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Free Radicals, Metal Ions and Oxidative Stress: Chemical Mechanisms of Damage and Protection in Living Systems

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Mecanismos químicos de danos biológicos ocasionados por radicais livres dependem da disponibilidade de oxigênio molecular. Na ausência de oxigênio, a reação direta dos radicais orgânicos com biomoléculas é a fonte principal dos danos. Na presença de oxigênio, o risco de danos é consideravelmente ampliado pela atividade redox de íons metálicos em relação aos intermediários da redução do oxigênio, os chamados "oxi-radicais". Vários sistemas de defesa foram desenvolvidos nas células para evitar danos oxidativos. Tais sistemas são tipicamente classificados de acordo com sua capacidade de ou interceptar radicais formados ou atuar na prevenção de sua formação. Enzimas como superóxido dismutases e hidroperoxidases são típicos interceptores. Por outro lado, a defesa preventiva baseia-se principalmente no controle da reatividade de íons metálicos redox, como ferro e cobre. Resultados recentes de nosso laboratório sugerem que em alguns casos a prevenção pela quelação do metal e pela remoção catalítica dos oxi-radicais pode ser efetuada por uma única molécula participando de reações químicas distintas para cada função. Neste contexto, dados referentes a cobre, superóxido dismutase e glutationa serão apresentados e discutidos.

The chemical mechanisms of biological damage by free radicals depend on the availability of dioxygen in the environment. In the absence of oxygen, the direct reaction of organic radicals with biomolecules is the major source of damage. In the presence of oxygen, the risk of damage is greatly amplified by the redox activity of metal ions with respect to intermediates of oxygen reduction or "oxyradicals". A number of defence systems have evolved in cells to counteract "oxidative" damage. Such systems are typically classified according to their capability to either "intercepting" formed radicals or acting at the prevention or the repair level. Typical "interceptors" are enzymes such as superoxide dismutases and hydroperoxidases. Preventive defence mostly relies on the control of the reactivity of redox metal ions, such as iron and copper. Recent results from our laboratory suggest that in some instances prevention by metal chelation and catalytic removal of oxyradicals can be brought about by a single molecule carrying out distinct chemical reactions for either function. In this regard, data concerning copper, superoxide dismutase and glutathione are presented and discussed.

Keywords: *oxygen free radicals, antioxidative defence, glutathione, opper, superoxide dismuase*

Free Radical Production and Oxidative Stress

Oxidative stress is a consequence of aerobic life. In fact, though molecular oxygen (O_2) availability allows to withdraw a higher energy yield from metabolism, danger lurks in its utilization. Detrimental effects may derive from the simultaneous generation, even under basal metabolic conditions, of potentially toxic reactive intermediates, resulting from the partial reduction of O_2: superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\bullet$). Both $OH^\bullet$ and O_2^- are "free radicals", since they have one unpaired electron in the outer orbital. In the ground state O_2 is itself a radical, with two unpaired electrons each located in a π^* antibonding orbital.

The reaction of O_2 with biomolecules is made difficult by its peculiar molecular configuration[1]. In fact, two main factors make oxygen kinetically inert: the spin restriction imposed by its triplet state, and the negative standard potential for the one electron reduction to O_2^-. However, spin restriction can be overcome by strategies which involve single electron exchange. Therefore, the activation of O_2 by specific enzymes is achieved by the presence at the active site of either flavins or reduced transition metals, such as iron or copper, which can perform single electron donation to O_2. In addition, in the case of metalloproteins, a varying degree of electron transfer from the metals to oxygen is possible. On this basis, metalloproteins can behave either as O_2 carriers (hemoglobin, hemocyanin, hemerythrin, myoglobin), where reversible interaction with O_2 occurs, or as O_2 reductants. Electron transfer to oxygen is catalyzed by oxidases for the production of chemical energy or for the oxidation of substrates, whereas oxygenases incorporate one or two oxygen atoms into substrates. These enzymes, located in different subcellular districts (mitochondria, endoplasmic reticulum, peroxisomes), may become potential sources of partially reduced copper derivatives in the biological milieu[2]. It has been demonstrated that O_2^- is formed upon the autoxidation of oxyhemoglobin. Cytosolic enzymes (xanthine oxidase, aldehyde oxidase) also produce O_2^- during their catalysis. The mitochondrial electron transport chain may occasionally reduce O_2 to O_2^- at the ubiquinone and NADH dehydrogenase sites. Microsomal cytochrome P450 and its reductase produce O_2^- during xenobiotic biotransformation[3].

