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A New Paradigm: Manganese Superoxide Dismutase Influences the Production of H_2O_2 in Cells and Thereby Their Biological State

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Abstract

The principal source of hydrogen peroxide in mitochondria is thought to be from the dismutation of superoxide *via* the enzyme manganese superoxide dismutase (MnSOD). However, the nature of the effect of SOD on the cellular production of H_2O_2 is not widely appreciated. The current paradigm is that the presence of SOD results in a lower level of H_2O_2 because it would prevent the non-enzymatic reactions of superoxide that form H_2O_2 . The goal of this work was to: a) demonstrate that SOD can increase the flux of H_2O_2 , and b) use kinetic modelling to determine what kinetic and thermodynamic conditions result in SOD increasing the flux of H_2O_2 . We examined two biological sources of superoxide production (xanthine oxidase and coenzyme Q semiquinone, $CoQ^{\bullet-}$) that have different thermodynamic and kinetic properties. We found that SOD could change the rate of formation of H_2O_2 in cases where equilibrium-specific reactions form superoxide with an equilibrium constant (K) less than 1. An example is the formation of superoxide in the electron transport chain (ETC) of the mitochondria by the reaction of ubisemiquinone radical with dioxygen. We measured the rate of release of H_2O_2 into culture medium from cells with differing levels of MnSOD. We found that the higher the level of SOD, the greater the rate of accumulation of H_2O_2 . Results with kinetic modelling were consistent with this observation; the steady-state level of H_2O_2 increases if $K < 1$, for example $CoQ^{\bullet-} + O_2 \rightarrow CoQ + O_2^{\bullet-}$. However, when $K > 1$, *e.g.* xanthine oxidase forming $O_2^{\bullet-}$, SOD does not affect the steady state-level of H_2O_2 . Thus, the current paradigm that SOD will lower the flux of H_2O_2 does not hold for the ETC. These observations indicate that MnSOD contributes to the flux of H_2O_2 in cells and thereby is involved in establishing the cellular redox environment and thus the biological state of the cell.

Keywords

superoxide dismutase; mitochondria; coenzyme Q; hydrogen peroxide; superoxide; free radical; redox environment

Introduction

Superoxide dismutase is an important antioxidant enzyme as it is found in nearly all organisms. In mammals there are at least three forms of SOD: a cytosolic (CuZnSOD), an extracellular (ECsOD), and a mitochondrial form (MnSOD). All SOD enzymes catalyze the dismutation of superoxide, Rxns 2-5 Table 1 [1,2]:

It has been shown that MnSOD-knockout mice die within 1–18 days after birth, depending on their genetic background [6,7]; thus, MnSOD is an essential enzyme. Changes in SOD levels inside cells can have opposing effects. High overexpression of SOD in *E. coli* has been found to increase sensitivity to paraquat and to hyperoxia [8], as well as ionizing radiation due to apparent increased production of H_2O_2 [9]. Human and mouse cell clones that overexpress human CuZnSOD appear to have higher levels of hydrogen peroxide [10,11]. However, transfection of V79 Chinese hamster cells to overexpress CuZnSOD resulted in a decrease in H_2O_2 in cells [12]. Overexpression of MnSOD in several human cell lines has provided indirect evidence that this increased expression leads to increases in the production of H_2O_2 [13,14,15,16]. However, the current paradigm in the research community is that SOD decreases the production of H_2O_2 [17,18,19]: “the proposal that SOD enhances H_2O_2 by catalyzing the dismutation reaction can be discounted” [17].

The current paradigm

The concentrations of $\text{O}_2^{\bullet-}$ and H_2O_2 in a cell are assumed to be in a quasi steady-state. These steady-state concentrations, $[\text{O}_2^{\bullet-}]_{\text{ss}}$ and $[\text{H}_2\text{O}_2]_{\text{ss}}$, reflect a balance between the rate of formation and the rate of removal. Thus, the steady-state level can change by either changing the rate of formation and/or the rate of removal. It is widely accepted that changes in levels of cellular SOD will result in:

- a. a change in $[\text{O}_2^{\bullet-}]_{\text{ss}}$, *e.g.* an increase in SOD would increase the rate of removal of $\text{O}_2^{\bullet-}$ and thereby lower $[\text{O}_2^{\bullet-}]_{\text{ss}}$;
- b. no change in the rate of production of $\text{O}_2^{\bullet-}$;
- c. a minor change in the rate of production of H_2O_2 , no more than a factor of two (This assumes that *Case 3* described below is not applicable.); this change in the rate of production of H_2O_2 could result in
- d. a minor change in $[\text{H}_2\text{O}_2]_{\text{ss}}$ [17].

This paradigm is based on the observation that the rate of production of H_2O_2 by the enzyme xanthine oxidase (XO) is not affected by SOD. XO is a well-studied enzyme that is widely used as a tool to generate superoxide and hydrogen peroxide in experimental systems [20].

A new paradigm

However, we propose that there are other superoxide-generating systems (*e.g.* the mitochondrial electron transport chain) that do not behave like the xanthine/xanthine oxidase (X/XO) system. In these systems, changes in levels of cellular SOD can result in:

- a. a change in $[\text{O}_2^{\bullet-}]_{\text{ss}}$;
- b. a change in the rate of production of $\text{O}_2^{\bullet-}$;
- c. a major change in the rate of production of H_2O_2 , more than a factor of two; this could result in
- d. a major change in $[\text{H}_2\text{O}_2]_{\text{ss}}$.

How could SOD change the level of H_2O_2 in cells?

There are various ways that superoxide can be converted to H_2O_2 .

Case 1: Dismutation reaction—Independent of how $O_2^{\bullet-}$ dismutates, *i.e.* with or without SOD, **two** $O_2^{\bullet-}$ will yield **one** H_2O_2 , net of Rxns 2-5 of Table 1.

Case 2: Reactions with electron donors—If $O_2^{\bullet-}$ reacts with an electron-proton/hydrogen-atom donor, then **one** $O_2^{\bullet-}$ will yield **one** H_2O_2 .

In addition to cellular reducing agents, the donor could be a transition metal such as Fe^{2+} yielding Fe^{3+} , for example with the 4Fe-4S cluster of aconitase.

Case 3: Chain reactions that produce superoxide—There are cases where one superoxide can result in the formation of considerably more of both superoxide and hydrogen peroxide, *i.e.*, **one** $O_2^{\bullet-}$ will yield **n** H_2O_2 , where n can be much greater than one. This scenario can occur in a chain reaction where $O_2^{\bullet-}$ is a product of the reaction and is also a chain-carrying radical, initiating a new chain [21,22,23,24].

