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COMMENTARY

The Role of Iron-Sulfur Clusters in *in vivo* Hydroxyl Radical Production

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The *in vivo* production of HO· requires iron ions, H_2O_2 and O_2^- or other oxidants but probably does not occur through the Haber-Weiss reaction. Instead oxidants, such as O_2^-, increase free iron by releasing Fe(II) from the iron-sulfur clusters of dehydratases and by interfering with the iron-sulfur clusters reassembly. Fe(II) then reduces H_2O_2, and in turn Fe(III) and the oxidized cluster are re-reduced by cellular reductants such as NADPH and glutathione. In this way, SOD cooperates with cellular reductants in keeping the iron-sulfur clusters intact and the rate of HO· production to a minimum.

O_2^- and other oxidants can release iron from Fe(II)-containing enzymes as well as copper from thionein. The released Fe(III) and Cu(II) are then reduced to Fe(II) and Cu(I) and can then participate in the Fenton reaction.

In mammalian cells oxidants are able to convert cytosolic aconitase into active IRE-BP, which increases the "free" iron concentration intracellularly both by decreasing the biosynthesis of ferritin and increasing biosynthesis of transferrin receptors.

The biological role of the *soxRS* regulon of *Escherichia coli*, which is involved in the adaptation toward oxidative stress, is presumably to counteract the oxidative inactivation of the iron clusters and the subsequent release of iron with consequent increased rate of production of HO·.

Keywords: Hydroxyl radical, superoxide radical, iron-sulfur cluster, superoxide dismutase, *soxRS* regulon, Haber-Weiss reaction

Abbreviations: IRE-BP, iron-responsive element binding protein; SOD, superoxide dismutase; FALS, familial amyotropic lateral sclerosis; PQ, paraquat (methyl viologen)

INTRODUCTION

We have proposed[1] that, *in vivo*, superoxide radical (O_2^-) increases the production of hydroxyl radical (HO·) by acting as an oxidant toward [4Fe-4S] clusters of dehydratases, rather than as a reductant toward ferric iron (Fe III). Since then data have accumulated which support this hypothesis, which now gains the stature of a theory of oxidative stress. It seems appropriate to now analyze the existing evidence and to expand this theory. A historical approach will be most convenient.

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ESTABLISHMENT OF THE TOXICITY OF O_2^- AND THE ROLE OF SODs

The discovery of an enzyme that catalyzes the disproportionation of O_2^- (reaction 1) over 25 years ago[2] suggested that O_2^- plays a major role in oxidative stress, while superoxide dismutase (SOD), was postulated to exert a defensive role.[2,3]

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

Although this view is now universally accepted, it was originally hotly disputed. Proposals that the real biological role of SOD is different from catalyzing reaction 1 were seriously discussed.[4,5,6] The Cu,ZnSOD does indeed have a limited ability to catalyze some O_2^--independent reactions including nitration and peroxidase-like action.[7,8] Moreover mutations in Cu,ZnSOD such as those found in FALS patients increase some of those activities.[9,10]

There are, however, no reliable data so far that the ordinary function of SODs is anything but catalyzing the dismutation of O_2^- and thus preventing damage toward O_2^--sensitive targets. The suggestions for alternative functions of SOD arose because there were questions about the toxicity of O_2^-.[4,5,11,12] It is in fact well known that O_2^- oxidizes epinephrine,[2] pyrogallol,[13] 6-hydroxydopamine[14] and glutathione.[15] Further, O_2^- oxidizes NADH bound to lactate dehydrogenase[16] and O_2^-, in the presence of vanadate, oxidizes NAD(P)H by a chain mechanism.[17] O_2^- inactivates several enzymes including catalase and glutathione peroxidase.[18,19] O_2^- is also a potent oxidant of [4Fe-4S] containing dehydratases;[1,20] and, of course, O_2^- reacts at a diffusion-limited rate with NO,[21] yielding peroxynitrite which can rapidly attack thiols,[22] [4Fe-4S] clusters,[23,24] unsaturated lipids,[25] proteins, and DNA,[26] and kills *E. coli*.[27]

The concentrations of the potential reactants for O_2^- in the cell are such that, in the absence of SOD, there is no possibility that O_2^- would disappear safely through dismutation. For example, let us suppose that the only reactant for O_2^- in the cell is reduced glutathione. The rate constant of the GSH/O_2^- reaction is within 10^2–10^3 M^{-1} s^{-1} range,[15] or about three orders of magnitude lower than the rate constant for the spontaneous dismutation of O_2^-, while the $[O_2^-]/[GSH]$ ratio is much lower than that. It follows that each O_2^- has a much better chance of reacting with GSH rather than with another O_2^-.

This being the case, what matters is not the intracellular $[O_2^-]$ but the intracellular rate of production of O_2^-, and the latter is really impressive. Imlay and Fridovich[28] estimated that several thousand molecules of O_2^- are generated in each aerobic *E. coli* cell every second! This is in spite of the fact that the O_2^- production in *E. coli* is only about 0.1% of the oxygen consumption.[28] Clearly the potential for damage is tremendous.

