An insect-specific P450 oxidative decarbonylase for cuticular hydrocarbon biosynthesis

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Insects use hydrocarbons as cuticular waterproofing agents and as contact pheromones. Although their biosynthesis from fatty acyl precursors is well established, the last step of hydrocarbon biosynthesis from long-chain fatty aldehydes has remained mysterious. We show here that insects use a P450 enzyme of the CYP4G family to oxidatively produce hydrocarbons from aldehydes. Oenocyte-directed RNAi knock-down of *Drosophila* CYP4G1 or NADPH-cytochrome P450 reductase results in flies deficient in cuticular hydrocarbons, highly susceptible to desiccation, and with reduced viability upon adult emergence. The heterologously expressed enzyme converts C_{18}-trideuterated octadecanal to C_{17}-trideuterated heptadecane, showing that the insect enzyme is an oxidative decarbonylase that catalyzes the cleavage of long-chain aldehydes to hydrocarbons with the release of carbon dioxide. This process is unlike cyanobacteria that use a nonheme diiron decarbonylase to make alkanes from aldehydes with the release of formate. The unique and highly conserved insect CYP4G enzymes are a key evolutionary innovation that allowed their colonization of land.

Insects are the largest group of extant terrestrial organisms. As their crustacean ancestors moved from aquatic to terrestrial environments, insects were confronted with a new, dry frontier. Insects solved the ecophysiological problem of how to restrict water loss to prevent dessication by depositing long-chain hydrocarbons as essential waterproofing components on their epicuticle (1). These diverse chemicals now serve many additional functions, particularly in defense, reproduction, and communication (2). In flies, cuticular hydrocarbons (CHs) are a complex blend of long-chain (~C21–C37+) alkanes and alkenes that serve as species- and sex-specific semiochemicals, and some components are sex pheromones. CHs also serve in nest mate recognition by social insects and as trail pheromones in ants; the complexity of their blend can be useful in taxonomic discrimination of mosquitoes. Much is known about the biosynthesis of CHs from fatty acids in insects, involving a complex network of fatty acid synthases, elongases, and desaturases, leading to very long-chain acyl-CoA thioesters. These are converted by acyl-CoA reductases to aldehydes that serve as substrates for the last oxidative decarbonylation step (2) (Fig. 1). The single carbon chain-shortening conversion of precursor aldehydes to hydrocarbons is catalyzed by a P450 enzyme, P450hyd, with release of CO_2 (3), but this enzyme has not yet been identified. The only known decarbonylase that has recently been described occurs in cyanobacteria that use a nonheme diiron enzyme (4–8) to catalyze the last step (Fig. 1). Insect CH are synthesized in large ectodermally derived cells (9) called oenocytes (10–12), and are then shuttled by hemolymph lipophorin (13, 14) to the cuticle, where they are deposited on the outer epicuticular layer. Here we identify P450hyd as CYP4G1 in *Drosophila melanogaster*, and show that the enzyme is massively coexpressed with NADPH cytochrome P450 reductase in oenocytes. A recombinant fusion protein of house fly CYP4G2 with P450 reductase catalyzes aldehyde decarbonylation by a radical mechanism that is unique and different from the cyanobacterial aldehyde decarbonylases.

Results

CYP4G1 and P450 Reductase in *Drosophila* Oenocytes. We searched for a candidate aldehyde oxidative decarbonylase P450 (CYP) gene in the large complement of CYP genes of insect genomes (range in sequenced genomes: 37–170) (15, 16). We focused on the CYP4G subfamily because it had at least one ortholog in all insect genomes (16), because no members of this subfamily were functionally characterized, and because microarray data showed that *Cyp4g1* is the most highly expressed of all 85 *D. melanogaster* P450 genes (17). Immunohistochemistry of CYP4G1 and of its obligatory redox partner, NADPH-cytochrome P450 reductase (CPR), in *D. melanogaster* abdomens showed that both proteins colocalized in very large subepidermal cells that were easily visible by confocal microscopy through the cuticle (Fig. 2). These cells are oenocytes, distributed as first described by Koch (18) in "abdominal belts" in each hemisegment. Our observations were consistent with the anatomical identification of oenocytes in adult *D. melanogaster* (19–21) and with high *Cyp4g1* expression levels in these cells (20, 22). High CPR expression in mosquito and *D. melanogaster* oenocytes has also been reported (23). Our colocalization of high levels of both CYP4G1 and CPR in oenocytes suggests a near optimal CYP4G1-CPR stoichiometry in those cells.