An example is the oxidation of 6-hydroxydopamine (6-OHDA) to its semiquinone ($SQ^{\bullet-}$) and quinone (Q):

Here, after initiation, $O_2^{\bullet-}$ is formed by electron transfer from the semiquinone radical of 6-OHDA ($SQ^{\bullet-}$) to O_2 . Superoxide then serves as a chain-carrying radical to initiate additional oxidation cycles, each forming another $O_2^{\bullet-}$ and H_2O_2 . Thus, the formation of one $O_2^{\bullet-}$ will lead to n H_2O_2 , where n is essentially the chain-length of this set of reactions. The chain termination reaction of $SQ^{\bullet-} + O_2^{\bullet-}$ is probably not of consequence. See Table 1. Iron(III) is shown as the initiating species above, but in higher pH environments metals may not be needed as true autoxidation can occur [25].

Case 4: Reactions with non-hydrogen atom electron acceptors/donors—If superoxide reacts with an electron-acceptor, such as Fe^{3+} cytochrome c, or covalently with an electron-donor, such as nitric oxide, then there will be no H_2O_2 produced. In this case, SOD would increase the rate of production of H_2O_2 . Although these reactions of superoxide are biologically important, they were not included in this study as they are typically minor sinks for superoxide. In addition, the reactions are not considered reversible, and the focus of this study is on the reversible formation of superoxide.

The current paradigm is that if SOD is present in *Cases* 2 and 3 above, its reaction with $O_2^{\bullet-}$ would dominate reactions of $O_2^{\bullet-}$ with donor-H or the chain-initiation by $O_2^{\bullet-}$, thereby converting these processes into *Case* 1, resulting in less H_2O_2 . In *Case* 2 the amount of H_2O_2 would decrease by no more than a factor of two; in *Case* 3 the decrease would be by a factor of approximately n. In these cases, SOD lowers the level of H_2O_2 . Translating this into a cellular setting would mean that overexpression of SOD in cells would result in lower levels of H_2O_2 . However, experimental data have provided indirect evidence that cells with increased levels of SOD can have increased levels of H_2O_2 [13–16]. This apparent contradiction can be rationalized if increasing SOD increases the rate of formation of $O_2^{\bullet-}$ and consequently

H₂O₂. The paradigm summarized above does not consider the possibility that SOD could change the rate of production of O₂^{•−} reversible reactions.

How could SOD change the rate of formation of O₂^{•−} and H₂O₂?

If the rate of production of O₂^{•−} in a system is constant and the amount of SOD varies, then as indicated above the rate of production of H₂O₂ will either remain the same, *Case 1*, or decrease, *Cases 2 and 3*. However, if Le Chatelier's principle¹ applies to the reaction forming O₂^{•−}, then the rate of production of O₂^{•−} could change with changing levels of SOD, resulting in an increase in the rate of production of H₂O₂.

For example, in the reaction below, if SOD is present it will compete with the reverse reaction and remove some of the O₂^{•−} and thereby “pull” the reaction to the right. SOD will have the most influence when the rate constant of the reverse reaction is greater than the rate constant of the forward reaction ($k_r > k_f$; $K = k_f/k_r < 1$).

Increased levels of SOD will pull the equilibrium of this reversible reaction to the right, and the rate of production of O₂^{•−} and subsequently H₂O₂ will increase. If the reverse reaction is slow ($k_r < k_f$; $K > 1$),

then only a very small fraction of the O₂^{•−} molecules will enter the reverse reaction and dismutation will occur with or without SOD. Changes in the amount of SOD would have little impact. In a cellular setting both scenarios can be found. In the case of XO ($K > 1$) we propose that the current paradigm applies, *i.e.* increasing SOD will either have no effect, *Case 1*, or decrease levels of H₂O₂, because the rate of the reverse reaction is slow compared to the forward reaction that produces O₂^{•−}, *i.e.* *Case 2*. However, there are reactions in the cell that have $K < 1$, for example CoQ^{•−} forming O₂^{•−}. We propose that when the reverse reaction is comparable to or faster than the forward reaction ($K < 1$), then Le Chatelier's principle may apply and the current paradigm no longer holds. Or stated another way, when the equilibrium constant (K) for the reaction forming O₂^{•−} is $\gg 1$, then SOD will have little effect on the rate of production of H₂O₂; but, when K is ≈ 1 or < 1 , then SOD can affect the rate of production of H₂O₂. We propose that the latter is the case in the CoQ^{•−} reaction, but not in the reaction of XO to form O₂^{•−}.

A. The xanthine oxidoreductase system, thermodynamics

The metalloenzyme xanthine oxidoreductase (XOR) is a dimer that contains two molybdenum (Mo) atoms and eight irons as four (2Fe-2S) moieties. In addition there are two FAD groups in the enzyme. Thus, each subunit contains, 1 Mo, 2 (2Fe-2S), and 1 FAD [26]. The oxidase form of XOR is a widely used tool to produce superoxide in the laboratory, but it can also be a major source of unchecked superoxide production *in vivo* [27,28]. The oxidase form (XO) oxidizes xanthine, hypoxanthine or other substrates using oxygen as the preferred electron-acceptor, producing O₂^{•−} and H₂O₂. Figure 1 [29,30,31].

¹Le Chatelier's Principle states that if the conditions of a system, initially at equilibrium, are changed, the equilibrium will shift in such a direction as to tend to restore the original conditions.

It is the radical of the flavin moiety (XO-FADH[•]) that interacts with O₂ forming O₂^{•-} and subsequently H₂O₂ [32]. (The direct two-electron reduction of oxygen to form H₂O₂ in general occurs through a fully reduced flavin, FADH₂.)

The equilibrium constant for this reaction can be estimated from the reduction potentials of the two redox couples:

$$E^{\circ'} (\text{XO} - \text{FAD} / \text{XO} - \text{FADH}^{\bullet}; \text{pH } 7.4) = -400 \text{ mV} [33,34]$$

$$E^{\circ'} (\text{O}_2 / \text{O}_2^{\bullet-}; \text{pH } 7.4) = -160 \text{ mV} [35]$$

Using the fundamental relationship connecting the Gibbs free energy of a reaction to its equilibrium constant, *i.e.* $\Delta G^{\circ'} = -nF\Delta E^{\circ'} = -RT \ln K$, the equilibrium constant for the formation of O₂^{•-} by the flavin radical in XO is $\gg 1$ ($K_{1a} = 10^4$). With this equilibrium constant, very little of the O₂^{•-} produced will enter into the reverse reaction; in the absence of SOD the O₂^{•-} formed will chemically dismutate. The addition of SOD would have little effect on the rate of production of H₂O₂. SOD would convert the chemical dismutation into a faster enzyme-catalyzed dismutation and thereby lower the steady-state level of O₂^{•-}. However, it will not have a detectable effect on the production of H₂O₂ because there is not much superoxide entering into the reverse reaction to draw from.

