Isolation and Structure of a Third Form of Liver Microsomal Flavin Monooxygenase[†,‡]

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ABSTRACT: Flavin-containing monooxygenases (FMOs) catalyze NADPH-dependent oxygenation of nucleophilic nitrogen, sulfur, and phosphorous atoms in various drugs, pesticides, and xenobiotics. Two forms of this enzyme have been isolated and characterized from rabbit liver microsomes [Ozols, J. (1989) *Biochem. Biophys. Res. Commun. 163,* 49–55]. The isolation and the structure of a third isoform (FMO3) is presented here. The isolation procedure for FMO3 included solubilization of liver microsomes with cholate, poly(ethylene glycol) precipitation, chromatography on anion- and cation-exchange and hydroxyapatite columns in the presence of nonionic detergents and glycerol. On sodium dodecyl sulfate–polyacrylamide gel electrophoresis, FMO3 exhibited a distinct, single band with a M_r higher than those of FMO1 and FMO2. FMO3 copurified with a polypeptide complex of high FMO activity. This complex consisted of three polypeptides, named FMO3, FMO1a, and FMO2a. The column chromatographic behavior of FMO1a and 2a was distinct from that of FMO1 and 2. The electrophoretic mobility of FMO1a was identical to that of FMO1. Automated sequence analysis of this polypeptide complex indicated the presence of only one predominant peptide with an open N-terminus. The derived N-terminal amino acid sequence of some 20 residues was identical to the N-terminus of FMO2. The FMO complex, however, did not contain a polypeptide corresponding to the electrophoretic mobility of FMO2. The N-terminus of FMO3 was blocked by an acetyl residue. Automated Edman degradation of peptides obtained from chemical and enzymatic digests established the amino acid sequence of some 514 residues. Rabbit FMO3 has 55% amino acid sequence identity to rabbit liver FMO1, FMO2, and the rabbit lung FMO forms. The amino acid sequence of proposed FAD- and NADP-binding segments in FMO3 align exactly with those described for other mammalian species. FMO3 is a labile form with activity toward thiourea lower than that of FMO1, 2, or its parent complex. These findings suggest that a form of FMO may also exist as a complex of related polypeptides and that FMO3 may be a subunit of such a complex.

The FMOs are microsomal membrane xenobiotic-metabolizing enzymes present in liver, kidney, and lung, as well as other tissues. An important role for this enzyme system is the NADPH-dependent oxygenation of nitrogen-, phosphorous-, and sulfur-containing antipsychotic drugs, chemicals, and pesticides (Ziegler, 1988). In earlier communications, I reported the isolation and the amino acid sequence of two structurally related FMOs from rabbit liver microsomes, designated FMO1 and FMO2 (Ozols, 1989a, 1990a, Ozols, 1991).[1] A form related to the liver FMO, but with distinct catalytic properties, has been isolated from rabbit lung (Williams et al., 1984). The cDNA and the chemical sequence of this form has been recently determined (Lawton et al., 1990; Guan et al., 1991). While only one form of FMO has been found in pig liver (Gasser et al., 1990), two forms, a 54-kDa and a 56-kDa, have been found in guinea pig (Yamada, 1990). While the N-terminus of the 56-kDa form was blocked, the N-terminal sequence of 19 residues of the 54-kDa form showed 90% identity to the rabbit FMO2. Molecular cloning of three distinct forms of human liver FMOs have now been reported (Lomri et al., 1992; Dolphin et al., 1992). The deduced amino acid sequence of one human FMO (HLFMO II) had 80% similarity with the rabbit liver FMO2 but only 52% identity with the rabbit FMO1 or the porcine form. The substrate specificity of only the porcine liver FMO and the rabbit lung FMO have been examined in detail. The expression and the substrate specificity of HLFMO II a human liver FMO was recently described (Lomri et al., 1993). An unexpected finding with regard to the FMO3 enzyme is that it copurifies as a highly active FMO preparation consisting of several related polypeptides. Furthermore, despite the high amino acid sequence similarity to other FMOs, FMO3 was found to be a labile form with rather low enzymatic activity. To increase our understanding of FMO forms that may exist as a complex of related polypeptides, I report here the isolation and the covalent structure of FMO3 present in rabbit liver microsomes.

EXPERIMENTAL PROCEDURES

Materials. Detergents, enzyme substrates, cofactors, chromatographic media, and chemicals, unless stated otherwise, were obtained from Sigma. Hydroxyapatite-agarose (HA-Ultragel) was product of IBF Biotech, Villeneuve-la-Garenne, France. Trypsin was obtained from Worthington. Endopeptidases Asp-N and Glu-C were from Boehringer Mannheim. *Achromobacter* peptidase Lys C was obtained from Biochemical Diagnostics, Edgewood, NY. Acylamino acid

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[1] Rabbit liver FMO1A, 2, and 3 or forms 1, 2, and 3 have also been designated as FMO1, FMO3, and FMO5, respectively. Human liver HLFMO II has been designated as FMO3. The FMO enzyme present in the lung has also been designated as FMO2 (Lawton et al., in press).

