Isolation of insecticide resistance-related forms of cytochrome *P*-450 from *Drosophila melanogaster*

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Significant purification of the ubiquitous cytochrome *P*-450-A and the strain-specific *P*-450-B from *Drosophila melanogaster* has been achieved by sequential chromatography on octylamino-agarose, DEAE-cellulose and hydroxyapatite. Preparations of *P*-450-A (specific contents of 7–9 nmol/mg) were homogeneous as determined by SDS/polyacrylamide-gel electrophoresis (PAGE) analysis. Preparations enriched for *P*-450-B (specific contents of 4–7 nmol/mg) contained significant amounts of *P*-450-A but were essentially free of other proteins as judged by SDS/PAGE. Partial reconstitution of 7-ethoxycoumarin demethylase activity was achieved using rabbit NADPH: cytochrome *P*450 reductase and purified preparations containing *P*450-B.

INTRODUCTION

The cytochrome *P*-450-dependent mixed function oxidase system in insects appears to be particularly important in insecticide resistance (Wilkinson, 1983). Because of the multiplicity of *P*-450 isoenzymes and their overlapping substrate specificities, qualitative and quantitative variations in the different forms of *P*-450 can influence both insecticide metabolism and normal metabolic processes such as hormone and pheromone biosynthesis and feeding habits (Hodgson, 1983). Resistance to various insecticides in *Drosophila* and houseflies has been directly associated with increased or unique mixed function oxidase activities (Vincent *et al.*, 1985; Waters & Nix, 1988).

Electrophoretic analysis of the microsomal proteins of over 20 *Drosophila* strains reveals that one haem-containing band, *P*-450-A, is present in all strains examined, whereas an additional band, *P*-450-B, is found in only a few strains including Hikone-R (BG) (Waters *et al.*, 1984a). [Consistent with earlier nomenclature for *Drosophila* *P*-450 (Waters & Nix, 1988), *P*-450-A indicates all *P*-450 forms with approximate molecular masses of 59 kDa that are ubiquitous among strains; *P*-450-B indicates all isoenzymes of approximately 56 kDa whose expression is strain-dependent.] Genetic analysis shows that the genes required for *P*-450-B expression are located on chromosome II, and that chromosome III loci are required for maximum expression. These loci map at or near major insecticide-resistance loci on the two chromosomes (Waters & Nix, 1988). *P*-450-B expression is correlated with high nitrosodimethylamine demethylase activity and with resistance to DDT, malathion and phenylurea among *Drosophila* strains (Waters & Nix, 1988; Sundseth, 1988).

Hallstrom and coworkers (Hallstrom *et al.*, 1984; Hallstrom, 1985; Hallstrom & Blanck, 1985) reported the association of a 54 kDa microsomal protein band, on

SDS/polyacrylamide-gel electrophoresis (PAGE), with *p*-nitroanisole demethylase and biphenyl-3-hydroxylase activities in *Drosophila*. Biphenyl-4-hydroxylase was associated with a 56 kDa protein. These 54 and 56 kDa proteins probably correspond to *P*-450-B. The 58 kDa protein band, which is relatively invariant among the strains examined, is probably identical to *P*-450-A. On the basis of the ability to metabolize benzphetamine and of size, the 54.8 kDa cytochrome *P*-450 III purified by Agosin and coworkers (Naquira *et al.*, 1980; Agosin, 1982) presumably corresponds to *P*-450-B (Waters & Nix, 1988). The number of isoenzymes in each of the two *Drosophila* *P*-450 subsets is not known; however, we have no evidence for *P*-450s that are larger than *P*-450-A or smaller than *P*-450-B.

The fact that a large number of substrates can be metabolized by *Drosophila* suggests that the *P*-450-A and *P*-450-B subsets consist of multiple isoenzymes. Purification and immunochemical methods are required to estimate the multiplicity of *P*-450 isoenzymes in these subsets. Although previous studies have demonstrated *P*-450 multiplicity in *Drosophila* (Naquira *et al.*, 1980) and the housefly (Ronis *et al.*, 1988), none of the forms described was correlated with resistance to insecticides. Therefore isolated components of the *Drosophila* *P*-450 system, especially the *P*-450-B subset, are needed to be used as markers and reagents with which to study the molecular mechanisms that account for resistance. Specifically, the *P*-450s can be used to prepare and characterize antibodies that are specific for resistance-related forms of *P*-450. In turn, the antibodies can be used to identify and characterize the *P*-450-specific cDNA clones which are necessary to advance the study of *P*-450-dependent insecticide resistance.

