Identification of the Interactions between Cytochrome P450 2E1 and Cytochrome b₅ by Mass Spectrometry and Site-directed Mutagenesis

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The reaction cycles of cytochrome P450s (P450) require input of two electrons. Electrostatic interactions are considered important driving forces in the association of P450s with their redox partners, which in turn facilitates the transfer of the two electrons. In this study, the cross-linking reagent, 1-ethyl-3-[3-dimethylamino propyl] carbodiimide hydrochloride (EDC), was used to covalently link cytochrome P450 2E1 (CYP2E1) with cytochrome b₅ (b₅) through the formation of specific amide bonds between complementary charged residue pairs. Cross-linked peptides in the resulting protein complex were distinguished from non-cross-linked peptides using an ¹⁸O-labeling method on the basis that cross-linked peptides incorporate twice as many ¹⁸O atoms as non-cross-linked peptides during proteolysis conducted in ¹⁸O-water. Subsequent tandem mass spectrometric (MS/MS) analysis of the selected cross-linked peptide candidates led to the identification of two intermolecular cross-links, Lys⁴²⁸(CYP2E1)-Asp⁵¹⁸(b₅) and Lys⁴¹⁵(CYP2E1)-Glu⁵⁶(b₅), which provides the first direct experimental evidence for the interacting orientations of a microsomal P450 and its redox partner. The biological importance of the two ion pairs for the CYP2E1-b₅ interaction, and the stimulatory effect of b₅, was confirmed by site-directed mutagenesis. Based on the characterized cross-links, a CYP2E1-b₅ complex model was constructed, leading to improved insights into the protein interaction. The described method is potentially useful for mapping the interactions of various P450 isoforms and their redox partners, because the method is relatively rapid and sensitive, and is capable of suggesting not only protein interacting regions, but also interacting orientations.

Cytochrome P450s (P450s) are a superfamily of b-type hemoproteins responsible for the metabolism of a wide variety of exogenous compounds such as drugs and carcinogens, and endogenous compounds such as prostaglandins and steroids (1). P450 reactions require input of two electrons (supplemental Fig. S1) (1, 2). The efficiency of electron transfer is one of the key determinants of the reaction kinetics. In microsomal systems, NADPH is the ultimate source of the two electrons, and NADPH-dependent cytochrome P450 oxidoreductase (P450 reductase) together with cytochrome b₅ (b₅) facilitates the electron transfer. Knowledge of the interactions between P450s and their redox partners is fundamental to a complete understanding of the mechanisms of P450 reactions.

The interactions between P450s and b₅ have drawn much attention because of variable effects b₅ has on different P450 isoforms and P450 reactions. It has been shown that b₅ may stimulate, inhibit or have no effects on P450-catalyzed reactions depending on the particular isoform of P450 and the substrate of the reaction (3–5). However, there is no consensus on whether b₅ transfers electrons to P450, or causes an allosteric effect on P450, or whether both mechanisms are simultaneously operative (6–10). In addition, the mechanism of substrate- and P450 isoform dependency is unknown (5, 11).

CYP2E1 is a P450 isoform whose reactions are highly stimulated in the presence of b₅. For example, we previously found that b₅ stimulates CYP2E1-catalyzed oxidation of acetaminophen to its toxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI), by 25-fold (12). For other CYP2E1-catalyzed reactions, such as aniline p-hydroxylation and 7-ethoxycoumarin O-deethylation, b₅ stimulates the reactions by 270-fold and 67-fold, respectively (5, 11). In contrast to its stimulating effects on CYP3A4, CYP3A5, CYP2C19, CYP2B6, and CYP2C8, apo-b₅ is unable to replace holo-b₅ in stimulating CYP2E1-catalyzed reactions (11, 12). The requirement for the heme group increases the probability that b₅ stimulates CYP2E1-catalyzed reactions by facilitating electron transfer rather than by only causing a positive allosteric effect. Because intermolecular complex formation immediately precedes electron trans-

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The identification of the protein interacting regions and orientations in the CYP2E1-b5 complex is an important goal in understanding the effects of b5 on CYP2E1-catalyzed reactions. It has been shown, by site-directed mutagenesis and chemical modification, that several cationic residues on the proximal face of P450, and several anionic residues on b5, surrounding the solvent-exposed heme edge, are important for the functional interaction between the two proteins (4, 14, 15). However, the protein interacting surfaces have not been fully characterized, and the protein interacting orientations have never been determined. One complex model of b5 and CYP101, a microbial P450, was proposed by Sligar and co-workers (16) based on visual optimization of the intermolecular electrostatic interactions and minimization of the distance between the redox centers of the two proteins. However, the protein interacting orientations in this model have not been substantiated by experiments. In this study, a complex model of b5 and CYP2E1, a microsomal P450, is proposed based on two chemical cross-links characterized using mass spectrometry.

Because electrostatic interactions are considered the major driving forces for the P450-b5 interaction (4, 14, 15), a water-soluble cross-linking reagent, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), was chosen to covalently link CYP2E1 with b5. EDC generates “zero-length” cross-links (amide bonds) between basic (Lys) and acidic (Asp or Glu) residues that come into very close proximity (supplemental Fig. S2) (17). The short length of EDC cross-links generally leads to specific intra- and intermolecular linkages without sampling multiple protein orientations (17). EDC was previously used to form a cross-link between CYP2B4 and b5 (18). However, this complex was not structurally characterized.

To identify the cross-links in the CYP2E1-b5 complex, the complex was digested and the generated peptides were isotopically labeled. It has been noted that during trypsin-catalyzed proteolysis, two oxygen atoms from solvent are incorporated into the α-carboxyl group of a peptide C-terminally ending with a lysine or an arginine residue (19). When the proteolysis is conducted in fully enriched 18O-water, the generated peptides are labeled with two 18O atoms at their C termini (Fig. 1) (19, 20). Recently, 18O-labeling has been used to identify cross-linked peptides in a digest mixture (21, 22). Through the comparison of peptide ions generated from two digestions, one conducted in 16O-water and the other in 18O-water, cross-linked peptides can be distinguished from non-cross-linked peptides by virtue of incorporating more than two 18O atoms during proteolysis. The structures of the selected cross-linked peptide candidates are subsequently characterized by tandem mass spectrometric (MS/MS) analysis.

Here we describe the characterization of two cross-links in the CYP2E1-b5 complex using 18O-labeling and mass spectrometry. The importance of the identified ion pairs in the interacting proteins was confirmed by site-directed mutagenesis. Finally, a model that sheds light on the CYP2E1-b5 interaction was constructed.

EXPERIMENTAL PROCEDURES

Materials—Escherichia coli strain DH5α containing the expression vector pCWhum3A4(His)b5 and strain BL21(DE3) containing the plasmid (His)₅HMwRat-b5 were provided by Dr. Ronald W. Estabrook (10, 23). E. coli strain DH5α containing the expression vector pCWhum2E1 was from Dr. Elizabeth M. J. Gillam (24). Restriction enzymes and other DNA-modifying enzymes were from New England Biolabs (Beverly, MA). Platinum Pfx DNA polymerase, T4 DNA ligase, E. coli DH5α/F'IQ Max Efficiency Competent cells, E. coli expressed histidine-tagged recombinant human b5 were purchased from Invitrogen (Carlsbad, CA). Bactotryptone, bactopeptone, and bactoyeast extract were obtained from BD Biosciences Clontech (Palo Alto, CA). Emulgen 911 was from Kao Chemicals (Tokyo, Japan). Ni-NTA Superflow was from Qiagen (Valencia, CA). The cross-linking reagent EDC was purchased from Pierce. Sequencing grade modified trypsin was from Roche Applied Science (Indianapolis, IN). 18O-labeled water (99 atom % 18O) was purchased from Isotec (Miamisburg, OH). IPTG, δ-ALA, thiamine, imidazole hydrochloride, protease inhibitor mixture, sodium cholate, Coomassie Brilliant Blue R, Copper II chloride dehydrate, DTT, and iodoacetamide were purchased from Sigma-Aldrich. HPLC solvents were of the highest grade commercially available and were used as received. All other reagents were analytical grade.

