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Update on Abiotic Stress

Oxygen Stress and Superoxide Dismutases[1]

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THE OXYGEN PARADOX

The accumulation of dioxygen in Earth's atmosphere allowed for the evolution of aerobic organisms that use O_2 as the terminal electron acceptor, thus providing a higher yield of energy compared with fermentation and anaerobic respiration. For example, in aerobic metabolism, the complete breakdown of one molecule of glucose yields a total of 38 molecules of ATP, whereas the anaerobic breakdown of this same glucose molecule to ethanol and CO_2 yields only 8 ATPs.

In its ground state, molecular O_2 (dioxygen) is relatively unreactive, yet it is capable of giving rise to lethal reactive excited states as free radicals and derivatives. Utilization of O_2 proceeds most readily via a complete stepwise, four-electron reduction to water during which partially reduced reactive intermediates are generated (Fig. 1). The reactive species of reduced dioxygen include the superoxide radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\cdot OH$). These and the physiologically energized form of dioxygen, singlet oxygen ($^1O_2$), are the biologically most important O_2 species. An activation energy of approximately 22 kcal/mol is required to raise molecular O_2 from its ground state to its first singlet state. In higher plants, this energy is readily obtained from light quanta via such transfer molecules as Chl (Foote, 1976). All of these activated oxygen species are extremely reactive and cytotoxic in all organisms. These highly reactive species can react with unsaturated fatty acids to cause peroxidation of essential membrane lipids in the plasmalemma or intracellular organelles. Peroxidation damage of the plasmalemma leads to leakage of cellular contents, rapid desiccation, and cell death. Intracellular membrane damage can affect respiratory activity in mitochondria, cause pigment breakdown, and cause loss of carbon-fixing ability in chloroplasts.

Several Calvin-cycle enzymes within chloroplasts are extremely sensitive to H_2O_2, and high levels of H_2O_2 (the product of superoxide dismutation) directly inhibit CO_2 fixation (Kaiser, 1979). H_2O_2 has also been shown to be active with mixed function oxidases in marking several types of enzymes for proteolytic degradation (Fucci et al., 1983). Superoxide and H_2O_2 can react in a "Haber-Weiss" reaction to generate the hydroxyl radical ($\cdot OH$), which is the most potent oxidant known. The hydroxyl radical indiscriminately and rapidly attacks virtually all macromolecules, leading to serious damage in cellular components, DNA lesions, and mutations, and often leading to irreparable metabolic dysfunction and cell death. Thus, O_2, although essential for the existence and survival of aerobic life, presents living organisms with a variety of physiological challenges collectively termed "oxidative stress." These challenges may be greater for plants relative to other eukaryotes because of their stationary lifestyle under constantly changing environments and because plants both consume O_2 during respiration and generate it during photosynthesis.

In plants, the superoxide radical and singlet oxygen are commonly produced in illuminated chloroplasts by the occasional transfer of an electron from an excited Chl molecule or PSI components under conditions of high NADPH/NADP ratios to molecular O_2. Carotenoids, which are essential components of thylakoid membranes, can effectively quench the excited triplet state of Chl and/or singlet oxygen (Knox and Dodge, 1985). However, various environmental perturbations (*e.g.* intense light, drought, temperature stress, herbicides, etc.) can cause excess reactive O_2 species, overwhelming the system and necessitating additional defenses. For example, stomatal closure resulting from drought conditions limits CO_2 availability for photosynthetic carbon assimilation. Under such conditions and in intense sunlight, excess superoxide production in the chloroplast can result in photoinhibition and photooxidation damage. In addition, enzymes such as xanthine oxidase, aldehyde oxidase, and other flavin dehydrogenases are capable of generating superoxide as a catalytic by-product (Fridovich, 1986). The dismutation of two superoxide anions produces H_2O_2, which is also a product of the microbody-associated β-oxidation of fatty acids and peroxisomal photorespiration reactions (Beevers, 1979; Tolbert, 1982). Furthermore, among all organisms, the cellular concentration of dioxygen is highest in plants. Compare the dioxygen concentration in the vicinity of mammalian mitochondria (0.1 μM) and that in leaf cells (over 250 μM). Thus, plants are potentially exposed to the most severe environmental conditions with respect to the production of active O_2 and ensuing oxidative damage.

