CHARACTERIZATION OF MULTICOPPER OXIDASE-RELATED PROTEIN AND MULTICOPPER OXIDASE-1 IN INSECTS

by

ZEYU PENG

B.A., Ocean University of China, 2008

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Abstract

Typical multicopper oxidases (MCOs) have ten conserved histidines and one conserved cysteine that coordinate four copper atoms, which are required for oxidase activity. During our studies of insect MCOs, we discovered a gene that we named multicopper oxidase-related protein (MCORP). MCORPs share sequence identity with MCOs, but lack many of the copper-coordinating residues. We identified MCORP orthologs in many insect species, but not in other invertebrates or vertebrates. We purified recombinant *Tribolium castaneum* (red flour beetle) MCORP. As expected, no oxidase activity was detected. We analyzed expression profiles of TcMCORP and *Anopheles gambiae* (African malaria mosquito) MCORP. They are constitutively expressed at a low level in many tissues, including ovaries. TcMCORP larval RNAi led to 100% mortality before adult stage. These deaths occurred during the larval to pupal and pupal to adult molts. Pharate pupal RNAi resulted in 20% mortality during the pupal to adult molt, and 100% mortality by one month after adult eclosion.In addition, knockdown of TcMCORP in females prevented oocyte maturation, thus greatly decreasing the number of eggs laid. These results indicate that TcMCORP is an essential gene and that its function is required for reproduction. An understanding of the role MCORP plays in insect physiology may help to develop new strategies for controlling insect pests.

A multicopper oxidase-1 (MCO1) ortholog has been identified in all insect species examined so far; thus, MCO1 probably has a conserved physiological function in insects. Most of the well-studied MCOs are laccases, ferroxidases, or ascorbate oxidases. Previously we found *Drosophila melanogaster* MCO1 has ferroxidase activity and we identified three putative iron binding residues in DmMCO1. Our kinetic analysis of recombinant MCO1 from *Drosophila melanogaster*, *Anopheles gambiae*, *Tribolium castaneum* and *Manduca sexta* showed that MCO1 orthologs are much better at oxidizing ascorbate than laccase substrates or ferrous iron, suggesting that MCO1 orthologs function as ascorbate oxidases. The putative iron binding residues are required for ascorbate oxidase activity but not ferroxidase and laccase activities. Ascorbate oxidases have been identified only in plants. This is the first identification of ascorbate oxidase in insects. Further studies are needed to understand their physiological function in insects.

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Dedication

I dedicate this work to my loving parents, Hua Peng and Ying Wang, for their love, encouragement and support.

Chapter 1 - Literature Review

Copper-containing Proteins

Copper is essential in all living organisms (Nakamura and Go, 2005). The redox property of copper (Cu^{+}/Cu^{2+}) is utilized in biological oxidation-reduction reactions (Solomon et al., 1996; Sakurai and Kataoka, 2007a). There are a variety of proteins that utilize copper as a cofactor to exert a biological function. (Nakamura and Go, 2005). Functions of these proteins include electron transfer, catalyzing redox reactions of diverse substrates, and transport of oxygen molecules (Sakurai and Kataoka, 2007a).

The copper sites in proteins are historically classified into three types according to their spectroscopic and magnetic features. The type 1 (T1) copper site has a strong absorption at ~600 nm, which is responsible for the blue color of the protein. The type 2 (T2) copper site is almost undetectable in the visible region. The type 3 (T3) copper site contains two coupled coppers, and shows an absorption maximum at ~330 nm. In the electron paramagnetic resonance (EPR) spectra, the T1 copper site and T2 copper site give distinctive signals. However, the T3 copper site shows no EPR signal due to strong antiferromagnetic interaction of the two coupled coppers (Solomon et al., 1996; Nakamura and Go, 2005; Shleev et al., 2005; Sakurai and Kataoka, 2007a; Sakurai and Kataoka, 2007b).

T1 Copper Proteins

T1 copper proteins contain only a T1 copper site, and they are termed blue copper proteins. This group of proteins has a relatively small molecular weight (9-23 kDa). Most proteins of this class function in intermolecular electron transfer (e.g., plastocyanin, umecyanin, and azurin) (Sakurai and Kataoka, 2007a), and they are known as mono-domain cupredoxins (Nakamura and Go, 2005). The T1 copper ion is strongly coordinated by imidazole groups of two histidines and a thiol group of one cysteine, and further ligand(s) (Sakurai and Kataoka, 2007a). Methionine is commonly utilized as the fourth ligand, and the thioether group of methionine binds to the T1 copper ion more weakly compared to the imidazole nitrogen and thiol sulfur. In some cupredoxins, methionine is replaced by a non-methionine ligand, such as glutamine. The amide group of glutamine binds to the T1 copper ion, with a stronger interaction

compared to the thioether group of methionine. Another class of cupredoxins contains two further ligands – a thioether group of methionine and a carbonyl group of glycine. Characteristics and examples of T1 copper proteins – plastocyanin (Colman et al., 1978), umecyanin (Koch et al., 2004), and azurin (Nar et al., 1991) are listed in Table 1.1.

All T1 copper proteins have a strong absorbance around 600 nm due to ligand-to-metal charge transfer transition (cysteine sulfur to Cu^{2+}), which causes the blue color (Solomon et al., 1996).

T2 Copper Proteins

In general, T2 copper proteins function as oxidoreductases. There are several well studied enzyme families of this group: amine oxidases, CuZn superoxide dismutases, galactose oxidases, monooxygenases. Characteristics and examples of T2 copper proteins are listed in Table 1.1.

Copper amine oxidases (CuAOs) catalyze the oxidation of amines by deamination and concomitant reduction of oxygen to hydrogen peroxide. CuAOs are identified in bacteria, fungi, plants and mammals (MacPherson and Murphy, 2007). Crystal structures of CuAOs from several species have been solved (Parsons et al., 1995; Kumar et al., 1996; Wilce et al., 1997; Duff et al, 2003; Lunelli et al., 2005; Airenne et al, 2005). The enzyme folds as a dimer, and each monomer has one T2 copper ion. The copper ion is coordinated by three histidines and two water molecules. Besides the copper ion, to function properly, CuAOs also contain a trihydroxyphenylalanine quinone (TPQ) cofactor formed by the post-translational modification of a tyrosine residue (MacPherson and Murphy, 2007).

Superoxide dismutases (SODs) catalyze the dismutation of superoxide to hydrogen peroxide and dioxygen. One Class of SODs is CuZnSODs, which are found in all eukaryotes and many prokaryotes (MacPherson and Murphy, 2007). Eukaryotic CuZnSOD is a homodimer, containing one copper ion and one zinc ion per monomer. The T2 copper ion is coordinated by four histidines and one water molecule. The zinc ion is coordinated by three histidines and one aspartic acid, and it is ~6.6 Å away from the T2 copper ion. One of the histidines coordinates both the copper and zinc ions. (Tainer et al., 1982; MacPherson and Murphy, 2007).

Galactose oxidases are secreted fungal enzymes that oxidize primary alcohols to aldehydes and reduce dioxygen to hydrogen peroxide. The enzyme contains a T2 copper ion coordinated by two histidines, two tyrosines, and one water molecule (Whittacker, 2003).

Among copper containing proteins, coordination of the copper ion by two tyrosines in galactose oxidases is unique. In addition to the copper ion, the X-ray structure reveals a protein-derived redox cofactor in the active site (Ito et al., 1991). The cofactor is formed by two amino acids - tyrosine and cysteine, crosslinking at the *ortho*-position to the hydroxyl group of tyrosine and creating a novel thioether bond. The tyrosine forming the cofactor is one of the two tyrosines coordinating the copper ion. Not only the copper ion, but also the cofactor is required for the reaction catalyzed by the enzyme (Shleev et al., 2005).

Copper monooxygenases include peptidylglycine α-hydroxylating monooxygenase (PHM) and dopamine β-monooxygense (DβM). The two enzymes are identified mainly in metazoans (MacPherson and Murphy, 2007). PHM catalyzes the hydroxylation of the α-carbon atom of a carboxyl-terminal glycine, which is the first reaction step of terminal amidation of many neuropeptides and hormones (Prigge et al., 1997; Rosenzweig and Sazinsky, 2006). DβM hydroxylates dopamine at β-carbon, and produces norepinephrine, a well known hormone and neurotransmitter. In the two hydroxylation reactions, molecular oxygen is the source of OH, and ascorbate is the source of electrons. Both enzymes contain two T2 copper ions (MacPherson and Murphy, 2007). In 1997, the crystal structure of PHM was solved. In PHM, there are two mononuclear T2 copper sites, CuA and CuB, separated by 11 Å of solvent. CuA ion is coordinated by three histidines, and CuB ion is coordinated by two histidines, one methionine, and a water molecule. The oxygen molecule and substrate bind at the CuB site (Prigge et al., 1997). Both copper sites are involved in oxygen reduction, and substrate-mediated electron transfer between the two copper sites has been proposed (Prigge et al., 1999).

T3 Copper Proteins

The T3 copper site consists of two coupled copper ions bridged by molecular oxygen or a water molecule and is termed binuclear site, whereas T1 and T2 sites are mononuclear sites (Shleev et al., 2005). Each of the T3 copper ions is coordinated by three conserved histidines (Aguilera et al., 2013). Well studied T3 copper proteins include tyrosinases, catechol oxidases and hemocyanins (Rosenzweig and Sazinsky, 2006). Tyrosinases can catalyze the hydroxylation of monophenols to *o*-diphenols, and the oxidation of *o*-diphenols to the corresponding *o*-quinones (Solomon et al., 1996). They are widely distributed among eukaryotes, and are also identified in a few eubacteria (Solomon et al., 1996). In fungi and animals, tyrosinase

hydroxylates tyrosine to dopa as the first step in the production of melanin (Solomon et al., 1996). In plants, tyrosinase can catalyze a wide range of phenols when tissues get injured, which possibly protects the wound against pathogens and insects (Solomon et al., 1996). Insect phenoloxidases and mammalian tyrosinases are copper containing enzymes that have similar activity but no homology. Phenoloxidases are located in insect hymolymph as zymogens. Upon wounding or infection, they get activated and lead to synthesis of melanin; thus, they play a role in the innate immune response (Kanost and Gorman, 2008). Catechol oxidases catalyze the oxidation of *o*-diphenols to *o*-quinones together with the reduction of oxygen to water. They do not catalyze the hydroxylation of monophenols, so they are different from tyrosinases. They are found in a variety of plants (Klabunde et al., 1998). Hemocyanins are oligomeric copper proteins, which can bind molecular oxygen reversibly at the active site that contains a pair of T3 copper atoms. They function as dioxygen carriers in the hemolymph of some molluscs and arthropods (Salvato and Beltramini, 1990). Characteristics and examples of T3 copper proteins – tyrosinase (Matoba et al., 2006; Sendovski et al., 2011), catechol oxidase (Klabunde et al., 1998), and hemocyanin (Cuff et al., 1998) are listed in Table 1.1. UV/vis spectroscopy of T3 copper proteins usually shows a strong absorption around 330 nm (Shleev et al., 2005).

Multicopper Proteins

Multicopper proteins include copper-containing nitrite reductases (NiRs), which catalyze the reduction of nitrite to nitric oxide. NiRs are found in bacteria, fungi and some species of amoeba (MacPherson and Murphy, 2007). Typically, NiR forms a homotrimer, and each monomer is composed of two cupredoxin-like domains (MacPherson and Murphy, 2007). A T1 copper site is located within a cupredoxin domain, and a T2 copper site is located between two cupredoxin domains (Nakamura and Go, 2005). The T1 copper is coordinated by two histidines, one cysteine, and one methionine. The T2 copper is coordinated by three histidines and one water molecule (Godden et al., 1991). The T1 copper site accepts an electron from a small electron transfer protein, and then the electron is transferred to the T2 copper site via a Cys-His linkage. Finally nitrite is reduced to nitric oxide at the T2 copper site (MacPherson and Murphy, 2007).

Among multicopper proteins, three-domain multicopper oxidases (MCOs) form the largest group. They contain three cupredoxin-like domains, and they are found in bacteria, fungi,

plants, and insects (Nakamura and Go, 2005). Typically, three-domain MCOs contain four copper ions: one T1 copper, one T2 copper, and two coupled T3 coppers. The four coppers form two copper centers. The T1 center consists of the T1 copper, and the T2/T3 trinuclear center consists of the T2 copper and the pair of T3 coppers (Kosman, 2010). The T1 center is located in domain 3, and the trinuclear center is located at the interface of domain 1 and 3 (Sakurai and Kataoka, 2007a). All typical three-domain MCOs have ten conserved histidines and one conserved cysteine that coordinate the four coppers. The residues normally coordinating the T1 copper are two histidines and one cysteine. Methionine often serves as the fourth residue that coordinates the T1 copper, but it is more distant and the interaction is weaker. Alternatively, this position can be occupied by different amino acids, such as leucine or phenylalanine without coordinating the T1 copper. The T2 copper is coordinated by two histidines and one water molecule, and each of the T3 coppers is coordinated by three histidines and they are bridged by molecular oxygen (Nakamura and Go, 2005; Sakurai and Kataoka, 2007b). The four copper ions buried within MCOs are essential for oxidase activity. One electron of the substrate is transferred to the T1 center near the substrate-binding pocket, and then the electron is passed via intramolecular residues, a conserved His-Cys-His triad, to the pair of T3 copper ions. Molecular oxygen binds between the two T3 copper ions and is reduced to water after the transfer of four electrons (Kosman, 2010). The His-Cys-His triad, which achieves electron transfer between the T1 center and the trinuclear center, are three of the copper-coordinating residues that are conserved in MCOs.

Six-domain MCOs, such as ceruloplasmin and hephaestin, are the two best-studied mammalian ferroxidases (Nakamura and Go, 2005). In mammals, ferrous iron is transported out of cells by ferroportin, the ferrous iron permease, and is then oxidized by an extracellular membrane-bound ferroxidase; finally, the produced ferric iron is loaded onto serum transferrin, the transport protein (De Domenico et al., 2007; Han, 2011). Ceruloplasmin is synthesized as two forms - GPI-anchored and circulating proteins (Hellman and Gitlin, 2002). GPI-anchored ceruloplasmin has been shown to physically associate with ferroportin (Jeong and David, 2003). Hephaestin is a membrane-bound protein that directly interacts with ferroportin on the basal portion of epithelial cells (Yeh et al., 2009). Circulating ceruloplasmin is able to oxidize ferrous iron to ferric iron in blood serum, so that the ferric iron can be bound to transferrin and transported to other tissues (Hellman and Gitlin, 2002). The crystal structure of human

ceruloplasmin has been solved (Lindley et al., 1997). Ceruloplasmin consists of six domains and contains six copper ions. The second, fourth, and sixth domains each contains a T1 copper, and the first and sixth domains share a T2/T3 trinuclear center including a T2 copper and a pair of T3 coppers. The T1 copper located on domain 4 or 6 (Cu 4D or Cu 6D) is coordinated by four residues - two histidines, one cysteine, and one methionine. The T1 copper located on domain 2 (Cu 2D) is permanently reduced, and it is coordinated by three residues - two histidines, and one cysteine. It lacks methionine as a coordinating residue, and the methionine is replaced by a leucine, which does not coordinate metals. The Cu 6D site is closer to the trinuclear center compared to the other two T1 copper sites, and the cysteine ligand of the Cu 6D site is near two histidines, each coordinates a copper ion of the trinuclear center. The other two T1 copper sites (Cu 2D and Cu 4D) are far away from the trinuclear center (Bielli and Calabrese, 2002). Characteristics and examples of multicopper proteins are listed in Table 1.1.

Proteins Containing Newly Discovered Copper Sites

In addition to the classic T1, T2 and T3 copper sites, there are several newly discovered copper sites, including Cu_A site, Cu_B site, and Cu_Z site. Characteristics and examples of proteins containing these copper sites are listed in Table 1.1.

Cytochrome *c* oxidase is the last enzyme in the electron transport chain, which converts oxygen molecule to water. It is a large transmembrane multidomain protein (Rubino and Franz, 2012). It contains two copper sites - Cu_A site and Cu_B site. The Cu_A site is composed of two coppers bridged by two cysteines. One of the coppers is also coordinated by a histidine and a methionine, and the other copper is also coordinated by a histidine and the carbonyl oxygen of a glutamic acid. The Cu_A site in large enzymes functions to aid entry of electrons. The Cu_B site is coordinated by three histidines. The Cu_B site is involved in the electron transport from heme and is part of an oxygen binding site (Tsukihara et al., 1995).

Nitrous-oxide reductase catalyzes the reduction of nitrous oxide to dinitrogen as the last step in bacterial denitrification (Berks et al., 1995). The enzyme contains two copper sites – Cu_A site and Cu_Z site (Pomowski et al., 2011). The Cu_A site is similar to that of cytochrome *c* oxidase, except for one coordinating ligand, methionine, replaced by tryptophan (Pomowski et al., 2011). The Cu_Z site is the most recently discovered copper site in the redox proteins. The Cu_Z site is composed of four coppers (Cu_{z1}, Cu_{z2}, Cu_{z3} and Cu_{z4}) coordinated by seven histidines.

Specifically, cach of Cu_{z1}, Cu_{z2} and Cu_{z3} is coordinated by two histidines, and Cu_{z4} is coordinated by one histidine (Pomowski et al., 2011). The four coppers are bridged by an inorganic sulfur ion. In addition, Cu_{z1} and Cu_{z4} are bridged by another inorganic sulfur ion (Pomowski et al., 2011).

Copper Trafficking Proteins

The group of proteins described above utilize copper as a cofactor to execute a biological function. Another group of proteins (copper trafficking proteins) functions to transfer and stabilize copper, including integral membrane copper transport proteins, copper chaperones, and copper resistance proteins (Rubino and Franz, 2012). Copper coordination environments are different between proteins that use the copper as a cofactor and proteins that transport copper.

Biological copper is mainly coordinated by three residues: histidine, cysteine and methionine. Histidine has the ability to strongly bind both Cu^{+} and Cu^{2+}, whereas cysteine and methionine bind Cu^{+} more effectively. Cysteine exhibits the strongest affinity for copper, but methionine has much weaker binding affinity (Rubino and Franz, 2012). The proteins that utilize copper as a cofactor contain copper-binding sites with high affinity and stable geometries to avoid the loss of copper during redox cycling (Rubino and Franz, 2012). As described above, it is easy to notice that copper-coordinating residues are dominated by histidines. This is important because histidine can bind both Cu^{+} and Cu^{2+} strongly during redox cycling to prevent the copper loss from the binding site. In addition, the coordination numbers of ligands are generally 4-5.

Copper trafficking proteins also contain copper-binding sites, but with low affinity or flexible geometries to promote transport of copper. Copper ATPases belong to the family of integral membrane copper transport proteins. Two cysteines of copper ATPases bind copper in high affinity, but flexible and almost linear geometry (S-Cu-S bond angles ranging 120-170°) (Banci et al., 2001; Banci et al., 2004; DeSilva et al., 2005). Most copper chaperones also use the same copper-binding sites (two cysteines that bind copper in almost linear geometry), and some copper chaperones have an extra histidine in their binding sites (Rubino and Franz, 2012). Thus, copper ATPases and copper chaperones contain copper binding sites with high affinity, but less coordinating binding sites. Copper resistance proteins function to remove excess copper and prevent accumulation of toxic copper. These proteins are mainly found in prokaryotes, particularly gram-negative bacteria (Rubino and Franz, 2012). Binding sites of copper resistance proteins (such as CusA and CusB) are dominated by methionine (Bagai et al., 2007; Long et al.,

2010), sometimes with incorporation of a single histidine (such as PcoC and CopC) (Peariso et al., 2003; Arnesano et al., 2003). Even though the coordination sites are 3-4, the binding sites are rich in methionines and exhibit relatively weak affinity for copper.

Multicopper Oxidases

The multicopper oxidase (MCO) family of enzymes includes laccases, ascorbate oxidases, bilirubin oxidases, a subgroup of metallooxidases (ferroxidases, cuprous oxidases, and manganese oxidases), as well as some oxidases involved in biosyntheses (Sakurai and Kataoka, 2007b). Laccases can oxidize a variety of natural substrates (Giardina et al., 2010), whereas the other oxidases tend to oxidize specific substrates (Solomon et al., 1996; Kosman, 2010). MCOs have been found in bacteria, fungi, plants, insects, and vertebrates (Nakamura and Go, 2005; Sakurai and Kataoka, 2007b).

Laccases

Laccases are the largest subgroup of MCOs, and can oxidize a broad range of aromatic substrates, including *ortho*- and *para*-diphenols, aminophenols, methoxyphenols, and aromatic diamines, with the concomitant reduction of molecular oxygen to water. In addition, laccases can also oxidize electron donor substrates such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Madhavi and Lele, 2009; Giardina et al., 2010). The first laccase was discovered from the Japanese lacquer tree *Rhus vernicifera* in 1883 (Yoshida, 1883). So far, laccases have been identified in bacteria, fungi, plants and insects (Madhavi and Lele, 2009; Giardina et al., 2010). In bacteria, laccases appear to function in morphogenesis and pigmentation (Sharma et al., 2007). In fungi, laccases play various physiological roles, including lignin degradation, morphogenesis, detoxification and pigment formation (Mayar and Staples, 2002). In plants, laccases are involved in lignin biosynthesis and wound healing (McCaig et al., 2005). In insects, laccases are known to play a role in cuticle tanning (Arakane et al., 2005; Dittmer et al., 2009).

Laccases and tyrosinases (described above) are two types of copper-containing phnoloxidases. They both can oxidize *o*-diphenols to corresponding *o*-quinones, but they are completely different in sequence. Tyrosinases catalyze both the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones, but they do not oxidize *p*-diphenols. Laccases oxidize both *o*- and *p*-diphenols, but do not hydroxylate monophenols. The two enzymes can be distinguished from each other based on their differences in activity to

monophenols and *p*-diphenols (Dittmer and Kanost, 2010). In addition, they can be distinguished by use of substrates such as ABTS that can be oxidized by laccases but not tyrosinases.

Among laccases, the fungal laccases are intensively studied. Most fungal laccases are extracellular monomeric proteins, and they are usually glycosylated. They typically have an isoeletric point ~ pH 4 (Rodgers et al., 2009; Giardina et al., 2010). Crystal structures of many fungal laccases have been solved so far. All these laccases have three cupredoxin-like domains, and contain four copper ions coordinated by conserved residues (ten histidines and one cysteine). The T1 copper is located in domain 3, and the T2/T3 trinuclear center is embedded between domain 1 and 3 (Hakulinen et al., 2002; Piontek et al., 2002; Bertrand et al., 2002; Garavaglia et al., 2004; Ferraroni et al., 2007; Matera et al., 2008). The crystal structure of a laccase from *Trametes versicolor* (PDB number: 1KYA) is shown in Figure 1.1. In fungal laccases, four loop regions involved in substrate binding have been identified. The substrate binding cavity is quite wide so that it can accommodate various substrates (Giardina et al., 2010). A phenolic substrate binds to at least one of the histidines that coordinate the T1 copper via hydrogen bond, which forms the electron pathway from the substrate to the T1 copper ion (Kosman, 2010). For example, 2,5-xylidine forms hydrogen bond with His458 in *T. versicolor* laccase (Bertrand et al., 2002).

In a laccase, the four copper ions are cupric in the resting form (Sakurai and Kataoka, 2007b). The T1 copper ion located near the substrate binding cavity is the first acceptor of the electron from the substrate. The electron is then transferred via the highly conserved His-Cys-His tripeptide to the trinuclear center where molecular oxygen binds. After transfer of four electrons, molecular oxygen is reduced to water. During the catalytic process, the T1 copper ion must be reduced and oxidized four times. The three copper ions in the trinuclear center are successively reduced (Rodgers et al., 2009; Giardina et al., 2010). The electron transfer from the substrate to the T1 copper ion is controlled by redox potential difference. A higher redox potential of a laccase (the T1 site) shows greater affinity for electrons, so that the T1 copper ion is easier to be reduced; thus, the substrate is oxidized faster by a laccase with a higher redox potential (Madhavi and Lele, 2009). The T1 copper of fungal laccases is normally coordinated by two histidines and one cysteine without the fourth ligand – methionine (Sakurai and Kataoka, 2007a). Compared to other laccases, fungal laccases display higher redox potential mainly due to the lack of the fourth coordinating residue (Sakurai and Kataoka, 2007a; Giardina et al., 2010). Phenols are common

laccase substrates because their redox potentials are lower than the T1 copper ion and the electron can be transferred from the substrate to the T1 center (Giardina et al., 2010).

Fungal laccases have been used in diverse biotechnological applications, such as wood pulp delignification in the paper industry and dye-bleaching in the textile and dye industries, because they have nonspecific oxidation capability and use easily obtainable molecular oxygen as an electron acceptor (Giardina et al., 2010).

The catalytic efficiency of laccases can be decreased by inhibitors. Since laccases are copper-containing proteins, agents that chelate or reduce copper may decrease their activity. A partial list of these agents includes cyanide, azide, and ethylenediamine tetraacetic acid (EDTA) (Couto and Toca, 2006). Cyanide has been proposed to dissociate the copper ion from the laccase so that the enzyme loses the activity (Ragusa et al., 2002). The effect of chelators on enzymatic activity differs among various fungal laccases. Generally cyanide and azide are inhibitors. Halides inhibit laccases because they bind at the T2/T3 trinuclear center and prohibit the reduction of oxygen to water (Branden et al., 1973; Couto and Toca, 2006). The experimental inhibition effect order is fluoride > chloride > bromide, which is probably due to the restricted accessibility of larger halides to the trinuclear center (Xu, 1996; Abadulla et al., 2000). Other inhibitors include heavy metals (Couto and Toca, 2006) and fatty acids (Kreuter et al., 1991), but their mechanisms of inhibition are still unclear. Sulfhydryl reagents (such as dithiothreitol, thioglycolic acid, and diethyldithiocarbamate) described as laccase inhibitors appear to reduce the reaction products, rather than interact with the enzyme directly (Johannes and Majcherczyk, 2000). So far, true competitive inhibitors of laccases have not been identified.

Ascorbate Oxidases

Ascorbate oxidases catalyze the oxidation of ascorbate to dehydroascorbate with concomitant reduction of oxygen to water (Shleev et al., 2005). Ascorbate oxidases are widely distributed in higher plants (Solomon et al., 1996). Ascorbate oxidase was crystallographically characterized earlier than other MCOs (Nakamura and Go, 2005). The plant ascorbate oxidases are homodimers, and each monomer is composed of three cupredoxin-like domains and contains one T1 copper and one T2/T3 trinuclear center. The T1 copper bound inside domain 3 is coordinated by two histidines, one cysteine and one methionine. The T2/T3 coppers located at the interface between domain 1 and 3 are coordinated by eight histidines. The T1 copper and the

two T3 coppers are connected by a His-Cye-His tripeptide, which is necessary for intramolecular electron transfer (Messerschmidt et al., 1992). The formation of a homodimer for ascorbate oxidase is required for its stability and function (Mei et al., 1997). Based on a docking study of ascorbate oxidase-ascorbate complex, the putative binding site is located in a cavity near the T1 site. The binding of the substrate is predicted to be facilitated by hydrogen bonding interactions between the substrate and at least five residues of the enzyme: His512 that coordinates the T1 copper, Gln353, Trp362, Glu443 and Arg285. In addition, the substrate may also be stabilized by a stacking interaction of the ascorbate ring and the aromatic ring of Trp163. The binding site was further confirmed by an inhibition study – a competitive inhibitor binds at the same binding site as ascorbate (Santagostini et al., 2004).

Although ascorbate oxidases have been known for a long time, their physiological roles in plants are not fully understood. Ascorbate oxidase is a secreted glycoprotein localized in the cell wall of plants (Smirnoff, 2000). Ascorbate oxidase is highly expressed at a stage when rapid growth is happening, indicating ascorbate oxidase is involved in cell growth (Lin and Varner, 1991; Esaka et al., 1992; Kato and Esaka, 1999). A plant growth hormone, auxin, greatly induces expression of ascorbate oxidase and stimulates *in vivo* oxidase activity (Esaka et al., 1992; Kisu et al., 1997). Ascorbate oxidase has been suggested to function in cell expansion by the reaction of dehydroascorbate and the cell wall molecules to cause cell wall "loosening" (Lin and Varner, 1991; Esaka et al., 1992). In addition, the expression of ascorbate oxidase is induced by mechanical wounding (Asao et al., 2003), which suggests it may be involved in defense against oxidants.

Bilirubin Oxidases

Bilirubin oxidases oxidize bilirubin to biliverdin and reduce oxygen to water concomitantly (Shleev et al., 2005). This enzyme has been mainly used in the detection of bilirubin in serum; therefore, it is important in the medical field – diagnostic of jaundice (Perry et al., 1986; Doumas et al., 1987; Mullon and Langer, 1987; Kirihigashi et al., 2000). Bilirubin oxidases have been identified in bacteria and fungi (Mano, 2012). The spore coat protein CotA from bacteria was initially classified as a laccase; to date, it has been characterized as a new bacterial bilirubin oxidase since it shows extremely strong bilirubin oxidase activity (Sakasegawa et al., 2006; Durand et al., 2012a). The amino acid sequences of bilirubin oxidases

contain the conserved copper-binding residues that are characteristic of MCOs (Mano, 2012). The purified bilirubin oxidase has the typical deep blue color of MCOs, and shows typical UV/vis spectrum of MCOs (a peak at ~600 nm and a shoulder at ~330 nm) (Durand et al., 2012b). Recently, the crystal structures of fungal bilirubin oxidases were solved (Mizutani et al., 2010; Cracknell et al., 2011; Kjaergaard et al., 2012). The catalytic site of bilirubin oxidase also contains four copper ions. Bilirubin oxidases show high stability and activity at neutral pH, high tolerance to chloride and chelating agents, as well as high thermal stability in some species, including bacterial CotA proteins and some fungal bilirubin oxidases (Mano, 2012).

Metallooxidases

A subgroup of MCO proteins, metallooxidases, show substrate specificity for ferrous ion, cuprous ion or manganous ion (Kosman, 2010).

Ferroxidases consist of three- and six-domain proteins (Kosman, 2010). Well studied ferroxidases include yeast Fet3p and human ceruloplasmin. Fet3p is a glycosylated protein located on the yeast plasma membrane that catalyzes the ferroxidase reaction – the oxidation of ferrous ion to ferric ion coupled with the reduction of oxygen to water (Taylor et al., 2005). The produced ferric ion is introduced to the cytosol by the permease Ftr1p (Singh et al., 2006; Philpott and Protchenko, 2008). The structure of Fet3p has been solved (Taylor et al., 2005). Fet3p has three cupredoxin-like domains and contains four copper atoms which are required for its activity (Taylor et al., 2005). Ten histidines and one cysteine are involved in coordinating the four copper atoms. The T1 copper atom is coordinated by two histidines and one cysteine, the T2 copper atom is coordinated by two histidines, and the two T3 copper atoms are coordinated by six histidines (Taylor et al., 2005). So far, the crystal structure of a Fet3p-Fe^{2+} complex has not been characterized, but spectral and kinetic data show that Glu185, Asp283 and Asp409 in Fet3p provide a binding site for the ferrous ion. The substrate binds to Fet3p through the carboxylate groups of the acidic residues. Two of the carboxylate groups form hydrogen bonds with the two histidine ligands that coordinate the T1 copper; thus, electron transfer occurs through the hydrogen bonds (Stoj et al., 2006; Kosman, 2010). The electron transfer between the T1 and T3 copper ions are achieved via the His-Cys-His tripeptide, which is conserved in all typical MCOs. The cysteine is one of the ligands that coordinate the T1 copper ion, and each of the two histidines coordinates one T3 copper ion. In Fet3p, the carbonyl group of Cys484 forms

hydrogen bonds with the amino group of His485 (coordinating T3α copper ion) and the imidazole group of His483 (coordinating T3β copper ion) (Kosman, 2010). Six-domain ferroxidases have been described in "Multicopper Proteins" section.

CeuO from *E.coli* is a well studied cuprous oxidase. CeuO catalyzes the oxidation of cuprous ion to cupric ion, which is proposed to function in copper resistance since cuprous ion is more harmful than cupric ion. Its cuprous oxidase activity is higher than that of any other known MCOs (Nakamura and Go, 2005). The crystal structure of CeuO is similar to those of other three-domain MCOs. The main difference is that CeuO has a methionine-rich region in domain 3. Nine of the methionines are located in a helix close to the T1 center (Roberts et al., 2002). In the presence of excess copper ions, the substrate (a copper ion) is bound near the T1 center in the methionine-rich region. The substrate-binding site consists of Met355 and Asp360 of the methionine-rich region, as well as Asp439, Met441 and a water molecule (Roberts et al., 2003).

Compared to other MCOs, manganese oxidases are poorly characterized. Manganese oxidases are identified in bacteria (Nakamura and Go, 2005). There is no crystal structure currently available for any manganese oxidase. CumA is identified in *Pseudomonas* strains and is require for the oxidation of manganous ion (Francis and Tebo, 2001). CumA is a three-domain MCO, similar to laccases (Nakamura and Go, 2005). MofA from *Leptothrix discophora* is able to oxidize environmental manganous ion and ferrous ion. Compared to other three-domain MCOs, *L. discophora* MofA has a large insertion (~500 amino acids) between domain 2 and 3. And the rest part of MofA shares approximately 20% sequence identity with three-domain MCOs (Corstjens et al., 1992; Nakamura and Go, 2005). MnxG from *Bacillus* SG-1 can oxidize manganous ion (van Waasbergen, et al., 1996; Francis and Tebo, 2002). Based on sequence alignment of MnxG with other MCOs (such AO, laccase, and human Cp), MnxG contains six cupredoxin-like domains, which is similar to Cp. MnxG has a T1 center in domain 2 and a T2/T3 trinuclear center between domain 2 and 3; thus, the copper binding sites are different from Cp (van Waasbergen, et al., 1996).

Atypical Multicopper Oxidases

Although MCOs usually contain four copper atoms coordinated by ten conserved histidines and one conserved cysteine, and exhibit a strong absorption peak at ~600 nm and an absorption shoulder at ~330 nm in the UV/vis spectra (Sakurai and Kataoka, 2007b), some

atypical MCOs have been identified. Among atypical MCOs, some were studied in-depth, but others were not. Most identified atypical MCOs and their features are listed in Table 1.2.

There have been several studies of atypical fungal laccases, which contain fewer than four copper atoms, but still exhibit laccase activity. POXA1 isolated from *Pleurotus ostreatus* contains one copper, one iron, and two zincs (Palmieri et al., 1997). Another laccase extracted from *Phellinus ribis* contains one copper, one manganese, and two zincs (Min et al., 2001). A laccase from *Phlebia radiata* contains one copper, one zinc, and two irons (Kaneko et al., 2009). One unusual laccase from *Trametes hirsuta* has three coppers and one manganese (Haibo et al., 2009). In addition, one laccase from *Marasmius sp.* contains only a single copper and no other metals (Schückel et al., 2011). These laccases were termed "white" laccases because they lack the blue color of most MCOs, and contain fewer than four copper atoms. These studies focused on the biotechnological applications of the unusual laccases rather than their biochemical features; therefore, the amino acid sequences, including the presence or absence of conserved copper-coordinating residues, were not reported.

In addition to "white" laccases, "yellow" laccases were reported (See Table 1.2). "Yellow" laccases do not show an absorption peak at ~600 nm, and they have a characteristic yellow color so that they were termed "yellow" laccases. Yellow laccases are capable of oxidizing non-phenolic substrates without mediators, so they are thought to have greater industrial applications compared to typical laccases (Leontievsky et al., 1997b; Edens et al., 1999; Pozdnyakova et al., 2006b). In terms of metal content, "yellow" laccases were not studied in detail compared to "white" laccases.

