A Proteolytically Sensitive Region Common to Several Rat Liver Cytochromes P450: Effect of Cleavage on Substrate Binding

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ABSTRACT: Limited proteolysis of rat liver microsomes was used to probe the topography and structure of cytochrome P450 bound to the endoplasmic reticulum. Three cytochromes P450 from two families were examined. Monoclonal antibodies to cytochrome P450 forms 1A1, 2B1, and 2E1 were used to immunopurify these proteolyzed cytochromes P450 from microsomes from rats treated with 3-methylcholanthrene, phenobarbital, and acetone, respectively. Electrophoretic and immunoblot analysis of tryptic fragments revealed a highly sensitive cleavage site in all three cytochromes P450. N-Terminal sequencing was performed on the fragments after transfer onto poly(vinylidene difluoride) membranes and showed that this preferential cleavage site is at amino acid position 298 of P450 1A1, position 277 of P450 2B1, and position 278 of P450 2E1. Multiple sequence alignment revealed that these positions are at the amino terminal of a highly conserved region of these cytochromes P450. The important functional role implied by primary sequence conservation along with the proteolytic sensitivity at its amino terminal suggests that this region is a protein domain. Comparison with the known structure of the bacterial cytochrome P450cam predicts that this proteolytically sensitive site is within an interhelical turn region connected to the distal helix that partially encompasses the heme-containing active site. Substrate binding to the cleaved cytochromes P450 was examined in order to determine whether the newly added conformational freedom near the cleavage site functionally altered these cytochromes P450. Cleavage of P450 2B1 abolished benzphetamine binding, which indicates that the cleavage site contains an important structural determinant for binding this substrate. However, cleavage did not affect benzo[*a*]pyrene binding to P450 1A1.

The cytochromes P450 are involved in the metabolism of a wide variety of xenobiotics such as drugs and carcinogens, as well as endobiotics such as steroids and prostaglandins (Lu & West, 1980; Ortiz de Montellano, 1986; Ryan & Levin, 1990). The individual forms of P450[1] exhibit unique catalytic activity profiles toward different substrates. The different substrate specificities of P450s derive from amino acid sequence differences. The P450 superfamily is classified into families such that P450s from different families exhibit limited (<36%) amino acid similarity with one another (Nebert et al., 1989). The families are further divided into subfamilies whose members exhibit a more substantial similarity (>59%) with one another.

The three-dimensional crystal structure of P450cam has been determined (Poulos et al., 1987) and has yielded valuable insights into structure–function relationships (Poulos et al., 1986; Raag & Poulos, 1989, 1991; Raag et al., 1990). Unfortunately, a corresponding crystal structure is unavailable for a mammalian P450. Construction of a mammalian P450 model via homology with P450cam is difficult because of the low sequence similarities between mammalian P450s and P450cam. However, since the tertiary structure is more conserved than the primary sequence for functionally similar proteins, mammalian P450s are believed to have similar structures that resemble P450cam. This supposition is supported by the results of hydropathy (Nelson & Strobel, 1988; Tretiakov et al., 1989) and secondary structure (Tretiakov et al., 1989; Nelson & Strobel, 1989; Edwards et al., 1989) prediction algorithms, which show similarities among different P450s.

Nevertheless, there is little experimental evidence for structural similarities among mammalian P450s in different families. We have thus utilized proteolytic sensitivity of native microsomal-bound P450 as a probe of structural similarity. While three P450s from two different families exhibited a similarly positioned sensitive site, the functional implications of proteolytic cleavage differed for these P450s.

MATERIALS AND METHODS

Preparation of Rat Liver Microsomes. Male Sprague-Dawley rats (6 weeks old) were injected intraperitoneally daily with phenobarbital (PB) (80 mg/kg of body weight for 3 days) to induce P450 2B1, or with 3-methylcholanthrene (MC) (40 mg/kg of body weight for 3 days) to induce P450 1A1. Rats were treated with 25% acetone (AC) in their drinking water for 3 days to induce P450 2E1. Liver microsomes were prepared by differential centrifugation and were suspended in 0.25 M sucrose and stored at −80 °C. The P450 concentration was determined by the method of Omura and Sato (1964) using a molar extinction of 91 mM^{-1} cm^{-1}. The protein concentration was determined by the BCA protein assay (Pierce) using bovine serum albumin as a standard.