B. The CoQ system, thermodynamics

The CoQ-system of the mitochondrial electron transport chain has a different equilibrium constant than the X/XO system for forming O₂^{•-}. Superoxide is formed by mitochondria [36, 37,38]; a major source is thought to be CoQ^{•-}, Rxn 1 (Table 1, Figure 2) [39,40]:

Literature values for this one-electron reduction potential vary considerably due to the difficulty of determining a true reduction potential while in the membrane. Values range from -40 mV to -230 mV [35,41,42,43], depending on solvent, environment [44], and whether CoQ is bound. Thus, the one-electron reduction potential for CoQ is estimated as:

$$E^{\circ'} (\text{CoQ} / \text{CoQ}^{\bullet-}; \text{pH } 7.0) = -40 \text{ to } -230 \text{ mV} [41,44,45]$$

Using this range of one-electron reduction potentials, the estimated equilibrium constant for Rxn 1b is $K_{1b} \approx 10^{-2} - 10^{+1}$, which is much smaller than $K_{1a} \approx 10^{+4}$ for the production of superoxide by the flavin radical in XO. Because of the relatively rapid reverse reaction for Rxn 1b, we propose that the current paradigm, that SOD has no influence on the rate of production, will not apply for the CoQ system. Modulating the level of SOD in the CoQ-system would result in a change in the net rate for the reaction forming O₂^{•-}; following Le Chatelier's principle, an increase in MnSOD would change the rate of production of O₂^{•-} and H₂O₂.

To examine how the value of the equilibrium constant, K_j , alters the rate of formation of O₂^{•-} and subsequent production of H₂O₂ and how SOD influences the system, we developed a kinetic model to simulate the production of superoxide and hydrogen peroxide by the mitochondrial CoQ system and the X/XO-system.

Materials and methods

Kinetic Model of the Superoxide-Peroxide System

Antioxidant enzymes are involved in a series of reactions, converting superoxide radical to hydrogen peroxide, and finally to water. Superoxide dismutases convert superoxide radical to hydrogen peroxide. Catalase and glutathione peroxidase on the other hand convert hydrogen peroxide to water. The reactions involved can be separated into three phases:

I. Superoxide Generation

II. Superoxide Dismutation

III. Peroxide removal

When considering the complete process, from the generation of superoxide radicals to the final conversion of hydrogen peroxide to water inside mitochondria, a detailed version can be written as in Table 1. From these reactions a rate expression can be written for time-dependent concentration changes for each species, Table 2. The subsequent sixteen non-linear ordinary differential equations (ODE) are shown in Equations (15) – (30). Evaluating the reaction rate constants, these equations represent a stiff system. Therefore, the modified Rosenbrock numerical method was selected to solve the transient expressions [46].

H₂O₂ measurement from cells

To determine if cells with varying levels of MnSOD produce H₂O₂ at different rates, we measured the accumulation of H₂O₂ in their growth medium. The steady-state level of H₂O₂ in cells has been estimated to be in the range of 10⁻¹⁰ – 10⁻⁷ M [47,48,49,50]; the levels are normally on the order of 10⁻⁹ – 10⁻⁸ M (1–10 nM), with higher levels associated with pathology [49]. However, intracellular determination of levels of H₂O₂ is problematic. We relied on the diffusion of H₂O₂ from cells into culture medium. The diffusion coefficient of H₂O₂ is nearly the same as H₂O, but more importantly, their permeability coefficients to cellular membranes are also nearly the same [47,51]. The time constant for the exchange of intracellular water for glioma cells in culture is on the order of 50 ms [52]. While the equivalent time constant in the fully functioning brain *in vivo* is an order of magnitude larger [53], this time constant is still < 1 s. Thus, intracellular H₂O₂ will rapidly appear in the extra-cellular medium. The rate of appearance will be proportional to the concentration gradient between inside and outside the cell. The levels of H₂O₂ were measured by the change in fluorescence of Amplex red, 10-acetyl-3,7-dihydroxyphenoxazine (ADHP) [54], using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Eugene, OR). Amplex red is considered to be a sensitive probe for H₂O₂, but can have interferences that can overwhelm the H₂O₂ signal of interest [55]. In the presence of H₂O₂ and horseradish peroxidase (HRP), ADHP generates a fluorescent oxidation product, resorufin, with an absorption maxima of approximately 563 nm and emission maxima around 587 nm [56]. In the assay exponentially growing cells had their growth medium replaced with a phosphate buffer and the appearance of H₂O₂ in the buffer was followed with time. The rates of appearance of H₂O₂, reflecting the intracellular [H₂O₂]_{ss}, are compared.

Results and discussion

MnSOD overexpression in cells results in an increase in H_2O_2

There are various cellular sources that can produce H_2O_2 . As a reliable measure of cellular production of H_2O_2 we have determined the rate of accumulation of extracellular H_2O_2 from several cell types. To determine if MnSOD influences the formation of H_2O_2 we used cells that had been transfected with MnSOD cDNA. This transfection provided clones with varying levels of MnSOD. These clones have been well characterized [57,58,59] and their antioxidant enzyme profile was verified for these experiments. The relative MnSOD activity for the different clones ranged from the same as wild type to a 19-fold increase in MnSOD activity. CuZnSOD activity in the transfected clones was comparable to parental cells; likewise peroxide-removing ability (catalase and glutathione peroxidase) was similar. We measured the appearance of H_2O_2 in media from dividing, unstressed cells. The rate of appearance of H_2O_2 in the media increased as the activity of MnSOD increased, Figure 3. Different MnSOD levels influenced the formation of H_2O_2 to different degrees. A 3-fold increase in MnSOD activity resulted in a 2-fold increase in the rate of accumulation of H_2O_2 in the medium, while a 19-fold increase in MnSOD, resulted only in a 3.5-fold increase in the rate of accumulation of H_2O_2 . Thus, in cells with low levels of MnSOD, modest increases in MnSOD will have a greater relative impact on the rate of formation of H_2O_2 than very large increases in MnSOD.

Similar measurements were done with wild type prostate cancer cells (PC-3) and an MnSOD-overexpressing clone (8.3-fold increase in MnSOD activity). The rate of appearance of extracellular H_2O_2 was 1.9-fold higher in the transfected clone compared to wild type PC-3 cells [59]. In a second study with PC-3 cells, we found that a clone with an 8.2-fold increase in MnSOD activity produced a 1.5-fold increase in the rate of appearance of H_2O_2 in the media compared to wildtype PC-3 cells [58]. Thus, an increase in MnSOD activity increases the rate of appearance of H_2O_2 in the extracellular medium, consistent with an increase in the rate of production of intracellular H_2O_2 .

Setting up the components of the kinetic model for the formation of H_2O_2

To understand how increasing the level of SOD increases the rate of production of H_2O_2 in cells (Figure 3), we set up a kinetic model. Because the experimental results above used cells with varying levels of the mitochondrial form of SOD, MnSOD, we chose as a focus the production of superoxide by the mitochondrial electron transport chain (ETC) and compared this to the formation of $O_2^{\bullet-}$ from the X/XO system.

