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REVIEW ARTICLE

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Oxidative stress in bacteria and protein damage by reactive oxygen species

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Summary The advent of O_2 in the atmosphere was among the first major pollution events occurred on earth. The reaction between ferrous iron, very abundant in the reductive early atmosphere, and oxygen results in the formation of harmful superoxide and hydroxyl radicals, which affect all macromolecules (DNA, lipids and proteins). Living organisms have to build up mechanisms to protect themselves against oxidative stress, with enzymes such as catalase and superoxide dismutase, small proteins like thioredoxin and glutaredoxin, and molecules such as glutathione. Bacterial genetic responses to oxidative stress are controlled by two major transcriptional regulators (OxyR and SoxRS). This paper reviews major key points in the generation of reactive oxygen species in bacteria, defense mechanisms and genetic responses to oxidative stress. Special attention is paid to the oxidative damage to proteins.

Key words Oxygen · Oxidative stress · Reactive oxygen species · Protein oxidation · Carbonyl groups

Introduction

Aerobic organisms use molecular oxygen (O_2) for respiration or oxidation of nutrients to obtain energy. Reactive by-products of oxygen, such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radicals ($^{\bullet}OH$), are generated continuously in cells grown aerobically. Most of such products derive from sequential univalent reductions of molecular oxygen catalyzed by several membrane–associated respiratory chain enzymes. Experimental data indicate that, in *Escherichia coli*, the respiratory chain can account for as much as 87% of the total H_2O_2 production [23]. The leakage of single electrons from the bacterial respiratory chain was observed at the NADH dehydrogenase and ubiquinone sites, and was similar to that observed in eukaryotic mitochondria. Environmental agents such as ionizing, near-UV radiation, or numerous compounds that generate intracellular O_2^- (redox-cycling agents such as menadione and paraquat) can cause oxidative stress, which arises when the concentration of active oxygen increases to a level that exceeds the cell's defense capacity. Some immune cells which use the NADPH oxidase enzyme, upon invasion by pathogenic bacteria, also exploit oxidative stress as a weapon during phagocytosis.

The biological targets for these highly reactive oxygen species are DNA, RNA, proteins and lipids. Much of the damage is caused by hydroxyl radicals generated from H_2O_2 via the Fenton reaction, which requires iron (or another divalent metal ion, such as copper) and a source of reducing equivalents (possibly NADH) to regenerate the metal. Lipids are major targets during oxidative stress. Free radicals can attack directly polyunsaturated fatty acids in membranes and initiate lipid peroxidation. A primary effect of lipid peroxidation is a decrease in membrane fluidity, which alters membrane properties and can disrupt membrane-bound proteins significantly. This effect acts as an amplifier, more radicals are formed, and polyunsaturated fatty acids are degraded to a variety of products. Some of them, such as aldehydes, are very reactive and can damage molecules such as proteins [28]. Unlike reactive free radicals, aldehydes are rather long lived and can therefore diffuse from the site of their origin and reach and attack targets which are distant from the initial free-radical event, acting as "second toxic messengers" of the complex chain reactions initiated. Among the many different aldehydes which can form during lipid peroxidation, the most intensively studied are malonaldehyde (MDA) and 4-hydroxyalkenals, in particular 4-hydroxynonenal (HNE) [17]. DNA is also a main target; active species attack both the base and the sugar moieties producing single- and double-strand breaks in the backbone, adducts of base and sugar groups, and cross-links to other molecules, lesions that block replication [41, 42]. The spectrum of adducts in oxidized DNA in vitro and in vivo includes more than 20 known products, including damage to all four bases and thymine-tyrosine cross-links [14]. The oxidation of proteins,

which has been traditionally less well characterized, is one of the aims of this review. Several classes of damage are documented [21, 44], including oxidation of sulfhydryl groups, reduction of disulfides, oxidative adduction of amino acid residues close to metal-binding sites via metal-catalyzed oxidation, reaction with aldehydes, modification of prosthetic groups or metal clusters, protein-protein cross-linking and peptide fragmentation. All these modifications are deleterious to the cell, since they lead to a loss of function of membranes and proteins, and block DNA replication or cause mutations.

