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Complete amino acid sequence and predicted membrane topology of phenobarbital-induced cytochrome P-450 (isozyme 2) from rabbit liver microsomes

(hydrophobic protein/sequence analysis/heme ligand/thiolate/protein secondary structure)

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ABSTRACT **The complete amino acid sequence of phenobarbital-induced isozyme 2 of rabbit liver microsomal cytochrome P-450 (P-450$_{LM2}$) is presented. The polypeptide consists of 491 residues with a calculated M_r of 55,755. The rabbit isozyme is 77% identical to the corresponding rat cytochrome, P-450b, as deduced from cDNA, with 96% of the hydrophobic, 88% of the anionic, and 83% of the cationic positions conserved. The secondary structure of isozyme 2 was predicted and a model was developed for the membrane topology of this cytochrome. Of the two highly conserved cysteinyl peptides in P-450$_{LM2}$, P-450b, and bacterial P-450$_{cam}$, we favor, on the basis of our model, the one nearer the NH_2 terminus (Cys-152 in P-450$_{LM2}$) as the source of the thiolate ligand to the heme iron atom. The recently reported sequence of the apparently identical protein [Heinemann, F. S. & Ozols, J. (1983) *J. Biol. Chem.* 258, 4195–4201] has two fewer residues and differs in 14 other amino acid assignments.**

After the isolation of P-450$_{LM2}$* in an electrophoretically homogeneous state (1), we reported that it contained a single NH_2-terminal amino acid sequence (2). The composition and sequence of this region provided an example of a mature protein having retained a "signal peptide" of the type ordinarily removed from preproteins by biological processing (3). More recently, we reported additional sequence data on rabbit P-450 isozyme 2 (4), which showed that it is about 80% identical to the corresponding rat protein, P-450b, the sequence of which was deduced from cloned cDNA (5), and we compared highly conserved cysteine-containing regions from rabbit isozyme 2, rat isozyme b, and P-450$_{cam}$ from *Pseudomonas putida* (6).

We report here the complete amino acid sequence of P-450$_{LM2}$, along with methodology that should be generally effective with other hydrophobic proteins. We include a correction to our previous report (4), in which Cys-436 was mistakenly identified as Glu, and offer compelling evidence for the identity of positions and residues at variance with the conclusions of Heinemann and Ozols (7) regarding this protein. A calculated model for membrane-bound P-450 isozyme 2 is also presented. Our results continue to support Cys-152 as the source of the axial thiolate ligand to the heme iron contrary to the proposals of others (5, 7).

MATERIALS AND METHODS

Materials. P-450$_{LM2}$ was purified to homogeneity from liver microsomes of male New Zealand White rabbits that had been treated with phenobarbital (1). CNBr, 4-vinylpyridine, acetic, succinic, and 1,2,4-benzenetricarboxylic anhydrides, and trifluoroacetic and heptafluorobutyric acids were obtained from Aldrich. Clostripain and proteinase A were from Sigma. *Staphylococcus aureus* V8 protease came from Miles, endoproteinase Lys-C was from Boehringer Mannheim, and L-1-tosylamido-2-phenylethyl chloromethyl ketone treated-trypsin and α-chymotrypsin were from Worthington. All HPLC solvents were obtained from Burdick and Jackson (Muskegon, MI).

Chemical Modification. In most cases, prior to protein fragmentation cysteine residues were reduced with 3 equivalents of dithiothreitol in 6 M guanidinium chloride/0.5 M *N*-ethylmorpholinium acetate, pH 8.2, and alkylated with a 1.4-fold excess of 4-vinylpyridine (8), with both reactions at 22°C for 4 hr. Several reagents were tested to provide stable groups to block enzymic digestion at Lys residues. The best results were obtained with acid anhydrides, which were added to a solution or fine suspension of the intact protein or fragment in *N*-methylmorpholine/H_2O, 1:1 at 0°C, followed by stirring at 22°C for 1 hr. The benzenetricarboxylic derivative of isozyme 2 was by far the most soluble at neutral pH, so it was used for the earlier tryptic digestions. Reaction with succinic anhydride tended to produce a more uniform product and a more easily analyzed Lys derivative, so it was employed for most of the later studies. With smaller peptides or when neutral solubility was unimportant, acetic anhydride was most convenient.

