The Chemistry of Antioxidants*

1. Metalloenzymatic Antioxidants

*K Hussain Reddy

Antioxidants play a vital role in the immunity system. In this three-part article, the chemistry of antioxidants, which plays a pivotal role in nature’s defense strategy, is described. In the first part, structural and mechanistic aspects and antioxidant properties of metalloenzymes are presented.

Introduction

Antioxidants are chemical compounds that inhibit oxidation. The ability of antioxidants to destroy free radicals protects the structural integrity of cells and tissues. Recent clinical trials indicate that antioxidant supplementation can dramatically improve certain immune responses. Antioxidants scavenge free radicals from the body cells and prevent or reduce the damage caused by oxidation.

Oxidation is a chemical process that produces free radicals that in turn cause chain reactions and damage the living cells. Antioxidants can prevent these chain reactions. To balance oxidative stress, plants and animals employ antioxidants. These antioxidants may be classified into two categories—enzymatic antioxidants and non-enzymatic antioxidants. The enzymatic antioxidants may further be divided into two categories—metalloenzymatic antioxidants and non-metalloenzymatic antioxidants. Classification of antioxidants and examples under each category are given in Figure 1. In this article, the structural, mechanistic, and antioxidant properties of metalloenzymatic antioxidants are discussed.

Throughout normal metabolic reactions reactive oxygen species

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Keywords
Antioxidants, metalloenzymes, SOD, peroxidases, catalase.
ROS are spontaneously formed due to the utilization of molecular oxygen (O₂) during normal cellular consumption. These are inherently unstable as they contain unpaired electrons. As a result, they become highly active and cause harmful effects to cell components, such as proteins, lipids, carbohydrates, and even deoxyribonucleic acid (DNA). Higher levels of ROS cause oxidative stress leading to cancer, neurological disorders, diabetes, hypertension, asthma, etc. The provenances of ROS are of two types—endogenous sources and exogenous sources.

1. **Endogenous Sources**: ROS are spontaneously formed due to the utilization of molecular oxygen (O₂) during normal cellular consumption. ROS may be classified into two types—free radicals and non-radicals. Species having one or more unpaired electrons and with increased reactivity are known as free radicals. When two free radicals stake their unpaired electron, a non-radical is formed. ROS having physiological significance are: superoxide (O₂⁻), hydroxyl radical (·OH) and hydrogen peroxide (H₂O₂). The reaction sources of endogenous oxidants are given in Table 1.

Mitochondria is the prime source for producing superoxide (O₂⁻). It arises when molecular oxygen gains one electron. The redox reaction is facilitated by nicotinamide adenine dinucleotide phos-
Table 1. Reaction sources of endogenous oxidants.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Name of the oxidant</th>
<th>Reaction that produce oxidant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Superoxide (O$_2^-$)</td>
<td>NADPH + 2O$_2$ → NADP$^+$ + 2O$_2^-$ + H$^+$</td>
</tr>
</tbody>
</table>
| 2.    | Hydrogen peroxide (H$_2$O$_2$) | (i) 2 O$_2^-$ + 2H$^+$ → O Hydrogen peroxide (H$_2$O$_2$)  
(ii) Hypoxantine + H$_2$O + O$_2$ → Xantine + H$_2$O$_2$  
(iii) Xantine + H$_2$O + O$_2$ → Uric acid + H$_2$O$_2$ |
| 3.    | Hydroxyl radical (·OH)       | Fe$^{II}$ + H$_2$O$_2$ → Fe$^{III}$ + ·OH + H$_2$O |
| 4.    | Peroxy radical (ROO·)        | R$^\cdot$ + O$_2$ → ROO· “the radical” (R$^\cdot$ is alkyl radical) |
| 5.    | Hydroperoxy radical          | O$_2^+$ + H$_2$O → HOO$^-$ + OH$^+$ |
| 6.    | Hypochlorous acid            | H$_2$O$_2$ + Cl$^-$ → HCl + H$_2$O |

The hydroxyl radical is the most reactive species, and it can easily damage proteins, lipids, carbohydrates, and even DNA. Hypochlorous acid (HOCI) is produced by the reaction of chloride ions with H$_2$O$_2$. HOCI is highly oxidative to DNA and adds chloride to DNA bases producing pyrimidine oxidation products. The peroxo and hydroperoxy radicals can activate lipid peroxidation chain reaction.

