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THE NATURE AND MECHANISM OF SUPEROXIDE PRODUCTION BY THE ELECTRON TRANSPORT CHAIN: ITS RELEVANCE TO AGING

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ABSTRACT

Most biogerontologists agree that oxygen (and nitrogen) free radicals play a major role in the process of aging. The evidence strongly suggests that the electron transport chain, located in the inner mitochondrial membrane, is the major source of reactive oxygen species in animal cells. It has been reported that there exists an inverse correlation between the rate of superoxide/hydrogen peroxide production by mitochondria and the maximum longevity of mammalian species. However, no correlation or most frequently an inverse correlation exists between the amount of antioxidant enzymes and maximum longevity. Although overexpression of the antioxidant enzymes SOD1 and CAT (as well as SOD1 alone) have been successful at extending maximum lifespan in *Drosophila*, this has not been the case in mice. Several labs have overexpressed SOD1 and failed to see a positive effect on longevity. An explanation for this failure is that there is some level of superoxide damage that is not preventable by SOD, such as that initiated by the hydroperoxyl radical inside the lipid bilayer, and that accumulation of this damage is responsible for aging. I therefore suggest an alternative approach to testing the free radical theory of aging in mammals. Instead of trying to increase the amount of antioxidant enzymes, I suggest using molecular biology/transgenics to decrease the rate of superoxide production, which in the context of the free radical theory of aging would be expected to increase longevity. This paper aims to summarize what is known about the nature and mechanisms of superoxide production and what genes are involved in controlling the rate of superoxide production.

INTRODUCTION

While most biogerontologists agree that reactive oxygen species play an important role in aging, there is great discord on the details. There is a large body of evidence supporting the role of free radical reactions in aging and longevity, which has been summarized extensively (1-3). The most pertinent and most often cited piece of evidence is the experiment of Orr and Sohal (4), in which overexpression of Cu,Zn-superoxide dismutase (SOD1) and Catalase (CAT) was found to increase maximum lifespan (MLSP) in *Drosophila melanogaster*. Although this experiment has been widely criticized, it has recently been vindicated by the fact that another independent investigator produced very similar results (5, 6). Overexpression of SOD1 by these investigators in *Drosophila* motor neurons did indeed increase MLSP (5). It should also be mentioned that several investigators who overexpressed SOD1 in *Drosophila* saw only an increase in average lifespan (7, 8), whereas even others saw no effect at all (9, 10). Nevertheless, considering the large number of variables involved in generating *Drosophila* lines overexpressing SOD1, the positive results outweigh the negative ones and it can be tentatively concluded that the free radical theory of aging has been proven correct in the case of *Drosophila melanogaster*. That is to say, free radicals have been shown to be a longevity-determining factor in *that organism*. Unfortunately, demonstrating such a relationship in mammals has proven a greater challenge. Strains of transgenic mice overexpressing SOD1 have been constructed but did not show increased longevity (11). Since most negative results go unpublished, it has been difficult to get a clear picture on the state of progress in this area. However, in a low $O_2^{\bar{}}\bullet$ producing organism (relative to fruit flies) such as the mouse (12), superoxide dismutase activity may not be the rate-limiting factor for the age-dependent accumulation of oxidative damage. This inductive observation stems from the fact that neither an SOD1 homozygous knockout (k.o.) mouse (13-15), nor an Mn-superoxide dismutase (SOD2) heterozygous k.o. mouse, exhibit significantly altered lifespans (16). In fact, the SOD2 heterozygote (+/-) is no more sensitive to hyperoxia than wild type (WT), suggesting that half the amount of Mn-SOD dismutase is sufficient for normal physiological function (17). Since 50% of the normal level of Mn-SOD

and 0% of the level of Cu,Zn-SOD is sufficient for normal longevity, how could one expect overexpression of either to increase maximum (or even mean) lifespan? In contrast, a null mutant of Mn-SOD is lethal, indicating that superoxide can reach critical levels in the total absence of this enzyme (18, 19). In light of interspecies correlational data, however, it should not be too surprising that overexpression of Cu,Zn-SOD does not increase MLSP in mammals. Interspecies variation in SOD activity is not correlated with MLSP in mammals (20) and the activity of other antioxidant enzymes negatively correlates with MLSP (21-24). A mouse and a pigeon, species that differ in MLSP by a factor of 10, essentially have the same level of SOD activity (25).

On the other hand, there is a strong correlation between longevity and rates of $O_2^{\bar{\bullet}}$ and H_2O_2 production by mitochondria isolated from diverse mammalian species (12, 26, 27), just as would be predicted by the rate of living relationship (12, 26, 28, 29). This correlation, that is, mitochondria of longer-lived animals producing less $O_2^{\bar{\bullet}}$/H_2O_2 than those of shorter lived ones, persists even in comparisons between organisms which cannot be explained by the rate of living relationship (25, 30-35). Assuming that the free radical theory holds true in mammals—and the literature taken as a whole clearly suggests so (1-3) —these data indicate that $O_2^{\bar{\bullet}}$ production, rather than $O_2^{\bar{\bullet}}$ scavenging, is the determining factor for age-related, steady-state accumulation of oxidative damage. Support for this concept is provided by the recognition that interspecies variation in free radical damage to mtDNA (36), appearance of oxidized bases in urine (37) and accumulation of lipofuscin pigments (38) also negatively correlate with MLSP. An additional line of evidence related to this idea comes from studies involving effects of caloric restriction on maximum lifespan. Caloric restriction (CR) of rodents is the only treatment that consistently increases MLSP in mammals in controlled studies (39-41). The CR model has provided very strong support for the free radical theory of aging, in that levels of oxidative damage are consistently lower in CR rodents vs. those fed *ad libitum* (39, 41). While there is no consistent effect of CR on SOD or other antioxidant enzyme levels (41, 42), there is a unanimous consensus (39, 41, 42) that CR decreases the rate of $O_2^{\bar{\bullet}}$ production and H_2O_2 release from mitochondria (43).

All the above data appear to confirm that lifespan determination is not due to expression of antioxidant enzymes but, rather, to levels of free radical generation. The counter-argument could be made, based on the observation that resistance to a wide variety of exogenous oxidative stressors (paraquat, hydrogen peroxide, arsenite) is correlated with maximum lifespan in a diverse series of mammals (44). While one could interpret these data as indicating that higher resistance to oxidative stress is due to higher levels of antioxidants, it is also possible that increased capacity for free radical generation can lead to increased sensitivity to external oxidative stress, such as in the case of the *C. elegans* mev-1 mutant (45).

If we accept that free radicals play a major role in the aging process (and the literature as a whole strongly suggests so (2)) then clearly it is the level of generation, rather than the level of scavenging, that contributes to the determination of MLSP in mammals*(23, 46, 47). How is it possible then, for $O_2^{\bar{\bullet}}$ production to be a determinant of lifespan while SOD levels are not? To explain how this is mechanistically possible, a brief review of mitochondrial sub-anatomy and of redox chemistry is necessary.

* *This sentence should not be interpreted as meaning that $O_2^{\bar{\bullet}}$ production is the only factor controlling lifespan, there is no question that there are many others (e.g. DNA repair).*

The mitochondrial electron transfer chain as the major source of $O_2^{\bar{\bullet}}$

As is widely known, it is the overexpression of cytoplasmic SOD (Cu,Zn-SOD or SOD1) that has been shown to extend lifespan in *Drosophila* (4, 48). This should not be interpreted as meaning that the source of $O_2^{\bar{\bullet}}$, in this case, is necessarily cytoplasmic. Although it is well established that Mn-SOD is in the mitochondrion and Cu,Zn-SOD is in the cytoplasm, there appears to be great overlap in the function of the two enzymes, at least in yeast (49, 50). In fact, it is the SOD1 k.o. that has the more severe phenotype in *Saccharomyces*, yet the source of $O_2^{\bar{\bullet}}$ is clearly mitochondrial since the phenotype can be "rescued" by eliminating mitochondrial respiration (49, 50). One possibility is that $O_2^{\bar{\bullet}}$ diffuses through the outer mitochondrial membrane in its protonated form or that it passes through anion channels to reach the cytoplasm (50, 51) (The long held notion that $O_2^{\bar{\bullet}}$ production occurs only towards the mitochondrial matrix is most likely not true; it will be treated in more detail, below). Denham Harman, the father of the free radical theory of aging (52), postulated that the mitochondrion is the main producer as well as the major target of oxidative damage as early as 1972 (53). Although this paradigm has been widely criticized, the idea is based on a sound theoretical basis (namely, that over 90% of $O_{2(g)}$ is consumed in the mitochondrion) and is supported by strong experimental evidence (16, 49, 50, 54-56). Recently, much talk in the aging community has focused on alternative sources of $O_2^{\bar{\bullet}}$ that may contribute to aging (57) and some have gone as far as rejecting the notion that the mitochondrion is the main source of $O_2^{\bar{\bullet}}$ (57). Because these views are widespread, it is important to reiterate what evidence supports the dogmatic paradigm. While there are indeed many sources of $O_2^{\bar{\bullet}}$ in the cytoplasm, estimates of their magnitude and biological significance are widely varying (the exception to the controversy is the macrophage oxidative burst, which is well-characterized and of widely-accepted biological significance (58)). Moreover, unless their damaging effects are somehow not preventable by SOD, these cytoplasmic sources of $O_2^{\bar{\bullet}}$ have very little contribution towards aging since a null mutant of Cu,Zn-SOD does

not show significantly decreased lifespan (15, 59). In mammals, besides the large amount of classical biochemical data (2, 56), the most convincing piece of evidence that the mitochondrion is the main source (as well as the main target) of $O_2^{\bar{\bullet}}$ is that an SOD2 k.o. (located in the mitochondrion) in mice is lethal (16, 18, 19), whereas the SOD1 k.o. (cytoplasmic) and SOD3 (extracellular) k.o. are not (15, 60, 61). Both SOD1 and 3 k.o.'s are superficially healthy although, on closer inspection, they do appear to have significant defects (13-15, 61-63) indicating that there is some physiological role for these enzymes. Lastly, doubt has been cast on the value of *in vitro* measurements of $O_2^{\bar{\bullet}}$ production on the *in vivo* situation (64). It has been pointed out that air-saturated buffers, as well as respiratory inhibitors (12, 56, 65) and saturating substrates, have to be used to obtain $O_2^{\bar{\bullet}}/H_2O_2$ production (66). While this criticism is indeed valid, isolated mitochondria supplemented with physiological levels of substrate (66) and without added respiratory inhibitors (25, 26, 32-35) still produce detectable levels of H_2O_2 (66). In addition, SOD k.o. experiments in *Saccharomyces cerevisiae* have shown that $O_2^{\bar{\bullet}}$ production occurs *in vivo* under normal conditions (49, 50, 54) and that these effects are lethal (49). Using these strains, it has also been demonstrated that the lethality of the SOD k.o. could be rescued by mutations that inhibit mitochondrial respiration, clearly demonstrating that this is one of the main sites of $O_2^{\bar{\bullet}}$ production *in vivo* (49, 54). In addition, inhibitors of the respiratory chain that decrease the rate of $O_2^{\bar{\bullet}}$ production *in vitro* were also found to prevent the loss of viability of an SOD2 k.o. yeast *in vivo* (67). This further confirms that $O_2^{\bar{\bullet}}$ production by the mitochondrial electron transport chain occurs *in vivo* as well as *in vitro*. Overall, the evidence supporting the dogmatic view is strong. Since the mitochondrial electron transport is the main site of superoxide production in the eukaryotic cell, decreasing $O_2^{\bar{\bullet}}$ production at this site is of great interest to test the free radical theory of aging. The following section explores the structural and chemical characteristics of the mitochondrial electron transport chain that explain why $O_2^{\bar{\bullet}}$ production occurs there and gives a hint as to how it may be modulated.

Thermodynamics of $O_2^{\bar{\bullet}}$ formation and biochemical characteristics of the mitochondrial electron transport chain

The mitochondrial electron transport chain is located in the inner mitochondrial membrane. It consists of four distinct electron transport complexes and the ATP synthase. These enzyme complexes are numbered in the order in which electrons pass through them from NADH. Complex I (also called NADH:ubiquinone oxidoreductase or NADH dehydrogenase (68, 69)), the largest and least understood of the electron transport complexes, catalyses the transfer of electrons from NADH to ubiquinone (CoQ) and translocates 4 protons (or more) per 2 electrons in that process (68). Complex II (also called succinate dehydrogenase (70)) catalyses electron transfer from succinate to ubiquinone and the succinate dehydrogenase activity is considered to be part of the citric acid cycle. It is a peripheral membrane protein and it does not translocate protons. Complex III (also called bc1 complex for its main redox groups (71)) is arguably the most-studied and best-understood of all electron transport complexes. It catalyzes the reduction of cytochrome c by ubiquinol and translocates 4 protons per 2 electrons transferred to cytochrome c ((71), see Figure 2). Complex IV catalyzes the oxidation of cytochrome c by O_2 and is often referred to as cytochrome oxidase (72). This enzyme also translocates 4 protons per 2 electrons in the process (72). Contrary to the other enzymes of the respiratory chain, the terminal reaction of this enzyme occurs through a 2-electron transfer step (73). The electron transport chain, as a whole, is one of the main subcellular locations for one-electron redox chemistry (in contrast to glycolysis or the Krebs cycle, which are 2-electron redox chemistry chains). Knowing nothing else of its mechanisms, this fact alone would make it the ideal candidate for the generation of $O_2^{\bar{\bullet}}$. To understand why this is the case, it is worthwhile to first consider a few fine points of O_2 chemistry.

