Modification of Cytochrome P450 1A2 Enzymes by the Mechanism-Based Inactivator 2-Ethynylnaphthalene and the Photoaffinity Label 4-Azidobiphenyl†

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ABSTRACT: 2-Ethynylnaphthalene (2EN) had previously been demonstrated to be a mechanism-based inactivator of rat cytochrome P450 (P450) 1A2 [Hammons, G. J., Alworth, W. L., Hopkins, N. E., Guengerich, F. P., & Kadlubar, F. F. (1989) *Chem. Res. Toxicol. 2*, 367–374]. In this work 2EN was also demonstrated to be a useful inactivator of rabbit P450 1A2 ($k_{inactivation}$ 0.094 min^{-1}, K_i 11 μM) but it did not inactivate human P450 1A2, although the sequences of the three proteins are ~80% identical. Rat and rabbit P450 1A2 were modified by incubation with NADPH–P450 reductase, NADPH, and [^{3}H]2EN to levels of 0.35 and 0.47 nmol of adduct (nmol of P450)$^{-1}$, respectively. In each case only a single tryptic peptide was labeled; recovery of labeled peptides was low under the acidic HPLC conditions. The rabbit P450 1A2 peptide FQELMAAVGR (positions 175–184) and the rat P450 1A2 peptide L(S)QQYGDVLQIR (positions 67–78) were identified. 4-Azidobiphenyl (4-N_3BP) was developed as a photoaffinity label for P-450 1A2 proteins because of its similarity to 4-aminobiphenyl, a known substrate for the enzymes. 4-N_3BP was shown to be photolyzed with 350-nm light and radioactive label could be incorporated into rat P450 1A2. Labeling of the protein was found to be saturable with increasing concentrations of 4-N_3BP and up to 0.59 nmol of label could be incorporated (nmol P450 1A2)$^{-1}$. The substrate 4-aminobiphenyl and the competitive inhibitor 7,8-benzoflavone blocked photolabeling of P450 1A2 with 4-N_3BP, and 4-N_3BP inhibited N-hydroxylation of 4-aminobiphenyl by P450 1A2 in the usual enzyme assay. SEEMLNLVK (positions 212–220) was isolated as the only labeled peptide from modified rat P450 1A2. With 4-N_3BP-modified rabbit P450 1A2, only a very hydrophobic portion was labeled and this binding was not attenuated by 4-aminobiphenyl. The results indicate quite different labeling patterns for three P450s having a high degree of primary sequence similarity. On the basis of the predictions of others about three-dimensional locations of the residues in P450 1A2 proteins, the regions 67–78 and 175–184 might respectively correspond to the so-called A and D helices of P450 101 [Poulos, T. L. (1988) *Pharm. Res. 5*, 67–75]. It is of interest that the region of rat P450 1A2 modified by 4-N_3BP (212–220) is near a corresponding site of mouse P450 2A5 where mutagenesis has been shown to modulate both catalytic specificity and heme distal ligation [Iwasaki, M., Juvonen, R., Lindberg, R., & Negishi, M. (1991) *J. Biol. Chem. 266*, 3380–3382].

P450[1] proteins are of interest because of their roles in catalyzing the oxidation of a great variety of drugs, chemical carcinogens, pesticides, steroids, vitamins, eicosanoids, and other substrates of these enzymes.[2] P450s seem to be found through the phylogenetic kingdoms and in each animal species there is evidence that >40 different P450 genes can be expressed (Nebert et al., 1991). Collectively the P450s are known to oxidize substrates ranging in size from ethylene to cyclosporin (Guengerich, 1989). The basic chemistry of catalysis is thought to be rather invariant among these P450s (Guengerich, 1990b) [possible exceptions can be considered such as the prostacyclin, thromboxane, and allene oxide synthases and chloroperoxidase (Ortiz de Montellano, 1986; Guengerich, 1991)]. The catalytic specificity is thought to be predominantly a function of binding forces in the transition state, which are presumably approximated in the geometry of the enzyme–substrate complex. Understanding catalytic specificity in these molecular terms is an extremely challenging but potentially very practical goal. In the case of the bacterial P450 101, much has been learned from the resolution of three-dimensional structures through X-ray diffraction (Poulos et al., 1987). With the eukaryotic P450s, which are all intrinsic membrane proteins, crystallization efforts have not yet been successful and essentially all of our present knowledge has come from studies using site-directed mutagenesis [for review see Johnson et al. (1992)], analysis of natural mutants (Matsunaga et al., 1990), and models based upon alignment of sequences with P450 101 (Kalb & Loper, 1988; Nelson & Strobel, 1989; Edwards et al., 1989; Gotoh & Fujii-Kuriyama, 1989; Gotoh, 1992; Poulos 1988, 1991).

One approach that has had remarkably little application toward this goal is that of localization of binding of affinity labels and mechanism-based inactivators. The apparent flexibility of many of the active sites of the P450, particularly those involved in oxidation of xenobiotic chemicals, offers

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[1] Abbreviations: P450, liver microsomal cytochrome P450 [also now referred to as heme-thiolate protein P450 (Palmer & Reedijk, 1991)]; 2EN, 2-ethynylnaphthalene; 4-N_3BP, 4-azidobiphenyl; HPLC, high-performance liquid chromatography. For reference to individual forms of P450 and nomenclature see Nebert et al. (1991).

[2] For insight into the current states of P450 knowledge and research problems see the entire January 1992 issue of *FASEB J.*

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options for attaching reactive moieties to substrates and inhibitors, many of which are known to bind tightly to P450s. There is an extensive literature on mechanism-based inactivators of P450s (Ortiz de Montellano & Correia, 1983; Ortiz de Montellano & Reich, 1986; Murray & Reidy, 1990). While many of these P450 inhibitors act by destroying the heme prosthetic group, there is also considerable information available on covalent modification of apoprotein as well. For instance, chloramphenicol and several steroid dichloromethane derivatives have been shown by Halpert and his associates to be oxidized and bind to P450s (Stevens et al., 1991). Our own laboratories have reported the covalent modification of P450s in processes involving either the oxidation of the prosthetic heme group (Guengerich, 1986) or activation of ethynyl substrate-inhibitors (Hammons et al., 1989) (the latter reference also reviews rates of inactivation of P450s by mechanism-based inactivators). Photolabeling approaches (Singh et al., 1962; Bayley & Knowles, 1977) have been used to attach various labels to P450s for a number of years; a list of compounds and P450s used includes 1-(4-azidophenyl)-imidazole/P450 101 (Swanson & Dus, 1979), 3-(trifluoromethyl)-3-(*m*-iodophenyl)diazirine/P450 1A1 and 2B1 (Frey et al., 1986), and 4′-azidowarfarin/(rat) P450 1A1 (Obach et al., 1992).

Nevertheless, in very few studies has the site (or sites) of labeling been identified. Jänig et al. (1984) used *N*-acetylimidazole and tetranitromethane to modify tyrosine residues of rabbit P450 2B4 and suggested that one of these was the distal ligand to the heme iron. Parkinson et al. (1986a,b) reported that the general electrophile 2-bromo-4′-nitroacetophenone reacted with 10 different rat P450s but only in the case of rat P450 1A1 was the enzyme inhibited. The site of attachment was identified as Cys 292 but the authors concluded that this residue was not close to either the heme or the bound substrate. Adamovich et al. (1989) modified bovine adrenal P450 11B ($P450_{scc}$) with succinic anhydride and suggested that the 11 Lys residues that had reacted are involved in binding adrenodoxin; however, Tsubaki et al. (1989) modified Lys residues in the same protein with pyridoxal phosphate and identified only Lys 377 and Lys 381 as being involved in adrenodoxin binding [neither had been implicated in the work of Adamovich et al. (1989)]. Apparently the only report of characterization of the site of labeling of a P450 with an *affinity* label is that of Onoda et al. (1987), who labeled hog testes P450 17 with 17-(bromoacetyl)progesterone and identified Cys 235 as the site of binding. The region in which this label was bound was suggested to be in "homology region 1" (Onoda et al., 1987), corresponding to the residues in helix D of P450 101 (Poulos, 1988).

