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OXYGEN: FRIEND OR FOE? ARCHAEAL SUPEROXIDE DISMUTASES IN THE PROTECTION OF INTRA- AND EXTRACELLULAR OXIDATIVE STRESS

Raffaele Cannio [1], Gabriella Fiorentino [2], Alessandra Morana [1], Mosè Rossi [2] and Simonetta Bartolucci [2]

[1]CNR, Istituto di Scienze dell'Alimentazione, Via Roma, 53 C/A-83100 Avellino, Italy, [2]Dipartimento di Chimica Organica e Biologica, Università degli Studi di Napoli "Federico II", Via Mezzocannone, 16-80134 Napoli, Italy

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1. ABSTRACT

Both "environmental chemistry" and metabolic biochemical reactions can constantly generate *in vivo* free radicals and other oxygen-derived species that can cause severe damage to almost all biomolecules, especially to DNA, proteins, and lipids. The superoxide anion has been shown to be the most readily generated and spread radical among organisms and it is a common intermediate of oxidative stress processes in the cells. The antioxidant defense system of superoxide dismutases (SOD) scavenges and minimizes the formation of this radical, and thus plays a major role in reducing cumulative oxidative damage in different cell compartments both in aerobic and anaerobic cells. In the cell, cytosol SODs are constitutively present and induced by many oxidative agents able to raise the superoxide concentrations. Presence of SODs, however, in extracellular cell-associated locations demonstrates how valuable they are in maintaining the integrity of cells against oxidative stress generated by the cell environment, particularly upon increased oxygenation. Because SODs have recently been found in Archaea, which are prokaryotes, sometimes living in extreme environments, even in anaerobic ones, these enzymes can be considered essential: they may have allowed the evolution of aerobic respiration starting from an ancient form of oxygen-insensitive life.

2. INTRODUCTION

When living organisms first appeared on Earth, they probably were essentially anaerobes since the atmosphere might have contained low amounts of O_2. The O_2 content in the atmosphere probably increased with the appearance and evolution of organisms endowed with photosynthetic ionolysis of water and thereby became prohibitive for the survival of many primitive organisms. Therefore, anaerobic microorganisms, that still exist today, must have followed a kind of evolutionary "adaptation" to the increasing atmospheric oxygen levels by restricting themselves to extreme environments, where O_2 does not penetrate in poisonous concentrations. Other organisms have generated antioxidant defenses to tolerate O_2 and/or to use it for metabolic purposes, such as energy production gained by the electron transport chains in which O_2 is the terminal electron acceptor.

It is interesting to note that O_2 can also be toxic for aerobic organisms at concentrations greater than 21%, the current atmospheric level, and the damaging effects vary considerably with the organism considered (1), and with exposure time and administration route.

Oxygen toxicity cannot be satisfactorily explained

by a mere inhibitory effect on enzymes, as earlier proposed, or by direct damage of a few biologically-active molecules. Fifty years ago, Gerschman proposed that the toxicity of O_2 could be attributed to the formation of oxygen radicals (2). This theory has been further developed very slowly and confirmed since the extreme instability of the short-lived radical species generated by dioxygen made the reaction mechanisms difficult to elucidate and rationalize. This "oxy-radical hypothesis" was fully developed by Fridovich into the "superoxide theory of O_2 toxicity", which states that the formation of the superoxide radical *in vivo* plays a key role in the toxicity of O_2 and that the SOD enzymes are important antioxidant defenses (2,3,4,5).

The superoxide substrate is an unstable free radical that is present in very small amounts and is barely detectable in both the living cells and in their environment. Moreover, the dismutation reaction occurs at a high rate even spontaneously, namely in the absence of the SOD enzyme. Despite its extremely short life, superoxide can exert a highly toxic effect, in the face of which SOD has been shown to be essential for the survival of all aerobic and some anaerobic cells.

Due to the current and widespread interest in stress by oxidative damage of living cells, this review will only deal with portions of this broad field. It will focus on the natural occurrence and generation of the superoxide radical, and the SODs as the main cellular antioxidant agents. The distribution of this enzyme in organisms of the three domains of life, Eukarya, Bacteria and Archaea, will also be presented focusing on the archaeal representatives that possess SODs with unique and interesting features at the molecular and evolutionary levels.

3. OXYRADICAL PRODUCTION

3.1. Oxygen free-radicals

A radical is defined as any chemical species that has one or more unpaired electrons. An unpaired electron is any electron that is alone in an orbital and a radical is very reactive because it aims to pair its electron with another of opposite spin present in a second radical. Oxygen radicals are produced during oxygen reduction to water in the intermediate steps:

1) $O_2 + 1\ e^- + H^+ \leftrightarrows HO_2^{\cdot} \leftrightarrows H^+ + O_2^-$ (superoxide)

2) $HO_2^{\cdot} + 1\ e^- + H^+ \leftrightarrows H_2O_2$

3) $H_2O_2 + 1\ e^- + H^+ \leftrightarrows H_3O_2 \leftrightarrows H_2O + OH^{\cdot}$ (hydroxyl radical)

4) $OH^{\cdot} + 1\ e^- + H^+ \leftrightarrows H_2O$

3.1.1. Superoxide

The superoxide radical (O_2^-) is the one-electron reduction product of the oxygen and it is formed in almost all aerobic cells (6). It can act either as reducing agent forming molecular oxygen or as oxidizing agent forming hydrogen peroxide.

