The world of multi-copper enzymes

A review about the functions and mechanism of multi-copper enzymes

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Contents

Abstract

In this review the natural functions and reaction mechanisms of some multi-copper enzymes are described. Three multi-copper oxidases (MCOs) are describes and one reductive enzyme, nitrite reductase. The structure that all these enzyme have in common is a cupredoxin-like folded beta barrel. They all contain a T1 copper site in this beta barrel. The MCOs all contain a trinuclear copper cluster (TNC) as well, consisting of a T2 copper and a T3 copper. Nitrite reductase contains only a T2 copper instead of a TNC. In all of them electrons are accepted at the T1 copper and transferred internally to the TNC/T2 site. Here oxygen is reduced to water, in the case of MCOs, or nitrite to nitric oxide, in the case of nitrite reductase. The MCOs described are laccases, ascorbate oxidase and human ferroxidases. Laccases are found in various organisms, such as plants, bacteria and fungi. It catalyzes the oxidation of lignin-related compounds and could be involved in the depolymerisation of lignin as well. Ascorbate oxidase is found in plants, near the cell walls. It catalyzes the oxidation of ascorbate to dehydroascorbate. This reaction might be involved in the wound healing of plants. There are three types of human ferroxidases: ceruloplasmin, hephaestin and zyklopen. All of these are found in different part of the human body. They are capable of the oxidation of iron and are responsible for the iron regulation. Nitrite reductases are found in bacteria. It is involved in the respiratory chain, where it can reduce nitrite to nitric oxide, as an anaerobic alternative for oxygen. This reaction is important for the terrestrial nitrogen cycle as well. These denitrifying bacteria can be used to remove nitrite from waste- and groundwater as well.

Introduction

Enzymes play an important role in life and are found in all organisms. They catalyze all kinds of reaction in the metabolism of cells. For example, they create building blocks of cell walls and membranes, are involved in energy production and help to maintain the homeostasis in the cell. Some enzymes contain metal ions as a cofactor, that can catalyze redox reactions. Metals that are found are for example iron, manganese, zinc, magnesium, cobalt and copper. The subject of this review is enzymes that contain copper. More specifically, enzymes that contain multiple copper ions: the multi-copper enzymes.

There are a lot of copper containing enzymes, which play an important role in biological processes. Their four basic functions are: 1) metal ion storage, transport and uptake; 2) electron transfer; 3) dioxygen storage, transport and uptake; 4) catalysis (Tepper, 2005). This review focuses on enzymes, which contain multiple copper sites. They are found in a wide variety of organisms: mammals, bacteria, fungi and plants for example. They are capable of catalyzing various redox reactions. Examples of multi-copper enzymes are multi-copper oxidases, such as laccases, ascorbate oxidase and some ferroxidases (Reiss et al, 2013). There are also multi copper enzymes known, that catalyze reductive reactions, for example nitrite reductases (Solomon et al, 1996). All of these enzymes have different biological functions, but they contain the same copper sites.

In this review I will explain about the general structure and mechanism of multi-copper enzymes. I will also have a more detailed look at some specific types of enzymes: laccases, ascorbate oxidases, ferroxidases and nitrite reductases. Of these enzymes the biological function, structure and catalyzed reaction will be described in more detail. If possible, the industrial applications will be looked into as well.

General structure and mechanism

The different copper sites

Based on their spectroscopic properties, the different copper sites were initially divided into three classes. Later more classes were defined, but in multi-copper enzymes only type 1 (T1), type 2 (T2) and type 3 (T3) sites are found. The T1 centres are also called blue copper centres, because of the blue colour of the oxidized state (Tepper, 2005). The T1 site shows high absorption in the visible light region, at a wavelength around 600 nm, resulting from a highly covalent bond between a sulphur (from cysteine) and the copper (Kjaergaard et al, 2015). This type of copper sites, contain one copper atom and are found in mononuclear copper proteins, where it is involved in intermolecular electron transfer, as well as in the multi-copper enzymes, where it functions in the intramolecular electron transfer. In MCOs the T1 site accepts electrons from the substrate and transfers them internally to another site (Solomon et al, 1996). T2 sites are similar to T1 sites, but colourless. These sites are found in enzymes that catalyze oxidation reactions. T3 sites consist of two closely spaced copper atoms. Proteins that are involved in oxygen transport and activation are known to have a T3 site (Tepper, 2005). T2 sited have no detectable absorption, but are distinguished from T1 sites by their EPR spectrum. T3 sites have an absorption peak near the ultra-violet region, at a wavelength around 330 nm (Messerschmidt et al, 1990). In MCOs the T2 and T3 site are combined in a trinuclear cluster (TNC). There is a low sequence homology among the different MCOs, but the overall structure and also the binding motifs of the copper are highly conserved. The T1 copper is bound by two histidines and one cysteine. It also has a methionine, leucine or isoleucine nearby. In the TNC the T2 copper is

bound by two histidines and the two T3 coppers by six histidines. The binding of copper is strictly conserved in four motifs in the amino acid sequence: HXHG, HXH, HXXHXH and HCHXXXHXXXXM/L/F (Reiss et al, 2013).

