

DNA Cell Biol, 2002, in press

HO₂•: the forgotten radical

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Running title: HO₂•: the forgotten radical

Abstract

HO₂•, usually termed either hydroperoxyl radical or perhydroxyl radical, is the protonated form of superoxide; the protonation/deprotonation equilibrium exhibits a pK_a of around 4.8. Consequently, about 0.3% of any superoxide present in the cytosol of a typical cell is in the protonated form. This ratio is rather accurately reflected by the published literature on the two species, as identified by a PubMed search; at the time of writing only 28 articles mention “HO₂”, “hydroperoxyl” or “perhydroxyl” in their titles, as against 9228 mentioning superoxide. Here it is argued that this correlation is not justifiable: that HO₂•’s biological and biomedical importance far exceeds the attention it has received. Several key observations of recent years are reviewed that can be explained much more economically when the participation of HO₂• is postulated. It is suggested that a more widespread appreciation of the possible role of HO₂• in biological systems would be of considerable benefit to biomedical research.

Introduction

The “bible” of free radical research is Halliwell and Gutteridge’s *Free Radicals in Biology and Medicine*, whose third edition was published in 1999 (27). As the originating reactive oxygen species formed by numerous metabolic processes, in particular by mitochondrial electron transport, superoxide is naturally discussed at great length there. By contrast, its protonated form, HO_2^\bullet or hydroperoxyl radical, is only mentioned briefly and does not even rate an entry in the index. The most prominent reference to HO_2^\bullet is the following sentence (page 61):

Although at the pH of most body tissues the ratio of $[\text{O}_2^{\bullet-}]/[\text{HO}_2^\bullet]$ will be large (100/1 at pH 6.8, 1000/1 at pH 7.8), the high reactivity of HO_2^\bullet and its uncharged nature, which might allow it to cross membranes more readily than the charged $\text{O}_2^{\bullet-}$, have combined to maintain interest in this species.

It is difficult to agree with either the beginning or the end of this statement. Firstly, a ratio of between two and three orders of magnitude is small when compared to the ratio in reactivity of HO_2^\bullet and $\text{O}_2^{\bullet-}$ with many biomolecules, so the chemistry of superoxide in living systems is in large part dominated by the reactions of HO_2^\bullet . Secondly, interest in HO_2^\bullet has conspicuously *not* been maintained: at the time of writing, just 28 articles listed in PubMed mention “HO2”, “hydroperoxyl” or “perhydroxyl” in their titles, of which the last study of the radical’s biological activity is from 1996 (19).

In this article we highlight a selection of significant findings from the literature of the past decade that can, it will be argued, be best understood by a consideration of the possible involvement of HO_2^\bullet , a scenario that has generally not been adequately discussed in relation to those observations. The examples chosen focus on the mitochondrion, but HO_2^\bullet ’s potential relevance elsewhere is also implied. It is hoped that these examples will inspire a restoration of interest in HO_2^\bullet ’s possible biological roles.

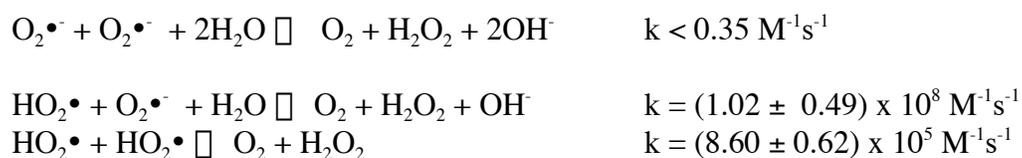
A challenge to delocalised chemiosmosis

Mitchell’s chemiosmotic theory was first published in 1961 (38). It was not until 1966, however, that in a much more detailed exposition of his model (39) Mitchell made explicit a component of it which is now widely considered indispensable: its spatially delocalised nature. The proton circuit that transfers energy from the electron transport chain to the ATP synthase involves the travel of protons through the aqueous medium on either side of the mitochondrial inner membrane; this has been robustly demonstrated by experiments involving external manipulation of the transmembrane proton gradient (31,54). What is less clear, however, is how constrained is the path that those protons take within the two aqueous compartments. The delocalised chemiosmotic theory states that the path is constrained only by the boundary of each aqueous compartment—in other words, the broadly proton-impermeant plasma membrane and inner mitochondrial membrane for the cytosolic compartment and the inner mitochondrial membrane alone for the matrix compartment. By this model, any two points within the same compartment are at equal electrochemical potential. (The speed of proton movement by the Grotthuss mechanism is so great that effects due to protons entering and exiting a compartment at distinct sites can be neglected.)

