Proposed Mechanism for the Cytochrome P450-Catalyzed Conversion of Aldehydes to Hydrocarbons in the House Fly, *Musca domestica*†

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ABSTRACT: Experiments were performed to elucidate the mechanism of hydrocarbon formation in microsomal preparations from the house fly, *Musca domestica*. Antibody to both house fly cytochrome P450 reductase and a purified cytochrome P450 (CYP6A1) from the house fly inhibited (*Z*)-9-tricosene (Z9−23:Hy) formation from [15,16-^{3}H]-(*Z*)-15-tetracosenal (24:1 aldehyde). Chemical ionization−gas chromatography−mass spectrometry (CI−GC−MS) analyses of the *n*-tricosane formed by microsomal preparations from [2,2-$^{2}H_2$,2-^{13}C]- and [3,3-$^{2}H_2$,3-^{13}C]tetracosanoyl-CoA demonstrated that the deuteriums on the 2,2- and 3,3-positions were retained in the conversion to the hydrocarbon product. Likewise, CI−GC−MS analysis of the Z9−23:Hy formed from [1-^{2}H]tetracosenal by microsomal preparations demonstrated that the aldehydic proton on the 1-carbon was transferred to the hydrocarbon product. Hydrogen peroxide, cumene hydroperoxide, and iodosobenzene were able to support hydrocarbon production from [^{3}H]24:1 aldehyde in place of O_2 and NADPH for short incubation times. From these data, a cytochrome P450 mechanism is proposed in which the perferryl iron−oxene, resulting from heterolytic cleavage of the O−O bond of the iron−peroxy intermediate, abstracts an electron from the C=O double bond of the carbonyl group of the aldehyde. The reduced perferryl attacks the 1-carbon of the aldehyde to form a thiyl−iron−hemiacetal diradical. The latter intermediate can fragment to form an alkyl radical and a thiyl−iron−formyl radical. The alkyl radical then abstracts the formyl hydrogen to produce the hydrocarbon and CO_2.

Hydrocarbons occur in many organisms and are abundant in plants and insects (Tulloch, 1976; Blomquist et al., 1987), where they are important constituents of cuticular lipids and function to prevent desiccation of the organism (Tulloch, 1976; Blomquist & Dillwith, 1985). In some insect species, hydrocarbons also function in chemical communication (Blomquist et al., 1987).

The cuticular lipids of the house fly, *Musca domestica*, contain a complex mixture of hydrocarbons, including n-alkanes, alkenes, and methyl-branched components (Nelson et al., 1981). The major constituent in the sex pheromone produced by the female house fly is (*Z*)-9-tricosene (Z9−23:Hy)[1] (Carlson et al., 1971), which functions as a short-range attractant and stimulant to males. The pheromone also contains an epoxide [(*Z*)-9,10-epoxytricosane], a ketone [(*Z*)-14-tricosen-10-one] (Uebel et al., 1976), and a variety of methylalkanes (Uebel et al., 1978). The epoxide and ketone constituents function as sex recognition factors (Uebel et al., 1976) and are synthesized from Z9−23:Hy by a cytochrome P450 which is present in the microsomes of both males and females (Uebel et al., 1976; Ahmad, 1987). The methylalkanes act as an arrestant in the pheromone blend (Adams & Holt, 1987).

A head-to-head condensation of fatty acids was proposed for hydrocarbon formation in the 1920s (Cannon & Chibnall, 1929). In the 1960s and 1970s, evidence was accumulated that fatty acids are elongated and then converted to hydrocarbons by the loss of the carboxyl group (Kolattukudy et al., 1976). More recently, a reductive decarbonylation mechanism was proposed for the synthesis of terminally saturated hydrocarbons from acyl-CoA precursors (Cheesebrough & Kolattukudy, 1984, 1988; Dennis & Kolattukudy, 1991). In this reaction, a fatty acyl-CoA is first reduced to an aldehyde and then decarbonylated to hydrocarbon and carbon monoxide (CO). The decarbonylation step requires no cofactors and often requires anaerobic conditions (Cheesebrough & Kolattukudy, 1984, 1988).

Recently, we have proposed a different mechanism for hydrocarbon production in the house fly (Reed et al., 1994). As in the reductive decarbonylation, the reaction is a two-step process and an acyl-CoA is reduced to an aldehyde intermediate in the first step. This reaction in the house fly requires NADPH (Reed et al., in press). In contrast to reductive decarbonylation, the conversion of aldehyde to hydrocarbon in the house fly requires O_2 and either NADH or NADPH (Reed et al., 1994) and releases the carbonyl group as CO_2. In addition, the reaction is inhibited by CO and by an antibody to the house fly NADPH-cytochrome P450 reductase (Reed et al., 1994). These results suggest that the conversion of aldehyde to hydrocarbon is catalyzed by a cytochrome P450 which, herein, is informally designated as "P450hyd".

This study examines the mechanism for hydrocarbon formation in the house fly in more detail. Data is presented which provides additional evidence that the reaction is

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[1] Abbreviations: chemical ionization−gas chromatography−mass spectrometry (CI−GC−MS); (*Z*)-9-tricosene (Z9−23:Hy); house fly cytochrome P450 (CYP6A1); hydrocarbon-producing cytochrome P450 (P450hyd); cytochrome P450 from *Pseudomonas putida* (P450*cam*); dithiothreitol (DTT); fatty acid methyl esters (FAME); aldehyde (ALD).