Although $O_{2(g)}$ (ground state diatomic oxygen, $^3\Sigma g^- O_2$) is a very strong oxidant, there are some restrictions in its chemistry that are strongly relevant to the present topic. Since diatomic oxygen in its triplet ground state has parallel spins, due to the Pauli principle, two-electron reductions of oxygen are only possible if both added electrons have the same spin (2). This is usually not the case in free solution, and anti-ferromagnetic coupling with metal ions is required to allow this to happen. One-electron reductions of oxygen on the other hand, are possible without violating any spin conversion rules. Hence, any one-electron transfer reaction in the electron transport chain can potentially be short-circuited by molecular oxygen, provided that it is accessible (by $O_{2(g)}$). This criterion explains why the mitochondrial electron transport chain (essentially a series of one-electron transfer steps) produces $O_2^{\bar{\bullet}}$ while other redox chains reactions, such as glycolysis and the Krebs cycle (which involve a series of 2-electron transfers) do not. However, a second thermodynamic requirement exists for superoxide production, which is that the electron donor must have a lower redox potential than the couple $O_{2(g)}/O_2^{\bar{\bullet}}$. Redox potential (E or E_m) is a measure of the tendency to accept or donate electrons. It is related to ΔG, the free energy of reaction, through the Nernst equation (71, 74). Very negative values of redox potential indicate strong reducing tendency while very positive values indicate strong oxidizing tendency. The redox midpoint potentials of oxygen in the standard state at pH 7 are traditionally quoted at $O_{2(g)}/O_2^{\bar{\bullet}}{}_{(aq)}$ $E^{o'}$= -330mV and $O_{2(aq)}/O_2^{\bar{\bullet}}{}_{(aq)}$ $E^{o'}$= −160mV (75). The latter figure ($O_{2(aq)}$) represents a non-standard situation, namely saturating amounts of $O_{2(g)}$ (75, 76), and it is incorrect to quote it as a substitute for $O_{2(g)}/O_2^{\bar{\bullet}}{}_{(aq)}$ (75). More recently, the figure for $O_{2(g)}/O_2^{\bar{\bullet}}{}_{(aq)}$ has been revised upwards from −330mV to $E^{o'}$= −140mV (76). The old values have assumed that the spontaneous dismutation

of $O_2^{\bar{\bullet}}{}_{(aq)}$ is irreversible, which is not the case (76). $O_2^{\bar{\bullet}}{}_{(aq)}$ is, in fact, in equilibrium with $H_2O_{2(aq)}$ and $O_{2(g)}$, although the equilibrium constant heavily favors $H_2O_{2(aq)} + O_{2(g)}$. It is this author's opinion that the argument made by Petlicki *et al* (76) is sound because it resolves discrepancies between National Bureau of Standards measurements of ΔG^o of formation (of $O_2^{\bar{\bullet}}$) and the values of E^o (of $O_2^{\bar{\bullet}}$) obtained through pulse radiolysis. Hence the old value should indeed be revised.

Even –140mV is a pretty low value for a redox potential and it indicates that a highly reducing substrate is required for $O_2^{\bar{\bullet}}$ production. All one-electron transfers having as a donor a redox pair with a midpoint potential of less than –140mV can thus be considered, with a good degree of certainty, as potential $O_2^{\bar{\bullet}}$ generators. In fact, the redox groups of well characterized $O_2^{\bar{\bullet}}$ producing systems all fall well below –140mV. Xanthine oxidase was one of the first enzymes to be shown to produce $O_2^{\bar{\bullet}}$ (56). This enzyme's redox centers are a molybdenum atom (Mo), a flavin and 2 iron sulfur clusters. Its redox components all have $E_{m's}$ between –302mV and –480mV (77). Paraquat, also known as methyl viologen, has a midpoint redox potential of –450mV (78). The neutrophil NADPH oxidase, which produces $O_2^{\bar{\bullet}}$ as a mechanism of innate immunity, uses as an electron donor a b-type cytochrome with a redox midpoint potential of E_m= -255 mV (79) which is a bit higher than –330mV, but substantially lower than -140mV.

There are important pitfalls that must be taken into account when using redox potential as a predictive tool for chemical reactions (80). These problems have been summarized elsewhere (78) and it is beyond the scope of this paper to do an in-depth analysis of these. Let it be said though, that redox potential is a measure of thermodynamics and not of kinetics. That is, a reaction might be thermodynamically possible and, yet, the rate (kinetic rate) at which this reaction occurs could be so slow that the reaction has no biological significance. However, free radical/free radical reactions tend to have very low energies of activation and are therefore very well suited for redox potential-based analysis. Moreover, the greater the difference in redox potential (ΔE) between two reaction couples, the stronger the confidence in the predicted reaction (78). Therefore, if a one-electron redox couple has a redox potential far below –140mV, there is a very good chance that it will produce $O_2^{\bar{\bullet}}$. With this knowledge in mind, let us examine why $O_2^{\bar{\bullet}}$ production is observed in some electron transport complexes but not in others.

Tables 1 and 2 give redox potentials for all documented one-electron transfers of the electron transport chain. The data, as a whole, indicate that Complex II and IV should not produce significant amounts of $O_2^{\bar{\bullet}}$. The data also suggest that Complex I and Complex III are the main producers of $O_2^{\bar{\bullet}}$ within the mitochondrial electron transport chain. These predictions match remarkably well with experimental observations.

Table 1

Component	E_m or E^o
NADH/NAD^+	-320mV
FMN/$FMNH_2$	-320mV
FMN/$FMNH^{\bullet}$	-380mV
$FMNH^{\bullet}$/$FMNH_2$	-260mV
N1a 2Fe-2S	-370mV
N1b 2Fe-2S	-250mV
N2 4Fe-4S	-150 to -20mV
N3 4Fe-4S	-225mV
N4 4Fe-4S	-225mV
Chemical analysis indicates that there are another 3-4 iron sulfur clusters but they are not EPR-detectable	
SQnf ubisemiquinone	?mV
SQns ubisemiquinone	?mV
Chemical analysis indicates 2 more quinones but they have not yet been detected or are EPR-silent	
Complex II (succinate dehydrogenase)	
ubiquinol/ubiquinone (in free solution)	90mV
succinate/fumarate	30mV
FAD/$FADH_2$	0 to -90mV
S-1 2Fe-2S	0mV
S-3 3Fe-xS (x=3,4)	130mV
Q_s/$Q_s^{\bullet}$ ubisemiquinone (TTFA-sensitive)	80mV
$Q_s^{\bullet}$/QH_2 (TTFA-sensitive)	140mV
There are 2 more prosthetic groups (an iron sulfur cluster and a b-type cytochrome) but neither is succinate- reducible, indicating that they are not involved in electron transport.	

Sources: (68, 69, 74, 98)

Table 2

Component	E_m or $E^{o'}$
Complex III (ubiquinol:cytochrome c oxidoreductase)	
ubiquinol/ubiquinone (in free solution)	90mV
$Q_o^{\bullet}$/QH_2	700mV to 300mV
Q_o/$Q_o^{\bullet}$ semiquinone	-300mV to -400mV
Q_i/$Q_i^{\bullet}$ semiquinone (yeast)	110mV
$Q_i^{\bullet}$/QH_2 (yeast)	200mV
Cytochrome b_l	-90mV
Cytochrome b_h	50mV
Riekse 2Fe-2S	290mV
Cytochrome c_1	220mV
Cytochrome c	260mV

Sources: (71, 74, 110)

Complex IV (cytochrome c oxidase)	
Cytochrome a	210mV
Cu_A	245mV
Cu_B	340mV
Cytochrome a_3	385mV
$O_{2(g)}$/$H_2O_{(l)}$	815mV

Sources: (187, 188)

To date, $O_2^{\bar{\bullet}}$ production (or indirectly via H_2O_2 production) has only been detected at Complex I (NADH ubiquinone reductase) and Complex III (ubiquinol-cyto-

chrome c reductase) (33, 35, 66, 81-84). It has not been detected at cytochrome c oxidase (35), where $O_{2(g)}$ gets reduced to H_2O. This can be explained by the high redox potential of its prosthetic groups but also by the fact that the terminal reaction with $O_{2(g)}$ to form H_2O occurs by a 2-electron transfer (73). Regarding Complex II, there is considerable evidence that suggests that it does not produce any $O_2^{\bar{\bullet}}$, foremost being that such has not been detected even under inhibitor treatment (33, 35, 56). Moreover, alkaline washing of sub-mitochondrial particles (SMPs), which remove Complex II, does not significantly affect H_2O_2 production (56), which further suggests that it contributes little, if anything, toward $O_2^{\bar{\bullet}}$ production. However, it has been recently published that an oxygen-sensitive mutant of *C. elegans* has a defect in the cytochrome b gene of Complex II (45), which shows elevated levels of oxidative damage. This mutant was isolated based on increased sensitivity to methyl viologen, a redox-cycling agent that, in the presence of a reducing substrate, generates high levels of $O_2^{\bar{\bullet}}$. It also has a reduced lifespan at high levels of atmospheric $O_{2(g)}$ but not under normal oxygen tension. The shortened lifespan and the detection of increased oxidative damage is interpreted by the authors as an indication that the mutant Complex II produces increased levels of $O_2^{\bar{\bullet}}$. While interesting and provocative, this data does not prove that $O_2^{\bar{\bullet}}$ production occurs in WT Complex II, and all other indications are that it does not. It should also be noted that Complex II has several low redox prosthetic groups (not listed in Table 1 because they do not take part in electron transfer) but these do not seem to participate in electron transfer because they are not reducible by succinate (their role being either structural or vestigial). There exists a closely related enzyme in bacteria (possibly ancestral), fumarate reductase, in which these centers are reduced during normal turnover. Incidentally, this enzyme also produces significant amounts of $O_2^{\bar{\bullet}}$ (85). If the mev-1 mutation somehow allowed these redox groups to be reduced (say by NADH for example), they could certainly produce $O_2^{\bar{\bullet}}$.

A few more ubiquinone-dependent enzyme systems may be significant producers of $O_2^{\bar{\bullet}}$ in the mitochondrion. Dihydroorotate dehydrogenase, a member of the pyrimidine synthesis pathway, is located in the outer leaflet of the inner mitochondrial membrane. This enzyme uses ubiquinone as an oxidant to create a double bond in the precursor of uracil and it has been reported that it produces $O_2^{\bar{\bullet}}$ *in vitro* (86). However, there are still questions as to whether it is within the enzyme itself that $O_2^{\bar{\bullet}}$ is produced or whether it is the product, ubiquinol, that produces $O_2^{\bar{\bullet}}$ in some other enzyme complex (such as Complex III). Another potential producer of $O_2^{\bar{\bullet}}$ is the glycerophosphate shuttle. This enzyme is located in the outer leaflet of the inner mitochondrial membrane and catalyses the oxidation of cytoplasmic sn-glycerol phosphate by ubiquinone, through which it feeds cytoplasmic reducing equivalents into the general electron transport chain. $O_2^{\bar{\bullet}}$ production has been experimentally measured at this location (87) in *Drosophila*, and it is likely that it is a significant source of $O_2^{\bar{\bullet}}$ production in insect flight muscles (87). However, its limited distribution in mammals makes it unlikely to be the main $O_2^{\bar{\bullet}}$ producer in the mitochondrion. On the other hand, because of their widespread distribution and high concentrations, Complex I and Complex III are probably the main $O_2^{\bar{\bullet}}$ generators within the mammalian mitochondrion. The next section will explore what is known about $O_2^{\bar{\bullet}}$ production in these two complexes and propose a mechanism based on published data.

$O_2^{\bar{\bullet}}$ production at Complex III

It is by now widely accepted that Complex III (also called cytochrome bc1-complex) produces $O_2^{\bar{\bullet}}$ *in vitro* (33, 46, 65, 88, 89). Although theoretical arguments have been made about why $O_2^{\bar{\bullet}}$ production (under normal conditions) at Complex III could not occur *in vivo* (90-93), experimental evidence clearly suggests that it does (49, 67).

The main experimental factors controlling the rate of $O_2^{\bar{\bullet}}$ production at Complex III *in vitro* are: 1) the presence of Q_i site respiratory inhibitors; 2) the partial pressure of $O_{2(g)}$ and 3) the strength of the membrane potential (56). These factors will be discussed at length in this section. While they can explain experimental variation, what is more relevant to the present discussion is what factors influence the rate of $O_2^{\bar{\bullet}}$ production *in vivo*. Since there is great interspecies variation in the rate of $O_2^{\bar{\bullet}}/H_2O_2$ production (12, 25-27, 30, 31, 33, 35), it is reasonable to infer that this rate is under genetic control. Some experiments have been carried out to determine what the molecular basis for the variation in $O_2^{\bar{\bullet}}/H_2O_2$ production might be, but so far have not led to a breakthrough. It has been shown that the cytochrome content of mammalian mitochondria is inversely correlated with longevity ((33) citing (94, 95)), thus one cannot exclude the possibility that a higher number of complexes account for higher $O_2^{\bar{\bullet}}$ production in the data reported by Sohal *et al* (12, 26, 27). It should be noted that the data were corrected for protein concentration (12, 26, 27). While, at present, there is very little known about what causes interspecies differences in the rate of $O_2^{\bar{\bullet}}/H_2O_2$ production, based on *in vitro* data and knowledge of redox chemistry, one can venture hypotheses as to what these factors might be and what genes might control them. Such a hypothetical list might include:

1. Concentration (partial pressure) and accessibility of $O_{2(g)}$
2. Membrane potential, $\Delta\Psi$
3. Redox potential of the redox active groups
4. Composition of the lipid bilayer
5. Conformation of the electron transport complex
6. State of reduction of the respiratory chain
7. Steady state concentration of high-energy intermediates

While all the factors listed above are very important, it is the author's opinion that the conformation of the complex (because it affects many other factors, such as redox potential of the prosthetic groups, $O_{2(g)}$ accessibility and perhaps steady state concentration of high energy intermediates) is one of the most important factors and therefore will be discussed at length. In other words, it is the properties of the individual electron transport complexes, rather than of the mitochondrion as a whole, that are responsible for the interspecies variation in the rate of $O_2^{\bar{\bullet}}/H_2O_2$ production observed by Sohal and Barja (12, 25-27, 30-33, 35).