Structure

As mentioned before, the structure of the binding sites of copper and the overall structure are highly conserved in MCOs. However, the quaternary structure of the protein differs in the different types of multi-copper enzymes. These proteins consist of two, three or six homologous domains. These domains are also homologous with cupredoxins, this are mono-domain copper proteins. Azurin and plastocyanin are enzymes that belong to this group (Nakamura et al, 2003). These proteins are folded in a eight stranded Greek key beta barrel, which consists of two beta sheets, containing four beta strands each (Reiss et al, 2013). In Figure 1 the structure of azurin is shown along with the general structure of a Greek key beta barrel. In the structure of azurin, the beta barrel is shown in yellow. The T1 copper is located inside this beta barrel, which is also shown in figure 1. The trinuclear T2/T3 cluster is located between the domains of multi-copper enzymes (Nakamura et al, 2003).

Figure 1. Left: Crystal structure of azurin II from PDB (Paraskevopoulos et al, 2006). Right: Greek key beta barrel.

Mechanism

The general reaction that multi-copper oxidases catalyze starts with a single electron oxidation of a substrate. Four of these reactions are coupled to the four electron reduction of dioxygen to water (Augustine et al, 2008). The first step in this reaction is the electron transfer from the substrate, which depends on the type of MCO, to the T1 copper. In this step the T1 copper is reduced. From here the electrons are transferred internally to the TNC, via a cysteine-histidine pathway of about 13 Å. This reduces the copper ions in the TNC and when four substrates are converted, all the copper in the enzyme is reduced (Solomon et al, 2008). The pathway of the general mechanism is shown in figure 2.

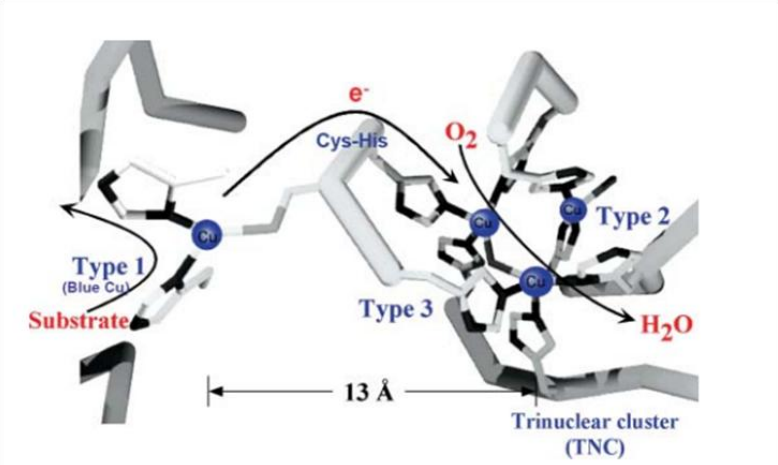

Figure 2. The structure of the active site of a MCO, with arrows indicating the flow of electrons, the substrate and oxygen. (Solomon et al, 2008).

The TNC of the fully reduced enzyme reacts with oxygen. The oxygen is reduced in two, two electron transfer steps. In the first step a peroxide intermediate is formed, followed by the formation of the native intermediate. In the native intermediate all coppers are oxidized and the reduced oxygen is still attached to the TNC. When the copper centres in the enzyme are reduced again, two water molecules are released and the fully reduced enzyme is regenerated. When no reducing substrate is present, the enzyme goes from the native intermediate to a resting state, where the enzyme remains oxidized, but one water is released. (Kjaergaard et al, 2015; Heppner et al, 2014). In figure 3 the mechanism for the reduction of oxygen is shown, including the different states of the enzyme.