For the initial conditions in the model we started with all the MnSOD in its resting state of $Mn^{III}SOD$ with no H_2O_2 present, Table 3. To investigate how the rate of formation of H_2O_2 changes with $[MnSOD]$, we varied the apparent equilibrium constant for the specific reaction that forms superoxide, Rxn 1, by changing the value of the reverse rate constant for Rxn 1, k_{-1} , and thereby changing the apparent value of the equilibrium constant for this reaction, $K_1 = 0.001 - 1000$. For each value of K_1 we varied the level of MnSOD from $0.7 \mu M - 10 \mu M$. We kept the capacity for the removal of peroxide constant. Once the system was allowed to evolve, it took on the order of $10 - 100$ s for H_2O_2 to reach observable levels, Figure 4. After a short time the level of H_2O_2 reaches a steady-state. The delay in the appearance of H_2O_2 is because H_2O_2 is only formed *via* the reaction of $O_2^{\bullet-}$ with $Mn^{II}SOD$, Rxns 4 and 5. Because the initial conditions had MnSOD in the oxidation state of Mn^{III} , it takes some time to convert a significant amount of $Mn^{III}SOD$ to $Mn^{II}SOD$, Rxns 2 and 3. As expected, this lag time increases with increasing initial concentration of $Mn^{III}SOD$.

For our kinetic model we have chosen concentrations of participants that are our best interpretation of the literature, Table 3. However, even if future research suggests

concentrations that differ by an order of magnitude or more, the principal findings of this model still hold.

If $K_I > 1$, e.g. XO, then SOD has no influence on the production of H_2O_2

When the rate constant for the forward reaction of Rxn 1 is greater than the rate constant for the reverse reaction, *i.e.* $k_f > k_r$ or $K_I > 1$, then $[H_2O_2]_{ss}$ does not change with varying concentrations of MnSOD, Figures 4A and 4B. When $K_I > 1$, *e.g.* $K_I = 1000$ (Figure 4A), the same steady-state level of H_2O_2 is achieved for all [MnSOD] examined. This is because little of the $O_2^{\bullet-}$ formed enters into the reverse reaction, so essentially all superoxide dismutates to give H_2O_2 , with or without SOD. This scenario applies for the XO-xanthine system.

The kinetics for superoxide production by X/XO is initiated by the formation of a XO-xanthine complex, Figure 1. The actual production of superoxide is accomplished by reaction of the flavin radical of FAD, $FADH^{\bullet}$, with O_2 . In the blood plasma of healthy humans typical levels of hypoxanthine are approximately 2 μM with xanthine levels on the order of only 1 μM or less [62]; these values can vary considerably. Using the rate constants of Figure 1, a pseudo first-order rate constant for X/XO complex formation will be about $10 s^{-1}$. These electrons are quickly transferred to FAD. Oxygen levels in blood of course vary, but if we assume a typical concentration to be $\approx 150 \mu M$, then the pseudo first-order rate constant for the oxidation of $FADH^{\bullet}$ by oxygen to form $O_2^{\bullet-}$ will be $10 s^{-1}$. This estimate shows that the rate of enzyme reduction and subsequent oxidation to form $O_2^{\bullet-}$ are about the same. Thus, SOD could have an effect on the rate of production of H_2O_2 because the oxidation of $FADH^{\bullet}$ is not necessarily rate-limiting. However, the thermodynamics of the $FADH^{\bullet} + O_2$ reaction to form $O_2^{\bullet-}$ has $K_{Ia} = 10^4$; see Introduction. The model predicts that SOD would have little or no effect on the rate of production of superoxide and hydrogen peroxide because of this high value for K_I .

When $K_I > 1$, to change the rate of production of $O_2^{\bullet-}$, and subsequently H_2O_2 , the concentration of the limiting reagents must be modulated. The limit for the rate of formation of $O_2^{\bullet-}$ in this setting is the rate of the forward reaction for Rxn 1. Thus, increasing the concentration of the radical source for $O_2^{\bullet-}$ ($FADH^{\bullet}$) or O_2 would increase the flux of $O_2^{\bullet-}$ and H_2O_2 as either $FADH^{\bullet}$ or O_2 , or both, are the limiting reagents. The level of MnSOD will affect $[O_2^{\bullet-}]_{ss}$. In this kinetic model, if [MnSOD] is increased by a factor of two, the steady state level of $O_2^{\bullet-}$ decreases by a factor two, *i.e.* $[O_2^{\bullet-}]_{ss}$ will be a direct function of $1/[MnSOD]$, assuming there are no other significant sinks for $O_2^{\bullet-}$. However, the level of MnSOD would not affect the flux of $O_2^{\bullet-}$ and H_2O_2 .

If $K_I < 1$, e.g. CoQ, then SOD can influence the production of H_2O_2

When $k_f < k_r$, $K_I < 1$, the steady-state level of H_2O_2 achieved is a function of the level of MnSOD, the higher [MnSOD] the greater $[H_2O_2]_{ss}$, Figures 4C and 4D. The steady-state level of H_2O_2 will change if either its rate of removal or rate of formation changes. In the model we kept the capacity to remove H_2O_2 constant; thus, the rate of production must change. The rate of production of H_2O_2 changes in the presence of SOD because the very fast reverse reaction for Rxn 1 gives little chance for chemical dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2 . The very fast reaction of SOD consumes the $O_2^{\bullet-}$ before it can enter the reverse reaction of Rxn 1. From an examination of the slopes of the lines at $\approx 1000 s$ ($\log t = 3$) in Figure 4D ($K_I = 0.001$), we can see that indeed the rate of formation of H_2O_2 increases with increasing MnSOD. Thus, the kinetic model predicts that a change in the level of MnSOD will change the rate of production of $O_2^{\bullet-}$ and subsequently the flux of H_2O_2 . This scenario applies to the CoQ-system.

The semiquinone radical ($CoQ^{\bullet-}$) of coenzyme Q (ubiquinone) of the ETC is thought to be the primary source of superoxide in most non-phagocytic cells, Figure 2 [63,64,65,66,67,68,69, 70,71]. A small percent (on the order of 1% or less) of the overall electron-flow through the

ETC in normal, healthy cells is thought to result in the production of superoxide [70,72,73]. Values for some of the parameters in the kinetic model are sparse. To ensure that the conclusions drawn from the model are reliable, we tested wide ranges of concentrations to determine when significant changes in the outcome occurred. In the kinetic model we assumed a constant level of $\text{CoQ}^{\bullet-}$ and dioxygen [74,75]. We reasoned that the level of $\text{CoQ}^{\bullet-}$ would be in a relative steady-state in an unchanging environment because of its formation as an intermediate in the protonmotive Q cycle [71,76]; in addition CoQ and CoQH_2 readily comproportionate to form $2\text{CoQ}^{\bullet-}$ ($k \approx 250 \text{ M}^{-1} \text{ s}^{-1}$; $K \approx 6 \times 10^{-5}$ [77]). Thus, any $\text{CoQ}^{\bullet-}$ lost to its reaction with O_2 to form $\text{O}_2^{\bullet-}$, Rxn 1, would be rapidly replaced.

