Determination of Lumenal Orientation of Microsomal 50-kDa Esterase/*N*-Deacetylase†

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ABSTRACT: The amino acid arrangements responsible for the insertion and specific lumenal orientation of proteins having an uncleaved signal-peptide-like anchor are poorly understood. A 50-kDa protein having a hydrophobic N-terminus similar to the lumenal glycoprotein 11β-hydroxysteroid dehydrogenase [Ozols, J. (1995) *J. Biol. Chem. 270*, 2305−2312] was identified in detergent-solubilized microsomes. The posttranslational modifications and the membrane orientation of the 50-kDa protein were investigated using the approaches of protein structure analysis. Sequence analysis of the entire 50-kDa protein showed a lack of structural relatedness to the steroid dehydrogenase beyond the membrane binding segment. Structure analysis of peptides revealed that carbohydrate is attached at Asn-77 and Asn-281, implying that these sites of the 50-kDa protein are oriented toward the lumenal side of the endoplasmic membrane (ER). Specific enzymatic deglycosylation on the intact protein identified the two glycans as high mannose carbohydrate rather than of the complex type, suggesting that the protein had not undergone further trafficking steps beyond the lumen of ER. Chemical modification of cysteinyl residues showed a lack of free thiols in the intact protein. Peptide mapping identified one disulfide bond between Cys-115 and -340 further restricting the bulk of the protein to the lumenal compartment. Proteolysis of intact and solubilized microsomes showed that the 50-kDa protein is resistant to fragmentation at the conditions which led to the removal of the membranous segments from cytochrome b_5 and the NADH−cytochrome b_5 reductase. The proposed model of the 50-kDa protein predicts one transmembrane segment at the N-terminus, flanked by positively charged residues on the cytosolic surface and negatively charged residues on the lumenal side of the hydrophobic domain, with most of the polypeptide projecting into the lumen of the ER. The stated similarities in the topology between 11β-steroid dehydrogenase and 50-kDa protein envision their transmembrane segment consisting of a basic residue(s) followed by an array of some 17 hydrophobic residues containing the Ala-Tyr-Tyr-X-Tyr cluster, where X represents a hydrophobic amino acid, which terminates with acidic residues. It is proposed here that such a motif may constitute a lumenal targeting signal for a set of single-membrane-spanning proteins that are otherwise structurally and functionally unrelated.

The structural features of ER proteins that are responsible for their insertion and orientation continue to be elucidated. Most microsomal proteins are targeted for membrane insertion by a hydrophobic signal peptide near the N-terminus (*1*). During translation, the signal peptide is recognized by a cytosolic RNA/protein complex called the signal recognition particle (SRP) (*2*). Upon binding a signal peptide, the SRP arrests protein synthesis until it has facilitated transfer of the nascent polypeptide−ribosome complex to the translocation channel (*2*). The signals that control the folding and eventual topology of the membrane proteins after the nascent chain is transferred to the translocation channel are unknown. One early critical event in the multistep folding process that defines the maturation of membranous proteins after translocation has begun is whether the signal peptide is removed. If the protein has a cleavable signal peptide, then the newly formed N-terminus is generally located in the lumen of the ER. In cases where the N-terminal signal peptide is not removed, such a segment functions as a membrane anchor, directing the transmembrane insertion of the nascent polypeptide. Membrane proteins with uncleaved signal peptide can have either cytosolic or lumenal orientation. Mutagenesis experiments on a number of eukaryotic plasma membrane proteins with a single N-terminal transmembrane segment have revealed that the charge distribution, the length, and the folding properties of the polar segments following the anchor peptide as well as the length of the transmembrane segment are some of the factors that determine the specific topology of such proteins (*3*). Detailed analysis of these factors has been complicated by a lack of agreement on the topology of the model proteins employed. Moreover, additional ambiguities are encountered in trying to overexpress eukaryotic membrane proteins in prokaryotic hosts. Nevertheless, it appears clear not only that a signal sequence mediates the recognition of a protein by the

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[1] Abbreviations: ER, endoplasmic reticulum; 11β-HSD, 11β-hydroxysteroid dehydrogenase; PEG, poly(ethylene glycol); DTT, dithiothreitol; HPLC, high-performance liquid chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; endo H, endo-*N*-acetylglucosaminidase H; DFP, diisopropylphosphofluoridate; DIP, diisopropylphosphoryl.

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membrane-linked transport system but also that it may function as a permanent transmembrane anchor. Whether a particular amino acid arrangement of a signal peptide results in a resistance to peptidase cleavage or carries information that is responsible for the specific orientation of proteins having an uncleaved signal peptide membrane anchor is the basis for this investigation.

In previous studies, we reported the amino acid sequence and identified the segments comprising the membrane-binding anchors of cytochromes b_5 reductase (*4*), cytochrome b_5 (*5, 6*), cytochrome P-450 (*7, 8*), epoxide hydrolase (*9*), Δ^9-stearoyl-coenzyme A desaturase (*10, 11*), and three forms of flavin containing monooxygenases (*12, 13*). Moreover, we provided evidence for the lumenal localization of several liver microsomal glycoproteins including two isoforms of carboxylesterases (*14, 15*), NADP−glucose-6-phosphate dehydrogenase (*16*), oligosaccharyltransferase (*17, 18*), and the 11β-hydroxysteroid dehydrogenase (11β-HSD) (*19*). Cytochrome b_5 is held in the ER by a hydrophobic segment of 30 residues at the C-terminus of the protein molecule with the N-terminal catalytic domain exposed to the cytosol (*6*). The anchor segment of cytochrome b_5 reductase consists of a myristoyl residue attached to a hydrophobic segment at the N-terminus of the molecule (*4*). Cytochromes P-450, epoxide hydrolase, desaturase, and the flavin monooxygenases are multispanning membrane proteins with unknown topology. Although there has been much debate over the orientation of the specific segments of cytochromes P-450 in the membrane, experimental evidence from different laboratories for a particular topology has been contradictory for this group of proteins. The microsomal carboxylesterases and the glucose-6-phosphate dehydrogenase lacked transmembrane segments. The microsomal carboxylesterases carry a C-terminal segment related to the Lys-Asp-Glu-Leu (KDEL), the ER lumen retention motif (*20*). While the evidence for a retrieving function of the KDEL receptor is solid, it is clear that additional mechanisms presently unknown are responsible for the retention of lumenal proteins devoid of KDEL-like sequences or hydrophobic anchors. Several signals for the retrieval of ER proteins have been recognized. A di-lysine motif at the cytoplasmic C-terminus of Type 1 ER membrane proteins and di-arginine sequences at the cytoplasmic N-terminus of Type 2 ER membrane proteins have been recognized as ER localization signals (*21, 22*).