Digestion. Standard methods were used for cleavage with CNBr, trypsin, chymotrypsin, *S. aureus* protease (9), proteinase A (10), and clostripain (11). For digestion with endoproteinase Lys-C an enzyme-to-substrate ratio of 1:50 (wt/wt) was used and the mixture was incubated in 0.1 M KPO_4 (pH 7.4) at 37°C for 24 hr. The digestion was quenched by acidifying with 88% formic acid. Specific cleavage at Cys was achieved with this enzyme by using succinylated protein under conditions analogous to those indicated for trypsin (12). A detailed account of this method will be published separately. Specific acid cleavage on the carboxyl side of Asp, mainly at Asp-Pro, was achieved by boiling the protein in 0.1% trifluoroacetic acid for 1 hr.

Peptide Purification. Peptide mixtures were fractionated by reversed-phase HPLC. The standard mobile phase was water vs. CH_3CN/2-propanol, 3:1 (vol/vol), with 0.1% trifluoroacetic acid throughout (13) and a gradient rate of 1% per ml. Vydac C_{18} (5 μm, 4.5 × 250 mm) and Waters μBondapak C_{18} (10 μm, 3.9 × 300 mm) columns proved most useful. Fractions were repurified on a Waters μBondapak phenyl column (10 μm, 3.9 × 300 mm) or with the substitution of heptafluorobutyric acid for trifluoroacetic acid. Tryptic peptides were separated on an Altex Ultrasphere-ODS (5 μm, 4.5 × 250 mm) column by using a gradient of water vs. 1-propanol with 0.1 M hexafluoroace-

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* The main phenobarbital-induced form of rabbit liver microsomal cytochrome P-450 is termed P-450$_{LM2}$ or isozyme 2.

tone/ammonia, pH 7.2, throughout (13). These peptides were rechromatographed as phenylthiocarbamoyl derivatives in the same system.

Other Methods. Amino acid compositions were determined either by conventional techniques (4) or by reversed-phase chromatography of phenylthiocarbamoyl amino acids (unpublished data; a brief description of the method appears in ref. 14). Peptides were subjected to sequence analysis by the method of Tarr (15), and the NH_2-terminal sequence of the protein was determined with a Beckman 890C Sequenator (16). Phenylthiohydantoin amino acids were analyzed and quantified as reported (4). Sequence analysis yields reported here have been corrected for background and out-of-phase.

A computer data base of sequence results was maintained for the purpose of accurate information retrieval and analysis. Various BASIC computer programs written in this laboratory—"PEPMP" for management of peptide sequence information, "CHOFAS" for secondary structure calculations, and "AAATEK" for amino acid analysis data—were used in conjunction with the data base.

RESULTS AND DISCUSSION

Primary Structure. In the course of determination of the primary structure of isozyme 2 both "small peptide" and "large peptide" strategies were pursued. Initial efforts centered mainly on the fractionation of large CNBr peptides by size exclusion in conventional systems such as 6 M urea/Sephadex or in novel systems such as formic acid/ethanol/Sephadex LH-60. All such efforts gave fractions that proved to be complex mixtures, often in poor yield. We explored with contrasting success the small peptides of tryptic digestion and their separation by reversed-phase HPLC. However, the sequence information obtained resided in many nonoverlapping fragments while about 30% of the protein was unaccounted for due to the extremely low recovery of large or hydrophobic peptides from the narrow-pore columns then used. Additional sequence information was obtained from the small peptides generated by *S. aureus* V8 protease, proteinase A, and chymotrypsin. Improvements in HPLC methodology, notably the use of wider-pore (120 or 300 Å) packings and more effective eluting solvents (13), permitted a return to large peptides generated by CNBr, endoproteinase Lys-C, and acid cleavage. Thus, the ordering of small fragments and extended sequence analysis through regions lacking favorable residues for specific cleavage was made possible. In the final phase a combination of both approaches established or confirmed the few remaining residues and overlaps; the protein was first fragmented into peptides of medium size (15–65 residues) by acid cleavage or by a tandem combination of acid and CNBr cleavages. The purified fragments were cleaved further with enzymes such as chymotrypsin and clostripain or with harsher acid treatment. The few small subfragments generated were easily purified in good yield in a single HPLC step. Small fragments also gave the clearest sequence analysis results because the partitioning/HCl method could be used, which gives high yields of Ser and Thr compared with the procedures required for large peptides or with automatic sequenators (17). A less obvious advantage of this approach is that regions contained in moderate-sized subfragments seemed more susceptible to enzymic digestion than the same regions of the intact protein.

