Current Topics

The 50th Anniversary and New Horizons of Cytochrome P450 Research:
Expanding Knowledge on the Multiplicity and Versatility of P450
and Its Industrial Applications

Recent Studies on Insect Hormone Metabolic Pathways Mediated by
Cytochrome P450 Enzymes

Masatoshi Iga and Hiroshi Kataoka*

Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo;
5–1–5 Kashiwanoha, Kashiwa, Chiba 277–8562, Japan.
Received January 16, 2012

In insects molting and metamorphosis are primarily under the control of two insect hormones, ecdysone
and juvenile hormone (JH). Physiological and biochemical studies of insect hormone metabolic pathways
suggested the involvement of P450 (CYP) enzymes in the pathways, but molecular details of the enzymes
were unclear. In recent years, the genome information and studies using molecular biology and genetics have
allowed us to understand enzymes in the ecdysteroid and JH metabolic pathways. Genome sequencing has
been accomplished in several insect species, and has shown the presence of 36–180 CYP enzymes. To date,
six and one CYP enzymes have been revealed in the biosynthesis and inactivation pathways of 20-hydroxyec-
dysone (20E), respectively. In the 20E biosynthetic pathway, correlation among the enzymes, substrates and
metabolites is elucidated in the late steps, but the enzyme(s) and intermediates in the early steps have not
been fully understood and are referred to as the ‘Black Box’. The gene expression of some CYP enzymes in
the 20E biosynthesis is modulated by neuropeptides and JH. Furthermore, involvement of a CYP enzyme is
found in both JH biosynthesis and inactivation pathways. Thus, recent studies have shown the importance of
CYP enzymes in insect hormone metabolisms.

Key words insect; P450; metamorphosis; ecdysteroid; juvenile hormone

1. INTRODUCTION

P450 (CYP) enzymes are heme-containing proteins that have monoxygenase activity, and they are involved in the
synthesis and inactivation of bioactive substances and xenobiotic metabolism. CYP enzymes constitute one of the largest
families and are distributed in a wide variety of organisms from bacteria to plants and animals, including insects.11 Genome
sequencing of many insect species has been accomplished over the past decade, following the first insect genome
sequencing project on the fruit fly Drosophila melanogaster in the year 2000. The number of CYP enzymes varies greatly in
insects, and 76 to 92 in flies, 105 to 180 in mosquitoes, 87 in the silkworm Bombyx mori, 134 in the red flour beetle Tribolium
castaneum, 46 to 106 in bees and wasps, 64 in the pea aphid Acyrthosiphon pisum and 36 in the body louse Pediculhus
humanus have been identified to date.2) The function of insect CYP enzymes is well studied in the field of xenobiotic
metabolism including their adaptation to plant chemicals and insecticide resistance.3) On the other hand, detailed function of
these enzymes involved in physiological phenomena was not fully understood until recently.

Molting and metamorphosis are essential for insect growth and development, and they are mainly controlled by two insect
hormones: a steroid hormone, 20-hydroxyecdysone (20E) and a sesquiterpenoid, juvenile hormone (JH). The functions of
these hormones are well studied, but the metabolic pathways were not fully understood. Numerous intensive physiological
and biochemical studies in insect hormone metabolism were made in the second half of the 19th century, but the molecular
detail of the enzymes was not elucidated. Recent updates using genome information, techniques of molecular biology
and genetics have allowed a better understanding of ecdysteroid and JH metabolic pathways. Here we describe that CYP
enzymes participate in the metabolic pathways of ecdysteroid and JH.

2. ECDYSTEROID BIOSYNTHESIS AND INACTIVA-
TION

During the postembryonic development in insects, ecdysone (E) is predominantly synthesized in the prothoracic glands
(PGs), and its biosynthesis and release are controlled by prothoracicotropic and prothoracostatic neuropeptides. The syn-
thesized E is secreted into hemolymph and converted to 20E in the peripheral tissues, such as fat body and midgut. Since
insects lack the genes encoding squalene synthase and other subsequent enzymes involved in the pathway of cholesterol
biosynthesis, they cannot synthesize cholesterol de novo.4,5) Therefore, the cholesterol used for the ecdysteroid biosynthe-
sis is derived from dietary cholesterol or phytosterols.

The ecdysteroid biosynthesis begins from conversion of cholesterol into 7-dehydrocholesterol (7dC). This step is medi-
ated by a Rieske oxygenase, designated as Neverland (Fig. 1A). The cholesterol 7,8-dehydrogenase activity of Neverland
has been confirmed by biochemical and genetic analyses.5,7)
The gene was first identified by a microarray analysis of *B. mori*, and the orthologs were identified in many species. Interestingly, the gene is evolutionarily conserved not only in insects but also in deuterostome species that do not produce ecdysteroids.  

