Electrospray Ionization Mass Spectrometric Analysis of Intact Cytochrome P450: Identification of Tienilic Acid Adducts to P450 2C9†

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ABSTRACT: A general scheme for the purification of baculovirus-expressed cytochrome P450s (P450s) from the crude insect cell pastes has been designed which renders the P450s suitable for analysis by high-performance liquid chromatography (HPLC) electrospray ionization mass spectrometry (ESI-MS). An HPLC/ESI-MS procedure has been developed to analyze small amounts of intact purified P450 (P450s cam-HT, 1A1, 1A2, 2A6, 2B1, 2C9, 2C9 C175R, 3A4, 3A4-HT) and rat NADPH cytochrome P450 reductase (P450 reductase). The experimentally determined and predicted (based on the amino acid sequences) molecular masses (MMs) of the various proteins had identical rank orders. For each individual protein, the difference between the experimentally determined (±SD, based on experiments performed on at least 3 different days) and predicted MMs ranged from 0.002 to 0.035%. Each experimentally determined MM had a standard deviation of less than 0.09% (based on the charge state distribution). Application of this HPLC/ESI-MS technique made the detection of the covalent modification to P450 2C9 following mechanism-based inactivation by tienilic acid possible. In the absence of glutathione, three P450 2C9 species were detected that produced ESI mass spectra corresponding to native P450 2C9 and both a monoadduct and a diadduct of tienilic acid to P450 2C9. In the presence of glutathione, only native P450 2C9 and the monoadduct were detected. Based on the observed mass shifts for the P450 2C9/tienilic acid adducts, a mechanism for the inactivation of P450 2C9 by tienilic acid is proposed.

Recent advances in the field of mass spectrometry have made the accurate mass determination of large biomolecules not only possible, but largely routine. Electrospray ionization mass spectrometry (ESI-MS)[1] has proved to be especially useful in this regard since it is a soft ionization technique that generates multiply charged gas-phase ions directly from biomolecules in solution (*1*). The cytochrome P450s (P450s) are a superfamily of hemoproteins (MM ≈ 55 kDa) that are important, particularly to drug metabolism, for the oxidation of a wide variety of exogenous and endogenous compounds. Advances in the field of recombinant protein chemistry have made the expression of individual P450s possible (*2*, *3*). As a result, P450s have been successfully expressed using a number of cell types including bacterial, yeast, insect, and mammalian cell lines, each of which possesses its own unique benefits and disadvantages. The baculovirus/insect cell system is especially attractive for the high-level expression of membrane-bound mammalian P450s and for subsequent purification and reconstitution. Because these enzymes are membrane-bound, the difficulty in obtaining an X-ray structure still has to be resolved while methodology to obtain mass spectrometric data of the intact proteins is just being realized.

Two methods that are used to identify amino acid residues critical to conferring a characteristic P450 activity are (i) site-directed mutagenesis followed by analysis of the functional consequence of the mutation (*4*, *5*), and (ii) sequencing of non-null allelic variants (*6*−*8*). Another potential technique for identifying these substrate recognition sites involves the use of mechanism-based inactivators (MBIs) (*9*−*14*). MBIs undergo catalytic processing by the P450, resulting

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[1] Abbreviations: ACN, acetonitrile; β-ME, β-mercaptoethanol; cytochrome b_5, human cytochrome b_5; DLPC, L-α-dilauroylphosphatidylcholine; DTT, dithiothreitol; FBS, fetal bovine serum; GSH, reduced glutathione; HPLC/ESI-MS, high-performance liquid chromatography electrospray ionization mass spectrometry; MBI, mechanism-based inactivator; MM, molecular mass; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PEG, poly(ethylene glycol); P450, cytochrome P450; P450 cam-HT, poly(His)-tagged P450 cam; P450 3A4-HT, poly(His)-tagged P450 3A4; P450 reductase, rat NADPH cytochrome P450 reductase; PI, post infection; PMSF, phenylmethylsulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

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in the generation of a reactive intermediate which modifies P450 apoprotein and/or heme (*15*) and leads to enzyme inactivation. Identification of the site of covalent modification has been approached using a radiolabeled MBI, isolating radiolabeled fragments of the covalently modified protein after proteolysis, and identifying the bound amino acid residue(s) chemically (Edman degradation) or by mass spectrometry. To obtain sequence information, identify the site(s) of covalent modification, and study the functional consequence of these covalent modifications, the proteins under investigation should be in a purified state. However, purified P450s usually require saturating amounts of NADPH cytochrome P450 reductase and, in some cases, cytochrome b_5 for full catalytic activity (*16*). Thus, to analyze the P450 after covalent modification by either chemical means or mass spectrometry, it is necessary to develop a technique that separates the P450 from the other proteins present in the incubation mixture.

Herein, we describe a generally applicable purification procedure for preparing baculovirus-expressed P450s for high-performance liquid chromatography (HPLC) ESI-MS analysis. The utility of this protocol is demonstrated by the use of HPLC/ESI-MS to separate the components of the reconstituted P450 system, to analyze intact purified P450s, and to detect the incorporation of the reactive electrophile of tienilic acid into the P450 2C9 apoprotein following mechanism-based inactivation.

MATERIALS AND METHODS

Materials. Trichoplusia ni (T. ni) cells (High Five cells) were from Invitrogen (San Diego, CA). Glycerol, sodium cholate, reduced nicotinamide adenine dinucleotide phosphate (NADPH), catalase, L-α-dilauroylphosphatidylcholine (DLPC), dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), hemin, β-mercaptoethanol (β-ME), trypsin (sequencing grade), and Octyl Sepharose CL-4B were purchased from Sigma (St. Louis, MO). EDTA, reduced glutathione (GSH), and glycerol were from Aldrich (Milwaukee, WI). Emulgen 911 was from Kao-Atlas (Tokyo, Japan). Tienilic acid was from laboratory stock. DEAE Sepharose Fast Flow (DEAE-FF) was from Pharmacia (Piscataway, NJ), and ceramic hydroxyapatite was from Bio-Rad Laboratories (Hercules, CA). Ni^{2+}-NTA Resin was from Qiagen (Chatsworth, CA). HyQCCM-3 media and fetal bovine serum (FBS) were from HyClone Laboratories, and 0.5−3 mL Slide-A-Lyzer dialysis kits were from Pierce (Rockford, IL). Trifluoroacetic acid (TFA) was from Applied Biosystems (Foster City, CA). HPLC solvents were of the highest grade commercially available and were used as received.