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[1] Abbreviations: P450, rat liver microsomal cytochrome P450; PB, phenobarbital; MC, 3-methylcholanthrene; AC, acetone; PB-, MC-, and AC-microsomes, liver microsomes from PB-, MC-, and AC-treated rats, respectively; mAb, monoclonal antibody; Bz, benzphetamine; BP, benzo[*a*]pyrene; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Proteolysis of Microsomal P450. Microsomes were suspended in 50 mM sodium phosphate (pH 7.4)/20% glycerol to a final protein concentration of 10 mg/mL and treated with trypsin (Sigma) at a trypsin:protein ratio (w:w) of 1:100 for PB- and MC-microsomes and 1:10 for AC-microsomes. Digestion generally proceeded at 30 °C for 30 min and was stopped by addition of a 2-fold molar excess of soybean trypsin inhibitor (Sigma). These conditions were determined from preliminary experiments to achieve limited (≈30–50%) digestion of the intact P450, as detected on immunoblots. The microsomal digests were then solubilized with 0.5% Emulgen 911 (Kao-Atlas), and P450s were immunopurified using Sepharose–mAb immunoadsorbents, essentially as previously described (Cheng et al., 1984). Monoclonal antibodies 1-36-1 to P450 1A1 (Park et al., 1982), 2-66-3 to P450 2B1 (Park et al., 1984), and 1-98-1 to P450 2E1 (Ko et al., 1987) were used to immunopurify these P450s from MC-, PB-, and AC-microsomes, respectively. These mAbs were obtained from Dr. S. S. Park (National Cancer Institute) and purified from mouse ascites fluid (Stanker et al., 1985).

Protein and Peptide Analyses. Immunopurified P450s and their peptides were separated by SDS–PAGE on a 10–20% gradient gel. Protein was visualized by Coomassie brilliant blue staining of the gel. Immunoblot analysis was performed as described (Alston et al., 1991). Densitometric analysis of stained bands was performed on either a Beckman DU-8 with scanning accessory or a Molecular Dynamics computing densitometer. The extent of P450 cleavage was quantitated from the integrated band intensities of the intact P450 and P450 fragments. For amino acid sequencing, separated peptides were electroblotted from the gel to poly(vinylidene difluoride) membranes (Immobilon, Millipore) (Matsudaira, 1987), using a transfer solution of 0.005% sodium dodecyl sulfate, 20% methanol, and 50 mM Tris–glycine (pH 8.2). Peptides were visualized with Coomassie brilliant blue, excised from the membrane, and sequenced on a Porton Model 2020 protein sequencer, using standard program number 1. Phenylthiohydantoin amino acids were analyzed on a Beckman system Gold HPLC.

Multiple alignment of protein sequences was performed with the MACAW program (Schuler et al., 1991), implemented on an IBM PS/2 Model 80 microcomputer under Windows 3.0. For visualization of the P450cam crystal structure (Poulos et al., 1987), its coordinates (data set 2CPP) were obtained from the Protein Data Bank (Brookhaven National Laboratories, Upton, NY) and displayed on a Silicon Graphics 4D/70G workstation using QUANTA molecular modeling software (Polygen Corp, Waltham, MA).

Substrate-Binding Spectra. Substrate-binding difference spectra (Schenkman et al., 1967) were obtained using an Aminco DW2000 spectrophotometer. Measurements were performed on the immunoadsorbed P450s because elution of P450 from the immunoadsorbent requires denaturing conditions (Cheng et al., 1984). The Sepharose–mAb–P450 complex was suspended in 0.1 M potassium phosphate (pH 7.4)/20% glycerol in the sample cuvette, and the buffer solution was added to the reference cuvette. Temperature was maintained at 25 °C with an external water circulator bath with constant stirring. The concentrations of immunopurified P450 from intact and digested microsomes were obtained from the heme absorbances at 417 nm, using an extinction coefficient of 107 $mM^{-1} cm^{-1}$ (Hashimoto-Yutsudo et al., 1980); spectra were obtained in the range of 350–600 nm and corrected for scatter (Leach & Sheraga, 1960). After a base line was recorded, a small volume of Bz (in water), BP (in DMSO), or aniline (in water) was added to both sample and reference cuvettes in the measurements on P450s from PB-, MC-, or AC-microsomes, respectively. The spectrum was then obtained, and the substrate-induced difference spectrum was calculated by subtraction of the base-line spectrum. Difference spectra of intact and digested P450s were compared after minor (<13%) correction for differences in P450 content. Binding of BP to P450 1A1 was also determined via fluorescence quenching measurements (Omata & Friedman, 1991) on an SLM Model 8000c spectrofluorometer, with excitation of BP fluorescence at 368 nm.

