Cell. Mol. Life Sci. 64 (2007) 2887 – 2899
1420-682X/07/222887-13
DOI 10.1007/s00018-007-7310-9

Cellular and Molecular Life Sciences

Review

Type-2 copper-containing enzymes

I. S. MacPherson[a] and M. E. P. Murphy[b,*]

[a] Department of Biochemistry and Molecular Biology, Life Sciences Institute, University of British Columbia, 2350 Health Sciences Mall, Vancouver, BC V6T 1Z3 (Canada)
[b] Department of Microbiology and Immunology, Life Sciences Institute, University of British Columbia, 2350 Health Sciences Mall, Vancouver, BC V6T 1Z3 (Canada), Fax: +1 604 822 6041,
e-mail: Michael.Murphy@ubc.ca

Received 6 July 2007; accepted 18 July 2007
Online First 17 September 2007

Abstract. Type-2 Cu sites are found in all the major branches of life and are often involved in the catalysis of oxygen species. Four type-2 Cu protein families are selected as model systems for review: amine oxidases, Cu monooxygenases, nitrite reductase/multicopper oxidase, and CuZn superoxide dismutase. For each model protein, the availability of multiple crystal structures and detailed enzymological studies provides a detailed molecular view of the type-2 Cu site and delineation of the mechanistic role of the Cu in biological function. Comparison of these model proteins leads to the identification of common properties of the Cu sites and insight into the evolution of the trinuclear active site found in multicopper oxidases.

Keywords. Nitrite reductase, amine oxidase, superoxide dismutase, dopamine monooxygenase, peptidyl amidating enzyme.

Introduction

Enzymes incorporate transition metal cofactors to perform essential metabolic reactions. Examples include proteins that comprise electron transport chains and catalyze difficult reactions such as nitrogen fixation and the reduction of ribonucleotides to deoxyribonucleotides. The most common metals employed are iron, zinc and copper; however, only iron and copper are suitable to perform redox reactions. The reduction potentials and coordination chemistries of both iron and copper are ideal for many biological processes, and thus these metals are found broadly in nature. Moreover, theses metals are able to bind and manipulate gaseous substrates such as oxygen and nitric oxide. For a particular system, the type of metal found is likely to be a consequence of its unique chemical properties and bioavailability in the course of evolution.

The bioavailability of copper and iron has changed dramatically over the geological history of the earth (for a review see Williams and Frausto de Silva) [1]. Before the advent of photosynthesis, an anaerobic earth favored reduced iron [Fe(II)] and sulfur (sulfides). Iron bioavailability was dominant since iron sulfides are much more soluble than copper sulfides. As the earth became oxygenated by early photosynthetic organisms, iron was oxidized to the less soluble Fe(III) form that precipitated as iron oxides. In contrast, as sulfides were oxidized to sulfates, copper was liberated since copper oxides are generally soluble, allowing organisms to incorporate this new metal into protein scaffolds to perform chemistry that previously was solely the domain of iron. The increased bioavailability of copper occurred at a time

* Corresponding author.

when organisms were adapting to a new oxic environment, which may explain why many copper proteins are found in systems that mediate oxygen chemistry. The Cu sites observed in proteins are classified into three types based on their structural and spectroscopic properties. Blue Cu proteins attracted attention as a consequence of their intense blue to green color [2]. Strong visible absorption is characteristic of type-1 Cu sites when in the oxidized [Cu(II)] state. The reduction potentials of most type-1 Cu sites are sufficiently low that the oxidized state is favored in the presence of ambient oxygen. Structurally, the Cu atom of a typical type-1 site is coordinated by a Cys and two His residues in a trigonal planar arrangement. Often the thioether of a Met coordinates axially, distorting the geometry towards tetrahedral. The color is due to charge transfer (LMCT) between the Cu and the S atom of the Cys ligand. EPR spectra of the oxidized site show unusually low coupling constants (A values) [3]. The function of these sites is exclusively single electron transfer reactions. Type-1 sites are found in small electron transfer proteins (cupredoxins) that ferry electrons between larger enzymes such as components of the denitrification pathway and photosynthesis. Also, they are found in the larger enzymes nitrite reductase and multicopper oxidase and function in intramolecular electron transfer to copper active sites. Within some of the large enzymes, a CuA site functions as an electron entry point. This site is an expansion of the type-1 site by a second Cu to form a metal-metal bond. Both type-1 and CuA sites are rigid and characterized by low reorganization energies to facilitate electron transfer [4, 5].

Type-2 is used to designate Cu sites with a variety of amino acid ligands and geometries. Most type-2 sites are three to four coordinate and one or more of the Cu ligands are the imidazole side chains of histidines. The coordination sphere may be completed by methionine, glutamate, glutamine or tyrosine. The absence of a thiol group results in weak visible absorption and thus no evident color. EPR spectra of type-2 sites are characterized by a weaker signal with larger A values and are clearly distinct from type-1 site spectra [3]. Coordination positions in type-2 Cu sites can either be vacant or occupied by exogenous ligands. Consequently, these sites can be catalytically active by interacting directly with enzyme substrates. When molecular oxygen is the substrate, type-2 sites may function as oxidases reducing oxygen to water or peroxide, monooxygenases where one oxygen atom is inserted into the substrate and the other is reduced to water, or dioxygenases where both oxygen atoms are incorporated into the substrate. In addition, type-2 sites are able to perform the dismutation of superoxide (SOD) and reduce nitrite to nitric oxide.

More complex Cu sites such as the CuA site mentioned above are constructed from multiple metal centers. Type-3 sites consist of two antiferromagnetically coupled Cu atoms bridged by molecular oxygen or a hydroxyl. The type-3 pair plus a third Cu (type-2) is part of the trinuclear cluster in multicopper oxidases [6]. A dinuclear type-3 Cu site is found in hemocyanins, which function as oxygen carriers in invertebrates [6]. A similar site is observed in tyrosinases; however, these enzymes function as monooxygenases and activate oxygen for insertion in phenolic substrates [6].

Recently, a broad review of copper proteins (mononuclear, dinuclear, polynuclear) and their mechanisms of oxygen activation has been published [7]. In the present review, we focus on the structure and function of the mononuclear type-2 Cu sites in four well characterized enzyme families: amine oxidases, copper monooxygenases, nitrite reductases and superoxide dismutases. Multiple crystal structures of each protein family in different physiological states combined with extensive spectroscopic and enzymology studies makes these enzymes excellent model systems for mechanistic understanding of type-2 Cu function. Furthermore, the crystal structures of these enzymes reveal common features of the Cu sites that give insight into how enzymes use the metal to achieve the intended chemistry required for biological function.

Amine oxidases

Copper amine oxidase (CuAO) belongs to a larger class of amine oxidases that catalyze the oxidative deamination of amines and concomitant reduction of oxygen to hydrogen peroxide by the reaction:

$$RCH_2NH_2 + H_2O + O_2 \rightarrow RCHO + NH_3 + H_2O_2$$

These ubiquitous enzymes are found in a large range of organisms, from microbes (including bacteria and fungi) to plants and mammals (for a review see Brazeau et al. [8]). In bacteria, CuAOs have well-established roles in providing carbon or nitrogen sources when primary amines are available. In plants, there is evidence for the role of CuAOs in defense responses [9] and a variety of developmental processes including lignification and the deposition of suberin in the formation of cork [10]. In mammals, CuAOs are found in a wide range of tissues, including the placenta, blood, muscle, and endothelium; however, their function in mammals are not well understood. One of the best characterized mammalian CuAOs is vascular adhesion protein-1 (VAP-1). Increased expression and translocation of VAP-1 to

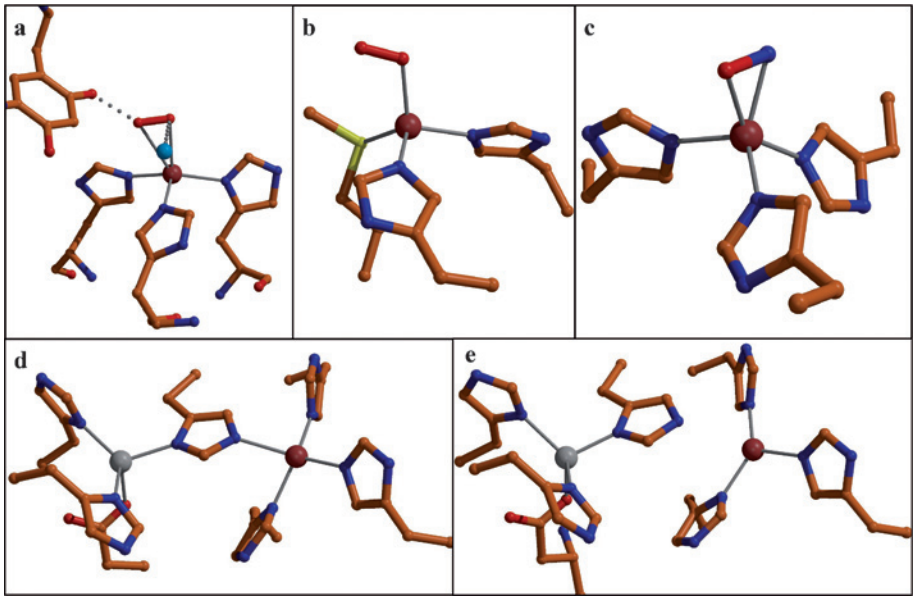

Figure 1. Mononuclear type-2 Cu sites in four enzyme families. In each panel, amino acid ligands are drawn in ball and sticks with C, N, O, S atoms in orange, blue, red and yellow, respectively. The Cu atoms (brown) and solvent atoms (cyan) are depicted as spheres. Gray spheres in superoxide dismutase (SOD) are zinc atoms. (*a*) Hydroperoxo-bound copper amine oxidase (CuAO; PDB entry 1D6Z). Part of the trihydroxyphenylalanine quinone (TPQ) cofactor is shown. (*b*) End-on superoxo-bound to peptidylglycine α-hydroxylating monooxygenase (PHM; PDB entry 1SDW). (*c*) NO bound to nitrite reductase (NiR; PDB entry 1SNR). (*d*) Bridged SOD (PDB entry 2SOD). (*e*) Bridge-broken SOD (PDB entry 2JCW).

