Cytochrome P450 (P450, CYP) 4A11 is a human fatty acid ω-hydroxylase that catalyzes the oxidation of arachidonic acid to the eicosanoid 20-hydroxyeicosatetraenoic acid (20-HETE), which plays important roles in regulating blood pressure regulation. Variants of P450 4A11 have been associated with high blood pressure and resistance to anti-hypertensive drugs, and 20-HETE has both pro- and antihypertensive properties relating to increased vasoconstriction and natriuresis, respectively. These physiological activities are likely influenced by the redox environment, but the mechanisms are unclear. Here, we found that reducing agents (e.g. dithiothreitol and tris(2-carboxyethyl)phosphine) strongly enhanced the catalytic activity of P450 4A11, but not of 10 other human P450s tested. Conversely, added H₂O₂ attenuated P450 4A11 catalytic activity. Catalytic roles of five of the potentially eight implicated Cys residues of P450 4A11 were eliminated by site-directed mutagenesis. Using an isotope-coded dimedone/iodomidedone-labeling strategy and mass spectrometry of peptides, we demonstrated that the heme–thiolate cysteine (Cys-457) is selectively sulfenylated in an H₂O₂ concentration-dependent manner. This sulfenylation could be reversed by reducing agents, including dithiothreitol and dithionite. Of note, we observed heme ligand cysteine sulfenylation of P450 4A11 ex vivo in kidneys and livers derived from CYP4A11 transgenic mice. We also detected sulfenylation of murine P450 4a12 and 4b1 heme peptides in kidneys. To our knowledge, reversible oxidation of the heme thiolate has not previously been observed in P450s and may have relevance for 20-HETE–mediated functions.

Human cytochrome P450 (P450 or CYP)⁴ enzymes catalyze oxidations of numerous endogenous substrates, including eicosanoids, steroids, and vitamins (1). At least 13 human P450 enzymes (2C8, 2C9, 2J2, 2U1, 4A11, 4F22, 4F12, 4V2, 4F2, 4F3, 4F8, 5A1, and 8A1) utilize fatty acids and/or eicosanoids as substrates (2). P450 4A11 is primarily an ω-hydroxylase, with its main endogenous role apparently being the conversion of arachidonic acid to 20-hydroxyeicosatetraenoic acid (20-HETE) (3). P450 4A11 can also catalyze oxidations of other fatty acid substrates, including lauric acid (4).

\[
\text{CH}_3\text{(CH}_2)_n\text{CO}_2\text{H} \rightarrow \text{HOCH}_2\text{(CH}_2)_n\text{CO}_2\text{H}
\]

Reaction 1

P450 4A11 is expressed mainly in the liver, kidney, and vasculature (5, 6), and subfamily 4A P450 enzymes have been associated with vascular dysfunction and hypertension in rodent models and humans (7). The P450 4A11 F434S variant has been associated with increased blood pressure in several populations studied (8–13). 20-HETE plays opposing roles, having both pro- and antihypertensive actions by promoting vasoconstriction or natriuresis, respectively (14). 20-HETE has been reported to induce transcription of NADPH oxidase, activating this enzyme complex through protein kinase C (15–18), and has also been reported to increase mitochondrial reactive oxygen species (ROS) production (19). Studies with P450 4A11 transgenic mice have identified P450 4A11 regulation via fasting and growth hormone (20) and shown that 20-HETE and P450 4A11 activity may uncouple the renal renin angiotensin system (21).

⁴The abbreviations used are: P450 (or CYP), cytochrome P450; b₅, cytochrome b₅; DLPC, L-α,1,2-dilauroyl-sn-glycerol-3-phosphocholine; HCD, higher-energy collisional dissociation; HEPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; 20-HETE, 20-hydroxyeicosatetraenoic acid; ICAT, isotope-coded affinity tagging; ICID, isotope-coded dimedone/iodomidedone; ROS, reactive oxygen species; TCEP, tris(2-carboxyethyl)phosphine; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-[hydroxymethyl]propane-1,3-diol.

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4 The abbreviations used are: P450 (or CYP), cytochrome P450; b₅, cytochrome b₅; DLPC, L-α,1,2-dilauroyl-sn-glycerol-3-phosphocholine; HCD, higher-energy collisional dissociation; HEPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; 20-HETE, 20-hydroxyeicosatetraenoic acid; ICAT, isotope-coded affinity tagging; ICID, isotope-coded dimedone/iodomidedone; ROS, reactive oxygen species; TCEP, tris(2-carboxyethyl)phosphine; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-[hydroxymethyl]propane-1,3-diol.
ROS, specifically H₂O₂, have recently become regarded as important secondary signaling molecules (22). H₂O₂ modifies epidermal growth factor receptor signaling (23) and regulates glycolysis through glyceraldehyde-3-phosphate dehydrogenase (24). Common targets of H₂O₂ are protein thiols, which it oxidizes to more reactive species, mainly sulfenic acids (-SOH). This transient posttranslational modification aids in the formation of inter- or intramolecular disulfide bonds, alters cellular signaling, and regulates enzymatic activity (25, 26).

In this study, we noted the stimulation of P450 4A11 activity by thiols and other reducing agents and further examined the ability of P450 4A11 to respond to perturbations in the redox environment in vitro. Ten other recombinant human P450s were tested for sensitivity to reductants, and no large changes in activity were noted. Five of the eight possible cysteine (to serine) mutants of P450 4A11 were expressed and were found to be catalytically active, reducing the possibilities for the sensitive thiol.

We observed that P450 4A11 is sensitive to oxidation and, interestingly, sulfenic acid formation was able to disrupt binding of Cys-457 to the heme iron and inactivate the enzyme, in a unique among human P450 enzymes examined here.

