Identification of the Heme-Modified Peptides from Cumene Hydroperoxide-Inactivated Cytochrome P450 3A4[†]

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ABSTRACT: Cumene hydroperoxide-mediated (CuOOH-mediated) inactivation of cytochromes P450 (CYPs) results in destruction of their prosthetic heme to reactive fragments that irreversibly bind to the protein. We have attempted to characterize this process structurally, using purified, ^{14}C-heme labeled, recombinant human liver P450 3A4 as the target of CuOOH-mediated inactivation, and a battery of protein characterization approaches [chemical (CNBr) and proteolytic (lysylendopeptidase-C) digestion, HPLC-peptide mapping, microEdman sequencing, and mass spectrometric analyses]. The heme−peptide adducts isolated after CNBr/lysylendopeptidase-C digestion of the CuOOH-inactivated P450 3A4 pertain to two distinct P450 3A4 active site domains. One of the peptides isolated corresponds to the proximal helix L/Cys-region peptide 429−450 domain and the others to the K-region (peptide 359−386 domain). Although the precise residue(s) targeted remain to be identified, we have narrowed down the region of attack to within a 17 amino acid peptide (429−445) stretch of the 55-amino acid proximal helix L/Cys domain. Furthermore, although the exact structures of the heme-modifying fragments and the nature of the adduction remain to be established conclusively, the incremental masses of ≈ 302 and 314 Da detected by electrospray mass spectrometric analyses of the heme-modified peptides are consistent with a dipyrrolic heme fragment comprised of either pyrrole ring A−D or B−C, a known soluble product of peroxidative heme degradation, as a modifying species.

The superfamily of hepatic microsomal hemoproteins cytochromes P450 (P450s)[1] plays an important role not only in the metabolism of xenobiotics and endobiotics but also in the bioactivation and/or detoxification of carcinogens and other toxic xenobiotics (*1*−*3*). These hemoproteins ($M_r \approx$ 50 kDa) are monomeric, containing one prosthetic heme (iron−protoporphyrin IX) moiety per molecule of enzyme. The heme−iron is coordinated to the thiolate of a cysteine residue, that endows the enzyme with its characteristic oxidative properties. Such coordination also allows the heme to exist in a dynamic equilibrium with the hepatic "free" heme pool.

Although no crystal structure is currently available for the eukaryotic membrane bound P450s, the known crystal structures of the prokaryotic P450s (P450s 101, 102, 108, and 107A1) have provided useful blueprints for homology modeling and/or comparative sequence alignment of the eukaryotic P450s (*4*−*14*). In particular, this combined exercise reveals that the heme-apoP450 active site domains of the membrane-bound P450s closely resemble those of the corresponding prokaryotic P450s. The common active site motif emerging from these analyses is that of a heme moiety well-sequestered within the protein interior and sandwiched by two helices *(proximal L and distal I in P450101)* that are highly conserved through evolution (*4*, *9*, *10*). Accordingly, in analogy to the bacterial P450s, the SH-donating cysteine residue is sequestered in the highly conserved pentadecapeptide region *(Cys-region in P450101)* in the COOH terminus and thus protected from facile oxidation. On the distal side, another helix *(corresponding to helix I in P450101)* containing a highly conserved threonine residue (Thr_{252} in P450 101) appears to contact the heme as well as substrates (*9*, *10*). The identification of this helix as the site of protein modification in CYP2B1 and CYP2B4 by suicide substrates (*15*, *16*) provides independent confirmation of its localization in the active site.

This architectural sequestration of the prosthetic heme protects it from externally generated reactive species (radicals). However, the hemoprotein is known to be inactivated by reactive species generated in the course of its oxidative metabolism of certain compounds (*17*; and references

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[1] Abbreviations: CuOOH, cumene hydroperoxide; CYPs, P450s, cytochromes P450; DDEP, 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine; DPs, dipyrrolic fragments; EDTA, ethylenediaminetetraacetic acid, ESMS; electrospray mass spectrometry; HA, hematinic acid; Lys-C, Lysylendopeptidase C; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MVM, methylvinylmaleimide; PDPs, propentdyopents; TFA, trifluoroacetic acid.

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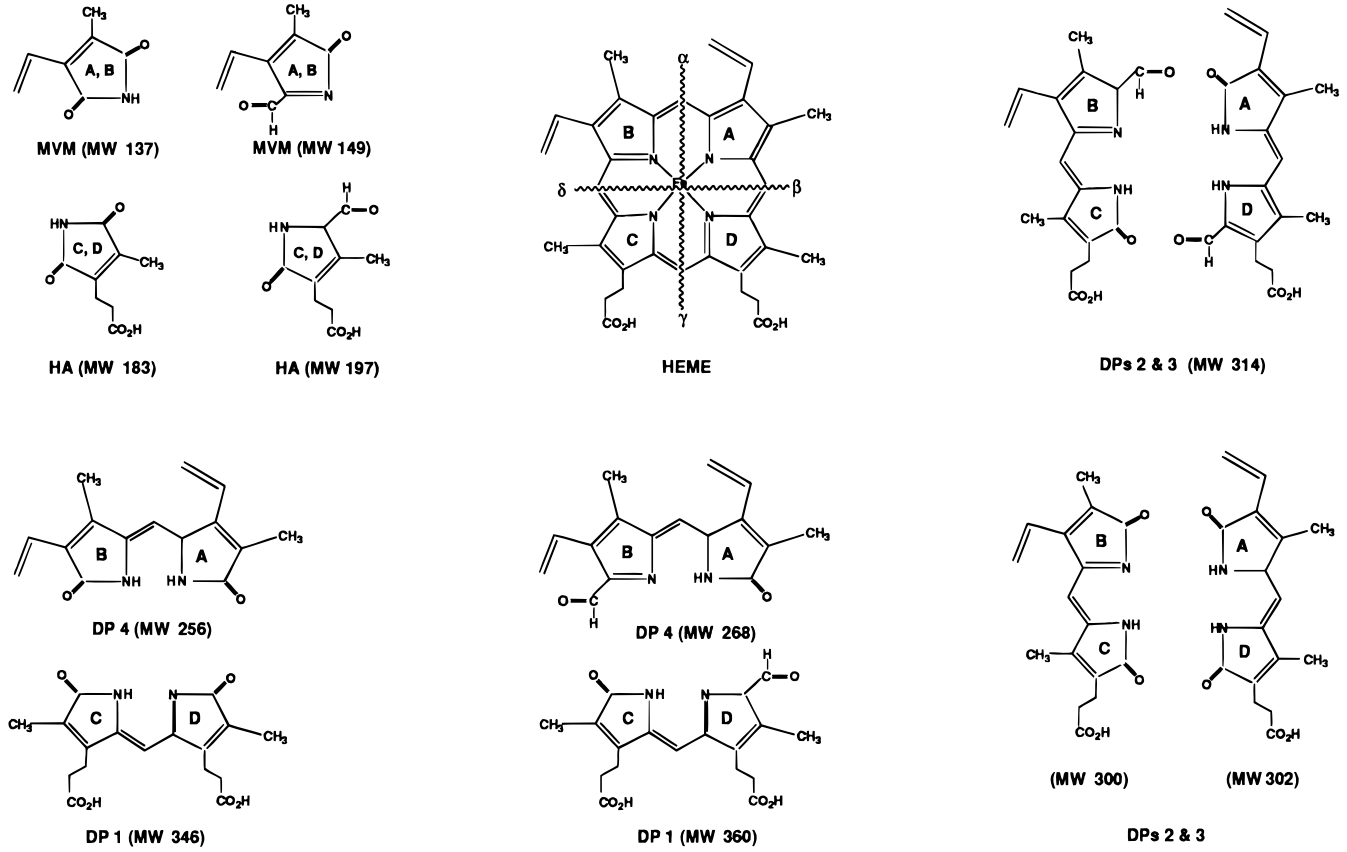

FIGURE 1: Oxidative heme fragmentation: Formation of mono- and dipyrroles. MVM, methylvinylmaleimide; HA, hematinic acid; DPs, dipyrrolic species. The masses (Da) of the deformylated and formylated species are shown.

therein). Such species may modify the heme and/or protein moiety, or even cause the heme to irreversibly modify the protein within the active site, in a process termed "suicide" or mechanism-based inactivation (*17, 18*).