A group of ascorbate oxidase homologs identified in plants share sequence similarity with ascorbate oxidases, but, they lack copper binding residues (Nakamura and Go, 2005). The best-studied of these ascorbate oxidase homologs, SKU5 from *Arabidopsis thaliana*, shares 23% sequence identity with ascorbate oxidases. The residues required for coordinating the T1 and T3 copper ions are absent in SKU5, but the two histidines required for coordinating the T2 copper are present. SKU is expressed in all tissues, most strongly in expanding tissues, and it is involved in directional root growth (Sedbrook et al., 2002). Bp 10 from *Brassica napus* shares 30% sequence identity with ascorbate oxidases, lacks most of the conserved copper-coordinating ligands, and is expressed in developing pollen (Albani et al., 1992). Whether SKU5 and Bp10 bind to copper and whether they have oxidase activity are unknown.

Insect Multicopper Oxidases

In insects, putative MCOs have been identified based on sequence information from insect genomes. Insect MCOs include MCO1 orthologs, MCO2 orthologs, and a mosquito specific group (MCO3, MCO4, and MCO5 orthologs) (Gorman et al., 2008; Dittmer and Kanost, 2010).

cDNAs for MCOs from different insect species have been cloned (Parkinson et al., 2003; Dittmer et al., 2004; Arakane et al., 2005; Gorman et al., 2008; Niu et al., 2008; Yatsu and Asano, 2009; Pan et al., 2009; Lang et al., 2012a). Based on the deduced amino acid sequences, all of the putative insect MCOs have the conserved copper-coordinating residues – ten histidines and one cysteine. Additionally, methionine serves as the fourth ligand that coordinates the T1 copper in insect MCOs. The domain architectures of insect MCOs are shown in Figure 1.2. There is a cysteine-rich domain after the amino-terminus in all insect MCOs, which is not present in MCOs from other organisms, but the function of this domain is unclear (Dittmer and Kanost, 2010). The *Anopheles gambiae* MCO1 (Dittmer et al., 2004) and *Drosophila melanogaster* MCO1 (Lang et al., 2012a) both contain additional amino-terminal von Willebrand factor domains, whose function is unknown. So far, only dipteran MCO1 orthologs are predicted to have these von Willebrand factor domains. The sequences of MCO2 orthologs from different insect species are highly conserved, which suggests a conserved function (Dittmer and Kanost, 2010). The MCO2 genes from three insect species (*Tribolium castaneum*, *A. gambiae* and *Bombyx mori*) have been shown to have alternatively spliced isoforms (MCO2A and MCO2B) derived from exon duplication (Arakane et al., 2005; Gorman et al., 2008; Yatsu and Asano, 2009). MCO1 and MCO2 have been identified in all examined insect species, and they formed two distinct clades in a phylogenetic analysis of insect MCOs; thus, MCO1 and MCO2 each may have a conserved physiological function in insects (Dittmer and Kanost, 2010). MCO3, MCO4 and MCO5 have been identified in several mosquito species but not other diptera, such as *D. melanogaster*. In a phylogenetic analysis, they form a mosquito-specific branch (Dittmer and Kanost, 2010). Among *A. gambiae* MCOs, except for MCO4 that contains a predicted amino-terminal signal anchor, the other MCOs have a putative signal peptide and thus are probably secreted proteins (Gorman et al., 2008). MCO1 sequences contain a putative carboxyl-terminal transmembrane region, predicted with TMPred software (Hofmann and Stoffel, 1993). MCO1 has been proposed to

enter the secretary pathway in cells and remain attached to the cell membrane by a carboxyl-terminal transmembrane region (Lang et al., 2012a).

MCO1 is expressed in all developmental stages and most tissues (Dittmer et al., 2004; Chintapalli et al., 2007; Gorman et al., 2008; Graveley et al., 2011). AgMCO1 was upregulated in the midgut and Malpighian tubules after a blood meal. In addition, AgMCO1 was upregulated in the adult abdominal carcass (the abdomen without midgut and Malpighian tubules, including fat body) 1 h after injection of a gram-positive bacterium, *Micrococcus luteus*, which suggests AgMCO1 may function in an immune pathway (Gorman et al., 2008). Purified recombinant *D. melanogaster* MCO1 can oxidize ferrous iron (Lang et al., 2012a). Immunohistochemistry experiments showed that DmMCO1 is located on the basal side of midgut and Malpighian tubules of the fly (Lang et al., 2012a). Knockdown of DmMCO1 is correlated with decreased iron accumulation in midguts and whole insects and with increased life span of flies fed on high-iron food. These results suggest that DmMCO1 has ferroxidase activity, and is involved in iron homeostasis *in vivo* (Lang et al., 2012a).

In *Manduca sexta*, MCO2 was found to be highly expressed in the epidermis right before the larval to pupal molt (Dittmer et al., 2004). In *B. mori*, MCO2 was found to be highly expressed in the epidermis right before the larval to larval and larval to pupal molts (Yatsu and Asano, 2009). In *T. castaneum*, the highest levels of MCO2 transcripts were detected during the larval to pupal and pupal to adult molts (Arakane et al., 2005). In *Monochamus alternates*, MCO2 was observed to reach the highest levels during the pupal to adult molt (Niu et al., 2008). These expression patterns are consistent with the role of MCO2 in cuticle tanning (Arakane et al., 2005; Niu et al., 2008). Insect MCO2 orthologs are laccases (Lac2), which are synthesized by epithelial cells and secreted into new cuticle before the start of sclerotization or pigmentation (Dittmer et al., 2009; Yatsu and Asano, 2009; Gorman et al., 2012). Diphenols such as dopamine, N-acetyldopamine (NADA) and N-β-alanyldopamine (NBAD) are oxidized by Lac2. The generated quinones react with cuticular proteins to form protein cross-links resulting in sclerotization, or the quinones undergo further reactions that lead to melanin synthesis resulting in pigmentation (Andersen, 2010; Futahashi et al., 2010). RNAi in *T. castaneum* showed that splicing isoform TcLac2A plays a more important role in cuticle tanning compared to TcLac2B. Knockdown of TcLac2A resulted in soft and unpigmented cuticle of beetles, whereas knockdown of TcLac2B only postponed cuticle tanning (Arakane et al., 2005). *A. gambiae*

Lac2B was upregulated in the ovaries after blood feeding, whereas Lac2A remained constant (Gorman et al., 2008). Therefore, the two isoforms may have non-redundant and different functions (Dittmer and Kanost, 2010). No major differences were observed in substrate specificity between the two Lac2 isoforms (Gorman et al., 2012); thus, the two splicing isoforms seem to oxidize the same substrates *in vivo*. The physiological functions of the two isoforms *in vivo* may be related to substrate concentration, environmental pH or specific tissues. Additional experiments are needed to further study isoform-specific functions (Gorman et al., 2012).

A. gambiae MCO3 is predominantly expressed in the pupal and adult stages. In adults, AgMCO3 is highly expressed in the midguts and Malpighian tubules, and also in the male reproductive tissues. The expression was upregulated in the midguts and Malpighian tubules, and downregulated in the ovaries after a blood meal. In addition, after bacterial challenge, the expression of AgMCO3 was induced in the abdominal carcass (Gorman et al., 2008). AgMCO3 is a laccase and is located in the peritrophic matrix, but its physiological function is still unclear (Lang et al., 2012b). In contrast to AgMCO3, AgMCO4 and 5 are highly expressed in the embryonic and larval stages, and predominantly expressed in the larval abdominal carcass (Gorman et al., 2008).

Except for the laccases that catalyze cuticle tanning (MCO2 orthologs), the enzymatic properties and physiological functions of the other insect MCOs are still poorly characterized. We have been interested in insect MCOs because they may function in other important physiological processes, such as iron homeostasis (Lang et al., 2012a). In addition, studies of insect MCOs may lead to the development of new insecticides (Prasain et al., 2012). During our studies of insect MCOs, we noticed that the *T. castaneum* and *A. gambiae* genomes each contained an unusual MCO-like gene, which we named multicopper oxidase-related protein (MCORP). The MCORP sequences share sequence identity with MCOs, but lack the conserved cysteine and many of the conserved histidines that coordinate copper ions (Figure 1.2). The goal of my first project was to investigate the biochemical features and possible physiological functions of MCORP (Chapter 2). To date, it is still unclear what kind of oxidases MCO1 orthologs are. Most of the well-studied MCOs can be categorized as laccases, ferroxidases, or ascorbate oxidases. The substrate specificity of MCOs determines their physiological function. The goal of my second project was to characterize substrate specificity of recombinant MCO1 isoforms (Chapter 3).

References

Aguilera, F., McDougall, C., and Degnan, B. M. (2013) Origin, evolution and classification of type-3 copper proteins: lineage-specific gene expansions and losses across the metazoa. *BMC Evol. Biol.* **13,** 96

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

Albani, D., Sardana, R., Robert, L. S., Altosaar, I., Arnison, P. G., and Fabijanski, S. F. (1992) A *Brassica napus* gene family which shows sequence similarity to ascorbate oxidase is expressed in developing pollen. Molecular characterization and analysis of promoter activity in transgenic tobacco plants. *Plant J.* **2,** 331-342

Andersen, S.O. (2010) Insect cuticular sclerotization: A review. *Insect Biochem. Mol. Biol.* **40,** 166-178

Arakane, Y., Muthukrishnan, S., Beeman, R. W., Kanost, M. R., and Kramer, K. J. (2005) Laccase 2 is the phenoloxidase gene required for beetle cuticle tanning. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 11337-11342

Arnesano, F., Banci, L., Bertini, I., Mangani, S., and Thompsett, A. R. (2003) A redox switch in CopC: An intriguing copper trafficking protein that binds copper(I) and copper(II) at different sites. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 3814-3819

Asao, H., Yoshida, K., Nishi, Y., and Shinmyo, A. (2003) Wound-responsive *cis*-element in the 5′-upstream region of cucumber ascorbate oxidase gene. *Biosci. Biotechnol. Biochem.* **67,** 271-277

Bagai, I., Liu, W., Rensing, C., Blackburn, N. J., and McEvoy, M. M. (2007) Substrate-linked conformational change in the periplasmic component of a Cu(I)/Ag(I) efflux system. *J. Biol. Chem.* **282,** 35695-35702

Banci, L., Bertini, I., Ciofi-Baffoni, S., Huffman, D. L., and O'Halloran, T. V. (2001) Solution structure of the yeast copper transporter domain Ccc2a in the apo and Cu(I)-loaded states. *J. Biol. Chem.* **276,** 8415-8426

Banci, L., Bertini, I., Del Conte, R., D'Onofrio, M., and Rosato, A. (2004) Solution structure and backbone dynamics of the Cu(I) and apo forms of the second metal-binding domain of the Menkes protein ATP7A. *Biochemistry.* **43,** 3396-3403

Berks, B.C., Ferguson, S.J., Moir, J.W.B., and Richardson D.J. (1995) Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. *Biochim. Biophys. Acta.* **1232,** 97-173

Bertrand, T., Jolivalt, C., Briozzo, P., Caminade, E., Joly, N., Madzak, C., and Mougin, C. (2002) Crystal structure of a four-copper laccase complexed with an arylamine: insights into substrate recognition and correlation with kinetics. *Biochemistry.* **41,** 7325-7333

Bielli, P., and Calabrese, L. (2002) Structure to function relationships in ceruloplasmin: a 'moonlighting' protein. *Cell. Mol. Life Sci.* **59,** 1413-1427

Brändén, R., Malmström, B. G., and Vänngård, T. (1973) The effect of fluoride on the spectral and catalytic properties of three copper-containing oxidases. *Eur. J. Biochem.* **36,** 195-200

Chen, S., Ge, W., and Buswell, J. A. (2004) Biochemical and molecular characterization of a laccase from the edible straw mushroom, *Volvariella volvacea*. *Eur. J. Biochem.* **271,** 318-328

Chintapalli, V.R., Wang, J., and Dow, J.A.T. (2007) Using FlyAtlas to identify better *Drosophila melanogaster* models of human disease. *Nat. Genet.* **39,** 715–720.

Colman, P.M., Freeman, H.C., Guss, J. M., Murata, M., Norris, V.A., Ramshaw, J.A.M., and Venkatappa, M.P. (1978) X-ray crystal structure analysis of plastocyanin at 2.7 Å resolution. *Nature.* **272,** 319-324

Corstjens, P. L., de Vrind, J. P., Westbroek, P., and de Vrind-de Jong, E. W. (1992) Enzymatic iron oxidation by *Leptothrix discophora*: identification of an iron-oxidizing protein. *Appl. Environ. Microbiol.* **58,** 450-454

Couto, S. R., and Toca, J. L. (2006) Inhibitors of laccases: a review. *Current Enzyme Inhibition.* **2,** 343-352

Cracknell, J. A., McNamara, T. P., Lowe, E. D., and Blanford, C. F. (2011) Bilirubin oxidase from *Myrothecium verrucaria*: X-ray determination of the complete crystal structure and a rational surface modification for enhanced electrocatalytic O_2 reduction. *Dalton Transactions.* **40,** 6668-6675

Cuff, M. E., Miller, K. I., van Holde, K. E., and Hendrickson, W. A. (1998) Crystal structure of a functional unit from *Octopus* hemocyanin. *J. Mol. Biol.* **278,** 855-870

Daroch, M., Houghton, C. A., Moore, J. K., Wilkinson, M. C., Carnell, A. J., Bates, A. D., and Iwanejko, L. A. (2014) Glycosylated yellow laccases of the basidiomycete *Stropharia aeruginosa*. *Enzyme Microb. Technol.* **58,** 1-7

De Domenico, I., Ward, D. M., di Patti, M.C.B., Jeong, S. Y., David, S., Musci, G., and Kaplan, J. (2007) Ferroxidase activity is required for the stability of cell surface ferroportin in cells expressing GPI-ceruloplasmin. *EMBO J.* **26,** 2823-2831

De Vries, O.M.H., Kooistra, W.H.C.F., and Wessels, J.G.H. (1986) Formation of an extracellular laccase by a *Schizophyllum commune* dikaryon. *J. Gen. Microbiol.* **132,** 2817-2826

DeSilva, T. M., Veglia, G., and Opella, S. J. (2005) Solution structures of the reduced and Cu (I) bound forms of the first metal binding sequence of ATP7A associated with Menkes disease. *Proteins: Struct., Funct., Bioinf.*. **61,** 1038-1049

Dittmer, N. T., Gorman, M. J., and Kanost, M. R. (2009) Characterization of endogenous and recombinant forms of laccase-2, a multicopper oxidase from the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol.* **39,** 596-606

Dittmer, N. T., and Kanost, M. R. (2010) Insect multicopper oxidases: Diversity, properties, and physiological roles. *Insect Biochem. Mol. Biol.* **40,** 179-188

Dittmer, N. T., Suderman, R. J., Jiang, H., Zhu, Y., Gorman, M. J., Kramer, K. J., and Kanost, M. R. (2004) Characterization of cDNAs encoding putative laccase-like multicopper oxidases and developmental expression in the tobacco hornworm, *Manduca sexta*, and the malaria mosquito, *Anopheles gambiae*. *Insect Biochem. Mol. Biol.* **34,** 29-41

Doumas, B.T., Perry, P., Jendrzejczak, B., and Davis, L. (1987) Measurement of direct bilirubin by use of bilirubin oxidase. *Clin. Chem*. **33,** 1349-1353

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

Durand, F., Gounel, S., Kjaergaard, C. H., Solomon, E. I., and Mano, N. (2012b) Bilirubin oxidase from *Magnaporthe oryzae*: an attractive new enzyme for biotechnological applications. *Appl. Microbiol. Biotechnol.* **96,** 1489-1498

Durand, F., Kjaergaard, C. H., Suraniti, E., Gounel, S., Hadt, R. G., Solomon, E. I., and Mano, N. (2012a) Bilirubin oxidase from *Bacillus pumilus*: A promising enzyme for the elaboration of efficient cathodes in biofuel cells. *Biosensors and Bioelectronics.* **35,** 140-146

Edens, W. A., Goins, T. Q., Dooley, D., and Henson, J. M. (1999) Purification and characterization of a secreted laccase of *Gaeumannomyces graminis* var. *tritici*. *Appl. Environ. Microbiol.* **65,** 3071-3074

Esaka, M., Fujisawa, K., Goto, M., and Kisu, Y. (1992) Regulation of ascorbate oxidase expression in pumpkin by auxin and copper. *Plant Physiol.* **100,** 231-237

Ferraroni, M., Myasoedova, N., Schmatchenko, V., Leontievsky, A., Golovleva, L., Scozzafava, A., and Briganti, F. (2007) Crystal structure of a blue laccase from *Lentinus tigrinus*: evidences for intermediates in the molecular oxygen reductive splitting by multicopper oxidases. *BMC Structural Biology.* **7,** 60

Francis, C. A., and Tebo, B. M. (2001) cumA multicopper oxidase genes from diverse Mn(II)-oxidizing and non-Mn(II)-oxidizing *Pseudomonas* strains. *Appl. Environ. Microbiol.* **67,** 4272-4278

Francis, C. A., and Tebo, B. M. (2002) Enzymatic manganese(II) oxidation by metabolically dormant spores of diverse *Bacillus* species. *Appl. Environ. Microbiol.* **68,** 874-880

Futahashi, R., Banno, Y., and Fujiwara, H. (2010) Caterpillar color patterns are determined by a two-phase melanin gene prepatterning process: new evidence from tan and laccase2. *Evol. Dev.* **12,** 157-167

Garavaglia, S., Cambria, M.T., Miglio, M., Ragusa, S., Iacobazzi, V., Palmieri, F., D'Ambrosio, C., Scaloni, A., and Rizzi, M. (2004) The structure of *Rigidoporus lignosus* laccase containing a full complement of copper ions, reveals an asymmetrical arrangement for the T3 copper pair. *J. Mol. Biol.* **342,** 1519-1531

Giardina, P., Faraco, V., Pezzella, C., Piscitelli, A., Vanhulle, S., and Sannia, G. (2010) Laccases: a never-ending story. *Cell. Mol. Life Sci.* **67,** 369-385

Godden, J. W., Turley, S., Teller, D. C., Adman, E. T., Liu, M. Y., Payne, W. J., and LeGall, J. (1991) The 2.3 angstrom X-ray structure of nitrite reductase from *Achromobacter cycloclastes*. *Science.* **253,** 438-442

Gorman, M. J., Dittmer, N. T., Marshall, J. L., and Kanost, M. R. (2008) Characterization of the multicopper oxidase gene family in *Anopheles gambiae*. *Insect Biochem. Mol. Biol.* **38,** 817-824

Gorman, M. J., Sullivan, L. I., Nguyen, T. D., Dai, H., Arakane, Y., Dittmer, N. T., Syed, L. U., Li, J., Hua, D. H., and Kanost, M. R. (2012) Kinetic properties of alternatively spliced isoforms of laccase-2 from *Tribolium castaneum* and *Anopheles gambiae*. *Insect Biochem. Mol. Biol.* **42,** 193-202

Graveley, B.R., et al. (2011) The developmental transcriptome of *Drosophila melanogaster*. *Nature.* **471,** 473–479.

Haibo, Z., Yinglong, Z., Feng, H., Peiji, G., and Jiachuan, C. (2009) Purification and characterization of a thermostable laccase with unique oxidative characteristics from *Trametes hirsuta*. *Biotechnol. Lett.* **31,** 837-843

Hakulinen, N., Kiiskinen, L., Kruus, K., Saloheimo, M., Paananen, A., Koivula, A., and Rouvinen, J. (2002) Crystal structure of a laccase from *Melanocarpus albomyces* with an intact trinuclear copper site. *Nat. Struct. Mol. Biol.* **9,** 601-605

Han, O. (2011) Molecular mechanism of intestinal iron absorption. *Metallomics.* **3,** 103-109

Hellman, N. E., and Gitlin, J. D. (2002) Ceruloplasmin metabolism and function. *Annu. Rev. Nutr.* **22,** 439-458

Hofmann, K., and Stoffel, W. (1993) TMbase - A database of membrane spanning proteins segments. *Biol. Chem. Hoppe-Seyler.* **347,** 166

Ito, N., Phillips, S. E.V., Stevens, C., Ogel, Z. B., McPherson, M. J., Keen, J. N., Yadav, K. D.S., and Knowles, P. F. (1991) Novel thioether bond revealed by a 1.7 Å crystal structure of galactose oxidase. *Nature.* **350,** 87-90

Jeong, S. Y., and David, S. (2003) Glycosylphosphatidylinositol-anchored ceruloplasmin is required for iron efflux from cells in the central nervous system. *J. Biol. Chem.* **278,** 27144-27148

Johannes, C., and Majcherczyk, A. (2000) Laccase activity tests and laccase inhibitors. *J. Biotechnol.* **78,** 193-199

Kaneko, S., Cheng, M., Murai, H., Takenaka, S., Murakami, S., and Aoki, K. (2009) Purification and characterization of an extracellular laccase from *Phlebia radiata* strain BP-11-2 that decolorizes fungal melanin. *Biosci. Biotechnol. Biochem.* **73,** 939-942

Kanost, M.R. and Gorman, M.G. (2008) Phenoloxidases in insect immunity. In: *Insect Immunology* (N. Beckage, ed.) Academic Press/Elsevier, San Diego, pp. 69-96.

Kato, N., and Esaka, M. (1999) Changes in ascorbate oxidase gene expression and ascorbate levels in cell division and cell elongation in tobacco cells. *Physiologia Plantarum.* **105,** 321-329

Kirihigashi, K., Tatsumi, N., Hino, M., Yamane, T., and Ohta K. (2000) Basic and clinical evaluation of a newly-developed enzymatic bilirubin assay. *Osaka City Med. J.* **46,** 55-70

Kisu, Y., Harada, Y., Goto, M., and Esaka, M. (1997) Cloning of the pumpkin ascorbate oxidase gene and analysis of a *cis*-acting region involved in induction by auxin. *Plant Cell Physiol.* **38,** 631-637

Kjaergaard, C. H., Durand, F., Tasca, F., Qayyum, M. F., Kauffmann, B., Gounel, S., Suraniti, E., Hodgson, K. O., Hedman, B., Mano, N., and Solomon E.I. (2012) Spectroscopic and crystallographic characterization of “alternative resting” and “resting oxidized” enzyme forms of bilirubin oxidase: implications for activity and electrochemical behavior of multicopper oxidases. *J. Am. Chem. Soc.* **134,** 5548-5551

Klabunde, T., Eicken, C., Sacchettini, J. C., and Krebs, B. (1998) Crystal structure of a plant catechol oxidase containing a dicopper center. *Nat. Struct. Mol. Biol.* **5,** 1084-1090

Koch, M., Velarde, M., Harrison, M. D., Echt, S., Fischer, M., Messerschmidt, A., and Dennison, C. (2005) Crystal structures of oxidized and reduced stellacyanin from horseradish roots. *J. Am. Chem. Soc.* **127,** 158-166

Kosman, D. J. (2010) Multicopper oxidases: A workshop on copper coordination chemistry, electron transfer, and metallophysiology. *J Biol Inorg Chem.* **15,** 15-28

Kreuter, Th., Steudel, A., and Pickert, H. (1991) On the inhibition of laccase by lower fatty acids. *Acta Biotechnol.* **11,** 81-83

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

Lang, M., Braun, C. L., Kanost, M. R., and Gorman, M. J. (2012a) Multicopper oxidase-1 is a ferroxidase essential for iron homeostasis in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A.* **109,** 13337-13342

Lang, M., Kanost, M. R., and Gorman, M. J. (2012b) Multicopper oxidase-3 is a laccase associated with the peritrophic matrix of *Anopheles gambiae*. *PloS one.* **7,** e33985

Leontievsky, A., Myasoedova, N., Pozdnyakova, N., and Golovleva, L. (1997b) 'Yellow' laccase of *Panus tigrinus* oxidizes non-phenolic substrates without electron-transfer mediators. *FEBS Lett.* **413,** 446-448

Leontievsky, A. A., Vares, T., Lankinen, P., Shergill, J. K., Pozdnyakova, N. N., Myasoedova, N. M., Kalkkinen, N., Golovleva, L. A., Cammack, R., Thurston, C. F., and Hatakka A. (1997a) Blue and yellow laccases of ligninolytic fungi. *FEMS Microbiol. Lett.* **156,** 9-14

Lin, L. S., and Varner, J. E. (1991) Expression of ascorbic acid oxidase in zucchini squash (*Cucurbita pepo* L.). *Plant Physiol.* **96,** 159-165

Lindley, P. F., Card, G., Zaitseva, I., Zaitsev, V., Reinhammar, B., Selin-Lindgren, E., and Yoshida, K. (1997) An X-ray structural study of human ceruloplasmin in relation to ferroxidase activity. *J. Biol. Inorg. Chem.* **2,** 454-463

Long, F., Su, C., Zimmermann, M. T., Boyken, S. E., Rajashankar, K. R., Jernigan, R. L., and Yu, E.W. (2010) Crystal structures of the CusA efflux pump suggest methionine-mediated metal transport. *Nature.* **467,** 484-488

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

MacPherson, I.S., and Murphy, M.E.P. (2007) Type-2 copper-containing enzymes. *Cell. Mol. Life Sci.* **64,** 2887-2899

Madhavi, V., and Lele, S.S, (2009) Laccase: Properties and applications. *BioResources.* **4,** 1694-1717

Mano, N. (2012) Features and applications of bilirubin oxidases. *Appl. Microbiol. Biotechnol.* **96,** 301-307

Mate, D. M., Garcia-Ruiz, E., Camarero, S., Shubin, V. V., Falk, M., Shleev, S., Ballesteros, A. O., and Alcalde, M. (2013) Switching from blue to yellow: altering the spectral properties of a high redox potential laccase by directed evolution. *Biocatalysis Biotransformation.* **31,** 8-21

Matera, I., Gullotto, A., Tilli, S., Ferraroni, M., Scozzafava, A., and Briganti, F. (2008) Crystal structure of the blue multicopper oxidase from the white-rot fungus *Trametes trogii* complexed with *p*-toluate. *Inorg. Chim. Acta.* **361,** 4129-4137

Matoba, Y., Kumagai, T., Yamamoto, A., Yoshitsu, H., and Sugiyama, M. (2006) Crystallographic evidence that the dinuclear copper center of tyrosinase is flexible during catalysis. *J. Biol. Chem.* **281,** 8981-8990

Mayer, A. M., and Staples, R. C. (2002) Laccase: new functions for an old enzyme. *Phytochemistry.* **60,** 551-565

McCaig, B. C., Meagher, R. B., and Dean, J. F.D. (2005) Gene structure and molecular analysis of the laccase-like multicopper oxidase (LMCO) gene family in *Arabidopsis thaliana*. *Planta.* **221,** 619-636

Mei, G., Di Venere, A., Buganza, M., Vecchini, P., Rosato, N., and Finazzi-Agró, A. (1997) Role of quaternary structure in the stability of dimeric proteins: the case of ascorbate oxidase. *Biochemistry.* **36,** 10917-10922

Messerschmidt, A., Ladenstein, R., Huber, R., Bolognesi, M., Avigliano, L., Petruzzelli, R., Rossi, A., and Finazzi-Agró, A. (1992) Refined crystal structure of ascorbate oxidase at 1.9 Å resolution. *J. Mol. Biol.* **224,** 179-205

Min, K., Kim, Y., Kim, Y. W., Jung, H. S., and Hah, Y. C. (2001) Characterization of a novel laccase produced by the wood-rotting fungus *Phellinus ribis*. *Arch. Biochem. Biophys.* **392,** 279-286

Mizutani, K., Toyoda, M., Sagara, K., Takahashi, N., Sato, A., Kamitaka, Y., Tsujimura, S., Nakanishi, Y., Sugiura, T., Yamaguchi, S., Kano, K., and Mikami, B. (2010) X-ray analysis of bilirubin oxidase from *Myrothecium verrucaria* at 2.3 Å resolution using a twinned crystal. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **66,** 765-770

Mullon C.J., and Langer R. (1987) Determination of conjugated and total bilirubin in serum of neonates, with use of bilirubin oxidase. *Clin. Chem.* **33,** 1822-1825

Nakamura, K., and Go, N. (2005) Function and molecular evolution of multicopper blue proteins. *Cell. Mol. Life Sci.* **62,** 2050-2066

Nar, H., Messerschmidt, A., Huber, R., van de Kamp, M., and Canters, G. W. (1991) Crystal structure analysis of oxidized *Pseudomonas aeruginosa* azurin at pH 5.5 and pH 9.0. A pH-induced conformational transition involves a peptide bond flip. *J. Mol. Biol.* **221,** 765-772

Niu, B., Shen, W., Liu, Y., Weng, H., He, L., Mu, J., Wu, Z., Jiang, P., Tao, Y., and Meng, Z. (2008) Cloning and RNAi-mediated functional characterization of MaLac2 of the pine sawyer, *Monochamus alternatus*. *Insect Mol. Biol.* **17,** 303-312

Palmieri, G., Giardina, P., Bianco, C., Scaloni, A., Capasso, A., and Sannia, G. (1997) A novel white laccase from *Pleurotus ostreatus*. *J. Biol. Chem.* **272,** 31301-31307

Pan, C., Zhou, Y., and Mo, J. (2009) The clone of laccase gene and its potential function in cuticular penetration resistance of *Culex pipiens pallens* to fenvalerate. *Pestic. Biochem. Physiol.* **93,** 105-111

Parkinson, N. M., Conyers, C. M., Keen, J. N., MacNicoll, A. D., Smith, I., and Weaver, R. J. (2003) cDNAs encoding large venom proteins from the parasitoid wasp *Pimpla hypochondriaca* identified by random sequence analysis. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **134,** 513-520

Parsons, M.R., Convery, M.A., Wilmot, C.M., Yadav, K.S.D., Blakeley, V., Corner, A.S., Phillips, S.E.V., 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

Peariso, K., Huffman, D. L., Penner-Hahn, J. E., and O'Halloran, T. V. (2003) The PcoC copper resistance protein coordinates Cu(I) via novel S-methionine interactions. *J. Am. Chem. Soc.* **125,** 342-343

Perry, B., Doumas, B.T., Buffone, G., Glick, M., Ou, C.N., and Ryder, K. (1986) Measurement of total bilirubin by use of bilirubin oxidase. *Clin. Chem.* **32,** 329-332

Philpott, C.C., and Protchenko, O. (2008) Response to iron deprivation in *Saccharomyces cerevisiae*. *Eukaryot. Cell.* **7,** 20-27

Piontek, K., Antorini, M., and Choinowski, T. (2002) Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90 Å resolution containing a full complement of coppers. *J. Biol. Chem.* **277,** 37663-37669

Pomowski, A., Zumft, W. G., Kroneck, P. M., and Einsle, O. (2011) N_2O binding at a [4Cu:2S] copper-sulphur cluster in nitrous oxide reductase. *Nature.* **477,** 234-237

Pozdnyakova, N. N., Rodakiewicz-Nowak, J., Turkovskaya, O. V., and Haber, J. (2006b) Oxidative degradation of polyaromatic hydrocarbons and their derivatives catalyzed directly by the yellow laccase from *Pleurotus ostreatus* D1. *J. Molec. Catal. B Enzymatic* **41,** 8-15

Pozdnyakova, N.N., Turkovskaya, O.V., Yudina, E.N., and Rodakiewicz-Nowak, Y. (2006a) Yellow laccase from the fungus *Pleurotus ostreatus* D1: purification and characterization. *Appl. Biochem. Microbiol.* **42,** 56-61

Prasain, K., Nguyen, T. D. T., Gorman, M. J., Barrigan, L. M., Peng, Z., Kanost, M. R., Syed, L. U., Li, J., Zhu, K. Y., and Hua, D. H. (2012) Redox potentials, laccase oxidation, and antilarval activities of substituted phenols. *Bioorg. Med. Chem.* **20,** 1679-1689.

