
Oxidative Processes and Antioxidative Metaloenzymes

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1. Introduction

Oxidative processes are necessary for life. They provide the energy necessary for many cellular functions. Most chemical energy in the body exists as ATP, produced during aerobic respiration. Nutrient oxidation is carried out by reduced coenzymes in the mitochondria, which are oxidized in the respiratory chain. The electrons are transferred to the oxygen created proton gradient that allows for ATP generation. One-electron transmission leads to the formation of reactive oxygen species (ROS). A series of oxidation processes take place in the peroxisomes. Hydrogen peroxide arises as a by-product of the oxidation of very long, long and branched-chain fatty acids, amino acids, synthesis and deamination of biologically active molecules (hormones, neurotransmitters, etc.), and biotransformation of xenobiotics. Oxidation reactions are also used for the degradation of unneeded molecules to excrement form, e.g. purines to form uric acid by xantine oxidase reaction, where hydrogen peroxide is also produced. The body also makes use of ROS also against the invasion of microorganisms, as neutrophils produce hypochloric acid from superoxide radicals via NADPH oxidase. ROS and reactive nitrogen species (RNS) may also be of exogenous origin. They can be taken up through diet or ventilation, or sometimes due to ionising radiation. Given that highly reactive substances could damage the cells and the whole organism, they must be inactivated by an antioxidative defence system. Metaloenzymes, which contain transition metals, and other antioxidant enzymes have an important role in this stage. Various endogenous substances, which are necessary for enzyme activity, form part of the antioxidant defence. Reduced glutathione is the most important of these endogenous substances and is functional both as a cofactor of other enzymes and for its reducing effects on oxidized molecules. NADPH is needed for the reduction of glutathione. The whole system is often referred as the glutathione defence system. The antioxidant defence system is very complicated. It is influenced by a number of other factors and circumstances, by both synthesis of endogenous and by intake of exogenous anti- or pro-oxidant substances. It also

includes the receipt, transport and binding of metals into organic compounds of the organism; not just those that are part of the redox phenomena but also toxic elements, e.g. those which show a high affinity for sulphur, such as Hg, Cd, Sb, As, and other. These can affect the whole defence system, but may also be disposed to integration into the metallothioneins, peptides with high content of cysteine (approximately 1/3 of amino acids).

It is necessary to realize that the whole system is inducible. The aim of this is to describe the important aspects of this system that are mediated via metalloproteins.

2. Sources of reactive oxygen species and free radicals in an organism

All aerobic organisms produce reactive oxygen species physiologically. The five most productive pathways are involved in regulating the production of ROS/RNS and the resulting effects on signalling cascades. The five mechanisms described produce ROS in a non-regulated mode. However, there are many sources within the cells that are only mentioned.

2.1. Regulated production of reactive oxygen and nitrogen species

2.1.1. Nitric oxide synthase (NOS)

Nitric oxide (NO[•]) is produced from a guanidine nitrogen of L-arginine via electron transfer from NADPH in two successive steps. The enzyme responsible for this exists in three isoforms: neuronal (nNOS, type I, NOS-I or NOS-1), endothelial (eNOS, type III, NOS-III or NOS-3) and inducible (iNOS, type II, NOS-II or NOS-2). nNOS and eNOS are constitutively expressed, but their activity is regulated by the intracellular Ca²⁺ concentration. nNOS exhibits NADPH-diaphorase (NADPH-d) activity. The NOS isoforms are homodimeric, bi-domain enzymes. Each monomer consists of a flavin-containing reductase domain linked to a heme-containing oxygenase domain by a calmodulin-binding sequence. Although it possesses very little structural resemblance to P450, the oxygenase domain of NOS is referred to as being "P450-like" due to the presence of iron protoporphyrin IX (heme), linked axially by a cysteine residue to the NOS protein, which carries out "P450-like" mono-oxygenation reactions [130]. The isoform iNOS is inducibly expressed in macrophages after stimulation by cytokines, lipopolysaccharides, and other immunologically relevant agents [21]. Expression of iNOS is regulated at the transcriptional and post-transcriptional level by signalling pathways that involve agents such as the redox-responsive transcription factor NF-κB or mitogen-activated protein kinases (MAPKs) [120]. NO[•] is a reactive and unstable free radical gas that can cross cell membranes easily by diffusion independent of any release or uptake mechanism [86].

The rate of NO[•] synthesis is affected to some extent by the availability of the substrate L-arginine and by the cofactor tetrahydrobiopterin (BH₄). The physiological function of NO[•] varies widely due to the diverse localization of isoforms within different cell populations of the body. In physiological concentrations, NO[•] functions as an intracellular messenger [88]. In pathophysiological situations where iNOS is upregulated, the most common RNS

generated are dinitrogen trioxide (N₂O₃) and peroxynitrite (ONOO⁻), both of which are able to induce nitrosative and oxidative stress [194]. Upon NOS activation in many inflammatory diseases nitrite (NO₂⁻), the major oxidation product from NO, is produced. In activated neutrophils, this can be oxidized by the effect of myeloperoxidase (MPO) to form either nitryl ion (NO₂⁺) or nitrogen dioxide (NO₂^{*}) [30].

2.1.2. NADPH oxidase

2.1.2.1. NADPH oxidase in phagocytic cells

Activated neutrophils and macrophages produce superoxide and its derivatives as cytotoxic agents forming part of the respiratory burst via the action of membrane bound NADPH oxidase on molecular oxygen. It is a heme-containing protein complex. Hydrogen peroxide (H₂O₂) is produced by activated macrophages in an inflammatory environment, at an estimated rate of 2-6 × 10⁻¹⁴ mol.l⁻¹.cell⁻¹ and may reach a concentration of 10-100 μM in the vicinity of these cells [46,106]. This multicomponent enzyme catalyzes the one-electron reduction of O₂ to superoxide (O₂⁻), using NADPH as the electron donor through the transmembrane protein cytochrome *b*₅₅₈. The transfer of electrons occurs from NADPH on the inner side of the plasma membrane to O₂ on the outer side. During phagocytosis, the plasma membrane is internalized as the wall of the phagocytic vesicle, with what was once the outer membrane surface now facing the interior of the vesicle. This targets the delivery of O₂⁻ and its reactive metabolites internally for localized microbicidal activity [11].

The massive production of antimicrobial and tumoricidal ROS in an inflammatory environment is called the “oxidative burst” and plays an important role as the first line of defence against environmental pathogens. The combined activities of NADPH oxidase and myeloperoxidase (MPO) in phagocytes leads to the production of hypochlorous acid (HClO), one of the strongest physiological oxidants and a powerful antimicrobial agent [76]. MPO is a heterodimeric, cationic and glycosylated heme enzyme. The enzyme is a 140-kDa dimer of identical halves, each consisting of two polypeptide chains of 108 and 466 amino acids. Each half contains a covalently attached heme [7]. Like the other heme peroxidases, MPO combines with hydrogen peroxide and in the presence of halide (chloride, bromide, or iodide) to form the highly reactive redox intermediate in the phagosomes of neutrophils.



The oxidation of iron-sulfur centres in micro-organisms by the myeloperoxidase-H₂O₂-halide system may contribute to the death of an organism. MPO also catalyzes the oxidation of tyrosine in organisms to form the toxic amino acid residue, tyrosyl radical (Tyr^{*}), involved in the activity of neutrophils. Activated neutrophils and macrophages also generate singlet oxygen (¹O₂) by reactions that involve either MPO or NADPH oxidase [23,46]. Neutrophils also produce RNS, which can react with superoxide (O₂⁻) to produce ONOO⁻, itself a powerful oxidant, which may decompose to form a hydroxyl radical (^{*}OH). The activation of phagocytic NADPH oxidase can be induced by microbial products such as

bacterial lipopolysaccharide, by lipoproteins, or by the cytokines interferon- γ , interleukin-1 β , and interleukin-8 [23]. Enzyme activation is mainly controlled by rac2 in neutrophils and rac1 in macrophages and monocytes [46,112].

2.1.2.2. NADPH oxidase in nonphagocytic cells

Fibroblasts, endothelial cells, vascular smooth muscle cells, cardiac monocytes and thyroid tissue nonphagocytic NAD(P)H oxidase (similar but not identical to phagocytic NADPH oxidase) produce $O_2^{\bullet-}$ and to regulate intracellular signalling cascades [66,208]. In most of these, rac1 is involved in the induction of NAD(P)H oxidase activity [91,210]. Muscle cells and fibroblasts account for the majority of $O_2^{\bullet-}$ produced in the normal vessel wall. The NAD(P)H oxidase isoforms of the cardiovascular system are membrane-associated enzymes that appear to utilize both NADH and NADPH [66]. The rate of $O_2^{\bullet-}$ production in nonphagocytic cells is only about one-third that of neutrophils. $O_2^{\bullet-}$ and H_2O_2 are mainly produced intracellularly in vascular smooth muscle cells, in contrast to neutrophils, endothelial cells, and fibroblasts. The cardiovascular NAD(P)H oxidase isoforms are induced by hormones, hemodynamic forces, or by local metabolic changes [66]. Mechanical forces stimulate NAD(P)H oxidase activity in endothelial cells and reoxygenation in cardiac myocytes. An NAD(P)H oxidase with low affinity for oxygen and high affinity for cyanide is believed to act as one of the sensors for oxygen tension in the carotid body, controlling the rate of ventilation [2]. The function of oxygen sensing is apparently shared by several proteins, including a nonmitochondrial cytochrome *b₅₅₈*, a mitochondrial protein, and possibly a third heme protein [105,209].