Defense mechanisms

The appearance of oxygen in the atmosphere led to the development of defense mechanisms that either kept the concentration of the O_2-derived radicals at acceptable levels or repaired oxidative damages. Iron plays a significant role in biology (transport, storage and activation of molecular oxygen, reduction of ribonucleotides, activation and decomposition of peroxides, and electron transport) and Fe^{2+} is required for the growth of almost all living cells. Due to its potential damaging effects, in bacteria, iron solubilization and metabolism is strictly regulated at two levels: (i) the entrance to the cell by specific membrane-bound receptors, and (ii) inside the cell, by two proteins, bacterioferritin and ferritin, very similar to the eukaryotic ferritin, but presenting ferroxidase activity. Some molecules are constitutively present and help to maintain an intracellular reducing environment or to scavenge chemically reactive oxygen. Among these molecules are nonenzymatic antioxidants such as NADPH and NADH pools, β-carotene, ascorbic acid, α-tocopherol, and glutathione (GSH). GSH, present at high concentrations, maintain a strong reducing environment in the cell, and its reduced form is maintained by glutathione reductase using NADPH as a source of reducing power. In addition, specific enzymes decrease the steady-state levels of reactive oxygen. Two superoxide dismutases (SOD), which convert O_2^- to H_2O_2 and O_2, have been described in *Escherichia coli*: an iron-containing enzyme, whose expression is modulated by intracellular iron levels [38], and a manganese-containing SOD, the predominant enzyme during aerobic growth, whose expression is transcriptionally regulated by at least six control systems [6]. A third SOD activity with properties like eukaryotic CuZn-SOD has been found in the *E. coli* periplasmic space [2]. In *E. coli*, H_2O_2 is removed by two catalases (yielding H_2O and O_2): hydroperoxidase I (HPI),which is present during aerobic growth and transcriptionally controlled at different levels [20], and hydroperoxidase II (HPII), which is induced during stationary phase [49]. Glutathione peroxidase and DT-diaphorase are also scavenging enzymes.

Secondary defenses include DNA-repair systems and proteolytic and lipolytic enzymes. DNA repair enzymes [reviewed in ref. 13] include endonuclease IV, which is induced by oxidative stress, and exonuclease III, which is induced in the stationary phase and in starving cells. Both enzymes act on duplex DNA cleaning up DNA 3' termini. Prokaryotic cells contain catalysts able to repair directly some covalent modifications to the primary structure of proteins. One of the most frequent modifications is the reduction of oxidized disulfide bonds: (i) thioredoxin reductase transfers electrons from NADPH to thioredoxin via a flavin carrier, (ii) glutaredoxin is also able to reduce disulfide bonds, but using GSH as an electron donor and, (iii) protein disulfide isomerase facilitates disulfide exchange reactions with large inactive protein substrates, besides having chaperone activity. Oxidation of methionine to methionine sulfoxide can be repaired by methionine sulfoxide reductase. Recent experimental data described that surface-exposed methionine residues surrounding the entrance to the active site are preferentially oxidized without loss of catalytic activity, and suggested that methionine residues could function as a “last-chance” antioxidant defense system for proteins [31].

Genetic responses

Genetic responses to oxidative stress occur in bacteria [extensively reviewed in ref. 19 and 26], yeast, mammalian cell lines and, in general, in all aerobic organisms. *E. coli* cells possess a specific defense against peroxides, mediated by the transcriptional activator OxyR, and another against superoxide, controlled by the two-stage SoxRS system. The SoxRS regulon contains at least ten genes, including those encoding the Mn-SOD, endonuclease IV, glucose-6-P DH, a fumarase, aconitase, ferredoxin reductase and micF RNA, which affects the expression of a major outer membrane protein. The *oxyR* gene controls, among others, the genes encoding the HPI catalase, glutaredoxin, glutathione reductase, NADPH-dependent alkyl hydroperoxide reductase, and a protective DNA-binding protein (Dps). The activation of these responses greatly increases cellular resistance to oxidative agents.