Organic molecules (xenobiotic, drugs) can be activated to free radicals during intracellular pathways of reductive biotransformation. The radical species can then be reoxidated by O_2 to the parent compound with concomitant formation of O_2^-. This mechanism is at the basis of both the noxious effects produced by several foreign compounds and of the pharmacological efficacy of antitumoral drugs[4,5].

H_2O_2, the two electron reduced derivative of O_2, may either derive from spontaneous or enzymatic dismutation of O_2^- or be formed directly through the catalysis of oxidases (monoamine oxidases, lysyl oxidase, diamine oxidase), in some instances located in peroxisomes (amino acid oxidase, urate oxidase)[3].

Possible sources of the reactive oxygen species are summarized in Fig. 1.

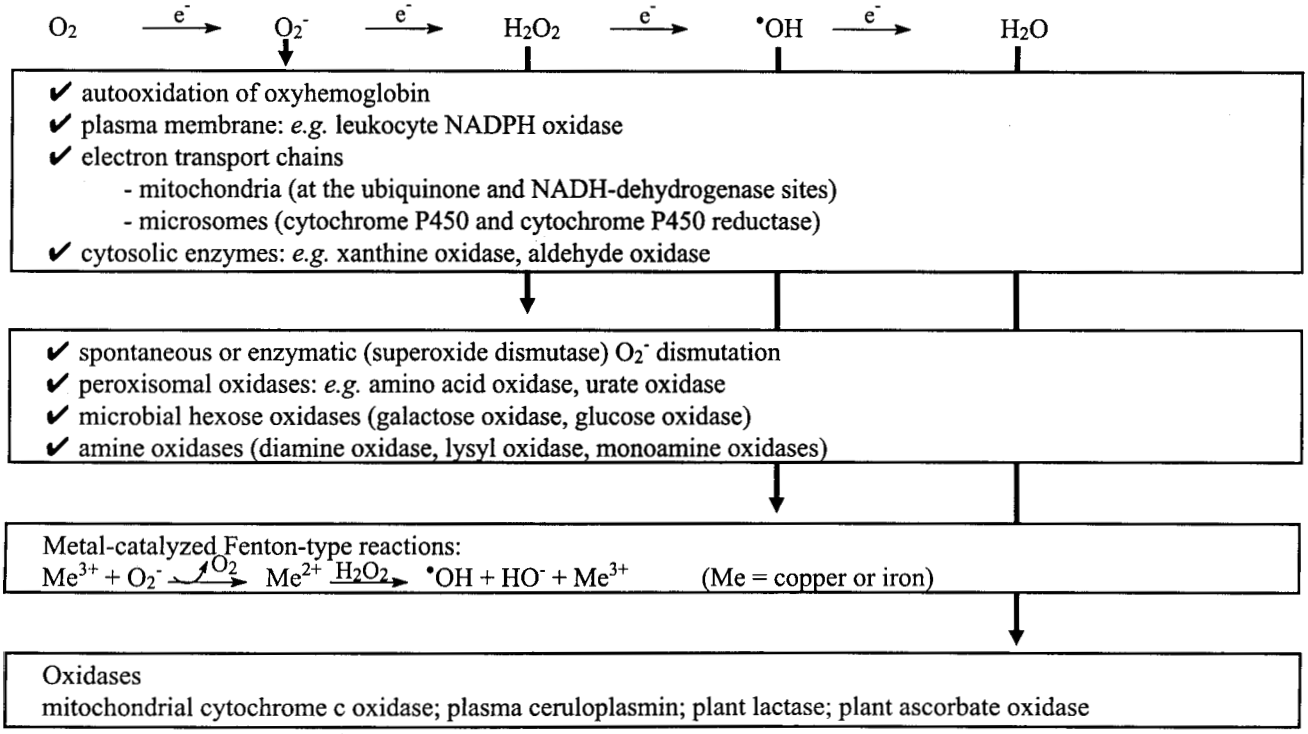

Figure 1. Sources of reduced oxygen species in biological systems.