A similar group of proteins was suggested to be involved as oxygen sensors in the regulation of erythropoietin production in human hepatoma cells [209]. A microsomal NADH oxidase was implicated as an oxygen sensor in bovine pulmonary and coronary arteries, where changes in oxygen tension regulate vascular relaxation through changes in $O_2^{\bullet-}$ production and cGMP formation [198]. Increased aortic adventitial $O_2^{\bullet-}$ production contributes to hypertension by blocking the vasodilatory effects of NO^{\bullet} [189]. There is a strong possibility that rac-like proteins also occur in plants [1,193], where they may be involved in the induction of NAD(P)H oxidase-like enzymes [167]. The oxidative burst in plants is an effective bactericidal mechanism.

2.1.3. Arachidonate cascade enzymes

2.1.3.1. 5-lipoxygenase (5-LOX)

The enzyme 5-LOX has been identified as an inducible source of ROS production in lymphocytes [23,118,126], but the evidence for its physiological role in redox signalling is still scarce. There are several lipoxygenases which differ by substrate specificity and optimum reaction conditions. Lipoxygenases in plants and animals are heme containing dioxygenases that oxidize polyunsaturated fatty acids at specific carbon sites to give enantiomers of hydroperoxide derivatives with conjugated double bonds. The number in specific enzyme names such as 5-LOX, 12-LOX, or 15-LOX refers to the arachidonic acid site

that is predominantly oxidized [202]. 5-LOX is best known for its role in biosynthesis of the leukotrienes A₄, B₄, C₄, D₄ and E₄. The oxidized metabolites generated by 5-LOX were found to change the intracellular redox balance and to induce signal transduction pathways and gene expression. 5-LOX was shown to be involved in the production of H₂O₂ by T lymphocytes after ligation of the CD28 costimulatory receptor [118] and in response to interleukin-1 β [23]. A lipid metabolizing enzyme in fibroblasts similar to 15-LOX has been shown to generate large amounts of extracellular O₂⁻ [168].

2.1.3.2. Cyclooxygenase (COX-1)

Cyclooxygenase-1 has been implicated in ROS production through formation of endoperoxides, which are susceptible to scavenging by some antioxidants in cells stimulated with TNF- α , interleukin-1, bacterial lipopolysaccharide, or the tumor promoter 4-O-tetradecanoylphorbol-13-acetate [48]. Cyclooxygenase participation in redox signalling remains scarce.

2.2. Non-regulated production of reactive oxygen species

2.2.1. Mitochondrial respiration

The four-electron reduction of oxygen occurs within the mitochondrial electron transport system of all cells undergoing aerobic respiration. It is estimated that 2-3% of O₂ consumed by mitochondria is incompletely reduced, yielding ROS [173] and 1-5% leads to H₂O₂ production [134]. It is well documented that mitochondria are a source of H₂O₂; however, the release of O₂⁻ from mitochondria into the cytosol has yet to be definitively established [77]. ROS are only produced at complexes I and II in the mitochondrial matrix, while complex III is capable of producing ROS on both sides of the mitochondrial inner membrane [135,173]. It is generally thought that the two major sites of mitochondrial ROS production are complexes I and III. NADH-ubiquinone oxidoreductase (complex I) is composed of ~45 subunits and is the site of NADH oxidation. The flavin mononucleotide (FMN) of complex I accepts the electrons from NADH and passes them through a series of eight iron-sulfur clusters to ubiquinone [84] to generate O₂⁻ in the presence of NADH. Complex I also generates ROS after the oxidation of succinate at complex II via a process referred to as reverse electron transport (RET). It is also hypothesized that ROS production from complex I during RET occurs from FMN as well [103,122]. Ubiquinol:cytochrome *c* oxidoreductase (complex III) has 11 subunits and contains 3 hemes and an Fe-S cluster center. Complex III plays an intricate role in passaging electrons from the ubiquinol generated by complexes I and II to cytochrome *c* [116]. Upon binding with the Q_o site, one electron from ubiquinol is transferred through the Rieske Fe-S cluster protein to the electron acceptor, cytochrome *c*. The resulting unstable semiquinone then donates the remaining electron to the heme groups on cytochrome *b*. The electron in cytochrome *b* is then used to re-reduce ubiquinone at the Q_i site to produce ubiquinol. Two electrons from semiquinones in Q_o are required for the reduction of ubiquinone to ubiquinol in the Q_i site. This process is referred to as the Q-cycle because lone electrons remaining in semiquinone are reused to reduce ubiquinone back to ubiquinol [35].

The mechanism of mitochondrial production and release of H_2O_2 and $O_2^{\bullet-}$ takes place in two steps. Firstly, part of $O_2^{\bullet-}$ generated during mitochondrial electron transfer is vectorially released into the intermembrane space [78]. The mechanism underlying the release of $O_2^{\bullet-}$ into the intermembrane space covers the formation of ubisemiquinone at two sites in the ubiquinone pool: the Q_1 site that lies near the matrix, and the Q_o site in the vicinity of the intermembrane space [154]. Autooxidation of ubisemiquinone at the Q_o site ($UQ_o^{\bullet-}$) results in the release of $O_2^{\bullet-}$ through the cytosolic side of the mitochondrial inner membrane. $O_2^{\bullet-}$ cannot cross membranes, except in the protonated form, which represents only a small fraction of the $O_2^{\bullet-}$ pool at physiological pH. Taken together, H_2O_2 is formed both at the intermembrane space and the matrix from $O_2^{\bullet-}$ generated towards the respective compartments [77]. Second, the release of $O_2^{\bullet-}$ into the intermembrane space would be in a functional relationship to the localization of a superoxide dismutase (SOD) activity in this compartment. The intermembrane space contains several $O_2^{\bullet-}$ scavenging pathways besides SOD, such as cytochrome *c* [179] as well as pores for $O_2^{\bullet-}$ diffusion across the outer membrane into cytosol, in particular the voltage-dependent anion channel [77].

$O_2^{\bullet-}$ released into the cytoplasm from mitochondria could play an important role in cell signalling, as $O_2^{\bullet-}$ has been implicated in several signalling events. In addition, cytoplasmic aconitase and other cytoplasmic enzymes susceptible to $O_2^{\bullet-}$ may be targets of $O_2^{\bullet-}$ released from mitochondria [61]. Another important decay pathway of $O_2^{\bullet-}$ at a diffusion-controlled rate may involve the reaction with NO^{\bullet} to yield $ONOO^{\bullet-}$ in the intermembrane compartment. This may be of some significance, as nitrosation of cytochrome *c* and proapoptotic caspases occurs prior to apoptosis [123].

2.2.2. Chloroplasts

The ability of phototrophs to convert light into biological energy is critical for life and therefore organisms capable of photosynthesis are especially at risk of oxidative damage, due to their bioenergetic lifestyle and the abundance of photosensitizers and oxidable polyunsaturated fatty acids in the chloroplast envelope. The presence of O_2 in the atmosphere enables respiratory metabolism and efficient energy generation systems which use O_2 as final electron acceptor, leading to the formation of ROS in cells [166]. The presence of ROS producing centres such as triplet chlorophyll, and ETC in PS I and PS II make chloroplasts a major site of ROS production in plants and algae [145]. Atmospheric oxygen is relatively non-reactive. It has been estimated that 1-2 % of O_2 consumed by plants is sidetracked to produce ROS in various subcellular loci [19].

Oxygen generated in chloroplasts during photosynthesis can accept electrons passing through the photosystems (PS). PS II is a multisubunit protein complex also present in cyanobacteria that use light energy for oxidation of water and reduction of plastoquinone [146]. Various abiotic stresses such as excess light, drought, salt stress and CO_2 limiting conditions, enhance the production of ROS. Under normal conditions, the electron flow from the excited PS centres is directed to $NADP^+$, which is reduced to NADPH. It then enters the Calvin cycle and reduces the final electron acceptor, CO_2 . In cases of ETC-

overloading, a part of the electron flow is diverted from ferredoxin to O_2 , reducing it to $O_2^{\bullet-}$ via the Mehler reaction. The acceptor side of ETC in PS II also provides sides (QA, QB) with electron leakage to O_2 producing $O_2^{\bullet-}$. On the external "stromal" membrane surface, $O_2^{\bullet-}$ is enzymatically dismutated to H_2O_2 [50,65,163]. 1O_2 is a natural byproduct of photosynthesis, mainly formed at PS II even under low-light conditions [29]. Generation takes place due to the excitation energy transfer from triplet chlorophyll formed by the intersystem crossing from singlet chlorophyll and the charge recombination of separated charges in the PS II antenna complex and reaction center of PS II [146]. In cases of insufficient energy dissipation, the chlorophyll triplet state becomes able to react with 3O_2 to give up 1O_2 [79].