It may initially seem hard to imagine how this could be wrong, but some remarkably direct evidence suggests that it is. In a series of studies between 1969 and 1984 [see ref. 52 for a review of the main work], the group of Tedeschi found that mitochondria could synthesize ATP despite generating no proton gradient whatever. Their technique for measuring the membrane potential was with microelectrodes in giant mitochondria, a method that can be argued to be less disruptive to the electrical properties of the organelle than the more common approach, introduction of lipophilic cationic dyes that equilibrate according to the Nernst equation. A model was proposed in 1979 (32) that went some way towards explaining the phenomenon; it was soon seen to possess an important flaw (40), but a refinement that “repairs” it was proposed recently (14).

A study involving superoxide dismutation (24) appears to shed new light on this issue and strongly to challenge delocalised chemiosmosis. Whatever the details of the mechanism, if the cytosolic component of the proton circuit is constrained to a region of the cytosol, the proton-pumping action of the electron transport chain will acidify that region relative to the rest of the cytosol. The smaller the volume of the region that contains the chemiosmosis-engaged protons, the greater this acidification will be. The models mentioned above (32,14) propose that it is a layer only ~1nm thick on the outside of the inner membrane, allowing the acidification to be considerable.

Guidot et al. (24) obtained a result that appears to imply that this acidification indeed occurs as a result of mitochondrial respiration. They isolated mitochondria from a strain of yeast that lacked the mitochondrial isoform of superoxide dismutase (SOD), and exposed them to an extramitochondrial source of superoxide. They then measured the rate of dismutation of that superoxide, which must be non-enzymatic unless there is SOD in the intermembrane space (a topic that will be discussed below). They found that the rate of dismutation was markedly greater when the mitochondria were actively respiring than when, by any of a variety of means, the formation of a proton gradient was prevented. The effect was also found in wild-type yeast (i.e. with mitochondrial SOD). Guidot et al. attributed this to a respiration-dependent acidification of the intermembrane space (into which extramitochondrially-generated superoxide would readily diffuse, on account of the pores in the outer membrane). This would produce the effect observed, because the lower pH would increase the proportion of superoxide that was protonated to HO₂• and would thus increase the rate of non-enzymatic dismutation (Figure 1), according to the rate constants derived by Bielski (6):



[These values, together with a pK_a of 4.69 for the equilibrium between O₂•⁻ + H⁺ and HO₂•, were derived (6) by measuring the second-order rate constant for the decay of O₂•⁻ as a function of pH. This also requires knowledge of the molar extinction coefficients of O₂•⁻ and HO₂•, whose less accurate measurements had previously implied slightly different values for the pK_a.]

However, Guidot et al. did not draw attention to the fact that this model flatly contradicts the generally accepted version of the chemiosmotic theory (namely, the delocalised model), and supports the heretical alternative (32,14) mentioned above. In consequence, attention has not yet been paid to this potentially pivotal result by bioenergeticists at large.

Endogenous superoxide in the mitochondrial intermembrane space

The previous section discussed an experiment involving *externally* generated superoxide diffusing into the mitochondrial intermembrane space; other work bears on the relevance of penetration into the intermembrane space of superoxide generated by the electron transport chain (ETC). It has been argued (56) that all the superoxide made by electron leak from the ETC emerges on the matrix side of the membrane, but the evidence cited in support of this conclusion only demonstrates that *some* does. Additionally, however, it has been noted (9) that superoxide reacts very rapidly with ferricytochrome c, whose millimolar concentration in the intermembrane space would seem sufficient to eliminate any superoxide there before it can undergo any potentially toxic reactions. (The reaction with ferricytochrome c restores the superoxide to molecular oxygen and the electron is simply transported to Complex IV, so any potential for toxicity is averted.)