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catalyzed by a cytochrome P450. Peroxide compounds which have been shown to substitute for NADPH and O_2 in P450 reactions (Rahimtula & O'Brien , 1974; Lichtenberger et al., 1976; Gelb et al., 1982) are also used in studying the conversion of [15,16-^{3}H]24:1-ALD to Z9−23:Hy. In addition, CI−GC−MS analyses of the hydrocarbons produced from [1-^{2}H]24:1-ALD, [2,2-$^2H_2$,2-^{13}C]tetracosanoyl-CoA, and [3,3-$^2H_2$,3-^{13}C]tetracosanoyl-CoA provide constraints on the biochemical reaction. From these data, a P450 mechanism for the conversion of aldehydes to hydrocarbons is proposed.

MATERIALS AND METHODS

Insects. Pupae of Fales 1958 strain T-II were supplied courtesy of S. C. Johnson and Sons, Racine, WI. Male and female house flies were separated within 24 h of emergence. All flies were maintained on a sucrose:low fat powdered milk diet (1:1 v/v) and water. Insects were held on a 12 h:12 h light/dark photoperiod.

Chemicals and Materials. NADPH, fumed silica, 2,5-diphenyloxazole (PPO), $MgCl_2$, EDTA, EGTA, hydrogen peroxide (H_2O_2) (30% solution), cumene hydroperoxide (95%), piperonyl butoxide, *n*-tricosane, (*Z*)-9-tricosene, and sodium ascorbate were obtained from Sigma, St. Louis, MO. Iodosobenzene (95%) was purchased from Pfaltz and Bauer, Inc., Waterbury, CT. Bio-Sil A was purchased from Bio-Rad, Richmond, CA. Dithiothreitol (DTT) was purchased from Boehringer Mannheim, IN. Triton X-100 (Triton) was purchased from Calbiochem, San Diego, CA. HPLC-grade hexane, diethyl ether, chloroform, toluene, and sodium sulfate were obtained from Fisher Scientific, Pittsburgh, PA, and the chloroform and hexane were redistilled prior to use. The antibodies to the house fly P450 reductase and to the house fly CYP6A1 were a gift from Dr. Rene Feyereison, University of Arizona, Tucson, AZ.

Labeled Substrates and Standards. [15,16-^{3}H]-(Z)-15-Tetracosenoate methyl ester (24:1-FAME) and [15,16-^{3}H]-(*Z*)-15-tetracosenal (24:1-ALD) were prepared as described (Reed et al., 1994). Methyl [2,2-$^2H_2$,2-^{13}C]- and [3,3-$^2H_2$,3-^{13}C]tetracosanoates were prepared, their structures were confirmed by NMR (Pomonis & Hakk, 1987), and they were hydrolyzed and converted to their CoA derivatives as described (Reed et al., 1994). [1-$^2H_2$]-(*Z*)-15-Tetracosenol, which was made and its structure confirmed via mass spectroscopy by Dr. Alfin Vaz (University of Michigan, Ann Arbor, MI) from unlabeled 24:1-FAME by reduction with $LiAlD_4$ in anhydrous diethyl ether, was converted to the [1-^{2}H]24:1-ALD as described (Reed et al., 1994).

[9,10-^{3}H]Z9-23:Hy was obtained by reduction of 9-tricosyne using tritium gas as described in Guo et al. (1991). The purity of radioactive compounds was determined by radio-TLC (Bioscan Imaging Scanning Systems, 200-IBM, Bioscan, Inc., Washington, DC) and by radio-HPLC to be >95%. The [9,10-^{3}H]Z9−23:Hy and the [^{3}H]24:1-ALD were used as standards in the radio-TLC of the products of enzyme assays (see below).

Preparation of Microsomes. For assays involving the conversion of acyl-CoAs to hydrocarbons, microsomes were prepared from the whole insect. Flies, which were immobilized with CO_2, were homogenized in a chilled no. 2 mortar and pestle with a homogenization buffer containing 0.1 M potassium phosphate, 25 mM sucrose, 2 mM $MgCl_2$, 2 mM DTT, 1 mM EDTA, and 1 mM EGTA at pH 7.2. The homogenate was centrifuged in a Beckman J2-21M/E centrifuge at 7740*g* for 5 min and 12 100*g* for 20 min at 4 °C. The pellet was discarded, and the supernatant was centrifuged at 46 500*g* for 15 s to ensure complete removal of all mitochondria. The supernatant was then centrifuged in a Beckman L8-55 ultracentrifuge at 165 000*g* for 70 min at 4 °C.

Microsomal pellets were resuspended with a Potter−Elvehjem homogenizer in a "storage" buffer containing 0.1 M potassium phosphate, 0.5 M NaCl, 2 mM DTT, 1 mM EDTA, 1 mM EGTA, and 10% (v/v) glycerol. Protein concentrations were assayed by the method of Bradford (1976), utilizing bovine serum albumin as a standard. The microsomal preparation was frozen with liquid nitrogen and stored at −70 °C.

The conversion of aldehydes to hydrocarbons was not detected in microsomes from the whole insect; therefore, when doing these assays, the microsomes were prepared from abdominal integument and fat body which were isolated as described in Wheelock and Scott (1989). Briefly, flies that were frozen with liquid N_2 were shaken vigorously in a 1000 mL Erlenmeyer flask to break apart the bodies of the insects. The body parts were then sieved through a 1.7 mm mesh sieve. The abdomens were separated and were placed in a 55 mL Potter−Elvehjem homogenizer containing approximately 20 mL of the homogenization buffer. The abdomens were homogenized by (<10) short pulses of a model 474, Rockwell drill (Pittsburgh, PA) which was attached to the pestle of the homogenizer. The end of the homogenizer was placed in ice while the abdomens were sheared with the drill. Microsomes were isolated from the homogenized preparation as described above.