Heme modification of the protein appears to be a common feature of the mechanism-based inactivation of P450s of the CYP3A subfamilies by radical-yielding agents such as 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine (DDEP) and spironolactone (*19*−*21*). This process is characterized by complete rupture of the tetrapyrrolic structure at the meso carbons and hence disruption of the heme chromophore, with concomitant generation of monopyrrolic fragments [hematinic acid (HA) and methylvinylmaleimide (MVM)] and dipyrrolic fragments [DPs; or the hydroxylated DPs, propentdyopents (PDPs)] and possibly other yet uncharacterized reactive species that irreversibly modify the protein (*19*). Accordingly, cleavage of the heme along its $\alpha-\gamma$ axis would yield equimolar amounts of the corresponding ring A−ring D (DP 3) and ring B−ring C (DP 2) dipyrroles, whereas cleavage along the $\beta-\delta$ axis would yield equimolar amounts of the corresponding ring A−ring B (DP 4) and ring C−ring D (DP 1) dipyrroles (Figure 1).

Such P450 heme degradation is also a characteristic feature of H_2O_2- or cumene hydroperoxide-mediated (CuOOH-mediated) inactivation of P450s (*22*−*24*). Indeed, our previous identification of a heme-modified peptide of CuOOH-inactivated 2B1 as a helix L domain, convincingly confines such inactivation to the active site (*25*). However, despite of our attempts, the structural characterization of the heme-modifying species remained elusive. To structurally characterize the modifying heme species, we examined the CuOOH inactivation of CYP3A4, the major human liver P450 that is responsible for the metabolism of over 60% of clinically relevant drugs (*26, 27*). In the present study, we describe the structural characterization of heme-modified peptides isolated from *E. coli*-expressed and purified P450 3A4 after its in vitro inactivation by CuOOH. We also provide some insight into the methodological conditions required for the successful mass spectrometric characterization of these otherwise recalcitrant hydrophobic P450 peptides.

MATERIALS AND METHODS

Materials. Restriction endonucleases, DH5αF′ competent *E. coli* cells and media for bacterial growth were purchased from GIBCO−BRL (Grand Island, NY). δ-[4-^{14}C]Aminolevulinic acid (ALA) hydrochloride was purchased from DuPont NEN (Boston, MA). δ-ALA hydrochloride, Lubrol (poly(oxyethylene 9 lauryl ether)) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Dilauroylphosphatidylcholine was obtained from Doosan Serdary Research Laboratories (Englewood Cliffs, NJ). Isopropyl-β-D-thiogalactopyranoside and sodium cholate (99% pure) were purchased from Calbiochem Corp. (La Jolla, CA). CuOOH was purchased from Aldrich Chemical Co. (Milwaukee, WI) and lysyl endopeptidase C (Lys-C) was obtained from Wako Chemicals USA, Inc. (Richmond, VA).

P450 3A4 Expression in E. coli. A full-length 3A4 cDNA, engineered to code for deletions of the N-terminal residues 3−12 and substitution of residue Ser18 with Phe, and

incorporated into the pCW vector was obtained from Dr. R. Estabrook (SW Medical School, Dallas), and expressed in *E. coli* DH5αF′ cells as previously described (*28*). The bacterial cell membranes containing the expressed P450 3A4 were harvested by centrifugation and solubilized with detergents. P450 3A4 was isolated from these membranes and purified to homogeneity by DEAE-Sephacel column chromatography, followed by detergent exchange by passage through hydroxylapatite. ^{14}C-Heme-labeled P450 3A4 was similarly obtained except that ^{14}C-ALA (0.25 mCi) was added along with cold ALA (4.2 mg), 8 h after the addition of the *tac* promoter isopropyl-β-D-thiogalactopyranoside (1 mM) and every 2 h thereafter, over the next 8 h for a total of five doses. The radiolabeled enzyme, isolated and purified as above, exhibited a specific activity of 44 450 dpm (40 000 cpm)/nmol P450.

CuOOH-Mediated Inactivation of P450 3A4. The purified P450 3A4 (1 nmol of ^{14}C-heme labeled and 4 nmol of unlabeled P450 3A4) was incubated with 0.5 mM CuOOH in 2.5 mL of 50 mM Hepes buffer containing 20% glycerol, 1 mM EDTA, and 2 mM GSH at 37 °C for 15 min. The extent of irreversible ^{14}C-heme binding to P450 3A4 protein was determined after the addition of carrier protein (10 mg of rat liver microsomes or bovine serum albumin), precipitation with 5% H_2SO_4/methanol, and subsequent extensive washing with organic solvents to remove the noncovalently associated radioactivity, as previously detailed (*25*).

Anaerobic Stopped-Flow Analysis. Mixtures of P450 3A4 (3 μM) and CuOOH (0.5−2.0 mM) in Hepes buffer were made anaerobic by several cycles of alternating argon and vacuum exchange for over 2 h in ice. Stopped-flow analysis was performed on a Hi-Tech SF-40 Stopped-Flow spectrophotometer (Salisbury, England) interfaced with a Compaq 386 IBM-compatible PC at room temperature (23 °C). The CuOOH-mediated inactivation of P450 3A4 was determined by the time-dependent decrease in its absolute absorbance at 400 nm, monitored by scanning between 300 and 600 nm.

HPLC Isolation of Heme-Modified P450 Protein. The CuOOH-inactivated P450 3A4 incubation mixture was subjected directly to HPLC on a Poros column (RH1, PerSeptive Biosystems), with a solvent system consisting of 0.1% TFA (A) and 45%/45%/0.1% acetonitrile/2-propanol/TFA (B) and a linear gradient of 30% B to 100% B over 20 min, and subsequently washed with 0.1% TFA at a flow rate of 3 mL/min. The protein and heme were monitored simultaneously at 214 and 400 nm, respectively. The radioactivity was measured by sequential scintillation counting of an aliquot from fractions collected every minute. The elution of P450 protein was monitored by SDS−PAGE analysis.

CNBr Digest of Heme-Modified apoP450 3A4. The ^{14}C-heme labeled P450 fraction (isolated after the Poros-HPLC) was concentrated by rotary evaporation in the presence of 50 μL of glycerol, dissolved in TFA (70%) and digested with CNBr (20 μL, 1 mg/μL in 70% of TFA) in the dark, overnight. The sample was then concentrated to near dryness, washed 3 times with 1 mL of water, solubilized in 0.1% TFA/water, and finally centrifuged at 14 000 rpm for 10 min. More than 70% of the ^{14}C-heme-modified peptide remained in the supernatant. Further digestion of the pellet released additional ^{14}C-heme-derived species into the supernatant, thereby indicating that the radioactivity in the pellet was due to incomplete digestion of the protein. The inclusion of glycerol into the Poros-HPLC fraction containing apoP450 not only improved the recovery of the protein during concentration of the sample but also enabled the solubilization of hydrophobic peptides due to its retention of considerable amounts of TFA.