Protein Expression. Rat NADPH cytochrome P450 reductase (P450 reductase) and human cytochrome b_5 (cytochrome b_5) were expressed and purified from bacterial cultures according to published procedures (*17*, *18*). Spectral P450 was determined by the method of Omura and Sato (*19*) and was used as a measure to establish optimal conditions for P450 expression. The P450 2C9 C175R point mutant was constructed using Transformer mutagenesis protocols and expressed in a baculovirus/*T. ni* insect cell system using protocols previously described (*5*). P450s 1A2, 2A6, 2C9, and 3A4 coexpressed with P450 reductase were expressed using methodology analogous to that previously described for P450 2C9 (*5*), except 150 × 25 mm culture dishes were used instead of suspension cultures. In all cases, cell culture medium was supplemented with 7.5% (v/v) FBS. Hemin (final concentration, 1 μg/mL) was added at 30, 60, 30, and 30 h post infection (PI) for optimal expression of P450 1A2, 2A6, 2C9, and 3A4, respectively. The insect cell pellets were harvested by centrifugation at 6000*g* for 10 min at 108, 120, 120, and 120 h PI for P450 1A2, 2A6, 2C9, and 3A4, respectively. The procedures used here for P450 expression generally resulted in yields of 5−10 nmol/plate which is equivalent to 200−400 nmol/L. The insect cell pellets were stored at −80 °C in buffer (50 mM potassium phosphate, pH 7.4) containing glycerol [20% (v/v)] and EDTA (1 mM) until further use.

Purification of Baculovirus-Expressed P450s. All steps were carried out at 4 °C, and all buffers were at pH 7.4. For the purification of P450 3A4, it was necessary to include ethylmorphine (50 μM) in all buffers until the final dialysis step. The crude insect cell pellet was homogenized in 1 mL of Solubilization buffer [20% (v/v) glycerol, 1 mM EDTA, 1 mM DTT, 0.4 mM PMSF, and 0.5% (w/v) cholate in 50 mM potassium phosphate buffer] per nanomole of P450 by making 5−10 passes with a glass/Teflon homogenizer. The P450 was solubilized by stirring the homogenized insect cell pellet for 45 min followed by removal of insoluble material by centrifugation at 100000*g* for 35 min. The yellow-brownish supernatant was applied to an Octyl Sepharose CL-4B column (1 mL/2 nmol of P450) preequilibrated with Buffer A [20% (v/v) glycerol, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, and 0.5% (w/v) cholate in 50 mM potassium phosphate buffer] at 60 mL/h. Once the protein was loaded, the column was washed with 4 column volumes of Buffer A + 0.15% (v/v) Emulgen 911. When the R_f was 0.5, the P450-enriched fraction was eluted with Buffer A + 1% (v/v) Emulgen 911.

The P450-containing fractions were diluted 2-fold with Buffer B [20% (v/v) glycerol, 1 mM EDTA, and 0.5 mM DTT in 5 mM potassium phosphate buffer] and loaded onto a DEAE-FF column preequilibrated with Buffer B + 0.5% (v/v) Emulgen 911 (3 mL/2 nmol of P450) at 80 mL/h. The flow-through was collected in fractions (5 mL), and their purity was analyzed by 9% SDS−PAGE. The fractions that were electrophoretically homogeneous were adsorbed onto a ceramic hydroxyapatite column (3 mL/50 nmol) preequilibrated with Buffer C [20% (v/v) glycerol, 1 mM EDTA, 0.5 mM DTT, and 0.5% (v/v) Emulgen 911 in 25 mM potassium phosphate buffer] at 80 mL/h. The column was washed with Buffer D [20% (v/v) glycerol, 1 mM EDTA, 0.5 mM DTT, and 0.1% (w/v) cholate in 25 mM potassium phosphate buffer] until $A_{276}-A_{350} < 0.01$ (≈400 mL of Buffer D). The P450 was eluted with Buffer E [20% (v/v) glycerol, 1 mM EDTA, and 0.5 mM DTT in 500 mM potassium phosphate buffer], and fractions (0.5 mL) were collected. The Emulgen-free P450 was dialyzed overnight against 2 × 100 volumes of Buffer F [20% (v/v) glycerol and 1 mM EDTA in 50 mM potassium phosphate buffer] using a 0.5−3 mL Slide-A-Lyzer dialysis kit. The purified P450 sample was divided into aliquots and stored at −80 °C until further use.

Purification of Non-Baculovirus-Expressed Proteins. Rat P450s 2B1 and 1A1 were purified from phenobarbital- and 3-methylcholanthrene-induced rat liver microsomes, respectively, according to published procedures (*20*). Poly(His)-

tagged P450 cam (P450 cam-HT) and P450 3A4 (P450 3A4-HT) were expressed in *E. coli* using previously described procedures (*21*, *22*) and purified using a Ni^{2+}-NTA column as follows. P450 3A4-HT was coexpressed with P450 reductase. All purification steps were performed at 4 °C. The poly(His)-tagged proteins were solubilized for 75 min in Binding buffer [20 mM potassium phosphate buffer (pH 7.4), 500 mM KCl, 0.1% Emulgen 911, and 20% (v/v) glycerol] + 0.4% (v/v) Emulgen 911 (1 mL/nmol). Insoluble material was removed by centrifugation at 100000*g* for 60 min. The supernatant was applied to a Ni^{2+}-NTA column (1 mL/35 nmol) preequilibrated in Binding buffer. Nonspecifically bound material was removed with Binding buffer + 75 mM imidazole. Binding buffer + 1 M imidazole was used to elute the P450 as a bright red fraction. This sample was dialyzed immediately against 2 × 100 volumes of Binding buffer + 0.2 mM DTT. Triton X-100 [0.5% (v/v)] or sodium cholate [0.2% (w/v)] could be substituted for Emulgen 911 in the purification of the poly(His)-tagged proteins. Nonionic detergent was removed using a ceramic hydroxyapatite column as described above for the purification of baculovirus expressed P450s.