Both OxyR and SoxR are present, but inactivated in “unstressed” cells. It was proposed that the activation of SoxR protein could result from reversible one-electron oxidation of its iron-sulfur centers [27]. More recently, it was reported that OxyR is reversibly activated by the formation of an intramolecular disulfide bond, resulting from the altered redox state of the cytosol. OxyR activation is reversed by cellular disulfide-reducing machinery, with particular dependence on glutaredoxin. The gene encoding glutaredoxin is regulated by OxyR, thus providing a mechanism for autoregulation [1, 50]. Using the formation and reduction of a disulfide bond as an “on-off” switch allows for rapid response to oxidative conditions (Fig. 1).

The response against H_2O_2 does not finish with the activation of OxyR. *Oxy*R defective mutants are able to induce around 20–30 proteins in response to H_2O_2. These mutants are, however, hypersensitive to H_2O_2 and have higher mutation rates even

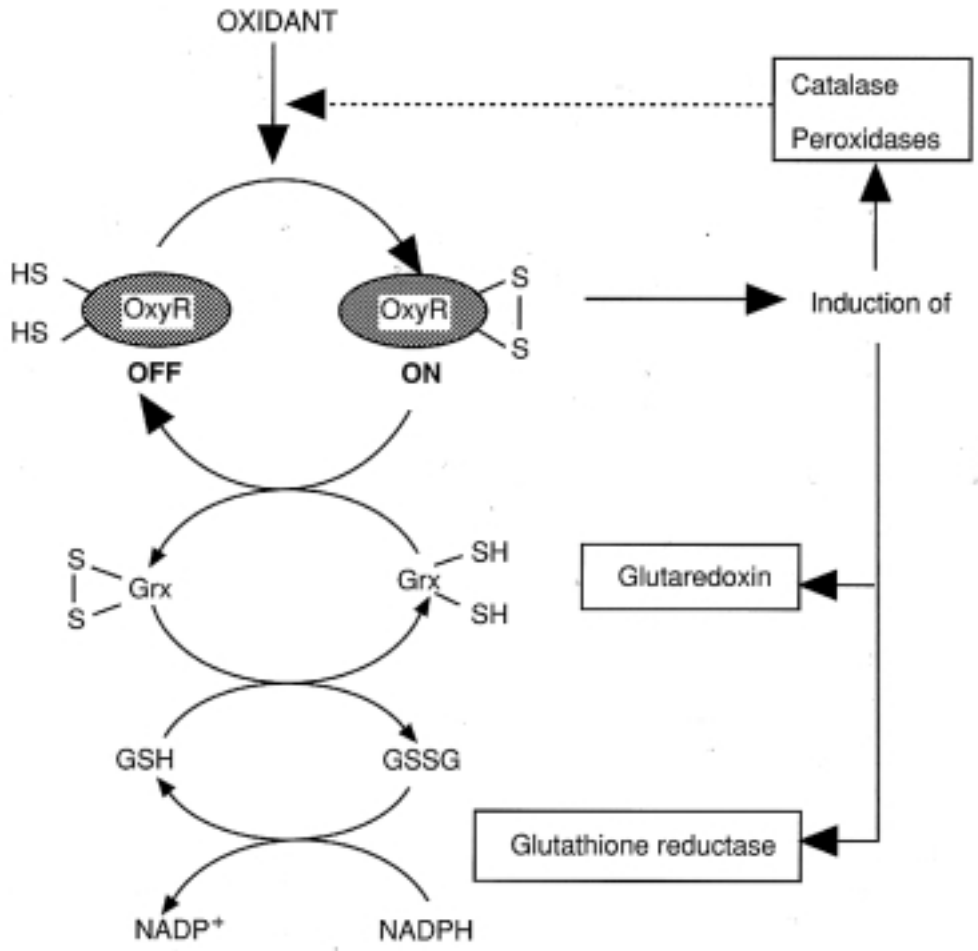

Fig. 1 Oxidative stress activates OxyR by formation of a disulfide bond. OxyR triggers the expression of reductive activities such as enzymes that degrade the oxidant and reduce disulfide bonds. Reduction of the disulfide bonds provides for negative feedback

