Identification of Active-Site Peptides from ^{3}H-Labeled 2-Ethynylnaphthalene-Inactivated P450 2B1 and 2B4 Using Amino Acid Sequencing and Mass Spectrometry†

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ABSTRACT: 2-Ethynylnaphthalene (2EN) is a mechanism-based inactivator of rat cytochrome P450 (P450) 2B1 with 1.3 mol of adduct bound per mole of P450 inactivated [Roberts, E. S., Hopkins, N. E., Alworth, W. L., & Hollenberg, P. F. (1993) *Chem. Res. Toxicol. 6*, 470–479]. Further studies have shown that 2EN is also an efficient mechanism-based inactivator of the 7-ethoxycoumarin *O*-deethylase activity of rabbit P450 2B4 with 0.83 mol of adduct bound per mole of P450. Cleavage of [^{3}H]2EN-inactivated 2B1 with cyanogen bromide, separation of the peptides by HPLC, and further purification of the radiolabeled fraction by Tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) led to the identification by autoradiography of a radiolabeled peptide ($M_r \approx 3000$). Amino acid sequence analysis of the first 12 N-terminal residues revealed the sequence ISLLSLFFAGTE corresponding to positions 290–301 in the protein. When the radiolabeled fraction from the HPLC separation was analyzed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS), peaks at m/z 2722.5 and 2890.6 were detected. The lower mass peak corresponds to the molecular ion (average mass) of the cyanogen bromide peptide Ile290 to Met314 (theoretical 2722.2), while the higher mass peak corresponds to the same peptide with a bound 2-naphthylacetyl group (theoretical 2890.4). When [^{3}H]2EN-inactivated 2B4 was treated with cyanogen bromide, the peptides were separated by HPLC, and the fractions were analyzed by Tricine–SDS–PAGE, two radiolabeled peptides ($M_r \approx 5000$ and 8000) were identified by autoradiography. Amino acid sequence analysis of the first 11 residues revealed identical N-termini with the sequence EKDKSDPSSEF corresponding to positions 273–283. When the fraction containing these peptides was analyzed by MALDI-MS, peaks at m/z 4730.4 and 4897.6 were detected. The lower mass peak represents the MH^+ for the peptide Glu273 to Met314 (theoretical 4729.3), while the higher mass peak corresponds to the MH^+ of the modified peptide (theoretical 4897.5). Two additional peaks were identified from this fraction at m/z 8603.7 and 8435.6 which could be explained by the presence of a microheterogeneous form of 2B4 with Met314 replaced by Leu. Again, the difference in mass between the two peaks (approximately 168) would correspond to the addition of a 2-naphthylacetyl group to the unmodified peptide.

The cytochrome P450 (P450)[1]-dependent mixed-function oxidases are involved in the metabolism of a wide variety of endogenous and xenobiotic compounds (Porter & Coon, 1991; Nelson et al., 1993). The common catalytic function of these enzymes is the two-electron reduction of molecular oxygen to form water and a reactive oxygen species. The products from these reactions can have more or less biological activity or toxicity than the parent compound. With those compounds which act as mechanism-based inactivators, catalysis produces a highly reactive intermediate that inactivates the enzyme. Three mechanisms for the inactivation of P450 by these compounds are known: (1) N-alkylation of the prosthetic heme group; (2) destruction of the heme, resulting in products that bind covalently to the apoprotein; and (3) covalent modification of the apoprotein by the reactive intermediate. While *N*-alkyl heme adducts have been isolated and fully characterized (Kunze et al., 1983; Ortiz de Montellano & Kunze, 1981; Ortiz de Montellano et al., 1982), and preliminary mass spectral data have been reported for the identification of an active-site heme-modified peptide (Yao et al., 1993), there has not been structural characterization by mass spectroscopy of a peptide covalently modified by a reactive intermediate. Compounds that bind to the apoprotein should be especially useful in the identification of peptides at the P450 active site.

Certain compounds containing the acetylenic functional group have been shown to be mechanism-based inactivators of the P450 enzymes [as reviewed in Murray and Reidy (1990)]. 2-Ethynylnaphthalene (2EN) has previously been

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[1] Abbreviations: P450, cytochrome P450; P450 2B1 and P450 2B4, major forms of P450 from liver microsomes of phenobarbital-treated rats and rabbits, respectively; P450 101, camphor monooxygenase P450 from *Pseudomonas putida*; P450 102, fatty acid monooxygenase from *Bacillus megaterium*; reductase, NADPH–cytochrome P450 reductase; 2EN, 2-ethynylnaphthalene; DLPC, L-α-phosphatidylcholine, dilauroyl; TFA, trifluoroacetic acid; HPLC, high-pressure liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry.