Cloning of Histidine-tagged Recombinant Human CYP2E1—The vector construct pCWhum3A4(His)b5 (10) was the source of the pCWori + expression vector for cloning the histidine-tagged CYP2E1 expression vector construct. The cDNA of human CYP2E1 (24) was used as the template for PCR. Amplifications were performed using Platinum Pfx DNA polymerase. Reactions were assembled and heated to 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 56 °C for 45 s, and extension at 72 °C for 1 min. Cycling was followed by a final extension at 72 °C for 5 min. DNA from the...
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reactions was purified using a Qiagen PCR purification kit and digested with NdeI and Sall. The digests were then electrophoresed on a 1.1% agarose gel and the CYP2E1 amplicons of expected size were gel-purified using a Qiagen Geneclean kit. The pCWhum3A4(His)6 plasmid was digested with NdeI and Sall to enable gel-purification of the linearized plasmid from which the CYP3A4 insert had been removed. The vector was treated with calf intestinal phophatase and the CYP2E1 fragment was ligated to the vector to generate the histidine-tagged CYP2E1 expression vector construct. Ligation reactions were used to transform DH5α cells. Positive clones containing the desired inserts were verified initially by colony PCR and restriction analysis, and finally by DNA sequencing using the dideoxy chain termination method.

Site-directed Mutagenesis of CYP2E1—The cloned plasmid pCWhum2E1(His)6, was used as the template for amplification reactions with the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. Oligonucleotide primers used in the generation of K428A and K434A single mutants plasmids were as follows (mismatches indicated by the underlined bases): K428A forward, 5’-GTGACTATTTTGCCTCATTTCAC-3’; K428A reverse, 5’-GTGGAATAATGGGCGGAAAAATGCATAC-3’. K434A forward, 5’-AAGCCATTTTCCA-CAGGAGCAGTGTGTGAGAAG-3’; K434A reverse, 5’-TTCTCCAGACACACCTCTGTCTCCTGAGGAT-GTC-3’. A double mutant of pCWhum2E1(His)6, containing both the K428A and the K434A replacements was constructed using the K434A single mutant plasmid and the K428A forward and reverse primers. DpnI-digested DNA was transformed into XL1-Blue cells, and DNA from several of the resulting colonies was isolated. The cDNA sequence was analyzed for the presence of the desired mutations and the absence of extraneous mutations (University of Washington Sequencing Facility).

Protein Expression and Purification—A single isolated colony of histidine-tagged CYP2E1 was used to inoculate 10 ml of LB-ampicillin media, which was cultivated with shaking at 37 °C overnight and then diluted 1:100 in Terrific Broth containing 100 mg of ampicillin liter⁻¹, 1.0 mM thiamine, and trace elements (25). The cultures were shaker (180 rpm) at 37 °C until the A₆₀₀ reached 0.4, after which isopropyl-1-thio-β-D-galactopyranoside (1.0 mM) and δ-ALA (0.5 mM) were added, and the cultures were shaken (160 rpm) at 28 °C for 36 h. Cells were harvested by centrifugation at 5000 × g (4 °C, 15 min), resuspended in storage buffer (50 mM KPi, pH 7.4, 20% glycerol, and 0.5 mM EDTA), pooled into 50-mL Falcon tubes, and re-centrifuged at 4000 × g (4 °C, 30 min). The supernatant was discarded, and the cell pellets were resuspended in resuspension buffer (100 mM Tris-HCl, pH 7.4, 20% glycerol, with the addition of 1 ml of protease inhibitor mixture per liter of the initial culture volume). After the addition of lysozyme (5 mg/L), the culture was stirred at 4 °C for 1 h. Cells were homogenized and spun at 150,000 × g. The pellets were resuspended in the resuspension buffer by homogenization and stirred at 4 °C for 1 h after the addition of 1% Emulgen 911. After centrifugation at 150,000 × g for 25 min, imidazole was added to the red/orange supernatant to a final concentration of 20 mM. The supernatant was applied to a Ni-NTA agarose column that had been pre-equilibrated with 15 column volumes of equilibrium buffer (50 mM KPi, pH 7.4, 20% glycerol, 0.5 mM KCl, 0.05% sodium cholate, 50 mM p-α-NF, and protease inhibitors). The column was washed with 20 column volumes of washing buffer A (50 mM KPi, pH 7.4, 20% glycerol, 40 mM imidazole, 0.05% sodium cholate, 0.02 mM DTT, and protease inhibitors). CYP2E1 enzyme was eluted with elution buffer A (50 mM KPi, pH 7.4, 20% glycerol, 350 mM imidazole, and 0.02% sodium cholate). The eluted fractions were dialyzed against dialysis buffer A (100 mM KPi, pH 7.4, 20% glycerol, 0.5 mM EDTA, and 0.1 mM DTT). The CYP2E1 enzyme was eluted with elution buffer B (300 mM KPi, pH 7.4, 20% glycerol) and dialyzed against dialysis buffer B (50 mM KPi, pH 7.4, 20% glycerol).

Rat b₅ expression plasmid was kindly provided by Dr. Ronald W. Estabrook and expressed in BL21-DE3 cells using previously described conditions (23). Expression and purification of P450 reductase was accomplished as previously described (26).

Cross-linking Reactions—All enzymes used for cross-linking reactions were dialyzed against dialysis buffer B. CYP2E1, b₅ (human b₅ or rat b₅) and DLPC were reconstituted with molar ratio of 1:1:500. The solution was gently stirred for 10 min and held at room temperature for 2 h. EDC was added to 8 mM final concentration from a 100 mM stock. The reaction was allowed to proceed at room temperature for 2 h.

Proteolytic Digestions—For in-gel proteolysis, the cross-linking reaction was quenched by the addition of an equal volume of 2 × SDS loading buffer containing 200 mM DTT. SDS-PAGE was performed, and the protein gels were stained by either Coomassie Blue or copper II chloride dihydrate. Copper staining was carried out by soaking the gel in 300 mM CuCl₂. When the desired degree of opacity was reached, the staining solution was removed, and the gel was kept in water. The band of interest was excised and washed three times with destaining buffer (25 mM Tris-HCl, 192 mM glycine, pH 8.3). After destaining, the gel piece was washed with 500 μl of 100 mM ammonium bicarbonate buffer (pH 8.5) for 10 min, dehydrated in 500 μl of acetonitrile at room temperature for 15 min, and dried in a Speed Vac for 20 min. Subsequently, the gel was rehydrated with 300 μl of 100 mM ammonium bicarbonate buffer containing 10 mM DTT, and incubated at 56 °C for 50 min to reduce the disulfides. Then the gel piece was washed with 500 μl of 100 mM ammonium bicarbonate buffer (pH 8.5) for 10 min, dehydrated in 500 μl of acetonitrile at room temperature for 15 min, and dried in the SpeedVac for 20 min. To alkylate cysteine residues, the gel piece was rehydrated with 200 μl of 100 mM ammonium bicarbonate buffer containing 60 mM iodoacetamide and incubated in the dark at room temperature for 50 min. The gel piece was cut into two halves and both pieces were then washed with 500 μl of 100 mM ammonium bicarbonate buffer (pH 8.5) for 10 min, dehydrated in 500 μl of acetonitrile at room temperature for 15 min and dried in the SpeedVac. The dried gel pieces were rehydrated with two digestion solutions (50 mM ammonium bicarbonate, pH 8.5, with sequencing grade trypsin at an enzymesubstrate ratio of 1:25 (w/w), prepared with ¹⁸O- and ¹⁸O-water respectively. The digestion was allowed to proceed.
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Acetaminophen (APAP) Oxidation Catalyzed by CYP2E1—Assays of APAP oxidation metabolites were performed with slight modifications to a previously described procedure (12). Briefly, purified CYP2E1 was reconstituted with recombinant rat P450 reductase, purified human cytochrome b5, D LPC, DOPC, and DLPS at a molar ratio of 1:2:2:250:250, respectively. The reconstituted complex was dialyzed twice against 1000 volumes of 50 mM potassium phosphate buffer, pH 7.4, for 4 h at 4 °C to remove residual glycerol, which was in the storage buffer. The incubation mixture containing 0.1 μM CYP2E1, selected concentrations of APAP (0.15, 0.3, 0.6, 1.2, 2.4, 4.8, 9.6, 15, and 19.2 mM), 10 mM GSH, and 50 mM potassium phosphate buffer (pH 7.4) was preincubated for 3 min at 37 °C. The reaction was initiated by the addition of an NADPH-generating system (0.4 mM NADP+, 10 mM glucose 6-phosphate, and 0.4 units of glucose-6-phosphate dehydrogenase). The final incubation volume was 100 μl. The assay was conducted at 37 °C for 10 min and terminated by addition of 10 μl of 33% sulfosalicylic acid (w/v). APAP metabolite 3-glutathione-S-yl-APAP (GS-APAP) was quantified on an Agilent 1100 series system. A Hewlett-Packard 1049A electrochemical detector was connected to the UV detector in tandem and was set at a constant voltage of 0.6 V. Separations were performed on a 3.5-μm Zorbax SB-C18 column (4.6 mm × 15 cm, Agilent). The isocratic mobile phase was composed of 25 mM ammonium phosphate buffer (pH 5.3) containing 10% methanol. A solvent flow rate of 1.0 ml/min was used. Nonlinear regression analysis was performed using the software GraphPad Prism 3.0 (GraphPad Software Inc.,

at 37 °C for 24 h, and the reaction was quenched with 0.1% trifluoroacetic acid.