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

Prigge, S. T., Kolhekar, A. S., Eipper, B. A., Mains, R. E., and Amzel, L. M. (1999) Substrate-mediated electron transfer in peptidylglycine alpha-hydroxylating monooxygenase. *Nat. Struct. Biol.* **6,** 976-983

Ragusa, S., Cambria, M. T., Pierfederici, F., Scirè, A., Bertoli, E., Tanfani, F., and Cambria, A. (2002) Structure–activity relationship on fungal laccase from *Rigidoporus lignosus*: a Fourier-transform infrared spectroscopic study. *Biochim. Biophys. Acta.* **1601,** 155-162

Roberts, S. A., Weichsel, A., Grass, G., Thakali, K., Hazzard, J. T., Tollin, G., Rensing, C., and Montfort, W. R. (2002) Crystal structure and electron transfer kinetics of CueO, a multicopper oxidase required for copper homeostasis in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 2766-2771

Roberts, S. A., Wildner, G. F., Grass, G., Weichsel, A., Ambrus, A., Rensing, C., and Montfort, W. R. (2003) A labile regulatory copper ion lies near the T1 copper site in the multicopper oxidase CueO. *J. Biol. Chem.* **278,** 31958-31963

Rodgers, C. J., Blanford, C. F., Giddens, S. R., Skamnioti, P., Armstrong, F. A., and Gurr, S. J. (2010) Designer laccases: a vogue for high-potential fungal enzymes? *Trends Biotechnol.* **28,** 63-72

Rosenzweig, A. C., and Sazinsky, M. H. (2006) Structural insights into dioxygen-activating copper enzymes. *Curr. Opin. Struct. Biol.* **16,** 729-735

Rubino, J. T., and Franz, K. J. (2012) Coordination chemistry of copper proteins: How nature handles a toxic cargo for essential function. *J. Inorg. Biochem.* **107,** 129-143

Sakasegawa, S., Ishikawa, H., Imamura, S., Sakuraba, H., Goda, S., and Ohshima, T. (2006) Bilirubin oxidase activity of *Bacillus subtilis* CotA. *Appl. Environ. Microbiol.* **72,** 972-975

Sakurai, T., and Kataoka, K. (2007a) Structure and function of type I copper in multicopper oxidases. *Cell. Mol. Life Sci.* **64,** 2642-2656

Sakurai, T., and Kataoka, K. (2007b) Basic and applied features of multicopper oxidases, CueO, bilirubin oxidase, and laccase. *The Chemical Record.* **7,** 220-229

Salvato, B., and Beltramini, M. (1990) Hemocyanins: molecular architecture, structure and reactivity of the binuclear copper active site. *Life Chemistry Reports.* **8,** 1-47

Santagostini, L., Gullotti, M., De Gioia, L., Fantucci, P., Franzini, E., Marchesini, A., Monzani, E., and Casella, L. (2004) Probing the location of the substrate binding site of ascorbate oxidase

near type 1 copper: an investigation through spectroscopic, inhibition and docking studies. *Int. J. Biochem. Cell Biol.* **36,** 881-892

Schückel, J., Matura, A., and van Pée, K. (2011) One-copper laccase-related enzyme from *Marasmius* sp.: Purification, characterization and bleaching of textile dyes. *Enzyme Microb. Technol.* **48,** 278-284

Sedbrook, J. C., Carroll, K. L., Hung, K. F., Masson, P. H., and Somerville, C. R. (2002) The arabidopsis SKU5 gene encodes an extracellular glycosyl phosphatidylinositol-anchored glycoprotein involved in directional root growth. *Plant Cell.* **14,** 1635-1648

Sendovski, M., Kanteev, M., Ben-Yosef, V. S., Adir, N., and Fishman, A. (2011) First structures of an active bacterial tyrosinase reveal copper plasticity. *J. Mol. Biol.* **405,** 227-237

Sharma, P., Goel, R., and Capalash, N. (2007) Bacterial laccases. *World J. Microbiol. Biotechnol.* **23,** 823-832

Shleev, S., Tkac, J., Christenson, A., Ruzgas, T., Yaropolov, A. I., Whittaker, J. W., and Gorton, L. (2005) Direct electron transfer between copper-containing proteins and electrodes. *Biosensors and Bioelectronics.* **20,** 2517-2554

Singh, A., Severance, S., Kaur, N., Wiltsie, W., and Kosman, D.J. (2006) Assembly, activation, and trafficking of the Fet3p.Ftr1p high affinity iron permease complex in *Saccharomyces cerevisiae. J. Biol. Chem.* **281,** 13355-13364

Smirnoff, N. (2000) Ascorbic acid: metabolism and functions of a multi-facetted molecule. *Curr. Opin. Plant. Biol.* **3,** 229-235

Solomon, E. I., Sundaram, U. M., and Machonkin, T. E. (1996) Multicopper oxidases and oxygenases. *Chem. Rev.* **96,** 2563-2606

Stoj, C. S., Augustine, A. J., Zeigler, L., Solomon, E. I., and Kosman, D. J. (2006) Structural basis of the ferrous iron specificity of the yeast ferroxidase, Fet3p. *Biochemistry.* **45,** 12741-12749

Tainer, J. A., Getzoff, E. D., Beem, K. M., Richardson, J. S., and Richardson, D. C. (1982) Determination and analysis of the 2 Å structure of copper, zinc superoxide dismutase. *J. Mol. Biol.* **160,** 181-217

Tamayo-Ramos, J. A., van Berkel, W.J., and de Graaff, L. H. (2012) Biocatalytic potential of laccase-like multicopper oxidases from *Aspergillus niger*. *Microb. Cell Fact.* **11,** 165

Taylor, A. B., Stoj, C. S., Ziegler, L., Kosman, D. J., and Hart, P. J. (2005) The copper-iron connection in biology: Structure of the metallo-oxidase Fet3p. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 15459-15464

Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1995) Structures of metal sites of oxidized bovine heart cytochrome c oxidase at 2.8 Å. *Science.* **269,** 1069-1074

van Waasbergen, L. G., Hildebrand, M., and Tebo, B. M. (1996) Identification and characterization of a gene cluster involved in manganese oxidation by spores of the marine *Bacillus* sp. strain SG-1. *J. Bacteriol.* **178,** 3517-3530

Whittaker, J. W. (2003) Free radical catalysis by galactose oxidase. *Chem. Rev.* **103,** 2347-2364

Wilce, M. C.J., 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

Wood, D.A. (1980) Production, purification and properties of extracellular laccase of *Agaricus bisporus*. *J. Gen. Microbiol.* **117,** 327-338

Xu, F. (1996) Oxidation of phenols, anilines, and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition. *Biochemistry.* **35,** 7608-7614

Yatsu, J., and Asano, T. (2009) Cuticle laccase of the silkworm, *Bombyx mori*: Purification, gene identification and presence of its inactive precursor in the cuticle. *Insect Biochem. Mol. Biol.* **39,** 254-262

Yeh, K. Y., Yeh, M., Mims, L., and Glass, J. (2009) Iron feeding induces ferroportin 1 and hephaestin migration and interaction in rat duodenal epithelium. *Am. J. Physiol. Gastrointest. Liver Physiol.* **296,** G55-65

Yoshida, H. (1883) Chemistry of lacquer (Urushi). Part I. Communication from the Chemical Society of Tokio. *J. Chem. Soc. Transact.* **43,** 472–486

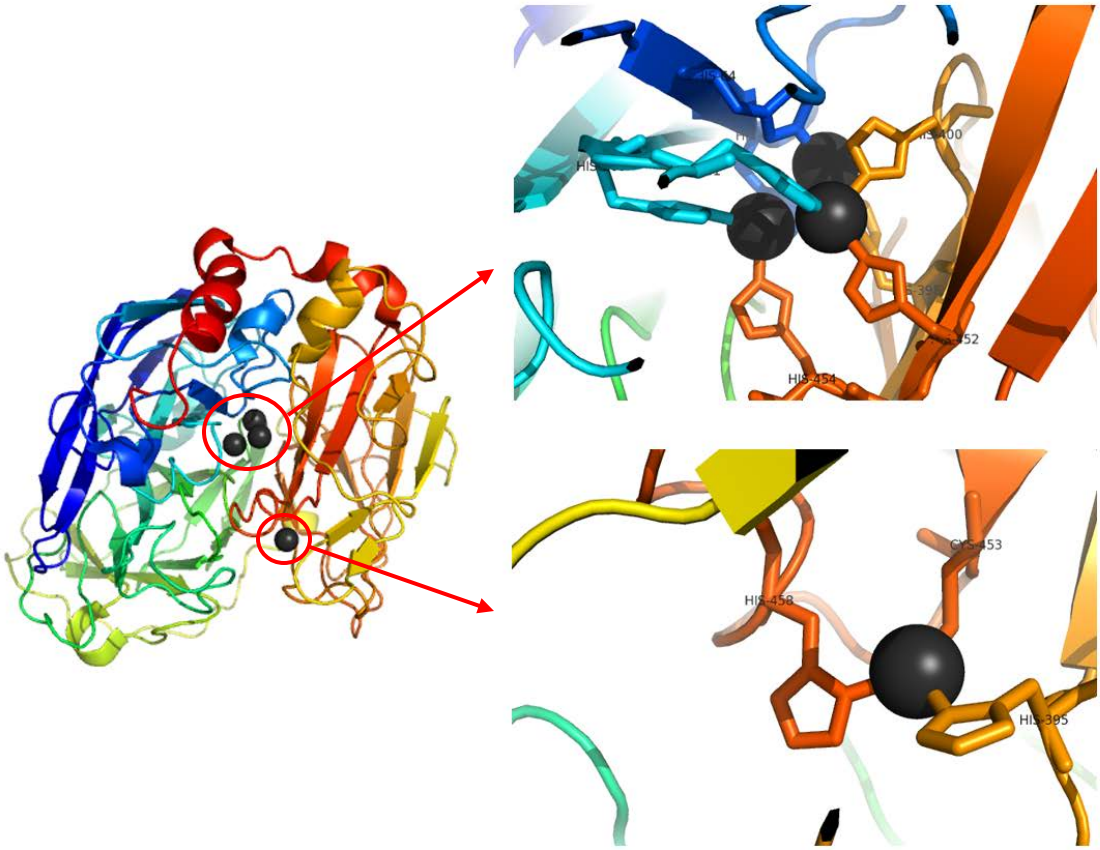

Figure 1.1 Crystal structure of a laccase from *Trametes versicolor.*

The fungal laccase contains four copper atoms, which are shown as black balls. The four copper atoms form two copper centers. The T1 center consists of one T1 copper atom coordinated by two histidines and one cysteine, which is shown in the lower right image. The T2/T3 trinuclear center consists of one T2 copper atom and a pair of T3 copper atoms, and the T2/T3 copper atoms are coordinated by eight histidines. The T2/T3 trinuclear center is shown in the upper right image. The ten histidines and one cysteine are conserved in all typical MCOs. PDB number of the fungal laccase is 1KYA. The images were produced by PyMOL software.

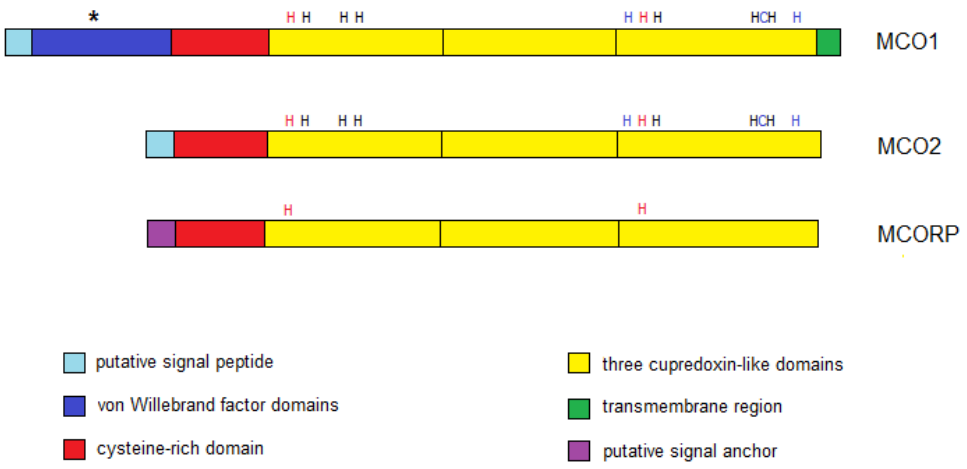

Figure 1.2 The Domain Architectures of Insect MCOs and MCORP.

MCOs found in most insect genomes include MCO1 orthologs, MCO2 orthologs, and MCORP orthologs. A mosquito specific group (MCO3, MCO4, and MCO5 orthologs) has domain architecture similar to MCO2. All insect MCOs have three cupredoxin-like domains. The dipteran MCO1 orthologs contain additional amino-terminal von Willebrand factor domains. MCO1 orthologs contain a carboxyl-terminal transmembrane region. A cysteine-rich domain is present after the amino-terminus in all insect MCOs but is not present in MCOs from other organisms. Except for MCO4 that contains a predicted amino-terminal signal anchor, the other MCOs have a putative secretion signal peptide. All insect MCOs have ten conserved histidines and one conserved cysteine that coordinate four copper atoms. MCORP, described in Chapter 2, contains a predicted amino-terminal signal anchor, a cysteine-rich domain and three cupredoxin-like domains. Only the two histidines that coordinate T2 copper atom remain in all identified MCORP orthologs. Note: stars indicate domains only found in dipteran MCO1 orthologs; residues in blue coordinate T1 copper atom; residues in red coordinate T2 copper atom; residues in black coordinate T3 copper atoms.

Table 1.1 Characteristics and Examples of Different Copper Types in Proteins

Type of Cu	Cu #	Example	Ligands for Cu	Function
Type 1 (T1)	1	Plastocyanin	2His, 1Cys, 1Met	Intermolecular electron transfer
	1	Umecyanin	2His, 1Cys, 1Glu	
	1	Azurin	2His, 1Cys, 1Met, 1Gly	
Type 2 (T2)	1	Amine oxidase	3His, $2H_2O$	Oxidation of primary amines (Reduction of O_2 to H_2O_2)
	1	CuZnSOD	4His, $1H_2O$	Oxidation of O_2^- to O_2 Reduction of O_2^- to H_2O_2
	1	Galactose oxidase	2His, 2Tyr, $1H_2O$	Oxidation of primary alcohols to aldehydes (Reduction of O_2 to H_2O_2)
	1+1	PHM	CuA – 3His CuB – 2His, 1Met, $1H_2O$	Hydroxylation of the α-carbon atom of a C-terminal glysine
Type 3 (T3)	2	Tyrosinase	6His	Hydroxylation of monophenols to *o*-diphenols Oxidation of *o*-diphenols to *o*-quinones (Reduction of O_2 to H_2O)
	2	Catechol oxidase	6His	Oxidation of *o*-diphenols to *o*-quinones (Reduction of O_2 to H_2O)
	2	Hemocyanin	6His	O_2 transport
T1+T2	1+1	Nitrite Reductase	T1 – 2His, 1Cys, 1Met T2 – 3His, $1H_2O$	Reduction of NO_2^- to NO
T1+T2/T3	1+1+2	Three-domain multicopper oxidase	T1 – 2His, 1Cys, (1Met)* T2 – 2His, $1H_2O$ T3 – 6His	Oxidation of small molecules and metals (Reduction of O_2 to H_2O)
	3+1+2	Ceruloplasmin	T1 (x2)** – 2His, 1Cys, 1Met T1 (x1)** – 2His, 1Cys T2 – 2His, $1H_2O$ T3 – 6His	Oxidation of Fe^{2+} to Fe^{3+} (Reduction of O_2 to H_2O)
Cu_A+Cu_B	2+1	Cytochrome *c* oxidase	Cu_A – 2His, 2Cys, 1Met, 1Glu Cu_B – 3His	Reduction of O_2 to H_2O
Cu_A+Cu_Z	2+4	Nitrous-oxide reductase	Cu_A – 2His, 2Cys, 1Met, 1Trp Cu_Z – 7His, 2S	Reduction of N_2O to N_2

Abbreviations: CuZnSOD, CuZn superoxide dismutase; PHM, peptidylglycine α-hydroxylating monooxygenase

Note: * The residues normally coordinating the T1 copper are two histidines and one cysteine. In some MCOs, methionine serves as the fourth residue. ** Ceruloplasmin contains six copper atoms. Two T1 copper atoms each is coordinated by 2His, 1Cys, 1Met. Another T1 copper atom is coordinated by 2His, 1Cys. The reference for each enzyme is cited in the text.

Table 1.2 Identified Atypical MCOs in Literature

Abbreviations: AAS, atomic absorption spectrometry; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); ICP-MS, inductively coupled plasma mass spectrometry; DMP, 2,6-dimethoxyphenol; CM, colorimetric method; DMPD, *N*,*N*-dimethyl-*p*-phenylenediamine; ADBP, 4-amino-2,6-dibromophenol; DMA, 2,5-dimethylaniline; DAB, diaminobenzidine; PAH, polyaromatic hydrocarbon; ICP-OES, inductively coupled plasma-optical emission spectrometry

Note: N/A, not determined or analyzed; *method that did not determine other metal content; **the number of copper atoms has not been determined; ***other conserved residues for coordinating T1 and T3 copper atoms are missing; ^only 1 conserved histidine remains

Types	Species	Metals	Method	600 nm Peak	Substrates	Cu Ligands	Reference
White laccases	*P. ostreatus*	1Cu; 1Fe; 2Zn	AAS	No (unknown conc.)	ABTS; Syringaldazine	N/A	Palmieri et al., 1997
	P. ribis	1Cu; 1Mn; 2Zn	ICP-MS	No (300 ug/mL)	ABTS; DMP; Syringaldazine	N/A	Min et al., 2001
	P. radiata	1Cu; 1Zn; 2Fe	ICP-MS	No (0.18 mg/mL)	ABTS; DMP; Guaiacol; Hydroquinone; Syringaldazine	N/A	Kaneko et al., 2009
	T. hirsuta	3Cu 1Mn	AAS	No (unknown conc.)	ABTS; DMP; Methyl Red	N/A	Haibo et al., 2009
	Marasmius sp.	1Cu	AAS	Weak (0.86 mg/mL)	ABTS; Guaiacol; Syringaldazine	N/A	Schückel et al., 2011
Yellow laccases	*A. bisporus*	2Cu	CM*	N/A (yellow at >10 mg/mL)	DMPD; Guaiacol; *p*-Phenylenediamine	N/A	Wood, 1980
	S. commune	2Cu	AAS*	N/A (yellow at 0.5 mg/mL)	ADBP; 4-Chloro-1-naphthol; DAB; Guaiacol; *p*-Phenylenediamine; Syringaldazine; Tetramethylbenzidine	N/A	De Vries et al., 1986
	P. tigrinus; P. radiata; A. bisporus	N/A	N/A	No (1.3 mg/mL)	ABTS	N/A	Leontievsky et al., 1997a
	P. tigrinus	4Cu	AAS	No (1 mg/mL)	Non-phenolic substrates	N/A	Leontievsky et al., 1997b
	G. graminis var. *tritici*	?Cu**	ICP-MS	No (unknown conc.)	Catechol; DMP; Guaiacol; Pyrogallol; Melanin precursor; Lignin-like polymer	N/A	Edens et al., 1999
	V. volvacea	N/A	N/A	No (unknown conc.)	ABTS; Syringaldazine	Ten His + one Cys	Chen et al., 2004
	P. ostreatus	?Cu**	CM	No (925 ug/mL)	ABTS; DMP; Catechol; Syringaldazine;	N/A	Pozdnyakova et al., 2006a
	P. ostreatus	N/A	N/A	N/A	PAHs; PAHs' derivatives; *p*-Phenylenediamine; *N*-Acetyl-*p*-phenylenediamine	N/A	Pozdnyakova et al., 2006b
	A. niger	N/A	N/A	Weak (~1 mg/mL; yellow at 10-15 mg/mL)	ABTS; ADBP; DMP; DMPD; Hydroquinone; Phenol; Ferulic acid; Syringic acid; Vanillic acid	N/A	Tamayo-Ramos et al., 2012
	S. cerevisiae	4Cu	ICP-OES	No (1.3 mg/mL)	ABTS; DMP; Guaiacol; Sinapic acid	T1 – two His + one Cys	Mate et al., 2013
	S. aeruginosa	N/A	N/A	No (unknown conc.)	ABTS; *o*-Dianisidine; Syringaldazine	Ten His + one Cys	Daroch et al., 2014
AO homolog	*A. thaliana*	N/A	N/A	N/A	N/A	T2 – two His***	Sedbrook et al., 2002
	B. napus	N/A	N/A	N/A	N/A	1 His^	Albani et al., 1992

Chapter 2 - A Multicopper Oxidase-related Protein Is Essential in Insect Molting, Life Span and Ovary Development

Introduction

The multicopper oxidase (MCO) family of enzymes includes laccases, ascorbate oxidases, bilirubin oxidases, a subgroup of metal oxidases (ferroxidases, cuprous oxidases, and manganese oxidases), as well as some oxidases involved in biosyntheses (Sakurai and Kataoka, 2007a). Laccases are the largest subgroup of MCOs, and can oxidize a broad range of aromatic substrates (Giardina et al., 2010). The other oxidases tend to oxidize specific substrates. MCOs have been found in bacteria, fungi, plants, insects, and vertebrates (Sakurai and Kataoka, 2007a; Nakamura and Go, 2005).

Enzymes in the MCO family typically contain four copper atoms: one type 1 (T1) copper, one type 2 (T2) copper, and two coupled type 3 (T3) coppers (Sakurai and Kataoka, 2007a). The types of copper sites in proteins are classified based on their spectroscopic and magnetic properties. The T1 copper has a strong absorption at ~600 nm, which is responsible for the deep blue color of the protein (Giardina et al., 2010; Shleev et al., 2005). The T2 copper is almost undetectable in the visible region (Giardina et al., 2010). The T3 copper site contains two coupled coppers, and shows an absorption maximum at ~330 nm (Shleev et al., 2005). In electron paramagnetic resonance (EPR) spectra, the T1 copper and T2 copper give distinctive signals (Sakurai and Kataoka, 2007a; Sakurai and Kataoka, 2007b). However, the T3 site shows no EPR signal due to strong antiferromagnetic interaction of the two coupled coppers (Sakurai and Kataoka, 2007a; Sakurai and Kataoka, 2007b). In MCOs, the four coppers form two copper centers. The T1 center consists of one T1 copper, and the trinuclear center consists of one T2 copper and the pair of T3 coppers (Kosman, 2010). Most MCOs contain three cupredoxin-like domains (Sakurai and Kataoka, 2007a; Sakurai and Kataoka, 2007b). The T1 center is located in domain 3, and the trinuclear center is located at the interface of domain 1 and 3 (Sakurai and Kataoka, 2007b).

All typical MCOs have ten conserved histidines (His) and one conserved cysteine (Cys) that coordinate the four copper atoms (Sakurai and Kataoka, 2007a). The residues normally coordinating the T1 copper are two histidines and one cysteine (Sakurai and Kataoka, 2007b). Methionine often serves as the fourth residue that coordinates the T1 copper, but it is more

distant and the interaction is weaker (Nakamura and Go, 2005; Sakurai and Kataoka, 2007b). Alternatively, this position can be occupied by different amino acids, such as leucine or phenylalanine without coordinating the T1 copper (Sakurai and Kataoka, 2007b). The T2 copper is coordinated by two histidines, and each of the T3 coppers is coordinated by three histidines (Sakurai and Kataoka, 2007a). The four copper ions buried within MCOs are essential for oxidase activity. One electron of the substrate is transferred to the T1 center near the substrate binding pocket, and then the electron is passed via intramolecular residues, a conserved His-Cys-His triad, to the pair of T3 copper ions (Kosman, 2010). Molecular oxygen binds between the two T3 copper ions and is reduced to water after the transfer of four electrons (Kosman, 2010).

In insects, putative MCOs have been identified based on sequence information from insect genomes. Insect MCOs include MCO1 orthologs, MCO2 orthologs, and a mosquito specific group (MCO3, MCO4, and MCO5 orthologs) (Gorman et al., 2008; Dittmer and Kanost, 2010). So far, it has been proven that insect MCO2 orthologs are laccases (Dittmer et al., 2009; Gorman et al., 2012) that play a role in cuticle tanning (Dittmer et al., 2004; Arakane et al., 2005). *Drosophila melanogaster* MCO1 has ferroxidase activity, and is involved in iron homeostasis *in vivo* (Lang et al., 2012a). *Anopheles gambiae* MCO3 is a laccase and is located in the peritrophic matrix, but its physiological function is still unclear (Lang et al., 2012b). Except for the laccases that catalyze cuticle tanning, the enzymatic properties and physiological functions of the other insect MCOs are still poorly characterized.

During our studies of insect MCOs, we noticed that the *Tribolium castaneum* (red flour beetle) and *A. gambiae* (African malaria mosquito) genomes each contained an unusual MCO-like gene, which we named multicopper oxidase-related protein (MCORP). The MCORP sequences share sequence identity with MCOs, but lack the conserved cysteine and many of the conserved histidines that coordinate copper ions. MCORP orthologs are novel proteins that have not been studied previously; therefore, their functions are unknown. The goal of this study was to investigate the biochemical features and possible physiological functions of MCORP. To accomplish these goals, we performed phylogenetic and sequence analyses of putative insect MCORPs, purified recombinant *T. castaneum* MCORP (TcMCORP) and evaluated copper content and oxidase activity, determined expression profiles of TcMCORP and *A. gambiae* MCORP (AgMCORP), and analyzed loss-of-function phenotypes.

Experimental Procedures

cDNA Cloning

To obtain a cDNA clone of TcMCORP, primers were designed based on a predicted sequence (NCBI accession number: XP_967121). To exclude the predicted signal anchor, the corresponding amino acid sequence began with P31. A cDNA pool from *T. castaneum* pupae was used as the template. To obtain cDNA clones of AgMCORP, Rapid Amplification of cDNA Ends (RACE) method was used. Primers for 5' and 3' ends were designed based on an EST consensus sequence (GenBank # BX611297), and 5' and 3' regions were cloned with a RACE kit (GeneRacer, Invitrogen). A cDNA pool from *Anopheles gambiae* pupae was used as the template. Using primers corresponding to 5' and 3' untranslated sequences, a cDNA containing the full coding region of AgMCORP was amplified. All primers are listed in Table 2.1. Nucleotide sequences of TcMCORP and AgMCORP were confirmed by DNA sequencing (NCBI accession number: TcMCORP, KJ500311; AgMCORP, KJ500312).

Sequence and Phylogenetic Analysis

Insect MCORP sequences were identified by performing BLAST searches of sequenced insect genomes using the AgMCORP sequence as the query. To identify any putative MCORP orthologs in vertebrates, a BLAST search against the non-redundant protein database for vertebrata was performed. To identify any putative MCORP orthologs in non-insect invertebrates, a BLAST search against the non-redundant protein database excluding sequences from vertebrates, plants, fungi and insects was performed. Phylogenetic analysis was performed using MEGA5 software (Tamura et al., 2011). The amino-terminal and carboxyl-terminal ends of the sequences, which are highly variable, were not included in the alignment. Sequences beginning with the cysteine-rich region (Dittmer and Kanost, 2010) were aligned by ClustalW in MEGA5 and mannually adjusted (Appendix A). Gaps were omitted from the phylogenetic analysis. The phylogenetic tree was constructed by the neighbor-joining method with a Poisson model. Statistical analysis was performed by the bootstrap method with 1000 repetitions. The sequences and their NCBI accession numbers are listed in Table 2.2.

To identify the presence or absence of putative copper-coordinating residues in MCORP sequences, the sequences of Fet3p (NCBI accession number: NP_013774) and each MCORP were aligned by ClustalW2 (EMBL-EBI), and the conserved residues coordinating four coppers

in Fet3p were compared with the corresponding residues in MCORPs. Signal anchors were predicted by SignalP 2.0 Server (Nielsen and Krogh, 1998). Subcellular localization was predicted with PSORT II (Nakai and Horton, 1999).

Production of Polyclonal Antiserum

The cDNA of TcMCORP, beginning with the codon for P31, and the cDNA for AgMCORP, beginning with the codon for V26, were cloned into an *E. coli* expression vector, pET-28a (Novagen). The truncated fusion protein was expressed in MAX Efficiency DH5α Competent Cells (Invitrogen), and purified by nickel affinity chromatography under denaturing conditions. The purified protein was confirmed to be TcMCORP by peptide mass fingerprinting analysis. Purified protein was subjected to SDS-PAGE. The gel was stained with E-Zinc Reversible Stain Kit (Thermo Scientific), and the protein band was excised and sent to Open Biosystems for the production of polyclonal antiserum in a rabbit. Antisera were used at a 1:2000 dilution for immunoblot analysis of TcMCORP and 1:1000 dilution for immunoblot analysis of AgMCORP.

Expression and Analysis of Recombinant AgMCORP

A truncated form of AgMCORP was expressed using a *Drosophila* S2 cell expression system (Invitrogen). The cDNA (NCBI accession number: KJ500312) beginning with the codon for A39 (without a predicted N-terminal signal anchor and several hydrophobic residues) was cloned into pMT/Bip/V5-His A (Invitrogen), which encodes a *D. melanogaster* Bip signal peptide. Primers are listed in Table 2.1. *D. melanogaster* S2 cells (4×10^6 cells/mL) were transfected with recombinant DNA together with pCoBlast using calcium phosphate, and the cells were selected in Schneider's *Drosophila* medium + 10% fetal bovine serum containing 25 μg/mL of blasticidin for two weeks. After selection, the stable cells were removed by centrifugation (500 × *g* for 15 min). To test whether AgMCORP aggregated in the medium, 10 mL medium was concentrated to 0.5 mL with the use of Amicon Ultracel 30 K centrifugal filter. Then 10 mL buffer (20 mM MES + 150 mM NaCl, pH 6.5) was added, and the sample was concentrated to ~ 1 mL. The sample was analyzed by chromatography using a Superdex 200 10/300 GL column (GE Healthcare Life Sciences). After the gel filtration, immunoblot analysis was used to detect the fractions containing AgMCORP.

Full length AgMCORP was first expressed using the *Drosophila* S2 cell expression system (Invitrogen). The full length AgMCORP cDNA was cloned into pMT/V5-His A vector (Invitrogen). Primers are listed in Table 2.1. After selection of stable cell lines, the protein expression was extremely low. The full length AgMCORP was also expressed using the Bac-to-Bac baculovirus expression system (Invitrogen). The full-length AgMCORP cDNA in pMT/V5-His A was cloned into pFastBac1 with the use of restriction sites SpeI and Xbal. Antartctic phosphatase (BioLabs) was used to avoid self-ligation of the plasmid after cutting by restriction enzymes. After the DNA sequence was confirmed to be correct, a recombinant baculovirus was generated. Plaque assays were used to determine titers of amplified virus stocks. For expression, Sf9 cells (2×10^6 cells/mL in SF900 II serum free medium supplemented with 0.1 mM copper sulfate) were infected with baculovirus at a multiplicity of infection of 2, and cells were incubated at 27°C with shaking at 140 rpm for 24 h. After incubation, cells were pelleted by centrifugation (250 × *g* for 5 min).

Membrane bound proteins were extracted using a DUALXtract total membrane protein extraction kit (Dualsystems Biotech), with minor modifications. Briefly, cells were washed with ice-cold Cell Wash Solution and permeabilized by ice-cold Cell Permeabilization Buffer (brief vortex and continuous rocking for 10 min at 4 °C). Cells were pelleted by centrifugation (16000 × *g* for 15 min) at 4°C. The supernatant was saved as a cytoplasmic protein fraction. Ice-cold Membrane Protein Extraction Buffer (containing CHAPS) was added to cell pellets. The cells and buffer were mixed by pipetting. The mixture was incubated for 30 min at 4°C with vigorous shaking, and then was centrifuged at 16000 × *g* for 15 min. Both the supernatant (membrane protein fraction) and the final cell debris were saved.

In addition, we used another method to extract membrane proteins without the use of Cell Permeabilization Buffer during the process, and, in addition to CHAPS, we tried to use another mild detergent octyl-β-glucoside. Briefly, cell pellets were resuspended in buffer (20 mM MES + 150 mM NaCl, pH 6.5). The sample was sonicated using 30 sec bursts, 4 times. Then, ultracentrifugation (100000 × *g* for 60 min) was performed to pellet membranes, membrane proteins and cell debris. The supernatant was saved as a cytoplasmic protein fraction. The membrane proteins were extracted using CHAPS or 100 mM octyl-β-glucoside. The cells and buffer were mixed by pipetting. The cells were incubated with CHAPS for 1 h or octyl-β-

glucoside for 30 min at 4°C with vigorous shaking, and then were centrifuged at 16000 × *g* for 15 min. Both the supernatant (membrane protein fraction) and the cell debris were saved.

Expression and Purification of Recombinant TcMCORP

TcMCORP was expressed using a baculovirus expression system (flashBAC, Oxford Expression Technologies). A cDNA containing the coding region of TcMCORP without a predicted N-terminal signal anchor was first cloned into pMT/BiP/V5-His A vector (Invitrogen), which encodes a *D. melanogaster* Bip signal peptide. The signal peptide-TcMCORP fusion cDNA was then cloned into the pOET3 baculovirus transfer vector. Primers are listed in Table 2.1. Sf9 cells were transfected, and recombinant baculovirus was generated. Plaque assays were performed to determine titers of amplified virus stocks. For expression, 2.8 L of Sf9 cells (2×10^6 cells/mL) were infected with baculovirus at a multiplicity of infection of 1, and cells were incubated at 27 °C with shaking at 140 rpm for 24 h. SF900 II serum free medium was used and supplemented with 0.1 mM copper sulfate. After incubation, cells were removed by centrifugation (500 × *g* for 15 min).

For purification, MCORP and other glycosylated proteins in the cell culture medium were bound to concanavalin-A-Sepharose and eluted with 0.5 M methyl-α-D-mannopyranoside in 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4 (4 °C). Then, eluted proteins were dialyzed against 50 mM sodium phosphate, pH 6.5 (4 °C) and loaded on a HiPrep SP FF 16/10 column (GE Healthcare Life Sciences). Proteins were eluted with a linear gradient of NaCl (0-1 M) in 50 mM sodium phosphate, pH 6.5. Fractions containing TcMCORP were pooled and concentrated to 5 mL with the use of Amicon Ultracel 30 K centrifugal filter. The final purification step was accomplished by a Superdex 200 HiLoad 16/60 column (GE Healthcare Life Sciences) using 50 mM sodium phosphate, 150 mM NaCl, pH 6.5. At each purification step, immunoblot analysis was used to detect the fractions containing TcMCORP. The concentration of purified recombinant TcMCORP was estimated as described previously (Lang et al., 2012b). The yield was ~1 mg per liter of cell culture. The purified protein was subjected to SDS-PAGE and analyzed by immunoblot. Purified TcMCORP was stored at 4 °C.

Spectral Properties and Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

For spectral properties, purified recombinant TcMCORP was concentrated to 4 μg/μL with the use of Amicon Ultracel 30 K centrifugal filter. The concentration was estimated as

described previously (Lang et al., 2012b). AgMCO3 (Lang et al., 2012b) (4 μg/μL) was used as a positive control. Absorbance spectra of TcMCORP and AgMCO3 were recorded on a Beckman DU-640 Spectrophotometer. Absorbance was read from 250 to 700 nm at 1 nm intervals.

For ICP-MS analysis, TcMCORP in 50 mM sodium phosphate, 150 mM NaCl, pH 6.5, was concentrated to 8 μg/μL (0.1333 mM). TcMCORP and the storage buffer were diluted 12-fold immediately prior to manual analysis through a peristaltic pump at 0.4 mL/min serving a Babbington nebulizer and cooled (2 °C) spray chamber serving a standard quartz torch and 1400W argon plasma. Data were recorded in 5 second replicates and manually processed for the small volume received. They were calibrated to NIST-traceable standards which are validated with NIST standard reference material solutions. The metal content of TcMCORP was calculated as the metal content of the TcMCORP sample minus the metal content of the storage buffer.

Activity Assays

The laccase substrates used were 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), syringaldazine, catechol, L-dopa, dopamine, *N*-acetyldopamine (NADA), *N*-β-alanyldopamine (NBAD), hydroquinone, methyl hydroquinone, *o*-phenylenediamine, 2-aminophenol, guaiacol, and *N,N*-dimethyl-*p*-phenylenediamine. Reactions to determine enzyme activity were made by mixing 5μg purified recombinant TcMCORP with 1 mM substrate (except for syringaldazine, which was used at 10 μM because of its low solubility in the assay buffer) in a total volume of 200 μL and observing the change in absorbance for 30 min to detect product formation using a microplate spectrophotometer. All assays were done in duplicate in citrate-phosphate buffer at pH 5, 6, or 7. Reactions with no TcMCORP were done to measure autooxidation of substrates and served as “blank” reactions. Wavelengths used for detecting the products of interest were: 414 nm for ABTS, 530 nm for syringaldazine, 450 nm for catechol, 475 nm for L-dopa and dopamine, 390 nm for NADA and NBAD, 250 nm for hydroquinone and methyl hydroquinone, 440 nm for *o*-phenylenediamine, 434 nm for 2-aminophenol, 436 nm for guaiacol, and 515 nm for *N,N*-dimethyl-*p*-phenylenediamine.

RNA Isolation, cDNA Synthesis and RT-PCR

For a developmental expression profile of AgMCORP, *A. gambiae* at different developmental stages (eggs from an overnight collection, older eggs that were between one and two days old, pharate larvae, 1^{st} – 4^{th} instar larvae, pupae, pharate adults, and adult females) were

collected for RNA isolation. For a tissue expression profile of AgMCORP, tissues from *A. gambiae* adult females and males were dissected as described previously (Gorman et al., 2008). For a tissue expression profile of TcMCORP, ovaries, guts and Malpighian tubules were removed from *T. castaneum* females (adult Day 6), and the remainder of the abdomen ("carcass") was saved. RNA isolation and cDNA synthesis were done as described previously (Lang et al., 2012a). Pools of cDNA from different developmental stages (eggs, younger larvae, older larvae, pharate pupae, pupae, and adults) of *T. castaneum* were kindly supplied by Dr. Yasuyuki Arakane. 1μL of cDNA was used for each RT-PCR. All primers are listed in Table 2.1.

Insect Culture

For mosquito culture, the G3 strain of *A. gambiae* was obtained from the Malaria Research and Reference Reagent Resource Center. The mosquitoes were reared as described previously (Gorman et al., 2008). For *T. castaneum* culture, the GA-1 strain was reared in whole wheat flour containing 5% brewer's yeast at 30 °C under standard conditions (Beeman and Stuart, 1990).

RNAi to Determine Loss-of-function Phenotypes

TcMCORP, *T. castaneum* vermillion (TcVer), AgMCORP, and GFP dsRNAs were synthesized by using the MEGAscript RNAi kit (Ambion). TcVer (Arakane et al., 2009) and GFP served as negative controls. Primers are listed in Table 2.1. dsRNAs were injected by using a microinjection system (Nanoliter 2000 and Micro4 controller) and a stereomicroscope.

T. castaneum pharate pupae were injected with TcMCORP dsRNA or TcVer dsRNA (400 ng per insect). For longevity assays, live insects were counted starting from adult day 0 (that is, the number of adults on day 0 was considered 100% survivorship). The insects were counted approximately every 3 days for one month. For assays relevant to reproduction, females and males were separated at the pupal stage. For tissue observation, on day 10 post adult eclosion, ovaries, male reproductive tissues, guts, and Malpighian tubules were dissected from the insects and observed. For determination of egg numbers, on day 4 post adult eclosion, single pair-based crosses were performed: dsTcMCORP ♀ × wild type (WT) ♂, dsTcVer ♀ × WT ♂, dsTcMCORP ♂ × WT ♀, and dsTcVer ♂ × WT ♀. After 7 days of rearing, the egg number from each pair was determined. Pairwise differences in egg numbers were assessed by performing a nonparametric analysis (Mann-Whitney test). In order to test the efficiency of TcMCORP

knockdown, total RNA was prepared from pupae (2 days post injection) with dsTcMCORP or dsTcVer treatment. RNA isolation and cDNA synthesis were done as described previously (Lang et al., 2012a). RT-PCR with gene specific primers was performed. Late larvae (larvae that have reached their maximum size – plump and long) were injected with dsRNA (400 ng per insect) to assess the effects of knockdown at an earlier stage.