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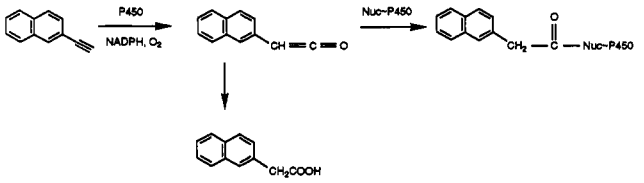
Scheme 1: Proposed Scheme for the Formation of 2-Naphthylacetic Acid and Labeling of P450



shown to be a mechanism-based inactivator of P450, and the radiolabeled compound was found to irreversibly bind to the protein moiety of rat 2B1 (Roberts et al., 1993), rat 1A1 and 1A2, and rabbit 1A2 (Hammons et al., 1989; Yun et al., 1992) with a binding stoichiometry of approximately 0.5–1.3 nmol of adduct/nmol of P450 after incubation in the presence of reductase, lipid, and reducing equivalents. The chemistry of inactivation by these acetylenic compounds is thought to proceed through the formation of a ketene intermediate (Scheme 1) within the active site of the enzyme (Ortiz de Montellano & Komives, 1985; Komives & Ortiz de Montellano, 1987; CaJacob et al., 1988; Hammons et al., 1989). The products observed from this reaction would result from uncatalyzed hydrolysis of the ketene intermediate to form acetic acid metabolites or reaction of the ketene with an active-site nucleophile to form modified protein. 2-Naphthylacetic acid has been identified as a product from the metabolism of 2EN in the reconstituted system containing rat 1A2 by comigration with a standard on HPLC (Hammons et al., 1989). It has also been shown by GC-MS to be a metabolite formed with 2B1 (Roberts et al., 1993). However, there has not been definitive structural evidence that the naphthylacetyl group is covalently attached to the protein. Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) has been used to determine the molecular weight of proteins and peptides with picomole sensitivities [as reviewed by Chait and Kent (1992), Biemann (1992), and Lewis et al. (1993)] and to characterize modifications of peptides and proteins (Wang et al., 1993). In this study, we inactivated the major phenobarbital-inducible P450s from rat and rabbit, 2B1 and 2B4, respectively, with [^{3}H]2EN and isolated radiolabeled peptides after cyanogen bromide digestion of the proteins. We used amino acid sequence analysis and MALDI-MS to identify the sequences and masses of the modified peptides.

EXPERIMENTAL PROCEDURES

Materials. 2EN and 2-ethynyl[*ring*-G-^{3}H]naphthalene (268 mCi mmol^{-1}) were prepared as described previously (Hall et al., 1990; Hammons et al., 1989). 2-([2′-^{3}H]Ethynyl)-naphthalene (acetylenic ^{3}H-labeled 2EN; 170 mCi mmol^{-1}) was prepared as described below. L-α-Phosphatidylcholine, dilauroyl (DLPC), NADPH, and catalase were purchased from Sigma Chemical Co. (St. Louis, MO). 7-Ethoxycoumarin, 7-hydroxycoumarin, and cyanogen bromide were purchased from Aldrich (Milwaukee, WI). Trifluoroacetic acid (TFA) was purchased from Pierce (Rockford, IL). Safety Solve was obtained from Research Products International Corp. (Mount Prospect, IL). Microconcentrators (Centricon-10, 10 000 MW cutoff) were obtained from Amicon (Danver, MA).

