Identification of a Common Cytochrome P450 Epitope near the Conserved Heme-Binding Peptide with Antibodies Raised against Recombinant Cytochrome P450 Family 2 Proteins†

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ABSTRACT: The cytochrome P450 (P450) proteins constitute a superfamily of enzymes involved in various oxidations and related activities. Polyclonal antibodies raised against bacterial recombinant human P450s varied in specificity, depending upon the individual rabbits used. Several of the antisera raised against P450s 2C10 and 2E1 recognized a number of P450 family 1, 2, and 3 proteins, and two of the less selective antibodies were used to identify cross-reactive epitopes. P450 2C10 peptides reacting with anti-P450 2E1 and P450 2E1 peptides reacting with anti-P450 2C10 were isolated after electrophoresis/immunoblotting and analyzed by Edman degradation. Several of these were in a region near the highly conserved Cys that is a putative axial ligand to the heme. Peptides corresponding to the most conserved regions in this area were synthesized. Anti-P450 2C10 sera did not recognize 14-mer peptides corresponding to the heme-binding region (2C10 410−423 or 2E1 409−422) or the 14-mer peptides immediately C-terminal to these (2C10 425−438 or 2E1 424−437), but anti-P450 2E1 sera showed weak reaction with the latter two synthetic peptides. A longer peptide (29-mer) of P450 2E1 containing parts of both regions (412−440) reacted with both anti-P450 2C10 and anti-P450 2E1 antisera. Antibodies raised against a conjugate of the 29-mer peptide (with hemocyanin) recognized this antigen, the more C-terminal 14-mer peptides (2C10 425−438 and 2E1 424−437), P450s 2C10 and 2E1, and P450s 1A1, 11A1, and 17A. The 29-mer peptide showed considerable α-helix structure as judged by CD spectroscopy, in contrast to any of the 14-mers. The cross-reactivity seen in immunoblotting was not observed in inhibition of catalytic activity. We conclude that the region immediately beyond the conserved Cys region is a common epitope for P450 2C10 and P450 2E1 and is a common epitope for the immunochemical cross-reactivity seen with some other P450 proteins. The region appears to correspond to the L-helix of bacterial P450s on the basis of sequence alignments and is probably exposed to the surface of the proteins.

P450[1] enzymes are involved in numerous oxidations of substrates of endobiotic and xenobiotic origin (Ortiz de Montellano, 1986; Guengerich, 1991, 1993; Porter & Coon, 1991; Waterman & Johnson, 1991) (see also the entire January 1992 issue of *FASEB J.*). The genes are classified in a superfamily, which is further divided into families and subfamilies on the basis of sequence identity (Nelson *et al.*, 1993). The most conserved region in these proteins is one near the C-terminus and contains Cys which serves as the axial ligand to the heme. The three-dimensional structures of four soluble bacterial P450 enzymes have been determined by X-ray diffraction studies (Poulos *et al.*, 1987; Ravichandran *et al.*, 1993; Hasemann *et al.*, 1994; Cupp-Vickery & Poulos, 1995). The general structural features may apply to the more intractable eukaryotic, membrane-bound P450s although prediction of fine details is probably not realistic (Poulos, 1991; Hasemann *et al.*, 1994). Numerous studies have been done using site-directed mutagenesis to define elements of P450s involved in aspects of catalysis, and some general patterns seem to be evolving (Johnson, 1992; Johnson *et al.*, 1992; Chen & Zhou, 1992; Morohashi *et al.*, 1993).

Antibodies to P450s have been used to study their biochemistry for a number of years (Dus *et al.*, 1974; Thomas *et al.*, 1976, 1977; Park *et al.*, 1980; Reik *et al.*, 1982; Gelboin, 1993). Some epitopes have been defined by the preparation of antibodies raised against selected peptides (Edwards *et al.*, 1988, 1990, 1993a,b; Sanghera *et al.*, 1991). The focus of such work has usually been the development of reagents that will discriminate among closely related structures. The consideration of epitopes that are common to many P450s is another matter and has not been studied systematically. Cross-reactivity of different P450s was sometimes seen in early immunochemical work (Dus *et al.*, 1974; Thomas *et al.*, 1976; Guengerich, 1978). However, when antibodies were raised against P450s isolated from animal tissues there were always concerns about contamination of the antigens used, in both antibody preparation and analysis. Cross-reactive antibodies are removed in cross-

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[1] Abbreviations: P450, cytochrome P450 [also termed heme−thiolate protein P450 (Palmer & Reedijk, 1992)]; $NaDodSO_4$, sodium dodecyl sulfate; KLH, keyhole limpet hemocyanin; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization/time-of-flight (mass spectrometry); IgG, immunoglobulin G (fraction of antisera); CD, circular dichroism. For reference to P450 nomenclature see Nelson *et al.* (1993).

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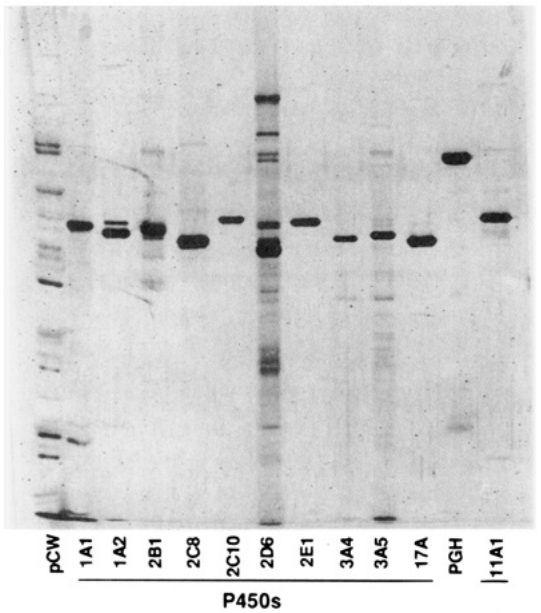