The toxicity of this radical depends mainly on its interaction with hydrogen peroxide leading to the formation of the hydroxyl radical, an extremely oxidant species.

3.1.2. Hydroxyl radical

Superoxide and hydrogen peroxide can react generating the hydroxyl radical ($OH^{\cdot}$) according to the *Haber-Weiss* reaction (7):

5) $O_2^- + H_2O_2 \rightarrow OH^- + OH^{\cdot} + O_2$

The hydroxyl radical reacts with high rate constants with almost every type of organic molecule present in living cells: amino acids, DNA bases, sugars, and phospholipids. The reactivity of the hydroxyl radical is so great that it reacts immediately with whatever biological molecule is in its proximity, producing secondary radicals of variable reactivity. The rate constant for this reaction in aqueous solution has been shown to be virtually zero (6) and it certainly could not occur at the low steady-state concentrations of superoxide and hydrogen peroxide present *in vivo*. Several scientists (6) have shown that hydroxyl radical formation can be accounted for if the *Haber-Weiss* reaction is catalyzed by traces of transition metal ions. Since iron salts are available *in vivo*, a plausible mechanism can be the following:

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

7) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$ (Fenton reaction)
Fe salt

8) $O_2^- + H_2O_2 \rightarrow O_2 + OH^- + OH^{\cdot}$
catalyst

All systems producing the superoxide also lead to the formation of hydrogen peroxide and, if these products are accumulated, they begin to produce the hydroxyl radical in the iron-catalyzed *Haber-Weiss* reaction. SODs that eliminate the superoxide, and catalases and peroxidases that decompose the hydrogen peroxide, avoid the production of the hydroxyl radical.

3.1.3. Hydrogen peroxide (a nonradical)

In aqueous solution, the superoxide undergoes the dismutation reaction to form hydrogen peroxide and oxygen. The overall reaction is as follows:

9) $2\ O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$

though it is the sum of several stages. The rate of dismutation is faster at acidic pH.

As well as arising from the superoxide, hydrogen peroxide is produced by the action of several oxidases *in vivo*, including amino acid oxidases and the enzyme xanthine oxidase (8). Hydrogen peroxide is able to oxidize many substances like sulfidrilic compounds and methionine residues of the proteins, but these oxidations only occur at concentrations higher than those present *in vivo*. Therefore, it is considered that the toxicity of the hydrogen peroxide depends on its capability to generate the hydroxyl radical in presence of superoxide or iron ions.

3.2. Oxyradical produced by metabolic reactions

It is now well confirmed that superoxide and hydrogen peroxide are produced in aerobes, although the precise amounts generated are still uncertain (9, 10). Generation of these species occurs in two types of processes.

3.2.1 Spontaneous generation

This includes mechanisms like "leakage" of electrons onto O_2 from the mitochondrial electron-transport chain and the microsomal cytochromes P450 and their electron-donating enzymes.

The two most probable sites involved in the generation of superoxide and hydrogen peroxide at the level of the electron-transport chain are the cytochrome b_{566}, which is considered an autoxidable compound and the ubiquinon ($Q_{10}H_2$).

Other autoxidable sites involved in the production of the superoxide are reduced flavines, catecholamines and tetrahydrofolates.

Many oxidative enzymes produce superoxide: dihydro-orotic dehydrogenase, aldehyde oxidase, xanthine oxidase.

3.2.2. Induced synthesis

The classic example of metabolic production of reactive oxygen species (ROS) is the production of O_2^- and H_2O_2 by activated phagocytes (neutrophils, monocytes, macrophages, and eosinophils) when they contact foreign particles or immune complexes (11). Hydrogen peroxide is additionally generated *in vivo* by several oxidase enzymes, such as glycolate oxidase, and D-amino acid oxidase (10, 11, 12). It resembles water in its molecular structure and is very well distributed within and between cells.

Evidence is accumulating that extracellular superoxide is also produced *in vivo* by several cell types other than phagocytes, including lymphocytes, fibroblasts and vascular endothelial cells (13, 14, 15). This superoxide may serve important biological functions, such as intercellular signaling and cell growth regulation (13, 14, 15, 16).

3.3. Environmental generation of free-radicals

When an ionizing radiation collides with a living organism, it can react with biological molecules or water. The interaction with the biological molecules generates free radicals and it is known as direct effect whereas the interaction with water is known as indirect effect.

Since water is present in the tissues of living organisms at levels of 70-80%, the indirect action contributes for the main part to biological damage through energy transfer from the free radicals deriving from water, to the target molecule.