The definition of conformation not only includes the three-dimensional structure of a protein but also the sequence composition of its amino acids. Regarding factors that are relevant to $O_2^{\bar{\bullet}}$ production, the conformation of a protein is known to affect O_2 accessibility and redox potential of prosthetic groups. Complex III from *Gallus gallus* is about 240 kD in size and is composed of 11 subunits, one of which (cytochrome b) is mtDNA-encoded (96, 97) (see Figure 1). The complex has 3 (4 if one includes cytochrome c) redox active subunits, the others being required for import, correct assembly and structural integrity of the complex (97). In addition, there are two solvent-exchangeable ubiquinone binding sites on distinct regions of the protein. As illustrated in Fig. 1, the functional redox groups of Complex III include the membrane-spanning cytochrome b, the Rieske Fe_2S_2 protein, and cytochrome c1 (more on these later). Table II gives the midpoint potentials of the prosthetic groups and the semiquinones at Q_o and Q_i sites. It should be noted that these values differ greatly from semiquinones in free solution. The binding pocket itself controls both the redox potential of the pair $CoQ/CoQH_2$ and the pair $CoQ^{\bullet}/CoQ$. The redox potential (midpoint potential) is determined by the intrinsic potential of the quinol/quinone pair (which is controlled by the substitutes on the aromatic ring) but also by the binding site on the protein itself (74). Despite having the same chemical composition, the ubisemiquinone at Q_o has a much lower redox potential (–400 to–300mV) than the ubisemiquinone at Q_i (+110mV). Conversely, the semiquinone at Complex II (which is detectable by electron paramagnetic spectroscopy (EPR) (98)), also has a very high redox potential (+80mV). How then, does the protein binding site control the redox potential of the semiquinone? It is now widely accepted that at least part of the catalytic activity of enzymes is due to the preferential binding of the transition state (99). By binding the transition state more tightly than either the product or the reactant, an enzyme can increase its stability and thereby lower the energy of activation of a chemical reaction (99). In a similar manner, a protein can modulate the redox potential of any semiquinone/quinone (or quinol) pair by preferentially binding one over the other (74). One limitation, however, is that in order not to violate the first law of thermodynamics, the arithmetic mean of the redox potential (E_m) of the first couple ($CoQH_2/CoQH^{\bullet}$) and the second couple

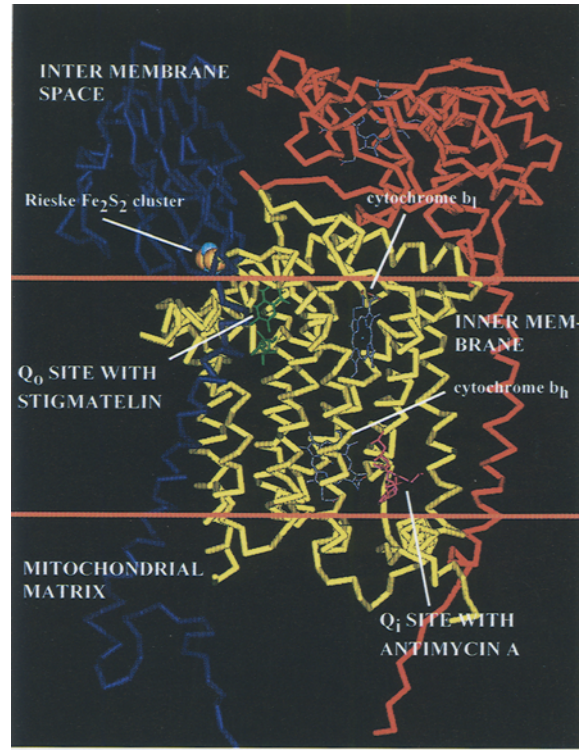

Figure 1. Structure of chicken Complex III with stigmatelin and antimycin A. In its native state, Complex III is a dimer of 11 subunits each. Only the 3 redox active subunits are shown (in the monomeric form); the others are omitted for clarity. Cytochrome c1 is in red, the Rieske iron sulfur protein is in blue and cytochrome b is in yellow. Stigmatelin, a Q_o site inhibitor, is in green. Antimycin A, a Q_i site inhibitor, is in fuschia. The membrane is also pointed out; it was delineated based on the hydrophobicity of the amino acids of cytochrome b. This picture is a rendition of the publicly deposited X-ray coordinates of Berry *et al* (97), using Weblabviewer lite® software.

($CoQH^{\bullet}/CoQ$) must be equal to that of the two electron transfers ($CoQ/CoQH_2$)(74). In this manner, a protein can control the redox potential of any semiquinone and adjust it to fit a particular bioenergetic need.

After this sidenote on ubisemiquinone redox potential, let us now resume the discussion on the other redox active subunits of Complex III.

In its mature form, the cytochrome b protein of *Gallus gallus* is made up of 379 amino acids and contains two b-type hemes, termed b_l (for cyt b low redox potential) and b_h (for cyt b high redox potential). The cytochrome b protein is predominantly made up of alpha helices, nine of which span the membrane and enclose the b-type hemes. The cytochrome b protein also contains the Q_o (ubiquinol oxidation site) and Q_i (ubiquinone reduction site) ubiquinone-binding sites on the cytosolic and matrix sides of the inner mitochondrial membrane, respectively. The Rieske iron sulfur protein (Fe_2S_2) is found on the cytosolic face of the inner mitochondrial membrane, although it is anchored to the rest of the complex via a transmembrane helix. The position of the Rieske iron sulfur protein shifts quite extensively between a cytochrome b-proximal position and a cyto-

chrome c1-proximal position. These positions depend on the presence and type of inhibitors (97). Most investigators argue that the motion of the Rieske iron sulfur protein is, in fact, an integral part of the normal turnover of the complex (96, 97, 100). Cytochrome c1 is about 30 angstrom from the Q_o site and it is to explain the motion of electrons across such a long distance that the movement of the Rieske iron sulfur protein is thought to occur. Cytochrome c is the final electron acceptor in Complex III and it links the electron flow to cytochrome c oxidase. The last two redox groups, cytochrome c1 and cytochrome c, are of only limited importance to this discussion because they are not directly involved in Q_o site semiquinone formation which, as will be explained later, is the crucial step in $O_2^{\bar{\bullet}}$ production. Let us now examine what the structure of the complex can tell us about the pathway of electron transfer through Complex III.

The X-ray structure of Complex III has been solved for both bovine and chicken bc1 complex (96, 97, 101). The positions and orientations of the two cytochromes were determined using EPR, which forced the revision of an early crystal structure (102). Although the structures were determined in two independent laboratories (96, 97), they match well, as would be expected from the high degree of sequence homology. The structure lends strong support to the "modified Q-cycle" hypothesis of electron transfer and confirms the existence of two distinct quinone binding sites (96, 97, 101). The structure reveals that the Q_o site is oriented towards the intermembrane space. Conversely, the Q_i site is located towards the matrix side of the inner mitochondrial membrane. The pathway of electrons is described in Figure 2. For every two electrons that enter Complex III, one is "recycled" and goes to Q_i by the intermediate of cytochrome b_l and b_h. There, it is used to reduce ubiquinone to ubisemiquinone. A second cycle is then needed to transfer a second electron through the same pathway (that is from Q_o to Q_i via the b_l and b_h cytochromes) to reduce the semiquinone to ubiquinol. Ubiquinol then diffuses out of the site and eventually is oxidized at Q_o. The other electron (in both cycles) goes to the Rieske iron sulfur protein, followed by cytochrome c1 and cytochrome c. Recycling of the second electron allows for the pumping of an extra proton per electron (103). While this general model is now accepted by most investigators, there is substantial discord regarding the mechanism of ubiquinol oxidation at the Q_o site. While numerous models have been proposed, most lack any real experimental support. The Crofts model however, stands out in that it both fits very nicely with X-ray crystallographic and thermodynamic data (71, 104). In addition, it agrees remarkably well with Marcus theory predictions (105). This model argues that ubiquinol, bound in the cytochrome b-distal pocket of the Q_o site, is first oxidized by the Rieske iron sulfur protein (which is the rate determining step), to form the semiquinone (104). A proton is then abstracted from the semiquinone by histidine 161 of the Rieske iron sulfur protein. As the semiquinone moves from the distal pocket into the cytochrome b_l proximal pocket, the Rieske iron sulfur protein moves from the Q_o site to cytochrome c1. The semiquinone is then oxidized by cytochrome b_l and the second proton is abstracted by glutamic acid 272 (of cytochrome b) (104). A Chime-animation of this model using the X-ray coordinates of Berry *et al* is available via the internet (100). The mechanism of $O_2^{\bar{\bullet}}$ production will be discussed in the framework of this model. Before discussing $O_2^{\bar{\bullet}}$ production at length, a short review on Complex III inhibitors is in order because inhibitors have very strong effects on the rate of $O_2^{\bar{\bullet}}$ production by Complex III.

Inhibitors of Complex III have been grouped into three categories based on competitiveness and effects on cytochrome b spectra. All three groups are competitive inhibitors with regards to ubiquinol (98) and hence were postulated to bind in the quinol binding pockets. A co-crystal of Complex III with stigmatelin and antimycin A confirmed that this is indeed the case (96, 97). Group I (stigmatelin) and group II inhibitors (myxothiazol) bind to the Q_o site. They are mutually competitive (as well as competitive with ubiquinol) and the only difference is that each binds the Q_o site in a slightly different manner (97). Group II inhibitors have a stronger effect on the spectral properties of cytochrome b_l and the X-ray structure shows myxothiazol binding closer to cytochrome b_l than stigmatelin (101). Hence, group II inhibitors are called "proximal" and group I are called "distal" Q_o site inhibitors (104). Group III inhibitors (antimycin A, funicoloscin) are not competitive with either group I or group II inhibitors and they affect the spectrum of cytochrome b_h. Group III inhibitors are thus Q_i site inhibitors. Q_i inhibitors such as antimycin A and funicoloscin (group III) drastically increase $O_2^{\bar{\bullet}}$ production in submitochondrial particles and reconstructed vesicles containing Complex III (56, 106, 107). Inhibitor studies suggest that the semiquinone located at Q_o is the main electron donor to form $O_2^{\bar{\bullet}}$ (108). When antimycin and, subsequently, myxothiazol are added to a mitochondrial preparation, superoxide production is negligible. Under those conditions, both cytochromes b_l and b_h are in the reduced state, yet $O_2^{\bar{\bullet}}$ production is negligible, excluding them as electron sources for the production of superoxide (108). When myxothiazol is added alone, superoxide production is also negligible, yet the semiquinone at Q_i is still EPR-detectable, excluding it as a source of electrons for the generation of superoxide (108). When antimycin A is added alone, $O_2^{\bar{\bullet}}$ production is drastically increased. Under those conditions, the semiquinone at Q_o is stabilized because cytochrome b_l is reduced and therefore cannot oxidize the semiquinone to form ubiquinone. Having excluded both cytochrome b_l and b_h as well as the Q_i semiquinone, the only possible electron donor to oxygen is the semiquinone at Q_o (108). Thus, taking into account the topology of Complex III (with Q_o facing the cytosol or intermembrane space (101)), in the presence of antimycin A, $O_2^{\bar{\bullet}}$ production at Complex III is directed toward the intermembrane space, although the evidence sug-

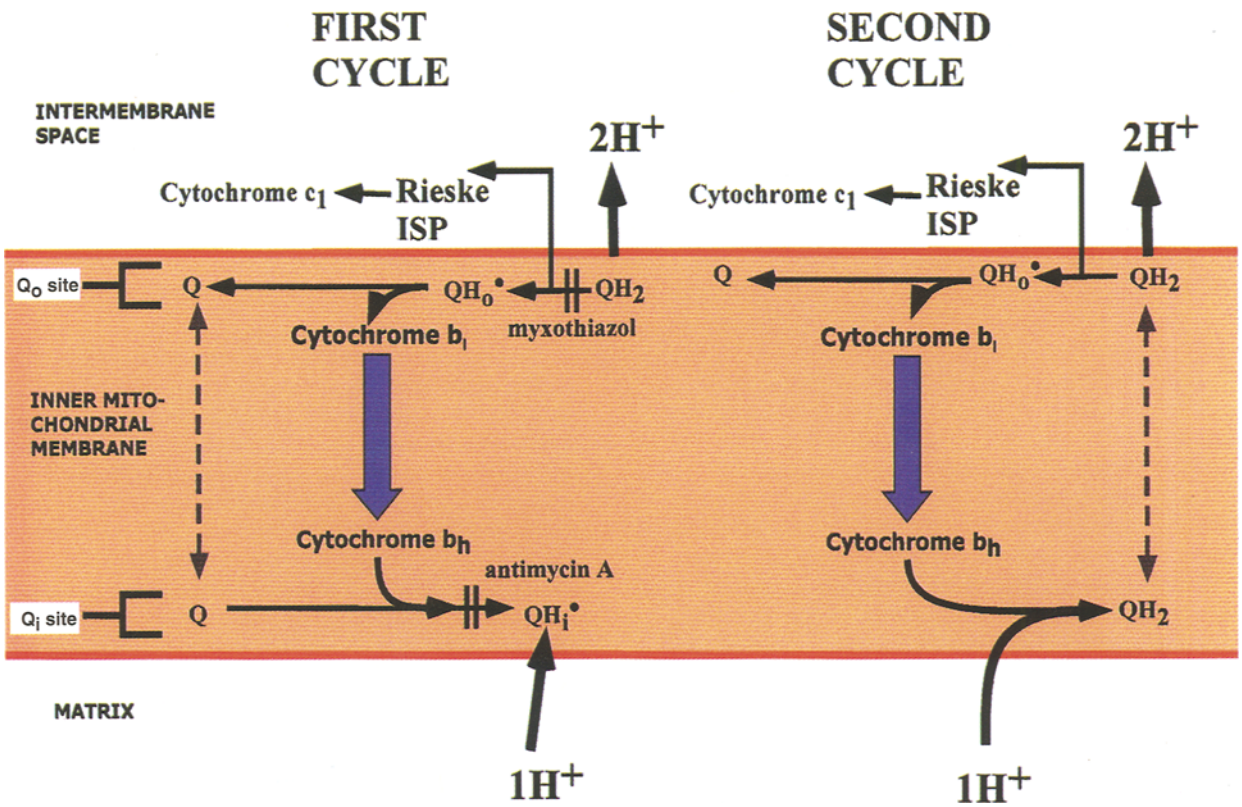

Figure 2. The modified Q cycle. Ubiquinol docks at the Q_o site where one electron is transferred to cytochrome c via the Rieske iron sulfur protein (high potential chain). The other electron is transferred to cytochrome b_l, cytochrome b_h (the low potential chain) and then reduces ubiquinone at the Q_i site to form the stable Q_i semiquinone (antimycin A-sensitive). The oxidation of ubiquinol at the Q_o site results in the release (pumping) of 2 protons. This cycle is repeated and the second electron that goes through the low potential chain reduces the Q_i semiquinone to ubiquinol. The very low redox potential of the Q_o semiquinone (which makes it susceptible to superoxide production) is required to efficiently reduce the low potential chain and pump one extra proton per electron transferred to cytochrome c. In this manner, oxidation of a single ubiquinol molecule results in the translocation of 4 protons instead of simply 2 protons. The large arrow between cytochrome b_l and b_h indicates that this is the major electrogenic step (and hence the step most slowed by increasing $\Delta\Psi$) in the turnover of the complex. Sketch based on Trumpower *et al* (186).

gests that it can exert deleterious effects on either side of the membrane (49, 67, 109). An obvious question that then arises is why the semiquinone at Q_o would produce $O_2^{\bar{\bullet}}$ while other semiquinones such as those of Complex II and Complex III-Q_i do not. This fundamental difference can easily be explained by the large redox potential difference between those semiquinones. The redox potential of the semiquinone at Q_o is in the order of –300mV to –400mV (71) while the redox potential of the semiquinone at the Q_i site is 110mV (110) and that of the semiquinone at Complex II is +80mV (98). The difference in redox potential (–400 to –300mV vs. +110mV, +80mV) can explain why $O_2^{\bar{\bullet}}$ production occurs at the Q_o semiquinone but not at the Q_i and Complex II semiquinones. It should be noted that the low potential of the Q_o semiquinone is necessary for the efficient reduction of the low potential chain (71). It is also worth remembering that the reduction of O_2 to $O_2^{\bar{\bullet}}$ requires a very strong electron donor because of the low redox potential of that couple (E_m $O_{2(g)}$/ $O_{2(aq)}^{\bar{\bullet}}$ = –140mV).