A. gambiae day 0 females were injected with dsRNA for AgMCORP or GFP (2 μg per insect). Injected females and WT males were mated for 7 days, and then given access to a blood meal. Females that had taken a blood meal were selected from the pool of females on day 8. On day 9, ovaries were dissected, observed, and used for testing the efficiency of knock down. Total RNA was isolated and cDNA was synthesized as described previously (Lang et al., 2012a). There were three biological replicates from dsAgMCORP and dsGFP treatments. Real-time PCR reactions were performed. Expression of AgMCORP data were normalized to RPS3 (ribosomal protein S3). Primers are listed in Table 2.1.

Results

A Gene Encoding an Unusual Multicopper Oxidase-related Protein Was Found in T. castaneum *and* A. gambiae *Genomes*

During our studies of *T. castaneum* and *A. gambiae* MCOs, we discovered that these two insect species have a gene encoding a multicopper oxidase-related protein. We used RT-PCR to clone cDNAs of these two MCORPs and analyzed their predicted amino acid sequences. The MCORPs have sequence identity with MCO sequences, but many of the residues that coordinate copper ions in MCOs are absent (Fig. 2.1). The MCORP sequences have a hydrophobic amino-terminus that resembles a signal peptide but is predicted to remain uncleaved (Fig. 2.1). This type of amino-terminus, referred to as a signal anchor, directs a protein to the secretory pathway, but tethers the protein to the membrane so that it is not secreted into the extracellular space (Sakaguchi et al., 1992). Subcellular localization prediction software predicts that both MCORPs are localized to the endoplasmic reticulum. The sequences also contain a cysteine-rich region that is present in all known insect MCOs (Dittmer and Kanost, 2010), and three putative cupredoxin-like domains (Fig. 2.1).

MCORP Orthologs Belong to a Distinct Clade in Relation to MCOs in a Phylogenetic Analysis

To determine whether MCORP is a conserved insect gene, we searched the genomes of many insect species representing six orders, including Diptera, Coleoptera, Lepidoptera, Hymenoptera, Hemiptera, and Anoplura. We identified putative MCORP orthologs in almost all of the genomes. MCORP orthologs were identified in three dipteran species (all mosquitoes), but not in other dipterans, including *Drosophila melanogaster*, other *Drosophila* species, *Musca domestica* (house fly), *Lutzomyia longipalpus* (sand fly), *Mayetiola destructor* (Hessian fly), or *Simulium damnosum* (black fly). We did not find MCORP orthologs in other invertebrates or vertebrates. Phylogenetic analysis of all identified MCORPs and some representative insect MCOs clearly show that MCORPs belong to a specific cluster with a bootstrap value of 100 (Fig. 2.2). Thus, MCORP is present in a diverse set of insect species, although it appears to have been lost in some dipteran lineages. Our phylogenetic analysis suggests that MCORPs may have a conserved function that differs from those of insect MCOs.

MCORPs Lack Many of the Conserved Copper-coordinating Residues in MCOs

Almost all MCOs have ten conserved histidines and one conserved cysteine that form two copper centers. Each MCORP ortholog was aligned with a well studied yeast ferroxidase, Fet3p, whose structure is solved (Taylor et al., 2005). The conserved copper-coordinating residues in Fet3p and the corresponding residues in MCORPs were analyzed (Fig. 2.3). It is obvious that all MCORPs lack many of the conserved residues that coordinate copper atoms. The two histidines that typically coordinate the T2 copper are present in the MCORP sequences, but most of the copper-coordinating residues for the other coppers are missing. In MCOs, an electron is transferred from the T1 copper to T3 coppers through the highly conserved His-Cys-His tripeptide (Giardina et al., 2010). Specifically for Fet3p, H485, C484, and H483 are involved in the electron transfer (Kosman, 2010). However, those residues are missing in MCORPs. Based on the alignment analysis, we conclude that copper binding and any electron transfer in MCORPs must be different from those of MCOs.

Recombinant AgMCORP Is Membrane-bound

In order to determine copper content and test oxidase activity of MCORP, we first tried to purify recombinant AgMCORP using an insect cell expression system. To simplify protein

purification, we replaced the putative signal anchor with a *D. melanogaster* Bip signal peptide so that the recombinant protein would be secreted. However, secreted recombinant AgMCORP formed aggregates and could not be purified. Then, we tried to purify full length AgMCORP. Full length AgMCORP was not secreted but remained associated with the cultured cells (Fig. 2.4A). We used mild detergents, CHAPS and octyl-β-gluciside, to extract membrane bound proteins. AgMCORP was not in the cytoplasmic protein fraction; it was not successfully extracted from the membrane, but was present in the cell debris that contains insoluble materials, unbroken cells and lipid rafts (Fig. 2.4A and B). Although we failed to purify full length AgMCORP, this result suggests that AgMCORP is membrane-bound, which is consistent with the presence of an amino-terminal signal anchor.

Recombinant TcMCORP Was Purified from Cultured Insect Cells

Unlike AgMCORP, secreted recombinant TcMCORP did not aggregate, and purification was successful. During expression, the serum-free medium was supplemented with 0.1 mM copper sulfate in order to supply copper atoms for copper loading of the protein. The recombinant TcMCORP was purified with the use of concanavalin-A affinity chromatography, SP cation exchange chromatography, and gel filtration chromatography. We purified about 1 mg TcMCORP per liter of cell culture. SDS-PAGE followed by Coomassie staining showed that the recombinant TcMCORP was approximately the expected mass (60 kDa) and was very pure (Fig. 2.5A). The identity of TcMCORP was verified by immunoblot analysis using antiserum against TcMCORP (Fig. 2.5B).

Recombinant TcMCORP Contains Little Copper

MCOs have a blue color due to the presence of a T1 copper (Giardina et al., 2010; Shleev et al., 2005). We noticed that highly concentrated (8 μg/μL = 0.1333 mM) recombinant TcMCORP was not blue, which is consistent with our prediction that MCORP does not contain a T1 copper. In addition, based on its absorbance spectrum (Fig. 2.5C), TcMCORP does not have the typical spectroscopic properties that MCOs possess. The characteristic absorption maximum at ~600 nm and the shoulder at ~330 nm were absent in the recombinant TcMCORP, but were present in recombinant AgMCO3 that served as a positive control (Fig. 2.5C). Because MCORPs contain the two conserved histidines that coordinate a T2 copper in typical MCOs, we predicted that each molecule of TcMCORP may bind to one copper atom; however, metal content analysis

using inductively coupled plasma mass spectrometry (ICP-MS) demonstrated that recombinant TcMCORP contains little copper (Table 2.3). The ratio of copper to MCORP was one to seventeen; therefore, if each MCORP can bind to one copper, this result would indicate that ~6% of the MCORP molecules contain one copper atom. Based on ICP-MS analysis, we do not know whether the copper is bound to the two histidines that are supposed to coordinate the T2 copper atom or other places, such as histidines on the surface of the protein.

Recombinant TcMCORP Does Not Have Laccase Activity

The apparent lack of T1 and T3 coppers would suggest that TcMCORP is not an active oxidase; however, some atypical laccases that have fewer than four copper atoms have been identified (Palmieri et al., 1997; Min et al., 2001; Kaneko et al., 2009; Haibo et al., 2009; Schückel et al., 2011); therefore, we decided to test whether TcMCORP has laccase activity. We used a broad range of laccase substrates, including several diphenols (catechol, hydroquinone, methyl hydroquinone, L-dopa, dopamine, *N*-acetyldopamine, and *N*-β-alanyldopamine), a diamine (*o*-phenylenediamine), a substituted monophenol (guaiacol), 2-aminophenol, and three substrates with complicated structures (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), syringaldazine, *N*,*N*-dimethyl-*p*-phenylenediamine). In each reaction, 5 μg recombinant TcMCORP was mixed with 1 mM substrate (except for syringaldazine, which was used at 10 μM). The amount of TcMCORP was ten times more than the amount we usually use to analyze insect laccase activity, and 1 mM substrate was used because insect laccases tend to have K_ms in the range of 0.1 - 10 mM (Dittmer et al., 2009; Gorman et al., 2012; Lang et al., 2012b). Activity was tested at pH 5, 6 and 7.The result was that no laccase activity of the recombinant TcMCORP was detected.

MCORP Is Expressed at Each Developmental Stage and in Many Different Tissues

To begin to learn about the biological function of MCORP, we first analyzed expression patterns of TcMCORP and AgMCORP. TcMCORP mRNA was detected in all developmental stages, including eggs, younger larvae, older larvae, pharate pupae, pupae, and adults (Fig. 2.6A). Likewise, AgMCORP was detected in all developmental stages, including eggs, pharate larvae, $1^{st} – 4^{th}$ instar larvae, pupae, pharate adults and adults (Fig. 2.6B). The expression levels varied among the stages, with significant expression during metamorphosis (pharate pupal, pupal, and pharate adult stages). The high number of PCR cycles (>30) required to generate detectable PCR

products suggest that MCORP is expressed at a relatively low level. In order to investigate the tissues in which MCORP is expressed, RNA was isolated from various adult tissues followed by RT-PCR. TcMCORP mRNA was detected in all of the tissues analyzed, with significant expression in ovaries (Fig. 2.7A). Similarly, AgMCORP mRNA was detected in midguts, Malpighian tubules, ovaries, male reproductive tissues, abdominal carcass, and thorax (Fig. 2.7B). In addition, AgMCORP transcripts have been detected by microarray studies in midguts, fat bodies, ovaries, heads, and hemocytes (Marinotti et al., 2006; Pinto et al., 2009). Taken together, the expression data indicate that TcMCORP and AgMCORP are constitutively expressed at a low level in many different tissues.

Knockdown of TcMCORP Results in Mortality

To detect MCORP loss-of-function phenotypes, we used RNAi-mediated knockdown. *T. castaneum* pharate pupae were injected with dsRNA that targets TcMCORP or TcVer. The insects injected with dsTcVer served as negative controls. Transcripts for TcMCORP were suppressed efficiently in the pupal stage, two days after dsRNA injections (Fig. 2.8A). Approximately 20% of insects died during the pupal-adult molting process after dsTcMCORP treatment, but only ~2% of insects died after dsTcVer treatment. Among the dead insects, some died very late in the pupal stage (just before eclosion), and some died at the time of adult eclosion, with incomplete shedding of the pupal cuticle. Successful eclosion occurred in the rest of the treated insects. During the adult stage, dsTcMCORP injected insects had a much shorter life span than control insects (Fig. 2.8B). Compared to the controls, mortality increased gradually in the first two weeks of adult life, and then increased drastically, resulting in almost 0% survivorship one month after eclosion. The external morphology of dead adults in the dsTcMCORP treatment group was similar to that of live adults in the dsTcVer treatment group. The midguts dissected from live dsTcMCORP injected adults contained food, which indicates that the insects could eat when they were alive and suggests that the dsTcMCORP insects did not starve. To observe the effect of knockdown at an earlier stage, late larvae were injected with dsTcMCORP. Insects died at either the pharate pupal stage (could not molt to pupae) or pharate adult stage (could not molt to adults), and none of them survived to the adult stage. These results indicate that TcMCORP is an essential gene.

Knockdown of TcMCORP in Females Reduces the Number of Eggs Laid

We observed that dsTcMCORP treated adults had very few progeny; therefore, we were interested in whether knockdown of MCORP had an effect on egg production. A single pair-based cross design was used to evaluate egg numbers from knockdown females mated to control males and from knockdown males mated to control females. The number of eggs produced from dsTcMCORP ♀ × wild type (WT) ♂ crosses was much less than that from dsTcVer ♀ × WT ♂ crosses (Fig. 2.9A). Eleven out of thirteen pairs of dsTcMCORP ♀ × WT ♂ produced 0-2 eggs, whereas only two pairs produced a significant number of eggs. The two outlier pairs might be the result of poor knockdown efficiency in those two pairs or the result of phenotypic variability. The number of eggs produced from dsTcMCORP ♂ × WT ♀ crosses was similar to that from dsTcVer ♂ × WT ♀ crosses (Fig. 2.9B). Thus, RNAi of TcMCORP in females caused a significant reduction in egg production, but knockdown in males had no observable effect on reproduction.

Knockdown of TcMCORP Severely Affects Oocyte Maturation

A decrease in egg production by females treated with dsTcMCORP suggested that MCORP may be required for ovary development. To test this hypothesis, we assessed the effect of TcMCORP knockdown on ovary morphology. *T. castaneum* pharate pupae were injected with dsRNA, and females and males were separated at the pupal stage so that no mating would occur. Ten days post adult eclosion, the insects were dissected, and ovaries, male reproductive tissues, guts, Malpighian tubules and fat bodies were observed. Knockdown of TcMCORP expression seriously impaired the maturation of the primary oocytes. As expected, TcVer RNAi did not block the maturation of the primary oocytes and the oocytes developed normally (Fig. 2.10A). TcMCORP knockdown had no visible effect on the reproductive tissues in males (Fig. 2.10B). These results are consistent with our observation that knockdown of TcMCORP in females greatly reduced egg production and that knockdown in males had no effect. In addition, there was no obvious difference in other tissues, such as guts, Malpighian tubules and fat bodies, between dsTcMCORP and dsTcVer treatments (data not shown). Therefore, the mortality observed in dsTcMCORP treated adults was not associated with any obvious morphological changes in the internal tissues.

We wondered whether knockdown of MCORP would also affect ovary development in *A. gambiae*. Injection of larvae or pupae leads to high mortality in *A. gambiae*; therefore, we injected day 0 females with dsRNA that targets AgMCORP or GFP (negative control). Injected females and WT males were mated and then given a blood meal, which triggers oocyte development in anopheline mosquitoes (Clements and Boocock, 1984). Mosquitoes subjected to AgMCORP RNAi had fully developed ovaries, similar to those subjected to GFP RNAi. One interpretation of this result is that MCORP is not required for ovary development in *A. gambiae*; however, it seems likely that the negative result is due to one of two technical difficulties: incomplete knockdown (65% based on qPCR), despite injecting the maximum amount of dsRNA per insect (2 µg), or dsRNA injection in the adult stage, after the ovaries were already partly developed. More sophisticated RNAi methods, which are currently unavailable, will be necessary to determine whether MCORP is required for oocyte development in mosquitoes.

Discussion

This study describes a novel protein, MCORP, which shares sequence identity with MCOs, but lacks most of the highly conserved residues necessary to coordinate the T1 and T3 coppers. Without the T1 and T3 coppers, we predicted that MCORP would not have oxidase activity; however, there have been several studies of atypical fungal laccases, which contain fewer than four copper atoms but still exhibit laccase activity (Palmieri et al., 1997; Min et al., 2001; Kaneko et al., 2009; Haibo et al., 2009; Schückel et al., 2011). These laccases were termed "white" laccases because they lack the blue color of most MCOs. Those studies focused on the biotechnological applications of the unusual laccases rather than their biochemical features; therefore, the amino acid sequences, including the presence or absence of conserved copper-coordinating residues, were not reported. We found that, unlike the atypical laccases, recombinant TcMCORP had no detectable laccase activity. MCORP may oxidize a natural substrate that we did not test, but we think this is unlikely because lacccases oxidize a broad range of substrates, and even MCOs with preferences for non-phenolic substrates (such as iron or ascorbate) can oxidize laccase substrates (Lang et al., 2012a; Dayan and Dawson, 1976; Quintanar et al., 2007).

Based on ICP-MS analysis, it is still unclear whether MCORP is a copper-binding protein. MCORP orthologs from different insect species have two conserved histidines that aligned with

the two histidines that coordinate the T2 copper atom in typical MCOs, so we hypothesized that MCORP may bind to one copper atom. However, ICP-MS analysis showed that TcMCORP contains very little copper. This could be due to unknown technical problems during recombinant protein expression, although we have used the same insect expression system to express some active insect MCOs (Dittmer et al., 2009; Gorman et al., 2012; Lang et al., 2012a; Lang et al., 2012b). The low copper content of recombinant TcMCORP might suggest that TcMCORP is a copper-binding protein that does not require copper as a cofactor. Perhaps MCORPs have a function related to copper homeostasis, such as copper sensing or copper transport; thus, we would expect that only some TcMCORP molecules contain copper. Very small amounts of zinc and iron were also detected in the TcMCORP sample, but it seems unlikely that the presence of these metals in such low content is biologically relevant.

MCORPs have not been described previously, but an analogous type of protein has been identified in plants. These proteins share sequence identity with ascorbate oxidases, but, like the insect MCORPs, lack copper-coordinating residues (Nakamura and Go, 2005). The best-studied of these ascorbate oxidase homologs, SKU5 from *Arabidopsis thaliana*, shares 23% sequence identity with ascorbate oxidases. The residues required for coordinating the T1 and T3 copper ions are absent in SKU5, but the two histidines required for coordinating the T2 copper are present. SKU is expressed in all tissues, most strongly in expanding tissues, and it is involved in directional root growth (Sedbrook et al., 2002). Bp 10 from *Brassica napus* shares 30% sequence identity with ascorbate oxidases, lacks most of the conserved copper-coordinating ligands, and is expressed in developing pollen (Albani et al., 1992). Whether SKU5 and Bp10 bind to copper and whether they have oxidase activity are unknown. These ascorbate oxidase homologs are evolutionarily related to ascorbate oxidases in plants (Nakamura and Go, 2005), but not to MCORPs.

We found that MCORP is expressed at each developmental stage and in many different tissues. *T. castaneum* larval RNAi led to 100% mortality prior to adult eclosion. These deaths were associated with larval-pupal molting and pupal-adult molting. Pharate pupal RNAi resulted in about 20% mortality during pupal-adult molting, and 100% mortality by one month after eclosion. We have been unable to determine the cause of the deaths, but they do not seem to be caused by starvation or gross morphological abnormalities. Given the expression pattern and

knockdown phenotypes of TcMCORP, it seems likely that MCORP is required in all life stages, and MCORP may play a role in a basic cellular process, especially at the time of molting.

In our study, knockdown of TcMCORP in females severely affected oocyte maturation and reduced the number of eggs laid. Female reproduction of insects includes vitellogenesis and oogenesis. During vitellogenesis, yolk proteins, such as vitelloginin (Vg), are synthesized in the fat body, secreted into the hemolymph and accumulate inside the oocyte. In addition to vitellogenesis, successful reproduction requires ovarian growth and oocyte maturation. Insect reproduction is regulated by complex molecular networks. For example, insect Vg and lipophorin (Lp) receptors play an important role in oocyte development by mediating endocytosis of Vg and Lp (Tufail and Takeda, 2009); hormones such as juvenile hormone (JH) and 20-hydroxyecdysone (20E) regulate insect reproduction (Parthasarathy and Palli, 2011). In *T. castaneum*, female reproduction is regulated by JH, 20E and nutritional signaling (Parthasarathy and Palli, 2011). JH regulates Vg synthesis in the fat body (Parthasarathy et al., 2010b), 20E regulates oocyte maturation (Parthasarathy et al., 2010a), and nutritional signals play key roles in both Vg synthesis and oocyte maturation (Parthasarathy and Palli, 2011). In addition to the studied hormones and nutritional signals, other genes might also regulate Vg synthesis and/or oocyte maturation (Parthasarathy et al., 2010a; Parthasarathy et al., 2010b). Currently we do not know the molecular mechanisms of TcMCORP involvement in insect reproduction, but our study adds new knowledge to this topic.

We discovered putative MCORP orthologs in insects, but not in other invertebrates or vertebrates. Because MCORP is required for insect viability and reproduction, and MCORP orthologs are only found in insects, MCORP might be a good target for insect control. It may become possible to develop a chemical method to target MCORP if the biochemical features of MCORP are known. In addition, an RNAi approach could be used to control plant pests even if the exact function of MCORP is unknown. Several studies have shown that dsRNAs can be successfully fed to insects through transgenic plants, which offers a new method for agricultural pest control (Baum et al., 2007; Mao et al., 2007; Zha et al., 2011; Zhu et al., 2012).

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References

Albani, D., Sardana, R., Robert, L. S., Altosaar, I., Arnison, P. G., and Fabijanski, S. F. (1992) A *Brassica napus* gene family which shows sequence similarity to ascorbate oxidase is expressed in developing pollen. Molecular characterization and analysis of promoter activity in transgenic tobacco plants. *The Plant Journal.* **2,** 331-342

Arakane, Y., Lomakin, J., Beeman, R. W., Muthukrishnan, S., Gehrke, S. H., Kanost, M. R., and Kramer, K. J. (2009) Molecular and functional analyses of amino acid decarboxylases involved in cuticle tanning in *Tribolium castaneum*. *J. Biol. Chem.* **284,** 16584-16594

Arakane, Y., Muthukrishnan, S., Beeman, R. W., Kanost, M. R., and Kramer, K. J. (2005) *Laccase 2* is the phenoloxidase gene required for beetle cuticle tanning. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 11337-11342

Baum, J.A., Bogaert, T., Clinton, W., Heck, G.R., Feldmann, P., Ilagan, O., Johnson, S., Plaetinck, G., Munyikwa, T., Pleau, M., Vaughn, T., and Roberts, J. (2007) Control of coleopteran insect pests through RNA interference. *Nat. Biotechnol.* **25,** 1322-1326

Beeman, R. W., and Stuart, J. J. (1990) A gene for lindane + cyclodiene resistance in the red flour beetle (coleoptera: Tenebrionidae). *J. Econ. Entomol.* **83,** 1745-1751

Clements, A., and Boocock, M. (1984) Ovarian development in mosquitoes: Stages of growth and arrest, and follicular resorption. *Physiol. Entomol.* **9,** 1-8

Dayan, J., and Dawson, C. R. (1976) Substrate specificity of ascorbate oxidase. *Biochem. Biophys. Res. Commun.* **73,** 451-458

Dittmer, N. T., Gorman, M. J., and Kanost, M. R. (2009) Characterization of endogenous and recombinant forms of laccase-2, a multicopper oxidase from the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol.* **39,** 596-606

Dittmer, N. T., and Kanost, M. R. (2010) Insect multicopper oxidases: Diversity, properties, and physiological roles. *Insect Biochem. Mol. Biol.* **40,** 179-188

Dittmer, N. T., Suderman, R. J., Jiang, H., Zhu, Y. C., Gorman, M. J., Kramer, K. J., and Kanost, M. R. (2004) Characterization of cDNAs encoding putative laccase-like multicopper oxidases and developmental expression in the tobacco hornworm, *Manduca sexta*, and the malaria mosquito, *Anopheles gambiae*. *Insect Biochem. Mol. Biol.* **34,** 29-41

Giardina, P., Faraco, V., Pezzella, C., Piscitelli, A., Vanhulle, S., and Sannia, G. (2010) Laccases: A never-ending story. *Cell Mol. Life Sci.* **67,** 369-385

Gorman, M. J., Dittmer, N. T., Marshall, J. L., and Kanost, M. R. (2008) Characterization of the multicopper oxidase gene family in *Anopheles gambiae*. *Insect Biochem. Mol. Biol.* **38,** 817-824

Gorman, M. J., Sullivan, L. I., Nguyen, T. D., Dai, H., Arakane, Y., Dittmer, N. T., Syed, L. U., Li, J., Hua, D. H., and Kanost, M. R. (2012) Kinetic properties of alternatively spliced isoforms of laccase-2 from *Tribolium castaneum* and *Anopheles gambiae*. *Insect Biochem. Mol. Biol.* **42,** 193-202

Haibo, Z., Yinglong, Z., Feng, H., Peiji, G., and Jiachuan, C. (2009) Purification and characterization of a thermostable laccase with unique oxidative characteristics from *Trametes hirsuta*. *Biotechnol. Lett.* **31,** 837-843

Kaneko, S., Cheng, M., Murai, H., Takenaka, S., Murakami, S., and Aoki, K. (2009) Purification and characterization of an extracellular laccase from *Phlebia radiata* strain BP-11-2 that decolorizes fungal melanin. *Biosci. Biotechnol. Biochem.* **73,** 939-942

Kosman, D. J. (2010) Multicopper oxidases: A workshop on copper coordination chemistry, electron transfer, and metallophysiology. *J. Biol. Inorg. Chem.* **15,** 15-28

Lang, M., Braun, C. L., Kanost, M. R., and Gorman, M. J. (2012a) Multicopper oxidase-1 is a ferroxidase essential for iron homeostasis in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A.* **109,** 13337-13342

Lang, M., Kanost, M. R., and Gorman, M. J. (2012b) Multicopper oxidase-3 is a laccase associated with the peritrophic matrix of *Anopheles gambiae*. *PLoS One.* **7,** e33985

Mao, Y.B., Cai, W.J., Wang, J.W., Hong, G.J., Tao, X.Y., Wang, L.J., Huang, Y.P., and Chen, X.Y. (2007) Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. *Nat. Biotechnol.* **25,** 1307-1313

Marinotti, O., Calvo, E., Nguyen, Q. K., Dissanayake, S., Ribeiro, J., and James, A. A. (2006) Genome-wide analysis of gene expression in adult *Anopheles gambiae*. *Insect Mol. Biol.* **15,** 1-12

Min, K., Kim, Y., Kim, Y. W., Jung, H. S., and Hah, Y. C. (2001) Characterization of a novel laccase produced by the wood-rotting fungus *Phellinus ribis*. *Arch. Biochem. Biophys.* **392,** 279-286

Nakai, K., and Horton, P. (1999) PSORT: A program for detecting sorting signals in proteins and predicting their subcellular localization. *Trends Biochem. Sci.* **24,** 34-36

Nakamura, K., and Go, N. (2005) Function and molecular evolution of multicopper blue proteins. *Cell Mol. Life Sci.* **62,** 2050-2066

Nielsen, H., and Krogh, A. (1998) Prediction of signal peptides and signal anchors by a hidden markov model. *Proc. Int. Conf. Intell. Syst. Mol. Biol.* **6,** 122-130

Palmieri, G., Giardina, P., Bianco, C., Scaloni, A., Capasso, A., and Sannia, G. (1997) A novel white laccase from *Pleurotus ostreatus*. *J. Biol. Chem.* **272,** 31301-31307

Parthasarathy, R., and Palli, S. R. (2011) Molecular analysis of nutritional and hormonal regulation of female reproduction in the red flour beetle, *Tribolium castaneum*. *Insect Biochem. Mol. Biol.* **41,** 294-305

Parthasarathy, R., Sheng, Z., Sun, Z., and Palli, S. R. (2010a) Ecdysteroid regulation of ovarian growth and oocyte maturation in the red flour beetle, *Tribolium castaneum*. *Insect Biochem. Mol. Biol.* **40,** 429-439

Parthasarathy, R., Sun, Z., Bai, H., and Palli, S. R. (2010b) Juvenile hormone regulation of vitellogenin synthesis in the red flour beetle, *Tribolium castaneum*. *Insect Biochem. Mol. Biol.* **40,** 405-414

Pinto, S. B., Lombardo, F., Koutsos, A. C., Waterhouse, R. M., McKay, K., An, C., Ramakrishnan, C., Kafatos, F. C., and Michel, K. (2009) Discovery of *Plasmodium* modulators by genome-wide analysis of circulating hemocytes in *Anopheles gambiae*. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 21270-21275

Quintanar, L., Stoj, C., Taylor, A. B., Hart, P. J., Kosman, D. J., and Solomon, E. I. (2007) Shall we dance? How a multicopper oxidase chooses its electron transfer partner. *Acc. Chem. Res.* **40,** 445-452

Sakaguchi, M., Tomiyoshi, R., Kuroiwa, T., Mihara, K., and Omura, T. (1992) Functions of signal and signal-anchor sequences are determined by the balance between the hydrophobic segment and the N-terminal charge. *Proc. Natl. Acad. Sci. U. S. A.* **89,** 16-19

Sakurai, T., and Kataoka, K. (2007a) Basic and applied features of multicopper oxidases, CueO, bilirubin oxidase, and laccase. *Chem. Rec.* **7,** 220-229

Sakurai, T., and Kataoka, K. (2007b) Structure and function of type I copper in multicopper oxidases. *Cell Mol. Life Sci.* **64,** 2642-2656

Schückel, J., Matura, A., and Van Pee, K. (2011) One-copper laccase-related enzyme from *Marasmius sp.*: Purification, characterization and bleaching of textile dyes. *Enzyme Microb. Technol.* **48,** 278-284

Sedbrook, J. C., Carroll, K. L., Hung, K. F., Masson, P. H., and Somerville, C. R. (2002) The Arabidopsis *SKU5* gene encodes an extracellular glycosyl phosphatidylinositol-anchored glycoprotein involved in directional root growth. *Plant Cell.* **14,** 1635-1648

Shleev, S., Tkac, J., Christenson, A., Ruzgas, T., Yaropolov, A. I., Whittaker, J. W., and Gorton, L. (2005) Direct electron transfer between copper-containing proteins and electrodes. *Biosens. Bioelectron.* **20,** 2517-2554

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28,** 2731-2739

Taylor, A. B., Stoj, C. S., Ziegler, L., Kosman, D. J., and Hart, P. J. (2005) The copper-iron connection in biology: Structure of the metallo-oxidase Fet3p. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 15459-15464

Tufail, M., and Takeda, M. (2009) Insect vitellogenin/lipophorin receptors: Molecular structures, role in oogenesis, and regulatory mechanisms. *J. Insect Physiol.* **55,** 88-104

Zha, W., Peng, X., Chen, R., Du, B., Zhu, L., and He, G. (2011) Knockdown of midgut genes by dsRNA-transgenic plant-mediated RNA interference in the hemipteran insect *Nilaparvata lugens*. *PLoS One.* **6,** e20504

Zhu, J.Q., Liu, S., Ma, Y., Zhang, J.Q., Qi, H.S., Wei, Z.J., Yao, Q., Zhang, W.Q., Li, S. (2012) Improvement of pest resistance in transgenic tobacco plants expressing dsRNA of an insect-associated gene EcR. *PLoS One.* **7,** e38572


TcMCORP ----------MSKTQNV 7
AgMCORP ----------MKTLQRIVLI 10
TcLac2A MDGTQRYLLIATAALFLFFDLCHGVRAPGAKKKVGPIDQSAAAASWHDFDNSDFFQSEHA 60
MLRVTIVMG----------MMVAVVAVLYLTPLP-EGTFLSCDRPCHDLDWPMI 50
VQRVLITVG----------VFLAAAVLIHFFAIDPTERAVSCDRVCNRADWPRI 54
TcLac2A VIQTHPSIGGGPRFSSGGGRKAWKHLDFRNSAAAELLKNPSLSSPDECARACREGEPPRI 120
CRIKIQIENK------RPCKDCSFNNDTSS----------EIIAVNGQSPGPAIQICQ 92
AgMCORP CRYELVVEKRTF----RPGSTPSTDNVTLTGGSKSTGQPITVSYYTVNGRYVGPTLTVCE 110
TcLac2A CYYHFTLELYTVLGAACQVCTPNATNTVWSHCQCVLADGVERGILTANRMIPGPSIQVCE 180
TcMCORP NDILVVDVVNKMPGHSLTIHWRGQANVEAPFMDGVPLVTQCPIFSYTTFQYKFRVTSPGT 152
AgMCORP NDFLVVDVENRIPGESITLHWTGQSQRRTPFMDGVPMITQCPIASFTRFQYKFQADRAGT 170
TcLac2A GDKVVIDVENHIEGNEVTLHWHGVWQRGSQYYDGVPFVTQCPIQQGNTFRYQWIAGNAGT 240
TcMCORP HLYQAFSDSELNRGLFGALIVRQAEKNDLQRKYYDVDSRNHIIMISERDSK--------- 203
AgMCORP HLYHGFAGSERTQGLLGAFVVRSAYEQ-RQSPVLSALHNDPVWLVTELNGM--------- 220
TcLac2A HFWHAHTGLQKMDGLYGSVVIRQPPAKDPNSHLYDYDLTTHVMLLSDWMHEDATERFPGR 300
TcMCORP ----------VLVNGKG----------SSEVTKFTVKRNKRYRFRVAFAGSNSGCPVT 241
AgMCORP ----------LGVNGQ----------RDWTQTLNGTLNLRARLIYAVAHCQHW 253
TcLac2A LAVNTGQDPESLLINGKGQFRDPNTGFMTNTPLEVFTITPGRRYRFRMINSFAS-VCPAQ 359
TcMCORP LTVDNHLIKVIALDGNLVFP---TEVTSVVLTKGERLDFVLK--TDQKIKRHYVR----- 291
AgMCORP LELEDHRLQVLALDGNVLDHNSTPSVSRILLHDGVRMDVALTRLAGDKIKRDYEIRFTPH 313
TcLac2A LTIQGHDLTLIATDGEPVHP---VRVNTIISFSGERYDFVIN--ADQTPGAYWIQLRGLG 414
TcMCORP -VKSCLGEGLALLNYELG----------DTTPTEKPQKREKEGRVLDTSLCHSQIGKV 338
AgMCORP EATSSCGRAVFRLHYDADRAAANDAESIKYNLITPPPPSPHRTEELRLDLTGTACSETML 373
TcLac2A ECGIRRVQQLGILRYAKG------PYQPSQAPPTYDYGIPQGVVLNPLDARCNEIRPDAI 468
TcMCORP CLSDLN-FVEKMPETVKTAPKT-VYLSLGSQIVNVSGNFGS----------RVFGV 382
AgMCORP CPQDLPGGRDQLPKDLQRGYDTRLVFTIGSRQQAGTGPFGEPTH----------ETVRSV 423
TcLac2A CVSQLKNALSIDKGILREKPDVKIFLPFRFHIYTPEDLFAPNTYNRHLVAPNGDHVISLI 528
TcMCORP NNLTFTYPSSPLLTQ-LDRVAMNSVCDG--RNVPEKCVG-KEVCECVHVEHIPLRAVAEI 438
AgMCORP NGITFAFPPVLMVREPLANGALGAHCCGGQRPLPRSCQPLTARCECVHVEHIEAGHRVEM 483
TcLac2A DEISYMAPPAPLISQ-YDDIDPQQFCNG--DNRPADCQQ---NCMCTHKVDIPLNAIVEI 582
TcMCORP VLINQDASQEE-HIFHLHGYRFYVVGFRHFEN---APSTDEIKLLDQENTLFQRNFNNPA 494
AgMCORP VLINADAAVD--YVYHLHGQSVFVVALATAG----RPGTALGALSGQAG----QRISAPL 533
TcLac2A VLVDEVQQPNLSHPFHLHGYAFNVIGIGRSPDQNVKKINLKHALDLDRQGLLHRQFNLPP 642
TcMCORP IKDTIRIPKNSVVALRFLADNPGFWMLRDEGSRGWTRGLDIVLQVGEPSDMVSTPTDFPT 554
AgMCORP QRDTIVVRRGSTVAVRFVANLAGLWLLRDIGSPGWSRGLDVVLSVGTPQ--PDIPRNFPA 591
TcLac2A AKDTIAVPNNGYVVLRLRANNPGFWLFHCHFLFHIVIGMNLVLQVGTHADLPPVPPNFPT 702
TcMCORP CGNYIGPDFFLM--- 566
AgMCORP CRHFAGPRYFLI--- 603
TcLac2A CGDHVPEINSNPNLV 717

Figure 2.1 Alignment of Amino Acid Sequences of TcMCORP, AgMCORP, and TcLac2A. The ten histidines and one cysteine that are expected to coordinate copper ions in TcLac2A (an MCO) are highlighted in black. Predicted signal anchors are in italicized text. A conserved cysteine-rich region is highlighted in gray. The three cupredoxin-like domains are indicated by dashed underlining (I), bold underlining (II), and wave underlining (III). NCBI accession numbers: TcMCORP, XP_967121; AgMCORP, KJ500312; TcLac2A, NP_001034487.