We previously reported that 2EN is a relatively selective mechanism-based inactivator of rat P450 1A2 and that label became irreversibly attached to the protein (Hammons et al., 1989). With this background information, we initiated a systematic characterization of such binding of 2EN to rat, rabbit, and human P450 1A2 enzymes. We also extended our study of these enzymes with 4-N_3BP, a photoaffinity analog of the substrate 4-aminobiphenyl (Butler et al., 1989a). These P450 1A2 enzymes are of particular interest because of their demonstrated roles in the oxidation of carcinogenic aryl amines and heterocyclic amines (Guengerich & Shimada, 1991).

EXPERIMENTAL PROCEDURES

Materials. 2EN was synthesized and [*ring*-^3H]2EN (202 mCi mmol^{-1}) was prepared from 2-acetyl[*ring*-^{3}H]naphthalene as described previously (Hammons et al., 1989). [*ring*-^{3}H]Phenacetin (55 mCi mmol^{-1}) and 4-amino[2,2′-^{3}H]-biphenyl (55 mCi mmol^{-1}) were purchased from Chemsyn Science Laboratories (Lenexa, KS). All amino acid sequencing reagents, including poly(vinylidene difluoride) membranes, were purchased from Applied Biosystems Inc. (Foster City, CA). 4-Aminobiphenyl was purchased from Aldrich Chemical Co. (Milwaukee, WI) and L-tosyl chlorophenyl ketone-treated trypsin was purchased from Worthington Biochemical Corp. (Freehold, NJ). ICH_2CO_2H was crystallized from hexane (for use in carboxymethylation). CF_3CO_2H was distilled from CrO_3 before use as an HPLC solvent (Shively, 1986).

Radiolabeled and unlabeled 4-N_3BP were prepared from 4-aminobiphenyl through the diazonium salt intermediate using the procedure described by Smith and Boyer (1963). 4-Aminobiphenyl (in a mixture of 1 M H_2SO_4 in glacial CH_3CO_2H) was incubated on ice with an equimolar amount of $NaNO_2$. After conversion of starting material to the diazonium salt, a 2-fold excess of NaN_3 was added slowly to the reaction mixture under subdued light. Incubation on ice was continued for 3 h. The pH of the mixture was adjusted to 5.0 and the precipitate was collected, washed with cold H_2O, dried by dissolving it in dry ethyl acetate and evaporating the solvent to dryness, and then purified by recrystallization from hexane: yield 84%, mp 71–73 °C. The product was found to be >98% pure by HPLC analysis and identified as 4-N_3BP: ^{1}H NMR (C^2HCl_3) δ 7.187–7.193 (d, 2 H), 7.344–7.373 (t, 1 H), 7.445–7.475 (t, 2 H), 7.645–7.663 (d, 2 H), 7.714–7.722 (d, 2 H); mass spectrum (electron impact, relative abundance and assignment in parentheses) m/z 195 (15, M^+), 167 (100, M − 28, loss of N_2); IR, 2138 and 2100 cm^{-1} (strong). The azide product was stored at −20 °C in the dark.

Other reagents were analytical grade.

Enzyme Sources. Human liver microsomes were obtained through Tennessee Donor Services (Nashville, TN) from organ donors who met accidental deaths. Rat P450 1A2 was purified from liver microsomes of isosafrole-treated animals as described previously (Guengerich et al., 1982). Rabbit P450 1A2 was purified from liver microsomes of 5,6-benzoflavone-treated animals as described elsewhere (Alterman & Dowgii, 1990). NADPH-P450 reductase was isolated from phenobarbital-treated rabbits by slight modification (Shimada et al., 1986) of the basic procedure of Yasukochi and Masters (1976).

HPLC of 4-N_3BP Products. Reaction products from the photoactivation of 4-N_3BP were analyzed by reverse-phase HPLC on a Beckman-Altex Model 100A instrument (Beckman, San Ramon, CA) equipped with a Waters Model 440 absorbance detector (Waters/Millipore, Medford, MA) and a Radiomatic Flo-One/Beta radioactivity flow detector (Radiomatic/Packard, Meredian, CT). Separations were performed on a Waters μBondapak C_{18} column (0.39 × 30 cm) using a gradient of 20 mM aqueous $(C_2H_5)_2NH$ (pH 6.2) and CH_3OH (Butler et al., 1989a). The solvent program consisted of 30–70% CH_3OH (0–20 min, linear gradient), 70–100% CH_3OH (20–22 min, linear gradient), and 100% CH_3OH (22–27 min). The flow rate was 2 mL min^{-1}.

Spectrometry. UV–visible spectra were obtained with a Beckman DU-65 spectrophotometer. ^{1}H NMR spectra were obtained on a Bruker Model AM500 Fourier-transform spectrometer (Bruker, Billerica, MA). Mass spectral data were obtained on a Finnigan 4000 instrument (Finnigan, Sunnyvale, CA) equipped with a direct exposure probe operating in the electron impact mode at 70 eV. IR spectra were obtained with a Bio-Rad FTS-40 instrument (Bio-Rad, Richmond, CA).

Protein Labeling with 2EN. A 10-mL mixture of rabbit or rat P450 1A2 (20 nmol) and rabbit NADPH–P450 reductase (24 nmol) was incubated at 37 °C for 30 min in 50 mM potassium phosphate buffer (pH 7.5) containing 30 μM L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine, 12 μM [*ring*-^{3}H]2EN (202 mCi mmol^{-1}), and an NADPH-generating system (Guengerich, 1989). The reaction was cooled to 4 °C, concentrated to <2 mL volume using an Amicon PM-30 membrane device (Amicon, Lexington, MA), and then applied to a 2′,5′-ADP–agarose column (Pharmacia, Bromma) of 1-mL volume, which had been previously equilibrated with 50 mM potassium phosphate buffer (pH 7.25) containing 0.1% sodium cholate (w/v), 1 mM EDTA, and 20% glycerol (v/v). The void fraction (obtained by washing with the equilibration buffer) contained only P450 as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli, 1970). The samples were dialyzed extensively against H_2O (at 4 °C) and concentrated by lyophilization. Bound adducts were estimated by comparison of radioactivity (determined by liquid scintillation spectrometry) and recovered protein. Protein concentrations were estimated using a bicinchoninic acid (BCA) method according to the manufacturer's instructions (Pierce, Rockford, IL).

Photolysis. Irradiations were performed at 4 °C in a Rayonet RMR-500 photochemical reactor fitted with four PRP 350-nm lamps and a Merry-Go-Round sample holder (Southern New England Ultraviolet, Hamden, CT). Samples in quartz tubes were placed in the sample holder. In photolabeling experiments, rat or rabbit P450 1A2 (0.25 μM) in 10 mM sodium phosphate buffer (pH 7.4) was incubated on ice for 10 min in the dark with varying concentrations (50–400 μM) on [2,2′-$^3H_2$]-4-N_3BP and then irradiated for various time intervals (0–20 min). Control experiments were conducted in the absence of UV radiation and with prephotolyzed azide compound. Protection experiments were also performed under conditions identical with those described above, except that the enzyme was preincubated with varying concentrations of 4-aminobiphenyl before the addition of the photoaffinity reagent.