The thermodynamics of the $\text{CoQ}^{\bullet-} + \text{O}_2$ reaction to form superoxide is considerably different than for the X/XO system. The range of ΔE for Rxn 1 with $\text{CoQ}^{\bullet-}$ as the electron source for $\text{O}_2^{\bullet-}$ yields an equilibrium constant in the range of 10^{-2} to 10^1 . This much smaller value for K_1 allows MnSOD to change the rate of production of superoxide and H_2O_2 , Figure 3.

A pool of electrons is essential

Another difference between the CoQ system and the XO system is the size of the electron pool. Only a very small percentage ($\approx 1\%$ or less) [70,72,73] of the electrons passing through the electron transport chain actually result in superoxide formation. This loss of electrons from the ETC due to the increase in the rate of superoxide formation with increasing levels of SOD is only a small perturbation on the overall electron-flow through the mitochondria. The ETC has at any time more than 40,000 redox active electrons, of which 60% reside in Complexes I, II and CoQH_2 , Table 4. This large pool of electrons in the ETC coupled with the mobility of CoQ in the mitochondrial inner membrane [78] will instantly re-establish the steady-state level of $\text{CoQ}^{\bullet-}$, because $\text{CoQ}^{\bullet-}$ is formed as an intermediate in the protonmotive Q-cycle [71,76] as well as through comproportionation of CoQ and CoQH_2 . The system is then poised to produce yet another molecule of superoxide. This large pool of electrons is a ready source for the production of $\text{O}_2^{\bullet-}$ and H_2O_2 that can be modulated by SOD, Figure 5.

In contrast to $\text{CoQ}^{\bullet-}$, the X/XO system has a very small pool of electrons. Each enzyme molecule can hold six electrons per subunit. X/XO makes both H_2O_2 and O_2 . The ratio of products depends on pH, substrate, and substrate concentration [79]. Superoxide is formed from $\text{FADH}^{\bullet}$; this moiety exists only when XO is relatively empty of electrons [80,81]. The nearer XO is to full capacity, the greater the proportion of H_2O_2 formed. SOD will have no effect on the direct formation of H_2O_2 by XO; it will also have no effect on the production of H_2O_2 *via* superoxide.

Even if the thermodynamics of a reaction are appropriate and there are no limiting reagents on the reactant side of Rxn 1, the rate of the process must be on the time-scale of the biochemistry of the cell for mass action to be of significance. Or stated another way, the magnitude of the activation energies must be appropriate to allow reactions to proceed on the time scale of the biochemistry of the cell. We evaluated the literature and chose to use a forward rate constant for the $\text{CoQ}^{\bullet-}$ system of $\approx 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for Rxn 1b, Table 1. If this rate constant were lower, *e.g.* $\approx 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$, and the equilibrium constant was ≈ 0.01 , as with Rxn 1b, then SOD would still “pull” the reaction as per Le Chatelier’s principle. However, in a biological setting and biological time scale there would be no detectable change in the flux of H_2O_2 . The rate of formation of H_2O_2 would be a million times slower and would not be of consequence; The curves of Figure 4 would be shifted from a sub- μM concentration-range to sub-pM, *i.e.* less than 1 molecule of H_2O_2 from this source in a typical cell. Thus, rate constants must be appropriate for the time scale of the process; the rate constants for Rxn 1b are appropriate for the biological time scale.

Biological implications: a new view on the function of MnSOD When $K_1 < 1$, SOD can change $[H_2O_2]_{ss}$

The experimental results shown in Figure 3 are consistent with our kinetic model. The MCF-7 wildtype cells have very low MnSOD activity. Increasing this activity modestly results in a large increase in the rate of appearance of H_2O_2 in the media, but additional increases result in a smaller relative increase in H_2O_2 .

Our kinetic model demonstrates that maximal $[H_2O_2]_{ss}$ is achieved when $K_1 > 1$. When $K_1 > 1$, the reverse reaction is relatively slow and the vast majority of the superoxide formed dismutates, Figures 4A and 4B, resulting in maximal flux of $O_2^{\bullet-}/H_2O_2$. Most interesting is that when $K_1 < 1$, the $[H_2O_2]_{ss}$ achieved is less than the maximum possible $[H_2O_2]_{ss}$ as most of the superoxide formed is lost in the reverse reaction, Figure 4D. Of special interest in Figure 4D is that even when $[MnSOD] = 10 \mu M$, the steady-state level of H_2O_2 is only 60% of that when $K_1 > 1$, e.g. Figures 4A and 4B. When $K_1 = 0.001$ (Figure 4D), it would take $[MnSOD] > 100 \mu M$ to approach the maximum achievable $[H_2O_2]_{ss}$. These findings on H_2O_2 are of considerable biological significance as they suggest that for cells with relatively low MnSOD, an increase in enzyme activity will produce a relatively large increase in the rate of formation of H_2O_2 and $[H_2O_2]_{ss}$. But for cells that have robust levels of MnSOD, more MnSOD will have only a minor effect on the rate of formation of H_2O_2 .

These findings establish a basis for a new paradigm for the biological function of SOD. The traditional view is that by controlling the steady-state level of superoxide, SOD protects cells and tissues from potential damage. Thus, SOD is a primary antioxidant enzyme. Here we have shown how SOD can alter the flux of H_2O_2 . Both, superoxide and H_2O_2 are signalling molecules. Superoxide is known to react with certain Fe-centers in enzymes while hydrogen peroxide is in general changing the sulfhydryl tone of the cell. SOD serves as a rheostat for each of these processes; an increase in MnSOD would lower $[O_2^{\bullet-}]_{ss}$ and increase $[H_2O_2]_{ss}$; thus, MnSOD could serve as the switching mechanism between one-electron signalling and two-electron signalling processes, Figure 6. Being able to modulate the intracellular H_2O_2 flux, positions SOD as a major player in establishing the redox environment of a cell. It is thought that the redox environment of the cell is tightly connected to the biological status of the cell. Changes in the redox environment from reducing to more oxidizing can move cells through biological states such as proliferation, differentiation, and cell death [92]. H_2O_2 has been implicated in bringing about these biological changes. Thus, MnSOD could be a regulator of the cellular redox environment and with this control in part the biological state of the cell. This could explain the role of $O_2^{\bullet-}$ and MnSOD in the basic biology of proliferating cells, such as cancer cells, as well as a role for SOD in the treatment of cancer. MnSOD may have an even larger influence in pathologies that affect mitochondria.