the vascular endothelial surface occurs during inflammation [11, 12]. VAP-1 amine oxidase activity has been attributed to the tight binding, rolling, and transmigration of lymphocytes on endothelial cells during inflammation [13–15]. Although the substrate or substrates of the enzyme are not known, H_2O_2 produced by VAP-1 has been suggested to play a signaling role [16]. Increased CuAO expression in humans is a marker of several diseases including cancer, diabetes, congestive heart failure, and liver cirrhosis [17, 18].

Crystal structures have been solved for CuAO from several organisms, including *E. coli* [19], *Arthrobacter globiformis* [20], *Pichia pastoris* [21], *Pisum sativum* (pea seedling) [22], *Bos taurus* (bovine) [23], and human [24]. CuAOs are invariably dimeric proteins, containing one Cu atom per monomer. A deep solvent-filled cleft formed by a β-sandwich domain in the protein leads to the active site, roughly 15 Å from the protein surface. The Cu atom is coordinated by three histidine residues and two water molecules (one axial and one equatorial to the Cu), in a nearly square pyramidal geometry. The Cu site with oxygen bound is shown in Fig. 1a. Two of the histidine residues that coordinate the Cu are derived from a single β-strand with one intervening residue (His-X-His), whereas the third histidine comes from an adjacent β-strand. CuAOs are unique in that they contain a trihydroxyphenylalanine quinone (TPQ) cofactor that originates from a tyrosine residue of the protein. The active site Cu as well as oxygen is required for tyrosine maturation into TPQ [25]. During this maturation process, two molar equivalents of dioxygen are consumed, one incorporated into the TPQ and another reduced to hydrogen peroxide [26]. Several intermediates have been trapped in crystals by controlling the availability of copper and oxygen, as well as the length of time for maturation to occur [27]. Since the mature enzyme has a distinct UV-visible spectrum, crystals can be monitored as a function of time and cryogenically preserved at different points of the maturation process. Throughout the proposed mechanism of TPQ biogenesis, the Cu plays a role in coordinating to the pre-TPQ tyrosine and its intermediates, *via* the aromatic oxygen atoms of the ring (Fig. 2). Also, the Cu atom is likely to play a role in activating molecular oxygen to a peroxo-bridged intermediate with position 3 of the tyrosine ring. Spontaneous splitting of the peroxo-bridge results in oxygen addition to the ring as well as the formation of a Cu-bound hydroxyl species, which attacks at position 6 (position 2 in the new species) on the ring. Finally, fully reduced TPQ bound to the Cu is oxidized, yielding a molar equivalent of hydrogen peroxide.

Activation of molecular oxygen in TPQ biogenesis is a process still under active investigation. Thus far, a reduced Cu(I) state is not observed. Replacement of Cu by other transition metals such as cobalt or nickel significantly lowers the rate of TPQ formation [28, 29]. However, these metals nonetheless catalyze the maturation process, implying that a metal redox cycle may be not essential for TPQ formation. In copper- or nickel-constituted protein, Klinman et al. [28] propose that the tyrosine reduction potential is lowered by tyrosinate coordination to the metal (Fig. 2c); thereby allowing for a reaction with O_2 to form the peroxo-bridged intermediate (Fig. 2d). Slower rates for nickel

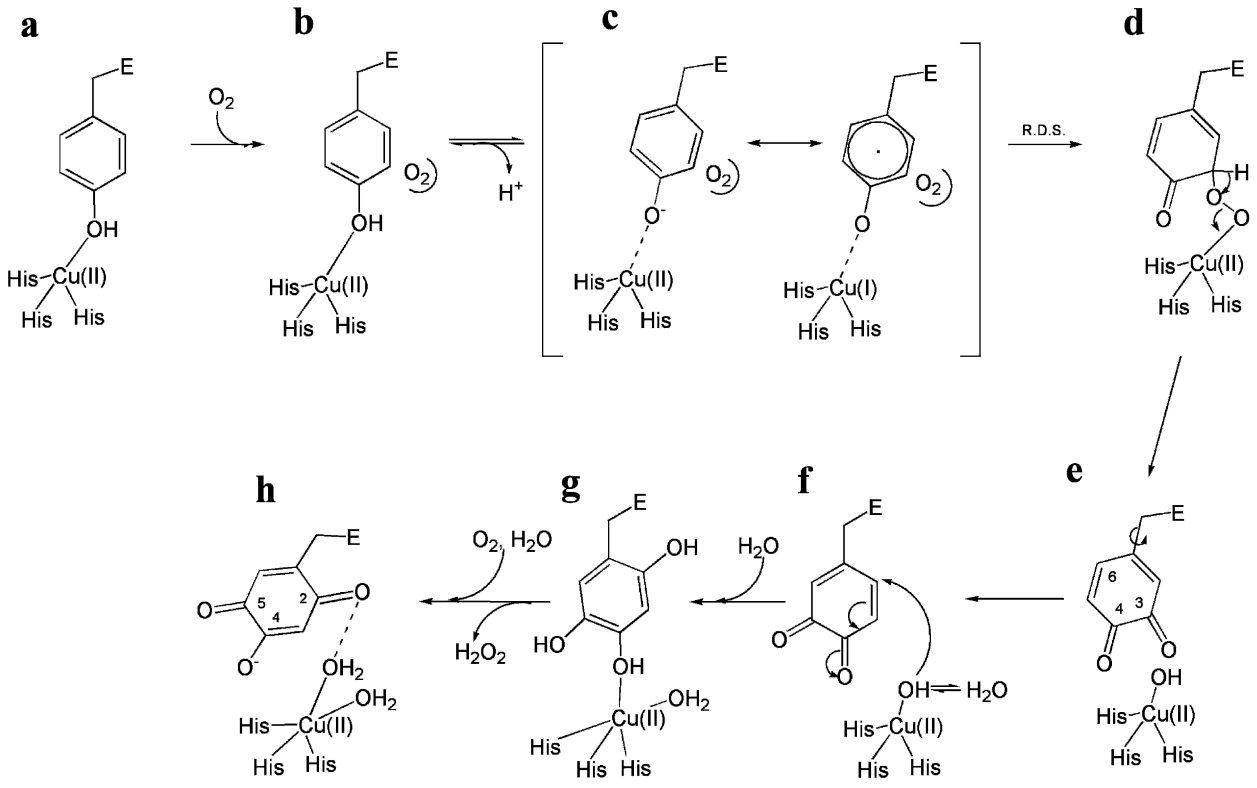

Figure 2. Mechanism of TPQ cofactor biogenesis in CuAO (reprinted from [8] with permission).

were attributed to differences in tyrosinate-metal reactivity with oxygen as well as the weaker Lewis acid properties of nickel, which could slow the addition of hydroxide to the TPQ precursor (Fig. 2f) [28].

Catalysis by CuAO can be described as two half-reactions. First is the reductive half-reaction, in which primary amines are oxidized to their respective aldehydes and the TPQ cofactor is reduced by a total of two electrons. The second half-reaction is the oxidative step, in which the TPQ is reoxidized by transferring two electrons to molecular oxygen, yielding hydrogen peroxide. The mechanism of CuAO has been studied extensively; however, the details of the mechanism of oxygen reduction are still not clear. For example, the role if any of the Cu atom in the transfer of electrons from the reduced TPQ oxygen is not defined. Replacement of the Cu in the mature enzyme with cobalt results in a nearly fully active enzyme, with a higher K_m for O_2, suggesting that the specific redox cycle of Cu is not necessary for amine oxidation [30]. Additionally, the TPQ is too far from the Cu atom for direct coordination, ruling out direct Cu reduction during catalysis. Further insight into the mechanism is provided by a crystal structure with oxygen bound to the active site [31]. The oxygen species was determined to be hydroperoxo, based on distance from the Cu (longer than expected for superoxo species) and its Cu-O-O coordination angle of 88°, characteristic of Cu-hydroperoxo species. Thus, the more likely role of Cu (and cobalt in cobalt-substituted CuAO) is to stabilize oxygen by coordination as it is being reduced. A two-electron reduction of oxygen by consecutive single-electron transfers must involve a superoxo (singly reduced) species. This reaction step has a standard reduction potential of –0.33 V *versus* NHE [32], which would be unfavorable for the overall amine oxidase reaction, given that the TPQ reduction potential is 0.102 V *versus* NHE [33]. However, side-on coordination of superoxide to Cu is favorable thermodynamically, and it is possible that the active site metal stabilizes the superoxo intermediate, poising it for the final reduction to hydrogen peroxide.

