Cytochrome $P450_{BM-3}$ (CYP102): Regiospecificity of Oxidation of ω-Unsaturated Fatty Acids and Mechanism-Based Inactivation†

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ABSTRACT: Cytochrome $P450_{BM-3}$ preferentially oxidized fatty acids with terminal double or triple bonds to the ω-2 hydroxylated fatty acids rather than, respectively, to the epoxide or diacid metabolites. The enzyme is inactivated during catalytic turnover of long, terminally unsaturated fatty acids but not by the analogous medium-length fatty acids. Enzyme inactivation by 17-octadecynoic acid and 16-hydroxy-17-octadecynoic acid is due to alkylation of the prosthetic heme group to give an adduct tentatively identified as *N*-(2-oxo-3-hydroxy-17-carboxyheptadecyl)protoporphyrin IX by its chromatographic and spectroscopic properties. Catalytic turnover of 17-octadecenoic acid also results in heme modification. Fatty diacid monoethyl thioesters are introduced as a new class of irreversible inhibitors that exploit the ω-2 oxidation specificity of cytochrome $P450_{BM-3}$. Catalytic oxidation of the monoethyl thioesters of dodecanedioic and hexadecanedioic acids results in enzyme inactivation and formation of the parent diacids as metabolites. Limited tryptic digestion of the enzyme after incubation with the monoethyl thioester of [^{14}C]hexadecanedioic acid shows that the inactivating agent binds covalently to both the heme and flavin domains. This finding, and the observation that glutathione prevents inactivation of the enzyme by the monoethyl thioesters, indicate that a diffusible metabolite, probably the sulfoxide, is responsible for enzyme inactivation. The strong preference for ω-2 allylic or propargylic hydroxylation over terminal π-bond oxidation is opposite to the usual cytochrome P450 pattern and requires that the enzyme actively suppress terminal π-bond oxidation. The inference that the enzyme binds and sequesters the terminal carbon in a lipophilic pocket is consistent with the crystal structure of the hemoprotein domain of cytochrome $P450_{BM-3}$.

Cytochrome $P450_{BM-3}$ (CYP102)[1] is an unusual cytochrome P450 enzyme produced by a strain of *Bacillus megaterium* (Fulco, 1991). It is a soluble protein like cytochrome $P450_{cam}$, a monooxygenase from *Pseudomonas putida*, but differs from that enzyme in that the electrons required for its catalytic turnover are provided by a flavoprotein rather than by an iron–sulfur protein (Narhi & Fulco, 1986, 1987). The flavoprotein appears to be quite similar to the cytochrome P450 reductase involved in the turnover of eukaryotic microsomal cytochrome P450 enzymes (Ruettinger et al., 1989; Oster et al., 1991). Cytochrome $P450_{BM-3}$ is unique among cytochrome P450 enzymes, however, in that the reductase and monooxygenase are part of a single polypeptide chain (Narhi & Fulco, 1986, 1987; Ruettinger et al., 1989). Cytochrome $P450_{BM-3}$ is unusual, furthermore, in that its synthesis is induced by phenobarbital at the transcription level even though phenobarbital and its congeners do not interact directly with the enzyme (Narhi et al., 1983; Wen & Fulco, 1987; He & Fulco, 1991; Shaw & Fulco, 1992; Ruettinger et al., 1984).

Cytochrome $P450_{BM-3}$ has been cloned (Ruettinger et al., 1989), sequenced (Ruettinger et al., 1989), and successfully expressed in *Escherichia coli* (Wen & Fulco, 1987; Li et al., 1992; Boddupalli et al., 1990). The enzyme exhibits higher sequence identity (25%) with the CYP4A class of mammalian enzymes than with other bacterial enzymes (Ruettinger et al., 1989). It is cleanly cut by trypsin into hemoprotein and reductase domains when a fatty acid is bound but suffers an additional cut between Lys-9 and Thr-10 in the absence of a bound fatty acid (Narhi & Fulco, 1987). The latter alteration prevents the low- to high-spin shift normally observed on substrate binding, suggesting that Lys-9 is protected by the substrate. The intact protein, and the individual heme and flavin domains, have been expressed in *E. coli* in catalytically active form (Oster et al., 1991; Boddupalli et al., 1992a; Miles et al., 1992). The cytochrome P450 domain has been crystallized in two different laboratories, and a high-resolution structure of the substrate-free form of the enzyme has been obtained (Li et al., 1992; Boddupalli et al., 1992b; Ravichandran et al., 1993).

The enzyme catalyzes ω-1, ω-2, and ω-3, but not ω, hydroxylation of medium- and long-chain fatty acids (Ho & Fulco, 1976; Ruettinger & Fulco, 1981; Boddupalli et al., 1992c). The K_m values descend from 110 μM for C_{12} to a minimum of 2 μM for C_{15} and C_{16} before rising again to 80 μM for C_{18} (Narhi & Fulco, 1986). The turnover number with these fatty acids is in the order of 4600 nmol/nmol of P450/min, a value orders of magnitude higher than is common for other cytochrome P450 enzymes (Narhi & Fulco, 1986).

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[1] Abbreviations: cytochrome $P450_{BM-3}$, CYP102 according to the nomenclature of Nebert *et al.* (1993); heme, iron protoporphyrin IX regardless of the iron oxidation or ligation state; 17-ODEA, 17-octadecenoic acid; 16-hydroxy-17-ODEA, 16-hydroxy-17-octadecenoic acid; 17-ODYA, 17-octadecynoic acid; 16-hydroxy-17-ODYA, 16-hydroxy-17-octadecynoic acid; TDDA, monoethyl thioester of dodecanedioic acid; THDA, monoethyl thioester of hexadecanedioic acid; HPLC, high-pressure liquid chromatography; CIMS, chemical ionization mass spectrometry; EIMS, electron impact mass spectrometry; MOPS, *N*-morpholinepropanesulfonic acid; THF, tetrahydrofuran.

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Recent work shows that cytochrome $P450_{BM-3}$ can catalyze sequential hydroxylations of the same fatty acid chain to give diol, ketone, and hydroxy ketone derivatives (Boddupalli et al., 1992c; Matson et al., 1980). The enzyme also catalyzes the epoxidation of fatty acids with an internal double bond, the percent of the total product represented by the epoxide decreasing from 35% for *cis*-9-hexadecenoic acid to 0.7% for *cis*-5-tetradecenoic acid (Ruettinger & Fulco, 1981). Epoxidation of these unsaturated fatty acids thus competes, with differing degrees of success, with the usual ω-1, ω-2, and ω-3 hydroxylations. Long-chain amides and alcohols are also substrates for the enzyme, but long-chain hydrocarbons and fatty acid methyl esters are not (Miura & Fulco, 1975). This suggests that a polar, hydrogen-bonding terminus is necessary for catalytic acceptance. On the basis of these specificities, it has been proposed that the terminal methyl and the carboxyl group bind to specific loci in the active site (Boddupalli et al., 1992c). Except for the fact that barbiturates are not substrates (Narhi et al., 1983; Ruettinger et al., 1984), little additional information is available on the substrate specificity of the enzyme (Fulco, 1991).

We report here a study of the regiospecificity of the cytochrome $P450_{BM-3}$-catalyzed oxidation of terminally unsaturated fatty acids and of the consequences of that specificity for mechanism-based inactivation of the enzyme. We also report catalysis-dependent inactivation of cytochrome $P450_{BM-3}$ by thioesters, a new class of irreversible inhibitors that are catalytically oxidized to reactive species that bind covalently to the protein.

EXPERIMENTAL PROCEDURES

Materials. Lauric acid was purchased from Nu Chek Prep, Inc. (Elysian, MN). Stearic acid, 1-[^{14}C]hexadecanedioic acid, 13-octadecyn-1-ol, glutathione, and NADPH were purchased from Sigma (St. Louis, MO). Deuterium oxide and hexadecanedioic acid were obtained from Aldrich (Milwaukee, WI). The fatty acid substrates dissolved in ethanol (4% final concentration) were suspended in 50 mM potassium carbonate immediately before use. The suspensions were kept at ~25 °C. All solvents and reagents were of the highest purity commercially available. Spectroscopic studies were carried out on a Hewlett Packard 8452 diode array spectrophotometer. HPLC was done on a Hewlett-Packard Model 1090 Series II instrument equipped with a diode array detector and interfaced to a Hewlett-Packard HPLC ChemStation. Gas chromatography was done on a Hewlett-Packard Model 5890 gas chromatograph equipped with a flame ionization detector and interfaced to a Hewlett-Packard 3365 ChemStation. Proton NMR spectra were obtained at 300 MHz on a GE QE-300 instrument. Mass spectra were measured with a VG-70 mass spectrometer equipped with a Hewlett-Packard 5890A gas chromatograph.

