SUPEROXIDE DISMUTASE: AN EMPHASIS ON NICKEL SUPEROXIDE DISMUTASE CHEMISTRY AND FUTURE PERSPECTIVES

by

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(Under the Direction of Todd Harrop)

ABSTRACT

Free radicals and reactive oxygen species can have adverse affects on human health. Many pathological conditions including cancer are related to free radical mediated cellular damage. Antioxidant molecules and enzymes in the body act as a defense system against the potential toxicity of these harmful molecules. Superoxide dismutases are special metallo-enzymes that protect cells from deleterious superoxide anion radical by a catalytic disproportionating mechanism. Four classes of superoxide dismutases based on the active site metal are known to date. A novel nickel containing superoxide dismutase has been characterized recently. The chemistry, enzymatic function, and the future perspective of this enzyme as a therapeutic agent in curing diseases are reviewed in this thesis.

INDEX WORDS: Free radicals, superoxide anion radical, antioxidants, oxidative stress, superoxide dismutase, SOD therapy

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To my beloved parents, my brother, my sister,

Manju, and Sandali

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CHAPTER 1

REACTIVE OXYGEN SPECIES

1.1. REACTIVE OXYGEN SPECIES

All aerobic organisms including humans consume oxygen for cellular respiration. This is a complicated cellular metabolic process occurring in mitochondria inside the cell where oxygen is ideally converted into water. However, at the end of the mitochondrial respiratory chain, a small percentage of oxygen (1% - 2%) undergoes partial reduction into superoxide anions and other byproducts. These small molecules are collectively known as Reactive Oxygen Species (ROS) and are characterized by their instability and high reactivity.

ROS exist in two forms, free radical and non-radical ROS. Free radical ROS include hydroxyl radical $^{\bullet}OH$, superoxide anion radical $O_2^{\bullet -}$, singlet oxygen $^1O_2$, peroxyl radicals $ROO^{\bullet}$, alkoxyl radicals $RO^{\bullet}$, thiyl radicals $RS^{\bullet}$, oxides of nitrogen, $NO^{\bullet}$ and $^{\bullet}NO_2$. Hydrogen peroxide H_2O_2 and hyperchloride HOCl are examples of two non-radical ROS (Halliwell 1999). Free radicals have one or more spin unpaired electrons, and usually they are intermediates of chemical reactions with a relatively short life time (10^{-11} – 10^{-9} sec). These unpaired electrons are highly unstable and tend to gain stability by pairing up with another electron from an adjacent molecule by donating, abstracting or sharing an electron. This reaction usually does not terminate in one single step, instead, initiates a chain reaction making the second molecule a radical.

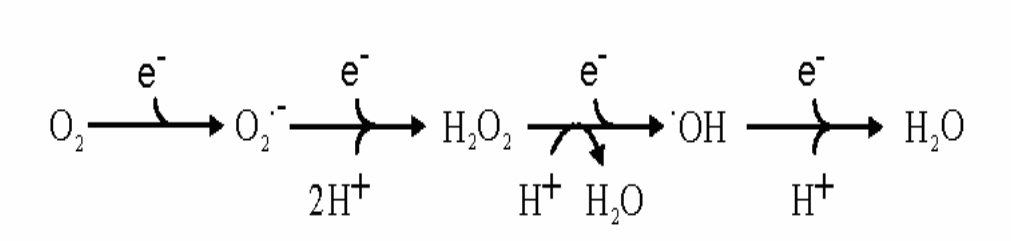
ROS play a crucial role in the chemistry of our body. In a regulated environment, these short-lived small molecules can function as intracellular signaling molecules. At the end of each mitochondrial respiration chain reaction, superoxides are formed and further converted into other

forms of ROS such as hydrogen peroxide and hydroxyl radical. Other sources of endogenous ROS include peroxisomes and cytochrome P450. Exogenous sources of generation of ROS include UV light, ionizing radiation, inflammatory cytokines, and various pathogens. In the defense mechanism of host microorganisms and pathogens, leukocytes produce large amount of superoxides. Cigarette smoke is highly saturated with free radicals, which is a very common source of external ROS. Ionization reactions from exposure to radiation such as radon, ozone, and UV light can also produce free radicals inside our body.

These ROS are in balance with biochemical antioxidants such as glutathione, α-tocopherol, and superoxide dismutases inside the body. Deviation from this nature-designed ROS-antioxidant balance is called "Oxidative Stress", which is one of the primary causes of many significant health problems. Mutations caused by damage to DNA by ROS are known to be one of the leading causes of tumorigenesis and cancer.

1.2. CHEMISTRY OF ROS AND FREE RADICALS

Superoxide is the one electron reduced form of molecular oxygen due to the direct reaction of electrons with oxygen during the mitochondrial respiratory chain (Fridovich 1997). Mitochondria generate energy in a four step reduction chain reaction (eq. 1) in which oxygen is reduced to water.



Eq.1: Mitochondrial oxygen reduction

Superoxide can also be produced in activated polymorphonuclear leukocytes in response to inflammations. It has been estimated that each cell in our body is exposed to about 10^{10} superoxide molecules each day and generates approximately 3 x 10^9 molecules of H_2O_2 per hour (Newcomb 1996). This molecule is the precursor ion for many other ROS such as hydroxyl radicals and singlet oxygen. Reaction with nitric oxide can yield peroxinitrite, which will further react with hydroxyl radicals causing NO-mediated cell injury. Hydroxyl radical $^{\bullet}OH$ is formed as a result of the reaction between superoxide anion and hydrogen peroxide in the presence of a catalytic metal such as copper or iron (eq. 2). It has the highest reactivity and the highest one-electron reduction potential (2310 mV versus Ag/AgCl) among all the other free radicals in the body. Hydroxyl radical can easily add to a double bond, hence reacting with lipids, proteins, peptides, and DNA.

$$\mathbf{O_2^{\bullet -} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + O_2} \qquad \mathbf{(2)}$$

Hydrogen peroxide is usually the product of dismutation of superoxide anion. It has the least reactivity among all ROS (eq. 3). However, it has a higher ability to penetrate the plasma membrane.

$$\mathbf{2\ O_2^{\bullet -} + 2H^+ \rightarrow\ H_2O_2 + O_2} \qquad \mathbf{(3)}$$

Hydrogen peroxide is a strong agent in lipid peroxidation which also can be decomposed into hydroxyl radical $^{\bullet}OH$, in the presence of transition metals such as iron and copper, according to the Fenton reaction (Fenton 1984) (eq.4). It can also react with hemoglobin releasing free iron.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-} \qquad (4)$$

Reaction of alkyl radicals and oxygen generates peroxyl radicals and the decomposition of alkyl peroxides (ROOH) yields both peroxyl ($ROO^{\bullet}$) and alkoxyl radicals ($RO^{\bullet}$). Homolysis of peroxides in the presence of UV light or transition metals yields peroxyl and alkoxyl radicals (eq. 5, 6).

$$ROOH \rightarrow ROO^{\bullet} + H^{\bullet} \qquad (5)$$

$$ROOH + Fe^{3+} \rightarrow ROO^{\bullet} + Fe^{2+} + H^{+} \qquad (6)$$

Both of these are good oxidizing agents and can easily abstract hydrogen from other molecules. Open chain peroxyl and alkoxyl radicals are more reactive than aromatic derivatives due to the stabilization of free radicals by electron delocalization by the aromatic ring. Nitric oxide $NO^{\bullet}$ is formed from the enzyme NO-synthase from L-arginine and dioxygen. It is an important chemical mediator generated by the endothelial cells, macrophages, neurons, and some other cell types. Excess $NO^{\bullet}$ is involved in ischemic reperfusion, neurodegenerative, and inflammatory diseases. Peroxynitrite $^{-}OONO$ is a highly toxic molecule which is formed by the reaction between superoxide anion and nitric oxide free radical (eq.7).

$$O_2^{\bullet -} + NO^{\bullet} \rightarrow {}^{-}OONO \qquad (7)$$

Tissue damage, protein oxidation, and DNA modifications are caused by the cytotoxicity of peroxynitrite.

The reaction of NO with peroxyl radicals yield nitric dioxide NO_2· radical. Peroxinitrite also can generate nitric dioxide radical (eq. 8).

$$ONOO^- + H^+ \rightarrow {}^{\bullet}OH + {}^{\bullet}NO_2 \qquad (8)$$

It also has several other effects such as the selective nitration of tyrosine residues in proteins including prostacyclin synthase and Mn-SOD.

1.3. ROS AND HUMAN HEALTH

Due to the high reactivity and extensive damaging nature to cells, ROS have been known to have a direct relationship to the etiology of a wide array of human diseases. Superoxide anion which is produced inside the body can be highly toxic if the intracellular concentration reaches nanomolar level. There is a large body of research and literature that support the concept of involvement of ROS and free radicals in human pathology. Radiation injury (damage caused by free radicals formed due to exposure to radiation) is one of the main forms of ROS mediated health problems that both cause acute toxicity and long term health risks. One extreme case is the thermonuclear explosion in Hiroshima and Nagasaki which killed thousands instantaneously and caused development of free radical mediated injuries such as leukemia and lymphoma after several years. Neurodegenerative disorders like Amyotrophic Lateral Sclerosis (ALS or Lou Gehrig's disease), Parkinson's disease, Alzheimer's disease, Prion diseases and other common cases like myocardial and cerebral ischemia, complicated forms of diabetes, vasospasm, hyperoxia, arthritis, dermatitis, cataractogenesis, retinal damage, liver injury, asthma, cancers of various kinds, and even aging have a physiopathological connection to ROS (figure 1.1). This damage can take place via many mechanistic pathways such as free radical mediated damage to

DNA, proteins and lipids and oxidation of lipoproteins, induction of oxidative stress, and disregulation of normal pathogenic defense system. This ROS mediated damage is primarily caused by a mechanism called lipid peroxidation.

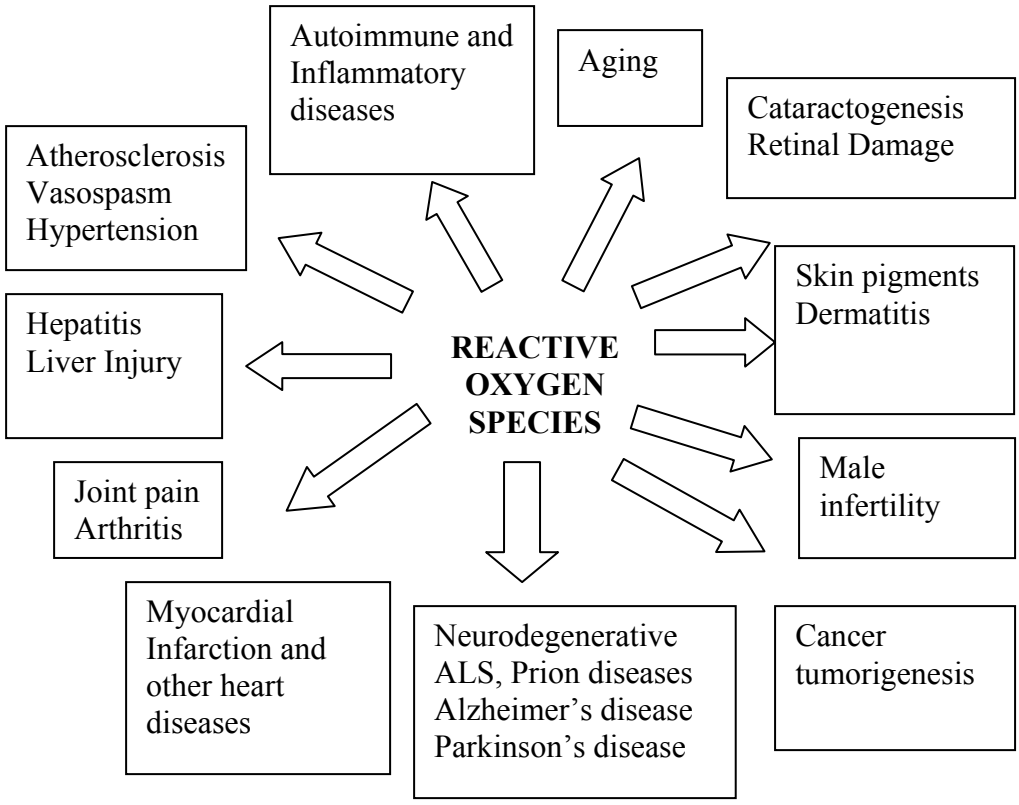


Figure 1.1. Implication of ROS in human health

LIPID PEROXIDATION

Hydroxyl radicals are very reactive and can abstract hydrogen from an unsaturated fatty acid producing a lipid radical, which in turn reacts further with molecular oxygen forming a lipid peroxyl radical (figure. 1.2).

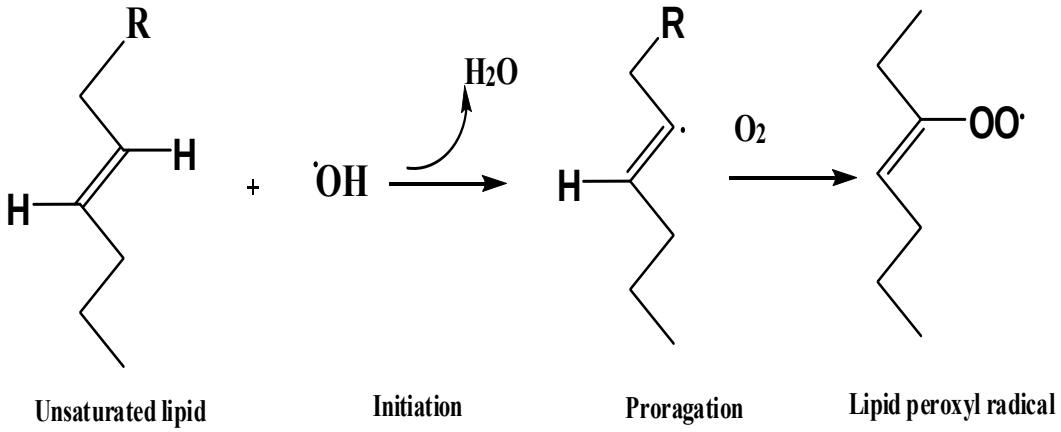


Figure 1.2. The mechanism and the products of lipid peroxidation

This is a chain reaction and eventually destructs the coherence and the integrity of the membrane structure. This oxidative "deterioration of polyunsaturated lipids" impairs the enzyme receptors, and transport proteins (Halliwell & Gutteridge 1999).

ALS- AMYOTROPIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease) is a very serious neurodegenerative disorder which causes the death of motor neurons in the brain, brain stem and the spinal cord. This disease is progressively fatal and usually the patient dies between 2-5 years after symptoms appear. "Amyotrophic" refers to the degradation of muscles and "Sclerosis" is a term which describes the degradation of neurons in the spinal column. French neurologist Jean-Martin Charcot first discovered the motor neurons in the spinal cord that get affected by ALS in 1869. Due to its deleterious and painful nature, the disease is even considered as a candidate for assisted suicide. According to the statistics from the National Institutes of Health, approximately 5000 new patients are diagnosed with ALS each year and it is becoming more prevalent in the United States. Both men and women between the ages of 40-70 are susceptible to this condition

and approximately 90% of these cases are sporadic (sALS). Other cases are related to gene alterations and categorized as familial (FALS) (Valentine, J.S., Doucette, P.A., Potter, S.Z., 2005). The defective gene is identified as SOD-1 and occurs in chromosome 21 (Rosen et al., 1993; Vijayvergiya 2005). The actual physiological cause is the damage of motor neurons by a free radical mechanism where SOD (Superoxide Dismutases) fail to neutralize the deleterious effect of free radicals (function of SOD will be discussed in the next section). Initial symptoms are weakness and cramps in muscles of both the hands and legs, difficulty of speaking, chewing, swallowing etc. In the later stages of the disease, patients suffer from breathing problems and usually the death is caused by respiratory failure (Valentine 2005). No permanent cure has been found for this tragic condition. However, certain medications such as Zyloprim (Allopurinol), Gabapentin (Neurontin), Myotropin, and Roluzole are in use in order to alleviate the pain associated with the disease.

ROS AND CANCER

The term cancer entails a wide range of conditions of uncontrolled cell growth and proliferation due to changes in the genetic environment of the cells. Cancer affects people of all ages and is the leading cause of death worldwide. Cancers can be localized or invasive. Most malignant cancers intrude on and destruct adjacent tissues. They can also spread to other locations in the body via lymph or blood, which is called metastasis. Nearly all cancers are due to abnormalities in the genetic material of the transformed cells. Various interactions of ROS with cellular components such as DNA base modification, DNA sequence changes, miscoding of DNA, gene duplication, and activation of oncogenes are implicated in the initiation of cancers (figure 1.3) (Waris 2006). Mutations caused secondary to prolonged oxidative stress is postulated to be one of the causes of tumorigenesis (Feig 1994).

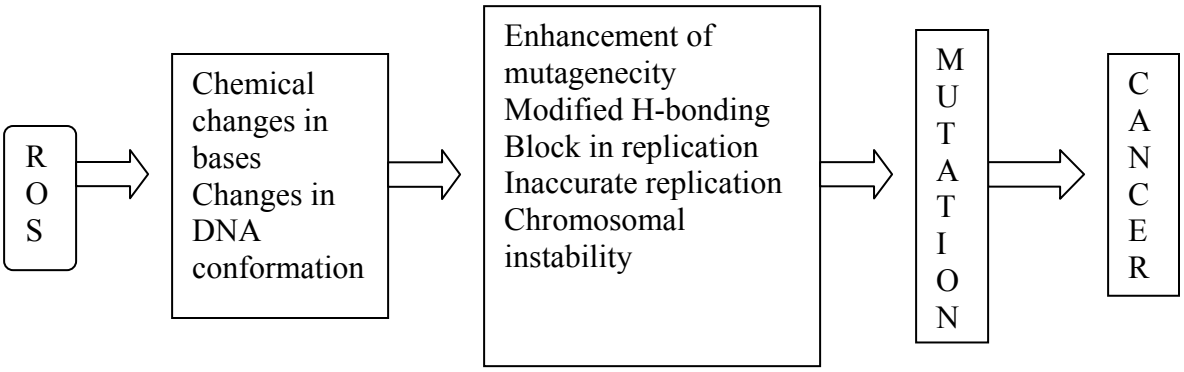


Figure 1.3. ROS leading to cancer

Patients having precursor diseases of cancer such as Fanconi anemia, chronic hepatitis, cystic fibrosis, and some auto-immune diseases have been routinely detected to have elevated levels of oxidative DNA damage, hence neoplastic transformations of cells leading to cancer. Chronic infection of the liver by hepatitis B or C viruses or ingestion of aflatoxins give rise to oxidative stress of the hepatic cells over time, thus initiating hepatic carcinoma. Another piece of evidence that ROS can be related to the initiation of cancer is the onset of prostate cancer in the later part of life where no direct correlation to carcinogens is found except the postulated endogenous cellular processes leading to the disease. No unambiguous data about the potential chemicals and environmental factors that can cause prostate cancer are established, therefore, it is accepted that cancer in the prostate is due to age related factors like accumulation of genotoxins from ROS. Thus, ROS play roles in both the initiation and the progression of malignancy. Cancerous cells lack oxygen, therefore, experience higher degree of oxidative stress. Moreover, tumors of all kinds and other types of cancers generate superoxide anions which in turn enhance further damage.