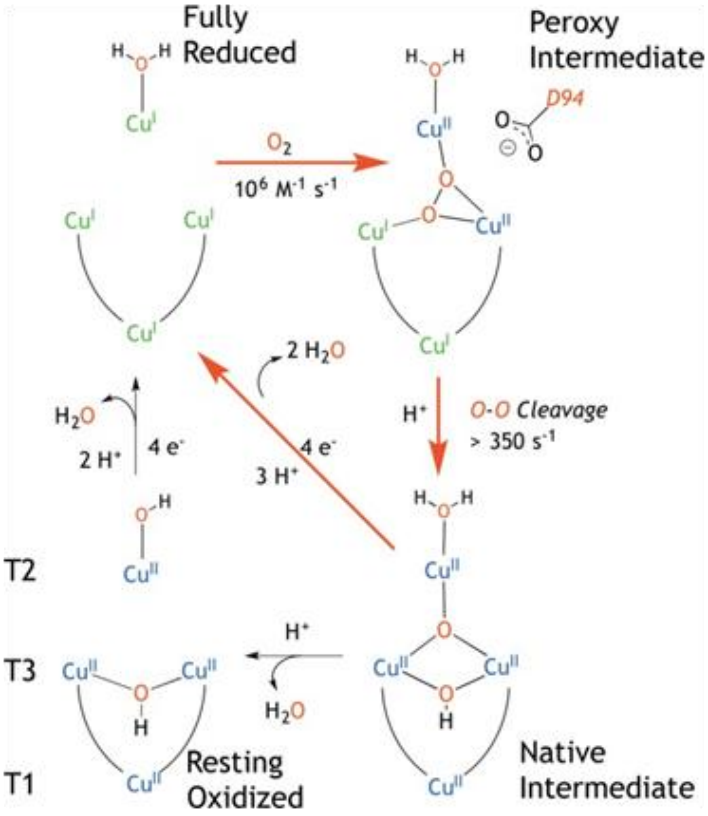

Figure 3. Mechanism of the reduction of oxygen by multi-copper oxidases. (Heppner et al, 2014).

Laccases

Natural occurrence and biological function

Laccases are the largest group of multi-copper oxidases and distributed widely in nature. It was first found in the sap of the japanese laquer tree *Rhus vernicifera* (Giardina et al, 2009). It is found in higher plants, some insects and a few bacteria as well. However, the largest and best known group are the fungal laccases (Hakulinen et al, 2002).

Plant laccases are found in wood and cellular walls of herbaceous species. They are involved in the biosynthesis of lignin, of which the cell walls are build up (Giardina et al, 2009). They are able to couple monolignols to dimers and trimers, where peroxidase has higher activity toward oligomers. It may be that laccases initiates the synthesis of lignin, by making oligomer, and peroxidases are responsible for making the actual lignin polymers (Solomon et al, 1996). The involvement of peroxidases and oxidases in lignin formation is well confirmed in the literature, but the role of laccase is still unclear, even in more recent publications (Berthet et al, 2012). Because some laccases are found extracellular or cannot oxidize monolignins, which both is the case for the laccase from *Rhus vernicifera*, it is also proposed that it is involved in the wound healing of plants (Solomon et al, 1996; Hakulinen et al, 2002).

The function of fungal laccases is better known than for plants. They are involved in various physiological processes, such as lignin degradation, detoxification, pigmentation, pathogenicity and stress defence (Giardina et al, 2009; Hakulinen et al, 2002). Laccase activity is found in many different fungi species. For example for wood-rotting fungi, where it is responsible for the lignin degradation in the wood (Baldrian, 2006). However, the role of laccase is not entirely clear, because other enzymes, lignin peroxidase and manganese-dependent peroxidase, are found to be able to degrade lignin, but this activity is lowered in the absence of laccase (Thurston, 1994). It is also proposed that laccase is excreted by some fungi to remove toxic phenols, which are formed during the lignin degradation of produced by other organisms (Solomon et al, 1996). This could be an explanation of the lowered ligninolytic activity, when no laccase is present. In later literature the theory that laccase is involved in lignin degradation, in combination with other enzymes, is described as well (Pardo et al, 2015). However, the only organisms that are capable of completely degrading lignin are white-rot basidiomycetes.

Structure

Laccases are monomers consisting of three cupredoxin-like folded domains, which are described earlier (Hakulinen et al, 2002). The T1 copper site is located in domain 3 and the TNC between domains 1 and 3, where both domains provide residues for the copper coordination. The structure is stabilized by multiple sulphur bridges. The amount and location of these bridges vary in different laccases. In basidiomycete laccases for example, two sulphur bridges are present: one between domains 1 and 3 and one between domains 1 and 2 (Giardina et al, 2009). In laccase from *M. albomyces* three sulphur bridges are found: located in domain A, between domains A and C and in domain B near the substrate-binding site (Hakulinen et al, 2002). Another property of laccases is that they are heavily glycosylated, with an extent of glycosylation ranging between 10 and 25% (Giardina et al, 2009).

