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Transfer and expression of an organophosphate insecticide-degrading gene from *Pseudomonas* in *Drosophila melanogaster*

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ABSTRACT **The organophosphorus acid hydrolases represent a distinct class of enzymes that catalyze the hydrolysis of a variety of organophosphate substrates, including many insecticides and their structural analogues. The plasmid-borne *opd* gene of *Pseudomonas diminuta* strain MG specifies an organophosphorus acid hydrolase, a phosphotriesterase, that has been well characterized and can hydrolyze a broad spectrum of insect and mammalian neurotoxins. The *in situ* functioning of this enzyme in the metabolism of organophosphates has been analyzed directly in insects by transferring the *opd* gene into embryos of *Drosophila melanogaster* by *P* element-mediated transformation. The chromosomal locations of this stably inherited transgenic locus differed from strain to strain and demonstrated various expressivity on the whole-insect basis. Transcriptional induction of *opd* in one of these strains under control of the *Drosophila* heat shock promoter, hsp70, resulted in the synthesis of stable active enzyme that accumulated to high levels with repeated induction. The heat shock-induced synthesis of organophosphorus acid hydrolases in transgenic flies conferred enhanced resistance to toxic paralysis by the organophosphate insecticide paraoxon.**

Organophosphates are neurotoxic in many biological systems by virtue of their acetylcholinesterase-inhibitory properties (1, 2). Although general metabolic systems for xenobiotic detoxication can be mobilized against organophosphates, such systems tend to be nonspecific and relatively ineffective against these intracellular neurotoxins (3, 4). It has been discovered that some of the organophosphate insecticides and their structural analogues can be selectively degraded by organophosphorus acid hydrolases (OPHs), a distinct class of phosphotriesterases of variable substrate specificity. These enzymes have been identified in such diverse organisms as squid, protozoa, mammals, and soil bacteria (5–10). The enzyme from *Pseudomonas diminuta* strain MG is a 39-kDa protein that can be processed to a mature 35-kDa form in both bacterial and eukaryotic host systems. The active enzyme is membrane-associated through a hydrophobic NH_2-terminal domain (C. Miller and J.R.W., unpublished observation). This enzyme has been shown to catalyze the single phosphate-transfer hydrolysis of the organophosphate paraoxon using a stereospecific S_N2 reaction mechanism (11). This enzyme is quite unusual due to its capability to hydrolyze a wide variety of organophosphate substrates from the insecticides parathion, coumaphos, and orthene to the mammalian nerve agents diisopropyl fluorophosphate (DFP) and sarin (11–15).

The native biological role of OPH is not well understood. OPH activity does not permit bacteria to use organophosphates as a nutritional substrate in pure culture, although some role in a complex ecosystem consortium cannot be eliminated (14). Furthermore, a logical role in a scheme of general metabolic detoxication is difficult to define because of the rarity of natural phosphotriesters or other apparent substrate analogues in biological systems. The recent isolation from *P. diminuta* MG of a plasmid-born gene, *opd* (organophosphate degrading), which specifies a broad-spectrum OPH (14, 15) provides an opportunity to investigate the biological consequences of OPH activity in an insect under neurotoxic challenge. The present studies describe transformation and expression of the *opd* gene of *P. diminuta* MG (12) in *Drosophila melanogaster*, an insect normally quite vulnerable to organophosphate toxicity. Although *Drosophila* populations can be genetically selected for increased resistance to organophosphate insecticides through elevated levels of NADPH-dependent oxidative metabolism (16, 17), OPH is not a native *Drosophila* enzyme and does not contribute to such multigenic resistance.

MATERIALS AND METHODS

The *opd* Gene and Enzyme. The *opd* gene was originally isolated on a 1.3-kilobase (kb) *Pst* I fragment from a nonessential 51-kb plasmid carried by *P. diminuta* MG (12). This fragment encodes an open reading frame of 325 codons that predicts the amino acid sequence of OPH as obtained from partial microsequencing (12). Catalytically active enzyme has been produced in transformed heterologous bacteria (12) and in baculovirus-infected insect cells in culture. The enzyme has been purified from both sources (15, 18).