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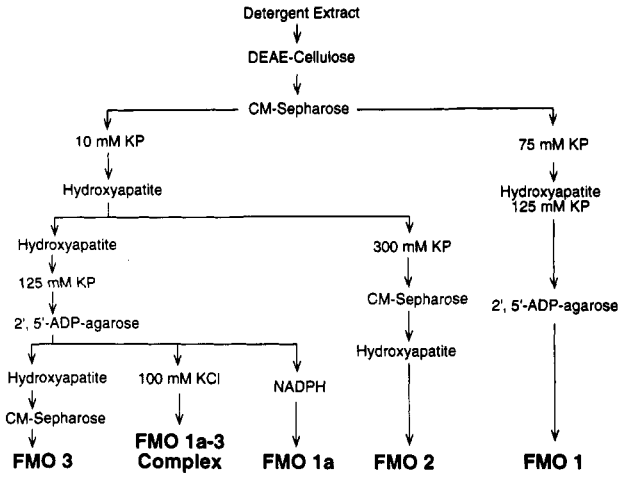

FIGURE 1: Summary of purification steps of FMOs from rabbit liver microsomes. KP denotes potassium phosphate.

releasing enzyme was from Takara Biochemicals Inc., Berkley, CA. Solvents for HPLC[2] and gel filtrations were from Burdick and Jackson.

Isolation of FMO3. Liver microsomes were isolated from New Zealand male rabbits and solubilized as described previously (Ozols, 1990b). Microsomes were suspended in 10 mM potassium phosphate, pH 7.4, containing 20% glycerol, 0.1 mM DTT, and 1 mM EDTA to a concentration of 15 mg/mL protein. Solubilization of the microsomes was achieved by the addition of sodium cholate, 1.2 mg/mg of protein. The solubilized material was fractionated with PEG as described in (Ozols, 1990b). The 6–12% PEG precipitate was solubilized with Tergitol NP-10, 1.3 mg/mg of protein. The solubilized material was applied to a DEAE-cellulose column (200 mL) equilibrated with 5 mM potassium phosphate, pH 7.4, containing 20% glycerol, 0.5% NP-10, 1 mM EDTA, and 0.1 mM DTT (Figure 1). All buffers used in the subsequent purification steps contained 20% glycerol, 1 mM EDTA, and 0.1 mM DTT, abbreviated as GED. Unbound material eluting from the DEAE-cellulose column was applied to a column containing 100 mL of CM-Sepharose equilibrated with 10 mM potassium phosphate, pH 7.4, containing 0.2% NP-40 and GED. The unbound material from the CM-Sepharose was applied to a column (100 mL) of hydroxyapatite, equilibrated with 10 mM potassium phosphate, pH 7.4, containing 0.2% NP-40, and GED (buffer A). The column was washed with 75 mL of buffer A, followed by 75 mL of buffer A containing 125 mM potassium phosphate, pH 7.4, and then with 75 mL of buffer A containing 300 mM potassium phosphate. FMO3-containing fractions were dialyzed against 1 L of 5 mM potassium phosphate, pH 6.8, containing 0.2% NP-40 and GED, and applied to a column (20 mL) of CM-Sepharose equilibrated with the same buffer. After the column was washed with 50 mL of the equilibration buffer, the column was developed with a linear gradient of increasing concentration of potassium phosphate, pH 7.4, 10–50 mM, 25 mL per each gradient chamber. Form 3 eluted at about 40 mM potassium phosphate. Fractions containing FMO3 were dialyzed against 1 L of 20 mM Tris-acetate, pH 8.1, containing 0.1% NP-40 and GED (buffer B), and applied to a 7 mL of 2′,5′-ADP-Sepharose equilibrated with buffer B. Form 3 is eluted after the column is developed with 20 mL of buffer B. FMO3–1a complex is eluted upon washing the column with buffer B containing 50 mM KCl. FMO1a is eluted with buffer B containing 100 mM KCl and 0.5 mM NADPH (Figure 1). In the last purification steps FMO3 containing fractions are applied directly to a column containing 15 mL of hydroxyapatite equilibrated with buffer A. The column was developed with a linear gradient of increasing concentration of potassium phosphate (10–150 mM, 75 mL of each). If the FMO3 preparation obtained from this column is not homogenous as judged by SDS–PAGE, then the FMO3 containing fractions are reapplied to a short CM-Sepharose column and eluted with a linear gradient of potassium phosphate as described above. The yield of homogeneous FMO3 was approximately 0.25–0.5 mg per rabbit liver.

Purification of the FMO Complex. The isolation of the active FMO complex containing related polypeptides was achieved by subjecting the fractions eluted by 125 mM potassium phosphate from the first hydroxyapatite column (after dialysis) to a 15-mL CM-Sepharose column. The column was developed with the equilibration buffer followed by the elution with a linear gradient of increasing concentration of potassium phosphate (10–125 mM, 50 mL each). The FMO complex eluted as a single peak at about 100 mM potassium phosphate concentration. Homogeneous FMO3 was obtained by fractionating the complex on the ADP-Sepharose column as described above. FMO3, FMO3–1a, and FMO1a were eluted as described above.