2.2.3. Xanthine oxidoreductase (XOR)

XOR exists as either an oxidase (XO) which transfers reducing equivalents to oxygen, or as a dehydrogenase (XDH) that utilizes NAD or oxygen as the final electron acceptor [17,59]. The enzyme is derived from xanthine dehydrogenase by proteolytic cleavage. It contains molybdenum in the form of molybdopterin, and two clusters with iron and sulfur compounds of FAD cofactor in both subunits. The enzyme catalyzes the production of uric acid with co-production of $O_2^{\bullet-}$. The physiological substrates, xanthine and hypoxanthine, bind with the oxidized enzyme and donate two electrons into the molybdenum cofactor reducing it from Mo^{6+} to Mo^{4+} . Substrates are hydroxylated by H_2O at the molybdenum site as the electrons travel via two iron-sulfide residues to flavine-adenine dinucleotide (FAD). Reduced FAD can be divalently reoxidized by oxygen to produce hydrogen peroxide, or univalently reoxidized in two steps to generate two equivalents of superoxide $O_2^{\bullet-}$ [17,82]. Under normal conditions, XOR accounts for only a minor proportion of total ROS production [46]. The release of $O_2^{\bullet-}$ results in the recruitment and activation of neutrophils and their adherence to endothelial cells, stimulating formation of XOR in the endothelium with further $O_2^{\bullet-}$ production. Therefore, it has been observed in TNF-treated endothelial cells [58] and has been implicated as a major source of oxidative stress under ischemia and reperfusion [46].

2.2.4. Dopamine (DA)

As a neurotransmitter, DA is stable in the synaptic vesicle. When an excess of cytosolic DA exists outside of the synaptic vesicle, DA is easily metabolized via monoamine oxidase (MAO) or by autooxidation to produce ROS, subsequently leading to the formation of neuromelanin [162]. During the oxidation of DA by MAO, H_2O_2 and dihydroxyphenylacetic acid are generated [67]. Spontaneously oxidized cytosolic DA produces $O_2^{\bullet-}$ and reactive quinones such as DA quinones or DOPA quinones. DA quinones are also generated in the enzymatic oxidation of DA by COX in the form of prostaglandin H synthase, LOX, tyrosinase and XOR. These quinones are easily oxidized to the cyclized aminochromes: DA-chrome and DOPA-chrome, and are then finally polymerized to form melanin, as reviewed in Miyazaki & Asanuma [132]. Although ROS from the autooxidation of DA show widespread toxicity not only in DA neurons but also in other regions, highly reactive DA quinone or DOPA quinone exert cytotoxicity predominantly in DA neurons and

surrounding neural cells. It is thought that DA acts as an endogenous neurotoxin, contributing to the pathology of neurodegenerative disorders and ischemia-induced damage in the striatum [24,124,201].

2.2.5. Photosensitization reactions

Photosensitization reactions involve the oxidation of organic compounds by atmospheric oxygen upon exposure to visible light. The photoexcited state, most often the triplet state of the sensitizer, is the key photoreactive intermediate and exerts photodamage through direct reaction with substrate molecules (type I photosensitization) or activation of molecular oxygen by energy transfer reactions (type II photosensitization) [199]. $^1\text{O}_2$ is an excited state molecule formed by direct energy transfer between the excited sensitizer and ground state $^3\text{O}_2$. Less than 1% of triplet oxygen is converted in parallel to superoxide anion (O_2^-). The formation of O_2^- as a precursor of H_2O_2 occurs via electron transfer via production of a sensitizer radical cation, or after an intermediate reduction of the sensitizer with a substrate followed by the single electron reduction of O_2 [38,99].

2.3. Other cellular ROS sources

The most studied producers of O_2^- by oxidizing unsaturated fatty acids and xenobiotics are cytochrome P450 and the b_5 family of enzymes [168]. Electrons leaking from nuclear membrane cytochrome oxidases and electron transport systems may give rise to ROS [75]. In addition to intracellular membrane-associated oxidases, aldehyde oxidase, dihydroorotate dehydrogenase, flavoprotein dehydrogenase and tryptofan dioxygenase can all generate ROS during catalytic cycling. pH-dependent cell wall peroxidases, germin-like oxalate oxidases and amine oxidases have been proposed as a source of H_2O_2 in the apoplast of plant cells [22]. Glycolate oxidase, D-amino acid oxidase, urate oxidase, flavin oxidase, L- α -hydroxy acid oxidase, and fatty acyl-CoA oxidase are important sources of total cellular H_2O_2 production in peroxisomes [168]. Auto-oxidation of small molecules such as epinephrine, flavins, and hydroquinones can also be an important source of intracellular ROS production [57].

3. Chemistry of reactive oxygen and nitrogen species

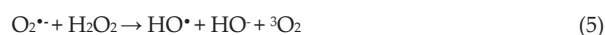
During plant photosynthesis and in analogous reactions of the respiratory chain, triplet oxygen is reduced to water (reaction 3). As a result of one-, two- and three- electron reduction, toxic forms of oxygen, free radicals and covalent compounds are produced as side products and oxidize additional biomolecules [181].



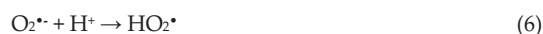
$^1\text{O}_2$ is the first excited electronic state of O_2 , and is an unusual ROS, as it is not related to electron transfer to O_2 . It is formed in photosensory reactions and is effectively quenched by β -carotene, tocoferols, plastoquinones and vitamin C. If not, $^1\text{O}_2$ can lead to gene upregulation, involved in the molecular defence responses against photooxidative stress

[102]. The lifetime of $^1\text{O}_2$ in a cell has been measured to be approximately 3 μs [79] and in this time, a fraction of $^1\text{O}_2$ may be able to diffuse over considerable distances of several hundred nanometers. Other studies have also found that $^1\text{O}_2$ can last for nearly 4 μs in H_2O and 100 μs in polar solvent [102].

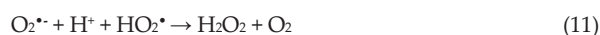
The monovalent reduction of molecular oxygen, the one-electron reduction of $^3\text{O}_2$ catalyzed by NADPH oxidases, gives rise to $\text{O}_2^{\bullet-}$ (reaction 4). $\text{O}_2^{\bullet-}$ has an approximate half-life of 2-4 μs and undergoes fast, non-enzymatic, one-electron reduction or dismutation in the Haber-Weiss reaction (reaction 5).



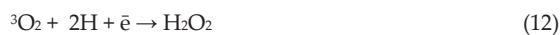
It has been noted that $\text{O}_2^{\bullet-}$ can undergo protonation to give up a strong oxidizing agent HO_2^{\bullet} (reaction 6) which directly attacks the polyunsaturated fatty acids (PUFAs) in negatively charged membrane surfaces [65]. The hydrogen donor for the reduction of PUFAs may well be ascorbic acid, forming H_2O_2 and a radical of ascorbic acid. Enzymatic dismutation to H_2O_2 is the most effective quenching mechanism (reactions 7, 8).



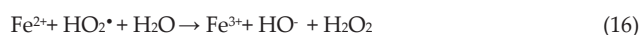
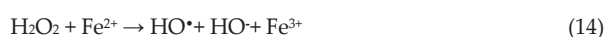
The interaction of O_2 with trace concentrations of redox-active transition metals leads to $\text{O}_2^{\bullet-}$ production (reaction 9) and the non-enzymatic reduction of $\text{O}_2^{\bullet-}$ in the presence of Fe forms $^3\text{O}_2$ (reaction 10). At low pH, dismutation of $\text{O}_2^{\bullet-}$ is unavoidable, with one $\text{O}_2^{\bullet-}$ giving up its added electron to another $\text{O}_2^{\bullet-}$, generating H_2O_2 following protonation (reaction 11) [181].



H_2O_2 is produced by the two-electron reduction of $^3\text{O}_2$ (reaction 12) and the univalent reduction of $\text{O}_2^{\bullet-}$. H_2O_2 is moderately reactive and has a relatively long half-life (1 ms) [19]. It is broken down partially enzymatically by catalase or glutathione peroxidase to water or in case of substrate peroxides to corresponding alcohols and water. In cases where the speed of its decomposition is not sufficient, it may lead to its one-electron reduction (reaction 13).



The reaction takes place similarly to the Haber-Weiss reaction in the presence of transition metals (Fenton reaction), producing the very reactive HO• and HO• (reaction 14) [181]. Instead of O₂^{•-}, HO₂[•] may arise, which is actually the H₂O₂ radical (reactions 15, 16). Common mechanisms involving the Fenton reaction, generation of the O₂^{•-} and HO• appear to be involved for Fe, Cu, Cr, V, Co primarily associated with mitochondria, microsomes and peroxisomes. However, a recent discovery, that the upper limit of free pools of Cu is far less than a single atom per cell casts serious doubt on the role of Cu in Fenton-like generation of free radicals [178].



HO• is also generated by the three-electron reduction of ³O₂ (reaction 17). It predominantly attacks the unsaturated fatty acids of membranes. The most effective protective mechanisms include reduction of HO₂[•] by tocopherols, taking the form of tocopherol radicals, for which the retroactive reduction of the reaction requires the oxidation of ascorbic acid. Resulting hydroperoxides (R-O-OH) are released by phospholipase A₂, which makes them available substrates for peroxidases. In the presence of suitable transition metals, especially Fe, HO• can also be produced from O₂^{•-} and H₂O₂ at neutral pH and ambient temperatures by the iron-catalyzed Fenton reaction [187].