Procedure for Preparation of 2-([2′-^{3}H]Ethynyl)naphthalene (Acetylenic Hydrogen-Labeled 2EN). 2EN (38 mg, 0.2 mmol), synthesized and purified as previously described (Hall et al., 1990), was placed in a flask that had been dried in an oven and then connected to a stream of dry nitrogen at atmospheric pressure before it cooled. Ten milliliters of dry tetrahydrofuran, distilled from sodium benzophenone dianion under nitrogen atmosphere, was added to the flask through a septum with an oven-dried syringe. The resulting solution of 2EN, under nitrogen atmosphere, was stirred with a magnetic stir bar and cooled to −78 °C. A solution of *n*-butyllithium in hexane (0.25 mL of a 11.6 M solution, Aldrich Chemical Co.) was added with an oven-dried syringe; after 10 min at −78 °C, the acetylide anion was quenched by the addition of a 10-fold excess of high specific activity ^3H_2O (2 mmol, 900 mCi/mmol; ICN Biomedicals, Inc., Irvine, CA). The stirred reaction, still under nitrogen atmosphere, was allowed to warm to room temperature over a 1-h period, 50 mL of ether was added, and the resulting solution was extracted 9 times with sodium bicarbonate solution to remove all traces of excess ^3H_2O. The ether solution was then extracted a single time with water, dried with anhydrous sodium sulfate, and evaporated to dryness *in vacuo*. The residue was purified by flash chromatography on silica gel with petroleum ether elution. The yield of 2-([2′-^{3}H]ethynyl)naphthalene was 29 mg; 170 mCi/mmol.

Isolation of Enzymes. P450 2B1 and reductase were purified as described by Saito and Strobel (1981) and Strobel and Dignam (1978), respectively, from microsomes prepared from the livers of male Long Evans rats (150–175 g) given 0.1% phenobarbital in the drinking water for 12 days. P450 2B4 was purified from microsomes prepared as previously described (Coon et al., 1978) from the livers of New Zealand White male rabbits given 0.1% phenobarbital in the drinking water for 7 days.

Enzyme Inactivation, Cyanogen Bromide Digestion, and Peptide Separation. In studies involving the inactivation of 7-ethoxycoumarin deethylase activity, incubation mixtures contained 0.1 μM 2B1 or 2B4, 0.1 μM reductase, 30 μg/mL DLPC, 110 units of catalase, 0.5 μM 2EN in 5 μL of methanol or methanol alone in the incubations without 2EN, and 50 mM potassium phosphate buffer (pH 7.7) in a total volume of 1.0 mL. The mixture was preincubated for 3 min at 30 °C before the addition of 0.15 mM NADPH to start the reaction. In control experiments that had no NADPH, water was added to the reaction mixture. At 0 and 10 min after initiation of the reaction, the 7-ethoxycoumarin deethylase activity was measured spectrofluorometrically as described (Ullrich & Weber, 1972; Roberts et al., 1993) after the addition of 500 μM 7-ethoxycoumarin.

In studies involving the isolation of the radiolabeled peptide from 2B1, the incubation mixtures contained 2.5 nmol of 2B1, 1.25 nmol of reductase, 220 units of catalase, 2 mM NADPH, 12 μM [*ring*-^{3}H]2EN, and 50 mM potassium phosphate buffer (pH 7.7) in a total volume of 2.0 mL. After incubation at 30 °C for 20 min, an aliquot was removed, and the 7-ethoxycoumarin deethylase activity was measured and determined to be less than 3% of that seen at zero time. The excess reagents and the metabolites were removed, and the protein was concentrated using microconcentrators as previously described (Roberts et al., 1993). The cyanogen bromide digestion and HPLC reverse-phase separation of the resulting peptides were as previously described (Roberts et al., 1993).

The incubation conditions for the inactivation of 2B4 with 2EN were as described for 2B1 except for the following substitutions: 3.0 nmol of 2B4 and 1.5 nmol of reductase for 2B1 and reductase, and 15 μM [^{3}H]2EN labeled on the acetylenic hydrogen or the ring-labeled compound. The two radioactive compounds, one labeled on the ring and the other labeled on the acetylenic hydrogen, gave similar results in both inactivation and labeling of P450 2B1 and 2B4 (data not

shown). After inactivation, the proteins were concentrated using microconcentrators and cleaved with cyanogen bromide. The resulting peptides were separated on a POROS RH column (Perseptive Biosystems, Cambridge, MA) with a solvent system consisting of buffer A (0.1% TFA) and buffer B (95%CH_3CN/5% H_2O/0.1% TFA). After maintaining initial conditions of 35% B for 2 min, the peptides were eluted with a linear gradient to 65% B in 13 min at a flow rate of 3.0 mL/min. The fractions containing the most radioactivity were pooled, concentrated, and treated with sample loading buffer, and the peptides were separated by Tricine–SDS–PAGE.