The irradiation of water with X- or gamma-rays, or with high-energy electrons, produces several reactive species represented by free radicals such as hydroxyl radical, hydrogen radical, and non radical forms such as hydrogen peroxide and hydrogen:

10) $H_2O \rightarrow H^{\cdot}, OH^{\cdot}, H_2, H_2O_2$

The free radicals generated from ionizing radiation diffuse in solution and react with the target molecules producing new chemical species. Among these products, hydrogen peroxide is particularly important since it is able to react with other radicals producing new hydroxyl radicals and the hydroperoxyl radical that corresponds to the protonated form of the superoxide:

11) $H^{\cdot} + H_2O_2 \rightarrow H_2O + OH^{\cdot}$

12) $OH^{\cdot} + H_2O_2 \rightarrow H_2O + HO_2^{\cdot}$ (hydroperoxyl radical)

4. SUPEROXIDE'S TARGETS AND SOD PREVENTION OF PEROXIDATION

The superoxide dismutase is able to scavenge superoxide anions generated in different environments, and together with its substrate is widespread among aerobic cells in different subcellular compartments. The first indirect proof of this parallel presence was provided by the study of erythrocyte metabolism and xanthine oxidase, which is able to produce O_2^-: the erythrocytes were found to contain SOD, namely an enzyme able to efficiently and specifically dismutate the radical (17).

Over the last 30 years it has been demonstrated that O_2^- attacks directly or through other species that it can generate, proteins, lipids, polysaccharides, and nucleic acids. Due to the extreme diversity of the organisms and natural habitats, O_2^- exerts both a reducing and an oxidizing action. The reactivity can be very high in organic solvents, but not in aqueous media where it acts mainly as a moderate reducing agent (18). Enzymes are the most obvious targets, especially those containing chelated metal cofactors (19). As an example, (4Fe-4S) clusters in the active sites of the dehydratase family, such as aconitase and fumarase, can undergo rapid oxidation by O_2^- with the release of the Fe (II) ion and hence inactivation. Moreover, it has been claimed that it can inactivate the NADH dehydrogenase complex of the human mitochondrial respiratory chain (20). Very recently the cell-bound glucose and succinate dehydrogenases were shown to be irreversibly inactivated by superoxide in the hyperthermophilic archaeon *Sulfolobus solfataricus* (21).

However, it has been proposed that O_2^- cannot be the most efficient oxidizing agent not only because of its usual short lived state, but also because its reactivity is rather low when compared to that of other oxygen radicals. As an example, peroxidation of polyunsaturated fatty acids can be initiated by the protonated form of O_2^- but not by O_2 itself although the latter occurs only in weakly acidic media in which the perhydroxil radical is more stable (22, 23). The superoxide toxicity is mainly dependent on its interaction with hydrogen peroxide (*Haber-Weiss* reaction, see reaction 5 (7)), formed by its own dismutation. $OH^{\cdot}$ can be also generated in the *Fenton* reaction in which Fe (II) (released from the enzyme clusters or other transition metals attacked by superoxide) reduces H_2O_2. The radical species $OH^{\cdot}$ is actually the one able to attack virtually all

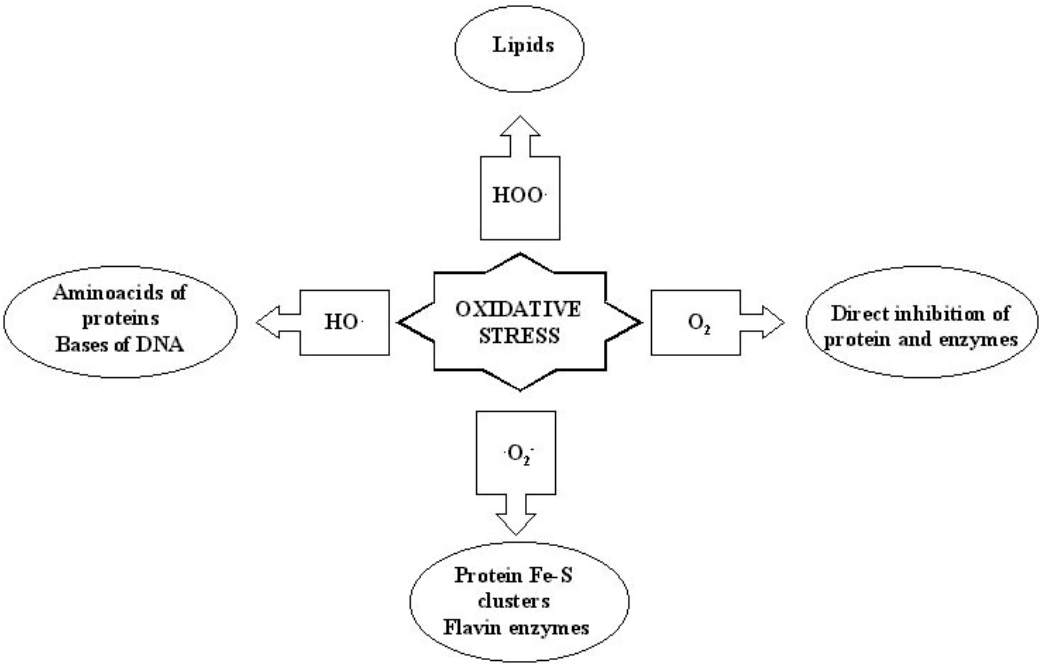

Figure 1. Oxygen radical species and their specific molecular targets in the cell under oxidative stress.