For post-proteolysis (a proteolysis step conducted in 16O-water followed by a post-digest labeling step carried out in 18O-water), subsequent to trypsin digestion in 16O-water, the peptide mixture was dried completely in the SpeedVac. Digestion solution prepared with 18O-water was then added, and the oxygen exchange reaction was allowed to proceed at 37 °C for 24 h. The reaction was quenched with 0.1% trifluoroacetic acid.

For in-solution proteolysis, the cross-linking reaction was quenched by the removal of EDC through dialysis against dialysis buffer B. Glycerol was removed by a second dialysis against dialysis buffer C (50 mM KPi, pH 7.4). The sample was dried completely in the SpeedVac and resuspended in 6 M urea, 100 mM Tris-Base, pH 8.0, to yield a protein concentration ~2 mg/ml. 50 μl of the protein sample was transferred to another microcentrifuge tube and reduced by adding 10 μl of 100 mM Tris-Base, pH 8.0, containing 100 mM DTT. The reaction was carried out at room temperature for 1 h. Subsequent alkylation reactions were initiated by adding 30 μl of 100 mM Tris-Base, pH 8.0, containing 500 mM iodoacetamide. The reactions were allowed to proceed in the dark at room temperature for 1 h. Samples were subsequently diluted with 50 mM ammonium bicarbonate and centrifuged using an Ultrafree-4 Centrifugal Filter Unit (Millipore, Billerica, MA). The dilution and centrifugation steps were repeated three times. Samples were then split into two equal aliquots, which were dried in the SpeedVac. The two dried peptide samples were reconstituted in two digestion solutions (prepared with 16O- and 18O-water respectively). The digestion was allowed to proceed at 37 °C for 24 h, and the reaction was quenched with 0.1% trifluoroacetic acid.

ESI-QTOF MS Analysis—Protein mass spectra were recorded on an API-US quadrupole/time-of-flight (QTOF) mass spectrometer (Micromass, Manchester, UK). Protein samples were injected on a 300 μm i.d. × 5 cm perfusion column, packed with 20 μm POROS R2 particles (PerSeptive Biosystems, Framingham, MA), operated at a flow rate of 20 μl/min and interfaced on-line with the QTOF mass spectrometer. Instrument parameters were as follows: source temperature, 100 °C; N2, drying gas, 50 liters/hr; electrospray voltage, 3.8 kV; and cone voltage, 60 V. Data acquisition was carried out from m/z 800–2400 using a 2.4 s scanning time. The gradient elution profile was set as follows: 5% solvent B for 2 min and 5–90% solvent B over the next 5 min. (solvent A, 5% acetonitrile, 0.1% trifluoroacetic acid; solvent B, 95% acetonitrile, 0.1% trifluoroacetic acid).

Peptide digests were analyzed using the QTOF mass spectrometer equipped with a CapLC system (Waters, Milford, MA). The stream select module was configured with a 5 mm × 300 μm i.d. trap column packed with 5-μm C18 particles (LC Packings, San Francisco, CA) connected by a ZU1XC metallic union (Valco, Houston, TX) to a 20 cm × 75 μm i.d. nanoscale analytical column packed in-house with 5-μm Jupiter C18 particles (Phenomenex, Torrance, CA) using the method described by Kennedy and Jorgenson (27). Peptide samples were injected onto the trap column at 10 μl/min, cleaned-up and back-flushed to the analytical column at 0.5 μl/min using gradient elution. Binary gradients of 5–60% solvent B were generated over 30 min, followed by 60% B for 5 min and 60–90% B for 5 min (solvent A, 3.3% acetonitrile, 1.7% 2-propyl alcohol, and 0.1% trifluoroacetic acid; solvent B, 63.3% acetonitrile, 31.7% 2-propyl alcohol, and 0.1% trifluoroacetic acid). QTOF parameters were set as follows: electrospray potential, 3.6 kV; cone voltage, 35 V; and source temperature, 100 °C. The instrument was operated at a mass resolving power of 6,000. For MS/MS, the scan time was set to 2 s, the precursor isolation width set to 4 Da, and the collision energy set to 25–45 eV according to the m/z of the precursor and the charge state.

IT-FT-ICR MS Analysis—Peptide digests were analyzed by electrospray ionization in the positive ion mode on a hybrid ion trap–Fourier transform ion cyclotron resonance mass spectrometer (Thermo Electron Corp., San Jose, CA). Nanoflow HPLC was performed using a similar approach to that described by Yi et al. (28). The electrospray voltage was applied via a liquid junction using a platinum wire inserted into a micro-tee union (Upchurch Scientific, Oak Harbor, WA). Ion source conditions were as follows: ESI voltage, 1.4 kV; capillary temperature, 200 °C; capillary voltage, 44 V; and tube lens voltage, 180 V. All other voltages were optimized using a tuning solution composed of caffeine (Sigma), MRFA (Bachem, King of Prussia, PA) and Ultramark 1621 (LC. Synthesis, Windham, NH). Injection waveforms for the LTQ-FT ion trap and ICR cell were kept on for all acquisitions. ICR resolution was set to 50,000 (m/z 400). ICR ion populations in the ICR cell were held at 1e6 and 5e5 for MS and MS/MS, respectively. For MS/MS, the precursor isolation width was set to 10 Da and the collision energy set to 40 and 55% for quintuply and quadruply charged precursor ions, respectively.
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San Diego, CA) with the Michaelis-Menten equation fitted to the kinetic data.

*p*-Nitrophenol (PNP) Hydroxylation Catalyzed by CYP2E1—Reconstituted CYP2E1 was prepared as described above. The incubation mixture containing 30 pmol of CYP2E1, 500 μM PNP, and 50 mM potassium phosphate buffer (pH 7.4) was preincubated for 3 min at 37 °C. The reaction was initiated by adding NADPH (1 mM final concentration). In all cases the final incubation volume was 250 μl. The assay was conducted at 37 °C for 10 min and terminated by the addition of 8 μl of trifluoroacetic acid. The formation of *p*-nitrocatechol was quantified using the same HPLC column and system as used for the APAP oxidation assay. The electrochemical detector was set at a constant voltage of 0.7 V. The isocratic mobile phase was as previously described (29) and was composed of 40 mM sodium phosphate buffer (pH 2.6) containing 1 mM heptane sulfonic acid, 80 μM sodium EDTA, and 20% methanol. A solvent flow rate of 1.0 ml/min was used.

Determination of the Apparent Equilibrium Dissociation Constant (Kd) for the CYP2E1-b5 Interaction—The apparent equilibrium dissociation constant was determined by measuring the formation of APAP oxidative metabolite (GS-APAP) at a constant concentration of CYP2E1 (0.1 μM), a constant concentration of P450 reductase (0.2 μM), and varying concentrations of b5 (0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1 μM). The quantitation of GS-APAP was performed as described for APAP oxidation. The Kd value was calculated using the software GraphPad Prism 3.0.

RESULTS

Characterization of Protein Molecular Masses—Molecular masses of the investigated proteins were measured using an ESI-QTOF mass spectrometer. The measured values matched the predicted values (supplemental Figs. S3, S4, and S5), which provided quality assurance for the protein samples used in the following cross-linking experiments.

