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Cytochrome P450 monooxygenases and insecticide resistance in insects

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Cytochrome P450 monooxygenases are involved in many cases of resistance of insects to insecticides. Resistance has long been associated with an increase in monooxygenase activities and with an increase in cytochrome P450 content. However, this increase does not always account for all of the resistance. In *Drosophila melanogaster*, we have shown that the overproduction of cytochrome P450 can be lost by the fly without a corresponding complete loss of resistance. These results prompted the sequencing of a cytochrome P450 candidate for resistance in resistant and susceptible flies. Several mutations leading to amino-acid substitutions have been detected in the P450 gene *CYP6A2* of a resistant strain. The location of these mutations in a model of the 3D structure of the CYP6A2 protein suggested that some of them may be important for enzyme activity of this molecule. This has been verified by heterologous expression of wild-type and mutated cDNA in *Escherichia coli*. When other resistance mechanisms are considered, relatively few genetic mutations are involved in insecticide resistance, and this has led to an optimistic view of the management of resistance. Our observations compel us to survey in more detail the genetic diversity of cytochrome P450 genes and alleles involved in resistance.

Keywords: cytochrome P450; insecticide metabolism; *Drosophila melanogaster*; overexpression; point mutations

1. GENERAL INFORMATION ON MONOOXYGENASE ACTIVITIES AND THE P450 CYTOCHROMES

The P450 monooxygenases are ubiquitous enzymes, found from bacteria to mammals. They are involved in endogenous metabolism as well as in the metabolism of xenobiotics. For example, in insects these activities are essential for the synthesis and the degradation of the steroid moulting hormones and juvenile hormones and also in the metabolism of pheromones. The P450 enzymes are also important for the adaptative mechanisms of insects to the toxic chemicals synthesized by their host plants (Gould 1984). This adaptation is notable for the fact that the biosynthesis of these enzymes can be induced by the presence of the toxins in the food (Frank & Fogleman 1992; Berenbaum *et al.* 1990; Hung *et al.* 1995). We also know that P450 monooxygenase activities can be involved in the metabolism of virtually all insecticides, leading to an activation of the molecule or, more generally, to a detoxification (Wilkinson & Brattsten 1972; Hodgson 1985; Agosin 1985). For some insects, this detoxification is so active (Taylor & Feyereisen 1996) that the insecticide does not reach its molecular target before being metabolized and degraded by these enzymes: such individuals are resistant to insecticides.

P450 enzymes bind molecular oxygen and receive electrons from NADPH to introduce an oxygen atom into the substrate and to form water with the other oxygen atom according to the reaction:

$$\text{substrate(S)} + (\text{NADPH} + \text{H}^+) + \text{O}_2 \rightarrow \text{S(O)} + \text{NADP}^+ + \text{H}_2\text{O}.$$

The electrons necessary for this reaction are transferred from nicotinamide-adenine dinucleotide phosphate (NADPH) on the 'substrate–P450' complex by an NADPH cytochrome P450 reductase, but this reaction can also be stimulated by cytochrome b_5 (Guzov *et al.* 1996; Megias *et al.* 1984; Zhang & Scott 1996). The stability of the initial product [S(O)] can vary, leading to final overall reactions as diverse as hydroxylation, epoxidation, O-, N-, and S- dealkylations, N- and S-oxidations and to such various chemical reactions, and products that these enzymes have been called 'diversozymes' (Coon *et al.* 1996). The key protein of this enzymatic system is in each case a cytochrome P450 that is responsible for the specificity of the reaction. This protein has an absorption at 450 nm when reduced and saturated with CO, hence its name (Omura & Sato 1964). Comprehensive reviews on P450 from insects have been published (Wilkinson & Brattsten 1972; Hodgson 1985; Agosin 1985), and an updated review will be published soon (Feyereisen 1999). If P450 monooxygenase activities are exerted on such a significant diversity of substrates (steroids, juvenile hormone, hydrocarbons, pesticides, etc.), it is because there is a high number of cytochromes P450 in each individual. The P450s certainly constitute one of the most important superfamilies of proteins, considering the large number of forms. To cope with such a diversity it was necessary to adopt a nomenclature based on sequences homologies of P450 and hence on phylogeny (Nelson *et al.* 1996). This nomenclature, now universally accepted, designates all gene members of the P450 superfamily with a CYP prefix, followed by a numeral for the

family, a letter for the subfamily, and a numeral for the individual gene. All members of a family share more than 40% identity at the amino-acid sequence level, and members of a subfamily share more than 55% identity. Genes are described in italics, whereas the gene product, mRNA, and enzyme are in capitals. To date, insect P450s have been assigned to six CYP families; five are insect-specific (CYP6, 9, 12, 18 and 28), and one, CYP4, is shared with sequences from other organisms. Extrapolations based on the known P450 and on a screen of the currently available single transcribed sequences (STSs) and expressed sequence tags (ESTs) of *Drosophila melanogaster* lead to the idea that the total number of P450s in this species will be between 60 and 100 (Feyereisen 1999).