Stimulation of P450 4A11 ω-hydroxylation activity by reducing agents

In previous work, the rates of ω-hydroxylation of lauric acid varied considerably, from 9 to 21 min⁻¹, under varying conditions (4). When P450 4A11 was preincubated with either DTT or tris(2-carboxyethyl)phosphine (TCEP) reagents in our preliminary experiments (Fig. 1), ω-hydroxylation activity was routinely increased 2–4-fold. Similar results were found with reduced glutathione (results not shown). The ratio of the two major products, 11- and 12-hydroxylauric acid, remained constant with this activation (results not shown). These results indicated that P450 4A11 has a thiol-dependent redox sensitivity, which was investigated further. Redox-dependent activation was also reflected in the increase in \( k_{cat} \) values for both the wild-type and F434S (rs1126742) (8) variants of P450 4A11 (21-fold) (Fig. 2, A and B).

Figure 1. Stimulation of lauric acid ω-hydroxylation activity by DTT and TCEP. P450 4A11 was preincubated with varying concentrations of either DTT (4) or TCEP (B), and rates of lauric acid ω-hydroxylation were measured. Error bars, S.D.

Figure 2. Stimulation of lauric acid ω-hydroxylation activity by DTT. A, wild-type P450 4A11 (CYP4A11*1); B, F434S variant (rs1126742).

DTT remained constant between the wild-type enzyme (63 ± 10 and 62 ± 15 µM, respectively) and the F434S variant (67 ± 7 and 58 ± 10 µM, respectively) (Fig. 2, A and B). Collectively, these results indicate that both wild-type and F434S variant P450 4A11 are activated in a reducing environment, which may have biological relevance in vivo.

Ten other human P450 enzymes were tested for thiol-dependent activation with their relevant substrates (Table 1). The highest stimulation observed among these was P450 2C9, with only a 33% increase. The redox sensitivity of P450 4A11 was unique among human P450 enzymes examined here.

Oxidative inhibition of P450 4A11

P450 4A11 hydroxylation activity was inhibited by H₂O₂. This inhibition occurred in a time-dependent manner with an 80% loss in activity occurring within 15 min (Fig. 3), a rate much higher than reported for uncatalyzed sulfenylation of thiols (27). Fifty percent activity loss occurred at a concentration of 140 µM H₂O₂ (Fig. 4).

Site-directed mutagenesis

P450 4A11 encodes eight cysteinyl residues in the translated protein, and five of these cysteines were mutated to serines. Cys-457 corresponds to the proximal heme ligand and could not be changed (28), and neither C347S nor C375S produced protein with the typical P450 difference spectrum (29). Steady-state kinetic assays were done with the remaining five cysteine mutants, both with and without DTT pretreatment (Fig. 5). The response to DTT was qualitatively the same as that of the wild-type P450 4A11 (Fig. 2A), except for C200S (Fig. 5C) and possibly C513S (Fig. 5, A and E), but these analyses did not implicate any of these five cysteines as being involved in the
modulation of hydroxylation activity observed in the wild-type enzyme. The major conclusion was that none of these five cysteines are critical to the normal activity of P450 4A11, leaving only the non-mutated Cys-347, Cys-375, and Cys-457 as candidates for the oxidation-reduction phenomenon.

Identification of a sulfenic acid

A portion of oxidized P450 4A11 treated with each of several different concentrations of H₂O₂ was treated with d₆-dime-done (co-incubation) and subsequently counter-alkylated with (d₆¢)-iodoiodomédone (30) (Fig. 6). The alkylated protein was digested with trypsin and analyzed by LC-MS/MS (Fig. 7); integrated areas of extracted ion chromatograms for the heavy (sulfenic acid-modified) and light (free thiol) were compared using the program Skyline (31). Sulfenylation of the heme–thiolate ligand Cys-457 coincided with the attenuation of enzymatic activity observed with increasing concentrations of H₂O₂ (Fig. 8 and supplemental Figs. S1 and S2). Oxidation of the heme–thiolate ligand would lead to an inability of the heme iron to effectively activate oxygen and carry out catalysis.

This sulfenylation, however, did not exclude the possibility for thiol oxidation to occur in the form of intra- or intermolecular disulfide bonds for other P450 4A11 thiols. An isotope-coded affinity tagging (ICAT) approach was developed using d₅/d₆¢-iodoacetanilide (32, 33) (Fig. 6). Briefly, samples were treated with varying concentrations of H₂O₂, and thiols were then trapped using trichloroacetic acid precipitation (34). Protein was then pelleted by centrifugation, resuspended, and treated with d₅-iodoacetanilide to alkylate free thiols (35). The protein was then precipitated and resuspended in reducing buffer to reduce disulfide bonds (and any unreacted sulfenic acids) and alkylated with d₆-iodoacetanilide. Tryptic peptides were analyzed via LC-MS/MS, and the area ratios of alkylated deuterated/non-deuterated thiol-containing peptides were analyzed. This experiment showed no differences in ratios between a reduced P450 4A11 protein control and up to 1 mM H₂O₂ (supplemental Fig. S2). Seven of the eight cysteines in P450 4A11 maintained at least 60% reduction (i.e. > 60% d₀ labeling) with only Cys-513, the carboxyl-terminal cysteine, being oxidized further with increasing H₂O₂.

Spectral analysis of heme–thiolate oxidation

Spectral properties of P450 4A11 were used to further address the proteomic results (i.e. to address the hypothesis that the sulfenic acid moiety disrupts the iron coordination of the heme). TCEP-treated P450 4A11 was treated with H₂O₂,

Table 1

Effects of DTT on catalytic activities of P450 4A11 and other human P450s

| Human P450 | Substrate | Reaction |
|------------|-----------|----------|
| 4A11       | Lauric acid | 12-OH    |
| 2C8        | Paclitaxel | 6-OH     |
| 2C9        | Tolbutamide | 1'-OH  |
| 2D6        | Bufuralol  | 4'-OH    |
| 3A4        | Nifedipine | Desaturation |
| 19A1       | Testosterone | Estrone formation |
| 21A2       | Progestrone | 21-OH     |
| 2A6        | Coumarin   | 6-OH     |
| 1A1        | Ethoxyresorufin | (Resorufin) |
| 1A2        | Ethoxyresorufin | (Resorufin) |
| 1B1        | Ethoxyresorufin | (Resorufin) |