However, Kerver et al. (33) demonstrated in an elegant histochemical study that singlet oxygen is formed in the intermembrane space. The relevance of this result is that, while the reaction of superoxide with ferricytochrome c (or with SOD in the oxygen-regenerating step of its cycle) produces ground-state oxygen, non-enzymatic dismutation is reported (11) to produce singlet oxygen. While this is of course not a proof that non-enzymatic dismutation of superoxide is taking place in the intermembrane space, it constitutes an argument by elimination, in that no alternative mechanism for the formation of singlet oxygen in that compartment has ever been suggested. Thus, whether or not some mitochondrially-generated superoxide emerges in the matrix, evidently some also emerges in the intermembrane space; additionally, the presence of ferricytochrome c is not sufficient to eliminate it. The essential role of HO_2^\bullet in non-enzymatic dismutation is evident from the rate constants mentioned in the previous section. However, Kerver et al. did not mention this, instead stating that the dismutation of superoxide anions was responsible.

Additionally, Balzan et al. (4) engineered a transgenic superoxide dismutase in yeast, with a leader sequence that caused it to be localised to the intermembrane space. This was shown to improve the resistance to hyperoxia of yeast that lacked the mitochondrial SOD, again suggesting that superoxide is present in the intermembrane space. However, it must be conceded that the absence of mitochondrial SOD might itself cause a rise in the level of intermembrane space superoxide, since matrix superoxide might have a longer lifetime and thus have greater potential to diffuse through the inner membrane. This possibility is made more plausible when one considers the protonated form, HO_2^\bullet , because as mentioned in the Introduction, HO_2^\bullet is uncharged and thus more membrane-permeant than superoxide.

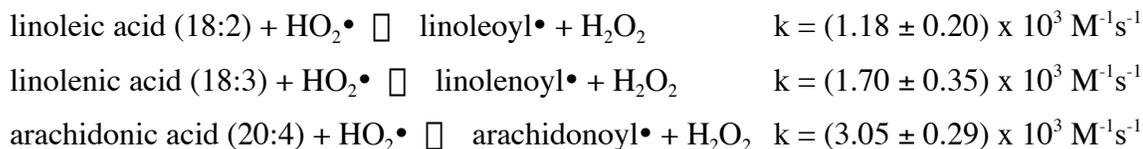
A recent report (20) suggests that mitochondria possess a superoxide dismutase isoform in the intermembrane space. If this is correct, it is difficult to understand why addition of transgenic SOD should be beneficial (4), or why the presence of a proton gradient should considerably accelerate dismutation (24). A possible explanation is that the intermembrane

space SOD does not exist in yeast, the organism used in both those studies; this is hinted at by the observation (24) that the respiration-dependent component of non-enzymatic dismutation was much greater in yeast mitochondria than in mitochondria isolated from two rat tissues. Kerver et al. (33) studied rat tissues, however.

Finally, it must be noted that the occurrence of non-enzymatic dismutation requires the collision of two superoxide molecules (one or both protonated), and thus their presence in the intermembrane space of the same mitochondrion at the same time. This is not in accordance with the classical estimate (10) of a concentration of superoxide in the intermembrane space of the order of 10^{-11} M. Thus, that estimate may need to be reconsidered.