Sources of O_2^- production include electron transport chains in mitochondria or *E. coli*, enzymes such as fumarate reductase and flavin reductase, flavin containing dehydrogenases and probably autoxidizable small molecules.[28–32]

Indeed O_2^- is far less indiscriminately reactive than HO· but it has been pointed out that this is rather an argument in favor of O_2^- toxicity since more molecules of the less reactive agent would survive to selectively damage critical targets.[33] A mathematical model supporting this idea was presented.[34] The demonstration that O_2^- is indeed very toxic has a long history[3,20,35,36] but has most dramatically been demonstrated by creating mutant organisms lacking SOD.[20,35,37–40]

Thus *sodAsodB E. coli* strains either do not grow or grow poorly under aerobic conditions and are extremely sensitive toward redox cycling compounds that produce O_2^- intracellularly.[37,38] Most recently a mitochondrial SOD deficient mouse was constructed and the homozygotes were severely impaired and died within ten days of birth.[40] The question then is not: Is O_2^- toxic; but, rather, why is it so toxic?

[4Fe-4S] CLUSTER-CONTAINING DEHYDRATASES AS CRITICAL TARGETS FOR O_2^-

Odixative stress in general and O_2^- in particular can cause two clearly distinguishable types of damage, namely metabolical slowdown and more or less permanent damage such as mutagenicity and death.[20,35,37–46] In *E. coli*, depending on the growth conditions, bacteriostatic and/or bactericidal effect could be seen.[37,38,43,45,46] The SOD double mutant *E. coli* not only does not grow well aerobically, even in rich medium, but exhibits enhanced mutagenicity and dies rapidly in the stationary phase.[37,38,41,43] The decrease of the growth rate is currently thought to be due to inactivation of enzymes, while mutagenicity and death are due to damage to DNA.[1,20,26,45]

Brown and coworkers observed, during studies on the mechanism of toxicity of hyperbaric oxygen and paraquat in *E. coli*, that this toxicity could be relieved by supplementation with branched chain amino aids and niacin.[47,48] Two enzymes were found to be sensitive to hyperoxia and paraquat, namely dihydroxy acid dehydratase involved in the branched-chain amino acid biosynthesis[47] and the A protein of the quinolinate synthase[49] which catalyzes the condensation of α-imino aspartate with dihydroxyacetone phosphate.

Kuo *et al.*[50] subsequently found that dihydroxy acid dehydratase is O_2^- sensitive, while Flint and Emptage proposed and later found that it contains [4Fe-4S] cluster.[51,52] Quinolinate synthase was also suggested to have [4Fe-4S] cluster.[49] Additional dehydratases were found to be both O_2^--sensitive and to contain [4Fe-4S] clusters, including 6-phosphogluconate dehydratase, aconitase and both fumarase A and fumarase B.[23,53–60] The rate constants for the reactions of O_2^- with *E. coli* dihydroxy acid dehydratase, fumarases A and B, and aconitase, as well as beef heart mitochondrial aconitase and the cytosolic aconitase/IRE-BP were determined and found to be in the range of 10^6–10^7 M^{-1} s^{-1}.[23,59] It was also repeatedly confirmed that several of these enzymes including fumarase A, aconitase and dihydroxy acid dehydratase are sensitive *in vivo* toward inactivation by O_2^-.[55,57,61]

Thus all of the enzymes mentioned above contain [4Fe-4S] clusters and, with the exception of quinolinate synthase,[49] are very sensitive to O_2^-. Not surprisingly, therefore, in the absence of branched-chain amino acids the SOD double mutant did not grow aerobically but grew when they were suppplemented.[38] These and similar studies made clear that much of the damage caused by O_2^- is due to damage of proteins and enzymes that contain [4Fe-4S] clusters. Yet, even if grown in the "most favorable" aerobic conditions such as in rich LB medium, the double SOD mutant still grows ~1.5 times slower than the parental strain and exhibits a markedly higher rate of spontaneous mutangenesis.[38,42] How is this portion of O_2^- toxicity to be accounted for? First as yet unidentified O_2^--sensitive FeS cluster-containing enzymes and proteins probably exist. Second, the probability that damage to small biomolecules also contributes cannot be excluded. Finally, the production of HO·, with ensuing DNA damage, occurs by a mechanism to be discussed.

The conclusion that [4Fe-4S] cluster-containing enzymes are major targets for O_2^- toxicity was recently extended to mammals. As discussed above, Li *et al.*[40] constructed a mouse totally lacking mitochondrial MnSOD and have observed significant reductions in the activity of the FeS cluster containing enzymes aconitase and succinate dehydrogenase.

THE HABER-WEISS REACTION

More than 60 years ago Haber and Weiss[62] studied the reaction of Fe(II) with H_2O_2 (reaction 2), also known as the Fenton reaction, and observed that more H_2O_2 is decomposed than Fe(III) formed.