Tricine–SDS–PAGE was carried out according to the method of Schägger and van Jagow (1987) with modifications (Roberts et al., 1993). For autoradiography, the stained and destained gels were treated with ENTENSIFY (Dupont NEN Research Products, Boston, MA), dried, and exposed to Hyperfilm-MP (Amersham Corp., Arlington Heights, IL) for 13 days at −70 °C. Alternatively, after electrophoresis, the peptides were electroblotted onto an Immobilon P^{SQ} poly(vinylidene difluoride) (PVDF) membrane (Millipore Corp., Milford, MA) as described (Roberts et al., 1993). The amino acid sequences of the PVDF membrane-bound peptides were determined in the Macromolecular Core Facility at Wayne State University (by J. Snow) by automated Edman chemistry on a Model 470 Applied Biosystems gas-phase sequenator, with an on-line Model 120 HPLC, a Nelson analytical chromatography data system, and a Model 900-A control/data system.

Mass Spectrometry. Labeled peptides were collected by HPLC, and concentrated in polypropylene tubes with the use of a Speed-Vac device (Savant, Farmingdale, NY). MALDI mass spectra were obtained on a VT-2000 (Vestec Corp., Houston, TX) linear time-of-flight mass spectrometer equipped with a nitrogen laser (337-nm, 3-ns pulse). The accelerating voltage in the ion source was 30 kV. Data were acquired using a transient recorder with 5-ns resolution. Each spectrum was produced by accumulating data collected from 64 laser shots. Time-to-mass conversion was achieved by internal calibration using sodium or matrix peaks, and/or peaks from bradykinin, insulin B chain, or insulin added as internal standards (peptide/protein standards were from Sigma Chemical Co.). The matrix used in all experiments was α-cyano-4-hydroxycinnamic acid (Aldrich Chemical Co.). Samples were prepared for MALDI-MS by adding 1 μL of matrix solution, prepared as saturated α-cyano-4-hydroxycinnamic acid in 1:1 acetonitrile/0.1% aqueous TFA, to an equal volume of the peptide sample (estimated to be 5–30 pmol/μL) collected by HPLC as described. The analyte–matrix solutions were then air-dried on stainless-steel probe tips prior to MALDI-MS analysis.

RESULTS AND DISCUSSION

Covalent Labeling of 2B1 by 2EN and Isolation of a Labeled Peptide. As shown previously, P450 2B1 was irreversibly modified after incubation with reductase and [^{3}H]2EN for 10 min in the presence of NADPH (Roberts et al., 1993). The labeled 2B1 and the reductase were cleaved with cyanogen bromide and the resulting peptides separated by reverse-phase HPLC with analysis by UV detection or liquid scintillation counting. The fractions containing the most radioactivity and eluting late in the gradient (65% B) were further analyzed by Tricine–SDS–PAGE. As shown in Figure 1, there was a single radiolabeled peptide. After the peptides were transferred to a PVDF membrane, the 3.0-kDa peptide was partially

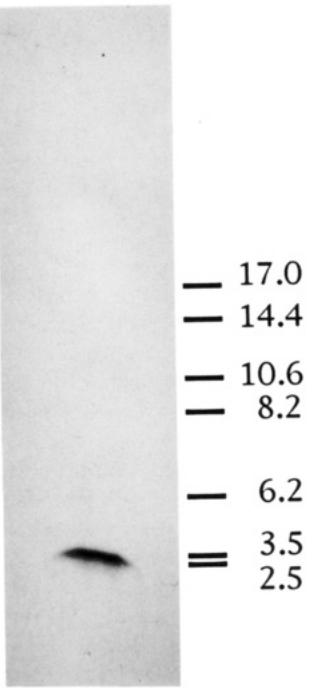


FIGURE 1: Autoradiogram of the fractions eluting at 63–64 min from the HPLC separation of cyanogen bromide-treated [^{3}H]2EN-inactivated 2B1 after Tricine–SDS–PAGE separation. The positions of the molecular mass markers (kDa) are indicated. The cleavage procedure, HPLC conditions, and Tricine–SDS–PAGE analysis were as described under Experimental Procedures.