Catalytic activity

|                  | -DTT | +DTT (1 mM) | Change |
|------------------|------|------------|--------|
| 4A11 (nmol product/min/nmol P450) % | 9.0 ± 1.1 | 38 ± 1 | +320 |
| 2C8 | 1.8 ± 0.1 | 1.9 ± 0.1 | +7 |
| 2C9 | 1.1 ± 0.1 | 1.5 ± 0.2 | +33 |
| 2D6 | 2.9 ± 0.1 | 3.2 ± 0.1 | +9 |
| 3A4 | 2.0 ± 0.6 | 1.8 ± 0.5 | -10 |
| 19A1 | 10.8 ± 0.2 | 10.9 ± 0.9 | +1 |
| 21A2 | 51 ± 10 | 68 ± 6 | +30 |
| 2A6 | NC | NC | -14 |
| 1A1 | NC | NC | +8 |
| 1A2 | NC | NC | +26 |
| 1B1 | NC | NC | -4 |

a NC, assayed using fluorescence methods, but absolute amounts of product not calibrated.
reconstituted with NADPH-P450 reductase, deaerated, and placed under an anaerobic atmosphere of CO. Following the addition of NADPH, spectra were recorded for TCEP-reduced (Fig. 9A) and H$_2$O$_2$-oxidized (Fig. 9B) P450 4A11. The H$_2$O$_2$-oxidized protein showed increased absorbance at 420 nm for the CO-bound form. Sodium dithionite was added to these samples, which increased the absorbance of the oxidized enzyme at 450 nm and decreased the absorbance at 420 nm. NADPH-P450 reductase is able to reduce ferric heme to the ferrous state (allowing CO binding) but cannot reduce the sulfenic acid, leading to an inactive protein due to the loss of the thiolate axial coordination. However, dithionite is able to reduce a sulfenic acid (36), allowing for the reduced thiolate to religand with the heme–iron (Fig. 10). The change in $A_{450}$ upon the addition of dithionite in Fig. 9B corresponds to an increase of $\sim 0.3\ \mu$M P450 ($\sim 30\%$ total, uncorrected for loss of P450 in the degassing procedure), somewhat less than predicted from the LC-MS labeling study (Fig. 8).

**Sulfenylation of family 4 P450 enzymes in a CYP4A11 transgenic mouse model**

To determine whether this oxidative modification of P450 4A11 occurs in vivo, a CYP4A11 transgenic mouse model was used (20). Liver and kidney tissues from four male mice were harvested, and subcellular fractionation was done immediately. Microsomes were obtained within 3 h of tissue harvesting via differential centrifugation and, as with the recombinant protein, incubated with either buffer, d$_6$-dimedone, or d$_6$-dimedone supplemented with 500 $\mu$M H$_2$O$_2$ (Fig. 6). These labeled samples were then treated with catalase, reduced, counteralkylated with ($d_0$)-iododimedone, and digested with chymotrypsin. Chymotrypsin was used to produce peptides containing the heme–ligand cysteine unique to both P450s 4A11 and 4a12 in kidneys of male mice (supplemental Fig. S3) (37). The microsomal chymotryptic peptides were analyzed via LC-MS/MS, and the resulting peak area ratios were quantitated. The heme–thiolate Cys-457 of P450 4A11 showed $\sim 75\%$ dimedone labeling (sulfenylation) in both the dimedone and H$_2$O$_2$-supplemented samples in the kidney microsomes of three mice and $\sim 40\%$ labeling in the liver microsomes (Fig. 11 (A and B) and supplemental Fig. S4). One mouse was considerably younger (age of 6 weeks at time of harvest) than the other three (two of which were 3 months old and one of which was 7 months old), and the expression of P450 4A11 was greatly diminished compared with that in the older mice, as judged by peptide recovery. Interestingly, murine P450s 4a12 (Fig. 11 (C and D) and supplemental Fig. S5) and 4b1 (Fig. 11E and supplemental Fig. S6) also

![Figure 5. Lauric acid $\omega$-hydroxylation activity of Cys $\rightarrow$ Ser mutants of P450 4A11. For experiments done at the same time with wild-type P450 4A11, see Fig. 2A. Assays were done in the absence (■) and presence (○) of 1 mM DTT, as indicated. A, C43S; B, C86S; C, C200S; D, C255S; E, C513S. Error bars, S.D.](image-url)
showed significant sulfenylation of the respective heme–thiol ligands. The younger mouse had consistently lower levels of sulfenylation of the heme-binding peptide for all of the family 4 P450s in the kidneys and lower levels of P450 4a12 in the liver, but the sulfenylation of P450 4A11 in the liver was not different from that of the older mice.

Discussion

We have shown that H₂O₂ reacts with the heme–thiolate cysteine of P450 4A11 to form a sulfenic acid and disrupt the iron-sulfur coordination. Five of the eight Cys residues in the protein were shown not to be essential for activity (Fig. 5), and the heme-proximal ligand Cys-457 was identified as the major site of oxidation by modification and proteomic analysis (Figs. 7 and 8). It is well-established that breaking this bond will also convert P450 to an inactive form, cytochrome P420 (38, 39). The accessibility of the heme thiolate ligand has been established in studies with mercurials, as well as the conversion to the inactive cytochrome P420 (40, 41).

The concentration of H₂O₂ required to inhibit P450 4A11 (Fig. 4) is within a physiological range (42) and was shown to be reversible via reduction by thiols (Fig. 1). P450 enzymes are known to produce H₂O₂ in conjunction with NADPH-P450 reductase and NADPH, with or without substrate present (43). H₂O₂ production was measured (43) for a typical reaction containing 0.2 μM P450 4A11, 0.4 μM NADPH-P450 reductase, the NADPH-generating system, and 100 μM lauric acid and determined to be 5 μM min⁻¹ (i.e. 25 nmol of H₂O₂ produced min⁻¹ (nmol of P450 4A11)⁻¹). Typical incubations with lauric acid or arachidonic acid are conducted for 2–3 times this long, and the concentration of H₂O₂ produced should be negligible in proportion to the amount needed to inhibit the enzyme (Fig. 4).