They therefore proposed a chain mechanism according to which the HO· formed in reaction 2 oxidizes H_2O_2 to HO_2 (the protonated form of O_2^-) which in turn reduces H_2O_2 in reaction 3 to produce HO·. Reaction 3 is now called Haber-Weiss reaction.

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + HO\cdot + OH^- \quad (2)$$

$$O_2^- + H_2O_2 \rightarrow HO\cdot + OH^- + O_2 \quad (3)$$

Later it was found that reaction 3 is actually iron mediated and reaction 3 is the net reaction of reaction 2 and reaction 4.[63,64]

$$Fe(III) + O_2^- \rightarrow Fe(II) + O_2 \quad (4)$$

The iron-mediated Haber-Weiss reaction has also been referred to as superoxide driven Fenton chemistry. It is noteworthy to point out that very similar chemistry is displayed by other transition metal ions, of which the chemistry of the vanadyl reaction with H_2O_2 is an interesting example.[65,66] FeO^{2+} might be the product of reaction 2 rather than HO· or at least the product that causes the damage in many cases. This controversy is ongoing in numerous articles, but is not relevant to the present discussion and the product of the Fenton reaction shall be referred to here as HO·.

Soon after the discovery of SOD, there were doubts about the toxicity of O_2^-. In 1970 Beauchamp and Fridovich[67] noticed that methional decomposition to ethylene, catalyzed by an enzymatical source of O_2^-, was strongly inhibited, not only by SOD, but also by catalase and by HO· scavengers such as benzoate and ethanol. They proposed that HO· produced via reaction 3 was the proximal cause of ethylene production. It was later established that iron was required[68,69] and in this way the Haber-Weiss reaction was introduced as the explanation of how the poorly reactive O_2^- can cause damage, via creating the very reactive HO·.

Despite some cautions expressed that the Haber-Weiss reaction cannot explain every case of O_2^- toxicity,[36] the overwhelming majority of research favored the iron-mediated Haber-Weiss reaction as the main, if not the exclusive, mechanism for O_2^- toxicity. We have cited previously a small part of this extensive literature (about 40 references), concerning the Haber-Weiss reaction both *in vivo* and *in vitro*.[1] Thus an impressive number of publications show that a very broad range of *in vitro* and *in vivo* effects including damage of proteins, DNA, other biomolecules and biomembranes, mutations and cell death, are due to HO· production due to the interplay of O_2^-, H_2O_2 and iron ions in a process interpreted as the iron-mediated Haber-Weiss reaction.

CRITICISM OF THE HABER-WEISS REACTION AND ALTERNATIVE EXPLANATIONS

The Haber-Weiss reaction obviously occurs *in vitro*. O_2^- can reduce Fe(III) to Fe(II) in a rapid reaction[64] and in a simple system consisting of Fe(III) complexes, O_2^--generating system and H_2O_2 there is no doubt that HO· is generated exactly because of the Haber-Weiss reaction.[1,67–69] However several authors[11,70–72] have argued that:

(a) O_2^- could not out compete cellular reductants such as GSH and ascorbate. As Czapski *et al.*[70] pointed out, if reaction 4 is the one responsible for the toxicity of O_2^-, it is rather puzzling how we survive in the presence of other reductants in the cell.

(b) Cells maintain highly reduced state even aerobically and any available iron therefore must be kept in its reduced form. It is difficult to see then, as Winterbourn[71] writes, how O_2^- will be able to drive the Fenton reaction, and SOD to prevent HO· formation by a Fenton mechanism. While the first part of this argument is very valid, an important objective of this review is to provide a correct explanation for how O_2^- can drive the Fenton reaction *in vivo*.

Arguments *a* and *b* belong to the category which Halliwell[73] characterized as "arguments

that do not negate the importance of O_2^-; they merely attack a particular explanation of its role in producing HO·." Indeed the evidence that O_2^- causes increased HO· production *in vivo* is overwhelming.

Free Fe(II) is undoubtedly a very dangerous species, yet Fe(II) is absolutely required for the maturation of many enzymes including those containing FeS clusters. Thus not Fe(II) itself but its misplacement is dangerous, as discussed below.

It has been argued that loosely bound iron is non-existent in cells. This argument is false as has been discussed by Halliwell.[73] Early results show that the killing of *Staphylococcus aureus* by H_2O_2 is due to HO· formation in proportion to the intracellular iron concentration.[74] More recently it has been shown[75,76] that if the regulation of iron uptake or the SODs of *E. coli* are impaired, the resulting increase of catalytically-active iron causes DNA damage and cell death through the Fenton reaction; while Böhnke and Matzanke[77] have discussed the nature of this iron pool. Further evidence for formation of HO·, presumably through the Fenton reaction, is provided by the characteristic pattern of oxidative DNA damage generated in cells subjected to oxidative stress.[26]

Attempts to provide an explanation for O_2^- toxicity, which does not depend on the reduction of "free" Fe(III), have been made:

(a) O_2^- not only reacts with "free" iron but also is able to release iron from ferritin, which then might react with H_2O_2 to produce HO·.[78–81] The problem with this explanation is that O_2^- again must act as a reductant and the same criticism as that applied to the Haber-Weiss reaction *in vivo* is relevant.