7dC is the substrate used for the following conversion step, but the responsible enzyme and the metabolite have not yet been identified (Fig. 1B). The identified intermediate of E next to 7dC is 2,22,25-trideoxyecdysone (ketodiol), and the conversion of 7dC into ketodiol is apparently multiple steps. However, none of the intermediates has been identified, and the unclear conversion step is the so-called 'Black Box' (Fig. 1B). It is believed that the reactions in the 'Black Box' proceed very quickly and/or that the intermediates must be much more labile than the known intermediates. Various scenarios, including the CYP enzyme(s) mediated catalytic reactions have been proposed for the reactions in the 'Black Box.' In fact, analysis of the embryonic lethal and low ecdysteroid mutant strain in *Drosophila* and differential display in *Bombyx* revealed the involvement of CYP307A1/A2 (Spook/Spookier) in the 'Black Box.' Furthermore, involvement of a short-chain dehydrogenase/reductase (Non-molting glossy: Nm-g) was identified by positional cloning of a non-molting larval arrest mutant of *B. mori*, and the homolog was identified in *Drosophila* (Shroud). The larval arrest phenotype of the loss of *cyp307a2* function and *nm-g* mutant is rescued by feeding of ketodiol or later intermediates of 20E, but is not rescued by 7dC. Thus, it is clear that both the enzymes participate in the 'Black Box,' but their responsible reactions, substrates and metabolites have not yet been elucidated.

On the other hand, the last four hydroxylation steps for synthesizing the 20E are well studied. The ketodiol is sequentially hydroxylated at C-25 (Fig. 1C, ketodiol into 2,22-dideoxyecdysone; ketotriol), C-22 (Fig. 1D, ketotriol into 2-deoxyecdysone; 2dE) and C-2 (Fig. 1E, 2dE into E), and these are catalyzed by CYP306A1 (Phantom), CYP302A1 (Disembodied) and CYP315A1 (Shadow) in the PGs, respectively. The last conversion step of E into 20E (hydroxylation at C-20)
is catalyzed by CYP314A1 (Shade) in the peripheral tissues\(^7\) (Fig. 1F). These CYP enzymes were first identified from the mutant strains in *Drosophila* (Halloween mutant) and by microarray analysis in *Bombyx*, and the homologs have been identified in many other species.\(^8\)–\(^22\) In addition to insects, all these enzymes are identified in the water flea *Daphnia pulex* and some of these are identified in the spider mite *Tetranychus urticae*.\(^23\),\(^24\)

The gene expression regulatory mechanisms of ecdysteroidogenic CYP enzymes were investigated in *B. mori*. Prothoracicotropic hormone (PTTH) up-regulates the transcriptional level of cyp307a1, cyp306a1 and cyp302a1 in the PGs.\(^1,\(^5\),\(^23\),\(^25\) In addition to PTTH, Bommo-FMRFamide (BRFa) and JH also modulate the transcriptional level of certain CYP enzymes.\(^25\) BRFa and JH suppress the PTTH induced transcription of cyp307a1. Furthermore, BRFa suppresses the transcriptional level of cyp306a1 and cyp302a1. Thus, multiple factors differentially regulate the transcriptional level of CYP enzymes in ecdysteroid biosynthesis.

Recently, CYP4G1 in *D. melanogaster* and the closely related homolog of *B. mori*, CYP4G25 were reported to be candidates regulating the function of PGs and controlling ecdysteroid production or metabolism.\(^26\) *Drosophila* cyp4g1 is highly expressed in the ring gland (including PG cells) and the expression pattern of *Bombyx* cyp4g25 was in concert with the hemolymph ecdysteroid titer. In addition, the expression of *Bombyx* cyp4g25 was up-regulated by PTTH. Since *Drosophila* CYP4G1 and *Bombyx* CYP4G25 did not convert the known edysone intermediate, these enzymes seem to be involved in the conversion steps of the ‘Black Box,’ but the details remain unclear. Furthermore, our group performed a global analysis of ecdysteroid biosynthesis-related genes in the *Bombyx* PGs by next generation sequencing, and we identified several candidate genes, including CYP enzymes (data not shown). The tissue distribution and developmental profile of these candidate genes strongly suggested the involvement in the ecdysteroid biosynthesis, but the detailed functional analysis remains to be determined. Thus, it has not been elucidated whether all these candidates directly contribute to ecdysteroid biosynthesis, but they do seem to have important roles in ecdysteroid metabolism.

When the insects need to decrease the level of 20E, the excess 20E is eliminated from the body by direct excretion and/or conversion of 20E into inactive forms.\(^23\) In contrast with the 20E biosynthesis pathway, little is known of the inactivation pathway of 20E. One of these pathways is oxidation at C-3 by edysone oxidase which mediates the conversion of 20E into 3-dehydro-20-hydroxy edysone\(^28\)–\(^29\) (Fig. 1G). CYP18A1 has long been suspected of catalyzing the inactivation by CYP18A1 for proper development was shown in *Drosophila*.\(^31\) The cyp18al encodes an ecdysteroid 26-hydroxylase and this is the key enzyme inactivating the insect steroid hormone. CYP18A1 catalyzes not only 26-hydroxylation but also further oxidation of 20E, and it converts 20E into 20-hydroxyecdysone acid (Figs. 1H, I). The timing of cyp18al expression is consistent with the decrease of ecdysteroid titer in *Drosophila*.\(^31\) The cyp18al is widely expressed in various ecdysteroid target tissues, such as epidermis, fat body, salivary glands and eye-antenna imaginal discs. Since loss-of-function mutants, RNA interference (RNAi) strains and ectopic over-expression of CYP18A1 showed a lethal phenotype during metamorphosis, proper reduction of ecdysteroid titer by CYP18A1 is crucial for insect metamorphosis.