RNAi Suppression of CYP4G1 and CPR. To show that *Cyp4g1* encodes an aldehyde oxidative decarbonylase, we first studied the effect of RNAi-mediated knock down of the gene on CH levels. We crossed oenocyte-selective GAL4-promoter lines with transgenic lines for UAS-driven dsRNA for *Cyp4g1* or CPR. RNAi suppression of either CYP4G1 or CPR in oenocytes produced identical phenotypes, with high mortality at the time of adult emergence (Fig. S1) and a dramatic decrease in cuticular alkane/alkene content of flies that survived (Table 1). GC-MS analysis of 5-min hexane washes of flies showed that although control flies had a normal, complex profile of alkanes and alkenes on day 1 posteclosion, the RNAi-suppressed flies had a significantly different pattern, with 12 different esters and fatty acids—not normally found on the epicuticle—predominating, and with alkanes/alkenes as minor components (Fig. 3 and Table S1). Principal component analysis of 38 peaks identified from the GC-MS profile showed that the effect was similar for CYP4G1- and CPR-suppressed flies, confirming that the pattern of CH depended on high expression of both genes in oenocytes (Fig. 3). Three longer-chain CH were still present on the cuticle

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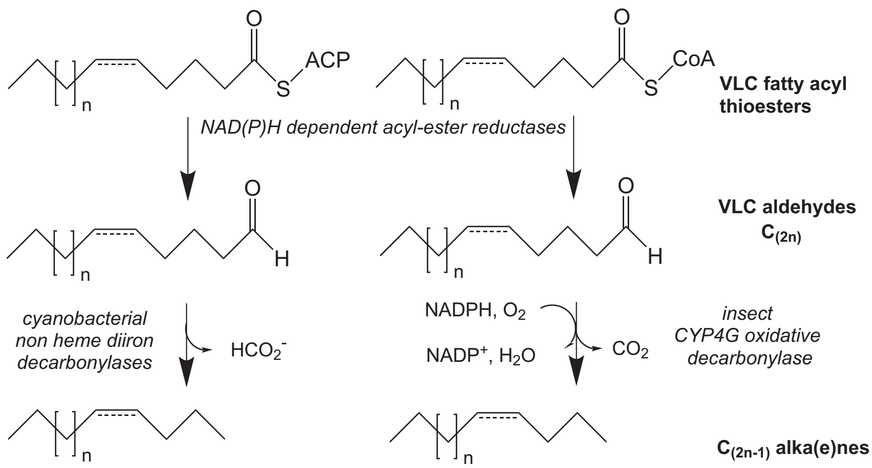

Fig. 1. Hydrocarbon biosynthesis from very long-chain fatty acyl thioesters in cyanobacteria and in insects. The decarbonylase enzyme from plants has not been formally identified to date. ACP, acyl carrier protein.

in day 1 flies, and CYP4G1-suppressed flies that survived to maturity on day 4 had an even more pronounced decrease in the level of all CH, including the long-chain CH (Tables S2 and S3). When measured on surviving mature flies on day 4, the deficit in CHs caused by RNAi suppression of CYP4G1 resulted in a significant decrease in desiccation tolerance, from a median lethal time (LT_{50} ± SEM) of 11.1 ± 0.3 h to 6.1 ± 0.3 h (males) and from 12.2 ± 0.5 h to 6.4 ± 0.2 h (females) (Fig. 4). The decrease in CHs by RNAi suppression of CYP4G1 in females also affected courtship behavior of control males, with decreases in the percentage of males courting and copulating, increases in the latency time of these behaviors, and severely reduced numbers of wing vibrations and copulation attempts (Fig. S2): all phenotypes that are typical of reduced CH levels in females (21).