Cross-linking Reactions—First, the cross-linking reaction was carried out with equimolar CYP2E1 and human b5. Based on the measurement of the protein molecular masses, the molecular mass of a 1:1 complex (CYP2E1-human b5) is ~70 kDa, and the molecular mass of a 1:2 complex (CYP2E1-human b5) is ~85 kDa. Both complexes were absent in the blank sample, a mixture of equimolar CYP2E1 and human b5 without the addition of the cross-linking reagent (Fig. 2, A and B, lane 3). Treatment of the mixture with EDC resulted in the formation of both complexes (Fig. 2, A and B, lane 2) in the Coomassie Blue-stained gel. The yields of the 1:1 and 1:2 complexes were ~35 and 10% with respect to CYP2E1 (Fig. 2A). The 1:2 complex was visible after the protein gel was destained for 2 h (Fig. 2A), and was invisible after 12 h (Fig. 2B), whereas the 1:1 complex remained visible even after the protein gel was destained for 24 h. Subsequent increases in the amount of human b5 to two, three and four times that of CYP2E1 did not generate additional detectable complexes in the protein gel. Therefore, the generation of cross-linked species with one molecule of CYP2E1 binding to more than two molecules of human b5 is unfavorable. CYP2E1 was found to interact with rat b5, in the same way as it did with human b5.

In-gel proteolysis and subsequent MS/MS analysis were conducted with all gel bands containing the complexes. The presence of both CYP2E1 and b5 polypeptide chains in the complexes was verified by data base searching with MASCOT (Matrix Science, London, UK).

**TABLE 1**

| Measured monoisotopic peak | Charge state | Measured peptide mass | Number of 18O atoms incorporated | Mass matches to intermolecular cross-linked peptides |
|---------------------------|-------------|-----------------------|---------------------------------|--------------------------------------------------|
| 803.65                    | +4          | 3210.60               | 3                               | (Human b5: M[^Oxidized^]AHHHIIM[^Oxidized^]AEQSDEAVK)-(CYP2E1: NYGMGKQGNEWSR) (calculated peptide mass: 3210.39 Da) |
| 1071.19                   | +3          | 3210.57               | 3                               | (Human b5: EQAGGDATAENFDVGHSTDAR)-(CYP2E1: YSDYFKPSTGKR) (calculated peptide mass: 3781.70 Da) |
| 757.37                    | +5          | 3781.85               | 4                               | (Human b5: EQAGGDATAENFDVGHSTDAR)-(CYP2E1: YSDYFKPSTGKR) (calculated peptide mass: 3625.59 Da) |
| 946.45                    | +4          | 3781.80               | 4                               |                                                   |
| 726.14                    | +5          | 3625.70               | 3                               |                                                   |
| 907.43                    | +4          | 3625.72               | 3                               |                                                   |
Isotopic Labeling and Mass Spectrometric Analysis of the Peptides—To achieve maximal incorporation of $^{18}$O atoms in the peptides, in-gel proteolysis, post-proteolysis, and in-solution proteolysis were conducted as described under “Experimental Procedures.” Extensive incorporation of $^{18}$O atoms was observed only through in-solution proteolysis (supplemental Fig. S6), which resulted in complete incorporation of $^{18}$O atoms in more than 95% of the peptides. Data analysis of the peptide ions generated from the in-solution proteolysis facilitated an extensive search of cross-linked peptide candidates. By comparing peptide masses in the $^{16}$O-digest and the $^{18}$O-digest, peptides with mass increases of more than 4 Da, that is, incorporation of more than two $^{18}$O atoms, were selected as cross-linked peptide candidates.

Masses of the $^{16}$O- and $^{18}$O-labeled peptides were then analyzed by ESI-QTOF mass spectrometry. Under electrospray ionization (ESI) conditions, peptide ions exist in multiple charge states. The values of monoisotopic peak shifts represent different peptide mass increases depending on the charge states of the ions. The QTOF mass spectrometer provided sufficient resolution to resolve the isotopic distribution of different charge states so that monoisotopic shifts were observed unambiguously.

Using DetectShift, a customized software program (30), fourteen peptide ions were selected as candidate ions that incorporated more than two $^{18}$O atoms. Out of the fourteen candidate ions, six were targeted for subsequent MS/MS analysis because the masses of the six ions matched three intermolecular cross-linked peptides (Table 1). The remaining eight ions were possibly generated from intramolecular cross-linked peptides or complex cross-linked peptides containing multiple cross-linked residue pairs. Such complex cross-linked peptides are not characterized in most current mass spectrometric studies because of the difficulties in predicting peptide masses and in assigning fragment ions. Here, we only characterize intermolecular cross-linked peptides containing one cross-linked residue pair and two linear peptides.

Characterization of the Intermolecular Cross-linked Peptide Candidates Using ESI-QTOF MS—The six ions representing three intermolecular cross-linked peptide candidates (Table 1) first underwent MS/MS characterization using the QTOF mass spectrometer.

The first cross-linked peptide candidate (Table 1), with its triply charged ion at $m/z$ 1071.19 and quadruply charged ion at $m/z$ 803.65, incorporated three $^{18}$O atoms as a result of $^{18}$O-labeling (supplemental Fig. S7). The measured mass of this peptide matched the calculated mass of an intermolecular cross-linked peptide (human $b_{5}$: EQAGGDATENFEDVGHSTDAR)-(CYP2E1: YSDYFKPFGKGR). The recorded QTOF MS/MS spectrum (supplemental Fig. S8) of the quadruply charged precursor ion confirmed the peptide sequence. The possible reason for the generation of the false positive is that, although the two precursor ions were not generated from a cross-linked peptide under investigation, their masses happened to match the mass of the intermolecular cross-linked peptide.

The second cross-linked peptide candidate (Table 1), with its quadruply charged ion at $m/z$ 946.45 and quintuply charged ion at $m/z$ 757.37, incorporated four $^{18}$O atoms as a result of $^{18}$O-labeling (Fig. 3). The measured mass of this peptide matched the calculated mass of an intermolecular cross-linked peptide (human $b_{5}$: EQAGGDATENFEDVGHSTDAR)-(CYP2E1: YSDYFKPFGKGR). Both precursor ions were targeted for MS/MS analysis. Unfortunately, their MS/MS spectra were not observable using the ESI-QTOF mass spectrometer.

Characterization of the Intermolecular Cross-linked Peptide Candidates Using IT-FT-ICR MS—To further characterize the two intermolecular cross-linked peptides, the protein complex
digests were subsequently analyzed on a hybrid ion trap-Fourier transform ion cyclotron resonance mass spectrometer (IT-FT-ICR MS).

For the second cross-linked peptide candidate, the measured masses of the quadruply charged ion at \(m/z\) 946.4325 (±0.9 ppm) and the quintuply charged ion at \(m/z\) 757.3460 (±0.8 ppm) (supplemental Fig. S9) precisely matched the calculated mass of the intermolecular cross-linked peptide (human \(b_5\): EQAGGDATENFEDVGHSTDAR)-(CYP2E1: YSDYFKPFSTGKR), further confirming the existence of the peptide.

Both precursor ions were targeted for MS/MS analysis. Peptide fragmentation was induced by collision-induced dissociation (CID). Under CID conditions, b- and y-type ions are predominant fragment ions (31), and these ions were used for peptide structure characterization. Fragment ions resulting from possible losses of \(H_2O, NH_3, CO,\) or \(CO_2\) were included in the fragment ion assignments. The nomenclature of the fragment ions follows that proposed recently (32). MS/MS spectrum assignments were performed using AssignXLink, a customized software program (30). The assignments of the MS/MS spectra of both the quadruply charged precursor ion at \(m/z\) 946.4325 (Fig. 5, Table 2) and the quintuply charged precursor ion at \(m/z\) 757.3460 (Fig. 6, Table 3) revealed the same cross-link, Glu\(^{56}(b_5)\)-Lys\(^{434}(CYP2E1).

For the third cross-linked peptide candidate, the measured masses of the quadruply charged ion at \(m/z\) 907.4068 (±2.5 ppm) and the quintuply charged ion at \(m/z\) 726.1260 (±1.1 ppm) (supplemental Fig. S10) precisely matched the calculated mass of the intermolecular cross-linked peptide (human \(b_5\): EQAGGDATENFEDVGHSTDAR)-(CYP2E1: YSDYFKPFSTGKR). The acquired MS/MS spectra (Fig. 7 and Fig. 8) and the fragment ion assignments using AssignXLink (Table 4 and Table 5) facilitated the identification of the cross-link, Asp\(^{53}(b_5)\)-Lys\(^{428}(CYP2E1).