during basal metabolism. All this suggested the presence of other unknown regulatory systems. Membrillo-Hernández et al. reported the first example of a gene, *hmp*, which encodes a soluble flavohemoglobin in *E. coli*, and is up-regulated by paraquat in a SoxRS-independent manner [37]. Induction of *hmp* by paraquat was demonstrated in aerobically grown cultures during exponential growth and in the stationary phase. The results obtained by Membrillo-Hernández et al. supports the role of σ^s (the global regulator of stationary phase gene expression, RpoS) in one of the regulatory mechanism. Mutations in *oxyR* or *rob*, known regulators of several stress promoters in *E. coli*, had no effect on the induction of *hmp* by paraquat.

Note that the magnitude of the H_2O_2 response depends not only on the magnitude and type of stimulus but also on the growth phase, the response being maximal at logarithmic phase and almost negligible during early stationary phase. Stationary phase cultures of *Bacillus subtilis* display complete viability after treatment with 10 mM H_2O_2, a concentration that reduces the viability of exponential phase cells to approximately 0.01% [15]. The same phenomenon has been observed in *E. coli*, where the response has been partly characterized and found to be quite separate from that occurring during exponential phase [16]. It has been suggested that starved cells might prepare in advance for the possibility of environmental stress, as their capacity for rapid response is severely compromised. Moreover, Dps (DNA protecting under stationary phase), which is induced during stationary phase (nutritional stress), and under oxidative stress, carries out two major functions: (i) it physically protects DNA from oxidative damage, and (ii) it maintains a low level of gene expression. The structure of Dps, which has been recently crystallized, has essentially the same fold as ferritin. This suggests that the mechanism for DNA protection is based on the sequestration of iron ions [24].

The genetic response allows microorganisms to better resist the damaging effects of a toxic agent when first preexposed to

low doses [7]. This is a widespread phenomenon that has been observed in prokaryotes, yeast, mammals, and plants. Among prokaryotes, *E. coli* [12] and *Salmonella typhimurium* [5] are capable of adapting to H_2O_2 in a similar manner. After 60 min treatment with 60 μM H_2O_2, *S. typhimurium* became resistant to killing by 10 mM H_2O_2 (80% viability) when compared with untreated cells (1% viability). *S. thyphimurium* became resistant also to other kinds of stresses, such as heat, *N*-ethylmaleimide, 1-chloro-2,4-dinitrobenzene and menadione, after pretreatment with non lethal levels of H_2O_2 [5]. The main physiologic benefit of adaptive response is clear: to protect cells and organisms from higher doses of a toxic agent. Such a protective response also indicates that the cell, once exposed to the toxin, expects, or at least is prepared for, a subsequent lethal dose.

Protein oxidation

Studies of free radical-mediated oxidation of proteins started at the beginning of the 20th century, when Dakin reported oxidation of amino acids by Fenton systems in 1906; in 1925, Hopkins described the role of glutathione as anti- and prooxidant, this latter activity depending on the presence of transition-metal ions [11]. At the cellular level, when proteins are exposed to reactive oxygen species, modifications of amino acid side chains occur and, consequently, the protein structure is altered. These modifications lead to functional changes that disturb cellular metabolism. The observed accumulation and damaging actions of oxidized proteins in several pathological states such as neurodegenerative diseases, diabetes and atherosclerosis, and during aging, highly increased the research in this field in the last decades of the 20th century [45].

