compounds, its specificity is poorly defined.

With all currently available probes for detecting cellular ROS, there is uncertainty as to the precise nature of the pathways involved in generating the fluorescent signals. This indicates that extreme care must be taken when interpreting the results obtained with these probes. At best they may be used as qualitative indicators of general oxidative stress, as opposed to the precise quantification of specific ROS. Ideally, experiments designed to compare levels of oxidative stress between one physiological condition and another should employ a variety of probes in conjunction with several positive and negative controls.

3. Superoxide Production by Isolated Mitochondria

3.1. Introduction

The interest in ROS production by mitochondria began over 35 years ago *(19–21)* and there are several reviews on the subject *(22–26)*. Typical experiments involve isolation of mitochondria from tissues or cells, followed by incubation under a defined set of conditions with the chosen detection system. **Table 11.1** shows a small selection of the absolute rates of hydrogen peroxide production obtained by various laboratories. Clearly the rates obtained from specific tissues are somewhat variable. There are many potential sources of this variability, such as the method employed for isolating mitochondria, which can impact on the overall purity and quality of the mitochondrial preparation. Another explanation is the differences in incubation conditions and application of appropriate controls, which vary considerably from laboratory to laboratory. Despite this variability in absolute rates, there are several consistent findings on mitochondrial superoxide production that have emerged, and it is on these findings that we focus in the following sections. We stress the sites of superoxide production and the topology of ROS production from these sites, since ROS produced at the matrix surface of the inner membrane, into the inner membrane itself, or on the outer surface of the membrane are likely to have very different physiological and pathological effects. This topology is likely to be important, given the location of mitochondrial DNA in the matrix and attached to the matrix surface of the inner membrane, and the presence in the inner membrane of polyunsaturated phospholipids that are susceptible to attack by ROS to produce lipid peroxides that can go on to generate further lipooxidative damage and produce other well-known markers of oxidative stress, such as hydroxynonenal, TBARS, and protein carbonyls.

3.2. Complex I

NADH–ubiquinone oxidoreductase (Complex I) is the first enzyme of the electron transport chain. It oxidizes NADH generated by the tricarboxylic acid cycle and reduces ubiquinone to ubiquinol with the concurrent pumping of four protons across the mitochondrial inner membrane. Relatively, the eukaryotic enzyme is huge, comprising some 46 subunits with a combined mass of almost 1 MDa. The enzyme's redox centers comprise a flavin mononucleotide (FMN), numerous iron–sulfur (Fe–S) clusters, and a ubiquinone (Q) binding site, with the accepted sequence of electron transfers being NADH → FMN → Fe–S → Q. Overall, Complex I has an L-shaped structure, with the hydrophobic arm (containing the Q-binding site) embedded in the membrane and the hydrophilic arm (containing the FMN and the iron–sulfur clusters) protruding into the matrix. The mechanism of Complex I (how the electron transfer is coupled to proton pumping) is unknown *(27)*.

The rate of superoxide production by Complex I can vary considerably. One of the main factors influencing the rate is which respiratory substrates are present. The highest rates are generally seen in the presence of succinate *(28–34)*. During succinate oxidation, electrons are transported down the electron transport chain, which results in proton pumping by Complexes III and IV (**Fig. 11.1A**). The resulting proton motive force and reduced Q pool drive electrons thermodynamically uphill into Complex I, reducing NAD^+ to NADH. When rotenone, a specific Complex I Q-site inhibitor, is added under these conditions, the rate of superoxide production is markedly diminished (*see* **Table 11.1** and **Fig. 11.1B**) *(28–34)*. The standard interpretation of this result is that superoxide is generated by Complex I during reverse electron transport from QH_2 to NAD^+. Relatively high rates of superoxide production are also obtained with glycerol-3-phosphate as substrate. In a similar fashion to succinate, these high rates are diminished by rotenone, indicating again that Complex I produces superoxide during reverse electron transport *(35)*.