MnSOD has been found to be low in many cancer cells [58,82,83,84,85,86], fetal cells [87, 88,89], as well as stem cells [90]. Forced overexpression of MnSOD slows the growth of cancer cells both *in vitro* and *in vivo* [58,85,86,91]. These and many other studies have clearly demonstrated that MnSOD suppresses cell growth. This growth suppression could be in part a result of increasing the flux of H_2O_2 and thereby pushing the redox status of the cell to a more oxidized state. This more oxidized redox environment will no longer support cell proliferation, but rather is associated with differentiation [92,93,94].

Summary

The results presented here indicate that:

- the flux of H_2O_2 increases in cells as MnSOD increases;

- SOD can modulate the flux of $O_2^{\bullet-}$ and H_2O_2 only when the equilibrium constant for the reaction forming superoxide is less than or equal to ≈ 1 , *e.g.* $CoQ_{10}^{\bullet-} + O_2$;
- in addition, for MnSOD to be able to influence the flux of H_2O_2 , a sufficient pool of electrons is needed; the ETC has such a pool;
- the rate constant for the reaction of $CoQ^{\bullet-}$ with O_2 to form $O_2^{\bullet-}$ is of a magnitude to yield a rate of formation of H_2O_2 appropriate for the time scale of the biochemistry of the cell;
- an increase in MnSOD will lower $[O_2^{\bullet-}]_{ss}$ in proportion to $1/[MnSOD]$;
- an increase in MnSOD will increase the relative flux of H_2O_2 and $[H_2O_2]_{ss}$ in cells with low MnSOD more, compared to those with robust levels of MnSOD.

The results presented here suggest an entirely new function for MnSOD. MnSOD not only controls the levels of $O_2^{\bullet-}$, but it can also be viewed as an enzyme that plays a role in establishing the flux of H_2O_2 in cells. Because H_2O_2 is a key to determining the redox environment of cells and tissues, MnSOD should now be viewed not only as an antioxidant enzyme but also as a key enzyme involved in the establishment of the cellular redox environment and thereby the biological status of cells and tissues.

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Figure 1. The flow of electrons through xanthine oxidase

Xanthine makes a complex with XO at the molybdenum site ($k_a = 6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, 37°C [116]); then xanthine is oxidized to urate with reduction of the molybdenum ($k_b = 15 \text{ s}^{-1}$). This is followed by transfer of electrons to the Fe/S centers ($k_c = 8.5 \times 10^3 \text{ s}^{-1}$ [117]); then to FAD ($k_d = 2 \times 10^2 \text{ s}^{-1}$ [117]), and finally production of O₂^{•-} from FADH[•] ($k_{1a} = 7 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$). The thermodynamics for the formation of O₂^{•-} from the FADH[•] indicate an equilibrium constant of $\approx 10^4$. Thus the back reaction would have a rate constant of $\approx 7 \text{ M}^{-1} \text{ s}^{-1}$ [30].

Figure 2. $\text{CoQ}^{\bullet-}$ of the ETC can make $\text{O}_2^{\bullet-}$, a source of H_2O_2
 $\text{CoQ}^{\bullet-}$ can be formed during electron/proton transfer during the protonmotive Q-cycle, from comproportionation of CoQ and CoQH_2 , and also by other one-electron oxidation-reduction processes. K_{1b} is the equilibrium constant highlighted in the model. (k_c and k_d are the rate constants for comproportionation and disproportionation, respectively).

Figure 3. The rate of accumulation of H_2O_2 in the media increases with the cellular MnSOD activity (♦) The H_2O_2 released from MCF-7 cells and several clones overexpressing MnSOD (1.1-, 3-, 6-, and 19-fold) in exponential growth was determined at several time points over a period of two hours. The concentrations of H_2O_2 in the medium were measured using Amplex red. The ordinate represents the change in the $[H_2O_2]$ in the media *versus* time compared to wildtype MCF-7 cells (1 on the left ordinate scale represents $2.8 \text{ zmol cell}^{-1} \text{ s}^{-1}$). Results are from three independent experiments $\pm$ standard deviation.

( ) The mitochondrial steady-state level of H_2O_2 (nM) from the kinetic model achieved with different levels of MnSOD (0.7–10 μM) as presented in Figure 4D.

Figure 4. The steady-state concentration of H₂O₂ in cells can be a function of the level of MnSOD
The kinetic model clearly demonstrates that when $K < 1$ for Rxn 1 the level of SOD will determine the flux of H₂O₂ as well as its steady-state level. (A) $K = 1000$; (B) $K = 10$; (C) $K = 0.1$; (D) $K = 0.001$. The different values for the equilibrium constant were achieved by varying the rate constant for the reverse reaction of Rxn 1, keeping the value of the forward rate constant at $8 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$. In the kinetic model, the capacity to remove H₂O₂ was kept constant.

Figure 5. The ability of SOD to modulate the flux of H_2O_2 and $[H_2O_2]_{ss}$ depends on the availability of a sufficient pool of electrons

The ETC has a large pool of electrons. The vast majority flow through complex IV to O_2 forming H_2O at the end of the ETC; a very small fraction form $O_2^{\bullet-}$. The CoQ-system has appropriate thermodynamics and access to a large pool of electrons so that MnSOD can modulate the flux of $O_2^{\bullet-}$ and subsequently H_2O_2 . XO has unfavorable thermodynamics, which do not allow SOD to modulate the flux of $O_2^{\bullet-}$; in addition there is a very limited pool of electrons to draw from. Although $CoQ^{\bullet-}$ is thought to be the principal source for formation of superoxide in mitochondria, other sites may also be sources of $O_2^{\bullet-}$.

Figure 6. MnSOD serves as a rheostat for both superoxide and hydrogen peroxide signalling
 By controlling $[O_2^{\bullet -}]_{ss}$ MnSOD influences certain reactive Fe-centers in enzymes and proteins that are sensitive to oxidation/reduction by $O_2^{\bullet -}$. By changing the flux of H_2O_2 , MnSOD will also modulate signalling processes that go through the thiol-system. Thus, MnSOD serves as a rheostat for both one-electron and two-electron signalling pathways. These signalling processes set the redox environment of cells, which in turn establishes the biological state of cells and tissues.

