Insect Biochemistry and Molecular Biology 38 (2008) 817–824

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Characterization of the multicopper oxidase gene family in *Anopheles gambiae*

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ARTICLE INFO

Article history:
Received 27 February 2008
Received in revised form 3 July 2008
Accepted 3 July 2008

Keywords:
Laccase
Multicopper oxidase
Cuticle tanning
Sclerotization
Detoxification
Immunity
Metal metabolism

ABSTRACT

The multicopper oxidase (MCO) family of enzymes includes laccases, which oxidize a broad range of substrates including diphenols, and several oxidases with specific substrates such as iron, copper or ascorbic acid. We have identified five putative MCO genes in the genome of *Anopheles gambiae* and have cloned cDNAs encompassing the full coding region for each gene. MCO1 mRNA was detected in all developmental stages and in all of the larval and adult tissues tested. We observed an increase in MCO1 transcript abundance in the midguts and Malphighian tubules of adult females following a blood meal and in adult abdominal carcasses in response to an immune challenge. Two alternatively spliced isoforms of MCO2 mRNA were identified. The A isoform of MCO2 was previously detected in larval and pupal cuticle where it probably catalyzes sclerotization reactions (He, N., Botelho, J.M.C., McNall, R.J., Belozerov, V., Dunn, W.A., Mize, T., Orlando, R., Willis, J.H., 2007. Proteomic analysis of cast cuticles from *Anopheles gambiae* by tandem mass spectrometry. Insect Biochem. Mol. Biol. 37, 135–146). The B isoform was transcriptionally upregulated in ovaries in response to a blood meal. MCO3 mRNA was detected in the adult midgut, Malpighian tubules, and male reproductive tissues; like MCO1, it was upregulated in response to an immune challenge or a blood meal. MCO4 and MCO5 were observed primarily in eggs and in the abdominal carcass of larvae. A phylogenetic analysis of insect MCO genes identified putative orthologs of MCO1 and MCO2 in all of the insect genomes tested, whereas MCO3, MCO4 and MCO5 were found only in the two mosquito species analyzed. MCO2 orthologs have especially high sequence similarity, suggesting that they are under strong purifying selection; the A isoforms are more conserved than the B isoforms. The mosquito specific group shares a common ancestor with MCO2. This initial study of mosquito MCOs suggests that MCO2 may be required for egg development or eggshell tanning in addition to cuticle tanning, while MCO1 and MCO3 may be involved in metal metabolism or immunity.

1. Introduction

The multicopper oxidase (MCO) family of enzymes includes laccases, ferroxidases, cuprous oxidases, ascorbate oxidases and bilirubin oxidases (Sakurai and Kataoka, 2007). Laccases have exceptionally broad substrate specificity whereas the other family members tend to oxidize a single substrate *in vivo*: iron, copper, ascorbic acid, and bilirubin, respectively. A partial list of laccase substrates includes diphenols, substituted monophenols, diamines, *N*-hydroxy compounds, and lignins (Thurston, 1994; Baldrian, 2006). Laccases have been discovered in bacteria, fungi, plants and insects. MCOs that function as metal oxidases have been identified from bacteria, fungi and plants but not insects; ascorbate oxidases have been found mainly in plants; and bilirubin oxidases are fungal enzymes (e.g., Huston et al., 2002; Quintanar et al., 2004; Larrondo et al., 2003; Hoopes and Dean, 2004; Sakurai and Kataoka, 2007). Some of the functions associated with multicopper oxidases include pigmentation, morphogenesis, detoxification, and lignin degradation (Hullo et al., 2001; Mayer and Staples, 2002).

Despite their diverse functions, MCOs are evolutionarily and structurally related (Nakamura and Go, 2005; Hoegger et al., 2006). Most MCOs bind four copper atoms within two highly conserved copper centers (designated T1 and T2/T3). The substrate is bound near the T1 center, and dioxygen is bound near the T2/T3 center. Oxidation of a substrate occurs when an electron is transferred from the substrate to the T1 copper, which transfers the electron to the T2/T3 copper center where dioxygen is reduced to water (Solomon et al., 1996). The copper binding residues (10 histidines and 1 cysteine) in the T1 and T2/T3 centers can be used as signature residues to identify putative MCO genes (Nakamura and Go, 2005).

Of the insect genomes that have been investigated, each contains at least two putative MCO genes, and some genomes contain more (Dittmer et al., 2004). The genome of *Anopheles gambiae*, a species of mosquito, encodes five putative MCO genes (Dittmer et al., 2004). At present, the function of just one type of insect MCO is known: cuticle tanning catalyzed by laccase 2 (Lac2)

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doi:10.1016/j.ibmb.2008.07.001

orthologs. In *Manduca sexta*, MsLac2 is expressed in cuticle undergoing sclerotization (Dittmer et al., 2004), and it oxidizes substrates that have been implicated in cuticle sclerotization (Dittmer and Kanost, unpublished data). In *Tribolium castaneum*, RNAi mediated silencing of TcLac2 causes newly synthesized larval, pupal and adult cuticle to remain soft and pale instead of undergoing the normal hardening and darkening process (Arakane et al., 2005).