(b) Czapski *et al.*[70] proposed that O_2^- might react not as a reductant but as an oxidant of Fe(III), resulting in a higher oxidation state (FeIV) which is then the agent that causes damage to critical biomolecules. Despite its interest, however, this explanation does not provide for the role of H_2O_2.

THE HYPOTHESIS

By 1993 it was reasonable to assume that O_2^- could not serve as an important reductant of Fe(III) *in vivo* but must rather generate Fe(II) by acting as an oxidant and that the reaction of O_2^- with the Fe(II) generating targets must be fast enough to compete with other pathways of disappearance of O_2^-.

Since O_2^- reacts with the [4Fe-4S] clusters of several dehydratases at a rate of ~10^7 M^{-1} s^{-1}[23,59] and since that reaction is an oxidation which causes release of Fe(II) from the cluster and the concomitant reduction of O_2^- to H_2O_2, we proposed[1] the following:

$$[2Fe(II)\,2Fe(III)-4S]+O_2^-+2H^+ \rightarrow [Fe(II)\,3Fe(III)-4S]+H_2O_2 \quad (5)$$

$$[Fe(II)\,3Fe(III)-4S] \rightarrow Fe(II)+[3Fe(III)-4S] \quad (6)$$

Fe(II) and H_2O_2 are the products of reactions 5 and 6 and they will react according to the Fenton reaction (reaction 2). Even if Fe should be released from the clusters as Fe(III), it will be rapidly reduced by the cellular reductants to Fe(II) in reactions such as reaction 7.

$$2GSH+2Fe(III) \rightarrow GSSG+2Fe(II)+2H^+ \quad (7)$$

The oxidatively inactivated Fe-S cluster-containing enzymes are subject to reductive reactivation both *in vivo* and *in vitro*.[49,55,57,82–84] *In vitro* reactivation can be achieved by thiols in the presence of iron ions[84] and there are data that glutathione plays some such role *in vivo*.[82]

Assuming that NADPH is a major, although indirect, reductant, the net reaction for the full cycle of O_2^--dependent inactivation (reactions 5 and 6) and the NADPH dependent reactivation, which includes reduction of Fe(III) and of the oxidized cluster, will be (reaction 8).

$$NADPH + O_2^- + 2H^+ \xrightarrow{\text{Fe S cluster}} NADP^+ + HO\cdot + H_2O \quad (8)$$

This process is in essence a FeS cluster-catalyzed oxidation of reductants by O_2^- which results in

HO· formation. Reductants will be consumed even more rapidly because of the cycle consisting of reactions 2 and 7 and the subsequent regeneration of reduced glutathione by NADPH via glutathione reductase.

Unlike the classical Haber-Weiss reaction, wherein O_2^- behaves as reductant, here it is an oxidant. Consequently SOD, in scavenging O_2^-, acts as an antioxidant sparing reductants and preventing HO· formation. Moreover SOD and reductants cooperate to keep FeS clusters intact and HO· production low and if this cooperation is impaired, with only one participant missing, strong oxidative stress should result.

The condition of oxidative stress will be characterized by decreased activity of FeS cluster-containing dehydratases, increased "free" iron concentration, increased rate of HO· production, and increased consumption of reductants.

We also proposed[1] an additional mechanism, which might operate in some eukaryotic cells. Thus O_2^- can increase the catalytically active iron and the rate of HO· production by converting the cytosolic aconitase into the form of IRE-BP which inhibits ferritin biosynthesis.[85–87] Hence sequestration of iron into this protein is diminished. Since O_2^- reacts with the active (as aconitase) form of IRE-BP with a rate constant of 2.7×10^7 M^{-1} s^{-1},[23] it is conceivable that this will contribute to an increase of [free iron].

CONFIRMATION AND EXPANSION OF THE HYPOTHESIS

O_2 as well O_2^-[42,57,59] is capable of inactivating susceptible FeS clusters and so is peroxynitrite.[23,24] Other biologically relevant oxidants might also do so leading to increased HO· production and lipid peroxidation.

Another way to decrease the level of intact FeS cluster-containing enzymes, and to increase free iron, and thus to increase the *in vivo* HO· production, might be to inhibit reactivation or assembly of the clusters. Thus in cell extracts from *E. coli*, under anaerobic conditions, the previously aerobically-inactivated fumarase A spontaneously reactivates and this reactivation is inhibited by EDTA, while the aerobic inactivation is not.[57] Moreover no reactivation occurs aerobically. Since EDTA-chelatable iron was required for the anaerobic reactivation, we concluded that the inhibition of reactivation by aerobic conditions was due to oxidation of Fe(II) to Fe(III) which then could not be used for cluster reassembly. It follows that any agent that inhibits the reactivation or reassembly reactions intracellularly will cause a decrease in the level of intact FeS clusters and an increase of "free" iron and, finally, of HO·.