sequenced by Edman degradation, and the amino acid yields (picomoles) were as follows: I (25); S (3.4); L (13.0); L (11.4); S (0.6); L (4.8); F (3.5); F (3.3); A (2.2); G (1.8); T (0.4); E (0.6). This peptide sequence corresponds to positions 290–301 of 2B1 (Fujii-Kuriyama et al., 1982; Yuan et al., 1983), and, as expected, the first residue follows a Met residue. Previously, we identified a radiolabeled peptide from 2B1, residues 290–297, using sequence data from three purification methods. In each experiment, at least two sequences were present, but the peptide containing residues 290–297 was common to every experiment. In this study, only one sequence was identified when the peptide was purified using a combination of HPLC and electrophoresis. We attempted to identify the amino acid that was modified by liquid scintillation counting of an aliquot of the sample collected from each sequencing cycle. In several experiments, the last residue identified was Glu301 (which would be followed by Thr302), and a majority of the counts remained on the membrane and sequencing filter. In one case, we were able to sequence 20 residues of the peptide corresponding to residues 290–309 of 2B1, and some radioactivity was released at cycle 13 (Thr302).

The HPLC fractions containing the labeled peptide from cyanogen bromide cleavage of 2B1 were also analyzed by MALDI-MS. Figure 2A shows the mass spectrum of the labeled fraction, and Table 1 lists the major ions of interest that were detected. From the known sequence of 2B1 and the peptide sequence identified by Edman degradation, the labeled peptide would be expected to include 25 residues (Ile290–Met314). The theoretical MH^+ (average mass) of the unmodified peptide would be 2722.2. In Figure 2A, there are peaks at m/z 2722.5 and 2890.6 (a difference of 168.1). When the reconstituted system was incubated with [^{3}H]2EN in the absence of NADPH, and cleavage and HPLC separation were performed as before, the molecular ion corresponding to the unmodified peptide of 2720.6 was present; however, there was no peak at m/z 2890.6 (Figure 2B). In addition, the

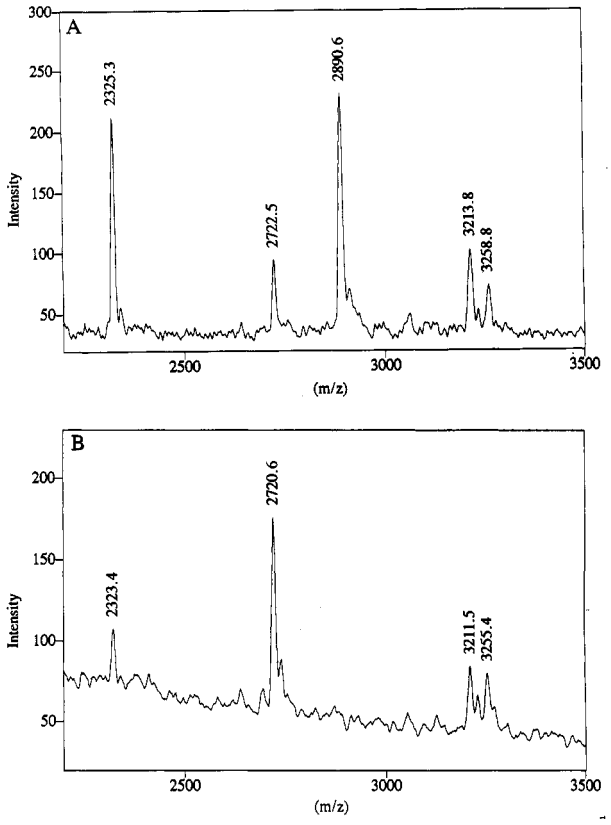


FIGURE 2: (A) MALDI mass spectrum of the fraction containing radioactivity from an HPLC separation of cyanogen bromide-treated [^{3}H]2EN-inactivated 2B1 as described under Experimental Procedures. (B) MALDI mass spectrum of an HPLC fraction eluting at 63–64 min from a separation of cyanogen bromide-treated proteins in which the reconstituted system was incubated with [^{3}H]2EN in the absence of NADPH.

Table 1: Theoretical and Observed Molecular Ions of Peptides after Modification of P450 2B1 and 2B4 with [^{3}H]2EN[a]

peptide	MH$^+$ average mass		
	theoretical	observed +NADPH[b]	observed −NADPH[c]
2B1			
residues 290–314	2722.2	2722.5	2720.6
modified with 2EN[d]	2890.4	2890.6	ND[e]
2B4			
residues 273–314	4729.3	4730.4	4731.8
modified with 2EN	4897.5	4897.6	ND
2B4			
variant 273–346[f]	8435.6	8435.6	8442.9
modified with 2EN	8603.8	8603.7	ND