FIGURE 1: $NaDodSO_4$−polyacrylamide gel electrophoresis of P450 and prostaglandin H synthase preparations. The P450s[2] are labeled with the systematic nomenclature. The pCW lane contained an aliquot of membranes prepared from *E. coli* transfected with the pCW vector but devoid of any P450 or other cDNA. "PGH" indicates prostaglandin H synthase. Approximately 1.0 μg of each protein was loaded. Electrophoresis was according to the method of Laemmli (1970) (10% acrylamide, w/v) as modified (Guengerich, 1994), using staining with ammoniacal silver (Wray *et al.*, 1981). Migration was from the top to bottom.

adsorption procedures, such as that developed by Thomas *et al.* (1979). Cross-reactive polyclonal antibodies recognizing rat P450s 1A1 and 1A2 could be isolated using such approaches (Reik *et al.*, 1982); the common epitopes were not identified. Most monoclonal antibody selection screens have been biased toward selection of specific antibodies and not cross-reactive ones [e.g., Gelboin (1993) and references therein]. Although there have been anecdotal reports of attempts to raise antibodies against the conserved heme peptide, no successful studies have been published.

To our knowledge, antibodies had not been raised against recombinant P450 proteins previously. We used this approach in order to rule out any possibility of antibody production due to contamination by other P450s. Several of the antisera showed considerable cross-reactivity among recombinant P450s, and we have characterized a major common epitope in the C-terminal region near the conserved heme-binding region.

EXPERIMENTAL PROCEDURES

P450s and Other Proteins. Human P450s 1A1 (Guo *et al.*, 1994), 1A2 (Sandhu *et al.*, 1994), 2C10 (Sandhu *et al.*, 1993), 2D6 (Gillam *et al.*, 1995a), 2E1 (Gillam *et al.*, 1994), 3A4 (Gillam *et al.*, 1993), and 3A5 (Gillam *et al.*, 1995b) were modified at the 5′-termini for expression in a pCW vector in *Escherichia coli* as previously described and purified.[2] $NaDodSO_4$−polyacrylamide gel electrophoresis results are shown in Figure 1. P450 2B1 was purified from liver microsomes isolated from phenobarbital-treated rats as described (Guengerich *et al.*, 1982a). Baculovirus recombinant P450 2C8 was a gift of Dr. D. Zeldin and Prof. J. H. Capdevila, Department of Medicine, Vanderbilt University. *E. coli* recombinant bovine P450s 11A1 (Wada *et al.*, 1991) and 17A (Barnes *et al.*, 1991) were gifts of Prof. M. R. Waterman of this department.

Rat liver epoxide hydrolase (Guengerich *et al.*, 1979) and rabbit liver NADPH−P450 reductase (Yasukochi & Masters, 1976; Guengerich, 1994) were purified essentially as described previously. *E. coli* flavodoxin reductase (Jenkins & Waterman, 1994) was a gift of C. M. Jenkins and Prof. M. R. Waterman of this department. Human cytochrome b_5 was isolated as described (Shimada *et al.*, 1986). Sheep prostaglandin H synthase (COX 1) was a gift of Prof. L. J. Marnett of this department. Other proteins used in immunochemical analyses were generally purchased from Sigma Chemical Co. (St. Louis, MO) or other commercial sources.

Antibodies. Antisera against *E. coli* recombinant human P450s were raised in rabbits using the following schedule, modified from previous work (Kaminsky *et al.*, 1981). Female New Zealand White rabbits (2.0−2.5 kg) were used. The P450 preparation (0.45 nmol) was diluted in a sterile aqueous NaCl solution (0.9% w/v) and mixed with an equal volume (1.0 mL) of Freund's complete adjuvant by repeatedly forcing the mixture through a 20 gauge needle fixed between two syringes. The material was injected in ~20 small injections under the skin over the (shaved) back. The process was repeated 1 week later. Four weeks later, each rabbit was given an intramuscular injection of 0.10 nmol of P450 (dissolved in 0.9% NaCl w/v). After another 1 week, blood was collected from the ear vein. Samples were allowed to clot for 1 h at room temperature and then overnight at 4 °C. Sera were recovered by centrifugation for 10 min (10^4g, 10 min) and heated at 56 °C for 20 min to inactivate complement. The centrifugation step was repeated, and the sera (supernatants) were stored in small aliquots at −20 °C.

A conjugate of a 29-mer peptide (designated V) and KLH was prepared according to the method of Bauminger *et al.* (1973) and used to raise antibodies in the same way, injecting each of three different rabbits with 0.2, 0.6, or 1.8 mg of the conjugate in each of the first two treatments and then 0.2 mg in the third. The rabbits were designated #633, #634, and #635, respectively.

IgG fractions were isolated using a protein G−agarose chromatography method described by the manufacturer (Pierce Chemical Co., Rockford, IL). Fractions eluted from the column were dialyzed twice *vs* 50 volumes of 25 mM Tris-HCl buffer (pH 8.0) and concentrated using an ultrafiltration device (PM30 membrane; Amicon, Danvers, MA).

Peptide Synthesis and Purification. Synthetic peptides (designated I, II, III, IV, and V) were prepared in the facility in the Department of Molecular Biology by J. Willis using an Applied Biosystems 431A peptide synthesizer (Foster City, CA) and (9-fluorenylmethoxy)carbonyl amino acid esters. The synthetic peptides were purified by HPLC using a Brownlee 2.1 × 100 mm Aquapore RP-300 reversed-phase column (7 μm packing, Applied Biosystems) and linear gradients of 4−80% CH_3CN in 0.10% aqueous CF_3CO_2H (v/v). The effluent was monitored at 214 nm, and the major peaks were concentrated using a Speed-Vac vacuum centrifuge (Savant, Farmingdale, NY). The identities of the

[2] The work done in the first portions of this study used a partially purified preparation of P450 2D6 (Figure 1). Later studies utilized an electrophoretically homogeneous preparation (Gillam *et al.*, 1995a), and similar results were obtained.

synthetic peptides were verified by Edman degradation (*vide infra*) or by mass spectrometry.

MALDI-TOF mass spectrometry was done by L. C. Bell in the Vanderbilt facility using a Kratos Kompact MALDI III instrument (Kratos Analytical Ltd., Manchester, U.K.). The matrix was sinapinic acid, and mellitin (M_r 2847.5) was added to each sample as an internal standard for calibration.