NO• is generated by specific NOSs, which metabolise arginine to citrulline via a five electron oxidative reaction [63]. NO• reacts with O₂^{•-} (reaction 18) in a reaction with the highest rate constants known (7.0 × 10⁹ m⁻¹.s⁻¹) [32]. ONOO• can be transformed into peroxynitrite acid and then to HO• (reaction 19). NO• binds certain transition metal ions; in fact, many effects of NO• are exerted as a result of its initial binding to Fe²⁺ heme groups. The most commonly seen product of such a reaction is [Fe³⁺ NO] [177].



4. Formation of radicals in biological systems and consequences of oxidation of biological molecules

4.1. Oxidation of lipids

This is considered to be the most damaging process known to occur in living organisms [62]. It includes a number of reactions leading to the development of oxidized lipids and fatty acids that give rise to free radicals. Oxidation products of lipids, particularly (2E)-4-hydroxyalk-2-enals and aldehydes such as malondialdehyde, as well as alkanes, lipid

epoxides and alcohols, react with proteins and nucleic acids. The overall effects of lipid oxidation are a decrease in membrane fluidity, an increase in the leakiness of the membrane to substances that do not normally cross it except through specific channels and damage to membrane proteins, and inactivation of receptors, enzymes, and ion channels.

4.1.1. Oxidation by $^3\text{O}_2$

The most common oxidation of fatty acids is by $^3\text{O}_2$ from the air. Oxidation of unsaturated fatty acids only occurs in three stages at normal temperatures. In the initiation stage, free hydrogen H^\bullet and fatty acid R^\bullet emerge as the C-H covalent bond of the hydrocarbon chain is split. The energy required to split bonds can come from ultraviolet radiation, radioactivity, and also visible light. In the latter case, it is a two-electron oxidation of $^1\text{O}_2$. A reaction also exists to break any binding with other free radicals or transition metals. During the second, propagation stage the reactive R^\bullet quickly merges with O_2 , and produces a peroxy radical (R-O-O^\bullet). As the hydrogen atom splits from the hydrocarbon chain, another molecule of unsaturated fatty acid forms hydroperoxide (R-O-OH) and another R^\bullet (reaction 20). The initiation rate of oxidation for the production of R-O-OH is slow (induction period) leading to a gradual accumulation of R-O-OH , followed by the creation of other radicals. As long as there is enough oxygen, the reaction takes place spontaneously, sharply rising to reach the maximum speed of reaction, in which reactive groups are diminished. The rate of this reaction then slows and starts to be overtaken by the degradation of R-O-OH . R-O-OH is very fragile and H^\bullet splits from the molecule, leaving R-O-O^\bullet (reactions 21, 22) or HO^\bullet . According to the current knowledge, R-O-OH degradation with conjugated double bonds leads preferentially to formation of the alkoxy radical (R-O^\bullet) (reaction 23) [36].

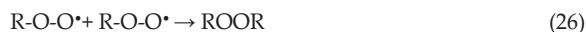


The reaction of R^\bullet with O_2 is much faster than with a hydrocarbon lipid chain. When the concentration of free radicals is high, it is likely that these will react together to form a nonradical product, which terminates the chain reaction. While R^\bullet is prevalent in the reaction system, hydrocarbon radical recombination is the major termination reaction. If, however, there is a preponderance of R-O-O^\bullet , the termination reaction leads either to recombination of R^\bullet with R-O-O^\bullet forming peroxide bridged dimers (reaction 24) or to the reciprocal recombination of R-O-O^\bullet (reaction 25). In the case of unsaturated fatty acids, H^\bullet splits from the methylene group near the double bond producing mainly R-O-OH . This reaction becomes easier as the number of double bonds increases. However, if the number of double bonds is unchanged, the double bond moves one carbon closer to the carboxyl or methylene end of the chain. By moving the double bonds, a double bond in the *cis* configuration is changed to more stable *trans* configuration.



4.1.2. Oxidation by R-O-OH

R-O-OH of fatty acids and their radicals may react in three ways. In the first case, there is no change in the number of carbon atoms in the molecule. R-O-OH species from polyunsaturated fatty acids (PUFAs) containing three or more double bonds in a molecule are unstable, and they tend to pass in 1,4 cyclization to the six-member peroxides derived from 1,2-dioxanes, which are also unstable compounds and decompose to low molecular active products. R-O-OH molecules by 1,3 cyclisation pass to five-member peroxides, 1,2-dioxolanes and endoperoxides. The main malondialdehyde precursors emerge from 1,2 dioxolane-type peroxohydroperoxides. R-O-OH and R-O-O \cdot , react very easily with the double bond of unsaturated fatty acids to generate epoxides. The addition of R-O-O \cdot across a double bond can take place intermolecularly. R-O-OH is oxidized by the nonradical mechanism and the resulting epoxide is immediately hydrolyzed to dihydroxyderivatives. Epoxides can arise even with the addition of R-O \cdot to PUFA by intermolecular reactions. An accrued radical of epoxy acid reacts with oxygen to give HO $_2\cdot$, from which R-O-OH is formed and subsequently, R-O \cdot . By the recombination of R-O \cdot with H \cdot , a competent hydroxyl acid or oxo acid arises by elimination of H $^+$. In the second case, the molecule breaks and gives volatile and sensory active substances with less carbon atoms. Breaking the molecule takes place both due to the R-O \cdot created (reaction 26) and depending on the position of the double bond in relation to the hydroperoxide group. From this, saturated and unsaturated aldehydes, saturated and unsaturated hydrocarbons, and oxo acids are formed. The most reactive compounds formed are aldehydes, which are further oxidized and react with the proteins. Malondialdehyde is an important product of this oxidation [125]. The third mechanism is oxypolymerization, in which the number of carbons in the molecule is increased due to the reduction of two radicals. Concerning R-O \cdot , radicals are condensed by a -C-C- bond, which is not frequent, because R-O \cdot is less available. Therefore, the majority of radicals combine through ether-like -C-O-C- or peroxide-like -C-O-O-C- bonds.



4.1.3. Oxidation by 1O_2

Excitation of the common 3O_2 leads to a reactive 1O_2 which may react with the double bond of unsaturated lipids and other unsaturated compounds. It reacts with the listed compounds because they are rich in electrons and are therefore able to fill its free molecular orbital [158]. The rate of reaction between common unsaturated acids and 1O_2 is at least 1450-fold higher in comparison to the reaction with triplet oxygen. It has been found that the PUFAs (linoleic acid 18:2 and linolenic acid 18:3) are particularly susceptible to attack from 1O_2 and HO \cdot [134]. Unstable cyclic peroxide compounds moloxides with four or six-member rings are

formed by adduction across double bonds. Intermediate products of the reaction decompose rapidly and give rise to respective hydroperoxides.

By the reaction with an atom in methylene groups on the carboxyl end of fatty acids, R-O-OH arises in a similar process to peroxide oxidation by $^3\text{O}_2$. However, the mechanism of primary production of hydroperoxides differs from the mechanisms of $^3\text{O}_2$ oxidation, therefore producing a different ratio of constitutional isomers.

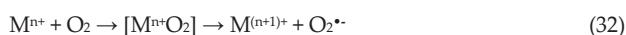
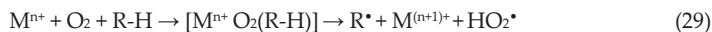
4.1.4. Oxidation catalyzed by metals

This type of oxidation is catalyzed by compounds of transition metals, especially Fe and Cu, which are present in tissues that are reduced by accepting an electron. They are involved directly or indirectly in initiation, propagation and termination reactions of radicals [181].

Metals in their higher oxidation state $\text{M}^{(n+1)+}$ are responsible for initiating oxidation reactions. The electron transfer in the reactions leads to the formation of R^\bullet (reaction 27)

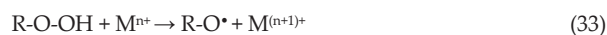


The initial reaction is also indirectly catalyzed by metals in the lower oxidation state M^{n+} , producing a transient complex with the metal, oxygen and R-H before decaying to R^\bullet , metals with higher oxidation state and ROS (reactions 28-32) [156,181].



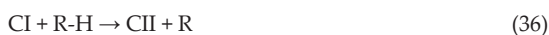
Subsequently, the oxidation reaction is catalyzed by the ROS produced. The reaction of HO_2^\bullet with unsaturated fatty acids is slow, while $\text{O}_2^{\bullet-}$ does not react at all. HO^\bullet is more reactive ($\text{R-H} + \text{HO}^\bullet \rightarrow \text{R}^\bullet + \text{H}_2\text{O}$), and is generated by the Fenton reaction.

Metals in a lower oxidation state, such as Fe and Cu catalyze decomposition of R-O-OH to R-O^\bullet (reaction 33) and, in their higher oxidation state, catalyze decomposition of R-O-OH to R-O-O^\bullet (reaction 34). These emerging radicals increase the reaction rate by increasing the propagation phase rate, as the metal-catalyzed R-O-OH disintegration is faster than the emergence of new radicals.