FMO activity was determined by monitoring thiourea-dependent NADPH oxidation at 340 nm as described by Ozols (1989a) or in 0.1 M Tricine, pH 8.4, 0.1 mM EDTA by the method of Dixit and Roach (1984). Absorption spectra of FMO3 was determined using a Shimadzu model UV-260 spectrophotometer. The flavin content of FMO3 was determined spectrally on the trichloracetic acid supernatant of precipitated FMO3 as follows: One milliliter of the solution of FMO3 (Figure 2D) was concentrated some 8× in the Amicon Centricon-30 microconcentrator, and the retentate was diluted to 1 mL with water. Following the addition of 200 μL of 100% TCA and centrifugation, spectra of the flavin in the supernatant were determined as indicated in the legend of Figure 2D. The blank cuvette contained 1 mL of buffer B concentrated and solubilized with TCA as described above.

Sequence Analysis. Protein/peptide hydrolyses were performed with 6 M HCl in the gas-phase, at 150 °C for 1 h as described (Ozols, 1990c). Reduction, carboxymethylation, succinylation, enzymatic, and chemical cleavages were performed as detailed previously (Ozols, 1990a). Peptide mixtures were first separated using a 1.5 × 100 cm column of LH-60 Sephadex equilibrated with formic acid/ethanol, 3:7 (vol/vol), as the solvent. Peptide mixtures from the gel-filtration column were further resolved by reverse-phase HPLC. The latter methodology has been described in detail (Ozols et al., 1980; Ozols, 1990a). Reverse-phase columns employed for HPLC separations include Vydac (Hesperia, CA) (4 × 0.46 cm, 15 × 0.46 cm) or Waters C18 Bondapac (30 × 0.39 cm). Peptide mixtures were dissolved in 88% (vol/vol) formic acid prior to injection on column. Solvent A was 0.1% trifluoroacetic acid, and solvent B was 0.1% trifluoroacetic acid in 75% (vol/vol) acetonitrile or *n*-propanol. A linear gradient from 0% to 100% of solvent B was applied at a flow rate of 1.0 mL/min. Examples of such separations are given in Figure 2D,E.

Sequence analysis of peptides was carried out on an Applied Biosystems model 470A sequencer equipped with model 120A

[2] Abbreviations: HPLC, high-pressure liquid chromatography; PEG, poly(ethylene glycol); DTT, dithiothreitol; TFA, trifluoroacetic acid; GED, buffer containing 20% glycerol, 1 mM EDTA, and 0.1 mM DTT; TDIM, peptides present in the tryptic digest insoluble material; TCA, trichloroacetic acid.