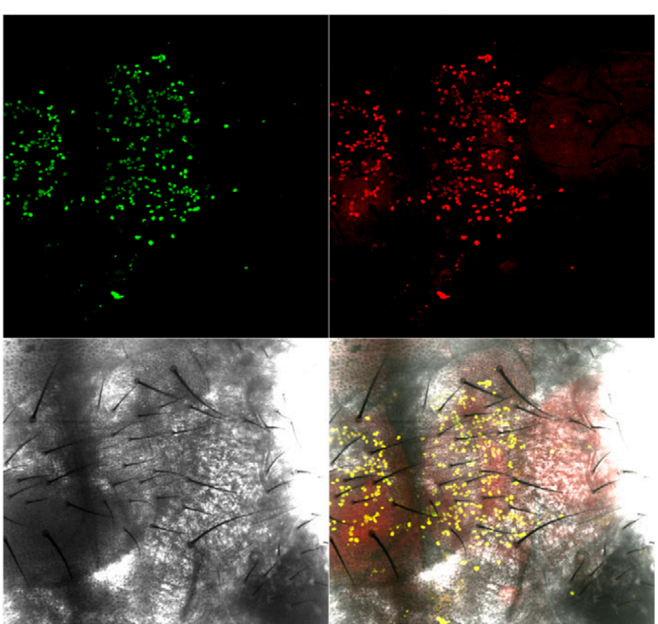

Fig. 2. Colocalization of CYP4G1 and CPR in oenocytes. Whole-mount immunocytochemistry of NADPH-cytochrome P450 reductase (*Upper Left*, FITC) and CYP4G1 (*Upper Right*, Alexa 633) in *Drosophila* abdomens. Confocal microscopy shows the bands of large oenocytes where both enzymes are colocalized (*Lower Right*, yellow). *Lower Left* is the bright field image showing bristles for scale.

Oxidative Decarbonylase Activity of CYP4G2. Direct evidence of the CYP4G activity as aldehyde oxidative decarbonylase was obtained by functional expression of the orthologous *Drosophila* (CYP4G1) and house fly (CYP4G2) enzymes. Preliminary assays using yeast (*Saccharomyces cerevisiae*) coexpressing CYP4G1 and CPR yielded low and erratic conversion of C24, C26, and C28 aldehydes to the respective C23, C25, or C27 alkanes. The abundance of both CYP4G1 and CPR in oenocytes suggested that aldehyde oxidative decarbonylase function in vivo depends on high levels of both P450 and CPR, perhaps associating physically with fatty acyl-CoA reductases as a substrate-channeling cellular metabolon. Such metabolons have been postulated for cyanogenic glucoside biosynthesis in plants (24). To ensure stoichiometric coproduction of both enzymes, we thus produced a CYP4G2-CPR fusion protein in *Sf9* (insect) cells using a baculovirus expression system (Fig. S3*A*). Microsomes from infected *Sf9* cells producing the fusion protein had a CO-difference spectrum with a peak at 450 nm, as expected (Fig. S3*B*). This enzyme preparation showed time- and NADPH-dependent oxidative decarbonylase activity on octadecanal (Fig. S3 *C* and *D*). To verify the identity of the reaction product, we incubated the enzyme with 18,18,18-$^2H_3$-octadecanal and analyzed the product by GC-MS. The heptadecane obtained was trideuterated with a mass spectrum shifted by three atomic mass units ($M^+ = 243$) compared with the heptadecane standard ($M^+ = 240$). Furthermore, the carbonyl carbon was released as CO_2, confirming experiments with unpurified native enzymes (25) (Fig. 5). The considerable hydrophobicity of substrates and products and sluggishness of the enzyme prevented us from obtaining dependable kinetic constants, and by these limitations, the insect oxidative decarbonylase is similar to the cyanobacterial aldehyde decarbonylases (4–8).

Discussion

Our data conclusively show that insect CYP4Gs function as oxidative decarbonylases, catalyzing the terminal step in insect hydrocarbon production. Our conclusion is based on the localization of CYP4G1 and CPR in oenocytes, the known site of CH biosynthesis; on the suppression of CH levels in flies made deficient in CYP4G1 or CPR by RNAi; and on the conversion of aldehyde to hydrocarbon by a recombinant CYP4G2-CPR enzyme in vitro.