HPLC Peptide Mapping of the CNBr Digest. The supernatant in 0.1% TFA was subjected to HPLC on a C4 column (Applied Biosystems, Aquapore butyl, 7 μm, 220 × 2.1 mm) using a solvent system consisting of 0.1% TFA (A) and 90%/0.1% acetonitrile/TFA (B) and a stepwise linear gradient of 0% B for 5 min, 0−20% B over 10 min, 20−60% B over 100 min, and 60−100% B over 5 min at a flow rate of 0.6 mL/min. The elution of the peptides was detected at 214 nm, and their radioactivity was monitored by sequential scintillation counting of 2 min fractions. To further purify the sample for mass spectrometric analysis, the CNBr digest was first subjected to a Poros column under conditions similar to those described above under HPLC isolation of the intact heme-modified P450 protein. The major ^{14}C-heme-labeled peak fraction was collected and repurified by HPLC on a C4 column under conditions similar to those described above.

Tricine SDS−PAGE and Amino Acid Sequencing Analyses of the ^{14}C-Heme-Modified Peptide. The ^{14}C-heme-labeled HPLC peptide fraction with retention time of 44−56 min was subjected to tricine SDS−PAGE, electrotransferred onto a polyvinylidene difluoride membrane (ProBlott) which was subsequently autoradiographed. The radiolabeled peptide band on the polyvinylidene difluoride membrane was subjected to amino acid sequencing by microEdman degradation as described previously (*15, 25*).

Lysyl Endopeptidase-C (Lys-C) Subdigestion, HPLC Purification, and Edman Sequencing of the Heme-Modified Peptide. The ^{14}C-heme-labeled HPLC fraction obtained after CNBr digestion was concentrated in the presence of glycerol (20 μL). The peptide was dissolved in 50 mM Tris-HCl (pH 9) buffer with 8 M urea and heated at 60 °C for 30 min. The solution was adjusted to pH 9 with 1 N NaOH and then diluted to 4 M urea with 50 mM Tris-HCl buffer, pH 9. The Lys-C digestion was carried out at 30 °C overnight, using a 1:20 molar ratio of Lys-C:peptide as described (*15*). The digest was subjected to HPLC on a C4 column with the same solvent system as that described above for peptide mapping of the CNBr digest, except that the stepwise gradient was changed to 0% B for 5 min, 0−40% B over 100 min, and 40−100% B over 15 min. The ^{14}C-heme-labeled HPLC fraction so obtained was subjected to microEdman sequencing as described previously (*15, 25*).

Modified Experimental Conditions for Mass Spectrometric Analyses of Heme-Modified Peptides. ^{14}C-heme-labeled P450 (1−2 nmol; 10 000 cpm) was incubated with CuOOH (1 mM), GSH (2 mM), at 37 °C for 15 min as described above. At the end of the incubation, the residual CuOOH was quenched by the addition of sodium bisulfite (5 mM). The incubation mixture was then acidified with 1 N HCl (100 μL) and extracted three times with 2 mL volumes of water-saturated butanone, to remove any adventitious intact heme or heme-derived fragments. The residual butanone was evaporated off under N_2, and TFA was added to the aqueous layer (≈1 mL; qs to 70%), and the solubilized protein was then treated with CNBr (20 mg) and digested overnight at

room temperature in the dark. The digests were dried down under N_2 to near dryness, washed with acetonitrile (50% in water, 400 μL), and redried. This process was repeated twice. The washed concentrated digests were then sequentially dissolved in TFA (25 μL), acetonitrile (50 μL), and water (qs 500 μL). They were then subjected to HPLC on a C4 column at 0.6 mL/min and a solvent system consisting of (A) 0.1% TFA in water and (B) 95%/0.08% acetonitrile/TFA and a linear gradient of 20−100% B over 60 min. Fractions at every 2 min were collected. Most of the ^{14}C-radioactivity eluted in the void peak. The void peak was collected, dried down, and dissolved in TFA (25 μL), acetonitrile (100 μL), and water (qs 500 μL). The radiolabeled peptides were further resolved by HPLC on a Rainin C18-MV column (300 Å), with a solvent linear gradient of 5−100% B over 60 min. Fractions at every 2 min were collected. Most of the radioactivity eluted broadly between 18 and 32 min. This fraction was dried down and when radioquantitated was found to contain 250 pmol of radiolabeled peptides (assuming quantitative retention of ^{14}C-heme radioactivity in the peptides). This fraction was subjected to MALDI-TOF and ESMS analyses.

Because of the myriad of peptides detected in this fraction by mass spectrometric analyses, subsequent experiments involved peptide mapping of CNBr digests using HPLC conditions further modified to improve the resolution of the peptides. For this purpose, the digests (obtained as described above) were subjected to C4-HPLC with a stepwise solvent gradient (flow rate, 0.6 mL/min) consisting of 0%B (for 5 min), 0−60% B (over 55 min), and 60−100% B (over 5 min). Two min fractions were collected. The major radiolabeled peak eluted at 24−36 min, corresponding to fractions 12−18. Two smaller peaks were also detected at ≈40 and ≈48 min, respectively. The major peak was further purified by HPLC on a C18 column with a solvent gradient of 0−100% B over 60 min at a flow rate of 1 mL/min. The major radioactive peak was found in fractions 10−14 eluting between 20 and 28 min, corresponding to 112 pmol (≈560 cpm) of radiolabeled peptides (assuming quantitative retention of ^{14}C-heme equivalents in the peptides). This peak fraction was then subjected to LC-ESMS.

Mass Spectrometric Analysis. The MALDI-TOF mass spectrometric analysis of the ^{14}C-heme-labeled fraction obtained from the C-18 HPLC-peptide mapping of the CNBr digest was carried out on a Voyager DE MALDI reflecting time-of-flight (TOF) mass spectrometer (PerSeptive Biosystems, Framingham, MA), equipped with delayed extraction. Samples were mixed with matrix solution (10 g/L of α-cyano-4-hydroxycinnamic acid in a 50:50 mixture of acetonitrile and 0.2% aqueous TFA) and allowed to air-dry on the target. LC-ESMS analyses were carried out by subjecting the sample to HPLC using a LC-Packings C18 column (0.18 mm × 150 mm; San Francisco, CA) and a linear gradient of solvent A (0.1% formic acid in double distilled water) and solvent B (0.05% formic acid in ethanol: propanol, 5:2, v/v) at a flow rate of 1.0 μL/min. The HPLC was online with a PerSeptive Biosystems Mariner Orthogonal-TOF electrospray ionization (ESI) mass spectrometer, with a nominal resolution of 5000 (fwhh), spray tip potential of approximately 3000 V, and nozzle potential of approximately 75 V. Spectra were acquired by summing for 5 s over the *m*/*z* range 360−2000.

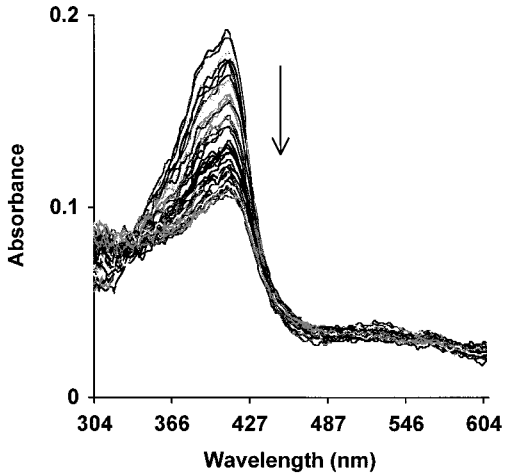

FIGURE 2: Time-dependent stopped-flow spectrophotometric analyses of CuOOH-mediated inactivation of purified P450 3A4. The spectra were obtained after repetitive scanning of the inactivation mixture under anaerobic conditions at 23.5 s intervals over the wavelength range of 300−600 nm over a 940 s period as described (Materials and Methods).