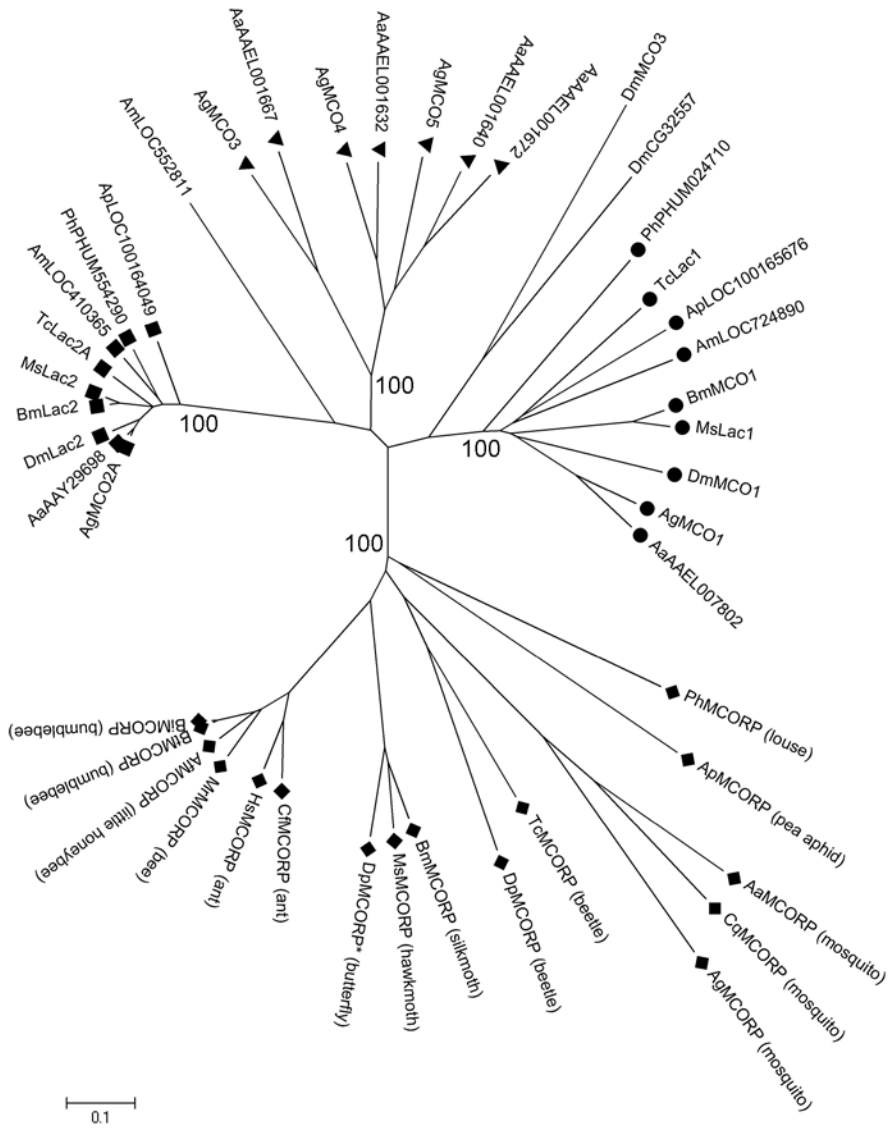

Figure 2.2 Phylogenetic Tree of Insect MCOs and MCORPs.

The tree was constructed by the neighbor-joining method. Numbers shown are bootstrap values expressed as a percentage. Bootstrap values are only shown for the MCORP branch (diamond), MCO1 branch (circle), MCO2 branch (square), and the mosquito-specific branch (triangle). All identified MCORPs were included in the analysis, and MCOs from insect species that represent different insect orders were included, Abbreviations used are: Ag, *Anopheles gambiae*; Aa, *Aedes aegypti*; Cq, *Culex quinquefasciatus*; Tc, *Tribolium castaneum*; Dp, *Dendroctonus ponderosae*; Ms, *Manduca sexta*; Bm, *Bombyx mori*; Dp*, *Danaus plexippus*; Cf, *Camponotus floridanus*; Hs, *Harpegnathos saltator*; Bt, *Bombus terrestris*; Bi, *Bombus impatiens*; Mr, *Megachile rotundata*; Af, *Apis florae*; Ap, *Acyrthosiphon pisum*; Ph, *Pediculus humanus corporis*; Dm, *Drosophila melanogaster*; Am, *Apis mellifera*. Accession numbers are listed in Table 2.2.

		T1			T2		T3α			T3β		
	Fet3p	H413	C484	H489	H81	H416	H83	H126	H485	H128	H418	H483
Diptera	AgMCORP	Y	D	G	H	H	T	H	I	F	H	R
	AaMCORP	Y	D	H	H	H	T	Y	L	F	H	R
	CqMCORP	H	D	H	H	H	T	H	L	F	H	R
Coleoptera	TcMCORP	H	D	G	H	H	R	Q	E	F	H	R
	DpMCORP	H	D	G	H	H	R	H	E	Y	H	R
Lepidoptera	MsMCORP	H	D	H	H	H	R	H	E	H	H	R
	BmMCORP	H	D	H	H	H	R	H	E	H	H	R
	DpMCORP*	H	D	H	H	H	R	H	E	H	H	R
Hymenoptera	CfMCORP	L	D	D	H	H	R	H	E	H	H	R
	HsMCORP	L	D	D	H	H	R	H	E	H	H	R
	BtMCORP	L	D	E	H	H	R	H	E	H	H	R
	BiMCORP	L	D	E	H	H	R	H	E	H	H	R
	MrMCORP	L	D	Y	H	H	R	H	E	H	H	R
	AfMCORP	L	D	E	H	H	R	H	E	H	H	R
Hemiptera	ApMCORP	H	S	E	H	Y	R	Q	E	L	H	R
Anoplura	PhMCORP	H	E	H	H	H	R	Q	E	N	H	D

Figure 2.3 Comparison of Copper-coordinating Residues in Fet3p with the Corresponding Residues in MCORPs.

Yeast ferroxidase, Fet3p, contains 10 histidines and 1 cysteine that coordinate to four coppers. T1 copper is coordinated with H413, H489 and C484, T2 copper is coordinated with H81 and H416, T3α copper is coordinated with H128, H418 and H483, and T3β copper is coordinated with H83, H126 and H485. Fet3p was aligned with each MCORP, and the corresponding residues in MCORPs are shown. Histidines conserved in MCORPs are highlighted in gray. Abbreviations used are the same as for Fig. 2.2.

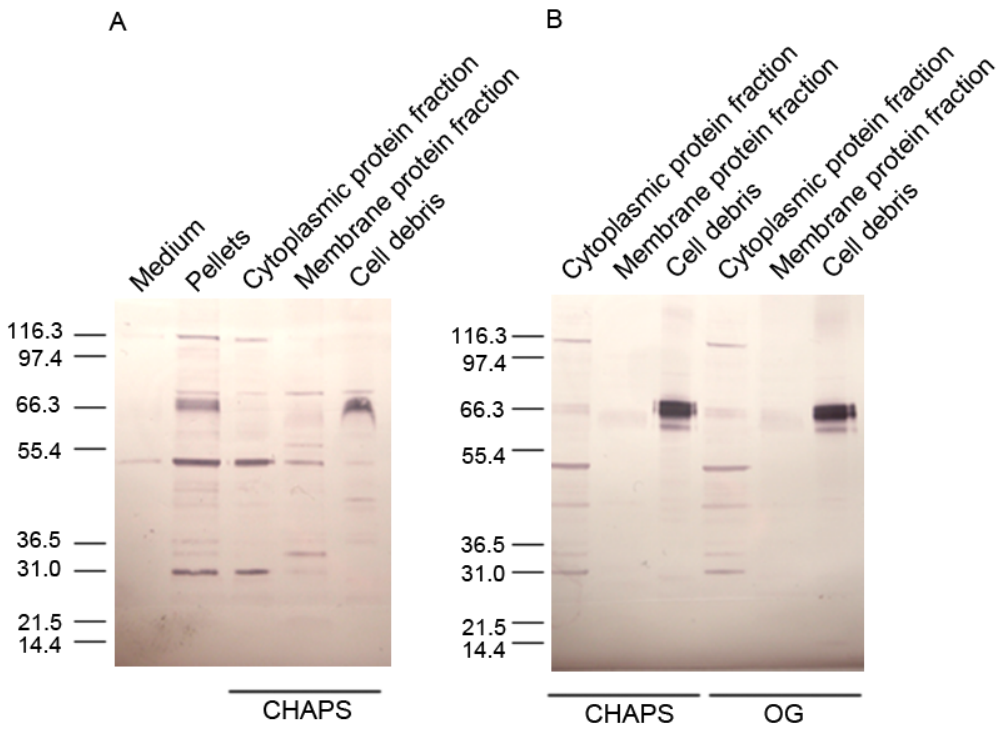

Figure 2.4 Expression and Immunoblot Analysis of Recombinant Full Length AgMCORP. A) Membrane proteins were extracted using DUALXtract total membrane protein extraction kit (Dualsystems Biotech). After 24 h expression, cells were pelleted by centrifugation. Both the supernatant and pellets were analyzed. Full length AgMCORP (66 kDa) was not detected in the medium, but was present in pellets. After extraction by CHAPS, AgMCORP was not in the cytoplasmic protein fraction or membrane protein fraction, but in the cell debris. B) Membrane proteins were extracted using a different procedure. The procedure included sonication and ultracentrifugation to separate cytoplasmic proteins and membrane proteins. Two mild detergents, CHAPS and OG (octyl-β-gluside), were used to extract the membrane fraction. AgMCORP was not extracted by the detergents and remained in the cell debris fraction.

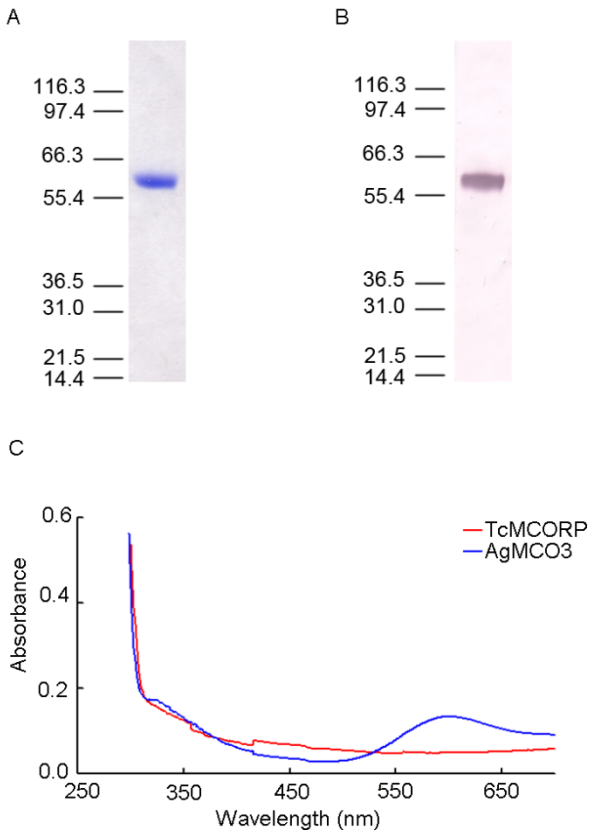

Figure 2.5 SDS-PAGE, Immunoblot Analysis and Absorbance Spectrum of Purified TcMCORP.

A) Purity was verified by Coomassie staining. B) The identity of the purified protein was confirmed by immunoblot analysis using antiserum against TcMCORP. C) UV/vis spectra of purified TcMCORP and AgMCO3 (positive control). The concentration used was 4 μg/μL.

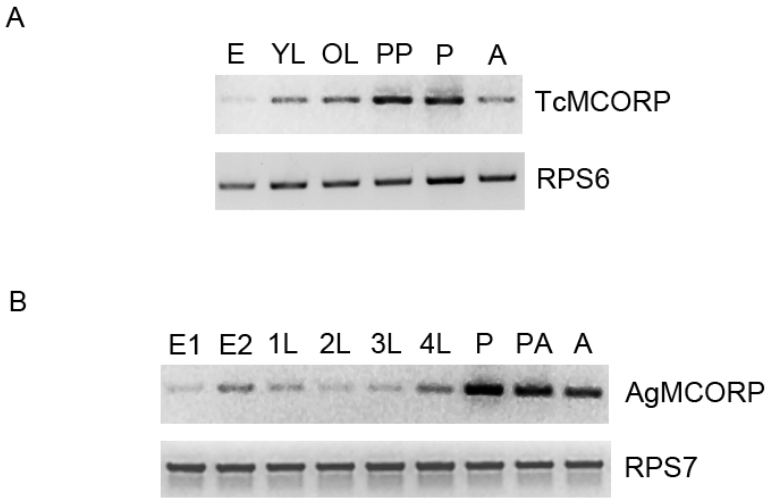

Figure 2.6 Developmental Expression Profiles of MCORP in *T. castaneum* and *A. gambiae*. RT-PCR was used to determine if a gene is expressed at a particular developmental stage. A) Primers specific to TcMCORP gene were used. The RPS6 (ribosomal protein S6) gene was used as a reference gene. 35 PCR cycles were used for TcMCORP reactions and 24 PCR cycles were used for RPS6 reactions. Abbreviations used are: E, eggs; YL, younger larvae; OL, older larvae; PP, pharate pupae; P, pupae; A, adults. B) Primers specific to AgMCORP were used. The RPS7 gene was used as a reference gene. 30 PCR cycles were used for AgMCORP reactions and 25 PCR cycles were used for RPS7 reactions. Abbreviations used are: E1, eggs; E2, older eggs and pharate larvae; 1L, 1st instar larvae; 2L, 2nd instar larvae; 3L, 3rd instar larvae; 4L, 4th instar larvae; P, pupae; PA, pharate adults; A, adult females.

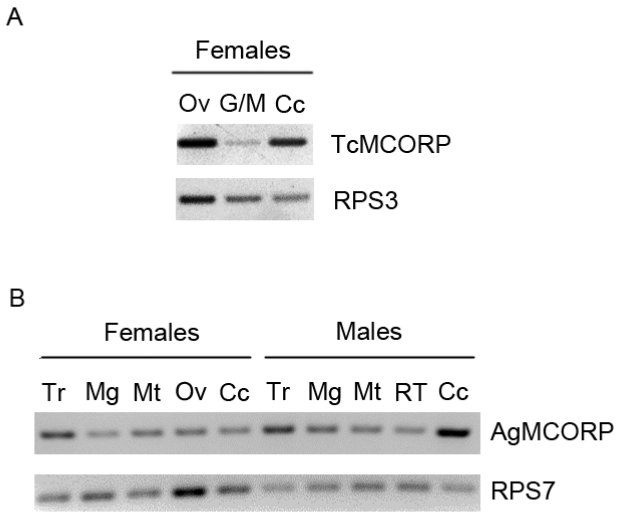

Figure 2.7 Expression of MCORP in Adult Tissues.

RT-PCR was used to determine if a gene is expressed in different tissues. A) Tissues from *T. castaneum* adult females were analyzed. The number of PCR cycles used was 32 cycles for TcMCORP reactions and 24 for the reference gene RPS3 reactions. Abbreviations used are: Ov, ovaries; G/M, guts and Malpighian tubules; Cc, carcass. B) Tissues from *A. gambiae* adult females and males were analyzed. The number of PCR cycles used was 35 for AgMCORP reactions and 25 for the RPS7 reactions. Abbreviations used are: Tr, thorax; Mg, midguts; Mt, Malpighian tubules; Ov, ovaries; RT, reproductive tissues; Cc, carcass.

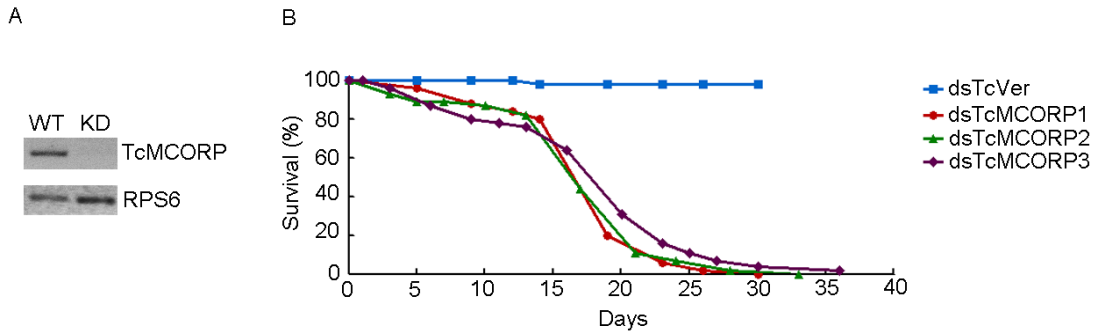

Figure 2.8 Efficiency of TcMCORP Knockdown and Effect of TcMCORP Knockdown on Life Span.

A) Whole bodies from WT *T. castaneum* pupae and from pupae with dsTcMCORP treatment (KD) 2 days post injection were analyzed. The number of PCR cycles used was 35 cycles for TcMCORP and 24 cycles for RPS6 reactions. B) *T. castaneum* pharate pupae were injected with TcMCORP dsRNA or TcVer dsRNA (400 ng per insect). Live insects were counted starting from adult day 0. The life span of TcMCORP knockdown beetles was shorter than that of control beetles (TcVer knockdown). For TcMCORP knockdown, data are from three biological replicates (n = 49, 45, or 55).

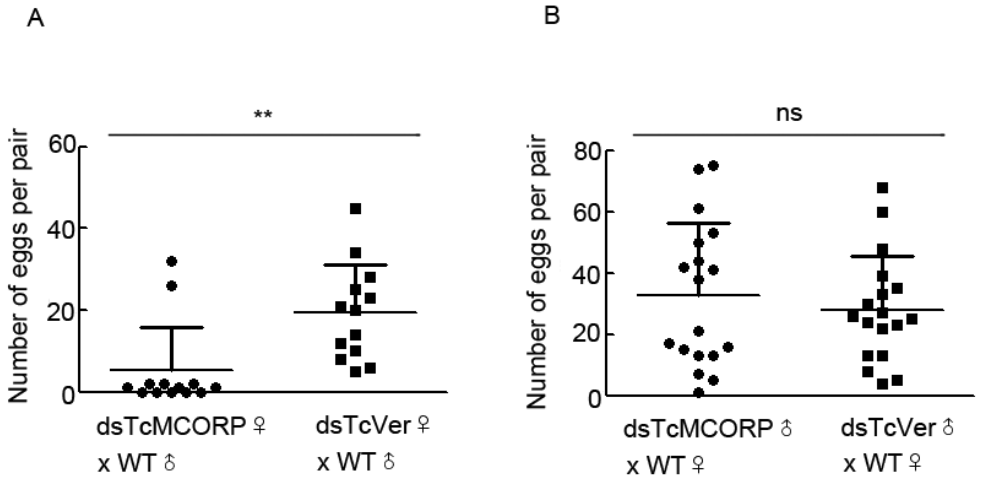

Figure 2.9 Effect of TcMCORP Knockdown on Egg Laying.

dsRNA for TcMCORP or TcVer was injected into *T. castaneum* pharate pupae. Injected females and males were sexed at the pupal stage. At 4 days after adult eclosion, the RNAi insects were mated with wild type (WT) insects on a single pair basis. The eggs produced from each pair were counted. Pairwise differences in egg numbers were assessed by performing a nonparametric analysis (Mann-Whitney test). Bars represent mean + standard deviation. A) Comparison of the number of eggs produced from dsTcMCORP ♀ × WT ♂ crosses and dsTcVer ♀ × WT ♂ crosses. The latter group served as a control. A significant difference was observed (** = $p<0.01$, n = 13). B) Comparison of the number of eggs produced from dsTcMCORP ♂ × WT ♀ crosses and dsTcVer × WT ♀ crosses. The latter group served as a control (ns = not significant, n = 18).

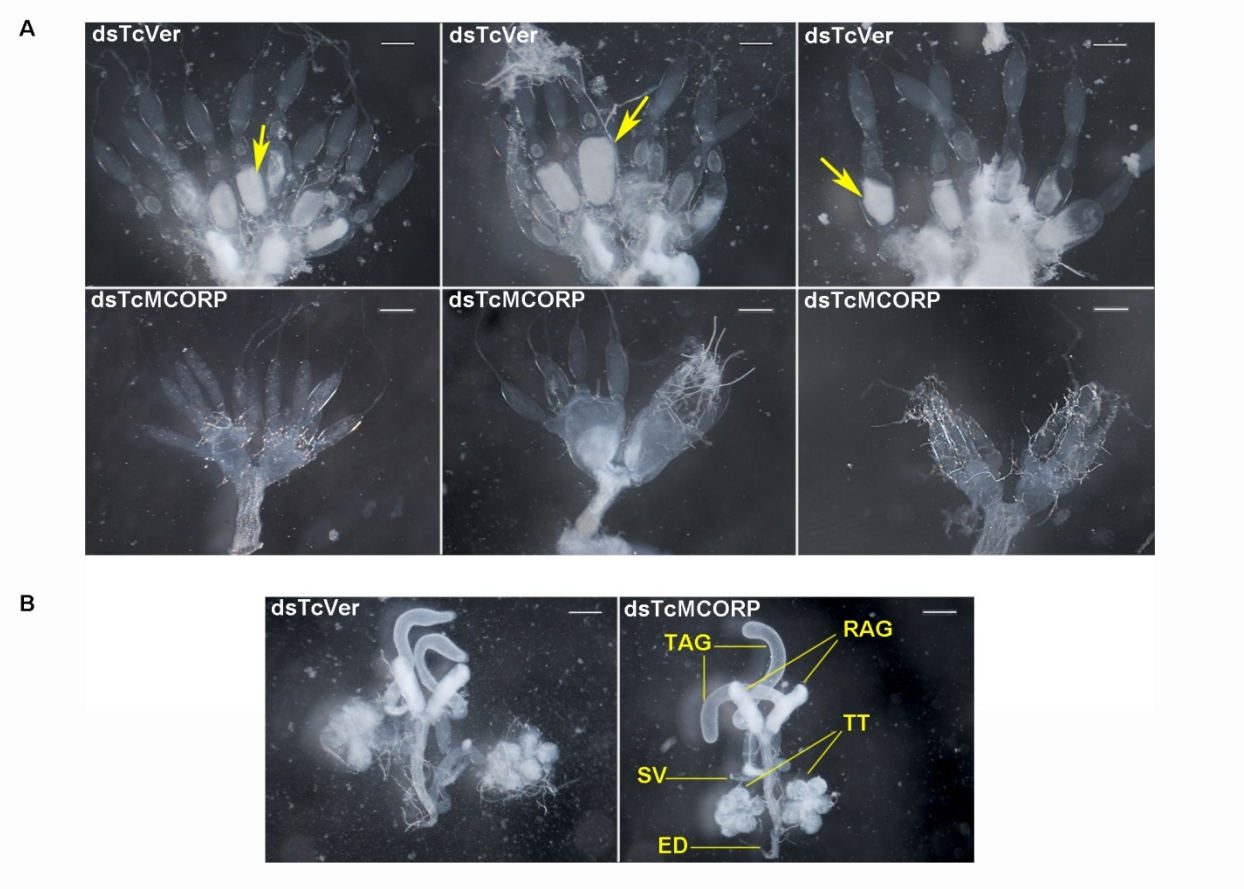

Figure 2.10 Effect of RNAi on the Reproductive Tissues in *T. castaneum*.

A) The top three images represent ovaries dissected from TcVer knockdown insects (control), and the bottom three images show ovaries dissected from TcMCORP knockdown insects. In each image, the ovary is oriented with nurse cells on top and oocytes at the bottom. The mature primary oocytes are marked by a yellow arrow. Note that the primary oocyte development was blocked in TcMCORP RNAi insects but not in TcVer RNAi insects. For dsTcVer treatment, all ovaries (n=12) were fully developed. For dsTcMCORP treatment, 14 out of 18 insects showed the absence of mature primary oocytes. Scale bar: 200 μm. B) The left image shows the male reproductive tissue dissected from a TcVer knockdown insects (control), and the right image shows the male reproductive tissue from a TcMCORP knockdown insect. Note that they are very similar. Abbreviations used are: TAG, tubular accessory glands; RAG, rod-shaped accessory glands; SV, seminal vesicle; TT, testes; ED, ejaculatory duct. Scale bar: 200 μm.

Table 2.1 Primers Used in This Work

Primer name	Primer sequence (5' – 3')	Primer usage
TcMCORP-F	GGATCCCCAGAGGGCACATTC	Cloning of TcMCORP to generate antiserum
TcMCORP-R	GTCGACTTACATCAGAAAGAAATC	
RACE 5' P	AGGTCCTGCGGGCACAGCATCGTTT	5' RACE of AgMCORP
RACE 5' nested P	AACTCCTCCGACCGATGGGGAAACG	
RACE 3' P	GGACGGCTTGCGGTGAAACGATGCT	3' RACE of AgMCORP
RACE 3' nested P	CAAAGGATCTGCAGCGCGGGTACGA	
fAgMCORP-F	GCGTATGCGAGTGCTGCTA	Cloning of full length AgMCORP
fAgMCORP-R	CAACTACTACCACCGCCACC	
AgMCORP-F	GAATTCGTTTTGATACACTTT	Cloning of AgMCORP to generate antiserum
AgMCORP-R	GTCGACTTAAATTAAAAAGTACCT	
tAgMCORPS2-F	AGATCTGCCGTTTCGTGCGAC	Cloning of truncated AgMCORP into pMT/Bip/V5-His A vector
tAgMCORPS2-R	GAATTCTTAAATTAAAAAGTACCT	
fAgMCORPS2-F	ACTAGTATGAAAACACTGCAG	Cloning of full length AgMCORP into pMT/V5-His A vector
fAgMCORPS2-R	GAATTCTTAAATTAAAAAGTA	
TcMCORPSf9-F	GTCGACATGAAGTTATGCATA	Cloning of Bip-TcMCORP to make recombinant protein
TcMCORPSf9-R	GAATTCTTACATCAGAAAGAAATC	
TcRTPCR-F	GCTCCAAAGACCGTGTA	RT-PCR of TcMCORP in developmental stages
TcRTPCR-R	TCTTGGCTCGCATCTTG	
TcRPS6-F	AGATATATGGAAGCATCATGAAGC	RT-PCR of TcRPS6
TcRPS6-R	CGTCGTCTTCTTTGCTCAAATTG	
qTcMCORP-F	CCAATGTCCAATACTTAGC	RT-PCR of TcMCORP in tissues
qTcMCORP-R	GCCTGATAGAGATGTGTT	
qRPS3-F	GCAGAGTCTCTTAGATTCA	RT-PCR of TcRPS3
qRPS3-R	ATTCCATGATGTACCTCAA	
AgRTPCR-F	CCTTCAGTCAGTCGCATCCT	RT-PCR of AgMCORP
AgRTPCR-R	TCTCGTGGGTCGGTTCTCC	
AgRPS7-F	CGCTATGGTGTTCGGTTCC	RT-PCR of AgRPS7
AgRPS7-R	TGCTGCAAACTTGGGCTAT	
dsRNATc-F	TAATACGACTCACTATAGGGAG	Synthesis of TcMCORP dsRNA
dsRNATc-R	TAATACGACTCACTATAGGGAG	
dsRNAVer-F	TAATACGACTCACTATAGGG	Synthesis of TcVer dsRNA
dsRNAVer-R	TAATACGACTCACTATAGGG	
dsRNAAg-F	TAATACGACTCACTATAGGGAG	Synthesis of AgMCORP dsRNA
dsRNAAg-R	TAATACGACTCACTATAGGGAG	
dsRNAGFP-F	TAATACGACTCACTATAGGGCGATGC	Synthesis of GFP dsRNA
dsRNAGFP-R	TAATACGACTCACTATAGGGCGGACT	
qAgMCORP-F	CATTACACTGCACTGGAC	qPCR of AgMCORP
qAgMCORP-R	TGGAATTTGTACTGGAACC	
qRPS7-F	GTGGTCGGCAAGCGTATCC	qPCR of AgRPS7
qRPS7-R	GGTGGTCTGGTTCTTATCC	

Table 2.2 NCBI Accession Numbers of Sequences Used for Phylogenetic Analysis

Species	Phylogenetic group	NCBI accession number
Anopheles gambiae	MCO1	AAN17505
A. gambiae	MCO2A	AAX49501
A. gambiae	MCO3	ABQ95972
A. gambiae	MCO4	ABY84643
A. gambiae	MCO5	ABY84644
A. gambiae	MCORP	KJ500312
Aedes aegypti	AAEL007802	XP_001652917
A. aegypti	AAY29698	AAY29698
A. aegypti	AAEL001667	XP_001653727
A. aegypti	AAEL001632	XP_001653728
A. aegypti	AAEL001640	XP_001653729
A. aegypti	AAEL001672	XP_001653730
A. aegypti	MCORP	XP_001652083
Culex quinquefasciatus	MCORP	XP_001862832
Drosophila melanogaster	MCO1	NP_609287
D. melanogaster	Lac2	NP_724412
D. melanogaster	MCO3	NP_651441
D. melanogaster	CG32557	NP_573249
Manduca sexta	Lac1	AAN17506
M. sexta	Lac2	AAN17507
M. sexta	MCORP	Msex2.04321*
Bombyx mori	MCO1	DAA06286
B. mori	Lac2	NP_001103395
B. mori	MCORP	XP_004930412
Danaus plexippus	MCORP	EHJ73418
Camponotus floridanus	MCORP	EFN64969
Harpegnathos saltator	MCORP	EFN82577
Apis mellifera	LOC724890	XP_001120790
A. mellifera	LOC410365	XP_006562317
A. mellifera	LOC552811	XP_625189
Apis florea	MCORP	XP_003695203
Bombus terrestris	MCORP	XP_003399857
Bombus impatiens	MCORP	XP_003486821
Megachile rotundata	MCORP	XP_003707167
Tribolium castaneum	Lac1	NP_001034514
T. castaneum	Lac2A	NP_001034487
T. castaneum	MCORP	KJ500311
Dendroctonus ponderosae	MCORP	ENN80477
Pediculus humanus corporis	PHUM024710	XP_002422943
P. h. humanus	PHUM554290	XP_002431869
P. h. humanus	MCORP	XP_002423995
Acyrthosiphon pisum	LOC100165676	XP_001948070
A. pisum	LOC100164049	XP_001950788
A. pisum	MCORP	XP_001946224

*Accession number is from Manduca Base (http://agripestbase.org/manduca/)

Table 2.3 ICP-MS Analysis of Recombinant TcMCORP

Metal	Concentration (ng/mL)	Molarity (mM)	Molar ratio (metal: TcMCORP)
Copper	498.4	0.0078	1:17
Zinc	165.8	0.0026	1:51
Iron	82.1	0.0015	1:89
Manganese	3.6	<0.0001	1: >1000

Chapter 3 - Multicopper Oxidase-1 Orthologs from Diverse Insect Species Have Ascorbate Oxidase Activity

Introduction

Insect multicopper oxidases (MCOs) include MCO1 orthologs, MCO2 orthologs, and a mosquito specific group (MCO3, MCO4, and MCO5 orthologs) (Gorman et al., 2008; Dittmer and Kanost, 2010). We have been studying insect MCOs for several years because they function in essential physiological processes such as cuticle sclerotization (Dittmer et al., 2004; Gorman et al., 2008; Dittmer et al., 2009; Gorman et al., 2012; Lang et al., 2012a; Lang et al., 2012b). Of the insect MCOs that have been identified, MCO1 and MCO2 orthologs are present in all of the insect genomes that we analyzed (Dittmer and Kanost, 2010). MCO2 orthologs are known as laccases (Dittmer et al., 2009, Gorman et al., 2012) that play a role in cuticle tanning (Dittmer et al., 2004, Arakane et al., 2005).

To date, it is still unclear what kind of oxidases MCO1 orthologs are. MCO1 is expressed in all developmental stages and most tissues (Dittmer et al., 2004; Chintapalli et al., 2007; Gorman et al., 2008; Graveley et al., 2011). MCO1 is located on the basal side of the digestive system and Malpighian tubules; thus, it might be able to oxidize substrates in the hemolymph (Lang et al., 2012a). Most of the well-studied MCOs can be categorized as laccases, ferroxidases or ascorbate oxidases. Laccases are able to oxidize aromatic compounds, such as diphenols and aromatic diamines (Giardina et al., 2010). *Drosophila melanogaster* MCO1 (DmMCO1) was found to have extremely low laccase activity (Lang et al. 2012a). The catalytic efficiencies (k_{cat}/K_m) of DmMCO1 using laccase substrates (Lang et al., 2012a) are much lower than those of two mosquito laccases, *Anopheles gambiae* MCO2A (Gorman et al., 2012) and MCO3 (Lang et al., 2012b). Thus, MCO1 orthologs are unlikely to be laccases.

Ferroxidases catalyze the oxidation of ferrous iron to ferric iron (Kosman, 2010). Well-studied ferroxidases include yeast Fet3p and human ceruloplasmin. The functions of Fet3p and ceruloplasmin are similar since they each work with an iron permease to transport iron across cell membranes; however; Fet3p is associated with a ferric iron permease and involeved in iron uptake, and ceruloplasmin interacts with a ferrous iron permease and involved in iron efflux (Jeong and David, 2003; Singh et al., 2006; Philpott and Protchenko, 2008; De Domenico et al., 2007; Han, 2011). Kinetic analysis of Fet3p showed that Asp283, Glu185 and Asp409 provide a

binding site for ferrous iron (Stoj et al., 2006). Ferroxidases are essential in iron metabolism, which is a crucial biological process in all eukaryotic organisms. So far, iron metabolism in insects is still poorly understood. Because ferroxidases are important in iron transport in other organisms, we hypothesize that a ferroxidase is also involved in iron transport in insects. Previously we found DmMCO1 has ferroxidase activity, and is involved in iron homeostasis *in vivo*, but we do not know its specific physiological functions (Lang et al., 2012a). In addition, we have not tested whether other MCO1 orthologs have the similar substrate specifivity; thus, MCO1 functions as a ferroxidase is not definitive.

Another possibility is that MCO1 is an ascorbate oxidase. Ascorbate oxidases catalyze the oxidation of ascorbate to semidehydroascorbate radical, which is in turn further oxidized to dehydroascorbate; concomitantly, oxygen, bound to the T2/T3 copper center of ascorbate oxidase, is reduced to water (Shleev et al., 2005). Ascorbate oxidases have been identified in plants, but not in animals (Nakamura and Go, 2005). The crystal structure of ascorbate oxidase was characterized over two decades ago (Messerschmidt et al., 1992). Although ascorbate oxidases have been known for a long time, their physiological roles in plants are still not defined. Ascorbate oxidase is a secreted glycoprotein localized in the cell wall of plants (Smirnoff, 2000). The expression and activity of ascorbate oxidase are closely correlated with rapid cell growth (Lin and Varner, 1991; Esaka et al., 1992; Kato and Esaka, 1999). The expression of ascorbate oxidase is greatly induced by auxin, a plant growth hormone (Esaka et al., 1992; Kisu et al., 1997). No mechanism that might explain the correlation between higher expression of ascorbate oxidase and rapid cell expansion has been established. In addition, the expression of ascorbate oxidase is induced by mechanical wounding (Asao et al., 2003), which suggests it may be involved in defense against oxidants.