Quantification of Incorporated Photoprobe. A filter precipitation method was used to quantitate the amount of radioactivity incorporated into P450 1A2 by irradiation with [2,2′-$^3H_2$]-4-N_3BP. Following photolysis, samples were removed and spotted on glass fiber filters (Whatman GC/C, Whatman, Piscataway, NJ). The filters were dipped in cold 5% aqueous CCl_3CO_2H (w/v) and washed under vacuum successively with cold 5% CCl_3CO_2H (w/v), cold C_2H_5OH, ethyl acetate, and hexane (the label was found to be stable in the Cl_3CCO_2H solution for 5 h at 4 °C but was released upon standing at room temperature). The filters were then dried in air and radioactivity was analyzed by liquid scintillation spectrometry. To quantitate background radioactivity on the filters not due to photoincorporation of [2,2′-$^3H_2$]-4-N_3BP, reactions were performed in which photolysis was conducted in the absence of enzyme (controls were done with prephotolyzed azide, vide supra).

Preparative Photolabeling with 4-N_3BP. Rat or rabbit P450 1A2 (2.5 nmol) in 10 mL of 10 mM sodium phosphate buffer (pH 7.4) was pre-incubated for 10 min on ice in the dark with [2,2′-$^3H_2$]-4-N_3BP (200 μM). The sample was photolyzed for 20 min as described above. The experiment was repeated under the same conditions except that photolysis was performed in the presence of 4-aminobiphenyl (200 μM). Each sample was then dialyzed exhaustively (72 h) against 10 mM sodium phosphate buffer (pH 7.4) to remove unincorporated radioactivity (no more detected after further treatment).

Separation and Analysis of Peptides. The labeled protein from each sample (labeled with [^{3}H]2EN or [2,2′-$^3H_2$]-4-N_3BP) was denatured and reduced and cysteine residues were carboxymethylated as described earlier (Ged et al., 1988; Srivastava et al., 1991); the proteins were digested with trypsin in a similar way as described. Peptides were separated with the use of a 2.1 × 100 mm Aquapore RP-300 octylsilane (C8) HPLC column (Brownlee/Applied Biosystems, Santa Clara, CA) using gradients of H_2O increasing to CH_3CN, with 0.1% CF_3CO_2H (v/v) present. In preliminary experiments, poor resolution of peptides occurred with ion-exchange and neutral pH/reverse-phase systems. For general information on practical aspects of peptide separation see Petrides (1986), Shively (1986), and Wilson (1988). The flow rate was usually 0.4 mL min^{-1}. Peptides were collected manually and aliquots were used for radioactivity measurements [measured by mixing with ACS cocktail (Amersham, Des Plaines, IL)].

Labeled peptides were collected in polypropylene tubes (previously washed with CH_3CN) and concentrated with the use of a Speed-Vac device (Savant, Farmingdale, NY); care was taken to avoid reducing the samples to dryness in order to avoid losses in handling. Further HPLC purification involved the same HPLC column and a more shallow gradient of CH_3CN (Petrides, 1986). The amino acid sequences of the purified peptides were determined in the Vanderbilt facility (by T. A. Porter) using an Applied Biosystems 470A instrument equipped with on-line HPLC, using Edman degradation chemistry optimized for the instrument. Recoveries of individual phenylthiohydantoin derivatives are based upon comparison with external standards. For the analysis of radioactivity, the HPLC was disconnected and samples were collected directly for analysis by liquid scintillation spectrometry after solution in ACS cocktail.

RESULTS

Inactivation of Rabbit P450 1A2 by 2EN. Loss of the phenacetin O-deethylation activity of rabbit P450 1A2 was examined using a general dilution procedure (Silverman, 1988; Hammons et al., 1989; Guengerich, 1990a). Increasing concentrations of 2EN yielded higher rates of inactivation (Figure 1); the competitive inhibition by 2EN is also reflected in the oridinate intercepts. Analysis of the data yielded values of $k_{inactivation}$ = 0.094 min^{-1} and K_i = 11 μM. These compare favorably with those previously reported for rat P450 1A2—$k_{inactivation}$ = 0.23 min^{-1} and K_i = 7.7 μM (Hammons et al., 1989).

These experiments were also carried out with human P450 1A2 (in liver microsomal preparations), using phenacetin O-deethylation as a specific assay (Distlerath et al., 1985). However, no appreciable time-dependent inhibition by 2EN was observed.

Covalent Labeling of Rabbit and Rat P450 1A2 by 2EN and Identification of Modified Peptides. Rabbit and rat P450 1A2 were labeled with 2EN by incubation in the presence of NADPH–P450 reductase and NADPH. The reductase was removed by affinity chromatography and the samples were concentrated and extensively dialyzed. The levels of binding were 0.47 and 0.35 nmol of 2EN adduct bound (nmol of P450)$^{-1}$ for rabbit and rat P450 1A2, respectively.[3]

[3] With the particular incubation conditions used ~2 half-lives of inactivation should have been finished (Figure 1) and typically, then, ~75% of the enzyme would have been inactivated at this point.

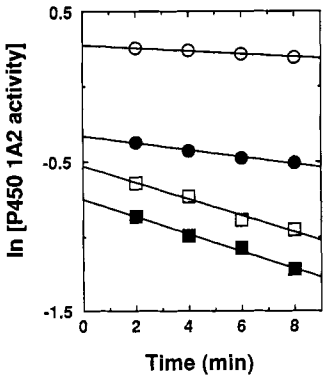


FIGURE 1: Inactivation of rabbit P450 during oxidation of 2EN. Rabbit P450 1A2 was incubated with NADPH–P450 reductase, L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine, and an NADPH-generating system in the presence of varying concentrations of 2EN at 37 °C, and at varying time intervals, aliquots were withdrawn and diluted into buffer containing 10 μM [*ring*-^{3}H]phenacetin and an NADPH-generating system, using the general approach described elsewhere (Silverman, 1988; Hammons et al., 1989; Guengerich, 1990a). The phenacetin reactions proceeded; rates of O-deethylation were measured using TLC analysis as described elsewhere (Larrey et al., 1984). Rates of inactivation of phenacetin O-deethylation activity were measured at varying concentrations of 2EN: 1 μM (○), 5 μM (●), 10 μM (□), and 20 μM (■). Nonlinear analysis of the rates (k-cat program, BioMetallics Inc., Princeton, NJ) yielded estimates of 0.094 min^{-1} for $k_{inactivation}$ and 11 μM for K_i.

The P450 1A2 samples were subjected to carboxymethylation and digestion with trypsin. In the cases of both rat and rabbit P450 1A2, a single labeled peptide was found after HPLC analysis and two cycles of HPLC were sufficient to achieve purity (Figures 2 and 3). The yields of recovered radioactive peptide were low in both cases after the first column (19% and 12% for rabbit and rat P450 1A2, respectively) and the second column (0.8% and 0.9%, respectively, based upon original radioactivity). However, no other distinct radioactive peptide peaks were identified, even in the void volume or the 80% CH_3CN fraction (Figures 2 and 3). The losses are considered to reflect the inherent instability of the adducts during exposure to the acidic conditions we found to be required for high-resolution separation of these peptides, as indicated in preliminary stability studies with the modified rat P450 1A2 protein.