While numerous works show that Complex III produces $O_2^{\bar{\bullet}}$ *in vitro*, what is the evidence that it also does so *in vivo*? Inhibitors that decrease $O_2^{\bar{\bullet}}$ production by Complex III *in vitro* were also found to prevent the loss of viability of a yeast SOD2 k.o. in the post-diauxic (respiratory) phase (67). Conversely, inhibitors (antimycin A) that increase the rate of $O_2^{\bar{\bullet}}$ production at Complex III *in vitro* also drastically decrease the growth rate of an SOD1 k.o. yeast on rich glucose medium and drastically increase the rate at which it loses viability in stationary phase (Muller F. and Kramer D., unpublished observations). These effects of antimycin A were not observed in WT cells. In addition, the deleterious phenotypes of an SOD1 k.o yeast can be substantially alleviated by knocking out COQ3, a gene required for ubiquinone biosynthesis (49). This is consistent with the notion that a large source of $O_2^{\bar{\bullet}}$ is due to ubisemiquinone radicals. Overall, the evidence strongly suggests that Complex III produces $O_2^{\bar{\bullet}}$ *in vivo*. Let us now consider what insights the structure of the Q_o binding site can give us about the mechanism of $O_2^{\bar{\bullet}}$ production.

The structure of the Q_o site (101) shows that there are two possible ways for $O_{2(g)}$ to come into contact with the semiquinone (see Figure 3). The first and most obvious

way is through the "hole" on top of cytochrome b, which makes contact with the aqueous phase. The second, less obvious way is through the lipid phase entry point to the Q_o site (Figure 3), along the ubiquinol tail. Based on these considerations, I propose two mutually non-exclusive mechanisms for $O_2^{\bar{\bullet}}$ production. The first mechanism is as follows: $O_2^{\bar{\bullet}}$ is produced in the aqueous phase by an electron transfer from ubisemiquinone to $O_{2(g)}$. This electron transfer goes downhill with the membrane potential, which is favorable in energy. Since the reduction of $O_{2(g)}$ would occur in the aqueous medium, the reduction potential would be $O_2/O_2^{\bar{\bullet}}$ E_m= -140mV. This is a plausible scenario yet it has its problems. The Q_o site is, in fact, a few angstroms within the membrane and the great majority of the amino acids that make up the site are hydrophobic (101). This suggests that the Q_o site itself is not in the aqueous phase. Nevertheless, there is an opening at the top of the Q_o site that is used by the Rieske iron sulfur protein to oxidize ubiquinol (111). This channel connects the Q_o site to the aqueous phase. It is conceivable that an electron could escape to $O_{2(g)}$ in the aqueous phase as the Rieske protein undergoes its rotation. Nevertheless, even through this channel the contact area between the semiquinone and the aqueous phase remains relatively small and, when combined with the low stability constant of the semiquinone (68, 71), the reaction between the semiquinone and $O_{2(g)}$ in the aqueous phase seems somewhat unlikely.

The alternative mechanism I propose is that $O_2^{\bar{\bullet}}$ is produced in the hydrophobic environment of the membrane. Production of $O_2^{\bar{\bullet}}$ in the hydrophobic phase is very attractive for several reasons, one of which is that $O_{2(g)}$ is, in fact, several-fold more soluble in the lipid phase, resulting in a steady state concentration of $O_{2(g)}$ about 8-fold higher in that environment *vs.* in the aqueous phase (80, 89). Also, the X-ray structure of Complex III shows that there is considerable space in the hydrophobic part of the Q_o docking site, such that $O_{2(g)}$ could easily diffuse into the Q_o site from inside the membrane along the ubiquinol tail. The cavity is so spacious (see Figure 3a) that it could not disprove a very exotic model for Q_o oxidation which postulated the existence of two quinones at the Q_o site (although not generally accepted by most investigators for lack of evidence (112)). Also, contrary to in the aqueous phase, a large proportion of the collisional surface of the semiquinone would be exposed to $O_{2(g)}$. In short, this model is very attractive and previous investigators have taken it for granted that $O_2^{\bar{\bullet}}$ production occurs in the lipid phase (89, 93). Recent

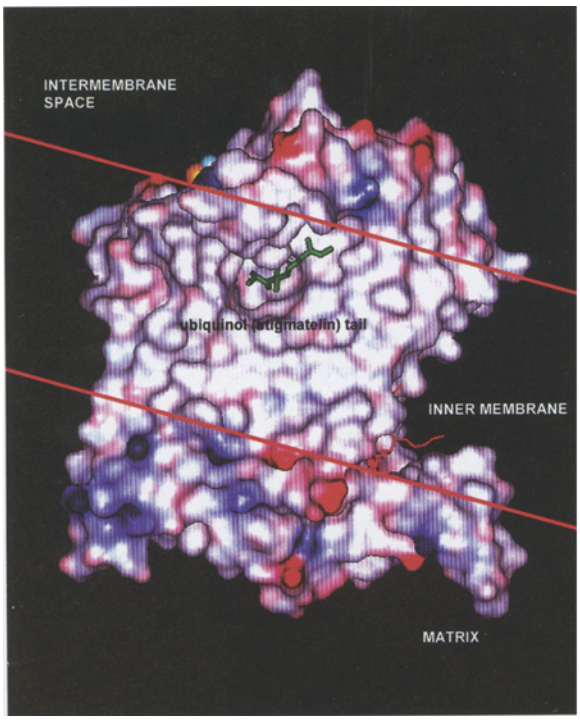

Figure 3A.

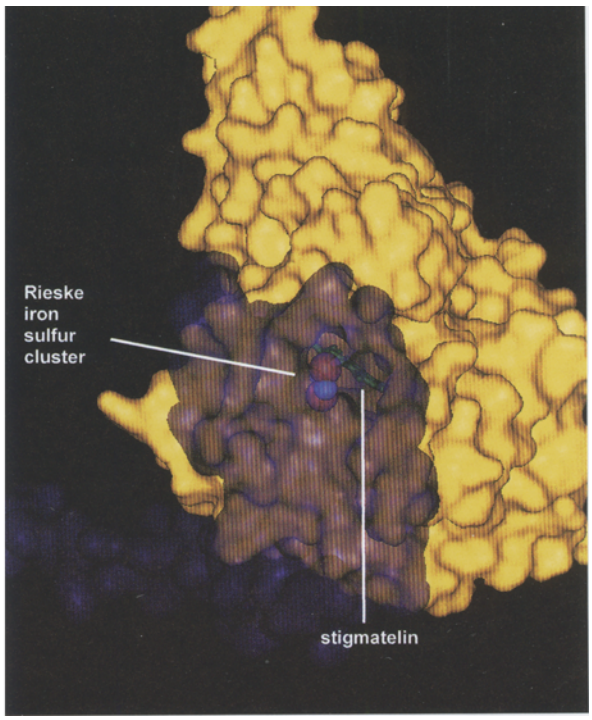

Figure 3B.

The two possible contact sites between $O_{2(g)}$ and the Q_o semiquinone. On the left, in Figure 3a, is a side view (from inside of the membrane) of a surface rendition of cytochrome b. The color coding is based on charge, white being absence of charge. The ubiquinol tail (in this case the stigmatelin tail) extends from the Q_o site. $O_{2(g)}$ could easily diffuse into this cavity from the membrane itself. Figure 3b shows the Rieske iron sulfur protein (transparent blue) docking with cytochrome b (yellow). This view is looking down on the Q_o site from the intermembrane space. Note how the Rieske iron sulfur protein shields stigmatelin (normally the Q_o semiquinone) from the aqueous phase. While it is certainly conceivable that $O_{2(g)}$ could manage to go past this barrier (to go on reacting with the semiquinone), taking into account the low concentration of $O_{2(g)}$ in the aqueous phase and the low stability constant of the semiquinone, its oxidation by $O_{2(g)}$ from the aqueous phase does seem somewhat improbable.

experimental evidence, using Complex III in reconstituted phospholipid vesicles, supports this assumption (107). However, there is a substantial problem with the above model which is that the reduction potential of $O_2^{\bar{\bullet}}$ in the hydrophobic phase (as opposed to aqueous phase) is in the order of –700mV (113). This very low reduction potential is due to the creation of a negative ion and its unfavorable solubility in an aprotic environment (113). Even if we assume the lowest value for the Q_o semiquinone, –400mV, it is still too high to reduce O_2 to $O_2^{\bar{\bullet}}$ in the hydrophobic phase. However, if instead of $O_2^{\bar{\bullet}}$, $HO_2^{\bullet}$ (the protonated version of $O_2^{\bar{\bullet}}$) were produced in the hydrophobic phase, the high redox barrier would be eliminated because no anion would be formed. The revised standard redox potential (E°) of $HO_2^{\bullet}$, derived from the standard free energy of formation (ΔG°) is +153mV (76). Since the standard free energy of formation (ΔG°) is measured at pH 0, this value has to be adjusted to pH 7 (74, 75). Using the Nernst equation, a value of –262 mV is obtained. This value is for aqueous solutions but since the reaction would occur in a low dielectric environment (the Q_o site), the number should be somewhat higher (perhaps around –200mV). $HO_{2\,(lipid)}^{\bullet}$ produced in the hydrophobic phase of the Q_o site could then diffuse to either side of the inner mitochondrial membrane, and the bulk would dissociate to $O_2^{\bar{\bullet}}$ (and H^+) as soon as it came in contact with water. From a thermodynamic perspective, is there data to suggest this is correct? Regarding protonation, it is well established that $O_2^{\bar{\bullet}}$ is a strong base (114) and the pKa of its conjugate acid, $HO_2^{\bullet}$, has been measured at 4.88 (115). This value indicates that $O_2^{\bar{\bullet}}$ could efficiently compete for protons inside the Q_o site (glutamic acid has a pKa of 3.97). The proton transfer could occur through a direct hydrogen atom transfer between the semiquinone and $O_{2(g)}$ or, after its formation, via protonation from either glutamic acid 272 or the resulting quinone cation. Regarding thermodynamics, there is also good evidence to indicate that, were the electron transfer to occur in the lipid phase, $HO_2^{\bullet}$ instead of $O_2^{\bar{\bullet}}$ would be produced. In a defined aprotic chemical system, ubiquinone can be completely reduced to the semiquinone anion by stoichiometric amounts of potassium superoxide (93). The equilibrium is on the side of the semiquinone anion and no superoxide is detected. When protons are added, the equilibrium is reversed, with the formation of superoxide and disappearance of the semiquinone (92, 93). This is consistent with the notion that protonation increases the rate of the reaction by increasing the redox potential of $O_2^{\bar{\bullet}}$ (that is, by forming $HO_2^{\bullet}$). Based on the above data and the incorrect assumption that there are no protons at the Q_o site, it has been repeatedly argued that production of superoxide in the lipid phase is thermodynamically impossible (90, 92, 93).