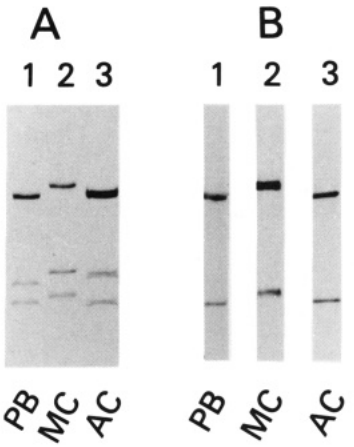

FIGURE 1: SDS–PAGE of immunopurified P450s and fragments. (A) Lanes 1–3 show a Coomassie brilliant blue stain of the P450 proteins and peptides immunopurified from proteolyzed PB-, MC-, and AC-microsomes, using mAbs 2-66-3, 1-36-1, and 1-98-1, respectively. The trypsin:protein ratios used for proteolysis were 1:100 for PB- and MC-microsomes and 1:10 for AC-microsomes. (B) Immunoblot of the samples in panel A. The mAb used for immunodetection was the same as that used for immunopurification.

RESULTS

Limited trypsin digestion was used to identify accessible and cytoplasmic-exposed regions of P450 in its native milieu in the endoplasmic reticulum. The experimental procedure entails (1) tryptic digestion of microsomes, (2) purification of P450 fragments, and (3) identification of proteolytically sensitive sites. We utilized mAbs for immunoaffinity purification of digested P450s from microsomes which had been enriched in mAb–specific P450s by pretreatment of rats with appropriate inducers. Thus, mAb 1-36-1 was used for immunopurification of MC-induced P450 1A1, mAb 2-66-3 for PB-induced P450 2B1, and mAb 1-98-1 for AC-induced P450 2E1.

Limited Proteolysis of P450. The immunopurified P450 digests are shown in Figure 1A. In addition to the intact P450s (highest molecular weight band), only two fragments were observed for each P450, which indicates a single proteolytically sensitive site. The apparent molecular weights of these fragments are shown in Table I. Our preliminary experiments established the amounts of trypsin required for this mild (30–50%) digestion of P450, which did not yield any additional, smaller peptides. Microsomal P450 2E1 was more resistant to digestion than P450 2B1 or P450 1A1, since P450 2E1 required a much higher ratio (1:10) of trypsin than P450 2B1 or P450 1A1 (1:100) during a 30-min digestion period, and was digested to a smaller extent (≈30% for 2E1 versus ≈50% for 2B1 and 1A1). This protease resistance of P450 2E1 is probably unrelated to its microsomal attachment since we previously observed that this purified cytochrome was also relatively protease-resistant (Robinson et al., 1989).

Table I: Size and Automated Sequence Determination of P450 Fragments[a]

P450 fragment	size[b]	cycle 1	2	3	4	5	6	7	8	9	10
P450 1A1	30.9 ± 0.3	Pro	Ser	Val	Tyr	Gly	Phe	Pro	Ala		
		20[c]	5	16	9	2	11	11	7		
	25.1 ± 0.6	Leu	Asp	Glu	Asn	Ala	Asn	Val	Gln		
		118	55	43	61	41	24	17	18		
P450 2B1	27.3 ± 0.6	Met	Glu	Pro	Ser	Ile	Leu	Leu			
		12	17	7	2	6	7	7			
	23.9 ± 0.4	Ser	Asn	His	His	Thr	Glu	Phe	His		
		106	105	130	161	12	20	72	44		
P450 2E1	28.1 ± 0.9	Ala	Val	Leu	Gly	Ile	Thr	Ile	Ala	Leu	Leu
		25	24	16	13	9	7	5	4	5	9
	23.4 ± 0.4	His	Ser	Gln	Glu	Pro	Met	Tyr	Thr	Met	Glu
		63	5	31	31	21	13	19	12	9	11

[a] P450 fragments were separated by SDS–PAGE, electroblotted to poly(vinylidene difluoride) membranes, and subjected to N-terminal sequence analysis. [b] Peptide sizes (in kilodaltons) of both fragments from each P450 were determined by SDS–PAGE. [c] Repetitive yield in picomoles.