Instrumentation. HPLC/ESI-MS was performed on a Fisons VG Quattro II triple quadrupole mass spectrometer fitted with a Megaflow Electrospray source. The instrument was controlled by a Digital DECpcLPx 466d2 computer running Fisons Mass Lynx 0.1 software. Instrument settings were as follows: source temperature, 100 °C (intact protein analysis) or 150 °C (tryptic digest analysis); N_2 drying gas = 150 L/h; nebulizing gas = 20 L/h; probe voltage = 3.8 kV; cone voltage = 42 kV (intact protein analysis), 30−35 kV (tryptic digest analysis); probe position, on axis. Acquisition was carried out from *m*/*z* 200−2000 Da or 1000−4000 Da over 4.5 s in the CENTROID or CONTINUUM scanning mode, with unit resolution up to at least 1500 Da based on the calibration and resolution optimization using a mixture of poly(ethylene glycol) (PEG) 300/600/1000/1500. The HPLC interface to the mass spectrometer consisted of Shimadzu LC10AD solvent delivery modules and a Shimadzu SPD-10AV module UV−Vis spectrophotometric detector with wavelength detection at 214 nm.

For intact protein analysis, each protein (200−300 pmol of spectrally detectable P450, in most cases) was injected onto the HPLC equipped with a POROS R2 perfusion column (4.6 × 100 mm) from Perseptive Biosystems (Cambridge, MA) at a flow rate of 3 mL/min with ≈2% (50 μL) directed to the mass spectrometer using a splitter. The solvent gradient consisted of buffer A [0.05% CF_3COOH (TFA) (pH 3.0)] and buffer B [0.05% TFA in 95:5 acetonitrile (ACN)/H_2O (pH 2.5)]. Proteins were separated using a gradient elution of 35−50% buffer B from 0 to 5 min, isocratic elution at 50% buffer B from 5 to 6.5 min, and gradient elution of 50−100% buffer B from 6.5 to 10 min. Under these conditions, heme eluted at 2.4 min, P450 reductase eluted at 4.0 (truncated form) and 5 min (full-length form), and P450 eluted at approximately 8 min. ESI mass spectra were collected, the individual scans across the HPLC peak were combined, and each spectrum was deconvoluted using the MaxEnt computer program.

Reconstitution Procedures. The desired amounts of P450 2C9, P450 reductase, and cytochrome b_5 were combined and dialyzed overnight against at least 100 volumes of buffer (50 mM potassium phosphate, pH 7.4). This initial dialysis step was performed in an attempt to remove any residual glycerol and/or detergent present in the purified protein samples. The reconstituted P450 2C9 system was composed of P450 2C9 (1 nmol), P450 reductase (3 nmol), cytochrome b_5 (1 nmol), and DLPC (50 μg) in buffer (50 mM potassium phosphate, pH 7.4) in relative molar amounts required for optimal (*S*)-warfarin 7-hydroxylase activity (*5*). Catalase (2000 units/mL) was added to each incubation to prevent inactivation of P450 by reactive oxygen species. P450, P450 reductase, cytochrome b_5, catalase, and DLPC were reconstituted in that order and allowed to mix for 60 min on ice. Tienilic acid (1 mM), buffer (50 mM potassium phosphate, pH 7.4), and nucleophilic trapping agents (where indicated) were added, and this mixture was preincubated at 30 °C for 2 min. NADPH (1 mM) was added to initiate the reaction (final incubation volume, 500 μL) which was allowed to proceed for 45 min to ensure complete enzyme inactivation. The inactivated enzyme mixture was kept on ice prior to injection onto the HPLC/ESI-MS system for determination of the MM of the intact protein as described above.

P450 2C9 Activity Assays. Reconstituted P450 2C9 (150 pmol) was preincubated with GSH (0−10 mM) and tienilic acid (100 μM) in buffer (50 mM potassium phosphate buffer, pH 7.4) for 3 min at 30 °C. The reaction was initiated by the addition of NADPH (1 mM) (final incubation volume, 300 μL) and terminated after 30 min by the addition of 1.2 N $HClO_4$ (50 μL). The samples were set on ice, and after centrifugation for 10 min at 2500 rpm (HNS II Centrifuge, International Equipment Co.), an aliquot (100 μL) of the supernatant was injected onto an HPLC equipped with a reverse-phase C8 column (Econosphere 5 μm, 150 mm × 4.6 mm) operating at a flow rate of 1 mL/min. Tienilic acid metabolite formation was monitored at 300 nm using wavelength detection. The solvent gradient consisted of buffer A [0.05% CF_3COOH (TFA) (pH 3.0)] and buffer B [0.05% TFA in 95:5 acetonitrile (ACN)/H_2O (pH 2.5)]. Gradient elution using buffer A and buffer B from 20 to 55% buffer B over 9 min yielded retention times of 7.5 and 9.3 min for 5-hydroxytienilic acid and the parent compound, respectively. 5-Hydroxytienilic acid was purified by HPLC after incubation and exhibited absorbance maxima at 346 (pH 2.5) and 384 nm (pH 11), in accord with maxima previously reported (*23*). There was no detectable conversion of tienilic acid to 5-hydroxytienilic acid after a 24 h exposure to 1 N $HClO_4$. Due to significant degradation of 5-hydroxytienilic acid in acidic solution, it was critical to analyze tienilic acid metabolite formation immediately.