TABLE 1

The reactions in the kinetic model

Rxn Nr.	Reaction	Rate Constant $M^{-1} \cdot s^{-1}$ or s^{-1}	Reference/comment
1a	$XO-FADH^{\bullet} + O_2 \rightarrow XO-FAD + O_2^{\bullet-} + H^+$	$k_{1a} = 7 \times 10^4$	[30]
-1a	$XO-FAD + O_2^{\bullet-} + H^+ \rightarrow XO-FADH^{\bullet} + O_2$	$k_{-1a} = 7$	Estimated here using $K_{1b} = 10^4$
1b	$CoQ_{10}^{\bullet-} + O_2 \rightarrow CoQ_{10} + O_2^{\bullet-}$	$k_{1b} = 8 \times 10^3$	[77,95,96,97] The actual value is uncertain, but an equilibrium constant appears to be $\approx 0.01-0.1$.
-1b	$CoQ_{10} + O_2^{\bullet-} \rightarrow CoQ_{10}^{\bullet-} + O_2$	$k_{-1b} = 8 \times 10^5$	[77,95,96]
2	$Mn^{III}SOD + O_2^{\bullet-} \rightarrow Mn^{III}SOD:O_2^{\bullet-a}$	$k_2 = 1.5 \times 10^9$	[98,99]
-2	$Mn^{III}SOD:O_2^{\bullet-a} \rightarrow Mn^{III}SOD + O_2^{\bullet-}$	$k_{-2} = 3.5 \times 10^4$	[99]
3	$Mn^{III}SOD:O_2^{\bullet-a} \rightarrow Mn^{II}SOD + O_2$	$k_3 = 2.5 \times 10^4$	[99]
-3	$Mn^{II}SOD + O_2 \rightarrow Mn^{III}SOD:O_2^{\bullet-a}$	$k_{-3} = 0$	[99]
4	$Mn^{II}SOD + O_2^{\bullet-} \rightarrow Mn^{II}SOD:O_2^{\bullet-b}$	$k_4 = 1.5 \times 10^9$	[98,99]
-4	$Mn^{II}SOD:O_2^{\bullet-b} \rightarrow Mn^{II}SOD + O_2^{\bullet-}$	$k_{-4} = 3.5 \times 10^4$	[99]
5	$Mn^{III}SOD:O_2^{\bullet-a} + 2H^+ \rightarrow Mn^{III}SOD + H_2O_2$	$k_5 = 2.5 \times 10^4$	[99]
-5	$Mn^{III}SOD + H_2O_2 \rightarrow Mn^{II}SOD:O_2^{\bullet-a} + 2H^+$	$k_{-5} = 300$	[99]
6	$Mn^{II}SOD:O_2^{\bullet-a} \rightarrow DEP^c$	$k_6 = 650$	[99]
-6	$DEP \rightarrow Mn^{II}SOD:O_2^{\bullet-a}$	$k_{-6} = 10$	[99]
7	$2H^+ + 2O_2^{\bullet-} \rightarrow O_2 + H_2O_2$	$k_7 = 2.4 \times 10^5$	[5]
-7	$O_2 + H_2O_2 \rightarrow 2H^+ + 2O_2^{\bullet-}$	$k_{-7} = 0$	[5]
8	$GPx_r + H_2O_2 + H^+ \rightarrow GPx_0 + H_2O^d$	$k_8 = 2.1 \times 10^7$	[100,101,102]
-8	$GPx_0 + H_2O \rightarrow GPx_r + H_2O_2$	$k_{-8} = 0$	[100,101]
9	$GPx_0 + GSH \rightarrow GSGPx + H_2O$	$k_9 = 4 \times 10^4$	[100,101]
-9	$GSGPx + H_2O \rightarrow GPx_0 + GSH$	$k_{-9} = 0$	[100,101]
10	$GSGPx + GSH \rightarrow GPx_r + GSSG + H^+$	$k_{10} = 1 \times 10^7$	[100,101]
-10	$GPx_r + GSSG + H^+ \rightarrow GSGPx + GSH$	$k_{-10} = 0$	[100,101]
11	$GSSG \rightarrow 2GSH$		
-11	$2GSH \rightarrow GSSG$		
12	$CoQ^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow CoQ + H_2O_2$	$k_{12(obs)} = 3 \times 10^6^e$	Estimated

^a $Mn^{III}SOD:O_2^{\bullet-}$ is a complex of $Mn^{III}SOD$ and $O_2^{\bullet-}$.

^b $Mn^{II}SOD:O_2^{\bullet-}$ is a complex of $Mn^{II}SOD$ and $O_2^{\bullet-}$.

^c DEP is dead end product.

^d Catalase was not included in the kinetic model because it is primarily a peroxisomal enzyme. We included only GPx1 as a sink for H_2O_2 . Because we kept the capacity of the system for removing H_2O_2 constant, the inclusion of catalase or peroxiredoxin-III as additional components of the H_2O_2 -removing system would make no difference in the final results of the model.

^e This is an estimate based on the non-enzymatic dismutation of superoxide. The rate constant will rely on the pH and pK_a of these two species. If we assume the maximum rate constant is parallel to that of superoxide, *i.e.* the reaction of protonated and unprotonated species, then we can assume that the fastest reaction will have either $O_2^{\bullet-}$ protonated or $CoQ^{\bullet-}$ protonated. If the effective pK_a of $CoQH^{\bullet}$ is 5.9 [103] then at pH 7.4 about 3% of $CoQ^{\bullet-}$ and 0.2% of $O_2^{\bullet-}$ will be protonated. With this, an estimate of an observed rate constant would be $k_{obs} \approx 3 \times 10^6 M^{-1} s^{-1}$. Once superoxide is formed, possible routes to formation of H_2O_2 , as a rate equation, are: $+d[H_2O_2]/dt = \{k_{SOD} [SOD] + k_{CoQ^{\bullet-}} [CoQ^{\bullet-}] + k_{dismut}[O_2^{\bullet-}] + k_{other}[other]\} [O_2^{\bullet-}]$. Estimating the contributions of each term $+d[H_2O_2]/dt = \{2 \times 10^4 s^{-1} + 3 \times 10^{-1} s^{-1} + 2.4 \times 10^{-4} s^{-1} \times k_{other}[other]\} \times [O_2^{\bullet-}]$, and assuming that $[O_2^{\bullet-}]$ is at most on the order of 1 nM, we see that the termination reaction in question is negligible, as well as the chemical dismutation of superoxide. Thus this reaction was not included in the kinetic model. Here, "other" refers to reactions of superoxide with substances such as aconitase, Fe^{3+} cytochrome c, nitric oxide *etc.* These represent a small fraction of the reactions of superoxide [104,105].

Table 2
The Differential Equations of the Model.