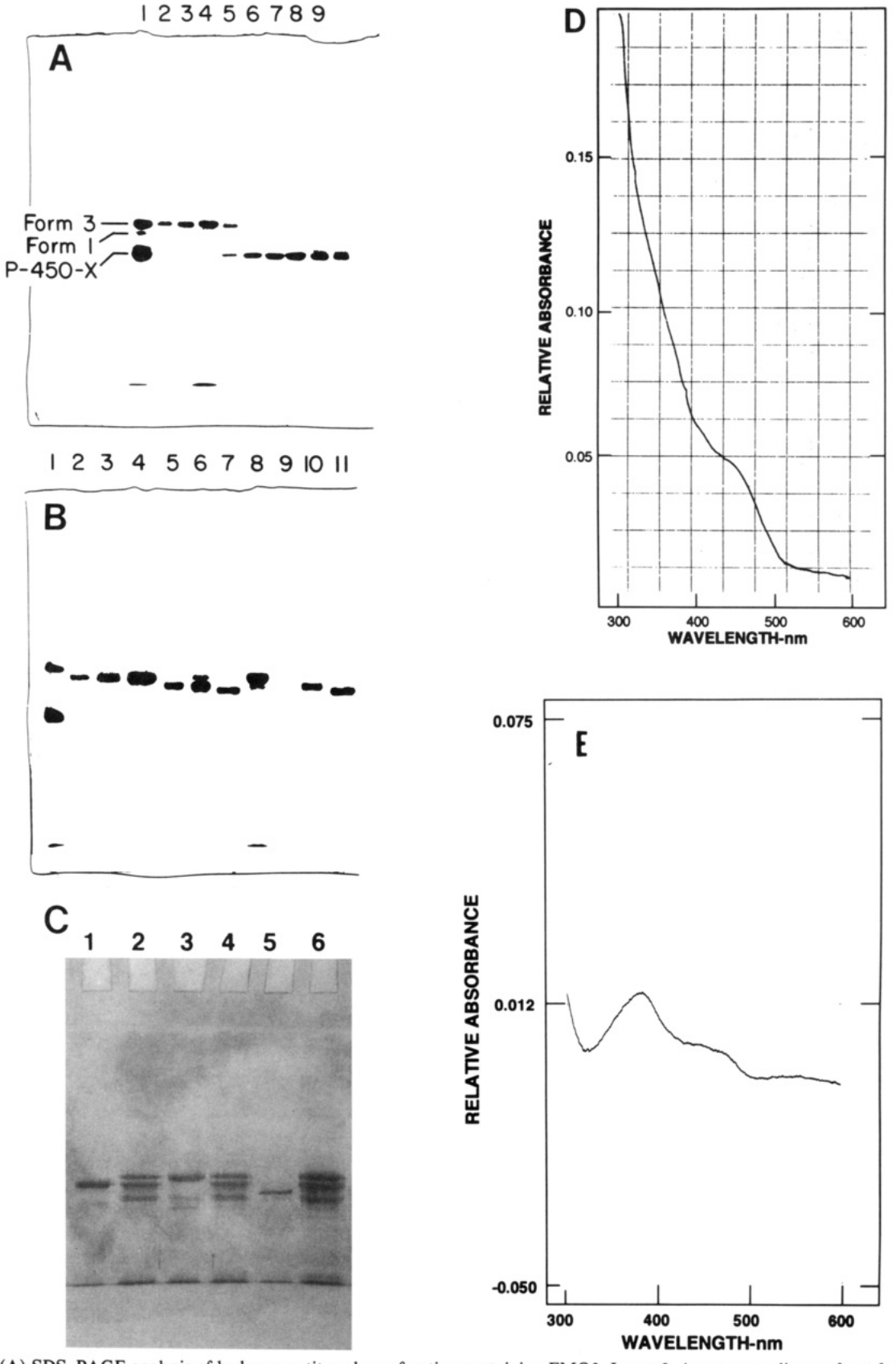

FIGURE 2: (A) SDS–PAGE analysis of hydroxyapatite column fraction containing FMO3. Lanes 2–4 represent aliquots from fractions 33, 34, and 36. Lanes 5–9 are from aliquots from fractions 38–43 containing an unknown cytochrome P-450 (P-450-X). (B) Lane 1, molecular weight standards; top band, microsomal esterase calculated M_r 58 478; lower band, microsomal epoxide hydrolase, calculated M_r 52 695 (Ozols, 1989). Lanes 2–4 and 8, FMO3. Lane 5, FMO1a. Lane 6, FMO1a–3 complex. Lanes 7 and 11, FMO2, M_r 60 096 (Ozols, 1991). Lane 10, FMO1, M_r 60 237 (Ozols, 1990a). (C) SDS–PAGE analysis of fractions obtained from CM-Sepharose column containing the FMO complex. Lane 1, FMO1a; lanes 2, 4, and 6, the FMO complex at increasing concentrations. Lane 3, FMO3. Lane 5, FMO2. (D) Absorption spectra of FMO3. The spectra was recorded as a solution containing 400 μg of protein/mL in 20 mM Tris, pH 8.1, containing 20% glycerol, 0.2% NP-40, 1.0 mM EDTA, 0.1 mM DDT, and NADPH. (E) Absorption spectra of TCA solubilized FMO3. TCA precipitation of FMO3 and recording of the spectra was performed as described under Experimental Procedures.