A peculiar system generating oxyradicals is represented by the electron transport chain located on the surface of polymorphonucleated leukocytes, which produces O_2^- as a defence against infective bacteria[6]. In this case, detrimental consequences of oxyradical production are changed into beneficial effects.

Oxidative Damage to Macromolecules

Oxygen intermediates are extremely reactive molecules. In biological systems they attack functional and structural macromolecules (lipids, nucleic acids, proteins), thereby modifying them[7]. Oxidative injury to polyunsaturated lipids gives rise to peroxidated molecules, which subsequently break down to form reactive metabolites. As a consequence, being lipids the major components of biological membranes, fluidity and permeability of these structures are severely affected, together with membrane protein functionality. Base modification, scission of deoxyribose rings, strand breaks and ultimately chromosomal aberration are the consequences of oxidative stress to nucleic acids. The oxidative challenge to proteins leads to the modification of the side chain of amino acids with the introduction of carbonyl groups, or oxidation of sulphydryl groups with consequent cross linking and aggregation[8]. The presence of oxidative modifications ultimately results in increased susceptibility of modified proteins to specific proteases, enzyme deactivation or, conversely, activation of silent harmful enzymes.

It has been proposed that oxidative modifications to macromolecules are implicated in several physiological and pathological processes, including ageing, cataract, arthritis, cancer, pulmonary diseases and ischemia-reperfusion syndromes[7,9] (Fig. 2).

Recently, in addition to the widely accepted concept that oxyradicals are dangerous species, hypotheses about their involvement in cellular signaling and regulation have been proposed. It has been suggested that reactive oxygen intermediates participate as chemical messengers in several physiological phenomena including cell growth, cell chemo-attraction, programed cell death, platelet aggregation, hormone-receptor interaction, protein binding to mRNA and binding of transcription factors to DNA[10-14].

The Role of Metals in the Production of Oxygen Radicals and in the Exacerbation of Damage

Transition metal ions are essential micronutrients, since they are present at the catalytic centers of several key enzymes of cellular metabolism. However, they stimulate free radical production and potentiate oxidative damage[15]. The formation of $OH^{\bullet}$, the most reactive among the partially reduced oxygen derivatives, occurs mainly upon the reaction of H_2O_2 with reduced metal ions (copper or iron) during the so-called "Fenton reaction" (Fig. 1). In particular, the binding of metals to proteins and nucleic acids promotes site-specific Fenton-type reactions, leading to the introduction of oxidized groups which can be hallmarks for oxidative modification[8]. Furthermore, autoxidation of reduced metals is itself a source of O_2^-.

Defence Systems Against Oxidative Stress

The survival of aerobic organisms depends on their capability to counteract oxidative damage. They have evolved a number of defence mechanisms against free radicals (see Fig. 3) which operate at three levels: prevention, interception and repair[3,16].

Radical sequestration by proteins which utilize O_2, such as cytochrome oxidase, ribonucleotide reductase and oxyhemoglobin is the main mechanism for the prevention of free radical formation.

Radical production is also prevented by the sequestration of redox active metals by specific proteins (metallothionein, ceruloplasmin, ferritin, lactoferrin, transferrin), which impedes their participation in Fenton reactions. For instance, copper distribution is finely tuned, so that this

✔ **Inflammatory-immune diseases**
Auto-immune diseases
Rheumatoid arthritis

✔ **Physical factors**
Radiation injury (radiotherapy, accidental exposure)
Solar radiation (porphyria)

✔ **Degenerative syndromes**
Ageing
Cataract
Retrolental fibrosis
Alzheimer's disease
Parkinson's disease
Atherosclerosis
Amyotrophic lateral sclerosis

✔ **Ischemia-reperfusion states**
Myocardial, brain, intestinal infarction
Organ transplantation