Reactions

Laccases catalyze the oxidation of a wide range of aromatic substrates, such as *ortho* and *para*-diphenols, aminophenols, polyphenols, polyamines and aryl diamines (Giardina et al, 2009). In this oxidation reaction a reactive radical in formed. This radicals can react with each other to form dimers, oligomers or polymers. In plants, the linkage of phenolic monomers is part of the lignification process. Laccases are also involved in the depolymerisation of complex natural polymers, such as lignin. Due to steric hindrance, the enzyme might not come into contact with the polymer directly, but it can oxidize small organic compounds, which mediate the radical-catalyzed depolymerisation (Claus, 2004). In figure 4 an example is shown of a reaction, where two molecules are coupled via a radical reaction, catalyzed by laccase.

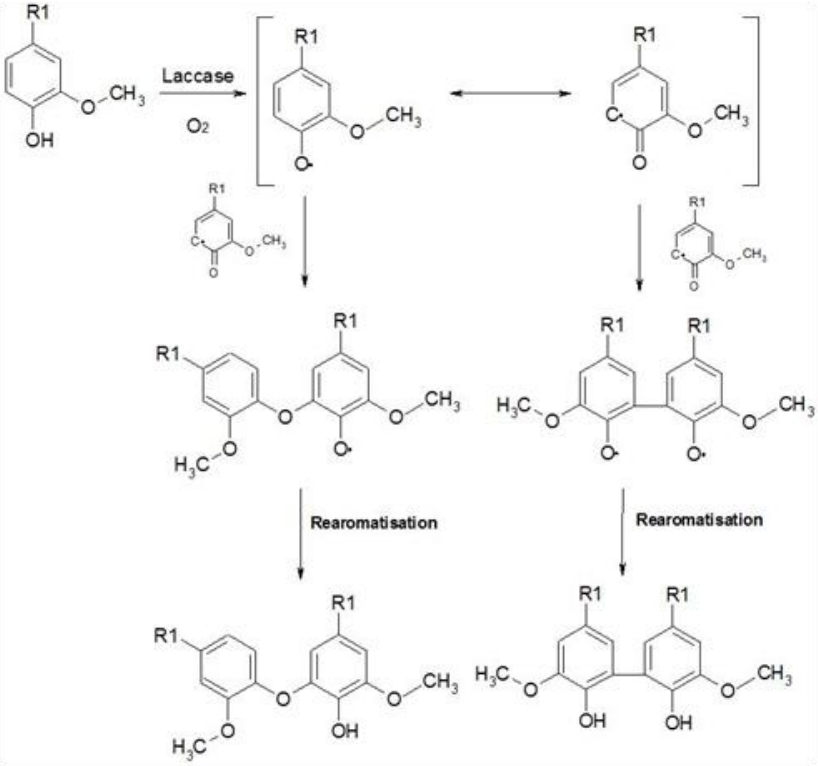

Figure 4. Radical coupling reaction, catalyzed by laccase. (Madad et al, 2013).

Industrial applications

Laccases are industrially very interesting. They can be used in various fields. In the food industry they can be applied in processes that enhance or modify the colour appearance of food or beverage. This process involves the elimination of undesirable phenolic compounds, which leads to browning, haze formation and turbidity development in clear fruit juice, beer and wine (Couto et al, 2006). They are also interesting for paper and pulp industries. The pressure from environtal protection agencies, to decrease the involvement of chlorine and chlorine-based chemical from the bleacing process, is increasing. Due to this, a lot of research has been done to replace these chemicals with bio-based alternatives. Lignin degrading enzymes, such as fungal laccases, can help to reduce the use of chlorine-based chemicals in the paper industries (Singh et al, 2015). The use of laccases in the textile industries is growing as well. Their decolourization abilities could be used for the treatment of dye wastewater, which is inefficient and not economical now. Due to their abilities to degrade various dyes, laccases seem like an attractive solution. Also, they are used for the bleaching of textiles and

even for the synthesis of dyes (Couto et al, 2006). There is research done about the use of laccases for the production of biofuels as well. The feedstock of the production of bio-ethanol is lignocellulose. The ability of laccases, in combination with mediator molecules, to degrade lignin makes them interesting for the biofuel industry. But their strong polymerization activity counters the decomposition of lignin by repolymerizing the degradation products. The challenge for the future is to shift the activity toward lignin cleavage (Roth et al, 2015). For the use in the industries mentioned before, the main challenge is the immobilization of the enzyme afterwards. The lack of sufficient enzyme stocks and the cost of redox mediators, are another important obstacle (Couto et al, 2006).