targets, including all components of DNA (24) and many amino acids in proteins, such as methionine (25, 26, 27). The widespread homolytic fission of O-H bonds in water provoked by ionizing radiation which also generates $OH^{\cdot}$, exposes every living organism to this radical; evolution has selected many specific repair systems to counterbalance this oxidative stress in addition to $OH^{\cdot}$ scavenging molecules. The impressive high-rate reactivity of the radical with practically all cell components probably explains the impossibility of a direct inactivation and the evolution of effective systems for the prevention of its generation. Besides many low molecular weight antioxidants, able to scavenge radicals, such as vitamin E (28), ascorbate (29), glutathione (8), polyphenols (30), and storage proteins, like ferritin (31) that specifically sequestrate transition metals rendering them less available for *Fenton*-like reactions, it has been proposed that enzymes are the main defense against oxygen radicals. Catalases and peroxidases catalyze the reduction of H_2O_2 while superoxide dismutases act as scavengers of O_2^- accelerating its conversion towards H_2O_2, so that the main reagents of the *Haber-Weiss* (7) reaction and their toxic product $^{\cdot}OH$ are kept in controlled non-lethal concentrations.

In conclusion, oxidative stress for living cells is created every time the equilibrium between ROS and antioxidants becomes unbalanced because of abnormal intracellular or environmental events. The most obvious reason is a low content of antioxidant molecules due to decreased nutrient uptake or to defective metabolism. On the other hand, overproduction of ROS by suddenly increasing levels of O_2 partial pressures, or induction of ROS-generating processes are also very common articularly for those organisms exposed to rapid variation of external conditions (Figure 1). Much experimental evidence, particularly that obtained with oxygen adaptation of mice and defective *sod*$^-$ mutant microorganisms, has shown that superoxide dismutases play an essential role in the defense against oxygen toxicity. The recent discovery of these enzymes also in anaerobic microorganisms, including archaeal representatives, may also provide new insights and perspectives for clarifying the evolution of aerobic respiration and the divergence of the three domains of life.

5. THE SOD FAMILY OF ENZYMES

SOD catalyses the dismutation of $O_2^{\cdot}$- by successive oxidation and reduction of the transition metal ion at the active site in a Ping-Pong type mechanism with remarkably high reaction rates (32).

Four classes of SOD have been identified based on the metal cofactor, which could be either a dinuclear Cu/Zn or mononuclear Fe, Mn or Ni (33). Fe- and Mn-types show homology and possess identical metal chelating residues at the active site, sharing substantial sequence and tertiary structure homology, while the other SODs are structurally unrelated. SODs are found in Eukarya as cytosolic Cu/Zn-SOD, mitochondrial Mn-SOD, and extracellular-SOD (EC-SOD) (34), and in Bacteria also as periplasmic **(**table 1).

5.1. Cu/Zn SOD

Copper and zinc containing SODs have been found in the cytosol of eukaryotes, in chloroplasts, and in the periplasm of some prokaryotes. The properties of these enzymes have been conserved throughout the evolution. In

Table 1. The SOD families

Metal cofactor		Localization	3D structure	Subunit	Reference
Cu/Zn	*Eukarya:*	Cytosol	Y[a]	2	38, 44
		Chloroplast	N[b]	N	
		Extracellular	N	4	73, 76
	Bacteria:	Periplasm	Y	2/1	36,49,52/37
Fe-	*Eukarya:*	Mitochondria	Y	4	62
Mn-		Chloroplast	N	N	
	Bacteria:	Cytosol	Y	2	54, 56, 57, 58, 59, 60, 61
		Extracellular	Y	2	80
	Archaea:	Cytosol	Y	4/2	63, 64, 66, 85, 86,90/84
		Extracellular	N	4	21
Ni	*Bacteria:*	Cytosol	N	4	67

[a]Y = determined, [b]N = not determined

fact, the classes found in fungi, plants, birds, and mammals, are distinguishable from each other due to a few differences in amino-acid composition and electron-paramagnetic resonance spectra (EPR) (35), while structural differences have emerged between eukaryotic and prokaryotic Cu/Zn SOD (36, 37). The eukaryotic SODs usually possess two identical subunits of 16 kDa each linked by two cysteines forming an intrasubunit disulfide bridge; each subunit contains a metal cluster at the active site with copper and zinc bridged by a common ligand that is the hymidazole ring of His61 (position referred to the superoxide dismutase from bovine erythrocytes (38)). NMR results indicate that Cu^{2+} is exposed to the solvent, while Zn^{2+} is buried inside the structure (39, 40). The other groups that co-ordinate Cu^{2+} are His44, 46, and 118, while for Zn^{2+} they are His69, 78 and Asp81. Spectroscopic techniques have clarified the ligand symmetry around the metals that is tetrahedrally distorted but forms a square plane for Cu^{2+}, and a trigonal pyramidal conformation for Zn^{2+} (41, 42, 43). More recent structural information (44, 45) has confirmed the previously investigated mechanism of action. There is general agreement that Cu^{2+} is alternatively reduced and oxidized by direct electron transfer during the dismutation of superoxide to hydrogen peroxide and molecular oxygen (46).