For example, quinolinate synthase is relatively insensitive to O_2^- and H_2O_2 but nevertheless the incubation of wild type *E. coli* with aerobic paraquat (PQ) caused a significant decrease in the activity of this enzyme.[49] It is reasonable to propose that PQ caused this effect by interfering with the reactivation. A similar conclusion can be drawn from the fact that incubation of wild type *E. coli* with aerobic PQ causes a decrease in the activity of the unstable fumarases,[57] while overproduction of SOD does not protect against PQ toxicity.[88,89] Since raising [SOD] above that normally present did not protect against PQ it follows that O_2^- was not the culprit in this case. We can suppose that diversion of electron flow by PQ decreased the supply of reductants which, in turn, prevented the reconstruction of oxidatively inactivated [4Fe-4S] clusters.

In accord with this view is the observation that while α,α-dipyridyl did not inhibit the quinolinate synthetase itself, it prevented the anaerobic reactivation of the enzyme.[49] Taken together with the similar ability of EDTA to prevent the reactivation of fumarase A[57] this strongly suggests that the aerobic inactivation of those enzymes creates a pool of "free" iron which then can be used either for reactivation or for the Fenton reaction. Similar experiments have been described for aconitase[55] and dihydroxy-acid dehydratase.[83]

It is possible to further expand this hypothesis by considering other enzymes that require Fe(II) for their activity. Catechol 2,3-dioxygenase has four identical subunits and contains one catalytically active Fe(II) per subunit. This enzyme can be inactivated by oxidants and reactivated by incubation with ascorbate plus Fe(II). Catechol 2,3-dioxygenase was inhibited by incubation of *Pseudomonas putida* with H_2O_2 and could be reactivated *in vivo* in a wild type strain, but not in a strain lacking ferredoxin.[90] It was concluded that the inactivation was due to an oxidation and/or removal of iron, while the ferredoxin-dependent reactivation was attributed to reduction of the oxidized iron cofactor.[90] Clearly inactivation of enzymes, requiring Fe(II) for their activity, by H_2O_2, O_2^- (which reacts with certain Fe(II) complexes very rapidly)[64] or other oxidants, would tend to increase the "free" iron content. Reactivation of enzymes that contain [Fe-S] clusters or Fe(II), may require ferredoxin or flavodoxin. Thus ferredoxin is required for the reactivation of catechol 2,3-dioxygenase of *P. putida*, while flavodoxin is involved in the reactivation of the anaerobic ribonucleotide reductase of *E. coli* through reduction of the FeS center of the enzyme.[91,92] Flavodoxin was found to be involved in the conversion of dethiobiotin into biotin in *E. coli* in a process requiring other components and enzymes such as NADPH, S-adenosyl-L-methionine, Fe(II) and probably ferredoxin-$NADP^+$ reductase.[93] Independently the product of the *bioB* gene, commonly known as biotin synthetase has been purified and characterized as homodimer having two $[2Fe\text{-}2S]^{2+}$ clusters.[94] It seems therefore that flavodoxin is involved in the cluster reduction.

The redox-cycling agent PQ is capable of causing O_2^--independent effects in *E. coli*.[56,89] Since it has been reported that *E. coli* possesses NAD(P)H:PQ oxidoreductase (PQ diaphorase), we reinvestigated the problem and found that there are at least 4 PQ-diaphorases in *E. coli*.[95,96] Three of them were identified as thioredoxin reductase,[95] ferredoxin:$NADP^+$ oxidoreductase[96] and sulfite reductase.[97,98]

In addition, pyruvate:ferredoxin (flavodoxin) reductase of *E. coli* is also capable of reducing PQ.[99] Consequently it could be concluded that PQ will exert its effects at least partly by diverting electron flow from flavodoxin and ferredoxin and thus decreasing the reactivation or reduction of Fe-S cluster containing enzymes. Moreover both PQ and O_2^- can oxidize flavodoxin, the latter via a chain reaction.[100,101]

Flavodoxin knockout mutations are thought to be lethal in *E. coli*[93] on the grounds that no such flavodoxin mutant is known. Nevertheless, because of the reductive manner through which flavodoxin works one wonders whether a mutant might survive anaerobically.

Further support for the role of flavodoxin and/or ferredoxin in the protection against oxidative stress is the report that ferredoxin (flavodoxin):$NADP^+$ oxidoreductase mutants of *E. coli* grow well anaerobically but are very sensitive to toxicity of aerobic PQ.[102] The *murA* mutation probably inactivates the ferredoxin $NADP^+$ oxidoreductase of *E. coli*, which results in dramatically increased sensitivity toward the toxicity of PQ, which a ferredoxin reductase transgene of plant origin reverses.[103] Other enzymes such as the NAD(P)H:flavin oxidoreductases may be involved in reducing Fe(III) to Fe(II) via a free flavin dependent mechanism.[104] These enzymes have the potential to potentiate the Fenton reaction or alternatively to be involved in FeS cluster reassembly.