There is very good indirect experimental evidence supporting the fact that $HO_2^{\bullet}$ can cross biological membranes and exist there in significant quantities. It has been documented that $O_2^{\bar{\bullet}}$ generated inside synthetic vesicles (at pH 7.3) can, in fact, reach the outside, although 90% spontaneously dismutates before doing so (116). Regarding quantification of $HO_2^{\bullet}$ inside membranes, no experiments have yet been carried out. However, the production of $HO_2^{\bullet}$ instead of $O_2^{\bar{\bullet}}$ at Complex III could explain a series of discrepancies in the measurements of the rate of $O_2^{\bar{\bullet}}$ production. In the traditional view, most $O_2^{\bar{\bullet}}$ is produced toward the matrix side (117). This is based on the observation that inverted SMPs (the matrix side faces the outside of the vesicle) treated with antimycin A produce large amounts of superoxide. It was assumed that, due to its charged nature, superoxide could not cross any membrane and therefore must originate from the matrix side (the outside for SMPs) (55, 117). However, these measurements fall about 50% lower than what would be expected from the measurement of H_2O_2 in SMPs and intact mitochondria (89). $HO_{2\,(lipid)}^{\bullet}$ could explain these inconsistencies. When one measures $O_2^{\bar{\bullet}}$ production in the presence of antimycin A in submitochondrial particles (under which conditions $O_2^{\bar{\bullet}}$ production occurs at the Q_o site (108)), if one accepts: a) the fact that those vesicles are inside out (118, 119)(see Figure 4), and b) the topology of Complex III as determined from X-ray crystallography (101), then one must accept that $O_2^{\bar{\bullet}}$ can diffuse through the inner mitochondrial membrane because under those conditions $O_2^{\bar{\bullet}}$ *is generated towards the inside of the vesicle* (cytochrome c, the probe used in these experiments, cannot cross that membrane). Could it be that $HO_{2\,(lipid)}^{\bullet}$ produced at the Q_o site diffuses through the membrane and reacts with cytochrome c outside the vesicle? Evidently, there are alternative explanations to account for this behavior. For example, it might be that $O_2^{\bar{\bullet}}$ travels through an anion transport channel (such as a chloride channel) or that not all SMPs are actually inverted. However, the production of $HO_{2\,(lipid)}^{\bullet}$ could explain the inconsistency in the measurements of the rate of H_2O_2 production and $O_2^{\bar{\bullet}}$ production in SMPs (89). Such an explanation would posit that a large proportion of $HO_{2\,(lipid)}^{\bullet}$ produced at Q_o dismutates (to H_2O_2) in the aqueous interior of the SMP, resulting in an underestimation of the rate of $O_2^{\bar{\bullet}}$ production. Moreover, there is additional evidence that $HO_2^{\bullet}$ may indeed be produced. When SMPs are treated with antimycin A and, subsequently, with α-tocopherol, the rate of $O_2^{\bar{\bullet}}$ production can be drastically decreased (120). The reaction of $O_2^{\bar{\bullet}}$ with α-tocopherol is very slow, if it occurs at all (115, 121). On the other hand, $HO_2^{\bullet}$ reacts very rapidly with α-tocopherol with a rate constant of around 10^5 M^{-1} s^{-1} (115, 121), which is comparable to the rate constant of cytochrome c reduction by $HO_2^{\bullet}$ (115). Hence, a plausible explanation is that $O_2^{\bar{\bullet}}$ production is decreased (in SMPs treated with antimycin A) by α-tocopherol treatment because it scavenges a significant fraction of $O_2^{\bar{\bullet}}$ (in the form of $HO_{2\,(lipid)}^{\bullet}$) while it is inside the membrane, preventing it from reacting with the probe. While this does not disprove that some $O_2^{\bar{\bullet}}$ may pass through chloride channels (or other alternatives), it suggests that a significant fraction of $O_2^{\bar{\bullet}}$ occurs in the form $HO_{2\,(lipid)}^{\bullet}$ at one point or another.

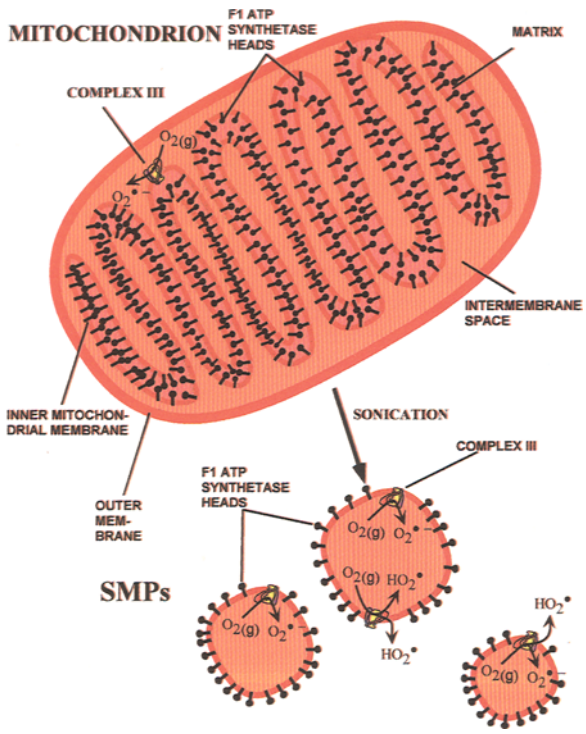

Figure 4. The topology of SMPs vs. intact mitochondria. In intact mitochondria, the Q_o site (where the superoxide-producing semiquinone is formed) is facing the intermembrane space. When mitochondria are sonicated, they are turned inside out such that the matrix-facing side of the inner membrane now faces the outside of the vesicle. Conversely, the Q_o site now faces the inside of the vesicle. Hence, in the presence of antimycin A, superoxide production is directed toward the inside of the vesicle. The topology (that is which side faces inward, and which faces outward) of SMPs was resolved by electron microscopy of the F_1 subunit of the ATP synthetase (the "lollipops")(118).

In short, there is robust evidence that the Q_o semiquinone at Complex III might actually produce $HO_2^{\bullet}$ instead of (or as well as) $O_2^{\bar{\bullet}}$. It is conceivable that this can be extrapolated to all low potential semiquinones (including those of Complex I).

Mechanistic aspects aside, no discussion of $O_2^{\bar{\bullet}}$ production at Complex III would be complete without mentioning the effect of the proton motive force (Δp) on the rate of $O_2^{\bar{\bullet}}$ production. In the absence of any inhibitors, Complex III $O_2^{\bar{\bullet}}$ production is highest under state 4 conditions. That is when the isolated mitochondria or SMPs are supplemented with excess substrate (in the case of Complex III, a Complex II linked substrate, succinate, is added at around 5mM) and no ADP+P_i is available. Under those conditions, the proton motive force (Δp) is at its highest, the electron transport chain components are in the reduced state and the electron transport complexes are in equilibrium with Δp. It is said that mitochondria *in vivo* are somewhere between state 4 and 3 (state 3 is defined as mitochondrial respiration with substrate and excess ADP+P_i available, (122)), but more closely toward state 4 (122). Δp is made up of two components, $\Delta\Psi$, the electrical membrane potential and ΔpH, the pH gradient. The strong positive correlation between proton motive force (Δp) and $O_2^{\bar{\bullet}}$ production has been documented by many investigators (66, 107, 123) and it is widely accepted that in the absence of respiratory inhibitors, the strength of Δp is one of the main determinants of the rate of $O_2^{\bar{\bullet}}$ production (56, 123). In fact, the addition of even very low levels of uncouplers can decrease $O_2^{\bar{\bullet}}$ production (124), an observation that has been recently confirmed *in vivo* (67, 125). Skulachev has done extensive work on the correlation between H_2O_2 production and Δp in isolated mitochondria (123, 126) and has formulated a kinetic model to explain this phenomenon (127).

It is the author's opinion that high membrane potential ($\Delta\Psi$) slows the oxidation of cytochrome b_l by cytochrome b_h and therefore increases the steady state concentration of the semiquinone species (at Q_o; see Figure 5). Although the difference between the redox midpoint potentials of b_l and b_h is high (-130 mV, in Complex III extracted from *Bos taurus* heart mitochondria (74)) the electron transfer crosses the 75% dielectric core of the inner mitochondrial membrane antiparallel to the electric field that spans that membrane. Hence, the electron has to move uphill in the electric field, which requires more energy with increasing strength of the field. In simpler terms, the higher the membrane potential, the slower the oxidation of cytochrome b_l by b_h, the slower the oxidation of the ubisemiquinone (at Q_o) by b_l, the higher its steady state concentration, the higher the rate of $O_2^{\bar{\bullet}}$ production. A similar explanation was given by Skulachev *et al* in their kinetic model of $O_2^{\bar{\bullet}}$ production (127), however they conclude that $\Delta\mu H^+$ (equivalent to Δp, which is equal to $\Delta\Psi+\Delta pH$) is responsible, irrespective of its composition. An analogous situation develops when mitochondria are treated with antimycin A, in that both cytochrome b_l and b_h are locked in the reduced state and the steady state concentration of the Q_o semiquinone is increased. Although this theoretical explanation is very appealing, there is currently little experimental evidence to support it. However, the prediction made by this hypothesis is very straightforward: if the redox potential difference between cytochrome b_h and b_l is decreased, the rate of $O_2^{\bar{\bullet}}$ production should increase. If this hypothesis turns out to be correct, it could provide a very reasonable explanation for the interspecies variation in the rate of $O_2^{\bar{\bullet}}/H_2O_2$ production observed by Sohal and Barja (12, 25-27, 30-33, 35).

$O_2^{\bar{\bullet}}$ production at Complex I

Lately there has been a renewed interest in Complex I because of the finding that several inherited diseases are caused by mtDNA mutations in genes coding for Complex I subunits (128). It has also been suggested that at least some of the phenotypes observed are due to increased production of $O_2^{\bar{\bullet}}$ (128). Anecdotally, it has recently been reported that a single nucleotide polymorphism in the ND2 subunit of Complex I is found at higher frequency in Japanese centenarians than in non-centenarian controls (129). These data could be interpreted

as indicating that this polymorphism results in greater longevity due to decreased rate of $O_2^{\bar{\bullet}}$ production. *In vitro*, $O_2^{\bar{\bullet}}$ production has been measured at Complex I by several investigators: in isolated Complex I (81, 130), in submitochondrial particles (82, 130, 131) and in intact mitochondria measuring H_2O_2 production (32, 35, 47, 66). However, as of yet, there is no evidence that it does so *in vivo*. Most *in vitro* work was done with partially purified Complex I (81, 130) or SMPs, and one of the main issues is what percentage of $O_2^{\bar{\bullet}}$ is actually produced by Complex I *vs* Complex III. Some authors have found that Complex I is the higher $O_2^{\bar{\bullet}}$ producer in brain or heart mitochondria (35, 66), while others have found equal contribution (in heart mitochondria, (82)) while even others have found that Complex III to be the major producer (81). This author is inclined to think that for equal stoichiometry between Complex I and Complex III (in state 4 conditions), Complex I will be the higher $O_2^{\bar{\bullet}}$ producer. The rationale behind this is that Complex I is located "higher" in the respiratory chain than Complex III and the midpoint redox potentials of its prosthetic groups are lower (in simplified terms, lower electron affinity, see Table 1). It follows from this, all things being equal, that $O_2^{\bar{\bullet}}$ production is expected to be higher at Complex I than at Complex III, since it is easier for $O_{2(g)}$ to oxidize electron carriers with lower redox potential.

There is presently no X-ray crystallographic picture of Complex I, although John Walker's group is actively working on it. However, there is substantial structural information based on electron microscopy and EPR (132-136). The general shape of the complex is known from electron microscopy and it resembles a shoe ((132-134) see Figure 6). It has a long protruding arm (protruding into the mitochondrial matrix) which is also called the "Hydrophilic fraction". Complex I is made up of 43 subunits (7 of which are mtDNA-encoded) and its molecular mass is close to 1000 kD, making it one of the most intricate and complex proteins known (134). The sequence of all subunits is known for *Bos taurus*, *Neurospora* and *Homo sapiens* (69). Elemental chemical analysis indicates that the prosthetic groups include one FMN, and perhaps as many as 9 iron sulfur clusters

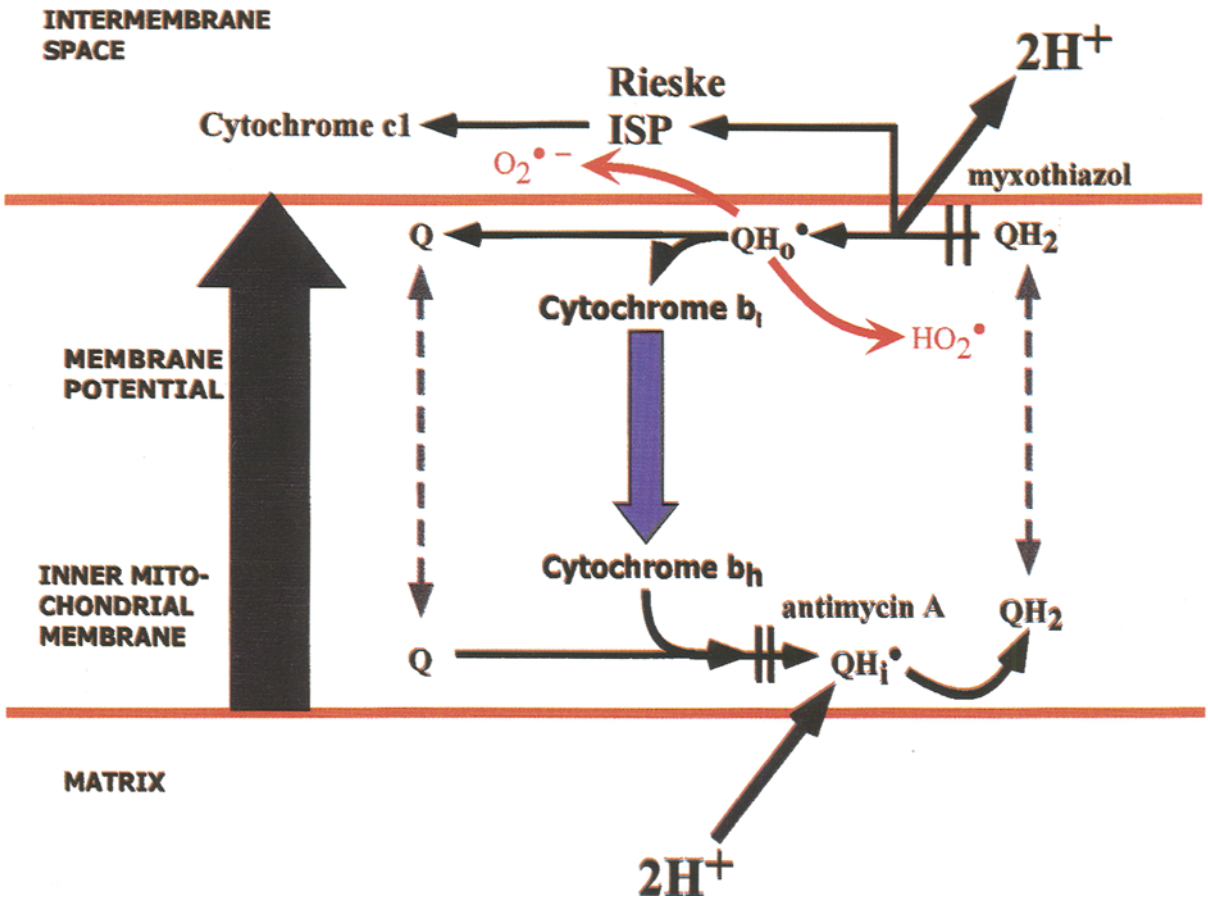

Figure 5. This diagram represents electron transfer through Complex III. The large arrow to the left indicates the direction of the membrane potential (from negative to positive). To the right, the electron transfer between cyt b_l and cyt b_h runs anti-parallel to the membrane potential. The drop in redox potential from cyt b_l to cyt b_h is quite considerable (–130mV) but at a critical value of Δp, the drop in redox potential will not be enough to compensate for the energy required to move uphill in the electric field. When this happens, cyt b_l will remain in the reduced state and will be unable to accept an electron from the Q_o site ubisemiquinone. This will increase the half-life of semiquinone allowing it to react with $O_{2(g)}$ to form $O_2^{\bar{\bullet}}$ and/or $HO_2^{\bullet}$