[a] The conditions for protein cleavage, purification, and mass spectrometry were as described under Experimental Procedures. [b] The values are from the spectra in Figures 2A and 5A for 2B1 and 2B4, respectively. [c] The values are from the spectra in Figures 2B and 5B for 2B1 and 2B4, respectively. [d] The modified peptide has the addition of a naphthylacetyl group as proposed in Scheme 1. [e] ND, not detected. [f] The variant would have a Met314 to Leu314 change.

other peaks seen with the complete incubation system are seen in this control (m/z 2325.2, 3213.8, and 3258.8). The chemistry of inactivation by these types of inactivators is thought to proceed through oxidation of the acetylene by P450 to a ketene as shown in Scheme 1 (Ortiz de Montellano & Kunze, 1980; Komives & Ortiz de Montellano, 1987; CaJacob et al., 1988). Attack by a nucleophilic residue, such as Thr, at the active site would yield a naphthylacetic acid ester, and this would result in the addition of 168.2 mass units to the unmodified peptide. The protein inactivation, peptide purification, and mass spectral analysis of the labeled peptide were repeated 4 times. The mean and standard deviation of the peptide mass determination from these experiments gave values of m/z 2723.5 ± 1.3 and 2890.0 ± 1.7 for the unmodified and modified peptides, respectively.

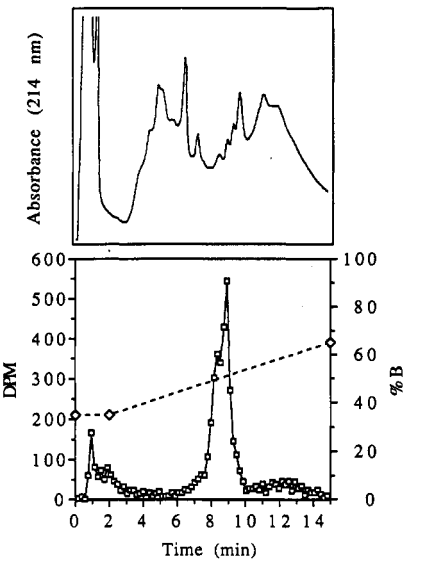


FIGURE 3: HPLC reverse-phase separation of the peptides generated by cyanogen bromide cleavage of a reaction mixture containing reductase and [^{3}H]2EN-inactivated 2B4. The cleavage procedure and HPLC conditions were as described under Experimental Procedures. The radioactive fractions eluting at 8.5–9.5 min were collected and analyzed by Tricine–SDS–PAGE (Figure 4) and MALDI-MS (Figure 5).

Covalent Labeling of 2B4. The 7-ethoxycoumarin *O*-deethylase activity of 2B4 was inactivated 30% after incubation with 2EN for 10 min in the presence of NADPH. Some inhibition of deethylase activity was seen without prior incubation (zero time controls) and is most probably due to a competitive inhibition as seen previously with 2EN (Roberts et al., 1993; Hammons et al., 1989; Yun et al., 1992; Hopkins et al., 1992). 2B4 was modified by [^{3}H]2EN after incubation with NADPH and reductase, and the stoichiometry of binding, determined by the method previously described for the inactivation and labeling of 2B1 (Roberts et al., 1993), was 0.8 nmol of adduct/nmol of 2B4 (data not shown). The [^{3}H]-2EN-inactivated 2B4 was cleaved with cyanogen bromide, and the resulting peptides were separated by HPLC and analyzed by UV detection or liquid scintillation counting (Figure 3). The fractions containing the most radioactivity (8.5–9.5 min) were combined and further analyzed by Tricine–SDS–PAGE and mass spectrometry. Figure 4A shows the pattern observed on the PVDF membrane after transferring from the Tricine–SDS–PAGE gel and staining with Coomassie Blue. If the gel was subjected to autoradiography, the peptide bands at approximately 6 and 8 kDa were radiolabeled (Figure 4B). The results from the partial sequencing of these two bands are given in Table 2. Surprisingly, the N-termini were identical for the two peptides, and the sequences correspond to positions 273–283 in 2B4 (Tarr et al., 1983) with the first residue following a Met.