This paper describes the use of methods traditionally used for mammalian *P*-450 purification (Imai & Sato, 1974; van der Hoeven & Coon, 1974; Guengerich, 1978) to isolate components of the two subsets of *Drosophila* *P*-

Abbreviations used: *P*-450, cytochrome *P*450; PAGE, polyacrylamide-gel electrophoresis; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride.

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450. By sequential chromatography on octylamino-agarose, DEAE-cellulose and hydroxyapatite, we have purified components of *P*-450-A to apparent homogeneity, as judged by SDS/PAGE, from both susceptible (Oregon-R) and resistant [Hikone-R (BG)] *Drosophila* strains. Samples substantially enriched for components of *P*-450-B, but containing some residual *P*-450-A, have also been prepared from Hikone-R (BG) microsomes. Results of attempts to reconstitute enzyme activity in these preparations are also presented. A preliminary report of some aspects of this work has been presented in abstract form (Waters & Nix, 1986).

MATERIALS AND METHODS

Drosophila strains

The wild-type strains used were Oregon-R, insecticide-susceptible, from the Oak Ridge Collection and Hikone-R (BG), insecticide-resistant, from the Mid-American Stock Center, Bowling Green, OH, U.S.A. Flies were raised on standard cornmeal agar medium at 22–23 °C.

Chemicals

Octylamino-agarose was from P.L. Biochemicals-Pharmacia. DEAE-cellulose (Bio-Gel A), hydroxyapatite (DNA-grade Bio-gel HTP) and SM-2 beads were obtained from Bio-Rad. Emulgen 913 was from Kao Chemicals Co. Lubrol-PX, sodium cholate, cytochrome *c* and NADPH were from Sigma Chemical Co. 7-Ethoxycoumarin and 7-hydroxycoumarin were from Aldrich Chemical Co. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were obtained from Avanti Polar Lipids, Inc. Purified rabbit NADPH:cytochrome *P*-450 reductase was a gift from Dr. D. Waxman, Dana-Farber Cancer Institute, Boston, MA, U.S.A., and its preparation and catalytic activity have been described elsewhere (Waxman & Walsh, 1982; Leblanc & Waxman, 1988). All other chemicals and reagents were of the highest quality available.

Microsome preparation and cytochrome *P*-450 purification

Microsomes were prepared from the whole body homogenates of adult flies (0–8 days old) as previously described (Waters *et al.*, 1983). The washed microsome pellets were stored at –80 °C until used. 1 g of flies (approx. 1000) yielded about 4.2 mg of microsomal protein.

All protein purification procedures were performed at 0–4 °C. Thawed microsome pellets were resuspended to a concentration of 3–5 mg of protein/ml in 100 mM-potassium phosphate (KPO₄) buffer (pH 7.25), containing 1 mM-EDTA, 1 mM-dithiothreitol (DTT), 0.4 mM-phenylmethanesulphonyl fluoride (PMSF) and 20% (v/v) glycerol (buffer A). An equal volume of buffer A containing 1% sodium cholate was added dropwise with stirring and stirred for an additional 30 min. The sodium-cholate-soluble cytochrome *P*-450 was recovered in the supernatant after centrifugation of the mixture at 45000 rev./min for 3 h in a Beckman 60 Ti rotor. The solubilized *P*-450 was applied at a flow rate of 1 ml/min to an octylamino-agarose affinity column (10 ml/ml of column bed) equilibrated with buffer A containing 0.5% sodium cholate. The column was washed with buffer A containing 0.4% sodium cholate until the absorbance at 417 nm equalled that of the wash buffer. *P*-450 was eluted with