Ascorbate oxidases

Natural occurrence and biological function

Ascorbate oxidase in a multi-copper oxidase that is found higher plants, such as pumpkin, cucumber and zucchini. It is localized in the apoplast of the plants (Pignocchi et al, 2003). Even though this enzyme is known for many decades and structures have been solved (Messerschmidt et al, 1992), the role of this enzyme is still not entirely clear. Manipulations have shown that ascorbate oxidase is involved in the regulation of the ascorbate pool, especially in the apoplast. Changes in activity had little effect on the overall ascorbate pool of the leaf, but dramatically changed it in the apoplast. Ascorbate in the apoplast is involved in the redox buffering (Pignocchi et al, 2003). It is also involved in the ozone detoxification in the leaves of plants (Luwe, 1993). So indirectly ascorbate oxidase could be responsible for these processes.

Structure

A crystal structure for ascorbate oxidase has been solved by Messerschmidt et al in 1992. It shows that the enzyme is dimeric. Each subunit consists of three domain. These domains are also cupredoxin-like folded. However, domains 2 and 3 have a slightly different beta-barrel than described before, containing additional beta sheets. The mononuclear copper site (T1) is located in domain three, and the THC is located between domains 1 and 3. The copper ions are coordinated by histidines and cysteine as described before. Each monomer contains three sulphur bridges: one between domains 1 and 3, one between domains 1 and 2 and one in domain 2. The structure of a monomer of ascorbate oxidase is very similar to laccase structures. A difference is that ascorbate oxidase is a dimer and also, it has a carbohydrate content of about 3%, which is significantly lower than for laccases.

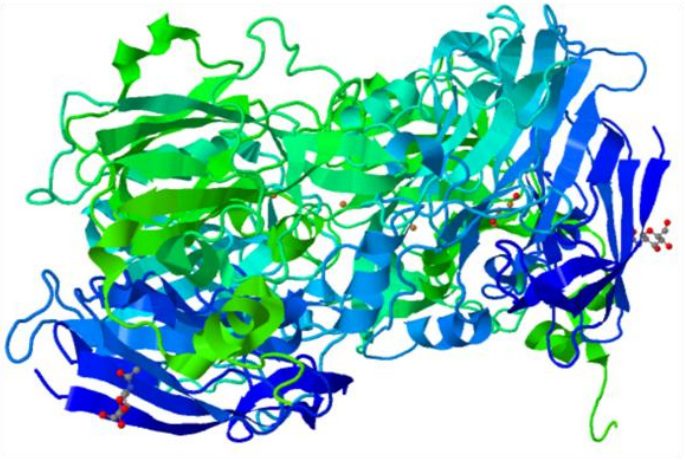

Figure 5. The crystal structure of ascorbate oxidase from the PDB (Messerschmidt et al, 1992).

Reaction

Ascorbate oxidase catalyzes one reaction, which is pretty straight forward: the oxidation of ascorbate. In this reaction dehydroascorbate is formed in combination with the reduction of oxygen to water (Pignocchi, 2003). The half reaction of the oxidation of ascorbate is shown in reaction 1. To accommodate the reduction of oxygen (reaction 2), two substrates have to be converted, leading to the net redox reaction shown in reaction 3. As described earlier, the substrates are oxidized first and the oxygen is reduced by the fully reduced enzyme. So the two half reaction shown below do not occur simultaneously.

Reaction 1: ascorbate ($C_6H_8O_6$) → dehydroascorbate ($C_6H_6O_6$) + 2 e^- + 2 H^+
Reaction 2: O_2 + 4 H^+ + 4 e^- → 2 H_2O
Reaction 3: 2 ascorbate ($C_6H_8O_6$) + O_2 → 2 dehydroascorbate ($C_6H_6O_6$) + 2 H_2O

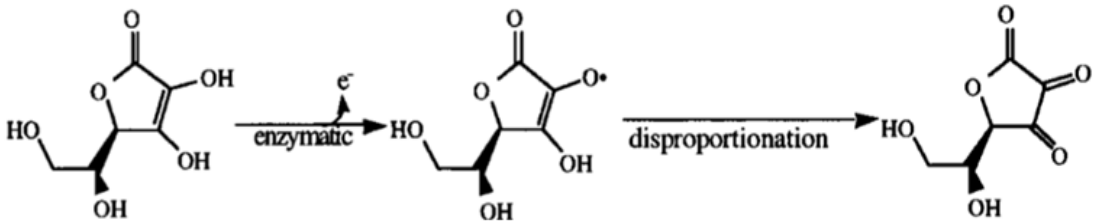

Figure 6. Reaction catalyzed by ascorbate oxidase (Solomon, 1996).