Assay for the Conversion of [15,16-^{3}H]24:1-CoA to [^{3}H]-Z9−23:Hy. All samples were assayed in triplicate. Final concentrations in the reaction mixture, except where otherwise indicated, were 16 μM [15,16-^{3}H]24:1-CoA, 1 mg of protein, 0.1 M potassium phosphate, 2 mM sodium ascorbate, 1 mM DTT, 2 mM NADPH, 1 mM EDTA, and 1 mM EGTA at pH 7.2. The total volume was 1 mL. Assays were incubated aerobically for 10 min at 30 °C and were stopped by the addition of 200 μL of 2 M HCl.

Lipid was extracted with chloroform (4 × 1 mL). The combined chloroform extracts were dried over anhydrous sodium sulfate, and the chloroform was evaporated under a gentle stream of N_2 gas. The dried samples were taken up in approximately 200 μL of hexane, and 40 μL was spotted on a silica gel G plate (Fisher, Pittsburgh, PA) for TLC. The samples were developed in hexane:diethyl ether (85:15). The locations of the radioactive hydrocarbons and aldehydes were identified with standards (described above). The plates were scraped to collect the two compounds, and the silica containing the samples of interest was added to a scintillation cocktail composed of 4% (w/v) fumed silica and 0.4% (w/v) PPO in a solvent of toluene. Radioactivity in the aldehyde and the hydrocarbon fractions was assayed by liquid scintillation counting.

For assays involving [2,2-$^2H_2$,2-^{13}C] and [3,3-$^2H_2$,3-^{13}C]-24:0-CoAs, hydrocarbons were isolated by Bio-Sil A column chromatography and were analyzed by CI−GC−MS using chemical ionization with methane on a Finnigan MAT ssQ710 mass spectrometer with a molecular weight scanning range of 320−330 at 70 eV. A Varian model 3400 gas

chromatograph containing a 30 m × 0.25 mm DB-5 column with a 0.25 μM film thickness was temperature programmed from 150−280 °C at 5 °C/min. The carrier gas was helium at 14 psi head pressure, flow rate 1 mL/min. In order to obtain enough product to analyze by CI−GC−MS, assays with each of the 24:0-CoAs were performed in 10 separate incubations, and the hydrocarbons that were recovered from these assays were combined. The incubation conditions were the same as those specified above except that 0.2 mg of microsomal protein and 30 μM of 24:0-CoA were used and the reaction time was 40 min.

Assay for the Synthesis of Hydrocarbon from Aldehyde. The reactions for the conversion of [15,16-^{3}H]24:1-ALD to [^{3}H]Z9−23:Hy were done in triplicate and, unless stated otherwise, in the presence of 5 μM [15,16-^{3}H]24:1-ALD, 0.5 mM NADPH, 0.5 mg of microsomal protein, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, and 0.33 mM Triton X-100 in 1 mL. The aldehyde was suspended in a 0.3 mM Triton X-100 solution by sonication for 30 s and was diluted 10-fold when added to start the assays. When antibody (Ab) was added to the reaction, 0.3 mg of Ab was added per mg of microsomal protein along with appropriate buffers and Triton X-100 and incubated at room temperature for 30 min before adding NADPH to start the reaction. All other incubation conditions and extraction procedures were the same as those described above.

When [1-^{2}H]24:1-ALD was used as a substrate, the reactions were done as described above except that the aldehyde concentration was 20 μM and the reaction time was 40 min. Hydrocarbons were isolated by Bio-Sil A column chromatography and were analyzed by CI−GC−MS as described above. The reactions were done in 12 separate incubations, and the hydrocarbon products were combined into triplicate samples which were each composed of the products from four different incubations. The products from unlabeled 24:1-ALD were assayed under the same conditions.

Assays for the Conversion of [15,16-^{3}H]24:1-ALD to [^{3}H]-Z9−23:Hy in the Presence of H_2O_2, Cumene Hydroperoxide, and Iodosobenzene. The reactions stimulated by the oxidizing compounds, hydrogen peroxide, cumene hydroperoxide, and iodosobenzene, were done under anaerobic conditions without NADPH. Anaerobic conditions were maintained by connecting stoppered 7 mL tubes in sequence by 5 inch sections of rubber tubing which were fitted with 21 gauge needles. The test tubes were then connected to an aspirator and evacuated for 2 min. Afterward, the tubes were connected to an N_2 tank, the aspirator was disconnected, and an outlet needle was placed in the last tube, and N_2 was flushed through the tubes for an additional 2 min. The aspirator was then reconnected, the outlet needle was removed, and the cycle was repeated four more times. At the conclusion of the last cycle, the N_2 and the outlet tube needle remained connected, the tubes were transferred to a water bath at 30 °C, and the reaction was initiated as described above.

The incubation conditions were as those described above except that the concentration of [15,16-^{3}H]24:1-ALD was 10 μM and the reactions did not contain NADPH. Hydrocarbon formation was not detected when iodosobenzene was used unless piperonyl butoxide was added to the incubations; therefore, all of the reactions contained 250 μM piperonyl butoxide. Even though piperonyl butoxide is a P450 inhibitor, it does not inhibit the P450hyd (data not shown).