Metals bound in complexes might or might not be effective depending on the environment. The addition of an iron complex to biological samples encouraged peroxidation by peroxide

decomposition, generating R-O• and R-O-O•. The rate constant for this reaction when ferrous ions are involved, has been given as $1.5 \times 10^3 \text{ mol}^{-1} \cdot \text{l}^1 \cdot \text{s}^{-1}$, which is higher than the rate constant for the reaction of ferrous ions with H₂O₂ in the Fenton reaction $76 \text{ mol}^{-1} \cdot \text{l}^1 \cdot \text{s}^{-1}$ [73]. The redox potentials of the metals Mn and Co are low and are therefore incapable of catalyzing the breakdown of R-O-OH in aqueous systems. In fats, however, they can catalyze the decomposition of R-O-OH through the transient hydroperoxide complexes to R-O-O•. It is not yet known whether the oxidation of lipids can also be catalyzed by complexes of Fe with oxygen (Fe³⁺-O₂-Fe²⁺) and hypervalent iron as ferryl cations FeO²⁺ and ferrate anions FeO₄²⁻, which are the active forms in the enzymes containing heme cofactors, e. g. catalases and cytochrome P450. However, it is known that (ferric; Fe³⁺-Px) peroxidases mediate one-electron oxidation of organic compounds with the concomitant reduction of H₂O₂ to H₂O. In this mechanism, peroxidase donates two electrons to H₂O₂ resulting in cleavage of H₂O₂ and formation of a redox intermediate of enzyme (I). This intermediate consists of an oxoferryl protein cation radical, in which one of the oxidation equivalents exists as the ferryl ion and the other as a porphyrin-centred cation radical (reaction 35). The enzyme intermediate reacts with reductants (R-H) to generate substrate free radicals and another redox intermediate (II), in which oxoferryl species remain intact but the cation radical is reduced. A one-electron reduction of II by a second molecule of reductant regenerates the ferric enzyme and forms a second equivalent of R (reaction 36). Another redox intermediate (III) is formed in the course of peroxidase catalytic cycle (reaction 37). It is catalytically inactive and exists as a resonance form between the Fe²⁺-O₂ and Fe³⁺-O₂ complexes [49,141].



Also the perferryl [Fe⁵⁺] radicals are catalytically active in numerous biological processes, and these ferryl/perferryl moieties, whether as components of enzymes or simple iron complexes, can be very powerful oxidants capable of abstracting hydrogen atoms in lipid peroxidation [20]. Some metal ions with a fixed oxidation number can affect the rate of peroxidation, e.g. Ca²⁺, Al³⁺, and Pb²⁺ ions can accelerate peroxidation stimulated by iron salts under certain conditions [72].

Exposure to heavy metals can change the composition of the reaction products. High concentrations of free radicals may outweigh termination reactions, where the metals inhibit the oxidation. Inhibition of oxidation may occur with higher concentrations of metal ions. It is supposed that Fe and Cu ions oxidize and reduce hydrocarbon free radicals to their corresponding anions (reaction 38) and cations (reaction 39) together with the emergence of free radical complexes (reaction 40). Other complexes are formed with Co (reactions 40-42). All of them break the radical chain reaction.





4.2. Oxidation of proteins

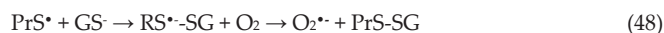
The principal agents for protein oxidation are atmospheric O_2 , 1O_2 , $O_2^{\cdot-}$, $HO\cdot$, $R-O-OH$ and H_2O_2 . Other agents that lead to protein oxidation include $HOCl$, xenobiotics, reduced transition metals, γ -irradiation in the presence of O_2 , activated neutrophils and oxidoreductase enzymes [153]. Free peroxy radicals react with proteins and produce protein radicals, which then react with other free protein radicals to form dimers, or with free lipid radicals to form copolymers. A protein radical arises most frequently when the more labile hydrogen atom on C_α splits from the protein. A hydroxyl acid is obtained from an alkoxy radical, and hydroperoxide from a peroxy radical. Recombination of protein radicals leads subsequently to protein oligomers.

Besides Trp and Tyr, sulfur-containing amino acids, Met ($-S-CH_3$), Cys ($-SH$) are also quite oxidizable in proteins. O_2 oxidation of thiol groups ($-SH$) leads to disulfide formation ($-S-S-$) and vice versa. Under normal conditions, dehydrogenases have the same effect in organisms, such as the oxidation of Cys to cysteine, for example. The first stage of oxidation is the emergence of alkylthiolate (RS^-) in the presence of the hydroxyl anion (HO^-) (reaction 44). Thiolate reacts with oxygen and produces a thyl radical ($RS\cdot$) (reaction 45) [85,94]. The second stage is the reactions with thiols and their emerging radicals (reaction 46, 47). As their quantity increases, the probability of them reacting to form a non-radical product also grows ($2 RS \rightarrow RSSR$).

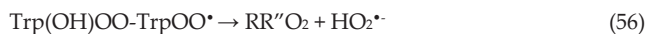
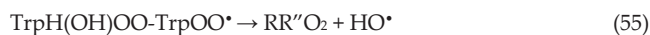


H_2O_2 and $R-O-OH$ are, however, more efficient oxidizing agents. In response to the reaction of protein thiols (PrS) with $R-O-OH$, atoms of sulfur are simultaneously oxidized (frequently those in Cys), forming corresponding monoxides (thiosulfonates) and, where appropriate, further oxidized products containing 2 sulfoxide groups (disulfoxide), sulfone moiety (dioxide, thiosulfonate), sulfoxide and sulfone moiety (sulfoxido sulfone, trioxides), and 2 sulfone groups (disulfonates, tetraoxides) [181]. Reactions with hydro and hydrogen peroxides convert thiol proteins also into sulfenic acids ($RSOH$), which can be further

oxidized to higher oxidation states such as sulfinic (RSO₂H) and sulfonic (RSO₃H) acids [85,169]. Oxidative modifications of critical amino acids within the functional domain of proteins may also occur by S-glutathionylation. Such alterations may alter the activity of an enzyme if the critical cysteine is located within its catalytic domain or the ability of a transcription factor to bind DNA if it is located within its DNA binding motif [14]. RS• then reacts with a glutathionylate anion (GS⁻) to form a radical mixed disulfide (RSSG•), which can lose an electron to oxygen to form O₂•⁻; leaving a mixed disulfide (reaction 48) [155,195]. Another route to mixed disulfides is through the two electron oxidation of a thiol to RSOH, which will then react with a thiolate anion to displace HO• (reaction 49). Exposure to NO during pathological conditions can lead to the formation of ONOO•, which can oxidize thiols to either RS• or RSOH and lead to protein glutathionylation. It is also possible that S-nitrosylation of PrSH to form PrSNO can lead to protein glutathionylation by the displacement of the NO• by glutathione (reaction 50) [26,55]. A study from Thannickal & Fanburg [168] confirms that cysteine modification involving S-glutathionylation is readily reversed to the active sulfhydryl group by thioltransferases. Met is oxidized to methioninsulfoxide. Further methioninsulfoxide oxidation produces methioninsulfone, which is unexploitable.



Trp is a very oxylabile compound, especially in an acidic environment. It is easily oxidized by O₂ on exposure to light, in a photooxidation reaction catalyzed by riboflavin. Oxidation occurs due to the action of sulfoxides, peroxyacids, H₂O₂, R-O-OH, but also undergoes autooxidation under γ-irradiation [90]. Autooxidation propagated by peroxy radicals is a chain reaction. The initial phase is the reaction of HO• with tryptophan across the double C=C bonds, yielding Trp-OH adducts [93]. These adducts react with oxygen to produce the corresponding peroxy radicals. H• reacts with Trp yielding the corresponding Trp-H adducts, while a small amount of the H-atoms react with oxygen yielding HO₂•. The following set of reactions according to Janković & Josimović [90] demonstrates initiation (reaction 51, 52), propagation (forming 2- and 3-adduct peroxy radicals) in reactions 53-56 and termination reactions (57) of Trp autooxidation:





Similarly, the preferred targets of radicals produced during γ -radiolysis in proteins are other hydrophobic amino acids such as Tyr, Phe, Val and Ile. In biological systems, the presence of $\text{HO}\cdot$ during radiolysis leads predominantly to extensive protein-protein crosslinkage via tyrosine-tyrosine (dityrosine) bonding and possibly other amino acid crosslinks as well [71]. The mechanism of dityrosine formation begins with the generation of a $\text{Tyr}\cdot$, radical isomerisation followed by diradical reaction, and finally enolization. The overall rate constant for this process was reported to be $4 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ [69]. $\text{Tyr}\cdot$ may dissipate by pathways other than those involving intermolecular diradical crosslinking of Tyr. Formation of Tyr oxidation products might involve cyclization, decarboxylation, and further oxidation steps on either the protein or fragments released from the protein [70].

The chelating amino acids in proteins, such as His, are most susceptible to oxidative attack due to their proximity to the radicals formed by binding transition metals [160]. Metal-catalyzed oxidation of histidine generally causes formation of oxo-His or Asp [16,114]. Other amino acyl moieties, especially Lys, Arg, Pro and Thr, incur formation of carbonyl groups (aldehydes and ketones) on the side chains [6,159].

