Resistance to juvenile hormone and an insect growth regulator in *Drosophila* is associated with an altered cytosolic juvenile hormone-binding protein

(insecticide resistance)

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ABSTRACT The *Met* mutant of *Drosophila melanogaster* is highly resistant to juvenile hormone III (JH III) or its chemical analog, methoprene, an insect growth regulator. Five major mechanisms of insecticide resistance were examined in *Met* and susceptible *Met*⁺ flies. These two strains showed only minor differences when penetration, excretion, tissue sequestration, or metabolism of [³H]JH III was measured. In contrast, when we examined JH III binding by a cytosolic binding protein from a JH target tissue, *Met* strains had a 10-fold lower binding affinity than did *Met*⁺ strains. Studies using deficiency-bearing chromosomes provide strong evidence that the *Met* locus controls the binding protein characteristics and may encode the protein. These studies indicate that resistance in *Met* flies results from reduced binding affinity of a cytosolic binding protein for JH III.

Juvenile hormone (JH) is a sesquiterpenoid involved in a variety of critical functions in insects, including development, reproduction, and morphological differentiation (1, 2). A number of chemical analogs have been synthesized, and many of them have potent JH activity as well as the ability to mortally disrupt development of some insect species (3). One of these analogs, methoprene, is a registered insecticide of the insect growth regulator class that is especially effective against dipteran insects (3).

Initially, it was thought that insects would have difficulty evolving resistance to a compound resembling one of their own hormones (4). However, resistance to methoprene was soon demonstrated in several species (5, 6). Recently, we have detected methoprene resistance in *Drosophila melanogaster* in strains having chromosomes derived from natural populations (7) or in susceptible laboratory strains following mutagenesis (8). The latter study identified and genetically characterized a semidominant mutation, *Met* (Methoprene-tolerant), that confers high (100-fold) resistance to methoprene or JH III either topically applied or incorporated into the diet (8). *Met* has been mapped by recombination and in deficiency heterozygotes, and the mutation has been cytogenetically localized to the 10C2–10D4 region of the X chromosome (8). An understanding of the mechanism of resistance of *Met* flies might shed light not only on the genetics of pesticide resistance but also on JH endocrinology.

Biochemical mechanisms of insecticide resistance have been found generally to fall into five categories (9). Each of these was investigated in *Met* and methoprene-susceptible *Met*⁺ flies. Only minor differences between resistant and susceptible flies could be detected for four of these mechanisms. However, a cytosolic JH-binding protein with a 10-fold lower affinity for JH III was detected in *Met* flies,

suggesting a target-site insensitivity mechanism of resistance.

EXPERIMENTAL PROCEDURES

JHs and Insects. JH III (Sigma) and [³H]JH III (New England Nuclear; specific activity, 11.9 Ci/mmol; 1 Ci = 37 GBq) were racemic mixes. [³H]Methoprene (*R* isomer, 83.9 Ci/mmol) was a generous gift of G. Prestwich (Stony Brook, NY). Each was stored in a stock solution in hexane at –20°C. Purity was monitored periodically by thin-layer chromatography. Breakdown was almost negligible over a 1-year period under these conditions. Concentrations were determined by radioactivity or UV spectroscopy.

Flies were raised at 25 ± 1°C on a cornmeal/agar/yeast/molasses diet supplemented with Tegosept or propionic acid to retard mold growth. Adults were collected from uncrowded cultures following eclosion. Third-instar larvae were selected several hours before pupariation from the walls of culture bottles.

Three alleles of *Met* were examined in this study (Table 1). Each was recovered from separate screens for methoprene-resistant mutants following ethyl methanesulfonate mutagenesis of susceptible strains. *Met* and *Met*² were recovered following mutagenesis of the Oregon-RC wild-type strain and *Met*³ of the yellow vermilion strain. Each has been maintained for several years as homozygotes; none has shown significant change in resistance to methoprene during this time. Two susceptible *Met*⁺ strains were examined. First Multiple Seven (*FM7*) is a laboratory balancer strain having a useful semidominant eye mutation (described in ref. 10). *FM7* flies are sensitive to methoprene, and this strain has been used as a *Met*⁺ strain in our previous studies (8, 11). For additional comparison, the wild-type *Ho-R* strain was also examined; these flies have been shown to be sensitive to methoprene (7).