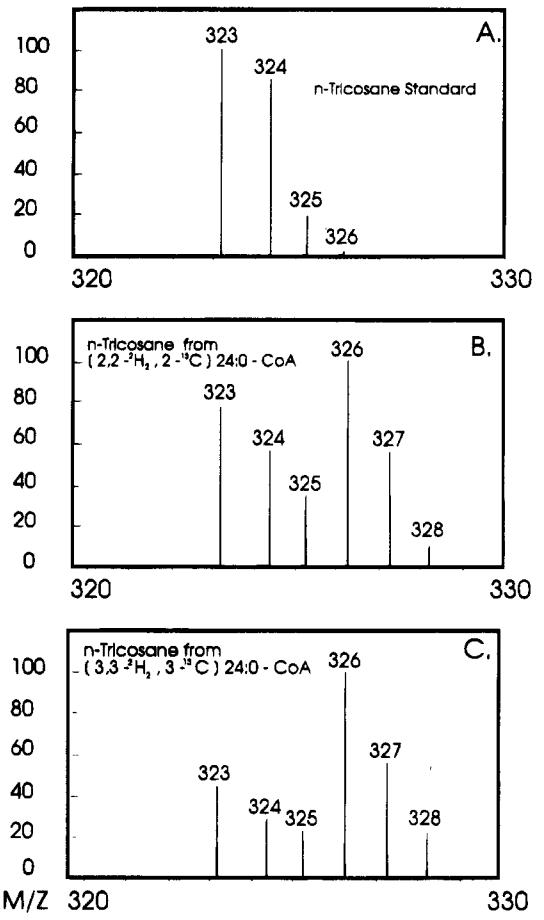

FIGURE 1: CI−GC−MS analysis of an *n*-tricosane standard (A) and *n*-tricosanes obtained from the microsomal incubation of [2,2-$^2H_2$, 2-^{13}C]24:0-CoA (B) and [3,3-$^2H_2$,3-^{13}C]24:0-CoA (C). Experiments were performed as described in Materials and Methods.

The reactions were also started with the oxidizing compounds and not with aldehyde. Cumene hydroperoxide and iodosobenzene were prepared in methanolic solutions, and 5 μL of each solution was added to reach the desired assay concentrations. In separate assays, it was found that this volume of methanol did not effect hydrocarbon production (data not shown). Hydrogen peroxide was diluted in an aqueous solution, and 100 μL of this solution was added to start the reaction. The concentrations tested were as follows: 1.0 mM hydrogen peroxide, 1.0 mM cumene hydroperoxide, and 0.125 mM iodosobenzene. The concentration of iodosobenzene was determined by titration with a standardized sodium thiosulfate solution as described (Lucas et al., 1955). Aerobic controls that were initiated by adding NADPH also were performed. Controls that contained no protein and which encompassed a 6 min time interval were also included when each of the oxidizing reagents was tested.

RESULTS

CI−GC−MS Analysis of Hydrocarbons Produced from [2,2-$^2H_2$,2-^{13}C]- and [3,3-$^2H_2$,3-^{13}C] 24:0-CoAs. Hydrocarbons synthesized by microsomes from 4 day old males from either [2,2-$^2H_2$,2-^{13}C] or [3,3-$^2H_2$,3-^{13}C]24:0-CoAs were analyzed by CI−GC−MS. An *n*-tricosane standard (Figure 1A) gave predominant M^+ (*m/z* at 324) and $(M - 1)^+$ (*m/z* at 323) ions and a smaller $(M + 1)^+$ (*m/z* at 325) ion under the conditions used. The C_{23} hydrocarbon from microsomes incubated with the deuterated ^{13}C precursors contained

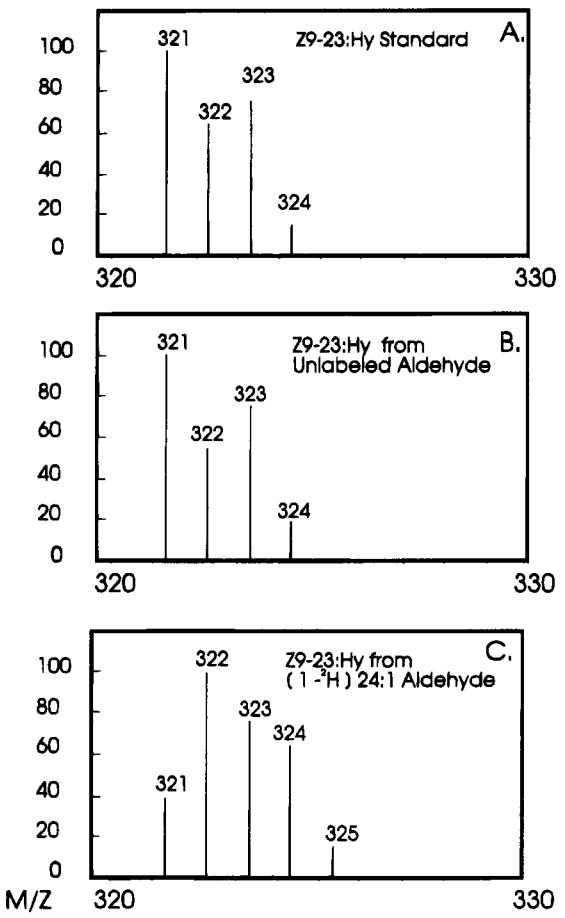

FIGURE 2: CI–GC–MS analysis of a Z9–23:Hy standard (A) and Z9–23:Hy derived from microsomal incubations with either unlabeled 24:1-ALD (B) or [1-^{2}H]24:1-ALD (C). Experiments were performed as described in Materials and Methods.

endogenous *n*-tricosane (ions with *m/z* at 323, 324, and 325). However, the major ions with masses corresponding to the $(M - 1)^+$, M^+, and $(M + 1)^+$ are 3 mass units larger (Figure 1B,C) than those respective ions in the standard. This indicates that the deuteriums at the 2,2- and 3,3-positions, along with the ^{13}C, were retained in the conversion of acyl-CoA to hydrocarbon.