CD spectra were obtained with a JASCO 720 instrument (Japan Applied Spectroscopy Co., Tokyo) in the Vanderbilt facility.

Proteolysis, Electrophoresis, and Immunostaining. For NaDodSO$_4$–polyacrylamide gel electrophoresis, the basic method of Laemmli (1970) was used with a 10% acrylamide (w/v) gel when intact P450 proteins were analyzed (a stacking gel was used in all cases). Resolved proteins were electrophoretically transferred to nitrocellulose sheets as described (Guengerich *et al.*, 1982b; Guengerich, 1994). The basic immunostaining procedure involved washing six times (5 min each) with 20 mM potassium phosphate buffer (pH 7.4) containing 150 mM NaCl and 0.05% Tween 20 (w/v), followed by incubation with a 1:10^3 dilution of each antiserum in the above buffer at 37 °C for 1 h for intact proteins or 4 h for peptides (all with orbital shaking). In the case of anti-peptide V, overnight incubation at room temperature was used with a 1:500 dilution of antisera #633. Unbound material was removed by six 5 min washes with the buffer. A 1:(2 × 10^3) dilution of goat anti-rabbit IgG/horseradish peroxidase (ICN Biochemicals, Cleveland, OH) in the buffer was used to treat the blots for 60 min at room temperature. The blots were washed again with six 5 min treatments with buffer, followed by three 30 min washes with the same buffer devoid of Tween 80 (to reduce problems with fading of the stain), and then color was developed by treatment with a mixture of 2.5 mM 4-chloro-1-naphthol and 15 mM H_2O_2 in the buffer.

P450s were treated with N^{α}-*p*-tosyl-L-lysine chloroketone-treated chymotrypsin, *Staphylococcus aureus* V8 protease, trypsin, or papain at the respective P450:protease (w/w) ratios of 10:1, 10:1, 10:1, and 200:1 for 30 min at room temperature to achieve partial cleavage. For the preparation of smaller peptides, chymotrypsin and *S. aureus* V8 protease were used at (w/w) ratios of 30:1 and 15:1 for 15 h at room temperature. In all cases the digestions were done in 125 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol (v/v), 0.5% NaDodSO$_4$, and 0.010% bromphenol blue (P450 samples were heated at 95 °C for 2 min prior to addition of proteases). After proteolytic digestion, NaDodSO$_4$ was added to 2% (w/v) and 2-mercaptoethanol to 5% (v/v); samples were heated again at 95 °C for 2 min.

For the isolation of small peptides, a 50–100 pmol aliquot of a P450 digest (based on original concentration) was applied to a gel using the method of Schagger and von Jagow (1987) (4% acrylamide in stacking gel, 10% in spacer gel, 16.5% in separating gel, all w/v). The resolved peptides were transferred to ProBlot poly(vinylidene fluoride) membranes (Applied Biosystems) using 10 mM sodium 3-(cyclohexylamino)-1-propanesulfonate buffer (pH 11.0) at a 100 mA current setting for 45 min. Portions were treated to develop immunoreactive bands as described above. For the detection of total peptides, an aliquot with 300 pmol equivalent of P450 was used in the electrophoretic and transfer procedures and staining was done for 5 min with a 0.10% solution (w/v) of Commassie Brilliant Blue R-250, followed by extensive destaining with a 50:40:10 mixture of H_2O, CH_3OH, and CH_3CO_2H (v/v/v). Appropriate bands were marked and then (after destaining) used directly for N-terminal amino acid sequence analysis.

In some cases proteins and peptides were applied directly to sheets of Immobilon poly(vinylidene fluoride) membranes (Waters-Millipore, Bedford, MA) using a "slot-blot" device (Hoefer, San Francisco, CA) and treated directly to bind antibodies, stain, etc.

N-Terminal Amino Acid Sequence Analysis. Edman degradation was done in the Vanderbilt facility by E. Howard using an updated Applied Biosystems 470A or a Beckman Porton LF-3000 G instrument (Beckman, Fullerton, CA). Samples were introduced from ProBlot membranes. Quantitations are based on external standards.

Catalytic Assays and Immunoinhibition. Assays for tolbutamide methyl hydroxylation (Knodell *et al.*, 1987) and chlorzoxazone 6-hydroxylation (Peter *et al.*, 1990) were done with human liver microsomes as described, using 50 pmol of P450 in each case. The uninhibited activities were 0.52 nmol of hydroxytolbutamide formed min^{-1} (nmol of P450)$^{-1}$ (liver sample HL 112) and 1.25 nmol of 6-hydroxychlorzoxazone formed min^{-1} (nmol of P450)$^{-1}$ (liver sample HL 100). Either 50, 250, or 500 μg of the appropriate IgG was incubated with the microsomes for 30 min at room temperature prior to addition of other assay components.

Other Methods. Protein sequences were retrieved from the SwissProt database using the Gened program of Intelligenetics. Sequence alignment was done using the Intelligenetics Genalign program.

P450 concentrations were estimated using the spectral method of Omura and Sato (1964, 1967).

Protein concentrations were estimated using a bicinchoninic acid method (Pierce Chemical Co.) according to the manufacturer's instructions. Concentrations of peptides were estimated from A_{214} measurements.