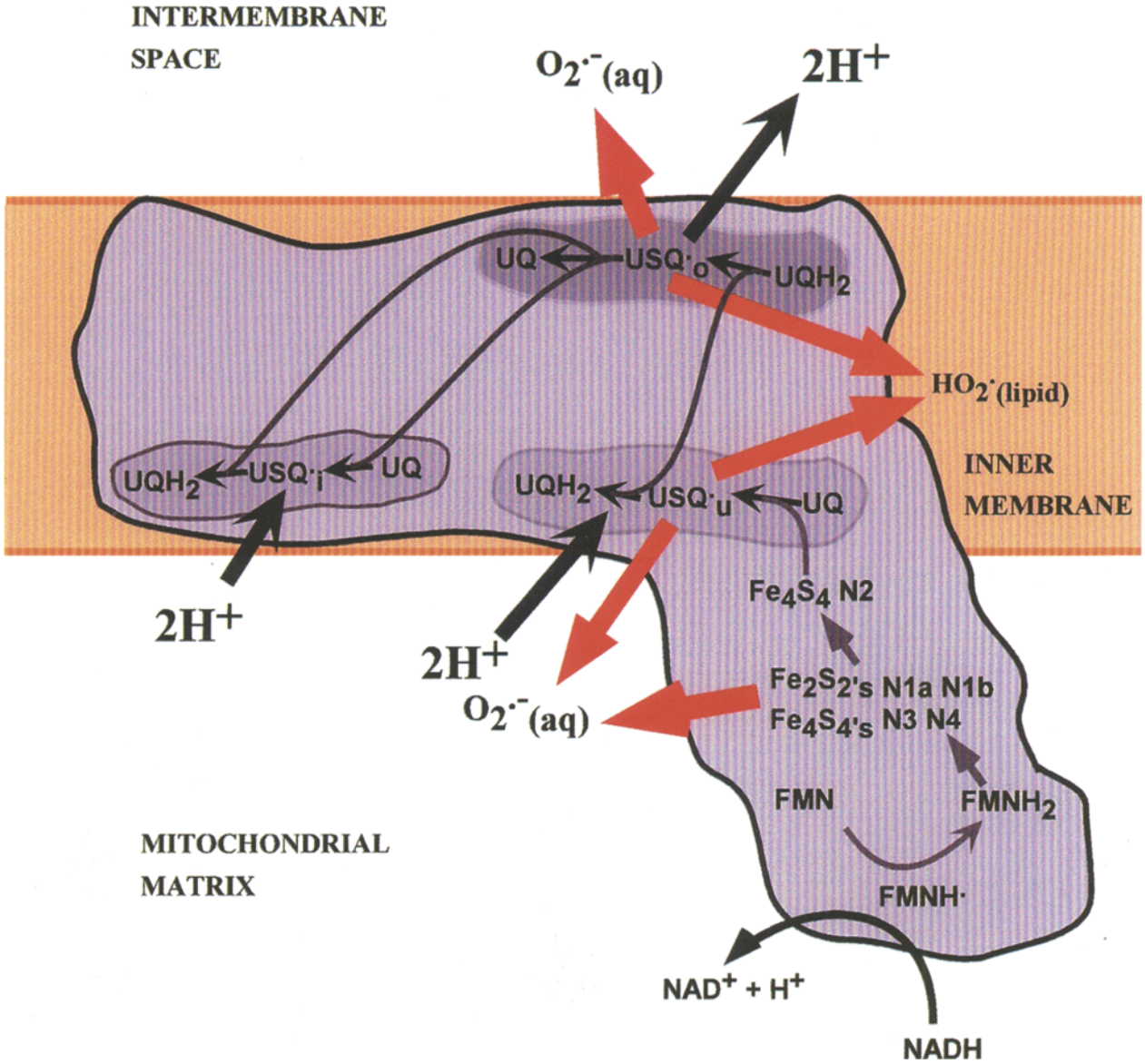

Figure 6. Electron transport through Complex I. The three quinone binding sites are circled. Both the Q_o-like and the Q_u semiquinones are expected to produce $O_2^{\bullet -}$ due to their low redox potential. As with the Q_o semiquinone of Complex III, it is expected that both can sustain $HO_2^{\bullet}$ as well as $O_2^{\bullet -}$ production. The iron sulfur clusters on the other hand, since they are located in the hydrophilic fraction, are expected to produce only $O_2^{\bullet -}$. For a detailed explanation of the mechanism, see (138). The structural information is based on electron microscopy studies, sequence homology, purification and EPR.

(69). The number of reported ubiquinone binding sites varies depending on the author but the consensus right now is 3 binding sites (69). Two semiquinones are readily detectable by EPR (135-137) and there may be as many as three operational Q cycles (68, 69, 135, 136, 138). Several mechanisms have been proposed to explain the $4H^+/2$ electron stoichiometry. The two models most consistent with the available evidence are the redox gated ligand conduction (138) and reduction-induced oxidation mechanisms (68). Both models postulate 3 semiquinones and both include the cluster N2 as a centerpiece in proton translocation. For the purposes of $O_2^{\bullet -}$ production, both models can be treated almost identically in that expected $O_2^{\bullet -}$ production sites are the same in both models. A general outline is given in Figure 6. This is a sketch by the author based on those of Brandt and Ohnishi (68, 138). The structural information, that is, the general shape of the complex and position of the iron sulfur clusters, is not arbitrary but based on experimental evidence. The distance relationships between the semiquinones and the iron sulfur proteins, as well as the general placement of the subunits, are based on EPR (68, 135-137). Both models predict the existence of Q_o-type sites, defined earlier as quinol oxidation sites facing the intermembrane space (139). Several authors have suggested their existence, mainly based on the fact that Complex III Q_o site inhibitors (myxothiazol, stigmatelin) inhibit Complex I activity at higher concentrations (139). There also exists sequence homology between a subunit of Complex I and

the Q_o binding region of cytochrome b (140). The most potent inhibitor of Complex I, piericidin A, also inhibits Complex III at a slightly higher concentration (139). This putative Q_o site is of great interest for the generation of $O_2^{\bar{\bullet}}$ because of the low redox potential semiquinone intermediate. Both the Ohnishi and Brandt models also postulate a Q_i type site. There is extensive sequence homology between subunit 6 of Complex I and the Q_i site of Complex III, suggesting that such a site is indeed present (141). This quinone binding site is of limited interest because the semiquinone there is expected to be stable (high redox potential, like the Q_i semiquinone of Complex III). The last quinone binding site is postulated to be unique to Complex I and reduction of ubiquinone by a high energy electron occurs there. This is the most interesting ubiquinone binding site for $O_2^{\bar{\bullet}}$ production because the semiquinone (termed SQ_u in Figure 6) there is expected to be extremely unstable because it receives low energy electrons directly from NADH (–330mV (68)).

Although no X-ray crystallographic picture exists, there is substantial structural information that has relevance for $O_2^{\bar{\bullet}}$ production. EPR coupled redox titrations have yielded redox potentials of the iron sulfur clusters that are well within the range required for $O_{2(g)}$ reduction (to form $O_2^{\bar{\bullet}}$, see Table 1). Moreover, it has also been established that the EPR detectable iron sulfur clusters, as well as FMN, are within the protruding arm of Complex I (Hydrophilic fraction). Because the long arm protrudes into the mitochondrial matrix, what this indicates is that, contrary to Complex III, $O_2^{\bar{\bullet}}$ produced by those sources would be directed toward the matrix rather then the intermembrane space. While the redox potentials indicate that most redox groups could produce $O_2^{\bar{\bullet}}$, the evidence thus far indicates that the semiquinones are the major $O_2^{\bar{\bullet}}$ producers in Complex I. This has been postulated by Britton Chance based on the rapid reaction of semiquinones with $O_{2(g)}$ in pulse radiolysis studies (56). However, it has often been ignored that there is a large body of evidence corroborating this postulate. As a result, many authors have dismissed this view and have looked for other potential donors for $O_2^{\bar{\bullet}}$ production (93). It has to be pointed out that removal of quinones with pentane drastically decreases $O_2^{\bar{\bullet}}$ production by Complex I (142) and that progressive re-addition of quinones linearly increases it. Furthermore, using analogues of ubiquinone (with different phytyl tail lengths Q_0, Q_1, Q_2, Q_6) as substrates in the re-addition experiments, the rate of H_2O_2/ $O_2^{\bar{\bullet}}$ production by Complex I can be significantly modulated (81). While this is good evidence that the ubisemiquinones are a significant producer of $O_2^{\bar{\bullet}}$, there is a significant residual level of $O_2^{\bar{\bullet}}/H_2O_2$ production that cannot be removed by ubiquinone extraction, which suggests that FMN and the iron sulfur clusters do contribute toward $O_2^{\bar{\bullet}}$ production (81). The FMN semiquinone is particularly attractive because of its very low redox potential (-380mV). In fact, in the *E. coli* NADH dehydrogenase, the majority of $O_2^{\bar{\bullet}}$ production can be ascribed to FMN and FAD semiquinones (143). Regarding the iron sulfur clusters, besides their low redox potential, there is evidence from inhibitor studies that they do produce $O_2^{\bar{\bullet}}$. In isolated mitochondria, in the presence of pyruvate and rotenone (this results in maximal stimulation of H_2O_2 production reported by these authors) H_2O_2 production can be decreased several fold by ethoxyformic anhydride and parachloromercuribenzoate, agents that inhibit the flow of electrons between the flavin and the ferricyanide reduction site (32, 33, 131), hence the flavin radical is not responsible for the vast majority of $O_2^{\bar{\bullet}}/H_2O_2$ (at least under rotenone treatment). However, it should be noted that destruction of the Rieske iron sulfur protein also eliminates $O_2^{\bar{\bullet}}$ production at Complex III (56, 89) but it does so because this prevents the formation of the semiquinone (when ubiquinone is absent and the cluster is reduced by chemical means, it does not produce $O_2^{\bar{\bullet}}$ itself). So these results do not disprove that the semiquinones at Complex I contribute significantly towards $O_2^{\bar{\bullet}}/H_2O_2$ production but do suggest that the flavin radical is not a significant source of superoxide. Taken together, the results of Chance and Barja suggest that both the semiquinones and the iron sulfur clusters contribute to $O_2^{\bar{\bullet}}$ production at Complex I.

What sets Complex I apart from Complex III in terms of $O_2^{\bar{\bullet}}/H_2O_2$ production is that even under state 3 respiratory conditions, $O_2^{\bar{\bullet}}/H_2O_2$ production at this complex is still very active (32, 34). This is particularly relevant to the exercise paradox (34) and in tissues that tend towards state 3 respiration (such as the liver of fed rats (122)). This makes Complex I $O_2^{\bar{\bullet}}$ production even more interesting for the free radical theory of aging.

The investigation of $O_2^{\bar{\bullet}}$ production at Complex III was greatly aided by the existence of very well defined inhibitor categories. The same cannot be said of Complex I. When looking at the effects of inhibitors on the rate of superoxide production by Complex I, it is important to bear in mind that they are by no means as clear cut as those of Complex III. For example, rotenone only partially inhibits Complex I (even at very large doses, the maximum inhibition is around 90%). Moreover, most inhibitors are actually competitive with one another and the only way to categorize them has been through different kinetics (139, 144). Even with these studies, it is not clear whether there exists a single binding site for all inhibitors or whether different inhibitors bind at different sites with different affinities. Common inhibitors of Complex I are rotenone, amytal, piericidin A and annonaceous acetogenins (145). Based on structural similarity, some inhibitors such as piericidin A are clearly ubiquinone (perhaps even semiquinone) analogues (139). Others, such as rotenone, have no structural similarity whatsoever with ubiquinone, but because they are competitive with piericidin A, they most likely act in a similar manner. Rotenone is the only inhibitor whose effect on $O_2^{\bar{\bullet}}/H_2O_2$ production has been thoroughly characterized (and it would perhaps be informative if the effect of other inhibitors was studied as well). Rotenone

significantly decreases or significantly increases $O_2^{\bar{\bullet}}$/H_2O_2 production depending on the source of electrons (that is Complex I or Complex II linked). With NADH linked substrates, rotenone increases $O_2^{\bar{\bullet}}$/H_2O_2 production (32, 33, 35, 66, 82, 84, 130), but with Complex II linked substrates (succinate) rotenone decreases it (33, 66). This may be due to the fact that when excess Complex II linked substrates are provided, reverse-electron transfer can occur through Complex I (66). Reverse electron transfer can be blocked by rotenone, suggesting that it binds close to the "exit" for electrons. That is, in the presence of NADH linked substrates (glutamate\malate) rotenone increases the reduction of the redox groups of Complex I and hence increases the rate of $O_2^{\bar{\bullet}}$/H_2O_2 production. However, in the presence of Complex II linked substrates, rotenone blocks the reverse entry of electrons, decreasing the reduction of the prosthetic groups of Complex I thus decreasing $O_2^{\bar{\bullet}}$/H_2O_2 production.

In conclusion, there is very little known about $O_2^{\bar{\bullet}}$ generation at Complex I save that a significant fraction is due to ubisemiquinone and iron sulfur clusters (33, 81, 84, 131) and that it is active both during state 3 and state 4 respiration (32, 34). A lot of fundamental work is still needed to understand the mechanism of normal electron transfer through Complex I, which will no doubt shed some light on the mechanism of $O_2^{\bar{\bullet}}$ production at that complex.

The rate of $O_2^{\bar{\bullet}}$ production controls lifespan independently of SOD

Now that we have explored mitochondrial molecular anatomy and the mechanism of $O_2^{\bar{\bullet}}$ production, let us consider how this may answer the question raised in the introduction. This question is, how is it possible for longevity to be controlled by the rate of $O_2^{\bar{\bullet}}$ production but not by the level of SOD?