Tenebrio molitor were maintained at room temperature on locally purchased chicken feed. Hemolymph was withdrawn with a microcapillary tube from 1- to 2-day pupae.

Penetration and Excretion. A quantity of 4 pmol of [³H]JH III (New England Nuclear) was topically applied in one dose in acetone solution to third-instar larvae as described (12). This dose was chosen as a physiological dose because the amount of hormone that penetrated the cuticle could be readily measured by its radioactivity. Treated larvae were held in glass scintillation vials for 1 hr at 25°C. They were then rinsed in acetone to remove unpenetrated hormone. Pene-

Abbreviations: JH, juvenile hormone; *Met*, methoprene-tolerant.

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Table 1. *Met* and *Met*⁺ strains used in this study

Genotype	Resistance to methoprene	Ref.
<i>Met</i>	Strong	8
<i>Met</i> ²	Moderate	8
<i>y v Met</i> ³	Strong	Unpublished
<i>FM7</i>	Susceptible	8
<i>Ho-R</i>	Susceptible	7

trated hormone was determined by homogenizing the rinsed larvae in 1 ml of ethyl ether/ethyl acetate (2:1) and assaying an aliquot of the homogenate for radioactivity in Aquassure (New England Nuclear). Excretion of the hormone was estimated by measuring the radioactivity remaining in the incubation vials.

Sequestration. [³H]JH III (4 pmol) was topically applied in acetone solution to third-instar larvae, and after 1 hr at 25°C unpenetrated hormone was removed as described above. Larvae were then dissected into six tissue fractions described in Table 2. Each fraction was solubilized in 0.5 ml of Protosol (New England Nuclear) and then assayed for radioactivity.

Metabolism. Metabolism was measured as described (12) with minor modifications. For hemolymph metabolism, 0.4 µl of hemolymph collected from 10–12 third-instar *Drosophila* larvae or from one *Tenebrio* pupa was incubated with 80 fmol of [³H]JH III for 1 hr at 25°C. *In vivo* metabolism by third-instar larvae was measured using the application and homogenization procedure described above for the penetration experiments and then extracting the radiolabel from the homogenate with diethyl ether. JH III metabolites were identified by running the hemolymph and homogenate extracts on silica gel thin-layer chromatography sheets (Baker) together with purified metabolites in two solvent systems: benzene/ethyl acetate (4:1) and benzene/propanol (9:1). The plates were cut into 1-cm strips and assayed for radioactivity directly in Aquassure.

Fat Body Cytosol Preparation and Binding Assay. Larval fat body cells from 0 to 4-hr-old adults, frozen at –80°C, were dissected by making a small tear in the abdomen and shaking the cells loose into TTgm buffer (13) on ice. The cells were washed four times in TTgm buffer and then placed in LS buffer (14) containing 5 µM 3-octylthio-1,1,1-trifluoro-2-propanone as an esterase inhibitor (15) and stored at –80°C until used. To make the fat body cytosol, cells were homogenized in a glass homogenizer and spun at 30,000 × *g* for 10 min. The supernatant was filtered through glass wool and then spun at 100,000 × *g* for 1.5 hr. The resulting supernatant was extracted with dextran-coated charcoal (DCC) to remove endogenous JH (13). This extraction procedure with DCC resulted in a 40% increase in specific binding of [³H]JH III. Extraction was carried out by incubating 0.25 ml of DCC (16) with 1 ml of cytosol for 2 min on ice and spinning at 5000 × *g* for 2 min. The cytosol was divided into aliquots and stored at –80°C until used in a binding assay.