The high level of CPR protein in oenocytes shown here and in a previous study (23) was somewhat surprising because CPR is

Table 1. **Effect of RNAi suppression of CYP4G1 and of CPR in oenocytes on cuticular lipids of *D. melanogaster***

	Males			Females		
	n	Esters and fatty acids	Alkanes and alkenes	*n*	Esters and fatty acids	Alkanes and alkenes
GAL4 parents	6	1.7 ± 0.2 a	427 ± 31 a	6	1.0 ± 0.1 a	475 ± 35 a
UAS parents	10	1.2 ± 0.4 a	537 ± 49 a	10	0.8 ± 0.2 a	452 ± 143 a
CYP4G1 RNAi crosses	20	695 ± 136 b	196 ± 7 b	19	943 ± 231 b	235 ± 9 b
CPR RNAi crosses	17	570 ± 121 b	172 ± 9 b	19	762 ± 137 b	214 ± 10 b

The average (nanogram per fly ± SEM) of total alkanes and alkenes (26 compounds) and of total esters and fatty acids (12 compounds) quantified by GC-MS on day one are shown for males and females. Average values in each column followed by the same letter are not statistically different (ANOVA; $P < 0.05$).

ubiquitously present in all cell types where P450 enzymes function and P450 levels are usually in excess of CPR levels (16). As P450 activity is determined by the concentration of the [P450-CPR] complex irrespective of which enzyme is present in excess (26), the high levels of both CYP4G1 and CPR in oenocytes must have a specific biochemical significance that we are currently exploring. Oenocyte-selective RNAi suppression of CYP4G1 and CPR considerably changed the profile of CH. Control profiles on day 1 were consistent with previous studies (27), but RNAi-suppressed flies had new lipids (midchain fatty acids and esters) and much reduced alkanes/alkenes. There are two possible explanations for the origin of the new lipids that we consider to be a pathological side-effect. On one hand, block of terminal decarbonylation may shunt precursors to lipophorin (13, 14) that would then deliver these abnormal lipids to the cuticle, and such retrafficking has been observed previously (14, 28). On the other hand, the hexane extraction designed to recover CH may be harsher on unprotected cuticle and extract lipids from now more accessible stores. More intriguing is the observation that although alkanes/alkenes of midchain length are virtually absent in young RNAi-suppressed flies, the longest-chain CH are still present on day 1 but are decreased drastically on day 4. We interpret this differential retention of long-chain hydrocarbons by a slower clearance of the CH pool in the body (hemolymph lipophorin and other stores) that results from their higher hydrophobicity. RNAi severely reduces biosynthesis in the oenocytes, but the CH measured on the epicuticle are drawn via lipophorin from the whole body pool of hydrocarbons and not directly from oenocytes (2, 13, 14, 29). A similar relative retention of the higher-chain CH was also observed in flies with genetically ablated oenocytes (21). It is still unclear how hydrocarbons produced in the oenocytes located within the epidermal cell layer and loaded onto hemolymph lipophorin cross the cuticle from the hemocoel to the outside epicuticle, but the pool of hydrocarbons in the body is considerable compared with the steady state levels of CH on the epicuticle (29). Taken together, our experiments show that CYP4G1 synthesizes all CHs, so that species, sex, and age-specific hydrocarbon composition (2, 30) is controlled by the combined upstream activities of elongases, desaturases, and acyl-CoA reductases, all converging to the aldehyde oxidative decarbonylation as the last step of alkane/alkene formation.