CI–GC–MS Analysis of the Hydrocarbon Formed from [1-^{2}H]24:1-ALD. Day 4 male microsomes were used for the reactions with the [1-^{2}H]24:1-ALD because it has been shown previously (Nelson et al., 1981) that these flies contain undetectable amounts of Z9–23:Hy. The predominant ion produced, as analyzed by CI–GC–MS, from the (Z)-9-tricosene standard and from the Z9–23:Hy produced from the unlabeled aldehyde was the $(M - 1)^+$ (*m/z* at 321) (Figure 2A,B). Strong peaks were also obtained from the M^+ (*m/z* at 322) and the $(M + 1)^+$ (*m/z* at 323) ions, and a less intense peak was obtained from the $(M + 2)^+$ (*m/z* at 324) ion which results from ^{13}C natural abundance.

The masses of the predominant ions [corresponding to the $(M - 1)^+$, M^+, and the $(M + 1)^+$] derived from the hydrocarbon synthesized from the [1-^{2}H]24:1-ALD are 1 mass unit larger (Figure 2C). This indicates that a significant proportion of the deuterium atoms were retained as the aldehyde was converted to the hydrocarbon that is one carbon shorter in chain length.

The proportion of deuterium retained by the Z9–23:Hy can be approximated. The ratio of the intensities of the M^+ peak to the $(M - 1)^+$ peak in the unlabeled hydrocarbons (R_u) was measured as 0.51 ± 0.05. If it is assumed that the ions with a mass of 321 in the samples with deuterated Z9–23:Hy were derived from non-deuterated hydrocarbon, the ratio, R_u, can be used to determine the fraction of ions with mass 322 that are contributed by unlabeled Z9–23:Hy (R_u × the intensity of the 321 ion in the "deuterated" sample/ intensity of the 322 ion in the "deuterated" sample). The percent retention of deuterium atoms in Z9–23:Hy as indicated by this calculation is 80% ± 1%. This value is a lower limit because it assumes that none of the deuterium atoms was lost in the chemical conversion of the methyl ester to the alcohol and of the alcohol to the aldehyde.

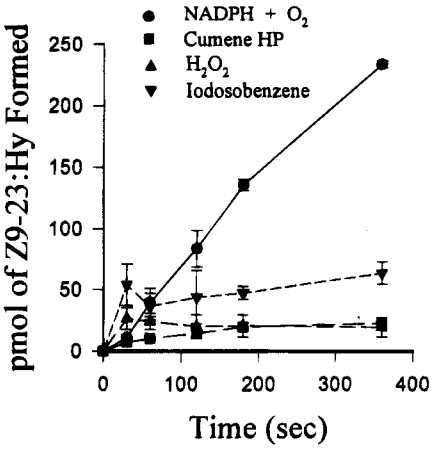

FIGURE 3: Conversion of [15,16-^{3}H]24:1-ALD to [^{3}H]Z9–23:Hy with selected oxidizing agents compared to NADPH and O_2. The conditions were as described in Materials and Methods. Controls containing no protein were incubated for 6 min with the different oxidizing compounds and were subtracted from each time interval of each reaction. The no-protein controls produced 25.5 ± 3 pmol of hydrocarbon over a 6 min incubation, and there was no increase in hydrocarbon production from 3–6 min in the no-protein control.

Substitution of Oxidizing Compounds for NADPH and O_2. All of the oxidizing compounds tested stimulated hydrocarbon production for short time intervals (Figure 3). Of the three oxidizing compounds tested, iodosobenzene stimulated Z9–23:Hy production most efficiently. Iodosobenzene and H_2O_2 resulted in a rapid burst of enzyme activity (relative to an NADPH plus O_2 control), but this was terminated by 30 s. Iodosobenzene has been shown to stimulate the rate of steroid α-hydroxylation compared to NADPH (Gustafsson et al., 1979) by bypassing the NADPH-supported reduction of P450. Iodosobenzene also has been shown to activate P450 by producing a transient enzyme intermediate containing one oxygen derived from iodosobenzene (Blake & Coon, 1989). This transient species was produced in two phases, from 0 to 15 s and from 200 to 1000 s, which is in the time frame of the results reported in Figure 3. The stimulation of hydrocarbon formation by cumene hydroperoxide was comparable to that observed with NADPH plus O_2 for the first 30 s. However, after this time interval, the rate of Z9–23:Hy synthesis from cumene hydroperoxide slowed markedly. Because cumene hydroperoxide is hydrophobic, it may diffuse into the membrane before exerting its inhibition, and the lipid may provide somewhat of a protecting phase for the P450 (Balvers et al., 1985). The rapid inactivation produced by all additions is within the time frame of inactivation of various P540s (Blake & Coon, 1989; Nordblom et al., 1976; Balvers et al., 1985). The rapid inactiva-