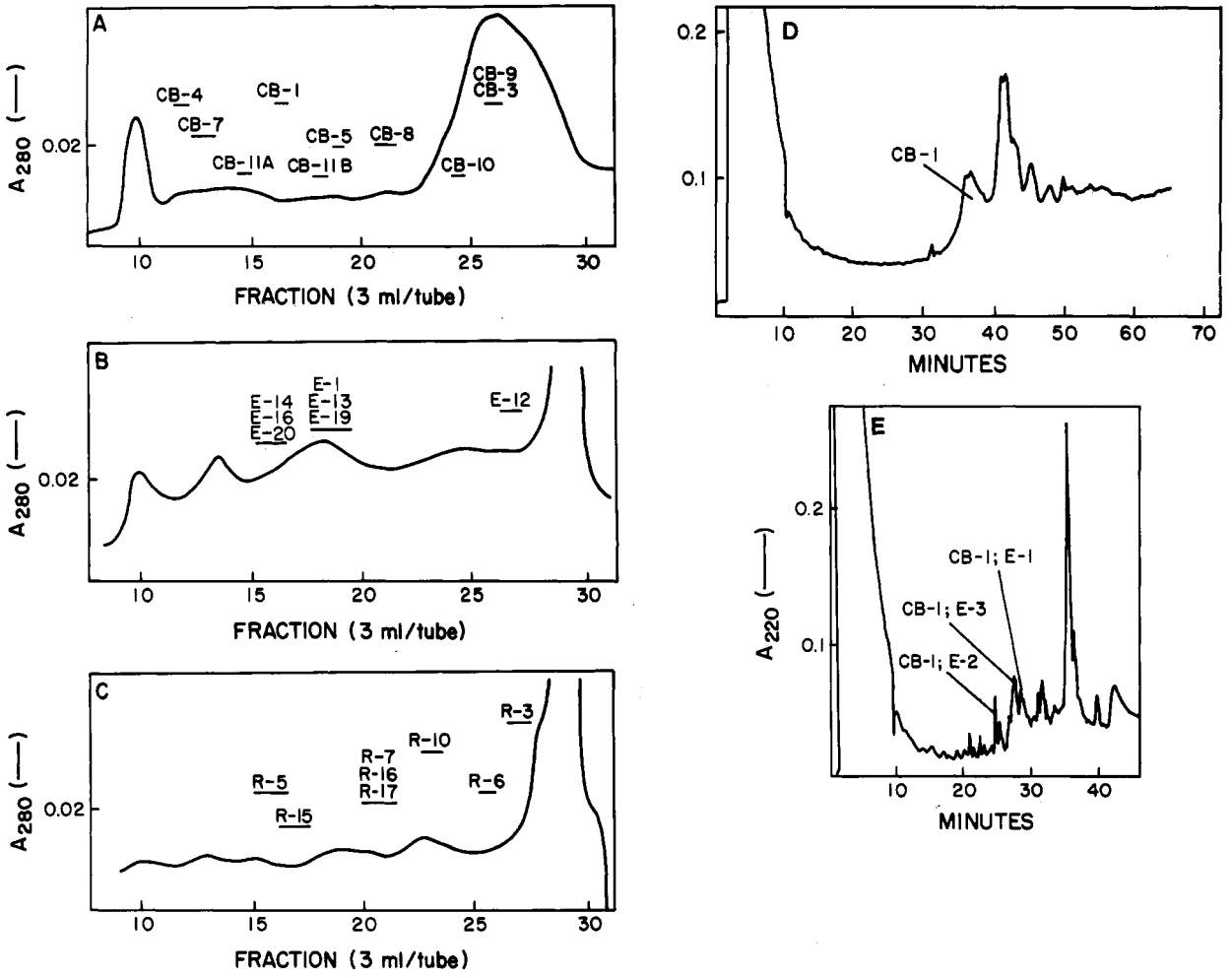

FIGURE 3: Gel filtration of carboxymethylated FMO3 digests on a column of LH-60 Sephadex (1.5 × 100 cm) equilibrated with formic acid/ethanol, 3:7 (vol/vol). Fractions (3 mL) were collected at a flow rate of 10 mL/h. Bars indicate the fractions of a particular peptide pooled for HPLC. (A) CNBr digest of 6 nmol of FMO3. (B) Endopeptidase Glu-C digest of 8 nmol of protein. (C) Tryptic digest of 6 nmol of succinylated protein. (D) Selected sample of peptide isolation by HPLC. Fractions indicated in panel A by a bar were resolved on a Vydac C4 column (15 × 0.46 cm). Solvent A was aqueous 0.1% TFA, and solvent B was 0.1% TFA in 75% acetonitrile. A linear gradient from 0% to 100% of solvent B in 70 min was performed at a flow rate of 1 mL/min. (E) Example of peptide isolation from subcleavage of CB-1 by Glu-C endopeptidase.

phenylthiohydantoin analyzer according to manufacturer's instructions. Solid-phase sequencing of peptides was carried out on a 6600 ProSequencer system (MiliGen/Biosearch, Novato, CA) as previously described (Ozols, 1990a). Data were collected and analyzed using the MiliGen/Biosearch PTH-Maxima chromatography package. Chromatograms were optimally aligned, using alignment algorithms, and then subtracted to give a difference trace for successive sequencer cycles.

Peptides with blocked N-terminus were heated for 15 min with 1.2 M HCl in an evacuated sealed tube at 107 °C or hydrolyzed with acylamino acid releasing enzyme according to manufacturer's instructions. Acylamino acid releasing enzyme hydrolysates of blocked peptides were subjected to HPLC prior to the sequence analysis.

The C-terminal peptide of FMO3 was purified as follows: 2 nmol of alkylated and succinylated FMO in a 1.5-mL Eppendorf tube was dissolved in 160 μL of 8 M urea. After addition of 50 μL of 1 M Tris-acetate, pH 8.1, the tube was heated at 100 °C for 1.5 min, and 50 μL of 2 M ammonium bicarbonate was added. To this solution, 300 μL of water containing 0.5 nmol of trypsin was then added. After 18 h at room temperature, 2 μg of endopeptidase Asp-N was added and the digest incubated at 37 °C for 18 h. Following centrifugation of the tube, the supernatant was removed and the insoluble material washed with 500 μL of 8 M urea, followed by two 700-μL water washes. The Eppendorf tube containing the sample was then lyophilized, and the pellet was transferred with microforceps to an arylamine membrane, coupled to the membrane, and sequenced according to the MiliGen/Biosearch 6600 ProSequencer instructions.

RESULTS

The purification procedure for FMO3 is summarized in Figure 1. All FMO forms eluted in the void volume of the DEAE-cellulose column. The first CM-Sepharose column retained only FMO1, and the third column, hydroxyapatite, effectively resolved FMO2 from FMO3 and the other FMO forms. Affinity chromatography of the 125 mM potassium phosphate fraction on 2′,5′-ADP-agarose resolved FMO3 from FMO1a. A homogenous preparation of FMO3 was obtained after further chromatography on hydroxyapatite and CM-Sepharose columns. Figure 2A shows the SDS–PAGE analysis of fractions containing FMO3 obtained from the second hydroxyapatite column. The FMO3 preparation migrates as a single band with molecular mass of ~60 kDa. An unidentified form of cytochrome P-450 (p-450-X) is eluted following FMO3. As shown in Figure 2B, the apparent molecular weight of FMO3 is slightly higher than that of FMO1 and 2. Alternatively, FMO3 may be isolated from the