Amino acid sequence analysis of the recovered peptides yielded good repetitive yields of sequences clearly identified in the two proteins (Table I). In each case the first residue follows a Lys or Arg residue predicted by the cDNA. The rabbit peptide FQELMAAVGR corresponds to positions 175–184 and the rat sequence L(S)QQYGDVLQIR corresponds to positions 67–78 (Gotoh & Fujii-Kuriyama, 1989).

In the rat peptide the expected Ser was not observed in the second cycle and the radioactivity was essentially all recovered in that fraction (efforts were not made to identify the small amount of the putative adduct available). However, a Ser ester formed with the putative ketene derived from 2EN (Scheme I, Komives & Ortiz de Montellano, 1987; Hammons et al., 1989) would be consistent with the data, and some of the loss of label from such an ester might be expected during processing. In the rabbit peptide FQELMAAVGR all of the radioactivity was released in the sequenator during the first cycle, indicative of instability. The only nucleophilic groups in this peptide are those of Glu, Met, and Arg, and none of these would be expected to form very stable adducts. For instance, attack of a Glu anion on a ketene would yield an anhydride (Scheme I) likely to decompose during purification.

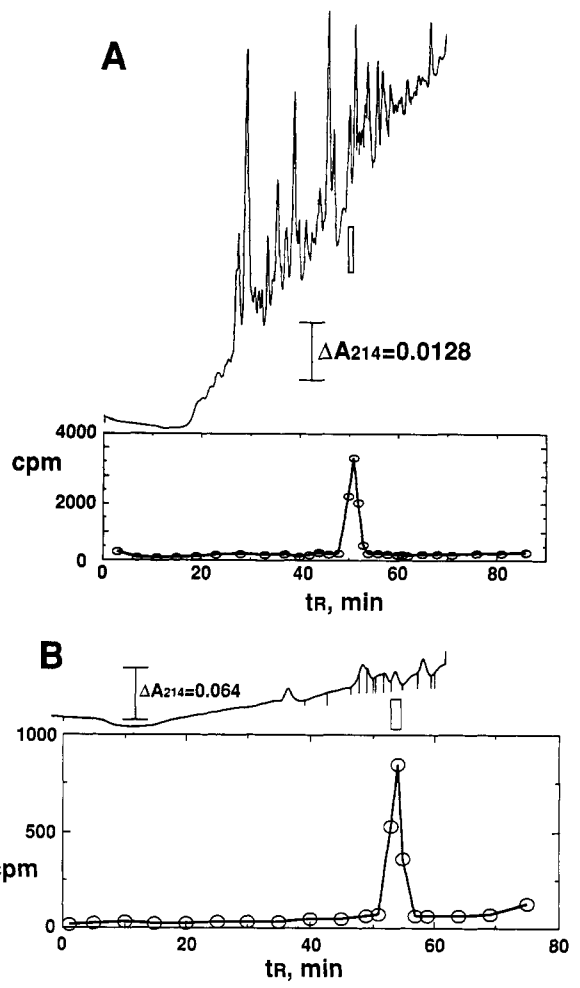


FIGURE 2: Isolation of a ^{3}H-labeled tryptic peptide from rabbit P450 1A2 modified by oxidation of 2EN. (A) Initial HPLC separation of tryptic peptides. Both A_{214} and cpm are shown. A linear gradient of 4–42% CH_3CN (v/v) in 0.1% (aqueous) CF_3CO_2H (v/v) over the time period of 5–105 min was used, with the gradient then going to 80% CH_3CN (v/v). The A_{214} peak indicated by the rectangle was collected. No other radioactive peaks were eluted when the column was washed with more 80% CH_3CN. (B) Second HPLC separation of tryptic peptides. The fraction recovered from part A was concentrated and reapplied to the same HPLC column, which was eluted with a linear 12–42% CH_3CN gradient (v/v) in 0.1% (aqueous) CF_3CO_2H (v/v). A_{214} and cpm profiles are aligned. The peak indicated by the rectangle was collected. No other radioactive peaks were eluted with further washing with 80% CH_3CN (v/v). (These chromatograms are not directly comparable to those in Figures 3, 6, and 7.) For amino acid sequence determination see Table I.

The low yields of label recovered are consistent with the instability of these adducts.

Photolysis of 4-N_3BP. Preparation of 4-N_3BP from 4-aminobiphenyl was readily accomplished using a standard synthetic approach. The photoactivation of 4-N_3BP by UV light was characterized by recording the spectral changes that occur after intervals of irradiation at 350 nm, and the band centered near 270 nm disappeared with time (results not shown). Analysis of photoactivation mixtures by reverse-phase HPLC after 20 min confirmed that 4-N_3BP was completely converted to a product with a retention time several minutes longer than that of the azide compound.

Photoaffinity Labeling of P450 1A2 Proteins. Photolysis of radiolabeled 4-N_3BP in the presence of rat P450 1A2 resulted in covalent modification of the enzyme. The extent of modification was determined by precipitating the P450 after photolabeling with [2,2′-$^3H_2$]-4-N_3BP and measuring the incorporated radioactivity. Control experiments were

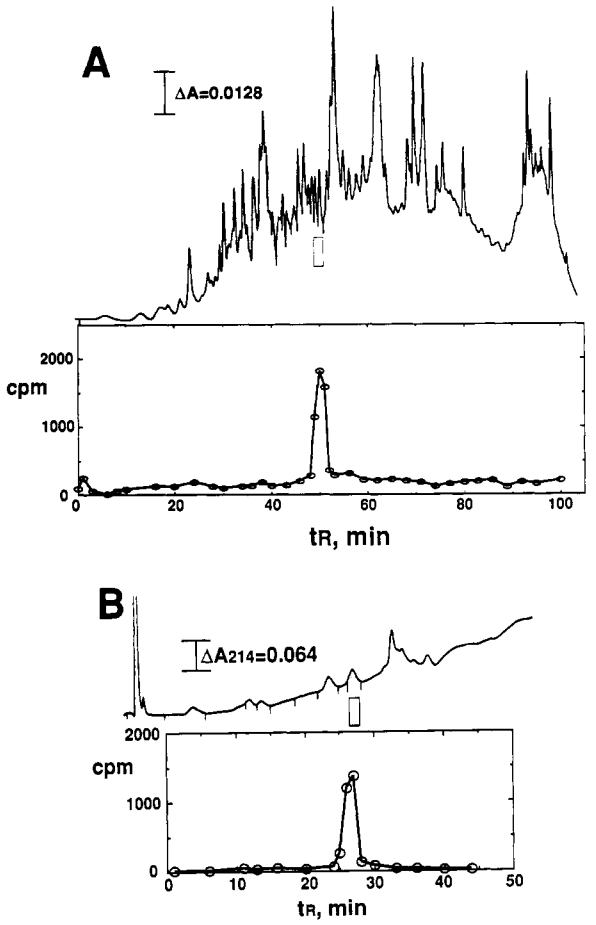


FIGURE 3: Isolation of a ^3H-labeled tryptic peptide from rat P450 1A2 modified by oxidation of 2EN. (A) Initial HPLC separation of tryptic peptides. Both A_{214} and cpm are shown. A linear gradient of 4–42% CH_3CN (v/v) in 0.1% (aqueous) CF_3CO_2H (v/v) over the time period of 5–85 min was used, with the gradient then going to 80% CH_3CN (v/v). The A_{214} peak indicated by the rectangle was collected. No other radioactive peaks were eluted from the column upon further washing with 80% CH_3CN (v/v). (B) Second HPLC separation of tryptic peptides. The fraction recovered from part A was concentrated and reapplied to the same HPLC column, which was eluted with a linear 12–42% CH_3CN gradient (v/v) in 0.1% (aqueous) CF_3CO_2H (v/v). A_{214} and cpm profiles are aligned. The peak indicated by the rectangle was collected. No other radioactive peaks were eluted with more CH_3CN. (These chromatograms are not directly comparable to those in Figures 2, 6, and 7.) For amino acid sequence determination see Table I.