ROS AND AGING

Aging is a process where cells in our body continuously undergo detrimental changes. Loss of mitochondrial function which is responsible for the generation of energy in the cell is thought to be the primary cause for aging. The antioxidant defense capacity in the body decays over aging, and therefore, cells experience oxidative stress (Halliwell 1997). Increase in various forms of oxidized lipids, nucleic acids, proteins, sugars, and sterols are generally associated with aging (Ashok and Ali 1999). ROS and free radicals are the main oxidizing agents that contribute to the elevation of oxidized species. High levels of accumulated ROS induce programmed cell death, hence aging. Free radical theory of aging (Harman 1983) describes that aging is either due to loss of mitochondria which produce energy or due to lesser number of mitochondria as a result of free radical damage. Research on this field has shown that the mutations in mitochondrial DNA can accelerate premature aging (Martin, G.M. 2005).

ROS AND REPRODUCTIVE HEALTH

Recently, more emphasis has been given to the role of ROS on human sperm cells and the etiology of male infertility. Alteration of the mobility of sperm cells and the decrease in the ability of sperm cells to bind with the zona pellucida and fuse with the oocyte membrane causing infertility are linked to the damage caused by ROS. Evidently, a higher number of ROS are routinely detected in semen samples of males having infertility (Iwasaki & Gagnon, 1992). Macleod first postulated the involvement of oxidative stress in sperm malfunction in 1943. Many years later Mann and Jones (1973) confirmed that lipid peroxidation and oxidative stress are inevitable factors for male infertility. Human sperm cells have a very complex lipid composition with each lipid having a distinct function (Sanocka, D. and Kurpisz, M., 2004). Hydrogen peroxide was identified as the main ROS in semen, which causes the peroxidation of membrane

lipids of spermatozoa (Le Lannou 1997). Low concentrations of ROS can act as mediators for the healthy function of sperm whereas excess of ROS can have adverse affects. ROS in human ejaculate come from many sources, semen itself, precursor germ cells, abnormal sperm cells, and leukocytes present in semen (Alvarez et al., 1978; Aitken, et al., 1994). In extreme conditions like genital track inflammation, excess amount of ROS are released to semen and greatly damage the healthy function of sperm cells.

PRION DISEASES

Prion diseases, a class of fatal neurodegenerative diseases also known as transmissible spongiform encephalopathies (TSE's) found in mammals including humans, are caused by the alteration of normal Prion protein Pr-PC into abnormal isoforms of infectious and pathogenic forms of Pr-PSc. The central nervous system is primarily affected and is characterized by neuron cell loss, vacuolation, astrocytosis, and the presence of amyloid plaques (Catellani 2004). Oxidative stress caused by ROS is believed to be the basis for these alterations, however, adequate proof has yet to be established and thus the need for further elucidation of the etiology of these diseases. The involvement of trace elements in prion diseases has also been reviewed recently (Leach 2007). Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker syndrome (GSS), Fatal familial and sporadic insomnia (FFI and sFI), kuru, new variant Creutzfelds-Jacob disease (nvCJD) are the five phenotypes of human prion diseases (Smith, M.A. 2004; Prusiner, S.B., 1991; Hsiao, K., 1990). In contrast to the infectious and transmissible animal forms, human Prion diseases are rather inherited (mutations in the PRNP gene) or sporadic. Prion diseases are not transmitted by casual means such as touching or sharing utensils. However, it can be transmitted by accidental exposure to prion-contaminated tissues or blood during a medical procedure.

1.4. IMPORTANT FUNCTIONS OF ROS AND FREE RADICALS

The potential harm of free radicals and other ROS of both endogenous and exogenous sources were discussed in the previous section. However, these chemical agents also play a very critical and highly specific function in our body under regulated conditions because free radicals meet many criteria for biological messengers such as short-life time, degradability, controllability and reusability. Signal transduction, apoptosis, differentiation and senescence, activation of nuclear transcription factor, gene expression, and defense against host microbial infections are recognized as some of the helpful functions of free radicals (Fruehauf 2007). They are also responsible for the control of vascular tone and neurotransmission. The importance of $^{\bullet}$NO in cardiovascular function and maintenance is discussed in many reviews. The depletion of bio-available $^{\bullet}$NO can cause many cardiovascular conditions such as heart failure, sepsis, coronary artery diseases (CAD), and myocardial infarction (MI) (Radi 2007). The role of $^{\bullet}$NO in penile erection is an undisputable example for the necessity of free radical intervention in bodily functions (Snyder 1992). The defensive role of singlet oxygen against various pathogens and other inflammatory agents has also been reported. $^{\bullet}$NO executes many important functions including neurotransmission and the immune response. Moreover, targeted and regulated free radicals from drugs can be administrated in chemotherapy of tumors to kill oxygen lacking cells.

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CHAPTER 2

SUPEROXIDE DISMUTASE (SOD)

2.1. SUPEROXIDE DISMUTASES [SOD]

Nature has provided an amazing way of scavenging poisonous free radicals and other ROS from our body in order to maintain the desired homeostasis of the cellular chemistry. Antioxidants and enzymatic-antioxidants neutralize the effect of free radicals and ROS in a variety of different mechanisms. Without these vital chemical agents, living organisms would not survive the deleterious effects of free radicals and ROS. Antioxidants are usually small molecules and are found in many fruits and vegetables in our daily consumption. Alpha tocopherol (vitamin E) Ascorbic acid (vitamin C), beta carotene, lycopene, lutein are some examples for antioxidants from food sources (figure 2.1, 2.2, 2.3.). Enzymatic antioxidants [Superoxide dismutases (SOD), Glutathione peroxidase (GPx), and Catalase (CAT)] are found in the body and they are usually macromolecules. Common antioxidants and enzymatic antioxidants that are found in our body are listed in table 1. Glutathione peroxidase is a selenium containing tetrameric glycoprotein. Glutathione peoxidase utilize GSH, the reduced monomeric glutathione, to donate hydrogen whereby GSH is oxidized to glutathione disulfide (eq. 9). The catalytic cycle is completed by glutathione reductase (GR) (eq. 10).

$$\mathbf{2GSH + H_2O_2 \rightarrow GS–SG + 2H_2O} \qquad \mathbf{(9)}$$

$$\mathbf{GS–SG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+} \qquad \mathbf{(10)}$$

Catalase is an enzyme which can be found in almost all organisms. It catalyzes the reduction of hydrogen peroxide into oxygen and water (eq. 11).

$$2H_2O_2 \rightarrow 2H_2O + O_2 \qquad (11)$$

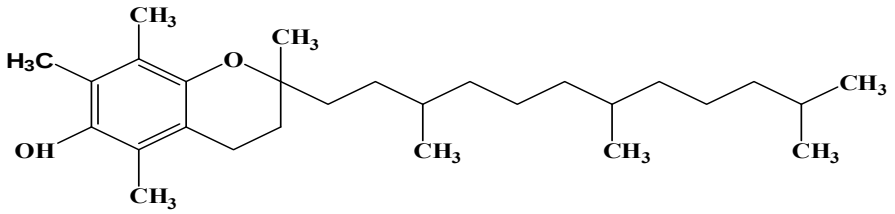


Figure 2.1. Alpha tocopherol (vitamin E)

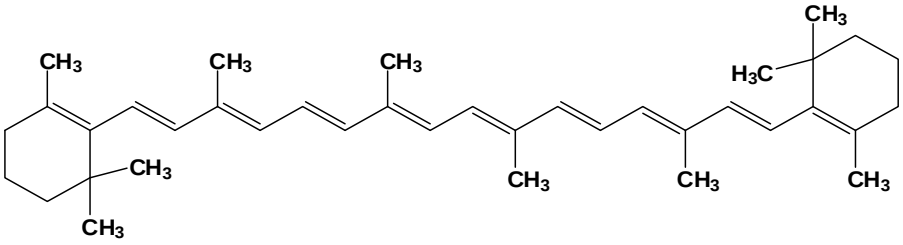


Figure 2.2. Beta carotene (vitamin A)

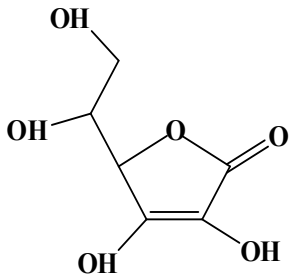


Figure 2.3. Ascorbic acid (vitamin C)

Table 1. Antioxidant defense system in the body

Antioxidant defense	Source/ occurrence
Antioxidants α– tocopherol (vitamin E)	Vegetables/ vegetable oil, meat, fish
Ascorbic acid (Vitamin C)	Citrus fruits, vegetables
B-Carotene	Yellow/ orange vegetables and fruits, leafs
Reduced glutathione GSH	Cytosol, Extracellular fluid
Uric acid	Cytosol, Extracellular fluid
Antioxidant enzymes	
Superoxide dismutase	
Cu/Zn-SOD	Cytosol and extracellular fluid
Mn-SOD	Mitochondria
Fe-SOD	Cytosol
Catalase	Peroxisomes
Glutathione Peroxidase	Cytosol, extracellular fluid

Superoxide dismutases convert highly reactive superoxide radical to lesser reactive H_2O_2 which will further be eliminated by catalases and peroxidases (eq.12).

$$\mathbf{2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2} \qquad \mathbf{(12)}$$

Three forms of SOD are present in the human body; SOD-1 (CuZn-SOD) in the cytoplasm, SOD-2 (Mn-SOD) in the mitochondria, and SOD-3 (EC CuZn-SOD) in the extracellular environment. Superoxide dismutases with different metal cofactors and their occurrence are shown in table 2.

Table 2. Superoxide dismutases and their occurrence

Metal cofactor	Name	Species	Location
Manganese	sodA	*E. coli*	Intracellular
	Sod-2 and Sod-3	*C. elegans*	Presumed mitochondrial
	CytMnSOD	*C. sapidus*	Cytosolic
	MtMnSOD	*C. sapidus*	Mitochondria
	SOD3	*C. albicans*	Cytosolic
	SOD2	*S. cerevisiae*	Mitochondrial matrix
		Mammals	
Iron	sodB	*E. coli*	Intracellular
Nickel	Ni-SOD	*S. seoulensis*	Intracellular
Copper/Zn	Sod-1 and Sod-5	*C. elegans*	Presumed cytosolic
	sodC	*E. coli*	Periplasmic
	SOD1	*S. cerevisiae*	Cytoplasm/mitochondrial
	SOD3	Mammals	Extracellular

Adopted from [Biochemica et Biophysica Acta, 1763, Culotta, V.C., Yang, M, and O'Halloran, Activation of superoxide dismutases: putting the metal to the pedal, page 748, Copyright (2006)] with permission from Elsevier.

The catalytic activity of SOD is given by the following chemical reaction (eq. 13, 14) where two molecules of superoxide anion are converted to one molecule of molecular oxygen and one molecule of hydrogen peroxide.

$$M^{ox} + O_2^{\bullet -} \rightarrow M^{red} + O_2 \quad (13)$$

$$M^{red} + O_2^{\bullet -} + 2H^+ \rightarrow M^{ox} + H_2O_2 \quad (14)$$

A large body of literature is available supporting the idea that SODs play a defensive role against many human diseases. SOD's defensive role against superoxide mediated cytotoxicity is multifaceted. SOD inhibits reactions such as the inactivation of mitochondrial proteins containing iron-sulfur (Fe-S) centers like aconitase and fumarase, which in turn inhibit the release of free iron and subsequent formation of hydroxyl radical. Decreased amounts of both manganese and copper-zinc containing SOD and increased amounts of superoxide radicals in most tumors provide evidence for the role of superoxides as defensive agents in the human body. Recently, many researches have been emerging to find the enormous value of superoxide dismutases in prevention and cure of diseases (McCord 2005).

2.2. SOD ACTIVITY

The enzymatic activity of SOD can be measured by several direct and indirect assays. The most common method is the spectroscopic monitoring of the reduction of cytochrome C by superoxide anion. xanthine oxidase, a complex molybdoflavoenzyme, containing molybdenum, flavin adenine dinucleotide (FAD), is used as the source of superoxide ion (figure 2.4). Cytochrome C is brightly colored because heme absorbs strongly in the visible range of the electromagnetic spectrum (red orange color). However, using cytochrome C is not good for low levels of SOD because of its high reactivity with superoxide anion. Kinetic data such as the first order rate constant (corresponding to the slowest step (k_{cat})) and enzyme dissociation constant/enzyme affinity for the substrate (K_M) are obtained by other methods such as stopped-

flow or pulsed radiolytic assays. Several other versatile commercial techniques are also available for measuring the SOD activity.

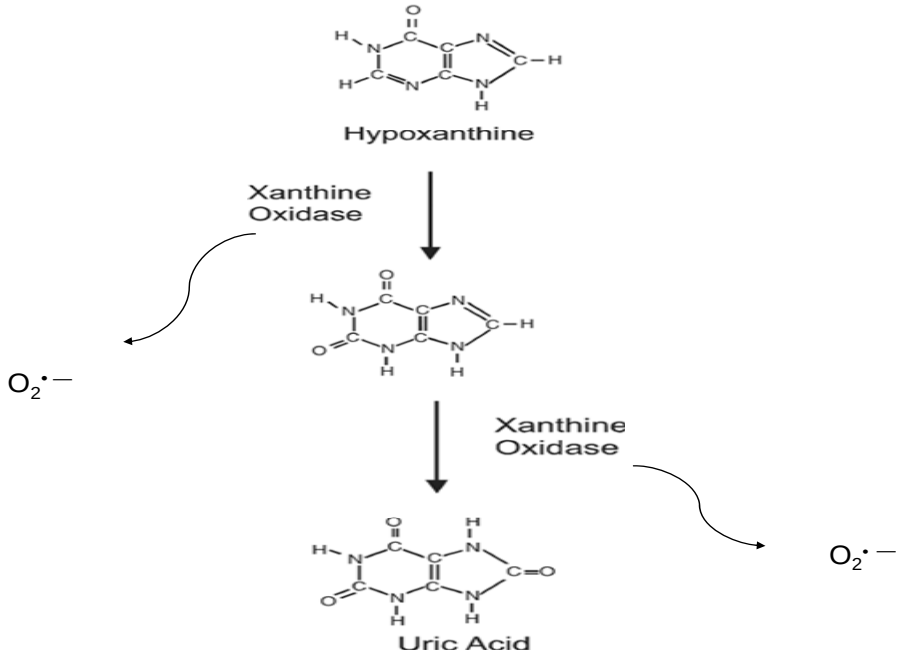
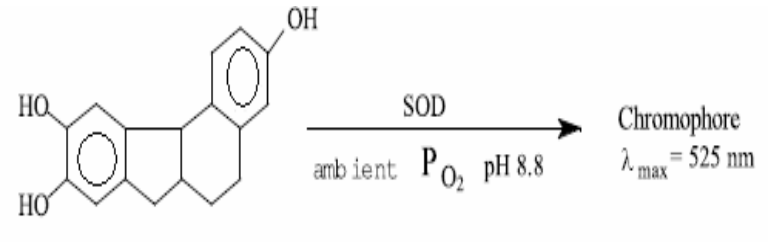


Figure 2.4. Generation of superoxide anion by xanthine oxidase.

In one of these methods, the molecule 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorene (eq. 15) is auto-oxidized in the presence of SOD and alkaline medium yielding a chromophore with λ_{max} = 525 nm. The resultant chromophore has not been isolated or identified yet. Samples have to be pre-treated with 1-methyl-2-vinylpyridinium to avoid the interference with mercapto compounds such as reduced glutathione. SOD activity is determined by comparing the rate of oxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorine in the presence (Vs) and absence (Vc) of SOD (eq.16).