The six fragments were identified by electroblotting to poly(vinylidene difluoride) membranes and automated amino acid sequence analysis. The N-terminal sequence data for the higher and lower molecular weight fragments from each P450 are shown in Table I. The sequences of the larger fragments corresponded to the known N-terminal sequences of P450 1A1 (Sogawa et al., 1984; Hines et al., 1985), P450 2B1 (Fujii-Kuriyama et al., 1982; Yuan et al., 1983), and P450 2E1 (Song et al., 1986). The N-terminal sequence of each smaller fragment was identical to an internal sequence of its parent P450, and corresponded to amino acid positions 298 of P450 1A1, 277 of P450 2B1, and 278 of P450 2E1. Immunoblot analysis of the P450 digests revealed that only the smaller fragments were immunoreactive (Figure 1B) and thus that the mAb-specific epitopes reside within the carboxy-terminal region. The immunopurification procedure yielded both this epitope-containing fragment and the fragment lacking this epitope (Figure 1A), indicating that both fragments remain associated as a stable complex after proteolytic cleavage.

For each P450, the sum of the masses of the two fragments was the same as the masses of the intact P450: the respective values are (in kilodaltons) 56.0 ± 0.5 and 56.6 ± 0.5 for P450 1A1, 51.2 ± 0.9 and 50.7 ± 0.7 for P450 2B1, and 51.0 ± 1.2 and 49.8 ± 0.7 for P450 2E1. This indicates that the two fragments comprise the entire P450 molecule and that mild tryptic treatment thus yields a complete, singly cleaved P450.

Position of Cleavage Site in Multiple Sequence Alignment of P450s. The sequences of these P450s, representing two distinct families, were then examined for similarities near the proteolysis site. We used the multiple alignment program MACAW, which employs a local alignment strategy to identify smaller, highly conserved regions within larger sequences which may otherwise display little homology (Schuler et al., 1991). We thus identified several regions of similarity in these P450s (Figure 2). The largest and most homologous region commences at relative sequence position 301, and its N-terminal corresponds to the preferable cleavage sites of the three P450s. This region of high similarity was also recently identified by pairwise alignment of multiple P450s (Kalb & Loper, 1988). Thus, the conservation of sequence of this region implies an important functional role, while common tryptic sensitivity at its N-terminus implies structural relatedness. These observations thus suggest that this region is the juncture of a protein domain.

Effect of Cleavage on Substrate Binding. We next examined whether the proteolytically sensitive region had a functional role by evaluating the cleaved P450s for their capability to bind known substrates. We utilized the substrate-induced difference spectrum to monitor benzphetamine binding to immunopurified P450 2B1 (Figure 3). It is evident from the reduced spectral change that cleavage of P450 2B1 reduces benzphetamine binding. In order to establish a clear relationship between decreased benzphetamine binding and single-point cleavage of P450 2B1, we examined P450 2B1

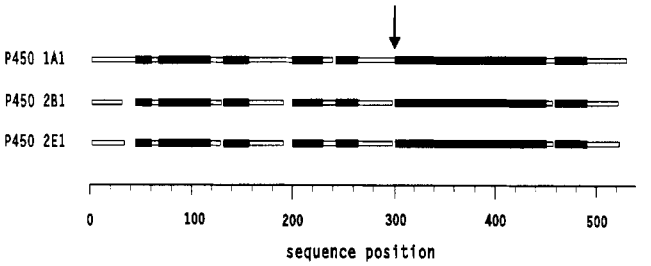

FIGURE 2: Sequence alignment of P450s. The sequences of the immunopurified P450s were subjected to multiple sequence alignment using the MACAW program. Regions with high sequence similarity are filled. The axis indicates the computer-generated relative sequence positions for the optimal alignment. The arrow indicates the common tryptic cleavage position.