✔ **Drug and toxin-induced reactions**
Cancer (smoke, chemicals, asbestos etc.)
Iron overload (from multiple transfusion)
Alcoholism
Hemolytic syndromes (favism, antimalarial drugs etc.)
Lung emphysema
Bleomycin toxicity
Paraquat toxicity
Adriamycin cardiotoxicity
Endotoxin liver injury
Halogenated hydrocarbon liver injury

Figure 2. Clinical conditions in which the involvement of oxidative stress has been suggested.

metal never exists in the free form: indeed, after intestinal uptake, copper is transported by ceruloplasmin in plasma and delivered to cells where it is complexed to amino acids and metallothionein and other copper proteins including superoxide dismutase[17].

The interception of oxyradicals is carried out by non-catalytic or catalytic molecules. Oxygen radical scavengers are represented by soluble antioxidants such as ascorbic acid, urate, glutathione, flavonoids or lipid-associated molecules such as vitamin E, ubiquinone and vitamin A[16]. Upon reaction with oxyradicals these molecules are transformed into radicals having a longer lifetime and low reactivity.

Catalytic defence is carried out by three enzymes which act in concert: superoxide dismutase, which produces H_2O_2 by dismutation of O_2^-, catalase and peroxidases which eliminate H_2O_2 and organic peroxides. Among these, of particular relevance are the glutathione peroxidases which utilize glutathione as a substrate[3].

Glutathione, a tripeptide composed of glycin, cysteine and glutamic acid, represents the most abundant sulphydryl-containing compound in cells[18]. It deserves particular mention because of the multiple roles it plays: as an interceptor of free radicals, as a co-factor of antioxidant enzymes (Fig. 4), and finally as a metal-chelator, a function which will be discussed below.

Despite the existence of systems directed to the prevention or to the interception of oxygen free radicals, the oxidative stress of macromolecules may still occur: therefore, as a third level of defence, oxidatively damaged molecules are repaired by specific enzymatic systems[16].

Novel Roles for Superoxide Dismutase and Glutathione in the Prevention of Oxidative Stress

In 1939 a copper protein was isolated from bovine erythrocytes and named erythrocuprein. A functional role for this protein was postulated by McCord and Fridovich[19] in 1969, when they discovered that it could act catalytically dismutating O_2^- to H_2O_2. This enzyme was then called superoxide dismutase and its characterization showed that it is organized as a homodimer, presenting two equivalents of copper per mole of enzyme, together with two equivalents of zinc, which is catalytically inert. This protein has been reported to be present mainly in the cytosol of eukaryotes. Later, other proteins containing different metals at the active sites were also shown to perform the dismutation of O_2^-: a form present in mitochondria and prokaryotes containing manganese, and a form exclusively present in bacteria identified as an iron-protein[20]. While for the manganese-containing enzyme a clear relationship between its occurrence and the presence of oxygen has been demonstrated[21,22], thus confirming its role as an antioxidative enzyme, the same cannot be said for the copper, zinc-containing enzyme (Cu, Zn SOD). In fact the intracellular level of this protein is not directly influenced by the oxygen tension of the environment.

Recent work in our laboratory has evidenced that copper can regulate the intracellular level of this enzyme: thus,