Partition ratios were calculated for the inactivation of P450 2C9 by tienilic acid using a substrate depletion method and the equation: partition ratio = amount of inactivator consumed ÷ spectrally detectable P450 2C9 present in the incubation (*24*). Metabolite formation was monitored by HPLC with wavelength detection as described above in addition to ESI-MS by diverting ≈5% (50 μL) of the flow to the mass spectrometer. The mass spectrometer was set to scan from 100 to 1000 Da over 2.5 s. Reconstituted P450 2C9 (187.5 pmol) was preincubated with GSH (10 mM) or β-ME (5 mM) and tienilic acid (100 μM) in buffer (50 mM potassium phosphate buffer, pH 7.4) for 3 min at 30 °C. The reaction was initiated by the addition of NADPH (1 mM) or buffer (50 mM potassium phosphate buffer, pH 7.4) (final

incubation volume, 300 μL) and terminated after 60 min by the addition of 1.2 N $HClO_4$ (50 μL). The samples were set on ice and centrifuged, and an aliquot (100 μL) was injected onto the HPLC and analyzed for metabolite formation as described above.

P450 2C9 spectral content was determined after exposure to NADPH in the absence or presence of tienilic acid. Reconstituted P450 2C9 (600 pmol) was preincubated with catalase (2000 units) and tienilic acid (100 μM) in the absence or presence of GSH (0 or 10 mM) in buffer (50 mM potassium phosphate buffer, pH 7.4) for 3 min at 30 °C. The reaction was initiated by the addition of NADPH (1 mM) or buffer (50 mM potassium phosphate buffer, pH 7.4) (final incubation volume, 600 μL). At various time points (0−15 min), an aliquot (100 μL) was removed and diluted 1:5 into Solubilization buffer [20% (v/v) glycerol, 1 mM EDTA, 1 mM DTT, 0.4 mM PMSF, and 0.5% (w/v) cholate in 50 mM potassium phosphate buffer] and set on ice. P450 2C9 spectral content was determined according to the method of Omura and Sato (*19*).

RESULTS AND DISCUSSION

Protein Expression and Purification. Purified proteins were obtained from a number of different sources including rat liver microsomes and insect and bacterial cell pastes. *T. ni* cells were infected with the homologous recombinant baculoviruses containing the human P450's, 1A2, 2A6, and 2C9, and 3A4 coexpressed with P450 reductase. The culture medium was supplemented with hemin at time points found to result in optimal P450 expression levels. This procedure resulted in the expression of sufficient quantities of P450 for purification using a minimal amount of cell culture media and reagents.

The purification of P450s was accomplished using procedures described previously (*5*, *25*) with the following modifications. The modifications are critical if high overall yields and purity of proteins suitable for HPLC/ESI-MS are to be obtained. The initial purification step consisted of using cholate to extract the P450 fraction from the insect cell pellet. Increasing the concentration of cholate did not result in a greater amount of extracted P450, and in the case of P450 3A4, a substrate, ethylmorphine, had to be included in the cholate buffer to prevent a severe loss in P450 spectral content. An initial PEG solubilization step has been used for the purification of baculovirus-expressed P450s (*5*, *25*). However, once PEG is added to the sample (even if as an initial step), it is virtually impossible to remove completely. Due to its facile ionization, the residual trace amount of PEG makes the purified P450 preparation totally unsuitable for ESI-MS analysis. Initial experiments indicated that the PEG precipitation step was unnecessary for obtaining a homogeneous sample. In the absence of PEG, the solubilized P450 was found to be highly purified after elution with Emulgen 911 on Octyl Sepharose chromatography. High salt concentrations, often used in hydrophobic interaction chromatographic steps, were avoided as significant losses (>75%) in P450s 1A2 and 2A6 spectral content were observed if salt was included. This omission also significantly decreased the time necessary for purification as it precluded the use of an overnight dialysis step prior to anion exchange chromatography. A yellow protein fraction, possibly insect cell flavoprotein, bound tightly to the DEAE column material while the P450 fraction was found to interact very weakly and eluted in the flow through. Finally, ceramic hydroxyapatite chromatography was used for detergent exchange with virtually no loss in spectrally detectable P450. SDS−PAGE analysis indicated that the P450s purified by this method were homogeneous (Figure 1), had specific contents greater than 14.3 nmol/mg, and were suitable for reconstitution studies (*26*, *27*). Moreover, the purified proteins could be fully characterized by ESI-MS analysis (see below), and the purification methodology was useful for purifying P450 3A4 which had been coexpressed with P450 reductase. The total time required for purification was on the order of 2 days.

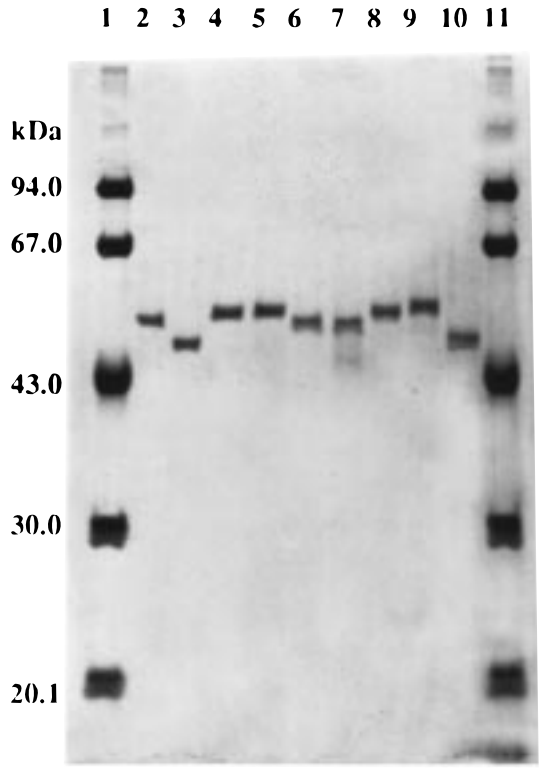

FIGURE 1: SDS−PAGE of purified P450s (20 pmol of spectrally detectable P450/lane) used in this investigation. Lanes 1 and 11, molecular mass markers (mass in kDa); lane 2, human P450 1A2; lane 3, human P450 2A6; lane 4, human P450 2C9; lane 5, human P450 2C9 C175R; lane 6, human P450 3A4; lane 7, human P450 3A4-HT; lane 8, rat P450 2B1; lane 9, rat P450 1A1; and lane 10, P450 cam-HT.