Finally, the products of two genes *nifS* and *nifU* from *Azotobacter vinelandii* have been implicated in the FeS cluster reassembly.[105] The *nifS* product is cysteine desulfurase which probably provides sulfide, and the *nifU* product is probably a Fe(III) reductase responsible for providing Fe(II) for cluster reassembly.[105]

The process of reassembly and reactivation of the FeS clusters and the enzymes that depend upon them is still not well understood. What is clear, however, is that O_2^- as well as other oxidants can influence the processes of reactivation in a variety of ways, thus changing the steady-

state activity of enzymes dependent upon [Fe-S] clusters.

Any hypothesis becomes theory when it explains the known facts and none are found to contradict it. In this regard the studies discussed so far confirm and expand the theory about the nature of the *in vivo* HO· production. These studies, however, were not especially designed *a priori* to test that particular hypothesis and the fact that they confirm it becomes clear only through post-facto analysis.

The only investigation so far performed with the intention of testing the *in vivo* hypothesis,[1] while regrettably failing to acknowledge it, was carried on recently by Imlay and coworkers.[76] It describes a crucial and convincing experiment.

Thus when *E. coli* were transferred from aerobic to anaerobic conditions O_2^- production will cease and the existing O_2^- will disappear in seconds due to its reactivity. Therefore one would anticipate that the classical Haber-Weiss reaction would also cease following the transition to anaerobiosis. On the contrary if O_2^- causes release of iron due to FeS clusters disassembly, there will be persistent increase in the "free" iron pool aerobically and therefore the Fenton reaction will continue for a considerable amount of time following the removal of O_2. Indeed the reactivation of FeS cluster-containing enzymes both *in vivo* and *in vitro* goes on for many minutes, not seconds.[57,82,83]

Imlay and coworkers used catalase deficient cells, some of which were constructed to be SOD deficient as well. They transferred both the SOD-proficient strains and the SOD-deficient strains from aerobic to anaerobic conditions and when, after several minutes, completely anaerobic conditions were achieved they challenged both strains with H_2O_2. The SOD deficient strain, but not the wild type continued to be killed anaerobically for a considerable time! Moreover the killing of the SOD-deficient strain by H_2O_2 was suppressed by iron chelators.[76]

The ability of oxidants such as peroxynitrite and O_2^- to increase the production of HO· might not be restricted to their attack on iron containing proteins. The rate constant for the reaction of O_2^- with Cu(I)-thionein is 7.5×10^6 M^{-1} s^{-1},[106] which is comparable with the rate constant for the reaction of O_2^- with FeS cluster containing enzymes and Cu(I), following the reduction of the released Cu(II), can participate in Fenton chemistry.[107]

The idea, about the involvement of O_2^- in the conversion of the cytoplasmic aconitase into an active IRE-BP, resulting in increase in the iron pool, and hence of HO· production, has also been supported recently. Thus, IRE-BP was activated in V79 Chinese hamster ovary cells incubated with H_2O_2.[108] It is concluded that IRE-BP senses Fe(II) and that the oxidation of Fe(II) to Fe(III) by H_2O_2 triggers a program for increasing iron uptake (and mobilization). This seems reasonable since Fe(II), and not Fe(III), is imperative for the reactivation (reassembly) of the iron cluster of IRE-BP. In agreement with such an interpretation, incubation of a catalase-negative strain of *E. coli* with paraquat[57] resulted in much greater inactivation of the unstable fumarases (Fum A and Fum B) than the inactivation seen in the parent catalase-proficient strain. This might reflect oxidation of the intraellular Fe(II) to Fe(III) by H_2O_2 resulting in an effective Fe(II) deficiency. Fe(II) should be an obligatory factor[57] for the reassembly of the oxidatively-damaged iron cluster of the unstable fumarases. Gardner *et al.*[109] have studied aconitase in mammalian cells and have concluded that it is modulated by both O_2^- and iron availability, and that it is subject to reactivation much as is *E. coli* aconitase. In the case of iron deficiency the activation of IRE-BP is the right strategy but in the case of oxidative stress it could be dangerous unless it is accompanied by adequate adaptation toward the oxidative stress. This adaptation has been examined in *E. coli*.

ADAPTATION TOWARDS OXIDATIVE STRESS

Two major systems are known to be involved in the adaptation of *E. coli* toward oxidative stress,

i.e. the *soxRS* and *oxyR* regulons.[110–112] We will discuss *soxRS* only, but clearly the major points of that discussion apply to *oxyR* as well.