The hydroxyapatite (HAP) binding assay was used as described (14), except that cytosol and [³H]JH III were incubated for 30 min before adding the HAP. Nonspecific binding was measured in the presence of a 100-fold excess of unlabeled JH III.

Binding Data Analysis. The data are graphically represented in Scatchard plots; however, the binding parameters *K*_d (dissociation constant) and *R*_t (binding capacity) were determined by nonlinear curve fitting analysis using LIGAND (17), as modified by McPherson (18). This analysis was done by treating each datum point separately and using a constant level of nonspecific binding, determined by averaging the nonspecific binding measured with the binding assay. Scatchard analysis of the same data indicated *K*_d and *R*_t values

approximately two times larger than those determined with LIGAND; however, the same differences in magnitude between *FM7* and *Met* strains were found. Binding parameters determined from LIGAND were selected over those from the Scatchard analysis because LIGAND provides a more objective and more exact analysis of binding data (17).

RESULTS

In the initial studies two alleles, *Met* and *Met*², were compared with methoprene-susceptible *FM7* flies. JH III, one of two naturally occurring juvenile hormones in *D. melanogaster* (19–21), is commercially available in radiolabeled form and was used in these studies; *Met* flies are as resistant to topical application of this hormone as to methoprene (8).

First, we examined reduced penetration of JH III through the cuticle of *Met* flies as a basis for the resistance. When [³H]JH III was topically applied to *FM7*, *Met*, or *Met*² larvae, little difference in the rate of penetration after 1 hr could be discerned among the three strains (Fig. 1 *Upper*). Therefore, it does not appear that penetration of hormone is sufficiently different among the strains to account for the high resistance seen.

Enhanced excretion (22) and sequestration (23) of insecticides seem to be responsible for the resistance of some insects. To check for these two mechanisms, larvae were topically treated with [³H]JH III. After 1 hr excretion and tissue sequestration of the penetrated hormone were measured. No strong difference was seen among *FM7*, *Met*, or *Met*² in either excretion (Fig. 1 *Lower*) or sequestration (Table 2).

A widespread mechanism of insecticide resistance is enhanced metabolism of the compound by insect tissues (9). Since enhanced methoprene metabolism was found in methoprene-resistant strains of houseflies (24) and mosquitoes

FIG. 1. JH III penetration into (*Upper*) and excretion out of (*Lower*) homozygous larvae. Four microliters (4 pmol) of [³H]JH III was topically applied to 12 larvae and afterward treated as described in the text. Ordinate values are the % of the total radiolabeled hormone applied to the larvae. Values are the averages of five replicates ± SD.

Table 2. Distribution of penetrated JH III in larvae

Genotype	% of penetrated [^3H]JH III					
	Hemo-lymph	Cuticle	Gut	Brain	Salivary glands	Fat body
<i>FM7/FM7</i>	19	10	50	3	3	16
<i>Met/Met</i>	22	14	44	3	4	14
<i>Met²/Met²</i>	25	11	42	2	3	16

One microliter (400 fmol) of [^3H]JH III was topically applied to four larvae and examined for sequestration. Some fractions contained more than one type of tissue: cuticle and trachea; gut and Malpighian tubules; brain and imaginal disks. The buffer in which the dissections were done was designated the hemolymph fraction. Each value is the average of six determinations on separate populations of larvae.

(25), this possible mechanism was of particular interest and was evaluated by examining the products of metabolism of JH III *in vitro* and *in vivo*. When hemolymph from *FM7*, *Met*, or *Met²* larvae was incubated *in vitro* with [^3H]JH III, little hormone breakdown was seen in any of the strains (Table 3). These results ruled out enhanced metabolism by a degradative enzyme in *Met* hemolymph, corroborating previous results from a study of genetic mosaics of *Met* flies that ruled out a circulating factor as the focus of resistance (8). *In vivo* metabolism of topically applied [^3H]JH III by third-instar larvae was greater than that in hemolymph (Table 3), as was expected from previous results with JH I metabolism in *D. melanogaster* (12). However, we could detect only minor differences in the amount of metabolism among *FM7*, *Met*, and *Met²* larvae.