Construction of pCHOPH. A 1.3-kb fragment carrying the *opd* gene of *P. diminuta* MG was isolated from pBR322-038 and inserted into the *Pst* I site within the 5′ untranslated leader sequence of *Drosophila* hsp70 (19) carried on plasmid pRN5HS. This insert and 0.5 kb of 5′-flanking hsp70 sequence were excised as a 1.8-kb *Xho* I fragment and spliced into the *Sal* I site of the *Drosophila P* element vector, pC20 (20), to construct pCHOPH-4, which carried a single hsp70–OPH insert, and pCHOPH-11, which carried two hsp70–OPH inserts in tandem (Fig. 1).

DNA and RNA Analysis. Ten micrograms of total DNA from adult transformants was digested with *Pvu* II and fractionated by electrophoresis through 0.7% agarose. Southern blots were hybridized with a ^{32}P-labeled *opd* DNA probe isolated from pBR322-038. Five micrograms of total RNA from adult transformants was denatured with glyoxal and dimethyl sulfoxide (22) and fractionated by electrophoresis through 1% acrylamide. RNA blots were hybridized with ^{32}P-labeled *opd* DNA probes isolated from pBR322-038 (14).

***In Situ* Hybridization.** The *opd* insert was isolated from pBR322-038, biotinylated by nick-translation, and hybridized

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Abbreviations: OPH, organophosphorus and hydrolases; kt_{50}, time required for toxic paralytic knock-down in 50% of the population.

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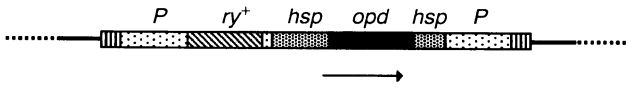

FIG. 1. Structure of pCHOPH. Solid bar, *opd* gene (and flanking sequences) of *P. diminuta* MG; heavily stippled bar, 5′ untranslated leader sequence of *Drosophila* hsp70; lightly stippled bar, *P* element vector, pC20; vertical hatching, terminal inverted repeat elements of pC20; diagonal hatching, ry^+ sequences of pC20. Solid lines indicate flanking *Drosophila* genomic DNA derived from isolation and construction of pC20 (21); dotted lines indicate plasmid vector sequences of pC20. Transcriptional direction is indicated by the arrow. Elements are not drawn to scale.

to larval salivary gland chromosomes as described in Kirkland and Phillips (23).

OPH Assay. Enzyme extracts resulted from homogenizing 0.1 g of flies in 2.0 ml of extraction buffer (10 mM Tris·HCl, pH 9.0/10 mM NaCl/1% Triton X-100/0.01% phenylthiocarbamide) in a glass/Teflon homogenizer. After centrifugation for 10 min at 12,000 × *g*, the supernatant was assayed immediately. All extraction steps were done at 0–5°C. OPH activity was determined by measuring the rate of *p*-nitrophenol production from paraoxon, as described (11). Fifty microliters of extract was incubated for 30 min at 37°C in a 1.0-ml reaction mixture containing 1 mM paraoxon. The production of *p*-nitrophenol was measured spectrophotometrically at 400 nm after stopping the reaction by adding SDS to 3.3%. Protein was measured by the method of Bradford (24) using the Bio-Rad protein assay. One unit of OPH activity is defined as the generation of 1 nmol of *p*-nitrophenol per min under these conditions. Specific activity is reported as units per mg of protein.

***P* Element Transformation.** The embryo microinjection method of Rubin and Spradling (21) was used to generate transformants. The plasmids pCHOPH-4, pCHOPH-11, and pC20 were injected at 300 μg/ml along with the helper plasmid pπ25.1 at 50 μg/ml into *ry cn b* embryos. Descriptions of these mutants can be found in Lindsley and Grell (25). Putative transformed lines ($ry^{\pm}$ *cn b*) were examined for chromosomally integrated *opd* sequences, the presence of *opd* transcripts, OPH activity, and resistance to paraoxon toxicity.

Heat Shock Induction of Gene Expression. The standard protocol for heat shock involved transferring adults of specified age in glass vials (10 adults per vial) into a 37°C incubator for 1.5 hr, followed by recovery at room temperature (≈22°C) for 2 hr.