SoxR is the sensor protein of the *soxRS* regulon. The activation of SoxR induces the transcription of *soxS* and the product of this gene in turn activates the transcription of the target genes and thus the induction of the corresponding enzymes and proteins, many of which have been identified.[56,96,113–116] The *soxRS* regulon has been thought initially to respond specifically to, and to ensure defense against, O_2^-.[111,113,114]

Later it was found that the *soxRS*- mediated inductions of fumarase C,[56,117] and possibly of SOD[118] can be modulated directly or indirectly by the NADPH/$NADP^+$ ratio.[96] PQ can induce *soxRS* dependent induction of fumarase C and of glucose 6-phosphate dehydrogenase both via O_2^--dependent and O_2^--independent pathways.[56,57,89] Moreover NO[119] or diamide[120] can induce *soxRS* even anaerobically.

Therefore O_2^- can be viewed as only one of the agents to which *soxRS* responds and defends the cell against. What is then the role of *soxRS*? We have discussed previously that some members of the *soxRS* might be involved in increasing the ability of the cell to reactivate FeS cluster containing enzymes, and even in replacing oxidatively unstable FeS cluster-containing enzymes with oxidatively stable isozymes.[1,56,96] Therefore based on the theory described above and on the accumulated data, it seems safe to take an additional step and to propose a specific role for *soxRS*.

The proposal is that the *soxRS* regulon evolved to protect FeS cluster-containing enzymes from oxidative inactivation and to repair the consequences of that inactivation.

Arguments in support of that view are:

1. The major targets in the cell for O_2^- are the FeS cluster-containing proteins and probably the cofactors involved in the FeS cluster-containing enzyme reassembly and reactivation. Therefore the induction of SOD through *soxRS*[113,114] makes sense.
2. In the case of an overwhelming flux of O_2^-, or in the case that the decreased activity of the FeS cluster-containing enzymes is caused by other oxidants, attempts to intensify the reactivation make sense. The role of ferredoxin and flavodoxin in that process was discussed above. It has been found that the NADPH:ferredoxin (flavodoxin) reductase of *E. coli* is a member of *soxRS*[96] and moreover it is stated[116] that pyruvate:ferredoxin (flavodoxin) reductase is *soxRS*-regulated as well.
3. According to our theory the *in vivo* O_2^--dependent HO· production causes extensive consumption of reductants, and it is desirable that they should be replenished. NADPH is probably used in more than one way through enzymes such as ferredoxin, glutathione and thioredoxin reductases. Therefore the up regulation of glucose 6-phosphate dehydrogenase by soxRS[113,114] is explained. The NADPH/$NADP^+$ ratio directly or indirectly modulates the activation of *soxRS* during oxidative stress and is likely to be important for the self-regulation of *soxRS*.[56,96] Increase of this ratio will indicate that the oxidative stress is diminished, that reactivation is successful and that reductants are being successfully replenished.
4. The reactivation of unstable FeS cluster-containing enzymes under oxidative stress is a difficult task and in many situations could not be completely accomplished. As discussed above the reactivation mechanism is very complex and might itself be a target for oxidants. Yet central metabolic pathways must be kept running. The citric acid cycle is such a central pathway yet four of the five aconitases plus fumarases in *E. coli* are oxidatively unstable enzymes. The problem is resolved in a radical way. Thus, while the unstable fumarases A and B were being inactivated in cells aerobically incubated with PQ, the stable fumarase C was induced, and this induction was mediated by *soxRS*.[56,57] With this, the problem is not yet resolved; since if the two aconitases are inactivated the citric acid cycle will still not be running. In fact one of the two aconitases was

also found to be a member of the *soxRS* regulon.[115] The induction of aconitase by the *soxRS* is an attempt to compensate for loss of aconitase during oxidative stress.[115]

5. The increased HO· production will damage many targets, and most importantly DNA.[1,20,76] This requires intensification of repair, which is accomplished by induction of endonuclease IV, again mediated by the *soxRS* regulon.[113,114]
6. SoxR, the sensor of *soxRS*, is a homodimer containing one [2Fe-2S] cluster per polypeptide and is a ferredoxin-like protein.[121,122] It seems to be stable to FeS cluster disassembly and does not lose iron easily.[121,122] The FeS cluster can be reduced by the strong reducing agent dithionite and the reduced cluster can be very easily oxidized by exposure to the air, the oxidized form of the cluster being $[2Fe\text{-}2S]^{2+}$.[121–123] The oxidized form activates transcription, while the results concerning the transcriptional activity of the reduced form are not conclusive due to its high oxidizability, but it is clear that the apoprotein is inactive.[121–123]

It has been suggested that SoxR is activated by oxidation of its reduced form or by compromising its FeS cluster reactivation (reduction of the oxidized form of SoxR).[96,123] This reduction of SoxR is proposed to be enzyme- and NADPH-dependent and mediated by "doxins" such as ferredoxin and flavodoxin.[96] While it seems well founded that SoxR is subject to redox control, an alternative hypothesis suggests that apoSoxR is the *in vivo* transcriptionally inactive form which becomes active by assembling a FeS cluster under conditions when other FeS clusters are being disassembled.[122]

Regardless of which of the two rival hypotheses will turn out to be the right one, the relevant point here is that SoxR is a FeS cluster-containing protein which is sensitive to conditions causing oxidative inactivation of other Fe-cluster enzymes and in fact those conditions are the signal to which SoxR is responding. If *soxRS* could speak it would probably tell us:

> "The nature of the oxidative stress that I recognize and fight against is oxidative inactivation of FeS cluster-containing proteins; which lead to release of iron followed by increased HO· production, via the Fenton reaction, and subsequent damage of DNA and other targets for HO·."