ONOO⁻ causes nitrosylation of Tyr residues and oxidative modification of other amino acid residues including Cys, Trp, Met and Phe [89] but it is a poor inducer of protein carbonyls [171]. The interaction of HOCl with Tyr, Trp, Lys and Met residues leads to the formation of chlorotyrosine, chloramines, aldehydes and methionine sulfoxide [80,97].

Metal-mediated formation of free radicals causes also various modifications to DNA bases, altered calcium and sulfhydryl homeostasis. Whilst Fe, Cu, Cr, V and cobalt Co undergo redox-cycling reactions, for a second group of metals, Hg, Cd and Ni, the primary route for their toxicity is depletion of glutathione and bonding to sulfhydryl groups of proteins. As is thought to bind directly to critical thiols, however, other mechanisms, involving formation of hydrogen peroxide under physiological conditions, have been proposed [178].

Indirect oxidative modification of protein amino acyl side chains occurs through the formation of adducts with products of oxidatively modified lipids, amino acids and sugars. Lipid peroxidation products such as hydroxynonenal, malondialdehyd and acrolein bind covalently to Lys, His and Cys residues, leading to the addition of aldehyde moieties to the protein [149,153,174]. α - β unsaturated alkenals may react with sulfhydryl groups of proteins to form stable covalent thioether adducts also containing carbonyl groups [69]. Products of free amino acid oxidation can also form covalent attachments to proteins [81]. Glutathiolation of Cys residues similarly, Schiff bases, obtained by the reaction of reducing sugars with an ϵ -amino group of lysyl residues in proteins may, upon Amadori rearrangement, yield ketoamine protein conjugates [71].

4.3. Oxidation of DNA

Reactions that alter DNA and other macromolecules in living systems are induced by oxidizing conditions resulting from normal metabolism or ionizing and ultraviolet

radiation. The most basic reaction is one-electron oxidation, the result of which is essentially independent of the process by which it is oxidized [110]. The loss of an electron converts DNA to its radical cation (an electron "hole"), which migrates reversibly through duplex DNA by hopping until it is trapped in an irreversible chemical reaction to form a structurally modified base [95]. The dominant mechanism for radical cation migration in DNA is multi-step hopping [113,172] where charge resides on a single base or on small number of adjacent bases and thermal fluctuations precipitate its movement from one base to another [96]. Superexchange is possible, but less effective for long distances, whereby charge is transported coherently in one step by tunnelling [13,157] from a donor to an acceptor through intervening bridging nucleobases [92]. An incoherent, multi-step, random passage from donor to acceptor consists of short-distance tunnelling intervals linked by base sequences that serve as resting sites for charges [64,115].

With respect to DNA, HO• oxidation is most prevalent. HO• reacts with DNA by addition across double bonds of DNA bases at or near diffusion-controlled rates with rate constants of 3 to $10 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$, the rate constant of H atom abstraction is $2 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ [186]. The addition of HO• to the C4, C5, and C8 positions of purines generates OH adduct radicals. C4-OH and C5-OH adduct radicals of purines dehydrate and are converted to an oxidizing purine(-H)• radical, which may be reduced and protonated to reconstitute the purine [142]. C4-OH adduct radicals possess oxidizing properties, whereas C5-OH and C8-OH adduct radicals are primarily reductants. On the other hand, different mesomeric structures of these radicals may have ambivalent redox states [182]. The rate constants of the dehydration of C4-OH adduct radicals of purines at neutral pH amount to $1.5 \times 10^5 \text{ s}^{-1}$ and $6 \times 10^3 \text{ s}^{-1}$. In contrast to C4-OH adduct radicals, the reaction of O₂ with C8-OH adduct radicals of purines is diffusion-controlled [182]. The one-electron oxidation leads to the formation of 8-hydroxypurines (7,8-dihydro-8-oxopurines) in DNA [25]. However, 8-hydroxypurines are also formed in the absence of O₂, but to a lesser extent. The oxidation of C8-OH adduct radicals competes with the unimolecular opening of the imidazole ring by scission of the C8-N9 bond at a rate constant of $2 \times 10^5 \text{ s}^{-1}$. The one-electron reduction of the ring-opened radical leads to the formation of 2,6-diamino-4-hydroxy-5-formamidopyrimidine from guanine and 4,6-diamino-5-formamidopyrimidine from adenine [25]. The one-electron reduction of C8-OH adduct radicals without ring-opening may also occur, resulting in the formation of 7-hydro-8-hydroxypurines, hemiorthoamides, which may be converted into formamidopyrimidines. The formation of 8-hydroxypurines is preferred in the presence of O₂.

The observation that DNA oxidation occurs predominantly at guanines has been attributed to the fact that this base has the lowest E_{ox} [31]. Similarly, it was found that GG steps are the preferred sites for reaction, with the 5'-G being especially reactive [147]. The relative reactivity of the guanines in a GG step is influenced by the surrounding bases. In particular, the reactivity of the 3'-G is reduced when flanked by pyrimidines, which has also been attributed to electronic effects [33]. The guanine radical cation (guanine^{•+}) is formed by elimination of HO• from the C4-OH adduct radical of guanine and may deprotonate depending on pH to give guanine(-H)•. Guanine^{•+} does not hydrate to form the C8-OH adduct radical or go on to form 8-hydroxyguanine (8-oxoguanine, 8-OH-Gua) by oxidation;

however, it may react with 2'-deoxyribose in DNA by H abstraction, causing DNA strand breaks [129]. On the other hand, the hydration of guanine^{•+} in double stranded DNA forms the C8-OH adduct radical, which gives rise to 8-OH-Gua upon oxidation [45]. The C4-OH adduct radical of guanine barely reacts with O₂; however, O₂ adds to guanine(-H)[•] with a rate constant of $3 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$. The reaction of guanine(-H)[•] with O₂ leads to imidazolone and oxazolone derivatives [34,47].

In the case of adenine, at least two OH adduct radicals are formed: C4-OH and C8-OH. The C4-OH adduct radical of adenine reacts with O₂ with a rate constant of $1.0 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$, giving rise to as yet unknown products [182]. 2-hydroxyadenine is also formed from adenine in DNA by a possible mechanism, such as HO[•] attack at the C2-position of adenine, followed by oxidation [136].

Addition of HO[•] across the C5-C6 double bond of pyrimidines leads to C5-OH and C6-OH adduct radicals and H atom abstraction from thymine, resulting in the formation of the allyl radical. The redox properties of adduct radicals differ; C5-OH adduct radicals are reducing while C6-OH adduct radicals are oxidizing [161]. In the absence of O₂, the oxidation of C5-OH adduct radicals, followed by addition of HO⁻ (or addition of water followed by deprotonation), leads to cytosine glycol and thymine glycol [25,85]. C5-OH-6-peroxyl radicals are formed by addition of O₂ to C5-OH adduct radicals at diffusion-controlled rates. C5-OH-6-peroxyl radicals eliminate O₂^{•-}, followed by a reaction with water by HO⁻ addition to yield thymine and cytosine glycols [34,85]. Oxygen reacts with the allyl radical, producing 5-hydroxymethyluracil and 5-formyluracil. Thymine peroxyl radicals are reduced, and then protonated to give hydroxyperoxides [189], which break down to yield thymine glycol, 5-hydroxymethyluracil, 5-formyluracil, and 5-hydroxy-5-methylhydantoin [189].

The products of cytosine oxidation undergo deamination and dehydration. Cytosine glycol deaminates to give uracil glycol, 5-hydroxycytosine, and 5-hydroxyuracil [25,44,188]. In the absence of O₂, C5-OH adduct radicals may be reduced, and subsequently protonated to give 5-hydroxy-6-hydroxypyrimidines. 5-hydroxy-6-hydroxycytosine readily deaminates into 5-hydroxy-6-hydroxyuracil. Similarly, C6-OH adduct radicals of pyrimidines may lead to the production of 6-hydroxy-5-hydroxypyrimidines. These products are typical of anoxic conditions because O₂ inhibits their formation by reacting with OH adduct radicals. By contrast, pyrimidine glycols and 5-hydroxymethyluracil are formed under both oxic and anoxic conditions. Further reactions of C5-OH-6-peroxyl and C6-OH-5-peroxyl radicals of cytosine result in formation of 4-amino-5-hydroxy-2,6(1H,5H)-pyrimidinedione and 4-amino-6-hydroxy-2,5(1H,6H)-pyrimidinedione, respectively, which may deaminate to give dialuric acid and isodialuric acid. Dialuric acid is oxidized in the presence of O₂ to alloxan [44]. C5-OH-6-hydroperoxide gives rise to trans-1-carbamoyl-2-oxo-4,5-dihydroxyimidazolidine as a major product of cytosine [34,188] but as a minor product from DNA [43].

4.4. Oxidation of saccharides

The functional group of carbohydrates is subject to oxidation. Auto-oxidation of carbohydrates is slow in neutral and faster in an acidic environments. D-glucose and D-

fructose form unstable hydroperoxides via 1-en-1,2-diols, which break down to form D-arabinonic and formic acids [181].