Site-directed Mutagenesis Evidence for the Importance of the MS-identified Ion Pairs in the CYP2E1-b\(_5\) Interaction—To confirm the importance of the two ion pairs identified in the CYP2E1-b\(_5\) interaction, Lys\(^{428}\) and Lys\(^{434}\) on CYP2E1 were mutated to alanine residues independently, and simultaneously. The equilibrium dissociation constants of \(b_5\) with wild-type CYP2E1, and with the CYP2E1 mutants were measured to assess the interactions, and thus the importance of the two lysine residues. In addition, a comparison of the catalytic activity of wild-type CYP2E1 to the catalytic activities of the CYP2E1 mutants in the presence of \(b_5\) was made to assess the stimulatory effect of \(b_5\).

Because the stimulatory effect of \(b_5\) is substrate-dependent, two probe substrates of CYP2E1, APAP and PNP, were selected to

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**FIGURE 4.** Isotopic distribution of the cross-linked peptide (\(b_5\)(Glu\(^{48}\)-Arg\(^{68}\)-CYP2E1(Tyr\(^{423}\)-Lys\(^{434}\)))) for its quadruply charged ion at \(m/z\) 907.43 (A) and quintuply charged ion at \(m/z\) 726.14 (B). The mass shifts of 1.5 for the quadruply charged ion and 1.2 for the quintuply charged ion indicate the incorporation of three \(^{18}O\) atoms at the C terminus of this peptide.

**FIGURE 5.** IT-FT-ICR MS/MS spectrum of the precursor ion \([M+4H]^{4+} = 946.4325\). See Table 2 for the MS/MS assignments.
provide a more complete assessment of the importance of the two ion pairs in the CYP2E1-b5 interaction. APAP oxidation showed Michaelis-Menten kinetics both in the presence and absence of b5. However, PNP hydroxylation exhibited substrate inhibition in the presence of b5, and nonsaturatable enzyme kinetics in the absence of b5, which agrees with previous reports (33, 34). Therefore, the catalytic efficiency (Vmax/Km) of APAP oxidation, and the rate of PNP hydroxylation at a concentration of PNP (500 μM) that saturates CYP2E1, were used to assess the interactions of b5 with CYP2E1 wild type and the mutants.

The most commonly used method to measure the equilibrium dissociation constants of P450 and its redox partners is to measure the spin state changes of the P450 by the addition of the redox protein. However, the addition of b5 did not alter the spin state of CYP2E1 significantly. Therefore, the apparent equilibrium dissociation constant of the CYP2E1-b5 interaction (Table 6) was determined by measuring the rate of CYP2E1-catalyzed APAP oxidation at constant concentrations of CYP2E1 and P450 reductase, and various concentrations of b5.

The catalytic activities of the wild-type CYP2E1, K428A single mutant, K434A single mutant, and K428A/K434A double mutant are shown in Table 6. In the absence of b5, the activities of the three mutants showed no significant differences from wild-type CYP2E1, indicating no significant changes of overall protein structure or local conformation in the three mutants. The addition of b5 to wild-type CYP2E1 increased the catalytic efficiency of APAP oxidation by 65-fold, and the rate of PNP hydroxylation by 8-fold. In comparison, the addition of b5 to the single mutants K428A and K434A stimulated the reactions to a lesser extent, indicating the involvement of the two lysine residues in the CYP2E1-b5 interaction, and the requirement of such interactions for maximal stimulation by b5. The substitution of alanine for both lysine residues in the K428A/K434A double mutant further reduced the stimulatory effect of b5, which, because the K434A mutant decreased the b5 stimulatory effect more than the K428A mutant. For APAP oxidation, the stimulatory effect of b5 on the K428A mutant (50-fold) was 77% of that of wild-type CYP2E1 (65-fold), whereas the stimulatory effect of b5 on the K434A mutant (21-fold) was only 32% of that of wild-type CYP2E1 (65-fold). The substitution of both lysine residues by alanine residues did not totally block, but further reduced the stimulatory effect of b5 on the metabolism of the two substrates. This indicates that although the two interacting sites are important, additional interactions are also involved.

The measured apparent equilibrium dissociation constants (Kd) satisfactorily complement the catalytic parameters. The Kd values of the mutants K428A, K434A, and K428A/K434A are 2-fold, 3-fold, and 13-fold of the Kd value of wild-type CYP2E1, indicating the importance of the two lysine residues, especially Lys434, for the CYP2E1-b5 interaction. The Kd values suggest that the two lysine residues work synergistically for the CYP2E1-b5 interaction, because the interruption of both ion pairs reduces the protein affinity by 13-fold, which is more than the effect of the interruption of each ion pair combined.

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| TABLE 2 |
| IT-FT-ICR MS/MS assignments for the precursor ion [M + 4H]4+ = 946.4325 |
|---------|---------|---------|---------|
| Labeling number | Measured m/z | Calculated m/z | Error ppm |
|---------|---------|---------|---------|
| 1 | 549.2639 | 549.2633 | 1.1 |
| 2 | 597.2646 | 597.2633 | 2.3 |
| 3 | 597.2646 | 597.2633 | 2.3 |
| 4 | 597.2646 | 597.2633 | 2.3 |
| 5 | 597.2646 | 597.2633 | 2.3 |
| 6 | 597.2646 | 597.2633 | 2.3 |
| 7 | 597.2646 | 597.2633 | 2.3 |
| 8 | 597.2646 | 597.2633 | 2.3 |
| 9 | 597.2646 | 597.2633 | 2.3 |
| 10 | 597.2646 | 597.2633 | 2.3 |
| 11 | 597.2646 | 597.2633 | 2.3 |
| 12 | 597.2646 | 597.2633 | 2.3 |
| 13 | 597.2646 | 597.2633 | 2.3 |
| 14 | 597.2646 | 597.2633 | 2.3 |
| 15 | 597.2646 | 597.2633 | 2.3 |
| 16 | 597.2646 | 597.2633 | 2.3 |
| 17 | 597.2646 | 597.2633 | 2.3 |
| 18 | 597.2646 | 597.2633 | 2.3 |
| 19 | 597.2646 | 597.2633 | 2.3 |
| 20 | 597.2646 | 597.2633 | 2.3 |
| 21 | 597.2646 | 597.2633 | 2.3 |
| 22 | 597.2646 | 597.2633 | 2.3 |
| 23 | 597.2646 | 597.2633 | 2.3 |
| 24 | 597.2646 | 597.2633 | 2.3 |
| 25 | 597.2646 | 597.2633 | 2.3 |
| 26 | 597.2646 | 597.2633 | 2.3 |
| 27 | 597.2646 | 597.2633 | 2.3 |
| 28 | 597.2646 | 597.2633 | 2.3 |
| 29 | 597.2646 | 597.2633 | 2.3 |
| 30 | 597.2646 | 597.2633 | 2.3 |
| 31 | 597.2646 | 597.2633 | 2.3 |
| 32 | 597.2646 | 597.2633 | 2.3 |
| 33 | 597.2646 | 597.2633 | 2.3 |
| 34 | 597.2646 | 597.2633 | 2.3 |
| 35 | 597.2646 | 597.2633 | 2.3 |
| 36 | 597.2646 | 597.2633 | 2.3 |
| 37 | 597.2646 | 597.2633 | 2.3 |
| 38 | 597.2646 | 597.2633 | 2.3 |
| 39 | 597.2646 | 597.2633 | 2.3 |
| 40 | 597.2646 | 597.2633 | 2.3 |

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The MS/MS spectrum is shown in Fig. 6. Ions marked with subscript /H11545 are from CYP2E1 peptide Tyr143-Arg145. Nomenclature of the fragment ions follows that proposed recently (31).

The MS/MS spectrum is shown in Fig. 6. Ions marked with subscript /H11545 are from CYP2E1 peptide Tyr143-Arg145. Nomenclature of the fragment ions follows that proposed recently (31).

