Protoplasma (2001) 217: 33–36

PROTOPLASMA

How superoxide radical damages the cell

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Received June 3, 2000
Accepted September 26, 2000

Summary. Superoxide is considered to be poorly reactive, and cell damage has been attributed to HO• generated via the Haber–Weiss reaction. The function of O_2^- in this reaction is only to reduce Fe^{3+} to Fe^{2+}. In vivo, however, superoxide could not out-compete cellular reductants such as glutathione, NADPH, and ascorbate, which makes the observed O_2^- toxicity rather puzzling. Little attention has been paid to the idea that, irrespective of its poor chemical reactivity, superoxide might be capable of interacting directly with specific intracellular targets; and that even the Haber–Weiss reaction might be a consequence of such direct interactions. This paper summarizes latest data that support the concept of such a mechanism.

Keywords: Oxidative stress; Hydroxyl radical; Superoxide dismutase.

Abbreviation: SOD superoxide dismutase.

Introduction

In 1969 McCord and Fridovich (1969) discovered that the blue copper-containing protein (erythrocuprein) in bovine erythrocytes catalyzes the disproportionation of the superoxide radical (O_2^-). The protein was named superoxide dismutase (SOD). Different types of superoxide dismutases were found in virtually all aerobic cells (McCord et al. 1971). All SODs are metalloenzymes and depending on the metal in their active site belong to one of the three groups: CuZn-SODs, FeSODs, or MnSODs (DiGuiseppi and Fridovich 1984). It was assumed that SODs play a major role in protecting the cells against oxidative stress, and their substrate, the superoxide radical, is a potent cell-damaging agent. This idea has remained controversial for a long time because O_2^- is not very reactive (Bielski et al. 1985, Bielski and Richter 1977, Sawyer and Valentine 1981, Fee 1982). It has been proposed that the superoxide radical causes cell damage not directly, but by participating in the so-called iron-mediated Haber–Weiss reaction or superoxide-driven Fenton chemistry to generate the highly reactive hydroxyl radical (HO•) (Halliwell 1978, McCord and Day 1978):

$$H_2O_2 + Fe^{2+} \rightarrow HO^\bullet + HO^- + Fe^{3+} \quad (1)$$

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2 \quad (2)$$

According to the above mechanism, the only role of O_2^- in the production of the hydroxyl radical is to reduce Fe^{3+} to Fe^{2+}. Obviously, O_2^- would be important for the Haber–Weiss reaction only if other iron reductants are absent. Several authors have pointed out, however, that O_2^- cannot out-compete cellular reductants such as glutathione, NADPH, and ascorbate (Czapski et al. 1988, Winterbourn 1982). Furthermore, as cells maintain a highly reduced state even aerobically, any available iron should exist only in its reduced form (Winterbourn 1993). It is really difficult to explain how in such a situation O_2^- would generate HO•. If O_2^- is not reactive enough to inflict direct damage and if it does not contribute in the Haber–Weiss reaction, then is O_2^- indeed harmful?

Oxidation of enzymes containing iron-sulfur clusters by superoxide radical

The first clear evidence that in vivo superoxide is toxic and deleterious was provided by creating an *Escherichia coli* mutant lacking cytoplasmic SODs (Carlioz and Touati 1986). This *sodAsodB* mutant was capable

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of aerobic growth only if branched-chain, sulfur-containing, and aromatic amino acids were present. It grew slower even in rich medium, required a fermentable carbon source, could not survive in stationary phase, and displayed a high rate of spontaneous mutagenesis (Far et al. 1986, Benov and Fridovich 1996). Each one of these phenotypic defects reflects a different way of damage by O_2^-.

The reason for the auxotrophy for branched-chain amino acids is the O_2^- sensitivity of dihydroxy acid dehydratase (Kuo et al. 1987, Flint et al. 1993). This [4Fe-4S]-containing enzyme catalyzes the penultimate step in the pathway of biosynthesis of branched-chain amino acids. O_2^- directly inactivates the enzyme and shuts down the entire pathway.

Aromatic-amino-acid auxotrophy is due to the inactivation of transketolase (Benov and Fridovich 1999). Transketolase catalyzes the production of erythrose-4-phosphate, which is essential for the first step of the aromatic biosynthetic pathway. An intermediate of the transketolase reaction (1,2-dihydroxyethyl thiamine pyrophosphate) is oxidized by O_2^-, which interferes with the production of erythrose-4-phosphate and inactivates the enzyme.

The auxotrophy for sulfur-containing amino acids is not related to a O_2^--sensitive enzyme but rather to membrane damage. This renders the cell membrane permeable for small molecules including sulfite (Benov and Fridovich 1997). Loss of sulfite interfered with the pathway from sulfate to cysteine (Benov et al. 1996).

The slow aerobic growth of the *sodAsodB* mutants is due to direct inactivation by superoxide of enzymes of central pathways, such as the aconitases and fumarases A and B of the citric acid cycle (Gardner and Fridovich 1991, Liochev and Fridovich 1993). This limits the supply of ATP and of reducing power (NADPH) needed for biosynthesis. The diversion of cell resources to the repair of oxidatively damaged cell components is another reason for slow growth, as is the leakage of metabolic intermediates through the damaged cell envelope.