Monooxygenases

Peptidylglycine α-hydroxylating monooxygenase (PHM) and dopamine beta-monooxygense (DβM) catalyze the hydroxylation of their respective substrates by the following reactions, respectively:

$$\mathrm{RCO\text{-}NH\text{-}CH_2\text{-}CO_2^- + O_2 + 2H^+ + 2e^-} \rightarrow \mathrm{RCO\text{-}NH\text{-}CHOH\text{-}CO_2^- + H_2O}$$

$$RCH_2\text{-}CH_2\text{-}NH_2 + O_2 + 2H^+ + 2e^- \rightarrow RCHOH\text{-}CH_2\text{-}NH_2 + H_2O$$

In both enzymes, two distinct Cu sites are used to split molecular oxygen, O_2, as the source of OH in the hydroxylation reaction. The external source of electrons required for this reaction is intracellular ascorbate.

PHM and DβM are found primarily in metazoa, and their functions *in vivo* are well established. PHM is one of two domains in peptidylglycine α-amidating monooxygenase (PAM), which is responsible for the activation of a variety of hormones by α-amidation, thereby improving hormone-receptor affinity [34]. The other domain of PAM is a peptidyl-α-hydroxyglycine α-amidating lyase, which removes a glyoxylate group formed after the PHM-catalyzed reaction. DβM catalyzes a similar reaction to PHM; however, the hydroxylation of dopamine is at the β-carbon. The product norepinephrine is a well-known hormone and the primary neurotransmitter in the peripheral sympathetic nervous system. Deficiency in DβM results in depressed serum levels of norepinephrine, resulting in severe orthostatic hypotension [35]. DβM is localized in chromaffin granules of adrenal glands, and some is also excreted into the plasma. Interestingly, monoamine oxidase, a FAD-containing amine oxidase rather than CuAO, further oxidizes norepinephrine to deactivate it as a neurotransmitter [36].

The aligned amino acid sequences of PHM and DβM proteins are 27% identical and 40% similar [37]. DβM contains large sections on its N and C termini that are not found in PHM. Nonetheless, both enzymes have a similar common core, containing two Cu sites, designated Cu_H and Cu_M. The crystal structure for PHM first solved in 1997 [38] has two domains, an N-terminal β-sandwich of two antiparallel β sheets that contains the Cu_H site, and a C-terminal β-sandwich consisting of antiparallel and mixed β sheets, that contains the Cu_M site. Cu_H is coordinated by three histidines, and has been determined to transfer electrons to the Cu_M site. Cu_M in its resting oxidized state is coordinated by two histidines, a methionine, and a water molecule (Fig. 1b). This is the site of oxygen and substrate activation. The two domains of PHM are separated by a wedge of solvent-filled space, and thus the Cu_H and Cu_M sites are separated by 11 Å of solvent, indicating an unprecedented mechanism of electron tunneling between the Cu sites.

Oxygen activation and catalysis

The mechanisms of both PHM and DβM have been studied extensively. For a more detailed discussion of the mechanism, refer to [37] and [39]. By studying deuterium isotope effects of PHM [40] and DβM [41], Klinman et al. showed that the reactions of both enzymes proceed *via* similar activated complexes. It had been shown that both Cu centers in DβM must be reduced (by ascorbate *in vivo*) for turnover to occur [42, 43]. The proposed mechanism involves abstraction of hydrogen from a substrate carbon to form a substrate radical. Electron transfer from Cu_H to Cu_M results in a Cu-oxo species, which donates an oxygen atom to the substrate followed by protonation to conclude the hydroxylation reaction (Fig. 3). In terms of the activated oxygen species, a primary suspect observed often in mononuclear iron monooxygenases is a hydroperoxo-bound intermediate. This species is feasible for mononuclear Cu sites and agrees well with the availability of two electrons from Cu_H and Cu_M. However, Klinman and colleagues [44] established that a hydroperoxo intermediate is not readily formed in PHM by showing that virtually no hydrogen peroxide is leaked into solution during DβM turnover. This observation also rules out the proposed shuttling of superoxide radicals between the Cu sites [45], since expelled peroxide resulting from spontaneous dismutation of superoxide is expected to be a minor product. Additionally, the Cu-peroxy anion (deprotonated form of Cu-hydroperoxo) was ruled out due to the absence of a Cu(II) EPR signal for non-reactive analogues [44]. Klinman therefore proposed the EPR-silent Cu-superoxo as the hydrogen abstracting species (Fig. 3).

Chen and Solomon [32] performed density functional theory calculations to model the activated oxygen species at the Cu_M copper site, leading to a proposal for the mechanism of PHM and DβM. In agreement with Klinman's proposed mechanism, they found that a side-on Cu-superoxo intermediate would be most energetically favorable for abstracting a hydrogen atom from substrate, the first major step in the reaction [32]. According to their calculations, the side-on oxygen species has the most accessible orbital available for bonding with the hydrogen for abstraction [32]. Crystallographic work done by Prigge et al. [46] sheds additional light onto the superoxide intermediate of PHM. Using a poorly catalyzed substrate analog, *N*-acetyl-diiodotyroxyl-D-threonine, the authors were able to observe a Cu-oxygen intermediate bound to the Cu_M active site. The refined O-O bond distance of 1.23 Å was characteristic of superoxide species. More surprising was the end-on orientation of the superoxide species, which is the first of its kind characterized crystallographically. The end-on nature of this intermediate orientates the superoxide ideally for H-atom abstraction from the physiological substrate.

Yet another Cu-oxygen species has been proposed to function in the hydrogen abstraction step in Cu

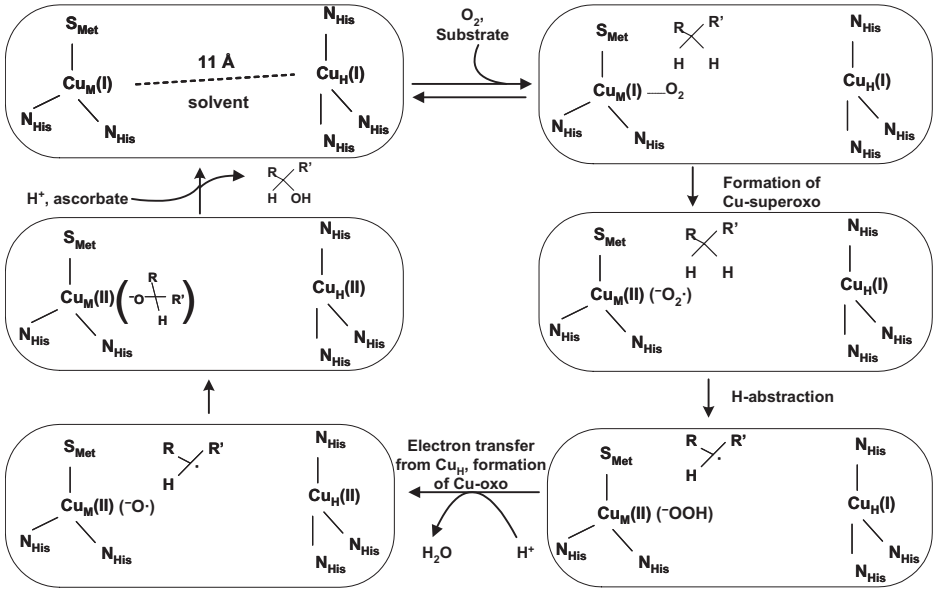

Figure 3. Catalytic mechanism of Cu monooxygenases (adapted from [37]).

monooxygenases. By QM/MM studies, Crespo et al. [39] and Yoshizawa et al. [47] found that a Cu(III)-oxo species is capable of H-atom abstraction with the lowest energy barrier. They suggest that a Cu-superoxo species is generated; however, acquisition of a non-substrate proton and an electron (from Cu_H) forms a Cu-hydroperoxo species. This species obtains another proton to generate H_2O plus Cu(III)-oxo, which then performs the substrate hydrogen abstraction step. The Cu(III) species has not been detected in Cu proteins, but is analogous to the activated oxygen species [Fe(IV)-oxo] proposed for cytochrome P450 enzymes [48].

Nitrite reductase

Copper-containing nitrite reductase (NiR) catalyzes the one-electron reduction of nitrite (NO_2^-) to nitric oxide (NO) by the following reaction:

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O$$

This process occurs during dissimilatory denitrification, in which nitrate and nitrite and their metabolites are used as electron acceptors when oxygen levels are low [49]. NiR is secreted in the periplasmic space between the inner and outer membrane of Gram-negative bacteria. Two types of NiRs are known, one that uses copper and the other heme iron cofactors [49]. By sequence analysis, copper-containing NiRs are found in unicellular organisms from all the main branches of life including some fungi. Most known examples are bacterial including all the lineages of proteobacteria, flavobacteria, eubacteria, as well as some archaea. In addition to fungi, the other eukaryotic examples are found in species of amoeba. The typical gene name for copper containing NiR is *nirK*. Due to the similarity between the multicopper oxidase family and NiR, many *nirK* sequences are misannotated in genome sequence projects; however, the sequences are easily distinguished by sequence motifs with the presence and absence of active site residues [50].