In mammalian cells the IRE-BP/cytosolic aconitase interconversion should be expected to be influenced by oxidants and other agents both through a direct attack of the [4Fe-4S] cluster or by interfering with FeS cluster (re)assembly, as does iron deficiency. Hence iron deficiency is not likely to be the only signal for this system. It is likely moreover that additional enzymes/proteins presumably involved in protection or reactivation of FeS clusters might be regulated by the same system. Similar views have been expressed recently (see references 124 and 125 and references cited therein). Thus it is an open question if the redox control of gene expression by iron-sulfur proteins is due to assembly/disassembly of FeS clusters[124] or to change of the oxidation-state of those clusters[125] or to both.

THEORIES OF OXIDATIVE STRESS

The theory described above does not pretend to be a TOE (theory of everything) in the field of oxidative stress. A number of other theories exist and the relationship between them is not simple. In some situations only one of them is enough to describe the effects under study, while in others the theories have to merge in order to explain the complex phenomena unfolding. The following few examples illustrate those complex relationships:

1. As already discussed the iron-mediated Haber-Weiss reaction is the mechanism which describes many situations *in vitro*. The same might be true for extracellular fluids. After all, the FeS cluster-containing enzymes discussed

above are intracellular. For example Carmine *et al.*[126] have observed an increase of iron, in a form able to catalyze free radical reactions, in the plasma of patients undergoing chemotherapy. It is conceivable that a source of O_2^- can trigger the Haber-Weiss reaction in that situation.

2. Rowley and Halliwell[127,128] noticed that SOD inhibits the formation of HO· in an *in vitro* system consisting of biological reductants such as NAD(P)H or thiols plus H_2O_2 and iron salts. They suggested that O_2^- recycles Fe(III) to Fe(II) and that an important physiological role of SOD *in vivo* may be that of decreasing O_2^--dependent formation of HO· from biological reductants in the presence of iron salts.

At first glance their proposal is analogous to the one described by net reaction 8. Indeed reaction 8 describes a process of HO· formation by O_2^- and biological reductants. In fact the two proposals differ mainly in the role that O_2^- is supposed to play, to reduce Fe(III) or alternatively to oxidize FeS clusters, but this is an important difference. While their proposal fairly well reflects the conditions of their *in vitro* experiment, it now seems more likely that the real *in vivo* mechanism is the one which involves FeS-clusters as presented earlier in this paper.

3. NO and O_2^- react to give peroxynitrite[21] whose reactivity has already been discussed. Recently Koppenol[129] proposed that the function of SOD is to prevent indirectly (by scavenging O_2^-) the formation of peroxynitrite. This hypothesis has several problems. One of them is that it implies that the only way O_2^- could be toxic is by combining with NO. This is not so and in practically all of the situations discussed in the present article only O_2^- but not NO can be implicated. Second, while explaining why SOD should inhibit, no role for iron and H_2O_2 is proposed.

Indeed in certain situations, for example when microbial cells are surrounded by activated macrophages,[130] enough NO might be produced to successfully compete for O_2^- with the other targets for O_2^-. SOD then might serve to minimize the ensuing peroxynitrite formation. Interestingly enough, peroxynitrite reacts rapidly with FeS-clusters[23,24] probably releasing "free" iron[24] and in such a situation one might expect toxicity of peroxynitrite which will involve, in addition to O_2^- and NO, also HO·, H_2O_2 and iron. A very good discussion of the interplay between O_2^-, NO and FeS clusters is made in the Castro *et al.*[24] paper. The role of SOD to prevent the reaction of O_2^- with NO is also discussed by Katusic.[131]

4. The elegant "free radical sink" hypothesis[71] postulates that a variety of oxidants will be neutralized producing, through a reaction with oxygen or glutathione, O_2^-. This will enable a single enzyme (SOD) to provide protection toward those oxidants. This theory does not contradict but supplements the FeS cluster-based theory, the latter explaining, among other things, why O_2^- is toxic.

CONCLUSION

The phenomenon of oxidative stress is due to a significant extent to inactivation of FeS center containing enzymes and the consequent increase of the *in vivo* production of HO· through the mechanisms described above. This view is presently well supported for the case of *E. coli*. It is reasonable to propose therefore that this is also true for eukaryotic cells.

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