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         1         2         3         4         5
2 4 6 8 Ø 2 4 6 8 Ø 2 4 6 8 Ø 2 4 6 8 Ø 2 4 6 8 Ø

MEFSLLLLLA FLAGLLLLLF RGHPKAHGRL PPGPSPLPVL GNLLQMDRKG   50
LLRSFLRLRE KYGDVFTVYL GSRPVVVLCG TDAIREALVD QAEAFSGRGK  100
IAVVDPIFQG YGVIFANGER WRALRRFSLA TMRDFGMGKR SVEERIQEEA  150
RCLVEELRKS KGALLDNTLL FHSITSNIIC SIVFGKRFDY KDPVFLRLLD  200
LFFQSFSLIS SFSSQVFELF PGFLKHFPGT HRQIYRNLQE INTFIGQSVE  250
KHRATLDPSN PRDFIDVYLL RMEKDKSDPS SEFHHQNLIL TVLSLFFAGT  300
ETTSTTLRYG FLLMLKYPHV TERVQKEIEQ VIGSHRPPAL DDRAKMPYTD  350
AVIHEIQRLG DLIPFGVPHT VTKDTQFRGY VIPKNTEVFP VLSSALHDPR  400
YFETPNTFNP GHFLDANGAL KRNEGFMPFS LGKRICLGEG IARTELFLFF  450
TTILQNFSIA SPVPPEDIDL TPRESGVGNV PPSYQIRFLA R  491
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FIG. 1. Complete amino acid sequence of rabbit liver microsomal cytochrome P-450 isozyme 2.

Several special techniques are worthy of emphasis here: (*i*) use of multiple-wavelength monitoring in HPLC to locate specific peptides, such as those containing pyridylethyl-Cys, which has high 254-nm absorbance, (*ii*) deformylation of Ser and Thr after CNBr digestion (13), (*iii*) side-chain modification of Lys to enhance solubility for enzymic digestion, (*iv*) use of the newly available endoproteinase Lys-C for specific cleavage at Lys (18) or suitably modified Cys, and (*v*) use of batchwise manual sequence analysis to screen HPLC fractions for peptides of particular interest (15). In retrospect it is clear that the entire sequence could have been established quickly with modest amounts of protein by using the fragmentation and HPLC separation methods developed in the course of this work.

The primary structure of isozyme 2 is shown in Fig. 1 and the overlap strategy in Fig. 2. The sequence is composed of 491 amino acid residues, with an average hydrophobicity of 37.3% (cf. ref. 16) and a polypeptide M_r of 55,755; 14.7% of the residues are basic, and 10.6% are acidic. Four reduced cysteine residues and a single tryptophan are present. Amino acid compositions obtained from the sequence and also determined analytically are in close agreement, as shown in Table 1.

Our primary structure indicates 16 differences with respect to the amino acid sequence recently reported by Heinemann

Table 1. Amino acid composition of rabbit P-450_{LM2}

Residue	Beckman 120C	Phenylthio-carbamoyl	Sequence
Cys	6	4	4
Asn	—*		15
Asp	39	39	24
Gln	—*		16
Glu	42	42	28
Arg	36	36	38
His	15	13	14
Ser	31	23	32
Gly	32	34	35
Thr	25	24	26
Pro	31	28	32
Ala	24	26	24
Tyr	12	13	12
Val	30	28	30
Met	7	8	8
Ile	23	24	27
Leu	57	63	64
Trp	1†		1
Phe	35	34	41
Lys	21	20	20

The values in the first column are time-extrapolated values from a Beckman 120C analyzer. The second column is the phenylthiocarbamoyl analysis from a single-point 150°C hydrolysis. The last column shows the true composition as derived from the sequence.
* Ammonia released indicated the presence of 32 amides.
† Determined by Haugen and Coon (1).