Cytochrome $P450_{BM-3}$ was expressed in *E. coli* and purified to homogeneity as previously described (Boddupalli et al., 1990). The enzyme was stored at −80 °C in 10 μL aliquots, each containing 3.4 nmol (417 μg) of enzyme in 50 mM MOPS buffer, 0.1 M KCl, and 1 mM dithiothreitol (pH 7.4). Each aliquot was only thawed once or twice to avoid activity losses caused by freeze-thawing. For metabolism studies, the concentrated enzyme was typically thawed on ice and diluted with 50 mM MOPS (pH 7.4) buffer to a concentration of 0.4 μM. TPCK–trypsin was purchased from Sigma and was dissolved in 0.2 M ammonium bicarbonate solution. Gel filtration of enzymes was performed on Sephadex G-25 resin (20–80 μm, Aldrich) equilibrated with 50 mM MOPS (pH 7.4) buffer. Incubations were carried out at 25–28 °C in 50 mM MOPS buffer freshly prepared with deionized, double-distilled water and neutralized to pH 7.4 with saturated potassium hydroxide solution.

Methyl 12,13-Epoxytridecanoate. A solution of *m*-chloroperbenzoic acid (173 mg, 1 mmol) and methyl 12-tridecenoate (226 mg, 1 mmol) in CH_2Cl_2 (8 mL) was stirred 14 h at room temperature. After washing sequentially with 10% sodium hydroxide and water and drying over anhydrous $MgSO_4$, the solvent was evaporated and the residue was purified by silica gel column chromatography (10:1 hexane–ethyl acetate). The title compound was obtained as a colorless oil (200 mg, 88.5%): ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.16–1.64 (m, 18H, H-3–H-11), 2.30 (t, 2H, H-2), 2.45 (dd, J = 3, 6 Hz, 1H, H-13), 2.74 (overlapping dd, J = 4 Hz, 1H, H-13), 2.89 (m, 1H, H-12), 3.66 ppm (s, 3H, OCH_3); EIMS m/z 243 (MH^+).

12,13-Epoxytridecanoic Acid. A mixture of methyl 12,-13-epoxytridecanoate (34 mg, 0.14 mmol), lithium hydroxide (16.8 mg, 0.7 mmol), tetrahydrofuran (3 mL), and water (1 mL) was stirred 16 h at room temperature. The organic phase was separated and poured into a mixture of ice and acetic acid. Extraction with ethyl acetate, drying over anhydrous $MgSO_4$, and solvent removal on a rotary evaporator yielded white crystals with no detectable impurities by NMR (29 mg, 90.7%): ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.25–1.65 (m, 18H, H-3–H-11), 2.33 (t, 2H, H-2), 2.48 (dd, J = 3, 6 Hz, 1H, H-13), 2.76 (overlapping dd, J = 5, 1H, H-13), 2.92 (m, 1H, H-12); CIMS (NH_3) m/z 228 (M^+), 246 ($M + NH_4^+$).

17-Octadecyn-1-ol. A suspension of sodium hydride (36 mg, 1.5 mmol) in 1,2-diaminopropane (0.5 mL) was heated at 50 °C under a positive argon atmosphere for 1 h. A solution of 13-octadecyn-1-ol (50 mg, 0.188 mmol) in 1,3-diaminopropane (0.7 mL) was then added, and the mixture was heated under argon at 80 °C for 18 h. After cooling to room temperature, the mixture was poured into water, extracted with ether, washed sequentially with 4 N HCl and brine, and dried over anhydrous $MgSO_4$. After evaporation of the solvent, the title compound was obtained as a white solid (28 mg, 56%). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.25–1.58 (m, 28H, H-2–H-15), 1.70 (s, br, 1H, OH), 1.94 (t, J = 3, 1H, HC≡C–), 2.18 (dt, J = 3, 7 Hz, 2H, C≡CCH_2), 3.63 (t, J = 6.5 2H, CH_2OH).

17-ODYA. To a solution of 17-octadecyn-1-ol (28 mg, 0.105 mmol) in acetone (5 mL), 5 mL of Jones reagent (prepared from 10.3 g of chromium trioxide, 8.7 mL of concentrated sulfuric, and 30 mL of water) was added at 5 °C. The mixture was stirred at room temperature for 30 min before it was diluted with 3 mL of water, extracted with ethyl acetate, dried over anhydrous $MgSO_4$, and taken to dryness on a rotary evaporator. Column chromatography on silica gel (80:20:1 hexane–ethyl acetate–acetic acid) provided 17-ODYA as a white solid (23 mg, 78.1%): ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.25–1.58 (m, 28H, H-2–H-15), 1.93 (t, J = 3, 1H, HC≡C), 2.20 (dt, J = 3, 7 Hz, 2H, C≡CCH_2), 2.34 (t, J = 7, 2H, CH_2COOH); EIMS m/z 281 (MH^+). The product was identical to material previously prepared by another route (Shak et al., 1985).

16-Hydroxy-17-ODYA. Lithium hexamethyldisilazide (1 M in THF, 0.3 mmol) was added at −78 °C to a solution of trimethysilylacetylene (27 mg, 0.28 mmol) in 3 mL of dry THF. The mixture was allowed to warm to room temperature and was stirred for 20 min before a solution of 16-oxohexadecanoic acid (20 mg, 0.11 mmol) in 1 mL of dry THF was added at −78 °C. The mixture was again allowed to warm

to room temperature and was stirred for 16 h before it was poured onto a mixture of ice and acetic acid. The mixture was extracted with ethyl acetate, the extract was dried over anhydrous $MgSO_4$, and the solvent was removed at a rotary evaporator. The crude product was stirred in 10 mL of methanol in the presence of potassium carbonate (30 mg) at ~25 °C for 20 h to desilylate the acetylenic group. The mixture was poured onto a mixture of ice and acetic acid. Extraction into ethyl acetate, drying over anhydrous $MgSO_4$, and solvent removal yielded a residue that was purified by flash column chromatography on silica gel (8:2:0.1 hexane–ethyl acetate–acetic acid). The title compound was obtained as a white solid (20 mg, 61%): ^{1}H NMR (300 MHz, $CDCl_3$) 1.17–1.48 (m, 22 H, CH_2 groups), 1.63 (m, 2 H, C*H*$_2$CH$_2$-CO), 1.68–1.77 (m, 2H, HOCHC*H*$_2$CH$_2$), 2.34 (t, J = 7 Hz, 2 H, C*H*$_2$COOH), 2.46 (d, J = 2 Hz, 1 H, C≡CH), and 4.37 ppm (td, J = 2 and J = 7 Hz, 1H, HOC*H*); ^{13}C NMR (75 MHz, $CDCl_3$) 26.1, 26.4, 30.5, 30.6, 31.0, 35.5, 39.0, 63.8/63.9 (HOCH), 74.2/74.3 (acetylene), 86.4 (acetylene), and 180.2 (COOH) ppm; CIMS (NH_3) m/z 296 (M^+), 314 (M + NH_4^+).

17-ODEA. A mixture of 17-ODYA (30 mg, 0.107 mmol), quinoline (25 mg), and Lindlar catalyst (5 mg) in acetone was stirred at ~25 °C under H_2 for 5 h. HCl (1 N) was then added until the solution was acidic. The mixture was extracted with ethyl acetate, and the combined extracts were washed twice with brine before they were dried over anhydrous $MgSO_4$. The residue obtained by solvent evaporation was purified by silica gel flash column chromatography (8:2:0.1 hexane–ethyl acetate–acetic acid). 17-ODEA was obtained as a white solid: ^{1}H NMR (300 MHz, $CDCl_3$) 1.25–1.40 (m, 24 H, –CH_2–), 1.63 (m, 2 H, H-3), 2.02 (m, 2 H, C*H*$_2$CH=C), 2.35 (t, J = 7 Hz, 2 H, C*H*$_2$COOH), 4.93 (d, J = 10 Hz, 1 H, *cis* *H*C=CHR), 4.99 (d, J = 18 Hz, *trans* *H*C=CHR), and 5.02–5.22 ppm (m, 1 H, H_2C=C*H*R); ^{13}C NMR (75 MHz, $CDCl_3$) 24.7, 29.0, 29.1, 29.2, 29.4, 29.5, 29.7, 31.9, 33.8, 34.1, 114.1, 139.3, and 180.0 ppm; CIMS (NH_3) m/z 282 (M^+), 300 (M + NH_4^+).