Thiol activation is common in in vitro (25). The activation seen in the case of P450 4A11 was considerably higher than with any other P450s tested (Table 1), indicating that this redox sensitivity is rather unusual among the human P450s tested. Although numerous P450 purification protocols include dithiothreitol or -mercaptoethanol (44, 45), it is not clear that this is necessary in most cases.

Oxidation of P450 4A11 with H₂O₂ led to an attenuation of enzymatic activity (Figs. 3 and 4). This loss of activity was correlated with an increased observation of dimedone alkylation (sulfenylation) at Cys-457, the heme–thiolate ligand (Figs. 7 and 8). Sulfenic acid modifications have been directly implicated in the regulation of numerous enzymatic processes, including glyceraldehyde-3-phosphate dehydrogenase, peroxiredoxin, epidermal growth factor receptor, and others (26). However, sulfenic acid formation involving disruption of heme–iron coordination is unprecedented to our knowledge. Choudhury et al. (46) mutated the cytochrome c peroxidase distal histidine ligand to a cysteine and observed a cysteic acid
(cysteine sulfonic acid) in a crystal structure of the cysteine mutant, with a sulfonate oxygen coordinated to the heme. They observed activity, but it was markedly lower than that of the unmodified enzyme. These results were interpreted as the heme iron itself oxidizing the cysteine, as a substrate. This is an appropriate conclusion, in that peroxidases generally have non-Cys heme ligands (47, 48). Nitrile hydratase enzymes are known to coordinate iron or cobalt with both sulfenylated and sulfinylated thiols; additionally, the sulfinic acid is essential for the formation of the carbamate product (49). Our results do not discern whether the sulfenylated thiol is still (weakly) coordinated to the heme or the coordination is completely disrupted in P450 4A11. Dimedone, however, can react with sulfenamides as well as sulfinic acids (50), and such reactions would provide similar results in these experiments as sulfinic acid. If a sulfenamide was indeed the cause of this redox modulation, generally thiol oxidation to sulfinic acid would occur first, followed by subsequent condensation with a backbone nitrogen to form the sulfinamide (50).

ICAT analysis with iodoacetanilide did not provide evidence for a disulfide bond. α-Halo ketones react with sulfinic acids to form sulfoxides (35), so these peptides would not be quantified in a direct analysis between d₀/d₅-alkylated peptides because the m/z would increase by 16, the mass of oxygen. Inaccuracy could also be due to further di- and trioxidation (-SO₂ and -SO₃, respectively) of cysteines. Cys-513 appeared to be the only thiol oxidized to a large extent in our analysis, although the exact nature is unknown. Additionally, Cys-347 and Cys-375 appear to have proximity to each other in the homology model (Fig. 12). The inability to express the mutated forms of these two cysteines indicates that they are probably important for structural and/or conformational integrity, but our ICAT analysis does not indicate that either thiol is oxidized.

Taken together, the mutant analysis, dimedone labeling, and ICAT labeling methods show that cysteines other than Cys-457 are oxidized in P450 4A11 but do not appear to directly inactivate the protein or are correlated with the loss of activity. The oxidation or mutation of these ancillary cysteines could produce slight conformational changes, but these changes would not produce the spectral change at 450 nm that sulfenylation of Cys-457 does.

Detection of sulfenylation in murine kidneys and livers provides a physiological context for this modification. The high percentage of dimedone alkylation seen in the P450 4A11, 4a12,
and 4b1 heme peptides was surprising in that this would imply that a large percentage of the family 4 P450 enzymes is constitutively inactive in the kidney. One possible explanation could be that, because 20-HETE is a very potent vasoconstrictor with submicromolar activity (52, 53), these enzymes are very tightly regulated, and sulfenylated enzymes could act as a reserve mechanism. Conversely, if an increase in vascular tone is required quickly, family 4 P450 enzymes could be reduced quickly and be available for production of 20-HETE. P450 4A11 sulfenylation was observed to a lesser degree in the liver. This may be due to the larger reducing potential in glutathione stores the liver has compared with the kidney (54).

One unique feature of subfamily 4A P450 enzymes is the covalent binding of the heme prosthetic group to the enzyme through a conserved glutamic acid residue (55, 56). This unique feature may allow for easier recoordination of the reduced thiol with an immobilized heme iron. In recombinant P450 4A11, it is reported that ~25% of the enzyme has covalently bound heme (56), but the fraction has not been determined in human samples. Rodent subfamily 4A P450 enzymes have higher percentages of heme covalently bound to the enzyme (56). LeBrun et al. (56) suggest that this covalent binding may contribute to differences in substrate binding rates and enzymatic regioselectivity.

A recent crystal structure of recombinant rabbit P450 4B1 shows complete adduction of the heme through a bond between a heme methyl group and Glu-310 (analogous to Glu-321 in P450 4A11) (57). In examining the alignment of mouse P450 4b1 and rabbit P450 4B1 (UniProt), these two enzymes share 86% identity and 95% similarity. A modeled structure shows a relatively open conformation on the proximal side of the protein (Fig. 12). Cysteines that were mutated in this study are located in the peripheries of the protein, away from the proposed active site, and probably do not influence enzyme function.