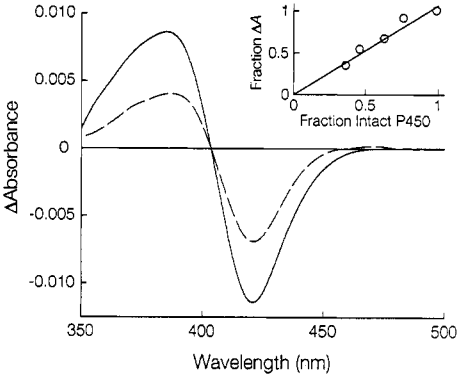

FIGURE 3: Benzphetamine-binding difference spectrum of P450 2B1. Immunoadsorbent-bound P450 2B1 was suspended in 0.1 M potassium phosphate (pH 7.4)/20% glycerol, and the benzphetamine-induced difference spectra were obtained as described under Materials and Methods. The concentrations of P450 2B1 and benzphetamine were 0.35 μM and 1 mM, respectively. Spectra are shown for intact P450 2B1 (—) from undigested PB microsomes and for a sample in which 54% of the P450 2B1 was cleaved (- - -), obtained from partially digested PB microsomes. The inset shows the relationship between P450 2B1 cleavage and binding of benzphetamine. Samples containing different ratios of cleaved to intact P450 2B1 were obtained by digesting PB microsomes for 0, 4, 15, 60, and 120 min. The Bz-induced difference spectra and amounts of intact P450 were determined as described under Materials and Methods, and the values at each time are presented as a fraction of the values obtained using intact P450 2B1 from undigested microsomes.

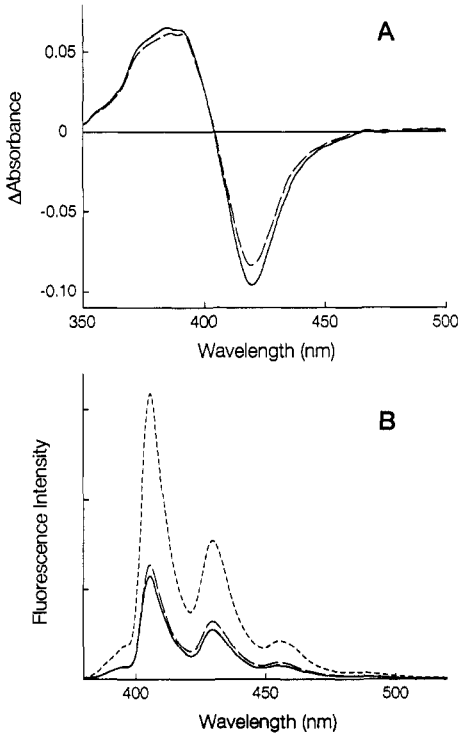

FIGURE 4: Benzo[*a*]pyrene-binding spectra of P450 1A1. Immunoadsorbent-bound P450 1A1 was suspended in 0.1 M potassium phosphate (pH 7.4)/20% glycerol, and spectra were obtained as described under Materials and Methods. (A) BP-induced absorbance difference spectra for intact (—) and partially (46%) cleaved (- -) P450 1A1. The P450 1A1 and BP concentrations were 2.7 and 2.5 μM, respectively. (B) Quenching of BP fluorescence upon binding to P450 1A1. Spectra are shown for BP alone (- - -) and in the presence of intact (—) or partially (62%) cleaved (- -) P450 1A1. The P450 1A1 and BP concentrations were 60 and 30 nM, respectively. BP fluorescence was excited at 368 nm.

samples with different ratios of cleaved to intact P450 2B1, which were produced by varying the proteolysis times. The fraction of intact P450 2B1 in each sample was densitometrically measured and compared to the fraction of ΔA, the Bz-induced absorbance change of the digested P450 2B1 mixture relative to that of intact P450 2B1. The results (Figure 3, inset) show a clear correlation between single-point cleavage and loss of benzphetamine-binding ability.

P450 1A1 was also examined for binding to its substrate, BP. We measured both the substrate-induced absorbance difference spectrum (Figure 4A) and the quenching of BP fluorescence that occurs upon binding to P450 1A1 (Figure 4B). There was no significant difference between intact and digested P450 1A1 in either spectral measurement. In contrast to P450 2B1, single-point cleavage of P450 1A1 thus does not inhibit substrate binding.

P450 2E1 binding to aniline was also detectable by difference spectroscopy, but the effect of proteolysis could not be assessed owing to the relatively large uncertainties in spectral measurements that resulted from the small amount of P450 2E1 along with its low (≈30%) proteolytic cleavage efficiency.