buffer A containing 0.4% sodium cholate and 0.1% Emulgen 913 or Lubrol PX. Elution of *P*-450 was monitored spectrophotometrically at 417 and 280 nm. Fractions containing *P*-450 as indicated by absorbance at 417 nm were pooled and dialysed against 1 litre volumes of 10 mM-KPO₄ buffer (pH 7.7) containing 0.1 mM-EDTA, 0.1 mM-DTT, 0.4 mM-PMSF, 0.2% sodium cholate, 0.1% Emulgen or Lubrol PX and 20% glycerol (dialysis buffer) until the conductivity of the sample matched that of the dialysis buffer. The dialysed sample was applied at 0.5 ml/min to a DEAE-cellulose anion exchange column (50 ml) equilibrated in dialysis buffer without PMSF. The column was washed with dialysis buffer and fractions were monitored as before. Fractions containing *P*-450 were pooled and applied to a hydroxyapatite column (20 ml) equilibrated with dialysis buffer at a pH of 7.25 (HA buffer) at a flow rate of 0.3 ml/min. In purifications using Emulgen 913, *P*-450-B bound to this column but *P*-450-A did not. The column was washed with HA buffer until absorbance at 417 nm reached the baseline value and was then washed with HA buffer without sodium cholate and Emulgen 913 to remove detergents. The hydroxyapatite-bound fraction was then eluted with 250 mM-KPO₄ (pH 7.25) containing 0.1 mM-EDTA, 0.1 mM-DTT, and 20% glycerol. To concentrate *P*-450-A, detergent was removed from the hydroxyapatite-flowthrough fraction by treating with SM-2 beads (15 ml/ml of beads), that had been rinsed with HA buffer without detergent, until the absorbance at 280 nm was near that of the HA buffer. The fraction was reappplied to a second hydroxyapatite column (10 ml) equilibrated with HA buffer without detergents. In the absence of detergents, *P*-450-A bound to hydroxyapatite and was eluted with 250 mM-KPO₄ buffer as described before. In purifications using Lubrol PX, detergents were removed from the DEAE *P*-450 pool with SM-2 beads as described and the sample was applied to a hydroxyapatite column (10 ml) equilibrated with HA buffer without detergents. The column was then eluted with 250 mM-KPO₄ buffer as previously described. The purified *P*-450 preparations were dialysed against 50 mM-NaPO₄ buffer (pH 7.4) containing 0.1 mM-EDTA and 20% glycerol and stored in small aliquots at –80 °C.

Reconstitution of cytochrome *P*-450 system

Reconstitution experiments were monitored by measuring the *O*-de-ethylation of 7-ethoxycoumarin in a final volume of 0.1 ml containing 50 mM-KPO₄ buffer (pH 7.4). PC or PE were prepared as 2 mg/ml solutions in 0.1 mM-EDTA and sonicated immediately before use. To reconstitute *P*-450 enzymic activity, 2 µg of PC or PE was preincubated for 10 min at room temperature with 0.05–0.1 nmol of purified *P*-450 preparation and 5–10 units of purified rabbit NADPH:cytochrome *P*-450 reductase. After preincubation, 7-ethoxycoumarin (0.5 mM), buffer and water were added to give a final volume of 95 µl. The reactions were initiated by adding 5 µl of 6 mM-NADPH and were incubated for 30 min at 27 °C. The reactions were stopped by adding 10 µl of cold 15% trichloroacetic acid, extracted with 1 ml of chloroform and centrifuged, and 0.9 ml of the chloroform layer was back-extracted with 2 ml of 0.01 M-NaOH/1 M-NaCl. The determination of the fluorescent product 7-hydroxycoumarin was performed at room temperature by excitation at 368 nm and emission at 456 nm (Greenlee & Poland, 1978). All assays were performed in duplicate,

and replicate tubes differed by less than 10 %. Controls were run simultaneously and omitted either NADP-H:cytochrome P-450 reductase, P-450 or NADPH. Extraction efficiency was monitored and fluorescence intensity was calibrated by concurrently incubating 7-hydroxycoumarin standards with all assay components except reductase and P-450.