2. Exogenous ROS Sources: Cigarette smoke, ozone exposure, higher O$_2$ levels, radiation exposure, heavy metal pollution, etc., are some important sources of exogenous oxidants. The sources of exogenous oxidants are given in Table 2.

Aerobic organisms have integrated antioxidant systems. Our body is provided with several types of antioxidants. They protect our bodies from the detrimental effects of oxidants. Antioxidants effectively scavenge ROS or block their harmful effects. In this

Our body is provided with several types of antioxidants. They protect our bodies from the detrimental effects of oxidants. Antioxidants effectively scavenge ROS or block their harmful effects.
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Source</th>
<th>Oxidant</th>
<th>Adverse effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cigarette smoke</td>
<td>$O_2^-$, NO</td>
<td>Accumulation of neutrophils and macrophages leading to oxidant injury</td>
</tr>
<tr>
<td>2.</td>
<td>Ozone exposure</td>
<td>—</td>
<td>Lipid peroxidation, release of inflammatory mediators, reduction of pulmonary function.</td>
</tr>
<tr>
<td>3.</td>
<td>Higher levels of oxygen</td>
<td>Hyperoxia, greater production of reactive oxygen and nitrogen species</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Exposure to ionizing radiation</td>
<td>OH, $O_2^-$ and organic hydro peroxides</td>
<td>UV radiation triggers oxidative reaction.</td>
</tr>
<tr>
<td>5.</td>
<td>Heavy metal ions, $O_2^-$, OH, $H_2O_2$, ROO, NO, $^{1}(CH_3)_3AsOO^-$</td>
<td>Inhibition of enzyme activity, lipid peroxidation, modify DNA bases.</td>
<td></td>
</tr>
</tbody>
</table>

^ Dimethylarsenic peroxo radical

Table 2. Reaction sources of exogenous oxidants.

The chemistry of metalloenzymatic antioxidants will be discussed.

**Metalloenzymatic Antioxidants**

The major metalloenzymes are (1) superoxide dismutase, (2) catalases, and (3) peroxidases. These enzymes play a vital role in the protection of antioxidants in the respiratory system and are involved in the defense mechanisms. The crucial reactions catalyzed by enzymatic antioxidants are given in Table 3.

1. **Superoxide Dismutases (SOD)**

The primary reactive species is superoxide ($O_2^-$), produced from a variety of sources. It undergoes dismutation\(^1\) by the catalytic action of SOD. Hence, the catalytic reaction is of vital importance for the survival of cells. Within a cell, the SODs are the first line of warriors in the defense against ROS. Irwin Fridovich and Joe McCord of Duke University discovered the enzymatic activity of superoxide dismutase in 1968. Superoxide dismutases were originally honored as the veterinary anti-inflammatory drug ‘Orgotein’.

\(^1\)Means disproportionation.
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Name of the enzyme</th>
<th>Reaction catalyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SuperoxideM^{(III)}^-SOD + O_2^2- → M^{II}_\text{SOD} + O_2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(SOD)</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Catalase (CAT)</td>
<td>M^{II}_\text{SOD} + O_2 + 2H_2O</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2H_2O_2 → O_2 + 2H_2O</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H_2O_2 + Fe(III)-E → H_2O + O - Fe(IV)-E(+)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H_2O_2 + O - Fe(IV)-E(-) → H_2O + Fe(III)-E + O_2</td>
</tr>
<tr>
<td>3.</td>
<td>Peroxidases</td>
<td>ROOR' + 2e^- + 2H^+ → R'OH + ROH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AH_2 + H_2O_2 → 2H_2O + A^3-</td>
</tr>
</tbody>
</table>

Cu, Zn-SOD, Mn-SOD, and EC-SOD are universally manifested in human respiratory organs. Mn-SOD is limited to the mitochondrial matrix, while EC-SOD (extracellular superoxide dismutase) is mainly located in the extracellular matrix. In a nutshell, these enzymes act as scavengers of superoxide radicals. EC-SOD is involved in lung matrix protection.

