

Chapter 2

The Discovery of Superoxide Dismutase and Its Role in Redox Biology

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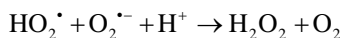
2.1 The Discovery of Superoxide Dismutase

When I began as a graduate student under the direction of Irwin Fridovich, I was assigned a “back burner” project regarding the mechanism of cytochrome *c* reduction by xanthine oxidase and xanthine. This reaction required the presence of oxygen, but little oxygen was consumed by the reaction. Fridovich and Handler had proposed that a bound oxygen molecule might serve as an “electron bridge” to facilitate the transfer of an electron from a reduced active site (e.g., Fe^{+2}) on xanthine oxidase to the heme of the cytochrome [1]. Fridovich had also observed that certain preparations of myoglobin [2] or carbonic anhydrase [3] could inhibit the transfer of this electron to cytochrome *c*, presumably by competing with the cytochrome for a common binding site on xanthine oxidase. It was a logical hypothesis supported by classical adherence to Michaelis–Menten kinetic behavior, but lacking physical evidence. My job was to demonstrate the physical binding of these competing proteins to xanthine oxidase. To make a long story short, no such evidence could be acquired using a variety of techniques. I began to rethink the hypothesis. Was there any other way that these players could interact that would produce kinetic behavior that was so seemingly consistent with Michaelis–Menten enzyme kinetics of saturation and competitive inhibition? An alternative possibility occurred to me in what can only be described as a “eureka” moment. If the proposed bridging oxygen and its single extra electron were released from xanthine oxidase as a superoxide radical ($\text{O}_2^{\cdot-}$), an unstable entity already known to and studied by radiation chemists, it seemed to me that all could be explained via a competition between two

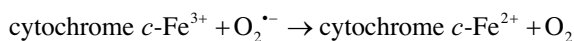
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reactions, the first being the spontaneous dismutation reaction which would occur in the absence of cytochrome *c*:



and the second being the reduction of cytochrome *c* by the superoxide radical, if the cytochrome were present:



The second reaction would obviously compete with the first, and would show saturation (i.e., the rate of cytochrome reduction would approach the rate of superoxide production) at very high concentrations of the cytochrome. If these reactions were taking place instead of the classical Michaelis–Menten enzyme-binding-substrate scenario, however, a very important distinction could be made. The Michaelis constant, K_m , reflects the binding constant between enzyme and substrate, and under conditions where the substrate concentration greatly exceeds the enzyme concentration, it is essentially constant. If, however, no binding were occurring then the concentration of cytochrome required to achieve half-maximal rate would be a function of enzyme concentration and steady-state concentration of superoxide achieved under those particular conditions—it would certainly not be a constant. The next day, I determined half-maximal rates of cytochrome reduction by xanthine oxidase over a 24-fold range of enzyme concentration, finding that the *apparent* K_m varied about 4.8 fold over this range. In fact, the half-maximal rates varied rather precisely with the square root of xanthine oxidase concentration, exactly as might have been predicted, given that the steady-state concentration of $\text{O}_2^{\bullet-}$ is determined by the spontaneous dismutation of the radical, a reaction that is second order in superoxide [4]. We concluded that superoxide was not an enzyme-bound intermediate but rather an actual product of the action of xanthine oxidase on xanthine, and was released into free solution where it could undergo spontaneous dismutation to produce hydrogen peroxide and oxygen, or where it could react with ferricytochrome *c*, reducing it to ferrocycytochrome *c*. This realization required an immediate rethinking of how the inhibitory proteins (misidentified at this point as myoglobin and carbonic anhydrase) could be producing their effects. The most likely possibility was that they were eliminating superoxide by catalyzing the dismutation reaction—i.e., they were *superoxide dismutases*.