Monosaccharide autooxidation is a metal-catalysed process. In the presence of transition metals, compounds with α -hydroxyaldehyde, such as glucose [196] and fructose [181] enolize and reduce transition metals and O_2 sequentially, forming α -ketoaldehydes as major products in the following reaction. Hydroxyaldehyde \rightarrow 1-en 1,2-diol (enediol) \rightarrow enediol radical anion \rightarrow α -dicarbonyl + $O_2^{\cdot-}$. H_2O_2 , formed by $O_2^{\cdot-}$ dismutation, regenerates the catalytic metal oxidation state and produces HO^{\cdot} . In the presence of HO^{\cdot} , hydroxyaldehyde hydrate is formed, which becomes a hydroxyalkyl radical (or alkyl radical, R^{\cdot}). Because glucose is an aldose and fructose is a ketose, it is expected that the extraction of hydrogen from the two kinds of saccharides by HO^{\cdot} would take place at different sites of the two kinds of saccharides, thus the alkyl/alkoxyl radicals they produce would have different properties [119]. Hydroxyalkyl radicals in the presence of O_2 give rise to dicarbonyls and HO_2^{\cdot} . Alternatively, peroxy radicals and HO_2^{\cdot} are formed, giving rise to α -hydroxyacids and further ketoaldehydes [196]. Similar products are also formed by oxidation of H_2O_2 alone. In the presence of Fe^{2+} ions, the decay of H_2O_2 generates free radicals and also oxidizes sugars to the glycosuloses.

The formation of $R-O-O^{\cdot}$ from lipid oxidation involves C-H bond cleavage at the C2 carbon of the carbohydrate. The resulting radical reacts with O_2 and the resulting peroxy radical spontaneously decays to the corresponding glycolysis and HO_2^{\cdot} , which is subject to further disproportionation to H_2O_2 and O_2 (reaction 58). The mechanism of HO_2^{\cdot} formation, which causes the transfer of H^+ in five members structures of the intermediate structure is not the only like that of carbohydrates by $R-O-O^{\cdot}$. The alternative is to induce the transfer of H^+ in the six-member structure of the intermediate. This creates aldonic acid, H_2O_2 , and glyoxal [181].



α -dicarbonyl compounds with the original number of carbon atoms are further oxidised and decomposed. The radicals that develop as intermediate products, α -dicarbonyl compounds and α -ketoaldehydes, react further with Lys and Arg in proteins and are involved in early glycosylation reactions. *N*-substituted 1-amino-1-deoxyfructose (Amadori product) appears as one of the early products of protein glycosylation [197]. Other important processes in these reactions are glycation, glycooxidation and the formation of advanced glycation by-products [191].

Reactions of HO^{\cdot} with the sugar moiety of DNA by H abstraction give rise to sugar modification and strand breaks. A unique reaction of the C5'-centered sugar radical is addition to the C8-position of the purine ring of the same nucleoside. This reaction leads to intramolecular cyclization, followed by 8,5-cyclopurine-2'-deoxynucleosides after oxidation [41,42]. Both 5'R- and 5'S-diastereomers of 8,5'-cyclo-2'-deoxyguanosine (cyclo-dG) and 8,5'-cyclo-2'-deoxyadenosine (cyclo-dA) are formed in DNA [41,42]. These compounds cause concomitant damage to both base and sugar moieties. O_2 inhibits their formation by reacting with C5'-centered sugar radical before cyclization is possible [34].

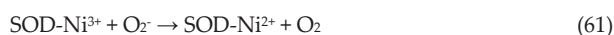
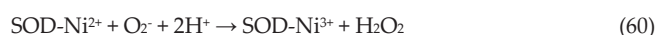
5. Antioxidant metalloenzymes

5.1. Superoxide dismutase (SOD)

Superoxide dismutase (EC 1.15.1.1) belongs to the group of oxido-reductases. SOD contributes significantly to protecting the organism from the toxic effects of $O_2^{\bullet-}$ [152]. In living systems, $O_2^{\bullet-}$ is capable of reacting with another molecule of $O_2^{\bullet-}$ (dismutation) or is also able to react with another radical, such as NO^{\bullet} . Formation of HO^{\bullet} from $O_2^{\bullet-}$ via the metal-catalyzed Haber-Weiss reaction has a reaction rate 10 000 times faster than that of spontaneous dismutation, so SOD provides the first line of defence against ROS [65].



These enzymes are present in almost all aerobic cells as well as in anaerobic organisms, in all subcellular compartments. The active site of the enzyme contains one or two different atoms of a transition metal in a certain oxidation state [148]. SODs are classified by their metal cofactors into known types: the Cu/ZnSOD and MnSOD, which are localized in different cellular compartments. Cu/Zn SOD is mainly extracellular and cytosolic, while MnSOD is a mitochondrial enzyme. Both types are also present in plants [131]. FeSOD isozymes, often not detected in plants, are usually associated with the chloroplast compartment [5]. The prokaryotic MnSOD, FeSOD and eukaryotic Cu/ZnSOD are dimers, whereas MnSOD of mitochondria are tetramers. NiSOD is the most recent class of SOD, which was discovered in *Streptomyces* [205] and cyanobacteria [144]. On the basis of amino acid sequence, metal ligand environment, and spectroscopic properties, NiSOD is distinct from other known SODs [28,87]. However, all SODs are known to have very similar catalytic rate constants, pH dependence, and catalytic functions [200]. Therefore, like its counterparts, the catalytic dismutation activity of NiSOD occurs through the oxidative and the reductive half-reactions, which can be described by the following two equations, where SOD-Ni²⁺ and SOD-Ni³⁺ represent the reduced and oxidized states of the metal center in the enzyme, respectively.



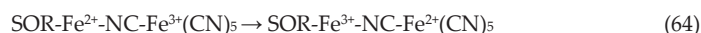
Formation of H_2O_2 in the absence of the enzyme, takes place with a dismutation rate $k = 10^5$ - $10^6 M^{-1}.s^{-1}$. This reaction is accelerated 10^4 -times by SOD [65].

5.2. Superoxide reductase (SOR)

(EC 1.15.1.2) is the second enzyme responsible for the detoxification of $O_2^{\bullet-}$; found only in prokaryotic cells, allowing them to survive in the presence of O_2 . SOR is a non-heme iron enzyme. The active site consists of a mononuclear ferrous ion in an unusual $[Fe^{2+} (N-His)_4(S-Cys)]$ square pyramidal pentacoordination complex [3,203]. The free, solvent-exposed, sixth coordination position is the site of $O_2^{\bullet-}$ reduction [104,139]. The reaction of SOR with $O_2^{\bullet-}$ may proceed through two reaction intermediates [117,140]. The first, presumably a Fe^{3+} -peroxo species, is formed by the almost diffusion-limited binding of $O_2^{\bullet-}$ to the ferrous

active site. This intermediate undergoes two sequential protonation reactions, first yielding a second intermediate, possibly a Fe³⁺-hydroperoxo species, and then the final reaction products, H₂O₂ and the ferric active site [140].

The active site of SOR binds ferrocyanide, also referred as hexacyanoferrate (II) or K₄Fe(CN)₆, at its sixth coordination position through a cyano bridge between the iron and the ferrocyanide molecule. The complex has both reduced and oxidized forms of iron in the active site [2]. Molina-Heredia et al. [133] proposed a mechanism for the reaction of SOR-Fe(CN)₆ complex with O₂^{•-}, forming weakly reactive components in comparison to H₂O₂, as they cannot be involved in the formation of HO[•].



5.3. Indole-2,3-dioxygenase (IDO)

Indoleamine 2,3-dioxygenase (deacyclizing) or indole:oxygen 2,3-oxidoreductase (EC 1.13.11.17) uses O₂^{•-} as cofactor in the initial step during the degradation of the indole ring of Trp to form kynurenine. This step involves two different enzymes, tryptophan-2,3-dioxygenase (TDO) and indoleamine-2,3-dioxygenase (IDO). IDO is a heme-dependent cytosol enzyme present predominantly in monocytes, macrophages, and microglial cells within the brain parenchyma. In liver hepatocytes, however, TDO is predominantly expressed (for more details see [37]) and as a proenzyme [51,52]. IDO does not show substrate specificity exhibited by TDO, catalyzing the oxygenative ring cleavage of various indoleamine derivatives. Even though it catalyzes the same dioxygenation reaction as classical hepatic TDO, it differs from the latter with respect to molecular size substrate specificity, cofactor requirements, and immunogenicity [18]. The enzyme scavenges O₂^{•-}, which increases only when SOD is inhibited. After conversion into 3-hydroxykynurenine, most of the kynurenine formed via IDO is metabolized into xanthurenic acid, rather than complete oxidation along the glutarate pathway or conversion into NAD [18]. IDO is stimulated by pro-inflammatory cytokines, especially IFN-γ [150,165], virus infection [204] and the administration of bacterial endotoxin [175]. The induction of IDO causes a marked increase in Trp catabolism in the body [164] causing kynurenine production and overall depletion of Trp in the cell. Trp is essential for the growth of bacteria and the growth of bacteria is suppressed by actively depleting Trp within infected cells and surrounding milieu [68,127]. IDO is down-regulated by NO as a consequence of the L-Arg metabolic pathway activation, which is also affected by IFN-γ [170]. Since IDO is expressed both in the periphery and in the central nervous system, it represents a possible link between the immune system and serotonergic pathway, as Trp availability controls the synthesis of serotonin [111]. Macrophages and dendritic cells, in particular plasma-cytoid cells, have been implicated in the IDO-mediated suppression of T-cells [10]. More recently, it has been established that IDO regulates maternal tolerance and possibly more general aspects of T-

cell tolerance [128]. The findings of Scott et al. [151] suggest that IDO modulates inflammatory responses, in particular those driven by B-cells.