NiR is typically a 110-kDa trimer, containing a type-1 (blue) Cu site within each monomer and a type-2 Cu site located between monomers. Each monomer is composed of two homologous domains with a Greek-key β-barrel (cupredoxin) fold [51]. The type-1 Cu site accepts electrons from a small protein electron donor. Electrons are subsequently transferred to the type-2 Cu site, where nitrite is reduced to NO gas [49]. The tetrahedral type-2 site is formed from three histidines, one of which is derived from an adjacent monomer (Fig. 1c). In the oxidized resting state, the fourth position is occupied by a water molecule. The type-1 and type-2 Cu sites are linked by a Cys-His bridge such that a cysteine coordinates to the type-1 Cu and an adjacent histidine on the protein chain coordinates to the type-2 Cu [51]. This bridge is believed to facilitate rapid rates ($>1000\ s^{-1}$) of electron transfer between the type-1 and type-2 Cu sites [52]. Recently, a variant of the typical NiR from *Hyphomicrobium denitrificans* was shown to have an additional N-terminal cupredoxin domain [53].

The catalytic mechanism of NiR proceeds in a random sequential order [54] to give a Cu-nitrosyl intermediate [49, 55]. Site-directed mutagenesis studies implicate Asp98 (numbering from *Achromobacter cycloclastes* NiR) and His255 directly in the catalytic mechanism [56, 57]. Crystallographic structures reveal that the Asp98 forms an H bond to both bound nitrite substrate and NO product [55, 56]. His255 is proposed to participate in proton transfer to a catalytic intermediate either directly or *via* Asp98 (Fig. 4). A large hydrophobic residue, usually an isoleucine (Ile257), partially occludes the type-2 active site, limiting the binding of larger substrates. Mutation of this residue results in alternate non-productive binding modes of nitrite to the Cu [58, 59]. If nitric oxide is not removed from the reaction vessel, NiR is able to produce nitrous oxide with a chemical reductant [49]. The concentration of nitric oxide reaches a steady state level of 80 nM, suggesting that it is a potent inhibitor [49]. In crystals of NiR exposed to NO, the diatomic molecule binds in a unique side-on fashion to the type-2 Cu [55]. Side-on binding of NO is supported by DFT calculations on both model compounds [60] and the enzyme active site [61].

NO is a structural analogue of O_2 and has been shown to bind O_2-reducing type-2 Cu enzymes such as amine oxidase [31] and laccase [62]. Thus, that NiR is able to reduce oxygen is not surprising [63], given the strong affinity of NO for the type-2 Cu site. The reduction product is believed to be hydrogen peroxide [63]. Continued reduction of hydrogen peroxide leads to the inactivation of NiR, suggesting that destructive hydroxyl radicals are being produced. This inactivation mechanism is supported by an abolition of enzyme inactivation when catalase is present in the reaction [63]. *In vivo*, the switch from anaerobic nitrate reduction to aerobic conditions in the absence of nitrite is shown to result in a rapid inactivation of NiR in *A. faecalis*, underlining the importance of oxygen reduction by NiR in nature [64]. The mechanism by which NiR reduces O_2 remains poorly understood.

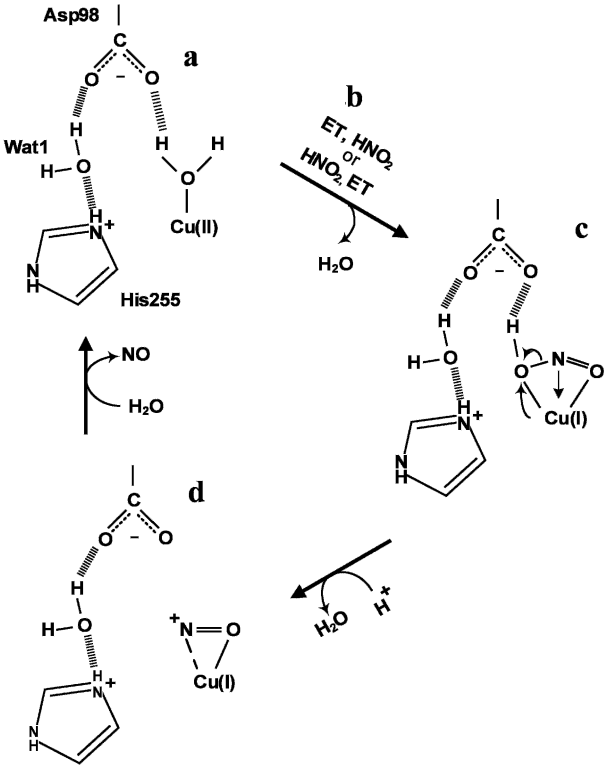

Figure 4. Mechanism of Cu containing NiR.

Multicopper oxidases (MCO) catalyze the oxidation of various small molecules and cations with the concomitant four-electron reduction of oxygen to water:

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$$

Some MCOs such as mammalian ceruloplasmin and yeast fet3p are ferroxidases, oxidizing Fe(II) to Fe(III). In contrast, laccases are MCOs that derive electrons from the oxidation of phenolic compounds. For a review of the mechanism of MCOs see [65]. Striking similarity exists between the Cu sites of NiR and those of the MCOs, both structurally and mechanistically. Furthermore, the fold and cupredoxin domain arrangement suggest that NiR and MCOs share a common ancestor [66, 67]. As noted, both enzymes are capable of reducing oxygen. While NiR releases hydrogen peroxide, MCOs form a peroxide-level intermediate on the pathway towards the full oxygen reduction to water. Reminiscent of hydroxyl radical formation by NiR, both laccase and fet3p are proposed to produce a hydroxyl radical when the type-1 Cu is made nonfunctional resulting in a three-electron reduction of oxygen [68, 69].

NiR and MCO share many structural features. A superposition of the amino acids that form the active Cu sites from *Alcaligenes faecalis* NiR (PDB entry 1SJM) and *Trametes versicolor* laccase (PDB entry 1GYC) is shown in Fig. 5. Firstly, in both NiRs and MCOs, the blue type-1 Cu sites are connected to a second Cu site *via* a Cys-His bridge. Paradoxically, the type-1 sites are derived from different domains, the N-terminal domain for NiR and the C-terminal domain for laccase. The oxygen-reducing site of MCO is a trinuclear copper cluster consisting of two antiferromagnetically coupled type-3 Cu atoms and one type-2 Cu. In the superposed structures, the type-2 Cu site of NiR overlaps with one of the type-3 Cu sites of laccase, including the positions of the three histidine residues coordinating to the respective Cu atoms (Fig. 5). Of

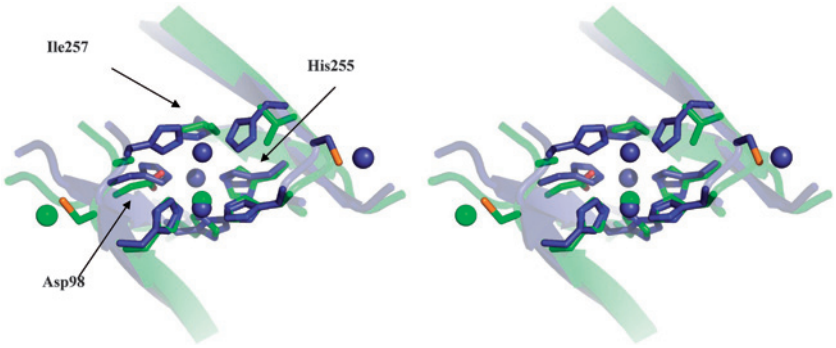

Figure 5. Stereo view of the superposition of the trinuclear cluster of the multicopper oxidases (MCO) laccase (colored blue, PDB entry 1GYC) with the type-2 site of NiR (colored green, PDB entry 1SJM). Note the opposite orientation of the type-1 Cu sites (far left and far right spheres). The NiR type-2 Cu and laccase type-3 Cu overlap. NiR residues Asp98, His255 and Ile257 are labeled.

the remaining five His ligands that coordinate the type-2 and second type-3 Cu atoms, one aligns with His255, an essential residue in NiR catalysis. Furthermore, the other two identified catalytically important NiR residues, Asp98 and Ile257, both superpose with MCO His ligands. Lastly, if Ala137 and Val304 were changed to His residues, an approximate MCO trinuclear cluster would be complete. Unfortunately, the trinuclear cluster is not achieved when these residues are simply changed to histidines (unpublished results). As mentioned, the type-2 Cu of NiR is located between monomers (His100 and 135 derived from one monomer, His 306 from an adjacent monomer). Considering that laccase is monomeric, the structural similarity between the active sites is remarkable. Multimeric small laccases have been characterized functionally and spectroscopically [70, 71], but await crystal structure determination. These laccases are proposed to be evolutionary links between NiR and the well known MCOs [67].