Cu/Zn SOD is believed to play a major role in antioxidant defense mechanisms; Cu/Zn SOD-gene knock out mice exhibited more susceptibility to paraquat toxicity, but remained alive (47), while transgenic mice with overexpressed Cu/Zn human SOD-gene were protected from post-ischemic injuries (48).

Bacterial Cu/Zn SODs are rare and poorly characterized compared to their eukaryotic counterparts.

They have been found in a few gram-negative Bacteria and have been shown to be periplasmic. These SODs were originally discovered in pathogens of humans (*Haemophilus parainfluenzae*) or domestic cattle (*Brucella abortus*) (49), which suggest that they have a potential role in bacterial virulence, *i.e.*, a defense against oxygen radicals produced by phagocytes (50). For non pathogenic bacteria (*Photobacterium leiognathi*, *Caulobacter crescentus*, *Escherichia coli*), a mechanism of cellular resistance to oxidative stress for cells entering in stationary phase has been postulated (51). Species containing periplasmic Cu/Zn SOD also contain intracellular Fe- or Mn- SOD. To date, several 3D structures of bacterial Cu/Zn SOD have been solved (36, 37, 49, 52). The structure shares the greek-key beta barrel fold common to the eukaryotic Cu/Zn SOD, but utilizes a different strategy for dimerisation. Comparisons with other known prokaryotic SODs, also including amino-acid sequences, showed that there is a water-mediated distribution of surface charged residues at the dimer interface (53). A unique monomeric Cu/Zn SOD from *E. coli* has been resolved and a net polar nature is displayed in the molecular surface region usually involved in subunit dimerisation (37).

No Archaea has yet been found with a Cu/Zn SOD.

5.2. Fe-SOD, Mn-SOD

Fe-SOD is present in both aerobic and anaerobic Bacteria, Archaea, and plants, whereas Mn-SOD is present in Bacteria, Archaea, mitochondria and chloroplasts. In comparison with the Cu/Zn protein, the Mn-SOD family has received less attention. Spectroscopic and magnetic studies revealed that the metals are tervalents and kinetic studies evidenced complexities in the catalytic mechanisms (54). Dismutases of the Fe- and Mn- type are closely related in sequence and structural homology suggesting a common ancestor. By contrast, they are folded differently with respect to the Cu/Zn family as is to be expected, considering that their encoding genes are unrelated. Recently, a homodimeric Fe-SOD with amino-acid composition, EPR spectra, and molecular weight (subunit of 22 kDa) similar to its aerobic counterparts has been isolated from the strictly anaerobic bacterium *Desulfovibrio gigas*, suggesting a role of this enzyme in combination with a catalase in the detoxification of oxygen by-products in anaerobic environments (55).

3D structures for Fe- and Mn-SOD have been reported for the enzyme from *E. coli* (54), *Bacillus stearothermophilus* (56), *Pseudomonas ovalis* (57), *Propionibacterium shermanii* (58), *Mycobacterium tuberculosis* (59), *Thermus thermophilus* (60), *Aquifex pyrophilus* (61), human mitochondria (62) and the Archaea *Sulfolobus solfataricus* (63) and *Sulfolobus acidocaldarius* (64). They are either homodimers or homotetramers (with subunit molecular weights of about 20 kDa). A high

conservation has been found both in the primary and tertiary structures of the enzymes compared (65, 54); residues essential for binding to metal cofactor near the subunit interface and residues forming a hydrophobic pocket around the active site are located in identical positions (66).

5.3. Ni-SOD

A novel type of cytosolic SOD containing nickel as a cofactor has recently been discovered in several *Streptomyces* species (67, 68, 69). Some of these strains also contain Fe-SOD. These enzymes are composed of four identical subunits of 13.4 kDa. The protein from *Streptomyces caelicolor* is processed *in vivo* (70). Transcriptional analyses revealed that Ni is also involved in activation of the gene encoding Ni-SOD (70) and in the repression of the gene encoding Fe-SOD (71). The amino-acid composition, N-terminal sequences, and immunological properties demonstrated that they are distinct from the Mn-, Fe- or Cu/Zn and thus represent a new class of SOD. X-ray spectroscopic studies of the Ni site demonstrated that the coordination environment is completely different from other SODs being composed largely of S-donor ligands (putative Cys and Met residues); kinetic investigations revealed that Ni-SOD catalyses the redox chemistry of superoxide with at least the same efficiency as that of other SOD families (72).