RESULTS

Specificity of Antibodies Raised against P450s. Antibodies were raised against P450s that had been expressed in *E. coli* in this laboratory. The specificity was somewhat variable depending upon the individual rabbit producing each antiserum, as judged by the results of electrophoresis/immunoblotting experiments (Figure 2). We concentrated our attention on the antisera raised against P450s 2C10 and 2E1, two proteins with 57% sequence identity. Antisera raised to either protein recognized both; the selectivity varied among individual rabbits even though a single antigen preparation was used. The selectivity was not simply due to the sensitivity seen at different antiserum dilutions (results not shown). Out of the six rabbits used for each antigen, roughly half yielded sera that were "more selective" (e.g., Figure 2A,C) and half yielded antisera that were "less selective" (e.g., Figure 2B,D). Even the more selective of these antisera showed some recognition of the other P450 2 family proteins under these immunoblotting conditions.[3] The less specific anti-P450 2C10 preparation (#485) recognized P450s 1A1, 1A2, 2B1, 2C8, 2C10, 2D6, 2E1, 3A4, and 3A5. The less specific anti-P450 2E1 preparation recognized all of the P450 family 2 proteins but not P450 subfamilies 1A or 3A. However, these antisera did not react strongly with P450 11A1 or P450 17A.[4]

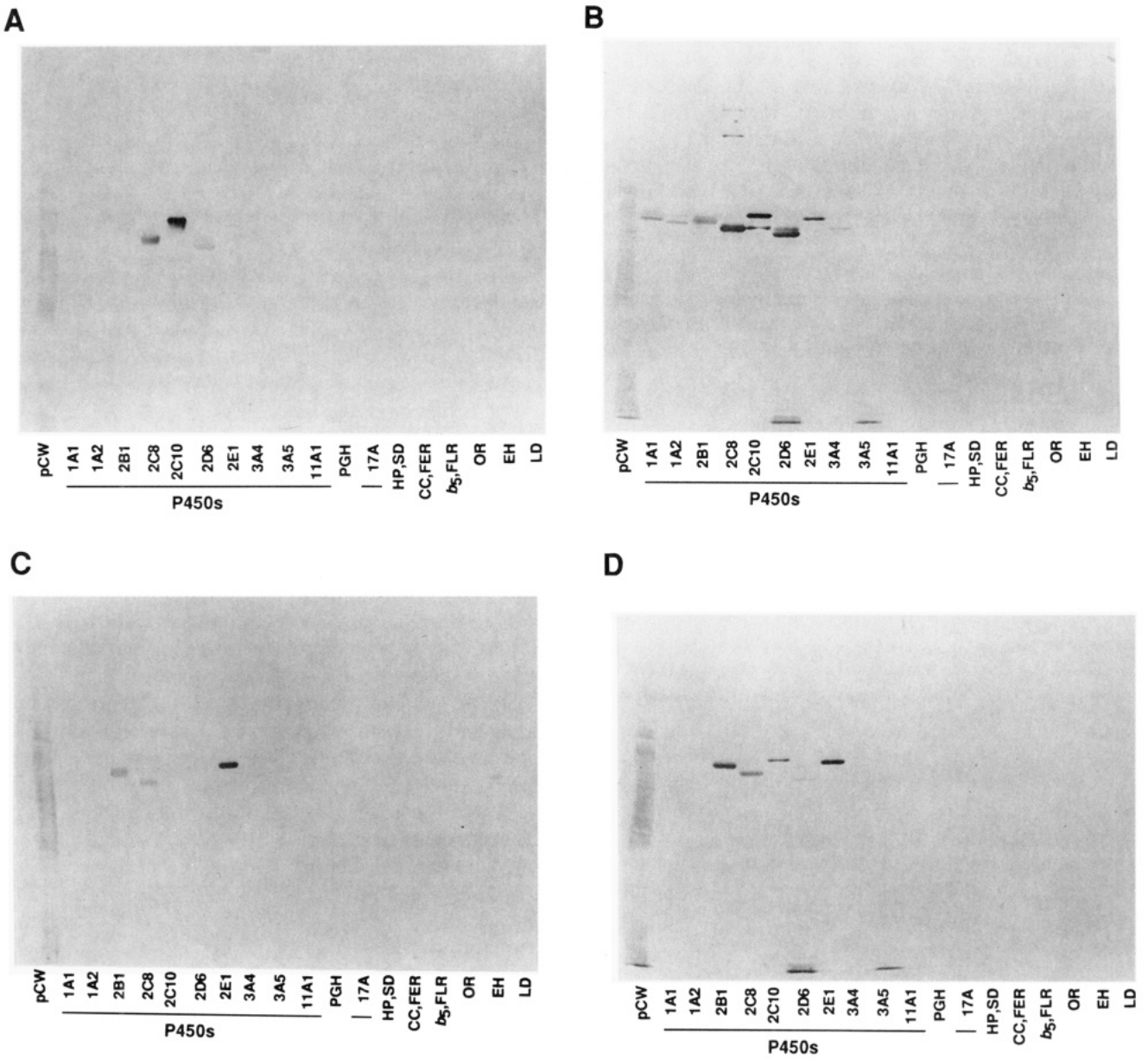

FIGURE 2: Comparison of electrophoresis/immunoblotting patterns of P450s with the more and less specific anti-P450 2C10 antisera. Each lane included *E. coli* (pCW) membranes (5 μg), the indicated P450 (2.0 pmol) or prostaglandin H synthase (PGH, 1.0 μg), horseradish peroxidase plus bovine erythrocyte superoxide dismutase (HP/SD, 1.0 μg each), horse heart cytochrome *c* (CC, 1.0 μg) plus spinach ferredoxin reductase (FER, 5 pmol), human cytochrome b_5 (b_5, 5 pmol) plus *E. coli* flavodoxin reductase (FLR, 5 pmol), rabbit NADPH−P450 reductase (OR), rat liver microsomal epoxide hydrolase (EH), and porcine heart lipoyl dehydrogenase (LD, 1.0 μg). Proteins were separated on a 10% acrylamide (w/v) gel, transferred to nitrocellulose, and visualized after treatment with a $1:10^3$ dilution of antisera. (A) Anti-P450 2C10 #481; (B) anti-P450 2C10 #485; (C) anti-P450 2E1 #161; (D) anti-P450 2E1 #158.

Isolation and Characterization of Cross-Reactive Epitopes. Preliminary immunoblotting studies with P450s 1A2, 2C10, 2E1, and 3A4, anti-P450 2C10 (#485), and anti-P450 2E1 (#158) and the proteases chymotrypsin, trypsin, papain, and *S. aureus* V8 protease indicated that the most promising cross-reacting peptides were those derived from P450s 2C10 and 2E1 with chymotrypsin and *S. aureus* V8 protease. These systems gave antibody-visualized bands that were small but still large enough to be retained on high acrylamide concentration gels.