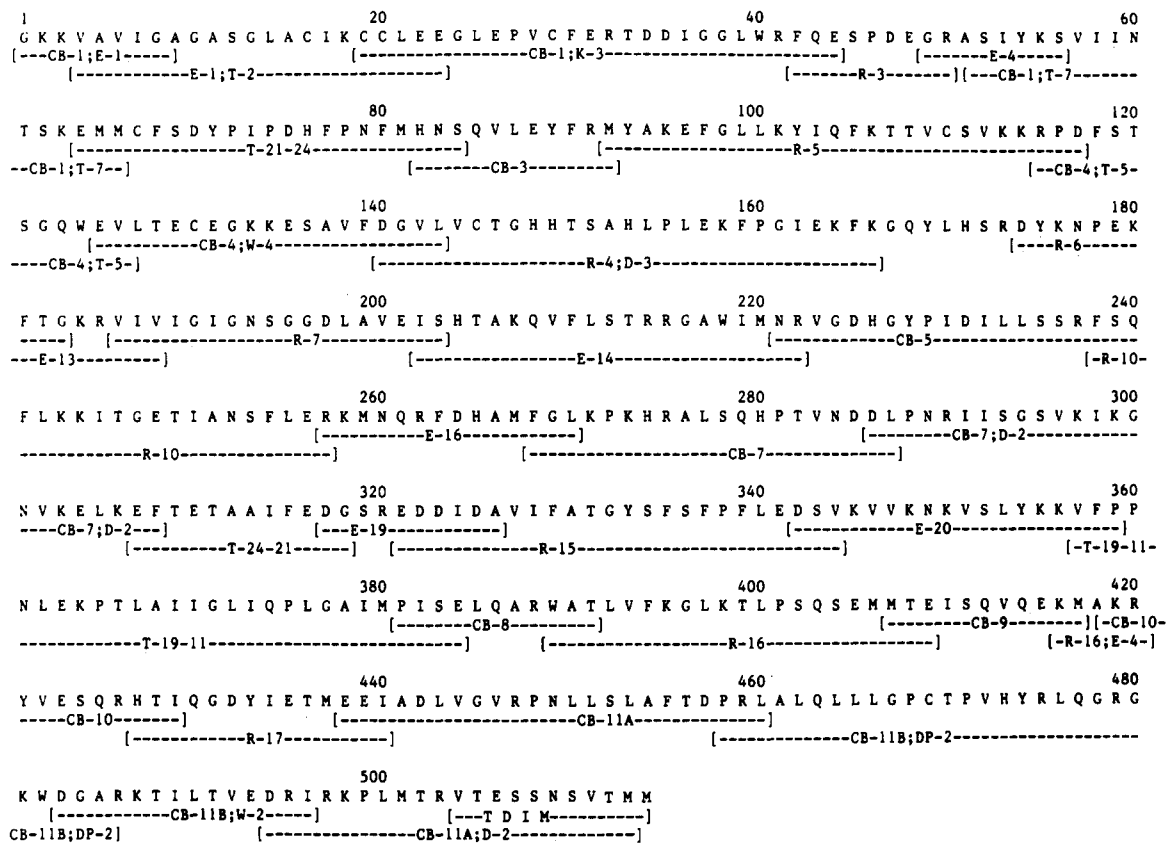

FIGURE 4: Amino acid sequence of FMO3 from rabbit liver microsomes. Peptides obtained by CNBr, endopeptidase Glu-C, trypsin, or trypsin cleavage of succinylated protein are designated by CB, E, T, and R, respectively. Subcleavage of peptides by endoproteinases Asp-N, Glu-C, *Achromobacter* protease Lys-C, or trypsin are designated by D, E, K, and T, respectively. The prefix DP indicates peptides obtained by cleavage with 88% formic acid at Asp–Pro bonds, and W marks peptides cleaved at tryptophan bonds by excess CNBr. TDIM indicates insoluble material obtained after tryptic cleavage, as described under Experimental Procedures. The glycyl residue at the N-terminus is acetylated. The amino acid sequence following residue 514 remains to be determined. The position of the variable residues is given in Table 1.

Table 1: Thiourea Oxidation Activities of FMO Complex, Its Components, and FMOs 1 and 2

enzyme	nmol/mg of protein	SDS–PAGE
FMO complex	400 ± 10	Figure 2C, lanes 2, 4, and 6
FMO3	5–10	Figure 2B, lane 2, and Figure 2C, lane 3
FMO3–1a	50–150	Figure 2B, lane 6
FMO1a	60 ± 5	Figure 2C, lane 1
FMO1	1400 ● 15	Figure 2B, lane 10
FMO2	200 ± 10	Figure 2B, lanes 7 and 11

purified FMO complex as described under Experimental Procedures. The polypeptide composition of the FMO complex is shown in Figure 2C. Automated sequence analysis (an aliquot of 60 μg of protein) of the fractions containing the FMO complex indicated the presence of only one predominant peptide with an open N-terminus. The derived N-terminal sequence of some 20 residues was identical to the N-terminus of FMO2. However, as seen in Figure 2C this preparation did not contain a polypeptide with electrophoretic mobility identical to FMO2.

As indicated in Figure 1, except for the final purification steps, FMO3 copurified with the other FMO forms. Since the latter were much more active FMO forms than FMO3, thiourea turnover measurements during the entire purification procedure were not of much value in monitoring the purification steps of FMO3.

The activity of FMO3 and the fractions containing FMO-related polypeptides using thiourea as the substrate is given in Table 1. The activity of FMO3 gradually decreases upon storage at 4 °C. Some protection was afforded by the inclusion of NADPH. The ratio of FMO3/FMO1a and the activity of the FMO3–1a complex varied from preparation to preparation. The spectra of FMO3 are shown in Figure 2D. Attempts to obtain spectra of the completely oxidized form by dialysis or the use of procedures that remove detergent led to a loss of the protein. That FMO3 contains flavin was confirmed by spectral analysis of the supernatant of trichloroacetic acid precipitated FMO3 (Figure 2E).