HPLC/ESI-MS Analysis of Purified Intact Baculovirus Expressed P450s. Purified proteins (P450s cam-HT, 1A1, 1A2, 2A6, 2B1, 2C9, 2C9 C175R, 3A4, 3A4-HT, and P450 reductase) were analyzed by HPLC/ESI-MS utilizing a reverse-phase POROS column with ≈2% of the flow directed to the mass spectrometer. At least 300 pmol of protein was used for the analysis of each purified P450, and the total run time was less than 10 min. Subsequent experiments demonstrated that the amount of P450 could be decreased to less than 100 pmol per analysis using a reverse-phase C4 column, but the total run time required to separate P450 reductase from P450 was longer than 30 min. We chose to use the POROS column for the experiments described here because it provided a shorter analysis time and the amount of sample was not a limiting factor. The separation of P450 and P450 reductase was of importance since both are the major components of the reconstituted systems used in covalent modification studies (see below). The cofactor(s)

Table 1: Yields of Baculovirus-Expressed P450 at Each Stage of Purification and Specific Content of the Final Purified P450 Preparation[a]

P450 isoform	CP	SOL	OS	DEAE	HAP	SC[b]
P450 1A2	235 (100)	165 (70)	85 (36)	45 (19)	38 (16)	15.1
P450 2A6	60 (100)	58 (97)	55 (92)	40 (67)	36 (60)	16.1
P450 2C9	200 (100)	200 (100)	170 (85)	162 (81)	154 (77)	16.6
P450 2C9 C175R	55 (100)	53 (96)	53 (96)	48 (87)	46 (84)	14.7
P450 3A4	90 (100)	81 (90)	72 (80)	54 (60)	50 (56)	14.3

[a] The amount of spectrally detectable P450 remaining after each step [insect cell paste (CP), cholate solubilization (SOL), Octyl Sepharose chromatography (OS), DEAE-FF chromatography (DEAE), and hydroxyapatite detergent exchange (HAP)] in the purification was determined using the method of Omura and Sato (*19*), and the percentage overall yield is given in parentheses. [b] The specific content (SC) of the final preparation is reported as nmol/mg.

Table 2: Cytochrome P450 and P450 Reductase ESI-MS Information[a]

protein	experimental MM (Da)	predicted MM (Da)	difference	% error
E. coli P450 cam-HT	47366.6 ± 2.0 (10)	47361.0 (10)	5.6	0.012
rat P450 1A1	59382.2 ± 14.1 (2)	59393.4 (2)	11.2	0.019
Bac. P450 1A2	58290.9 ± 5.5 (3)	58294.5 (3)	−3.6	0.006
Bac. P450 2A6	56544.4 ± 3.2 (6)	56541.6 (6)	2.8	0.005
rat P450 2B1	55935.7 ± 7.3 (7)	55933.8 (7)	1.9	0.003
Bac. P450 2C9	55578.6 ± 0.5 (9)	55575.1 (9)	3.5	0.006
Bac. P450 2C9 C175R	55633.9 ± 3.9 (8)	55628.1 (8)	5.8	0.010
Bac. P450 3A4	57313.9 ± 7.6 (4)	57299.3 (4)	14.6	0.025
E. coli P450 3A4-HT	57137.7 ± 0.3 (5)	57143.1 (5)	−5.4	0.009
E. coli rat P450 reductase	77716.8 ± 4.3 (1)	77714.9 (1)	1.9	0.002

[a] Experimentally determined and predicted MMs of purified rat and baculovirus (Bac.) and *E. coli* expressed proteins. Proteins which have a poly(His) tag at the C-terminus are also included. The average experimentally determined MMs (±SD) were calculated after injection on at least 3 separate days, and the rank order based on MM is included in parentheses. The average predicted MMs were calculated based on the amino acid sequences. The standard deviation associated with each individual determination of MM based on the observed charge state distribution was less than 0.09% for all proteins. The rank order of the proteins based on MM is included in parentheses. The MM range for the 8 mammalian P450s was 3818.3 Da.

(FAD and FMN for P450 reductase and heme for P450) was (were) stripped from each protein under the acidic conditions of the HPLC elution and could be individually detected by ESI-MS. For each P450, an experimentally determined MM was calculated (Table 2) using the observed charge state distribution, or ion envelope (usually made up of 30−40 charge states), that possessed a sample-to-sample standard deviation of less than 0.09%. Subsequent experiments showed that the signal-to-noise ratio at the higher *m*/*z* values could be significantly increased by using a cone voltage gradient (25−60 kV) over the scan range (200−2500 Da). The difference between the average of the experimentally determined MMs (±SD, based on experiments performed on at least 3 different days) and the predicted average MMs (based on the amino acid sequences) of the P450s and P450 reductase ranged from 0.002 to 0.035%. For example, the experimentally determined MM calculated for P450 2C9 differed by 3.5 Da, or 0.006%, from the MM of P450 2C9 predicted based on the amino acid sequence (Figure 2). The MM range of the eight mammalian P450s used in these experiments was only 3818.3 Da, but the rank order of the experimentally determined and predicted MMs of the P450s correlated exactly. The accuracy and precision afforded by this methodology should make it useful for the detection of covalent modifications incorporated into P450 and/or P450 reductase by site-directed mutagenesis and/or following mechanism-based inactivation.