The amino acid sequence of the microsomal glycoprotein, glucose-6-phosphate dehydrogenase, failed to identify sequence segments or a motif that would be responsible for its lumenal localization or retrieval, but displayed a segment of some 300 additional residues as compared to the cytosolic enzyme (*16*). The 50-kDa protein component of the oligosaccharyltransferase complex contained a single stretch of some 25 hydrophobic residues between positions 380 and 405 near the C-terminus, and, therefore, a lumenal orientation for this protein has been proposed (*17, 18*).

In an earlier report, we determined the structure and topology of the first ER lumenal protein having only one membrane-spanning domain at the N-terminus (*19*). In this report, we identified the second lumenal protein in the ER having a single N-terminal transmembrane segment with the bulk of the molecule oriented toward the lumenal side of the ER. A sequence motif for the lumenal localization of ER proteins with a single transmembrane segment is proposed.

EXPERIMENTAL PROCEDURES

Materials and Methods. Detergents, enzyme substrates, cofactors, chromatographic media, and chemicals, unless stated otherwise, were obtained from Sigma. Hydroxyapatite (HA-Ultragel) was a product of IBF Biotech, Villeneuve-la-Garenne, France. [^{3}H]Diisopropylphosphorofluoridate (DFP) was purchased from DuPont NEN. Trypsin and chymotrypsin were obtained from Worthington. Endopeptidases Asp-N, Arg-C, Glu-C, and endo-*N*-acetylglucosaminidase H (endo H) were from Boehringer Mannheim. *Achromobacter* peptidase Lys-C was obtained from Biochemical Diagnostics, Edgewood, NY. Cyanogen bromide was obtained from Pierce Chemicals. Solvents for HPLC and gel filtrations were from Burdick & Jackson. Molecular weight standards for SDS−PAGE were from Bio-Rad. The 66-kDa marker bovine serum albumin (BSA) in the premixed "Low Range" standard set occasionally precipitated upon storage and therefore resulted in a poor staining band in some of the standard lanes. ^{14}C-Methylated protein standards were from Sigma.

Isolation of 50-kDa Proteins. Liver microsomes were isolated from New Zealand male rabbits and solubilized as described previously (*23, 11*). 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 14 mg/mL of protein. Solubilization of microsomes was achieved by the addition of sodium cholate, 1.2 mg/mg of protein. The solubilized material was fractionated with poly(ethylene glycol) (PEG) as described in (*23*). 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 (250 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. All buffers used in the subsequent purification steps contained 20% glycerol, 1 mM EDTA, and 0.1 mM DTT, abbreviated as GED. The unbound material eluting from the DEAE column was applied to a column containing 100 mL of CM-Sepharose equilibrated with 10 mM potassium phosphate, pH 7.2, containing 0.2% NP-40 and GED. The column was washed with 200 mL of the equilibration buffer. The void volume of the CM-Sepharose column was applied to a column (100 mL) of hydroxyapatite, equilibrated with 10 mM potassium phosphate, pH 7.4, containing 0.1% NP-40 and GED. The unretained material contained highly purified 50-kDa protein which was collected in three 250 mL volumes and designated void volumes 1, 2, and 3. Each 250 mL aliquot of the void volume was dialyzed against 2 L of 10 mM Tris-HCl, pH 8.1, containing 0.1% NP-40 and GED (buffer B). The dialyzed material was applied to a column (75 mL) of Q-Sepharose equilibrated with buffer B. After washing the column with 200 mL of buffer B, the column was developed with a linear gradient of increasing concentration of sodium chloride, 10−300 mM, 100 mL per each gradient chamber.

A second form of the 50-kDa protein eluted upon the development of the hydroxyapatite column with 125 mM potassium phosphate, pH 7.4, containing 0.1% NP-40 and

GED. This form coeluted with a complex mixture of proteins including the lumenal glucose-6-phosphate dehydrogenase (*16*). It was purified to homogeneity by subjecting this sample to the CM-Sepharose, DEAE, and hydroxyapatite chromatography steps as described above. A third form of the 50-kDa protein was purified from the effluent fractions of the first DEAE column. It eluted upon developing the DEAE column with the equilibrating buffer containing 25 mM sodium chloride. The 50-kDa protein eluted in about 45 6 mL fractions shortly before the elution of the glycoprotein esterase 1 and cytochrome P-450 isoforms as described previously (*14*). The three forms of the 50-kDa proteins were purified to homogeneity by repetition of the above steps as summarized in Figure 1.