Age-related mitochondrial dysfunction

A number of groups, working with various tissues of various species, have reported that mitochondrial superoxide (or hydrogen peroxide) production rises with age (48,50,51) [though the robustness of this finding has also been questioned (5)]. It has also been reported that the mitochondrial proton gradient declines with age (47,26). This combination of changes is highly paradoxical at first sight, because superoxide production is found to rise with increasing membrane potential (29). (A mechanism explaining this last result in the case of Complex III concerns the ease of movement of electrons between the hemes of cytochrome b (18); a corresponding model for Complex I must await more detailed understanding of its enzymatic mechanism than is yet available.) An explanation is proposed here that revolves around the role of $\text{HO}_2\bullet$ in initiating lipid peroxidation, something that it (but not superoxide) can do even in the absence of pre-existing lipid hydroperoxides (7):



[Note that, as shown by the above results and others in the same study (7) and since, $\text{HO}_2\bullet$ abstracts only bisallylic H atoms of fatty acids.]

In exploring how this combination of changes can come about, one must examine in more detail what factors determine the proportion of electrons that escape from the respiratory chain to form superoxide. In addition to the membrane potential, this proportion is affected by the proportion of the time that the “leaky” sites in the respiratory chain possess an electron—the degree of reduction of those sites. This is in turn determined by the efficiency of electron flow both upstream and downstream of those sites. The classic example is that antimycin A blocks electron transfer on the matrix-side ubiquinol-binding site of cytochrome b (49) and thus increases the reduction of the cytosolic-side ubiquinol-binding site, which is where superoxide production by Complex III seems mainly to occur (28), whereas myxothiazol blocks electron flow upstream of this site (58); correspondingly,

antimycin A increases and myxothiazol decreases the rate of superoxide production at Complex III (57).

The efficiency of the respiratory chain enzymes can be hypothesised to alter as a function of age due to at least two factors: oxidative damage to its constituent proteins and changes to their environment. Since superoxide production rises with age, oxidative damage occurs at a higher rate; moreover, the half-life of mitochondrial turnover, and thus the lifetime of the average mitochondrial membrane protein, is increased in older individuals (46). However, some enzymes are much more sensitive than others to this damage: those containing iron-sulphur clusters are particularly susceptible (22,2). The major microenvironmental change with age may be the reduced level of cardiolipin in the inner membrane (44). Again, this affects some proteins more than others; Complex IV is particularly dependent on cardiolipin as a cofactor (21,44).

The sensitivity of Complex IV to cardiolipin levels provides a plausible explanation for increased superoxide production: since it is the terminal enzyme of the respiratory chain, its loss of efficiency can be predicted to increase the degree of reduction of the earlier enzymes that are responsible for superoxide production. This effect may outweigh the inhibitory effect of the lowered membrane potential on superoxide production. But what remains to be explained is why the cardiolipin level falls in the first place, since the enzymes necessary for its synthesis are present and the required rate of synthesis is presumably very low (being set by the rate of mitochondrial turnover).

Again, a possible answer may be found in the role of $\text{HO}_2\bullet$ (Figure 2). Cardiolipin is the only negatively-charged phospholipid present in significant quantity in the inner mitochondrial membrane: the other phospholipids present there, phosphatidylcholine and phosphatidylethanolamine, are zwitterionic (30). Negative fixed charges on the surface of a membrane cause a surface potential (an attraction of cations and a repulsion of anions), which in this case has been estimated to be about 60mV; this results, in turn, in a reduction of pH at the membrane of about one unit (37). Thus, lower cardiolipin levels will result in a less acid aqueous phase at the membrane surface and hence in a reduction in the proportion of superoxide released that is converted to $\text{HO}_2\bullet$. This is likely to reduce the rate of peroxidation of mitochondrial lipids and proteins, even if there is a higher rate of production of superoxide, since it has been shown that $\text{HO}_2\bullet$ is responsible for the vast majority of initiation of lipid peroxidation reactions in the inner membrane (3,34). The effect is independent of the transmembrane proton gradient, though the lowering of that gradient with age (47,26) also inhibits $\text{HO}_2\bullet$ formation as explained previously (Figure 1).