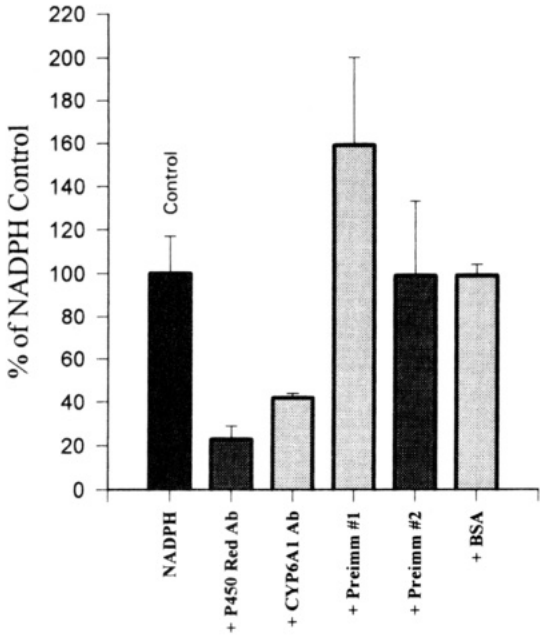

FIGURE 4: Effect of antibody on the production of hydrocarbon from aldehyde. The values are expressed as the percentage relative to those from an incubation with only NADPH. Male microsomes were used, and the NADPH concentration was 0.5 mM. The amount of the antibody protein and the preimmune sera added was 0.3 mg/mg of microsomal protein for the reductase and CYP6A1. BSA was added at 0.6 mg/mg of microsomal protein. The antibodies and/or preimmune serum was incubated for 30 min at room temperature before NADPH was added to start the reaction. Each value represents the mean ±SD from three assays.

tion also could be explained by the very low levels of the P450hyd present in house fly microsomes.

Effects of Antibody on NADPH Stimulated Hydrocarbon Production. The production of [^{3}H]Z9−23:Hy from [15,-16-^{3}H]24:1 Ald was measured in the presence of several antibodies (Figure 4). Previously, we have shown that polyclonal antibodies to the house fly P450 reductase inhibited hydrocarbon production (Reed et al., 1994). In this study, polyclonal antibodies against the house fly CYP6A1 inhibited the reaction by about 60% when about 0.15 mg of the partially purified IgG fraction containing the CYP6A1 antibody was incubated with 0.5 mg of house fly microsomal protein. The effect of the house fly P450 reductase antibody is shown for comparison. Four other polyclonal antibodies to the CYP6A1 were tested on this reaction; however, none of these other antibodies inhibited hydrocarbon formation (data not shown). The preimmune serum from the rabbits that made the inhibitory CYP6A1 antibody was not available, but two different preimmune sera and BSA were tested against the activity of hydrocarbon production. Again no decrease in activity was observed.

DISCUSSION

The objective of this study was to gain insight into the mechanism of hydrocarbon formation in the house fly. Previous work (Reed et al., 1994) showed that the mechanism must allow for the formation of CO_2 and Z9−23:Hy from the 24:1-ALD. CYP2B4 has been shown to produce olefins from aldehydes via a deformylation mechanism (Vaz et al., 1991; Roberts et al., 1991). A minor side reaction in this conversion was the formation of terminally saturated hydrocarbons. One major difference between these two reactions is that the CYP2B4 required aldehyde substrates with branching at the α-carbon, whereas the P450hyd uses straight chain aldehydes as substrates. Another discrepancy arises from the fact that the CYP2B4 deformylation mechanism requires an unsaturated intermediate. However, the data from the CI−GC−MS analyses of *n*-tricosanes derived from either the [2,2-$^2H_2$,2-^{13}C]- or the [3,3-$^2H_2$,3-^{13}C]24:0-CoAs show that the P450hyd reaction does not involve an unsaturated intermediate because the deuterium atoms at the 2,2- and 3,3-positions are retained in the hydrocarbon product (Figure 1). In addition, the results in this study show that cumene hydroperoxide, iodosobenzene, and hydrogen peroxide can substitute for NADPH and O_2 in hydrocarbon formation in the house fly, whereas only H_2O_2 was effective in the CYP2B4 reaction. This suggests that, unlike olefin formation by CYP2B4, the conversion by the P450hyd does not require the direct attack of the peroxy−iron intermediate on the aldehyde substrate. Finally, analysis of the Z9−23:Hy formed from [1-^{2}H]24:1-ALD demonstrates that the hydrogen atom on the carbonyl group of the aldehyde is also retained in the hydrocarbon product. The retention of the aldehydic proton on the hydrocarbon product is consistent with the results of Kolattukudy and co-workers for the plant *Pisum sativum* (Cheesebrough & Kolattukudy, 1984).

Most evidence indicates that homolytic cleavage of the oxygen−oxygen (O−O) bond of a peroxy−iron intermediate occurs when alkyl hydroperoxides are substituted for NADPH and O_2 in P450-catalyzed oxidations (White et al., 1980; Blake & Coon, 1981). The reaction sequence that is often believed to result from cumene hydroperoxide-stimulated P450 oxidation (Gelb et al., 1982; White & Coon, 1980; White, 1991) is demonstrated on the right side of Figure 5. After homolysis of the O−O bond, the alkoxy radical abstracts a hydrogen from the substrate to form an alkyl radical and an alcohol. The alkyl radical then recombines with the iron−oxene radical to form the oxidized product.