Automated Edman degradation of FMO3 indicated a blocked N-terminus. The general strategy of sequence analysis of FMO3 involved the reduction and carboxymethylation of the protein, followed by chemical and enzymatic cleavages. Resolution of the digests were first accomplished on a LH-60 column followed by HPLC of individual fractions (Figure 3). The N-terminus of peptide CB-1 was blocked. Digestion of this peptide with endopeptidase Glu-C yielded a 22-residue peptide (CB-1;E-1) with a blocked N-terminus. Partial acid cleavage or digestion with acylamino acid releasing enzyme of CB-1;E-1 removed the blocking group, and sequence analysis of the digest revealed a Gly-Lys-Lys-Val-Ala-Val-Ile sequence. The order of peptides obtained from CNBr cleavage was determined from isolation and sequence analysis of peptides obtained by cleavage of the reduced and alkylated protein with endopeptidase Glu-C, trypsin, and succinylated protein with trypsin. Some 20% of glutamyl bonds were resistant or only partially cleaved by endopeptidase Glu-C. Endopeptidases Asp-N and Lys-C were effective in cleaving the large peptides obtained from the primary digests. Solid-phase and gas-phase Edman degradation of the purified peptides established the complete amino acid sequence of 514 residues as summarized in Figure 4. The failure to isolate the expected

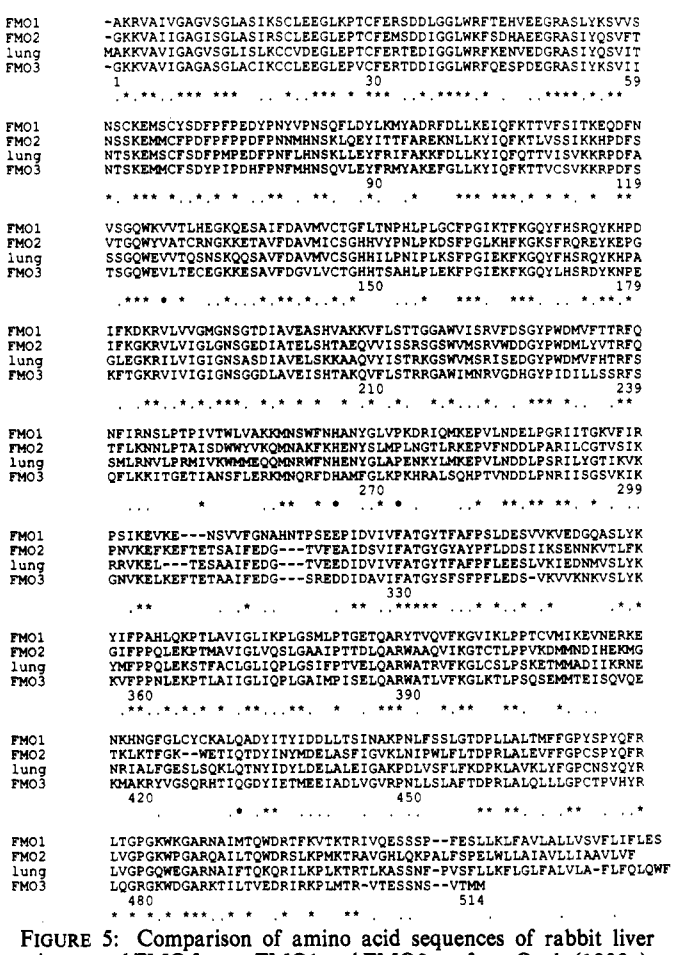

FIGURE 5: Comparison of amino acid sequences of rabbit liver microsomal FMO forms. FMO1 and FMO2 are from Ozols (1990a). The rabbit lung sequence is from Lawton et al. (1990) and Guan et al. (1991). Residues that are identical in all FMOs are denoted by asterisks.

tryptic peptide beginning with Val-505, or Glu-C peptide starting with Ser-508, in pure form, implied a modified C-terminus for this protein. Sequence analysis of the insoluble material (TDIM) obtained after tryptic digestion and 8 M urea wash of the pellet showed, in addition to other peptides, the presence of amino acid sequence identical to the C-terminus of peptide CB-11A;D-2, residues 505–514 (Figure 4). After 11 cycles of automated Edman degradation on this material, the assignment of residues removed, however, was not possible due to the low yield of the sequencer product. Attempts to purify TDIM by HPLC using columns and solvents, including those that yielded the C-terminal peptides for FMO1 and 2, failed to give the expected C-terminal peptide. Thus, the identity of some 15–18 residues at the C-terminus of FMO3 remains to be elucidated.

DISCUSSION

The primary object of this study was to isolate FMO3 in a form that is suitable for sequence analysis and to determine its primary structure. Description of enzymatic properties of FMO3 as well as attempts to reconstitute the proposed FMO complex is beyond the scope of this study.

The comparison of the FMOs 1, 2, 3 and the lung enzyme is shown in Figure 5. The four forms share approximately 55% identity. The least conserved segments are confined to residues 245–260, 404–419, and 421–440. The postulated FAD- and NADP-binding sites, positions 8–14 and 189–194, are well conserved in FMO3. A potential glycosylation site Asn-60 is not glycosylated in FMO3. Similar to FMOs 1 and 2, sites of variant residues were also found in FMO3. Table 2 indicates the positions that contain variant residues within each FMO gene product. Of interest is that all rabbit liver microsomal FMOs contain a consensus sequence common to serine proteases and esterases (Table 3). In addition, the amino acid sequence of FMO3 shows the presence of the consensus sequence containing the histidinyl residue of the catalytic triad of the diisopropylphosphoryl-sensitive carboxylesterases and thioesterases (Table 4). Such pattern of consensus sequences may indicate that the liver microsomal FMOs may have evolved from the serine proteases.