Paraoxon Toxicity Determination. Adult males (1–10 days of age; 10 flies per vial; 10 vials) were given three successive heat shocks at 2-hr intervals and then allowed to recover for 10 hr at room temperature. Flies taken before and after heat shock were transferred to vials (10 flies per vial) containing Whatman 1 filter discs saturated with paraoxon (25 ppm in 1% sucrose) and maintained at room temperature (≈22°C). "Knock-down" was scored when a fly was unable to stand or to right itself. The time required to knock down 50% of the population (kt_{50}) under these conditions is used to measure resistance.

RESULTS

Isolation of OPH Transformants. Transformants were generated with pCHOPH-4, pCHOPH-11, and pC20 by using the embryo microinjection method of Rubin and Spradling (21). Each transformed line was analyzed by Southern blot analysis (Fig. 2) and *in situ* hybridization to larval polytene chromosomes (Table 1). Each strain evaluated has been shown to carry the *opd* inserts at a single chromosomal location, which differs from strain to strain. Independent insertions were identified at four different locations in chromosome 2 and at a single position on the X chromosome. The level of heat shock-inducible OPH activity in these strains ranged from <1 unit per mg of protein (opd^{328}) to >22 units per mg of protein (opd^{82}) (Fig. 4). Because of its inducibility to high levels of OPH expression, strain opd^{82} was used for all subsequent characterizations.

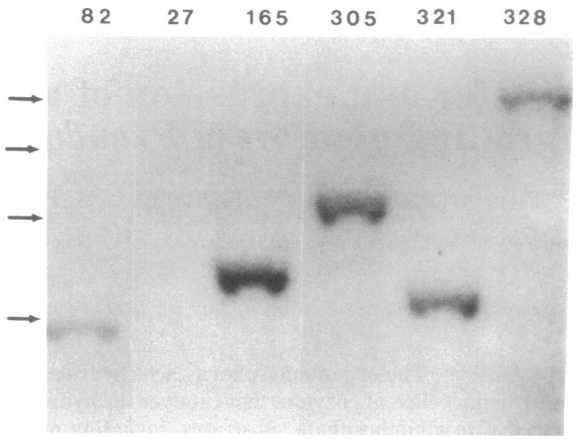

FIG. 2. Identification of *opd* sequences in ry^+ transformants. Southern blot analysis of genomic DNA from adult transformants. Arrows indicate positions of 23.8-, 9.45-, 6.67-, and 2.26-kb markers. See Table 1 for further molecular description of the strains.

Heat Shock-Induced Synthesis of OPH Transcripts. Transcription of *opd* in pCHOPH transformants would be expected to initiate at the hsp70 promoter, extend through the *opd* insert, and terminate 0.4–0.5 kb downstream in the 3′ region of the *P* element to produce a transcript of ≈2.0 kb. Northern (RNA) blot analysis of RNA from opd^{82} before and after heat shock demonstrated the heat shock-dependent synthesis of an appropriate *opd*-hybridizing transcript of 2.0 kb (Fig. 3). Two minor transcripts of ≈1.5–1.6 kb, possibly arising from premature termination within the *opd* insert, were also detected.

Appearance and Stability of OPH Activity After Heat Shock Induction. OPH activity in opd^{82} was detectable at low levels in the absence of heat shock (0.5 unit per mg of protein) and increased ≈40-fold in response to heat shock induction (Fig. 4). OPH activity was not detectable either before or after heat shock induction in control strain $C20^{27}$, which carries the Carnegie 20 vector without the hsp70–*opd* insert. OPH activity began to increase within 2 hr of induction (Fig. 5) and reached a maximum level ≈10 hr after heat shock induction. Thus, it should be noted that the assay of enzymatic activity immediately after a standard 2-hr recovery period as in Fig. 4 would overestimate base level of activity and underestimate maximum level of activity induced by a single 1.5-hr heat shock.