Analytical methods

Proteins were measured using the method of Lowry *et al.* (1951) or for samples containing detergents, the method of Bramhall *et al.* (1969), both with bovine serum albumin as the standard. Cytochrome P-450 contents were determined from difference spectra using a double-beam spectrophotometer (Hitachi Model 110) according to the method of Omura & Sato (1964) with the addition of 50 % glycerol to the buffer as described by Brattsten *et al.* (1980). NADPH:cytochrome P-450 reductase activity was assayed at room temperature using cytochrome *c* as an artificial electron acceptor according to Masters *et al.* (1967), with reducing equivalents supplied by a NADPH-regenerating system. One unit of reductase activity is defined as an absorbance change at 550 nm of 1.0 min per ml of enzyme solution at 24 °C. This corresponds to the reduction of 0.0476 μ mol of cytochrome *c*/min per ml.

Proteins in microsomal and purified P-450 preparations were analysed electrophoretically using the discontinuous system described by Laemmli (1970). Proteins were revealed by staining with Coomassie Blue.

RESULTS

P-450 purification

Purification of *Drosophila* P-450 was performed by sequential chromatography on octylamino-agarose, DEAE-cellulose and hydroxyapatite. Results of P-450 purification from *Drosophila* strains Hikone-R (BG) and Oregon-R are shown in Table 1. SDS/PAGE analysis of different P-450 purification fractions is shown in Fig. 1.

About 60 % of the microsomal P-450 was recovered upon solubilization with sodium cholate, but there was no apparent selectivity for either P-450-A or P-450-B (Fig. 1, lane 2). Solubilized microsomes were applied to the affinity column, and the column was washed to

remove non-specifically bound proteins and eluted with non-ionic detergent. This fraction was greatly enriched for P-450, but still contained significant amounts of contaminating protein as judged by SDS/PAGE (Fig. 1, lane 3). The eluate from the affinity column was dialysed

Fig. 1. SDS/PAGE of fractions obtained during purification of *Drosophila* cytochrome P-450 from Hikone-R (BG)

Samples were: microsomes (20 μ g), lanes 1, 5, and 8; sodium-cholate-soluble (10.1 μ g), lane 2; octylamino-agarose pool (9.5 μ g), lane 3; DEAE-cellulose pool (2.5 μ g), lane 4; hydroxyapatite-bound (1.9 μ g), lane 6; hydroxyapatite-flowthrough (1.0 μ g), lane 7; standard protein markers, (92.5, 66.2, 45.0 and 31.0 kDa), lane 9.

Table 1. Purification of cytochrome P-450 from *Drosophila* Hikone-R (BG) and Oregon-R strains

H, Results obtained with Hikone-R (BG); 0, results obtained with Oregon-R.

Fraction	Total Protein (mg)		Total P-450 (nmol)		Specific content (nmol/mg)		Yield of P-450 (%)	
	H	(0)	H	(0)	H	(0)	H	(0)
Microsomes	770	(713)	274	(210)	0.36	(0.29)	100	(100)
Solubilized microsomes	200	(139)	165	(124)	0.82	(0.89)	60	(59)
Octylamino-agarose eluate	28.1	(25.8)	109	(96.6)	3.88	(3.74)	40	(46)
DEAE-cellulose eluate	8.4	(9.0)	57.5	(42.6)	6.84	(4.73)	21	(20)
Hydroxyapatite-bound (P-450-A and P-450-B)	0.8	(-)	3.5	(-)	4.38*	(-)	1.3	(-)
Hydroxyapatite-flowthrough (P-450-A)	3.2	(2.7)	22.1	(23.5)	6.91	(8.70)	8.1	(11)

* In a separate purification a specific content of 7.4 was obtained for this fraction.