16-Hydroxy-17-ODEA. To a solution of vinylmagnesium bromide (1.0 M in THF, 0.5 mmol) in 10 mL of dry THF a solution of 10-oxohexadecanoic acid (50 mg, 0.185 mmol) in 2 mL of dry THF was added at –78 °C. The mixture was stirred at ~25 °C for 3 h. Acetic acid, ethyl acetate, and water were then added to the reaction mixture, and the organic phase was isolated and dried over anhydrous $MgSO_4$. After solvent evaporation, the residue was purified by column chromatography (silica gel, 7:3:0.1 hexane–ethyl acetate–acetic acid). The title compound was obtained as a white solid (15 mg, 27%): ^{1}H NMR (300 MHz, $CDCl_3$) 1.25–1.40 (m, 26 H, H-3–H-15), 2.35 (t, J = 7 Hz, 2 H, C*H*$_2$COOH), 4.10 (q, J = 7 Hz, 1 H, HC=CHC*H*OH), 5.10 (d, 1 H, J = 10 Hz, *cis* HC=CHR), 5.22 (d, J = 18 Hz, *trans* *H*C=CHR), and 5.82–5.93 ppm (m, 1 H, H_2C=C*H*R); EIMS m/z 294 ($M^+ - H_2O$); CIMS (NH_3) m/z 312 (M^+).

Methyl 17,18-Epoxyoctadecanoate. A solution of ODEA (3 mg, 10.6 μmol) and *m*CPBA (80%, 5 mg) in 2 mL of CH_2Cl_2 was stirred at 0 °C for 2 h and at ~25 °C for 6 h. Acetic acid and ethyl acetate were added, and the organic phase was separated and dried over anhydrous $MgSO_4$. After evaporation of the solvent, the residue was taken up in a small amount of CH_2Cl_2, and a solution of diazomethane in ether was added. After 20 min, the solvent was evaporated. The title compound was obtained as an oil: ^{1}H NMR (300 MHz, $CDCl_3$) 1.25–1.40 (m, 26 H, H-3–H-15), 1.45–1.63 (m, 4 H, H-2, H-15), 2.30 (t, J = 7 Hz, 2 H, C*H*$_2$COOCH$_3$), 2.46 (m, 1 H, epoxy), 2.75 (overlapping dd, 1 H, J = 4 Hz, epoxy), 2.91 (m, 1 H, epoxy), and 3.67 ppm (s, 3 H, OCH_3); ^{13}C NMR (75 MHz, $CDCl_3$) 24.9, 26.0, 29.1, 29.3, 29.5, 29.6, 32.5, 34.1, 47.2, 51.4, and 52.4 ppm; CIMS (NH_3) m/z 313 (MH^+), 330 (M + NH_4^+).

TDDA. Ethanethiol (62 mg, 1 mmol) was added at 0 °C to a solution of dodecanedioyl chloride (534 mg, 2 mmol) in 5 mL of THF, and the solution was stirred at ~25 °C for 2 h. Water and ethyl acetate were added, and the organic phase was separated and dried over anhydrous $MgSO_4$. After evaporation of the solvent the residue was purified by silica gel column chromatography (100:20:1 hexane–ethyl acetate–acetic acid). TDDA was obtained as a white solid (145 mg, 52.8%): ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.22–1.28 (m, 15H, CH_3, and part of CH_2), 1.61–1.67 (m, 4H, COC*H*$_2$C*H*$_2$), 2.35 (t, 2H, HOOCC*H*$_2$), 2.53 (t, 2H, EtSOCC*H*$_2$), 2.89 (q, 2H, CH_2S); CIMS (NH_3) m/z 275 (MH^+), 292 (M + NH_4^+).

THDA. A mixture of thionyl chloride (1 mL) and hexadecanedioic acid (573 mg, 2 mmol) was heated under reflux for 1 h. The excess of thionyl chloride was removed under vacuum, and THF (5 mL) was added. Ethanethiol (74 μL, 1 mmol) and pyridine (1 mL) were then added with stirring at 0 °C, and the mixture was stirred at ~25 °C for 1 h before water and ethyl acetate were added. The organic phase, after sequential washing with 1 N HCl and brine, was dried over anhydrous $MgSO_4$. Removal of the solvent at a rotary evaporator and column chromatography on silica gel (8:2:0.1 hexane–ethyl acetate–acetic acid) yielded THDA as a white solid (52 mg, 15.7% yield): ^{1}H NMR (300 MHz, $CDCl_3$) 1.25–1.28 (m, 23H, CH_3 and CH_2 groups), 1.61–1.67 (m, 4H, C*H*$_2$CH$_2$CO), 2.35 (t, J = 7 Hz, 2 H, C*H*$_2$COOH), 2.53 (t, J = 7 Hz, 2 H, C*H*$_2$COS), and 2.87 ppm (q, J = 7 Hz, 2 H, SCH_3); ^{13}C NMR (75 MHz, $CDCl_3$) 15.1, 22.8, 23.2, 24.7, 25.7, 29.0, 29.2, 29.4, 29.6, 34.0, 44.1, 179.9 (CO_2H), 199.9 (COS–) ppm; CIMS (NH_3) m/z 330 (M^+), 348 (M + NH_4^+).

[^{14}C]THDA. Oxalyl chloride (80 μL of a 2.0 M solution in CH_2Cl_2, 0.16 mmol) was added to a stirred solution of 1-[^{14}C]hexadecanedioic acid (500 μCi, 6.5 mCi/mmol, 23.1 mg, 0.08 mmol) in 2 mL of dry THF. Dry dimethylformamide (0.2 mL) was then added, and the mixture was stirred at ~25 °C for 2 h before 80 μL (0.08 mmol) of a 1 M solution of ethanethiol in CH_2Cl_2 was added. The mixture was then cooled to 0 °C, 0.2 mL of dry pyridine was added, and the mixture was stirred at ~25 °C for 1 h before HCl (1 N) and ethyl acetate were added. The organic phase was washed with 1 N HCl and brine, dried over anhydrous $MgSO_4$, and taken to dryness by solvent evaporation. Thin-layer chromatography on silica gel (7:3:0.1 hexane–ethyl acetate–acetic acid) indicated that the dithioester, monothioester, and diacid were obtained in a 30:50:20 ratio. This radiolabeled material, which was employed without further purification, was dissolved in ethanol (4% concentration in final enzyme solution) and suspended in 50 mM potassium carbonate buffer immediately prior to use.

Cytochrome P450$_{BM-3}$ Inactivation. Oxygen consumption was measured with a Clarke-type oxygen electrode (Gilson Oxygraph Model 5/6). The oxygen electrode was immersed in a 1.6-mL closed reaction chamber with a magnetic stirrer containing cytochrome P450$_{BM-3}$ (0.4 μM) in 1.6 mL of 50 mM MOPS buffer (pH 7.4). The desired amount of the test compound in 50 mM potassium carbonate solution was added by syringe, and the resulting mixture was preincubated at 25–28 °C for 3 min before adding NADPH (600 μM). After

incubation for 15 min, lauric acid (100 μM) was added and the rate of oxygen consumption during the first 3 min was recorded. Rates were estimated from the initial linear portion of the oxygen consumption trace obtained in these incubations. The amount of oxygen consumed was calculated from the percent of the maximum instrument response obtained when oxygen was completely consumed in calibration experiments by the addition of sodium dithionite, taking the oxygen concentration at 28 °C to be 228 μM (Lessler & Brierley, 1969). In control experiments, the test compound was replaced by lauric acid (amount equivalent to that of the test compound). The rate of oxygen consumption with lauric acid as the first substrate was 227 nmol/min/nmol of $P_{450BM-3}$, but the rate of oxygen consumption for lauric acid in the assay (second) incubation was approximately 29 nmol/min/nmol of $P450_{BM-3}$. The reason for the slower oxidation observed in the assay incubation is unclear, but the rate is reproducible. Inhibition percent was calculated from the rate of oxygen consumption in the assay incubation observed in the test versus control (lauric acid) experiments.

Protection from Inactivation by Glutathione. Incubation mixtures (1.6 mL volume) containing 0.4 μM cytochrome $P450_{BM-3}$, 50 μM inhibitor, and either no glutathione or 1 mM glutathione were placed in the stirred reaction chamber of a Gilson oxygen electrode and allowed to equilibrate for 3 min. The reaction was then initiated by adding NADPH (600 μM final concentration), and oxygen consumption was quantitated. After 15 min, 100 μM lauric acid was added and the rate of oxygen consumption over the first 3 min was determined. In another set of experiments, the initial incubation was carried out without glutathione. Glutathione (1 mM) was then added, and the mixture was incubated for 5 min before 100 μM lauric acid was added and oxygen consumption was measured.