TABLE 3

| Labeling number | Measured m/z | Calculated m/z | Error ppm | Assignments |
|-----------------|--------------|----------------|-----------|-------------|
| 1               | 246.1561     | 246.1566       | -2.2      | y10          |
| 2               | 540.2063     | 540.2054       | 1.7       | b12-H2O      |
| 3               | 543.7458     | 543.7447       | 2.0       | y12          |
| 4               | 611.2439     | 611.2425       | 2.2       | b12-H2O      |
| 5               | 617.2807     | 617.2789       | 3.0       | y12          |
| 6               | 665.7856     | 665.7871       | -2.2      | b12-NH4      |
| 7               | 674.3021     | 674.3004       | 2.6       | y12          |
| 8               | 676.2624     | 676.2619       | 0.9       | b12          |
| 9               | 679.3731     | 679.3685       | 5.8       | y14          |
| 10              | 789.7047     | 789.7028       | 2.3       | y10          |
| 11              | 807.1315     | 807.1278       | 4.6       | y12          |
| 12              | 809.8850     | 809.8808       | 5.2       | y12          |
| 13              | 835.8896     | 835.8845       | 6.1       | y12          |
| 14              | 841.3569     | 841.3328       | 4.9       | b12-int(12-19)-H2O |
| 15              | 844.7242     | 844.7196       | 5.5       | b12-H2O      |
| 16              | 844.7242     | 844.7317       | -8.9      | b12-int(22-19)-CO2 |
| 17              | 844.7242     | 844.7317       | -8.9      | b12-int(22-19)-CO2 |
| 18              | 850.1432     | 850.1399       | 3.9       | y17          |
| 19              | 864.3986     | 864.3953       | 3.9       | y17          |
| 20              | 870.6471     | 870.6413       | 6.7       | y16          |
| 21              | 879.4070     | 879.4033       | 4.2       | y16          |
| 22              | 882.1581     | 882.1545       | 4.0       | y16          |
| 23              | 885.1460     | 885.1426       | 3.8       | y16          |
| 24              | 893.7455     | 893.7424       | 3.5       | b14          |
| 25              | 901.1646     | 901.1614       | 3.6       | b14          |
| 26              | 936.7601     | 936.7566       | 3.7       | b14          |
| 27              | 975.1041     | 975.0989       | 5.4       | b14          |
| 28              | 981.1065     | 981.1024       | 4.1       | b14          |
| 29              | 987.7871     | 987.7840       | 3.2       | b14          |
| 30              | 994.1220     | 994.1182       | 3.8       | b14          |
| 31              | 1004.8185    | 1004.8222      | -3.4      | b14          |
| 32              | 1090.4890    | 1090.4856      | 3.1       | b14          |
| 33              | 1094.1601    | 1094.1538      | 5.7       | b14          |
| 34              | 1120.4505    | 1120.4441      | 5.8       | b14          |
| 35              | 1114.1663    | 1114.1646      | 1.5       | b14          |
| 36              | 1119.4986    | 1119.4963      | 2.1       | b14          |
| 37              | 1209.5613    | 1209.5540      | 6.0       | y18          |
| 38              | 1209.5613    | 1209.5556      | 4.7       | y18          |
| 39              | 1209.5613    | 1209.5556      | 4.7       | y18          |
| 40              | 1266.5816    | 1266.5755      | 4.8       | b14          |

FIGURE 6. IT-FT-ICR MS/MS spectrum of the precursor ion [M + 5H]+ = 757.3460. See Table 3 for the MS/MS assignments.

DISCUSSION

Chemical cross-linking in combination with mass spectrometry has developed into a powerful method for mapping low resolution three-dimensional protein structures, and for investigating molecular interfaces in protein complexes. Among several strategies (39) developed to identify cross-linked peptides in a digest mixture, 18O-labeling (21, 22) is an attractive one because it is suitable for all cross-linking reactions and, once optimized for maximal incorporation, is easy to conduct.

Compared with 18O-labeling, the commonly used method (40–43) of selecting cross-linked candidates based on peptide mass matching shows two disadvantages. First, many false signals are generated when deconvoluted masses are acquired from ESI MS data using the MaxEnt 3 function in MassLynx software (Micromass, Manchester, UK). When such deconvoluted masses are used for peptide mass matching, false cross-linked candidates are selected and true candidates potentially missed. Furthermore, it is time-consuming to assign the deconvoluted masses to the corresponding ions in the spectrum because of the multiple charge distribution of the ions. Second, certain proteolytic rules have to be designated to generate a...
predicted cross-linked peptide data base with a reasonable size. Therefore, peptides generated from abnormal cleavages are excluded from the data base. In contrast, in \(^{18}\text{O}\)-labeling experiments, the accurately measured monoisotopic and isotopic signals, instead of the software-generated deconvoluted masses, are analyzed, and cross-linked candidates caused by any type of proteolysis are selected.

In our study, mass shifts of peptides serve as a filter to select cross-linked peptide candidates that incorporate more than two \(^{18}\text{O}\) atoms. To select as many candidates as possible, experimental conditions were optimized for maximal isotopic incorporation. Both proteolysis and post-proteolysis conditions have been used to incorporate \(^{18}\text{O}\) atoms from \(^{18}\text{O}\)-water into peptides (44). However, we found that proteolysis resulted in more complete isotopic incorporation than post-proteolysis in comparative experiments with both the CYP2E1-\(b_5\) complex and bovine serum albumin. In addition, conducting \(^{18}\text{O}\)-labeling during in-gel proteolysis resulted in lower isotopic incorporation than during in-solution proteolysis. For in-gel proteolysis, complete dryness of gel pieces is difficult to achieve, even though the gel pieces are dried for a prolonged period of time (\(\sim 4\) h) before reconstitution in digestion solutions prepared with \(^{18}\text{O}\)-water. The retained \(^{16}\text{O}\)-water in the gel pieces can cause lower than maximal incorporation of \(^{18}\text{O}\) atoms. Complete incorporation of \(^{18}\text{O}\) atoms was observed for \(\sim 60\%\) of peptides obtained from in-gel proteolysis. In comparison, complete incorporation was observed for \(>95\%\) of peptides obtained from in-solution proteolysis, which facilitated an extensive search for the cross-linked peptide candidates.

Subsequent MS/MS analysis of the three selected candidates led to the identification of two intermolecular cross-linked peptides, whose structures were unambiguously characterized by IT-FT-ICR MS. IT-FT-ICR MS provides two major advantages over other available MS techniques in peptide structure elucidation. First, in IT-FT-ICR MS, less abundant precursor ions can accumulate to a desired ion population through the use of an automated gain control in the linear ion trap cell (45). Precursor ions of cross-linked peptides are generally less abundant than those of non-cross-linked peptides because of their lower stoichiometry. Moreover, MS data acquired from ESI mass spectrometers yield a wide charge distribution of peptide ions, further reducing the abundance of individual precursor ions. Without a precursor ion accumulation function, the QTOF MS/MS did not generate enough fragment ions (supplemental Fig. S8) to locate the cross-linked sites, and the fragmentation of our third cross-linked peptide (Table 1) was not achieved at all. In comparison, MS/MS analysis by IT-FT-ICR MS (Figs. 5–8) provided sufficient fragment ions for peptide structure elucidation. Second,
Identification of Interactions between CYP2E1 and b$_5$