Because of the limitations imposed on the P450hyd mechanism by the CI−GC−MS analyses of the deuterated hydrocarbon products, it seems unlikely that the cumene hydroperoxide-stimulated reaction can proceed by the homolytic sequence described above. It is improbable that the initial activation of the substrate (aldehyde) occurs by hydrogen abstraction when all of the hydrogens at the 1-, 2-, and 3-positions are retained in the hydrocarbon product. In addition, an aldehydic radical (resulting from hydrogen abstraction) would combine with the iron−oxene radical (resulting from homolytic cleavage of the O−O bond of the peroxy−iron) to form a stable oxidized product (fatty acid) (right side of Figure 5). Similarly, if the attack on the substrate is initiated by the iron−oxo radical resulting from homolytic cleavage, it is difficult to imagine how an alkoxy radical can interact with a likely intermediate to produce the hydrocarbon.

Z9−23:Hy can be formed from the H_2O_2-stimulated P450hyd activity by the direct attack of the peroxy−iron intermediate on the aldehyde which is followed by homolysis (left side of Figure 5). However, bicarbonate and Z9−23:Hy are formed by the reaction shown. It has not been determined if the reaction with H_2O_2 occurs by this type of mechanism. However, because of both the problem with a homolytic mechanism and the fact that the reaction is not limited to direct attack of a peroxy−iron intermediate, it seems that the NADPH-O_2-stimulated reaction involves a

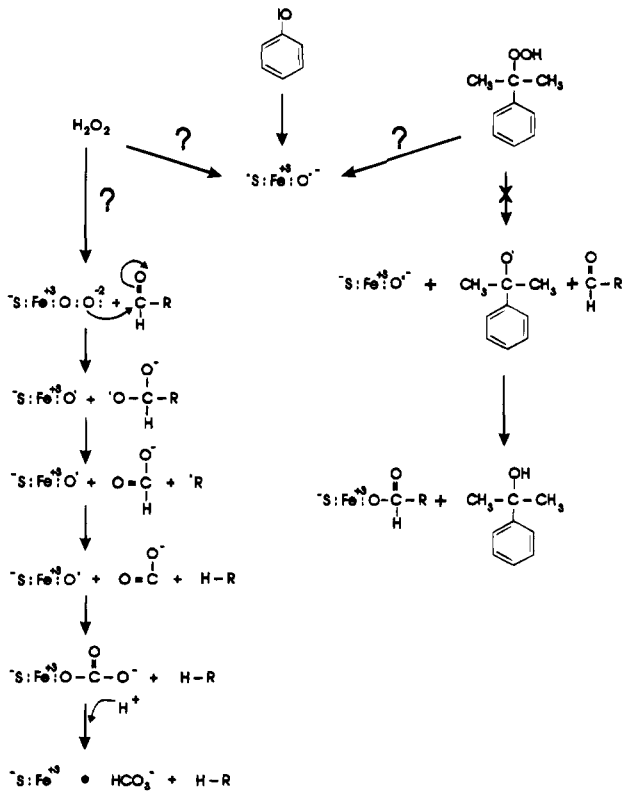

FIGURE 5: Two mechanisms by which an aldehyde could possibly interact with an active intermediate of a cytochrome P450. The pathway on the left side of the figure shows the formation of hydrocarbon and bicarbonate following the direct attack of the iron–peroxy intermediate on the aldehyde as may occur upon H_2O_2 stimulation (Vaz et al., 1991; Roberts et al., 1991). The reaction sequence on the right side of the figure shows the conventional mechanism invoked for homolytic cleavage following cumene hydroperoxide stimulation (Gelb et al., 1982; White & Coon, 1980; Groves et al., 1978). The "X" is drawn through the first reaction arrow because the pathway does not result in hydrocarbon production. The middle reaction sequence shows the formation of the perferryl intermediate resulting from iodosobenzene stimulation (Lichtenberger et al., 1976; Blake & Coon, 1989). Finally, it is shown by the diagonal arrows that all three oxidizing compounds may form the perferryl intermediate.

perferryl intermediate which is believed to result from heterolytic cleavage of a peroxy-iron precusor (Lichtenberger et al., 1976; White, 1991; Blake & Coon, 1981, 1989; White, 1991). A possible resonance form of this complex is shown at the top of the middle pathway in Figure 5. The complex is drawn as a diradical because of the proposed ability of the P450 thiolate ligand to donate a single electron to unstable intermediates of the catalytic cycle (White & Coon, 1980). The mechanisms presented herein rely on this property of the thiolate ligand.

In one of the possible mechanisms, the perferryl intermediate abstracts an electron from the C=O double bond of the aldehyde carbonyl group (left side of Figure 6). In abstracting the electron, a bond is formed between the 1-carbon of the aldehyde and the iron–oxene to produce a thiyl–iron–hemiacetal diradical. The reaction, as depicted in the diagram (Figure 5), shows that an aldehyde carbocation radical and a nucleophilic iron–oxo intermediate are formed after the electron is removed by the perferryl complex. These intermediates may actually never exist but are shown to more clearly demonstrate how it is proposed that the bond between the aldehyde and the iron–oxene is formed. The thiyl–iron–hemiacetal diradical subsequently forms a thiyl–iron–