When the HPLC fractions containing radioactivity were analyzed by mass spectrometry, the spectrum in Figure 5A was obtained. The theoretical MH$^+$ of the unmodified peptide corresponding to residues 273–314 is given in Table 1 as 4729.3. As shown in Figure 5A, there are two components at m/z 4730.4 and 4897.6 that differ by a mass of 167.2. In addition, there are peaks at m/z 8435.6 and 8603.7 that differ in mass

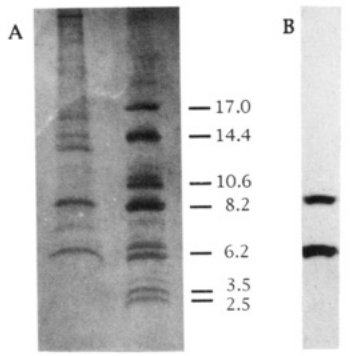


FIGURE 4: Tricine–SDS–PAGE separation of the labeled fraction from an HPLC separation of cyanogen bromide-treated [^{3}H]2EN-inactivated 2B4 followed by transfer to a PVDF membrane and staining with Coomassie Blue (A) or analysis by autoradiography (B). The positions of molecular mass markers (kDa) are indicated. The cleavage procedure, HPLC conditions, Tricine–SDS–PAGE analysis, and electroblotting were as described under Experimental Procedures.

Table 2: Amino Acid Sequence Determinations of Cyanogen Bromide-Generated Peptides Derived from Modification of 2B4 with [^{3}H]2EN[a]

	6 kDa		8 kDa	
cycle	amino acid	pmol	amino acid	pmol
1	E[b]	49	E[b]	9.2
2	K	24	K	8.0
3	D	43	D	7.2
4	K	21	K	7.0
5	S	6	S	0.1
6	D	28	D	4.4
7	P	16	P	4.4
8	S	4	S	0.4
9	S	5	S	0.5
10	E	12	E	1.2
11	F	11	F	1.7

[a] The cyanogen bromide cleavage, HPLC analysis, electrophoresis, transfer, and sequencing of the peptides on the PVDF membranes were as described under Experimental Procedures and in Figures 3 and 4. The amount of amino acid at each cycle has been corrected for background. The values for serine appear low but the serine adduct that arises from sequencing chemistry was very clear at each cycle that a serine was identified, but no quantitation of the adduct was available. [b] The peptides correspond to positions 273–283 in 2B4.

(168.1). Given the limited resolution and mass accuracy of MALDI-MS, these differences likely correspond to the same esterification as proposed in Scheme 1. If the reaction is carried out in the absence of NADPH, the peaks corresponding to the unmodified peptides are present whereas the peaks corresponding to the peptide adducts are not seen (Figure 5B). When the inactivation, peptide purification, and mass spectral analysis were repeated, peaks at m/z 4732 and 8441.8, for the unmodified peptides, and at m/z 4901 and 8607.5, for the modified peptides, were identified. Since the N-termini are the same for both peptides, we believed that the peptide with the greater mass resulted from either incomplete cleavage at Met314 or the presence of a variant protein. Since the observed mass of the unmodified peptide (8435.6) was different from the calculated mass of the peptide that would result from incomplete cleavage at Met314 (8453.6), we investigated the possibility of microheterogeneity of our 2B4 preparation. Komori et al. (1988) and Gasser et al. (1988) identified not only the cDNA corresponding to the 2B4 sequence as determined by Tarr et al. (1983) but also other cDNA clones from rabbit livers that would code for proteins with a high homology to 2B4. Reported variants that would yield a cyanogen bromide peptide with Leu at residue 314 instead of