| Labeling number | Measured m/z | Calculated m/z | Error (ppm) | Assignments |
|-----------------|--------------|----------------|-------------|-------------|
| 1               | 549.2642     | 549.2633       | 1.6         | y$_5$-H,O$^+$ |
| 2               | 618.3269     | 618.3251       | 2.9         | y$_5$-H$_2$O$^+$ |
| 3               | 636.3372     | 636.3357       | 2.3         | y$_5$-H$_2$O$^+$ |
| 4               | 648.2709     | 648.2670       | 6.1         | b$_8$-H$_2$O$^+$ |
| 5               | 678.6269     | 678.6219       | 1.5         | y$_5$-H$_2$O$^+$ |
| 6               | 686.3241     | 686.3228       | 2.8         | y$_5$-H$_2$O$^+$ |
| 7               | 694.2806     | 694.2796       | 1.4         | b-int(a13-a19)-H$_2$O$^+$ |
| 8               | 734.3461     | 734.3436       | 3.4         | y$_5$-H$_2$O$^+$ |
| 9               | 816.1273     | 816.1240       | 4.0         | y$_5$-H$_2$O$^+$ |
| 10              | 823.3247     | 823.3222       | 3.0         | b-int(a12-a19)-H$_2$O$^+$ |
| 11              | 824.8672     | 824.8641       | 3.8         | y$_5$-H$_2$O$^+$ |
| 12              | 842.4153     | 842.4120       | 3.9         | y$_5$-H$_2$O$^+$ |
| 13              | 844.8842     | 844.8807       | 4.1         | b$_8$-H$_2$O$^+$ |
| 14              | 939.4036     | 939.4030       | 0.6         | b$_8$-b$_6$-CO$^+$ |
| 15              | 957.4333     | 957.4390       | 4.6         | b$_8$-b$_6$-CO$^+$ |
| 16              | 965.9186     | 965.9107       | 8.2         | b$_8$-b$_6$-SO$_3$ |
| 17              | 980.9455     | 980.9398       | 5.9         | y$_5$-H$_2$O$^+$ |
| 18              | 980.9455     | 980.9398       | 5.8         | b$_8$-b$_6$-CO$^+$ |
| 19              | 984.4564     | 984.4521       | 4.4         | y$_5$-H$_2$O$^+$ |
| 20              | 984.4564     | 984.4527       | 0.8         | y$_5$-H$_2$O$^+$ |
| 21              | 989.9509     | 989.9450       | 5.9         | b$_8$-b$_6$-SO$_3$ |
| 22              | 991.7609     | 991.7573       | 3.6         | b$_8$-b$_6$-CO$^+$ |
| 23              | 997.7662     | 997.7608       | 5.4         | b$_8$-b$_6$-CO$^+$ |
| 24              | 1000.1125    | 1000.1096      | 2.9         | b$_8$-b$_6$-SO$_3$ |
| 25              | 1000.1125    | 1000.1096      | 2.9         | b$_8$-b$_6$-SO$_3$ |
| 26              | 1026.7900    | 1026.7850      | 4.9         | b$_8$-b$_6$-CO$^+$ |
| 27              | 1026.7900    | 1026.7887      | 1.2         | y$_5$-H$_2$O$^+$ |
| 28              | 1026.7900    | 1026.7958      | 5.6         | b$_8$-b$_6$-CO$^+$ |
| 29              | 1030.4350    | 1030.4320      | 3.0         | b$_8$-b$_6$-SO$_3$ |
| 30              | 1033.4810    | 1033.4749      | 5.9         | y$_5$-H$_2$O$^+$ |
| 31              | 1044.4615    | 1044.4554      | 5.8         | y$_5$-H$_2$O$^+$ |
| 32              | 1102.4474    | 1102.4441      | 3.0         | b-int(a10-a19)-$^{+1}$ |
| 33              | 1121.8215    | 1121.8170      | 4.0         | b$_8$-b$_6$-CO$^+$ |
| 34              | 1127.8237    | 1127.8205      | 2.8         | b$_8$-b$_6$-CO$^+$ |
| 35              | 1131.5078    | 1131.5035      | 3.9         | b$_8$-H$_2$O$^+$ |
| 36              | 1197.5322    | 1197.5302      | 2.2         | b$_8$-b$_6$-CO$^+$ |
| 37              | 1233.5573    | 1233.5500      | 5.9         | y$_5$-H$_2$O$^+$ |
| 38              | 1271.0659    | 1271.0644      | 1.2         | y$_5$-H$_2$O$^+$ |
| 39              | 1347.5998    | 1347.5929      | 5.1         | y$_5$-H$_2$O$^+$ |
| 40              | 1353.6074    | 1353.5999      | 5.5         | y$_5$-H$_2$O$^+$ |
| 41              | 1364.5610    | 1364.5577      | 3.0         | b$_8$-b$_6$-SO$_3$ |
| 42              | 1393.1062    | 1393.0992      | 5.0         | b$_8$-b$_6$-CO$^+$ |
| 43              | 1403.5788    | 1403.5715      | 5.2         | b-int(a7-a19)-$^{+1}$ |
| 44              | 1403.5788    | 1403.5715      | 5.2         | b-int(o8-a20)-$^{+1}$ |
| 45              | 1442.6149    | 1442.6390      | 2.0         | b$_8$-b$_6$-SO$_3$ |

The importance of the two ion pairs, Lys$^{428}$(CYP2E1)-Asp$^{53}(b_5)$ and Lys$^{434}$(CYP2E1)-Glu$^{56}(b_5)$, identified by mass spectrometry in the CYP2E1-b$_5$ interaction was confirmed by site-directed mutagenesis, and by the measurement of protein apparent equilibrium dissociation constants ($K_D$) and catalytic activities. The $K_D$ values confirmed the involvement and suggested a synergistic effect of the two ion pairs for the CYP2E1-b$_5$ interaction. The disruption of either ion pair reduced the interaction and thus, the stimulatory effect of b$_5$. Simultaneous interruption of the formation of both ion pairs further diminished the interaction, resulting in approximately a 43% decrease in the stimulatory effect of b$_5$ observed in the K428A/K434A double mutant on PNP hydroxylation, and approximately an 82% decrease on APAP oxidation, compared with the stimulatory effect of b$_5$ in wild-type CYP2E1. The results indicate that the two ion pairs, Lys$^{428}$(CYP2E1)-Asp$^{53}(b_5)$ and Lys$^{434}$(CYP2E1)-Glu$^{56}(b_5)$, in conjunction with
other intermolecular interactions, are structurally responsible for the stimulatory effect of \( b_5 \) on CYP2E1 oxidation rates.

In agreement with these results, a model constructed on the basis of the two identified “zero-length” cross-links, suggests nine ion pairs (Table 7) and seven H-bonds (supplemental Table S1) as part of the CYP2E1-\( b_5 \) interacting surface. Compared with Lys 428, Lys 434 appears to play a more significant role because the \( K_d \) value of the K434A mutant is higher than that of the K428A mutant, and because there is less stimulatory effect in the K434A mutant. This result agrees with the model, as well, in that among all proposed ion pairs, Lys434 is involved in two ion pair interactions, Lys434-\( (\text{CYP2E1}) \)-Glu434(\( b_5 \)) and Lys434-\( (\text{CYP2E1}) \)-Asp484(\( b_5 \)), whereas, Lys428 is involved only in one interaction, Lys428(\( \text{CYP2E1} \))-Asp428(\( b_5 \)) (Table 7).

In the study, two ion pairs, Lys428(\( \text{CYP2E1} \))-Asp428(\( b_5 \)) and Lys434(\( \text{CYP2E1} \))-Glu434(\( b_5 \)), were identified by mass spectrometry and seven more ion pairs were proposed by the constructed model (Table 7). Because EDC covalently links basic (Lys) and acidic (Asp or Glu) residues that come into proximity, a question arises why three of the proposed ion pairs containing lysine residues, Lys434(\( \text{CYP2E1} \))-Glu434(\( b_5 \)), Lys432(\( \text{CYP2E1} \))-Glu432(\( b_5 \)), and Lys434(\( \text{CYP2E1} \))-Asp434(\( b_5 \)), were not identified by mass spectrometry. There are four possible reasons. First, the competition for ion pair formation, caused by the proximity of one charged residue to several others with

### Table 6
Characterization of wild-type CYP2E1 and the mutants K428A, K434A, and K428A/K434A

| Mutant     | PNP hydroxylation rate \(+ b_5\) nmol/nmol/min | Ratio of activity \(+ b_5 / - b_5\) | APAP oxidation kinetic parameters | Catalytic efficiency \(+ b_5\) | Catalytic efficiency \(- b_5\) | Ratio of catalytic efficiency \(+ b_5 / - b_5\) | \( K_d \) (app) of CYP2E1-\( b_5 \) complex µM |
|------------|-----------------------------------------------|-------------------------------|-----------------------------------|-----------------------------|-----------------------------|----------------------------------------|---------------------------------|
| WT         | 23.13 ± 1.96                                 | 8.0                           |                                    | 9.11 ± 0.09                | 1.75 ± 0.07                | 5.21                                   | 60.0 ± 0.01                     | 79.9 ± 0.29                | 0.88                                   | 65                          | 0.03 ± 0.01                 |
| K428A      | 18.44 ± 0.86                                 | 6.5                           |                                    | 6.98 ± 0.07                | 2.32 ± 0.09                | 3.01                                   | 52.0 ± 0.01                     | 81.9 ± 0.32                | 0.06                                   | 50                          | 0.06 ± 0.01                 |
| K434A      | 17.23 ± 1.09                                 | 5.6                           |                                    | 4.77 ± 0.06                | 3.31 ± 0.14                | 1.44                                   | 73.0 ± 0.07                     | 98.7 ± 0.48                | 0.07                                   | 21                          | 0.09 ± 0.01                 |
| K428A/K434A| 12.39 ± 1.05                                 | 4.6                           |                                    | 2.65 ± 0.05                | 3.79 ± 0.22                | 0.70                                   | 47.0 ± 0.01                     | 73.0 ± 0.37                | 0.06                                   | 12                          | 0.38 ± 0.06                 |