Fragments identified as cross-reactive (designated peptides 1−8, Figure 3) were cut from blots, destained, and submitted

[3] The antibodies (IgG fractions) were examined for immunoinhibition of characteristic marker activities of P450 2C10 [tolbutamide methyl hydroxylation (Knodell *et al.*, 1987)] and P450 2E1 [chlorzoxazone 6-hydroxylation (Peter *et al.*, 1990)] in human liver microsomes, using increasing levels of IgG up to 10 mg (nmol of P450)$^{-1}$. The more selective anti-P450 2C10 (#481) inhibited tolbutamide hydroxylation (≥75%), but neither the less selective anti-P450 2C10 (#485) nor either of the two anti-P450 2E1 preparations was very inhibitory (≤15%). Both anti-P450 2E1 preparations inhibited chlorzoxazone 6-hydroxylation (≥85%), but neither anti-P450 2C10 antibody did (≤10%).

[4] These polyclonal antibodies had not been adsorbed to remove antibodies directed to bacteria present in the animals. Some trace bacterial protein contaminants may be responsible for minor bands visualized on the blots (e.g., those near the migration front in Figure 2B,D). However, the lanes loaded with *E. coli* membranes (left of each gel) did not produce prominent bands migrating with the P450s under consideration (i.e., 50 kDa region). A reaction with ovalbumin was seen with several antisera and seems to be an artifact; bovine serum albumin did not show this interaction (results not presented). A number of other proteins were also tested with these antisera; of these, the only unexpected positive response was seen with rat microsomal epoxide hydrolase and anti-P450 2E1 #161.

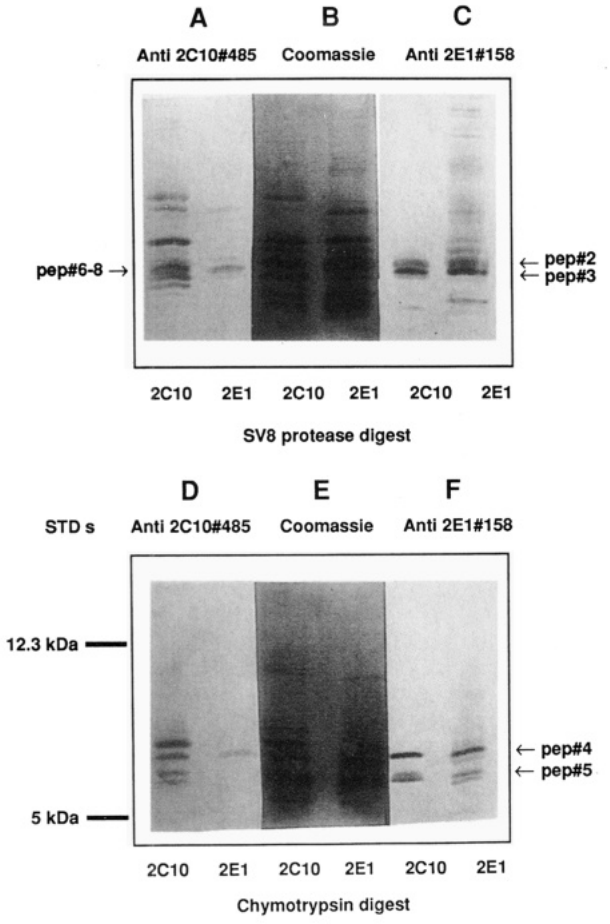

FIGURE 3: Electrophoretic separation of cross-reacting peptides for sequence analysis. Parts A−C: P450s 2C10 and 2E1 were digested with *S. aureus* V8 protease, and the resulting peptides were separated by $NaDodSO_4$−polyacrylamide gel electrophoresis and transferred to a ProBlot membrane. In part B, a segment of the blot was stained with Commassie Blue R-250 to visualize the peptides. In parts A and C, segments were treated with $1:10^3$ dilutions of anti-P450 2C10 #485 and anti-P450 2E1 #158, respectively. In parts D−E the entire procedure was repeated with chymotrypsin, and the peptides were visualized using either anti-P450 2C10 #485 (part D), Commassie Blue R-250 (part B), or anti-P450 2E1 #158. The indicated peptides (designated "pep") were cut out for N-terminal amino acid sequence analysis. Peptides 6, 7, and 8 were isolated from three separate digests and yielded the same sequence (Table 1).

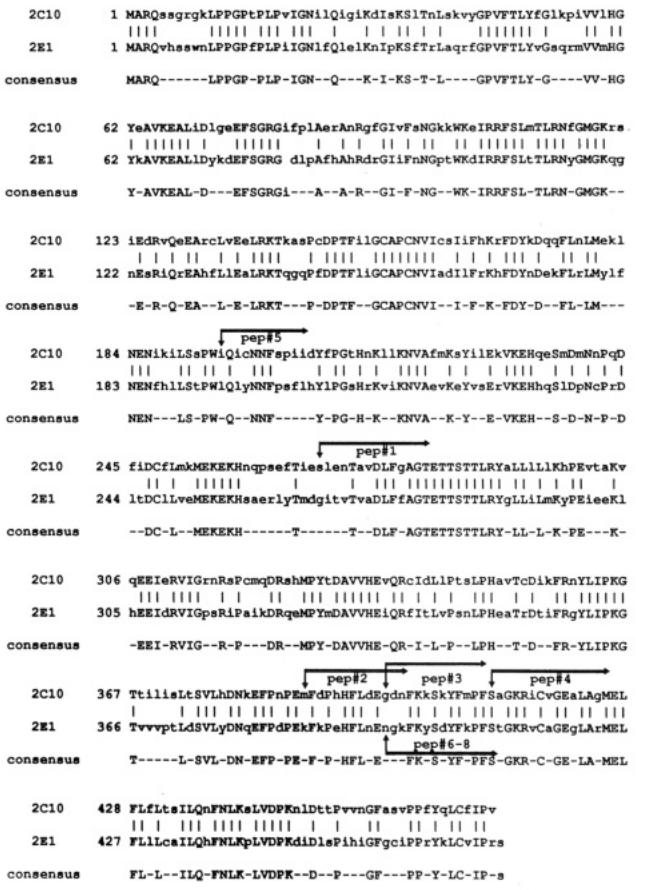

FIGURE 4: Alignment of human recombinant P450 2C10 and 2E1 sequences and positions of cross-reactive peptides. The N-terminal regions of the peptides recovered from the digestions shown in Figure 4 and analyzed in Table 1 are shown. Numbering is done on the basis of the constructs used for expression. These are shorter than the sequences in computer banks; the native sequence of P450 2C10 (and 2C9) is 18 residues longer and the native sequence of P450 2E1 is 12 residues longer (Sandhu *et al.*, 1993; Gillam *et al.*, 1994).