A final mechanism examined was "target-site insensitivity" (27). This type of insecticide resistance is usually due to an altered ligand receptor that has lower affinity for the insecticide, thus resulting in decreased perception at the target tissue site (9). Target-site insensitivity has become increasingly important as a demonstrated mechanism of resistance to insecticides (9, 27). If the resistance of *Met* is due to this mechanism, then one might expect to find an altered cellular JH-binding protein or cellular response to JH in these flies.

Since a high-affinity JH-binding protein had been previously detected in *D. melanogaster* hemolymph (14), it was not appropriate to examine whole flies for a cellular JH-binding protein. However, we detected specific JH III binding in the cytosol of larval fat body tissue from newly enclosed adults. This tissue appears to be a JH target tissue since its histolysis during the first 2 days of adult life is regulated by JH (28). When fat body cytosol was examined for [^3H]methoprene binding, no specific binding could be

Table 3. Metabolism of JH III by *Drosophila* larvae

Genotype	Metabolite, % of total			Unmetabolized JH III
	Diol	Acid	Acid-diol	
Larval hemolymph				
<i>FM7/FM7</i>	1.9	1.9	1.0	94.7
<i>Met/Met</i>	1.2	2.7	1.1	94.9
<i>Met²/Met²</i>	1.2	1.0	0.8	96.6
<i>Tenebrio</i>	17.5	73.7	2.8	5.6
Whole larvae				
<i>FM7/FM7</i>	9.2	7.4	53.0	22.9
<i>Met/Met</i>	6.8	5.0	55.6	26.0
<i>Met²/Met²</i>	6.0	4.4	55.8	26.2

Since JH metabolism by hemolymph is less pronounced in *Drosophila* (12) and other dipteran insects (26) than in insects from other orders, hemolymph from the beetle *T. molitor* was included as a positive control. Each value is the average of two determinations on separate populations of larvae.

detected. This result is not surprising since, based on the ability of methoprene to compete for [^3H]JH III binding (11), methoprene would have a very low binding affinity ($K_d = 10 \mu\text{M}$). Because nonspecific binders of JH have comparable K_d values and much larger binding capacities (29), any specific binding of methoprene would be obscured by the nonspecific binding. Therefore, [^3H]JH III was used as the ligand for the binding studies.

JH III binding was found to be saturable, specific for JH III, and associated with a protein; a full characterization is presented elsewhere (11). The binding data were plotted using the method of Scatchard (30), which allows an estimate of the K_d and the R_t . K_d can be determined from the slope of the line; a steeper slope indicates a higher affinity for the ligand. R_t can be determined from the x-axis intercept. These values can also be determined by nonlinear curve fitting using the LIGAND program, which we used. Analysis of the specific binding from *FM7* homozygotes revealed a single binding component with a K_d of 4.5 nM (Fig. 2 Upper), well within the range found for cytosolic JH-binding proteins from other insects (31). A similar K_d was found for the methoprene-susceptible wild-type *Ho-R* strain (Table 4). However, a much higher value of 50 nM was found for *Met* homozygotes (Fig. 2 Upper). The difference in K_d between *FM7* and *Met* was highly significant (*F* test, $P < 0.001$). *Met²*, a weaker allele (8), had a K_d of 23 nM (Table 4) and *Met³*, another strong allele (unpublished data), had a K_d of 58 nM (Table 4). Since *Met³* was induced in a strain (yellow vermilion) different from that of *Met* and *Met²* (Oregon-RC), variations in the

FIG. 2. Scatchard plots of JH III specific binding by larval fat body cytosol from *FM7/FM7* (●) and *Met/Met* (○) homozygotes (Upper) and from *Met/FM7* heterozygotes (Lower). Lines on Scatchard plots are regression lines; lines S1 and S2, shown for the heterozygotes, were computer-generated by LIGAND. Each point on the Scatchard plots is the average of one to three determinations in separate experiments with separate fly cultures.