Disruption of the iron-sulfur coordination would alter the absorbance properties of P450 4A11 (Fig. 10). Under an anaerobic CO atmosphere, NADPH-P450 reductase can reduce ferric heme to its ferrous state, allowing CO to bind. This CO-bound state has absorbance with a maximum at 450 nm if the heme–thiolate ligand is intact. When this coordination is disrupted (via sulfenylation in this case), the CO-binding spectrum is shifted to a maximum at 420 nm (Figs. 9 and 10). Interestingly, the addition of dithionite after reduction with NADPH-P450 oxidoreductase reduces the sulfenic acid (36) and allows for recoordination of the thiolate–heme complex. The observation of a sulfenylated heme–thiolate ligand has probably been overlooked previously because of the common laboratory practice of determining P450 concentration using dithionite as the reducing agent for obtaining P450 difference spectra.
Chemical synthesis

**Iododimedone**—Iododimedone was prepared from dime-
dione (4 mmol scale) by iodonation with N-iodosuccinimide
(59). Yield 48%, mp 145–146 °C; high-resolution mass spec-
trometry for C$_8$H$_{12}$O$_2$I $m/z$ 266.9882 (MH$^+$), found 266.9881
(Δ 0.4 ppm); $^1$H NMR (400 MHz, CDCl$_3$) δ 1.04 (s, 6H, (CH$_2$)$_2$),
2.50 (d (splitting due to I), 4H, CH$_2$), 6.35 (s, 1H, CHI) (litera-
ture mp 155 °C (60)).

**d$_4$-Dimedone**—A modification of the basic procedure of Seo
and Carroll (30) was used. Diethyl malonate and mesityl-
oxide were condensed in an ethanolic solution of NaOC$_2$H$_5$
under reflux, followed by decarboxylation with NaOH under
reflux. Following neutralization, the filtrate was collected and
extracted into ethyl acetate. The product was concentrated by
partial removal of solvent in vacuo and trituration with hexanes
to yield crystalline d$_4$-dimedone in 85% yield. mp 147–148 °C;
high-resolution mass spectrometry for C$_8$H$_{12}$O$_2$I $m/z$ 147.1292
(MH$^+$), obs 147.1284 (Δ 0.4 ppm); $^1$H NMR (400 MHz, CDCl$_3$)
δ 2.52 (s, 5H, -CH$_2$-), 3.33 (s, 2H, CO-CH$_2$-CO) (61) (literature
mp 145–147 °C (61)).

**d$_5$-Iodoacetanilide**—Iodoacetanilide was prepared from
idoacetic acid (2.53 mmol) and aniline (2.53 mmol) by amide
 coupling with dicyclohexylcarbodiimide (2.53 mmol) (62). Yield 70%,
$^1$H NMR (600 MHz, acetone-$d_6$) δ 9.51 (bs, 1H, NH)
7.64 (d, 2H, o-Ar), 7.31 (t, 2H, m-Ar), 7.02 (t, 1H, p-Ar), 3.90 (s,
2H, CH$_2$I), $^{13}$C NMR (150 MHz, acetone-$d_6$) 167.0, 140.1,
129.7, 124.6, 120.0, 0.72.

**d$_6$-Iodoacetanilide**—d$_6$-Iodoacetanilide was prepared from
idoacetic acid (2.53 mmol) and d$_6$-aniline (2.53 mmol) by amide
coupling with dicyclohexylcarbodiimide (2.53 mmol) (62). Yield 89%,
$^1$H NMR (600 MHz, acetone-$d_6$) δ 9.53 (bs, 1H, NH)
7.64 (d, 2H, o-Ar), 7.31 (t, 2H, m-Ar), 7.02 (t, 1H, p-Ar), 3.90 (s,
2H, CH$_2$I), $^{13}$C NMR (150 MHz, acetone-$d_6$) 167.0, 139.8,
124.1, 124.1, 119.6, 0.75.

Enzymes

Human P450 4A11 (wild-type and F434S, C52S, C85S,
C199S, C255S, and C512S mutants) was expressed and purified
as described previously (4, 63). *Escherichia coli* recombinant rat
NADPH-P450 reductase and human liver cytochrome $b_5$ ($b_5$)
were prepared as described by Hanna et al. (64) and Guengerich
(65), respectively.

Tissue samples

All experiments using mice were conducted with approved
protocols by the Institutional Animal Care and Use Committee
of Vanderbilt University and in accordance with the National
Institutes of Health Guide for the Care and Use of Laboratory
animals. 129Sv mice carrying one copy of the human cyto-
chrome P450 4A11 gene (CYP4A11) (under control of its native
promoter, generated as described previously (21)) were pro-
vided a normal chow diet (Laboratory Rodent 5001, Purina, St.
Louis, MO) with free access to water and were housed in an
Association for the Assessment and Accreditation of Labora-
tory Animal Care (AAALAC)-accredited, temperature-con-
trolled facility with a 12-h light/dark cycle. All studies were
conducted in mice 6–28 weeks of age. CYP4A11 transgenic
mice were crossed with pure Sv129 wild-type mice, and off-
spring were genotyped for the presence of a single copy of the

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**P450 4A11 sulfenic acid and inhibition**

![Absorbance graph](image)

Figure 9. Reduction of P450 4A11 by NADPH-P450 reductase and sodium
dithionite in the presence of CO. Reduced protein (A) and protein oxidized
with 500 μM H$_2$O$_2$ (B) was deaerated and placed under an anaerobic CO atmos-
phere. NADPH was then added, and absorbance spectra were recorded (blue).
Dithionite was then added (red).

In summary, P450 4A11 seems to show a unique activation
under reducing conditions compared with other P450 enzymes
tested. Conversely, H$_2$O$_2$ inhibits the enzymatic activity of the
enzyme. Sulfenic acid formation of the heme thiolate ligand was
detected by labeling and LC-MS analysis (Figs. 7 and 8) and was
verified spectrally (Fig. 9). The physiological relevance of the
modification was demonstrated with the identification of sul-
fenylated P450s in freshly isolated murine kidneys and livers.
This modification is a potentially important observation that
may have implications in other P450s, as shown by our results
with the mouse P450 4a and 4b enzymes. Sulfenylation could
provide for biological regulation of P450 4A11 and its produc-
tion of the potent vasoconstrictor 20-HETE. As mentioned pre-
viously, 20-HETE has been shown to induce ROS formation as
well as to play an important role in blood pressure control,
which is known to be mediated by ROS production (58). The
extent of sulfenylation of 4A/a P450 enzymes in tissues may
provide a mechanism to reduce the production of 20-HETE.