Column fractions were monitored by SDS−PAGE using 4% acrylamide stacking and 8, 10, or 12% acrylamide resolving gels (8 × 7 cm, 1.0 mm). Electrophoresis was performed in the BioRad minicell at room temperature for 40 min at 180 V. Gels were stained with 0.25% (w/v) Coomassie blue in 4% (v/v) methanol/10% (v/v) acetic acid, and destained in 10% (v/v) acetic acid/40% (v/v) methanol.

The esterase activity of the 50-kDa proteins was determined with a microplate reader using 4-methylumbelliferyl acetate as substrate (*15*). A 20 μL aliquot of a column effluent fraction was applied to a well of a 96-well UV transparent microplate containing 200 μL of 0.1 M sodium phosphate buffer, pH 6.3. The reaction was initiated by the addition of 10 μL of 1 mM 4-methylumbelliferyl acetate dissolved in acetone/ethanol (3:1) mixture. The plate was read at 350 nm in the SPECTRAmax Model 340 microplate reader. The K_m of the 50-kDa proteins for this substrate is about 30 μM, and the V_{max} about 10 μM min^{-1} mg^{-1}. Preparations free of the esterase 1 were also assayed for hydrolysis of *o*-nitrophenyl acetate in the microplate reader. The reaction mixture of the disposable microplate well consisted of 20 μL of sample and 200 μL of 0.1 M sodium phosphate, pH 7.4. The reaction was started by the addition of 20 μL of 10 mM *o*-nitrophenyl acetate dissolved in ethanol. The plate was read at 405 nm. DFP at a concentration of 2 μM completely inhibited the esterase activity of the enzyme. Deacylation of 2-acetylaminofluorine was performed as previously described (*24*).

Sequence Analysis. Protein/peptide hydrolysis for amino acid analysis was performed with 6 M HCl in the gas phase, at 150 °C for 1 h (*25*). Reduction, carboxymethylation, succinylation, and enzymatic and chemical cleavages were performed as described previously (*25*). Purification peptide mixtures on a large scale were first separated using a 1.5 × 100 cm column of LH60−Sephadex equilibrated with formic acid/ethanol, 3:7 (v/v), as the solvent. Peptide mixtures from the gel filtration column were resolved by reverse-phase HPLC. The latter methodology has been described in detail (*26*). Reverse-phase columns employed for HPLC separations include C4, Vydac (Hesperia, CA) (4 × 0.46 cm or 15 × 0.46 cm), and Waters C18 Bondapac (30 × 0.39 cm). Peptide mixtures were dissolved in 88% formic acid prior to injection on the column. Solvent conditions for the reverse-phase columns were as follows: Solvent A was 0.1% trifluoroacetic acid, and solvent B was 0.1% trifluoroacetic acid in 75% (v/v) acetonitrile. A linear gradient from 0% to 100% of solvent B was applied at a flow rate of 1.0 mL/min. Sequence analysis was carried out on an Applied Biosystems Model 470A sequencer equipped with a Model 120Z PTH-analyzer according to the manufacturer's instructions. Solid-phase sequencing was carried out on a 6600 ProSequencer system (MilliGen/Biosearch, Novato, CA) as previously described (*12*).

DFP-labeled 50-kDa protein was prepared by addition of a 1 mM mixture of [^{3}H]DFP and cold DFP and incubated overnight at 4 °C. The reaction mixture was then subjected to Centricon-10 filtration until the radioactivity was absent in the filtrate. Partially labeled DFP microsomes were prepared shortly before use by addition of 1 μL of [^{3}H]DFP (8.4 Ci/mmol) in propylene glycol to 30 μL of intact microsomal preparation. The integrity of microsomal preparations was estimated by determining the degree of latency of the lumenal glucose-6-phosphate dehydrogenase in the presence and absence of detergents (*16*). The latency of glucose-6-phosphate dehydrogenase in radiolabeled preparations was identical to the intact microsomal preparations. Proteolysis of microsomes was performed with radiolabeled preparations (10−15 mg of protein/mL in 0.25 M sucrose containing 10 mM Tris−acetate, pH 8.1). Samples were incubated with chymotrypsin at a protein to enzyme ratio of 200:1 at 25 °C for 30 min. The reaction was terminated by the addition of 3 volumes of SDS−PAGE sample loading buffer followed by electrophoresis and immunoblotting of 10 μL aliquots.

Specific labeling of the cysteine residues was as follows: A 50-kDa preparation was dialyzed against 100 volumes of buffer (degassed with nitrogen) containing 20 mM Tris−acetate, pH 8.1, 20% glycerol, 0.1% NP-40, and 1 mM EDTA. To 10 nmol of 50-kDa protein in 10 mL of dialysis buffer was added 15 mL of neutralized 8 M guanidine hydrochloride solution, followed by 50 μCi of [^{14}C]iodoacetamide (specific radioactivity 55 mCi/mmol). After 10 min at room temperature, 100 μL of 1 M iodoacetamide was added and the reaction mixture incubated for 1 h at 37 °C. The reaction mixture was then reduced by the addition of 250 μL of 1 M DTT. After 1 h at 37 °C, 700 μL of 1 M iodoacetic acid was added and the alkylation continued for 1 h at 37 °C. Following dialysis against 5 mM ammonium bicarbonate containing 0.1% β-mercaptoethanol, the preparation was lyophilized and subjected to trypsin or Lys-C protease hydrolysis. The enzymatic digests was first resolved on a column of LH60−Sephadex or directly by HPLC. Aliquots of peptide-containing material were withdrawn to measure the ^{14}C content using a Packard scintillation counter. The remaining peptide fraction was used for sequence analysis. Carboxymethyl- and carboxyamidomethyl-PTH-cysteine derivatives were distinguished by their characteristic elution positions on the PTH analyzer. Enzymatic deglycosylation of HCl/acetone-precipitated protein with endo H was performed as described by the manufacturer's instructions. Acid−acetone precipitation of protein solutions was performed by the addition of 20 volumes of cold (−20 °C) acetone containing 0.2% (v/v) HCl. Endoglycolytic cleavage of native 50-kDa protein was conducted in 20 mM sodium phosphate buffer, pH 6.7, containing 15 milliunits of endo H for 1−3 h, at room temperature (*19*). Aliquots of the digest were analyzed by SDS−PAGE.