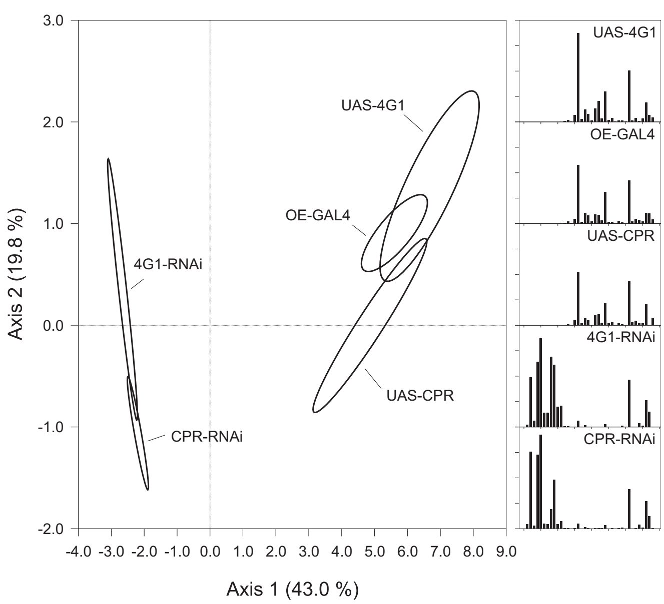

Fig. 3. Effects of RNAi suppression of CYP4G1 and CPR on cuticular hydrocarbons. Principal component analysis (PCA) of the peaks identified from the GC-MS profile of cuticle hexane washes of 1-d posteclosion flies. The correlation matrix of the values in nanograms per fly for 38 peaks and 107 profiles corresponding to five genotypes was analyzed using the PRINCOMP procedure in SAS (v9). The first two axes of the PCA account for 62.8% of the total variance in the data. Each ellipse represents the 2D 95% confidence interval of the mean of data for five fly genotypes. The ellipses on the right are the three parental genotypes (OE-GAL4, oenocyte GAL4 driver; UAS-4G1, UAS-dsRNA for CYP4G1; UAS-CPR, UAS-dsRNA for CPR) (n = 12, 10, and 10, respectively). On the left are the two GAL4-UAS offspring genotypes, 4G1-RNAi and CPR-RNAi (n = 39 and 36, respectively), that are similar to each other and nonoverlapping with their parents. The histograms on the right are the mean value of each peak for the five genotypes on the same scale. The peaks shown are 12 esters and fatty acids to the left and 26 hydrocarbons to the right, ranked by molecular weight (details in Table S1).

Data presented here are consistent with the results of several previous studies, if not with all their conclusions. The lethality of *Cyp4g1*-null mutants after larval life (20, 22) is consistent with the transition out of a humid environment, and death through desiccation of adults with severely reduced CH levels has been noted before (31). Larval oenocytes were thought to perform the same lipid-processing functions as the mammalian liver, leading to misidentification of CYP4G1 as a lipid ω-hydroxylase (20). A feedback effect of genetic oenocyte ablation on hemolymph lipid accumulation, and the role of oenocytes in scavenging lipids from fat body stores during starvation, were thought to model hepatic steatosis (20). A more parsimonious explanation is that oenocytes, as the site of hydrocarbon biosynthesis, are a sink for lipid precursors of CH, and that their genetic ablation profoundly disturbs the organismal lipid balance. Adult insects carry considerable amounts of CH on the epicuticle, between 2 μg (*D. melanogaster*) and 100 μg (*Drosophila mojavensis*) (30), and have equivalent stores of internal hydrocarbons (29). These high amounts can be rationalized by the high surface-to-volume ratio of insects that makes desiccation resistance a major challenge (1). Significantly, insects entering diapause, and hence prolonged periods of metabolic quiescence, overexpress CYP4G (32).

The biosynthesis of hydrocarbons has been convincingly shown to occur only in insects, plants, and bacteria; little information is available for other organisms. In particular, it is not clearly established whether terrestrial arthropods other than insects are capable of de novo hydrocarbon biosynthesis. Some bacteria make long-chain alkenes by a nondecarboxylative Claisen

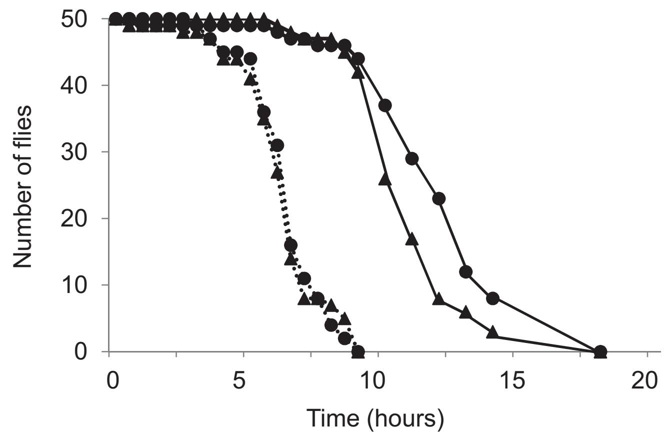

Fig. 4. Desiccation resistance of adult *D. melanogaster*. The time course of adult male (▲) and female (●) fly survival in dry conditions is shown for control insects (full lines) and for flies with RNAi-suppressed CYP4G1 expression (stippled lines). n = 20 for each condition.