2. CYTOCHROME P450 AND THE RESISTANCE OF INSECTS TO INSECTICIDES: HIGHLIGHTS

It is well established that many cases of metabolic resistance of insects to insecticides are the result of enhanced P450 activities. The involvement of P450 enzymes in resistance can be shown by several methods. P450 monooxygenase inhibitors such as piperonyl butoxide are most commonly used. Treatment of resistant insects by piperonyl butoxide can result in a complete loss of resistance, indicating that resistance is due only to P450 activity. Such a conclusion assumes that the effects of piperonyl butoxide are restricted to P450 inhibition, an assumption that is not always correct. Confirmation with other P450 synergists chemically unrelated to piperonyl butoxide (e.g. imidazole- or propynylether-type synergists) is usually in order. In the majority of the cases, however, resistance is due to several mechanisms and the treatment with the P450 synergist does not restore complete susceptibility. Thus in LearnPyrR, a pyrethroid-resistant strain of house flies, the resistance factor is 6000, but when the flies are treated with piperonyl butoxide this factor decreases to no more than 32 owing to residual resistance that involves a decrease in the penetration kinetics and modification of the target (Scott & Georghiou 1986). A more direct way to show the intervention of P450 in resistance to insecticides is to compare directly the NADPH-dependent metabolism of the insecticide in resistant and susceptible strains. This direct method is not often used because it requires radiolabelled molecules that are not always available to the researchers. In the case of the DDT-resistant strain RDDT^{R} of *Drosophila*, the NADPH-dependent metabolism of DDT is ten times higher than that of a susceptible strain (Cuany *et al*. 1990). Whatever the method of characterizing resistance, one can say that P450-dependent resistance has been reported for most insecticide classes and in most arthropod pest species, highlighting the need to obtain a good knowledge of this resistance mechanism.

3. P450 MONOOXYGENASE ACTIVITIES AND CONTENT OF P450 IN RESISTANT STRAINS

The total P450 content can be measured by optical spectroscopy by means of the absorption spectrum of P450 reduced and saturated with CO (Omura & Sato 1964). This content has been compared in several resistant and susceptible strains of the house fly (Scott *et al*. 1990). For all the strains that have resistance synergized by piperonyl butoxide there is an increase in the total content of cytochrome P450. This phenomenon has been known for a long time (Hodgson 1985; Agosin 1985; DeVries & Georghiou 1981; Vincent *et al*. 1985; Dyte 1972; Cohen 1982). Interestingly, it has been observed that, in addition to the increase in P450, there is also an increase in P450 reductase and cytochrome b_5 in some resistant strains (Scott & Georghiou 1986; Scott *et al*. 1990). This measurement of the total increase in P450 is an underestimate of the increase in specific forms of P450. The P450 overproduced in the resistant house fly strain LearnPyrR was purified and a specific antibody was produced (Wheelock & Scott 1990). An immunoassay of the overproduced P450 from LearnPyrR (called P450lpr) has shown that it accounts for 68% of the total P450, 44 times more than the level of P450lpr in the susceptible strain. Similar results were obtained on the RDDT^{R} strain of *Drosophila*, which has 40 times more CYP6A2 protein than the susceptible strain (M. Amichot and A. Brun, unpublished results).