Copper, zinc superoxide dismutase (Cu, Zn-SOD) is a unique metalloprotein in biology. The protein is abundant in all eukaryotic cells and functions as a catalyst of superoxide (O_2^-) disproportionation (i.e., superoxide dismutation). The observation of this activity led investigators to study the structure and mechanism of action of this distinct enzyme.

**Structure:** The structure of oxidized Cu, Zn-SOD isolated from bovine erythrocyte was determined by Richardson and his co-workers. Structural studies of different eukaryotic Cu, Zn-SOD revealed that the order of amino acids is highly preserved, particularly in the metal-binding region. Consequently, the spectroscopic properties of the enzyme isolated from different sources are also very similar. X-ray studies revealed that the protein consists of two identical subunits. Each subunit looks like a cylindrical barrel with β-pleated sheet made up of 8 antiparallel chains.

The Cu(II) and Zn(II) ions are in close proximity in each subunit. The chemical structure of the active site of bovine Cu, Zn-SOD is manifested in *Figure 2.*

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**Table 3.** Reaction catalyzed by enzymatic antioxidants.

Copper, zinc superoxide dismutase (Cu, Zn-SOD) is a unique metalloprotein in biology. The protein is abundant in all eukaryotic cells and functions as a catalyst of superoxide (O_2^-) disproportionation.
Cu(II) is coordinated by four imidazole nitrogen atoms coming from His-44, His-46, His-61, and His-118 amino acid residues of the protein chain. Further, one H_2O molecule occupies the 5th coordination site of Cu(II) to give a distorted square-pyramidal geometry to the coordination unit. On the other hand, the Zn^{2+} ion is coordinated by imidazole nitrogen from His-61, His-69, and His-78. The 4th coordination site of the metal ion is occupied by the carboxylate group of Asp-81 to give an approximately tetrahedral geometry. The unique structural features of this enzyme are as follows.

1. It has two different metal centers—Cu(II) and Zn(II). Hence it is regarded as an enzyme containing heteronuclear coordination unit.

2. It has reactive 5-coordinate Cu(II) center.

3. The most unusual aspect of the structure of this enzyme is that the different metal ions [Cu(II) and Zn(II)] share a common ligand (His-61) which holds the metal ions in the deprotonated form at a distance of 6.3 Å apart.

4. Zn(II) ion does not bind with exogenous ligands. On the other hand, Cu(II) can bind a variety of small anionic ligands, such as CN^−, N_3^−, halides.
**Mechanism of Action**: Superoxide dismutases have been considered as ubiquitous metalloenzymes. The only reaction catalyzed by these enzymes is given below.

\[
O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2
\]

The mechanism involves the alteration of oxidation states between Cu(I) and Cu(II). The peroxide species is formed due to the reaction between the reduced form of SOD with superoxide as shown below.

\[
Cu(I) + O_2 \rightarrow Cu(II) + O_2^{2-}
\]

In the above redox reaction, \(O_2^-\) gains electron from Cu(I). Since the peroxide species is extremely basic, it needs a proton prior to its dissociation in the domain of the Cu(II) ion. It has been suggested that the imidazolate ion of bridging His-61 is the source of this proton. The mechanism for superoxide disproportionation is shown in **Figure 3**.