OPH activity in *Drosophila* transformants was extremely stable *in vivo*. Greater than 50% maximum activity produced by a single heat shock of opd^{82} remained after 24 hr; 25% still remained after 40 hr (Fig. 5). By 50 hr after induction, the base-line-activity level had been reestablished. Such stability would contribute to the observed accumulation of OPH

Table 1. Structure and chromosomal localization of OPH constructs in transformed *Drosophila*

Strain	Construct	Cytology
$C20^{27}$	pC20	Not determined
opd^{82}	pCHOPH-4	60A (2R)
opd^{165}	pCHOPH-11	16A (X)
opd^{305}	pCHOPH-11	46B (2R)
opd^{321}	pCHOPH-11	58EF (2R)
opd^{328}	pCHOPH-11	41F (2R)

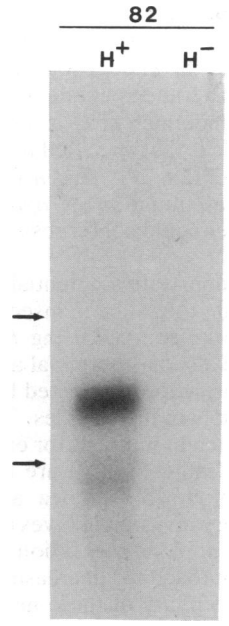

FIG. 3. *opd* transcripts in the opd^{82} ry^{+} transformant. Northern (RNA) blot analysis of total RNA from opd^{82} adult transformants. RNA was isolated before (H^{-}) and after (H^{+}) 1.5-hr heat shock followed by 2-hr recovery. Arrows indicate positions of mouse 18S and 28S mRNA.

activity upon repeated induction (Fig. 6). Three successive rounds of induction of opd^{82} at 2-hr intervals accumulated enough activity in each fly to hydrolyze ≈100 μg of paraoxon per hr at 25°C [(308 units per mg of protein) × (0.276 μg of paraoxon per min per unit) × (0.068 mg of protein per fly) × (60 min per hr) × (temperature correction factor, 37°/25°C; 0.33) = 0.115 mg of paraoxon per hr per fly at 25°C].

OPH Activity Enhances Resistance to Paraoxon. Like many insects, *D. melanogaster* is normally quite susceptible to the neurotoxic action of organophosphate insecticides. However, OPH induction in the transgenic insects significantly delayed the onset of toxic paralysis in opd^{82} adults acutely exposed to paraoxon (Fig. 7). Probit analysis of the data in Fig. 7 revealed the kt_{50} (time required to induce toxic paralytic knock-down in 50% of the population) for paraoxon exposure under these conditions changed from 3.95 ± 0.25 hr before induction to 7.12 ± 0.85 hr after induction, an increase of 180%. Adults flies from control strain $C20^{27}$ showed no changed in kt_{50} the same regimen of heat shock induction.

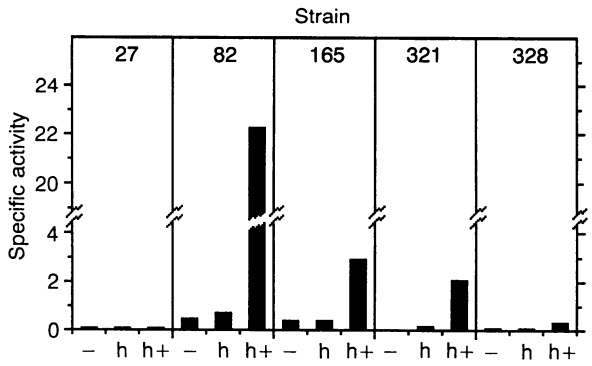

FIG. 4. OPH activity in ry^{+} transformants. Extracts were made from adult male transformants before heat shock (−), immediately after heat shock (h), and after 2-hr recovery (h+) and assayed for OPH activity as described. Assays were done at least twice in triplicate with essentially identical results. See Table 1 for further molecular description of the strains.

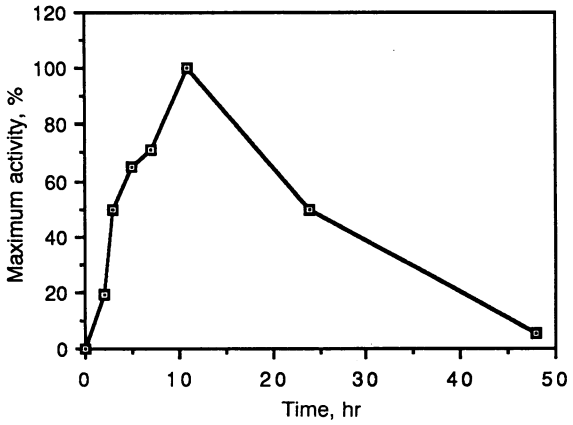

FIG. 5. Appearance and stability of OPH activity after heat shock induction. Extracts were made from 10 opd^{82} adult males at increasing intervals after a single 1.5-hr heat shock and assayed for OPH activity as described. Experiments were done at least twice in triplicate with essentially identical results.