5.4. Extracellular SOD

Extracellular SOD has been isolated first from mammalian sources. It is a tetrameric and glycosylated Cu/Zn SOD enzyme in humans, mice, and rats (73, 74, 75). The amino-acid sequence of the human enzyme has been deduced from the gene and contains a 18 amino-acid signal peptide, residues 96-193, with strong homology to the cytosolic Cu/Zn SOD, and residues 1-95 with no homology. Residues of the active site are conserved. Although if this enzyme has been widely studied, its 3D structure is still unknown. The protein is involved in protection against oxygen free radicals. Transgenic mice secreting human EC-SOD in milk have been obtained (76) and its use as powerful pharmaceutical to reduce hypertension has been postulated (76). Extracellular SODs have also been found in non-mammalian sources like *Schistosoma mansoni* (77), *Onchocerca volvulus* (78), where it appears to be a pathogenicity factor. Inactivation of secreted SOD with specific antibodies increases the susceptibility of the parasite to be killed by leukocytes (79). A homodimeric Mn-SOD was also purified from culture supernatant of the Gram-positive bacterium *Streptococcus pyogenes* (80). The Archaeon *Sulfolobus solfataricus* also possesses an extracellular Fe-SOD (21) identical in N-terminal sequence and molecular mass to the cytosolic protein isolated from the same source (81), demonstrated to be involved in defense of cell bound enzymes against superoxide radicals generated outside the *S. solfataricus* cytoplasm (21).

6. ARCHAEAL SOD

Archaea occupy all the deepest and shortest branches of the universal phylogenetic tree (82). As such, they can be expected to show the most archaic features in their adaptation to an oxygen containing environment, namely primitive characteristics similar to those of the common ancestor of life on earth.

Nevertheless, the characterization of SODs from extremophiles is interesting not only in terms of evolution; they exhibit unique properties of stability to extreme conditions and hence they represent also good model systems for structure-stabilization studies.

6.1. Thermophilic SOD

To date, only a few Fe-Mn-type SODs have been isolated and characterized from hyperthermophilic Archaea, with the enzymes from *Sulfolobus* species being the most extensively studied both at the structural and functional levels.

SOD from *Sulfolobus acidocaldarius* (SASOD) is an iron-dependent enzyme which shares about 35% sequence identity with bacterial enzymes (64). This SOD is one of the most thermostable enzymes known. In fact, no significant loss of activity has been observed after 24 hours incubation at 95°C (melting temperature of 125 °C). In addition, this enzyme is extremely resistant to high concentrations of denaturant agents like guanidinium hydrochloride, detergents like SDS, as well as to various proteases (64, 83). Moreover the crystal structure refined at 2.2 A resolution has been determined. The enzyme shows a dimeric structure at room temperature with a specific activity typical for dismutases (64, 83). However, it has been demonstrated that it can form tetramers at high temperature and that its specific activity has a 2.1-fold increase. The tetrameric structure is shared among archaeal SODs and the crystal SASOD confirms this feature showing a tightly packed tetramer. The structure of the monomer and its secondary elements show the general fold of Fe/Mn-dependent dismutases with the residues His33, His84, His174, Asp70 and a water molecule (most probably present as a hydroxyl ion) coordinating the iron atom.

In contrast with the often reported high-ion pair content of enzymes from hyperthermophiles SASOD has, despite its extraordinary stability, a surprisingly low number of intrasubunit ion pairs. Comparison with other mesophilic and thermophilic dismutase structures has also shown that the stabilization of the native state is probably based on the significant reduction of solvent accessible hydrophobic surfaces, as well as an increase in the percentage of buried hydrophobic residues.

A superoxide dismutase from the thermoacidophilic *S. solfataricus* has also been purified and characterized, and its crystal structure determined at 2.3 Å resolution by molecular replacement (63). Due to the high identity score between SASOD and SsSOD almost no difference could be detected at the structural level, with the general extremely tight compact arrangement of the subunit. This enzyme shows insensitivity to the general SOD inhibitor cyanide, but it can be inactivated by H_2O_2. Very recently it has been shown that this enzyme does not only have a cytosolic localization. It was also found as oxygen-induced in the culture fluid of *S. solfataricus* during growth on glucose rich medium (21).

The same protein was also identified as cell surface-associated with a 4-fold higher specific activity when compared to the released SOD, thus suggesting that cell adhesion is a major feature of the extracellular enzyme.

Surprisingly, a structural characterization in comparison with the cytosolic SOD revealed identical aminoterminal sequences, electrophoretic mobility, isoelectric point, and molecular mass for all three differently located enzymes.

In order to clarify the physiological role of the cell-associated SOD, the prevention of cell-bound protein deactivation by oxyradicals was investigated. A direct protective effect was demonstrated on the glucose dehydrogenase, located on the cell surface and inactivated by potassium superoxide and also in *in vitro* assays on the free released glucose dehydrogenase. The integral-membrane enzyme succinate dehydrogenase on the intact cultured cells could also be protected by SOD. The SOD added to cells was proven to reduce the critically damaging peroxidation and, hence, play a major role in maintaining the integrity of the outer cell-envelope components. The authors argued that, since the archaeal domain of life is considered the closest to the "first form of life", this Fe-, Mn-class of SODs and its two different cellular localization forms appeared during evolution in an aerobic ancestor before the other families that show a more specific type/compartmentalization relationship in the cell (21).