In addition to normal metabolic activity, reactive O_2 species can result from cellular exposure to various environmental stimuli (Fig. 2). Such factors as UV light and other forms of radiation, herbicides (e.g. paraquat, diquat), pathogens (e.g. *Cercospora*), certain injuries, hyperoxia, ozone, temperature fluctuations, and various other stresses are known to induce

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Abbreviations: Abbreviations: CAT, catalase; SOD, superoxide dismutase protein; *Sod*, superoxide dismutase gene/transcripts.

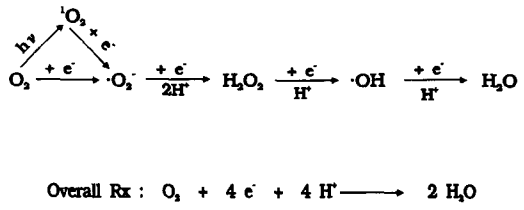

Figure 1. Pathways in the reduction of O_2 to water leading to the formation of various intermediate reactive O_2 species.

free radical formation in most aerobic organisms (Scandalios, 1992).

Reactive O_2 species are produced in significant quantities in various subcellular compartments or organelles. Each organelle has potential targets for oxidative stress as well as mechanisms for eliminating the noxious oxyradicals (Table I).

PROTECTIVE ANTIOXIDANT DEFENSES

Among the special problems incurred by aerobic organisms is the need to effectively eliminate the toxic O_2 intermediate species generated during normal metabolic activity or as a consequence of various exogenous environmental insults. Through selective pressure and evolution, numerous defense mechanisms, both enzymic and nonenzymic, have emerged to protect cells against oxidative injury. Among the latter are GSH, cysteine, hydroquinones, mannitol, vitamins C and E, flavonoids, some alkaloids, and β-carotene (Ames, 1983; Larson, 1988). The enzymic antioxidant defenses include enzymes capable of removing, neutralizing, or scavenging free radicals and oxyintermediates. Without these defenses, plants could not efficiently convert solar energy to chemical energy.

Examples of enzymic antioxidant defenses include ascorbate peroxidase and GSH reductase, which are believed to scavenge H_2O_2 in chloroplasts and mitochondria, respectively (Foyer and Halliwell, 1976); the CATs and peroxidases that remove H_2O_2 very efficiently (Scandalios, 1993); and SODs that scavenge the superoxide anion. The CATs and SODs are the most efficient antioxidant enzymes. Their combined action converts the potentially dangerous superoxide radical ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2) to water (H_2O) and molecular oxygen (O_2), thus averting cellular damage:

$$O_2 + e^- \rightarrow \cdot O_2^- \quad (1)$$

$$\cdot O_2^- + \cdot O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \quad (K_2 = 2.4 \times 10^9\ m^{-1}\ s^{-1}) \quad (2)$$

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

$$H_2O_2 + H_2O_2 \xrightarrow{\text{CAT}} 2H_2O + O_2 \quad (K_1 = 1.7 \times 10^7\ m^{-1}\ sec^{-1}) \quad (4)$$

Thus, the combined action of SOD and CAT abate the formation of the most toxic and highly reactive oxidant, the hydroxyl radical (·OH), which can react indiscriminately with all macromolecules. Although there are no known direct scavengers of singlet oxygen ($^1O_2$) or the hydroxyl radical (·OH), SOD is believed to function in their elimination by chemical reaction (Matheson et al., 1975).

It is clear that defenses to cope with oxidative stress exist in all aerobes. What is still needed, however, is a thorough understanding of the underlying mechanisms utilized by plants and other organisms to respond to oxidative stresses. Aside from numerous correlative responses (i.e. increases in oxy-stress resulting in increased levels of some antioxidant defenses), little is currently known as to how the genome perceives oxidative insult and mobilizes a response to it. Such information is interesting in and of itself, but it is also essential in any future attempts to raise tolerance to environmental oxidative stress in organisms and to reduce cellular damage by active O_2. To understand these mechanisms, it is essential to identify the responsive genes and to understand their structure, regulation, and expression. The molecular biology of various antioxidant defenses in different organisms was recently discussed in some detail (Scandalios, 1992). Herein, I will discuss in an abbreviated form some highlights of our current knowledge of plant SODs and their role(s) in providing antioxidant defenses.