RESULTS AND DISCUSSION

^{14}C-Heme Labeled P450 3A4 Expression in E. coli. The N-terminus (amino acids 3−12) truncated ^{14}C-heme-labeled P450 3A4 was expressed in *E. coli* at a spectrally detectable P450 concentration of 400−600 nmol/L (40 000 cpm/nmol). The P450 3A4 was purified from the detergent solubilized membranes by DEAE-Sephacel column chromatography with a recovery of about 50%.

CuOOH Inactivation of P450 3A4. As in the case of P450 2B1 (*25*), incubation of P450 3A4 with CuOOH resulted in complete loss of the P450 chromophore, although ≈10% of the Soret absorbance of the heme still remained. Approximately, 70% of the ^{14}C-heme was found covalently bound to the P450 protein. Stopped-flow spectrophotometric analysis revealed that the CuOOH-elicited spectral loss of P450 3A4 monitored at 417 nm, followed pseudo-first-order kinetics at 23 °C with a $K_{\text{inactivation}}$ of 0.05 min^{-1} under aerobic conditions and $K_{\text{inactivation}}$ of 0.02 min^{-1} under anaerobic conditions, respectively (Yin, H., He, K., and Correia, M. A., *unpublished observations*). Thus, the inactivation rate was lowered under anaerobic conditions, thereby revealing a role for O_2 in CuOOH-mediated P450 heme destruction. This was also the case when CuOOH was replaced by iodosobenzene (5 mM) as the surrogate oxygen donor, with the inactivation rate (K_{apparent}, 0.002 min^{-1}) under anaerobic conditions slowed to one-third of that (K_{apparent}, 0.006 min^{-1}) in the presence of O_2 (Yin, H., and Correia, M. A., *unpublished observations*). In an effort to trace the chemical trajectory of the CuOOH-mediated heme destruction, the course of the reaction was also monitored by scanning stopped-flow spectrophotometry. A time-dependent loss of heme absorbance at 417 nm was observed, although no spectral intermediates in the range of 350−600 nm were detected (Figure 2). However, a gradual increase in the 300 nm absorbance was found with longer incubations *(not shown)*.

Isolation of ^{14}C-Heme-Modified P450 Protein. The intact heme-modified P450 was completely separated from heme and heme fragments by HPLC (Figure 3). However, it was found to bind very tightly to the HPLC column such that

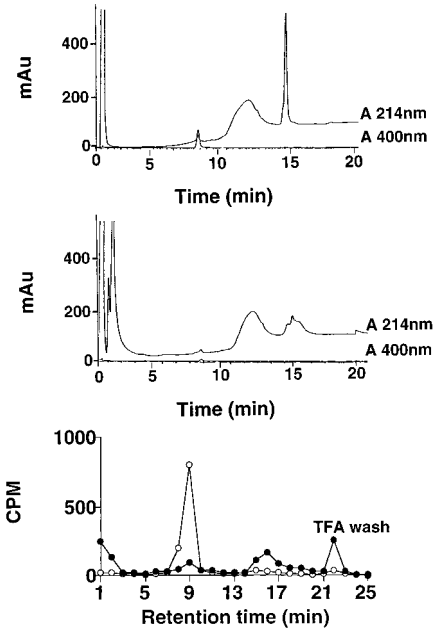

FIGURE 3: Isolation of CuOOH-inactivated ^{14}C-heme labeled P450 3A4. The incubation mixtures containing native and CuOOH-inactivated ^{14}C-heme-labeled P450 3A4 were subjected to HPLC on a hydrophobic Poros column to separate the protein from noncovalently bound heme and/or heme derived products. Top panel, shows the HPLC profile with spectrophotometric monitoring at 400 nm for the intact heme and 214 nm monitoring for the protein of the native P450 3A4. Middle panel depicts the HPLC profile with spectrophotometric monitoring at 400 nm for the intact heme and 214 nm monitoring for the protein of the CuOOH-inactivated P450 3A4. Bottom panel depicts the corresponding radioactivity of the fractions obtained after HPLC of the native (−○−) and CuOOH-inactivated (− ● −) ^{14}C-heme-labeled P450 3A4. When immunoblotted, the only material that cross-immunoreacted with anti-3A IgGs was found in the 14−17 and 21−23 min peak fractions.

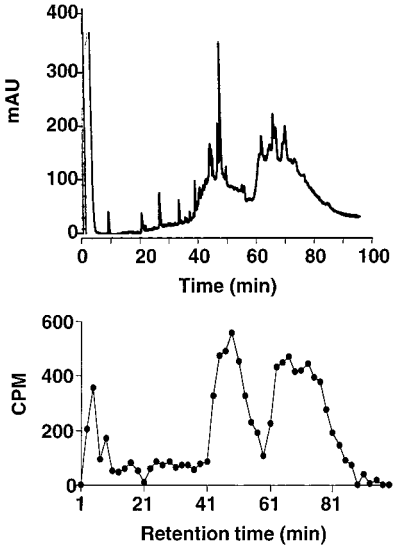

FIGURE 4: C4-HPLC peptide mapping of CNBr digest of CuOOH-inactivated ^{14}C-heme-labeled P450 3A4. The top panel is the HPLC profile monitored at 214 nm over 100 min. The bottom panel depicts the corresponding radioactivity profile determined from liquid scintillation counting of an aliquot of the 2 min fractions.

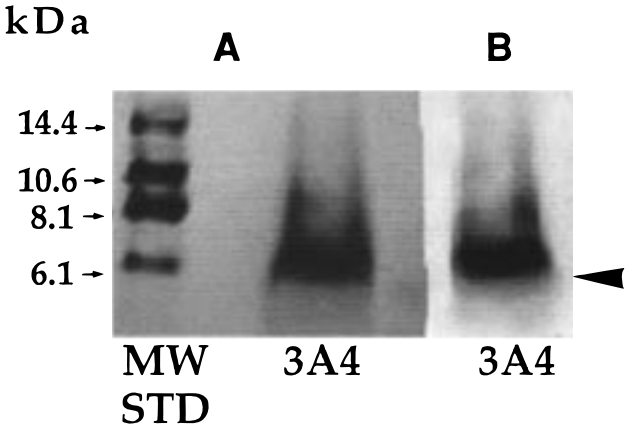

FIGURE 5: Tricine-SDS−PAGE and autoradiography of the 44−56 min peak fraction from C4/HPLC peptide mapping of CNBr digests. An aliquot of the 44−56 min fraction was subjected to Tricine-SDS−PAGE (A) followed by autoradiography (B). The band was also electrotransferred onto ProBlott membrane and subjected to micro-Edman degradation that yielded: I_{396}PSYALHRPKYWTEPEKFLPERFSKKNKDNIDPYIYTPFGSGPRNCIGMRFALM$_{450}$ as the primary sequence. Sequence termini K_{454}LALI$_{457}$ and V_{359}VNET$_{363}$ corresponding to 3A4 peptides 454−502 and 359−428, respectively, were also detected.