Eq.15. [Source: Spectrometric Assay for Superoxide Dismutase Product No. FR10, www.oxfordbiomed.com]

$$\frac{Vs}{Vc} = 1 + \frac{[SOD]}{\alpha \bullet [SOD] + \beta}$$

Eq. 16. (Vs = rate of sample containing SOD, Vc = Average rate of blank sample (SOD = 0), SOD = SOD activity in SOD units, α = Dimensionless coefficient, β = coefficient in SOD units [Source: Spectrometric Assay for Superoxide Dismutase Product No. FR10, www.oxfordbiomed.com]

One SOD activity unit is defined as the SOD activity required to double the rate of autooxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorine. The direct calculation of SOD activity is possible when α and β are substituted by numerical values 0.073 and 0.93 respectively (eq. 17)

$$[SOD] = \frac{0.93 \bullet \left(\frac{Vs}{Vc} - 1\right)}{1.073 - 0.073 \bullet \left(\frac{Vs}{Vc}\right)}$$

Eq. 17. [Source: Spectrometric Assay for Superoxide Dismutase Product No. FR10, www.oxfordbiomed.com]

2.3. COPPER/ZINC SUPEROXIDE DISMUTASE

CuZn-SOD (SOD1) is an important antioxidant enzymatic protein present in many organisms. CuZn-SOD has been purified from both eukaryotes (mammalian tissues, *Neurospora crassa*) and prokaryotes (*Coulobacter cresentus, Haemophilus influenzae*). In eukaryotic cells, CuZn-SOD is present in the cytosol, nucleus, peroxisomes, and mitochondrial intermembrane, and in bacteria it is found in the periplasmic space. It was first discovered in mammals in 1969 (Fridovich 1969). The human form of CuZn-SOD is a 32 kDa homodimer with one copper and one zinc at the active site (figure 2.5 and 2.6; Copper and zinc ions are shown in blue and orange colors respectively and the intra-subunit disulfide bond is shown in red) [Valentine, J.S., Doucette, P.A., Potter, S.Z. (2005)]. Each monomer is composed of a β-barrel arrangement and contains an imidazolate bridging ligand between Cu and Zn centers. CuZn-SOD exists as homotetramers in the chloroplast of higher plants and as monomers in some gram-negative bacteria (Bertini 1997). Trafficking factors called copper chaperones which are highly specific for copper ion transport deliver copper to the target active site guided by protein-protein interactions (Rosenzweig 2001). This enzyme utilizes the Cu center as the redox active site where copper undergoes oxidation and reduction during the dismutation of superoxide into oxygen and hydrogen peroxide (eq. 18, 19). CuZn-SOD dismutates superoxide at a fairly high rate of catalytic conversion (2×10^9 $M^{-1}s^{-1}$) and is active in a wide (5.0 -9.5) pH range (Cabelli, 2000).

$$O_2^{\bullet -} + Cu^{2+}\text{Zn-SOD} \rightarrow O_2 + Cu^{+}\text{Zn-SOD} \qquad (18)$$

$$O_2^{\bullet -} + 2\,H^{+} + Cu^{+}\text{Zn-SOD} \rightarrow H_2O_2 + Cu^{2+}\text{Zn-SOD} \qquad (19)$$

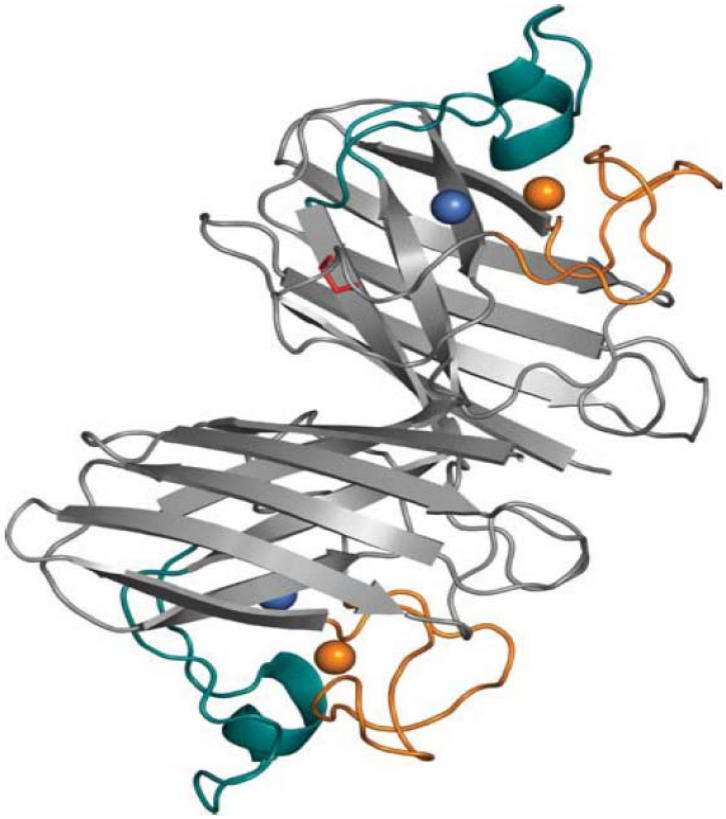


Figure 2.5. Crystal structure of dimeric human CuZn-SOD [Annual Review Biochem. 74,Valentine, J.S. et al., Copper-Zinc superoxide dismutase and Amyotrophic Lateral Sclerosis, Page 566, Copyright (2005)] with permission from Annual Review of Biochemistry.

CuZn-SOD is linked to Familial Amyotrophic Lateral Sclerosis (ALS) and more than one hundred point mutations in the enzyme have been identified in affected patients to date (Cleveland 2001). However, the histopathology of these point mutations causing the disease has not been clearly understood. Recent observations suggest that what cause the disease are not the mutations of the protein but rather gaining of some toxicity of the protein due to misfolding and aggregation (Oliveberg 2006). Immature forms of the disulfide-reduced CuZn-SOD tends to misfold and oligomerize under oxidative stress and is believed to be the basic form of mutated SOD protein aggregation causing neurodegeneracy (Oliveberg 2006). This gain of toxicity

hypothesis was supported by the observation of elevated H_2O_2 and ·OH levels in transgenic mice with mutated SOD1. Furthermore, it was evident that the mutations of SOD1 disrupts the normal detoxifying capacity of other defense enzymes leading to motor neuron death in ALS. CuZn-SOD is also crucial in vascular physiology. Inadequate levels of CuZn-SOD result in increased levels of vascular superoxides and peroxynitrite. Vascular tone is affected by alteration of the enzyme causing conditions such as increased myogenic tone, augmented vasoconstrictor response, vascular permeability after ischemia, and hypertrophy.

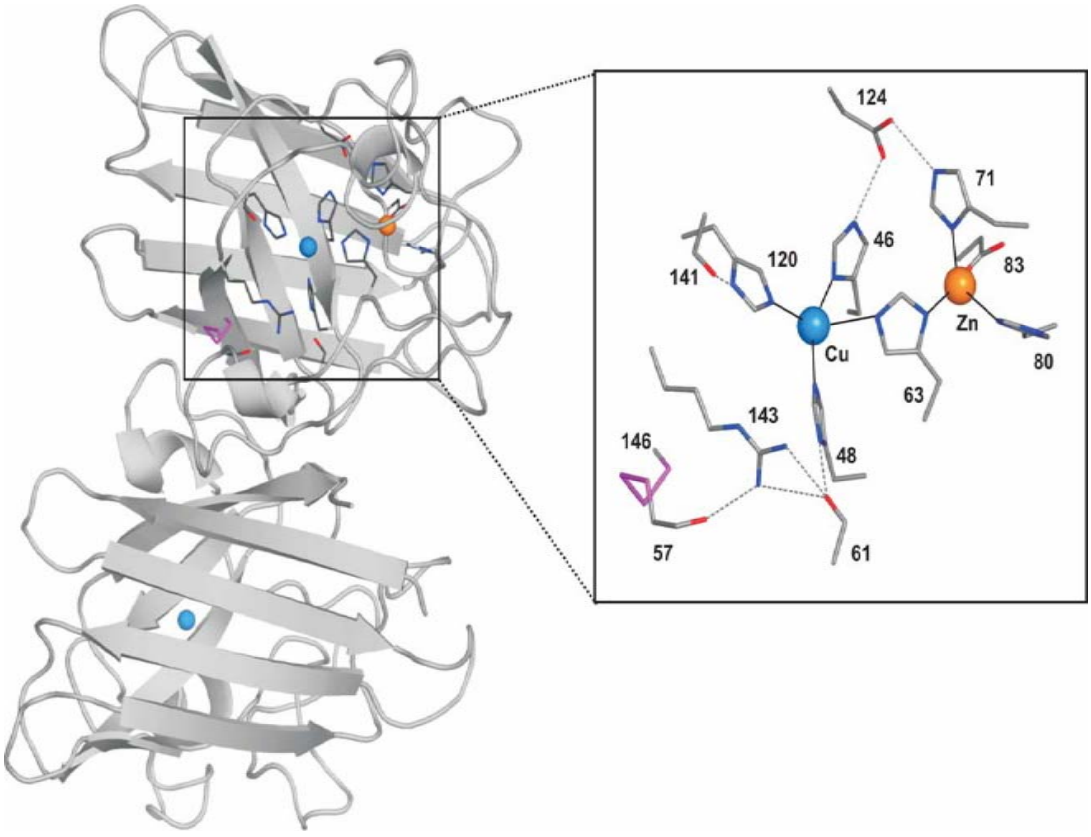


Figure 2.6. Active site and hydrogen bonding network of human CuZn-SOD. [Annual Review Biochem., 74, Valentine, J.S. et al., Copper-Zinc superoxide dismutase and Amyotrophic Lateral Sclerosis, page 567, Copyright(2005)] with permission from Annual Review of Biochemistry.

CuZn-SOD is also related to a number of red blood cell diseases such as anemia (both iron deficient and oxidative hemolytic types), thalassemia, sickle cell anemia, molecular dystrophy and cystic fibrosis (Pan Chenko, L.F., et al. 1979; Mavelli, I., et al. 1984; Concetti et al. 1976; Muzuno, Y., 1984). Alzheimer's disease is characterized by many deleterious neurological changes in the brain which is partly due to altered SOD activity. CuZn-SOD activity, among other SOD's, found to be elevated in both dementia of the Alzheimer type and vascular dementia. The aggregation of higher amounts of the enzyme was observed in large pyramidal neurons in the brain. This lead to the hypothesis that CuZn-SOD can be used as an early diagnosis marker of Alzheimer's disease because alteration of this enzyme activity is inevitably related in the early oxidative stress stages of the disease. Patients with Down's syndrome have an extra copy of the CuZn-SOD gene and therefore possess 50% more SOD activity compared to normal diploid population (De la Torre 1996). This increased SOD activity results in elevated production of H_2O_2 causing toxic activity.

Another form of this enzyme is the EC-SOD (Extracellular SOD; SOD3), which has the same active site metals but exists as a homotetramer of molecular weight 130 kDa. EC-SOD has been purified and characterized from the human lung (Marklund 1982). Similar to SOD-1, EC-SOD also has one atom of Cu and Zn in each monomer 30 kDa subunit. This enzyme is generated in fibroblasts and glial cells and the matured form is then transported into the extracellular fluid (Marklund 1990).

2.4. MANGANESE SUPEROXIDE DISMUTASE

Mn-SOD (SOD2), the primary mitochondrial SOD located exclusively in the mitochondria, is found in all eukaryotic cells and in bacteria (*E. coli, T. thermophilus, E. coli, C. elegans, C. sapidus, C. sapidus, C. albicans, S. cerevisiae*).This was the second mammalian SOD

to be discovered by Weisiger and Fridovich in 1973. It is transcribed in the nucleus and has a mitochondrial targeting sequence, thus the mature form of the enzyme is found in the mitochondrial matrix. Due to its sub-cellular localization, Mn-SOD is considered to be the front line defense against superoxides. However, the expression of Mn-SOD is considerably varied depending on the tissue type, age, sex, disease states, etc. For example, cerebral arteries have higher Mn-SOD than the carotid artery or aorta. Estrogen seems to increase vascular Mn-SOD and ovariectomy decrease the level of Mn-SOD which can be elevated by estrogen replacement. It has been found that Mn-SOD is expressed in bacteria under cellular stress, which is not commonly observed for other SODs (Cu-ZnSOD and Fe-SOD).

The eukaryotic Mn-SOD exists as a homotetramer (dimer of dimers) of 96 kDa with one Mn atom per each subunit. In prokaryotes, Mn-SOD is usually a dimer. The human form of Mn-SOD subunit is 22 kDa with 198 residues. The metal content of the enzyme can be quantitatively detected by atomic absorption spectroscopy, which gives a characteristic absorption at 480 nm. Mn can access a range of oxidation states (-3 to +7). However, in Mn-SOD, the metal cofactor cycles between its divalent and trivalent oxidation states. This SOD also shows high structural homology to Fe-SOD. The trigonal bipyramidal geometry at the Mn(II) center is obtained by the coordination of two histidines and an aspartic acid in the equatorial plane with a histidine and solvent molecule in the axial positions (figure 2.7.). Two hydrogen bonds are formed by the solvent molecule with the equatorial aspartate and the amide side chain of the glutamate Q146. Superoxide activity can be measured by the rate of disappearance of superoxide anion at $\lambda = 250$ nm. The catalytic rate constants measured by stopped-flow spectrophotometry shows similar values for both human and *T. thermophilus* ($k_{cat} = 4 \times 10^4$ s $^{-1}$ for human and $k_{cat} = 1.3 \times 10^4$ s $^{-1}$ for *T. thermophilus* Mn-SOD).

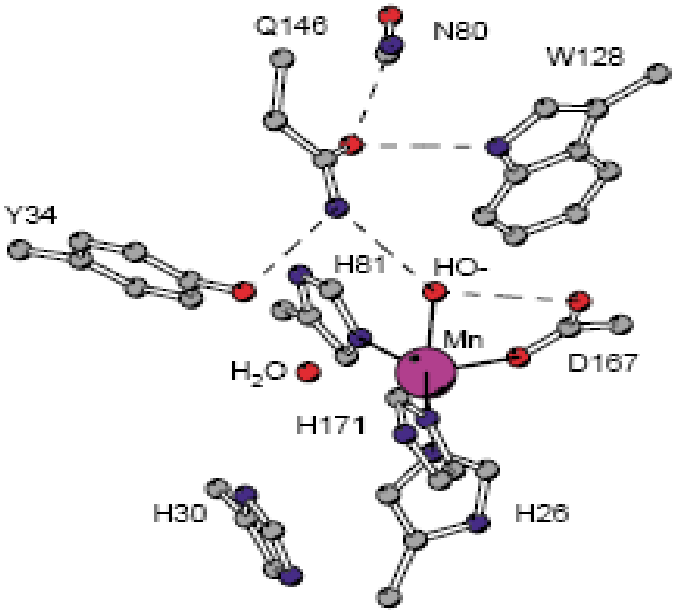


Figure 2.7. The active site of Mn-SOD from *E. coli.* [Current opinion in chemical biology, 8, Miller, A., Superoxide dismutases: Active site that save, but a protein that kills, 164, Copyright (2004)] with permission from Elsevier

Mn-SOD is undoubtedly related to the pathology of mammals including humans. Genetically altered mice lacking SOD-2 manifested various phenotypes such as neonatal or embryonic lethality, cardiomyopathy, hemolytic anemia, seizure, elevated cancer occurrence, spongiform encephalopathy, etc (Li 1995; Melov et al., 1998). It also plays a major role in protecting cells from hyperoxia-induced pulmonary toxicity (Wispe et al, 1992). Overexpression of Mn-SOD has been clinically proven to suppress tumors and various cancers including breast cancer by suppressing the HER2/neu oncogene (Chuang 2007). Therefore, researches are emerging regarding the utilization of Mn-SOD as a therapeutic agent for the treatment of HER2/neu mediated tumorigenesis. Nitration of Mn-SOD occurs at Tyr34 and consequently, diminished enzymatic activity was revealed suggesting that there is a direct link between the nitration and the inactivation of Mn-SOD.

2.5. IRON SUPEROXIDE DISMUTASE

Iron containing superoxide dismutase (Fe-SOD) has been purified from several prokaryotes such as *Escherichia coli, Sulfolobus solfataricus,* and *Methanobacterium brayantti*. It is also found in more primitive types of bacteria, chloroplasts of plants, and some eukaryotes as well. It is located in the periplasmic region of the cell and serves as a defense against oxidative stress. Fe-SOD exists as a dimer or tetramer of ~ 22 kDa monomers each with two domains around the active site (figure 2.8).

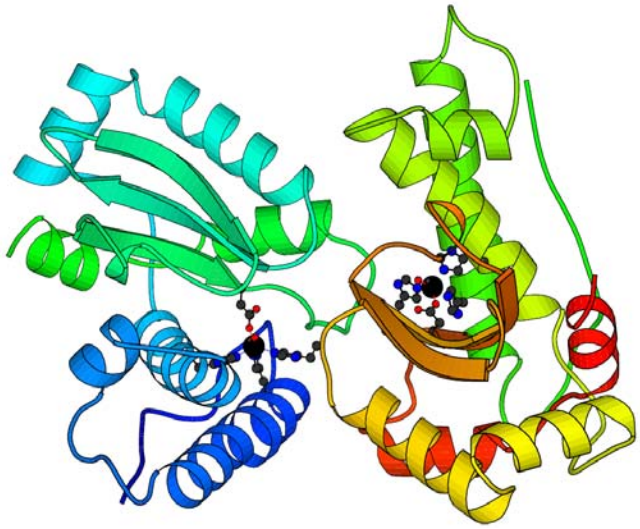


Figure 2.8. Fe-SOD from *E. coli* [Fe-superoxide dismutase, Miller, Anne-Frances., Hand book of Metalloproteins, 2001, Copyright: John Willey & sons, Chichester]

The active site of Fe-SOD exists in trigonal bipyramidal geometry with His26 and solvent molecules in the axial positions (figure 2.9). Broad overlapping bands at 350 nm in the UV/Vis spectrum of Fe-SOD are due to ligand-to metal charge transfer transition. The N-terminal domain (residue 1-80) makes up two helices with highly conserved Tyr34, His30, His26, and His73. The C-terminal domain that make up the Fe-binding unit (residue 89-192)

contains three-stranded β-sheets which provides ligation from Asp156 and His160. The fifth ligand comes from a coordinated solvent molecule (OH^- in the oxidized and H_2O in the reduced states). The N terminal consists of two long antiparallel αhelices. The Fe atom is ligated by two residues from each of N terminal helices and two residues from the C terminal loop.