Another example worthnoting of SOD from thermophiles is provided by that from *Aeropyrum pernix*, which is the first strict aerobic heterotrophic neutrophile found among the Crenarchaeota (84). The cloned gene was expressed in *E. coli* and the gene product characterized as a homodimer. The sequence shows an overall similarity to the sequences of known Fe/Mn SODs, especially with the SODs from *Sulfolobales*, confirming that this class of enzymes is common to hyperthermophilic Archaea as a unique type and probably the most archaic in evolution. The metal content varies according to the conditions for gene expression and culture, both Mn and Fe having been found in the native protein. Metal reconstitution experiments actually showed that *A. pernix* SOD is cambialistic, *i.e.* active with either Fe or Mn, with the Mn form showing about 5-fold higher catalytic efficiency. It also shows extraordinary resistance, with the Mn-reconstituted enzyme retaining full activity after 5h incubation at 105 °C and exhibiting complete insensitivity to sodium azide even at a concentration up to 400 mM. Given the facts that the Mn is more active than the Fe form in *A. pernix*, and that it is distributed mostly among aerobes or facultative anaerobes as opposed to the Fe type SODs, which are mostly found in microaerophiles and anaerobes, it is likely that microorganisms prefer Mn-SOD to Fe-SOD in aerobic environments and that cambialistic SODs bind Mn in aerobic environments, but bind Fe in anaerobic ones (84). Yet it is not possible to correlate the metal type content to specific chelating amino-acid residues since no conservation and apparent phylogenetic relationship are observed among cambialistic SODs and also no clear-cut difference can be deduced from the comparison with enzymes showing metal restricted specificity.

6.2. Halophilic SOD

SODs have been purified and extensively characterized from the halophilic Archaea *Halobacterium cutirubrum* and its immediate relative *Halobacterium halobium* (85, 86). They contain Mn as the metal cofactor, their subunit molecular weights are 38 kDa. They are resistant against cyanide and hydrogen-peroxide inhibitors as other archaeal SODs and their N-terminal sequences are homologous to the Mn/Fe family of Bacteria. The SOD enzyme and mRNA levels increase in cells exposed to paraquat, an oxygen radical generator, and in cell cultures grown with higher O_2 tension, indicating that the *sod* gene is basally expressed in the absence of oxygen (87). Since in both types of expression the same initiation site is used, it seems that the same promoter is responsible for basal and induced expression of the gene (87). Induction of SOD in *H. halobium* by heat shock has also been reported (88). Five additional genes of the *sod* family from other halophiles have been cloned, characterized, and the deduced protein sequences compared with those of other Mn- and Fe-SODs (66). The alignment revealed that residues involved in metal binding and residues of the active site were conserved with those in identical positions of other Fe/Mn SODs but revealed unique amino-acid residues, mostly acidic, conserved in the halophilic family but not found in any other SOD. A role in the general adaptation of the enzyme to high salt content has been postulated for such amino acids generally found at sites able to influence the hydrophobic and surface-hydration properties of the protein (89), but the authors also suggest that halophilic SODs, considered as a group, are unique and different from other SOD proteins (66).

6.3. SOD in methanogens

Despite their catalytic capacity for producing molecular oxygen, SODs have been identified also in the strictly anaerobic methanogens *Methanobacterium bryantii* (90) and *Methanobacterium thermoautotrophicum* (91). Both proteins are tetrameric and the EPR spectra of the former enzyme were typical of a Fe-SOD with Fe located in a rhombic structure (90). The deduced amino-acid sequence of the latter resembled more the archaeal and eukaryal Mn-SOD than the bacterial Fe-SOD, but analysis of metal content and the effects of inhibitors on the recombinant protein demonstrated it was a peculiar Fe-SOD as it was only partially inhibited by cyanide and resistant against hydrogen peroxide (92). These features were also observed in *M. bryantii* SOD (90). The lower redox potential of Fe^{2+}/Fe^{3+} compared with Mn^{2+}/Mn^{3+} may favour Fe-SOD in anaerobic environments.

The strategy of discriminating between Fe-SOD and Mn-SOD by analyzing residues at particular sites in their sequences was not applicable to the *M. thermoautotrophicum* SOD. Recently, it was proposed that the physiological role of these proteins in anaerobic microorganisms could be the superoxide reduction with consequent production of hydrogen peroxide, rather than a dismutation reaction (93). This would also explain the

resistance to hydrogen peroxide produced in the redox reaction which must afterwards be removed by specific enzymes.