to N-terminal amino acid sequence analysis (Table 1).[5] Even with <10 pmol, the sequences could be readily identified and fit into the context of the sequences. Peptides 6, 7, and 8 were taken from repeated blots because of concern about separation in the region of interest; all three gave the identical sequence.

Two of the peptides (1, 5) are near the middle of the protein (Figure 4). Because the length of these peptides is not known (as these are relatively large peptides), the amount of information in these two sequences is limited. However, the sequences of the other peptides place them in the C-terminus (six out of the total of eight analyzed), and it thus appears that the last one-fifth of the proteins contain major cross-reactive epitopes, as judged by the immunoblot intensity in these gels (Figure 3). Further, peptides 2, 3, and 4 are overlapping (and overlap peptides 6−8 in the other protein). Since peptides 3 and 4 appear to be of similar immunoreactive intensity, then it would appear that the cross-reactive epitope region should be part of the P450 2C10/2E1 sequence beginning at residues 410−411, the last 13% of the proteins which includes the conserved heme-binding region, the most highly conserved part of all P450s (Nelson *et al.*, 1993).

Recognition of Synthetic Peptides by Anti-P450 Antibodies. Consideration of the results of the peptide amino acid sequence determination studies and the sequence comparisons in Figures 4 and 5 suggested that either of two regions might be expected to contain the cross-reactive epitope. We synthesized 14-mer peptides corresponding to both segments and verified their identities by MALDI-TOF mass spectrometry (Table 2). Of these four peptides (I−IV), only II and IV showed any reaction with antisera (only anti-P450 2E1) (Figure 6). The possibility was considered that an epitope involved a combination of parts of the two peptide regions, but mixtures of the peptides were not recognized to a greater extent.

[5] Several attempts were made to recover cross-reactive peptides by applying P450 2C10 digests to columns of immobilized anti-P450 2E1 (#158) (IgG fraction coupled to Reacti-gel, using method of Pierce Chemical Co.) and eluting the bound peptides. Attempts were also made with P450 2E1 digests and anti-P450 2C10 #485 IgG columns. For several technical reasons, this approach was not very successful. Several isolated peptides were identified as IgG fragments upon sequence analysis.

Table 1: N-Terminal Amino Acid Analysis of Cross-Reactive P450 2C10 and P450 2E1 Peptides[a]

	peptide 1		peptide 2		peptide 3		peptide 4		peptide 5		peptide 6		peptide 7		peptide 8	
cycle	amino acid	pmol	amino acid	pmol	amino acid	pmol	amino acid	pmol	amino acid	pmol	amino acid	pmol	amino acid	pmol	amino acid	pmol
1	S	16.3	M	7.8	G	6.2	S	10.5	I	3.4	N	3.0	N	1.9	N	4.9
2	L	6.1	F	4.7	D	3.5	A	27.4	Q	4.5	G	4.9	G	5.5	G	8.5
3	E	4.8	D	2.7	N	3.3	G	20.5	I	6.3	K	3.6	K	2.3	K	3.7
4	–	–	P	2.5	F	4.8	K	12.6	–	–	F	4.5	F	5.6	F	5.9
5	T	3.9	–	–	K	2.7	–	–	N	2.1	K	3.3	K	2.8	K	3.9
6	A	6.7	–	–	K	2.9	I	11.7	N	3.4	Y	2.5	Y	4.5	Y	5.7
7	V	7.3	F	2.2	S	2.3	–	–	F	2.0	S	1.7	S	2.9	S	2.5
8	D	2.8	L	2.4	K	0.7	V	9.7	S	2.2	D	1.6	D	2.2	D	1.3
9	L	7.8	D	1.0	Y	2.4	G	8.9	P	1.6	Y	1.8	Y	3.3	Y	3.4
10	F	6.0	E	1.2	M	3.3	E	7.4	I	2.9	–	–			F	4.7
11	G	10.0	G	3.7	P	1.5	A	7.9	I	4.1	–	–			K	1.8
12	A	8.7	D	2.0	F	3.8	L	13.0			P	1.8			P	2.7
13	G	11.2	N	1.0	S	2.3	A	9.4			F	1.5			F	3.9
14	T	4.5			A	1.1	G	7.4			S	0.7			S	1.2
15						1.6	M	3.3			T	2.0			T	2.6

[a] See Figure 3 for electrophoresis of peptides and Figure 4 for placement in sequence. A blank place indicates no definite identification was made.