Since zinc is near the copper site, one would expect the role of for-
mer metal ion in $O_2^-$ disproportionation. However, the zinc free enzyme shows almost full activity. Zn(II) ion has not only a structural role but also facilitates the release of the proton (from imidazole/imidazolate ion). The two metal ions ($Cu^{2+}$ and $Zn^{2+}$) can be pulled out to produce an apoprotein, which in turn can again accept different metal ions to produce a large variety of metallo-substituted superoxide dismutases. Only Ag(I) and Co(II) have been lodged in place of the native Cu(II), whereas, many metals have been positioned in place of the native zinc ion.

2. Catalase

Catalases are commonly found in all organisms which depend on oxygen such as bacteria, plants, animals, and humans. It is found mainly in the peroxisomes, cytosol of erythrocytes, and mitochondria. It is present in higher concentrations in the liver of animals. Catalases protect the cell from oxidative damage caused by reactive oxygen species (ROS). This enzyme catalyzes the decomposition of hydrogen peroxide to give water and dioxygen as represented below.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

The products of the above reaction are essential to living organisms. In the above reaction, the toxic product ($H_2O_2$) of SOD is converted into useful products. Hence this enzyme acts as a scavenger and catalyze the oxidation of various metabolites and toxins. The general reaction is shown below.

$$H_2O_2 + H_2R \rightarrow 2H_2O + R$$

Structure: It is a heme-containing enzyme. Catalases have molecular weights of about 2,40,000 Daltons. It is a tetramer comprising 4 identical subunits, each containing one heme group and one polypeptide chain of over 500 amino acids long. The heme unit has high spin iron (III). In the equatorial plane, the four nitrogen atoms of porphyrin occupy four coordination sites. The 5th and
6th coordination sites (Figure 4) are respectively occupied by an amino acid (tyrosine) residue and an oxygen donor ligand, presumably water.

**Mechanism of action:** The reaction is known to occur in two steps.

\[
H_2O_2 + Fe(III) - E \rightarrow H_2O + O = Fe(IV) - E(+) \\
H_2O_2 + O = Fe(IV) - E(+) \rightarrow H_2O + Fe(III) - E + O_2
\]

\[
2H_2O_2 \rightarrow 2H_2O + O_2
\]

In the above reaction, Fe(III)-E means enzyme-containing heme unit, the Fe(IV)-E(+) is a mesomeric state of Fe(V)-E, indicating the iron is not completely oxidized to +V but gains some electron density from the heme unit. Hence it is shown as a cation radical (+·).

First, H₂O₂ reaches the reactive site and then interacts with the amino acids, viz., asparagine-148 and histidine-75, prompting H⁺ to transfer between the oxygen atoms. The free oxygen atom binds to iron, producing Fe(IV)=O cation radical and water molecule. The mono-oxygenated species reacts with a second H₂O₂ molecule to produce water and oxygen to restore the original enzyme (Fig-
Figure 5. Mechanism of action of catalase for disproportionation of hydrogen peroxide [The first $H_2O_2$ triggers two electron oxidation and the second molecule of $H_2O_2$ triggers two electron reduction].

Thus the reaction cycle is completed. The catalytic reaction follows first-order kinetics, and the rate is proportional to the $H_2O_2$ concentration. The enzyme is used in dairy, food, and textile industries for removing hydrogen peroxide.

Recent findings suggested that catalase can offer an effective therapeutic solution for hyper inflammation occurring due to the SARS-Cov-2 virus. Tests revealed that the enzyme can regulate the production of cytokines, a protein produced in the white blood cells. Cytokines are an important part of the human immune system required to combat the Covid-19 virus. It is hoped that the low-cost catalase enzyme may help to treat Covid-19.