Analysis of Fatty Acid Metabolites. Incubations (2 mL) contained cytochrome $P450_{BM-3}$ (0.5–2.0 μM), substrate (30–300 μM), and NADPH (300–600 μM) in 50 mM MOPS buffer (pH 7.4). Reactions were initiated by adding the NADPH to the other components after they had been preincubated for 3 min at 25–28 °C and were stopped after incubation for 10 min at the same temperature by adding 250 μL of 1 N HCl (final pH ~1.4). After vigorous mixing, the mixtures were extracted twice with a total of 3 mL of CH_2Cl_2 (ethyl acetate was used when 16-hydroxy-17-ODYA was the substrate) and the solvent was removed from the combined extracts under a stream of nitrogen gas. The residues were methylated by dissolving them in 1 mL of a solution of diazomethane (~0.5 M) in diethyl ether and allowing the mixtures to stand for 1 h at ~25 °C before removing the solvent and excess diazomethane under a stream of nitrogen (hood!). The residues were dissolved in 50 μL of $CHCl_3$, 2 μL of which was injected into a gas chromatograph equipped with a DB-1 capillary column (30 m × 0.25 mm, 0.25 μm i.d.). A DB-5 column was used for the 17-ODEA metabolites because they were not well resolved on the DB-1 column. In all cases, the injector and detector temperatures were set at 250 and 300 °C, respectively, and the column was raised isocratically from 200 to 250 °C at a rate of 5 °C/min. The flow rate of helium gas was 95 mL/min. The retention times of the products were compared with those of authentic standards.

The MOPS buffer and all the stock solutions (except for the enzyme stock solution) were prepared with 99.8% deuterated water for experiments intended to test whether deuterium is incorporated into the product during the conversion of THDA to hexadecanedioic acid. The reaction system (3.2 mL) contained 3.4 nmol of cytochrome $P450_{BM-3}$ (2.5 μL), 1.5 μmol of THDA, and 4.5 μmol of NADPH. The amount of normal water in the system was calculated to be 0.28%.

Extraction and Analysis of N-Alkylprotoporphyrin IX Adducts. To 2.5 nmol of cytochrome $P450_{BM-3}$ in 500 μL of 50 mM MOPS buffer (pH 7.4) was added 156 nmol of 17-ODYA or 16-hydroxy-17-ODYA. After 2 min, 60 nmol of NADPH was added and the mixture was incubated for 15 min at 28 °C. The reaction was then quenched by adding 5 mL of a freshly prepared 5:95 sulfuric acid (18 M)–acetonitrile solution and allowing the mixture to stand at 4 °C for 1 h before removing the organic solvent on a rotary evaporator. The residue was redissolved in 2 mL of 0.9 M aqueous sulfuric acid, and the mixture was extracted twice with a total of 3 mL of CH_2Cl_2. The combined organic extracts were concentrated under a stream of nitrogen, the residue was dissolved in 150 μL of solvent A (6:4:1 methanol–water–acetic acid), and a 100-μL aliquot was subjected to HPLC analysis on a 5-μm Partisil ODS-3 (Alltech) column. The column was eluted isocratically for 30 min with a 5.5:4.5 mixture of solvents A and B (solvent B is 10:1 methanol–acetic acid) followed by a 1-min gradient to 100% solvent B and 5-min isocratic elution at 100% solvent B. The column effluent was monitored at 416 nm with the diode array detector bandwidth set to 4 nm and the reference absorption measured at 600 nm. The *N*-biphenylprotoporphyrin IX isomers obtained from the reaction of biphenylhydrazine with equine myoglobin were used as the reference standards (Tuck & Ortiz de Montellano, 1992).

Limited Tryptic Digestion of Inactivated Cytochrome $P450_{BM-3}$. LImited proteolysis was performed at 0–4 °C by adding 430 ng of trypsin to 215 μg of cytochrome $P450_{BM-3}$ in 0.1 MOPS buffer (pH 7.2) in the presence of 0.1 mM dithiothreitol. Aliquots were removed at appropriate intervals for assay.

SDS–PAGE and Autoradiography. Polyacrylamide gel electrophoresis of protein fractions and protein standards was carried out on 12.5% polyacrylamide slab gels with a 4.75% polyacrylamide stacking gel according to the procedure of Laemmli (1970). Protein bands were visualized by staining with Coomassie blue. Autoradiograms were obtained after exposure of the dried SDS–PAGE gels to X-Omat AR film (Kodak) for 2 days at –80 °C.

Isolation, Digestion, and Analysis of [^{14}C]THDA-Inactivated Cytochrome $P450_{BM-3}$. A solution of cytochrome $P450_{BM-3}$ (3.4 mmol) and [^{14}C]THDA (~320 nmol) in 500 μL of buffer was preincubated for 2 min at 25 °C before NADPH (1.5 mmol) was added to initiate the reaction. A control incubation was carried out without the addition of NADPH. After 15 min, the reaction mixtures were filtered through a Sephadex G-25 column and the enzyme thus obtained was digested with TPCK-treated trypsin in 0.2 M ammonium carbonate buffer at 37 °C for 24 h. The trypsin-to-protein ratio (w/w) was 50. An aliquot of the tryptic digest was chromatographed on an Alltech 5-μm Partisil ODS-3 column. The column was eluted with a 90-min gradient from 0 to 70% solvent B in solvent A at a flow rate of 0.7 mL/min, where solvent A is water containing 0.1% trifluoroacetic acid and solvent B is acetonitrile containing 9.9% water and 0.1% trifluoroacetic acid. Radioactivity was monitored with a Radiomatic Flo-One radioactivity detector.

Table I: Oxidation of Terminally Unsaturated Fatty Acid Analogues by Cytochrome $P450_{BM-3}$ and Inactivation of the Enzyme[a]

compds	concn (μM)	inhibition (%)[b]	O_2 consumption (nmol)[c]
lauric acid	100	ND[d]	170
11-dodecenoic acid	100	ND	101
12-tridecenoic acid	100	ND	160
10-undecynoic acid	100	ND	10
2,2-dimethyl-11-dodecynoic acid	100	ND	37
17-ODEA	100	100	208
	25	66	90
	6.25	33	29
17-ODYA	25	100	53
	6.25	94	35
	1.56	52	24
16-hydroxy-17-ODYA	25	100	40
	6.25	95	30
	1.56	60	18

[a] The initial incubations (15 min) contained 0.64 nmol enzyme, the indicated concentration of lauric acid or inhibitor, and 960 nmol NADPH in 1.6 mL MOPS buffer. [b] Percent loss of ability to oxidize lauric acid caused by incubation with the indicated agent for 15 min versus similar incubation with lauric acid. [c] Total oxygen consumption during the incubation with the indicated agent. [d] ND = not detectable.

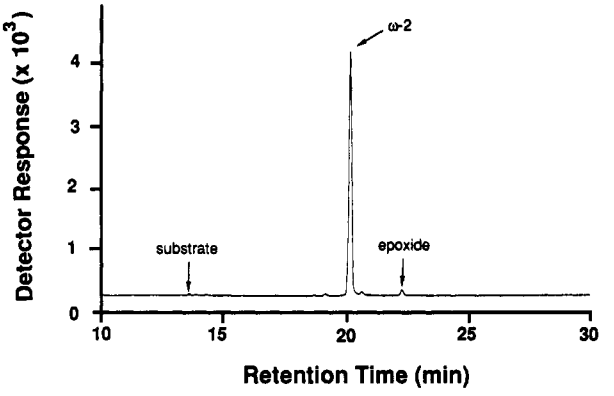

FIGURE 1: Gas–liquid chromatographic analysis of the products formed in the reaction of cytochrome $P450_{BM-3}$ with 12-tridecenoic acid. The incubation, carried out at 28 °C for 10 min, contained cytochrome $P450_{BM-3}$ (0.4 μM), NADPH (600 μM), and 12-tridecenoic acid (300 μM) in MOPS buffer (50 mM, pH 7.4). The extraction, methylation, and chromatography conditions are given in the Experimental Procedures.

RESULTS

Oxidation of Fatty Acids with Terminal Double or Triple Bonds. Oxygen consumption measurements indicate that cytochrome $P450_{BM-3}$ oxidizes terminally unsaturated fatty acids (Table I). Oxygen consumption appears to be a true measure of substrate turnover because the formation of H_2O_2 by uncoupled turnover is not significant (not shown) and high conversion of substrate to oxidized products is observed for all the substrates except 10-undecynoic acid (see below). This is readily confirmed for substrates such as lauric acid and 12-tridecenoic acid, for which oxygen consumption equals the amount of substrate oxidized (Table I; Figure 1). In other instances, oxygen consumption exceeds the amount of substrate present because substrates can undergo more than one oxidation event (see below). Oxidation of the fatty acids is length-dependent, as shown by the fact that 11-dodecenoic acid is a poorer substrate than 12-tridecenoic acid or 17-ODEA and that 10-undecynoic acid is a much poorer substrate than 2,2-dimethyl-11-dodecynoic acid or 17-ODYA. Interestingly, 11-dodecenoic acid is not as good a substrate as lauric acid, the saturated analogue.