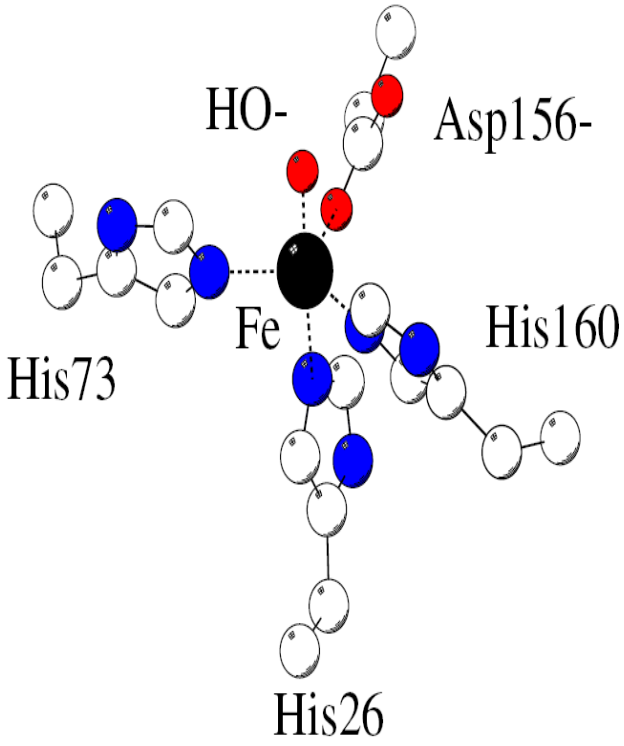


Figure 2.9. The active site model of Fe-SOD from *E. coli.* [Source; Fe-superoxide dismutase, Miller, Anne-Frances., Hand book of Metalloproteins, 2001, Copyright: John Willey & sons, Chichester]

Kinetic studies of Fe-SOD purified from *E. coli* shows catalytic activity characterized by k_{cat} = 2.6 x 10^4 s^{-1} and K_M = 80 for superoxide radical at pH 8.4 and 25 ^oC. It has been identified that the dismutation of superoxide by Fe-SOD occurs via a two step mechanism (eq. 20, 21).

$$O_2^{\bullet -} + Fe(III)SOD \rightarrow O_2 + Fe(II) \quad (20)$$

$$O_2^{\bullet -} + 2H^+ + Fe(II)SOD \rightarrow H_2O_2 + Fe(III)SOD \quad (21)$$

Structurally and functionally, Fe-SOD is very similar to Mn-SOD and a highly conserved amino acid sequence around the active site is observed in Fe-SOD.

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CHAPTER 3

BIOCHEMISTRY OF NICKEL

3.1 CHEMISTRY OF NICKEL

Transition metal ions at low concentrations are essential for all living organisms since they serve as cofactors for proteins such as in electron transfer, dioxygen binding, gene regulation, and catalysis. However, excess concentrations can be harmful to proteins, DNA, and lipids especially due to the production of metal catalyzed reactive oxygen species according to the Fenton reaction described in chapter 1 (Fenton 1894). A brief overview about the importance of Ni in biology is reviewed in the following paragraph.

Ni is the 7^{th} most abundant transition metal (relative atomic mass 58.69, first transition series group VIIIb) and it occurs as a silver white crystalline material. ^{58}Ni (68.27%) and ^{60}Ni (26.10%) are the two most abundant among the five known Ni isotopes. Ni can exist in one of several oxidation states, -1 to + 4, in which + 2 being the most stable and the predominant state. Nickel is soluble in dilute nitric acid but not in concentrated nitric acid. This is due to the passivation of the metal surface after introducing to the acid. The ability of Ni to absorb carbon monoxide forming $Ni(CO)_4$, is one of the interesting properties of nickel. Ni(II) can form many stable complexes including a wide range of cyano complexes. Ni can show anomalous behavior in the coordination environment depending on the number of CN ligands. Four CN^- ligands afford square-planer complexes whereas five CN^- ligands generate both square-pyramidal and trigonal-bipyramidal complexes. The most favorable coordination geometry is square-planar. Ni has been in use even before it was isolated and identified. Historical evidence has shown that

Syrian bronze contained trace amount of Ni (~ 3500 BC) and the Chinese have used Ni for minting coins (~ 235 BC). German miners discovered the metal along with some other metals and they first thought that it was some form of impure copper, hence naming it "Kupfernickel" which means "false or bad copper". Swedish scientist Axel Fredrik Cronstedt isolated pure Ni and identified its properties in 1751. Ni became a very popular metal in the alloy industry due to high strength, corrosion resistance, and hardness of such alloys. Stainless steel is a great example for one of the most valuable materials from the Ni industry. Other applications include electroplating, manufacturing nickel-cadmium batteries, medical prosthesis, and electronic devices. Canada is the world's largest Ni producer. Other countries that mine Ni in large scale are Russia, Australia, and France/New Calidonia.

Table 3. Nickel containing enzymes

Nickel containing Enzyme	Biological function
1.Urease	Hydrolysis of urea
2.Hydrogenase	Reversible oxidation of hydrogen
3.Carbon monoxide dehydrogenase/ Acetyl Coenzyme-A synthase	Interconversion of carbon monoxide and carbon dioxide/formation of acetyl coenzyme
4. Methyl-S-Coenzyme M reductase	Reduction of methyl-CoM in methanogenesis
5. Superoxide Dismutase	Disproportionation of superoxide

In the late 1800's, the therapeutic benefits of Ni salts in humans were investigated and found to have properties as analgesics, antidiarrheal agent, and antiepileptic drugs. However, its biological importance was not elucidated until the discovery of the Ni containing enzyme urease in the 1920's (Summer 1926). Six Nickel dependent enzymes have been identified with distinct metallocenters, namely urease, monofunctional carbon monoxide dehydrogenase, bifunctional carbon monoxide dehydrogenase/acetyl coenzyme-A synthase, [NiFe] hydrogenase, methyl-coenzyme M reductase, and finally the latest member, nickel superoxide dismutase (Ni-SOD). The biological functions associated with each of these enzymes are given in table 3.

3.2. UREASE

Urease is a biologically very important enzyme involved with nitrogen metabolism in many biological systems (bacteria, fungi, and plants). Urea is excreted by mammals as a detoxification product of their metabolism and can also be formed by the catabolism of environmental uric acid. This enzyme also serves as a virulence factor for a number of pathogens. Urease activity associated with microbes can give rise to several medical conditions such as the development of urinary stones, acute pyelonephritis, urinary catheter obstruction, hepatic coma and peptic ulcers. Urease converts urea into ammonia and carbamate (which spontaneously decomposes into carbonic acid) in a two step mechanism (eq. 22, 23). Without the catalytic activity of urease, urea can be transformed to ammonia and cyanic acid by an extremely slow (~ 10^{14} times slower) process.

$$\mathbf{H_2N\text{-}CO\text{-}NH_2 + H_2O \rightarrow NH_3 + H_2N\text{-}COOH} \qquad \mathbf{(22)}$$

$$\mathbf{H_2N\text{-}COOH + H_2O \rightarrow NH_3 + H_2CO_3} \qquad \mathbf{(23)}$$

Urease contains 840 amino acid residues and was first isolated and crystallized from jack beans (*Canavalia ensiformis*) by Summer JB (1926) and confirmed to have Ni by Dixon NE (1975). The enzyme consists of a dinuclear Ni active site where the two Ni atoms are separated by a Ni-Ni distance of 3.5 Å. The composition and the quaternary structure of urease can vary from species to species. Urease from *K. aerogenes* has approximately 250 kDa multisubunit complex arranged as trimer of trimers (i.e. $(\alpha\beta\gamma)_3$). The plant urease is a homohexamer of approximately 550 kDa. However, both possess a well established conserved region of greater that 50% in the protein sequence. It has been identified that the active site of urease contains a "solvent-filled pocket" which serves as the urea binding site. The two nickel ions, Ni-1 and Ni-2 are coordinated by two histidine residues and two histidine and one lysine residues respectively and bridged by a carbamylated lysine (Lys217) (figure 3.1).

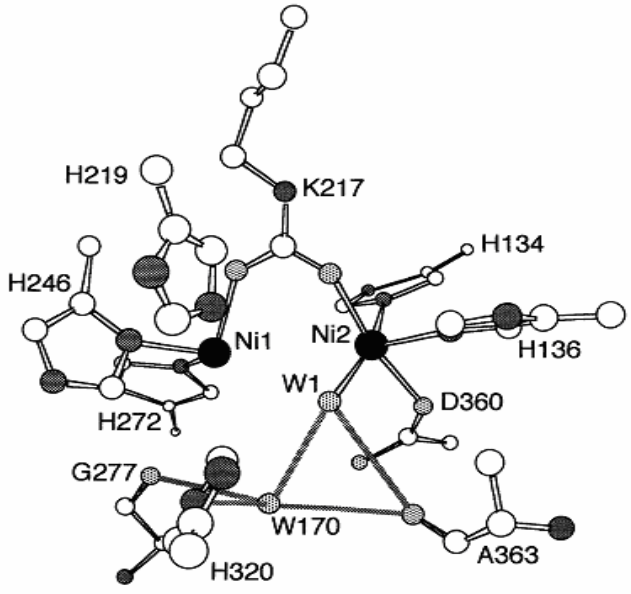

Figure 3.1. A model of the active site of urease from *Klebsiella aerogenes.* [Coordination Chemistry Reviews, 190-192, Ciurli, S., et al., Structural properties of the nickel ions in urease: novel insights into the catalytic and inhibition mechanisms, 335, Copyright (1999)] with permission from Elsevier.

Ni-1 is pentacoordinate in a distorted square-pyramidal geometry whereas N-2 is hexacoordinate in distorted octahedral geometry. This unusual carbamylated bridging unit has been identified as a key element for the catalytic activity of urease. The active site accessibility is controlled by a mobile α-helical flap which can open and close accordingly.

3.3 CODH AND ACS

Both carbon monoxide dehydrogenase (CODH) and acetyl coenzyme-A synthase (ACS) play an important role both in microorganisms and in the global carbon cycle. Utilizing these enzymes, carbon monoxide is converted to carbon dioxide releasing electrons which are then used to generate energy in organisms (eq. 24).

$$\mathbf{CO + H_2O \leftrightarrow CO_2 + 2H^+ + 2e^-} \qquad \mathbf{(24)}$$

CODH helps microbes to obtain cell carbon and energy solely utilizing carbon monoxide. This microbial conversion of carbon monoxide is highly beneficial as it removes approximately 10^8 tons of carbon monoxide annually from the lower atmosphere (Bartholamew 1979). The high energy electrons generated in the reaction contribute to the energy processing of the microbes whereas carbon dioxide is fixed into cellular carbon by CO_2 reductive pathways. CODH exist as two major classes of enzymes; aerobic and anaerobic. Nickel containing CODH are anaerobic and classified further into two classes (monofunctional and bifunctional) based on the active site metal cluster composition. Monofunctional Ni-CODH (*Rhodospirillum rubrum, C. hydrogenoformans*) contains 10 Fe and 1 Ni per monomer (Figure 3.2), whereas bifunctional Ni-CODH (*M. thermoacetica, M. thermophila*) has 14 Fe and 3 Ni per monomer. Ni-CODH has five metal clusters; two B clusters, two C clusters, and one D cluster. However, the catalytic site for

the reversible oxidation of CO occurs in the C-cluster. Acetyl-Co-A synthase (ACS) is found as a complex with CODH. Its biological function is the catalysis of the synthesis of acetyl-Co-A from CO. It contains a metal cluster composed of Ni, S, and Fe [4Fe-4S] classified as the A-cluster (figure 3.2).

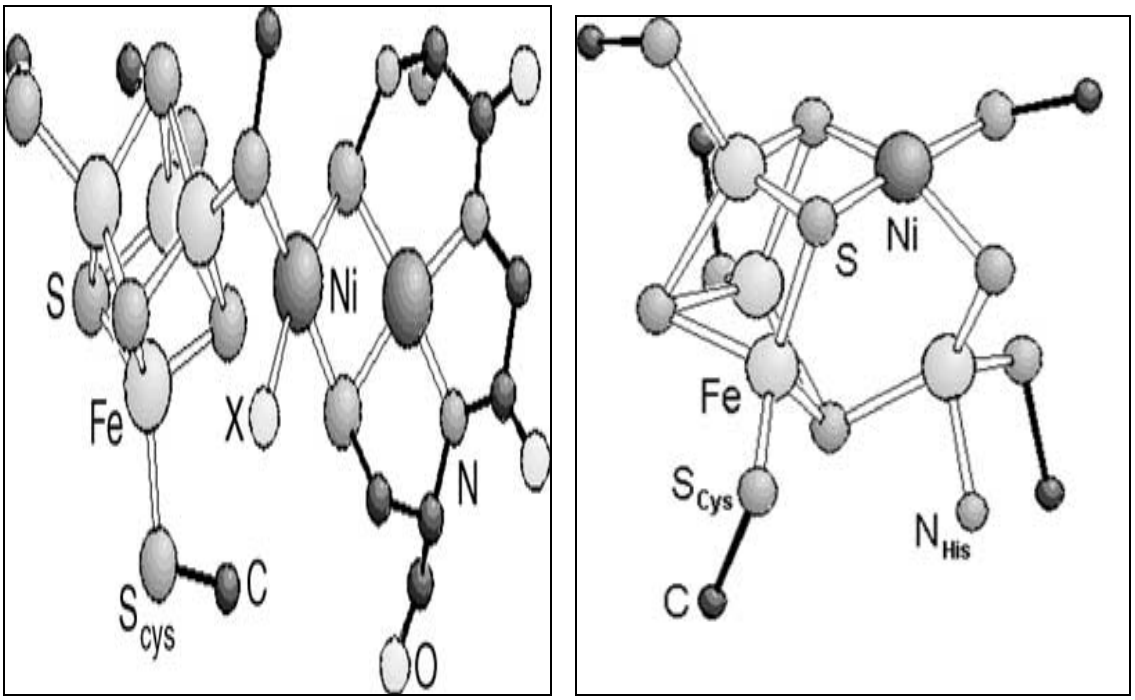

Figure 3.2. The C-cluster in CODH from *C. hydrogenoformans* (left). The A cluster of ACS from *C. hydrogenoformans* (right). [Coordination Chemistry Reviews, 249, Evans, D. J., Chemistry relating to the nickel enzymes CODH and ACS, page 1583, Copyright (2005)] with permission from Elsevier

3.4. METHYL-COENZYME M REDUCTASE

Methyl-CoM reduatase (MCR) catalyzes the reduction of methyl-CoM, one of the intermediates in methanogenesis to methane by a complex mechanism. This is an energy conserving mechanism by which methanogenic archaea (*Methanosarcina barkeri, Methanopyrus*

kandleri, Methanobacterium thermoautotrophicum) grow anaerobically. It is an enzyme of molecular mass of 300 kDa with an $(\alpha\beta\gamma)_2$ subunit structure. Recently, it has been elucidated that the overall reaction occurs via a three step mechanism in which coenzyme A (CoM, 2-mercaptoethane-sulfonate) and coenzyme B (CoB) are oxidized to a heterodisulphide molecule CoM-S-S-CoB (also known as HS-HTP, 7-mercaptoheptanoylthreonine phosphate). The MCR enzyme is isolated in two inactive forms namely $MCR_{ox1/silent}$ and $MCR_{silent,}$ both possessing five modified amino acids; 1-N-methyl-Hisα257, 5-(S)-methyl-Argα271, 2-methyl-Glnα400, S-methyl-Cysα452 and Glyα445. Ni is embedded in a tetrapyrrole structure called coenzyme F_{430} which lies in the active site of MCR (Figure 3.3).

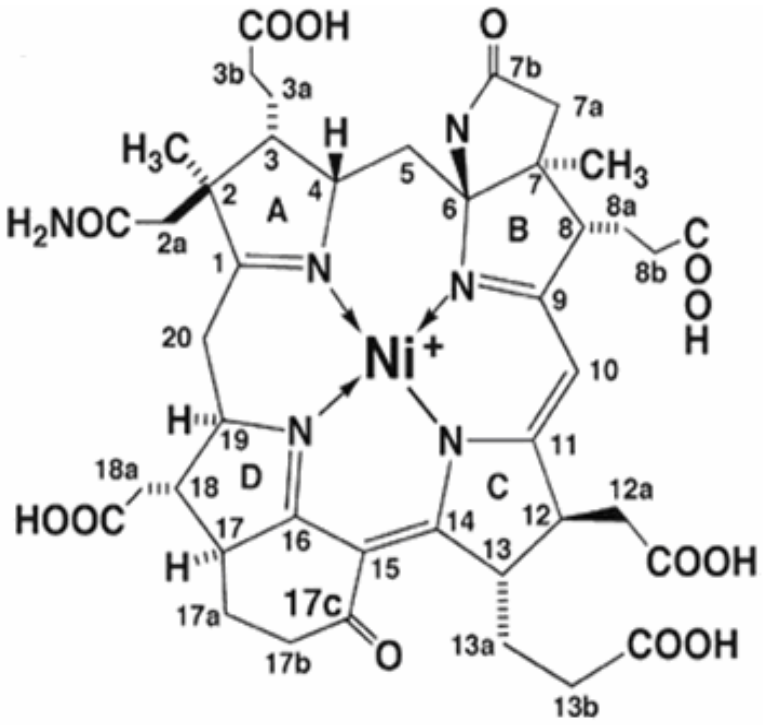

Figure 3.3. Ni-tetrahydrocorphin (F430) of methyl-CoM reductase [Journal of Inorganic Biochemistry, 101, Nickel and the carbon Cycle, Ragsdale, S.W., page 1658, Copyright (2007)] with permission from Elsevier

In order to participate in the catalytic reaction, Ni(II) has to be reduced to Ni(I). This reduction step is fostered by an ATP-driven electron transfer from an Fe-S cluster protein. After each catalytic cycle, Ni(I) is regenerated. The proposed three step mechanism, nucleophilic Ni(I) binds to the methyl group of methyl-CoM converting Ni(I) in to a Ni(III) intermediate. During the reduction of Ni(III) into Ni(II), methane and CoM thiyl radical are generated. In the final step, a heterodisulphide is formed by fusing the thiyl radical and the thiolate sulfur of CoB. Ni(II) returns to its reduced form Ni(I) by gaining an electron from the heterosulfide anion radical (scheme 1). This highly reactive radical based mechanism is confirmed to be a plausible one because of its hydrophobic active site pocket.

(i) $\mathbf{Ni(I) + CH_3{-}S{-}CoM \rightarrow Ni(III){-}CH_3 + {}^{-}S{-}CoM}$

$\mathbf{{}^{-}S{-}CoM + HS\text{-}CoB \rightarrow HS{-}CoM + {}^{-}S{-}CoB}$

(ii) $\mathbf{Ni(III){-}CH_3 + HS{-}CoM \rightarrow Ni(II){-}CH_3 + HS - CoM^{+} \rightarrow Ni(II) + CH_4 + S^{\bullet} - CoM}$

(iii) $\mathbf{S^{\bullet} - CoM + {}^{-}S{-}CoB \rightarrow CoM{-}S{-}S^{\bullet} - CoB^{-}}$

$\mathbf{Ni(II) + CoM{-}S{-}S^{\bullet} - CoB^{-} \rightarrow Ni(I) + CoM - S - S - CoB}$

Scheme 1: Three step mechanism involving methyl-CoM reductase. [Current Opinion in Structural Biology, 8, Ermler, U., et al. Active sites of transition–metal enzymes with a focus on nickel, page 751, Copyright (1998)] Current Biology Ltd.