These observations demonstrated that the induced resistance seen in opd^{82} did not arise from a heat shock response *per se*. Further, opd^{82} increased in kt_{50} value almost 2-fold over the $C20^{27}$ control without heat shock induction. This result probably reflected the response to constitutively expressed, low levels of OPH activity in opd^{82} (Fig. 4).

DISCUSSION

These studies describe the generation and characterization of transgenic strains of *D. melanogaster* that possess the *opd* gene of *P. diminuta* MG under transcriptional control of the hsp70 promoter. *opd* inserts at discrete, single-gene loci have been identified in several different transgenic strains. Consistent with the general pattern of *P*-element transformation, neither multiple-site nor obvious-tandem insertions were detected. The heat-shock-induced expression of one of the transgenic strains (82) has been analyzed in detail, and the induction of heterologous OPH activity in the *Drosophila* transformants conferred enhanced resistance to organophosphate toxicity.

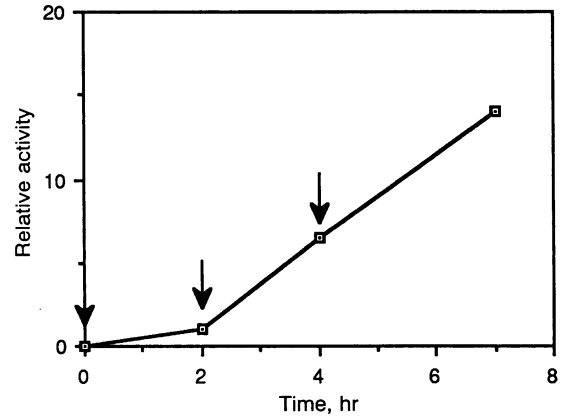

FIG. 6. Accumulation of OPH activity by serial induction. opd^{82} adult males, 1–10 days of age, were heat shocked three successive times at intervals of 2 hr (arrows). Flies were removed from the population at the indicated times and assayed for OPH activity as described. Activities are reported as values relative to the activity at 2 hr (22 units per mg of protein). Extracts were made from two independent experiments and assayed in triplicate with essentially identical results.

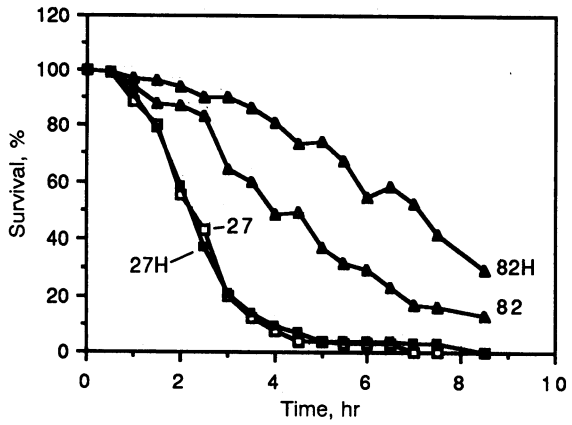

FIG. 7. Heat shock-dependent resistance to paraoxon. Adult males of strains opd^{82} and $C20^{27}$ were examined for resistance to paraoxon before (27 and 82) and after (27H and 82H) three consecutive heat shocks as described. Survival is plotted as a percentage of the total starting population (100 flies) and typifies results from several repeated experiments.

The synthesis of stable enzyme in induced *Drosophila* transformants provides a valuable system for evaluating the potential of OPH as an agent for *in vivo* detoxication of organophosphate insecticides. Paraoxon is a model organophosphate for such studies because (*i*) it is highly water soluble (unlike many organophosphates), (*ii*) it is a preferred substrate of *Pseudomonas* OPH (15), and (*iii*) it is the active (toxic) metabolite formed *in vivo* from several commercial organophosphate insecticides (2, 26). The viability and fertility of various *opd* transformants shows that heterologous OPH activity, even at induced levels, does not interfere with vital metabolism in *D. melanogaster*.