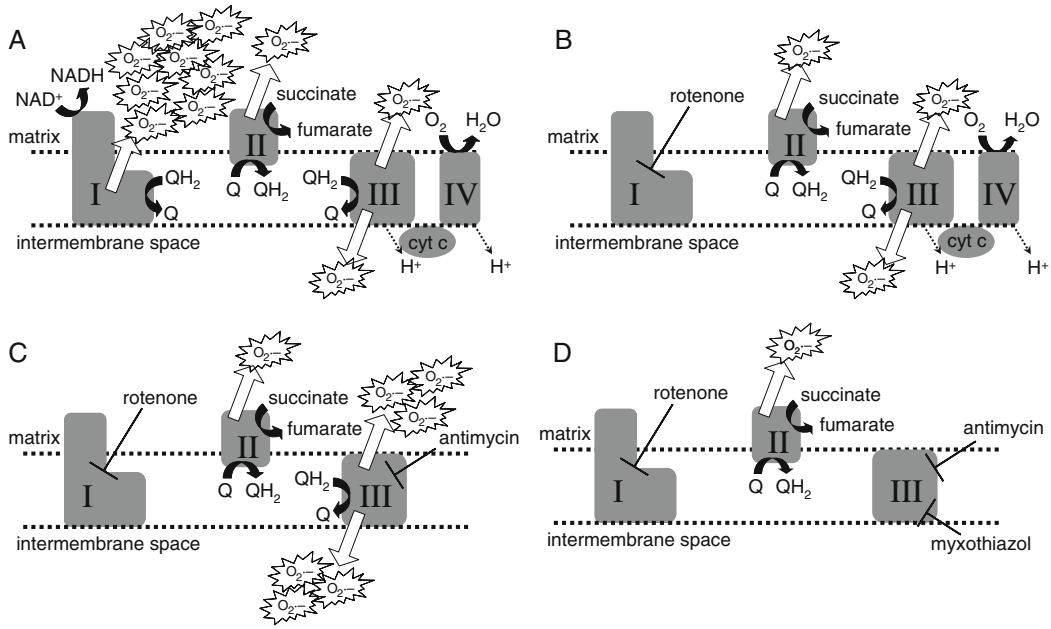

Fig. 11.1 Modes of superoxide production by the mitochondrial electron transport chain with succinate as substrate. Mitochondria oxidizing succinate produce a high proton motive force by proton pumping at Complexes III and IV. The superoxide production rate is high, mostly directed toward the matrix and almost exclusively from Complex I during reverse electron transport (**A**). Addition of the Complex I inhibitor rotenone inhibits this high rate; superoxide production at other sites is relatively low (**B**). In the presence of the Complex III center *i* inhibitor antimycin, there is no proton pumping and proton motive force is zero. Superoxide production rates are high, directed both to the matrix and intermembrane space in equal proportions, and located primarily at center *o* of Complex III (**C**). Addition of the Complex III center *o* inhibitor myxothiazol blocks center *i* and diminishes the high rate seen with antimycin (**D**).

High rates of superoxide production during reverse electron transport are apparent in mitochondria from several different tissues (brain, heart, skeletal muscle, and kidney) *(28–32, 36)*. Most of the work has been performed on rat mitochondria, but the observation also applies to mitochondria from a variety of vertebrate species and *Drosophila (35, 37)*. This strongly suggests that high superoxide production rates from Complex I during reverse electron transport are a universal property of isolated mitochondria, however, it is unknown if this mode of superoxide production occurs in whole cells. Some reports indicate that in intact cells, rotenone decreases ROS formation *(38–41)*, suggesting that the superoxide-producing site during reverse electron transport in Complex I is active in cells. Other studies report an increase in ROS production in cells treated with rotenone *(42–45)*, suggesting that the forward mode of superoxide production is in operation (see below).