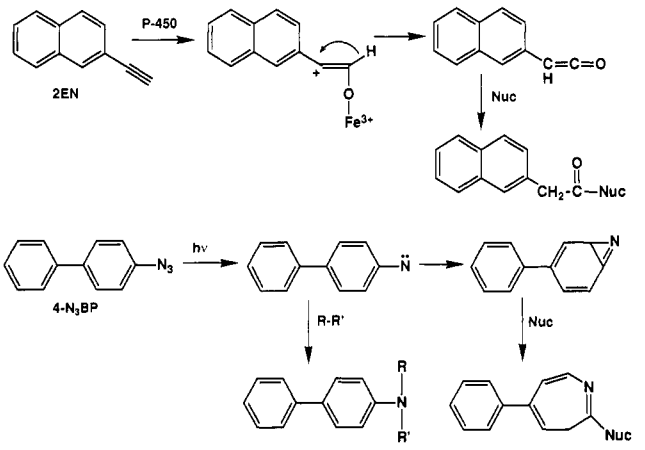
performed with unphotolyzed samples and with reactions photolyzed before addition of P450. Essentially no incorporation of radioactivity into the protein was found to occur in either of these control experiments. Using this technique, photoincorporation of radioactivity into the enzyme was found to increase with time of irradiation and was essentially complete within 20 min (Figure 4A). To determine if photolabeling of the enzyme with the photoanalog was saturable, reactions were performed with increasing concentrations of [2,2′-$^3H_2$]-4-N_3BP. Data in Figure 4B illustrate that photoincorporation was maximal at ~200 μM [2,2′-$^3H_2$]-4-N_3BP. Under these conditions 0.59 nmol of label (nmol of P450)$^{-1}$ was incorporated into the enzyme. In order to determine the extent of modification at the catalytic site of P450 1A2, experiments were conducted in the presence of 4-aminobiphenyl, which is a substrate for this enzyme (Butler et al., 1989a). At saturating photoprobe concentrations, labeling by [2,2′-$^3H_2$]-4-N_3BP was decreased ~70% by 200 μM 4-aminobiphenyl (Figure 4C). The protection by the substrate 4-aminobiphenyl is consistent with the observed inhibitory effect of 4-N_3BP on the catalytic

Table I: Amino Acid Sequence Determination of Tryptic Peptides Derived from Modification of P450 1A2 Proteins with [*ring*-^3H]2EN and [2,2′-^3H]4-N_3BP[a]

	rabbit P450 1A2 + 2EN			rat P450 1A2 + 2EN			rat P450 1A2 + 4-N_3BP		
cycle	amino acid	pmol	cpm	amino acid	pmol	cpm	amino acid	pmol	cpm
1	F[b]	9.9	83	L[c]	7.1	9	S[d]	8.5	721
2	Q	9.5	8	(S)[e]	*f*	380	E	6.5	46
3	E	9.5	8	Q	5.6	38	E	6.6	20
4	L	7.3	7	Q	5.4	11	M	3.2	46
5	M	2.8	6	Y	4.0	8	L	2.5	11
6	A	4.0	15	G	5.3	7	N	2.3	9
7	A	6.1	8	D	4.8	7	L	1.8	12
8	V	4.7	7	V	5.0	6	V	1.6	38
9	G	2.0	8	L	3.3	7	K	1.0	10
10	R	1.7	7	Q	3.1	14			
11			8	I	2.3	9			
12				R	1.1	18			

[a] For details of analysis, see Experimental Procedures and Figures 2, 3, 6, and 7. [b] The peptide corresponds to positions 175–184 of the protein. [c] The peptide corresponds to positions 67–78 of the protein. [d] The peptide corresponds to positions 212–220 of the protein. [e] Predicted by cDNA sequence. [f] No distinct residue was recovered that could be identified.

Scheme I: Postulated Reactions of 2EN and 4-N_3BP Relevant to P450 Labeling[a]



[a] Nuc = nucleophile.

activity of the P450 enzyme in the reconstituted system in the absence of photoactivation (Figure 5). In addition, a similar decrease in binding was observed with the competitive inhibitor 7,8-benzoflavone (100 μM; Butler et al., 1989a). A role for the potential scavenging action of 4-aminobiphenyl (Figures 6 and 7) is also precluded by the lack of any effect of 200 μM 4-aminobenzoic acid on incorporation (results not shown). 4-Aminobiphenyl N-oxidation catalyzed by reconstituted P450 1A2 was found to be inhibited ~85% by the azide analog, suggesting that the substrate and azide analog compete for the same binding site on the enzyme. In similar photolabeling experiments with rabbit P450 1A2, covalent modification of this enzyme was also found to occur to the extent of 0.34 nmol of label incorporated (nmol of P450)$^{-1}$ (data not shown).

Analysis of Peptides of Rabbit and Rat P450 1A2 Labeled with 4-N_3BP. Rat and rabbit P450 1A2 proteins labeled with 4-N_3BP were subjected to carboxymethylation, digestion with trypsin, and HPLC (Figure 7). In the case of rat P450 1A2 a single, relatively hydrophilic peptide was labeled, and the labeling was not observed in the tryptic digest prepared in the presence of the competitor 4-aminobiphenyl (Figure 6C). The yield of radiolabel recovered after the HPLC step was ~40% (compared to the labeled protein before digestion). Recovery