CuZn superoxide dismutase

Superoxide (O_2^-) results from one-electron reduction of molecular oxygen, and is a common byproduct of respiration and photosynthesis, as well as a killing agent produced by activated phagocytes. While O_2^- is a potent radical, protonation to form the hydroperoxyl radical ($HO_2^\bullet$) is implicated in lipid peroxidation [72], and O_2^- reacts with NO at near diffusion-controlled rates to form peroxynitrite ($ONOO^-$), one of the strongest physiological oxidants [73]. To counteract the damaging effects of superoxide, organisms have evolved superoxide dismutases, which function as O_2^- scavengers, catalyzing the dismutation of O_2^- to H_2O_2 and O_2 by the reaction:

$$2\,O_2^- + 2\,H^+ \rightarrow O_2 + H_2O_2$$

Note that this reaction occurs spontaneously at rates roughly 10^4 times slower than the enzyme catalyzed rates. Designated by their active site metals, three unrelated classes of SODs are defined. CuZnSOD, the focus of this review, constitute one class. FeSOD, MnSOD, and Fe/MnSOD form the second class, and NiSOD comprises the third class. All characterized eukaryotes and many prokaryotes express CuZnSOD [74]. Most of the mechanistic and crystallographic studies have focused on enzymes from eukaryotic sources, namely yeast, bovine, and human. Point mutations in human CuZnSODs have been linked to 20 % of cases of familial amylotrophic lateral sclerosis (FALS), a fatal neurogenerative disease characterized ultimately by paralysis [75, 76].

Eukaryotic CuZnSOD is a 32 kDa homodimer, each monomer containing one copper and one zinc atom. The overall fold of the CuZnSOD monomer is a flattened eight-stranded antiparallel Greek-key β-barrel. Using the numbering for the human and yeast proteins, the active site type-2 Cu of oxidized CuZnSOD is coordinated by four histidine residues (His46, His48, His63, and His120) in distorted square-planar geometry. A weakly coordinating water is bound at an axial position, 2.5 Å from the Cu. The zinc atom is located ~6.6 Å away from the Cu, and is coordinated by His63, His71, and His80 as well as Asp83. Of important note is the bridging coordination by His63 that ligates the copper and zinc atoms by the imidazolate Nε2 and Nδ1 atoms, respectively (Fig. 1d). The coordination sphere of the zinc atom is saturated, and this metal plays an indirect structural role in catalysis.

Spectroscopic findings have suggested that upon reduction the bridging imidazolate of His63 is protonated and loses Cu coordination [77–80]. Reduction is accompanied by the loss of the weakly coordinating water to generate a tricoordinate Cu site (Fig. 1e). However, Cu coordinations observed in crystallographic studies are not consistent with solely an oxidation state dependence (Figs. 1d, e). Both bridge-broken [81] and bridge-intact [82] states are observed for yeast Cu(II)ZnSOD. A bridge-broken and a bridge-intact state is observed in each monomer

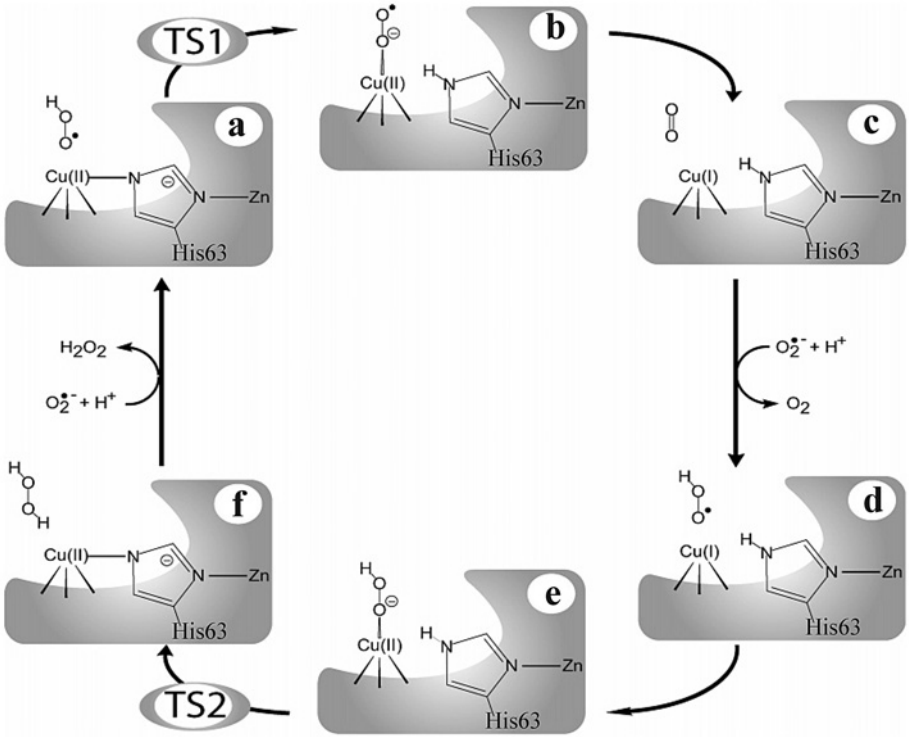

Figure 6. Mechanism of CuZnSOD (reprinted from [74] with permission, copyright 2002 American Chemical Society). This mechanism includes nonspecific protonation of superoxide before docking at the type-2 Cu active site, as well as oxidative coordination during the oxidative phase.

in the asymmetric unit of the same crystal of bovine Cu(II)ZnSOD [83]. The bridge-intact state is observed for reduced bovine Cu(I)ZnSOD [84]. Yet another study on a G37R mutant of human Cu(II)ZnSOD shows a bridge-broken and bridge-intact state for each monomer in the asymmetric unit [85]. Eisenberg and colleagues [86] suggested a "delicate balance" exists between the free energies of the reduced and oxidized enzymes. Thus, the protein environment in crystal, determined by the number and placement of crystal contacts, could favor the bridge-intact over bridge-broken state or *vice versa*, regardless of Cu oxidation state.

The dismutation reaction can be described as two half-reactions related by classical ping-pong mechanism. First, one molecule of O_2^- reduces the active site Cu to yield Cu(I)ZnSOD:

$$O_2^- + \text{Cu(II)ZnSOD} \rightarrow O_2 + \text{Cu(I)ZnSOD}$$

Second, another molecule of O_2^- acquires one electron from the Cu site and two protons to form hydrogen peroxide:

$$O_2^- + \text{Cu(I)ZnSOD} + 2\,H^+ \rightarrow H_2O_2 + \text{Cu(II)ZnSOD}$$

A well-supported mechanism for CuZnSOD remains elusive. In a working model (Fig. 6), the first or reductive phase of the enzyme mechanism is suggested to involve direct binding of superoxide to the active site Cu. Direct binding of superoxide is presumed since anions such as azide, cyanide, and fluoride bind directly to the Cu of Cu(II)ZnSOD [86]. Transfer of the electron from superoxide to the Cu is proposed to be accompanied by protonation of His63 and loss of its Cu coordination, followed by the release of O_2.

FTIR studies with azide as a superoxide mimic showed that this molecule does not directly coordinate the Cu in Cu(I)ZnSOD [87]. Leone et al. [87] suggest that the second or oxidative phase involves second-shell electron transfer, in which the superoxide radical docks near but not directly coordinating the active site Cu. The authors propose electrostatic binding to Arg143, a highly conserved residue in the CuZnSOD active site [87]. Arg143 has been shown to be important for catalysis by a 90% loss in activity when mutated to isoleucine [88]. Consistent with the FTIR studies but contradicting the proposed second shell electron transfer mechanism, theoretical work by Pelmenschikov and Siegbahn [74] suggest oxidative coordination by the superoxide species to Cu(I)ZnSOD (Fig. 6). Thus, the superoxide rapidly oxidizes Cu(I)ZnSOD to Cu(II)ZnSOD, while binding to the Cu(II) site to give a hydroperoxo species. Further experimental work is required to support either an oxidative coordination or second shell electron transfer mechanism during the oxidative phase of Cu(I)ZnSOD.

Protons must be shuttled to the active site to generate H_2O_2 in the SOD reaction. Ordered water molecules

in and around the active site are suggested to conduct both protons [86]. An agreed-upon proton source is the protonated, bridge-broken His63, but the second proton is the cause of some debate. Eisenberg and colleagues [86] suggest an invariant water molecule bound near the active site as the direct source of the second proton. Theoretical analysis by Pelmenschikov and Siegbahn [74] suggest an alternative proton source. They postulate that in both the reductive and oxidative reactions, superoxide is nonspecifically protonated (pKa = 4.8) due to a negative electrostatic field around the active site. In the reductive phase, the hydroperoxyl radical binds, yielding an electron to the Cu and a proton to His63. The oxidative phase would require only His63 as an explicit proton source to yield H_2O_2. Little experimental data has been acquired to support or refute this hypothesis.

Conclusions

The four enzyme families detailed in this review share many common features. Each enzyme is able to use oxygen or oxygen radicals as substrates by direct interaction with a mononuclear type-2 Cu site. The Cu atom cycles between the Cu(I) and Cu(II) oxidation state in the catalytic cycle except for the case of amine oxidase, in which the Cu likely stabilizes a superoxide intermediate before final reduction to hydrogen peroxide, or destabilizes the TPQ precursor tyrosine by direct coordination during cofactor biogenesis. Each Cu is coordinated by three or four protein ligands of which at least two are imidazolates of histidine residues and at most one is the thioether of a methionine residue. In addition, each Cu has an additional vacant coordination site to bind substrate. Furthermore, the substrate binding site is solvent exposed to allow substrate and products to freely diffuse to the catalytic center.