Providing mitochondria with substrates that result in forward electron transport, such as pyruvate plus malate to generate NADH, results in relatively low rates of superoxide production (**Table 11.1** and **Fig. 11.2A**) *(28, 29, 31, 32, 34, 36, 46, 47)*. Under these conditions, it is not known where in the mitochondria the superoxide originates. However, in the presence of Complex I inhibitors, this low rate can be increased several fold, suggesting that Complex I is capable of significant superoxide production during forward electron transport (**Table 11.1** and **Fig. 11.2B**). This mode of superoxide production is apparent in mitochondria from various tissues from a variety of species *(28, 29, 31, 32, 34, 36, 37, 46–50)*.

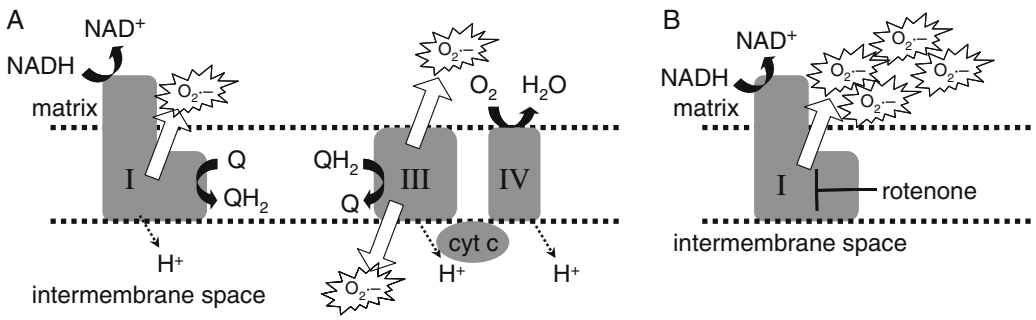

Fig. 11.2. Modes of superoxide production by the mitochondrial electron transport chain with pyruvate and malate/glutamate as substrates. The substrates generate NADH, and a high proton motive force results from proton pumping by Complexes I, III, and IV. Superoxide production rates are relatively low (**A**). Addition of the Complex I inhibitor rotenone to the system results in elevated superoxide production rates in the matrix from Complex I (**B**). Proton pumping is inhibited and proton motive force is zero, however, imposition of a proton motive force under these conditions doubles superoxide production *(47)*.

In addition to which substrates are present, there are several other factors that influence the rates of superoxide production by Complex I in isolated mitochondria. In particular, the magnitude

of the components of proton motive force (ΔpH and $\Delta\psi$) across the mitochondrial inner membrane has a strong influence during both forward and reverse electron transport *(28, 30–32, 34, 47, 51, 52)*. This phenomenon has led to the concept of "uncoupling to survive" that suggests that lowering proton motive force by mild uncoupling will lower superoxide production while still allowing for sufficient ATP production. One of the effects of this will be lowered oxidative damage and increased lifespan *(53, 54)*.

The actual sites of superoxide production within Complex I are unresolved. Any of the electron-carrying centers (FMN, iron–sulfur clusters, Q) is in theory capable of donating an electron to oxygen to yield superoxide. In the isolated enzyme, there is a strong evidence that superoxide is produced at the flavin site, by the reaction of the reduced form of the flavin ($FMNH_2$) with oxygen by a bi-molecular ping–pong mechanism *(55)*. Other authors have also concluded that the FMN site is where superoxide originates in Complex I *(31, 56)*. However, there are arguments that it is the iron–sulfur clusters that produce superoxide, in particular, clusters N1-a and N2 have been proposed *(29, 57)*, as well as iron–sulfur clusters in general *(58)*. The Q site has also been suggested as the main site of superoxide production *(33, 47)*. This lack of agreement may reflect the differences in methodology: as discussed above, the rate (and the site) of superoxide production by Complex I will vary depending on the conditions employed. In addition, some of the inhibitors employed to dissect out the sites of production are poorly characterized and non-specific. The current working model of Complex I superoxide production in this laboratory is that in energized mitochondria (i.e., in the presence of proton motive force), the majority of superoxide originates from the Q site. The other sites (particularly FMN) produce superoxide at modest rates that only become apparent in de-energized mitochondria when the flavin is kept reduced *(47)*.