to reduce the ionic strength and applied to DEAE-cellulose, where *Drosophila* P-450 bound slightly, but was easily removed by continued washing with the equilibration buffer. Further washing of the DEAE column with 0.2 M-NaCl eluted a large protein-containing peak that had material absorbing at 417 nm. Analysis of this peak by SDS/PAGE showed little material staining with Coomassie Blue in the P-450-containing region of the gel (50–60 kDa). After DEAE chromatography, the P-450 sample appeared to be essentially free of other proteins (Fig. 1, lane 4), but there was no separation of P-450-A and P-450-B. However, on hydroxyapatite in the presence of Emulgen 913, P-450-A was not retained and flowed unimpeded through the column (Fig. 1, lane 7). Another fraction bound tightly to the top of the column as a reddish-brown band and was eluted from the hydroxyapatite with 250 mM-KPO₄. On SDS/PAGE, this fraction was shown to be enriched for P-450-B, but still contained detectable amounts of P-450-A (Fig. 1, lane 6). A second passage of P-450-A over hydroxyapatite in the presence of Emulgen 913 removed residual P-450-B and produced a preparation of P-450-A, apparently free of other proteins from both Hikone-R (BG) and Oregon-R (results not shown). The contaminating band between P-450-A and P-450-B (Fig. 1, lane 7) is probably a degradation product of P-450-A. In smaller-scale purification experiments utilizing single microsomal pellets, this band was absent, even after incubation of the purified fractions at 27 °C for 15 min. These degradation products are presumably caused by endogenous proteinases present in one or more of the microsome pellets pooled for large-scale purification experiments. In the purified preparation, P-450-A and P-450-B had apparent molecular masses of 59.3 and 55.8 kDa respectively, identical to the values found on SDS/PAGE of microsomal samples.

Reconstitution of P-450-dependent enzyme activity

Reconstitutions were done with both PC and PE as the lipid components. Rabbit NADPH:cytochrome P-450 reductase was used in the reconstitution assays. The hydroxyapatite-bound fraction containing both P-450-A and P-450-B was the only purified preparation demonstrating any catalytic activity with 7-ethoxycoumarin. This activity was 0.0105 nmol of 7-hydroxycoumarin/min per nmol of P-450.

DISCUSSION

In this report we describe the purification of *Drosophila* cytochrome P-450 using techniques traditionally used for mammalian P-450. Affinity chromatography on octylamino-agarose gave a 10–12-fold increase in specific content. The DEAE-cellulose step improved the purity of P-450 by separating the fraction containing P-450 from another fraction containing significant amounts of both haem and protein that was eluted from DEAE-cellulose at a higher salt concentration. This fraction probably contained cytochrome *b₅*, because insect cytochrome *b₅* has been shown to co-elute with P-450 from affinity columns and to bind strongly to DEAE-cellulose (Ronis *et al.*, 1988). No significant amounts of either P-450-A or P-450-B were detected in this fraction by analysis on SDS/PAGE.

Drosophila P-450 was eluted from the affinity column

with either Emulgen 913 or Lubrol PX, and behaved identically on octylamino-agarose and DEAE-cellulose with either non-ionic detergent. With buffers containing Lubrol PX, however, neither P-450-A nor P-450-B would bind to hydroxyapatite. Both P-450-A and P-450-B bound equally well to hydroxyapatite in the absence of non-ionic detergents; thus the use of Lubrol PX eliminated the ability of this step to discriminate between P-450-A and P-450-B. In purifications using Emulgen 913, however, P-450-A could be resolved from P-450-B on hydroxyapatite, but the P-450-B fraction still contained significant amounts of P-450-A. Whereas the SDS/PAGE gels indicate excellent purity of the P-450 fractions, this is not evident in the spectrally determined measurements of specific content (Table 1). Since no conversion of P-450 to P-420 was noted with purified P-450 preparations, this discrepancy is still unresolved. The possibility that this represents a partial loss of haem was not investigated. Nonetheless, the specific contents of the purified P-450s were in the same range as those reported for other purified *Drosophila* (Naquira *et al.*, 1980), and housefly (Ronis *et al.*, 1988) P-450 preparations.