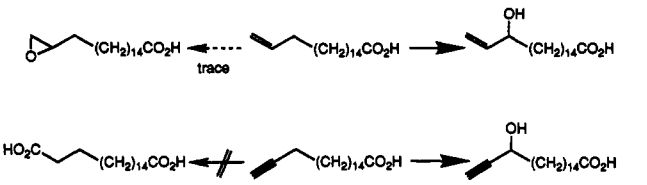

FIGURE 2: Structures of the products formed in the metabolism of 17-ODEA and 17-ODYA by cytochrome $P450_{BM-3}$.

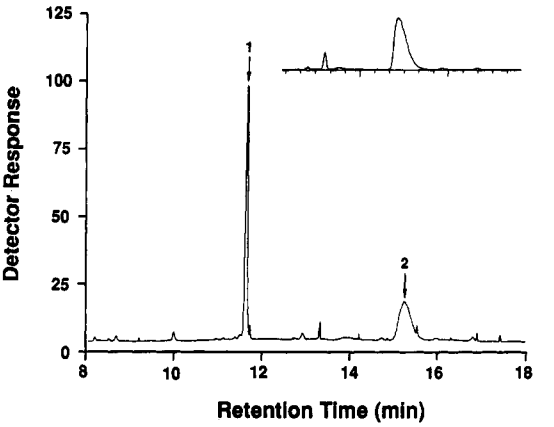

FIGURE 3: Gas–liquid chromatographic analysis of the product formed in the incubations of 17-ODYA with cytochrome $P450_{BM-3}$. The labeled peaks are 17-ODYA (1) and 16-hydroxy-17-ODYA (2). The chromatogram of authentic 16-hydroxy-17-ODYA is shown in the inset The incubation contained cytochrome P450BM-3 (0.5 μM), NADPH (313 μM), and 17-ODYA (31 μM) in MOPS buffer (50 mM, pH 7.4). The extraction, methylation, and chromatography conditions are given in the Experimental Procedures.

The oxidation of 12-tridecenoic acid, a typical terminally unsaturated fatty acid, by cytochrome $P450_{BM-3}$ yields two products in a 97:3 ratio (Figure 1). The substrate is essentially completely consumed in the reaction. The major product, as shown by mass spectrometry and ^1H NMR, is 11-hydroxy-12-tridecenoic acid, the ω-2 hydroxylation product. The minor product, as shown by direct comparison with a synthetic standard, is 12,13-epoxytridecanoic acid. Oxidation of 17-ODEA similarly provides 16-hydroxy-17-ODEA and the 17,-18-epoxide in a 95:5 ratio (Figure 2). The identities of the 17-ODEA metabolites were established by direct chromatographic and spectroscopic comparison with synthetic samples.

2,2-Dimethyl-11-dodecynoic acid is oxidized by cytochrome $P450_{BM-3}$ to a single detectable product (not shown). ^1H NMR and mass spectrometry unambiguously identify this single product as 10-hydroxy-2,2-dimethyl-11-dodecynoic acid, again the ω-2 hydroxylation product. No trace is detected of 2,2-dimethyldodecanedioic acid, the derivative expected from oxidation of the triple bond (Ortiz de Montellano, 1985). In accord with these findings, 17-ODYA is oxidized to 16-hydroxy-17-ODYA without the detectable formation of octadecanedioic acid, the product expected from oxidation of the triple bond (Figure 3) (Ortiz de Montellano, 1985; CaJacob et al., 1988). The structure of this metabolite (Figure 2), after methylation with diazomethane, was established by direct chromatographic and mass spectrometric comparison with an independently synthesized sample. Substrate consumption in incubations of synthetic 16-hydroxy-17-ODYA with cytochrome $P450_{BM-3}$ and NADPH indicates the alcohol is further metabolized (Table I). The products of this metabolism have not been definitively identified but include a compound that co-elutes with authentic 16-oxo-17-octadecynoic acid and other more polar, possibly dihydroxylated, products (not shown).

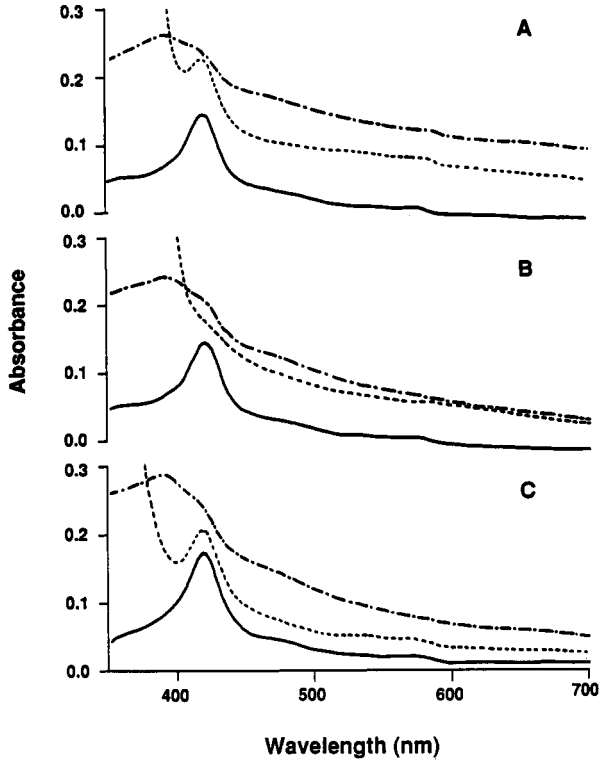

FIGURE 4: The absorption spectrum of cytochrome P450$_{BM-3}$ in the absence of substrate (—) and after extended incubation with stearic acid (A), 17-ODYA (B), and THDA (C) in the absence (- - -) and presence (- - -) of excess NADPH.

Inactivation of Cytochrome P450$_{BM-3}$ by Terminally Unsaturated Fatty Acids. Incubation of cytochrome P450$_{BM-3}$ and NADPH with 11-dodecenoic acid, 12-tridecenoic acid, or 10-undecynoic acid does not result in detectable time-dependent loss of the ability of the enzyme to subsequently oxidize lauric acid (Table I). Incubation of cytochrome P450$_{BM-3}$ with 17-ODYA and NADPH, however, results in inactivation of the enzyme. Catalytic activity is not recovered if 17-ODYA-inactivated enzyme is passed through a G-25 column prior to carrying out the lauric acid hydroxylation assay. Control experiments in which the enzyme was preincubated with lauric acid, passed through the G-25 column, and re-assayed with lauric acid showed that these manipulations do not cause loss of enzyme activity. Furthermore, addition of stearic acid or 17-ODYA to cytochrome P450$_{BM-3}$ causes a shift of the Soret band from 416 to 390 nm consistent with conversion of the enzyme from low- to high-spin state. Addition of excess NADPH to the enzyme-stearic acid mixture results in eventual reappearance of the Soret band at 416 nm due to complete oxidation of the substrate to less readily bound products. In contrast, the Soret band simply disappears with time when a complex of cytochrome P450$_{BM-3}$ with 17-ODYA is incubated with NADPH (Figure 4). Inclusion of glutathione in the incubation mixture does not prevent loss of the chromophore or catalytic activity of the enzyme (Table II). 17-ODYA thus inactivates the enzyme by a mechanism that involves destruction of the heme chromophore. Catalytic oxidation of 16-hydroxy-17-ODYA, the principal metabolite produced from 17-ODYA, causes similar concentration- and NADPH-dependent inactivation of cytochrome P450$_{BM-3}$ and heme chromophore loss (Table I). Incubation of cytochrome P450$_{BM-3}$ with 17-ODEA also results in inactivation of the enzyme and loss of the heme chromophore (Table I). Inclusion of glutathione in the 17-ODEA incubation mixture again does not prevent chromophore loss or inactivation of the enzyme (Table II).

Table II: Effects of Glutathione on the Inactivation of Cytochrome P450$_{BM-3}$

inhibitor	concn (μM)	glutathione (1 mM)	inhibition (%)[a]
17-ODYA	25	no	100
	25	yes	100
17-ODEA	100	no	100
	100	yes	100
TDDA	100	no	87
	100	yes	2
THDA	50	no	95
	50	yes	10

[a] Percent loss of the ability to oxidize lauric acid caused by incubation with the indicated agent versus incubation with lauric acid, which causes no inactivation.