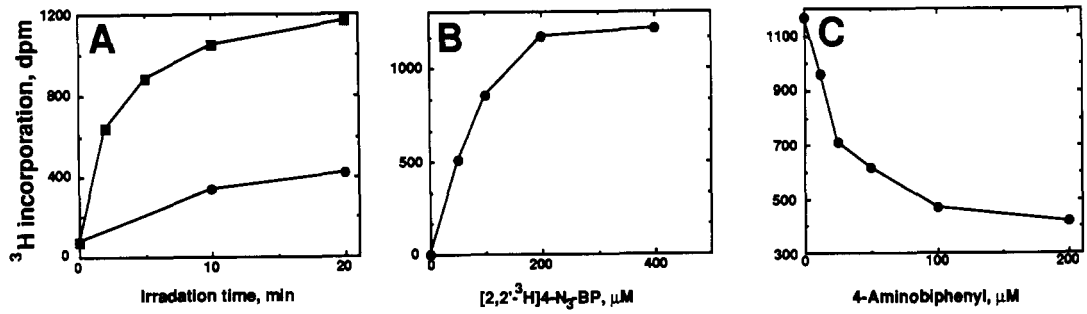


FIGURE 4: (A) Time dependence of photoincorporation of 4-N_3BP into P450 1A2. Rat P450 1A2 (0.25 μM) and [2,2'-$^3H_2$]-4-N_3BP (200 μM) in 10 mM sodium phosphate buffer (pH 7.4) were irradiated at 350 nm for various lengths of time at 4 °C. After each interval, the enzyme was precipitated and covalent incorporation of ^{3}H was determined by scintillation counting. (■) Experiments without 4-aminobiphenyl; (●) experiments in the presence of 200 μM 4-aminobiphenyl. (B) Effect of concentration of 4-N_3BP on photoincorporation into P450 1A2. Rat P450 1A2 (0.25 μM) in 10 mM sodium phosphate buffer (pH 7.4) was irradiated at 350 nm for 20 min at 4 °C in the presence of 50, 100, 200, or 400 μM [2,2'-$^3H_2$]-4-N_3BP and covalent incorporation of ^{3}H was determined. (C) Protection of 4-N_3BP photoincorporation into P450 1A2 by 4-aminobiphenyl. Rat P450 1A2 (0.25 μM) in 10 mM sodium phosphate buffer (pH 7.4) was irradiated at 350 nm for 20 min at 4 °C with [2,2'-$^3H_2$]-4-N_3BP (200 μM) in the presence of varying concentrations of 4-aminobiphenyl and covalent incorporation of ^{3}H was determined.

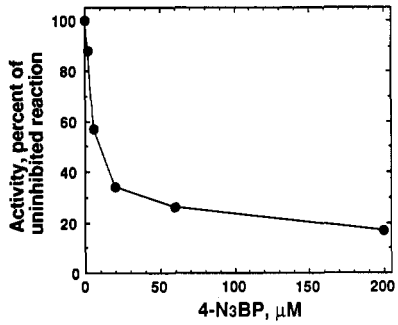


FIGURE 5: 4-N_3BP inhibition of P450 1A2-catalyzed 4-aminobiphenyl N-oxidation activity. Rat P450 1A2 was used in a reconstituted system and 4-aminobiphenyl N-oxidation activity was measured (Butler et al., 1989a). Activity was measured in the presence of 2–200 μM 4-N_3BP and expressed as the percentage of activity without added 4-N_3BP. These experiments were conducted in the absence of photoactivation.

of the peptide decreased with time as the sample was stored at 4 °C, indicating that the adduct is not completely stable (no other peaks were recovered after HPLC, either in the void volume or the 80% CH_3CN wash). Some of the loss may be due to general factors related to the peptide itself (adsorption, etc.) even though several precautions were taken to reduce losses (Petrides, 1986; Wilson, 1988). In studies in which the intact labeled protein was bound to filters, we found that the acidic conditions of the HPLC solvent gradually eluted bound radioactivity. With rabbit P450 1A2 the only ^{3}H label appeared in a nondescript region of hydrophobic peptides eluted with high CH_3CN concentration (Figure 6D); moreover, a similar level of label was found in the preparation subjected to photolysis in the presence of the competitor 4-aminobiphenyl (Figure 6E).

The peptide from rat P450 1A2 was isolated using two rounds of HPLC. Analysis of the labeled peptide derived from rat P450 1A2 yielded a single sequence with high repetitive yield (Table I). This peptide corresponds to residues 212–220 (Gotoh & Fujii-Kuriyama, 1989). Essentially all of the radioactivity was recovered in the first cycle. This result is consistent with either a Ser adduct that decomposed during the cycle or another unstable adduct in the peptide. The ratio of radioactivity to recovered amino acid phenylthiohydantoin and consideration of the specific radioactivity (55 mCi mmol^{-1}) indicates that the ratio of adduct to peptide was near unity in this experiment.

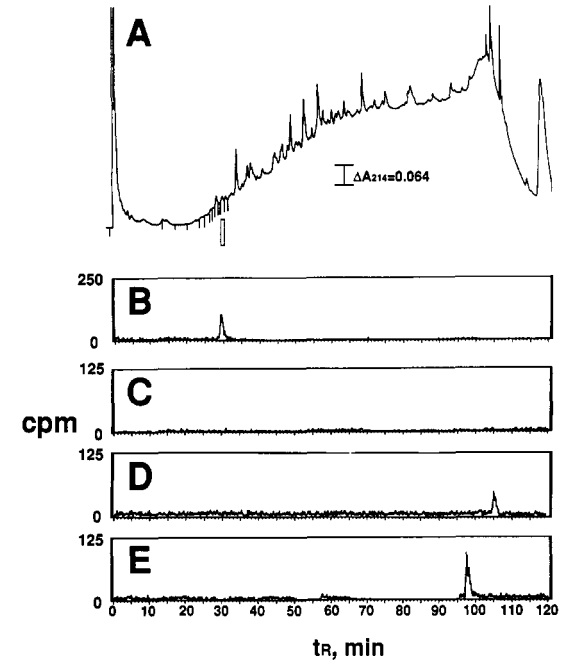


FIGURE 6: HPLC of tryptic peptides isolated from 4-N_3BP labeling of rat and rabbit P450 1A2. In all cases the peptides (corresponding to equivalent amounts of P450 1A2 used in the photoaffinity labeling) were applied to the column and eluted with a linear 4–42% gradient of CH_3CN (v/v) in 0.1% aqueous CF_3CO_2H (v/v). (A) A_{214} trace of rat P450 1A2 peptides after modification of P450 1A2 with 4-N_3-BP. The rectangle indicates the recovered fraction used for further studies. (B) Radiochromatogram corresponding to the sample used in part A. (C) Same as part B except that photoaffinity labeling of rat P450 1A2 was done in the presence of 200 μM 4-aminobiphenyl. (D) Radiochromatogram from HPLC of tryptic peptides from rabbit P450 1A2 subjected to photoaffinity labeling with 4-N_3BP. (E) Same as part D except that labeling of rabbit P450 1A2 was done in the presence of 200 μM 4-aminobiphenyl. (These chromatograms are not directly comparable with those in Figures 2 and 3.)

DISCUSSION

The rat, rabbit and human P450 1A2 proteins are highly similar proteins as judged by their primary sequences (Gotoh & Fujii-Kuriyama, 1989), their catalytic activities (Butler et al., 1989a, 1989b), and even some of their physical properties—e.g., their tendency to adopt a high-spin iron configuration and their notorious precipitation in the absence of buffer salts. Both rat and rabbit P450 1A2 were inactivated during the incubation with 2EN, with inactivation of the former protein being ~2-fold faster (Figure 1). However, we were

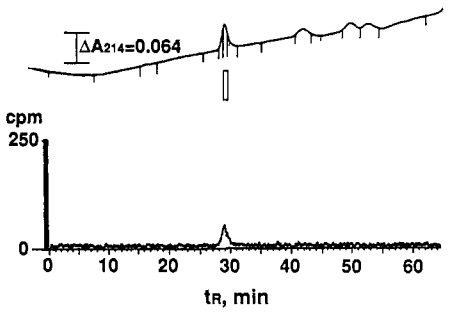


FIGURE 7: Second HPLC of rat P450 tryptic peptides modified by photoaffinity labeling in the presence of 4-N_3BP. The peptide fraction recovered the first HPLC (Figure 4A) were concentrated and further HPLC was done with a 4–11% linear gradient of CH_3CN (v/v) in 0.1% aqueous CF_3CO_2H (v/v). The fraction indicated by the rectangle was used for analysis (Table I).

unable to demonstrate inactivation of human P450 1A2 under the same conditions we have used with rat liver microsomes (Hammons et al., 1989) and did not pursue further characterization studies. Further, rat and rabbit P450 1A2 clearly differed in their sites of labeling with 2EN and 4-N_3BP (Figures 2, 3, 6, and 7 and Table I). The differences in the radioactivity elution patterns provide evidence for covalent attachment, even in the light of low recoveries.