100% TFA was required to fully dissociate it. Since native (non-CuOOH-inactivated) P450 could be easily eluted from this column, this implied that the CuOOH inactivation had resulted in the exposure of the hydrophobic groups of the P450 protein. Furthermore, the complete HPLC separation of ^{14}C-heme from the P450 protein of the non-CuOOH-inactivated sample documented that the radioactivity was exclusively associated with the heme moiety (eluting between 7.5 and 9 min). This result not only indicates that the P450 protein is free of radioactivity derived from any ^{14}C-ALA decomposed during the *E. coli* expression but also provides evidence that the covalently bound radioactivity of the CuOOH-inactivated P450 3A4 protein was derived entirely from its ^{14}C-labeled prosthetic heme. Furthermore, the failure of the CuOOH-inactivated P450 fraction to exhibit any 400 nm absorbance revealed that the modifying species was neither the intact heme nor its tetrapyrrolic skeleton (Figure 3).

Isolation and Characterization of the Heme-Modified Peptide after CNBr Cleavage. C4-HPLC peptide mapping of the CNBr digest of the heme modified P450 revealed two major radioactive peptide peaks, with retention times of 44−56 and 60−80 min, respectively (Figure 4). Further CNBr digestion of the peptide fraction with a retention time of 60−80 min resulted in shifting some of its radioactivity to the 44−56 min peak and some to the void peak. This result indicated that the 60−80 min peak contained incompletely digested peptides. The poor HPLC resolution could not be improved by changing the buffer composition or replacing the HPLC column (with a Poros RH1 or a C8 HPLC column). Tricine SDS−PAGE analysis of the heme modified peptide in the 44−56 min HPLC fraction yielded a somewhat smeared radiolabeled peptide band with approximate $M_r \approx 6$ kDa (Figure 5). Edman sequencing[2] of the first five amino acid residues after electrotransfer of this band to Problott membrane yielded two major sequences I_{396} PSYA$_{400}$ and K_{454}LALI$_{457}$, consistent with the presence of

[2] MicroEdman degradation gave the following amino acid yields (pmol) through five sequential cycles of sequencing: I (≈70), P (45), S (35), Y (12), A (39); K (≈31), L (>44), A (44) L (≈40) I (>7); and V (≈ 20), V (nc), N (6.3), E (>6), T (>7); nc, not calculated.

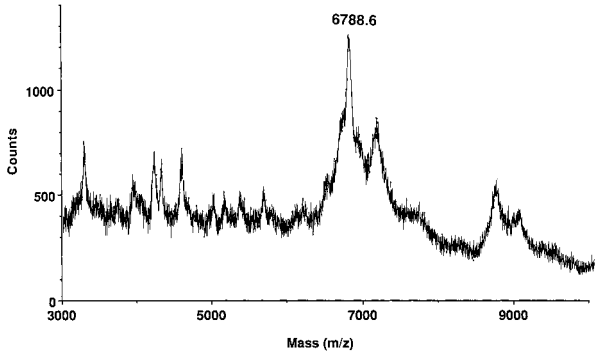

FIGURE 6: MALDI-TOF mass spectrum of the ^{14}C-heme-labeled peptide fraction purified from CNBr digest by HPLC. CNBr digest was first subjected to HPLC on a Poros column. The ^{14}C-heme-labeled fraction eluting between 11 and 12 min (*not shown*) was further purified by HPLC on a C4 column. The major ^{14}C-heme-labeled peptide fraction eluting between 34 and 36 min was analyzed by MALDI-TOF mass spectrometry.

CNBr cleaved P450 3A4 peptides 396−450 and 454−502, respectively, and a minor sequence V_{359}VNET$_{363}$, corresponding to P450 3A4 peptide 359−428. However, the mass spectrometric analyses of this crude 44−56 min fraction were unsuccessful.

In an attempt to increase the chances of successful mass spectrometric characterization of the ^{14}C-heme-labeled peptide fraction, the CNBr digests were first subjected to HPLC peptide mapping on a Poros RH-1 column, with further purification of the relevant fraction eluting between 11 and 12 min, by HPLC on a C4 column *(not shown)*. MALDI-TOF analyses of this purified fraction yielded a prominent peak at *m*/*z* 6788.6 (Figure 6), which we believe corresponds to the 3A4 peptide domain $I_{396}-M_{450}$ [mass 6474.3 (MH^+)], modified by a heme fragment of 314.3 Da. This mass[3] corresponds to a 2-formylated dipyrrolic (DP) species consisting of either ring A-ring D (DP 3) or ring B-ring C (DP 2), that is created by scission of the heme moiety along its $\alpha-\gamma$ axis *(see below)*. The peptide domain modified is identical to one of the major peptides identified by Edman sequencing after electrotransfer of the radiolabeled peptide band isolated by Tricine-PAGE (Figure 5). Although other peaks were detected in this spectrum (Figure 6), the structural identification of the corresponding peptides was not successful.

Lys-C Subdigestion and Identification of the ^{14}C-Heme-Modified Peptide. To facilitate their structural characterization, the CNBr cleaved peptides (in the 44−56 min peak fraction) were subdigested with Lys-C and subsequently separated by HPLC (Figure 7). One major ^{14}C-heme-labeled peak was found with a retention time of ≈100 min. Edman sequencing[4] of the first 10 amino acid residues of this HPLC fraction yielded three major peptides, V_{359}-VNETLRLFP$_{368}$, R_{372}LERV(C)(K)$_{378}$, and D_{425}NIDPYIYTP$_{434}$. Peptide sequence $D_{425}-P_{434}$ is a subset of ^{14}C-heme-modified

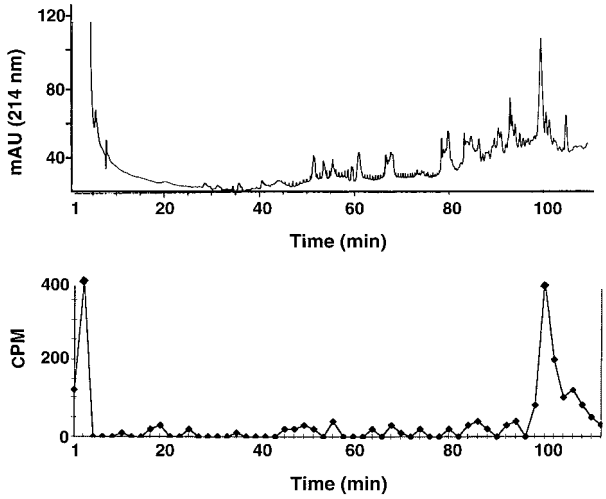

FIGURE 7: HPLC-peptide mapping of the Lys-C subdigests of the 44−56 min peptide fraction from CNBr-cleaved P450 3A4. The fraction eluting at 44−56 min after peptide mapping of CNBR digests of ^{14}C-heme-labeled P450 3A4 was subdigested with Lys-C (Materials and Methods) and resubjected to HPLC−peptide mapping on a C4 column. The HPLC profile of the peptides monitored at 214 nm is shown in the top panel and the corresponding radioactivity determined by scintillation counting of aliquots is shown in the bottom panel.

peptide $I_{396}-M_{450}$, identified by Edman sequencing of the 44−56 min peak peptide fraction from the CNBr digests (Figure 5), after Tricine SDS−PAGE and electrotransfer to Problott membrane and confirmed by MALDI-TOF analyses (Figure 6).

Comparative sequence alignment of this heme-modified peptide ($D_{425}-M_{450}$) with the sequences of P450s 101, 102, 108, and 107A1 with known crystal structures indicates that it aligns with the domain corresponding to their proximal L helix/Cys region. This domain contains the conserved cysteine (Cys_{442} in P450 3A4) that provides the essential SH ligand for the heme iron and thus is a very plausible candidate for heme modification. These results are consistent with our previous finding that this peptide was also identified as a site of heme modification of CuOOH-inactivated P450 2B1 (*25*). Our attempts to structurally characterize the modified peptides in the entire 100 min Lys-C fraction by mass spectrometric analyses were however unsuccessful.