**FIGURE 9. CYP2E1-\( b_5 \) complex model.** CYP2E1 is colored yellow with its heme group colored black; \( b_5 \) is colored gray with its heme group colored green. The interacting residues on CYP2E1 and \( b_5 \) are colored blue and red, respectively. A, interacting regions on CYP2E1 and \( b_5 \) are colored brown and purple, respectively. Protein regions far away from the interacting surfaces are truncated. B, molecule of \( b_5 \) in the complex is rotated 180 degrees to the right to display the interacting surfaces on CYP2E1 and \( b_5 \) completely. Nitrogen atoms of the side chains of the positively charged interacting residues on CYP2E1 are colored blue; oxygen atoms of the side chains of the negatively charged interacting residues on \( b_5 \) are colored red. Atom radii are decreased to 1.2 Å so that CYP2E1 heme can be seen.
Identification of Interactions between CYP2E1 and b5

TABLE 7
Summary of intermolecular electrostatic interactions in the CYP2E1-b5 complex model

| CYP2E1 residue   | b5 residue   | Intermolecular distance (Å) |
|------------------|--------------|-----------------------------|
| Lys334 (β-bulge region) | Glu56 (α4-helix) | 3.148                       |
| Lys120 (meander region) | Asp53 (loop region between α3- and α4-helices) | 6.023                       |
| Lys42 (J-helix) | Glu43 (α2-helix) | 6.675                       |
| Arg334 (J-helix) | Glu56 (α3-helix) | 6.376                       |
| Lys122 (meander region) | Glu43 (α3-helix) | 6.366                       |
| Arg334 (J-helix) | Glu56 (α3-helix) | 2.772                       |
| Arg444 (L-helix) | Glu56 (α3-helix) | 3.031                       |
| Arg126 (C-helix) | Asp56 (α4-helix) | 3.520                       |
| Lys334 (β-bulge region) | Asp56 (α4-helix) | 3.453                       |

opposite charge, may decrease the abundance of each ion pair, therefore preventing it from being identified by mass spectrometry. For example, Lys42 (CYP2E1)–Glu43 (b5), an ion pair predicted by the model, was not identified by mass spectrometry as a cross-link. An examination of the model reveals that Glu56 (b5) interacts with two other residues, Arg334 (CYP2E1) and Arg444 (CYP2E1), whereas Lys42 (CYP2E1) forms an intramolecular ion pair with Asp53 (CYP2E1). All the above interactions decrease the effective local charges of Lys42 (CYP2E1) and Glu43 (b5), thus reducing the abundance of the ion pair between the two residues. In agreement with our experimental results, the model shows that the two ion pairs identified by mass spectrometry are more specific because of less local distortion. Second, peptide yields from proteolysis vary, and the ability of peptides to form multiply charged molecular ions under the same ESI conditions differ. As a result, peptides with low yields, and those that do not efficiently generate ions, may not be observed. Third, the actual intermolecular atomic distances of the three undetected ion pairs may deviate from the predicted values and may be beyond the distance range of EDC-mediated cross-linking reactions, because neither protein flexibility nor protein local conformational changes induced by protein-protein interactions can be predicted by a rigid complex model (46). Fourth, lacking an x-ray crystallographic structure of CYP2E1, a homology model was used in this study to construct the complex model. The possible deviation of the atomic coordinates in the homology model leads to a less accurate prediction of the intermolecular atomic distances.

In agreement with previous proposals, our model (Fig. 9) constructed on the basis of the two cross-links shows that electrostatic interactions (Table 7) are the main stabilizing forces for the protein-protein interaction and contribute to the proper relative orientations of the prosthetic groups, which may result in a change of dielectric constant and facilitate the subsequent electron-transfer process. For all the proposed electrostatic interactions (Table 7), the negatively charged residues are contributed by b5. These residues are located on the surface region where the b5 heme group protrudes toward the solvent. The positively charged residues contributed by CYP2E1 are distributed across the proximal face of CYP2E1 where the buried CYP2E1 heme group comes closest to the solvent.

The surface region on b5 has previously been reported for its interactions with several redox proteins, such as P450, NADH-b5 reductase, cytochrome c, NADPH-P450 reductase and stearyl coenzyme A desaturase (4). The negatively charged residues on this surface region are conserved across species (3). In our study, the analysis of the complexes of CYP2E1-rat b5 and CYP2E1-human b5 identified the same cross-links, suggesting that the evolutionary conservation of the negatively charged residues is related to their functions in protein-protein interactions.

Previous studies, using geometric fit algorithms for protein docking, have shown three surface regions on CYP2B4 that can possibly bind to b5 (47). The first surface region is located on the distal face of CYP2B4 and includes residues in the A-helix, the E*-helix, the F-helix, the I-helix, and the β4-sheet. The second region is located on a face perpendicular to the heme plane of CYP2B4 and includes residues in the B’-helix. The third region is located on the proximal face of CYP2B4 and includes residues in the C-C*-helices, the β5-sheet, the K-helix, the meander region, the β-bulge and the L-helix. Studies with CYP2B4 and several other P450 isoforms, which used site-directed mutagenesis, synthetic peptide binding and chemical modification, suggest important roles for several basic residues on the third surface region in b5 binding (3, 4, 14, 48). In the CYP2E1-b5 interaction, most interacting residues on CYP2E1 (Table 7), such as Lys42 and Lys43 in the meander region, Lys334 in the β-bulge region, Arg126 in the C-helix, and Arg444 in the L-helix, are located on the third surface region. However, different from the third surface region, the CYP2E1-b5 model shows the involvement of the J’-helix of CYP2E1, instead of the β5-sheet, for b5 binding.

Compared with the CYP101-b5 complex model constructed in 1989 (16), our model shows the same interacting surface on b5 and some overlapped regions on P450. The main difference in the two models is that the β-helix of CYP101 is part of the β5 binding region, whereas the J’-helix of CYP2E1 is part of the β5 binding region. Furthermore, the protein docking orientations in the two models differ by about 90 degrees. In the CYP101-b5 model, the α3-helix of b5 is close to the B-helix, the meander region and the β-bulge of CYP101; whereas the α4-helix of b5 is in the proximity of the C-helix of CYP101; and the b5 heme propionate protrudes toward the L-helix of CYP101. In the CYP2E1-b5 model, the α3-helix of b5 is close to the L- and the J’-helices of CYP2E1; whereas the α4-helix of b5 apposes the meander region and the β-bulge of CYP2E1; and the b5 heme propionate protrudes toward the end of the C-helix of CYP2E1. Because of the structural differences in P450 isoforms, it remains unclear whether the protein interacting orientations are P450 isoform-dependent.

The generation of a CYP2E1-(b5)2 complex (Fig. 2) suggests more than one b5 binding region on CYP2E1. As mentioned, three surface regions on CYP2B4 were proposed for their possible involvement in b5 binding (16). The homology model of CYP2E1 shows three similar surface regions. Other than the predominant interaction (Fig. 9), b5 may approach the other surface regions, yielding the CYP2E1-(b5)2 complex. In addition, it has been shown that there are at least two positively charged regions on P450, one for the binding of b5 and the other for the binding of P450 reductase (18, 49, 50). In the absence of P450 reductase, the second molecule of b5 probably occupies the reductase binding region. However, the association of CYP2E1 to the second molecule of b5 appears to be much less favorable because the CYP2E1-(b5)2 complex band
was very weak and disappeared in the protein gel destined for more than 12 h (Fig. 2). It is possible that the less favorable interaction between CYP2E1 and $b_5$ does not provide as many stabilizing forces as the primary interaction does. Ion pairs in the less favorable protein complex were not identified by mass spectrometry because of their low abundance.

In conclusion, two intermolecular ion pairs in the CYP2E1-$b_5$ complex were characterized using chemical cross-linking, isotopic labeling and mass spectrometry, and the biological importance of the ion pairs was confirmed by site-directed mutagenesis. This study reveals the protein interacting surfaces and provides the first direct evidence to support protein orientations in a P450-$b_5$ complex. Inasmuch as some P450 isoforms (e.g. CYP1A2 and CYP2D6) have a more positively charged surface and are not affected by $b_5$, additional studies are underway to assess the specificity of this cross-linking method.

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