Cytosolic proteins have evolved to maintain their cysteines reduced in the native form, whereas many secreted proteins have evolved to be more stable when their cysteines are joined in disulfide bonds. Thus, changes in the reducing environment of the cytosol can have profound effects on protein folding and activity. Although it has not been directly demonstrated, it seems likely that unwanted disulfide bonds are generated in the normal resident proteins of the cytosol during oxidative stress, a situation referred as "disulfide stress" [1]. This is a reversible oxidation mechanism, because bacterial cell response can restore the redox homeostasis of the cytosol and eliminate harmful oxidant by activation of the transcription factors SoxRS and OxyR.

Irreversible oxidation of amino acid residues in a protein can be exerted by two major mechanisms: ionizing radiation and metal ion-catalyzed oxidation reactions. The effects of ionizing radiation on proteins are attributable to the hydroxyl radicals formed by the radiolysis of water; this radical reacts with an α-hydrogen atom of an amino acid residue to form a carbon centered radical. In the presence of oxygen, an alkoxyl radical is obtained which leads to the peptide bond cleavage. This cleavage is assumed to occur by either the diamide or the α-amidation pathway [3]. If oxygen is absent, the carbon-centered radical may react with another one to form protein-protein cross-linked derivatives [46].

From the studies of Stadtman, Levine and collaborators, using glutamine synthetase from *E. coli* [18, 21, 32–34], it was clear that metal-catalyzed oxidation (MCO) of proteins is a mechanism of post-translational modification that binds a cation capable of redox cycling (such as Fe^{2+}/Fe^{3+}) to a metal-binding site on the protein. The reaction with molecular oxygen or H_2O_2 generates active oxygen species which oxidize amino acid residues at or near that cation-binding site. Evidence suggests that these products react in a site-specific manner, and the reaction is viewed as a "caged" process in which the active oxygen species is not released into the surrounding medium; it preferentially reacts with functional groups of amino acid residues at the metal-binding site. In this mechanism, it is assumed that the hydroxyl radical *(˙OH)* is the reactive oxygen species formed by the reaction of Fe^{2+} with H_2O_2 (Fenton reaction). However, other active forms of oxygen (ferryl ion, perferryl ion, peroxyradical) may also form [45].

Many proteins can be modified both in vivo and in vitro by any of the enzymatic or non-enzymatic MCO systems [4, 43–45]. The main protein modifications observed are: loss of catalytic activity, amino acid modifications (Table 1), carbonyl group formation, increase in acidity, decrease in thermal stability, change in viscosity, change in fluorescence, fragmentation, formation of protein-protein cross-links, formation of S–S bridges, and increased susceptibility to proteolysis.

Table 1 Amino acid residues of proteins that are oxidized and products formed [3, 45]

Amino acid	Oxidation products
Arginine	Glutamic semialdehyde
Cysteine	Disulfides, cysteic acid
Glutamyl	Oxalic acid, pyruvic acid
Histidine	2-Oxohistidine, asparagine, aspartic acid
Lysine	2-Aminoadipic semialdehyde
Methionine	Methionine sulfoxide, methionine sulfone
Phenylalanine	2,3-Dihydroxyphenylalanine, 2-, 3-, and 4-hydroxyphenylalanine
Proline	2-Pyrrolidone, 4- and 5-hydroxyproline pyroglutamic acid, glutamic semialdehyde
Threonine	2-Amino-3-ketobutyric acid
Tryptophan	2-, 4-, 5-, 6-, and 7-hydroxytryptophan, nitrotryptophan, kynurenine, 3-hydroxykynurenine, formylkynurenine
Tyrosine	3,4-Dihidroxyphenylalanine, Tyr-Tyr cross-linkages, Tyr-O-Tyr, cross-linked nitrotyrosine

With the finding that some amino acid residues (including lysine, arginine, proline and threonine) are oxidized to carbonyl derivatives [3], several methods to detect the carbonyl content of proteins were developed and used to measure protein damage [35]. However, since not all oxidative modifications lead to carbonyl derivatives, these are minimal values. Note that carbonyl groups may also be introduced into the protein by mechanisms that do not involve oxidation of amino acid residues. Thus, α, β-unsaturated alkenals such as 4-hydroxynonenal produced during the peroxidation of polyunsaturated fatty acids have been shown to react with the sulfhydryl groups of proteins to form stable covalent thiolether adducts carrying a carbonyl function [28, 47]. Carbonyl groups are also introduced into proteins making them react with carbonyl derivatives (ketoamines, ketoaldehydes, deoxyosones) generated as a consequence of the reaction of either reducing sugars or their oxidation products with lysine residues of proteins.