Of the four enzyme families, the similarity between the reduced type-2 Cu sites of the NiR and the bridge-broken form of CuZnSOD has been noted [89]. Both are coordinated by three histidine ligands, although the geometries of the sites do differ. The histidine ligands and Cu are coplanar in CuZnSOD (Fig. 1) and thus this site is described as trigonal planar. Upon reduction, the type-2 site in NiR loses the solvent ligand but the geometry of the site remains otherwise the same [90], thus this site may be described as pyramidal or tetrahedral with a vacant coordination site. Nevertheless, significant SOD activity has been measured in NiRs isolated from *Rhodobacter sphaeroides* [91] and *Alcaligenes xylosoxidans* [89]. In both CuZnSOD and NiR, superoxide could bind and reduce an oxidized type-2 site in the first half-reaction, and produce H_2O_2 *via* a second shell mechanism not involving direct coordination to the Cu sites in the second half-reaction.

No evidence for the reverse reaction in NiR, the one-electron reduction of O_2 to superoxide, is described in the literature, but instead the enzyme catalyses the two-electron reduction to H_2O_2 [63]. The presence of two linked Cu sites in NiR (Fig. 5) would allow for a rapid sequential two-electron reduction of O_2 with a superoxo intermediate. However, considering the anology to CuZnSOD, NiR may produce superoxide in the presence of a reductant and then generate H_2O_2 by a second shell mechanism. The interaction of NiR with O_2 is relevant to facultative organisms when they switch from nitrite to oxygen as a terminal electron acceptor. In the cell, NiR would continue to consume electrons generated from intermediary metabolism and a catalase could quickly convert the H_2O_2 end product to H_2O and O_2.

Like NiR and bridge-broken CuZnSOD, the metal site in CuAO is coordinated by three histidines (Fig. 1). The geometry is described as square pyramidal as in bridged CuZnSOD due to the presence of a labile equatorial water molecule. Dioxygen is observed to bind weakly in the axial position [31]. The product is H_2O_2; however, the two electrons are proposed to be derived from the TPQ cofactor in both cofactor biogenesis and turnover. In one mechanism of cofactor formation, the Cu is proposed to stabilize a superoxide ion intermediate [92], although data to describe O_2 intermediates used by CuAO remain elusive. In contrast, more is known about O_2 activation in the Cu monooxygenase family [37]. The Cu_M site is proposed to coordinate both superoxo and oxo intermediates in the reaction cycle (Fig. 3). Of the enzymes families presented, only the Cu monooxygenases have a thioether (from Met) in the coordination sphere (Fig. 1). The protein ligands and Cu are approximately planar as in CuZnSOD and CuAO but not NiR. Theoretical calculations implicate the Met ligand in facilitating hydrogen abstraction [32], a unique feature of the monooxygenase mechanism.

For each of the type-2 Cu protein families discussed, alternative non-copper-containing enzymes exist in nature. A non-homologous family of metal independent flavin-containing amine oxidases exists [17]. Distinct SODs are described with at least three different metal combinations (CuZn, Fe, Mn, FeMn) [74]. Nitrite reduction to NO may be performed by an enzyme containing two distinct heme groups [49]. A given bacterium is known to express either heme-containing or copper-containing NiR. Both types of NiRs are widely distributed and two strains of the same bacterial species may express different types of NiR. In addition to Cu monooxygenases, both heme

and non-heme iron monooxygenases are well known in biology [48]. The use of Cu over alternatives to catalyze these reactions may be evolutionary responses to metal availability and protein localization. For example, bacteria may substitute a Cu NiR for the heme NiR when Fe is limiting.

Acknowledgements. We thank Elitza Tocheva for her helpful discussions. This work was supported by a Discovery Grant from the National Science and Engineering Research Council to M.E.P.M. and I.S.M. received a University Graduate Fellowship from The University of British Columbia.

1 Williams, R. J. P. and Frausto da Silva, J. J. R. (1996) The Natural Selection of the Chemical Elements: The Environment and Life's Chemistry. Oxford University Press. Oxford.
2 Nersissian, A. M. and Shipp, E. L. (2002) Blue copper-binding domains. Adv. Protein Chem. 60, 271 – 340.
3 Lippard, S. J. and Berg, J. M. (1994) Principles of Bioinorganic Chemistry. University Science Books, Mill Valley.
4 Wijma, H. J., MacPherson, I., Farver, O., Tocheva, E. I., Pecht, I., Verbeet, M. P., Murphy, M. E. and Canters, G. W. (2007) Effect of the methionine ligand on the reorganization energy of the type-1 copper site of nitrite reductase. J. Am. Chem. Soc. 129, 519 – 525.
5 Farver, O., Hwang, H. J., Lu, Y. and Pecht, I. (2007) Reorganization energy of the Cu(A) center in purple azurin: Impact of the mixed valence-to-trapped valence state transition. J. Phys. Chem. B 111, 6690 – 6694.
6 Solomon, E. I., Chen, P., Metz, M., Lee, S. K. and Palmer, A. E. (2001) Oxygen binding, activation, and reduction to water by copper proteins. Angew. Chem. Int. Ed. Engl. 40, 4570 – 4590.
7 Rosenzweig, A. C. and Sazinsky, M. H. (2006) Structural insights into dioxygen-activating copper enzymes. Curr. Opin. Struct. Biol. 16, 729 – 735.
8 Brazeau, B. J., Johnson, B. J. and Wilmot, C. M. (2004) Copper-containing amine oxidases. Biogenesis and catalysis; a structural perspective. Arch. Biochem. Biophys. 428, 22 – 31.
9 Rea, G., Metoui, O., Infantino, A., Federico, R. and Angelini, R. (2002) Copper amine oxidase expression in defense responses to wounding and *Ascochyta rabiei* invasion. Plant Phys. 128, 865 – 875.
10 Moller, S. G. and McPherson, M. J. (1998) Developmental expression and biochemical analysis of the *Arabidopsis atao1* gene encoding an H_20_2-generating diamine oxidase. Plant J. 13, 781 – 791.
11 Salmi, M., Kalimo, K. and Jalkanen, S. (1993) Induction and function of vascular adhesion protein-1 at sites of inflammation. J. Exp. Med. 178, 2255 – 2260.
12 Jaakkola, K., Nikula, T., Holopainen, R., Vahasilta, T., Matikainen, M. T., Laukkanen, M. L., Huupponen, R., Halkola, L., Nieminen, L., Hiltunen, J., Parviainen, S., Clark, M. R., Knuuti, J., Savunen, T., Kaapa, P., Voipio-Pulkki, L. M. and Jalkanen, S. (2000) *In vivo* detection of vascular adhesion protein-1 in experimental inflammation. Am. J. Pathol. 157, 463 – 471.
13 Salmi, M., Yegutkin, G. G., Lehvonen, R., Koskinen, K., Salminen, T. and Jalkanen, S. (2001) A cell surface amine oxidase directly controls lymphocyte migration. Immunity 14, 265 – 276.
14 Lalor, P. F., Edwards, S., McNab, G., Salmi, M., Jalkanen, S. and Adams, D. H. (2002) Vascular adhesion protein-1 mediates adhesion and transmigration of lymphocytes on human hepatic endothelial cells. J. Immunol. 169, 983 – 992.
15 Koskinen, K., Vainio, P. J., Smith, D. J., Pihlavisto, M., Yla-Herttuala, S., Jalkanen, S. and Salmi, M. (2004) Granulocyte transmigration through the endothelium is regulated by the oxidase activity of vascular adhesion protein-1 (VAP-1). Blood 103, 3388 – 3395.
16 Salmi, M. and Jalkanen, S. (2005) Cell-surface enzymes in control of leukocyte trafficking. Nat. Rev. Immunol. 5, 760 – 771.
17 Pietrangeli, P. and Mondovi, B. (2004) Amine oxidases and tumors. Neurotoxicology 25, 317 – 324.
18 Boomsma, F., Hut, H., Bhaggoe, U., van der Houwen, A. and van den Meiracker, A. (2005) Semicarbazide-sensitive amine oxidase (SSAO): From cell to circulation. Med. Sci. Mont. 11, 122 – 126.
19 Parsons, M. R., Convery, M. A., Wilmot, C. M., Yadav, K. D., Blakeley, V., Corner, A. S., Phillips, S. E., McPherson, M. J. and Knowles, P. F. (1995) Crystal structure of a quinoenzyme: copper amine oxidase of *Escherichia coli* at 2 Å resolution. Structure 3, 1171 – 1184.
20 Wilce, M. C., Dooley, D. M., Freeman, H. C., Guss, J. M., Matsunami, H., McIntire, W. S., Ruggiero, C. E., Tanizawa, K. and Yamaguchi, H. (1997) Crystal structures of the copper-containing amine oxidase from *Arthrobacter globiformis* in the holo and apo forms: Implications for the biogenesis of topaquinone. Biochemistry 36, 16116 – 16133.
21 Duff, A. P., Cohen, A. E., Ellis, P. J., Kuchar, J. A., Langley, D. B., Shepard, E. M., Dooley, D. M., Freeman, H. C. and Guss, J. M. (2003) The crystal structure of *Pichia pastoris* lysyl oxidase. Biochemistry 42, 15148 – 15157.
22 Kumar, V., Dooley, D. M., Freeman, H. C., Guss, J. M., Harvey, I., McGuirl, M. A., Wilce, M. C. and Zubak, V. M. (1996) Crystal structure of a eukaryotic (pea seedling) copper-containing amine oxidase at 2.2 Å resolution. Structure 4, 943 – 955.
23 Lunelli, M., Di Paolo, M. L., Biadene, M., Calderone, V., Battistutta, R., Scarpa, M., Rigo, A. and Zanotti, G. (2005) Crystal structure of amine oxidase from bovine serum. J. Mol. Biol. 346, 991 – 1004.
24 Airenne, T. T., Nymalm, Y., Kidron, H., Smith, D. J., Pihlavisto, M., Salmi, M., Jalkanen, S., Johnson, M. S. and Salminen, T. A. (2005) Crystal structure of the human vascular adhesion protein-1: unique structural features with functional implications. Protein Sci. 14, 1964 – 1974.
25 Cai, D. and Klinman, J. P. (1994) Evidence of a self-catalytic mechanism of 2,4,5-trihydroxyphenylalanine quinone biogenesis in yeast copper amine oxidase. J. Biol. Chem. 269, 32039 – 32042.
26 Ruggiero, C. E. and Dooley, D. M. (1999) Stoichiometry of the topa quinone biogenesis reaction in copper amine oxidases. Biochemistry 38, 2892 – 2898.
27 Kim, M., Okajima, T., Kishishita, S., Yoshimura, M., Kawamori, A., Tanizawa, K. and Yamaguchi, H. (2002) X-ray snapshots of quinone cofactor biogenesis in bacterial copper amine oxidase. Nat. Struct. Biol. 9, 591 – 596.
28 Samuels, N. M. and Klinman, J. P. (2005) 2,4,5-Trihydroxyphenylalanine quinone biogenesis in the copper amine oxidase from *Hansenula polymorpha* with the alternate metal nickel. Biochemistry 44, 14308 – 14317.
29 Okajima, T., Kishishita, S., Chiu, Y. C., Murakawa, T., Kim, M., Yamaguchi, H., Hirota, S., Kuroda, S. and Tanizawa, K. (2005) Reinvestigation of metal ion specificity for quinone cofactor biogenesis in bacterial copper amine oxidase. Biochemistry 44, 12041 – 12048.
30 Mills, S. A., Goto, Y., Su, Q., Plastino, J. and Klinman, J. P. (2002) Mechanistic comparison of the cobalt-substituted and wild-type copper amine oxidase from *Hansenula polymorpha.* Biochemistry 41, 10577 – 10584.
31 Wilmot, C. M., Hajdu, J., McPherson, M. J., Knowles, P. F. and Phillips, S. E. (1999) Visualization of dioxygen bound to copper during enzyme catalysis. Science 286, 1724 – 1728.
32 Chen, P. and Solomon, E. I. (2004) Oxygen activation by the noncoupled binuclear copper site in peptidylglycine alpha-hydroxylating monooxygenase. Reaction mechanism and role of the noncoupled nature of the active site. J. Am. Chem. Soc. 126, 4991 – 5000.