3.5. NICKEL CONTAINING HYDROGENASE

Many microorganisms use hydrogenase (most of these having Ni in the active site) for their energy metabolism. It was first discovered by Stephenson & Stickland in the 1930's. Hydrogenase has been isolated from several photosynthetic bacteria such as, *Chromatium*

vinosum and *Thiocapsa roseopersicina, Desulfovibrio fructosovorans, Desulfovibrio desulfuricans* and *C. hydrogenoformans*. These enzymes exist as multi-metal protein domains with some having Fe in its active site. Three classes of hydrogenase have been identified based on which active site metal it contains; Fe-only, Ni-Fe, and FeS free (no inorganic sulfide). The enzyme reversibly catalyzes the redox equilibrium of H_2 and H^+ (eq. 25) with a suitable electron donor.

$$\mathbf{H_2 \leftrightarrow 2H^+ + 2e^-} \qquad \mathbf{(25)}$$

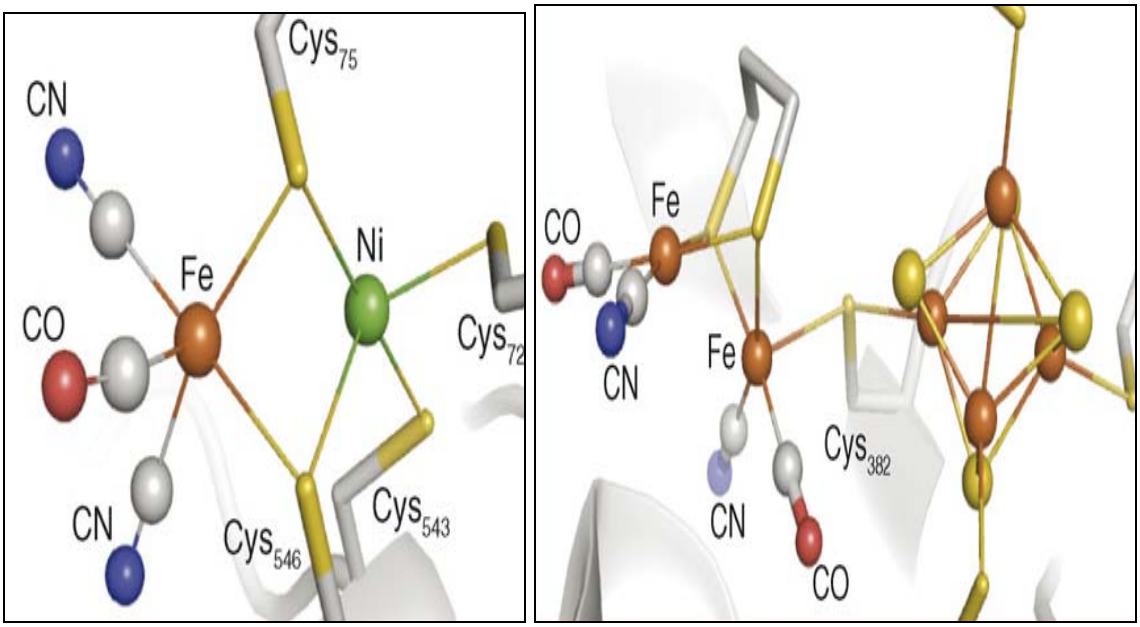

Figure 3.4. The structures of [NiFe] hydrogenase from *Desulfovibrio fructosovorans* (left). [FeFe] hydrogenase from *Desulfovibrio desulfuricans* (right). [Current opinion in Chemical Biology, 11, Metallocenter assembly of the hydrogenase enzymes, Leach, M.R. and Zamble, D.B., 160, Copyright (2007)] with permission from Elsevier.

Nickel-iron hydrogenase contains two subunits; one with molecular weight ~50-70 kDa and a smaller one with ~ 20-40 kDa. The quaternary structure of hydrogenase is very complex and

varies among different bacteria. *D. gigas*, the most common Ni-Fe hydrogenase has a globular heterodimer structure with a radius approximately 30 Å (figure 3.4).

3.6. TOXICITY OF NICKEL

As with most other transition metals, Ni can be toxic to humans at high concentration (Poulik, Z., 1997). The first observation of the toxicity of Ni was recorded as early as the 1500's. Many studies were done on the toxicity of Ni in the late 1800's and the assessment of the lethal dose of various Ni salts was established. It was found that the gaseous nickel compound [$Ni(CO)_4$] is highly toxic to animals in 1891. Two years later, the toxicity of Ni on plants was confirmed by Haselhoff (1893). The first animal study on the carcinogenicity of Ni was carried out by Campbell in 1943 and in the succeeding years to date, many other studies were done in this regard.

The toxicity of nickel to humans is also evident. Most inevitable medical conditions are skin allergies and contact dermatitis, lung fibrosis, and cancer (Denkhaus 2002). The exposure to nickel has been drastically increased due to the accelerated consumption of nickel especially in industrial areas. The primary source of exposure is the inhalation of nickel from anthropogenic sources (mainly in occupational settings) and ingestion via food. Atmospheric nickel suspensions are mainly due to the combustion of fossil fuel in industries. Non anthropogenic sources include direct leaching of the metal into water from exposed ores or rocks containing nickel and suspended particles from volcanic emissions. The International Agency for Research on Cancer (IARC) has assessed the carcinogenicity of nickel and confirmed that all nickel compounds except metallic nickel are potential carcinogens (1990). The threshold value for potential carcinogenesis for nickel was established as 1 mg/m^3 or above (for soluble forms of nickel) and 10 mg/m^3 or above (for less soluble forms of nickel) by the International Committee on Nickel

Carcinogenesis in Man in 1990 (Doll, R., 1990). Thus, many researches have emerged to work on the current state in the field. Viable ways of how nickel compounds can be carcinogenic include DNA damage, inhibition of DNA repair activity, and nickel induced oxidative stress. Though nickel itself is weakly mutagenic, it has been shown that nickel enhances DNA damage via a synergetic mechanism with other mutagens.

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CHAPTER 4

NICKEL SUPEROXIDE DISMUTASE

4.1 THE DISCOVERY OF NICKEL-SOD

Ni containing superoxide dismutase has been purified from several cyanobacteria and streptomyces (*Streptomyces seoulensis, Streptomyces coelicolor, Streptomyces spp*.) species (Kim, E.J. et al 1996, Chun, J. et al. 1997; Youn, H.D., et al. Youn, D.H. et al. 1996). Ni-SOD displays unique properties compared to other SOD families in terms of its amino acid sequence, spectroscopic properties, immunological properties, and the active site metal-ligand environment. No amino acid sequence homology was found to the other two classes of SOD, namely Cu-Zn and Mn-SOD. Ni-SOD is a mononuclear metalloenzyme and during the dismutation of superoxide, the nickel center cycles between the divalent and trivalent oxidation states.

4.2. STRUCTURE OF NICKEL-SOD

Crystallographic studies have been carried out by several research groups in order to elucidate the overall structural architecture and active site geometry of Ni-SOD. Ni-SOD extracted from *Streptomyces seoulensis* was crystallized for crystallography studies using ammonium sulfate as the precipitant (Wuerges, J., 2002). The hanging-drop vapor-diffusion method (McPherson, 1999) was utilized at 277 and 293 K to carry out crystallization of the purified samples. Multiple crystalline morphologies were obtained after crystallization; however, only forms that displayed high quality diffraction data were used for further studies (crystal forms I, II and III). Crystal form I was plate like and grew rapidly (1-2 weeks) in a medium

containing ammonium sulfate and 5% 2-propanol at pH 5.25. Approximate dimensions of these crystals are; 0.21 x 0.21 x 0.05 mm (figure 5.5). Crystal form II appeared after 8-10 days and was needle shaped with approximately 0.3 mm in length and 0.03 mm in diameter at the same pH. Growth of crystal form III was induced by macroseeding and crystals were obtained after 5-6 weeks. These were rod shaped crystals with dimensions 0.5 x 0.2 x 0.2 mm (figure 4.1).

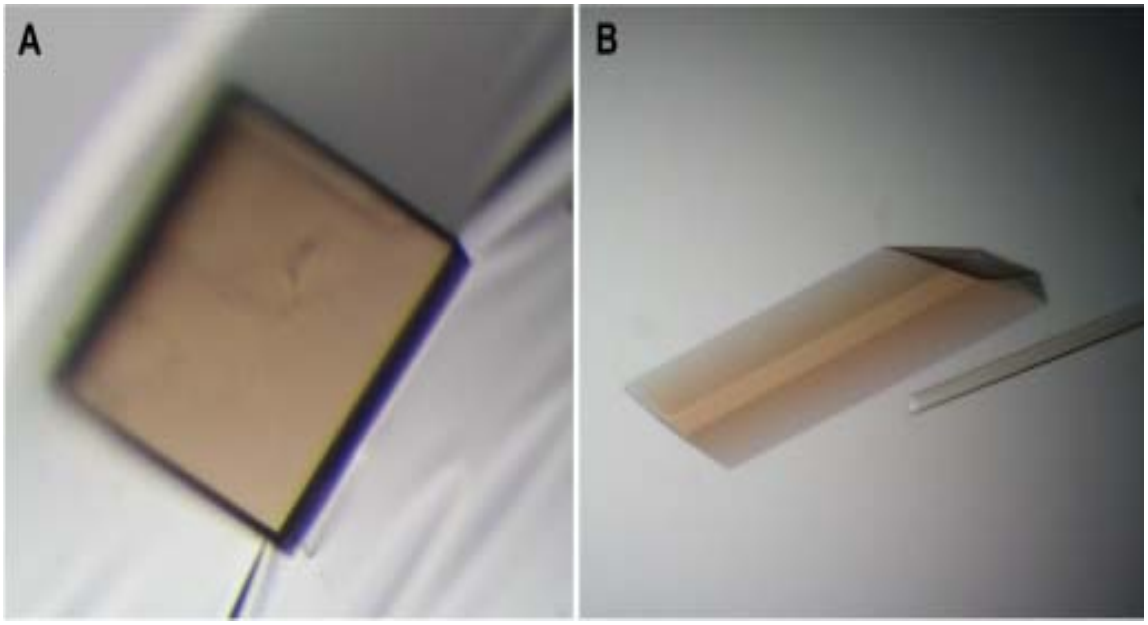

Figure 4.1. Crystal forms of Ni-SOD from *Streptomyces seoulensis*; plate-like crystals (left), rod-like and needle like crystals (right) [Acta. Crystallographica., D58, Crystallization of a nickel-containing superoxide dismutase and preliminary phase determination by MAD at the Ni K edge, Wuerges, J., et al., 1221, copyright (2002)] with permission.

All three of theses crystals were subjected to Multiple Anomalous Dispersion (MAD) analysis at 100 K. A summary of crystallographic data obtained from this experiment is given in the table 4. Crystallized Ni-SOD revealed three crystalline forms; two from space group $P2_12_12_1$ and one from space group R3. Data from Multiple wave-length Anomalous Dispersion (MAD) analysis

revealed that the functional unit of the Ni-SOD has a hexameric globular shape (figure 4.2) in which all the protein molecules are confined in a sphere of outer diameter of 72 Å and inner diameter of 23 Å (Wuerges, J., et al 2002).

Table 4. Crystallography data from MAD study

Crystal form	Form I	Form II	Form III
X-ray Source	ESRF, BM14	EMBL c/o DESY BW7A	ESRF, ID14-4
Space Group	$P2_12_12_1$	R3	$P2_12_12_1$
Unit-cell parameters			
a	112.3	189.4	65.2
b	113.8	189.4	119.3
c	128.6	159.9	121.0
Number of molecules per AU	12	18	6
V_M * (Å Da^{-1})	2.6	2.3	3.0
Solvent content (%)	51	46	57

* Using the molecular mass of Ni-SOD of 13.2 kDa and a molecular density value of 1.3 g cm^{-3} to estimate the solvent content of the crystal form. Adopted from [Acta. Crystallographica, D58, Crystallization of a nickel-containing superoxide dismutase and preliminary phase determination by MAD at the Ni K edge, Wuerges, J., et al., 1221, copyright (2002)] with permission.

According to some other studies, the hexameric structure is composed of four identical helix bundle subunits of 13.4 kDa each and possesses a three-fold symmetry (Youn, H.D., et al 1996).

The subunit is made up of 117 amino acid residues. Subunits are arranged in an anti-parallel topology forming a four-helix bundle structure and display mainly hydrophobic interactions. The topological view of this three dimensional arrangement is shown in figure 4.2. Each subunit contains one nickel atom which is surrounded by the N terminus of each monomer unit and ligated in a core of a special structural feature called the "Ni binding hook" (figure 4.3). The Ni binding hook consists of the first nine residues from the N-terminus.

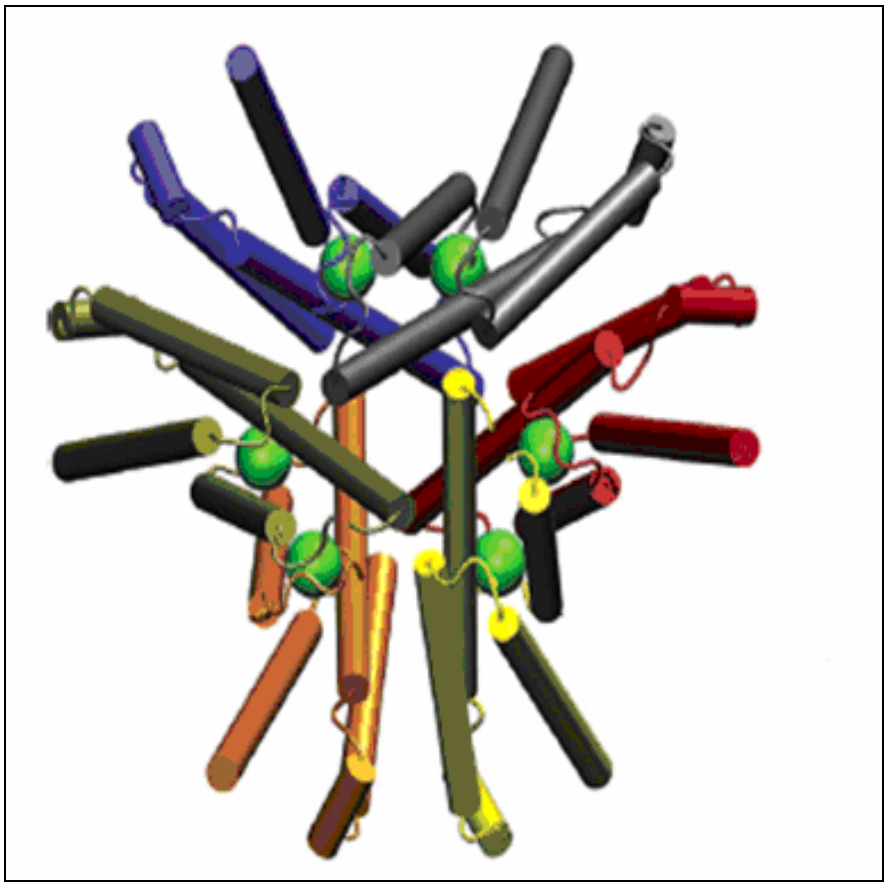

Figure 4.2. The homohexamer overall structure of Ni-SOD. [Journal of the American Chemical Society, 128, Nickel superoxide dismutase reaction mechanism studied by density functional methods, Pelmenschikov, V., and Siegbahn, P.E.M., 7467, Copyright (2006)] with permission from the American Chemical Society.

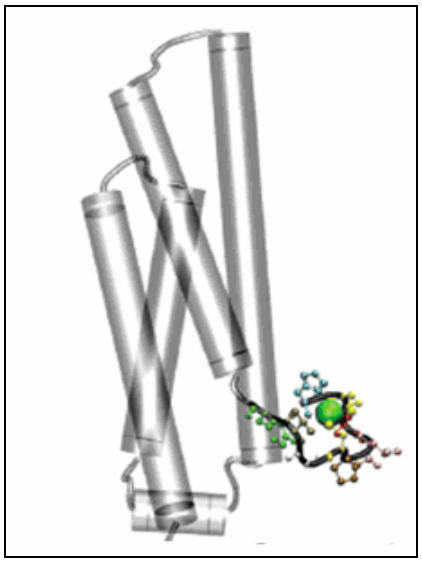

Figure 4.3. The monomer subunit of Ni-SOD. [Journal of the American Chemical Society, 128, Nickel superoxide dismutase reaction mechanism studied by density functional methods, Pelmenschikov, V., and Siegbahn, P.E.M., 7467, Copyright (2006)] with permission from the American Chemical Society.

The Ni atom is surrounded by the coplanar backbone of nitrogen from His-1, Asp-3, Cys-6, and Gly-7 along with Pro-5 and Tyr-9 creating a narrow entrance (bottle neck configuration) towards the active site. This unique structural design at the active site reveals relevance to the chemistry of superoxide dismutation. This small pocket volume contains only two molecules of solvent water and the bottle neck entrance selectively prohibits the entrance of larger molecules. Moreover, the catalytic rate of the enzyme is enhanced by long range electrostatic attraction due to the presence of positively charged amino acids in the proximity of the active site.

4.3. BONDING AND LIGATION

Two independent X-ray crystallography studies on Ni-SOD from *Streptomyces coelicolor and Streptomyces seoulensis* revealed a square-planar geometry with N_2S_2 coordination to the metal in the reduced state of Ni-SOD. X-ray structural studies reveal that in the reduced state of

the enzyme, Ni(II) possesses a tetra-coordinate square-planar geometry whereas in the oxidized Ni(III) state it changes to a penta-coordinated square-pyramidal geometry (Wuerges, J., et al. 2004; Barondeau, D.P., et al. 2004) (figure 4.4).