DISCUSSION

The position of the proteolytically sensitive site on the P450 surface can be predicted by using P450 models constructed on the basis of secondary structure algorithms and the known structure of P450cam (Nelson & Strobel, 1989; Edwards et al., 1989). The site is thus situated in a region corresponding to an interhelical turn between helices I and H of P450cam (arrow, Figure 5). The proposed cleavage region is on the

FIGURE 5: α-Carbon map of bacterial P450cam. The arrow indicates the predicted proteolytically sensitive region in mammalian P450s. Both the I helix which adjoins this region and the heme moiety are highlighted. Coordinates were obtained from data set 2CPP of the Protein Data Bank.

P450 surface and should be readily accessible to trypsin. This site is also probably exposed on the surface of the lipid bilayer and not embedded in the membrane.

Molecular dynamics simulation of P450cam furthermore identifies this region as one of several highly mobile interhelical turn regions (Paulsen & Ornstein, 1991). Such highly mobile regions in proteins have been correlated with preferential proteolysis (Fontana et al., 1986). This flexibility probably facilitates binding of the target peptide by the protease since adjoining residues are relatively free to adopt conformations which allow efficient binding. A similar rationale may be applied to the observation that in antibody–antigen interactions antibodies preferentially bind antigenic regions of high mobility (Geysen et al., 1987): peptide flexibility allows adoption of a conformation with optimal binding properties. One can perhaps generalize that for protein–protein interactions which require the highest degree of specificity, a relatively rigid framework is required to ensure complementary amino acid interactions, but some peptide flexibility may be required to optimize other interactions, such as antibody–antigen and protease–peptide.

We have demonstrated a single-point cleavage of P450 2B1 which eliminates Bz binding, for which there are several possible explanations. First, the cleaved region may include amino acid residues that directly bind Bz; this would be unlikely in the P450cam model, however, because of its great distance from the active-site heme. Second, changes in the structure/dynamics of the cleaved region can affect the substrate-binding region. For example, the increased mobility of the cleaved region may in turn enhance the mobility of the I helix which is found on one side of the heme (Figure 5); if this helix includes residues important for binding to Bz, their positions would then fluctuate rather than maintain the proper orientation for effective binding. Such fluctuations are an important factor in substrate binding: for P450cam, substrate binding reduces the dynamic fluctuations of several amino acids but does not alter their average positions (Poulos et al., 1986). Third, in addition to a direct effect on substrate-binding residues, cleavage may also influence substrate binding by altering heme–water ligation and thus the heme low–high-spin equilibrium; a similar situation has been shown to occur with mouse P450coh and $P450_{15\alpha}$, in which mutations at residue 209

markedly alter the spin equilibrium (Iwasaki et al., 1991).

In contrast to P450 2B1, cleaved P450 1A1 retained the capability to bind BP substrate. Although proteolytic disruption of the local structure and/or dynamics of the same region of two P450s thus has different consequences for substrate binding, it is unknown whether this differential effect is confined to the substrate-binding region or applies to larger structural elements. A detailed interpretation of this result must await the experimental determination of a detailed structure for a mammalian P450. Although P450cam structure has sometimes been used as a model for mammalian P450s, the structural homology between these two classes of P450 is questionable owing to the low sequence similarities (<15%), and the requirement for a minimum of 25% sequence similarity in order to infer structural similarity (Sander & Schneider, 1991).

Relatively few experimental studies have evaluated the tertiary structural similarities among mammalian P450s, or between mammalian P450s and P450cam. However, a rabbit P450 was recently compared to P450cam with respect to binding to bifunctional compounds composed of a pyridine heme ligand connected to an anionic diphosphinic acid via a variable-length linker (Krainev et al., 1991); the results imply a distance of ≈17 Å between the heme iron and a charged surface residue (Lys or Arg) in both P450s. Such experiments yield structural information that provides constraints for any proposed model of a mammalian P450. We have likewise utilized the criterion of limited proteolysis to evaluate the similarity among P450s from three subfamilies. While this supports a common structural model, care must be exercised in extrapolating structure–function information from one P450 to another, as the functional implications of perturbing a common region in P450s 2B1 and 1A1 were different.

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