condensation reaction of fatty acids "head to head" (33). A bacterial P450 uses a mechanism related to β-hydroxylation to make 1-alkenes (34). The recently described cyanobacterial aldehyde decarbonylases are members of the nonheme diiron oxygenase family (4, 7) and function by cleaving a fatty aldehyde with release of formate (5, 6, 8). Plants appear to use a putative metalloenzyme thought to release CO from the aldehyde (35), although the likely candidate (36) has not been biochemically characterized. We now confirm that insects have solved the difficult chemical problem of terminal carbon-carbon bond cleavage in a different way, with release of CO_2 rather than formate, and with NADPH consumption (Fig. 1). P450 enzymes are known to oxidatively cleave C-C bonds as in cholesterol side-chain cleavage (CYP11A) or in aromatase (CYP19) (37), but the CYP4G reaction is unique and may involve the formation and retention of radicals within the active site (25, 37). A characteristic Asp/Glu-rich insertion of ~35 aa between helix G and helix H is unique to CYP4G P450s and may contribute to this ability (Fig. S4). The insertion may push the F and G helices over the top of the active site, to seal it and allow the radical chemistry to occur.

CYP4G enzymes are members of an insect P450 subfamily that is remarkable because CYP4G genes are among the very few P450s with orthologs distributed across the Insecta. The ecdysone-biosynthetic genes that belong to the Halloween group of genes in *Drosophila*, and the juvenile hormone epoxidase gene are the other best known examples of conserved orthologs across insects (16, 38). By comparison, the larger CYP4 clan, to which the CYP4G subfamily belongs, is highly diversified in each insect genome, with 20–50 genes originating from recent series of duplications (16, 38). However, within the CYP4 clan there are

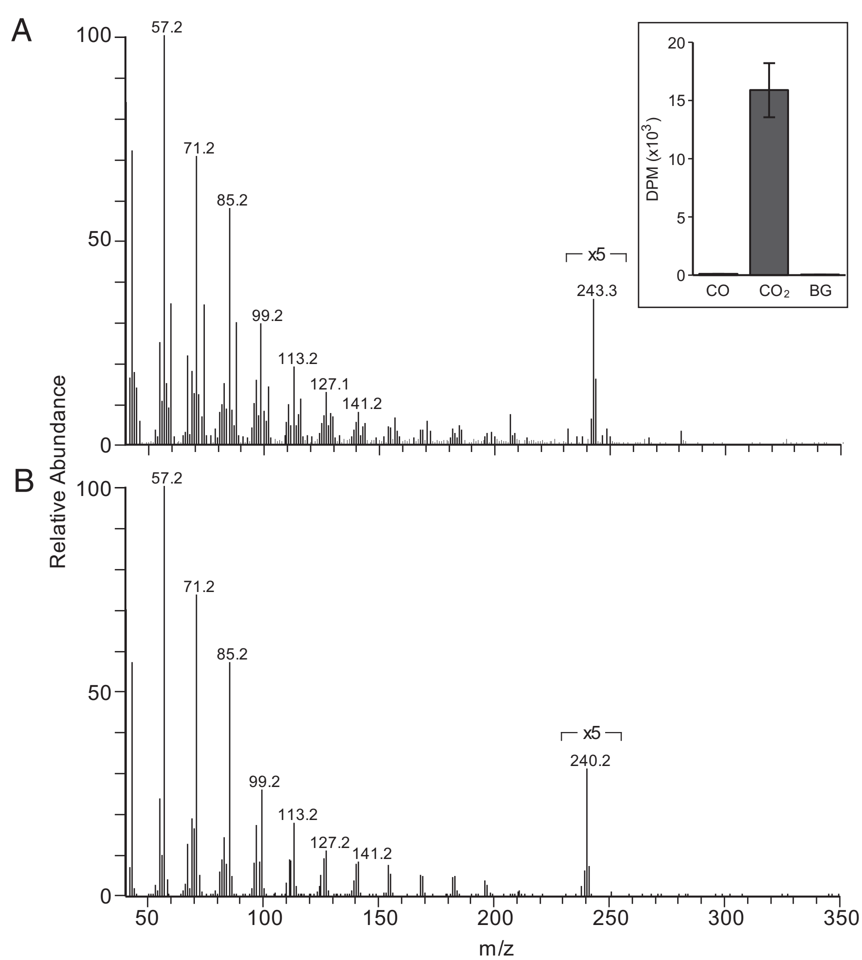

Fig. 5. Metabolism of aldehyde to alkane by recombinant CYP4G2/CPR fusion enzyme. Mass spectrum of trideuterated reaction product from [18,18,18-$^2H_3$] octadecanal (*A*) and of reference heptadecane (*B*). (*Inset*) Trapping assays show the carbonyl-carbon of [1-^{14}C]-C18 aldehyde is released as CO_2. Values are means ± SEM, n = 3. BG, background.