The first cloning of a P450 cDNA from insects, CYP6A1 from the house fly, was via an expression library of cDNA obtained from the resistant Rutgers strain overexpressing P450 (Feyereisen *et al*. 1989). By means of several PCR methods many other P450s have been cloned, making probes available to show that the mRNA of several P450s is constitutively overproduced in resistant strains: CYP6A1 is overproduced in the resistant Rutgers strain (Carino *et al*. 1992, 1994) and CYP6D1 in LearnPyrR (Scott *et al*. 1996); CYP6A2 is overproduced in resistant strains of *Drosophila* (Waters *et al*. 1992; Brun *et al*. 1996), whereas CYP6A9 is overproduced in other resistant strains of this species (Maitra *et al*. 1996); CYP6B2 is overproduced in *Helicoverpa* (Xiao-Ping & Hobbs 1995), as is CYP4G8 (Pittendrigh *et al*. 1997) and CYP9A1 in *Heliothis virescens* (Rose *et al*. 1997). In fact, several P450s can be overproduced together in an individual, e.g. CYP6A2 and CYP4E2 in the RDDT^{R} strain of *Drosophila* (Amichot *et al*. 1994), and CYP6A1 and CYP6D1 in LearnPyrR house flies (Carino *et al*. 1992). This overproduction can be due to an overexpression of the gene encoding these proteins, but a stabilization of the corresponding mRNA or protein cannot be excluded; to date, no gene amplification has been demonstrated in strains overproducing P450. It has been shown by mRNA *in situ* hybridization (Brun *et al*. 1996) that overproduction does not modify the spatial- and tissue-specific expression of CYP6A2, which is specifically expressed in the proximal gut, in the Malpighian tubules and in the subcuticular fat bodies. This overproduction of P450 must be involved in the resistance; indeed, when CYP6A1, CYP12A1 (Feyereisen 1999) and CYP6A2 (Dunkov *et al*. 1997) were expressed in *E. coli* or in baculovirus-infected cells, these P450 could cleave oxidatively the ester bond of diazinon, a reaction that represents a detoxification of the molecule.

The genetic mechanism responsible for P450 constitutive overproduction is not well understood; however, it is known that in the overproducing strains of the house fly and *Drosophila* there is an interference with the process of induction of these proteins by phenobarbital (Carino *et al*. 1994; Brun *et al*. 1996). At present, the best-characterized model is that of the Rutgers house fly strain, resistant to

organophosphates. In this strain, resistance is at least associated with chromosome 2 (Plapp 1984), but it is known that the structural gene for CYP6A1 is on chromosome 5 (Cohen *et al*. 1994). Similar results were obtained with CYP6D1, which is located on chromosome 1, whereas its expression is regulated by a factor on chromosome 2 (Liu & Scott 1996*a*), on which a factor regulating sensitivity to phenobarbital has also been reported (Liu & Scott 1997). Thus, at least in the house fly, there would be obviously a *trans* genetic factor relative to CYP6A1 that would control its expression and whose modification would be at the origin of the switch from low constitutive expression in the insecticide-susceptible flies to a constitutive overproduction in the resistant flies. In *Drosophila*, the data are less clear, but genetic data suggest that there is also a *trans* regulation of the overexpression of P450 (Waters & Nix 1988; Houpt *et al*. 1988). Molecular data lead to the same conclusion concerning the overexpression of CYP6A2, CYP4E2 and CYP6A9 (Maitra *et al*. 1996; Amichot *et al*. 1994).

This increase in the content of a component of the P450 system results in an increase in the enzymatic activity for insecticides in resistant insects. However, at least in *Drosophila*, one can also observe an increase in activity on substrates as varied as ethoxycoumarin, ethoxyresorufin, ecdysteroids, testosterone, and lauric acid and some of its unsaturated derivatives (Cuany *et al*. 1990). This diversity of substrates metabolized in resistant insects suggests that several P450s are overproduced in the resistant strains of *Drosophila*. However, we cannot eliminate the possibility that an overproduced P450 in the resistant strain has, in addition, a broader substrate specificity when compared with that of the allele present in the susceptible strain. A practical and rapid system to measure P450 activity via O-de-ethylation of 7-ethoxycoumarin (ECOD) in a single fruit fly was developed (deSousa *et al*. 1995). This technique, applied to determine ECOD activity in individuals from wild populations of *Drosophila* (Bride *et al*. 1997) and *Cydia pomonella* (Sauphanor *et al*. 1998), showed that this activity is well correlated with resistance level and that, compared to strains selected in laboratory, the standard deviation of measurements is much more important in wild populations than in the populations reared for a long time in the laboratory. In these wild populations there are probably various types of individuals, homozygotes and heterozygotes for the overexpression of this activity.