Table 4. Binding parameters of JH III specific binding to larval fat body cytosol

Strain	K_d , nM	R_t , fmol per fly
<i>Ho-R</i>	4.0	4.6
<i>Met²/Met²</i>	23.0	12.0
<i>Met³/Met³</i>	58.0	21.2
<i>FM7/Df(1)N71</i>	8.0	5.6
<i>Met/Df(1)N71</i>	49.0	20.0
<i>FM7/Df(1)M13</i>	18.0	7.0
<i>Met/Df(1)M13</i>	59.0	30.4

background genomes of *Met* and *FM7* are not responsible for the binding differences seen.

The change in binding affinity might be due to *Met* control of either the binding protein itself or of some modification of the binding protein, perhaps through a posttranslational modifying enzyme. If a modifying enzyme is defective in *Met* flies, then *Met/Met⁺* flies would be expected to have sufficient wild-type modifying enzyme to result in a binding protein having an essentially wild-type binding affinity. However, if *Met* directly controls the binding protein, one would expect two binding components in *Met/FM7* flies, each binder derived from the respective *Met* or *Met⁺* allele. This proved to be the case. Analysis of binding from *Met/FM7* heterozygotes showed a biphasic binding curve, with binding affinities characteristic of *FM7* and *Met* homozygotes (Fig. 2 Lower). LIGAND analysis found these data to fit two sites with the indicated binding parameters significantly better than one site (*F* test, *P* < 0.005).

These results correlate the *Met* mutation with the reduced binding affinity. To determine if *Met* controls this change in K_d , JH III binding was measured in heterozygotes of *Met* or *Met⁺* with either of two deficiency (*Df*) chromosomes that uncover the *Met* phenotype in *Met/Df* heterozygotes (8) and thus lack the *Met* gene. The K_d values for the *FM7/Df* flies were similar to those from the susceptible strains (Table 4). The *Met/Df* flies, unlike the *Met/FM7* heterozygotes, showed a single binding component with the characteristic *Met* K_d (Table 4). Therefore, neither of the deficiency chromosomes contributes a *Met⁺* binding component to *Met/Df* flies, presumably because the *Met⁺* binder is encoded by a gene located within the deleted portion of each deficiency chromosome. Since the region common to both deficiencies is only 4 bands (out of a total of 5000 in the *Drosophila* genome), this finding provides strong evidence that the *Met* mutation is the cause of the reduced binding affinity seen in the resistant strains.

DISCUSSION

Contrary to initial predictions (4), insect populations are capable of evolving high resistance to JH analog insecticides. Although methoprene-resistant field populations resulting from direct methoprene exposure have not been reported to date (probably because of the limited use of methoprene), cross-resistance of insect populations resistant to other classes of insecticides has been documented (5, 6, 32). Moreover, populations of *Culex* mosquitoes resistant to methoprene have been selected in the laboratory (33, 34). In none of these studies has the gene(s) responsible for resistance been identified. The ease of *Drosophila* genetic manipulations allowed us to identify the first gene, *Met*, involved in methoprene and JH III resistance (8). In the present work we have determined that the resistance apparently results from an altered cytosolic JH-binding protein in a JH target tissue. Previous studies of methoprene resistance have found enhanced metabolism to be most important in metho-

prene resistance (24, 25). The target-site insensitivity mechanism in *Met* flies represents a resistance mechanism to an insect growth regulator that has been previously unreported.

It is possible that the binding protein altered in *Met* flies is a JH receptor, especially considering that *Met* mutes a variety of responses to methoprene or JH III (8). We have also established a more direct relationship between the binding protein and a biochemical response to JH (11), but we cannot say with certainty that the binding protein is a JH receptor. Regardless of whether the binding protein is directly or indirectly involved in JH reception, the biphasic binding curve of *Met* heterozygotes suggests that the *Met⁺* locus encodes the binding protein. If the function of the *Met⁺* product were to modify the binding protein instead of encoding it, one would expect only one binding protein (and having *Met⁺* binding affinity) in heterozygotes.