Preparation of Antibodies and Immunoblotting. Anti-b_5 and anti-reductase antibodies were raised against the polar moieties of rat cytochrome b_5 and cytochrome b_5 reductase

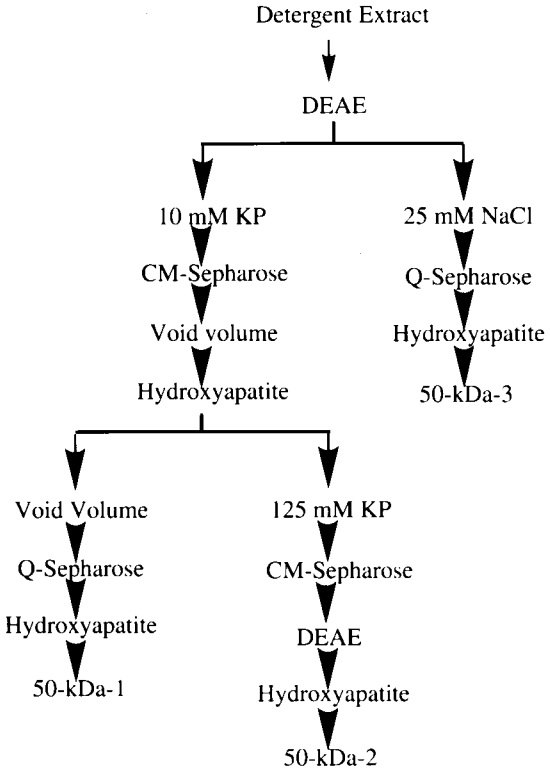

FIGURE 1: Summary of the purification steps of the 50-kDa proteins from rabbit liver microsomes. KP denotes the potassium phosphate concentration at which the fractions eluted from the column.

(*6*). Chickens were immunized by injection of the antigen as an emulsion with complete Freund's adjuvant. About 0.7 mg of cytochrome and 0.75 mg of reductase were injected in each chicken during a period of 3 months. IgG immunoglobulin was partially purified from serum by precipitation with ammonium sulfate. Proteolysis of cytochrome b_5 and reductase degradation were determined by immunoblotting (I-Blot) of [^{3}H]DFP-labeled microsomes. After incubations, samples were subjected to SDS−PAGE using a 12 or 10% acrylamide gel under reducing conditions in triplicate. One gel was subjected to autoradiography. Two identical gels were electrotransferred to Immobilon-P transfer membranes for immunoblotting. The membranes were reacted with chicken anti-cytochrome b_5 or anti-reductase antibody, which was then complexed with anti-chicken IgG−alkaline phosphatase (Sigma product A-9171). Molecular weight markers for I-Blots were prestained proteins (Bio-Rad Product 161-0305). Immunoreactive cytochrome or reductase bands were visualized using a phosphatase substrate system detection kit (Kirkegaard & Perry Laboratories Inc.).

RESULTS

The purification procedure for the 50-kDa proteins is outlined in Figure 1. Two forms of the enzyme eluted in the void volume of the first DEAE column. A third form eluted upon developing the column with 25 mM sodium chloride (Figure 2A−C). Chromatography of the DEAE void volume on a CM-Sepharose column yielded the 50-kDa protein exclusively in the void volume. Chromatography of the void volume of the CM-Sepharose column on a hydroxyapatite column gave 50-kDa-1 in the void volume and 50-kDa-2 upon elution with an increased phosphate concentration. The latter form coeluted with the previously described 90-kDa lumenal glucose-6-phosphate dehydrogenase (Figure 2B). Subsequent purification steps by ion-exchange and hydroxyapatite chromatography yielded preparations that were homogeneous upon SDS−PAGE. The SDS−PAGE analyses of the three 50-kDa proteins were identical (Figure 2A−C). The complete amino acid sequence was determined from 50-kDa-2 of the enzyme.

The amino acid sequence analysis of the 50-kDa protein involved the reduction and carboxymethylation of the protein, followed by chemical and enzymatic cleavages. Resolution of the digests initially was achieved by gel filtration on an LH-60 Sephadex column followed by HPLC of the individual fractions. The resolution of the CNBr and Lys-C digests by the LH-60 Sephadex column is seen in Figure 3A,B. The complete amino acid sequence and the order of all peptides obtained by the primary cleavages were determined by isolation and sequence analysis of peptides subcleaved with Asp-N and Arg-C endoproteases as shown in Figure 4. An abnormal Lys-C cleavage occurred at the Thr−Asn bond at residue 142, yielding peptide K-8 in good yield. The site labeled by [^{3}H]DFP was identified by sequence analysis of radiolabeled peptide K-10 isolated from the Lys-C digest (Figure 3C). The location of the label at Ser-188 was unambiguously determined by the radioactivity present at cycle 7 upon sequence analysis of peptide K-10 subcleaved by Arg-C peptidase (peptide K-10;R-2) (Table 2). Although automated sequence analysis of the parent peptide K-10 identified the label at cycle 14, the breakdown of the DIP-Ser adduct during the 13 cycles of degradation was evident. The signal versus background ratio at cycle 14 was about 4:1 when peptide K-10 was sequenced as compared to 8:1 for the sequence analysis of the shorter fragment of the same peptide.