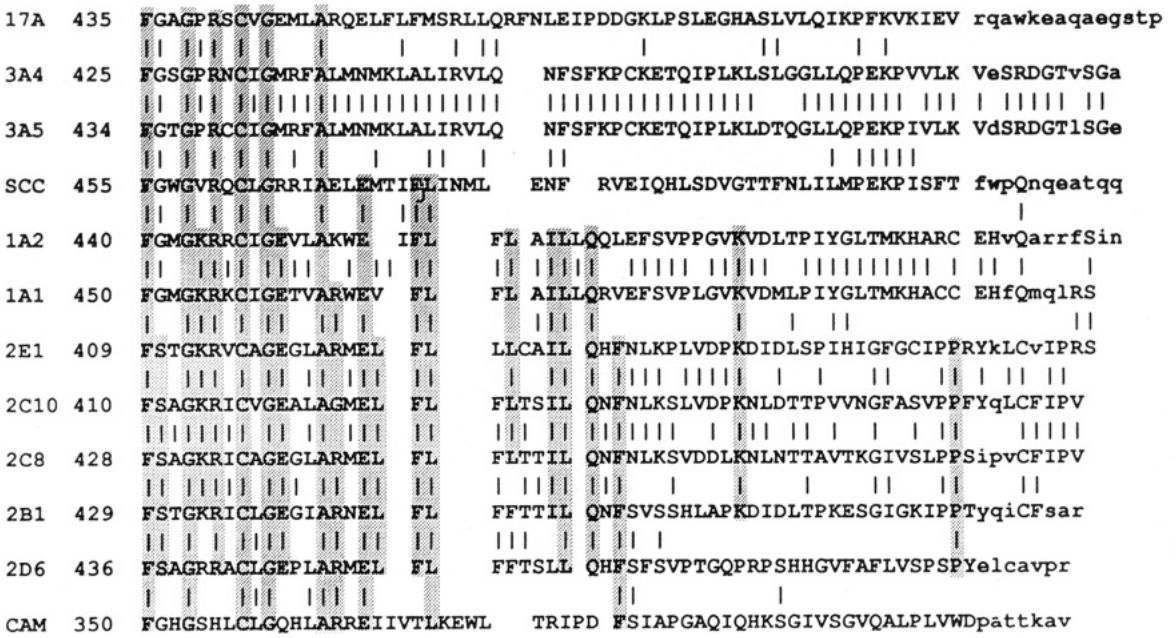

FIGURE 5: Sequence alignment of P450 proteins near the heme peptides. The segments extending from a conserved Phe (F) to the C-terminus are shown. P450 11A1 = SCC; P450 101 = CAM (*Psuedomonas putida*); P450s 11A1 and 17A are bovine; all others except 101 are human. See legend to Figure 4 for information relevant to the numbering of residues of P450s 2C10 and 2E1.

Table 2: Synthetic Peptides Used for Analysis

peptide designation	sequence	location	M_r expected	M_r found[a]	solubility
I	FSAGKRICVGEALA	P450 2C10/410−423	1420.8	1421.9	10% CH_3CO_2H
II	MELFLFLTSILQNF	P450 2C10/425−438	1716.0	1715.9	10% NH_4OH
III	FSTGKRVCAGEGLA	P450 2E1/409−422	1394.1	1394.0	H_2O
IV	MELFLLLCAILQHF	P450 2E1/424−437	1691.5	1691.4	CH_3OH
V	GKRVCAGEGLARMELFLLLCAILQHFNLK	P450 2E1/412−440	3161.8	*b*	H_2O

[a] MALDI-TOF mass spectrometry. [b] MALDI-TOF mass spectrometry was unsuccessful. The identity of the synthetic peptide was confirmed by Edman degradation (starting with 200 pmol of peptide). The initial yield was 70%, and the calculated repetitive yield was 94.8%. All of the first 27 residues were clearly identified except at cycles 3 (R), 5 (C), 12 (R), 20 (C), and 25 (H).

A longer peptide (V) (29-mer) containing most of both conserved regions (Figure 5) was prepared (Table 2).[6] This peptide (corresponding to a segment of P450 2E1) was recognized by both anti-P450 2C10 (#485) and anti-P450 2E1 (#158) (Figure 6).

Secondary Structure of Synthetic Peptides. CD spectroscopy of the synthetic peptides was utilized to gain estimates of the secondary structure. Peptides I and III, corresponding to the region containing the conserved Cys, appeared to be

[6] We were unsuccessful in obtaining mass spectra of this peptide, but its identity was verified by Edman degradation.

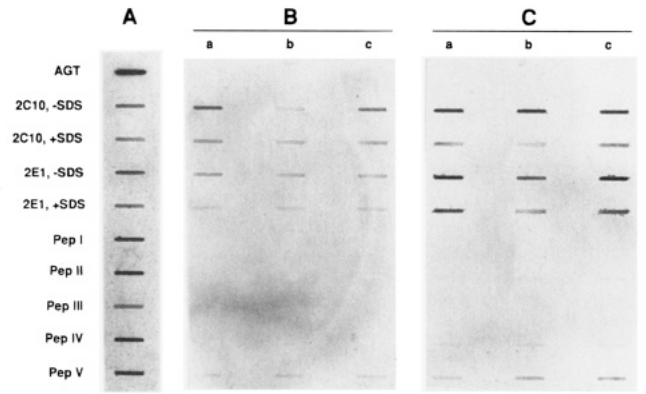

FIGURE 6: Reaction of antibodies with synthetic peptides corresponding to the region near the heme-binding Cys. Aliquots of recombinant P450 2C10 or 2E1 (2, 4, and 8 pmol each in parts a, b, and c, respectively), angiotensin (AGT), or the synthetic peptides (~0.5, 1.0, and 2.0 μg in parts a, b, and c, respectively) were spotted onto Immobilon membranes using a Hoeffer "slot-blot" apparatus and treated with (A) Commassie Blue R-250 or a 1:10^3 dilution of either (B) anti-P450 2C10 #485 or (C) anti-P450 2E1 #158.

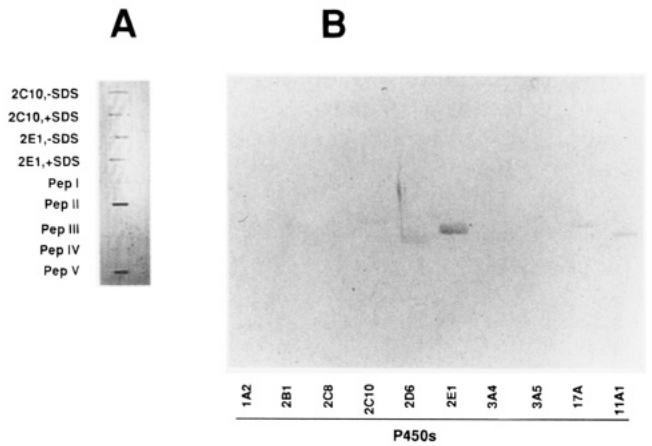

FIGURE 7: Reactions of anti-peptide V. Antisera from a rabbit immunized with peptide V: KLH conjugate (#633) were diluted and used to visualize various blots. (A) Slot-blot analysis with P450s 2C10 and 2E1 (±$NaDodSO_4$) and peptides I−V, as in Figure 6 (1:10^3 dilution). (B) Immunoelectrophoresis of various P450s (in the presence of $NaDodSO_4$). A 1:500 dilution of antisera was used with 5 pmol of each P450.

primarily random coils (<5% α-helix). Peptides II and IV had some β-sheet nature (but still <10% α-helix). The longer peptide V had considerably more α-helical character [46% as calculated from $[\theta]_{208}$ (Greenfield & Fasman, 1969; Chen & Yang, 1971)].