A possible answer to this question is that antioxidant enzyme levels are already optimal but that the free radical damage that leads to aging is somehow not preventable by these antioxidants. The idea that free radical production (more specifically $O_2^{\bar{\bullet}}$ production) is more important for the determination of lifespan than free radical scavenging has been formulated by three previous authors (12, 23, 24, 46, 146, 147). Sohal *et al* collected the initial correlational data and formulated the concept that rate of $O_2^{\bar{\bullet}}$ production is a lifespan determinant (12), however these authors placed equal emphasis on free radical scavenging and since then have tended to focus on increasing free radical scavenging as a way to test the free radical theory of aging (48, 148, 149). Since he observed that endogenous antioxidants are negatively correlated with maximum longevity, Barja proposed that it is oxygen free radical generation in mitochondria, not antioxidants, that determines, in part, aging (21, 22, 24, 25, 146). Barja argues that mitochondrial DNA, due to its proximity to the source of free radical production, the electron transport chain, suffers a high level of damage due to increased local concentration of oxygen free radicals. Due to the fact that this damage is very local, Barja argues that it cannot be prevented by general antioxidants such as SOD, CAT and glutathione peroxidase (21, 22, 24, 25, 46, 47, 146). This argument is strengthened when considering that, due to the high surface density of the inner mitochondrial membrane, most macromolecules of the mitochondrial matrix are in fact adjacent to the inner mitochondrial membrane (150). On the other hand, de Grey argues that oxidative damage in the intermembrane space cannot be controlled by SOD (because there is no SOD there) and lipid peroxidation initiated in this organellar compartment can damage mitochondrial DNA (147, 151, 152) with possible important consequences for aging even in other cells (152). He notes the role that may be played by $HO_{2\,(lipid)}^{\bullet}$ (the protonated version of $O_2^{\bar{\bullet}}$) in initiating lipid peroxidation. These views are not mutually exclusive and I agree with the arguments made by both authors. Nevertheless, I wish to elaborate certain points as well as state my own views. I propose that a significant fraction (between 10% and 50%) of $O_2^{\bar{\bullet}}$ is not actually produced as $O_{2\,(aq)}^{\bar{\bullet}}$ but instead is produced as $HO_{2\,(lipid)}^{\bullet}$ in the inner mitochondrial membrane, where it can undergo a set of reactions that are not competitively inhibited by SOD. These reactions include the initiation of lipid peroxidation and the formation of peroxynitrous acid.

A very important point of $O_2^{\bar{\bullet}}$ chemistry that is often overlooked is that it can act as a base, that is, it can be protonated. The pKa of the couple $O_2^{\bar{\bullet}}$/$HO_2^{\bullet}$ is about 4.88 (115). At pH 7, the ratio $HO_2^{\bullet}$/ $O_2^{\bar{\bullet}}$ would then be at around 1/100. In its protonated form, superoxide can diffuse through membranes (116). In synthetic vesicles at pH 7.3, the permeability coefficient of $O_2^{\bar{\bullet}}$ has been calculated at 2.1×10^{-6} cm/s (116). Through linear regression between the permeability coefficient and the partition coefficient between oil and water, this value yields a partition coefficient between oil and water of 0.1 to 0.3% (153). At pH 7.3, using the Henderson-Hasselbach equation, 0.3% of $O_2^{\bar{\bullet}}$ is in the form $HO_2^{\bullet}$. From these values, taking into account the pKa of $O_2^{\bar{\bullet}}$ and assuming that the contribution of $O_2^{\bar{\bullet}}$ itself is negligible, we can roughly estimate that the partition coefficient of $HO_2^{\bullet}$ is between 0.33 and 1. Other authors have estimated the partition coefficient for $HO_2^{\bullet}$ at 1 (154). A partition coefficient of 1 would indicate that $HO_2^{\bullet}$ is equally soluble in the membrane and the aqueous phase. Note how the rate constant of dismutation of $HO_2^{\bullet}$ compares with that of $O_2^{\bar{\bullet}}$ in aqueous solutions (all data from (115, 155)).

(1) $HO_{2\,(aq)}^{\bullet} + HO_{2\,(aq)}^{\bullet} \rightarrow H_2O_{2(aq)} + O_{2(g)}$ $\quad k=8.6\times10^{5}\ M^{-1}\ s^{-1}$

(2) $O_{2\,(aq)}^{\bar{\bullet}} + HO_{2\,(aq)}^{\bullet} + H^{+}_{(aq)} \rightarrow H_2O_{2(aq)} + O_{2(g)}$ $\quad k=1.0\times10^{8}\ M^{-1}\ s^{-1}$

(3) $O_{2\,(aq)}^{\bar{\bullet}} + O_{2\,(aq)}^{\bar{\bullet}} + 2H^{+}_{(aq)} \rightarrow H_2O_{2(aq)} + O_{2(g)}$ $\quad k<0.35\ M^{-1}\ s^{-1}$

(4) Catalyzed by SOD1 $\quad k\leqq10^{10}\ M^{-1}\ s^{-1}$

That is, at pH 7, the dismutation reaction overwhelmingly occurs by mechanism (2). The rate constant of the dismutation of $O_2^{\bar{\bullet}}$ is pH dependent (115) from 4.9 to 10, reaching a peak at pH 4.9, and decreasing at higher or lower pH. At physiological pH, it is close to $k=8\times10^5$ M^{-1} s^{-1}. The spontaneous dismutation of $HO_2^{\bullet}$ inside membranes would occur through mechanism (1) with a rate constant comparable to physiological pH. The rate constant given for the spontaneous dismutation of $HO_2^{\bullet}$ was determined from measurements in aqueous solution at low pH. Since the dismutation of $HO_2^{\bullet}$ involves proton transfers (113, 114), its rate constant is lower in low dielectric media (such as the inner mitochondrial membrane) because proton transfers are energetically unfavorable in that environment. To that effect, the rate constant of the dismutation of $HO_2^{\bullet}$ in the aprotic medium dimethylsulfoxide has been measured at 1.7×10^4 M^{-1} s^{-1}, 15 times slower than in water (114). All of this taken together suggests that it is possible for significant amounts of $HO_2^{\bullet}$ to exist in the membrane, because its rate of dismutation is relatively slow and no SOD is present.

The main experimental evidence suggesting the production of $HO_{2\,(lipid)}^{\bullet}$ by the electron transport chain is that in enclosed vesicles in which $O_2^{\bar{\bullet}}$ production is directed towards the center, it can still be detected on the outside (see Figure 4). Moreover, this reaction can be prevented by the addition of α-tocopherol, an antioxidant that reacts very rapidly with $HO_2^{\bullet}$ but not with $O_2^{\bar{\bullet}}$ (115, 121).

Once inside the membrane, $HO_2^{\bullet}$ can undergo a variety of fates. As with $O_2^{\bar{\bullet}}$, all reactions compete with one another and with the spontaneous dismutation. However, since the dismutation is not catalyzed within the membrane environment (and might be considerably slower than in aqueous solution), it is less effective at competing with damaging reactions of $HO_2^{\bullet}$. $HO_2^{\bullet}$ can initiate lipid peroxidation, reacting with a variety of polyunsaturated fatty acids (PUFAs) with rate constants of around $k=10^3$ M^{-1} s^{-1} (156-158). Using computational and mathematical models, it has been argued that $HO_2^{\bullet}$ is the main cause of lipid peroxidation in the inner mitochondrial membrane *in vivo* (154). Although the rate constant for the reaction of $HO_2^{\bullet}$ with PUFAs might not seem very large, the concentration of PUFAs is relatively high, so the reaction can be quite significant. $HO_2^{\bullet}$ can also react with NO to form peroxynitrous acid, the protonated (and more reactive) form of peroxynitrite (80). The rate constant of the reaction of $O_2^{\bar{\bullet}}$ with NO is 1.9×10^{10} M^{-1} s^{-1} (159, 160). We can assume that the rate constant for reaction of $HO_2^{\bullet}$ with $NO_{(g)}$ is roughly the same, because both are free radical annihilation reactions which have a very low energy of activation and their rate is essentially diffusion-controlled. As with $O_{2(g)}$, $NO_{(g)}$ is more soluble in the lipid phase than in the aqueous phase, resulting in steady state concentrations that are several fold higher in the membrane (80). Hence, it is possible for significant amounts of peroxynitrous acid to be formed because the concentration of $NO_{(g)}$ is relatively high (as compared to the aqueous phase) and because the competing reaction (dismutation) is relatively slow. $HO_2^{\bullet}$ could also damage the low redox potential iron sulfur clusters of Complex I and II, some of which are buried in the hydrophobic interior of the membrane. It is worth bearing in mind that (as stated before) reactions of $HO_2^{\bullet}$ inside the membrane can be highly significant because they do not have to compete with SOD (since it is a water-soluble enzyme). One might believe that the incidence of $HO_2^{\bullet}$ inside the membranes is a trivial matter, because membranes only occupy a small amount of volume in the cell. In the mitochondrion, however, due to the folding of the cristae, the inner mitochondrial membrane has an extremely high surface density (150) and occupies a considerable volume. The inner membrane of heart mitochondria has a surface density of 61$\mu m^2/\mu m^3$ (150) and it has been proposed that membranes might account for about 50% of volume in heart and brown fat mitochondria (150). Based on these same numbers, it has been estimated that a majority of matrix proteins is in fact adjacent to the inner membrane (150).

Under these circumstances, if $HO_2^{\bullet}$ is indeed produced in the inner mitochondrial membrane, taking into account the considerable volume these membranes occupy (150) and the fact that most molecules in the intermembrane space are adjacent to the membrane (150), there would exist a constant source of free radicals (ONOOH, LOO•), whose toxic effects are not preventable by antioxidant enzymes. It is the author's opinion that the amount of this damage is small but chronic.

The SOD unpreventable damage initiated by $HO_2^{\bullet}$ inside the membrane might explain why it is possible for longevity to correlate with the rate of $O_2^{\bar{\bullet}}$ production but not with the levels of SOD. In the light of the failure of SOD-overexpression to yield any significant increase in longevity in mammals (11), perhaps genetic manipulations aimed at decreasing the rate of $O_2^{\bar{\bullet}}$ production, rather than at increasing the levels of SOD, should be undertaken.

Tests for the postulated hypothesis

What factors control the rate of $O_2^{\bar{\bullet}}$ production and how can these be manipulated? The main experimentally-determined factors that control the rate of $O_2^{\bar{\bullet}}$ production *in vitro* are phylogeny (i.e. what species the mitochondria/SMPs are isolated from), strength of the membrane potential, the state of reduction of the respiratory chain and $pO_{2(g)}$. To the *in vivo* situation, this translates into the standard metabolic rate (SMR), state of mitochondrial respiration (state 3 *vs* state 4), strength of the membrane potential and structure of the electron transport complexes (which in turn controls redox potential and $O_{2(g)}$ accessibility of the high energy intermediates). Hence, in order to test the said hypothesis, manipulation of any one of these factors could be undertaken. However, before going into a deep discussion of how to decrease the rate of $O_2^{\bar{\bullet}}$ production, let us consider a set

of experiments aimed specifically at disproving the hypothesis that the rate of $O_2^{\bar{\bullet}}$ production is a determinant of MLSP.

The easiest way to disprove the present hypothesis would be to increase the rate of mitochondrial $O_2^{\bar{\bullet}}$ production throughout the tissues of an organism and fail to see a reduction in lifespan. This could be done by mutating any one of the respiratory complexes in a manner similar to Ishii *et al* (45). The ideal mutant would be one whose gene product is expressed in all cells and that has a 2- to 3-fold increase in the rate of mitochondrial superoxide production, but has otherwise minimally altered electron transfer activity. The cytochrome b and the Rieske iron sulfur genes would make excellent candidates for such mutation studies, because they are expressed in virtually all mammalian tissues. Both genes have been extensively mutated in *Saccharomyces cerevisiae* (161, 162) and these mutants have been thoroughly characterized with respect to sequence, redox potential and kinetic parameters of the enzyme. Surprisingly, $O_2^{\bar{\bullet}}$ production has not been measured in any of them. However, the author has learned privately that there is at least one investigator who is working on doing just that. Once a specific mutant with increased $O_2^{\bar{\bullet}}$ production has been isolated in *Saccharomyces*, ideally a residue conserved between yeast and mammals, the mutation could be produced in mice by a gene knock-in (although with cytochrome b this does pose a technical challenge since this gene is mitochondrial). After determining the rate of $O_2^{\bar{\bullet}}$ production *in vitro* and *in vivo*, the lifespan would be measured and if it failed to decrease (despite increasing the rate of mitochondrial $O_2^{\bar{\bullet}}$ production throughout the animal), the hypothesis would be proven wrong.

Alternatively, one could manipulate other factors besides the amino acid sequence of the respiratory complexes, to increase $O_2^{\bar{\bullet}}/H_2O_2$ production. Recent examples in the literature include manipulation of the strength of the membrane potential and the state of mitochondrial respiration (163, 164).

It has been demonstrated that knocking out the heart/muscle specific isoform of the adenine nucleotide translocator (ANT1) in mice results in cardiohypertrophy, ragged-red muscle fibers, mitochondrial proliferation and exercise intolerance (165). The function of this protein is to export ATP from the mitochondrial matrix (where it is generated) back into the cytoplasm, while at the same time importing ADP back into the mitochondrial matrix. Deleting this protein should have two effects. The availability of ATP in the cytoplasm would decrease, and the mitochondrial (depending on the flux conditions *in vivo*) electron transport chain would be locked in state 4 respiration. As mentioned previously, under state 4 conditions, the electron transport chain is in the reduced state, and Δp is in equilibrium with both the hydrolysis of ATP (164) and the electron transfer chain (and $O_2^{\bar{\bullet}}/H_2O_2$ production is highest). *In vitro*, mitochondria isolated from these mutant mice indeed show significantly increased H_2O_2 production during state 4 respiration (164). In addition, increased mtDNA rearrangements were also reported in the heart of these animals when compared to age-matched controls (164). Based on the *in vitro* evidence of increased H_2O_2 production, the present theory would predict that the ANT1 (-/-) mice have a decreased MLSP. However, the ANT1 (-/-) mutants do not fullfil the requirements listed in the previous paragraph. First of all, ANT1 is not expressed in all tissues of the body. The second isoform of the adenine nucleotide exchanger (ANT2) is distributed more widely than ANT1. It is present in the brain, heart and liver as well as other internal organs. Second, it has not been shown that H_2O_2 production, nor the steady state level of oxidative stress is in fact increased *in vivo*. H_2O_2 production was reported using a single substrate (succinate) at a single concentration (6.5 mM) strictly during state 4 respiration. Based on these considerations, the author does not venture a prediction on the longevity of these animals at this time.