The substrate specificity of MCOs determines their physiological function. In this study, we characterized the substrate specificity of recombinant MCO1 isoforms. We analyzed the kinetic properties of MCO1 from four insect species, *D. melanogaster*, *Anopheles gambiae*, *Tribolium castaneum* and *Manduca sexta*, using two laccase substrates (hydroquinone, dopamine), ferrous iron, and ascorbic acid as substrate. Our goal was to determine whether insect MCO1 orthologs have similar substrate specificity, which would suggest that they have similar physiological functions. In addition, we evaluated putative iron-binding residues in DmMCO1. MCO1 sequences have three highly conserved residues that were predicted to be the binding

sites of ferrous iron (Lang et al., 2012a): two acidic residues (Asp380 and Glu552) that align with Glu 185 and Asp283 in yeast Fet3p and a histidine residue (His374) that is present in the putative substrate binding pocket. We purified several mutant forms of DmMCO1 and determined kinetic constants using substrates mentioned above. We compared the kinetic propreties of the mutant isoforms with those of the wild type isoform to understand if these residues are important for the enzymatic activity of DmMCO1.

Experimental Procedures

Identification of Signal Peptides, Carboxyl-terminal Transmembrane Regions

Signal sequences were predicted by Signal P (Bendtsen et al., 2004), and putative transmembrane regions were predicted with TMPred software (Hofmann and Stoffel, 1993).

cDNAs Used for Recombinant Protein Expression

The cDNA used for expression of DmMCO1 isoforms was described previously (Lang et al., 2012a). Briefly, to circumvent problems associated with low protein expression and protein aggregation, we generated a truncated DmMCO1 cDNA that encoded only the cysteine-rich region and cupredoxin-like domains (and excluded the native signal peptide, amino-terminal von Willebrand domains, and carboxyl-terminal hydrophobic region). The truncated cDNA was cloned in-frame with the *D. melanogaster* Bip signal sequence so that the recombinant protein would be secreted. The recombinant enzyme that this cDNA encodes was designated DmMCO1T (Figure 3.1). We found that recombinant DmMCO1T was cleaved by an unknown protease in the cell culture medium; therefore, we used site-directed mutagenesis to create an Arg454Ala mutant isoform. Mutant versions of the DmMCO1T[R454A] cDNA were also made: DmMCO1T[R454A, H374S], DmMCO1T[R454A, D380A], DmMCO1T[R454A, D380A, E552A], and DmMCO1T[R454A, H374S, D380A, E552A]. The amino acid sequence of AgMCO1 was described previously (Gorman et al., 2008). The cDNAs used to express AgMCO1T and AgMCO1T[R542S] were analogous to the ones used to express DmMCO1T and DmMCO1T[R454A], that is, the cysteine-rich region and cupredoxin-like domains were cloned in-frame with the *D. melanogaster* Bip signal peptide, and the Arg542Ser mutation was made to prevent proteolytic cleavage at residue 542 (Figure 3.1). The amino acid sequence of TcMCO1 (synonym = TcLac1) was described previously (Arakane et al., 2005). We used RT-PCR to clone

a cDNA that encodes full-length TcMCO1. Two truncated forms of the TcMCO1 cDNA were made: a shorter form, TcMCO1Ts, which encodes Met1 through Val629, and a slightly longer form, TcMCO1Tl, which encodes Met1 through Ser658 (Figure 3.1). The amino acid sequence of MsMCO1 (synonym = MsLac1) was described previously (Dittmer et al., 2004). We used RT-PCR to clone a cDNA that encodes full-length MsMCO1. Two truncated forms of the MsMCO1 cDNA were made: a shorter form, MsMCO1Ts, which encodes Met1 through Gln724, and a slightly longer form, MsMCO1Tl, which encodes Met1 through Ser751 (Figure 3.1). The cDNA used for TcLac2A expression was described previously (Gorman et al., 2012). Mutagenesis was accomplished with the use of the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene).

Recombinant Baculovirus Production

Production of recombinant baculoviruses was done as described previously (Lang et al., 2012a). Briefly, cDNAs were inserted into the pOET3 transfer plasmid, and the flashBACGOLD system (Oxford Expression Technologies) was used to generate recombinant baculoviruses. The baculovirus used for AgMCO3 expression was described previously (Lang et al., 2012b).

Enzyme Purification

Recombinant multicopper oxidases were expressed in cultured insect cells and purified as described previously (Lang et al, 2012a). Briefly, recombinant baculoviruses were used to infect cultured Sf9 cells, and the secreted enzymes were purified from the culture medium by concanavalin-A-Sepharose, Q-Sepharose, and Superdex 200 chromatography. The concentration of recombinant enzymes was determined as described previously (Lang et al., 2012b). Yields ranged from 0.4 - 2.2 mg per liter of cell culture. Laccase-α from *Trametes versicolor* (TvLac-α) was purified from a commercial preparation of fungal laccases (Sigma). The sample was dialyzed against 20 mM Tris, pH 8.0 (4 ºC), and purified by Q-Sepharose and Superdex 200 chromatography. The Proteomics Center of the University of Nevada used peptide mass finger printing to identify the protein as TvLac-α (GenBank Accession Number = Q12718; Necochea et al., 2005). The yield was 0.26 mg from 100 mg starting material.

Laccase Assays

Laccase activity assays, determination of pH optima, and calculation of kinetic constants were done as described previously (Gorman et al, 2012). Briefly, reactions were done by mixing

enzyme with substrate in a total volume of 200 µl and detecting product formation with a microplate spectrophotometer. All of the MCO1 isoforms had the highest activity at pH 6.0 - 8.0 (data not shown); therefore, pH 7.0 (approximately the pH of insect hemolymph) was used for determining kinetic constants.

Ferroxidase Assays

The apo-transferrin-based assays were done as described previously (Osaki et al., 1966; Johnson et al., 1967) with minor modifications. Human apo-transferrin (Sigma) was dissolved in 50 mM Tris, 150 mM sodium chloride, 20 mM sodium bicarbonate, pH 7.3. Reactions (200 µl) contained 20 - 80 µg enzyme, 780 µg apo-transferrin, and ferrous ammonium sulfate (0 - 240 µM) in 100 mM sodium acetate, pH 6.0. The formation of holo-transferrin from apo-transferrin was determined by measuring absorbance 460 nm and using a molar extinction coefficient of 2,500 cm^{-1} M^{-1}. Kinetic curves were made by plotting activity versus substrate concentration. The data were fit to the Michaelis-Menten equation by non-linear regression using GraphPad Prism software, and kinetic constants were estimated from the fitted data.

Ascorbate Oxidase Assay

A spectrophotometric assay was used to detect ascorbate oxidase activity. Ascorbate concentration is commonly monitored by measuring absorbance at 265 nm (Oberbacher and Vines, 1963); however, to avoid excessively high absorbance readings when the concentration of ascorbate was greater than 500 µM, we monitored ascorbate concentration at 275 nm. We confirmed a linear relationship between absorbance at 275 nm and ascorbate concentration (from 5 - 600 µM, $R^2 = 0.9997$), and calculated a molar extinction coefficient of 12,000 M^{-1} cm^{-1}. Reactions were done by mixing 0.1 or 0.2 µg MCO1 with ascorbic acid in 0.1 M sodium phosphate, pH 7.0, in a total volume of 100 µl and observing a decrease in substrate over time with a microplate spectrophotometer. Blank reactions containing no enzyme measured the rate of non-enzymatic oxidation. MCO1 was replaced with 0.0012 µg ascorbate oxidase from *Cucurbita* sp. (Sigma) as a positive control or with 0.2 µg AgMCO3 (Lang et al, 2012) or TvLac-α, two laccases that functioned as negative controls. Assays were done in duplicate. Kinetic curves were made by plotting activity versus substrate concentration. The data were fit to the Michaelis-Menten and allosteric sigmoidal equations by non-linear regression using GraphPad Prism.

Akaike's Information Criteria was used to determine which equation fit the data better. Kinetic constants were estimated from the fitted data.

Results

MCO1 Isoforms Used to Study Enzymatic Activity

MCO1 is common to all examined insect species and MCO1 orthologs belong to a distinct clade in a phylogenetic analysis (Dittmer and Kanost, 2010); thus, it is possible that MCO1 orthologs have conserved and essential functions. Our previous studies of *D. melanogaster* MCO1 (DmMCO1) showed that DmMCO1 is able to oxidize ferrous iron. To further study the enzymatic activity of MCO1, we purified recombinant MCO1 isoforms from *D. melanogaster, A. gambiae, T. castaneum* and *M. sexta*. Alignment of MCO1 orthologs from the four species is shown in Figure 3.1. Both DmMCO1 and *A. gambiae* MCO1 (AgMCO1) proteins contain an additional region at their amino-terminal ends. DmMCO1 contains two von Willebrand factor C (vWFC) domains, and AgMCO1 contains two von Willebrand factor B (vWFB) domains and two vWFC domains. Only dipteran MCO1 orthologs are found to contain these domains. However, the function of this region is unknown (Dittmer and Kanost, 2010). These von Willebrand factor domains were omitted for making the recombinant proteins. MCO1 orthologs from the four species all contain a carboxyl-terminal transmembrane region, which was also omitted for making the recombinant protein. Both recombinant DmMCO1 and AgMCO1 were cleaved by an unknown protease present in the cell cultures used for protein expression. Based on amino-terminal sequencing results, we determined that the proteins were cleaved after an arginine residue that is conserved in dipteran MCO1 sequences. The arginine was mutated in DmMCO1 and AgMCO1 to generate uncleaved recombinant protein (Figure 3.1). MCO1 sequences have three highly conserved residues that were predicted to be the binding sites of ferrous iron (Lang et al., 2012a): two acidic residues (Asp380 and Glu552) that align with two studied iron binding residues of yeast Fet3p (Stoj et al., 2006) and a histidine residue (His374) that is present in the putative substrate binding pocket. The three putative iron-binding residues of MCO1 are shown in Figure 3.1. To test if these residues are necessary for oxidation of iron, several DmMCO1 mutants were purified. TcMCO1 and MsMCO1 were purified as shorter forms (with a carboxyl-terminus that corresponds to the DmMCO1 and AgMCO1 isoforms) and longer forms (that are wild-type except for the exclusion of the carboxyl-terminal

hydrophobic region) (Figure 3.1). In sum, MCO1 isoforms used to detect enzymatic activity are: DmMCO1T (cleaved form), DmMCO1T[RA] (uncleaved form), mutants to test putative iron binding residues (DmMCO1T[RA HS], DmMCO1T[RA DA], DmMCO1T[RA DA EA], DmMCO1T[RA HS DA EA],), AgMCO1T (cleaved form), AgMCO1T[RS] (uncleaved form), TcMCO1Ts (shorter form), TcMCO1Tl (longer form), MsMCO1Ts (shorter form) and MsMCO1Tl (longer form).

MCO1 Orthologs Are Not Laccases

Detectable but very low activity of all MCO1 isoforms was found with hydroquinone or dopamine as substrate, strongly suggesting that none of MCO1 isoforms function biologically as laccases. The kinetic constants of MCOs using laccase substrates are listed in Table 3.1.

MCO1 Orthologs Do Not Function as Ferroxidases

DmMCO1 does have ferroxidase activity (Lang et al., 2012a), but based on its K_m, it appears that its affinity for iron is much lower (10 - 100 fold) than that of known ferroxidases (Table 3.2). The ferroxidase activity of AgMCO1 is much lower than that of DmMCO1 with a more than ten-fold lower catalytic efficiency (Table 3.2). Both cleaved and uncleaved DmMCO1 and AgMCO1 give consistent results in measurements of enzymatic activity (Table 3.2). The other two MCO1 orthologs, TcMCO1 and MsMCO1, have even less ferroxidase activity, similar to that of insect laccases (Table 3.2). Both shorter and longer forms of TcMCO1 and MsMCO1 have similar activity (Table 3.2). These results indicate that the ferroxidase activity of MCO1 orthologs may not be a physiological function. To test whether the three putative iron binding residues are required for ferroxidase activity of MCO1, kinetic analysis of several DmMCO1 mutants using ferrous iron as substrate was carried out. Surprisingly, the mutations have no effect on oxidation of iron (Table 3.2). They also have no effect on oxidation of the laccase substrates, hydroquinone and dopamine (Table 3.1).

MCO1 Orthologs May Function as Ascorbate Oxidases

Most of the well-studied MCOs are laccases, ferroxidases or ascorbate oxidases. Ascorbate oxidases have been identified in plants, but not in animals (Nakamura and Go, 2005). Since MCO1 orthologs do not seem to be laccases or ferroxidases, we decided to test whether they can oxidize ascorbic acid. *Cucurbita sp.* ascorbate oxidase from Sigma served as a positive

control. Based on our spectrophotometric assay, kinetic constants of *Cucurbita sp.* ascorbate oxidase are K_m = 170 ± 10 µM and k_{cat} = 354 ± 17 s^{-1}. The values are very close to published kinetic constants of *Cucurbita sp.* ascorbate oxidase from Sigma using an oxygen monitor to assay activity, K_m = 204 ± 26 µM, k_{cat} = 682 ± 34 s^{-1} (Wimalasena and Dharmasena, 1994). Kinetic analysis of MCO1 orthologs from the four insect species showed that MCO1 orthologs oxidize ascorbic acid with high efficiency, and their K_ms are similar to that of *Cucurbita sp.* ascorbate oxidase (Table 3.3). They are much better at oxidizing ascorbic acid than diphenols (Table 3.1) or ferrous iron (Table 3.2). An insect laccase and a fungal laccase, serving as negative controls, have little ascorbate oxidase activity (Table 3.3). In addition, DmMCO1T[RA HS] and DmMCO1T[RA DA EA] have less ascorbate oxidase activity than DmMCO1, and DmMCO1T[RA DA EA HS] does not show any detectable activity (Figure 3.2; Table 3.3). The kinetic properties of the DmMCO1 mutant isoforms indicate that Asp380, Glu552, and His374 are required for the ascorbate oxidase activity of MCO1 but not its ferroxidase and laccase activities.

Discussion

Based on our data, we propose that insect MCO1 orthologs function mainly as ascorbate oxidases. The kinetic properties of the DmMCO1 mutant isoforms indicate that Asp380, Glu552, and His374 are required for the ascorbate oxidase activity of MCO1. However, the K_ms of DmMCO1T[RA DA EA] and Dm[RA HS] are similar to that of wild type DmMCO1. Therefore, the three putative iron binding residues do not seem to affect the binding affinity of ascorbic acid. More studies will be needed to determine how the D380A, E552A, and H374S mutations affect k_{cat}.

Currently we do not know the physiological functions of MCO1. Plant ascorbate oxidase was discovered 80 years ago (Dawson et al., 1975), but the physiological role of this enzyme is still unclear. No ascorbate oxidase has been identified in animals; thus, there are no animal models of ascorbate oxidase function to guide our thinking about MCO1 function.

MCO1 is expressed throughout development, and is expressed in most tissues with highest expression in the digestive system and Malpighian tubules (Dittmer et al., 2004; Chintapalli et al., 2007; Gorman et al., 2008; Graveley et al., 2011). MCO1 is upregulated upon blood feeding and immune challenge (Gorman et al., 2008). MCO1 is located on the basal

surface of tissues, which strongly support the hypothesis that MCO1 is positioned to oxidize substrates in the insect hemolymph (Lang et al., 2012a).

Our previous study of DmMCO1 demonstrated that recombinant DmMCO1 was able to oxidize ferrous iron. Strong knockdown of MCO1 led to pupal lethality, suggesting that MCO1 is an essential gene. Weak knockdown of MCO1 increased longevity of flies reared on food supplemented with 10 mM iron, which indicates MCO1 knockdown protects the flies from iron toxicity. In addition, knockdown of DmMCO1 led to a decrease in iron accumulation. Given these results, originally we concluded that MCO1 orthologs probably function as ferroxidases (Lang et al., 2012a). In this study, kinetic analysis of MCO1 isoforms clearly show that their K_m values are greater than those of known ferroxidases – Fet3p and human curoloplasmin, suggesting their affinity for iron is much lower. The K_m of DmMCO1 for ferrous iron is ~0.2 mM, and the K_m values of the other three MCO1 orthologs in our study were even higher (0.11 - 1.7 mM). The concentration of ferrous iron in insect hemolymph is unknown, but given the extremely low concentration of ferrous iron in mammalian extracellular fluids, we would expect that ferrous iron in hemolymph would be much lower than 0.1 mM. A low concentration of ferrous iron in hemolymph and a low affinity of MCO1 for ferrous iron would suggest that MCO1orthologs probably do not function as ferroxidases.

Now we propose MCO1 orthologs may function as ascorbate oxidases. L-ascorbic acid was found to be present in the insect hymolymph at a concentration of ~2.5 mM that is consistent with the K_ms we determined (Kramer, 1982). Generally, diatery L-ascorbic acid has a positive effect on insect growth and development. *Manduca sexta* cultured on an ascorbate-deficient diet showed reduced size and abnormal cuticle (Kramer, 1982). L-ascorbic acid is a prevalent antioxidant (Smirnoff, 2010), and is present in various tissues in insects (Kramer, 1982). The mechanism by which L-ascorbic acid acts in insects is unknown. There are several hypotheses about the functions of ascorbate in insects, including detoxification reactions (detoxifying superoxide and hydrogen peroxide), tyrosine metabolism and collagen formation. The function of L-ascorbic acid in the hemolymph is unknown; perhaps the hemolymph serves as a reservoir of L-ascorbic acid for other tissues (Kramer, 1982).

In mammals, no ascorbate oxidase has been identified. Ascorbate acts as an electron donor for enzymes involved in the biosynthesis of many essential biochemicals, and it is a higly effective antioxidant that reduces oxidative stress (Padayatty et al.,2003; Linster and Schaftingen,

2007). Ascorbate is a stong reducing agent; its one- and two-electron oxidized forms are semidehydroascorbate radical and dehydroascorbate (Padayatty et al.,2003; Linster and Schaftingen, 2007). Under physiogical conditions, ascorbate interacts with cellular oxidants to produce semidehydroascorbate radical and dehydroascorbate (Padayatty et al., 2003). Within cells, dehydroascorbate is rapidly reduced by glutathione and enzymatic reactions using glutathione and NADPH (Linster and Schaftingen, 2007).

It is possible that MCO1 is involved in regulation of oxidative stress, which could explain that MCO1 knockdown protects flies from a high iron diet. Specifically, a decrease in ascorbate oxidase activity may cause an increase in the concentration of ascorbate, a prevalent antioxidant (Smirnoff, 2000; Corti er al., 2010); thus, knockdown of MCO1 may protect flies from reactive oxygen species resulting from high levels of iron. In addition, studies have established that there is a relationship between ascorbate metabolism and iron homeostasis (Lane and Lawen, 2008; Grillet et al., 2014). Thus, the effect of MCO1 knockdown on iron homeostasis in *D. melanogaster* may be direct (Lang et al., 2012b). For example, erythroleukemia K562 cells incubated with dehydroascorbate took up more iron (Lane and Lawen, 2008); therefore, perhaps knockdown of MCO1 leads to less dehydroascorbate in the hymolymph, which in turn results in less iron uptake by cells and, thus, decreased iron content.

In addition to the above described possible physiological functions of MCO1 that might explain our previous observations, MCO1 may play other primary roles related to dehydroascorbate. Ascorbate is transported into mammalian cells by a sodium-dependent transporter, whereas dehydroascorbate is transported into cells by glucose transporters (such as GLUT1) followed by rapid intracellular reduction to ascorbate (Sagun et al. 2005; Corti et al., 2010). The latter transporters are more general and found in most cells. Thus, dehydroascorbate can be used to provide ascorbate to cell types that do not contain an ascorbate transporter (Huang et al., 2001; Heaney et al., 2008). In addition, dehydroascorbate has been proposed to have stronger antiviral effect than ascorbate and has a different mechanism of action compared with ascorbate (Furuya et al., 2008). Thus, MCO1 may mediate defense responses through the production of dehydroascorbate. This hypothesis is consistent with the upregulation of MCO1 orthologs in response to infection (Gorman et al., 2008).

So far, ascorbate oxidases have been identified only in plants (Nakamura and Go, 2005). This is the first identification of ascorbate oxidase in insects. Further studies are needed to understand their physiological functions in insects.

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References

Arakane, Y., Muthukrishnan, S., Beeman, R. W., Kanost, M. R., and Kramer, K. J. (2005) *Laccase 2* is the phenoloxidase gene required for beetle cuticle tanning. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 11337-11342

Asao, H., Yoshida, K., Nishi, Y., and Shinmyo, A. (2003) Wound-responsive *cis*-element in the 5′-upstream region of cucumber ascorbate oxidase gene. *Biosci. Biotechnol. Biochem.* **67,** 271-277

Bendtsen, J. D., Nielsen, H., von Heijne, G., and Brunak, S. (2004) Improved prediction of signal peptides: signalP 3.0. *J. Mol. Biol.* **340,** 783-795.

Chintapalli, V.R., Wang, J., and Dow, J.A.T. (2007) Using FlyAtlas to identify better *Drosophila melanogaster* models of human disease. *Nat. Genet.* **39,** 715–720.

Corti, A., Casini, A.F., and Pompella, A. Cellular pathways for transport and efflux of ascorbate and dehydroascorbate. *Arch. Biochem. Biophys.* **500,** 107-115

Dawson, C.R., Strothkamp, K.G., and Krul, K.G. (1975) Ascorbate oxidase and related copper proteins. *Ann. N. Y. Acad. Sci.* **258,** 209-220

De Domenico, I., Ward, D. M., di Patti, Maria Carmela Bonaccorsi, Jeong, S. Y., David, S., Musci, G., and Kaplan, J. (2007) Ferroxidase activity is required for the stability of cell surface ferroportin in cells expressing GPI-ceruloplasmin. *EMBO J.* **26,** 2823-2831

Dittmer, N. T., Gorman, M. J., and Kanost, M. R. (2009) Characterization of endogenous and recombinant forms of laccase-2, a multicopper oxidase from the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol.* **39,** 596-606

Dittmer, N. T., and Kanost, M. R. (2010) Insect multicopper oxidases: Diversity, properties, and physiological roles. *Insect Biochem. Mol. Biol.* **40,** 179-188

Dittmer, N. T., Suderman, R. J., Jiang, H., Zhu, Y. C., Gorman, M. J., Kramer, K. J., and Kanost, M. R. (2004) Characterization of cDNAs encoding putative laccase-like multicopper oxidases and developmental expression in the tobacco hornworm, *Manduca sexta*, and the malaria mosquito, *Anopheles gambiae*. *Insect Biochem. Mol. Biol.* **34,** 29-41

Esaka, M., Fujisawa, K., Goto, M., and Kisu, Y. (1992) Regulation of ascorbate oxidase expression in pumpkin by auxin and copper. *Plant Physiol.* **100,** 231-237

Furuya, A., Uozaki, M., Yamasaki, H., Arakawa, T., Arita, M., and Koyama, A.H. (2008) Antiviral effects of ascorbic and dehydroascorbic acids *in vitro*. *Int. J. Mol. Med.* **22,** 541-545

Giardina, P., Faraco, V., Pezzella, C., Piscitelli, A., Vanhulle, S., and Sannia, G. (2010) Laccases: a never-ending story. *Cell. Mol. Life Sci.* **67,** 369-385

Gorman, M. J., Dittmer, N. T., Marshall, J. L., and Kanost, M. R. (2008) Characterization of the multicopper oxidase gene family in *Anopheles gambiae*. *Insect Biochem. Mol. Biol.* **38,** 817-824

Gorman, M. J., Sullivan, L. I., Nguyen, T. D., Dai, H., Arakane, Y., Dittmer, N. T., Syed, L. U., Li, J., Hua, D. H., and Kanost, M. R. (2012) Kinetic properties of alternatively spliced isoforms of laccase-2 from *Tribolium castaneum* and *Anopheles gambiae*. *Insect Biochem. Mol. Biol.* **42,** 193-202

Graveley, B.R., et al. (2011) The developmental transcriptome of *Drosophila melanogaster*. *Nature.* **471,** 473–479

Grillet, L., Ouerdane, L., Flis, P., Hoang, M.T., Isaure, M.P., Lobinski, R., Curie, C., Mari, S. (2014) Ascorbate efflux as a new strategy for iron reduction and transport in plants. *J. Biol. Chem.* **289,** 2515-2525

Han, O. (2011) Molecular mechanism of intestinal iron absorption. *Metallomics.* **3,** 103-109

Heaney, M.L., Gardner, J.R., Karasavvas, N., Golde, D.W., Scheinberg, D.A., Smith, E.A., O'Connor, O.A. (2008) Vitamin C antagonizes the cytotoxic effects of antineoplastic drugs. *Cancer Res.* **68,** 8031-8038

Hellman, N. E., and Gitlin, J. D. (2002) Ceruloplasmin metabolism and function. *Annu. Rev. Nutr.* **22,** 439-458

Hofmann, K., and Stoffel, W. (1993) TMbase - A database of membrane spanning proteins segments. *Biol. Chem. Hoppe-Seyler.* **347,** 166

Huang, J., Agus, D.B., Winfree, C.J., Kiss, S., Mack, W.J., McTaggart, R.A., Choudhri, T.F., Kim, L.J., Mocco, J., Pinsky, D.J., Fox, W.D., Israel, R.J., Boyd, T.A., Golde, D.W., and Connolly E.S. Jr. (2001) Dehydroascorbic acid, a blood–brain barrier transportable form of vitamin C, mediates potent cerebroprotection in experimental stroke. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 11720-11724

Jeong, S. Y., and David, S. (2003) Glycosylphosphatidylinositol-anchored ceruloplasmin is required for iron efflux from cells in the central nervous system. *J. Biol. Chem.* **278,** 27144-27148

Johnson, D. A., Osaki, S, and Frieden, E. (1967) A micromethod for the determination of ferroxidase (ceruloplasmin) in human serums. *Clinical Chemistry* **13,** 142-150.

Kato, N., and Esaka M. (1999) Changes in ascorbate oxidase gene expression and ascorbate levels in cell division and cell elongation in tobacco cells. *Physiologia Plantarum* **105,** 321–329

Kisu, Y., Harada, Y., Goto, M., and Esaka, M. (1997) Cloning of the pumpkin ascorbate oxidase gene and analysis of a *cis*-acting region involved in induction by auxin. *Plant. Cell. Physiol.* **38,** 613-617

Kosman, D. J. (2010) Multicopper oxidases: A workshop on copper coordination chemistry, electron transfer, and metallophysiology. *J. Biol. Inorg. Chem.* **15,** 15-28

Kramer K.J. (1982) Ascorbic acid and the growth and development of insects. *Advances in Chemistry Series.* **200,** 275-291

Lane, D.J., and Lawen, A. (2008) Non-transferrin iron reduction and uptake are regulated by transmembrane ascorbate cycling in K562 cells. *J. Biol. Chem.* **283,** 12701-12708

Lang, M., Braun, C. L., Kanost, M. R., and Gorman, M. J. (2012a) Multicopper oxidase-1 is a ferroxidase essential for iron homeostasis in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A.* **109,** 13337-13342

Lang, M., Kanost, M. R., and Gorman, M. J. (2012b) Multicopper oxidase-3 is a laccase associated with the peritrophic matrix of *Anopheles gambiae*. *PLoS One.* **7,** e33985

Lin, L. S., and Varner, J. E. (1991) Expression of ascorbic acid oxidase in zucchini squash (*Cucurbita pepo* L.). *Plant Physiol.* **96,** 159-165

Linster, C.L., and Van Schaftingen, E. (2007) Vitamin C. Biosynthesis, recycling and degradation in mammals. *FEBS J.* **274,** 1-22

Messerschmidt, A., Ladenstein, R., Huber, R., Bolognesi, M., Avigliano, L., Petruzzelli, R., Rossi, A., and Finazzi-Agró, A. (1992) Refined crystal structure of ascorbate oxidase at 1.9 Å resolution. *J. Mol. Biol.* **224,** 179-205

Nakamura, K., and Go, N. (2005) Function and molecular evolution of multicopper blue proteins. *Cell. Mol. Life Sci.* **62,** 2050-2066

Necochea, R., Valderrama, B., Diaz-Sandoval, S., Folch-Mallol, J. L., Vazquez-Duhalt, R., and Iturriaga, G. (2005) Phylogenetic and biochemical characterization of a recombinant laccase from *Trametes versicolor*. *FEMS Microbiology Letters* **244,** 235-241.

Oberbacher, M. F., and Vines, H. M. (1963) Spectrophotometric assay of ascorbic acid oxidase. *Nature.* **197,** 1203-1204.

Osaki, S. (1966) Kinetic studies of ferrous iron oxidation with crystalline human ferroxidase (ceruloplasmin). *J. Biol. Chem.* **241,** 5053-5059.

Padayatty, S.J., Katz, A., Wang, Y., Eck, P., Kwon, O., Lee, J.H., Chen, S., Corpe, C., Dutta, A., Dutta, S.K., and Levine, M. (2003) Vitamin C as an antioxidant: evaluation of its role in disease prevention. *J. Am. Coll. Nutr.* **22,** 18-35

Philpott, C.C., and Protchenko, O. (2008) Response to iron deprivation in *Saccharomyces cerevisiae*. *Eukaryot. Cell.* **7,** 20-27.

Sagun, K.C., Cárcamo, J.M., and Golde, D.W. (2005) Vitamin C enters mitochondria via facilitative glucose transporter 1 (Glut1) and confers mitochondrial protection against oxidative injury. *FASEB J.* **19,** 1657-1667

Singh, A., Severance, S., Kaur, N., Wiltsie, W., and Kosman, D.J. (2006) Assembly, activation, and trafficking of the Fet3p.Ftr1p high affinity iron permease complex in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **281,** 133355-13364.

Shleev, S., Tkac, J., Christenson, A., Ruzgas, T., Yaropolov, A. I., Whittaker, J. W., and Gorton, L. (2005) Direct electron transfer between copper-containing proteins and electrodes. *Biosensors and Bioelectronics.* **20,** 2517-2554

Smirnoff, N. (2000) Ascorbic acid: metabolism and functions of a multi-facetted molecule. *Curr. Opin. Plant. Biol.* **3,** 229-235

Stoj, C. S., Augustine, A. J., Zeigler, L., Solomon, E. I., and Kosman, D. J. (2006) Structural basis of the ferrous iron specificity of the yeast ferroxidase, Fet3p. *Biochemistry.* **45,** 12741-12749

Stoj, C., and Kosman, D.J. (2003) Cuprous oxidase activity of yeast Fet3p and human ceruloplasmin: implication for function. *FEBS Lett.* **554,** 422-426

Wimalasena, K., and Dharmasena S. (1994) Substrate specificity of ascorbate oxidase: unexpected similarity to the reduction of dopamine β-monooxygenase. *Biochemical and Biophysical Research Communications.* **203,** 1471-1476.


DmMCO1
AgMCO1
TcMCO1
MsMCO1

Figure 3.1 Alignment of MCO1 Orthologs.

MCO1 orthologs from *Anopheles gambiae* (AgMCO1), *Drosophila melanogaster* (DmMCO1), *Tribolium castaneum* (TcMCO1) and *Manduca sexta* (MsMCO1) were aligned. Predicted signal peptides are highlighted in gray. Von Willebrand factor domains are highlighted in cyan. Cysteine-rich regions are highlighted in green. The ten histidines and one cysteine that are expected to coordinate copper ions are highlighted in yellow. Predicted transmembrane regions at the carboxyl-terminal ends of MCO1 orthologs are in blue color. Three cupredoxin-like domains (after cysteine-rich region) are divided by orange lines. The sequence that was used for making recombinant protein of each isoform is between two red lines. The extra residues in longer version of TcMCO1 and MsMCO1 recombinant proteins are in red color. Three conserved putative iron binding residues are highlighted in pink. The arginine residue that was mutated in AgMCO1 and DmMCO1 to generate uncleaved recombinant protein is highlighted in black. NCBI accession numbers: DmMCO1, NP_609287; AgMCO1, AAN17505; TcMCO1, NP_001034514; MsMCO1, AAN17506.

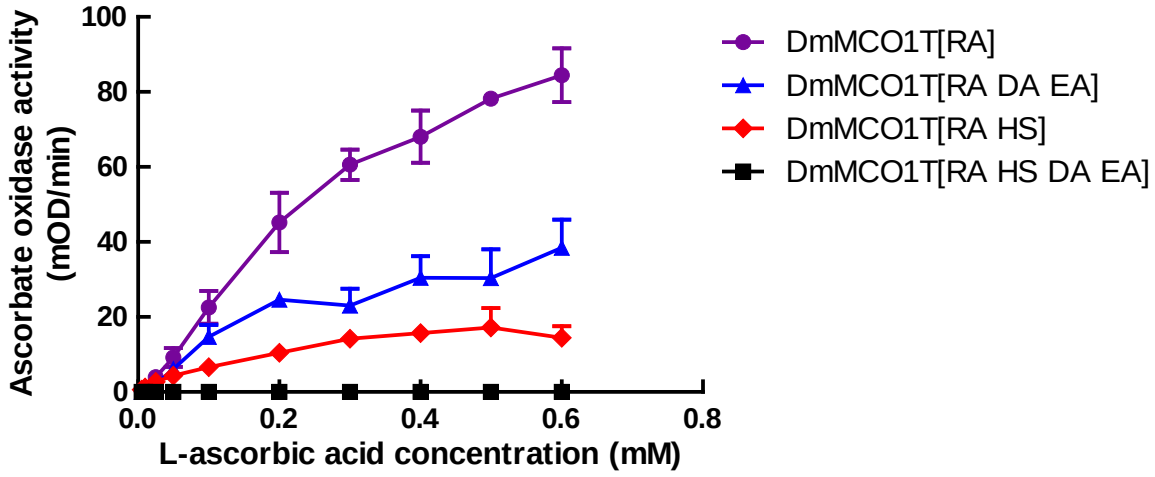

Figure 3.2 Comparison of Ascorbate Oxidase Activity of Different *Drosophila melanogaster* MCO1 Isoforms.

The amount of each MCO1 isoform used in the reaction was 0.2 µg.