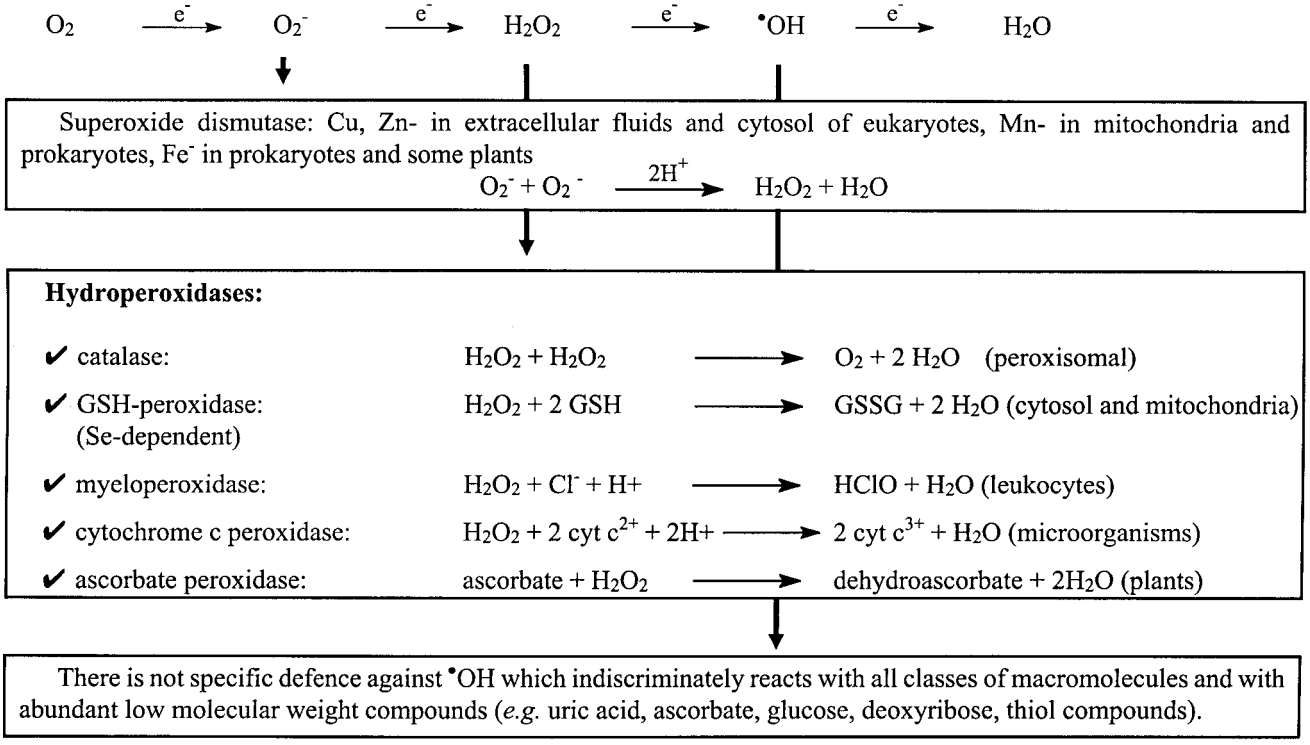

Figure 3. Defence against oxygen species in biological systems.

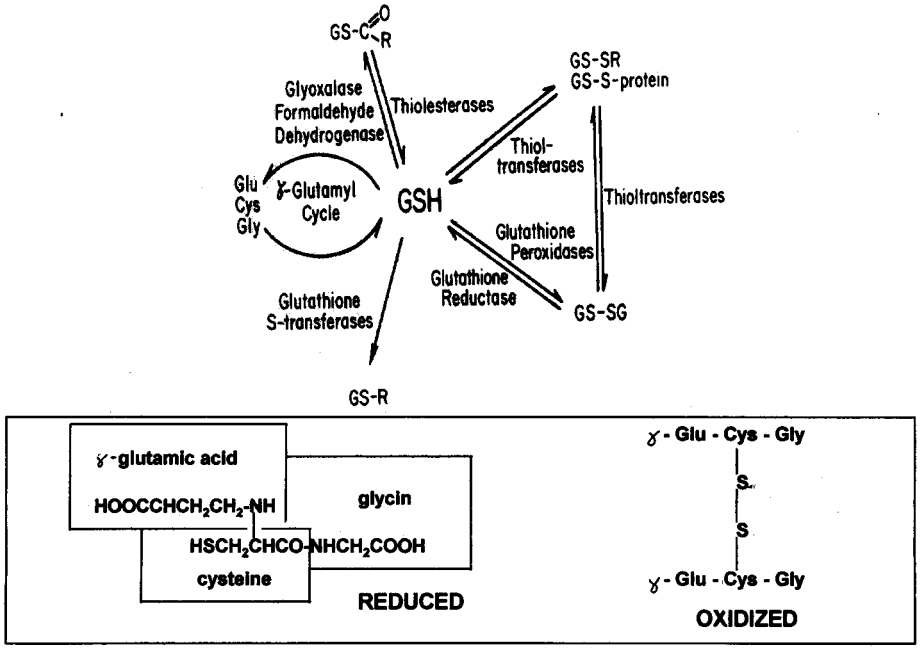

Figure 4. Intracellular glutathione (GSH) functions (*top*) and its reduced and oxidized states (*bottom*).

a novel physiological role of the Cu, Zn protein in copper metabolism has been postulated, *i.e.* as a chelator of potentially toxic transition metals, protecting the cells from their noxious effects.