In another study, it was shown that knocking out uncoupling protein 3 (UCP3) in mice leads to increased production of $O_2^{\bar{\bullet}}$ *in vitro* and *in vivo* (163). The UCPs are a family of proteins whose function is to dissipate the mitochondrial membrane potential to generate heat (instead of ATP). In doing so, the UCPs push mitochondrial respiration toward state 3. It has long been held that the physiological function of these proteins is to regulate body temperature in endothermic animals. More recently it has been proposed that the specific role of these proteins is, in fact, to control the mitochondrial membrane potential in order to minimize $O_2^{\bar{\bullet}}$ production (163). The author agrees with this notion but it is also his opinion, that, contrary to common chemical uncouplers, the uncoupling proteins are in fact "gated"; that is, their conductance to protons is dependent on the strength of the membrane potential. Since increased $O_2^{\bar{\bullet}}$ was observed, Vidal-Puig *et al* have indicated that they are planning to determine the maximum lifespan of these animals (163). Unfortunately, UCP3 expression is restricted to skeletal muscle (163). So this mutant does not satisfy the requirements (ubiquitous increase in superoxide production) in the above paragraph either. However, another uncoupling protein, UCP2, is expressed in nearly all tissues of mice. If a knock-out of this gene were available and it was shown that $O_2^{\bar{\bullet}}$ production is increased in all major tissues, it could be used to disprove the present hypothesis. If there is no lifespan reduction in either ANT1 or UCP3 k.o mice, the results would certainly weaken the present hypothesis. However, since neither k.o. leads to increased rate of $O_2^{\bar{\bullet}}$ throughout the tissues of the organism, it would not provide decisive proof against the hypothesis either.

Alternatively, one could use sublethal doses of antimycin A to increase the rate of mitochondrial $O_2^{\bar{\bullet}}$ production. As mentioned before, antimycin A increases $O_2^{\bar{\bullet}}$ production by Complex III through inhibition of the respiratory electron transport chain. Independent of its effects on free radical generation, at sufficiently high doses it is lethal. However, top-down metabolic analysis

reveals that Complex III does not exert great flux control in mammalian species (166). Therefore, low levels of antimycin A should have relatively little effect on energy metabolism while still causing increased generation of $O_2^{\bar{\bullet}}$. In other words, the experiment that is suggested is to essentially titrate MLSP with chronic sublethal levels of antimycin A. The expected outcome is that lifespan would be substantially decreased. Unfortunately, this experiment would not be as clean as having a high $O_2^{\bar{\bullet}}$ producing mutant because, as with any complex chemical, issues of tissue distribution and liver P450 processing could occur.

While such experiments could refute the stated hypothesis, consistent results would not be conclusive proof that the hypothesis is actually correct. It is far easier to disrupt something in biological systems than it is to improve on it. For this reason, to definitively prove the hypothesis, one would wish to decrease the rate of free radical generation and see an increase in longevity. This can be achieved by manipulating any one of the factors mentioned at the beginning of the section ($pO_{2(g)}$, state of mitochondrial respiration, structure of the electron transport complexes).

Since pO_2 is one of the most important determinants of the rate of $O_2^{\bar{\bullet}}$ production *in vitro* (56), it is reasonable to suggest that this is also the case *in vivo*. pO_2 is the partial pressure of $O_{2(g)}$ and it is the standard measure of gas concentration. In isolated mitochondria, increase of pO_2 to 1.92 Mpa (0.1 Mpa is equal to atmospheric pressure at sea level) results in a 4 fold increase in H_2O_2 production by pigeon mitochondria and a 15 to 20 fold increase in H_2O_2 release by rat mitochondria (167). In insects, whose cells are, in effect, in direct contact with the atmosphere (through tracheal tubes), intracellular pO_2 can be manipulated experimentally. Results from these studies dramatically show the importance of pO_2 in modulating oxidative damage and maximum lifespan. In both *Drosophila* and *C. elegans*, average as well as maximum lifespan can be decreased as well as increased by modulating oxygen tension (168-172). It is the author's opinion that these results are in fact some of the strongest evidence supporting the free radical theory of aging (and more specifically the free radical generation hypothesis) in invertebrates. In vertebrates, the *in situ* pO_2 is determined by several physiological factors including SMR. In its earliest form, the free radical theory of aging was proposed to explain the inverse correlation between SMR and maximum lifespan (in eutherian mammals) on which aging research had dwelt for half a century. Unfortunately, SMR is rather difficult to manipulate in mammals, partly because it is linked to body mass. Nevertheless, SMR can be manipulated with thyroid hormone treatment. As would be expected from the rate of living theory (173, 174), it was determined that hyperthyroidism (high metabolic rate) decreases longevity in rats (175), while hypothyroidism increases it (175). More recently, it was determined that Ames dwarf mice, which have a maximum longevity about 40% higher than normal, also have a body temperature that is 1.6 degrees lower than WT (176). This drop in body temperature could indicate a decrease in metabolic rate and oxidative processes (177). All these data clearly suggest that SMR is an important factor in the lifespan determination of mammals. However, it should not be overemphasized. The fact that animals with similar SMR can have very different lifespans suggests that it is only one of many factors controlling longevity. Regarding oxygen radical production, it has to be pointed out that animals with similar SMR can also have very different rates of $O_2^{\bar{\bullet}}/H_2O_2$ production (23, 25, 30) and that the rate of $O_2^{\bar{\bullet}}$ production correlates better with longevity than does SMR (23, 35). Thus, it was first shown (25) in birds, animals much longer lived than mammals of similar size and metabolic rate, that the fraction of electrons out of sequence which reduce O_2 to $O_2^{\bar{\bullet}}$ at the respiratory chain (the free radical leak (33)) can be lower than in mammals (25, 32, 33, 35). The lower free radical leak of birds can partially explain the paradox that they are almost the only animals on earth simultaneously showing high oxygen consumption rates and high maximum longevity (25, 46, 47). Nevertheless, taken as a whole, results from pO_2 manipulation studies strongly support the hypothesis that the rate of oxygen free radical production contributes to the determination of MLSP.

Since the main sources of $O_2^{\bar{\bullet}}$ are Complexes I and III, the most straightforward way of testing the hypothesis that the rate of mitochondrial $O_2^{\bar{\bullet}}$ generation is a lifespan determining factor in mammals would be to transfer the 43 genes for Complex I and the 11 genes for Complex III from a long-lived species such as *Homo* to a short-lived one such as *Mus* (mouse). Anyone who is vaguely familiar with molecular biology knows that this task is close to impossible at present. Before even contemplating such a demanding endeavor, a question has to be conclusively answered first. This question is whether the interspecies variation in the rate of $O_2^{\bar{\bullet}}/H_2O_2$ production reported by Barja and Sohal (12, 25-27, 30, 32, 33, 35, 47, 146, 178) is due to variation in the proprieties of individual Complexes (Complex I and III) or whether this is due to variation in the properties of the whole mitochondria (or even the organism as a whole). For example, it is possible that the steady state strength of the membrane potential is lower in birds than in mammals. It is also possible that the state of reduction of the mitochondrial electron transport chain is kept lower (this was indeed argued by Barja (47), although, as far as this author knows, it has not actually been measured). In the case of the data of Sohal *et al* (12, 26, 27), the interpretation does not have to be very complicated since all species followed the rate of living very closely. It has been shown that increasing metabolic rate with thyroxine leads to an increase in the cytochrome b, a, a3, and c1 content of mitochondria (even when the preparations are adjusted per mg protein, (179)). In addition, it has also been documented that the content of cytochrome b, a3 and c1 is inversely correlated with metabolic rate and MLSP in mammalian

mitochondria ((33) citing (94, 95)). Since cytochromes are exclusively (except for cytochrome b, which also occurs in proteins not part of the electron transport chain) associated with mitochondrial electron transport complexes (cyt a3 with Complex IV, cyt b with Complex II and III, cyt c1 with Complex III) their content is directly proportional to the content of electron transport complexes per mitochondria. Thus, an explanation for the variation in the rate of superoxide production reported by Sohal *et al* is that the mitochondrial preparations from species with a high metabolic rate have a higher content in respiratory complexes leading to an overall higher consumption of oxygen and higher production of $O_2^{\bar{\bullet}}$/ H_2O_2 (i.e., the free radical leak is essentially constant) (27). Therefore there is little reason to suggest that some intrinsic property of Complex I or Complex III accounts for the difference in the rate of oxygen free radical production in that particular case. However, the more interesting question is what controls (properties of the mitochondrion or properties of the individual electron transfer complex) the difference in "free radical leak" in species that do not follow the rate of living (such as birds, bats and primates (25, 173, 174)). For this question to be answered with definite certitude, the interspecies variation in the rate of $O_2^{\bar{\bullet}}/H_2O_2$ production reported by Sohal and Barja between pigeon and rat (25, 30, 32, 33, 35) would have to be reproduced in biochemically isolated Complex I and Complex III. Complex I has proven very difficult to isolate because of its fragility and it is not routinely done, work being instead conducted in SMPs (136). Regarding Complex III, there are several protocols available for large scale biochemical purification and these have been optimized to be applicable to a wide range of phyla (180). Therefore, measuring the interspecies variation in the rate of $O_2^{\bar{\bullet}}$ production in isolated Complex III is a feasible first step. The author is currently exploring the possibility of conducting such experiments.

Another way to decrease the rate of $O_2^{\bar{\bullet}}$ production is to decrease the state of reduction of the respiratory chain. In plants and in many fungi, there are enzymes that do just that. This family of enzymes, termed the alternative oxidases, bypass Complex III and IV and oxidize ubiquinol to H_2O (181). The first alternative oxidase was discovered in plant species which exhibit large antimycin A and cyanide resistant respiration. Its function was originally unknown but the consensus now is that its main function is protection against environmental stress-induced oxidative damage (182). Transcription of the alternative oxidase is increased by hydrogen peroxide and antimycin A (183). By short-circuiting the classical respiratory pathway, this enzyme minimizes state 4 respiratory conditions, lowering the reduction state of both Complex I and Complex III. In fact, multiple investigators have shown that expression of the alternative oxidase decreases the rate of H_2O_2 production by both Complex I and Complex III *in vitro* (181-184). More recently, it has been shown that overexpression of the alternative oxidase results in a decreased level of steady state oxidative stress (as measured by dichlorofluorescein) *in vivo* (182). Conversely, antisense inhibition of the alternative oxidase drastically increased steady state levels of oxidative stress (182). Multiple alternative oxidases have been cloned and one gene is sufficient for this enzymatic activity (182). An extremely interesting experiment would be to express the alternative oxidase in *Drosophila*, *C. elegans* or in mice and note effects on longevity. A potential problem should be noted which is that expression of the alternative oxidase will decrease the P/O ratio (by bypassing Complex III and IV) and this could ultimately kill the organism. However, this potential problem can be circumvented by conducting such an experiment with an inducible promoter whose expression can be tightly regulated, thus being able to express the alternative oxidase at high enough levels so as to reduce the state of reduction of the respiratory chain but low enough not to decrease the P/O ratio enough to kill the organism.

Besides state of reduction, membrane potential is also known to have a large effect on the rate of $O_2^{\bar{\bullet}}$ production. Membrane potential can be decreased by overexpression of uncoupling proteins (UCP, (185)). The same argument could be made as with overexpression of the alternative oxidase, that this would decrease the P/O and ultimately kill the animal. However, transgenic mice overexpressing UCP3 (13 fold higher than WT!) in skeletal muscle are viable, display hyperphagia and have decreased body weight compared to WT (185). Oxygen consumption was also found to be increased, and there should be little doubt that *in vivo* membrane potential, as well as the reduction state of the electron transport chain, are considerably decreased in these mice. To that effect, it would be extremely interesting to measure the lifespan of these animals. However, this protein was overexpressed using a skeletal muscle specific promoter. Therefore it is unlikely (in the author's opinion) to have a radical effect on MLSP, except perhaps through a mimicking of CR. This experiment does show, however, that it is possible to increase uncoupling in mammals without killing the organism. By overexpressing UCP3 throughout the body it should be possible to decrease $O_2^{\bar{\bullet}}$ production, and if the presented hypothesis is correct, increase maximum lifespan.

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ABBREVIATIONS

$O_2^{\bar{}}\bullet$: Superoxide, oxygen anion;
H_2O_2: Hydrogen peroxide;
$HO_2\bullet$: Hydroperoxyl or perhydroxyl radical;
$O_{2(g)}$: Ground-state molecular oxygen in the gas phase;
$O_{2(aq)}$: Oxygen in saturated aqueous solution;
SOD1 or Cu,Zn-SOD: cytoplasmic Cu,Zn-superoxide dismutase;
SOD2 or Mn-SOD: mitochondrial Mn-superoxide dismutase;
SOD3: extracellular Cu,Zn-superoxide dismutase;
CAT: Catalase;
WT: wild type;
k.o.: gene knockout or null mutant;
CR: caloric restriction;
bc1: cytochrome b and c1-complex a.k.a. Complex III;
Q_i: mitochondrial matrix facing ubiquinone binding site of Complex III;
Q_o: Intermembrane space facing ubiquinol binding site of Complex III;
mtDNA: mitochondrial DNA;
PUFA: polyunsaturated fatty acids;
SMPs: sub-mitochondrial particles;
kD: kilodalton;
mV: milli-Volts;
CoQ or Q: Ubiquinone, oxidized coenzyme Q;
$CoQH_2$ or QH_2: Ubiquinol or reduced coenzyme Q;
EPR: Electron paramagnetic resonance spectroscopy;
ONOOH: Peroxynitrous acid;
$LOO^{\bullet}$: Lipid hydroperoxy radical;
LOOH: Lipid hydroperoxyde;
MLSP: Maximum lifespan;
$\Delta\Psi$: Membrane potential;
ΔpH: pH gradient;
ψp or $\Delta\mu H^+$: Proton motive force;
SMR: Standard metabolic rate;
ANT: Adenine nucleotide translocator;
UCP: Uncoupling protein

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