Identification of Covalently Modified P450 2C9. The HPLC/ESI-MS technique described above was used to detect the covalent modification incorporated into P450 2C9 following mechanism-based inactivation by tienilic acid. Tienilic acid (Figure 3) has been shown to be a highly selective MBI of human liver microsomal P450 2C9 with a K_I and k_{inact} of 4.3 μM and 0.22 min^{-1}, respectively, calculated using yeast expressed P450 2C9 (*28*). It was shown that a large portion of the covalent binding of a reactive tienilic acid metabolite to P450 2C9 could be prevented by GSH, which is presumably acting as a scavenger of the electrophilic species formed (*28*−*30*). One of the criteria for a compound to be classified as an MBI is that the inactivation must be due to covalent modification of the active site (*24*). For P450s, GSH is a common additive used to ensure that the reactive species generated from a potential MBI is not inactivating the enzyme by modifying a site on its exterior (i.e., not in the active site). Thus, according to the classical definition, tienilic acid cannot be strictly categorized as a

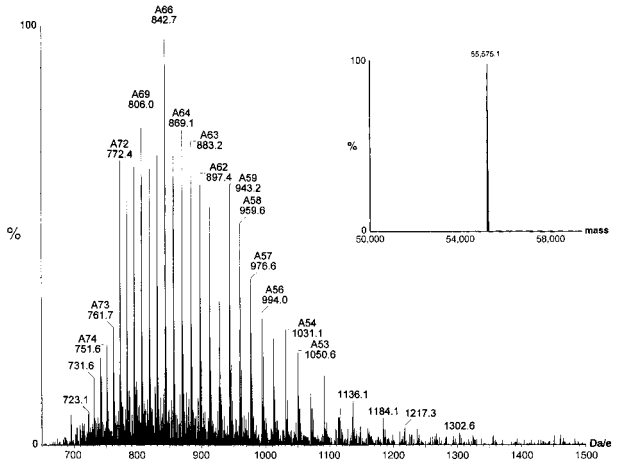

FIGURE 2: ESI mass spectrum of P450 2C9. The peaks are labeled with the protonation state (A*n*) and the number of protons (*n*) attached to the protein molecule. Inset: Deconvolution of the ESI mass spectral data which gives a representative profile of the P450 2C9 species present.

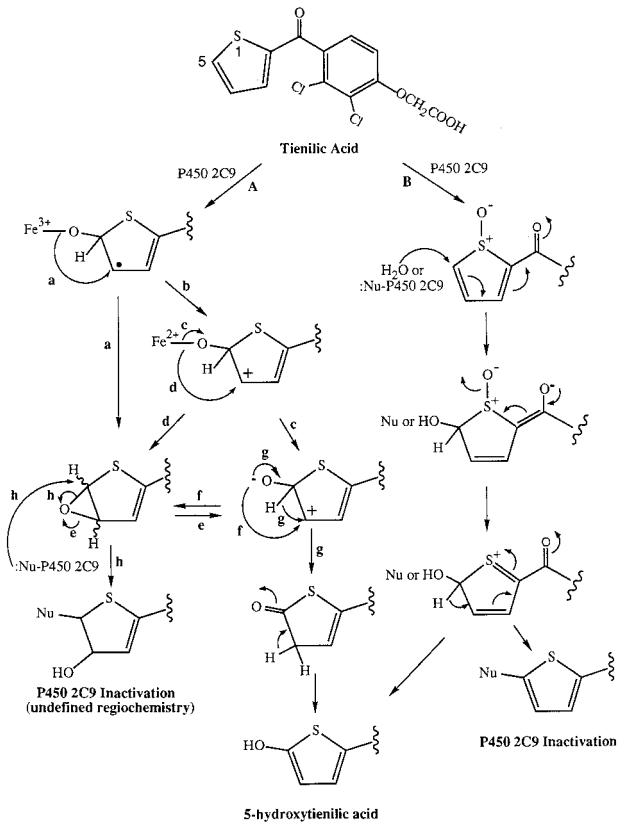

FIGURE 3: Chemical structure of tienilic acid and proposed mechanism for product formation and P450 2C9 inactivation: (A) via oxene addition to the thiophene ring followed by rearrangement and (B) via thiophene sulfur oxidation followed by nucleophilic attack at the thiophene ring.

pure MBI of P450 2C9. Despite the semantic ambiguity, a mechanism-based reactive intermediate is clearly formed from tienilic acid. Moreover, the structure of the reactive species has been postulated as being an electrophilic sulfoxide, although the possibility of a thiophene epoxide fulfilling this role has not been eliminated (Figure 3) (*28*).

P450 2C9 was reconstituted, exposed to tienilic acid and NADPH, and the mixture was analyzed by HPLC/ESI-MS for covalent modification of the reconstituted proteins. Three protein species were detected in the absence of GSH that had MMs corresponding to native P450 2C9, a monoadduct of P450 2C9 and tienilic acid, and a diadduct that incorporated two molecules of tienilic acid into P450 2C9 (Figure 4). It was necessary to inject a greater amount of the labeled P450 2C9 mixture, in comparison to the native P450 2C9 (1 nmol vs 0.3 nmol), to obtain reasonable mass spectral data due to the presence of these multiple species. The specificity of P450 2C9 apoprotein alkylation was confirmed by the complete lack of observable high mass shifts, indicative of adduct formation, in the ESI mass spectra of P450 reductase, cytochrome b_5, or the heme from P450 2C9 in any of the experiments. The average MMs of P450 2C9, the monoadduct of P450 2C9, and the diadduct of P450 2C9 were experimentally determined to be 55 578.6 ± 0.5, 55 923.0 ± 1.1, and 56 273.0 ± 4.4 Da, respectively. No modification was detected in the absence of NADPH, and the ratio of protein products was not altered when catalase was added to the incubation. A partition ratio of 32.0 ± 0.7 was calculated for the inactivation of P450 2C9 by tienilic acid based on the total amount of inactivator consumed

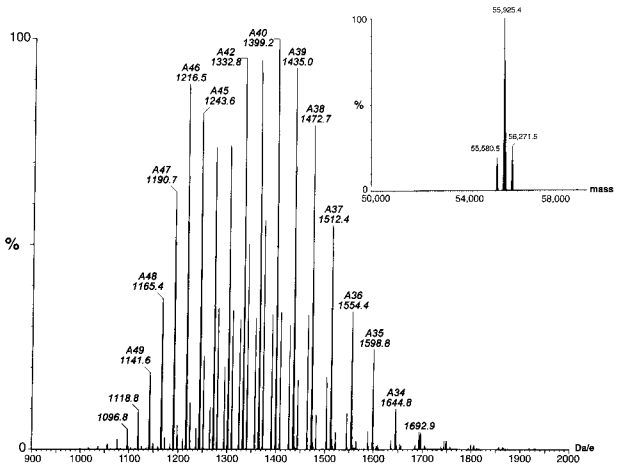

FIGURE 4: ESI mass spectrum of P450 2C9 after exposure to the MBI tienilic acid and NADPH. The peaks are labeled with the protonation state (A*n*) and the number of protons (*n*) attached to the protein molecule. Inset: Deconvolution of the ESI mass spectral data which gives a representative profile of the P450 2C9 species present.