The functions of laccase-1 type enzymes and other insect MCOs are still unknown. In *T. castaneum*, silencing of TcLac1 had no effect on cuticle tanning or viability (Arakane et al., 2005). The putative Lac1 ortholog in *Drosophila melanogaster* (predicted gene CG3759) is expressed in Malpighian tubules (Wang et al., 2004) and is slightly upregulated in unspecified tissues in response to immune challenge suggesting a possible immune function (De Gregorio et al., 2001). MsLac1 is expressed in the midgut and Malpighian tubules of feeding stage larvae but not in later, non-feeding stage larvae or pupae. This expression pattern may indicate a role in detoxification of phenolic plant compounds or in metabolizing iron or copper in the larval diet (Dittmer et al., 2004).

Several additional functions of insect laccases have been proposed. One of the first suggested roles for an insect laccase was sclerotization of the egg case of a cockroach species, *Periplaneta americana* (Whitehead et al., 1960). A laccase-like activity in the saliva of a species of leafhopper, *Nephotettix cincticeps*, may detoxify monolignols found in plants by oxidizing the monolignols, which may then form nontoxic polymers prior to ingestion by the leafhopper (Hattori et al., 2005). It has been suggested that a laccase present in the midgut of *Anopheles stephensi* may play a role in defense against parasites (Sidjanski et al., 1997), that a laccase in parasitoid wasp venom may affect host responses to the parasitoid's eggs (Parkinson et al., 2003), and that laccases may be involved in hemolymph coagulation (Theopold et al., 2002). Finally, an uncharacterized phenoloxidase that could be a laccase has been implicated in mating plug formation in *Drosophila nasuta* (Asada and Kitagawa, 1988). It is important to note that these hypotheses of laccase function, except for catalyzing cuticle tanning, remain unproven.

The aim of this study was to characterize MCO genes in *A. gambiae*. Based on our understanding of the functions of non-insect MCOs and on the preliminary observations of insect MCOs described above, we predicted that mosquito MCOs may be involved in one or more of the following: cuticle sclerotization, pigmentation, eggshell tanning, mating plug formation, immune processes, detoxification of phenolics, or metal metabolism. Three approaches were used to identify potential functions of the *A. gambiae* MCOs. First, we analyzed the predicted amino acid sequences to identify similarities between the mosquito MCOs and MCOs from other organisms. Then, orthologs were identified by determining the phylogenetic relationships of MCO genes from seven insect species. Finally, mRNA expression profiles were analyzed by developmental stage, tissue specificity, response to blood feeding and response to immune challenge.

2. Materials and methods

2.1. 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 by Benedict (1997) with minor modifications. Briefly, larvae were reared at 27 °C and were fed a mixture of baker's yeast and ground fish food (Vitapro Plus Staple Power Flakes, Mike Reed Enterprises). Adults were maintained at 27 °C with 85% relative humidity and were fed 10% sucrose. Adult females were fed heparinized equine blood with a membrane feeder (Hemotek).

2.2. Tissue preparation for RNA isolation

2.2.1. Developmental profile

Eggs from an overnight collection were combined with eggs that were between one and two days old. Two day old larvae were a mix of first and second instar larvae; six day old, third and fourth instar larvae; and nine day old, a mix of fourth instar larvae and pharate pupae. The pupae collected were tan or black (pharate adults). Female and male adults were two days old.

2.2.2. Tissue profiles

Insects were dissected in phosphate buffered saline (PBS, 10 mM sodium phosphate, 175 mM sodium chloride, pH 7.2), and tissues were placed in tubes on dry ice until all samples were collected. The samples were stored at −80 °C until RNA isolation was done. Midguts and Malpighian tubules were removed from the abdomen of fourth instar larvae, and the remainder of the abdomen ("carcass") was retained. Midguts, Malpighian tubules, ovaries, and testes plus accessory glands were dissected from 29 adult males and 17 adult females (nine days old).

2.2.3. Blood meal profiles

Adult females (4–6 days old) were fed heparinized equine blood with a membrane feeder. Mosquitoes that took a full blood meal were maintained at 27 °C and 85% relative humidity, and they had access to sucrose solution but no dishes for egg laying. After 3, 6, 12, 24, 48, and 72 h, mosquitoes were chilled on ice and dissected in PBS. The midguts (with the gut contents removed), Malpighian tubules, and ovaries were collected. Each sample represented 18 insects.

2.2.4. Immune challenge profile

Adult females 3 days old were chilled on ice to immobilize them, then pricked with a minuten pin dipped in a paste of freeze-dried *Micrococcus luteus*, a Gram positive species of bacteria. Control mosquitoes were not pricked. After 1, 3, 6, and 12 h, mosquitoes were chilled on ice and dissected in PBS. The spermatheca, midgut, Malpighian tubules, ovaries and crop were removed from each mosquito, and the remaining abdominal tissue, which we refer to as the abdominal carcass, was retained for RNA isolation. Each sample represented six insects. The experiment was done twice.

2.3. RNA isolation and cDNA synthesis

Total RNA was isolated using the Ultraspec reagent method (Biotecx) or Tri reagent method (Sigma), and genomic DNA was removed with DNAseI. Pools of cDNA were synthesized with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) and oligo(dT) primers. One microlitre of cDNA was used for each PCR. Typically, 30 PCR cycles were done. For semiquantitative analysis of the effect of blood feeding or immune challenge on expression, the number of cycles required to produce faint but visible bands was empirically determined for each set of primers. The ribosomal protein S7 gene (Salazar et al., 1993) was used as a reference gene. At the start of this study, PCR products obtained from reactions using gene specific primers were cloned and sequenced to verify that the expected gene products had been amplified.

2.4. cDNA cloning

The cloning of a full-length MCO1 cDNA was described previously (Dittmer et al., 2004). (Note that we have changed the name