only one (honey bee, aphid) or very few (two in *Drosophila* and most other species) CYP4G genes in each species. We did not find CYP4G genes in the genomes of Crustacea (the water flea *Daphnia pulex* or the salmon louse *Lepeophtheirus salmonis*), nor in the genomes of Chelicerates (the spider mite *Tetranychus urticae* or the tick *Ixodes scapularis*). Furthermore, no CYP4G-like sequence can be found in any other organism. CYP4G genes are therefore insect-specific and may have arisen in the Silurian, at the time of land colonization by the hexapod ancestor of insects (39). Their function in insects being hydrocarbon biosynthesis, CYP4G genes can be considered a key evolutionary innovation that contributed to the success of Insecta in moving out of the water environment of their Pancrustacean relatives.

The sustainable production of alkanes by biotechnological means in a world beyond fossil fuels requires a diverse enzymatic toolkit. Optimization of the CYP4G enzymes for biotechnological use in biofuel production is a considerable challenge, but the complexity of the reaction and its uniqueness in insects may make the CYP4G enzymes an interesting target for biorational insecticides.

Materials and Methods

Immunohistochemistry. The abdominal integument of adult Canton S flies with attached epidermis and oenocytes was treated as previously described (40). The primary antibodies were mouse monoclonal antibody to CYP4G1 diluted 1:2,000, and rabbit polyclonal antibody to CPR diluted 1:200 (41). The secondary antibodies were Alexa Fluor 633-coupled goat anti-mouse Ab (dilution 1:1,000) and FITC-coupled sheep anti-rabbit Ab (dilution 1:160). The tissues were observed on a Zeiss Axioplan 2 with the 24 FITC/Rhodamine filter and Plan-Neofluar 5×/0.15 objectives. Images were obtained with an HRc AxioCam camera.

RNAi Crosses, CH Extraction, and Analysis. UAS-dsRNA lines were obtained from the Vienna Drosophila RNAi Center (VRDC) stock center (42). Two lines each were used for CYP4G1 (102864KK and 30207GD) and for CPR (107422KK and 46715GD). These lines were crossed with the GAL4 driver lines RE Gal4F8 and RE Gal4F6. The crosses were made in both directions and offspring were analyzed separately by sex. Three replicates of six flies were dipped in 400 μL hexane for 5 min and the hexane extracts with an internal standard of hexacosane were analyzed by GC-MS on a RTX-5MS column (30 m × 0.25 mm ID 0.25-μm film thickness; Restek), on a Trace GC Ultra coupled to a DSQII single quadrupole MS detector (ThermoFisher). The carrier gas was hydrogen with a flow of 1.2 mL/min. The injector port was set at 230 °C and the temperature was held at 180 °C for 3.8 min, ramped to 270 °C at a rate of 5 °C/min, and held at 270 °C for 5 min. Mass detection was in EI mode with an ionization potential of 70 eV, a scan range of 45–500 atomic mass units, and five scans per second.

Desiccation Assay. The experimental and control flies were issued from the cross between the oenocyte GAL4 driver 1407-Gal4/Cy (19) and UAS-CYP4G1-RNAi (102864KK). The progeny were used as control (Cy) or experimental (RNAi). Adults were separated by sex at emergence and kept in groups of 20 in fresh-food vials at 25 °C for 4 d. Desiccation assay and LT_{50} calculations were as described in ref. 43. In control experiments, flies were starved at the same temperature with access to water, and mortality recorded over 10 d. There was no statistical difference in LT_{50} (days ± SEM) between parents and RNAi offspring for males (3.8 ± 0.3 in RNAi flies vs. 4.0 ± 0.3 in control flies) or females (4.8 ± 0.3 in RNAi flies vs. 4.9 ± 0.2 in control flies) (each $n = 50$), and little or no mortality during the first 15 h. This result indicates that mortality seen in the desiccation assay was not caused by lack of access to food.