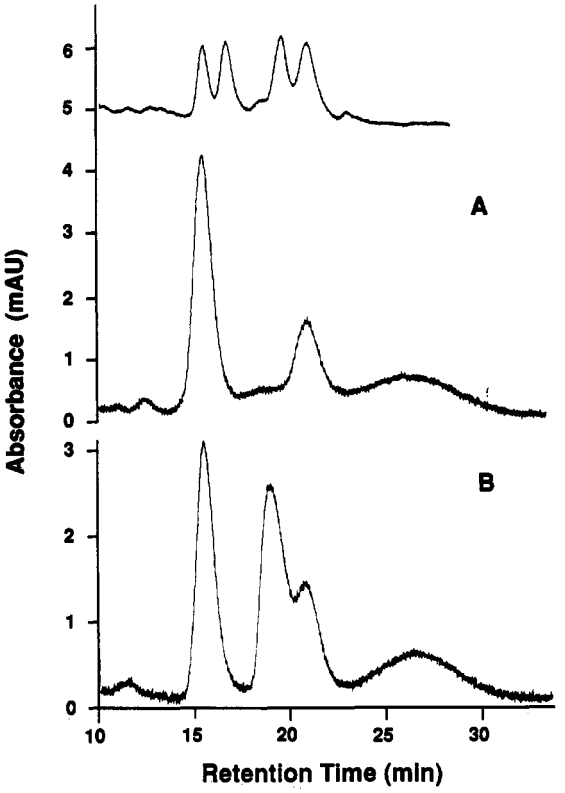

FIGURE 5: HPLC of the heme adducts extracted with dichloromethane from cytochrome P450$_{BM-3}$ inactivated with (A) 17-ODYA and (B) 16-hydroxy-17-ODYA. The retention times of the *N*-biphenylprotoporphyrin IX isomers obtained from reaction of myoglobin with biphenylhydrazine are shown at the top for comparison. The HPLC conditions are given in the Experimental Procedures.

Formation of Heme N-Alkyl Adducts with 17-ODYA and 16-Hydroxy-17-ODYA. Concurrent loss of both the heme chromophore and the catalytic activity in incubations with 17-ODYA or 16-hydroxy-17-ODYA (Figure 4; Table I) suggests that these compounds inactivate the enzyme by modifying the prosthetic heme group. Most terminal acetylenes inactivate cytochrome P450 by N-alkylating the prosthetic heme group, although 11-dodecynoic acid inactivates hepatic lauric acid ω-hydroxylase (CYP 4A1) by binding covalently to the protein (Ortiz de Montellano, 1985; CaJacob et al., 1988; Ortiz de Montellano & Reich, 1986). The prosthetic groups extracted from cytochrome P450$_{BM-3}$ after inactivation by 17-ODYA and 16-hydroxy-17-ODYA were analyzed by HPLC. As shown in Figure 5, similar but not identical porphyrin mixtures were obtained with both agents. Modified porphyrins with retention times of 15.8, 19.2, 20.9,

and 26.4 min were obtained with 16-hydroxy-17-ODYA and, except perhaps for the 19.2-min peak, with 17-ODYA. The porphyrins have Soret maxima at approximately 410 nm and have retention times in the same range as those (15.1, 16.4, 19.0, and 20.3 min) of the four authentic isomers of *N*-biphenylprotoporphyrin IX under the same chromatographic conditions. LC–MS analysis of the porphyrin products, which elute as a single peak with a retention time of 3 min in the chromatographic system used for the mass spectrometric analysis, yields a molecular ion at $m/z = 875.8$ (not shown). Under the same conditions, the protonated molecular ion of protoporphyrin IX is found at $m/z = 563.4$. The molecular ion of the modified porphyrin mixture is therefore exactly that expected for protoporphyrin IX bearing a $CH_2COCH(OH)(CH_2)_{14}CO_2H$ function on one of the pyrrole nitrogens. This is consistent with N-alkylation of the heme group during catalytic oxidation of the 16-hydroxy-17-ODYA metabolite formed by initial hydroxylation of 17-ODYA. The formation of three or four porphyrin adducts not only could be due to alkylation of more than one of the four possible pyrrole ring nitrogens but could also reflect the formation of diastereomeric products due to the chiral alcohol center in the side chain. The difference between the porphyrin patterns obtained with 17-ODYA and 16-hydroxy-17-ODYA as the inactivating agent may be due to the fact that the synthetic sample of 16-hydroxy-17-ODYA is a racemic mixture, whereas unequal amounts of the two enantiomers of 16-hydroxy-17-ODYA are likely to be formed metabolically from 17-ODYA. It is also possible that some heme alkylation occurs during catalytic turnover of 17-ODYA itself and not only during subsequent oxidation of the 16-hydroxy-17-ODYA metabolite.

Inactivation of Cytochrome $P450_{BM-3}$ by TDDA and THDA. Inactivation of cytochrome $P450_{BM-3}$ by 17-ODYA involves alkylation of the heme rather than the protein. Heme rather than protein alkylation is consistent with the bias against ω-oxidation by cytochrome $P450_{BM-3}$ because ω-1 oxidation of a terminal triple bond leads to heme alkylation whereas ω-oxidation, which produces a ketene intermediate, is required for protein modification (Ortiz de Montellano, 1985; CaJacob et al., 1988; Ortiz de Montellano & Reich, 1986). In an effort to develop a mechanism-based agent that would bind covalently to the protein when oxidized at the ω-2-position, we synthesized the monoethyl thioesters of dodecanedioic and hexadecanedioic acids. Although thioesters have not been investigated previously as mechanism-based inactivating agents for cytochrome P450 enzymes, it has been shown that thioester oxidation produces species that react with water to give the corresponding acids or with gutathione to give glutathione conjugates (Chen et al., 1979; Feng & Solsten, 1991).

Incubation of the enzyme with NADPH and one of the two thioesters results in time-, co-factor-, and concentration-dependent loss of lauric acid hydroxylase activity (Table III). THDA, the longer analogue, is a more effective inactivating agent than TDDA. Lauric acid hydroxylase activity is not recovered when the inactivated enzyme is passed through a G-25 column, in accord with an irreversible inactivation mechanism. Furthermore, the chromophore of the enzyme is not lost in the reactions with the thioesters (Figure 4) and no *N*-alkyl heme adducts are detected when the prosthetic groups of the inactivated proteins are extracted and analyzed (not shown). Control experiments demonstrate that dodecanedioic and hexadecanedioic acids, the two metabolites (vide infra), do not inactivate the enzyme (Table III). It appears, therefore, that the inactivation reflects covalent modification of the protein by catalytically activated species that precede the diacid metabolites.

Table III: Oxidation of Fatty Diacid Thioesters by Cytochrome $P450_{BM-3}$ and Inactivation of the Enzyme[a]

compds	concn (μM)	inhibition (%)[b]	O_2 consumption (nmol)[c]
lauric	100	ND[d]	170
$CH_3CH_2SCO(CH_2)_{10}CO_2H$(TDDA)	100	82	174
	50	61	80
	25	42	51
	6.25	ND	10
$CH_3CH_2SCO(CH_2)_{14}CO_2H$(THDA)	100	100	146
	50	94	106
	25	52	86
	6.25	18	37
THDA (no NADPH)	100	ND	
$HO_2C(CH_2)_{10}CO_2H$	100	7	10
$HO_2C(CH_2)_{14}CO_2H$	100	2	34
$CH_3CH_2SCO(CH_2)_{14}COSCH_2CH_3$	100	9	19

[a] The initial incubations (15 min) contained 0.64 nmol enzyme, 160 nmol lauric acid or inhibitor, and 960 nmol NADPH in 1.6 mL of MOPS buffer. [b] Percent loss of the ability to oxidize lauric acid caused by incubation with the indicated agent for 15 min versus similar incubation with lauric acid. [c] Total oxygen consumption during the 15 min incubation of the compound with cytochrome $P450_{BM-3}$. [d] ND = not detectable.

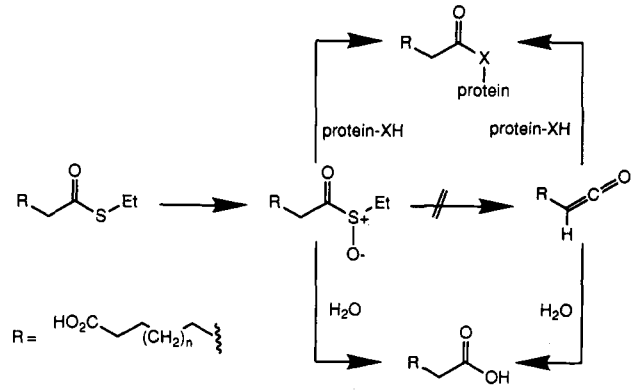

FIGURE 6: Mechanism for the oxidation of THDA and TDDA to species that (a) yield the corresponding dicarboxylic acids as unique metabolites and (b) inactivate cytochrome $P450_{BM-3}$ by covalently binding to the protein. The ketene intermediate is ruled out by the absence of deuterium incorporation into the diacid metabolite when the reaction is carried out in deuterated water.