The R_t is also altered in *Met* flies when R_t is normalized on a per-fly basis. Another basis for R_t , protein concentration, could not be used since in larval fat body tissue the rates of protein accumulation during larval development and of protein loss following eclosion vary widely according to hormonal, genetic, and environmental factors (35). *Met* strains had a larger R_t than did *Met⁺* strains. We believe that this increase in R_t represents a failure of JH-driven down-regulation of the binding protein in *Met* flies. Down-regulation of hormone receptors is a widespread phenomenon in vertebrates (36, 37) and has been demonstrated for ecdysone in the *Drosophila* K_c cell line (38). If down-regulation normally occurs in *Met⁺* flies, then the lower binding affinity of *Met* tissue for endogenous JH III might result in an insufficient cellular signal for normal down-regulation, resulting in an increased amount of binding protein in the mutant. To what extent the increase in R_t offsets a lowered K_d value for target-tissue responses to JH in *Met* flies is unclear. However, since *Met* females are nearly as fecund as *Met⁺* (39), perhaps the offset is appreciable in some presumed target tissues, like the ovary, but not in others, including those manifesting the resistance phenotypes (8).

Recent work in *Manduca* has demonstrated that JH and a JH analog, iodovinylmethoprenol, bind to separate sites in target tissue nuclei (40), suggesting that methoprene and JH may have separate binding proteins. This result is consistent with earlier work in other insects, including *Drosophila*, showing that methoprene is a poor competitor for JH III binding to an intracellular binding protein (31). We have found that fat body cytosolic extracts will bind labeled methoprene, but a large amount of nonspecific binding obscures any specific binding (unpublished data). Therefore, the relationship between JH III and methoprene binding in *Drosophila* is unclear. However, since *Met* as a single gene mutation confers resistance to both hormones, it would seem that some feature of the binding is common to both hormones. Perhaps they share a common protein (controlled by *Met*) with distinct binding sites for the two hormones.

It is unexpected that a mutant fly possessing such high resistance to JH would have high fitness (39). Although no role for JH has been detected in preadult *Drosophila*, it is clear that JH has roles in oogenesis (reviewed in ref. 41) and male accessory gland functioning (11, 42). If *Met* flies have a reduced ability to bind their endogenous hormone, one would expect a phenotype of at least partial sterility. This is in fact seen early in adult vitellogenic oocyte development, when *Met* females show resistance to JH-induced vitellogenic oocyte development (8). However, after several days oogenesis in *Met* and *Met⁺* females proceeds at nearly equivalent rates. Several explanations are possible: (i) the tissue-specific down-regulation response has been mentioned above; (ii) the newly discovered JH bisepoxide (21) may be the primary gonadotrophic hormone in *Drosophila* and its binding may be less affected by the *Met* mutation than is that

of JH III; or (iii) the *cricket* gene product (43) may be more important in JH signal reception in adult flies than in larvae. In support of the last possibility, *cricket* flies are female sterile (43); further speculation must await JH-binding studies in these flies.

Recently, binding of the inducer 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in *Heliothis zea* extracts has been shown to be competitively inhibited by DDT [1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane], JH I, or methoprene, and these authors postulate that JH receptors may be involved in resistance to a variety of insecticides (44). *Met* flies are not cross-resistant to other types of insecticides (8). Thus, we have no evidence that resistance resulting from the *Met* mutant extends beyond JH III and its analogs.

Insect growth regulators are attractive insecticides due to low mammalian toxicity (45). Presumably, target-tissue binding is involved in the action of each. Here, we have demonstrated that alteration of this binding can result in high resistance, yet retention of fitness. If target-tissue insensitivity proves to be a widespread mechanism of resistance to these compounds, then based on the characteristics of *Met*, resistance would be expected to evolve rapidly in pest populations.

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