Ferroxidases

Natural occurrence and biological function

Three multi-copper oxidases have been found in human: ceruloplasmin, hephaestin and zyklopen. Ceruloplasmin is found in the blood plasma, hephaestin in multiple organs, but in high abundance in the small intestine, and zyklopen in the placenta. All of these have high specificity towards iron, which leads to ferroxidase activity (Vashenko et al, 2013). These enzymes are all involved in the iron metabolism of the body. Ceruloplasmin is essential for iron binding to transferrin (the main iron-transporting protein), as well as to ferritin (the main iron-storage protein) (Wierzbicka et al, 2014). It is not only known to have ferroxidase activity, but can catalyze other oxidation reactions as well. It is for example capable of oxidizing organic amines and physiologically relevant substrates (biogenic amines), such as hormones (adrenaline, noradrenaline) and neurotransmitters (serotonin, dopamine) (Vashenko et al, 2013). Hephaestin is essential for iron binding to apotransferrin in the mucous layer of the small intestine. This process is important for the iron transport to the liver via the portal vein (Wierzbicka et al, 2014). In the heart, brain and pancreas, it can protect these tissues against Fe(II) toxicity. It is suggested that hephaestin in the placenta facilitates the iron transfer between mother and fetus (Vashenko et al, 2013). Zyklopen is also related to the iron transport in the placenta (Wierzbicka et al, 2014). So these three enzymes have very similar functions, but in different parts of the human body. Experiments in mice have even shown that hephaestin is increasingly expressed in the brain of mice with aceruloplasminaemia. This is a disease caused by ceruloplasmin deficiency, leading to iron accumulation in various tissues, including the brain. The increased expression of hephaestin makes that the iron level remains normal (Cui et al, 2009). This indicates that the human multi-copper oxidases are even able to take over tasks from each other.

Structure

Ceruloplasmin contains six cupredoxin-like folded domains. The T1 copper sites are located in domains 2, 4 and 6. It contains one trinuclear copper cluster, which is located between domains 1 and 6. The TNC is not only necessary for the catalytic activity, but also hold together the N- and C-terminal domains, providing stability to the enzyme. The iron binding sites, which are composed of two glutamate, one aspartate and one histidine residue, are located near the T1 sites in domains 4 and 6. It is located at the end of a narrow channel, which limits the accessibility for organic compounds (Vashenko et al, 2013). However, binding sites have been identified for organic substrates at domain 4 and for biogenic amines in domain 6 (Zaitsev et al, 1999).

Hephaestin is a transmembrane protein, in contrast to the soluble ceruloplasmin. The amino acid sequence of hephaestin is 68 % similar to the sequence of ceruloplasmin. No crystal structure of hephaestin could be found in the PDB, but it is proposed to be similar to the structure of ceruloplasmin, with the addition of a C-terminal transmembrane domain. This is also supported by the molecular mass of the proteins, where hephaestin (130 kDa) is slightly larger than ceruloplasmin (120 kDa). The structure of zyklopen is not deposited in the PDB either, but is expected to be closely related to hephaestin (Vashenko et al, 2013).

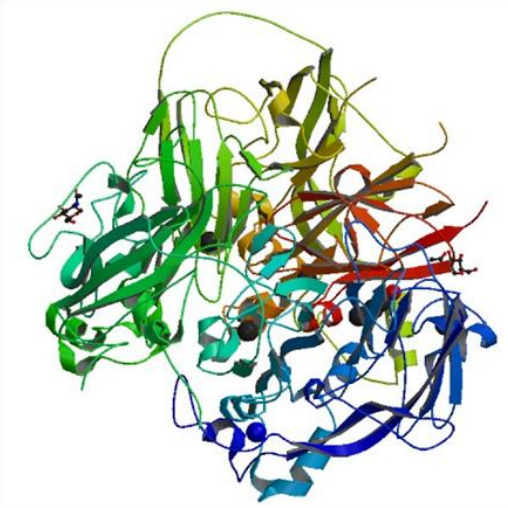

Figure 7. The crystal structure of ceruloplasmin from the PDB (Samygina, 2013).