Fusion Protein Design and Construction. The 4G2/CPR fusion protein consists of the full-length CYP4G2 protein fused via a Ser-Ser dipeptide to the catalytic domain of housefly cytochrome P450 reductase (amino acids 51–671, GenBank AAA29295.1). The details of the fusion protein construction are described in *SI Materials and Methods*.

Recombinant Protein Production. Recombinant 4G2/CPR was produced in *Sf9* cells using the Baculo-Direct expression system (Invitrogen). Briefly, the insert was transferred from pENTR4(NcoI-) into the linearized BaculoDirect C-term vector by LR recombination. A high-titer P3 viral stock was prepared by successive amplifications of P1 and P2 stocks. Serum-adapted cells were infected (multiplicity of infection = 0.02) for 72 h in 50-mL cultures (10^6 cells/mL) in Sf900 II serum-free media (Invitrogen) supplemented with 10% (vol/vol) FBS (Atlas Biologicals), 0.3 mM δ-aminolevulinic acid, and 0.2 mM ferric citrate. Recombinant HF-CPR was produced from the pFastBac1 viral stock in *Sf9* cells under similar conditions. Cells were then harvested with Cell Lysis Buffer (100 mM sodium phosphate, pH 7.6, 20% (vol/vol) glycerol, and 1.1 mM EDTA) supplemented with 100 μM DTT, 200 μM PMSF, and Protease Inhibitor Mixture (Sigma). Cells were lysed and microsomes prepared as per ref. 44. Production of P450-CPR fusion protein was determined by Western blotting using a polyclonal anti-CPR antibody (40) diluted 1:1,000 with a 1:5,000 dilution of HRP-conjugated goat anti-rabbit secondary antibody (BioRad) and SuperSignal West Pico (Pierce) chemiluminescent substrate (Fig. S3*A*). Functional 4G2/CPR was quantitated by CO-difference spectrum analysis (45) (Fig. S3*B*).

Microsome Preparation. Four-day-old male flies were immobilized at −20 °C but not frozen. Microsomes from abdomens were prepared as previously described (46).

Functional Assay. Octadecanal (substrate) was prepared and oxidative decarbonylase assays were performed essentially as described in ref. 3. Briefly, [9,10-^{3}H]stearic acid (Movarek Biochemicals), [18,18,18-$^2H_3$]stearic acid (CDN isotopes), and [1-^{14}C]stearic acid (ARC) were reduced to alcohol with $LiAlH_4$ in anhydrous diethyl ether. The alcohol product was then oxidized to the aldehyde with pyridinium chlorochromate. The aldehyde was dissolved in 0.05 mM Triton X-100 detergent before addition to the assay mixtures (3). Assays were performed in 0.5-mL volumes containing 1.0 mg of microsomal protein, 0.02 M potassium phosphate, 0.05 mM sucrose, 0.4 mM $MgCl_2$, 1 mg/mL BSA, 0.4 mM DTT, and a NADPH regeneration system kit (AAT Bioquest). The reactions were incubated at 30 °C for 1 h and stopped by the addition of 200 μL of 2 M HCl. The product was extracted six times with 1 mL $CHCl_3$. The combined chloroform extracts were dried over anhydrous Na_2SO_4, and the chloroform was evaporated under a gentle stream of N_2. The hydrocarbon fraction was isolated by column chromatography on 6 cm × 0.5 cm i.d. Florisil columns eluted with 5 mL of hexane. Radioactivity was assayed by a liquid scintillation counting. The product of the deuterated aldehyde was analyzed by GC-MS. Briefly, a trace gas chromatograph containing a 60-m, 25-μm film thickness DB-5 capillary column (Agilent) was programmed with the following parameters: initial temperature of 150 °C with no hold, programmed at 5 °C per min to 300 °C. The detector was a Finnigan Polaris Q ion trap with a molecular weight scanning range of 40–400 atomic mass units and an ionization potential of 70 eV. ^{14}CO and $^{14}CO_2$ were trapped and assayed by liquid scintillation counting, as previously described (3).

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