The chemistry postulated to occur in the labeling reactions is shown in Scheme I (Komives & Ortiz de Montellano, 1987; Hammons et al., 1989; Bayley & Knowles, 1977). With the amounts of purified peptides available (Table I) it has not been possible to characterize the structures and there are several possibilities. Considerable losses of radioactivity were experienced after each step in the purification procedure, probably due to the instability of the derivatives in the presence of CF_3CO_2H required for high resolution of the peptides. In the case of rat P450 1A2 modified by 2EN, the most likely adduct is a Ser ester derived from the ketene (Scheme I), since all of the radioactivity was recovered in the putative Ser cycle of the sequenator (predicted by the cDNA-derived sequence) but no Ser (or Ser ester) was recovered (Table I). In the case of rabbit P450 1A2 labeled with 2EN, the only nucleophilic residues in the peptide are Glu, Met, and Arg (Table I). All radioactivity was released in the first cycle, indicative of an unstable adduct. The adduct formed by reaction of a ketene with Glu or Met would be highly unstable and possibly not able to survive the isolation. The other possibility is Arg. Although we are unaware of precedent for products formed by reaction of a ketene with Arg, such an adduction is considered to be possible on the basis of the known reaction of Arg with glyoxal. Modification of the Arg would probably block the action of trypsin, although we cannot prove that cleavage at the Arg actually occurred on the basis of the sequence data (Table I).

With regard to the identity of the residue of rat P450 1A2 modified with 4-N_3BP, several possibilities involving both nitrene and aziridine chemistry can be considered (Scheme I) (Bayley & Knowles, 1977). However, the instability during purification and the loss of all radioactivity in the first cycle of the sequenator (Table I) might indicate that an unstable adduct formed by aziridine chemistry may be more likely than a stable insertion product. A possibility is a derivative of Ser (the only nucleophiles in the peptide are Ser and Lys and the Lys adduct might be expected to be stable). Although there was considerable loss of radiolabel at each HPLC step, it should be reemphasized that in all cases only a single labeled peptide was recovered (Figures 6 and 7) and that the sequence

Chart I: Alignment of P450 1A2 and P450 101 Sequences[a]

	67		78	174		183	212		220
Rat P-450 1A2	...KLSQQYGDVLQIR...			KFQKLMAEVGH...			KSEEMLNLVK...		
	68		79	175		184	213		221
Rabbit P-450 1A2	...RLSQRYGDVFQIR...			RFQELMAAVGR...			GSEEMLDVVR...		
	45		55	140		145	160		164
P-450 101	...VLQESNVPDL		VW...	SLR		PQGQ ...	RI		FMLL....

[a] Gotoh & Fujii-Kuriyama (1989).

analysis produced high yields of residues that exactly match those expected in the sequences.

One interesting exercise involves the alignment of the labeled peptides with P450 101 (Chart I) (Haniu et al., 1982; Gotoh & Fujii-Kuriyama, 1989). The three-dimensional structure of P450 101 is known (Poulos et al., 1987) and can provide the basis for speculation. By using the alignment scheme proposed by Edwards et al. (1989) it is possible to suggest that the rat P450 1A2–2EN adduct is in the A helix and the rabbit P450 1A2–2EN adduct is in the D helix. The prediction could be made that the rat P450 1A2–4-N_3BP adduct is found in or just prior to the start of the E helix. Most of the site-directed changes made to data in the rat P450 1A2 protein have been made in the region of the I helix (residues 316–322) thought to be near the distal ligand region of the heme (Shimuzu et al., 1988, 1991a–c; Sadeque et al., 1988; Furuya et al., 1989a,b). These modifications, which dramatically affect both the spectrally observed ligand binding properties and catalytic activity of rat P450 1A2, are thought to occur in the putative I helix of the model fitted to P450 101 (Poulos, 1988; Edwards et al., 1989). However, in other P450 proteins changes in regions outside of this area have also been found to have considerable effects; e.g., mutation of residue 209 in mouse P450 2A5 can dramatically influence both the heme spin state and catalytic specificity (Lindberg & Negishi, 1989; Iwasaki et al., 1991; Juvonen et al., 1991). This residue (mouse P450 2A5 residue 209) can be modeled to reside in the E helix of the three-dimensional P450 101 structure.

Different kinds of information may be expected from the use of different approaches to the analysis of enzyme active sites. Characterization through the localization of sites of labeling with mechanism-based inactivators is an established method of identifying catalytic sites. Caveats are required in that (i) the recovery of label in the peptides in these studies was low and (ii) there is a general concern that a reactive product generated in the reaction might migrate before reacting, with binding being influenced by nucleophilicity of residues instead of proximity. However, the very nature of the adducts under consideration in this study—quite unstable and each in only one specific region of the particular P450 1A2 enzyme under consideration—is not what would be predicted from a reactive intermediate reacting randomly with good nucleophilic amino residues. It is of interest that different peptides were modified in two enzymes with such similar primary sequences. Further, the highly related human P450 1A2 did not appear to be inactivated by 2EN.

REFERENCES

Adamovich, T. B., Pikuleva, I. A., Chashchin, V. L., & Usanov, S. A. (1989) *Biochim. Biophys. Acta 996*, 247–253.

Alterman, M. A., & Dowgii, A. I. (1990) *Biomed. Chromatog. 4*, 221–222.

Bayley, H., & Knowles, J. R. (1977) *Methods Enzymol. 46*, 69–112.

Butler, M. A., Guengerich, F. P., & Kadlubar, F. F. (1989a) *Cancer Res. 49*, 25–31.