Thus, cardiolipin decline may be a regulated, compensatory response to age-related deterioration of other cellular components, such as the lysosomal apparatus that is responsible for mitochondrial turnover (23). If accumulating undegradable material such as lipofuscin impairs lysosomal function, as has been postulated by Brunk (8,53), that could explain the slowing of mitochondrial turnover with age (46). A longer-lived mitochondrion must be adjusted to inflict free radical-mediated membrane damage upon itself more slowly, or else it will become unable to support a proton gradient and will be a drain on cellular resources (15). Thus, production of the free radicals that are responsible for this damage must be inhibited. In support of this hypothesis, restoration of cardiolipin levels in old rats

by dietary supplementation with acetyl-L-carnitine causes a rise in the rate of oxidative damage (25).

Superoxide production by Complex III

Another intriguing observation with relevance to the role of oxidative damage in aging is the absence of a correlation, across homeotherm species, between maximum lifespan and antioxidant enzyme levels (adjusted for specific metabolic rate). Early work by Cutler suggested that such a correlation did exist for superoxide dismutase but not for other antioxidant enzymes (55,13); even SOD is now known, however, to exhibit if anything a *lower* level in longer-lived species of comparable metabolic rate if birds are included in the calculation (35,45). Evolution of slower aging is accompanied by the expected changes in two other parameters related to free radical damage, namely the rate of production of superoxide as a proportion of oxygen consumption (45) and the degree of unsaturation of membrane (especially mitochondrial inner membrane) phospholipids (43), which determines their susceptibility to free radical-mediated peroxidation (12). But the natural expectation is that the level of antioxidant enzymes, which is just as much under genetic control as are the two parameters just mentioned, should also be adjusted in the appropriate direction when evolutionary pressure for increased longevity is present.

It was recently proposed (16) that this has failed to occur because the location in which the lifespan-limiting oxidative damage occurs is one in which such enzymes are ineffective, because they are absent: namely, the mitochondrial intermembrane space. Whereas superoxide generated in the mitochondrial matrix may be easily and completely detoxified by mitochondrial SOD, any that is generated on the outside of the inner membrane will have a longer lifetime and—in view of the more acidic environment there than in the matrix—will be more likely to become protonated to $\text{HO}_2\bullet$ and react with a phospholipid of the membrane. This may initiate a chain reaction that propagates through the membrane and eventually damages the mitochondrial DNA, which is attached to the membrane (1).

This particular model (16) is seriously challenged by the recent report (20) of an intermembrane space SOD, and might in any case be considered fragile in view of its reliance on the assumption that evolution has, for some reason, been unable to evolve a SOD isoform directed to the intermembrane space. But recently a variation on the hypothesis has been put forward (41) that escapes both of these objections. The cytosolic-side quinone-binding site of Complex III is not at the membrane surface but slightly buried within the membrane, and a glutamic acid residue lies very close to it. Muller (41) proposed that, on those occasions when an electron passing from ubiquinol to the heme group goes astray and becomes attached to a molecule of oxygen, forming superoxide, a proton from the glutamic acid residue is likely also to be transferred to the oxygen. In other words, $\text{HO}_2\bullet$ may be formed *within the inner membrane itself*. Such a scenario allows the possibility of initiation of lipid peroxidation before any antioxidant enzymes, even including a putative intermembrane space SOD, have had access to the originating free radical. It may thus, especially if increasing knowledge of the mechanism of superoxide production by Complex I leads to a similar model, be regarded as a highly plausible explanation for the ostensibly paradoxical failure of homeotherm evolution to use regulation of antioxidant enzyme levels as one of its strategies to select for long life.

Conclusion

The evidence surveyed in this article demonstrates that HO_2^\bullet probably plays a central role in mediating the toxic side-effects of aerobic respiration, due to its high reactivity with biomolecules and its membrane-permeance. Additionally, inclusion of HO_2^\bullet in the analysis of the chemiosmotic proton circuit is seen to offer a valuable insight into a controversy that has simmered for decades due to the absence of any conclusive experimental data. Since HO_2^\bullet is present in any compartment where superoxide is present and the pH is physiological, biochemical reactions and pathways that produce extramitochondrial superoxide—such as plasma membrane electron transport (42,17,36)—should also be carefully analysed in the context of the effects that HO_2^\bullet may have.