A subsequent study clarified things even more [5]. Superoxide dismutase (SOD) activity did not belong to carbonic anhydrase nor to myoglobin, but rather belonged to a minor impurity present in those preparations at a fraction of a percent. When isolated based on its activity from bovine erythrocytes, SOD was quickly identified as a previously studied copper-containing protein of unknown function that had been variously named hemocuprein, hepatocuprein, cerebrocuprein, erythrocuprein, and cytocuprein. We were able to demonstrate the activity of SOD in systems that involved neither xanthine oxidase nor cytochrome *c*, definitively ruling out protein–protein interactions of any kind. We also showed that hydrogen peroxide was

the product of the SOD-catalyzed reaction, establishing that it was indeed a dismutation rather than something more complex, such as a four-electron reduction of oxygen to form water as the product, analogous to the reaction catalyzed by cytochrome oxidase.

2.2 The Existence of SOD Raised a Number of Questions

The discovery of SOD, a free radical-scavenging enzyme, was viewed by some as a biochemical curiosity—perhaps as “a solution in search of a problem.” Why did this enzyme exist? Free radicals were known to be a generally reactive class of molecules, but superoxide seemed able to take care of its own destruction with a spontaneous dismutation rate of around $10^6 \text{ M}^{-1} \text{ s}^{-1}$ at cytosolic pH. Is spontaneous dismutation not fast enough? Is superoxide toxic in biological systems? What are the biological targets of superoxide? Do all organisms living in oxygen require SOD? Are there pathological consequences to unscavenged superoxide?

2.3 SOD Was Everywhere

How widely was SOD distributed? It was the summer of 1968 and fellow graduate student Chuck Beauchamp had a vegetable garden, so every day it seemed there was a new vegetable in the lab blender. They all contained SOD. When microbiologist Bernie Keele joined the lab as a postdoc, he acquired microorganisms from the collections of all of his colleagues. They all had SOD—all except the strict anaerobes. Unlike the facultative organisms that can live with or without oxygen, unlike the microaerophiles that require oxygen (just not too much), and unlike the aerotolerant anaerobes that can't utilize oxygen but can tolerate it, the strict anaerobes quickly die in oxygen, and are the ones without SOD [6]. Not only was SOD activity universally present in oxygen-metabolizing organisms, its concentration was found in a remarkably narrow range, whether in human brain or a tomato or *Escherichia coli*. This implied two things: that the metabolism of oxygen inevitably leads to superoxide production, and that the free radical, if left unscavenged, must be seriously toxic. It also quickly became apparent that not all SODs were the same. When we isolated the enzyme from *E. coli* it was pink, rather than the blue color of the Cu/Zn-SOD from bovine erythrocytes. While the native pink enzyme gave no EPR signal, the boiled enzyme showed the very characteristic signal of manganese [7]. This Mn-SOD was found to be structurally and genetically related to the avian and mammalian mitochondrial Mn-SODs [8], as well as to the iron-containing SODs isolated from bacteria such as *E. coli* [9] and from spinach [10]. The last genetically distinct family of SOD to be described in certain bacteria contains nickel at the active site [11].

2.4 Biological Toxicity and Chemical Reactivity Are Not Necessarily Related

While $O_2^{\cdot-}$ certainly meets the chemical definition of a free radical, it is rather mild mannered as free radicals go—a fact that chemists understood quite well. A few chemists offered the opinion that superoxide is so unreactive that it should not pose a problem [12], and that “superoxide dismutase” might be an incidental property of these metalloproteins whose real functions remained to be discovered [13]. At the same time, many biologists were struck by the novelty that biological systems could generate these dangerous-sounding “free radicals,” and assumed them to be indiscriminately reactive and damaging to biological systems. Neither view proved to be correct because biological toxicity is not always related to broad chemical reactivity. Many lethal biological toxins act with surgical precision, sometimes reacting with a single specific target. Cyanide toxicity, e.g., is due to its ability to block mitochondrial electron transport by inhibition of the cytochrome *c* oxidase. Ricin catalytically depurinates ribosomes, halting protein synthesis. A number of specific biological targets have been shown to be inactivated by superoxide including the citric acid cycle enzyme aconitase, certain dehydratases in *E. coli* such as the α,β -dihydroxyisovalerate dehydratase and 6-phosphogluconate dehydratase, and many other important enzymes including catalase, glyceraldehyde-3-phosphate dehydrogenase, ornithine decarboxylase, glutathione peroxidase, myofibrillar ATPase, adenylate cyclase, creatine phosphokinase, and glutamine synthase.