Table 3: Partition Ratio Calculations for the Inactivation of Reconstituted P450 2C9 by Tienilic Acid

incubation components	partition ratio (% tienilic acid consumed)
complete system[a] − 1 mM NADPH	−(0)
complete system	32.0 ± 0.9 (100)
complete system + 10 mM GSH	28.9 ± 0.5 (90)
complete system + 5 mM β-ME	53.8 ± 7.5 (168)

[a] The complete system consisted of reconstituted P450 2C9 (187.5 pmol), tienilic acid (100 μM), and NADPH (1 mM) in buffer (50 mM potassium phosphate, pH 7.4).

(Table 3). These results suggest that the covalent modification of P450 2C9 by tienilic acid is dependent upon catalysis and is not due to reactive oxygen species generated via P450 uncoupling mechanisms. HPLC separation and UV monitoring at 346 nm of the peptides generated by digesting a substantial amount of labeled P450 2C9 (1 nmol) with trypsin were used in an attempt to identify peptides modified by tienilic acid. However, these attempts failed. Possible explanations for the failure of these experiments include: (a) the chromophore of the tienilic acid adduct was too weak to allow recognition of an active site peptide(s) bound to tienilic acid, (b) the tienilic acid adduct(s) was (were) not stable to the enzymatic digest conditions and was (were) hydrolyzed, and/or (c) the modified peptide(s) was (were) extremely hydrophobic and, as a result, selectively lost on the walls of the tubes and/or head of the HPLC column. Identification of a modified peptide using ESI-MS and the mass shift expected due to attachment of the electrophile to the unmodified peptide was obscured and complicated by the saturating amounts of P450 reductase present in the reconstituted enzyme system. We were unable to obtain radiolabeled tienilic acid for use in the covalent modification experiments. Separation of P450 2C9 from P450 reductase by POROS HPLC after inactivation by tienilic acid resulted in a significant loss in the amount of covalently modified P450 2C9. This finding suggests that the P450 2C9/tienilic acid adduct(s) is (are) unstable, and is (are) likely to be hydrolyzed, under the acidic conditions of the HPLC gradient elution. Several attempts to separate P450 reductase from

Table 4: Effect of Tienilic Acid on Reconstituted P450 2C9 Spectral Content after 0 and 15 min Incubations

incubation components	% P450 2C9 spectral content[b] 0 min	15 min
complete system	98	90
complete system + NADPH	100	71
complete system + tienilic acid	98	90
complete system + 10 mM GSH	94	86
complete system + NADPH + GSH	94	65
complete system + NADPH + tienilic acid	98	48
complete system + NADPH + tienilic acid + GSH	90	36

[a] The complete system consisted of reconstituted P450 2C9 (600 pmol) and catalase (2000 units) in buffer (50 mM potassium phosphate, pH 7.4) (final incubation volume, 600 μL). Tienilic acid (100 μM), NADPH (1 mM), and/or GSH (10 mM) were added to the complete system and incubated for 0 and 15 min at 30 °C. [b] P450 2C9 spectral content was determined using the method of Omura and Sato (19).

P450 2C9 in the reconstituted system by affinity chromatography using a 2′,5′ADP Sepharose column or affinity chromatography using an anti-P450 reductase column failed.

The formation of the tienilic acid metabolite, 5-hydroxytienilic acid, has been proposed to arise as a result of initial P450 2C9 catalyzed S-oxidation followed by a 1,6-Michael addition of H_2O at the 5-position and dehydration of the resultant thiophene sulfoxide to the corresponding thiophene (Figure 3, pathway B) (*28*). If the covalent modification of P450 2C9 by tienilic acid proceeds via the same mechanism, then the monoadduct of P450 2C9 and tienilic acid should exhibit a mass shift of 331.2 Da. Alternatively, initial oxene addition to the thiophene ring (Figure 3, pathway A) followed by ring closure, which could occur directly (pathway a) and/or after electron transfer to the porphyrin ring (pathway b + d), would result in the formation of a reactive thiophene epoxide. Nucleophilic ring opening of the thiophene epoxide by an active site residue would produce an adduct of P450 2C9 with an observed mass shift of 349.0 Da from native P450 2C9. In this mechanism (pathway A), 5-hydroxytienilic acid formation would result from a branched pathway leading from the oxene−thiophene ring addition product. Rather than ring closing to the thiophene epoxide, it could rearrange to a keto intermediate (pathway b + c + g) and then rearomatize to a hydroxylated thiophene ring. The mass shifts for both the monoadduct and the diadduct of P450 2C9 and tienilic acid, 344.4 ± 1.1 and 694.4 ± 4.2 Da, respectively, are 16 mass units higher than would be expected from simple coupling of tienilic acid and P450 2C9. This finding suggests that each tienilic acid that is covalently adducted to P450 2C9 contains a hydroxyl group, a result that is only consistent with initial thiophene ring oxidation (pathway A) and/or with initial sulfoxide formation provided the attached sulfoxide does not dehydrate.