SODs

SOD (EC 1.15.1.1) was first isolated from bovine blood as a green copper protein (Mann and Keilin, 1938) whose biological function was believed to be copper storage. Over the years, the enzyme has been variably referred to as erythrocuprein, indophenol oxidase, and tetrazolium oxidase. The catalytic function of the enzyme was discovered by McCord and Fridovich (1969). The enzyme is ubiquitous, being widely distributed among O_2-consuming organisms, aerotolerant anaerobes, and some obligate anaerobes (Fridovich, 1986).

All SODs, irrespective of source, are multimeric metalloproteins that are very efficient at scavenging the superoxide radical. The Cu/ZnSODs, as well as most prokaryotic Mn-SODs and FeSODs, are dimeric, whereas the MnSODs from

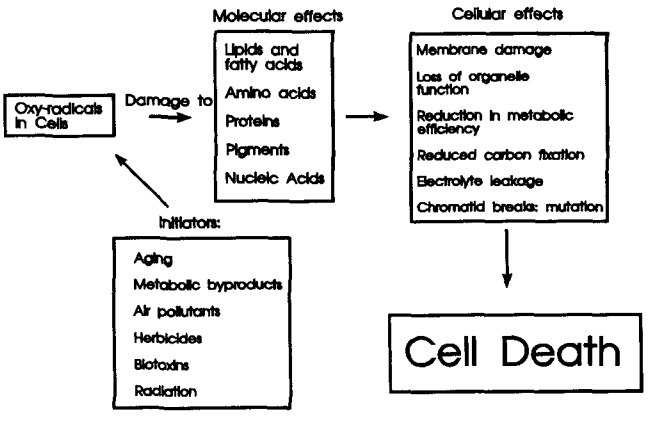

Figure 2. Scheme showing some initiators of oxyradicals and the biological consequences leading to biological dysfunctions and cell death.

Table I. *Antioxidant enzyme systems in higher plants*

Subcellular Location	Type of Active O_2 Species	Source of Active O_2 Species	Enzymic Scavenging Systems	Products	Nonenzymic Scavenging Systems
Chloroplast	Superoxide H_2O_2	PSII Enzymic	SOD Ascorbate peroxidase	H_2O_2 Dihydroascorbate GSH $NADP^+$	Fd Carotenoids Xanthophylls
Mitochondria	Superoxide H_2O_2	Electron transport and enzymic	SOD Peroxidase CAT (CAT-3, maize)	H_2O_2 H_2O H_2O, O_2	
Cytosol	Superoxide H_2O_2	Enzymic	SOD CAT Peroxidase	H_2O_2 H_2O, O_2 H_2O	
Glyoxysome and peroxisome	H_2O_2	β-Oxidation (G) Photorespiration (P)	CAT	H_2O, O_2	

mitochondria and certain thermophilic bacteria are tetrameric. SODs catalyze a disproportionate reaction at a rate very near that of diffusion (McCord and Fridovich, 1969). To accomplish this reaction, the mechanism employs an alternating reduction/oxidation of the respective metal associated with the enzyme. Three distinct types of SODs, based on the metal ion in their active sites, have been observed from a wide range of organisms examined. Thus, there are SODs that contain copper and zinc (Cu/ZnSOD), manganese (MnSOD), or iron (FeSOD). With a few exceptions, Cu/ZnSODs are generally found in the cytosol of eukaryotic cells and chloroplasts; the MnSODs are found in the matrix of mitochondria and in prokaryotes; the FeSODs are generally found in prokaryotes and have been reported to exist in some plants (Duke and Salin, 1985). A membrane-associated MnSOD has been reported in chloroplasts of some plants (Hayakawa et al., 1985), and a tetrameric glycosylated Cu/ZnSOD has been reported in mammalian extracellular fluids (Marklund, 1982); no extracellular SODs have been reported in plants.