This aspect has been investigated in several experimental model systems, the most extensively studied being the yeast *Saccharomyces cerevisiae*[23].

We have observed that in this organism Cu, Zn SOD activity increases with changes in the copper concentration in the medium[24], whereas manganese-SOD activity does not[25]. The effect on Cu, Zn SOD cannot be explained only on the basis of oxygen-dependent redox cycling of the metal ion, because a copper-dependent increase of Cu, Zn SOD is also observed under anaerobic conditions. As an explanation for this phenomenon we found that in *S. cerevisiae* the synthesis of Cu, Zn SOD is regulated by copper at the gene level, by the transcription factor ACE1, which was already known to regulate metallothionein expression[26]. The ACE1 factor is codified by the CUP1 gene, which is involved in the protection from copper toxicity. In fact, when copper is available, the protein ACE1 folds in an active form capable of binding to the promoter region of the metallothionein gene, causing a many-fold induction of the transcription. We found that this region is highly homologous to the promoter of the gene codifying for Cu, Zn SOD, and we demonstrated that yeasts lacking ACE1 for mutation of the CUP1 gene do not increase the synthesis of Cu, Zn SOD in response to copper challenge[27]. Therefore, in *S. cerevisiae* copper regulates Cu, Zn SOD and metallothionein *via* the same molecular mechanism. The data described above are summarized in Fig. 5.

The presence of copper at the catalytic site of Cu, Zn SOD suggests that copper availability can also limit the enzymatic activity, thus exerting a regulation at the post-transcription level. Indeed, we demonstrated the presence of a copper-free proenzyme in *S. cerevisiae* growing under anaerobic conditions, which can be reactivated upon copper addition[24]. On the basis of these data, we speculate that Cu, Zn SOD plays a role in the homeostasis of copper and other metal ions which are harmful to yeast. This hypothesis was recently confirmed by the identification and purification of an Ag, Zn superoxide dismutase, with the silver located at the copper site, from *S. cerevisiae* growing in the presence of $AgNO_3$[28].

Another experimental model where copper regulates Cu, Zn SOD at both the transcription and post-translation

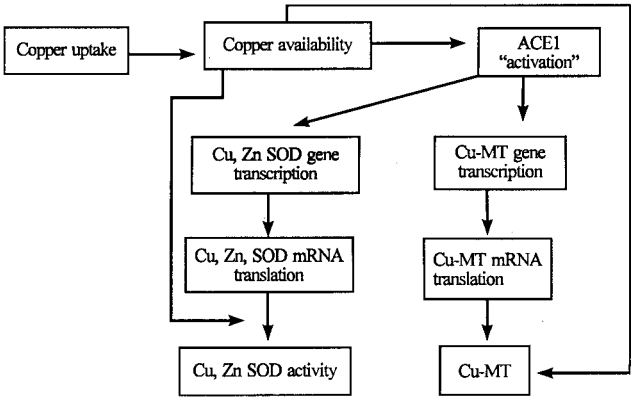

Figure 5. Copper metabolism in *Saccharomyces cerevisiae* and regulation of superoxide dismutase (SOD) and metallothionein (MT).

levels uses the liver of rats made copper-deficient by dietary manipulation. In this system, copper-deficiency produces a loss of SOD activity in the liver only that is higher than the loss of SOD as a protein, because of the existence of a stable form of enzyme void of copper, and therefore inactive[29]. This apo-form can be reactivated upon addition of $CuSO_4$ to liver homogenates (Fig. 6). Since the residual activity is close to that measured in heart and brain of copper-deficient rats, it is possible that rat liver SOD may act as a pool consisting of two fractions: a major one which serves as a copper-binding protein, sensitive to copper availability, and a smaller one essential for antioxidant defence.