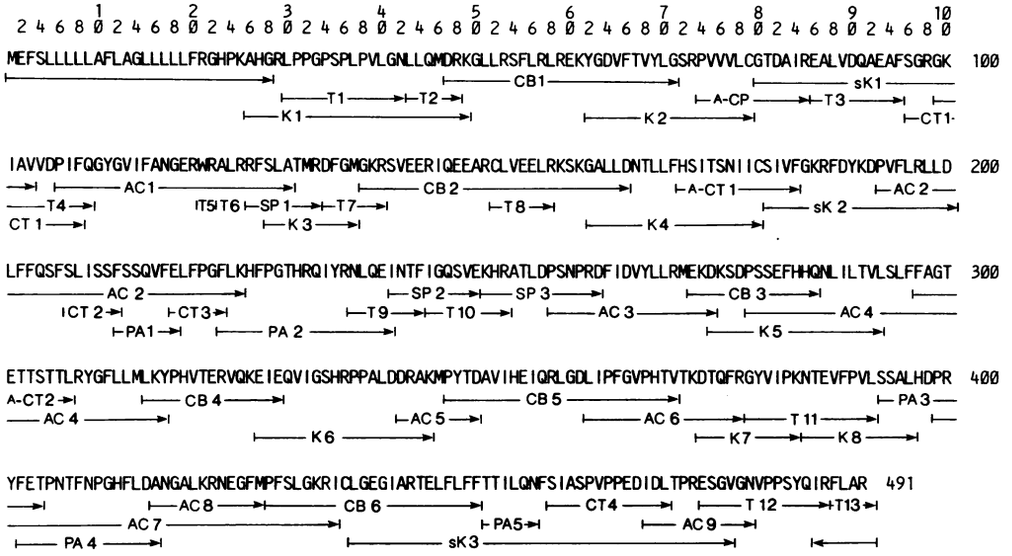

FIG. 2. Overlap strategy for the determination of the complete sequence of P-450 isozyme 2. The arrows indicate residues determined by manual sequence analysis of peptides obtained from cleavage with CNBr (CB), endoproteinase Lys-C on alkylated substrate (sK), trypsin (T), clostripain on acid- and CNBr-cleaved fragments (A-CP), endoproteinase Lys-C (K), chymotrypsin (CT), chymotrypsin on acid- and CNBr-cleaved fragments (A-CT), *S. aureus* V8 protease (SP), and proteinase A (PA). The arrow at the NH_2 terminus indicates residues determined from automated sequence analysis and the reverse arrow shows residues determined by carboxypeptidase Y.

and Ozols (7). Data indicating these differences are summarized in Table 2. For example, we find Gln, Phe, and Ser at positions 91, 95, and 96, respectively, instead of Glu, Ser, and Phe. Evidence is also presented for two amino acids in addition to the 489 residues reported by the other laboratory; as a result of the Gly at position 99 and Lys at 100, alignment of residues beyond this point in the reported sequences requires an offset of 2. By using our numbering system, the remaining differences given as "our assignment/their assignment" are: 135 Phe/Gly, 136 Gly/Tyr, 141 Ser/Gly, 193 Pro/Lys, 221 Pro/Ser, 303 Thr/Ala, 461 Ser/Gly, 462 Pro/Asn, 463 Val/Leu, 464 Pro/Ser, and 465 Pro/Leu. Although it is possible that the cytochrome preparation of Heinemann and Ozols represents a structural variant, this seems unlikely because both laboratories prepare isozyme 2 from outbred male New Zealand White rabbits by the same method (1, 7) and because we could verify only a single residue at each position over the 5 years of this investigation.

Comparison with Other P-450s. Our calculations indicate that the complete sequence of isozyme 2 is 77% identical to that of rat P-450b, as determined by Fujii-Kuriyama *et al.* (5). Furthermore, 96% of the hydrophobic, 88% of the anionic, and 83% of the cationic positions are conserved. Clearly, these phenobarbital-inducible enzymes show remarkable evolutionary conservation of size and hydrophobicity. However, when run side by side on calibrated $NaDodSO_4$/polyacrylamide gel electrophoresis (4), the rat P-450b appears to be 4,100 larger in molecular weight than isozyme 2. Furthermore, isozyme 2 elutes earlier than P-450b on reversed-phase HPLC. Previously published work rules out the possibility of carbohydrate as a significant modification (1, 19). We have examined the rabbit enzyme for secondary modifications and found none, but undetected covalent attachments to one or both proteins might explain these findings.