Table 3.1 Laccase Activity of MCOs

Enzyme	Hydroquinone			Dopamine		
	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m
DmMCO1T	62 ± 2.1	14.4 ± 0.91	4	19.0 ± 1.1	7.0 ± 0.93	3
DmMCO1T[RA][a]	63 ± 2.1	10.0 ± 0.60	6	13.9 ± 0.8	7.1.± 0.91	2
DmMCO1T[RA HS]	238 ± 7.8	13.9 ± 0.85	17	55.5 ± 1.9	7.1 ± 0.59	8
DmMCO1T[RA DA]	81 ± 2.9	12.9 ± 0.87	6	8.4 ± 0.8	4.4 ± 1.15	2
DmMCO1T[RA DA EA]	102 ± 3.1	9.6 ± 0.63	11	20.4 ± 0.8	14.3 ± 1.00	1
DmMCO1T[RA HS DA EA]	91 ± 4.5	10.5 ± 1.07	8	8.3 ± 0.3	5.0 ± 0.42	2
AgMCO1T	95 ± 7.8	31.9 ± 3.75	3	15.5 ± 1.1	35.2 ± 3.59	0.4
AgMCO1T[RS]	53 ± 2.4	34.2 ± 2.16	2	6.0 1.2	37.8 ± 10.48	0.2
TsMCO1Ts	311 ± 31.0	10.8 ± 2.19	29	19.2 ± 3.1	47.2 ± 9.97	0.4
TsMCO1Tl	301 ± 12.6	12.3 ± 1.00	24	5.1 ± 0.4	20.6 ± 2.38	0.2
MsMCO1Ts	44 ± 2.7	53.2 ± 4.19	0.8	31.9 ± 1.2	37.6 ± 2.01	0.8
MsMCO1Tl	48 ± 4.5	62.1 ± 7.10	0.8	30.8 ± 2.7	32.7 ± 4.18	0.9
AgMCO3[b]	1571 ± 123	5.2 ± 0.57	302	274 ± 4.0	1.6 ± 0.08	171
TcLac2A[c]	213 ± 5.9	1.0 ± 0.11	213	41 ± 1.0	0.8 ± 0.06	51
TvLacα	1963 ± 73.4	0.038 ± 0.006	51661	692 ± 10.8	0.055 ± 0.005	12597
Fet3p[d]	131 ± 5.0	25.5 ± 2.5	5	ND	ND	ND

Note: k_{cat} in min^{-1}; K_m in mM; k_{cat}/K_m in min^{-1} mM^{-1}; [a]data are from (Lang et al., 2012a); [b]data are from (Lang et al., 2012b); [c]data are from (Gorman et al., 2012); [d]data are from (Stoj et al., 2006); ND, not determined

Table 3.2 Ferroxidase Activity of MCOs

Enzyme	Method	k_{cat}	K_m	k_{cat}/K_m
DmMCO1T	apoTf	40 ± 2.4	0.26 ± 0.2	156
DmMCO1T[RA]	apoTf	42 ± 2.7	0.12 ± 0.02	359
DmMCO1T[RA HS]	apoTf	35 ± 4.8	0.10 ± 0.03	350
DmMCO1T[RA DA]	apoTf	54 ± 14.3	0.38 ± 0.15	139
DmMCO1T[RA DA EA]	apoTf	39 ± 1.8	0.13 ± 0.01	295
DmMCO1T[RA HS DA EA]	apoTf	40 ± 3.3	0.21 ± 0.03	191
AgMCO1T	apoTf	3 ± 0.2	0.12 ± 0.01	30
AgMCO1T[RS]	apoTf	2 ± 0.2	0.11 ± 0.03	16
TcMCO1Ts	apoTf	3 ± 0.7	0.45 ± 0.16	7
TsMCO1Tl	apoTf	9 ± 6.8	1.71 ± 1.49	5
MsMCO1Ts	apoTf	3 ± 0.2	0.21 ± 0.02	16
MsMCO1Tl	apoTf	9 ± 1.9	1.41 ± 0.35	6
AgMCO3	apoTf	0.4 ± 0.04	0.09 ± 0.02	5
TcLac2A	apoTf	1 ± 0.1	0.33 ± 0.05	3
TvLac-α	apoTf	372 ± 6.6	0.15 ± 0.005	2407
Human ceruloplasmin[a]	O_2 uptake	30.3 ± 1.6	0.0083 ± 0.0015	3650
Fet3p[b]	O_2 uptake	40.1 ± 1.4	0.0049 ± 0.0008	8000

Abbreviation: apo Tf, apo-transferrin

Note: k_{cat} in min^{-1}; K_m in mM; k_{cat}/K_m in min^{-1} mM^{-1}; [a]data from (Stoj and Kosman, 2003); [b]data from (Stoj et al., 2006); ND, not determined

Table 3.3 Ascorbate Oxidase Activity of MCOs

Enzyme	K_m or K_{half}	k_{cat}	k_{cat}/K_m
DmMCO1T*	0.29±0.05	2570±315	8806
DmMCO1T[RA]*	0.27±0.05	1480±170	5516
DmMCO1T[RA HS]**	0.20±0.06	294±34	1487
DmMCO1T[RA DA EA]**	0.36±0.14	765±145	2104
DmMCO1T[RA DA EA HS]	No detectable activity		
AgMCO1T*	0.12±0.02	916±84	7559
AgMCO1T[RS]**	0.20±0.04	568±43	2785
TcMCO1Ts*	0.32±0.01	12912±492	39766
TcMCO1Tl*	0.42±0.02	10541±504	25357
MsMCO1Ts*	0.49±0.28	3295±1322	6742
MsMCO1Tl*	0.57±0.29	2342±830	4074
Cucurbita sp. AO*	0.17±0.01	21264±996	127558
AgMCO3 **	0.64±0.26	148±36	233
TvLac-α**	0.21±0.06	29±3.43	135

Abbreviation: AO, ascorbate oxidase

Note: *Allosteric sigmoidal model (K_{half}); **Michaelis-Menten model (K_m)

Chapter 4 - Conclusions and Future Prospects

Multicopper Oxidase-related Protein

This study describes a novel protein, multicopper oxidase-related protein (MCORP), which shares sequence identity with MCOs, but lacks most of the highly conserved residues necessary to coordinate the T1 and T3 coppers. The goal of this study was to investigate biochemical features and possible physiological functions of MCORP.

We identified MCORP orthologs in many insect species, but not in other invertebrates or vertebrates. MCORP is predicted to have an amino-terminal signal anchor, which makes the protein membrane-bound, and it is predicted to be located in the endoplasmic reticulum. Expression of full length *Anopheles gambiae* MCORP (AgMCORP) in insect cells supported that it is membrane-bound. Sequence analysis suggested that each MCORP molecule binds to one copper atom. However, ICP-MS analysis of recombinant *Tribolium castaneum* MCORP (TcMCORP) demonstrated that the recombinant protein contains very little copper. The low copper content of recombinant TcMCORP might suggest that TcMCORP is a copper-binding protein that does not require copper as a cofactor. As expected, recombinant TcMCORP had no detectable laccase activity.

TcMCORP and AgMCORP are constitutively expressed in many different tissues at a low level. RNAi for TcMCORP resulted in lethality at the time of molting and decreased longevity of adults, which indicated that TcMCORP is an essential gene. In addition, knockdown of TcMCORP in females led to severe developmental defects of the ovary and reduced the number of eggs laid; therefore, TcMCORP function is required for reproduction. MCORP might be used as a target for pest control.

Although TcMCORP does not have laccase activity, we did not test whether it can oxidize other substrates of MCOs, such as ascorbate, iron and copper. However, MCOs with preferences for non-phenolic substrates (such as iron or ascorbate) are able to oxidize laccase substrates (Lang et al., 2012; Dayan and Dawson, 1976; Quintanar et al., 2007). Thus, it is unlikely that MCORP can oxidize a natural substrate that we did not test.

During expression of recombinant TcMCORP, we supplemented the medium with 0.1 mM copper sulfate for copper loading onto the protein, but we did not add other metals into the medium. It is possible that MCORP needs other metal(s) to mediate the oxidation of a substrate.

In future experiments, other metal(s) could be added to the medium during expression, followed by analysis of metal content and oxidase activity of the purified protein.

Based on amino acid sequence alignment of each MCORP ortholog and yeast Fet3p, MCORP orthologs from different insect species have two conserved histidines that correspond to the two histidines that coordinate the T2 copper atom in Fet3p, so we hypothesized that MCORP may bind to one copper atom. However, we do not know how similar the structure of MCORP is to typical MCOs. For example it is not know whether the two histidines are indeed buried in the protein so that they can coordinate copper, or they are just located on the protein surface, or if other cysteine or histidines buried in the protein could possibly coordinate copper. If we can purify extremely pure and a sufficient quantity of recombinant MCORP, it is possible to get it crystallized. Once we have the crystal structure, we would understand the structural features of the protein better.

Currently we do not know the molecular mechanisms of TcMCORP's involvement in insect reproduction. The only conclusion we made is that TcMCORP is required for reproduction. Female reproduction of insects includes vitellogenesis and oogenesis. During vitellogenesis, yolk proteins, such as vitelloginin, are synthesized in the fat body, secreted into the hemolymph and transported inside the oocyte by vitelloginin receptor (Tufail and Takeda, 2009). In *T. castaneum*, female reproduction is regulated by juvenile hormone, 20-hydroxyecdysone, and nutritional signaling (Parthasarathy and Palli, 2011). We could test whether knockdown of TcMCORP influences the expression of vitellogenin, vitellogenin receptor, or genes coding for proteins involved in juvenile hormone and 20-hydroxyecdysone action. Such experiments might help to provide clues to the biological aspects of egg development that are affected by TcMCORP.

Given the constitutive, ubiquitous expression of MCORP and its lethal knockdown phenotype, MCORP may play a role in a basic cellular process, especially at the time of molting. Thus, we can perform RNAi in insect cells, such as *T. castaneum* cell lines, to see if it causes cell death. If so, it would suggest MCORP possibliy functions in a basic cellular process. If not, it would suggest MCORP functions in processes that are not carried out at the cellular level.

MCORP orthologs were only identified in insect species but not vertebrates, possibly because vertebrates do not molt during their development, and, they do not need such a gene. MCORPs were found in most insect species with sequenced genomes but not some dipterans,

such as *D. melanogaster*. MCORP and the need for its function appear to have been lost in some dipteran lineages in the course of evolution.

Multicopper Oxidase 1

Multicopper oxidase 1 (MCO1) orthologs from four insect species, *Drosophila melanogaster, A. gambiae, T. castaneum* and *Manduca sexta*, have much lower laccase activity than the known insect laccases, suggesting MCO1 orthologs are unlikely to be laccases. *D. melanogaster* MCO1 (DmMCO1) does have ferroxidase activity, but it has much lower affinity for iron than known ferroxidases. A low concentration of ferrous iron in hemolymph and a low affinity of MCO1 for ferrous iron would suggest that MCO1orthologs probably do not function as ferroxidases. MCO1 orthologs from the other three insect species have even less ferroxidase activity, similar to that of insect laccases. These results suggest that MCO1 orthologs do not function as ferroxidases.

All MCO1 orthologs tested oxidized ascorbic acid with high efficiency, and their K_ms are similar to that of known ascorbate oxidase. They are much better at oxidizing ascorbic acid than diphenols or ferrous iron. Laccases serving as negative controls have little ascorbate oxidase activity. Mutations of putative iron-binding residues affect the ascorbate activity of MCO1but not its ferroxidase activity. Thus, insect MCO1 orthologs may function as ascorbate oxidases.

The biological roles for ascorbate in insects are not well understood, and there is no clear indication from previous research how dehydroascorbate might function in insect biology. One possibility is that dehydroascorbate may participate in iron metabolism or transport. A previous study (Lane and Lawen, 2008) showed that incubation of human K562 erythroleukemia cells with dehydroascorbate resulted in accumulation of ascorbate in the cells, and consequent release of ascorbate into the medium. In addition, incubation of the cells with dehydroascorbate led to a dose-dependent stimulation of iron reduction and uptake from radiolabeled [^{55}Fe] ferric citrate. It is a very nice report showing ferric iron reduction and uptake is causally linked to ascorbate cycling across the plasma membrane. MCO1 function could be studied in insect cell lines (e.g. *D. melanogaster* or *T. castaneum* cell lines). MCO1 expression in the cell lines could be decreased by RNAi to determine whether this changes the amount of ascorbate or dehydroascorbate in the medium and in the cells. Knockdown experiments could also be performed to test whether MCO1 expression affects iron reduction and uptake in insect cells.

We can also try to test if MCO1 knockdown results in a change in the amount of ascorbate or dehydroascorbate *in vivo* in *D. melanogaster*, *T. castaneum*, or *A. gambiae*. I hypothesize that knockdown of MCO1 would result in more ascorbate and less dehydroascorbate; if so, the result would support MCO1 functions as an asorbate oxidase.

Immunohistochemistry data of tissues from *D. melanogaster* and *A. gambiae* indicate that MCO1 is located on the basal side of epithelial cells in midgut and Malpighian tubes, but not from *T. castaneum* and *M. sexta*. We can try immunohistochemistry with tissues from the two species and determine if MCO1 orthologs are all located in the basal side of the cells. If MCO1 has the same functions in different insect species, the cellular location is expected to be conserved in other insect orders.

We have spent several years to study insect MCOs, and the focus of most insect MCO studies has been cuticle sclerotization. We have identified five MCO genes in insects, and we hypothesized that insect MCOs have functions in addition to their role in cuticle tanning. MCO1 seems to be common in insects, and its functions are unkncown. Based on our activity study of insect MCO1, MCO1 orthologs may function as ascorbate oxidases. So far, ascorbate oxidases have not been identified in other organisms except for in plants. This is the first report on identification of ascorbate oxidase in insects. During our study of insect MCOs, we identified MCORP in some insect genomes. Because MCORP is not present in other invertebrates and vertebrates, and because MCORP function is essential for insect viability and reproduction, MCORP might be useful as a new target for insect control.

References

Dayan, J., and Dawson, C. R. (1976) Substrate specificity of ascorbate oxidase. *Biochem. Biophys. Res. Commun.* **73,** 451-458

Lane, D.J., and Lawen, A. (2008) Non-transferrin iron reduction and uptake are regulated by transmembrane ascorbate cycling in K562 cells. *J. Biol. Chem.* **283,** 12701-12708

Lang, M., Braun, C. L., Kanost, M. R., and Gorman, M. J. (2012a) Multicopper oxidase-1 is a ferroxidase essential for iron homeostasis in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A.* **109,** 13337-13342

Parthasarathy, R., and Palli, S. R. (2011) Molecular analysis of nutritional and hormonal regulation of female reproduction in the red flour beetle, *Tribolium castaneum*. *Insect Biochem. Mol. Biol.* **41,** 294-305

Quintanar, L., Stoj, C., Taylor, A. B., Hart, P. J., Kosman, D. J., and Solomon, E. I. (2007) Shall we dance? How a multicopper oxidase chooses its electron transfer partner. *Acc. Chem. Res.* **40,** 445-452

Tufail, M., and Takeda, M. (2009) Insect vitellogenin/lipophorin receptors: Molecular structures, role in oogenesis, and regulatory mechanisms. *J. Insect Physiol.* **55,** 88-104