Characterization of the Heme-Modified Peptides. Successful mass spectrometric analyses required modification of the above approaches. The goal was (i) to improve the recovery of the heme-modified P450 3A4 protein, (ii) to minimize extraneous reactions due to residual CuOOH, and (iii) to increase the relative abundance of heme-modified P450 peptides, by circumventing the natural tendency of these hydrophobic peptides to aggregate and become insoluble. The radiolabeled peak fraction eluting between 18 and 32 min (*not shown*) after HPLC on a C18-MV column (Modified Procedure, Methods) was subjected to MALDI-TOF mass spectrometric analysis. Masses for a myriad of peptides were detected (*not shown*). MALDI/Post source decay structural analyses revealed that some of these peptides were glycerolated (addition of 74 Da to acidic residues and/or terminal carboxyl group of the peptide, and/or conceivably to the propionic acid moiety of the heme pyrrole ring C or D), an artifact introduced by the high glycerol concentrations attained on prolonged evaporation of the samples in acidic

[3] The masses of the unesterified deformylated and 2-formylated DPs 2 and 3, respectively (Figure 1), were estimated from the reported masses of the corresponding esterified PDP products determined by CI (CH_4) mass spectrometry.

[4] MicroEdman degradation gave the following amino acid yields (pmol) through 10 sequential cycles of sequencing: V (30), V (nc), N (15), E (11), T (≈ 7), L (13.9), R (9), L (14), F (6), P (14); D (≈10), N (14), I (31), D (9), P (10), Y (8.1), I (9), Y (>6), T (5.3); and R (50), L (≈26), E (>9), R (18), V (nc); nc, not calculated.

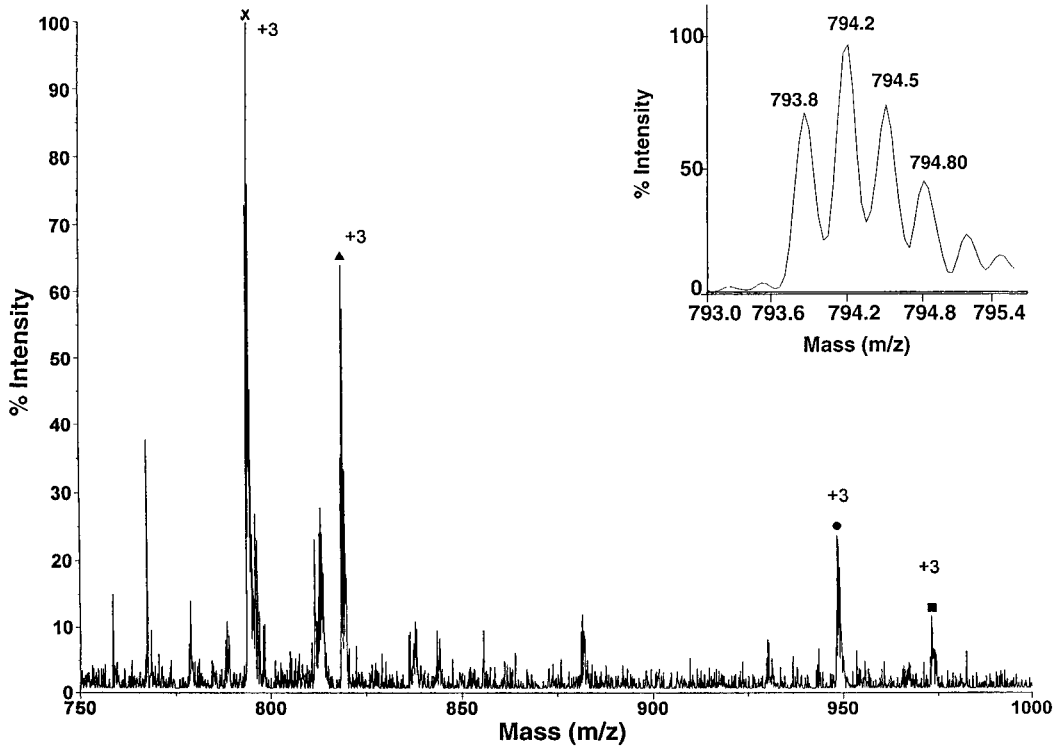

FIGURE 8: LC-ESMS analyses of CuOOH-inactivated 3A4 after HPLC peptide mapping using the modified protocol. For experimental details see Materials and Methods. Peaks marked ×, ▲, ●, and ■ are 3+ ions corresponding to species with neutral MW of 2378.4, 2452.5, 2841.6, and 2915.6, respectively (see Table 1). The isotopic peak spacing of the triply charged ion peak detected between *m*/*z* 793 and 795.5 which yielded the monoisotopic mass of 2378.4 Da is also shown (inset).

conditions under N_2 (Medzihradszky, and Burlingame, *personal communication*).[5] In the case of at the least one peptide (I_{396}PSYALD), the glycerolation was confirmed by high-resolution elemental composition analyses. Furthermore, CNBr digestion under the conditions employed not only generated peptide species containing homoserine (m) and homoserine lactone (m′) at the C-terminus, but also apparently resulted in cleavage at the Asp-Pro linkage of the peptide backbone, a site known to be cleaved at low pH.[6]

These combined features together with the detection of heme modified species containing the equivalent of one or two pyrrole rings (corresponding to any one of the possible 12 different chemical structures) largely contributed not only to the broadness of their HPLC elution profiles, but also to the multiplicity of masses detected and the consequent complexity of structural analyses. Nevertheless, the prominent peaks detected in the MALDI-TOF spectra are consistent with the presence of three predominant peptides (V_{359}VNETLRLFPIAm/m′$_{371}$, R_{372}LERVCKKDVEINGm-FIPKGWVVm′$_{395}$, and P_{429}YIYTPFGSGPRNCIGm/m′$_{445}$, not only variously modified by one or more heme monopyrrolic and/or dipyrrolic fragments but also frequently glycerolated (*not shown*). We found it noteworthy, however, that these peptides contain the same sequence stretches as those *(see above)* identified by Edman microsequencing of HPLC fractions from Lys-C/CNBr subdigests of heme modified 3A4. However the low mass accuracy (±5 Da) of these MALDI-TOF analyses led us to seek a more accurate alternative.

To gain more conclusive evidence, CNBr digests were subjected to sequential C4/C18 HPLC conditions further modified to improve the resolution of the peptides (Materials and Methods). The major radiolabeled fraction eluting between 20 and 28 min from the C18 column (*not shown*), was subjected to LC-ESMS. Several prominent peaks were detected in the mass spectra (Figure 8, Table 1). Two major species (mass 2378.4 and 2452.5 Da) were detected, which we believe correspond to the peptide fragment E_{354}-YLDmVVNETLRLFPIAm′$_{371}$ (mass 2076.1 Da), modified by a heme fragment with the mass of 302.5 Da, corresponding to either deformylated dipyrrolic DP 2 or 3 and its glycerolated (+74 Da) species, respectively. ESMS analyses also revealed a species at mass 2056.8 Da which is consistent with the heme-modified peptide being R_{372}LERVCKKD-VEINGm′$_{386}$ (mass 1741.9 Da) with a modifying species of mass 314.9 Da (Table 1).