At present, the evolutionary origins of the various SODs are not clear. However, sequence data suggest that the three types of SOD fall into two phylogenetic families, the Cu/ZnSODs and the Fe-/MnSODs. The FeSODs and MnSODs are closely related because they share a high degree of amino acid sequence and structural homologies, but are unrelated to Cu/ZnSODs (Stallings et al., 1984). The available data suggest that the two families of SOD must have evolved independently and were selected in response to a common environmental stress, the oxygenation of the biosphere by photosynthetic organisms.

Cu/ZnSODs for which complete amino acid sequences have been determined show that the metal-binding sites are highly conserved. The Cu is ligated to histidines 48, 50, 77, and 135; the Zn is ligated to histidines 77, 86, 95, and to aspartate 98. The Cu is exposed to solvent. Cyanide reversibly inhibits the enzyme, whereas H_2O_2 causes irreversible inhibition. Both cyanide and H_2O_2 were shown to interact with the Cu. The sensitivity of Cu/ZnSODs to cyanide has been used as a diagnostic tool to distinguish Cu/ZnSODs from Fe-/MnSODs that are unaffected by cyanide. Likewise, FeSODs are irreversibly inactivated by H_2O_2, whereas MnSODs are unaffected (Baum and Scandalios, 1979 and refs. therein).

DISSECTING THE PHYSIOLOGICAL FUNCTION(S) OF SODs

The biological role and significance of SODs as protective enzymes against O_2 toxicity are borne out in numerous studies with prokaryotes and lower and higher eukaryotes, including higher plants (Fridovich, 1986; Hassan and Scandalios, 1990; Scandalios, 1990, 1992; Bowler et al., 1992; Gralla and Kosman, 1992). Although convincing, the evidence until recently had been largely correlative. However, with the advent of recombinant DNA techniques, it became possible to analyze the genes responsible for coding the various SOD types in different organisms. Cloning and sequencing the *Sod* genes will provide significant knowledge and information toward understanding the structure and regulation of these genes. In addition, the *Sod* clones provide the opportunity for producing large quantities of active enzymes for detailed enzymic analyses, structural and evolutionary comparisons, analysis of functional domains, and commercial applications. The clones can be used further as probes for isolating homologous genes from various species and for in vitro (deletions or site specific) induction and isolation of mutants. Isolation of genomic clones provides the opportunity to determine intron-exon boundaries, start and stop sites of transcription, *cis*-regulatory regions, and to identify signal-responsive elements (*i.e.* sequences responsive to imposed oxidative stress signals) that might explain how these genes are regulated to respond and protect cells against oxidative damage.

PLANT SODs EXIST IN MULTIPLE FORMS (ISOZYMES)

Unlike most other organisms, plants have multiple enzymic forms (isozymes) of SOD. The existence of SOD isozymes in plants and their genetic basis was first demonstrated in maize (Baum and Scandalios, 1979, 1982), and the first plant *Sod* gene to be cloned was from maize (Cannon et al., 1987). The basis for the multiplicity of SOD in other plant species continues to be investigated, and indirect evidence suggests that multiple genes for SOD exist in most plants. The existence of multiple molecular forms of SOD, their location within cells, tissues, or organelles (Table I), and any changes they may undergo during development or in response to

various signals imply separate metabolic roles for each of the SOD isozymes.