It is unclear why the C-terminal peptides of FMO3, in contrast to those of FMO1 and 2, could not be recovered from reverse-phase HPLC. The absence of an acidic residue in the

Table 2: Variant Sites in Rabbit Liver Isoforms

Position	FMO 1[a]	FMO 1A[b] cDNA	FMO 2[c]	FMO 3	Position
19	Ser	Cys			19
98	Asp	Ser			98
102	Glu	Ser			102
115	Glu	Cys			115
125	Lys	Glu			125
130				Cys/Val	130
239				Ser/Gln	239
278	Leu/Met	Leu	Met/Arg		278
339	Ser	Phe			339
392				Val/Thr	392
404			Met/Val		404
405	Cys	Ser			405
421				Arg/Phe	421
453				Ser/Pro	453
454	Ser	Leu			454
456	Gly	Leu			456
				Ser/Leu	512

[a] Ozols (1990a). [b] Lawton et al. (1990). [c] Ozols (1991).

Table 3: Sequence Homology Between FMOs and the Active Site Serine Residue in Serine Proteases and Esterases

	194	
liver microsomal FMO 1	Gly Asn Ser Gly Thr	rabbit
liver microsomal FMO 2	Gly Asn Ser Gly Glu	rabbit
liver microsomal FMO 3	Gly Asn Ser Gly Gly	rabbit[a]
	195	
serine proteases	Gly Asp Ser Gly Gly	mammalian
V8 protease	Gly Asn Ser Gly Ser	*Staphylococcus aureus*[b]
	195	
liver microsomal esterases	Gly Glu Ser Ala Gly	rabbit[c]
	200	
acetyl cholinesterase	Gly Glu Ser Ala Gly	*Torpedo* eel[d]

[a] This study. [b] Drapeau (1978). [c] Ozols (1989b). [d] Schumacher et al. (1986).

Table 4: Consensus Sequence Around the Active Site Histidine in Various Esterases

	226	
liver microsomal FMO 1	Phe Asp Ser Gly	rabbit
liver microsomal FMO 2	Trp Asp Asp Gly	rabbit
liver microsomal FMO 3	Gly Asp His Gly	rabbit[a]
	226	
thioesterase II	Gly Asn His Phe	duck uropygial[b]
	237	
thioesterase II	Gly Asp His Phe	rat mammary[c]
	231	
thioesterase	Gly Asp His Phe	*Streptomyces*[d]
	441	
liver microsomal esterase I and II	Gly Asp His Gly	rabbit[e]
	276	
fatty acid synthesis thioesterase	Gly Asp His Arg	rat[f]

[a] This study. [b] Paulose et al. (1985). [c] Rhandawa and Smith (1987). [d] Witkowski et al. (1991). [e] Ozols (1989b). [f] Naggert et al. (1988).

segment 504–514, which is present in FMOs 1 and 2, may lead to an irreversible association of the peptide with the reverse-phase column support. Alternatively, it may be possible that the C-terminus of FMO is modified by a posttranslational reaction occurring in the microsomal membrane. For example, the cleavage of some 15 C-terminal hydrophobic residues, followed by the addition of a glycosyl phosphatidylinositol anchor (Ferguson & Williams, 1988), cannot be ruled out. Peptides containing such modifications have not been observed in the eluate from columns and conditions used in this study.

The unexpected low stability and enzyme activity of FMO3 toward some of the FMO1 substrates, despite the high sequence identity to other FMOs is of interest. FMO3 has electrophoretic mobility that is distinct from that of FMOs 1 and 2 (Figure 2B). FMO1a possesses different chromatographic and enzymatic activities than FMO1 (Figure 1 and Table 1). The structural features of polypeptides FMO1a and 2a remain to be elucidated. The finding that FMO3 coelutes with FMO1a from an ion-exchange column, upon increasing salt concentration, in a gradient rather than stepwise form, implies that FMO3 may exist in the membrane as a complex of related polypeptides. SDS–PAGE analysis of column fractions during the isolation steps of FMO1 and 2 showed an absence of polypeptides with the electrophoretic mobility of FMO3, inferring that FMO3 does not associate with FMO1 or 2.

The amino acid sequence determination of the third form of FMO from liver microsomes indicates that the extent of FMO multiplicity in liver microsomes may be greater than previously suspected. The chromatographic and enzymatic properties of FMO3 imply that a functional FMO may also exist in the microsomal membrane as a complex of related polypeptides rather than a single polypeptide.

ADDED IN PROOF

During the submission of this manuscript, the cloning and expression of FMO1C1 was reported by Atta-Asafo-Adiei et al. (1993). The amino acid sequence of FMO1C1 is very similar to that of FMO3, except that the former contains 19 additional residues at the C-terminus, a segment which could not be identified in FMO3. The cDNA-expressed protein was inactive with methimizole as substrate but active with *n*-octylamine.

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