Regarding topology, there is no detectable superoxide production by Complex I to the intermembrane space, because addition of SOD to mitochondria producing superoxide at Complex I does not increase the rate of hydrogen peroxide formation *(11, 59)*. Therefore, Complex I probably delivers superoxide exclusively to the mitochondrial matrix. From the structure of the complex, this is inevitable when the superoxide is produced at the flavin site. If superoxide is produced at the Q site of Complex I, this site must deliver the superoxide to the matrix and not to the intermembrane space.

3.3. Complex III

Ubiquinol (QH_2) is generated by Complex I and by other oxidoreductases that do not pump protons, in animals chiefly Complex II, glycerol-3-phosphate dehydrogenase, and the electron transferring flavoprotein ubiquinone oxidoreductase (ETF-QOR).

Ubiquinone–cytochrome *c* oxidoreductase (Complex III) transfers electrons from this ubiquinol (which is present in the lipid bilayer of the inner membrane) to cytochrome *c* in the intermembrane space. Two protons are pumped (and two more protons are dumped non-electrogenically into the intermembrane space) for every pair of electrons transferred; the mechanism of the coupling of electron transfer to proton pumping is described by the relatively well-understood Q-cycle mechanism *(60)*.

The native rates of superoxide production by Complex III are relatively low compared to the rates from Complex I during reverse electron transport. For example, during succinate oxidation in the presence of rotenone (where Complex I is functionally removed) the rates of hydrogen peroxide production are low (**Table 11.1** and **Fig. 11.1B**) *(28–34)*. Since it is diminished by myxothiazol, an inhibitor of Complex III, much of this low rate is from Complex III, and little (if any) is from other producers, such as ubiquinone or Complex II *(11, 35)*. The main evidence that Complex III can produce superoxide at high rates comes from the marked increase in the rate of production observed in the presence of antimycin *(11, 31, 34, 36, 61–63)*. This inhibitor is specific for center *i* of Complex III, where it blocks electron transfer from the *b* hemes to the quinone. This results in an increase in the formation of a semiquinone at center *o* in the complex, which can donate an electron to oxygen yielding superoxide at considerable rates (**Fig. 11.1C**) *(64)*. Center *o* inhibitors of Complex III (such as myxothiazol or stigmatellin), prevent electrons from accessing center *o*, and lower the high rates of superoxide production observed in the presence of antimycin (**Fig. 11.1D**) *(11, 28, 34, 61, 62, 65)*. This is taken as evidence that the main site of superoxide production within Complex III is the semiquinone at center *o*. Like Complex I, superoxide production by Complex III apparently exhibits dependence on the membrane potential *(30, 51, 66)*, but this effect and relative importance of ΔpH and $\Delta\psi$ have not been investigated in detail.

In terms of topology, addition of SOD to mitochondria that are producing superoxide at Complex III results in an increase in the rate of hydrogen peroxide production *(10, 11, 59)*. This indicates that Complex III is capable of producing superoxide toward the intermembrane space. Studies in mitoplasts (mitochondria with the outer membrane removed) also indicate that there is superoxide production directed outwards from the mitochondria *(67)*. The current consensus view is that about 50% of the superoxide from center *o* of Complex III is matrix directed, and the other 50% is released into the intermembrane space. Superoxide from center *o*, which is near the intermembrane surface of the inner membrane, is presumably produced in the protonated form as the hydroperoxyl radical into the membrane bilayer, and subsequently deprotonates and escapes as superoxide to both sides of the membrane *(59)*.