Reactivity of an Antibody Raised against Synthetic Peptide V. Peptide V appears to contain the cross-reactive epitope and was coupled to KLH for use as an antigen. Treatment of rabbits with this hapten complex produced antibodies that recognized peptide V and both P450s 2C10 and 2E1, in the absence or presence of $NaDodSO_4$ (Figure 7A).[7] The antibody also recognized peptides II and IV, but reaction with peptides I and III was weak. Thus, it would appear that the C-terminal portion of peptide V contains the epitope.

This antiserum reacted with P450 2E1 following $NaDodSO_4$−polyacrylamide gel electrophoresis (Figure 7B). Reactions was also seen with P450s 2C10, 2D6, 11A1, and 17A and, to a lesser extent, P450 1A1. In other experiments with native proteins in the absence of $NaDodSO_4$, reaction with P450s 2B1, 3A4, and 3A5 was also seen, but this reaction was not seen when the proteins were first heated with $NaDodSO_4$.[8]

DISCUSSION

Antibodies raised against a single P450 protein can recognize several P450s, as clearly shown here. The biological response is variable, as shown in the results obtained with different rabbits injected with a single antigen preparation (e.g., Figure 2). A number of potential artifacts can be ruled out, including contamination of antigens with other P450s and any bacterial proteins that would yield a confounding response. The cross-reaction seemed to be greatest among the P450 family 1, 2, and 3 members.

The cross-reactivity of P450s 2C10 and 2E1 was studied in detail. Analysis by protease digestion and Edman degradation localized a common epitope to the C-terminal region of these P450s. This epitope appears to be a major one, as judged by the intensity of the immunoblotted peptides. Consideration of the amino acid sequence analyses indicates that the common epitope region must begin near the conserved Cys (heme axial ligand), since peptide 4 has as much blot intensity as slightly longer peptides (Figures 3 and 4). We synthesized peptides corresponding to the most conserved two regions in this area and found that only the C-terminal of these reacted with the antibodies, and then weakly (Figure 6). However, a peptide containing both regions of P450 2E1 reacted better with both anti-P450 2C10 and anti-P450 2E1. On the basis of the results available, we conclude that the region immediately beyond the conserved Cys region contains at least part of a common epitope for P450s 2C10 and 2E1 and may be at least partially responsible for the immunochemical cross-reactivity seen with some other P450s. Elements of both of the conserved regions seem to be involved in this common epitope. Simply having a mixture of the two individual peptides present did not enhance reaction with the antibodies (results not shown). It is conceivable, however, that the region linking the conserved peptides is involved in the epitope. However, a more likely explanation is that the shorter peptides were not long enough to generate requisite secondary structure but that the longer peptide (V) was. Evidence for this view comes from the CD spectra, which show substantial α-helix only for peptide V.

When comparisons are made among P450 sequences and the experimentally-defined three-dimensional structures of bacterial P450s (Poulos *et al.*, 1987; Ravichandran *et al.*, 1993; Hasemann *et al.*, 1994), the region of the common epitope defined here corresponds to the "L-helix", which begins just beyond the Cys axial ligand and runs along the back of the heme (Poulos, 1988; Edwards *et al.*, 1989; Gotoh, 1992). This helix is exposed to the solvent in P450 101 and might be expected to in other P450s. Reaction of the cross-reactive antisera was seen with nondenatured P450s (Figure 6). Anti-peptide antibodies against this region were generated by conjugating peptide V with KLH and immunizing rabbits; these reacted with peptides II, IV, and V, as well as P450s 2C10 and 2E1 (Figure 7). The length of peptide

[7] Similar results were obtained with sera from rabbits that had been immunized with the different amounts of antigen.

[8] Some reaction with prostaglandin synthase was detected, even in the presence of $NaDodSO_4$.

V appears to be necessary to generate enough secondary structure, but the reactive epitope appears to be localized in the region shared by peptides II and IV. These antibodies also recognize some other P450s (Figure 7B).

The cross-reaction seen in immunoblotting studies does not seem to be manifested in the inhibition of catalytic activity. Thus it would appear that binding of antibodies to the conserved region in the C-terminal region does not inhibit catalytic activity. Shimizu and his associates have done site-directed mutagenesis studies on the Lys residues in this region of rat P450 1A2, expressed in *Saccharomyces cerevisiae* (Shimizu *et al.*, 1988, 1991; Furuya *et al.*, 1989; Kandala *et al.*, 1990). On the basis of their results, they have postulated that these Lys residues in this region are involved in binding NADPH−P450 reductase (Shimizu *et al.*, 1991). Most of the P450s under consideration here have a pair of basic residues (5th and 6th residues from left in Figure 5) and another conserved basic residue (15th from left in Figure 5, adjacent to the shaded, conserved Ala). However, in some of the P450s, only one of the first pair is basic (e.g., P450s 3A4, 5) and the later residue is not basic (P450s 3A4, 3A5, and 2C10).

Antibodies that recognize many P450s may be of practical value. For instance, it would appear that there are still more P450s in family 2 to be identified. Cross-reactive antibodies could be utilized in the screening of expression libraries or perhaps in immunohistochemical localization work done in developmental biology, etc. Such a reagent could provide a useful preliminary screen. These cross-reactive polyclonal antibodies seem to be easy to produce with the recombinant bacterial proteins. Our work also suggests caution in the direct use of antibodies without adequate screening and, if necessary, immunoadsorption techniques.

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