Our previous comparison of the partial structure of rabbit isozyme 2 with rat isozyme b and $P\text{-}450_{cam}$ revealed a highly conserved region around Cys-152 in the rabbit protein (4). A second highly conserved region was detected but discounted as containing the heme ligand because the Cys at position 436 was misidentified as a Glu due to a transient shift in the elution po-

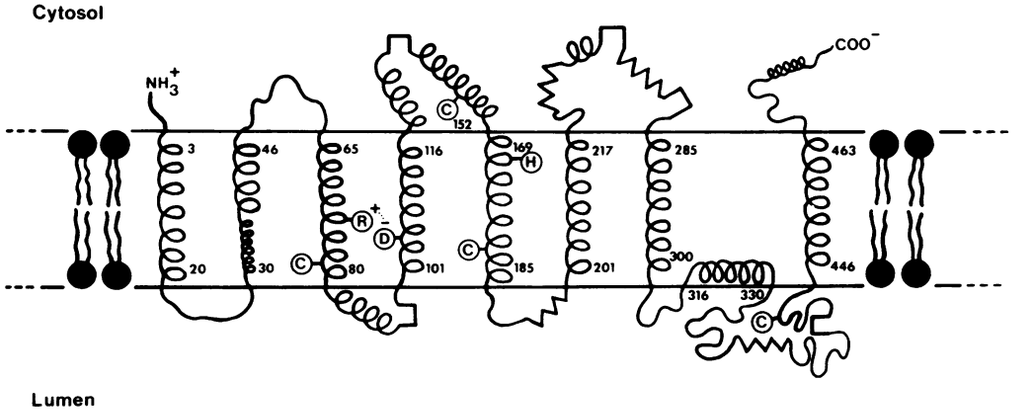

FIG. 3. Proposed topology of P-450 isozyme 2 in the bilayer membrane of the hepatic endoplasmic reticulum. Wide and narrow spiral segments indicate regions of predicted α and 3_{10} helix, respectively. Predicted β turns(⊏) and regions of β sheet (zigzag lines) are also shown. This model represents the most probable topology (see text for details).

Table 2. Sequence data for peptides at selected positions of rabbit P-450$_{LM2}$

Position	Residue	Yield, pmol			
		T 3	CT 1	CT 29.4	sK 1
88	Leu	(3) 1,140			(9) 52
89	Val	(4) 2,446	(1) 615		(10) 75
90	Asp	(5) 2,836	(2) 492		(11) 51
91	Gln*	(6) 1,562	(3) 522		(12) 48
92	Ala	(7) 1,786	(4) 562		(13) 33
93	Glu	(8) 1,752	(5) 446		(14) 30
94	Ala	(9) 778	(6) 532		(15) 31
95	Phe*	(10) 150	(7) 318		(16) 31
96	Ser*			(1) 767	(17) 4
97	Gly	T 4		(2) 579	(18) 18
98	Arg			(3) 368	(19) 16
99	Gly*	(1) 2,068		(4) 616	(20) 22
100	Lys*	(2) 600		(5) 897	(21) 4
101	Ile	(3) 928		(6) 951	(22) 18
102	Ala	(4) 812		(7) 748	(23) 18
103	Val	(5) 970		(8) 636	(24) 21
104	Val	(6) 716		(9) 676	
		T 7	K 3	CB 2	CT 27.4
132	Met		(5) 675		
133	Arg		(6) 212		
134	Asp	(1) 900	(7) 671		
135	Phe*	(2) 530	(8) 388		
136	Gly*	(3) 448	(9) 191		
137	Met	(4) 416	(10) 82		
138	Gly	(5) 338		(1) 589	
139	Lys	(6) 80		(2) 422	
140	Arg	(7) 60		(3) 661	
141	Ser*			(4) 292	(1) 473
142	Val			(5) 596	(2) 389
143	Glu			(6) 644	(3) 273
144	Glu			(7) 781	(4) 303
		T 24-3	sK 2	CT 25.4	AC 2
188	Phe	(1) 920	(8) 331		
189	Asp	(2) 658	(9) 325	(1) 599	
190	Tyr	(3) 480	(10) 257	(2) 381	
191	Lys	(4) 250	(11) 224	(3) 434	
192	Asp	(5) 400	(12) 179	(4) 393	
193	Pro*	(6) 262	(13) 174	(5) 359	(1) 1,830
194	Val	(7) 262	(14) 172	(6) 188	(2) 1,420
195	Phe	(8) 60	(15) 170	(7) 133	(3) 2,900
196	Leu	(9) 50	(16) 149	(8) 23	(4) 1,630