The effect of tienilic acid on the spectral content of reconstituted P450 2C9 was examined in the absence and presence of NADPH and/or GSH (Table 4). A modest (≈10%) decrease in P450 2C9 spectral content was observed over the course of the 15 min incubation in the absence of NADPH presumably due to thermal denaturation. Inclusion of NADPH or GSH in the reconstituted P450 2C9 incubation in the absence of substrate resulted in an additional 19 or 4% decrease in spectral content, respectively. Thus, it appears that even in the presence of catalase, NADPH is able to catalyze

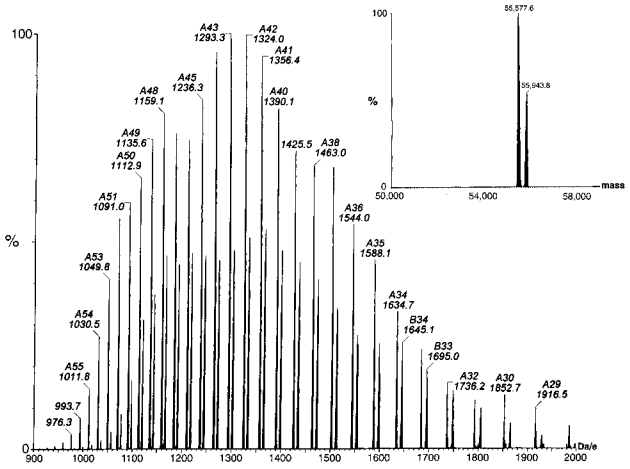

FIGURE 5: ESI mass spectrum of P450 2C9 after exposure to the MBI tienilic acid and NADPH in the presence of GSH (10 mM). The peaks are labeled with the protonation state (A*n*) and the number of protons (*n*) attached to the protein molecule. Inset: Deconvolution of the ESI mass spectral data which gives a representative profile of the P450 2C9 species present.

the formation of reactive oxygen species capable of inactivating a significant portion of P450 2C9. Inclusion of both NADPH and tienilic acid in the incubation led to a 52% decrease in P450 2C9 spectral content that could not be prevented by the addition of 10 mM GSH. This demonstrates that (a) the binding of CO to ferrous P450 2C9 can be modulated by exposure to tienilic acid even though inactivation proceeds exclusively via protein alkylation and (b) the binding of the reactive intermediate of tienilic acid to the exterior of the protein is not responsible for enzyme inactivation. Observation (a) has been shown to occur in other studies of the mechanism-based inactivation of P450 (*26*, *27*, *31*−*33*).

In the presence of GSH, native P450 2C9 was the predominant protein present in the incubation mixture while the active site monoadduct of P450 2C9 was the only other protein species observed (Figure 5). In the absence of GSH, the monoadduct, here composed of both active site and externally modified species, was the predominant form. In contrast, under similar conditions, the binding of several furanocoumarins to P450 2A6 and P450 2B1 after mechanism-based inactivation was not prevented by addition of GSH (*27*, *33*). Thus, it appears that, unlike other MBIs, the majority of the reactive intermediate formed from tienilic acid reacts with the exterior of P450 2C9 after escaping the P450 active site. Several GSH concentrations (0−10 mM) were used, but the greatest effect on the modification of intact P450 2C9 by tienilic acid was observed at 10 mM.

In the absence of GSH, a single tienilic acid metabolite was observed that had an $[M + H]^+$ ion of 347.0 Da corresponding to addition of an oxygen atom to the parent compound. The UV spectrum of the HPLC-purified metabolite was identical to that reported for 5-hydroxytienilic acid (*23*). In the presence of increasing amounts of GSH (0−10 mM), the amount of 5-hydroxytienilic acid formed from tienilic acid by reconstituted P450 2C9 decreased; however, there was only a modest (10%) decrease in the partition ratio (Table 3). This latter effect is probably a reflection of the inactivating effect of GSH itself on P450 2C9 (Table 4). Inclusion of β-ME in the incubations with P450 2C9 and tienilic acid led to the formation of a tienilic

acid/β-ME conjugate that was characterized by an $[M + H]^+$ ion of 407.0 as well as a significant increase (168%) in the partition ratio. The increase in partition ratio is likely a result of the much smaller size and increased lipophilicity of β-ME, relative to GSH. These properties would increase the accessibility of β-ME to the active site of P450 2C9 and allow it to scavenge the reactive species responsible for P450 inactivation. This in turn would lead to an extended lifetime for P450 2C9 and a corresponding increase in the amount of tienilic acid consumed. The $[M + H]^+$ ion of 407.0 for the tienilic acid/β-ME conjugate does not contain an additional hydroxyl group, unlike that of the tienilic acid/P450 2C9 adduct. Assuming the mass spectral interpretation regarding the presence of the hydroxyl group in the protein adduct is correct, the difference between the two adducts can be explained in one of two ways: (a) the tienilic acid/β-ME conjugate dehydrates to rearomatize the thiophene ring, but the tienilic acid/P450 2C9 adduct does not; or (b) the thiophene ring hydroxyl group, generated upon epoxide ring opening, is attacked from the opposite side and displaced by the β-hydroxy group of the bound β-ME to form a new stucture, a 1,4-thiopyran ring bound to a dihydrothiophene. Since there appears to be no reason the two conjugates should behave differently toward dehydration, explanation (a) seems unlikely. In contrast, explanation (b) does account for the observations as only the tienilic acid/β-ME conjugate has the possibility of forming the new ring system. These results indicate that although the reactive intermediate of tienilic acid can be trapped by GSH and β-ME, the actual events responsible for enzyme inactivation take place at the active site.

CONCLUSIONS

Our findings demonstrate that a three-column purification procedure can be used for the isolation of baculovirus-expressed P450s from insect cells and results in purified P450s suitable for analysis by HPLC/ESI-MS. HPLC/ESI-MS analysis of these P450s results in ion envelopes comprised of greater than 30 charge states from which very accurate and precise average MMs can be calculated. The method was also successfully used to detect covalent modification(s) made to P450 2C9 following mechanism-based inactivation by tienilic acid and to demonstrate that the majority of the reactive intermediate formed escapes the active site of P450 2C9 in the reconstituted enzyme system. The results suggest that this method offers a general procedure for obtaining useful mass spectra of intact, modified, and mutant P450s and related proteins.

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