7. COMPARISON OF THE FAMILIES OF SUPEROXIDE DISMUTASES

SODs have been grouped on the basis of the metal cofactor, which can be Cu/Zn or Fe, Mn or Ni (33). Different evolutionary strategies have led to an unexpected flexible structure where the dimer assembly seemed to be crucial for the optimization of biological function and substrate recognition. Two unrelated proteins have evolved independently from each other to exert the same function, *i.e.*, the protection of organisms from chemical damage caused by the reactive superoxide anion: Fe- and Mn-types show homology and possess identical metal-chelating residues at the active site, sharing substantial sequence and tertiary structure homology, while the Cu/Zn family is unrelated in sequence and structure to the previous one.

Conserved structural and functional features of Cu/Zn SODs have been studied by comparison of the known 3D structures and analysis of available amino-acid sequences. Alignment of 38 SODs from various sources and the deduced evolutionary tree indicated that cytosolic and extracellular enzymes followed independent evolutionary paths (94).

Cytosolic eukaryotic Cu/Zn superoxide dismutases share with prokaryotic enzymes a stable and flexible beta-barrel fold, a conserved ligand stereochemistry, the two-metal ions-active site, and a typical dimer assembly (44). However, recently, it has been shown that in the prokaryotic SODs the beta barrels elements responsible for the dimer interface, the strategy utilized for the electrostatic recognition of the superoxide radical, and the formation of the intersubunit disulfide bridge, are quite dissimilar from those which are highly conserved in their eukaryotic counterparts (53). These observations tend to disprove the previous hypothesis of a lateral *sod* gene transfer from eukaryotic to prokaryotic species (95). In homologous eukaryotic Cu/Zn SODs, local electric fields and electric-field flux in the proximity of the active site were found to be constant, suggesting that the spatial relationship of charges on the protein surface has also been conserved during evolution (96). The only monomeric variant of Cu/Zn SOD found in *E. coli* (37) displays an antiparallel beta-barrel structure, in which disulfide bridge connections are modified, functional electrostatic residues are absent, and the molecular surface region involved in dimer formation is structurally altered displaying a net polar nature. These data suggest a modified control of substrate-steering toward the catalytic center (37).

On the basis of these observations, it has been proposed that the putative Cu/Zn SOD precursor existed before the origin of eukaryotes but evolved divergently to form distinct eukaryotic and prokaryotic Cu/Zn classes (53).

The Fe- Mn family of proteins is completely unrelated to the Cu/Zn family. The Fe-protein is in fact a two-domain structure with the first domain organized in alfa-helices and the second displaying a three layer mixed alfa/beta structure. (54). There are also differences in the relative positions of the metal binding sites, quite close to the subunit interface and to each other in the Fe-protein, while far from the subunit interface and from each other in the Cu/Zn protein (54). Superposition of the Mn-enzyme (60) with the Fe-enzyme showed that the spatial arrangement of the active center and of metal-binding residues is very similar in both enzymes. To date, there is not yet a structural explanation for the selectivity of metal binding in these enzymes (60). Cambialistic Fe- Mn SOD has been recently discovered in *A. pernix* (84) and resolution of its structure may help in clarifying this point.
Alignments of Fe- and Mn-SOD sequences from halophilic and methanogenic Archaea with the *E. coli* enzyme identified *M. thermoautotrophicum* as the outgroup (66). Thus, assuming that an anaerobic preceded the aerobic environment on Earth, it was speculated that the Mn-enzyme differentiated at a secondary stage during evolution from the anaerobic Fe-SOD (92), while the Archaea-Bacteria divergence is still controversial (87, 92). Alignments of archaeal SODs performed by Dello Russo *et al*. (81) and the deduced dendrogram, confirmed the ancestor and supported the hypothesis that archaeal SODs evolved independently from their counterparts in thermophilic bacteria.

8. CONCLUDING REMARKS

Superoxide is generated by many spontaneous reactions and enzymatic oxidations within all respiring organism as well as some anaerobic cells, both intracellularly and extracellularly. Similarly, superoxide dismutases are widespread among living organisms so that we can conclude that O_2^- has driven the evolution of defenses, among which the SODs are prominent. Their involvement does not only consist of maintaining the cell integrity and viability, but of protecting single cell components assuring their functionality against the damages by oxidative stress. For this reason SODs may be examined not only for their biological importance, but also for their possible biotechnological applications. Indeed, protection of high-value biomolecules, such as lipids and proteins in the packaging and storage of food and pharmaceuticals, is only one of the attractive goals of modern biotechnology in which they could find useful applications.

The recent discovery and characterization of stable enzymes able to keep their structures and functions unaltered even under extreme conditions, comprising low-water and high-organic solvent compositions of the media, render SODs even more attractive for these purposes. This review has dealt mostly with the enzymes isolated from Archaea solely because the extraordinary SODs they express may have important impact both for the study of structure-function-stability relationships and evolutionary considerations. Nevertheless, the state of the art is only in the early stages and we confidently expect that it will progress in the future.

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Send correspondence to: Dr Simonetta Bartolucci-Dipartimento di Chimica Organica e Biologica, Università degli Studi di Napoli "Federico II" - Via Mezzocannone, 16-80134 Napoli, Italy, Tel: +39- 081 7041269, Fax: +39- 081 5521217, E-mail: bartoluc@unina.it

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