Metabolism of TDDA and THDA. Incubation of cytochrome $P450_{BM-3}$ and NADPH with the monothioesters of dodecanedioic and hexadecanedioic acids results in the consumption of molecular oxygen (Table III). The two compounds are therefore oxidized by the enzyme. A single metabolite of each of the two monoethyl thioesters is detected when the incubation products are extracted, methylated with diazomethane, and analyzed by gas–liquid chromatography (not shown). Direct chromatographic and ^{1}H-NMR comparisons with authentic samples identify the products formed from the monoethyl thioesters as, respectively, dodecanedioic and hexadecanedioic acids. Oxidation of the thioester group in each substrate thus converts it to a carboxylic acid function, either via direct reaction of the unstable sulfoxide product with water or via elimination of CH_3CH_2SOH to give a ketene (Figure 6). To determine whether hydrolysis or ketene formation is involved in conversion of the monoethyl thioester to a carboxylic acid function, oxidation of the monoethyl thioester of hexadecanedioic acid was carried out in highly deuterated medium and the diacid metabolite was isolated. The identity of the mass spectrum of the isolated metabolite

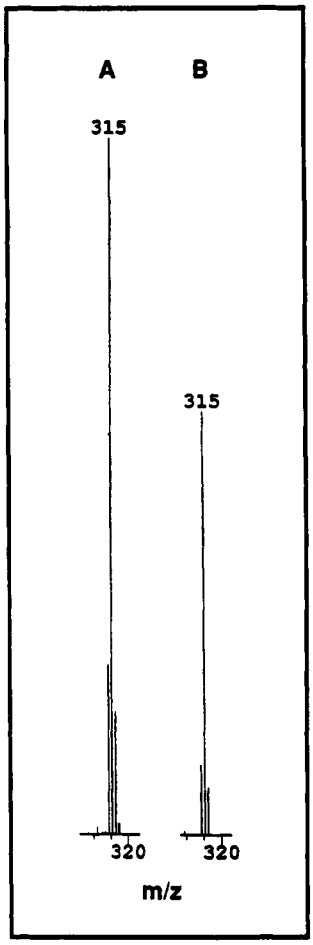

FIGURE 7: GC–EIMS of the product isolated from incubation of THDA with cytochrome P450$_{BM-3}$ in deuterated medium. The molecular ion region on the left is that for an authentic standard of the dimethyl ester of hexadecanedioic acid (B), and that on the right is for the product obtained in deuterated medium (A).

with that of the authentic diacid indicates that no deuterium is incorporated into the fatty acid chain during conversion of the thioester to the carboxylic acid (Figure 7). This rules out the ketene pathway because it requires incorporation of 1 equiv of deuterium into the fatty acid chain when the ketene is hydrated to the carboxylic acid (Figure 6).

Protection from Inactivation by Glutathione. In order to determine if a diffusible reactive species is responsible for inactivation of the enzyme, the inactivation of cytochrome P450$_{BM-3}$ by TDDA and THDA in the presence and absence of glutathione was examined. As already noted, glutathione does not protect the enzyme from inactivation by 17-ODYA or 17-ODEA (Table II). In contrast, glutathione provides essentially complete protection from inactivation by the two thioesters (Table II). Addition of glutathione to the enzyme after it was inactivated by THDA did not result in recovery of catalytic activity (not shown). The effect of glutathione is therefore not due simply to reduction of oxidized sulfhydryl groups on the protein. The glutathione protection experiments indicate that the thioesters are not true suicide substrates even if they must be catalytically activated because the enzyme is inactivated by metabolites that diffuse out of the active site. The thioesters thus differ from 17-ODEA and 17-ODYA, which are true mechanism-based inactivating agents.

Protein Labeling by [^{14}C]THDA. The radiolabeled monoethyl thioester of hexadecanedioic acid was synthesized to investigate whether the enzyme is inactivated by protein acylation by a catalytically generated reactive metabolite.

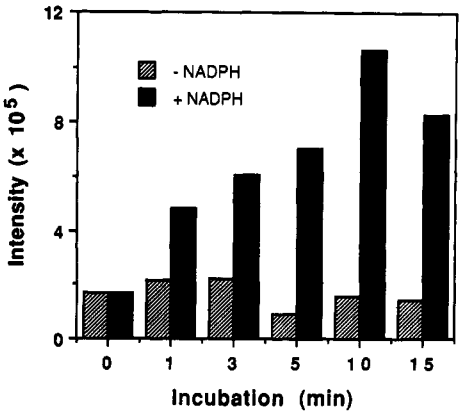

FIGURE 8: Time course of labeling of cytochrome P450$_{BM-3}$ by incubation with [^{14}C]THDA in the presence or (control) absence of NADPH.

The radiolabeled sample contained [^{14}C]hexadecanedioic acid (30%) and its diethyl thioester (20%) in addition to THDA, but control experiments show that the diacid and the diethyl thioester do not inactivate the enzyme (Table III).

Incubation of cytochrome P450$_{BM-3}$ with [^{14}C]THDA and NADPH for periods of 1–15 min followed by SDS–PAGE of the protein, autoradiography, and quantitation of the radioactivity in the protein by densitometry indicate that protein labeling occurs and reaches a maximum after 10 min (Figure 8). The time course of labeling of the protein is consistent with the fact that the enzyme is fully inactivated within 15 min of incubation with THDA. A control experiment done by exactly the same procedure except that the incubation was carried out in the absence of NADPH shows that some radiolabeling occurs without catalytic turnover of the enzyme but the extent of this background label incorporation is not time-dependent (Figure 8). Limited tryptic digestion of [^{14}C]-THDA-inactivated cytochrome P450$_{BM-3}$, which cleaves the 119 kD enzyme into a 55 kD hemoprotein domain and a 66 kD flavoprotein domain (Narhi & Fulco, 1986), followed by SDS–PAGE and autoradiography, indicates that the radioactive label is bound to both domains of the protein (Figure 9).

DISCUSSION

The ω-1, ω-2, and ω-3 monohydroxylated fatty acids are produced by cytochrome P450$_{BM-3}$ from lauric acid in a 36:30:34 ratio, tridecanoic acid in a 17:65:18 ratio, and octadecanoic acid in a 39:47:14 ratio (Miura & Fulco, 1975). Terminal (ω) hydroxylation was not observed with any of the substrates. Even greater selectivity for the ω-2 position is reported here for terminally unsaturated fatty acids. Thus, oxidation of tridecenoic acid, the olefin equivalent in length to tridecanoic acid, produces 11-hydroxy-12-tridecenoic acid with no more than a trace of the 12,13-epoxide (Figure 2), and oxidation of 17-ODEA, equivalent in length to octadecanoic acid, similarly produces 16-hydroxy-17-ODEA and a trace of the corresponding epoxide. Oxidation of 2,2-dimethyl-11-dodecynoic acid, which corresponds in length to lauric acid, and 17-ODYA, which corresponds to octadecanoic acid, yields 10-hydroxy-2,2-dimethyl-11-dodecynoic acid and 16-hydroxy-17-ODYA, respectively, as the only detectable metabolites. Oxidation at the ω-1 and ω-3 positions is thus virtually suppressed, leaving ω-2 hydroxylation as the single dominant pathway, when a terminal double or triple bond is present.

The relationship between C–H bond strength and intrinsic susceptibility to cytochrome P450-catalyzed hydroxylation

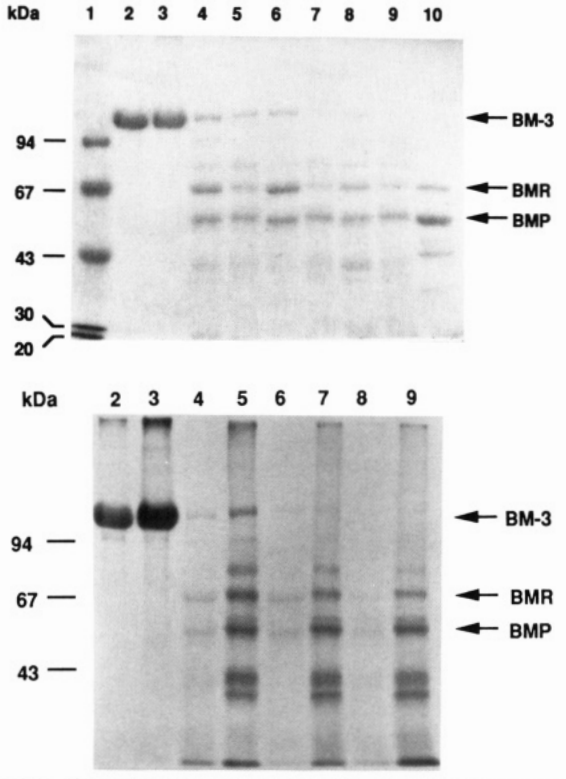

FIGURE 9: SDS–PAGE analysis of [^{14}C]THDA-inactivated cytochrome P450$_{BM-3}$: SDS–PAGE gel stained with Coomassie blue (A, top) and autoradiogram of the SDS–PAGE gel (B, bottom). Key: Lane 1, molecular weight marker proteins; lane 2, cytochrome P450$_{BM-3}$ incubated with [^{14}C]THDA but no NADPH; lane 3, cytochrome P450$_{BM-3}$ incubated with [^{14}C]THDA and NADPH; lanes 4, 6 and 8, 15-, 30-, and 60-min tryptic digests, respectively, of enzyme incubated with [^{14}C]THDA but no NADPH; lanes 5, 7, and 9, 15-, 30-, and 60-min tryptic digests, respectively, of enzyme incubated with [^{14}C]THDA and NADPH.