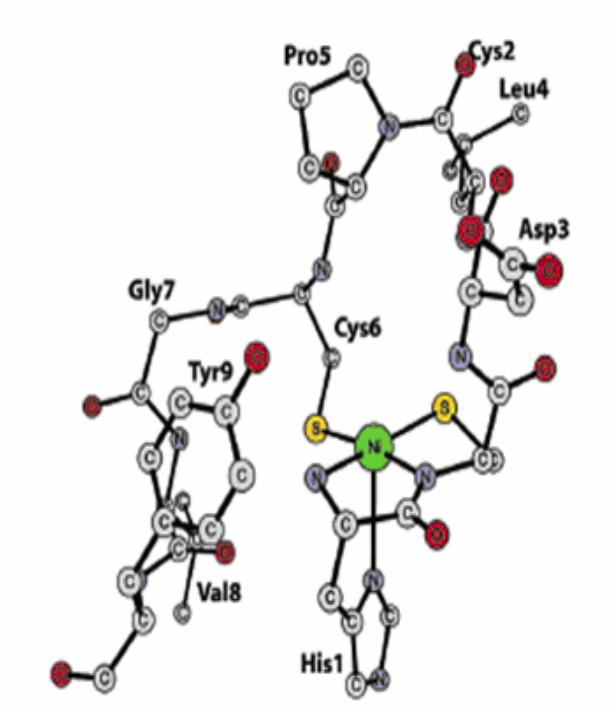

Figure 4.4. Amino acid residues comprising the nickel hook of Ni-SOD. [Journal of the American Chemical Society, 128, Nickel superoxide dismutase reaction mechanism studied by density functional methods, Pelmenschikov, V. and Siegbahn, P.E.M., 7467, Copyright (2006)] with permission from the American Chemical Society.

Square-planar geometry is due to the ligation of the metal ion by two cysteinate sulfurs and two nitrogens, one from the N terminal primary amine and the other from a deprotonated peptide originating from the N terminus. Imidazole ligation in the axial position is observed upon oxidization affording square-pyramidal geometry. This change in the ligand environment occurs during the oxidation of the enzyme when Ni(II) donates an electron to the superoxide substrate

and becomes Ni(III). This oxidation is accompanied by the addition of an axial ligand through the N of the His1 residue resulting in stabilization of the Ni(III) oxidation state (figure 4.5). It has been shown that the axial H(1) imidazole ligation is crucial to gain a maximum efficiency in the disproportionation mechanism. In the reduced state Ni(II) contains two Ni-S bonds with the Cys6 and Cys2 and Ni-N bond with the amide nitrogen of His1 (figure 4.5). The coordination environment of Ni-SOD is distinct from other SOD classes due to the presence of many S-donor ligands and the deprotonated peptide nitrogen. Spectroscopic studies have shown that the thiolate ligation is essential for SOD catalytic activity.

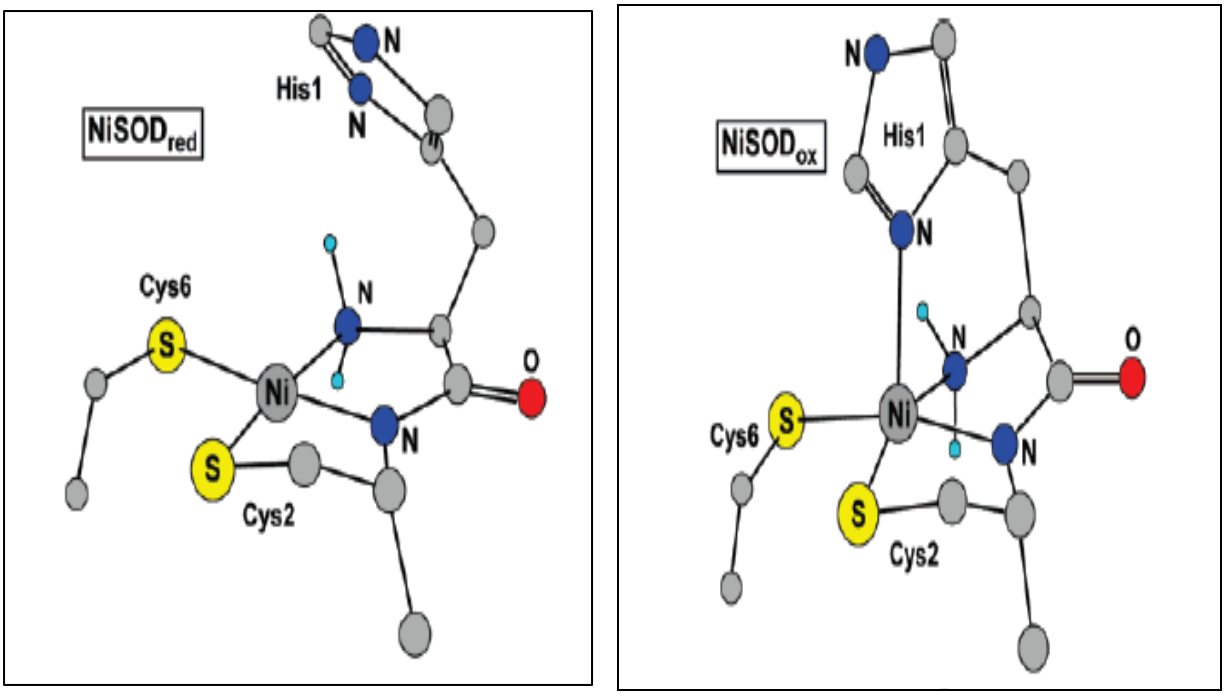

Figure 4.5. Active site of Ni-SOD in the oxidized (right) and reduced (left) states from *Streptomyces coelicolor/Streptomyces seoulensis*. [Journal of the American Chemical Society, 127, Spectroscopic and computational studies of Ni superoxide dismutase: Electronic structure contributions to enzymatic function, Fiedler, et al., 5450, Copyright (2005)] with permission from the American Chemical Society.

4.4. ACTIVE SITE CHEMISTRY AND REACTION MECHANISM

The dismutation of superoxide takes place via a catalytic pathway in which the enzyme shifts between oxidized and reduced states as represented below (eq. 26, 27) where E-Ni(II) and E-Ni(III) represent the reduced and oxidized state of the enzyme respectively.

$$\mathbf{E–Ni(II) + O_2^{\bullet -} + 2H^+ \rightarrow E–Ni(III) + H_2O_2} \qquad \mathbf{(26)}$$

$$\mathbf{E\text{-}Ni(III) + O_2^{\bullet -} \rightarrow E\text{-}Ni(II) + O_2} \qquad \mathbf{(27)}$$

It has been suggested that the catalytic cycle of Ni-SOD may take place via a four step mechanism. In the first step, superoxide binds to the vacant axial site of the Ni(II) metal center in trans position to the His1 side chain. The entrance of the anionic superoxide to the active site is guided by three conserved lysine residues. In step 2, superoxide is reduced through an electron donated by the Ni(II) ion and two protons possibly from the axial His1 residue or protonated thiol (Cys2 or Cys6) to produce hydrogen peroxide. Other proposed hydrogen donors are the backbone amide groups of Asp3, Cys6 and the hydroxyl group of Tyr9. The second molecule of superoxide then binds to the unoccupied axial position of the Ni(III) in step 3, followed by the electron transfer from superoxide to Ni(III) producing molecular oxygen in the final step (figure 4.6). Examination of the structure of Ni-SOD provides some evidence to its unique functionality. The Ni-hook which encapsulates the active site promotes selectivity of the dismutation reaction. Even though Ni is bonded by sulfur ligands, oxidation of sulfur is thought to be inhibited by the inaccessibility of substrates to the thiol ligands This preservation of vulnerable oxidation of the thiolate ligands contributes to the stability of the molecule.

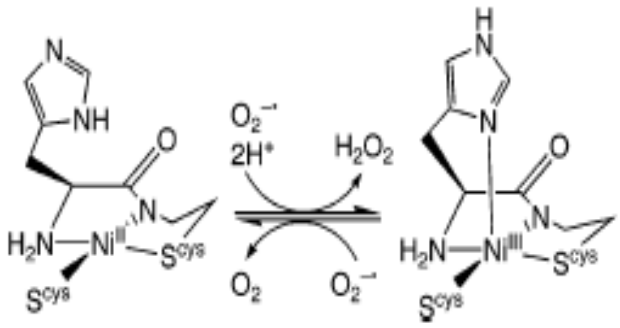

Figure 4.6. Proposed mechanism for the redox reaction of Ni-SOD [Inorganic Chemistry, 45(24), $(Me_4N)[Ni^{II}(BEAAM)]$: A synthetic model for nickel superoxide dismutase that contains Ni in a mixed Amine/Amide coordination environment, Shearer, J. and Zhao, N., 9637 Copyright (2006)] with permission from the American Chemical Society.

Szilagyi and coworkers (2004) investigated the active site chemistry of Ni-SOD utilizing S K-Edge X-ray absorption spectroscopy. Ni K-edge EXAFS provided evidence of sulfur coordination at the Ni center (Choudhury 1999). Coordination of sulfur at Cys-2 and Cys-6 to the Ni center makes up two ligands. The remaining three ligands in the five coordinated Ni(III) was revealed by hyperfine EPR studies. Further elucidation of the structure/mechanism of Ni-SOD was done by sulfur K-Edge XAS which directly probes the S coordination. A series of S K-Edge spectra were obtained for both S-ligated model compounds and Ni-SOD (figures 4.7). The main finding from this study was the presence of terminal and absence of bridging thiolate ligands in the oxidized form of Ni-SOD and the absence of terminal thiolate coordination in the reduced form of Ni-SOD. These changes in the ligation in oxidized and reduced form of Ni-SOD undoubtedly suggest a rearrangement mechanism at the active site. The proposed mechanism illustrating the findings from the XAS studies is shown in figure 4.8. However, later studies confirmed that both oxidation states have terminal thiolates (Fiedler, et al., 2004).

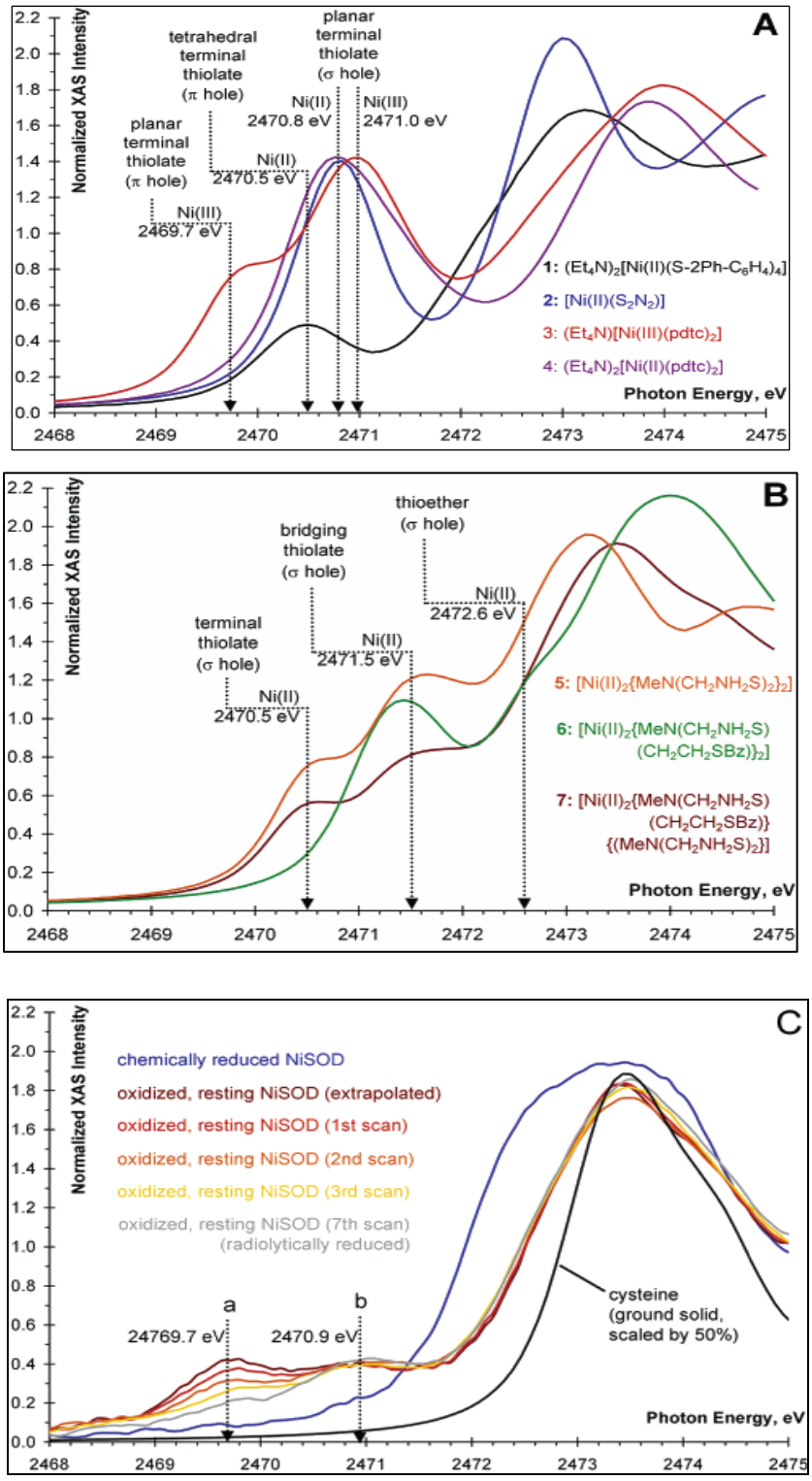

Figure 4.7. S K-edge XAS spectra of some mononuclear Ni compounds (A), binuclear Ni compounds (B) oxidized and reduces Ni-SOD (C). [Journal of the American Chemical Society, 126, Szilagyi, R.K. et al., page 3018, Copyright (2004)] with permission from the American Chemical Society.

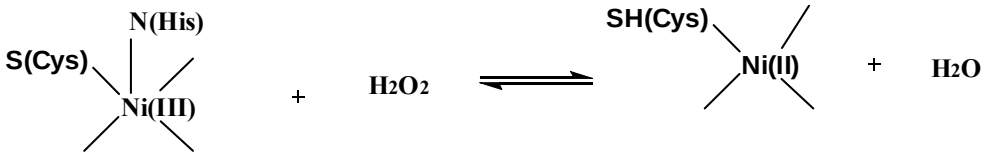

Figure 4.8. Proposed mechanism for the redox reaction of Ni-SOD [Journal of the American Chemical Society, 126, Szilagyi, R.K. et al., S K-Edge X-ray absorption spectroscopic investigation of the Ni-containing superoxide dismutase active site: New structural insight into the mechanism, page 3019, Copyright (2004)] with permission from the American Chemical Society.

4.5. KINETIC STUDIES OF NICKEL-SOD MODEL COMPOUNDS

A growing number of studies have emerged to investigate the kinetics of SODs. Kinetic studies performed on Ni-SOD isolated from *Streptomyces seoulensis* and *Streptomyces coelicolor* revealed that the enzyme's catalytic activity k_{cat} is greater than 2 x 10^9 M^{-1} s^{-1} which is comparable to other known SODs (Bryngelson 2004; Coudhurey 1999). Neupane and co-workers have used the Ni-SOD maquettes [$Ni^{II}(SOD^{M1})$] and [$Ni^{II}(SOD^{M2})$] described in 4.7 to probe the SOD activity for these model compounds using a modified xanthine/xanthine oxidase assay. In this method a steady-state of superoxide anion is generated via the reaction between xanthine oxidase and xanthine. Upon reduction, the superoxide anion reduces nitro blue tetrazolium (NBT) (figure 4.9), a colorless compound, to a blue colored compound. The SOD concentration which is required to effect 50% of this reduction reaction is referred to as IC_{50} and this value for [$Ni^{II}(SOD^{M2})$] was found to be 1 x 10^{-6} M. A much lower value of IC_{50} was recorded for CuZn-SOD (4 x 10^{-8} M) suggesting low SOD activity for the model compound. Other derivatives of this maquette also showed reduced SOD activity. The acylated form of $Ni^{II}(SOD^{M1})$ had a higher IC_{50} value (3 x 10^{-5} M) compared to the non-acyated form (2 x 10^{-7} M) (Neupane, 2006).

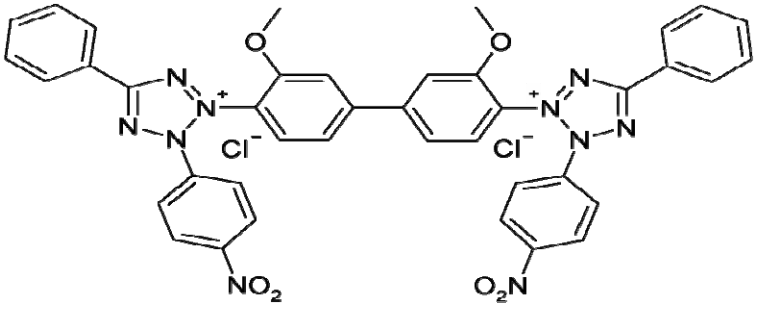

Figure 4.9. The structure of nitro blue tetrazolium (NBT)

4.6. OUTER SPHERE VS INNER SPHERE MECHANISM

The catalytic disproportionation mechanism of Ni-SOD involves electron transfer as in any other redox reaction. Such reactions can occur via two pathways namely inner-sphere mechanism and outer-sphere mechanism. In principle, an outer-sphere mechanism involves transfer of electron(s) beyond the coordination sphere of one molecule to the other during the electron transfer. Both species remain separate before and after the electron transfer. This type of mechanism is usually facilitated when the electron transfer is rapid. An outer-sphere mechanism can occur between two chemically different entities or the same type but with different oxidation states. Outer-sphere mechanism is entropically more favorable as it does not require special rearrangement during the process. In contrast, an inner sphere mechanism is characterized by the formation of a bridge between the redox couple during the electron transfer. In metalloenzymes, this covalently linked bridge is formed by coordination of a ligand to the metal center. The formation of such bridges is only possible with minimum steric hindrance; hence an inner sphere mechanism is not very common among biological systems due to bulky ligands. According to the studies done on Ni-SOD structure and reactivity, an outer-sphere mechanism is the most agreeable mechanism for the catalytic activity of the enzyme.