It appears that irrespective of its poor reactivity, in vivo O_2^- is capable to react with and to damage specific intracellular targets. Superoxide inactivates enzymes including catalase and glutathione peroxidase and oxidizes epinephrine, glutathione, 6-hydroxydopamine, pyrogallol, hydroxylamines, etc. (DiGuiseppi and Fridovich 1984). Not all phenotypic defects in the *sodAsodB* cells, however, can be attributed to direct damage by O_2^-. For example, the high rate of spontaneous mutagenesis by the SOD-deficient cells must be due to DNA damage and incomplete repair. Since O_2^- does not attack DNA directly, but $HO^\bullet$ does, the explanation had to be in the role for O_2^- in the production of $HO^\bullet$. As pointed out, O_2^- could not act as a reductant for iron in the Haber–Weiss reaction. At the same time, there is convincing evidence that in vivo O_2^- contributes to the production of $HO^\bullet$ from H_2O_2 (Liochev 1996). Recently Liochev and Fridovich (1994) proposed that O_2^- generates $HO^\bullet$ not by reducing Fe^{3+} to Fe^{2+} but by oxidizing [4Fe-4S]-cluster-containing proteins. Reaction (1) shows that $HO^\bullet$ cannot be generated if "free" or "loose" iron is not available to decompose H_2O_2. According to the above mentioned hypothesis, the interaction of O_2^- with the FeS-cluster-containing proteins provides the "free" iron for the Haber–Weiss reaction. O_2^- inactivates FeS-containing enzymes such as dihydroxy acid dehydratase, 6-phosphogluconate dehydratase, aconitase, fumarases A and B as well as mammalian mitochondrial and cytosolic aconitases (Gardner and Fridovich 1991a, b; Liochev and Fridovich 1993; Flint et al. 1993). The inactivation is rapid, with rate constants ranging from 10^6 to $10^7/M \cdot s$ (Hausladen and Fridovich 1994, Flint et al. 1993), and is associated with oxidation of the iron-sulfur cluster:

$$[2Fe^{2+}2Fe^{3+}\text{-}4S] + O_2^- + 2H^+ \rightarrow [Fe^{2+}3Fe^{3+}\text{-}4S] + H_2O_2. \quad (3)$$

The oxidized cluster then loses iron, because the sulfide ligand binds Fe^{3+} much stronger than Fe^{2+} (for details see Flint et al. 1993):

$$[Fe^{2+}3Fe^{3+}\text{-}4S] \rightarrow Fe^{2+} + [3Fe^{3+}\text{-}4S]. \quad (4)$$

Reactions (3) and (4) produce Fe^{2+} and H_2O_2 which combine in the Haber–Weiss reaction (1) to generate $HO^\bullet$. Several lines of evidence support this mechanism. Thus, the *sodAsodB* strains are more susceptible to killing by H_2O_2 than the respective SOD-proficient strains (Carlioz and Touati 1986). By applying whole-cell electron paramagnetic resonance Keyer and Imlay (1996) have demonstrated that the level of loose iron in SOD-deficient cells significantly exceeds that in the parental cells. Furthermore, overproduction of enzymes containing [4Fe-4S] clusters dramatically increases the "free"-iron content and the rates of spontaneous mutations (Keyer and Imlay 1996). On the

other hand, Marignati and Imlay (1999) have shown that mutations causing intracellular accumulation of an iron chelator suppress the phenotypic defects of *sodAsodB* cells.

It is important to stress that DNA damage by O_2^- is a consequence of the direct interaction of superoxide with a specific class of proteins, but damage due to liberation of iron from the FeS clusters is not limited to DNA only. Detailed studies of the oxidative modification of proteins in *E. coli* established that oxidation is dependent upon NAD(P)H, O_2, and Fe(III) (Stadtman and Oliver 1991). As with DNA this is a site-specific process involving the interaction of H_2O_2 and Fe(II) at metal binding sites on the proteins. Accumulation of oxidatively modified proteins rises sharply when strains lacking cytoplasmic SODs reach stationary phase (Dukan and Nystrom 1999). Such strains die rapidly in stationary phase if kept aerobic (Benov and Fridovich 1995).

One important consequence of the oxidative modification of proteins is that they become susceptible to proteolytic degradation (Davies and Lin 1988a, b). Davies and co-workers have demonstrated that the degradation of proteins in *E. coli* is greatly enhanced following the exposure of the cells to O_2^- or H_2O_2. Due to proteolytic degradation, the cellular content of some proteins was decreased by more than 50% (Davies and Lin 1988a).

Due to its low chemical reactivity superoxide can interact with a limited number of cellular targets. This interaction damages specific cellular constituents and at the same time provides substrates for the Haber–Weiss reaction. The hydroxyl radical, a product of this reaction, in turn damages molecules that are superoxide resistant. Thus, irrespective of its low chemical reactivity, superoxide is capable to inflict a profound cell damage.

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