Appendix A Alignment of Insect MCORPs and MCOs for Phylogenetic Analysis

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                  ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      5         15         25         35         45         55
AgMCO1            CKRACTLGRK PETCYYRFRL EWYRTLSKAC YN---CPYNA TDCERPHCIT GDGVRRNVAV
AgMCO2A           CARACREGEP PRICYYHFTV EYYTVLGAAC QVC-TPNATN TVWSHCQCVL ADGVERGILT
AgMCO3            CLRECDNTQP -RICHFSWTM EHYHVMGPAC RDC-AKG-NH TDCYHPACIT ADGVERGVMS
AgMCO4            CDRICTEREA PRVCYFRWIA EHYAAMGSAC GDC-RWG-NR SHCFHPQCIT ADGMERGVLA
AgMCO5            CDRHCVPGDP PLTCHFRWKL ENYATMGSAC WDC-RLG-NR AHCFHPQCIT ANGLERGVFA
AaAAEL007802      CRRECMRGRK PMNCYYRFKM EWYETLSKAC YE---CPYNV TDCERPHCIA ADGVSRSVIV
AaAAY29698        CARACREGEP PRICYYHFTA EYYTVLGAAC QVC-TPNATN TVWSHCQCVL ADGVERGILT
AaAAEL001667      CMRSCEDTQP -RVCYFKWVL EHYHSMGPAC KRC-ADG-VH SDCYLPACLT ADGFERGVMS
AaAAEL001632      CDRVCNATET PRICYFSWVA ENYAAMGSAC KDC-RWG-NH TDCSHPQCIT ADGMERPVVS
AaAAEL001640      CDRTCSVDEP SRICYYKWVL ESYAAMGSAC WDC-IRG-NR THCFHPQCVT ANGMERSIVS
AaAAEL001672      CDRKCTVGEP SRVCYFHWVL ENYSAMGSAC WDC-LRG-NR AHCFHPQCIT ANGMERAMVA
AmLOC724890       CRRNCVDEAP PMQCHYIFRL EAYHTMSKAC YD---CPFNV TDCFRKHCIP ADGIERSILV
AmLOC410365       CARACRENEP PRICYYHFTL EYYTVLGAAC QIC-TPNATN VVWSDCQCVL ADGVERGILT
AmLOC552811       CVRNCTDNEQ PKICYYFFHI EFYTTVGPAC DIQ-GSN--- ------QCIL ADGIEKTLIP
DmMCO1            CNRDCQVGAE PMTCRYKFVV EWYQTFSKAC YD---CPRNL TDCSRPHCVM GDGLERSITV
DmLac2            CARACREGEP PRICYYHFTL EYYTVLGAAC QVC-TPNATN TVWSHCQCVL ADGVERGILT
DmMCO3            CRRVCQQG-Q SQNCYYQLVV HNYQRLGPEC QR---CQFDE RACASEHCIY GDGVANPVMA
DmCG32557         CRRDCADK-Q PMTCYYYMVV HYDDTMAETC KRYDGIALAT QLAANDDCKY ADGLESEVMV
BmMCO1            CERECKEGED PMVCYYHFNL EWYQTMSKAC FN---CPFNE TDCFRPDCIP ADGMNRPLNV
BmLac2            CARACRENEP PRICYYHFTL EFYTVLGAAC QVC-TPNATN VVWSHCQCVL ADGVERGILT
MsLac1            CERECKEGEE PMVCYYHFNL EWYQTMSKAC YN---CPFNE TDCSRPDCIP ADGMNRALSV
MsLac2            CARACRENEP PRICYYHFTL ELYTVMGAAC QVC-APNATN VVWSHCQCVL ADGVERGILT
TcLac1            CARKCVKDSV PMTCRYTFLL EWYHTLSKAC YD---CPYNT QDCYREDCIP GDGNKRSIIV
TcLac2A           CARACREGEP PRICYYHFTL ELYTVLGAAC QVC-TPNATN TVWSHCQCVL ADGVERGILT
ApLOC100165676    CQRECRAGEP PKTCEYRFKV EWYYTMSKAC YD---CPYNI TDCYRPDCVP ADGVAKPIIV
ApLOC100164049    CARACREGEP PRICYYHFTA ELYNVLGAAC QVC-TPNATN TLWSHCQCIL ADGVERGMLA
PhPHUM024710      CERPCKKG-K LMTCKYNFVI EYYSVMGKGC VD---CPFNK SHCSLKNCIP GDGIKRTVLV
PhPHUM554290      CARACREGEP SKICYYHFTL ELYNVLGAAC QVC-TPNATN TIWSNCQCVL ADGVERGILT
AgMCORP           CDRVCNRADW PRICRYELVV EKRTFRPGST PST-----DN VTLTGGSKST EQPITVSYYT
TcMCORP           CDRPCHDLDW PMICRIKIQI E----NKRPC KDC------- ---------- ---TSSEIIA
AaMCORP           CKRPCNRADW PRICRYRLVI EKISLPKFLM NHK-ISNENG AATPDGSGKL SRQVESHYFL
CqMCORP           CDRPCKRADW PRICRYRLVI EKRLMPKSLL ATT---DQAA ATTTGGHLAW QHQQHRHYFL
BmMCORP           CDRECHELDW PMICRVKLVI EVYKTLSKSC GNC------- -GECPSTCIS ADGRERGVLT
MsMCORP           CDRECHELDW PMICRVKLVI EVYKTLSKSC GNC------- -GLCPPTCIS ADGRERGVLT
CfMCORP           CDRPCHHLDW PMICRVKLTL EVFQSLSKSC GDC------- -ACLAKHCVS TDGQRRGILT
MrMCORP           CDRPCHHLDW PMICRLKLTL EVFQSLSKSC GDC------- -ACLHNHCIT ADGQRRGILT
PhMCORP           CSRPCHELDW PMICRIKLTL ERFQTLGNPC KG---CPGNI SDCYINGCIT VDGQSKGILT
BtMCORP           CDRPCHHLDW PMICRLKLTL EVFQSLSKSC GDC------- -ACLSDHCVS ADGQRRGILT
BiMCORP           CDRPCHHLDW PMICRLKLTL EVFQSLSKSC GDC------- -ACLSDHCVS ADGQRRGILT
ApMCORP           CDRECHELDW PLICRYKIVL ETQKIQNNCQ KCS-----SN NQTECQHDIY CDRFSEKIIT
HsMCORP           CDRPCHHLDW PMICRVKLTL EVFQSLSKSC GDC------- -ACLANHCVA ADGQRRGILT
DpMCORP*          CDRECHELDW PMICRVKLVI EVYKTFSKSC NSC------- --ECPAMCIT ADGRERGVLS
AfMCORP           CDRPCHHLDW PMICRVKLTL EVFQSLSKSC GDC------- -ACLADHCVS ADGQRRGILT
DpMCORP           ---------- ------MTEA S----NPR-- ---------- ---------- ------TIIT
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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     65         75         85         95        105        115
AgMC01           INRMMPGPAI EVCENDIIVV DVENHLMGES TTIHWHGLHQ RRTPYMDGVP HVSQCPISPG
AgMC02A          VNRMIPGPSI QVCENDRVVI DVENHMEGME LTIHWHGIWQ RGTQYYDGVP FVTQCPIQQG
AgMC03           LNRKIPGPTI SVCRHDLIVV DITNAMAGTS AAIHWHGLHQ RATPYMDGVP FITQCPIGFG
AgMC04           LNRRIPGPTI HVCRHDLIVV DVVNHMEGLE STIHWHGAHQ YDTPWMDGVP MITQCPIPNG
AgMC05           INRRVPGPPI HVCKHDSIVV DVENQLEGLG STIHWHGFHQ KATPWMDGVP MVTQCPIPQD
AaAAEL007802     INRMMPGPSI EVCENDIITV DVENHLMGDS TTIHWHGLHQ KRTPYMDGVP HISQCPISPG
AaAAY29698       INRMIPGPSI QVCENDRVVI DVENHMEGME LTIHWHGIWQ RGTQYFDGVP FVTQCPIQQG
AaAAEL001667     INRQVPGPAI QVCKDDLIVV DMTNAMGGTA TAMHWHGLHQ RDTPHMDGVP FVTQCPIEFM
AaAAEL001632     LNRQMPGPAV IVCRNDIIVI DLLNHMEGSS TTIHWHGMHQ TQTPWMDGVP MVTQCPIPAG
AaAAEL001640     INRKMPGPLI FVCQGDTIVV DVSNEMEGMS ATIHWHGFRQ MQSPWMDGVP MVTQCPIAPS
AaAAEL001672     INRKMPGPPI FVCRGDTIVV DVSNEMEGMS NTIHWHGFHQ LKSPWMDGVP MLTQCPIAPS
AmLOC724890      VNRQMPGPAI EVCQGDRITV DVINLLHSES TTMHWHGQHH VKTPYMDGVP YVSQCPILPG
AmLOC410365      ANRMIPGPSI QVCQGDKVVI DVENHIEGME VTIHWHGVWQ RGSQYYDGVP FVTQCPIQEG
AmLOC552811      INRQLPGPPI EVCLNDRVVV DVQNAAMGME ATIHWHGLFQ NGFQYYDGVP YVTQCPIASS
DmMC01           VNRMMPGPAI EVCEGDEIVV DVKNHLLGES TSIHWHGLHQ KKTPYMDGVP HITQCPITPH
DmLac2           ANRMIPGPSI QVCENDKVVI DVENHMEGME VTIHWHGIWQ RGSQYYDGVP FVTQCPIQQG
DmMC03           VNRMVPGPSI ELCENDTVVV DVLNYLS-EP TTMHWHGVHM HRTPEMDGAP FITQYPLQPG
DmCG32557        VNGQLPGMNI EVCYGDTVVA DVINSMH-ET TTIHWHGMHQ RLTPFMDGVP HVTQYPIEAG
BmMC01           INRKMPGPAI EVCQHDRVIV DVENDLMTEG TTVHWHGQHQ KGTPYMDGTP YVTQCPIPPE
BmLac2           ANRMLPGPSI QVCENDKVVI DVENHMEGME VTIHWHGIWQ RGSQYYDGVP FVTQCPIQQG
MsLac1           VNRKMPGPAI EVCQDDRIIV DVENDLMTEG TTVHWHGQHQ RGTPYMDGTP YVTQCPILPE
MsLac2           ANRMLPGPSI QACENDKVVI DVENHMEGME VTIHWHGIWQ RGSQYYDGVP FVTQCPIQQG
TcLac1           VNRKMPGPSV EVCLGDEVII DVVNHLSSDS TTIHWHGHHQ KNSPYMDGVP FVTQCPIHPG
TcLac2A          ANRMIPGPSI QVCEGDKVVI DVENHIEGNE VTLHWHGVWQ RGSQYYDGVP FVTQCPIQQG
ApLOC100165676   INRSLPGPSI QVCLGDTVMV DVENAMMEES TSVHWHGHHQ RNSPYMDGVP YVTQCPVPPH
ApLOC100164049   VNRMLPGPSI QVCEGDKVVI DVLNHMHGME LVIHWHGIHQ KGTQYYDGVP YVTQCPIHEG
PhPHUM024710     VNRQMPGPRI DVCHGDTVEV KVTNKLMDIS TTIHWHGILQ KETPYMDGVP HVSQCPIGPQ
PhPHUM554290     ANRMIPGPSI QVCEGDKVVI DVENRMEGQA ASIHWHGVWQ RGTQYSDGVP FVTQCPIQEG
AgMCORP          VNGRYVGPTL TVCENDFLVV DVENRIPGES ITLHWTGQSQ RRTPFMDGVP MITQCPIASF
TcMCORP          VNGQSPGPAI QICQNDILVV DVVNKMPGHS LAIHWRGQPN VEAPFMDGVP LVTQCPILSY
AaMCORP          VNGHHTGPTL EVCKNDFLVI DVENRIPGRS ISLHWTGQTQ KRTPFMDGVP MISQCPITSY
CqMCORP          VNGRHTGPAL TVCERDFIVI DIVNRIPGQS IAIHWTGQSQ RRTPFMDGVP MITQCPIASY
BmMCORP          ANRALPAPTL HVCHNDILVV DVVHRAPAHA LSIHWRGQPQ KETPFMDGAP MLTQCPQPAY
MsMCORP          ANRALPAPPL HVCHNDILVV DVVHRAPAHT LSIHWRGQPQ KETPFMDGAP MLTQCPQPAY
CfMCORP          ANRQMPGPSI QVCENDILVI DVINRLPGKA MAIHWRGQTQ VEMPYMDGAP LITQCPIPSY
MrMCORP          ANRQLPGPTI QVCENDILVI DVINRLPGKA AAMHWRGQSQ VESPFMDGAP LITQCPIPSY
PhMCORP          VNRQFPGPTL EVCQYDIVLV DIVNRIPGQS FGVHWRGQSQ SETPFMDGVP MITQCPIPSL
BtMCORP          ANRQLPGPII QVCENDILVV DVINRIPGKT AAMHWRGQTQ IETPHMDGAP LVTQCPIPSY
BiMCORP          ANRQLPGPII QVCENDILVV DVINRIPGKT AAMHWRGQTQ IETPHMDGAP LVTQCPIPSY
ApMCORP          ANRQVPGPSI RVCENDIMVI DIVNRIPGHS VSVHWRGQWQ KETPVMDGAP MVTQCPILPH
HsMCORP          ANRQMPGPSI QVCENDILVV DVINRLPGKA TAVHWRGQTQ LEMPYMDGAP LVTQCPIPSY
DpMCORP*         ANRELPAPAF HVCQNDILVV DVVHRAPAHA LSIHWRGQPQ KETPFMDGAP MLTQCPQPAY
AfMCORP          ANRQMPGPTI QVCENDILVV DVINRLPGKA AAIHWRGQSQ LETPYMDGSP LVTQCPIPSY
DpMCORP          ANRQIPGPPI QVCQNDILIV DVINRVPGKS VTLHWRGQPN HEAPFMDGVP MVTQCPIPSY
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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     125        135        145        155        165        175
AgMC01           TTFRYTFRAD NPGTHFWHSH TGMQRGDGAF GALIIRK--D NDIQELLYDH DLSEHVITVQ
AgMC02A          NTFRYQWTG- NAGTHFWHAH TGLQKLDGLY GSIVVRQPPS RDPNSHLYDF DLTTHIMLVS
AgMC03           NTFRYAFLAT EPGTQFYHSH SGHHKVNGHY GALIVREPKR VDPNGDLYHY DTPAHVILGS
AgMC04           AAFRYAFNAS EPGTQLYHSH SGHQKANGHY GLFVIRSP-- TDINRHLYDY DLSEHHIITS
AgMC05           TTFRYQFTAV EAGTQFYHSH AGFQKANGHY GMVVVRDP-- SDLNQAHYDY DLSEHRIIIA
AaAAEL007802     TTFRYTFKAD NAGTHFWHSH TGMQRGDGAF GPLIIRR--D NDPQQILYDH DLSEHVITVQ
AaAAY29698       NTFRYQWTG- NAGTHFWHAH TGLQKLDGLY GSIVVRQPPS RDPNSHLYDF DLTTHIMLVS
AaAAEL001667     STFRYAFWAT EPGTQFYHSH AGHHKVNGHY GAMIIRQPEV NDPNAKLYDF DLPEHLIVAS
AaAAEL001632     NTFRYVFNAS EHGTQFYHSH AGHQKANGHF GLLVVRHP-- TDLNMNLYDY DLSEHHIIIA
AaAAEL001640     TTFRYRFVAE EAGTHWYHSH SGYHMANGHL GAAVVRNP-- LDVNMALYDF DLSEHVMLIS
AaAAEL001672     SSFRYTFQAE EPGTQWYHSH AGYHMANGHL GVAVVRNP-- LDVNADLYDF DLSEHVILLS
AmLOC724890      STFRYDFIAT EAGTHFWHSH SGFQRGDGVF GPLIVRTPPK ANWHKDLYDI DE--HIIQIS
AmLOC410365      STFRYQWTAG NEGTHFWHAH TGLQKMDGLY GSIVIRQPPS KDPNSNLYDY DLTTHVVLIS
AmLOC552811      STFRYDFVVK NSGTHFYHSH ISTHMLDGQI GSFIVKDPPR KNPHRDLYDK DE--IVIFLS
DmMC01           ATFRYSFPAD LSGTHFWHSH TGMQRGDGVF GALIIRKPKT AEPHGGLYDF DLSEHVMIVQ
DmLac2           NTFRYQWTG- NAGTHFWHAH TGLQKLDGLY GSVVVRQPPS RDPNSHLYDF DLTTHIMLIS
DmMC03           EVQRHEFKVD RSGSLWYHSH VGWQRGFGVA GAFVVRQTSQ ENQHSQLYDY DLVEHTLMIQ
DmCG32557        QAFRYRFEVD HGGTNWWHSH TEHQRAFGLA GPLVVRMPPK LNPHAHLYDF DMSEHVIMIQ
BmMC01           TTFRYQFNAT HTGTHFWHSH SGMQRADGAA GAFIVRKPKS QDPHGHLYDY DRTDHVMIVT
BmLac2           NTFRYQWQG- NAGTHFWHAH TGLQKLDGLY GSIVVRQPPS KDPNSHLYDY DLTTHVMLIS
MsLac1           TTFRYQFTAR HSGTHFWHSH SGMQRADGAA GAFIIRKPKS QEPYESLYDY DRSDHVMIVT
MsLac2           NTFRYQWQG- NAGTHFWHAH TGLQKLDGLY GSIVVRQPPS KDPNSHLYDY DLTTHVMLIS
TcLac1           MTFRYHFNVH NSGTHFWHSH SGFQRSDGTF GPFIVRVPEE DNPHAKLYDY DLSSHVITIL
TcLac2A          NTFRYQWIAG NAGTHFWHAH TGLQKMDGLY GSVVIRQPPA KDPNSHLYDY DLTTHVMLLS
ApLOC100165676   SSFRYVYLAD NEGTHFWHSH SGCQRGDGAF GSFVVRAPKS RDVHRDMYDV DV--HVITVT
ApLOC100164049   NTFRYQFDT- NSGTHFWHAH SGLQKIDGIY GSIVVRQPPS QDPNSHLYDY DLTTHVVLLS
PhPHUM024710     SSFLYKFYAD SPGTHIWHAH SAFQRGDGIY GGLVVRVPPE ENRHLSLYDF ELSEHVFTVM
PhPHUM554290     NTFRYQWNAE NAGTHFWHAH TGLHKLDGLY GSIVIRQAPS KDPNSHLYDY DLTTHVMLLS
AgMCORP          TRFQYKFQAD RAGTHLYHGF AGSERTQGLL GAFVVRSAYE QRQSPVLSAL -HNDPVWLVT
TcMCORP          TTFQYKFRVT SPGTHLYQAF SDSELDRGLF GALIVRQAEK NDLQRKYYDV DSRNHIIMIS
AaMCORP          TTFQYKFQAN RVGTHLYYGF SNDERKLGLI GALLVRSVHE QSQHPLTSQC -QDDLIWLIS
CqMCORP          TTFQYKFQAD HVGTHLYHGF SAEERGLGLV GAFVVRSEHE QRIHPVTSGC HQQELVWIIA
BmMCORP          TTFQYKFRAS AVGTHMYHAH SAADAADGLA GALVVRQSPR QDPLRKLYDT DASEHTIYVS
MsMCORP          TTFQYKFRAS AVGTHMYHAH SAADAADGLA GALIVRQSKR MDPLSKLYDI DSTEHTIFVS
CfMCORP          TTFQYKFRAS MPGTHLWHAH AGADITNGIF GALIVKQADL REPHRALYDI DDPNHVVLVT
MrMCORP          TTFQYKFRAS VAGTHLWHAH AGADVTNGIF GALIVKQADI KDPHRSLYDI DDSNHVVLVS
PhMCORP          TTFQYKFRAS EPGTHIWQVN TGEEYLDTLF GPLIVKKPYS KEINKNYYDT DDKKNVVVIH
BtMCORP          TTFQYKFRAS SAGTHLWHAH AGADVTNGIF GALIVKQADI KDPHRALYDI DDSDHVVLVS
BiMCORP          TTFQYKFRAS SAGTHLWHAH AGADVTNGIF GALIVKQADI KDPHRALYDI DDSNHVVLVS
ApMCORP          TTFQYKFRAA QAGTHWWQIL SGDELSDRVY GSFIVKQSKR REPHASIYDY DEIPHVLLVE
HsMCORP          TTFQYKFRAS VPGTHLWHAH AGADVSNGIF GALVVKQADL REPHRALYDI DDLNHVVLVT
DpMCORP*         TTFQYKFRAS AVGTHMYHAH SAADAADGLA GAFIVRQSPR LDPLASLYDV DATDHTIFVA
AfMCORP          TTFQYKFRAS AAGTHLWHAH AGDDVSNGIF GALIVKQADI RDPHRALYDI DDPSHVILVS
DpMCORP          TTFQYKFRAS KPGTHFYHAY MDADRSNGLF GALIVRKSDR TEPSKKMYDV DSKDHYILIS
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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    185        195        205        215        225        235
AgMC01           DLLINGRGKH VDKGRRYRFR LINAEFLNCP VELSIENHNL TVIASDGFGI QPLGSFVSYA
AgMC02A          DLLINGKGQT ITPGRRYRFR MINAFASVCP AQVTIEGHAL TVIATDGEPV HPVNTIISFS
AgMC03           DLLINGKGTT VRRGARFRFR FINAASHVCP LQLQIEDHMM EVIASDSFHL QPVDTLVSTS
AgMC04           DILINGRGRR VKKGYRYRFR LVSSGSQFCP FQLQIEKHRM QLIVTDGGAV QPVDTLISTS
AgMC05           DILINGRGRQ VEYGKRYRFR LISSGSQYCP FQMQIQNHSM LIISTDGGTV QPVDTLVSIS
AaAAEL007802     DILINGRGKH VEPKKRYRFR LINAEFLNCP VELSVEGHNL TVISSDSFDI NPLASIVSYA
AaAAY29698       DLLINGKGQT ITPGRRYRFR MINAFASVCP AQVTIEGHGL TVIATDGEPV LPVNTIISFS
AaAAEL001667     DLLINGRGTR VRKGGRYRFR FINAASHVCP LELQIANHTL EIIASDSYNL QPANTLVTTS
AaAAEL001632     DILINGRGRR VEQWKRYRFR MISSGSQFCP FQLQIEAHRM QIISTDGGAV QPVDTVISTS
AaAAEL001640     DILINGRGRR VRRNYRYRFR LISGGSQYCP FQLQIENHRM LVISTDGGAV KPVDTLISIS
AaAAEL001672     DILVNGRGR- ---------- ---------- ---NIEKHQK TIISTDGGAV KPVDTLISIS
AmLOC724890      DILINGLGRT VKQNTRYRFR LVNAEFLNCP IEISIDNHTM RVISSDGRDI EAAESLVSYA
AmLOC410365      DVLINGKGQT ITPGRRYRFR LINSFGSVCP SQITFEGHSL TIIATDGEAV QPVDTIISFS
AmLOC552811      DILINGLGNT VKKGERHRIR MINSFSTVCL TELRIEKHKL IIIAQDGENV KPVDKIVTST
DmMC01           DLLVNGKGRT VRRGFRYRFR IINAEYLNCP IVVSIDGHNL TAINSDGFDI EAVGSIVTYS
DmLac2           DMLINGKGQT ITPGRRYRFR MINAFASVCP AQVTIEGHGM TVIATDGEPV HPVNTIISFS
DmMC03           DILVNGKGRR VTPGYRYRMR VILNGIANCP VEFSIEQHRL LMISTDGNDI EPADGFFLTS
DmCG32557        DILINGRGRP VVRGGRYRFR VIFNGVSNCP ISFSIDKHDL VVIASDGNDI EPVQRIMFHG
BmMC01           DLLINGVGRN VEQGHRYRFR VINAEFLNCP IELSVDGHNI TVISSDGYDL EPATSLVTYA
BmLac2           DVLINGKGQT ITPGRRYRFR MINAFASVCP AQITFEGHNL TVIATDGEPV QPVNTIISFS
MsLac1           DLLINGVGRN VEQGYRYRFR VINAEFLNCP IEMSVDGHNI TVIASDGYDL EPATSLVTYA
MsLac2           DVLINGKGQT ITAGRRYRFR MINAFASVCP AQVTFEGHNL TVIATDGEPV QPVNTIISFS
TcLac1           DILVNGFGRT VEQGYRYRFR VINAGFLNCP IEVSIDNHTL SVISTDGSDF NAVDSLVTYA
TcLac2A          DLLINGKGQT ITPGRRYRFR MINSFASVCP AQLTIQGHDL TLIATDGEPV HPVNTIISFS
ApLOC100165676   DILINGRGRN VTRGKRYRFR LINAGFLNCP ISMSIDNHTF TVIATDGYNV QPVDSFVSYA
ApLOC100164049   DLLINGKGQT ITPGRRYRFR MINALASVCP AQITIQGHPL VLIATDGEPI QPVNTIISFS
PhPHUM024710     DLLINGKGRT VKQGYRYRFR LMNTGFLNCP IEMSIDEHNI TVISSDGEDL QPTVSLVSLA
PhPHUM554290     DLLINGKGQT ITPGRRYRFR MINSMASVCP VQLTIQGHSL ILIATDGEPV HPVNTIISFS
AgMCORP          ELGVNGQRD- LNGTLNLRVR LTYAV---CQ HWLELEDHRL QVLALDGNVL DHVSRILLHD
TcMCORP          EVLVNGKG-T VKRNKRYRFR VAFAGNSGCP VTLTVDNHLI KVIALDGNLV FPVTSVVLTK
AaMCORP          EFLINGNRS- VQPKSRYRLR VAYAANH-CQ RWLEIQDHNL TVIALDGNLV EPVERVALSD
CqMCORP          ELSINGRRS- AKPGLRYRLR VAFVSPEGCQ HWLEVDQHRL TVIALDGNLL EPVERVALSD
BmMCORP          ELLINGKGKS VEYGKRYRFR LAYGGSKSCP IQFSIEKHVL TLVALDGNGI EPVNSIELGR
MsMCORP          ELLINGKGRT VEHGKRYRFR LGYGGSKSCP IRFSIEKHVL ELVALDGNRI STVNSIGLGR
CfMCORP          HLLINGRGRT VIPGRRHRFR VANAGAGACP VTLFIDSHTL LLIALDGHPI EPVTSITLAK
MrMCORP          QLLINGRGRT VVPGRRHRFR IANAGAGSCP ITISVDAHPM LLIALDGQPV EPVTSITLAK
PhMCORP          TLLINGKTQN VTAGKRHRFR VIYVGEKNCQ IRFSIDEHKF FVIGFDGKSI QPVTSVKLFP
BtMCORP          QLLVNGRGRT VIPGRRHRFR VANAGAGSCP ITISVDAHPL LLIALDGQPV EPIASITLAK
BiMCORP          QLLVNGRGRT VIPGRRHRFR VANAGAGSCP ITISVDAHPL LLIALDGQPV EPIASITLAK
ApMCORP          YMRINGVEST VQSNSKYRFR TINTGVSQCP IEIKVHKHHL TVIAIDGHAI EPVDVIQVEP
HsMCORP          QLLINGKGRT VVPGRRHRFR VANAGAGACP VTLFIDGHSL LLIALDGHPI EPVTSITLAK
DpMCORP*         ELLINGKGKN VEYGKRYRFR LAYGGFKSCP INFSIDKHAI KLVALDGHII QTVTSIELGR
AfMCORP          QLLVNGRGRT VLPGRRYRFR LANAGAGSCP ITVLLDAHPL LLIGLDGQPV EPVASITLAK
DpMCORP          ELLVNGKA-N VKPGRRHRFR VAYTSLSGCP VNLTVDNHLL KIIELDGNPT NPVSSIRISK
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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    245        255        265        275        285        295
AgMC01           GERFDFIVKA NQPIGNYLIR FRGLMDCDER FTSAYQFAVL RYRGAPT--A PGVQLNSLNR
AgMC02A          GERYDFVITA DQPVGAYWIQ LRGLGECG-- IKRAQQLAIL RYARGP---P QGVVMNPLD-
AgMC03           GERYDFVLEA NGVKDTYWVR LRSLGPCA-- DLQLEQFAVL RYTTG--PFR NVATANHPN-
AgMC04           GERYDFVLSA NQKPGTYWVR VRAIGFCN-- IERREEFAVL SYEDEAHHVP SGTVLNNPN-
AgMC05           GERYDFVLTA NQPPGNYWVR VRGIGFCD-- QMRVEDFAIL SYRTPETAIP DGIVFNHQT-
AaAAEL007802     GERFDFILRA NQPVGNYLMR FRGLMDCDER FTSAYQVAVL RYKGAP---L EGMQLNSLNK
AaAAY29698       GERYDFVISA DQQVGAYWIQ LRGLGECG-- IKRAQQLAIL RYARGP---P QGVVLNPLD-
AaAAEL001667     GERYDFVVNA DQPTDDYWIR LRAIGPCD-- YRQISQVAVL SYQPMSVPEL NDVYVNHPN-
AaAAEL001632     GERYDFVLHA DQKPGDYWVR VRAVGFCN-- IQRKEEFAVL SYRSSSE-IP DGMTLNHPN-
AaAAEL001640     GERYDFVINA DQPVGNYWVR VRGAGFCS-- TLSVETFAIL SYADPSISTP MGQTLNEQT-
AaAAEL001672     GERYDFVLTA NQPPGNYWVR VRGIGFCN-- SQRVEGLAIL SYADSSIPTP LGRTLNHHM-
AmLOC724890      GERFDFVVET SQNIDNFWIR FRGLMDCDER FTKAYQVAIL RYEGATN--T DGQRINALNE
AmLOC410365      GERYDFVINA DQPVGAYWIQ VRSLGECG-- IPRAQQLGIL RYARGP---P QGVVLNPLD-
AmLOC552811      GERVDFILVA NQSVDSYWIQ ARGLGECA-- TTFMQQLAIL KYENGP---- DGVIYNGLNG
DmMC01           GERFDFVLNA NLEVGNYWIR LKGLMDCSEV FTSAFQVAIL RYEGAPD--- EGIELNVMNR
DmLac2           GERYDFVISA DQPVGAYWIQ LRGLGECG-- IRRAQQLAIL RYARGP---P QGVVMNPLD-
DmMC03           AERFDFVLEA NQYKKNYWIR IKGYEQCE-- NRNIYQGAVL SYRGSARS-E DLILVNDFRF
DmCG32557        AERFDFVLHA NQEVSNYWIR VKGYSFCA-- KNQLHQEAVL HYRDADTR-A PGKTLNELGD
BmMC01           GERYDFILDA NNEIDNYWIR FRGLMDCDEV FTKAKQVGVL HYEGAME--N EGLQLNALNK
BmLac2           GERYDFVIEA NNIPGAYWIQ VRGLGECG-- IKRAQQLGIL RYARGP---P QGVVMNPLD-
MsLac1           GERYDFILDA NNEIDNYWIR FRGLMDCDEI YTRAKQVAVL HYEGAMD--N EGLQLNALNK
MsLac2           GERYDFVIEA NNIPGAYWIQ VRGLGECG-- IKRAQQLGIL RYARGP---P QGVVMNPLD-
TcLac1           GERFDFIVTA DQPQDVYWMH FRGLMDCDER FTRAYQVAVL EYKGTQT--R EGKQLNPLNK
TcLac2A          GERYDFVINA DQTPGAYWIQ LRGLGECG-- IRRVQQLGIL RYAKGP---P QGVVLNPLD-
ApLOC100165676   GERWDFVVEA TANVGNYWMR FRGLMDCDER FTKAFEVSIL HYDGAGD--H SGIQLNALNK
ApLOC100164049   GERYDFIINA EQPVGAYWIQ VRGLGECG-- NKRVQQLAIL RYARGP---P QGVVMNPLD-
PhPHUM024710     GERFDFVVEA NKKIDNYWIR FKGLLDC--K FKEAYQTAIL HYDGADID-R TGLQVNKVNS
PhPHUM554290     ---------- -----AYWIQ ARGLGECG-- IARAQQLAVL RYARGP---P QGVVLNPLD-
AgMCORP          GVRMDVALTR L---RDYEIR FTPHGTTSSC GRAVFRLHYD AERAAANDAH RSEELQLDLT
TcMCORP          GERLDFVLKT DQKIKRHYVR VKS--CLG-- ----EGLALL NYELG----- -------RVL
AaMCORP          GNRVDLIVHT N---QDYEIR LVATGDSPRS -------VYT GYNSNECHLG STNNFRLKYV
CqMCORP          GDRIDLILET SGAGQEYEIR LVSVAS---- --------YN G-SCEQEHL- SSSRFKLKYV
BmMCORP          GERVDFILEA KRAPGVYKMS VVAHPDCQD- -N-LKGVAEL VYTNK----L NKVYRKFSTV
MsMCORP          GERADFVLEA KMIPGVYKIS VIADESCQN- -N-LEGIAEL VYYKN----N EHVTREFTTV
CfMCORP          GLRADFVLKA SKHVASYWMN VHTTKECGT- -STINGAAIL NYKGS-SEII GMNQLAAMTN
MrMCORP          GERADIVLKA NKRVASYWMH VHTSKECGT- -SPINGAAIL SYKGSTMEST S--RVTMTTN
PhMCORP          GERFDFVLSA NQKMASYSMK ATPVQCLN-- --LLSTSALI QYEGNEKKNP EFFEISTSFD
BtMCORP          GERADFVLKA NKRVASYWMN VHTSKECAV- -SPINGAAIL EYKGSSTKDV G---VAMTTN
BiMCORP          GERGDFVLKA NKRVASYWMN VYTSKECAI- -SPINGAAIL EYKGSSTKDV G---VAMTTN
ApMCORP          GETLDFILTT IKNQGIYDMT VTSEGHCK-- DSNHTHTLYI HYNSTLYN-- ---TISDTDN
HsMCORP          G-RADFVLRA SQRIGSYWMH VHTAKECGT- -STVNGAAVL NYKGS-TEVV ETTRLAATTN
DpMCORP*         GERADFILDA NQAIGVYKIR VVADKSCQD- -D-LEGEAEL IYKNQ----D STINRIFSTV
AfMCORP          GNRADFVVKA NKRIASYWIN VHASKECGT- -SPINGAAIL SYKGSSTEDS ND-RVAMTTN
DpMCORP          GERIDFILKA SQEIGAYYLS VKS--SCES- -SDLHGLAVI NYEGR----G EKNTKLKRHF
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   305        315        325        335        345        355
AgMC01          GP--GAENVI TIAETSALDQ ED--LLLLRN ETDYKFYVYY DFYGKDNPHQ QVVNNTNRLY
AgMC02A         AQCNVQRDD- AICVSQLKNA KE-DRALLQD KPDVKIFLPF RFYLYRPEEF LVAPTG-DHV
AgMC03          ATCGRPEFG- DYCITDFQAY DT-DEDVING VPDHQLTFGF YNYPVSFESY MNIYGS-VMM
AgMC04          ATCYVPGDD- DLCVADLESH EVHDDDLIDA APNKTFRILF NTFTADPAVY MTVVLT-LNN
AgMC05          APCYTPNDT- YICAADLEAY EVFDTGLIDA VPDRTFFLGF HVIEANNSLY ATVREG-FNT
AaAAEL007802    GT--GHSDTM SIAETSSRDQ ED--LLLLRE KTDFKFYVYY DFYAKDNPHK DVINNTNRLF
AaAAY29698      AQCNVQRDD- AICVSQLKNA KD-DRALLQE KPDVKIFLPF RFYLYRPEEF LVAPTG-DHV
AaAAEL001667    TTCGVSKP-- DVCITDFQAY ET-DDDVING VPDMQFILGF ENYPMKFEDF MNIHDD-IVL
AaAAEL001632    STCYQPGDQ- FVCASDLEAH EVQDDALIDA VPDKKFYVAF NTFTADTSLY MTVALT-LNN
AaAAEL001640    ATCYNSEDQ- FTCAADLETH EAHDDKLINA TPDVRLFLGF KMMHPDNRWF ITVRED-FNT
AaAAEL001672    TECYKEGDD- FTCAADLEAH EVHDHELIDS HPDVRLFLGF KVIEANNSMY ATVRNK-FNT
AmLOC724890     GT--ETNNSI SIPLLKAMDK ND---KSNTA DPDYQFYVSY DFYKKDNPHN QVKN-TKQVL
AmLOC410365     AICNRQRED- AICVSQLKNA RQ-DQGILQQ RPDVKIFLPF RFLFYRPEEF LVAPTG-DHV
AmLOC552811     TLCNTNITEP VLCINQLESL ES-ND-LLKV EPDERHILPF WFFNYTDTSF FNANDR-SQL
DmMC01          GP--GYPDTK TVAEMRALPI YDHDHDTLKP EADYKFFIYY DFYTKNNPDD MEMTQQNRLY
DmLac2          AQCNRQRND- AICVSQLKNA LE-DRGILAE KPDVKIFLPF RFFVYRAEDF LVAPTG-DHV
DmMC03          KP----ANST AISSLRQSLD KD-------N NVGTVALRSV DPVPWTRYTG SRTAPNGE-V
DmCG32557       DA----SGAR AG-------- ---------N SISLANLNAQ RPEPEVAPSN AFEVRQGEGF
BmMC01          GE--EEDETI SVAEMKSLDG YD---ESLKE IADYQFYVAY DFYAKNNSHY QVPEKVNRLY
BmLac2          ARCNISRND- AICVSQLKNA QN-DPAILQE RPDVKIFLPF RFFVYRPEMY LVAPGG-DHV
MsLac1          GE--EENETI SVAEMRSLAG YD---DSLKE IADYQFYIAY DFYAKNNSHY QVPEQVNRLY
MsLac2          ARCNILRND- AICVSQLKNA KH-DPAILQE RPDIKIFLPF RFFVYGPETY LVAPSG-DHV
TcLac1          GT-EADSSFV TLPQLHSLDE WD---DTLKE KADFQYYVSY DFYKMNHPVH NVTNTTLQNL
TcLac2A         ARCNEIRPD- AICVSQLKNA LS-DKGILRE KPDVKIFLPF RFHIYTPEDH LVAPNG-DHV
ApLOC100165676  GSGLMDTATV SELEDATPPK ND---LRLEK KPDVTLFMSY DFYSLDNPHK QVTHRSEQVY
ApLOC100164049  AVCDRPRTD- AICVNQLKNA KV-DKGLLQE RPDVKIFLPF KFLFYRPDEY LVAPGGGDHV
PhPHUM024710    RG--GHENYL IISELSSLPD KYQ--PALKP EPDFRFYIPF DFYPLDSDYP ----GTQRPG
PhPHUM554290    AVCNRERSD- AVCVSQLKNA KP-DEGILQQ RPDVKIFLPF RFLFYRPEEY LVAPSG-DHV
AgMCORP         GTACGDTMLC P------QDL PG-LPKDLQH GYDTRLEFTI GTRQQAGTG- --FGEPTHET
TcMCORP         DTSLCHSQIG KVCLSDLNFV EKM-ETVK-- TAPKTVYLSL GSQIVN---- --VSGNFGSR
AaMCORP         RSETADSTLP P------SGA TGDFPPELRE -VERQISLVL -TKRKVERQ- --FGERFYDV
CqMCORP         GTEAAGTQIL MKEPDDAGDA RGTFPAELRD -VDKTIRLVL -TKRAVQRE- --LGELFQDI
BmMCORP         ISVQCEDE-S VLCLTEARGY EQM-SELTKT -PDRTLYVPF NYSTRRISA- -RVESWGQS-
MsMCORP         FSDRCTAE-N VLCLTDVHSL DKL-ADLVSY -PDKIIYVPF NYSTRQISA- -RVESWGQT-
CfMCORP         PIEKCGGQ-E SLCVMDMQNI RKM-RALAMP KMDVTLYLPI NYKM-QATE- -IGNRGTETR
MrMCORP         PAEKCENP-E SLCVTEIQAL RKI-SVLAKP RTDVTIRLPI NYKF-QAND- -VVN-GAETR
PhMCORP         EDCKSDEFS- RICIQNIRSY EKI-KELAIE KPDFKYYLII DSTMSTNHN- --IGMKETVR
BtMCORP         PAAKCENP-E SLCVTELHAL RKI-AALGQP KTDVTIRLPI NYKL-QTSD- -VGNSGVEMR
BiMCORP         PAAKCENP-E SLCVTELHAL RKI-AALGKP KTDVTIRLPI NYKL-QTND- -VGNSGVEMR
ApMCORP         LAKPAEYGER HLSITSLDSL PY---ELSAV ELKNTIYLGF SSIKYQLGE- -------SWS
HsMCORP         PVDKCGGR-E NLCVMDVQNV RKM-HVLAKP RMDVTMYLPI NYRVNQETE- -IDN-GGATR
DpMCORP*        ASDNCVSD-T VLCLDEIHGA EKL-SELAEP -VDEVLYVPF NYSTRQMSA- -RFESWGQT-
AfMCORP         PADKCGNP-G SLCVTELHSL RKM-SGLGKQ KTDVTVRLPI NYKL-QTSD- -VGERFVGTR
DpMCORP         DTSLCRTESG KVCLGDVKSL DKM-KELRKE TVDRNIFLAI DYKYGERET- -EQYADLRKK
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                    365        375        385        395        405        415
AgMC01           TPQLNHISMR MPPVPFLP-C SHVLQIPLHA TVEMVMIDNH PFHLHGHAFR VVGMDRVSRN
AgMC02A          ISLIDEISYL SAPAPLLSQC THKVDIPLNA IVEVVLVDSH PFHLHGYAYN VVGIGR-SPD
AgMC03           QGAINNISLA YPPFSLLTQC THRVKINLGD IVELYILDNH PFHLHGYQMF VMEMSQDRRV
AgMC04           IGVTNNISMV FPDFPLLTQC LHRLKVALND VVEMSLIDYH PFHLHGHRFI VTGMGQLP-Q
AgMC05           IGATNMISFV PPSFPLLIQC THRMKVKHND VIEIVLYDYH PFHLHGHRFI VTDSGSFPSD
AaAAEL007802     TPQLNHISMR MPKIPMMP-C SHVVQVPLNS TVEMVLIDNH PFHLHGHAFR VVGMERLAGN
AaAAY29698       ISLIDEISYL SAPAPLLSQC THKVDIPLNA IVEVVLVDSH PFHLHGYAYN VIGIGR-SPD
AaAAEL001667     QGAINNISFT YPPFSLLTQC IHRLKIPLHA LVELYILDNH PFHLHGYQMY VMEMGQDRST
AaAAEL001632     IGITNNISMV YPSFPPLTQC VHRLKVDLND IVEMSLIDYH PFHLHGHRFI VTGMGQLPTR
AaAAEL001640     IAAANNISFR YPSFPLLIQC THRIKVKLND IVELTLYDYH PFHIHGHRFI ITDMGRLPES
AaAAEL001672     IGVANNISFI SPSFPLLIQC THRLKVKHNV LVEFVLYDYH PFHLHGHRFI ITDMGMLPDE
AmLOC724890      TPQLNHISMK LPPMPLLS-C THVLRVNLDS VVEIILVDNH PFHLHGYQFR VIAMERIGEN
AmLOC410365      ISLVDEISFT FPPAPPLSQC THQVDIPHNA VVEVVLVDSH PFHLHGYAFN VIGIGR-SPD
AmLOC552811      LSIFNDIAYE NPASNLLTQC AQIIKTKLNN VVELVMYDDH PFHLHGFAFQ VFSVGQFWPI
DmMC01           TPQLNHITLN FPSLALLP-C HHVLQVPLGA VVEMIIVDNH PFHLHGNAFR VMGLERLGEN
DmLac2           ISLIDEISYL SAPAPLTSQC THKIDIPLNA IVEVVLVDSH PFHLHGYGFS VIGIGR-SPD
DmMC03           LFQISDISYN SPGISLLQGC VNVMRLPAYR PLEMVVANTH PFHIHGFTFR LVGQGVLGNL
DmCG32557        RFQMDDISFS MPKMSLLQTC SNVIQVPANQ QVEFVISSPH PIHLHGYTFR VVGMGVLGEQ
BmMC01           TPQLNHISMK MPSSPLLV-C SHVLSVKLNS VVEVVIVDNH PFHLHGHSFR VVGLRRLASD
BmLac2           ISLIDEISYM SPPAPLISQC THKVDIPLNA VVEIVLVDSH PFHLHGYSYN VIGIGR-SPD
MsLac1           TPQLNHISMK MPTSPLLI-C PHVLSVKLNA IVEVIIVDNH PFHLHGHSFR VVGLRRLNRT
MsLac2           ISLIDEISYM SPPAPLLSQC THKVDIPLNA VVEIVLVDSH PFHLHGYAYN VIGIGR-SPD
TcLac1           TPQLNYISMK LQSFPLLS-C THVVNIPLGT VVEMVLIDNH PFHLHGHSFR VVAMERVGSH
TcLac2A          ISLIDEISYM APPAPLISQC THKVDIPLNA IVEIVLVDSH PFHLHGYAFN VIGIGR-SPD
ApLOC100165676   TPQINKMSFK LPSFPLLS-C TNIIKVPLGS IVELFLIDNH PFHLHGHPFR VVAMERVGNH
ApLOC100164049   ISLVDEISYT SPGSPMISQC SHKVDIPRHA VVEVVLVDSH PFHLHGYSFN VIGMGR-SPD
PhPHUM024710     TPQLNHISME FPSFPIMT-C IHVLQVPIDS VVEIVLIDNH PFHLHGIKFR VLGMDRLGNN
PhPHUM554290     ISLVDEISYV APPAPPLSQC THKVDIPLNA VVEVVLVDSH PFHLHGYAFN VIGIGR-SPD
AgMCORP          VRSVNGLTFA FPPVLMVRDC VHVEHIEAGH RVEMVLINDY VYHLHGQSVF VVA---LAGR
TcMCORP          VFGVNNLTFT YPSSPLLTQC VHVEHIPLRA VAEIVLINEH IFHLHGYRFY VVGFRHFENA
AaMCORP          SYSVNGFSFI YPSVLMLQRC VHVESVELGH RVELVIVNAY TYHLHGYSFF VIASTRTTQH
CqMCORP          SYSVNGFSFV FPTAVMLRKC VHVEHVEAGH RVELVLINDH SYHLHGHSFY LVG--AAHFE
BmMCORP          ----DGHRFT YPASPLLTQC VHVKNIPLYS TVEIVMFDDH IFHLHGYGFY VTGVREFNRS
MsMCORP          ----DGHRFT YPASPLLTQC VHVKNIPLHS TVEIVMFDDH IFHLHGNSFY VTGIREFNTS
CfMCORP          VLNVNNVTFT YPSSPLLTQC VHVRHVPLGA TVEIILLDDL VYHLHGYSFY IVGARQFGRS
MrMCORP          ILNVNNVTFT YPSSPLLTQC VHVRYIPLDS TVEIILLDDL VYHLHGYNFY IVGARKFDRS
PhMCORP          IKQINNLTML LPSSPLVSQC VHVVKIPLNS VVELVLVNDH VFHLHGYSFR IVGISKVPTF
BtMCORP          VLNVNNATFT YPSSPLLTQC VHVRYIPLGA TVEIILLDDL VYHLHGYTFY VVGARKFGRS
BiMCORP          VLNVNNATFT YPSSPLLTQC VHVRYIPLGA TVEIILLDDL VYHLHGYTFY VVGARKFGRS
ApMCORP          LPNFNNMTMV LPSAPLLLQC THIIDLPLGS ATELVIFDSH SFYLHGHSFY VVGQKSKAFV
HsMCORP          VLNVDNATFT FPSSPLLTQC VHVRHVPLGA TVEIILLDDL VYHLHGYSFY VVGARQFGRS
DpMCORP*         ----DGHRFT YPASPLLTQC VHVKYIPLHS TVELIMFDDH IFHLHGYSFY VTDVRQMDTK
AfMCORP          VLNVNNATFT YPSSPLLSQC VHVRYIPLGA TVEIILLDDL VYHLHGYTFY VVGARKFGRS
DpMCORP          IYRINNLTFS YPASPLLTQC VHLEAVDLGT SVEIMIVEEH ILHFHGNHFY IVGSRQFERP
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                    425        435        445        455        465        475
AgMC01           TTIEDIRRMD EAPIKDTVTI PDGGYTIIRF IANNPGYWLF H-CHIEFHAE IGMSLVLKVG
AgMC02A          SNVKKINLKH APPLKDTIAV PNNGYVVLRF RADNPGFWLF H-CHFLFHIV IGMNLILQVG
AgMC03           PITLEIAQNI APPRKDTVSI PSRGYARVRF RADNPGFWLM H-CHYEWHTA VGMALVLQVG
AgMC04           FGTQSEKVDF VPPYKDTVSV PSRGYTRIRF RADNPGFWLV H-CHFEWHLG IGMSFVLQVG
AgMC05           ILT--DQIAY LPPYKDTQSI PNRGYVRIRF RADNPGFWLV H-CHFEWHLA DGMGLVLQIG
AaAAEL007802     ITAEEVKRLD EAPIKDTVTI PDGGYTIIRF IANNPGYWLF H-CHIEFHAE IGMSLVLKVG
AaAAY29698       SNVKKINLKH APPLKDTIAV PNNGYVVLRF RADNPGFWLF H-CHFLFHIV IGMNLILQVG
AaAAEL001667     PITMERAQKI APPKKDTVSV PSKGYTRVRF VADNPGFWLM H-CHYEWHTA VGMVLVLQVG
AaAAEL001632     IKHSSEKLRW IPPYKDTVSI PSRGYTKIRF RADNPGFWLV H-CHFEWHLG IGMSFILQVG
AaAAEL001640     AIN--SRLKY LPPFKDTISI PNEGFVKTRF RASNPGFWFV H-CHFEWHLG TGMGLVLQVG
AaAAEL001672     AKT--LRLKY LPPYKDTISI PNEGYVKVRF RANNAGFWLV H-CHFEWHLG TGMGLVLQVG
AmLOC724890      VTVDKVKALD KAPLKDTVTV PDGGYTVVRF HANNPGYWLF H-CHIEFHAE VGMSLIFKVG
AmLOC410365      KNVKKINLKH APPAKDTIAV PNNGYVIFRF RADNPGYWLF H-CHFLFHIL IGMNLILHVG
AmLOC552811      RNISRQDINE VPPGKDTAKI PMGGYVIVRF KADNPGWWLL H-CHFSWHHI TGMELVILVG
DmMC01           VTVEMIKQLD QPPVKDTVTI PDGGYTIIRF EASNPGYWLF H-CHIEFHAE IGMALVFKVG
DmLac2           SSVKKINLKH APPTKDTIAV PNNGYVVLRF RADNPGFWLF H-CHFLFHIV IGMNLILQVG
DmMC03           NDLRNIQELD RAVAKDTVQI PGQGYIIVRF ISNNPGFWLY H-CHVEAHAV QGMVAVLKIG
DmCG32557        -KIGQIEQID KAPLKDSVQV PAFGYTILRF YSNSPGYWMF H-CHISPHSE NGMAAVVRVG
BmMC01           TTIEEVKAFD EAPIKDTVTV PDGGYTVIRF KADNPGYWLF H-CHIEFHVE VGMALVFKVG
BmLac2           QNVKKINLKH APPAKDTIAV PNNGYVILRF RATNPGFWLL H-CHFLFHIV IGMSLVLQVG
MsLac1           TTIEEIKAFD EAPIKDTVTV PDGGYTVIRF KADNPGYWLF H-CHIEFHVE VGMALVFKVG
MsLac2           QNVKKINLKH APPAKDTIAV PNSGYVILRF RATNPGFWLL H-CHFLFHIV IGMSLVLQVG
TcLac1           VNVSEILKMD QAPLKDTVTV PDGGFTIIRF KATNPGYWLF H-CHIEFHVE VGMALVFKIG
TcLac2A          QNVKKINLKH APPAKDTIAV PNNGYVVLRL RANNPGFWLF H-CHFLFHIV IGMNLVLQVG
ApLOC100165676   TTVEEIEQMD RAPLKDTVTV PDGGFTILRF LADNPGYWLF H-CHIEFHVE VGMATVFKIG
ApLOC100164049   KNVKKINLKH APPLKDTIAV PNNGYVVFRF RADNPGYWLF H-CHFLFHIV IGMNLVLHVG
PhPHUM024710     TSEDLVRRLD KPPIKDTVTT PDGGYSILRF HAINPGYWMF H-CHIDFHVE MGMVLLFKVG
PhPHUM554290     RNIKKINLKH APPAKDTIAV PNNGYVVLRF RADNPGYWLF H-CHFLFHIV IGMNLVVHVG
AgMCORP          PGTALGALGG QPLQRDTIVV RRGSTVAVRF VANLAGLWLL RDIGSP-GWS RGLDVVLSVG
TcMCORP          PSTDEIKLLD QPAIKDTIRI PKNSVVALRF LADNPGFWML R-DEGSRGWT RGLDIVLQVG
AaMCORP          ENWQTQLLN- -PVLLDTVRI ESNSMVVLRF VASNAGLWML RDLDAEHEWS RGLDVLLNVR
CqMCORP          EGWQHQQLDR LPVRRDTVRI GRRSLVVLRF VASHAGLWLL RDLGAEHGWS RGLDVLLVVH
BmMCORP          LSKETVIKMN EPVLKDTIVI PKFGVVALRF KADNPGYWMM R-DERSTHWT RGLDFILKVG
MsMCORP          LAKEDVIKMN EPVIKDTIVI PKFGAVSLRF KADNPGYWMM R-DERSTHWT RGLDFILKVG
CfMCORP          VSLQEVKKLD KPPIKDTVIV PKFGVVALRF KADNPGYWML R-DEHAADWT RGLDVVLQVG
MrMCORP          VSLQELKSLD DTVAKDTVVV PKFGAVAIRF KANNPGYWML R-DEHSPYWT RGLDVILQVG
PhMCORP          VATKEQIKQF DPVVKDTLAL SSGYLYVIRF KADNPGYWLF D-EENSSHFS KGLSLVFKVG
BtMCORP          VSLHELKSLD DTVAKDTVVV PKFGAVALRF KADNPGYWML R-DEHAAEWT RGLDVILQVG
BiMCORP          VSLQELKSLD DTVAKDTVVV PKFGAVALRF KADNPGYWML R-DEHAAEWT RGLDVILQVG
ApMCORP          KSADHAKKLD SSVLKNTVVV PAAGVSVVRF IADNPGYWLF R-SEKTSEWS SGLSLIFRVS
HsMCORP          MSLQNIRNLD KAPAKDTIVV PKFGAVALRF KADNPGYWML R-DEHAADWT RGLDVILQVG
DpMCORP*         LEKETVMKMN QPVRKDTIVI PKFGVAALRF KADNPGYWMM R-DERSAHWT RGLDFVLKVG
AfMCORP          VSLQELKSMD DTVAKDTVVV PKFGAVALRF KANNPGYWML R-DEHAAEWT RGLDVILQVG
DpMCORP          MSRKEIRELD QPVLKDTIRI PRFGVVILRF LAKNPGIWML R-DENSHGWS KGLDLAIEVG
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                  ....|....| ..
                      485
AgMC01            EMLPAPANFP TC
AgMC02A           DLPPVPPNFP TC
AgMC03            EMVKAPADFP KC
AgMC04            EMKQAPKDFP RC
AgMC05            EMLKPPANFP RC
AaAAEL007802      EMVAAPHNFP TC
AaAAY29698        DLPPVPPNFP TC
AaAAEL001667      SFVKPPAGFP TC
AaAAEL001632      QMIKTPPGFP TC
AaAAEL001640      QMLKAPPGFP RC
AaAAEL001672      QMLKAPPDFP RC
AmLOC724890       DMLPVPRNFP LC
AmLOC410365       DLPPIPPNFP RC
AmLOC552811       DLPPIPKNFP KC
DmMC01            QMVPVPENFP TC
DmLac2            DLPPVPPGFP TC
DmMC03            QMKNIPARVR C-
DmCG32557         EMKMCPVSN- C-
BmMC01            DMPPVPREFP KC
BmLac2            DLPPVPPNFP TC
MsLac1            DMAPLPRDFP TC
MsLac2            DLPPVPPGFP TC
TcLac1            EMPPVPKDFP QC
TcLac2A           DLPPVPPNFP TC
ApLOC100165676    EMPPPPPGFP KC
ApLOC100164049    DLPPVPENFP RC
PhPHUM024710      MFKLPPDNFP KC
PhPHUM554290      DLPPVPPNFP RC
AgMCORP           PQPDIPRNFP AC
TcMCORP           DMVSTPTDFP TC
AaMCORP           TEINFPDDFP KC
CqMCORP           DEFDIPGDFP TC
BmMCORP           DLVQAPPDFP KC
MsMCORP           DFVQAPADFP KC
CfMCORP           DMIPPPQDFP KC
MrMCORP           DMASAPQDFP KC
PhMCORP           DFPNVPDNFP KC
BtMCORP           DMVPAPEDFP KC
BiMCORP           DMVPAPEDFP KC
ApMCORP           SFPQVPEDFP KC
HsMCORP           DMVAAPQDFP KC
DpMCORP*          DFVKAPADFP KC
AfMCORP           DMVPAPEDFP KC
DpMCORP           QMVSTPSNFP TC
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Figure A.1 Alignment of Insect MCORPs and MCOs for Phylogenetic Analysis.

The amino-terminal and carboxyl-terminal ends of sequences, which are highly variable, were left out of the alignment. Sequences beginning with the cysteine rich region (Ditmmer and Kanost, 2010) were aligned by ClustalW in MEGA5 and adjusted by eye. Gaps were omitted in the alignment for phylogenetic analysis. Abbreviations used are: Ag, *Anopheles gambiae*; Aa, *Aedes aegypti*; Cq, *Culex quinquefasciatus*; Tc, *Tribolium castaneum*; Dp, *Dendroctonus ponderosae*; Ms, *Manduca sexta*; Bm, *Bombyx mori*; Dp*, *Danaus plexippus*; Cf, *Camponotus floridanus*; Hs, *Harpegnathos saltator*; Bt, *Bombus terrestris*; Bi, *Bombus impatiens*; Mr, *Megachile rotundata*; Af, *Apis florae*; Ap, *Acyrthosiphon pisum*; Ph, *Pediculus humanus corporis*; Dm, *Drosophila melanogaster*; Am, *Apis mellifera*.