Position	Residue	Yield, pmol	
		CT 3	
218	Glu	(1) 244	
219	Leu	(2) 88	
220	Phe	(3) 92	
221	Pro*	(4) 101	
222	Gly	(5) 86	
223	Phe	(6) 74	
224	Leu	(7) 29	
		A-CT 2	AC 4
297	Phe	(1) 338	(19) 686
298	Ala	(2) 318	(20) 631
299	Gly	(3) 334	(21) 473
300	Thr	(4) 210	(22) 122
301	Glu	(5) 186	(23) 417
302	Thr	(6) 205	(24) 103
303	Thr*	(7) 160	(25) 108
304	Ser	(8) 95	(26) 70
305	Thr	(9) 105	(27) 53
306	Thr	(10) 113	(28) 52
307	Leu	(11) 122	(29) 139
		CT 4	sK 3
458	Ser	(1) 271	(22) 42
459	Ile	(2) 242	(23) 106
460	Ala	(3) 336	(24) 164
461	Ser*	(4) 181	(25) 9
462	Pro*	(5) 347	(26) 204
463	Val*	(6) 281	(27) 194
464	Pro*	(7) 300	(28) 167
465	Pro*	(8) 331	(29) 146
466	Glu	(9) 179	(30) 65
467	Asp	(10) 161	(31) 89
468	Ile	(11) 104	(32) 57
469	Asp	(12) 179	(33) 54

The numbers in parentheses indicate manual Edman cycle number. Phenylthiohydantoin amino acids marked with * indicate positions where differences are found with Heinemann and Ozols (7). Peptides not shown in Fig. 2 retain their original designation. See legend to Fig. 2 for abbreviations.

sition of phenylthiohydantoin-pyridylethyl-Cys. Later analyses reliably resolved these two phenylthiohydantoin amino acids and reinvestigation of this part of the sequence clearly showed a Cys.

Membrane Topology. The secondary structure of isozyme 2 was predicted by using the method of Chou and Fasman (20). In the development of a model for the binding of the isozyme in the membrane, areas of predicted β sheet were assumed to be α helical when in a lipid bilayer (21, 22). In addition, membranous segments should have at least 16 residues, a negative "hydrophobic free energy" of partition (23), maximal charge neutralization through interaction with other segments within the bilayer, and a halt-transfer signal at the COOH-terminal end (3). In accord with currently proposed theories for the insertion of proteins into membranes, the NH_2 terminus should be located on the cytosolic side (3). The model presented in Fig. 3 shows eight hydrophobic α-helical segments that span the phospholipid bilayer. Charge neutralization is predicted to occur between Arg-73 and Asp-105 so that the net charge within the membrane will be 0 or +1, depending upon the protonation state of His-172. This model predicts Cys-152, found within a long segment of α helix, to be the only cysteine residue available to function as the fifth ligand to the heme iron atom, because of its location on the cytoplasmic side. An additional interesting feature of the model is the occurrence of 3_{10} helices at the beginning of the second hydrophobic segment, within the "proline cluster" (4), and near the COOH terminus; it is not yet clear if this helical form has any special structural significance. Finally, an amphipathic helix (24) occurs at residues 316–330. As substantiation for the model, we have found that rapid proteolysis does not occur within regions predicted to be in the membrane. Although we fully expect that modification of this

and other models (7, 25) may be required, the present formulation is in agreement with all available data and predictive parameters and should provide a useful source of hypotheses for further experimentation.

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