Solid-phase and gas-phase Edman degradation of the purified peptides failed to identify a residue at positions 77 and 181. The peptides comprising these segments contained Asp/Asn residues by amino acid composition, and since these positions contained Ser/Thr residues two residues downstream, glycosylated Asn residues were assigned in this segment.

The amino acid sequence of the 50-kDa protein lacks relatedness to the 11β-HSD beyond the N-terminus. Sequence search of the data banks, however, revealed 79% identity and some 90% homology to the recently molecularly sequenced human liver 2-acetylaminofluorine *N*-deacetylase (*24*) (Figure 4B). A striking similarity between the 50-kDa protein and the 60-kDa glycoprotein esterases (*14*, *15*) in the region of residues 109 and 191 as well as the residues comprising the "active" serine was also observed.

To identify the state of the two cysteinyl residues, the protein was denatured with guanidine hydrochloride and treated with iodoacetamide or [^{14}C]iodoacetamide followed by reduction and alkylation with iodoacetic acid. This carboxymethylation protocol was designed to label free (reduced) cysteine residues with [^{14}C]iodoacetamide and disulfide-linked (oxidized) cystines following reduction with iodoacetic acid. The modified protein was fragmented, and the resulting digest was resolved by HPLC. No radioactivity was present in peptides corresponding to segments containing Cys-115 or Cys-340. Furthermore, cross-linked peptides containing Cys-115 and Cys-340 were isolated from CNBr

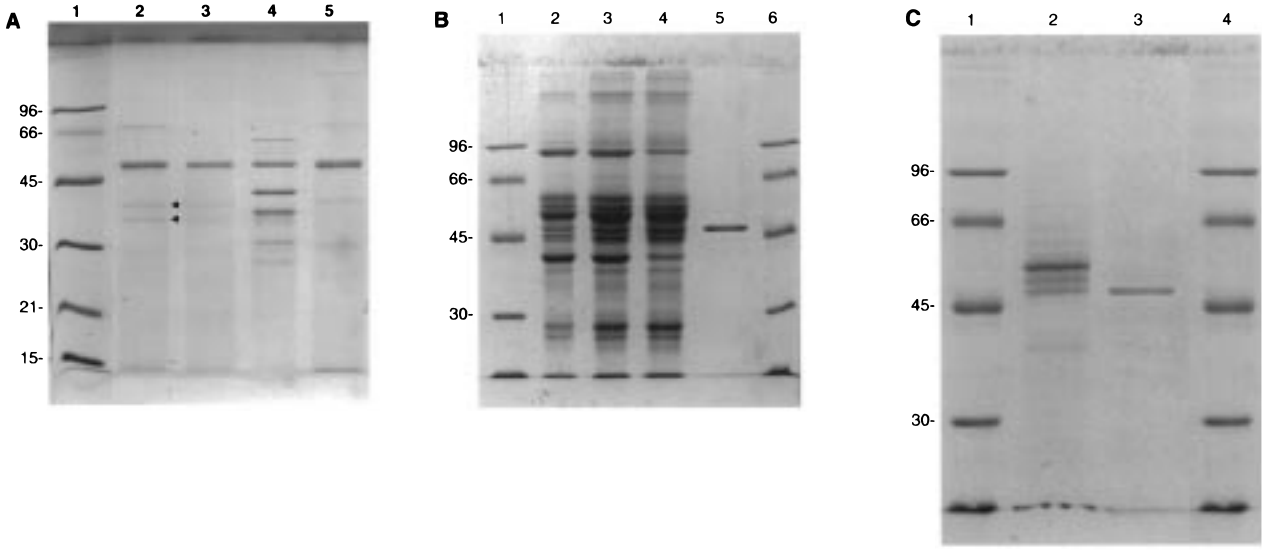

FIGURE 2: SDS−PAGE analysis of column fractions containing 50-kDa proteins. (Panel A) Lanes 2 and 3 represent purified 50-kDa-1. Lane 4 represents the proteins eluted in the void volume of the hydroxyapatite column of Figure 1. Lane 5 represents purified 50-kDa-2. The arrowheads shown next to lanes 2 and 3 represent fragments from autolysis of the 50-kDa-1 protein. Lane 1 denotes molecular weight standards with the mass marked on the left of the panel. (Panel B) Purified 50-kDa-2, lane 5. Lanes 2, 3, and 4 represent 125 mM potassium phosphate eluted fractions of the hydroxyapatite column shown in Figure 1 from which 50-kDa-2 was purified. The 90-kDa band in lanes 2, 3, and 4 is the lumenal glucose-6-phosphate dehydrogenase (*16*) which copurifies with 50-kDa-2. (Panel C) DEAE column fractions from which 50-kDa-3 was isolated. Lane 3 represents purified 50-kDa-3.