33 Hess, C. R., Juda, G. A., Dooley, D. M., Amii, R. N., Hill, M. G., Winkler, J. R. and Gray, H. B. (2003) Gold electrodes wired for coupling with the deeply buried active site of *Arthrobacter globiformis* amine oxidase. J. Am. Chem. Soc. 125, 7156 – 7157.
34 Prigge, S. T., Mains, R. E., Eipper, B. A. and Amzel, L. M. (2000) New insights into copper monooxygenases and peptide amidation: structure, mechanism and function. Cell. Mol. Life Sci. 57, 1236 – 1259.
35 Biaggioni, I., Goldstein, D. S., Atkinson, T. and Robertson, D. (1990) Dopamine-beta-hydroxylase deficiency in humans. Neurology 40, 370 – 373.
36 Herrmann, N., Lanctot, K. L. and Khan, L. R. (2004) The role of norepinephrine in the behavioral and psychological symptoms of dementia. J. Neuropsychiatry Clin. Neurosci. 16, 261 – 276.
37 Klinman, J. P. (2005) The copper-enzyme family of dopamine beta-monooxygenase and peptidylglycine alpha-hydroxylating monooxygenase: Resolving the chemical pathway for substrate hydroxylation. J. Biol. Chem. 281, 3013 – 3016.
38 Prigge, S. T., Kolhekar, A. S., Eipper, B. A., Mains, R. E. and Amzel, L. M. (1997) Amidation of bioactive peptides: the structure of peptidylglycine alpha-hydroxylating monooxygenase. Science 278, 1300 – 1305.
39 Crespo, A., Marti, M. A., Roitberg, A. E., Amzel, L. M. and Estrin, D. A. (2006) The catalytic mechanism of peptidylglycine alpha-hydroxylating monooxygenase investigated by computer simulation. J. Am. Chem. Soc. 128, 12817 – 12828.
40 Francisco, W. A., Merkler, D. J., Blackburn, N. J. and Klinman, J. P. (1998) Kinetic mechanism and intrinsic isotope effects for the peptidylglycine alpha-amidating enzyme reaction. Biochemistry 37, 8244 – 8252.
41 Miller, S. M. and Klinman, J. P. (1985) Secondary isotope effects and structure-reactivity correlations in the dopamine beta-monooxygenase reaction: Evidence for a chemical mechanism. Biochemistry 24, 2114 – 2127.
42 Brenner, M. C. and Klinman, J. P. (1989) Correlation of copper valency with product formation in single turnovers of dopamine beta-monooxygenase. Biochemistry 28, 4664 – 4670.
43 Kizer, J. S., Bateman, R. C. Jr., Miller, C. R., Humm, J., Busby, W. H. Jr. and Youngblood, W. W. (1986) Purification and characterization of a peptidyl glycine monooxygenase from porcine pituitary. Endocrinology 118, 2262 – 2267.
44 Evans, J. P., Ahn, K. and Klinman, J. P. (2003) Evidence that dioxygen and substrate activation are tightly coupled in dopamine beta-monooxygenase. Implications for the reactive oxygen species. J. Biol. Chem. 278, 49691 – 49698.
45 Jaron, S. and Blackburn, N. J. (1999) Does superoxide channel between the copper centers in peptidylglycine monooxygenase? A new mechanism based on carbon monoxide reactivity. Biochemistry 38, 15086 – 15096.
46 Prigge, S. T., Eipper, B. A., Mains, R. E. and Amzel, L. M. (2004) Dioxygen binds end-on to mononuclear copper in a precatalytic enzyme complex. Science 304, 864 – 867.
47 Yoshizawa, K., Kihara, N., Kamachi, T. and Shiota, Y. (2006) Catalytic mechanism of dopamine beta-monooxygenase mediated by Cu(III)-oxo. Inorg. Chem. 45, 3034 – 3041.
48 Decker, A. and Solomon, E. I. (2005) Dioxygen activation by copper, heme and non-heme iron enzymes: Comparison of electronic structures and reactivities. Curr. Opin. Chem. Biol. 9, 152 – 163.
49 Averill, B. (1996) Dissimilatory nitrite and nitric oxide reductases. Chem. Rev. 96, 2951 – 2964.
50 Boulanger, M. J. and Murphy, M. E. (2002) Crystal structure of the soluble domain of the major anaerobically induced outer membrane protein (AniA) from pathogenic Neisseria: A new class of copper-containing nitrite reductases. J. Mol. Biol. 315, 1111 – 1127.
51 Godden, J. W., Turley, S., Teller, D. C., Adman, E. T., Liu, M. Y., Payne, W. J. and LeGall, J. (1991) The 2.3 Ångstrom X-ray structure of nitrite reductase from *Achromobacter cycloclastes*. Science 253, 438 – 442.
52 Wherland, S., Farver, O. and Pecht, I. (2005) Intramolecular electron transfer in nitrite reductases. Chemphyschem 6, 805 – 812.
53 Yamaguchi, K., Kataoka, K., Kobayashi, M., Itoh, K., Fukui, A. and Suzuki, S. (2004) Characterization of two type 1 Cu sites of *Hyphomicrobium denitrificans* nitrite reductase: A new class of copper-containing nitrite reductases. Biochemistry 43, 14180 – 14188.
54 Wijma, H. J., Jeuken, L. J., Verbeet, M. P., Armstrong, F. A. and Canters, G. W. (2006) A random-sequential mechanism for nitrite binding and active site reduction in copper-containing nitrite reductase. J. Biol. Chem. 281, 16340 – 16346.
55 Tocheva, E. I., Rosell, F. I., Mauk, A. G. and Murphy, M. E. (2004) Side-on copper-nitrosyl coordination by nitrite reductase. Science 304, 867 – 870.
56 Boulanger, M. J., Kukimoto, M., Nishiyama, M., Horinouchi, S. and Murphy, M. E. (2000) Catalytic roles for two water bridged residues (Asp-98 and His-255) in the active site of copper-containing nitrite reductase. J. Biol. Chem. 275, 23957 – 2364.
57 Kataoka, K., Furusawa, H., Takagi, K., Yamaguchi, K. and Suzuki, S. (2000) Functional analysis of conserved aspartate and histidine residues located around the type 2 copper site of copper-containing nitrite reductase. J. Biochem. (Tokyo) 127, 345 – 350.
58 Boulanger, M. J. and Murphy, M. E. (2003) Directing the mode of nitrite binding to a copper-containing nitrite reductase from *Alcaligenes faecalis* S-6: Characterization of an active site isoleucine. Protein Sci. 12, 248 – 256.
59 Zhao, Y., Lukoyanov, D. A., Toropov, Y. V., Wu, K., Shapleigh, J. P. and Scholes, C. P. (2002) Catalytic function and local proton structure at the type 2 copper of nitrite reductase: The correlation of enzymatic pH dependence, conserved residues, and proton hyperfine structure. Biochemistry 41, 7464 – 7474.
60 Wasbotten, I. H. and Ghosh, A. (2005) Modeling side-on NO coordination to type 2 copper in nitrite reductase: Structures, energetics, and bonding. J. Am. Chem. Soc. 127, 15384 – 15385.
61 Silaghi-Dumitrescu, R. (2006) Copper-containing nitrite reductase: A DFT study of nitrite and nitric oxide adducts. J. Inorg. Biochem. 100, 396 – 402.
62 Torres, J., Svistunenko, D., Karlsson, B., Cooper, C. E. and Wilson, M. T. (2002) Fast reduction of a copper center in laccase by nitric oxide and formation of a peroxide intermediate. J. Am. Chem. Soc. 124, 963 – 967.
63 Kakutani, T., Watanabe, H., Arima, K. and Beppu, T. (1981) A blue protein as an inactivating factor for nitrite reductase from *Alcaligenes faecalis* strain S-6. J. Biochem. (Tokyo) 89, 463 – 472.
64 Kakutani, T., Beppu, T. and Arima, K. (1981) Regulation of nitrite reductase in the denitrifying bacterium *Alcaligenes faecalis* S-6. Agric. Biol. Chem. 45, 23 – 28.
65 Bento, I., Carrondo, M. A. and Lindley, P. F. (2006) Reduction of dioxygen by enzymes containing copper. J. Biol. Inorg. Chem. 11, 539 – 547.
66 Murphy, M. E., Lindley, P. F. and Adman, E. T. (1997) Structural comparison of cupredoxin domains: domain recycling to construct proteins with novel functions. Protein Sci. 6, 761 – 770.
67 Nakamura, K. and Go, N. (2005) Function and molecular evolution of multicopper blue proteins. Cell. Mol. Life Sci. 62, 2050 – 2066.
68 Shin, W., Sundaram, U. M., Cole, J. L., Zhang, H. H., Hedman, B., Hodgson, K. O. and Solomon, E. I. (1996) Chemical and spectroscopic definition of the peroxide-level intermediate in the multicopper oxidases: Relevance to the catalytic mechansim of dioxygen reduction to water. J. Am. Chem. Soc. 118, 3202 – 3215.
69 Palmer, A. E., Quintanar, L., Severance, S., Wang, T. P., Kosman, D. J. and Solomon, E. I. (2002) Spectroscopic characterization and O2 reactivity of the trinuclear Cu cluster of