RESPONSE OF SOD TO ENVIRONMENTAL STRESS

Numerous studies indicate that in both prokaryotes and eukaryotes, oxidative stress induces or enhances the activity of SOD (Scandalios, 1990; Bowler et al., 1992; Gralla and Kosman, 1992). Increases in SOD activity have been observed in response to treatment with herbicides that serve as preferred terminal electron acceptors at the reducing site of PSI (e.g. diquat, paraquat) or that are known to block electron transport (e.g. atrazine, diuron). Temperatures and light conditions leading to sunscald in vegetables, fruits, and flowers lead to increased levels of SOD (Rabinowitch and Sklan, 1980). Increases in SOD have been observed in response to ozone and SO_2. In addition, such environmental conditions as drought, chilling, anoxia, and pathogenic injury have been correlated with SOD activity (Monk et al., 1989). Poplar trees exposed to relatively low levels of SO_2 proved more resistant to subsequent damage on exposure to high levels of SO_2; the increased resistance was correlated with increased SOD activity (Tanaka and Sugahara, 1980). Paraquat-resistant varieties of various species including tobacco, ryegrass, and horseweed correlate with increased activities of SOD and other antioxidant enzymes (Harper and Harvey, 1978; Matters and Scandalios, 1986). However, the mechanisms for the observed increases in antioxidant enzymes in response to oxidative stress have yet to be resolved. The maize SOD gene-enzyme system, composed of at least six genetically and biochemically distinct isozymes (Scandalios, 1990), provided an opportunity to study the response of specific *Sod* isozyme genes to imposed environmental stresses. Changes in individual *Sod* genes in response to environmental stresses had not been examined in detail, nor had the responses to different stress factors within a single SOD multienzyme system been previously studied.

The herbicide paraquat produces its cytotoxic effects via a free radical mechanism, and although banned as an herbicide, it is used as an indicator in O_2 toxicity studies in many organisms. In maize, the increase observed in total SOD activity is correlated with increased levels of specific isozymes (Matters and Scandalios, 1986). SOD-1 (chloroplastic), SOD-2, and SOD-4 (both cytosolic) increased significantly; SOD-3 (mitochondrial) increased only slightly in activity. The increases observed were due to enhanced synthesis, and polysomal mRNA for SOD-4 and SOD-3 also increased following treatment with 10^{-5} M paraquat, suggesting increased transcription for the respective genes. Also, a significant increase in total SOD activity resulted upon exposure of maize to hyperoxia. This increase was shown to be due to increases in the protein and mRNA for the cytosolic Cu/ZnSODs, SOD-2 and SOD-4; SOD-1 and SOD-3 were unaffected.

Ethylene increases in plants under oxidative stress. Ethephon (2-chloroethylphosphoric acid) is metabolized by plants to ethylene and phosphoric acid. Following hydroponic uptake of ethephon by maize seedlings, *Sod4* RNA levels and protein increased significantly and in an organ-specific manner (Scandalios, 1992), whereas none of the other SODs were affected. This stem-specific *Sod4* mRNA induction is a novel phenomenon and provides a significant starting point toward unraveling the differential biological roles for the different cytosolic Cu/ZnSOD forms in maize. In particular, the differential response to ethephon of *Sod4* and *Sod4A* is quite intriguing, considering the strong sequence and structural conservation of these two genes and proteins (Cannon and Scandalios, 1989; Scandalios, 1992). The localization of pertinent regions of DNA responsible for providing the *Sod4* versus *Sod4A* organ-specific differential response to ethephon may provide valuable, biologically relevant information toward understanding both the differential regulation of and the need for multiple cytosolic Cu/ZnSODs.

Fungi of the genus *Cercospora* produce a light-induced, photoactivated polyketide toxin, cercosporin. By itself, the toxin does no damage to the host plant; however, in the presence of light, an excited triplet form of cercosporin reacts directly with molecular O_2 to produce singlet O_2 and/or superoxide radicals, depending on the redox potential of the environment (Daub and Hangarter, 1983). It was recently demonstrated in maize that applications of photoactivated cercosporin resulted in parallel increases in total CAT activity, protein, and RNA steady-state levels. In contrast, although accumulation of several *Sod* transcripts increased dramatically in response to cercosporin treatment, both total SOD activity and individual SOD isozyme protein levels remained constant in all toxin treatments (Williamson and Scandalios, 1992). This suggests that, among other possible mechanisms, protein turnover might play a key role in the response of the various SODs to activated O_2 species.

Plants completely deprived of O_2 survive a period of anoxia but perish on reexposure to air, suggesting oxidative damage during the recovery phase. In *Iris pseudacorus*, a significant increase in SOD activity was observed during 28 d of anoxia, which suggests a protective role for SOD in the postanoxic phase (Monk et al., 1989).