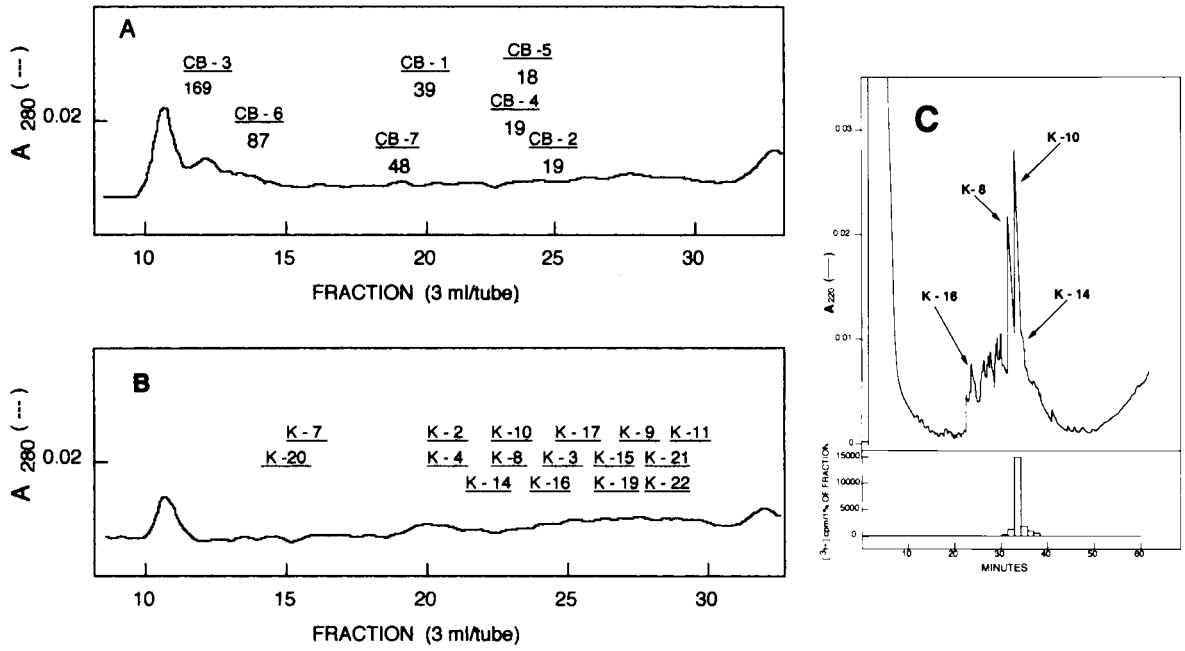

FIGURE 3: Gel filtration of 50-kDa protein digests on a column of LH-60 Sephadex (1.5 × 100 cm) equilibrated with formic acid/ethanol (3:7 (v/v). Effluent fractions were collected at a flow rate of 10 mL/h. Bars indicate the fractions of a particular peptide pooled for HPLC, and the number below the bar indicates the number of residues of the peptide. (Panel A) CNBr digest of approximately 2.5 nmol of protein. (Panel B) Gel filtration of the Lys-C endoprotease digest of 2.5 nmol of 50-kDa protein. (Panel C) HPLC isolation of peptide K-10 containing the [^{3}H]DFP-labeled active site serine from the Lys-C digest resolved as shown in panel B. The distribution of radioactivity in each peak is shown below the HPLC profile.

and Lys-C digests; thus, a disulfide bond was assigned between these residues.

The carbohydrate status of the enzyme was examined by deglycosylation with endo H followed by SDS−PAGE. Treatment of the 50-kDa protein with endo H resulted in a decrease of its apparent molecular mass. The deglycosylation reaction of the 50-kDa protein was complete as seen by a single band with an increased electrophoretic mobility on SDS−PAGE (Figure 5). To confirm the proposed lumenal orientation of the 50-kDa protein, intact microsomes were digested with chymotrypsin in the presence and absence of detergents. As seen in Figure 6A, chymotrypsin treatment of intact microsomes did not alter the mobility of the 50-kDa protein. Fragmentation of the labeled esterases was observed upon proteolysis of microsomes solubilized with TX-100/sodium cholate. Cytochrome b_5 and NADH cytochrome b_5 reductase were clearly fragmented in intact and solubilized microsomes (Figure 6B,C).