Reaction

The ferroxidase reaction that these human multi-copper oxidases catalyze is fairly simple. Fe(II) is oxidized to Fe(III), while oxygen is reduced to water. Fe(II) is oxidized by the T1 copper via the following reaction: Fe(II) + Cu(II) → Fe(III) + Cu(I). The electrons are transferred inside the enzyme to the TNC as described before, where oxygen is reduced. As four electrons are needed to reduce oxygen to water, four iron ions have to be oxidized as well, which leads to the following total reaction: 4 Fe(II) + O_2 + 4 H^+ → 4 Fe(III) + H_2O (Osaki, 1966).

As an example of the oxidation reaction of biogenic amines, 6-hydroxydopamine will be used. The oxidation of 6-hydroxydopamine can occur spontaneously, where a quinoidal product is formed, as well as several forms of reduced oxygen, such as H_2O_2 and oxygen radicals. These are considered to be toxic and responsible for degeneration of adrenergic nerve terminals. When this reaction is catalyzed by ceruloplasmin, these products will not be formed, as the enzyme reduces the oxygen to water. The fact that the same product can be formed, without the formation of side product, makes the activity of ceruloplasmin towards this type of molecules very important (Floris et al, 2000).

Nitrite reducatase

Natural occurance and biological function

Nitrite reductases are slightly different than the ones described before, as it catalyzed the reduction of its substrate, instead of oxidation. There are two main classes of nitrite reductase: one containing hemes, with iron, and one containing copper ions. Both are able to catalyze the reduction of nitrite to nitric oxide (Sundararajan et al, 2007). In nature nitrite reductases are found in denitrifying bacteria, which live in soil, water, foods and the digestive tract (Averill, 1996). Denitrification is an important biological process in which nitrite is reduced to nitric oxide, followed by the conversion to molecular nitrogen (Sundararajan et al, 2007). In these bacteria denitrification is invloved in the respiratory chain, where nitrogen oxide species are the terminal electron acceptor. It is the anearobic alternative of oxygen. In this process large amounts of fixed nitrogen are released into the atmosphere, which is important to complete the terestrial nitrogen cycle (Averill, 1996).

Structure

The structure of nitrite reductase has similarities to the ones of multi-copper oxidases, but is slightly different. A structure for nitrite reductase from *Achromobacter cycloclastes* is proposed by Godden et al in 1991 and is supported by more recent research (Fukuda et al, 2016). These domains have a cupredoxin-like fold. Each monomer contains two copper sites, one T1 and one T2. These copper ions are bound by the usual residues for each site: 2 histidines, one cysteine and one methionine for T1 and 3 histidines for T2. All these residues are provided by domain 1, except for one histidine that is bound to the T2 copper. This one is provided by domain 2 of a different monomer, so the T2 copper is only properly bound when the enzyme is a trimer (Godden et al, 1991). The T1 site is located in domain 1, near the protein surface, and the T2 site between two adjecent monomers, at the end of a solvent channel of about 12 Å. The two copper sites are about 12.5 Å apart and connected by a Cys-His bridge (Fukuda et al, 2016). This structure and distance is the same as the connection between the T1 and TNC of multi-copper oxidases.

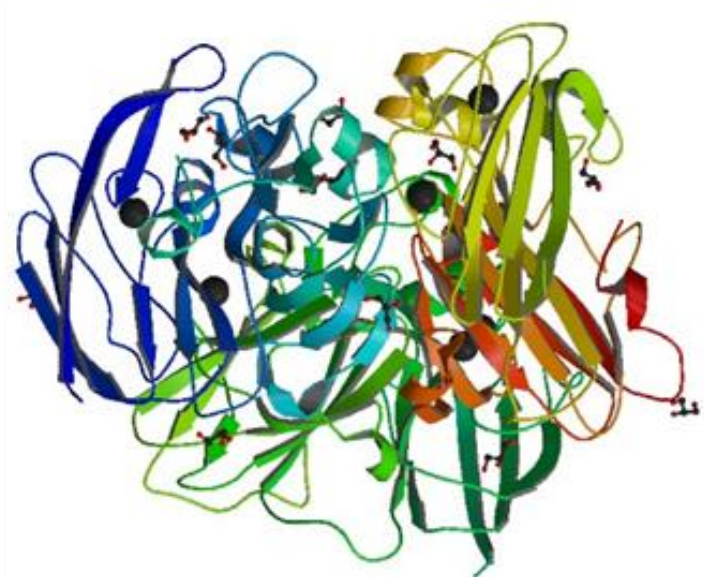

Figure 8. The crystal structure of nitrite oxidase from *Nitrosomonas europaea* from the PDB (Rozenzweig et al, 2013)