4. RESISTANCE TO INSECTICIDES AND AMINO-ACIDS SUBSTITUTIONS IN CYP6A2

The P450-dependent resistance cannot always be fully accounted for by an increase in the content of cytochrome P450. For example, no increase in CYP6A1 mRNA content has been observed in some strains of flies resistant to insecticides by a mechanism that can be inhibited by piperonyl butoxide (Carino *et al*. 1992). In the strain LearnPyrR, it is also impossible to correlate the piperonyl butoxide-dependent resistance to permethrin (resistance factor greater than 1000) (Sauphanor *et al*. 1998) with the ninefold overproduction of CYP6D1 protein (Scott *et al*. 1996). These observations, and the fact that in the RDDT^{R}-resistant strain of *Drosophila* the resistance is polygenic, led us to attempt a separation of the various resistance factors via backcrosses between the resistant strain RDDT^{R} and the susceptible strain 88100. The progeny of each backcross was selected by DDT by tarsal contact at 50 nmol cm^{-2}. After 15 selective backcrosses the strain obtained (called 152) metabolized DDT more intensely than the susceptible strain, and this metabolism is NADPH-dependent. The 152 strain has a monogenic P450-dependent resistance to DDT, with an LC50 of 60 nmol cm^{-2} for DDT (RDDT^{R} has an LC50 higher than 1 mmol cm^{-2}). Moreover, the 152 strain does not overproduce CYP6A2. Using this strain, the resistance factor was mapped to the approximate chromosome location 55–56. Owing to the imprecision of the correspondence between the genetic localization and the mapping determined via *in situ* hybridization, the 55–56 locus could well correspond to the 43A band on which CYP6A2 has been localized on polytene chromosomes. CYP9B1, which could be another candidate for resistance, also maps in this region but it is not overexpressed in the RDDT^{R} strain. Moreover, the homology between house fly CYP6A1 and *Drosophila* CYP6A2 is shown by the following facts (Dunkov *et al*. 1997).

1. There is a high degree (49%) of sequence identity for these members of the CYP6A subfamily.
2. They are localized on homologous chromosomes.
3. They have only one intron located at the same place.
4. They are both induced by phenobarbital and their promoter has characteristic BARBIE box sequences.
5. They are overexpressed in resistant strains; this is not the case for other CYP6As in the house fly.
6. Their expression is under the control of a factor found on chromosome II for the house fly and chromosome 3 for *Drosophila*, which are homologous.
7. They both metabolize diazinon and cyclodienes.

This probable orthology reinforces the idea that CYP6A1 and CYP6A2 are both contributing to resistance. The comparison of the sequences between CYP6A2 from a susceptible strain of *Drosophila* and CYP6A2 from RDDT^{R} or strain 152 shows that there are three amino-acid substitutions, R335S, L336V and V476L. Modelling of CYP6A2 based on sequence homologies with several crystallized P450s revealed that these three mutations may have an effect on the structure of the active site of CYP6A2. We thus expressed in *E. coli* a wild-type CYP6A2 and this same P450 mutagenized in order to introduce the mutations alone or in combination. The results to date show that these mutations do not modify the activity of CYP6A2 for testosterone, but that there is an increase in activity for 7-ethoxycoumarin, 7-benzoyloxycoumarin and especially DDT, hydroxylated to the non-insecticidal dicofol.

5. QUESTIONS AND WORKING HYPOTHESES AS CONCLUSIONS

It seems that CYP6A1 and CYP6A2 are very significant factors for P450-dependent insecticide resistance in house flies and *Drosophila*, respectively. In the latter case, resistance probably would results from a combination of overproduction and amino-acid substitution, which would lead to an overproduced cytochrome P450 with a good

catalytic efficiency with respect to the insecticide. However, many questions still remain outstanding. The overproduction of P450 appears unstable, relatively easily lost in the absence of selection, and one wonders why this is so? Does this reflect the relative importance in resistance of mutations causing overproduction and amino-acid substitution mutations? Some studies suggest that P450 overproduction decreases the fitness of individuals, which is logical as it is known that the overproduced P450 can metabolize hormonal endogenous molecules (Cuany *et al*. 1990). It is possible that amino-acid substitutions may involve less disturbances to the fitness of the individual that carries them. Once fixed in populations, these substitutions, if they confer significant resistance, would facilitate the loss of overproduction, a form of genetic succession (Taylor & Feyereisen 1996). What is the gene regulating the overexpression of P450 in the resistant insects? This is still unknown. Finally, it remains unclear how many different P450s participate in resistance in a given strain, and how many amino-acid substitutions of importance in resistance will be found in P450s?

We now have many tools that should enable us to answer these questions. In any event, it is only then that we will be able to consider seriously the possibilities of monitoring accurately each resistant allele of P450 and of managing their spread in wild populations of agricultural pests or vectors of disease.

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