4.7. NICKEL-SOD MODEL COMPOUNDS

A detailed study of macromolecules like SODs is always challenging due to their inherent complexity. Although the structures of both oxidized and reduced forms of Ni-SOD have been elucidated, a number of questions regarding the functionality and the mechanism of superoxide dismutation remain unanswered. One reasonable and practical way to circumvent this issue is to design SOD mimetics or maquettes that exhibit close structural resemblance to the original SOD enzyme. Metalloprotein maquettes are small metallopeptides that mimic the biological functionality of the parent metalloprotein. Some of these models directly represent the ligand environment of Ni-SOD while others are designed for specific ligand environment investigations.

$[NI^{II}(SOD^{M1})]$ AND DERIVATIVES

Neupane and coworkers have synthesized and carried out extensive studies on Ni-SOD model compound $[Ni^{II}(SOD^{M1})]$ (2006). $Ni^{II}(SOD^{M1})$ is a synthetic maquette of Ni-SOD from *Streptomyces coelicolor* based on the first 12 residues that make up the N-terminus. The objective of this work was to investigate how the primary coordination sphere of Ni-SOD contributes to the stability and reactivity of the metallocenter. The influence of amine/amide versus bis-amide coordination of Ni-SOD was probed utilizing $[Ni^{II}(SOD^{M1})]$ and its acylated form $[Ni^{II}(SOD^{M1}\text{-Ac})]$. The apopeptide of $Ni^{II}(SOD^{M1})$ [H_2N-HCDLPCGVYDPA-COOH] was synthesized via standard solid phase synthetic methods and characterized spectroscopically and electrochemically. It was revealed that the reduced Ni(II) form possesses a NiN_2S_2 square-planar geometry at the metal site with Ni-N distance of 1.846 Å and Ni-S distance of 2.174 Å. In contrast, the acylated form, $Ni^{II}(SOD^{M1}\text{-Ac})]$, revealed a decrease in the Ni covalency compared to the non-acylated form.

$[Ni^{II}(SOD^{M2})]$ AND DERIVATIVES

In order to probe the contribution of the axial ligand towards the reactivity and stability of Ni-SOD, Neupane and coworkers utilized metallopeptide maquette $[Ni^{II}(SOD^{M2})]$ and several of its derivatives (figure 4.10). $[Ni^{II}(SOD^{M2})]$ as model compounds containing only the residues involved in the Ni-binding hook (H_2N-HCDLPCG). All the investigations were done based on the assumption that this minimum configuration maintains identical properties of the original $Ni^{II}(SOD^{M2})$ maquette. Several derivatives were synthesized in order to assess the variation of spectroscopic, structural and reactive properties upon changing the axial ligation in $SOD^{M2}H(1)X$ where X is A or D.

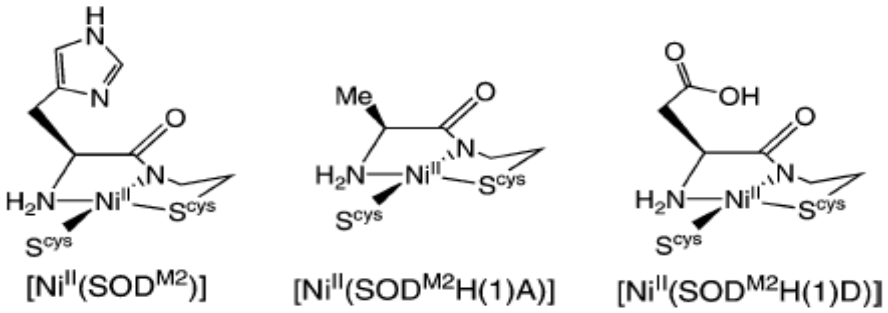

Figure 4.10. $Ni^{II}(SOD^{M2})$ and variants. [Journal of the American Chemical Society, 129, Neupane, K.P. et al., Probing variable axial ligation in nickel superoxide dismutase utilizing metallopeptide-based models: insight into the superoxide disproportionation mechanism, 14610, Copyright (2007)] with permission from the American Chemical Society.

All of these metallopeptides were prepared according to the standard Fmoc/^{t}Bu-based protection strategies on Wang resin with HBTU/HOBt/DIEPA coupling method. Metallation of the peptides was done by adding 1 equivalent of $NiCl_2$ to the apopeptide. Electronic and CD spectra obtained for Ni-SOD model compound $[Ni^{II}(SOD^{M2})]$ and its derivatives are shown in figure 4.11

(Neupane 2006). [$Ni^{II}(SOD^{M2})$] shows a prominent peak at 21 000 cm $^{-1}$ (457 nm, ε = 345 M^{-1} cm $^{-1}$) in the electronic absorption spectrum which is similar to other square-planar $Ni^{II}N_2S_2$ complexes from previous studies (Neupane, K.P. and Shearer, J., 2006; Fielder, A.T., et al., 2005). The negative signed feature of [$Ni^{II}(SOD^{M2})$] in the CD spectrum at 22 220 cm $^{-1}$ (Δε = -1.5 M $^{-1}$ cm $^{-1}$) is characteristic because this peak appears at 21 830 for [$Ni^{II}(SOD^{M1})$].

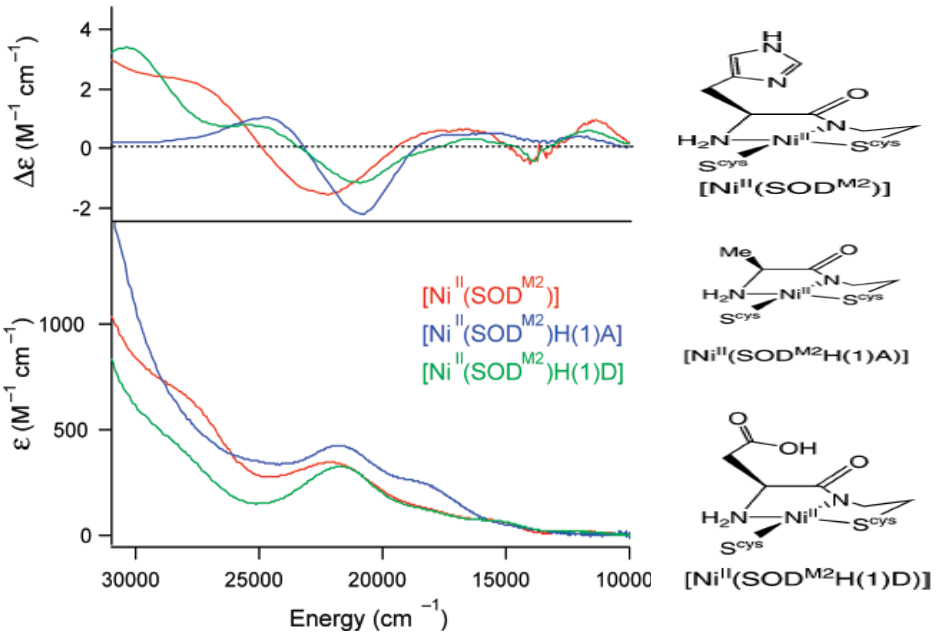

Figure 4.11. Electronic absorption (bottom) and CD spectra (top) of Ni-SOD model compounds (pH 7.4, 50 mM NEM). [Journal of American Chemical Society, 129, Neupane, K.P. et al., Probing variable axial ligation in nickel superoxide dismutase utilizing metallopeptide-based models: insight into the superoxide disproportionation mechanism, 14610, Copyright (2007)] with permission from the American Chemical Society.

This shift of 370 cm^{-1} is inadequate to form an acyclized geometry suggesting that both [$Ni^{II}(SOD^{M2})$] and [$Ni^{II}(SOD^{M1})$] possess similar coordination environments. Nearly similar spectral features indicate that the residues outside the nickel-hook do not contribute to much of the electronic features of the molecule.

Probing the electrochemical behavior of a redox system is crucial to understand its underlying mechanism. Several electrochemical studies have been done for the Ni-SOD model compounds along with other spectroscopic and computational analyses. Neupane and coworkers (2007) utilized the model compound [$Ni^{II}(SOD^{M2})$] to investigate various electrochemical parameters of this model to gain insight about Ni-SOD. A quasi-reversible behavior at 0.52 V versus Ag/AgCl electrode (100 mM $NaClO_{4\ (aq)}$) for Ni^{II}/Ni^{III} redox system was observed in the cyclic voltammograms of [$Ni^{II}(SOD^{M2})$] (figure 4.12).

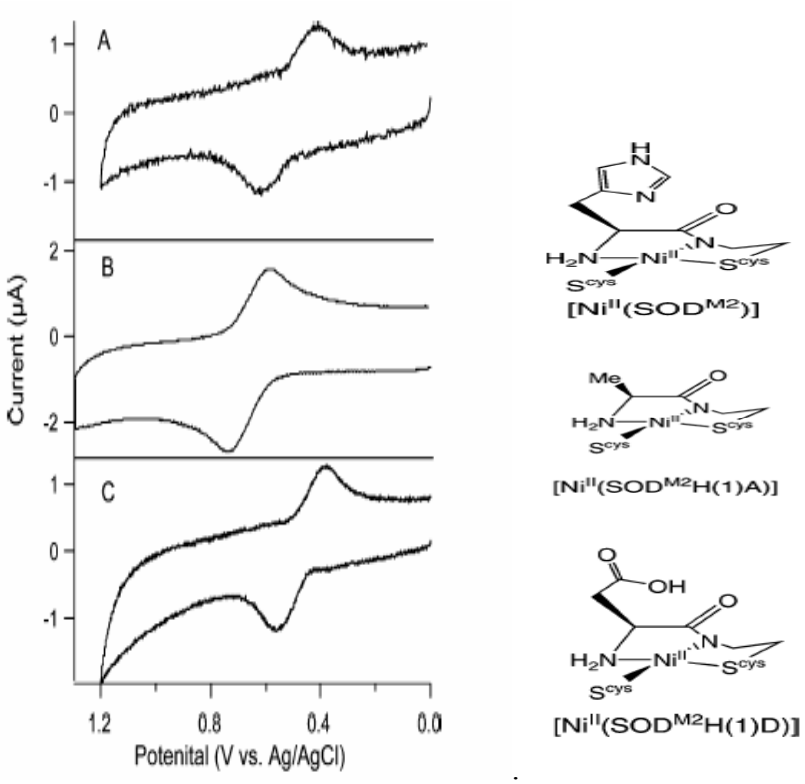

Figure 4.12. Cyclic voltammograms for $Ni^{II}(SOD^{M2})$ and variants. 1 mM solution of [$Ni^{II}(SOD^{M2})$] (A), of [$Ni^{II}(SOD^{M2}$ H(1)A] (B), and [$Ni^{II}(SOD^{M2}$ H(1)D)] (C) at 25 ^{o}C, 100 mM $NaClO_{4(aq)}$. (Scan velocity 100 mV s^{-1}). [Journal of American Chemical Society, 129, Neupane, K.P. et al., Probing variable axial ligation in nickel superoxide dismutase utilizing metallopeptide-based models: insight into the superoxide disproportionation mechanism, 14611, Copyright (2007)] with permission from the American Chemical Society.

$(Me_4N)[Ni^{II}(BEAAM)]$

$(Me_4N)[Ni^{II}(BEAAM)]$ is the first NiN_2S_2 synthetic model compound representing mixed amine/amide coordination to nickel (Shearer J. and Zhao N. 2006). Coordination bond distances of this compound are comparable to that of the actual enzyme (Ni-S of 2.177 Å, 2.137Å, and Ni-N of 1.989Å, 1.858 Å. The ligand BEAAM was prepared and metalated using $NiCl_2$ under anaerobic conditions. The synthesis of Ni^{II}(BEAAM) anion is illustrated in figure 4.13.

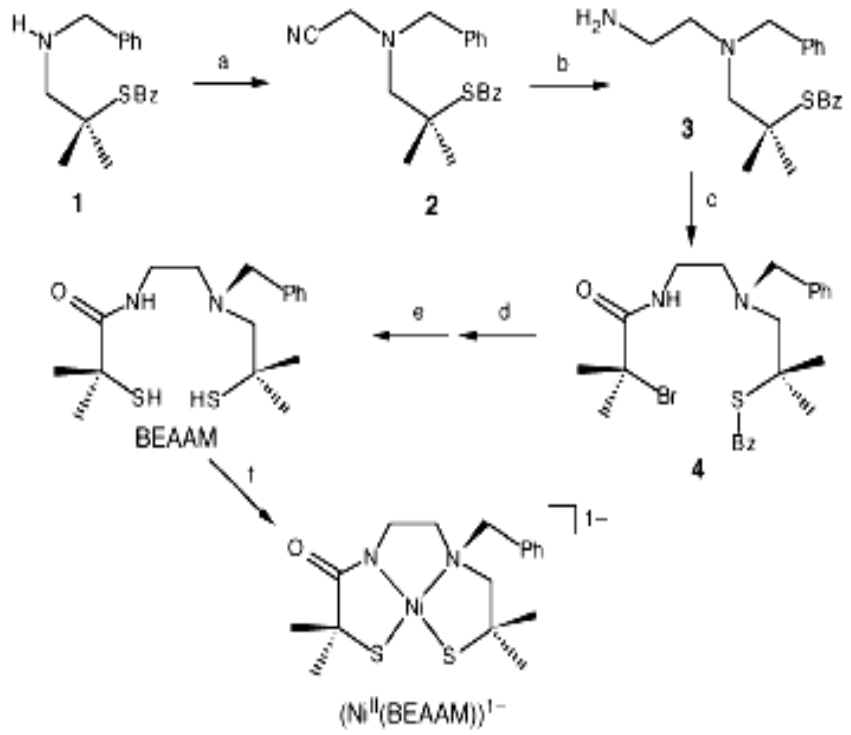

Figure 4.13. Synthesis of Ni^{II}(BEAAM). [(a). $BrCH_2CN$, K_2CO_3, NaI, MeCN, 8h reflux; (b)AlH_3, THF, 12h reflux; (c) 2-bromo-2methylpropionyl bromide, Et_3N, CH_2Cl_2, 12h room temperature; (d) benzylmercaptan, KOH, EtOH, 12h reflux; (e) Na, NH_3, 1h -78 ^oC; (F) $NiCl_2$, Me_4NCl, NaOMe, MeOH, 12h room temperature] [Inorganic Chemistry, 45(24), Shearer, J. and Zhao, N., $(Me_4N)[Ni^{II}(BEAAM)]$: A synthetic model for nickel superoxide dismutase that contains Ni in a mixed amine/amide coordination environment, 9638, Copyright (2006) with permission from the American Chemical Society.

This compound is an excellent model for studying the effects of the combination of amide/amine and sulfur ligation on Ni centers. The electronic absorption spectrum of $(Me_4N)[Ni^{II}(BEAAM)]$ show two defined peaks at 21 690 cm^{-1} (ε = 290 M^{-1} cm^{-1}) and 37 450 cm^{-1} (ε = 21 500 M^{-1} cm^{-1}) These feature correspond well with the absorption spectra of both Ni-SOD and the model compound $[Ni^{II}(SOD)^{M1}]$ (reduced Ni-SOD shows a peak at 22 240 cm^{-1} and a shoulder at 17 770 cm^{-1} ; $[Ni^{II}(SOD)^{M1}]$ shows a peak at 21 800 cm^{-1} and 18 100 cm^{-1}. Therefore, the electronic character of $(Me_4N)[Ni^{II}(BEAAM)]$ molecule is analogous to both Ni-SOD and $[Ni^{II}(SOD)^{M1}]$ which contain Ni(II) in a $N^{amine}N^{amide}S_2$ environment. Also, a quasireversible redox couple was observed for $(Me_4N)[(Ni^{II}(BEAAM)]$ at 0.12 V (versus Ag/AgCl, MeCN, 0.1 M Bu_4NPF_6) (figure 4.14).

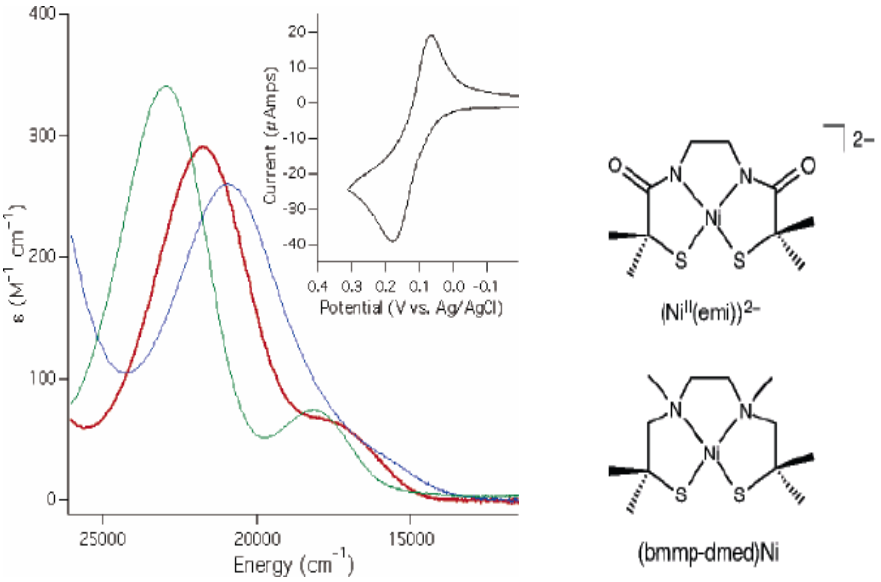

Figure 4.14. Electronic absorption spectra for $(Me_4N)[Ni^{II}(BEAAM)]$ (red bold line), $(Et_4N)_2(Ni^{II}(emi)]$ (green line), and (bmmp-dmed)Ni (blue line). The inset depicts the cyclic voltammograms obtained for $(Me_4N)[Ni^{II}(BEAAM)]$ (MeCN; 0.1 M Bu_4NPF_6; scan speed=100 mV s^{-1}; room temperature). [Inorganic Chemistry, 45(24), Shrearer, J. and Zhao, N., $(Me_4N)[Ni^{II}(BEAAM)]$: A synthetic model for nickel superoxide dismutase that contains Ni in a mixed Amine/Amide coordination environment, 9639, Copyright (2006)] with permission from the American Chemical Society.