### 3. JUVENILE HORMONE BIOSYNTHESIS AND IN-ACTIVATION

Juvenile hormone (JH) is another important hormone controlling insect development and metamorphosis. The JH is synthesized in the corpora allata and secreted into the hemolymph. Several forms of JH (e.g. JH I, JH II, JH III and JH III bisepoxide) are identified in insects, and JH III is the most ubiquitous product.\(^33\) Here we describe the synthetic pathways of the JH III from acetyl-CoA and its inactivation pathways. The early steps of JH III biosynthesis pathway are conversion of acetyl-CoA to farnesyl pyrophosphate via mevalonate pathway (Fig. 2A). In some species, propionyl-CoA is used as substitute for acetyl-CoA and is converted into homofarnesyl pyrophosphate. The mevalonate pathway is conserved in both vertebrates and invertebrates, and is in common with a part of the cholesterol and steroid hormone biosynthesis pathways in vertebrates. On the other hand, the latter steps from farnesyl pyrophosphate to JH are insect specific pathways. Farnesyl pyrophosphate is converted into farnesonic acid via esteratic cleavage and following oxidation (Fig. 2B). An esterification by methyltransferase and an epoxidation of C-10, 11 by a CYP enzyme are required for the synthesis of JH from farnesonic acid. The order of reactions is different among the species. In *Lepidoptera*, epoxidation by CYP15C1 precedes esterification by JH acid methyltransferase (JHAMT)\(^34\)–\(^35\) (Figs. 2C, D). On the other hand, epoxidation by CYP15A1 follows methylation by JHAMT in *Orthoptera* and *Dictyoptera*.\(^36\) (Figs. 2E, F). Interestingly, JHAMT was identified in *Drosophila* but a clear ortholog of CYP15A1 was not identified, therefore the possibility of using a different pathway remains in the *Drosophila* JH biosynthesis.\(^37\) In fact, a global analysis of CYP enzymes in *Drosophila* revealed specific expression of CYP6G2 in corpora allata, but the detailed function of the enzyme has not been elucidated yet.\(^38\) JH biosynthesis is mainly regulated by two neuropeptides, allatotropin and allatostatin.\(^39\) Recently, a neurotransmitter, glutamate mediated regulation of JH biosynthesis was suggested in *Drosophila*.\(^40\) Glutamate may stimulate the expression of JHAMT via N-methyl-d-aspartate subtype of glutamate receptors mediated signaling pathway and following transforming growth factor (TGF)-β signaling cascade.

JH esterase (JHE) and JH epoxide hydrolase (JHEH) are the two most important enzymes for the JH inactivation.\(^33\) The JHE is predominantly present in the hemolymph and converts JH III into JH III acid by hydrolyzation of the ester (Fig. 2G). JHEH is present in the cells and converts JH III into JH III diol (Fig. 2I) or JH III acid into JH III acid diol (Fig. 2H). The JH III diol is further hydrolyzed by JHE and JH III acid diol is generated (Fig. 2J). Alternatively, JH III diol is further phosphorylated by JH III diol kinase and JH III diol phosphate is generated\(^40\) (Fig. 2K). In addition, a CYP enzyme mediated JH inactivation pathway is reported in a cockroach, *Dicrotopus punctatus*.\(^42\) CYP4C7 converts JH III into 12-trans-hydroxy JH III and metabolizes other JH-like sesquiterpenoids as well (Fig. 2K). This ω-hydroxylation may
have an important function in the JH inactivation at the end of the gonotrophic cycle, but the details remain unclear.

4. CONCLUSION AND OUTLOOK

Recent studies using genome information, molecular biology and genetics revealed the importance of CYP enzymes in insect hormone metabolisms. Most of the insect hormone metabolic pathways has been elucidated, but still the early steps of ecdysteroid biosynthesis referred to as the ‘Black Box’ and the inactivation pathway of insect hormones are not fully understood. Involvement of several CYP and other enzymes is reported/assumed in the ‘Black Box,’ but their enzymatic functions, substrates and metabolites remain to be determined. As reported in E and JH biosynthesis pathways, neuropeptides, hormones and neurotransmitters regulate insect hormone metabolisms. Therefore, further study of the regulatory network among the hormones, neuropeptides and neuro-modulation is required to fully understand insect development and metamorphosis. We firmly believe the entire metabolic pathway and regulatory network of insect hormones will be elucidated in the near future.

Acknowledgment A part of this research was supported by the Programme for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry.

REFERENCES

2) Feyereisen R. Arthropod CYPomes illustrate the tempo and mode