3. Peroxidases

These are also heme containing enzymes. Since they break up peroxides, these enzymes are so named peroxidases. Peroxidases customarily catalyze the oxidation of organic peroxides, including $H_2O_2$:

$$ROOR' + 2e^- + 2H^+ \rightarrow R'OH + ROH$$

Peroxidases and catalases show several similar properties. Horseradish roots and the sap of fig trees are the richest sources of plant peroxidases. The properties of peroxidases are given in Table 4.
Most peroxidases are glycoproteins. Horseradish peroxidase (HRP) is the most well-studied peroxidase. Several peroxidases from animal tissues have also been studied, including thyroid peroxidase (which is fairly similar to HRP), lactoperoxidase, myeloperoxidase, and glutathione peroxidase. Lactoperoxidase and myeloperoxidase contain a formyl type of porphyrin, while glutathione peroxidase is unique in that it contains one selenium (Se) atom per subunit.

Cytochrome c peroxidases isolated from baker’s yeast also contain ferriproto porphyrin IX. Many of these enzymes are mixtures of isoenzymes differing in physical properties but with similar catalytic characteristics. Thus isoenzymes of HRP contain Ca^{2+}, which appears to be involved in the stabilization of protein conformation as evidenced by the lower thermal stability of the calcium free isoenzymes. These isoenzymes differ substantially in their ability to exchange Ca^{2+}.

In horseradish peroxidase (HRP), histidine residue and an aqua (H_{2}O) group are thought to occupy respectively the 5th and 6th coordination sites (Figure 6) of Fe(III)-heme. The H_{2}O group is thought to bind a second histidine via a hydrogen bond. The spin state of iron depends on the pH. At low pH, it is high spin, but it goes to a low spin state at high pH. Several studies revealed that the complex may be 5-coordinate under acidic and neutral conditions. In the basic medium, another protein ligand binds with iron to give six-coordinate species. This view has been accepted unanimously.

<table>
<thead>
<tr>
<th>Name of the peroxidase</th>
<th>Molecular weight</th>
<th>Prosthetic group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horseradish peroxidase</td>
<td>40,500</td>
<td>Ferrirypo porphyrin IX</td>
</tr>
<tr>
<td>Cytochrome c peroxidise</td>
<td>34,100</td>
<td>Ferrirypo porphyrin IX</td>
</tr>
<tr>
<td>Chloroperoxidase</td>
<td>40,200</td>
<td>Ferrirypo porphyrin IX</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>76,500</td>
<td>Derivative of mesoheme IX</td>
</tr>
<tr>
<td>Thyroid peroxidise</td>
<td>62,000</td>
<td>—</td>
</tr>
<tr>
<td>Japanese radish peroxidise</td>
<td>55,200</td>
<td>Ferrirypo porphyrin IX</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>1,49,000</td>
<td>Ferrirypo porphyrin IX</td>
</tr>
<tr>
<td>NADH peroxidase</td>
<td>12,000</td>
<td>FAD</td>
</tr>
<tr>
<td>Turnip peroxidase</td>
<td>49,000</td>
<td>Ferrirypo porphyrin IX</td>
</tr>
<tr>
<td>Glutathione peroxidise</td>
<td>90,000</td>
<td>One atom of selenium per sub-unit</td>
</tr>
</tbody>
</table>

**Table 4.** Properties of peroxidases.

Horseradish peroxidase (HRP) is the most well-studied peroxidase. Several peroxidases from animal tissues have also been studied, including thyroid peroxidase (which is fairly similar to HRP), lactoperoxidase, myeloperoxidase, and glutathione peroxidase.
**Figure 6.** Structure of active site in HRP.

**Function:** Peroxidases typically catalyze oxidation of organic compounds as shown below.

\[ AH_2 + H_2O_2 \rightarrow 2H_2O + A^{2-} \]

The non-metallo enzyme, glutathione peroxidase (GSH) plays a major role in the protection of red blood cells (RBC) from the toxic effects of hydrogen peroxide. The chemistry of this enzyme will be discussed in the next article.

**Conclusions**

The structure, functions, and mechanism of action of metalloenzymatic antioxidants viz., Cu, Zn-superoxide dismutase, catalase, and peroxides are focused in this article. The unique features of these enzymes are highlighted.

**Suggested Reading**


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