Compared with values reported for various mammalian P-450s, there was a disappointing lack of activity of the purified *Drosophila* P-450 in a reconstituted system. Only preparations containing both P-450-A and P-450-B showed any catalytic activity in the sensitive fluorimetric 7-ethoxycoumarin de-ethylase assay. Attempts to detect reconstituted activity for benzo[a]pyrene hydroxylation were unsuccessful. Although PE is known to be the major phospholipid in dipterous insects (Bieber *et al.*, 1961), either PE or PC performed equally well for the low level of reconstituted activity seen in our experiments. Moldenke *et al.* (1984) reconstituted aldrin and heptachlor epoxidase activities with crude partially purified housefly P-450, and found no absolute catalytic requirement for phospholipid. They concluded that residual detergent in the samples may have compensated for the absence of phospholipid. The *Drosophila* P-450 samples in these experiments had detergent removed by chromatography on hydroxyapatite and required either PC or PE for reconstitution of catalytic activity. To explore the possibility that purification procedures had selectively removed some P-450 isoenzymes with specific enzymic activities, sodium cholate-solubilized Hikone-R (BG) microsomes and partially purified fractions from octylamino-agarose were assayed for reconstituted activity after detergent removal on hydroxyapatite. None of these fractions had any detectable activity, even though intact microsomes from both Hikone-R (BG) and Oregon-R exhibit 7-ethoxycoumarin de-ethylase and benzo[a]pyrene hydroxylase activities (Waters *et al.*, 1984b; Waters & Nix, 1988).

Even though rat reductase was somewhat effective in the reconstitution of housefly P-450 activity (Ronis *et al.*, 1988), we were concerned that the fruit fly reductase might be required for *Drosophila* P-450 reconstitution. In an effort to test this, *Drosophila* reductase was partially purified by affinity chromatography on NADP-agarose. The preparation gave a single band on SDS/PAGE with an apparent molecular mass of 65 kDa. We realize that this is the molecular mass of a degraded form of housefly reductase that reduces cytochrome *c* but not P-450 (Vincent *et al.*, 1983; Ronis *et al.*, 1988). Two observations suggest, however, that the 65 kDa form might be the native *Drosophila* reductase. First, the enzyme was iso-

lated by a rapid one-step procedure, and there was no evidence for a larger form of the enzyme on SDS/PAGE. Second, our preparation was as effective as native rabbit reductase (74 kDa) in reducing *Drosophila* cytochrome P-450, i.e. approx. 20 % of the value obtained by chemical reduction with dithionite. These *Drosophila* reductase preparations did not, however, promote reconstitution of P-450-dependent activities. Further studies are required to determine whether the 65 kDa form is the native reductase in *Drosophila*.

Ronis *et al.* (1988) were also relatively unsuccessful in reconstituting housefly P-450-dependent activities. The housefly experiments and those reported here used methods traditionally employed for the reconstitution of mammalian P-450 enzymic activity. The optimum conditions for the reconstitution of insect P-450 activities may be considerably different than those customarily used for mammalian reconstitutions, and this may explain the low levels of activity measured with purified *Drosophila* P-450 preparations. Regardless, the reconstituted 7-ethoxycoumarin de-ethylase activity in the purified fraction containing both P-450-A and P-450-B is about 7 % of that in intact microsomes, and shows that this preparation contains P-450-dependent enzyme activity.

In summary, we have partially purified components of the two major subsets of *Drosophila* P-450: P-450-A, found in all strains, and P-450-B, only present in strains with unique enzyme activities and increased insecticide resistance. We have also performed some preliminary characterization of the catalytic activities of the purified P-450s in a reconstituted system. A major obstacle in the study of P-450-dependent resistance has been the lack of defined, isolated components of the P-450 system. Purified P-450-A and P-450-B represent such components and can be used to generate molecular probes, i.e. antibodies and DNA clones, to study the expression of specific P-450 isoenzymes. The recent isolation and characterization of a cDNA clone of a housefly P-450 represents a major advance in the study of insect P-450 (Feyereisen *et al.*, 1989). Additional cDNA probes, especially those for P-450s associated with resistance, will be useful to study the regulation of P-450 expression and the molecular mechanisms of insecticide resistance. Toward these goals we have used the purified P-450 preparations to generate P-450-A and P-450-B specific monoclonal antibodies and are using them to characterize the multiplicity and expression of P-450s in *Drosophila* and other insects (Sundseth *et al.*, 1987, 1988; Sundseth, 1988; Sundseth *et al.*, 1989) and to isolate the corresponding cDNAs from a *Drosophila* expression library.

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