The biological targets that lead to significant increases in lipid peroxidation deserve special mention due to the self-propagating nature of lipid peroxidation, once initiated, to the widespread nature of the damage that results when membrane integrity is breached, and to the large number of pathological states characterized by increased lipid peroxidation. Among the major classes of biological molecules (proteins, polysaccharides, nucleic acids, and lipids), the polyunsaturated fatty acid moieties of lipids are perhaps the most easily oxidized, and superoxide has been shown capable of initiating and promoting the propagation of the process. The perhydroxyl radical (HO_2^{\cdot}) is the conjugate acid of superoxide ($O_2^{\cdot-}$) and is present at lower concentrations whenever superoxide is generated in biological systems. Furthermore, it is uncharged and quite lipid soluble. It can initiate lipid peroxidation by abstraction of a *bis*-allylic hydrogen atom from a polyunsaturated fatty acid [14]. A second mechanism has been described wherein the perhydroxyl radical can also abstract a hydrogen atom from a lipid peroxide molecule (LOOH) to create a lipid peroxy radical (LOO^{\cdot}), effectively creating a branch point in the otherwise linear propagation sequence [15]. Thus, it appears that unscavenged superoxide production does indeed pose a serious toxic threat to virtually all organisms.

2.5 It's Not All Bad: The Importance of Redox Balance and Bell-Shaped Curves

One of the characteristics of evolution is the ability to make the best of a bad situation, to make a silk purse from the proverbial sow's ear. Thus, there are clear examples of how superoxide can actually be put to constructive uses. A good example is the evolution of our phagocytic NADPH oxidase. When first recognized as a biologic metabolite, it appeared that the superoxide radical was simply a noxious cytotoxic by-product that served no good purpose. That view changed when Bernard Babior and colleagues [16] realized that superoxide plays a crucial role in our defense against invading microbes. Precisely because superoxide is cytotoxic, this NADPH oxidase of phagocytes has evolved to purposefully generate superoxide radical on the membrane surface lining the contained microenvironment of the phagolysosome, providing chemical destruction for the ingested microbes. In effect, superoxide acts as an extremely broad spectrum antibiotic. The neutrophil is also sacrificed in the process, in a kind of Kamikaze mission. Surrounding healthy host cells may be injured or even killed through collateral damage in this system that errs on the side of vigilance. This ability to fend off microbial invaders as injured tissues are repaired is what links superoxide production to the inflammatory/immune system.

Ironically, one of the "good deeds" that can be attributed to the superoxide radical goes back to its roles in lipid peroxidation, a free radical chain reaction process that requires a free radical to initiate the process, and another free radical to terminate the chain. It appears that $O_2^{\cdot-}$ (or its conjugate acid HO_2^{\cdot}) can do both [17]. At high concentrations of $O_2^{\cdot-}$ (little SOD) initiation events would be maximal, but so would termination events, resulting in a large number of short chains. At low concentrations of the radical (high SOD) initiation events would be far fewer but the chain length would be quite long (limited only by other terminators such as vitamin E, or by the mutual annihilation of propagating radicals). Somewhere in the middle is a "sweet spot" at which net lipid peroxidation is minimal at one optimal concentration of SOD. This behavior can be observed experimentally and can be predicted by mathematic modeling [17].

Considerable evidence suggests that our bodies regulate redox balance, just as we regulate pH, body temperature, rates of respiration and oxygen delivery, blood glucose levels, and numerous other factors to achieve homeostasis. SOD is by no means the sole determinant of physiological redox balance; rather, it results from a network of interactive antioxidant enzymes, regulatory kinases, and cascading transcription factors. The balance is readily upset by injury, disease, and by aging itself. The future of redox biology will, no doubt, have much more to tell us.

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