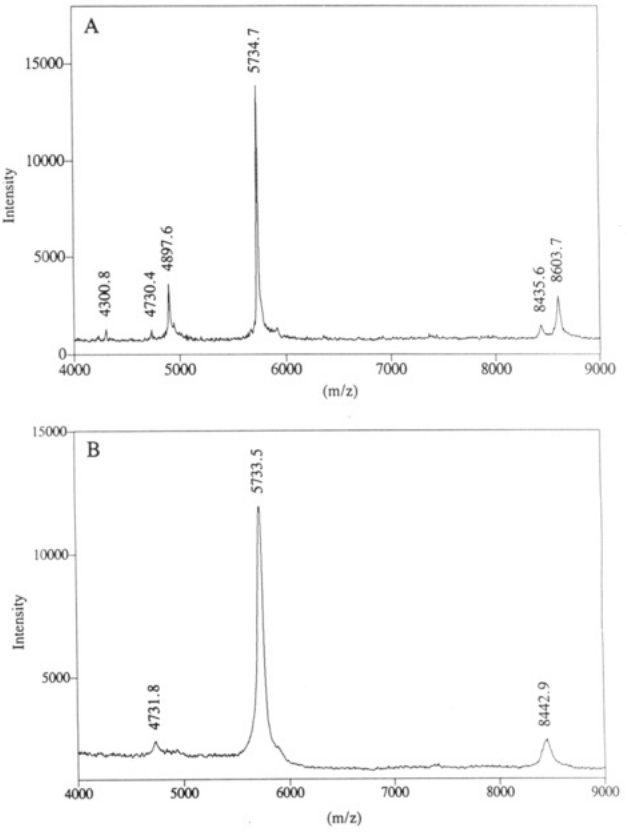


FIGURE 5: (A) MALDI mass spectrum of the fraction containing radioactivity from an HPLC separation of cyanogen bromide-treated [^{3}H]2EN-inactivated 2B4 as described in Figure 3. Insulin (5733.5 Da) was included as an internal standard. (B) MALDI mass spectrum of an HPLC fraction eluting at 8.5–9.5 min from a separation of cyanogen bromide-treated proteins in which the reconstituted system was incubated with [^{3}H]2EN in the absence of NADPH.

Met and thus would have a theoretical mass of 8435.6 identical to the observed mass include B1 (Gasser et al., 1988), b14, and b46 (Komori et al., 1988). B1 also has another change in this segment, but the Leu to Ile change at residue 290 would not result in a difference in mass (Gasser et al., 1988). Thus, the peaks at m/z 8435.6 and 8603.7 would appear to result from the inactivation of a form of P450 with a sequence very similar to 2B4. The committee which developed the nomenclature for P450 has arbitrarily assigned proteins having $\leq$3% divergence as being derived from two alleles of the same gene (Nelson et al., 1993). Since the variant clones were characterized from cDNA libraries derived from rabbit liver (Gasser et al., 1988; Komori et al., 1988), screening of rabbit genomic libraries followed by structural analysis of positive clones would be necessary to determine the origin of the microheterogeneity.

Conclusions and Implications. The rat and rabbit phenobarbital-inducible P450s, 2B1 and 2B4, respectively, are very similar proteins based on their primary sequences. Since there is no crystal structure available for these P450s, the sequences have been aligned with the known structure of P450 101 (Edwards et al., 1989; Gotoh & Fujii-Kuriyami, 1989) or P450 102 (Ravichandran et al., 1993). The labeled peptides from 2B1 and 2B4 would correspond to helix I, one of the regions that exhibits a high degree of homology among the proteins (Poulos, 1991; Ravichandran et al., 1993). In P450 101 and 102, the I-helix is located on the distal side of the heme surface and centers around Thr252 and Thr268, respectively. It has been proposed that these residues are essential parts of a proton relay pathway that donates protons

to dioxygen bound to the reduced heme during catalysis (Gerber & Sligar, 1992; Raag et al., 1991; Ravichandran et al., 1993). In several of our experiments with inactivated proteins, we were not able to sequence through the residue that corresponds to Thr252 or Thr268. The last residue sequenced was Glu301 which precedes Thr302. In one experiment, we were able to sequence the peptide from 2B1 for 20 cycles corresponding to residues 290–309 of 2B1, and some radioactivity was released at the cycle that corresponds to Thr302. We would suggest that the ketene intermediate derived from oxidation of the acetylene by P450 (Scheme 1) may be oriented in the active site in a position that facilitates nucleophilic attack by the hydroxyl group of Thr302.

We have demonstrated that the inactivation of 2B1 and 2B4 by [^{3}H]2EN results in covalent modification of the protein. The equivalent labeling of 2B4 with 2EN labeled on the ring or the acetylenic hydrogen is evidence that the ketene intermediate, postulated in Scheme 1 to be the source of labeling, must be derived from a P450-catalyzed 1,2-hydrogen shift as proposed by Ortiz de Montellano and Komives (1985) and Komives and Ortiz de Montellano (1987). The results from the MALDI-MS analysis give experimental structural evidence to support the previously proposed mechanism for inactivation of P450 by acetylenic compounds that bind to the protein. Recently, MALDI-MS has been used in combination with several different enzymatic digestions to unequivocally determine the phosphorylation sites of a signal transduction protein, Op18 (Wang et al., 1993), and future work will focus on applying this technique to the modified peptides from 2B1 and 2B4.

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