Experimental procedures

**Chemicals**

[1-14C]Lauric acid was obtained from American Research
Chemicals (St. Louis, MO). Lauric acid, TCEP, H$_2$O$_2$ (30%,
w/v), paclitaxel, tolbutamide, bufuralol, nifedipine, testos-
terone, progesterone, coumarin, and ethoxyresorufin were
obtained from Sigma-Aldrich.

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**J. Biol. Chem. (2017) 292(27) 11230–11242**
human CYP411 gene as described previously (21). Organs were collected from male transgenic mice immediately after sacrifice and used for microsomal preparations as described below.

**Site-directed mutagenesis**

P450 4A11 was subcloned into a pBlueScript vector to perform site-directed mutagenesis. Residues from each site were converted to serine by using the QuikChange II site-directed mutagenesis kit (Agilent Technologies). The resulting plasmids were sequenced to confirm successful mutation and subcloned into a pCW expression vector.

**Enzymatic assays**

Assays were done as described previously (4) with minor changes. Typical incubations included 0.2 μM P450 4A11, 0.4 μM NADPH-P450 reductase, 0.4 μM bab, 150 μM 1-α-dilauroyl-sn-glycero-3-phosphocholine (DLPC; Sigma-Aldrich), 100 mM potassium phosphate buffer (pH 7.4), and the indicated concentration of lauric acid ([1-14C]lauric acid, usually added as an aqueous 10 mM solution of sodium laurate) in a final volume of 0.25 ml. bab was included because it stimulates the catalytic activity (4). Following temperature equilibration to 37 °C for 5 min, reactions were initiated by the addition of an NADPH-regenerating system consisting of 0.5 mM NADP+, 10 mM glucose 6-phosphate, and 1 IU ml⁻¹ yeast glucose 6-phosphate dehydrogenase.

![Figure 10. Scheme of Cys-457 sulfenic acid formation and reduction in P450 4A11.](image)

In the presence of H₂O₂, the heme–thiolate ligand becomes oxidized and loses its coordination with the heme iron. The sulfenic acid is not reduced by NADPH-P450 reductase but can be reduced by dithionite, reforming the heme coordination.

![Figure 11. Analysis of human and mouse P450 peptides in tg4A11 mice.](image)

Microsomes (prepared from kidney and liver tissues of CYP4A11 transgenic mice within 3 h after sacrifice) were treated with either vehicle (control), d₆-dimedone, or d₆-dimedone supplemented with 500 μM H₂O₂. Integrated areas for ions corresponding to peptides of the heme–thiolate for P450s 4A11 (A and B), 4a12 (C and D), and 4b1 (E) were compared and reported as percent sulfenylation (percent sulfenylation = area of d₆-dimedone-labeled peptide/area of d₆-dimedone-labeled peptide plus area of d₀-dimedone-labeled peptide). See “Experimental procedures” and Fig. 6. The p values using Student’s t test comparing control- and dimedone-treated tissue were all < 0.03 except for the P450 4a12 kidney (C) (p = 0.058). The mouse indicated with the inverted triangle was 6 weeks old, much younger than the other three animals (two were 3 months old, and one was 7 months old).

**Enzymatic assays**

Assays were done as described previously (4) with minor changes. Typical incubations included 0.2 μM P450 4A11, 0.4 μM NADPH-P450 reductase, 0.4 μM bab, 150 μM 1-α-dilauroyl-sn-glycero-3-phosphocholine (DLPC; Sigma-Aldrich), 100 mM potassium phosphate buffer (pH 7.4), and the indicated concentration of lauric acid ([1-14C]lauric acid, usually added as an aqueous 10 mM solution of sodium laurate) in a final volume of 0.25 ml. bab was included because it stimulates the catalytic activity (4). Following temperature equilibration to 37 °C for 5 min, reactions were initiated by the addition of an NADPH-regenerating system consisting of 0.5 mM NADP+, 10 mM glucose 6-phosphate, and 1 IU ml⁻¹ yeast glucose 6-phosphate dehydrogenase.

![Figure 12. Homology model of P450 4A11 based on crystal structure of rabbit P450 4B1.](image)

A, all Cys residues are shown; B, positions of Asn-456, Lys-460, and Phe-462 near the heme and Cys-457 (yellow sphere).
dehydrogenase (66). Reactions generally proceeded at 37 °C for 2 min and were terminated with 1.0 ml of ethyl acetate containing 0.1% CH₃CO₂H (v/v), and, following mixing with a vortex device, the mixtures were centrifuged (10³ × g for 10 min). A 0.8-ml aliquot of the ethyl acetate layer (upper phase) was transferred to a clean tube, and the solvent was removed under an N₂ stream.

The dried extracts were dissolved in 200 µl of a 1:1 mixture of H₂O/CH₃CN containing 0.1% CH₃CO₂H (v/v) and 10 µM butylated hydroxytoluene, and aliquots were analyzed on a reversed-phase (octadecylsilane, C₁₈) HPLC column (5 µm, 2.1 × 100 mm (Waters, Milford, MA)) coupled with a radioactivity detector (β-RAM, IN/US Systems, Tampa, FL). Reaction products and substrate were eluted at a flow rate of 0.6 ml min⁻¹ using an increasing linear gradient of CH₃CN (including 0.1% (v/v) HCO₂H) from 35 to 95% (v/v) over 30 min.

Assays with P450s other than P450 4A11 were performed as described previously, with the modification of either preincubation with DTT (1 mM) for 10 min or not (the DTT remained in the reactions): P450 2C8-paclitaxel as substrate (67), P450 2C9–tobutamidine as substrate (68), P450 2D6–bufuralol as substrate (69), P450 3A4–nifedipine as substrate (70), P450 19A1–testosterone as substrate (71), P450 21A2–progestosterone as substrate (72), P450 2A6–coumarin as substrate (73), and P450s 1B1–, 1A1–, and 1A2–7–ethoxyresorufin as substrate (74).