3.4. Succinate Dehydrogenase

Succinate dehydrogenase (SDH, succinate–ubiquinone oxidoreductase, Complex II) faces the inner surface of the mitochondrial inner membrane and reduces Q to QH_2while converting succinate to fumarate during the operation of the tricarboxylic acid cycle. Mutations in this enzyme are associated with a variety of diseases in humans, and although the mechanistic details of the pathways are still being elucidated, overproduction of ROS has been implicated *(68)*. In *Caenorhabditis elegans*, defects in the enzyme result in elevated superoxide production rates and abnormal energy metabolism *(69)*. SDH appears to produce only superoxide, as no hydrogen peroxide generation was detected, at least in the *Escherichia coli* enzyme *(70)*. Compared to the high rates seen from Complex I during reverse electron transport, or Complex III in the presence of antimycin, the rates of superoxide production by SDH in mammalian mitochondria are very low. For example, in the presence of succinate, rotenone, and stigmatellin or myxothiazol, where electrons cannot enter either Complex I or Complex III, most of the superoxide presumably originates at SDH. Under these conditions, only very low rates of hydrogen peroxide generation are observed *(28, 34, 61, 62, 65)*. SDH contains an FAD site, three iron–sulfur clusters, a heme group, and a Q-binding site. It appears that it has evolved to produce very little ROS under normal conditions, by suppressing the formation of a flavin radical in the active site; the rates during succinate oxidation by fumarate reductase, which normally catalyzes the same reaction in reverse in *E. coli* and lacks the suppression mechanism, are very much greater *(70)*.

3.5. Glycerol-3-Phosphate Dehydrogenase

The glycerol-3-phosphate (G3P) shuttle provides a mechanism for the transport of electrons from cytosolic NADH to the mitochondrial electron transport chain. Two isoenzymes of glycerol-3-phosphate dehydrogenase (GPDH) participate in this shuttle. The cytosolic form catalyzes the oxidation of NADH by dihydroxyacetone phosphate and the mitochondrial form (whose active site faces the outer side of the mitochondrial inner membrane) is a G3P–ubiquinone oxidoreductase that oxidizes G3P and reduces Q to QH_2, thereby feeding electrons into the electron transport chain. G3P-dependent hydrogen peroxide production has been reported in mitochondria from various sources, including heart, brain, and kidney from mouse; brown adipose tissue from hamsters and rats; and flight muscle from *Drosophila (34–36, 71–73)*. This implies that G3PDH is capable of superoxide production. The rate of superoxide production by G3PDH in isolated *Drosophila* mitochondria was estimated to be about 70% of the total rate, the other 30% coming from Complex I *(35)*. Unlike Complexes I and III, superoxide production by GPDH is not very sensitive to membrane potential *(35)*. Like Complex III, the production is sensitive to externally added SOD, leading to the

conclusion that about 70% of the superoxide production is directed to the intermembrane space and 30% to the mitochondrial matrix *(10)*. The site of superoxide production within the enzyme in unknown, but it is likely to be the flavin.

3.6. Other ROS-Producing Enzymes in Mitochondria

During β-oxidation of fatty acids, the electron transferring flavoprotein ubiquinone oxidoreductase (ETF-QOR) catalyzes the oxidation of electron transferring flavoprotein with the concomitant reduction of Q to QH_2. The enzyme is located in the mitochondrial inner membrane and possesses an FAD group and an iron–sulfur cluster. The ETF-QOR appears to be capable of producing superoxide, as measurable rates of hydrogen peroxide production are obtained in the presence of palmitoyl carnitine. This rate is unaffected by externally added SOD, indicating that production occurs in, or is directed to, the matrix *(11)*.

A number of other sites within mitochondria have emerged as capable of ROS generation. These include dihydrolipoyl dehydrogenase-containing enzymes such as α-ketoglutarate dehydrogenase and pyruvate dehydrogenase *(74, 75)*, and NADH–ubiquinone oxidoreductases other than Complex I *(76)*. The production of ROS by these sites is not as well characterized and understood as production from the enzymes discussed above.

4. Summary

In isolated mitochondria a reasonably clear picture of the substrate and inhibitor dependence of superoxide production has emerged. High rates are seen with succinate, and the superoxide is most likely to originate from Complex I during reverse electron transport. During forward electron transport into Complex I, superoxide production rates are low, but they can be increased by Complex I inhibitors. Relatively low rates are seen from Complex III; these can be increased by antimycin and subsequently lowered by myxothiazol. It is unclear which sites of mitochondrial superoxide production are active in vivo, whether or not they are the same in all tissues under all conditions, and how they are regulated, and more work is required in this area.

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