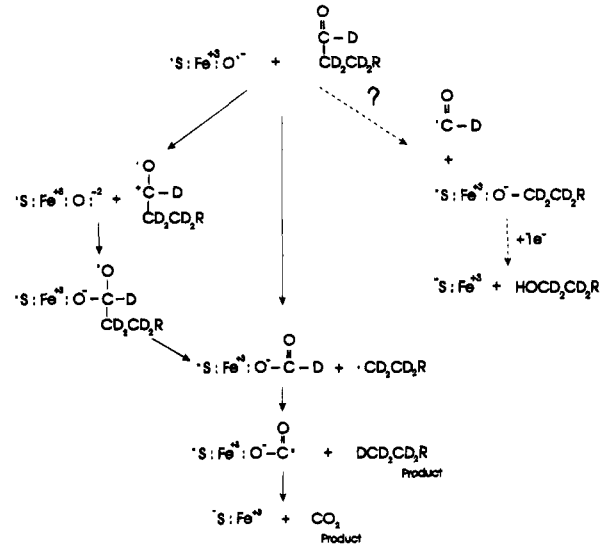

FIGURE 6: Proposed mechanisms for hydrocarbon biosynthesis from an aldehyde in the house fly. Because the proposed mechanisms are supported by the experimental data (see Results) showing that the deuterium atoms are retained in the reaction, they are represented on the aldehyde molecules by "D". Both mechanisms invoke the perferryl intermediate shown as a diradical at the top of the figure. The mechanism in the center of the figure. shows the direct formation of a thiyl–iron–formyl radical and an alkyl radical by abstraction of a CHO radical from the aldehyde. The mechanism to the left shows the abstraction of an electron from the C=O double bond of the aldehyde carbonyl group by the perferryl and the subsequent attack of the reduced perferryl on the resulting carbocation to form a thiyl–iron–hemiacetal diradical. The bond between the perferryl and the aldehyde may form as the electron is abstracted. Thus, the carbocation and the reduced perferryl may never exist as separate entities. The diradical fragments form the thiyl–iron–formyl and alkyl radicals. From this point, the two possible mechanisms are identical and involve the abstraction of the formyl hydrogen by the alkyl radical to form hydrocarbon and CO_2. The pathway to the right shows the possible formation of an alcohol which is one carbon shorter than the parent aldehyde. This pathway can result if the perferryl intermediate abstracts an alkyl radical instead of the CHO radical from the parent aldehyde (see text for details).

formyl radical and an alkyl radical by fragmenting in the manner proposed previously (Vaz et al., 1991; Roberts et al., 1991).

In the other possible mechanism, the perferryl intermediate forms a thiyl–iron–formyl radical and an alkyl radical directly by abstracting the CHO group of the aldehyde as a radical (center of Figure 6). Thus, from this point, both of the proposed mechanisms (left and center of Figure 6) are identical. Because the thiolate is donating an electron to stabilize the formyl moiety, it remains bound to the P450hyd heme group and allows the alkyl radical to abstract the formyl hydrogen (formyl deuterium in Figure 6) in the final step of the reaction. Thus, the final products, hydrocarbon and CO_2, are formed, and the ferric iron of P450 is regenerated.

It is not possible to distinguish between the two mechanisms proposed. However, the mechanism involving the formation of the thiyl–iron–hemiacetal radical (left, Figure 6) is favored because the perferryl intermediate can abstract an electron from the π bond of the C=O double bond to form the thiyl–iron–hemiacetal radical. Alternatively, in the other mechanism, the perferryl complex may react with one of two radicals formed in breaking a stronger σ bond. If this reaction occurred, one could expect that [^{3}H]tricosenol might form as a side reaction in the conversion of [15,16-^{3}H]24:1-ALD to [^{3}H]Z9-23:Hy. The reaction products from

these incubations have been analyzed by radio-HPLC, and no [^{3}H]tricosenol was detected (data not shown). These data also argue against any other mechanism which has the fragmentation of aldehyde preceding bond formation with the active iron−oxo intermediate.

The P450s of the hepatic microsomal system, which are most commonly studied, hydroxylate a wide variety of substrates with varying regiospecificity (Gelb et al., 1982; Groves et al., 1978). Most P450 enzymes do not catalyze reactions when H_2O_2 is substituted for NADPH and O_2 (White & Coon, 1980; Hrycay et al., 1975; Coon et al., 1979), and the reaction sequence that commonly occurs when alkyl hydroperoxides are substituted for the latter probably involves a different active iron−oxo intermediate which results from homolytic cleavage (White et al., 1980; Blake & Coon, 1981). The P450 from the soil bacterium *Pseudomonas putida* (P450*cam*) has characteristics that are unlike those described above for the hepatic P450s. More specifically, P450*cam* and the P450hyd catalyze their respective reactions when relatively low concentrations of H_2O_2 (<5 mM) are substituted for NADPH and O_2. Furthermore, it is possible that in both the P450hyd and the P450*cam* reactions the active iron−oxo intermediate is probably the perferryl intermediate for both peroxide- and NADPH-O_2-stimulated reactions (Gelb et al., 1982). Interestingly, it also has been shown that P450*cam* exhibits strict regiospecificity via an attack by hydroxylating camphor only at the 5-exo position (Gelb et al., 1982). This, in turn, implies a very rigid spatial orientation between the active iron−oxo intermediate and the substrate. The mechanism proposed in this study also requires rigid alignment between the perferryl complex and the substrate in order to prevent the initial attack on the substrate from being either the abstraction of a hydrogen or the formation of an alkyl hydroperoxide.

Work is in progress both to purify the P450hyd and to clone the gene for the enzyme. By comparing the active sites of the purified P450hyd and the P450*cam*, it may be possible to learn if there is a connection between the tight control of the orientation of the oxidative attack on a substrate and the ability to form common intermediates from peroxide- and NADPH-O_2-stimulated reactions.

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