DOES SOD PROTECT AGAINST OXY-STRESS?

The available data from all organisms suggest that SODs scavenge O_2^- in vivo as they do in vitro and that they protect against the damaging effects of active O_2. The protective role of SOD in bacteria has been supported by studies employing a variety of mutants (Touati, 1988). More recently, transgenic experiments demonstrated that plant SODs can be effectively utilized to rescue SOD-deficient yeast under oxidative stress (Bowler et al., 1992; Zhu and Scandalios, 1992).

Maize MnSOD-3 is synthesized as a precursor with a cleavable amino-terminal extension of 31 amino acids and is posttranslationally imported into the mitochondrial matrix. Pre-SOD-3 is targeted, imported, and processed in isolated maize mitochondria (White and Scandalios, 1989).

Full-length *Sod3* cDNA and its deletion mutants were used to transform a yeast MnSOD-deficient mutant strain, which is hypersensitive to O_2. The maize SOD-3 protein is effectively synthesized in the yeast cells, is properly targeted, imported, and processed in the yeast mitochondria in vivo, and sufficiently complements the MnSOD deficiency in the mutant yeast cells (Zhu and Scandalios, 1992). In addition, the transformed yeast containing active maize MnSOD-3 in

its mitochondria regains its resistance to oxidative stress. The ability of the mutant yeast cells to become resistant to oxidative stress is dependent on targeting the maize pre-SOD-3 into the yeast mitochondria. These findings support the notion that MnSOD functions to protect cells from the lethal effects of O_2 and that this functional role is conserved among various species. It is encouraging that SOD-deficient mutants of readily transformable organisms may serve as useful bioassays for identifying and differentiating the functional roles for distinct SOD isozymes.

CONCLUDING COMMENTS

In view of the current data, it seems reasonable to conclude that SODs play a significant role in protecting living cells against the toxicity and mutagenicity of active O_2 species by virtue of their capacity to scavenge the $\cdot O_2^-$. Whether SOD has other biological functions remains an open question.

It is clear that living cells must maintain a delicate balance between the rates of $\cdot O_2^-$ generation and removal. To maintain such a balance, organisms evolved elaborate regulatory mechanisms to control the synthesis of SODs in response to different oxidative stimuli. However, at present we have little information and understanding of the underlying molecular mechanisms for the mobilization of the antioxidant defenses in aerobic organisms. The differential responses observed for the different SOD isozymes to given stressors or signals may shed light upon the reason for the existence of multiple forms of these enzymes, and the absence of null mutants in all plant populations screened to date points to the critical role(s) for SOD.

An intriguing observation from several investigations is that, although the accumulation of several *Sod* transcripts changed significantly in response to a given stressor (e.g. cercosporin), the levels of the corresponding isozymes remained constant; whereas, for example, changes in steady-state levels of the various CAT isozymes and their corresponding transcripts were parallel (Williamson and Scandalios, 1992). Because the $\cdot O_2^-$ and the products of its peroxidation of the lipid bilayer are highly and immediately toxic to the cell, maximal steady-state levels of the appropriate SODs might be required at all times to provide adequate protection. It is conceivable that high levels of oxidative stress may result in high SOD protein turnover, resulting in the requirement for new SOD enzyme synthesis to maintain SOD levels sufficient for effective protection.

The identification and characterization of *cis*-acting elements and *trans*-acting factors involved in *Sod* gene regulation and expression will provide some depth in our understanding of the entire signal transduction pathway during oxidative stress and will enhance our efforts toward engineering organisms to better cope with oxidative insult. The available information suggests that any efforts to engineer more oxytolerant plants should perhaps focus less on over- or underexpressing SOD levels and more on maintaining the endogenous optimal levels of all SOD isozymes when the plant is under oxidative stress and in keeping a well-balanced and coordinated expression of all essential antioxidant enzymes in the various cell compartments. Molecular analyses of *Sod* gene structure and expression, particularly promoter analysis, along with quantitative expression mutant analyses, may help unravel the mechanisms regulating the expression of the individual *Sod* genes at the appropriate location and time in the plant.

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