**Protein oxidation**

Purified recombinant P450 4A11 (0.4 ml of a 10 µM stock solution, stored at −80 °C in 50 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol, 1 mM DTT, and 0.1 mM EDTA) was thawed on ice and reduced for 30 min by the addition of 1 mM TCEP in the dark. Protein was precipitated again using the same method as above to remove heavy reagent. Reduced protein was then diluted to a concentration of 500 µM using oxidation buffer. Aliquots were treated with varying amounts of H₂O₂ or TCEP. Aliquots for the activity assay (175 pmol; described above) were incubated with H₂O₂ or TCEP for 15 min at 37 °C. Human erythrocyte catalase (10 units) was added to each and incubated at 25 °C for 10 min to remove H₂O₂. The enzymes in the samples were pelleted by centrifugation (12,000 × g, 15 min, 4 °C). The supernatant was removed from each sample, and the pellet was washed with ice-cold CH₃CN. The pellet was resuspended in 50 µl of 100 mM HEPPS buffer (pH 7.4) containing 2% (w/v) SDS and 1 mM TCEP, and reduction proceeded for 30 min at 50 °C. d₄-Iodoacetanilide (100 µM, in acetone) was added to a final concentration of 10 µM, and incubation was done at 23 °C in the dark for 30 min, with shaking. Samples were subjected to SDS-PAGE (10% BisTris, NuPAGE, Invitrogen) separation and stained with SimplyBlue SafeStain (Invitrogen). The Mᵣ region corresponding to P450 4A11 was excised, digested with trypsin (8 ng/µl) for 16 h in 25 mM NH₄HCO₃ (pH 7.8) at 37 °C, and subjected to LC-MS/MS analysis.

**ICAT labeling of recombinant P450 4A11**

Additional aliquots of reduced, buffer-exchanged protein (from above; 100 pmol) were incubated with H₂O₂ or TCEP for 15 min. Human erythrocyte catalase (10 units) was added, and incubations proceeded at 23 °C for 5 min to remove H₂O₂. Tri-chloroaacetic acid was then added to a final concentration of 10% (v/v), and the samples were incubated on ice for 15 min. The enzymes in the samples were pelleted by centrifugation (10,000 × g, 15 min, 4 °C). The supernatant was removed from each sample, and the pellet was washed with ice-cold CH₃CN. The pellet was resuspended in 50 µl of 100 mM HEPPS buffer (pH 8.0) containing 2% (w/v) SDS and 1 mM TCEP, and reduction proceeded for 30 min at 50 °C. The mixtures were centrifuged (104,000 × g, 15 min, 4 °C). The supernatant was removed from each sample, and the pellet was washed with ice-cold CH₃CN. The pellet was resuspended in 50 µl of 100 mM HEPPS buffer (pH 8.0) containing 2% (w/v) SDS and 1 mM TCEP, and reduction proceeded for 30 min at 50 °C. Additional aliquots of oxidized protein (from above; 100 pmol) were incubated with H₂O₂ or TCEP for 15 min. Human erythrocyte catalase (10 units) was added, and incubations proceeded at 23 °C for 5 min to remove H₂O₂. Tri-chloroaacetic acid was then added to a final concentration of 10% (v/v), and the samples were incubated on ice for 15 min. The enzymes in the samples were pelleted by centrifugation (12,000 × g, 15 min, 4 °C). The supernatant was removed from each sample, and the pellet was washed with ice-cold CH₃CN. The pellet was resuspended in 50 µl of 100 mM HEPPS buffer (pH 8.0) containing 2% (w/v) SDS and 1 mM TCEP, and reduction proceeded for 30 min at 50 °C. The mixtures were centrifuged (104,000 × g, 15 min, 4 °C). The supernatant was removed from each sample, and the pellet was washed with ice-cold CH₃CN. The pellet was resuspended in 50 µl of 100 mM HEPPS buffer (pH 8.0) containing 2% (w/v) SDS and 1 mM TCEP, and reduction proceeded for 30 min at 50 °C. Additional aliquots of oxidized protein (from above; 100 pmol) were incubated with H₂O₂ or TCEP for 15 min. Human erythrocyte catalase (10 units) was added, and incubations proceeded at 23 °C for 5 min to remove H₂O₂. Tri-chloroaacetic acid was then added to a final concentration of 10% (v/v), and the samples were incubated on ice for 15 min. The enzymes in the samples were pelleted by centrifugation (12,000 × g, 15 min, 4 °C). The supernatant was removed from each sample, and the pellet was washed with ice-cold CH₃CN. The pellet was resuspended in 50 µl of 100 mM HEPPS buffer (pH 8.0) containing 2% (w/v) SDS and 1 mM TCEP, and reduction proceeded for 30 min at 50 °C. d₄-Iodoacetanilide (100 µM, in acetone) was added to a final concentration of 10 µM, and incubation was done at 23 °C in the dark for 30 min, with shaking. Samples were subjected to SDS-PAGE (10% BisTris, NuPAGE, Invitrogen) separation and stained with SimplyBlue SafeStain (Invitrogen). The Mᵣ region corresponding to P450 4A11 was excised, digested with trypsin (8 ng/µl) for 16 h in 25 mM NH₄HCO₃ (pH 7.8) at 37 °C, and subjected to LC-MS/MS analysis.

**Preparation of microsomes**

Microsomes from freshly excised tissues were prepared with slight modifications of published methods (66). For ICDID labeling studies, the buffer (0.10 M Tris acetate (pH 7.4) containing 0.10 M KCl, 1.0 mM EDTA, and 20 µM butylated hydroxytoluene) was sparged with argon before use. Tissue samples were homogenized using a Teflon-glass Potter-Elvehjem device and centrifuged at 104,000 × g for 10 min. The supernatant was removed from each sample, and the pellet was washed with ice-cold CH₃CN. The pellet was resuspended in 50 µl of 100 mM HEPPS buffer (pH 8.0) containing 2% (w/v) SDS and 1 mM TCEP, and reduction proceeded for 30 min at 50 °C. d₄-Iodoacetanilide (100 µM, in acetone) was added to a final concentration of 10 µM, and incubation was done at 23 °C in the dark for 30 min, with shaking. Samples were subjected to SDS-PAGE (10% BisTris, NuPAGE, Invitrogen) separation and stained with SimplyBlue SafeStain (Invitrogen). The Mᵣ region corresponding to P450 4A11 was excised, digested with trypsin (8 ng/µl) for 16 h in 25 mM NH₄HCO₃ (pH 7.8) at 37 °C, and subjected to LC-MS/MS analysis.
in 25 mM NH₄HCO₃ (pH 8.0) at 25 °C, and subjected to LC-MS/MS analysis as described below.