A less active copper-deficient enzyme is also present in human tumor cells, specifically erythroleukemia cells belonging to the line K562, when they are in the fully undifferentiated state[30] (Fig. 7). However, when these cells undergo differentiation along a pseudoerythroid pathway, where inhibition of cell growth and synthesis of hemoglobin occur, despite the constant levels of mRNA and protein, the dismutating activity increases. Again we demonstrated that the rise in activity is due to a higher availability of copper which fills up the proper sites of the protein.

In this model system reduced glutathione is likely to play a role in delivering copper to the copper deficient protein, since differentiated K562 cells, when depleted of glutathione, were not able to accumulate copper and recover Cu, Zn SOD activity[30].

Glutathione, beside its remarkable function in intracellular antioxidant defence, has been recently discovered to be involved in metal ion intracellular traffic. Freedman *et al.*[31] demonstrated that the resistance of hepatoma cells to

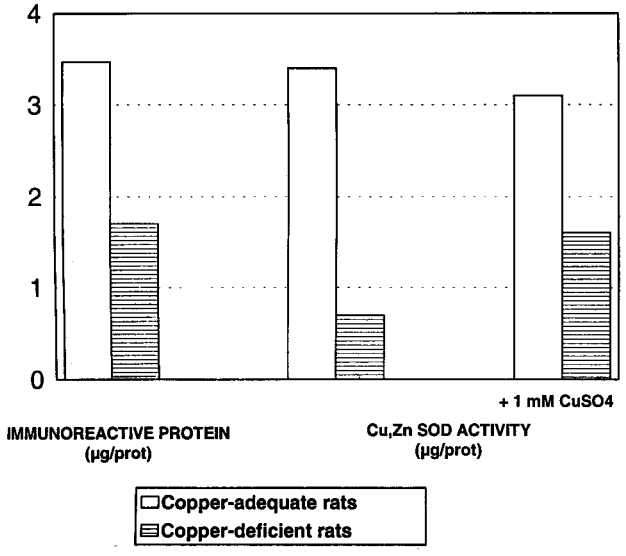

Figure 6. Comparison between Cu, Zn superoxide dismutase activity and immunoreactive protein in the liver of copper-deficient rats.

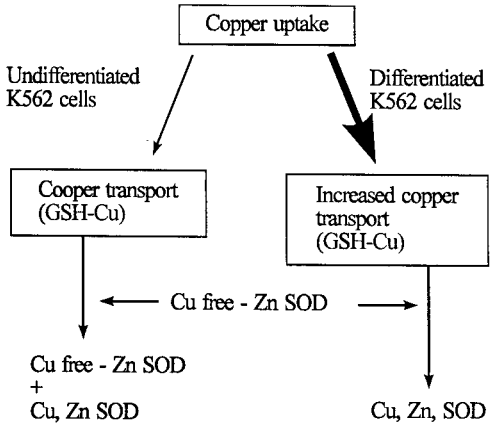

Figure 7. Copper metabolism in K562 erythroleukemic cells.

copper was associated not only with an increase of metallothionein but also with an increase of intracellular glutathione. Furthermore, radioactive copper supplied to hepatoma cells was found to be transiently bound to glutathione, before being associated to metallothionein or superoxide dismutase. This suggested that glutathione could be an important aid in delivering copper to apo-copper proteins *in vivo*. This was confirmed by the *in vitro* formation and characterization of the complex between reduced copper and glutathione[32]. This complex was found to be the most efficient metal donor for purified copper-free proteins, namely Cu, Zn SOD[32] and metallothionein[33].

In conclusion our results demonstrate that both SOD and reduced glutathione appear to exert their antioxidant activity by controlling not only the lifetime of oxyradicals but also metal binding and transport. This property results in a synergetic effect which optimizes their biological function as a major antioxidant. From an evolutionary point of view, it is likely that their metal-binding function could have been predominant in anaerobic life under reducing conditions, and the antioxidant activity could have emerged from their redox-properties in the presence of oxygen.

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