(Frommer et al., 1970; White et al., 1984b) readily explains the decrease in ω-2 oxidation because the ω-2 secondary allylic ($DH°_{298}$ = ~83 kcal/mol) or propargylic ($DH°_{298}$ = <94 kcal/mol) C–H bonds are weaker than the secondary C–H bonds at the ω-3 position ($DH°_{298}$ = ~98 kcal/mol) (Egger & Cocks, 1973). The minor extent of saturated chain ω-hydroxylation catalyzed by cytochrome P450 enzymes not specifically designed to promote ω-oxidation also follows from the relationship between C–H bond strength and susceptibility to hydroxylation. Primary C–H bonds are stronger than secondary C–H bonds and, in the absence of overriding steric factors, are more slowly oxidized. A terminal double bond, however, is easily oxidized and should be the favored site of oxidation. For example, rat liver microsomes oxidize 1-octene more than 40-times faster to 1,2-oxidooctane than to oct-1-en-3-ol (White et al., 1986; Ortiz de Montellano et al., 1983). Less information is available on triple bond oxidation, but oxidation of terminal acetylenes to carboxylic acids takes precedence over ω-2 oxidation to give propargylic alcohols (Ortiz de Montellano et al., 1985; CaJacob et al., 1988; White et al., 1984a). Furthermore, epoxidation of *cis*-9-hexadecenoic acid shows that there is no intrinsic mechanistic impediment to cytochrome P450$_{BM-3}$-catalyzed epoxidation reactions (Ruettinger & Fulco, 1981). In view of these facts, the virtual absence of terminal π-bond oxidation clearly indicates that the ω-1, ω-2, and ω-3 specificity of cytochrome P450$_{BM-3}$ is not simply determined by the electronic preference for oxidation of secondary over primary C–H bonds. The enzyme structure must actively suppress oxidation of the terminal double bond.

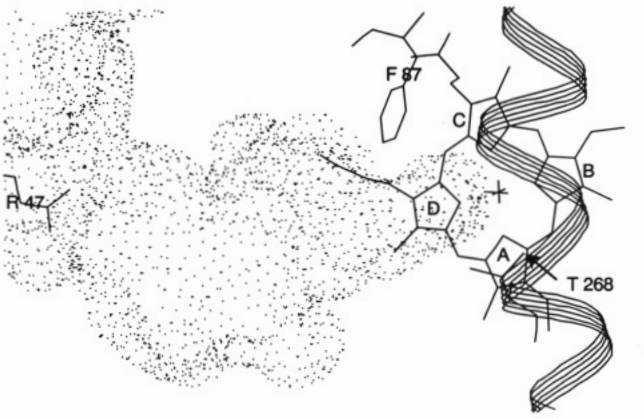

FIGURE 10: Substrate accessible surface of the hydrophobic access channel and the active site of cytochrome P450$_{BM-3}$. Residue Phe-87 is almost perpendicular to the plane of the porphyrin and is in van der Waals contact with it, forming a lipophilic pocket that may sequester the terminal carbon of the fatty acid chain (Ravichandran et al., 1993). Interaction of Arg-47, which is near the entry to the access channel, with the fatty acid carboxyl group may play a role in defining the chain-length specificity of the enzyme.

The inefficiency of cytochrome P450$_{BM-3}$ toward terminal π-bond oxidation can be rationalized by the crystal structure of the hemoprotein domain. The active site is a long channel that tapers down near the heme iron to a narrow funnel into which the fatty acid "tail" is inserted for oxidation (Figure 10). Of particular interest is the presence of a lipophilic pocket at the end of this funnel region that could serve as a binding site for the terminal carbon of the substrate (Ravichandran et al., 1993). Sequestration of the terminal methyl group in this pocket would reinforce the intrinsic preference for oxidation of secondary over primary C–H bonds. Sequestration of the olefinic and acetylenic termini could similarly prevent oxidation of the terminal π-bonds despite their high intrinsic reactivities.

Triple bond oxidation can result in cytochrome P450 inactivation via heme (Ortiz de Montellano et al., 1985; CaJacob et al., 1988) or protein (CaJacob et al., 1988; Yun et al., 1992) modification. The structures of the heme adducts obtained by administration of acetylenes to rats indicate that heme alkylation follows delivery of the activated oxygen to the internal carbon (ω-1) of the triple bond (Ortiz de Montellano, 1985; Ortiz de Montellano & Reich, 1986) and protein alkylation delivery of the oxygen to the terminal (ω) carbon (CaJacob et al., 1988). For example, inactivation of cytochrome P450 4A1, a lauric acid ω-hydroxylase, by 11-dodecynoic acid involves protein rather than heme modification (CaJacob et al., 1988). Inactivation of cytochrome P450$_{BM-3}$ by 17-ODYA, however, is mediated by prosthetic heme alkylation (Figures 4 and 5). The mass spectrometric molecular ion of the adduct is precisely that expected for addition of an oxygen to the internal carbon and a nitrogen of the porphyrin to the terminal carbon of the triple bond if the triple bond in question is that of 16-hydroxy-17-ODYA, the metabolite of 17-ODYA. The molecular weight of the *N*-alkyl group thus corresponds to $-CH_2COCH(OH)(CH_2)_{14}CO_2H$ rather than $-CH_2CO(CH_2)_{15}CO_2H$. This is consistent with the fact that 17-ODYA is rapidly oxidized to 16-hydroxy-17-ODYA and with the fact that authentic 16-hydroxy-17-ODYA inactivates the enzyme and gives similar modified porphyrins. Although the number of substrate molecules oxidized per enzyme molecule inactivated is high because the enzyme only poorly oxidized the triple bond, the partition ratio is approximately one if only the ratio of triple bond oxidation to enzyme inactivation is considered because the

dicarboxylic acid metabolite is not detected. 17-ODEA also inactivates the enzyme by heme modification, a result consistent with the finding that a small amount of the epoxide is formed because heme alkylation requires delivery of the oxygen to the internal (ω-1) carbon of the double bond (Ortiz de Montellano, 1988).

It is of interest that 11-dodecenoic, 12-tridecenoic, and 2,2-dimethyl-1-dodecynoic acids are oxidized by cytochrome $P450_{BM-3}$ but do not detectably inactivate it. The inactivity of the dodecynoic acid is particularly surprising because terminal acetylenes are more effective than olefins as mechanism-based inactivating agents. For example, the partition ratio for inactivation of cytochrome 4A1 by 11-dodecynoic acid is 2 (CaJacob et al., 1988), and that for cytochrome P450 2B1 by phenylacetylene is 31 (Komives & Ortiz de Montellano, 1987), whereas partition ratios for inactivation of cytochrome P450 2B1 by olefins are in the order of 200–500 (Ortiz de Montellano, 1988). Furthermore, the rate of oxidation of 2,2-dimethyl-11-dodecynoic acid, which does not inactivate the enzyme, is comparable to that of 17-ODYA, which does (Table I), and the only metabolite detected in both cases is propargylic alcohol. The role of chain length in inactivation of cytochrome $P450_{BM-3}$ is therefore unclear.

The efficient inactivation of cytochrome P450 4A1 by 10-undecynoic and 11-dodecynoic acids (CaJacob et al., 1988), a reaction attributed to acylation of the protein by a ketene metabolite, led us to search for approaches to the formation by ω-2 oxidation of comparable acylating species. The ethyl thioester function satisfies these requirements. Oxidation of the thioester is required to inactivate the enzyme, and inactivation involves protein modification. However, the thioester sulfoxide, the proposed reactive metabolite, appears to diffuse out of the active site before it inactivates the enzyme because (a) inactivation is prevented by glutathione and (b) the radiolabeled agent binds covalently to both the heme and flavin domains. Either the thioester sulfoxide is not sufficiently reactive or the active site of cytochrome $P450_{BM-3}$ does not have an appropriately located nucleophile. TDDA and THDA can therefore not be used as active site probes for cytochrome $P450_{BM-3}$ but are the first examples of a novel approach to the inactivation of cytochrome P450 enzymes.

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