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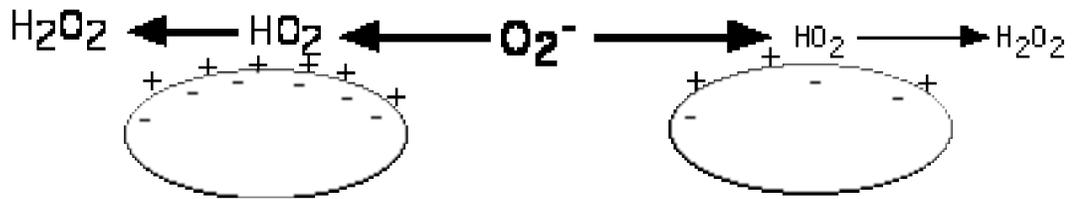


Figure 1. According to the surface-restricted (32,14), but not the textbook delocalised (39), version of the chemiosmotic theory, mitochondria with a substantial proton gradient (left) acidify the outer surface of the inner membrane, leading to greater protonation of superoxide and thus faster dismutation, than when the proton gradient is weak (right). This is in accordance with observed rates (24).

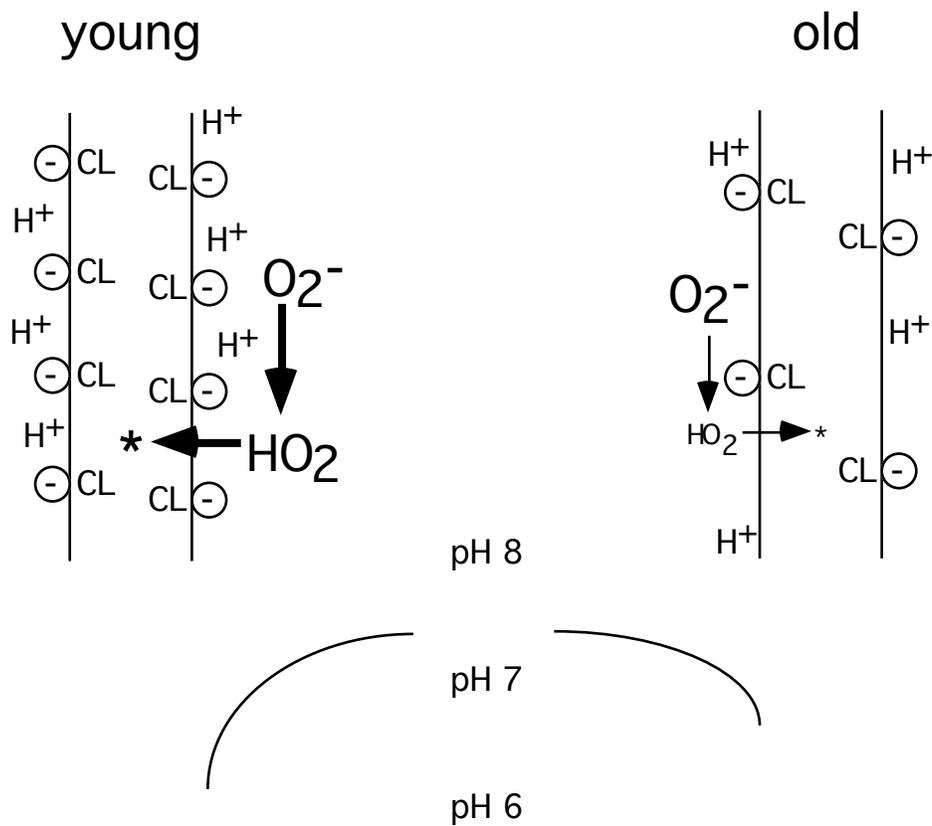


Figure 2. Cardiolipin in the mitochondrial inner membrane (left) causes a substantial acidification of the surface aqueous phase (independent of transmembrane proton gradient), due to its fixed negative charge. This increases $HO_2\cdot$ levels and consequently lipid peroxidation rates, relative to a mitochondrion with a depleted cardiolipin concentration (right). CL, cardiolipin.