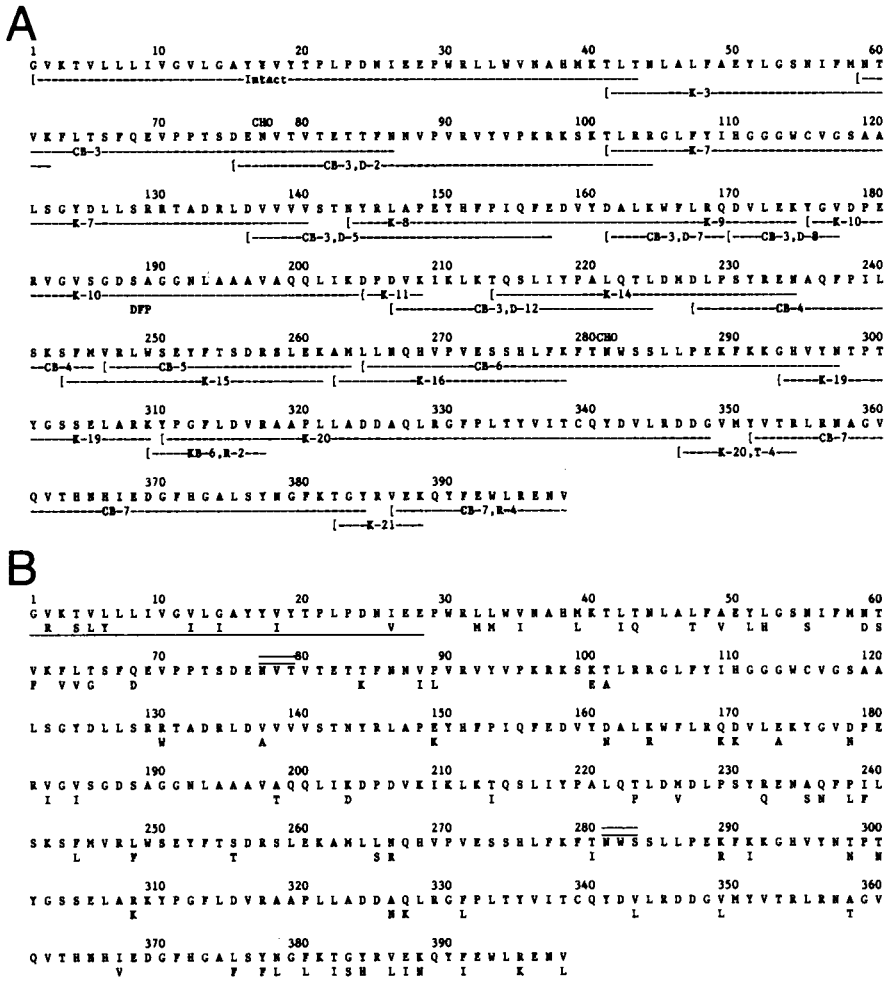

FIGURE 4: (A) Complete amino acid sequence of rabbit liver microsomal 50-kDa protein. Peptides obtained by Lys-C endoprotease and CNBr are designated by K- and CB-, respectively. Subcleavage of peptides by endoprotease Asp-N or Arg-C is designated by D- and R-, respectively. An abnormal Lys-C cleavage occurred at the Thr−Asn bond at position 142, yielding peptide K-8. Residues identified by automated Edman degradation are underlined, except for the Asn residues binding carbohydrate (CHO) at positions 77 and 281 where a PTH derivative could not be identified. The residue binding diisopropyl phosphate is Ser-188 and is denoted by DFP below the residue. (B) Alignment of the amino acid sequence of rabbit microsomal 50-kDa protein with the human liver 2-acetylaminofluorine *N*-deacetylase (*24*). The top sequence shows the rabbit protein, and the amino acid residues in the human protein different from the rabbit are shown beneath. The putative ER lumenal targeting sequence is underlined with a solid line. The two N-linked glycosylation sites are overlined by two solid lines. A disulfide bond links Cys-115 and Cys-340.

Table 1: Characteristics of the Termini of the 50-kDa and 11β-HSD Proteins

N-terminal

	1	28
50-kDa	GVKTVLLLIVGVLGAYYVYTPLPDNIEE	
	1	26
11β-HSD	AFMKK YLLPLLGLFL AYYYYSANEE	

C-terminal

		398
50-kDa	GFKTGYRVEKQYFEWLRENV	
11β-HSD	KRL IE FLHLRKFD I SKLVNN	
		293

Table 2: Sequence Analysis of the [^{3}H]DFP-Labeled Peptide of the 50-kDa Protein[a]

cycle	amino acid	yield	cpm
1	Val	38	969
2	Gly	22	686
3	Val	13	630
4	Ser	9	557
5	Gly	11	370
6	Asp	4	462
7	DIP-Ser	−	3584
8	Ala	2	1779
9	Gly	3	464
10	Gly	3	130
11	Asn	1	230
12	Leu	1	103

[a] Peptide K-10;R-2, residues 182−203 (100 pmol, 560 000 cpm), was applied to the sequencer. An 25% aliquot from each cycle was collected for radioactivity determination.

DISCUSSION

The 50-kDa protein described in this report was first detected in partially purified preparations of 11β-HSD (*19*). Subsequently, two additional forms of the 50-kDa protein were identified (Figure 1). Since all 3 forms of the 50-kDa protein had identical 10 N-terminal residues as well as similar electrophoretic mobility (Figure 2), the observed differences in their chromatographic properties most likely represent differences in the protein−protein interactions that occur during protein purification. A similar phenomenon was also observed during the purification of 11β-HSD (*19*). The

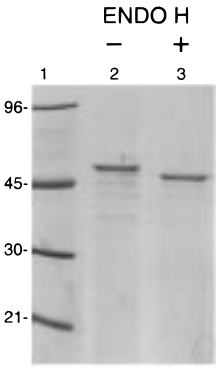

FIGURE 5: SDS−PAGE analysis of denatured 50-kDa protein before (lane 2) and after deglycosylation with endo H (lane 3). Lane 1 represents molecular weight markers with the mass indicated on the left of the panel.

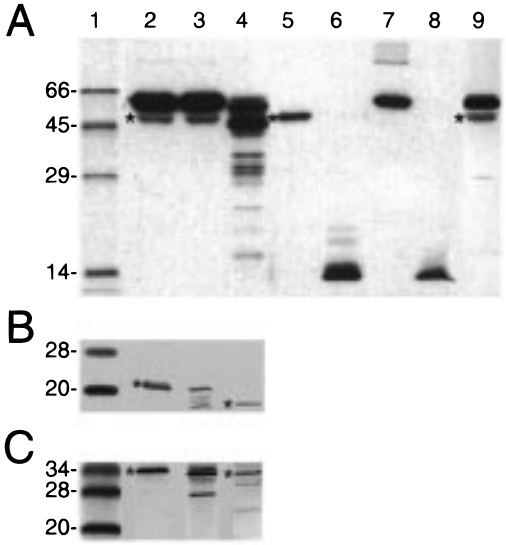

FIGURE 6: Stability of the 50-kDa protein, cytochrome b_5, and NADH−cytochrome b_5 reductase toward proteolysis in intact and detergent-solubilized microsomes. (Panel A) Autoradiogram of [^{3}H]-DFP-labeled microsomes. The position of the 50-kDa protein is indicated by an asterisk. Lanes 2 and 3 represent microsomes without and with chymotrypsin (200 μg/mL). Lane 4, chymotrypsin digest of Tx-100/sodium cholate-solubilized microsomes. Lanes 5 and 6 represent purified 50-kDa protein in 0.2% Tx-100 without and with chymotrypsin (200 μg/mL). Lanes 7 and 8, purified microsomal esterase I in the absence and presence of chymotrypsin (200 μg/mL). Lane 9, Lys-C endoprotease digest (300 μg/mL) of Tx-100/sodium cholate-solubilized microsomes. (Panel B) Immunoblot analysis of the presence of cytochrome b_5 in the above samples. The intact and the polar domains of the protein are indicated by asterisks. (Panel C) Immunoblot analysis of the presence of NADH−cytochrome b_5 reductase in the above samples. Asterisks indicate the native and the polar forms of the reductase. The protein bands were visualized with the phosphatase substrate detection system described under Experimental Procedures. Molecular weight markers for panel A are ^{14}C-methylated proteins with the molecular weight indicated on the left. For panels B and C, the molecular weight markers are prestained protein standards.