The *in vivo* stability and heat shock-inducibility of OPH combined to permit the accumulation of high levels of activity as the result of multiple inductions. After three successive rounds of induction, each fly had a level of OPH activity adequate to hydrolyze ≈0.115 mg of paraoxon per hr at 25°C. This *de novo* metabolic potential should have been sufficient to hydrolyze an amount of paraoxon equivalent to 5 ml of 25 ppm solution per hr; uptake of this level of insecticide would be a formidable task for a 1-mg fly. The modest level of paraoxon resistance resulting from transgenic induction indicates that the existing enzyme could not detoxify paraoxon at the expected levels. Therefore, the accessibility of the insecticide to the enzyme must be constrained in some fashion in *Drosophila*. In the native *Pseudomonas* and in transformed *Escherichia coli* strains, OPH activity is largely membrane-associated through an NH_2-terminal hydrophobic domain of the protein (14, 27). Consistent with these observations, the OPH activity in extracts of *Drosophila* transformants appeared associated with the particulate, membrane fractions of whole fly. In addition, the catalytic efficiency of the enzyme increased several-fold upon standing after cell fractionation. In addition, none of the OPH activity in serially induced transformants is detectable in hemolymph (J.H.X. and J.P.P., unpublished observations). These preliminary observations are consistent with a membrane association of OPH in *Drosophila* transformants and suggest that the enzyme is confined within the cells in which it is made. As such, OPH may be relatively unavailable to intercept the organophosphate before the latter reaches the synaptic site of its neurotoxic action. Further work is needed to understand the possible association of OPH with membranes in *Drosophila* as this association relates to paraoxon resistance.

Recent studies on genetic manipulation of the primary structure of OPH indicate that membrane association of the enzyme in bacteria is due to a hydrophobic NH_2-terminal signal sequence (27). Thus, it may be possible to enhance detoxication efficacy of OPH in transgenic *Drosophila* by deleting all or part of the endogenous signal sequence or by its replacement with a transit peptide involved in transport of proteins into the hemolymph of *Drosophila*. A candidate for such a transit peptide is that specified by the *Drosophila* yolk protein genes, YP1-2 (28, 29). The *in vivo* stability of OPH further suggests that initiation of *opd* transcription by a constitutive promoter could confer resistance throughout the insect life cycle.

These studies demonstrate a potential route for the appropriation of insecticide tolerance in insects due to a vectorial transfer from bacterial sources using *P*-element transposition. This technique also has potential application in conferring resistance or tolerance to selected beneficial insects for specific organophosphate insecticides. This type of application could be of particular interest, for example, in control of Varroa and tracheal mites, which are threatening the beekeeping industry in North America and Europe through destructive, predatory invasion of hives (30). In addition, and perhaps contrary to popular perception, this work identifies another potential approach for decreasing insecticide use by increasing target specificity of these neurotoxic agents. Organophosphate resistance in selected beneficial insects (e.g., pollinators, predatory, and parasitic insects) would allow targeting insecticide application to the most vulnerable stage of the pest insect life cycle, regardless of the overlapping presence of beneficial species. Moreover, resistant pollinators and beneficial insect predators would remain to do their work, thus further decreasing dependence on chemical control. Finally, organophosphate resistance conferred by the *opd* gene would not be expected to suffer from the genetic instability typical of most naturally selected multigenic resistance and should be stable during intermittent selection. *Drosophila* will be a useful host species in which to examine these and other aspects of the population dynamics of the heterologous *opd* gene.

The continued use of chemical insecticides, especially in agriculture, burdens the environment in a way that clearly cannot be sustained. In addition to direct toxic effects on numerous nontarget species, toxic insecticide residues persist and accumulate in a variety of unexpected ecological niches, affecting the health of an ever-expanding circle of inhabitants. The need to reduce our dependence on chemical insecticides is urgent, not only for these biological reasons, but because the continued high-level use of insecticides adds an enormous direct cost to the price of agricultural commodities.

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