**LC-MS/MS analysis**

Extracted peptides were analyzed on a nanoLC Ultra system (Eksigent Technologies, Dublin, CA) interfaced with a LTQ Orbitrap XL mass spectrometer (Thermo Scientific, San Jose, CA). Approximately 5 pmol of peptides was reconstituted in 0.1% HCO₃H in H₂O (solvent A) and 0.1% HCO₃H in CH₂CN (solvent B) (all v/v) by increasing from 2 to 45% B (v/v) over a period of 0–45 min at a flow rate of 500 nl min⁻¹. The spray voltage was set to 2.0 kV, and the heated capillary temperature was set to 200 °C. Higher-energy collisional dissociation (HCD) MS/MS spectra were recorded in the data-dependent mode using a top-2 method with an inclusion list containing m/z values corresponding to P450 4A11 tryptic peptides, which included both d₅- and d₆-dimedone cysteines, determined in silico with Skyline software (75). MS1 spectra were measured with a resolution of 70,000, an AGC target of 1e6, and a mass range from m/z 300 to 1,500. HCD MS/MS spectra were acquired with a resolution of 7,500, an AGC target of 1e5, and a normalized collision energy of 35. Peptide m/z values that triggered MS/MS scans were dynamically excluded from further MS/MS scans for 20 s, with a repeat count of 1.

**Peptide data analysis**

Raw data files were analyzed using MyriMatch software (76) against a decoy protein database consisting of a forward and reverse human UniProt/SwissProt database (version 20160620). Precursor ion mass tolerance was set at 10 ppm, and precursor ion mass tolerance was set at 10 ppm, and pressure-loaded (1.5 μl min⁻¹) onto a 360-μm outer diameter × 100-μm inner diameter microcapillary analytical column packed with Jupiter octadecylsilane (C18) (3 μm, 300 Å; Phenomenex) and equipped with an integrated electrospray emitter tip. Peptides were then separated with a linear gradient formed with 0.1% HCO₂H (v/v) and pressure-loaded (1.5 μl min⁻¹) onto a 360-μm outer diameter × 100-μm inner diameter microcapillary analytical column packed with Jupiter octadecylsilane (C18) (3 μm, 300 Å; Phenomenex) and equipped with an integrated electrospray emitter tip. Peptides were then separated with a linear gradient formed with 0.1% HCO₂H in H₂O (solvent A) and 0.1% HCO₃H in CH₂CN (solvent B) (all v/v) by increasing from 2 to 45% B (v/v) over a period of 0–45 min at a flow rate of 500 nl min⁻¹. The spray voltage was set to 2.0 kV, and the heated capillary temperature was set to 200 °C. Higher-energy collisional dissociation (HCD) MS/MS spectra were recorded in the data-dependent mode using a top-2 method with an inclusion list containing m/z values corresponding to P450 4A11 tryptic peptides, which included both d₅- and d₆-dimedone cysteines, determined in silico with Skyline software (75). MS1 spectra were measured with a resolution of 70,000, an AGC target of 1e6, and a mass range from m/z 300 to 1,500. HCD MS/MS spectra were acquired with a resolution of 7,500, an AGC target of 1e5, and a normalized collision energy of 35. Peptide m/z values that triggered MS/MS scans were dynamically excluded from further MS/MS scans for 20 s, with a repeat count of 1.

**Spectroscopy**

P450 4A11 was oxidized with H₂O₂ as above, but the procedure was adapted slightly for spectroscopic assays. The enzyme was diluted with oxidation buffer to 1 μM before the introduction of 1 mM TCEP or 500 μM H₂O₂. After oxidation, 30 units catalase was added, and the sample was incubated at 23 °C for 5 min. To this solution, final concentrations of 1 μM NADPH-P450 reductase, 150 μM DLPC, and 0.1 unit/ml protocatechu-e dioxide oxygenase (Sigma) were added to an anaerobic cuvette, with NADPH (300 nmol, aqueous) in a side arm of the cuvette. Samples were degassed, 20 μM 3,4-dihydroxybenzoate (Sigma; substrate for protocatechu-e dioxide oxygenase) was added to remove oxygen (78), and samples were further degassed using a manifold attached to both vacuum and purified argon (51, 79) and placed under an anaerobic CO atmosphere. The valves of the cuvettes were sealed, and multiple UV-visible absorbance spectra were recorded using an OLIS/Hewlett Packard 8452 diode array spectrophotometer (On-Line Instrument Systems, Bogart, GA). Spectra were collected from 380 to 600 nm before and after the addition of NADPH/sodium dithionite.

**Author contributions**—M. E. A. synthesized d₅- and d₆-idoacetanilide and did the dimedone and iodoacetanilide labeling experiments, LC-MS assays, spectroscopic anaerobic reduction assays, and assays of catalytic activity and analyzed results. D. K. did the site-directed mutagenesis studies. F. P. G. synthesized d₅-dimedone and (d₆)jiododimtedone. L. D. N., D. K., and F. P. G. did the catalytic assays with human P450s (other than P450 4A11). C.-H. Y. did the preliminary thiol activation assays. U. S., E. F. J., and A. P. developed and raised the transgenic P450 4A11 mice. M. E. A. and F. P. G. wrote most of the manuscript. F. P. G. oversaw the project. All authors contributed to the conclusions.

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