$\frac{d[CoQ_{10}^{\bullet-}]}{dt} = k_{-1}[CoQ_{10}][O_2^{\bullet-}] - k_1[CoQ_{10}^{\bullet-}][O_2]$	(15)
$\frac{d[CoQ_{10}]}{dt} = k_1[CoQ_{10}^{\bullet-}][O_2] - k_{-1}[CoQ_{10}][O_2^{\bullet-}]$	(16)
$\frac{d[O_2]}{dt} = k_{-1}[CoQ_{10}][O_2^{\bullet-}] + k_3[Mn^{III}SOD:O_2^{\bullet-}] + 2k_7[O_2^{\bullet-}]^2 - k_1[CoQ_{10}^{\bullet-}][O_2]$	(17)
$\frac{d[O_2^{\bullet-}]}{dt} = k_1[CoQ_{10}^{\bullet-}][O_2] + k_{-2}[Mn^{III}SOD:O_2^{\bullet-}] + k_{-4}[Mn^{II}SOD:O_2^{\bullet-}] - k_{-1}[CoQ_{10}][O_2^{\bullet-}] - k_2[Mn^{III}SOD][O_2^{\bullet-}] - k_4[Mn^{II}SOD][O_2^{\bullet-}] - 2k_7[O_2^{\bullet-}]^2$	(18)
$\frac{d[Mn^{III}SOD]}{dt} = k_{-2}[Mn^{III}SOD:O_2^{\bullet-}] + k_5[Mn^{II}SOD:O_2^{\bullet-}] - k_2[Mn^{III}SOD][O_2^{\bullet-}] - k_{-5}[Mn^{III}SOD][H_2O_2]$	(19)
$\frac{d[Mn^{III}SOD:O_2^{\bullet-}]}{dt} = k_2[Mn^{III}SOD][O_2^{\bullet-}] - k_{-2}[Mn^{III}SOD:O_2^{\bullet-}] - k_3[Mn^{III}SOD:O_2^{\bullet-}]$	(20)
$\frac{d[Mn^{III}SOD]}{dt} = k_3[Mn^{III}SOD:O_2^{\bullet-}] + k_{-4}[Mn^{II}SOD:O_2^{\bullet-}] - k_4[Mn^{II}SOD][O_2^{\bullet-}]$	(21)
$\frac{d[Mn^{II}SOD:O_2^{\bullet-}]}{dt} = k_4[Mn^{II}SOD][O_2^{\bullet-}] + k_{-5}[Mn^{III}SOD][H_2O_2] + k_{-6}[DEP] - k_{-4}[Mn^{II}SOD:O_2^{\bullet-}] - k_5[Mn^{II}SOD:O_2^{\bullet-}] - k_6[Mn^{II}SOD:O_2^{\bullet-}]$	(22)
$\frac{d[H_2O_2]}{dt} = k_5[Mn^{II}SOD:O_2^{\bullet-}] + 2k_7[O_2^{\bullet-}]^2 - k_{-5}[Mn^{III}SOD][H_2O_2] - k_8[GPx_r][H_2O_2]$	(23)
$\frac{d[DEP]}{dt} = k_6[Mn^{II}SOD:O_2^{\bullet-}] - k_{-6}[DEP]$	(24)
$\frac{d[GPx_r]}{dt} = k_{10}[GSGPx][GSH] - k_8[GPx_r][H_2O_2]$	(25)
$\frac{d[GPx_o]}{dt} = k_8[GPx_r][H_2O_2] - k_9[GPx_o][GSH]$	(26)
$\frac{d[H_2O]}{dt} = k_8[GPx_r][H_2O_2] + k_9[GPx_o][GSH]$	(27)
$\frac{d[GSH]}{dt} = -k_9[GPx_o][GSH] - K_{10}[GSGPx][GSH]$	(28)
$\frac{d[GSGPx]}{dt} = k_9[GPx_o][GSH] - k_{10}[GSGPx][GSH]$	(29)
$\frac{d[GSSG]}{dt} = k_{10}[GSGPx][GSH]$	(30)

Table 3

The species in the model and their initial concentrations

Species	[Species] _i /M ^a	Comment/Reference
CoQ + CoQH ₂	5×10^{-4}	This concentration is based on data on rat liver mitochondria (120,000 total molecules/mitochondrion [78]; volume = $0.42 \mu\text{m}^3$ [106]), but assumes a homogeneous distribution. If considering only the lipid phase of mitochondria, then [CoQ ₁₀] is estimated to be 10 times this concentration (5 mM) in this phase [107] This is comparable to information on heart mitochondria [108,109,110]
CoQ	4.5×10^{-4}	90% of coenzyme Q, ubiquinone, is estimated to be in the oxidized form [78], however this can be expected to vary [111].
CoQH ₂	0.5×10^{-4}	10% of coenzyme Q is estimated to be in the reduced form [78,112].
CoQ ^{•+}	1×10^{-7}	This estimate assumes that 1 part in 5,000 of CoQ + CoQH ₂ exists as the radical [77,113].
O ₂	25×10^{-6}	
O ₂ ^{•-}	0	
Mn(III)SOD	Varied in the model	The resting state of MnSOD
Mn(III)SOD:O ₂ ^{•-}	0	An intermediate in the reduction of Mn(III)SOD
Mn(II)SOD	0	The reduced form of MnSOD. The rate production of H ₂ O ₂ by MnSOD is maximized when [Mn(II)SOD]:Mn(III)SOD is 1:1
Mn(II)SOD:O ₂ ^{•-}	0	An intermediate in the oxidation of Mn(II)SOD
H ₂ O ₂	0	
DEP	0	Is a dead end product, <i>i.e.</i> inactivation of MnSOD
GPx _r	1×10^{-6}	The reduced form of GPx that reacts with hydroperoxides
GPx _o	0	The oxidized form of GPx that enters into reactions with GSH to recycle back to GPx _r
H ₂ O ₂	0	
GSH	1×10^{-3}	Glutathione
GSGPx	0	An intermediate in the reaction of GSH with GPx _o
GSSG	0	Glutathione disulfide

^aThis is the initial concentration at time zero.

Table 4

The pool of electrons in the ETC is large.

Redox component	Molecules per inner membrane[78] ^a	% reduced/oxidized of redox partners at steady state[78, 114]	Redox e ⁻ [115]	Number of redox electrons in pool per mitochondrion
Complex I	2,000	I(5 _{red}) → CoQ(90 _{ox}) ^b	2; 8 ^{cd}	800
Complex II	3,800	II(5 _{red}) → CoQ(90 _{ox})	2; 4	800
CoQ	120,000	CoQ(10 _{red}) → III(84 _{ox})	2; 2	24,000
Complex III	5,700	III(16 _{red}) → c(89 _{ox})	1; 3 ^e	2,700
Cytochrome c	17,000	c(11 _{red}) → IV(80 _{ox})	1; 1	1,900
Complex IV	13,000	IV(20 _{red})	4; 4	10,600
				Total = 40,800
				Total in Complexes I, II and CoQ = 25,000

^aThese estimates from [78] have been rounded to two-significant digits.

^bThese estimates are for uncoupled mitochondria from rat liver. The percent of reduced species will vary considerably, being greater for active mitochondria.

^cnumber of electrons transferred in turnover in the ETC.

^dmaximum number of redox electrons that could in principal be held in each complex or redox species.

^eThis does not include CoQ.