Butler, M. A., Iwasaki, M., Guengerich, F. P., & Kadlubar, F. F. (1989b) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7696–7700.
Distlerath, L. M., Reilly, P. E. B., Martin, M. V., Davis, G. G., Wilkinson, G. R., & Guengerich, F. P. (1985) *J. Biol. Chem. 260*, 9057–9067.
Edwards, R. J., Murray, B. P., Boobis, A. R., & Davies, D. S. (1989) *Biochemistry 28*, 3762–3770.
Frey, A. B., Kreibich, G., Wadhera, A., Clarke, L., & Waxman, D. J. (1986) *Biochemistry 25*, 4797–4803.
Furuya, H., Shimizu, T., Hatano, M., & Fujii-Kuriyama, Y. (1989a) *Biochem. Biophys. Res. Commun. 160*, 669–676.
Furuya, H., Shimizu, T., Hirano, K., Hatano, M., Fujii-Kuriyama, Y., Raag, R., & Poulos, T. L. (1989b) *Biochemistry 28*, 6848–6857.
Ged, C., Umbenhauer, D. R., Bellow, T. M., Bork, R. W., Srivastava, P. K., Shinriki, N., Lloyd, R. S., & Guengerich, F. P. (1988) *Biochemistry 27*, 6929–6940.
Gotoh, O. (1992) *J. Biol. Chem. 267*, 83–90.
Gotoh, O., & Fujii-Kuriyama, Y. (1989) in *Frontiers in Biotransformation* (Ruckpaul, K., & Rein, H., Eds.) Vol 1, pp 195–243, Akademie-Verlag, Berlin.
Guengerich, F. P. (1986) *Biochem. Biophys. Res. Commun. 138*, 193–198.
Guengerich, F. P. (1989) in *Principles & Methods of Toxicology* (Hayes, A. W., Ed.) pp 777–814, Raven Press, New York.
Guengerich, F. P. (1990a) *Chem. Res. Toxicol. 3*, 363–371.
Guengerich, F. P. (1990b) *Crit. Rev. Biochem. Mol. Biol. 25*, 97–153.
Guengerich, F. P. (1991) in *Monitoring People Exposed to Carcinogens: Analytical, Epidemiological, & Ethical Issues* (Groopman, J. D., & Skipper, P., Eds.) pp 27–51, CRC Press, Boca Raton, FL.
Guengerich, F. P., & Shimada, T. (1991) *Chem. Res. Toxicol. 4*, 391–407.
Guengerich, F. P., Dannan, G. A., Wright, S. T., Martin, M. V., & Kaminsky, L. S. (1982) *Biochemistry 21*, 6019–6030.
Hammons, G. J., Alworth, W. L., Hopkins, N. E., Guengerich, F. P., & Kadlubar, F. F. (1989) *Chem. Res. Toxicol. 2*, 367–374.
Haniu, M., Armes, L. G., Tanaka, M., Yasunobu, K. T., Shastry, B. S., Wagner, G. C., & Gunsalus, I. C. (1982) *Biochem. Biophys. Res. Commun. 105*, 889–894.
Iwasaki, M., Juvonen, R., Lindberg, R., & Negishi, M. (1991) *J. Biol. Chem. 266*, 3380–3382.
Jänig, G. R., Makower, A., Rabe, H., Bernhardt, R., & Ruckpaul, K. (1984) *Biochim. Biophys. Acta 787*, 8–18.
Johnson, E. F., Kronbach, T., & Hsu, M.-H. (1992) *FASEB J. 6*, 700–705.
Juvonen, R. O., Iwasaki, M., & Negishi, M. (1991) *J. Biol. Chem. 266*, 16341–16435.
Kalb, V. F., & Loper, J. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7221–7225.
Komives, E. A., & Ortiz de Montellano, P. R. (1987) *J. Biol. Chem. 262*, 9793–9802.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Larrey, D., Distlerath, L. M., Dannan, G. A., Wilkinson, G. R., & Guengerich, F. P. (1984) *Biochemistry 23*, 2787–2795.
Lindberg, R. L. P., & Negishi, M. (1989) *Nature (London) 339*, 632–634.
Matsunaga, E., Zeugin, T., Zanger, U. M., Aoyama, T., Meyer, U. A., & Gonzalez, F. J. (1990) *J. Biol. Chem. 265*, 17197–17201.
Murray, M., & Reidy, G. F. (1990) *Pharmacol. Rev. 42*, 85–101.
Nebert, D. W., Nelson, D. R., Coon, M. J., Estabrook, R. W., Feyereisen, R., Fujii-Kuriyama, Y., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Loper, J. C., Sato, R., Waterman, M. R., & Waxman, D. J. (1991) *DNA Cell Biol. 10*, 1–14.
Nelson, D. R., & Strobel, H. W. (1989) *Biochemistry 28*, 656–660.
Obach, R. S., Spink, D. C., Chen, N., & Kaminsky, L. S. (1992) *Arch. Biochem. Biophys. 294*, 215–222.
Onoda, M., Haniu, M., Yanagibashi, K., Sweet, F., Shively, J. E., & Hall, P. F. (1987) *Biochemistry 26*, 657–662.
Ortiz de Montellano, P. R., & Correia, M. A. (1983) *Annu. Rev. Pharmacol. Toxicol. 23*, 481–503.
Ortiz de Montellano, P. R., & Reich, N. O. (1986) in *Cytochrome P-450* (Ortiz de Montellano, P. R., Ed.) pp 273–314, Plenum Press, New York.
Palmer, G., & Reedijk, J. (1992) *J. Biol. Chem. 267*, 665–677.
Parkinson, A., Ryan, D. E., Thomas, P. E., Jerina, D. M., Sayer, J. M., van Bladeren, P. J., Haniu, M., Shively, J. E., & Levin, W. (1986a) *J. Biol. Chem. 261*, 11478–11486.
Parkinson, A., Thomas, P. E., Ryan, D. E., Gorsky, L. D., Shively, J. E., Sayer, J. M., Jerina, D. M., & Levin, W. (1986b) *J. Biol. Chem. 261*, 11487–11495.
Petrides, P. E. (1986) in *Methods of Protein Microcharacterization: A Practical Handbook* (Shively, J. E., Ed.) pp 3–39, Humana Press, Clifton, NJ.
Poulos, T. L. (1988) *Pharm. Res. 5*, 67–75.
Poulos, T. L. (1991) *Methods Enzymol. 206*, 11–30.
Poulos, T. L., Finzel, B. C., & Howard, A. J. (1987) *J. Mol. Biol. 195*, 687–700.
Sadeque, A. J., Shimizu, T., Hirano, K., & Hatano, M. (1988) *Inorg. Chim. Acta 153*, 161–164.
Shimada, T., Misono, K. S., & Guengerich, F. P. (1986) *J. Biol. Chem. 261*, 909–921.
Shimizu, T., Hirano, K., Takahashi, M., Hatano, M., & Fujii-Kuriyama, Y. (1988) *Biochemistry 27*, 4138–4141.
Shimizu, T., Ito, O., Hatano, M., & Fujii-Kuriyama, Y. (1991a) *Biochemistry 30*, 4659–4662.
Shimizu, T., Sadeque, A. J. M., Sadeque, G. N., Hatano, M., & Fujii-Kuriyama, Y. (1991b) *Biochemistry 30*, 1490–1496.
Shimizu, T., Tateishi, T., Hatano, M., & Fujii-Kuriyama, Y. (1991c) *J. Biol. Chem. 266*, 3372–3375.
Shively, J. E. (1986) in *Methods of Protein Microcharacterization: A Practical Handbook* (Shively, J. E., Ed.) pp 41–87, Humana Press, Clifton, NJ.
Silverman, R. B. (1988) *Mechanism-based Enzyme Inactivation: Chemistry & Enzymology*, CRC Press, Boca Raton, FL.
Singh, A., Thornton, E. R., & Westheimer, F. H. (1962) *J. Biol. Chem. 237*, 3006–3008.
Smith, P. A. S., & Boyer, J. B. (1963) *Organic Syntheses*, Collect. Vol. IV, pp 75–78, Wiley, New York.
Srivastava, P. K., Yun, C.-H., Beaune, P. H., Ged, C., & Guengerich, F. P. (1991) *Mol. Pharmacol. 40*, 69–79.
Stevens, J. C., Jaw, J. Y., Peng, C. T., & Halpert, J. (1991) *Biochemistry 30*, 3649–3658.
Swanson, R. A., & Dus, K. M. (1979) *J. Biol. Chem. 25*, 7238–7246.
Tsubaki, M., Iwamoto, Y., Hiwatashi, A., & Ichikawa, Y. (1989) *Biochemistry 28*, 6899–6907.
Wilson, K. J. (1988) in *Protein/Peptide Sequence Analysis: Current Methodologies* (Brown, A. S., Ed.) pp 1–33, CRC Press, Boca Raton, FL.
Yasukochi, Y., & Masters, B. S. S. (1976) *J. Biol. Chem. 251*, 5337–5344.

Registry No. 2EN, 2949-26-0; 4-N_3BP, 31656-91-4; P450, 9035-51-2; phenacetin *O*-deethylase, 67724-61-2.