A species of mass 2841.6 Da, corresponding to the heme-modified and end glycerolated P_{429}YIYTPFGSGPRNCIGm$_{445}$-RFALm′$_{450}$ peptide fragment (mass 2452.5 Da) was also detected. After subtraction of the glycerol moiety (74 Da), it yielded a heme fragment with a mass of 314.1 Da, corresponding to the 2-formylated DP 3 or DP 2. A species of mass 2915.6 Da, corresponding to the additionally glycerolated species of the heme modified peptide (mass 2841.6 Da) was also detected in this mixture, indicating that in addition to the terminal COOH of the peptide, the propionic acid moiety of the heme fragment also is possibly

[5] Using model peptides containing one to five acidic amino acid residues of known masses, we have confirmed by mass spectrometric analyses that glycerolation of all of these acidic groups occurs during evaporation under N_2 under acidic but not neutral conditions, and in the presence of as little as 5 μL of glycerol.

[6] We believe that the exhaustive conditions used in the CNBr digestion result in cleavage at sites additional to the canonical Met residue. It also appears that internal homoserine residues are detected due to incomplete cleavage of corresponding methionine residues (*44*, *45*).

Table 1: LC-ES Mass Spectrometric Analyses of CNBr Digests of CuOOH-Inactivated P450 3A4

3A4 peptide	theor. mass (MW)	mass found[a] (MW)	glycerol	modifying heme fragment (ΔMW)
E_{354}YLDmVVNET···Am′$_{371}$	2076.1	2378.4		302.3
E_{354}YLDmVVNET···Am′$_{371}$	2076.1	2452.5[b]	74	302.4
R_{372}LER···Gm′$_{386}$	1741.9	2056.8		314.9
P_{429}YIY···Gm$_{445}$	1863.1	2134.2	74	197.1
P_{429}YIY···Gm$_{445}$RFALm′$_{450}$	2452.5	2841.6[c]	74	314.1
P_{429}YIY···Gm$_{445}$RFALm′$_{450}$	2452.5	2915.6	74, 74	314.1

[a] These monoisotopic masses (MW, Da) were calculated on the basis of the triply charged ion peaks in the mass spectra. The charge state of each species was determined from the isotope peak spacing. [b] It is conceivable that this mass corresponds to the unmodified peptide P_{429}YIY-Lm′$_{450}$. [c] A mass of 2841.6 Da consistent with a glycerolated and heme modified P_{429}YIY-Lm′$_{450}$, and masses corresponding to the glycerolated and/or heme-modified peptides V_{359}VNET...Am$_{371}$, and R_{372}LER....VVm′$_{395}$ were also detected by MALDI-TOF MS analyses. However, the mass accuracy of ±5 Da prevented unambiguous interpretation of the data.

glycerolated. We found it noteworthy that a peak at the same mass was also detected by MALDI-TOF analyses of the crude mixture *(not shown)*. A species of mass 2134.2 Da was also detected in this peptide fraction, which we believe corresponds to the glycerolated peptide fragment P_{429}-YIYTPFGSGPRNCIGm$_{445}$ (mass 1863 + 74 Da), modified by a monopyrrolic fragment (2-formyl hematinic acid, mass 197 Da), derived from either ring C or D (Table 1).

Collectively, these findings using different approaches to processing and HPLC peptide mapping of the CuOOH-inactivated P450 (Materials and Methods) have once again identified all of the three initially identified peptides as the targets of CuOOH-induced heme modification of 3A4 and have narrowed down the region of modification to: P_{429}-YIYTPFGSGPRNCIGm$_{445}$RFALm′$_{450}$, E_{354}YLDmVVNETL-RLFPIAm′$_{371}$, and R_{372}LERVCKKDVEINGm′$_{386}$. As discussed earlier, the identification of peptide P_{429}−M_{450} corresponding to P450 3A4 helix L/Cys region, is not surprising, considering its strategic location and its role in providing the essential thiolate (Cys_{442}) for heme ligation, a feature that confers structural viability and catalytic reactivity on the enzyme. Residues 434−437, 440−444, and 447−448 are within 4−5 Å of the heme (Szklarz, G., *personal communication*) and thus plausible targets. The identification of E_{354}YLDmVVNETLRLFPIAm′$_{371}$ as a target peptide is equally plausible given that this segment represents the K-region in P450 sequence alignments (*9, 10*). A recently built homology model of P450 3A4 places some of the K-region residues in the active site (*14*), with residues 364, 369, and 370 within 5 Å of the heme (Szklarz, G., *personal communication)*, and thus within striking distance of heme fragments. Furthermore, site-directed mutation of Ile_{369} to Val results in dramatically reduced progesterone 16α-hydroxylase activities (*30*), as well as aflatoxin 3α-hydroxylase and exo-8,9 epoxidase activities (*31*), thereby confirming its strategic position in the active site of the enzyme. Similarly, the identification of R_{372}LERVCKKDVEINGm′$_{386}$ is not surprising given that residues 372, 373, and 375 appear to be within 5 Å of the heme moiety (Szklarz, G., *personal communication*) and thus plausible targets. Although, the precise residues modified in these peptides remain to be identified, the structural characterization of these peptides as active site domains attests to the fact that the CuOOH-induced heme modification is an active site event, which meets several of the criteria for a classical suicide inactivation. In theory, active site residues of helix I which are in close proximity to the heme would also be likely targets. Unfortunately, CNBr cleavage of this domain results in peptides of >4.7 kDa, which also being highly hydrophobic are not amenable to mass spectrometric analyses, without further size reduction.

CuOOH-Mediated P450 Heme Destruction: Mechanistic Implications. As discussed earlier, destruction of P450 heme by peroxides (CuOOH or H_2O_2) or by radical yielding agents (DDEP, spironolactone or CCl_4) is known to result predominantly in irreversible heme modification of the protein, with a minor fraction of the heme converted to polar monopyrrolic [HA and MVM] and hydroxylated dipyrrolic fragments [propentdyopents (PDPs)] (*19*−*24*). This result indicates that the heme is ruptured at each of the four methene bridges, but unlike the heme oxidation catalyzed by heme oxygenase, no biliverdin IXα and CO are detected as major products (*32*−*35*). On the other hand, studies with model heme degradation systems[7] wherein hemin (free or as methemalbumin) was incubated with either exogenous H_2O_2 or NADPH and NADPH-P450 reductase (which generate H_2O_2 in situ), reveal that PDPs along with HCOOH are the major products (*22*−*24, 32*−*37*). When ^{14}C-heme specifically labeled at the four meso carbons is used, H^{14}COOH recovered from the reaction is found to exceed the yield expected from the oxidation of just one meso-carbon, and together with the minor amounts of ^{14}CO and $^{14}CO_2$ concomitantly formed, accounted for nearly the yield expected from the oxidation of two meso carbons (*33, 34*). Together, these findings reveal that during peroxidative degradation of hemin, the tetrapyrrolic skeleton is ruptured at each of the meso carbons to yield the monopyrroles (HA, MVM) and/or along its α−γ or β−δ axis to yield the dipyrrolic fragments PDPs along with HCOOH (*22*−*24, 33*−*37*).