mutants of the multicopper oxidase Fet3p. Biochemistry 41, 6438 – 6448.
70 Endo, K., Hayashi, Y., Hibi, T., Hosono, K., Beppu, T. and Ueda, K. (2003) Enzymological characterization of EpoA, a laccase-like phenol oxidase produced by *Streptomyces griseus*. J. Biochem. (Tokyo) 133, 671 – 677.
71 Machczynski, M. C., Vijgenboom, E., Samyn, B. and Canters, G. W. (2004) Characterization of SLAC: A small laccase from *Streptomyces coelicolor* with unprecedented activity. Protein Sci. 13, 2388 – 2397.
72 Aikens, J. and Dix, T. A. (1991) Perhydroxyl radical (HOO.) initiated lipid peroxidation. The role of fatty acid hydroperoxides. J. Biol. Chem. 266, 15091 – 15098.
73 Huie, R. E. and Padmaja, S. (1993) The reaction of NO with superoxide. Free Radic. Res. Commun. 18, 195 – 199.
74 Pelmenschikov, V. and Siegbahn, P. E. (2005) Copper-zinc superoxide dismutase: Theoretical insights into the catalytic mechanism. Inorg. Chem. 44, 3311 – 3320.
75 Rosen, D. R., Siddique, T., Patterson, D., Figlewicz, D. A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J. P., Deng, H. X. et al. (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 362, 59 – 62.
76 Stathopulos, P. B., Rumfeldt, J. A., Scholz, G. A., Irani, R. A., Frey, H. E., Hallewell, R. A., Lepock, J. R. and Meiering, E. M. (2003) Cu/Zn superoxide dismutase mutants associated with amyotrophic lateral sclerosis show enhanced formation of aggregates *in vitro*. Proc. Natl. Acad. Sci. USA 100, 7021 – 7026.
77 Murphy, L. M., Strange, R. W. and Hasnain, S. S. (1997) A critical assessment of the evidence from XAFS and crystallography for the breakage of the imidazolate bridge during catalysis in CuZn superoxide dismutase. Structure 5, 371 – 379.
78 Bertini, I., Luchinat, C. and Monnanni, R. (1985) Evidence of the breaking of the copper-imidazolate bridge in copper/cobalt-substituted superoxide dismutase upon reduction of the Cu(II) centers. J. Am. Chem. Soc. 107, 2178 – 2179.
79 Blackburn, N. J., Hasnain, S. S., Binsted, N., Diakun, G. P., Garner, C. D. and Knowles, P. F. (1984) An extended-X-ray-absorption-fine-structure study of bovine erythrocyte superoxide dismutase in aqueous solution. Direct evidence for three-co-ordinate Cu(I) in reduced enzyme. Biochem. J. 219, 985 – 990.
80 Banci, L., Bertini, I., Bruni, B., Carloni, P., Luchinat, C., Mangani, S., Orioli, P. L., Piccioli, M., Ripniewski, W. and Wilson, K. S. (1994) X-ray, NMR and molecular dynamics studies on reduced bovine superoxide dismutase: Implications for the mechanism. Biochem. Biophys. Res. Commun. 202, 1088 – 1095.
81 Ogihara, N. L., Parge, H. E., Hart, P. J., Weiss, M. S., Goto, J. J., Crane, B. R., Tsang, J., Slater, K., Roe, J. A., Valentine, J. S., Eisenberg, D. and Tainer, J. A. (1996) Unusual trigonal-planar copper configuration revealed in the atomic structure of yeast copper-zinc superoxide dismutase. Biochemistry 35, 2316 – 2321.
82 Djinovic, K., Gatti, G., Coda, A., Antolini, L., Pelosi, G., Desideri, A., Falconi, M., Marmocchi, F., Rotilio, G. and Bolognesi, M. (1992) Crystal structure of yeast Cu,Zn superoxide dismutase. Crystallographic refinement at 2.5 Å resolution. J. Mol. Biol. 225, 791 – 809.
83 Hough, M. A. and Hasnain, S. S. (1999) Crystallographic structures of bovine copper-zinc superoxide dismutase reveal asymmetry in two subunits: Functionally important three and five coordinate copper sites captured in the same crystal. J. Mol. Biol. 287, 579 – 592.
84 Rypniewski, W. R., Mangani, S., Bruni, B., Orioli, P. L., Casati, M. and Wilson, K. S. (1995) Crystal structure of reduced bovine erythrocyte superoxide dismutase at 1.9 Å resolution. J. Mol. Biol. 251, 282 – 296.
85 Hart, P. J., Liu, H., Pellegrini, M., Nersissian, A. M., Gralla, E. B., Valentine, J. S. and Eisenberg, D. (1998) Subunit asymmetry in the three-dimensional structure of a human CuZnSOD mutant found in familial amyotrophic lateral sclerosis. Protein Sci. 7, 545 – 555.
86 Hart, P. J., Balbirnie, M. M., Ogihara, N. L., Nersissian, A. M., Weiss, M. S., Valentine, J. S. and Eisenberg, D. (1999) A structure-based mechanism for copper-zinc superoxide dismutase. Biochemistry 38, 2167 – 2178.
87 Leone, M., Cupane, A., Militello, V., Stroppolo, M. E. and Desideri, A. (1998) Fourier transform infrared analysis of the interaction of azide with the active site of oxidized and reduced bovine Cu,Zn superoxide dismutase. Biochemistry 37, 4459 – 4464.
88 Beyer, W. F. Jr., Fridovich, I., Mullenbach, G. T. and Hallewell, R. (1987) Examination of the role of arginine-143 in the human copper and zinc superoxide dismutase by site-specific mutagenesis. J. Biol. Chem. 262, 11182 – 11187.
89 Strange, R. W., Murphy, L. M., Dodd, F. E., Abraham, Z. H., Eady, R. R., Smith, B. E. and Hasnain, S. S. (1999) Structural and kinetic evidence for an ordered mechanism of copper nitrite reductase. J. Mol. Biol 287, 1001 – 1009.
90 Murphy, M. E., Turley, S. and Adman, E. T. (1997) Structure of nitrite bound to copper-containing nitrite reductase from *Alcaligenes faecalis*. Mechanistic implications. J. Biol. Chem. 272, 28455 – 28460.
91 Michalski, W. P. and Nicholas, D. J. D. (1985) Molecular characterization of a copper containing nitrite reductase from *Rhodopseudomonas sphaeroides forma* sp. *denitrificans*. Biochim. Biophys. Acta 828, 130 – 137.
92 Dubois, J. L. and Klinman, J. P. (2005) Mechanism of post-translational quinone formation in copper amine oxidases and its relationship to the catalytic turnover. Arch. Biochem. Biophys. 433, 255 – 265.