Reaction and mechanism

As mentioned before, the reaction catalyzed is a reduction of nitrite to nitric oxide. The chemical equation for this reactions is: $NO_2^- + 2\ H^+ + e^- \rightarrow NO + H_2O$. Recently a mechanism has been proposed for this reaction (Fukuda et al, 2016). Initially a water molecule is bound by the T2 copper. When nitrite binds, it replaces the water and forms a Cu(I)-NO_2^-. Then a electron is transferred internally from the T1 copper to the T2 copper. One of the oxygens accepts a proton from a nearby histidine residue. Then the electron transfer from the T2 copper to the nitrogen happens. This can go via two possible routes, end-on or side-on. This routes and the complete mechanism are shown in figure 9. In the last step nitric oxide is released and the enzyme goes back to its initial state. The electron that is necessary is received from the electron transport chain.

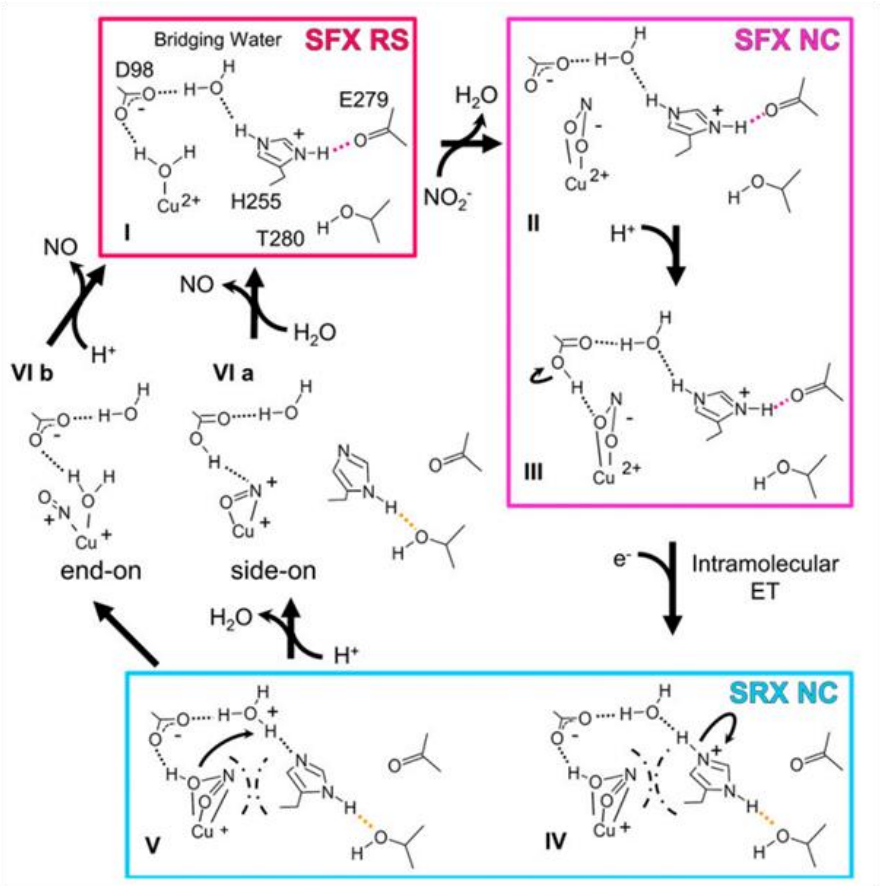

Figure 9. The mechanism for the reduction of nitrite. The coloured boxes indicate the method that was used to solve the structures (Fukuda et al, 2016).

Industrial applications

No industrial applications could be found for the nitrate reductase enzyme. But denitrifying bacteria, in which they naturally occur, have potential. They could be used for example in wastewater remediation (Averill, 1996). Pilot scale experiments have been done with denitrifying bacteria, to remove nitrogen from wastewater. In a process called nitrite-dependent anaerobic methane oxidation, nitrite is reduced to molecular nitrogen, with methane as electron donor (He et al, 2015). Research has been done about the removal of nitrite from ground water as well. Acetate and glucose were added to stimulate the indigenous denitrifying bacteria. This tactic worked bested under anaerobic conditions, but in an aerobic environment it worked as well (Calderer et al, 2010).

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