Unlike the findings in model heme systems, the fraction of the P450 heme converted to HA, MVM, and PDPs has been estimated to account for only 15−20% of the heme lost in purified P450/P450 reductase/NADPH incubations (*23, 24*). However, we have found that this can be as low as 2.5% in P450 3A-enriched rat liver microsomes incubated with DDEP/NADPH or CuOOH/NaN_3 (*19*). Furthermore, this small fraction is predominantly comprised of HA, with traces of MVM and little or no PDPs (*19*). When PDPs were appreciably detected in such microsomal systems, they were largely, if not solely, comprised of PDP 2 and/or PDP 3 (comprised of rings B and C and/or A and D of heme) (*23*). It could be argued on these grounds that either heme is only susceptible to cleavage along its α−γ axis or that if it is cleaved along its β−δ axis, then the corresponding

[7] Chemical studies of reactions with model meso-substituted tetraarylmetalloporphyrins and *tert*-butyl hydroperoxide (*t*-BuOOH) reveal the formation of the corresponding isoporphyrins wherein *tert*-butyl hydroperoxy moiety is bound to a meso-substituted carbon, whereas reactions of the ferric tetraphenylporphyrin with *t*-BuOOH apparently yields the corresponding ferric *tert*-butyl hydroperoxy complex (Fe^{3+}−OO−*t*-Bu) (*41*; and references cited therein). It has been suggested that the latter species might in fact be the precursor of the isoporphyrin (*41*). If such species are formed during CuOOH-mediated P450 inactivation, they appear to be too transient to be detected.

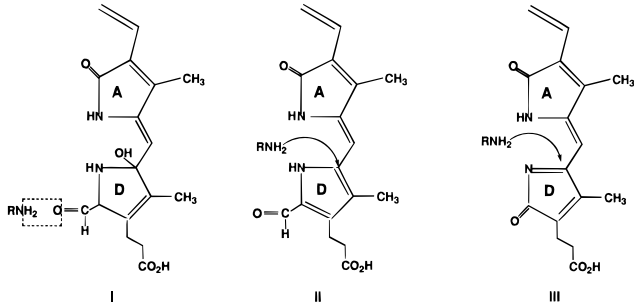

FIGURE 9: Possible mechanisms for DP-protein adduct formation. These include (I) the formation of a Schiff base between a hydroxy DP 2 or 3 and an amino group of a peptide residue or (II) the direct attack of a peptide nucleophilic residue on 2-formyl DP 2 or 3, either of which could account for the mass of 314 Da detected for the heme-modifying species. The corresponding formation of a peptide adduct of a heme-modifying species (DP 2 or 3) with the detected mass of 302 Da is also shown (III).

dipyrrolic products (PDP 4 and PDP 1) are irreversibly trapped by the protein and thus never detected.

Although the heme tetrapyrrolic skeleton is known to be ruptured beyond spectroscopic recognition during CuOOH-mediated P450 inactivation, the nature of the heme fragments (monopyrrolic and/or dipyrrolic?) that irreversibly modify the protein remained however to be elucidated. Our identification of radiolabeled heme-modified peptides from CuOOH-inactivated P450 3A4 with masses of 302 and 314 Da for the modifying species implicate the generation of deformylated and formylated dipyrrolic species DPs 2 and/or 3, respectively. Furthermore, the detection of a mass of 197 for a heme species modifying the same peptide P_{429}-m_{445} would not only argue for the existence of a monopyrrolic species, 2-formyl HA, derived from further meso carbon rupture of DP 2 or 3, but would also imply that the HA moiety is involved in peptide adduction (Figure 9).

Although not specifically detected in our studies, it is also quite conceivable that protein adducts of monopyrrolic MVMs and DPs 1 and 4 are also generated. The formation of GSH and mercaptoethanol adducts of MVM has been reported (*38*), and at the least one of the peptides (I_{396}−M_{450}) that we identified contains a Cys residue. The verification of this possibility and others awaits isolation of additional peptides, from the I-helix domain for instance, after further size reduction by proteolytic digestion.

The elucidation of the precise chemical nature of the adduct requires additional structural characterization. In principle, the formyl group generated from the cleavage of a heme methene bridge could form a Schiff base[8] with the ϵ-NH_2 of a vicinal Lys or a NH_2 donor residue, to yield an imine adduct of the dipyrrole and the protein (Figure 9). Since the formyl O would be lost during such Schiff base formation, the retention of 16 Da in the 314 heme fragment mass argues that if this occurs, the resulting species is a hydroxylated DP (PDP), a species previously reported as a product of peroxidative P450 degradation (*23*, *35*−*37*). Alternatively, an adduct could be generated through the attack of an amino group (or another suitable nucleophilic moiety) of a vicinal amino acid residue, much the same way that a PDP, a hydroxylated DP, is engendered (Figure 9). Finally, although to our knowledge, no DP modified at its vinyl substituent has been detected during peroxidative heme degradation, the formation of such protein adducts is conceivable should a protein radical be generated during this process. In any event, the resulting structure of the 2-formyl DP-protein adduct thus would retain at the least two meso carbons: one intact (bridging the two pyrrole rings) and the other in the form of a formyl/imine group. This proposal is consistent with our previous findings of no significant differences between the levels of irreversible heme−protein adducts observed in DDEP- or CuOOH-inactivated P450 3A-enriched rat liver microsomes containing heme radiolabeled with either 4-^{14}C-ALA (which labels carbons other than the heme meso carbons) or 5-^{14}C-ALA (which specifically labels the meso carbons) (*19*). However, we have also detected DP masses of 302 Da, corresponding to deformylated DP 2 or 3, that retain only one meso carbon.

Although the cleavage of P450 heme to products that irreversibly modify its protein is known to entail metabolic activation of the substrate/oxidant to radical products, either through one-electron oxidations, as in the case of DDEP and spironolactone, or through homolytic cleavage of the hydroperoxides (H_2O_2, CuOOH), the precise underlying mechanism involved in such an oxidative cleavage remains to be elucidated. Our finding that CuOOH-mediated P450 destruction is inhibited under anaerobic conditions[9] provides evidence that molecular O_2 plays a critical role in this destruction. While it is understandable that exclusion of molecular O_2 would impair normal P450-dependent one electron oxidations of drugs to radical products, it should not impair the P450-dependent heterolytic/homolytic cleavage of hydroperoxides. The requirement for molecular O_2, thus reveals that a peroxyl radical might be involved in the cleavage of prosthetic heme to products that irreversibly modify the P450 protein. Although the known inhibition of the NADPH/NADPH-P450 reductase mediated degradation of hemin by catalase confirms an intermediary role for H_2O_2 in this process (*33*−*35*), the corresponding ineffectiveness of superoxide dismutase ruled out a role for the superoxide anion. We have shown that neither catalase nor superoxide dismutase affects DDEP-dependent destruction of rat liver microsomal P450 3A, thereby excluding a role for external peroxide or superoxide anion in this process (*19*).

In conclusion, CuOOH-mediated inactivation of P450 3A4, results in irreversible binding of several prosthetic heme fragments to the protein at the active site, unlike the peroxidative inactivation of hemoproteins such as myoglobin and hemoglobin (*39*−*41*) or the reductive $CBrCl_3$-induced inactivation of myoglobin (*42*, *43*), wherein the protein is modified by the intact prosthetic heme moiety. The heme adducts isolated after CNBr/Lys C digestion of P450 3A4 pertain to two distinct active site domains. One of the peptides isolated corresponds to the proximal helix L/Cys-region peptide 425−450 domain and the other to the K-region. Although the precise residue targeted, the exact structures of the heme-modifying fragments and the nature

[8] The chemical instability of Schiff base adducts under the alkaline conditions (pH ≈ 9.0) of Lys-C digestion might account for our inability to detect heme-modified Lys-C peptides by mass spectrometric analyses.

[9] It is to be noted that rigorous exclusion of O_2 in the system by prolonged (>2 h in ice) alternate cycles of degassing/evacuation and argon is required, given that even traces of O_2 are sufficient for P450 heme destruction.

of the adduction remain to be definitively established, the masses of 302 and 314 Da detected in our studies suggest that DP 2 or 3 might be a modifying species.

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