Gardner et al. described that a number of enzymes with active-site iron-sulfur clusters are highly sensitive to inactivation by O_2^-. One of these enzymes is *E. coli* aconitase [22]. The mechanism of aconitase inhibition by O_2^- involves the release of free iron from the enzyme, which exacerbates oxygen stress, and it has been proposed that superoxide's genotoxicity could be a function of its ability to liberate protein-bound iron [36].

It is now clear that oxidized proteins become better substrates for proteolytic digestion and that a proteolytic pathway could provide a valuable line of "secondary antioxidant defense". This proposal was made independently by Davies [8] and by Stadtman [43] in 1986. A strong correlation was demonstrated between increased hydrophobicity on the protein surface and the recognition and proteolytic degradation of oxidatively modified proteins; besides, other variables may also be significant. *E. coli* seems to have specific proteinases that selectively degrade oxidized proteins in an ATP-independent pathway [9, 10]. In eukaryotic cells, many intracellular proteins are degraded by the multicatalytic proteinase complex, also called proteasome, in a nonlysosomal pathway [39]; it has been suggested that protein oxidation could predispose to ubiquitination, which in turn would be a target for proteasomal degradation [29]. It seems that after a certain degree of oxidative damage, further damage causes a decrease in proteolytic susceptibility. Several studies [11, 25] revealed that heavily oxidized proteins, extensively cross-linked and aggregated, are not only poor substrates for degradation but can also inhibit proteases to degrade other oxidized proteins. The removal of damaged proteins is necessary to prevent their accumulation, which could compromise the correct metabolism of any cell exposed to oxidative stress. This phenomenon has been related with aging in higher organisms [3, 45].

Various shock or stress proteins, both the constitutive and the inducible forms, can act as chaperones for the reconstitution of the tertiary structure of proteins. The induction of various shock proteins also occurs during oxidative stress. Recently, the activation of chaperone Hsp33 by disulfide bond formation induced by oxidative stress has been described. This chaperone is important for protection against oxidative stress and may play a major role for keeping oxidized proteins soluble, allowing them to either be reduced and regenerated or be degraded by cellular proteases [30].

Although several mechanisms of protein oxidation are known in detail, most of the studies were done in in vitro systems. In vivo modifications of proteins due to oxidative stress have been much less studied, especially because of the difficulty to identify modified proteins in a whole extract. With this aim, a Western blot technique was developed [40], taking advantage that carbonyl groups generated react with dinitrophenylhydrazine (DNPH), and that the derivatized proteins can be separated by SDS-PAGE and then analyzed for carbonyl content by immunoassay with anti-DNP antibodies. This technique was employed to test the relative susceptibility of proteins to oxidative modification promoted when *E. coli* cells growing aerobically were exposed to H_2O_2 or menadione (a superoxide generator compound) [48]. Proteins involved in different cell processes such as glucose catabolism (enolase), chaperone function (DNA K), protein synthesis (EF-G), outer membrane proteins (OmpA), and the β-subunit of ATPase were identified as major targets. Among these proteins, EF-G and the β-subunit of ATPase were strongly oxidized when menadione was used. The inactivation of these two enzymes could be a crucial step to stop growth and consequently to minimize cell damage, the process having the same goal that the well known induction of GADD (growth arrest and DNA damage) genes under oxidative stress. From this point of view, oxidative modification and inactivation of some proteins could not only be the result of the inescapable price that cells pay to use oxygen, but they might have evolved in this way because that would represent the best option for the cell to overcome stress.

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