4.8. COMPUTATIONAL STUDIES

Crystallographic studies have greatly contributed to the knowledge of the structural, functional, and mechanical aspects of the newly identified Ni-SOD. However, certain phenomena such as protection of the thiolate ligands from oxidation, rearrangements in the coordination environment upon oxidation and reduction of Ni-SOD require further means of investigation. Also, one disadvantage from crystallography studies is that all of the data and interpretations are confined to the resting state of the metalloenzyme. In order to gain insight for these questions, several computational studies have been done in the past few years. Density Functional Theory (DFT) is one of the most versatile methods in probing the molecular electrostatic potentials of these complex systems.

DFT STUDIES ON NiN_2S_2 REACTIVITY

Theoretically, nickel thiolates are prone to oxidation in the presence of dioxygen, hydrogen peroxide, and superoxide (Mullins 2006). Reactivity of sulfur in nickel thiolates has been well established by Busch and other workers (Busch D.H. et. al. 1962). However, sulfur-based oxidation is not observed in Ni-SOD. This lack of sulfur oxidation has stirred up curiosity among researchers. C.S. Mullins and coworkers (2006) have performed DFT calculation on a series of square-planar NiN_2S_2 complexes to investigate the sulfur based oxidation reactivity reaction of these models. These compounds comprise various nitrogen donor types including sulfur oxygenated derivatives and hydrogen bonded derivatives (figure 4.15 top). The goal of these DFT calculations was to identify the effects of nitrogen donor type and hydrogen bonding that may contribute to the protection of sulfur from oxidation. Molecular electrostatic potential diagrams provided insight about the reactivity of each of these models (figure 4.15 bottom). It showed that considerable changes occur in the nucleophilicity of sulfur in different environment.

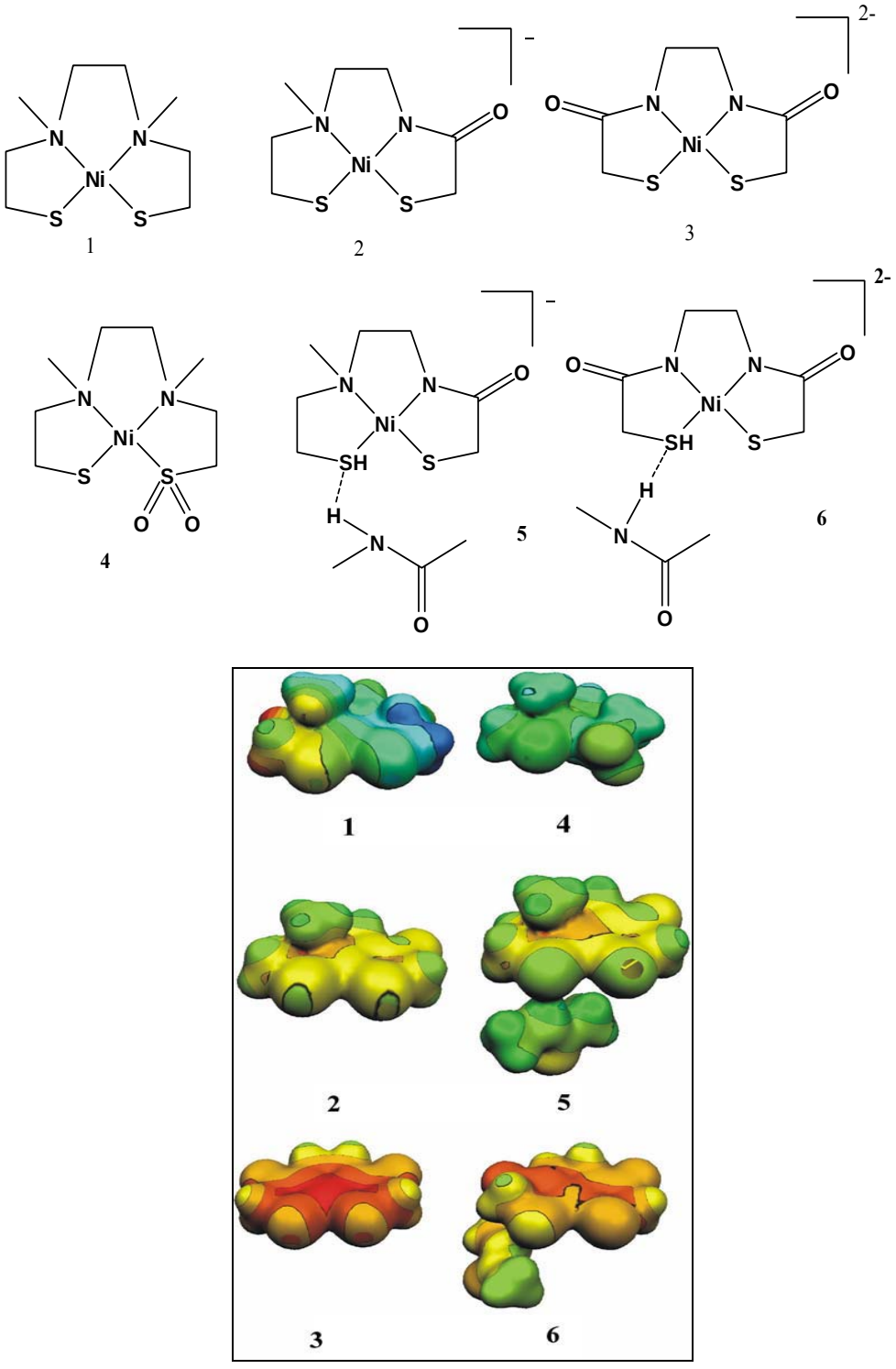

Figure. 4.15. A series of square-planar NiN_2S_2 complexes (top), Electrostatic potential diagrams of square-planar NiN_2S_2 complexes (bottom) [Journal of Biological Inorganic Chemistry, 11, Mullins, C.S. et al., Density functional theory investigation of NiN_2S_2 reactivity as a function of nitrogen donor type and N-H-S hydrogen bonding inspired by nickel-containing superoxide dismutase, 618, 623 Copyright (2006)] with permission from the American Chemical Society.

In diamido compounds, a significant shift in the negative potential from sulfur to nickel was observed (from structure 1 to 3, the negative potential moves from sulfur to Ni center). Also, hydrogen bonding at the active site further reduces the susceptibility of sulfur from oxidation due to the shift in reduction potential to lower values resulting in the deactivation of the nucleophilicity of both thiolates (in 4, both sulfur donors have decreased negative potential, in 5 and 6, electron density has shifted to Ni center). With the aid of molecular orbital analyses, atomic charges, and electrostatic potential diagrams, it was concluded that the nickel dithiolate complexes with a mixed amino/amido nitrogen ligand environment facilitate the inhibition of sulfur oxidation compared to diamido complexes.

FINAL REMARKS

The knowledge about structure, chemistry, mechanism, and biochemistry of this novel Ni-SOD enzyme continues to expand. According to the most recent experimental evidence, the axial imidazole is very likely to be remained ligated during the disproportionation mechanism portraying a new mechanistic scheme. The relatively fast kinetics is attributed to the axial ligation because the presence of imidazole ligand minimizes structural rearrangement during the catalytic process. The hypotheses of an outer-sphere electron transfer mechanism and electrostatic steering of the substrate towards the metal center are supported by many studies. However, the mechanism of how this unique ligand environment of Ni-SOD tunes its reactivity so efficiently still remains unclear. The discovery of Ni-SOD has stirred up a curiosity in chemists and biologists and continues to be a perplexing and fascinating question as to why nature has chosen such a distinct and unique enzyme even though it performs the same function as other commonly known SOD's.

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CHAPTER 5

NICKEL SOD AND FUTURE PERSPECTIVE

5.1 SOD THERAPY

SODs are of prime interest in the treatment of various diseases. Elevated amounts of inrtracellular SODs were found to be effective in reducing the possible damage caused by free radicals (Gregory and Fridovich 1973). Overexpression of manganese or copper-zinc SOD has shown properties that can inhibit the growth of breast cancer cells (Weydert, C.J., 2006). The anti-inflammatory effect of exogenously administrated SODs in reducing the damages related to ischemia and reperfusion was also observed (Klein et al., 2003; Jolly et al., 1984). SODs are also used in the treatment of arthritis and alleviation of the side effects of cancer. SOD is also administrated during surgical procedures in order to prevent injury to transplanting organs. Recent work has demonstrated that antioxidant treatment can attenuate or avoid the development of several pathological conditions particularly diseases related to mitochondrial dysfunction. Also, administration of SOD antioxidants have demonstrated prolonged lifetime of animal models subjected to various studies. Several age related diseases such as cancer, Alzheimer's disease, and Parkinson's disease, and type 2 diabetes are directly linked to mitochondrial dysfunction. A synthetic antioxidant EUK189 (Hinerfeld, et al 2004; Morten, et al., 2005) has been effectively used to treat mitochondrial dysfunctions such as depletion of complex II. These promising findings prompted the idea that SOD therapy can open a new page in medical history.

Although administration of SOD has shown potential therapeutic activity, clinical usage is being challenged by various factors including cellular accessibility, immune response to non-

human enzymes, and impenetrability through various cells or the blood-brain barrier. In order to overcome these challenges, SOD mimetics are taken into consideration. The inability to penetrate the cell membrane is a major disadvantage of SODs and in order to circumvent this problem, synthesizing low molecular weight and membrane permeable SOD mimetics is widely considered and studied. These substitutes should possess certain basic characteristics such as a reduction potential of -0.16 V < E_o < 0.89 V, low reactivity towards oxygen, water solubility, non-toxic, membrane permeability, non-immunogenecity, and stability to withstand cell metabolism to be used as successful SOD mimetics

5.2. SOD MIMETICS

M40403

M40403 is a nonpeptidyl SOD mimetic molecule of native Mn-SOD with added functionalities (Salvemini et al., 1999; Samlowski et al. 2003). It is a low molecular weight manganese complex with a bis(cyclohexylpyridine)-substituted (1,4,7,10,13-pentaazacyclopentadecane) macroligand (molecular weight 484.4) (figure 5.1). It is both chemically and biologically stable and functions kinetically very similar to native SOD. It catalyzes the dismutation of superoxide anion with a rate 2 x 10^7 M^{-1} s^{-1} at pH ~6, which is comparable to the native enzyme. Moreover, it has accessibility to intracellular and subcellular location reacting only with superoxide anion giving advantages over the native SOD. M40403 demonstrated protection of tissue from damage in rat models with ischemia-reperfusion injury (Salvemini et al 1999; Samlowski et al., 2003). M40403 has been successfully administrated as a combination drug for several cases such as post-surgical pain relief (with morphine) and collagen-induced arthritis (with dexamethasone) (Cuzzocrea et al., 2005). Thus, it shows promising therapeutic and clinical applications in the future.

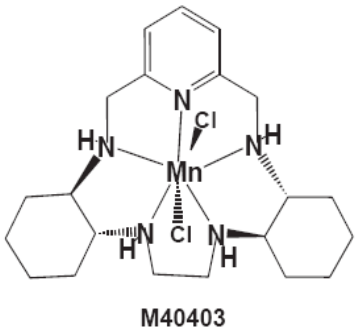


Figure 5.1. The structure of M40403 [Nature Medicine, 9, Samlowski, W.E., Petersen, R., et al., A non peptidyl mimic of superoxide dismutase, M40403, inhibits dose-limiting hypotension associated with interleukin-2 and increase its antitumor effects, 751, Copyright (2003)] with permission from Nature publishing group.

EUK-8 and EUK-134

EUK-8 and EUK-134 are novel synthetic SOD mimetics also known as synthetic catalytic scavengers (SCS) that exhibit both SOD and catalase activity. They are low molecular weight salen-manganese complexes (figure 5.2).

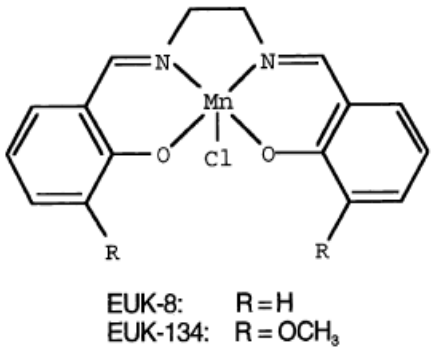
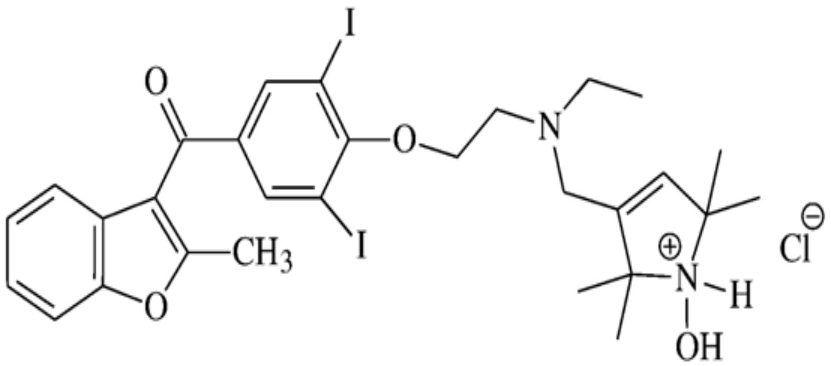


Figure 5.2. The structures of EUK-8 and EUK-134. [The Journal of Investigative Dermatology, 122, Decraene, D., et al., A synthetic superoxide dismutase/catalase mimetic (EUK-134) inhibits membrane-damage-induced activation of mitogen-activated protein kinase pathways and reduces p53 accumulation in ultraviolet B-exposed primary human keratinocytes., page 485, Copyright (2004)] with permission from Elsevier.

It has been shown that the treatment with EUK-8, especially at high doses, greatly reduced the LPS related (lipopolysaccharide) induced adult respiratory distress syndrome (Gonzalez 1995). EUK-134 has proven to be effective in preventing UV-induced oxidative damage (Decraene 2004).

HO-3538

Mitochondrial permeability transition (mPT), an opening of a massive pore in the mitochondrial membrane, induced by reactive oxygen species plays a key role in cell death by ischemia and reperfusion during myocardial infarction (Bogner, Z., et al 2006). It is crucial to inhibit mPT in order to minimize cellular injury. HO-3538 is a novel compound having properties similar to SOD which can inhibit mPT (figure 5.3). It has also shown that HO-3538 exhibits properties like minimizing lipid peroxidation and protein oxidation, recovery of mitochondrial metabolism, and diminished infarct size during myocardial infarction (Bogner, Z. et al. 2006).



HO-3538/OH/HCl

Figure 5.3. HO-3538 SOD mimetic compound. [Free Radical Biology and Medicine, 41, Bognar, Z. et al., A novel SOD-mimetic permeability transition inhibitor agent protects ischemic heart by inhibiting both apoptotic and necrotic cell death, page 836, Copyright (2006)] with permission from Elsevier.

FULLERINE DERIVATIVE C_3

Another molecule that exhibits SOD like properties is C_3. C_3 is a tris-malonic acid derivative of fullerene capable of scavenging superoxide anion (figure 5.4). However, the catalytic conversion rate is about 100 times slower than that of the native enzyme (2×10^{6} mol^{-1} s^{-1}) (Ali S.S., et al. 2004). Clinical trials with mice lacking mitochondrial Mn-SOD showed that administration of C_3 increased the lifespan of these mice suggesting that C_3 can replace the function of Mn-SOD (Quick et al., 2004).

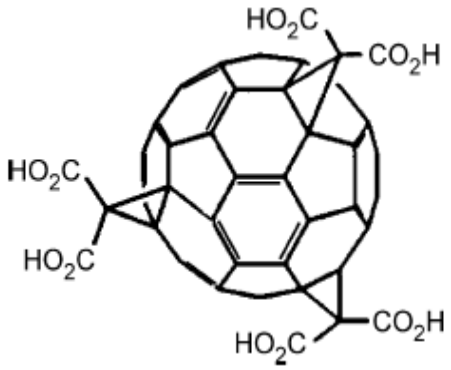


Figure 5.4. The structure of C_3 [Free radical Biology and Medicine, 37(8), Ali, S.S., et al., A biologically effective Fullerine (C_{60}) Derivative with superoxide dismutase mimetic properties, page1192, Copyright (2004)] with permission from Elsevier.

5.3. FUTURE OF SOD

Increasing evidence shows that SOD's and synthetic mimetics of SOD's demonstrate pharmacological efficacy. The root cause of most diseases is the oxidative stress or the homeostatic imbalance of ROS/free radicals versus immunological defense system. Therefore, treatment of these causes is more promising in preventing diseases than the treatment of the disease at its onset or progression. There are several possible ways that SOD can be

administrated therapeutically. Augmentation of the endogenous antioxidant defense system is one of them. Another postulate is that the genetic engineering of the SOD-genes can be used either to enhance the antioxidant role of SOD or compensate for any lack of SOD in the body. The use of SOD mimetic synthetic analogs in the cosmetic industry is also a fascinating area of research. Skin conditions due to both aging and exposure to sun/UV light can be effectively treated using agents that minimize oxidative stress in the skin. Several products are currently in the market. A more clinically important goal is developing drugs that can effectively alleviate and minimize damage to the myocardium during myocardial infarction. The mPT inhibiting SOD mimetics will be an ideal candidate for this. The potential use of SOD mimetics in cardiovascular therapy has also been reviewed recently (Webber 2003).

Ni-SOD or its mimetics have not yet been tested or administrated as therapeutic agents. However, its compatibility with other SOD's in terms of efficiency in catalyzing the dismutase reaction promises that Ni-SOD too can be a potential candidate for future pharmacological uses. With the current rate of research outcome in SOD therapy, we can anticipate a future, though quite contrary to the conventional practice, where patients will be tested for "oxidative stress" and administrated "antioxidants" for treatment. In conclusion, SOD therapy will certainly shed a new light on medical and pharmacological applications in the future.

5.4. REFERENCES

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