5.4. Catalase (CAT)

Catalase (H₂O₂:H₂O₂ oxidoreductase, EC 1.11.1.6) is a heme-containing enzyme that is present in virtually all aerobic organisms tested to date [4,12]. In the cell, it is localized predominantly in the peroxisomes [15], where it is important in the removal of H₂O₂ generated by oxidases involved in β -oxidation of fatty acids, respiration, and purine catabolism [9]. CATs from many species are known to be tetramers of 60-65 kDa subunits with each subunit containing 1 Fe-protoheme IX moiety (4 heme groups per tetramer). Each tetrameric molecule of mammalian CATs contains four molecules of tightly bound NADPH, which does not seem to be essential for the enzymatic conversion of H₂O₂ to H₂O and O₂, but rather protects CAT against inactivation by H₂O₂ [98]. CAT has the highest turnover rate among all enzymes, one molecule of CAT can convert approximately 6 million molecules of H₂O₂ to H₂O and O₂ per minute [62] and the pH optimum obtained from different sources is 6.8-7.5. The enzyme can function in 2 ways: α and β phases [107,108].

The α -phase works catalytically (reactions 65, 66), breaking H₂O₂ down into H₂O and O₂ without the production of free radicals. The reaction takes place in two two-electron reactions. In the first, a H₂O₂ molecule oxidizes the heme to compound I (CI), removing one oxidation equivalent from the ferric iron, generating the oxoferryl species, and the other from the porphyrin ring, generating a porphyrin cation radical. The second H₂O₂ then reduces CI to regenerate the resting (ferric) enzyme while releasing H₂O and molecular O₂.



At limiting H₂O₂ concentrations, catalases may undergo a one-electron reduction (reactions 67, 68) to an inactive intermediate, compound II (CII), which can be subsequently converted to another inactive form, compound III (CIII) [138].



The β phase works peroxidatively (reactions 69-71), by eliminating H₂O₂ with oxidizing alcohols, formate (RH₂) or nitrate as described in Aksoy et al. [4], thereby releasing O₂^{•-} and the natural enzyme.



The CAT reaction has evolved in at least three phylogenetically unrelated protein types: the monofunctional or “classical” CAT, the bifunctional catalase-peroxidase (KatG; EC 1.11.1.7), and the non-heme, Mn-containing catalase [138]. Generally, rate constants for the formation of CI from peroxidases and catalases were calculated to be in the range of 10^6 to $10^8 \text{ M}^{-1} \text{ s}^{-1}$ [48]. A distal His-Asn pair has been shown to be essential for CI formation in classical CATs, while a distal His-Arg has the same function in peroxidases [48,54]. The main difference in the enzymatic mechanism between CAT and peroxidases is CI reduction. In a catalase cycle, a second H_2O_2 molecule is used as a reducing agent for CI. This two-electron reduction completes the cycle forming ferric-CAT and O_2 (for details see [207]). With most substrates in a peroxidase cycle, CI is reduced back to the ferric enzyme in two consecutive one-electron steps via CII. KatGs can be viewed as a molecular fossil revealing the common phylogeny of catalytic and per-oxidative activity during evolution [206]. It has been proposed that KatG is responsible for the catalytic oxidation of H_2O_2 in a two-electron oxidation step with both oxygen atoms being derived from the same H_2O_2 molecule. This non-scrambling mechanism is independent of pH and is not affected by manipulation of highly-conserved and important catalytic residues. Principally, there are two possible mechanisms for the formation of O_2 following this retention mechanism: an ionic mechanism, via initial proton abstraction with the help of an acid-base catalyst followed by a hydride-ion removal from H_2O_2 and release of O_2 ; and hydrogen atom transfer from H_2O_2 to the ferryl species to yield a radical intermediate [185]. Until now, the complete gene sequences of KatGs were characterised only from prokaryotes (both from archaeobacteria and eubacteria) although several reports describe the presence of KatGs in lower eukaryotes [56]. It was shown phylogenetically that the closest neighbours of KatGs are eukaryotic ascorbate peroxidases and yeast cytochrome *c* peroxidase [192]. So far, KatGs are the only peroxidases known with both catalase activity comparable with catalases and typical peroxidase activity with broad specificity.

The variable response of CAT activity has been observed under metal stress [65], while all ions of heavy metals are non-competitive inhibitors of CAT. Cyanides are strong inhibitors of CAT as they form a strong bond with the heme of CAT and stop its catalytic activity [184]. Some studies have shown that CAT is effective in the degradation of H_2O_2 present only in mmol.l^{-1} , while glutathione peroxidase is effective in peroxide degradation at concentrations lower than $100 \mu\text{mol.l}^{-1}$ [39].

5.5. Glutathione peroxidase (GPx)

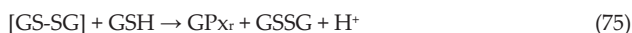
Glutathione peroxidase (EC 1.11.1.9) has eight known isoenzymes that, in active positions, may contain co-factors, such as heme and residues of cysteine or selenocysteine. GPx1-4 and GPx 6 are selenoenzymes, which contain the non-metal selenium [27]. Selenocysteine participates directly in the transfer of electrons to a peroxide substrate, thereby oxidizing it. However, the pathophysiological role of these isoenzymes in antioxidant defence is of substantial importance [100,101,180]. CAT is found in many types of cells and scavenges H_2O_2 as its sole substrate, GPx scavenges various peroxides. The expression of CAT in most cells is lower than that of GPx, with the exception of hepatocytes and erythrocytes. The K_m

value of CAT for H_2O_2 is higher than that of GPx, implying the primary importance of GPx in most tissues [8].

Like all peroxidases, they mediate the one-electron oxidation of organic compounds (reduced glutathione, GSH) with a concomitant reduction of H_2O_2 (for more detailed mechanisms of peroxidase action see 4.1.4 and 5.4). The activity of GPx is affected by the presence of another important antioxidant enzyme, glutathione reductase, which continuously recycles the oxidised glutathione to the reduced state. Lawrence et al. [109] described that non-selenium dependent GPx activity contributes to glutathione-S-transferase B (G-S-T) activity in mechanisms analogous to the G-S-T mechanism. Thus, an enzyme bound to GSH may attack the electrophilic oxygen of the peroxide and a second molecule of GSH may react in a non-enzymatic fashion similar to the reaction with organic nitrates, or by another enzyme catalyzed step to yield the glutathione disulphide (GSSG). Non-selenium dependent GPx also has the ability to reduce phospholipid hydroperoxides, without G-S-T activity [53]. Kinetic analysis of GPx activity indicated a *tert*-uni ping-pong mechanism similar to that described for other GSH peroxidases [176].



Kinetic behaviour of the overall reaction is discussed in detail in Ng et al. [137].



Hall et al. [74] showed that an epididymis-specific, secretory GPx has very little activity towards H_2O_2 or organic hydroperoxides. Instead, it binds to lipid peroxides.

Virtually all known peroxidases are inactivated by H_2O_2 and other hydroperoxides at relatively high concentrations [83]. This substrate inactivation leads to modification of the heme prosthetic group and the formation of a verdohemoprotein as the final product. The existence of CIII as the peroxy iron prophyrin free radical resonance form can facilitate the transfer of electrons from the ferrous state to an extra H_2O_2 molecule, thereby generating HO^\bullet . This highly reactive species has the propensity to attack the heme porphyrin ring and lead to irreversible inactivation [60].

5.6. Heme oxygenase (HO)

An iron-containing decyclizing oxygenase (EC 1.14.99.3) can be legitimately considered a part of the phase 2 response [40]. HO catalyzes the first, rate-limiting step of heme degradation. HO cleaves the α -meso carbon bridge of b-type heme molecules via oxidation to yield equimolar quantities of biliverdin IXa, CO and free iron. Biliverdin is subsequently converted to bilirubin via the action of biliverdin reductase, while the free iron is promptly sequestered into ferritin. To date, three isoforms (HO-1, HO-2 and HO-3) have been identified. Under physiological conditions, HO activity is highest in the spleen where

senescent erythrocytes are sequestered and destroyed [143]. HO-1, can be induced by a variety of non-heme products including ultraviolet irradiation, endotoxins, heavy metals, and oxidants as well as H₂O₂ [121,183]. The production of bilirubin/biliverdin and carbon monoxide from heme catabolism is capable of exerting protection against toxic compounds in the cell. Indeed, in a variety of cells and tissues, inducible oxidative stress represents part of an adaptive cellular response to inflammation.

6. Conclusion

Oxidative processes are essential to life, particularly for obtaining the energy needed for various metabolic processes, but they also serve as a source of ROS. Oxidation and reduction processes are inseparable. Given that transition metals readily accept or give away electrons, they play an important role in the oxidoreduction processes and are constituents of various proteins and enzymes. In fact metalloenzymes participate significantly in the antioxidant protection of the body as phase I and II antioxidants. It is important to understand and study the antioxidant defence system of the organism so that one can use this knowledge to prevent and treat diseases in which it has been proven to participate.

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7. References

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