primary objective of this study was to determine the structure and the membrane topology of this protein and its possible relationship to 11β-HSD. Initial structural analysis of the 50-kDa protein suggested that it might be related to 11β-HSD and therefore may have catalytic properties similar to the clinically important steroid dehydrogenases (Table 1). Both proteins have the sequence Ala-Tyr-Tyr-X-Tyr near the C-terminus of their N-terminal hydrophobic segment. Repeats of Tyr residues (A-Y-P-Y-Y-A) are found in the membranous domain of the 48-kDa subunit of microsomal oligosaccharyltransferase (*17*), and the T-cell receptor delta chain displays G-Y-Y-Y-Y-V sequences in their membrane binding segment (*27*). That the tyrosine-containing motif mediates ER retention of CD3-ϵ has been reported (*28*).

The complete amino acid sequence of the 50-kDa protein is shown in Figure 4. The 50-kDa protein is most likely an orthologous form of the human protein (*24*). The key structural features such as the N-terminus, active site serine, N-glycosylation sites, and the disulfide bonds are shared by the human form. The sequence similarity with 11β-HSD is limited to the N-terminal membrane-spanning segment (Table 1). The C-termini of the 50-kDa protein and 11β-HSD are unrelated. The 50-kDa protein did not display the pyridine nucleotide binding motif, Gly-X-Gly-X-X-X-Gly, or the Tyr-X-X-X-Lys sequence commonly found in the family of so-called short-chain dehydrogenases which includes 11β-HSD (*19*). The 50-kDa protein had no corticosteroid dehydrogenase or hydrocortisone reductase activity. Interestingly, column fractions containing 50-kDa-1 and 50-kDa-3 copurified with native and partially fragmented epoxide hydrase, and the flavin monooxygenase forms, hinting the presence of protease activity. Therefore, studies with the protease inhibitors were initiated. The resulting study indicated that the 50-kDa proteins labeled with [^{3}H]diisopropylphosphofluoridate. The DFP binding site was identified as Ser-188 located in the Gly-X-Ser-X-Gly segment, which is the motif for the active site of serine proteases, esterases, and lipases. Contrary to the fractions containing the partially purified 50-kDa protein, the purified enzyme did not fragment the above-mentioned microsomal proteins. Whether the 50-kDa protein is a subunit of a dormant membrane protease remains to be determined. The 50-kDa enzyme being a "serine" esterase certainly has the structural features related to the proteases. The purified 50-kDa enzyme, however, retained esterase and *N*-deacetylase activities. The esterase and deacetylase reactivities were completely inhibited by 2 μM DFP. Nonpermeating probes such as the proteolytic enzymes in conjunction with analysis of the integrity of the microsomal vesicles have been useful tools to investigate the orientation of the ER proteins. Chymotrypsin treatment of intact microsomal membranes did not affect the mobility of the 50-kDa protein on SDS−PAGE (Figure 6). Antibodies against cytochrome b_5 and the NADH−cytochrome b_5 reductase were used to monitor the extent of proteolysis. Cytochrome b_5 and its reductase are integral membrane proteins oriented toward the cytosolic side of the ER. As shown in Figure 6B, proteolysis of microsomes in the absence of detergents resulted in conversion of the cytochrome and the reductase to their hydrophilic domains. The native cytochrome b_5 form is bound to the ER membrane by a single hydrophobic segment at the C-terminus, whereas the native cytochrome b_5 reductase is anchored to the membrane by an N-myristoylated hydrophobic segment at the N-terminus of the protein (*4*). Limited proteolysis of microsomal membranes converted the native forms of cytochrome b_5 and the reductase to their lower molecular weight derivatives (Figure 6B,C, lane 3). The lower molecular weight, hydrophilic derivative of cytochrome b_5 lacks the C-terminal membranous segment of approximately 35 residues, whereas the hydrophilic domain of the reductase

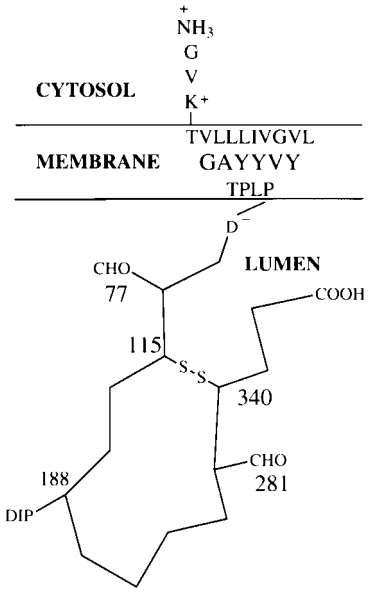

FIGURE 7: Proposed orientation of the 50-kDa protein in the membrane. CHO denotes the carbohydrate binding sites. The residue labeled by [^{3}H]diisopropylphosphofluoridate is designated by DIP.

is devoid of the N-myristoylated membrane binding domain of 28 residues. The resistance of the 50-kDa protein toward proteolysis in microsomes suggests that the domains of the 50-kDa protein reside in the lumen of the ER which is not accessible to proteolytic enzymes (Figure 6A, lane 3).

The proposed topology for the 50-kDa protein in membrane is depicted in Figure 7. This orientation is based on the identification of high-mannose carbohydrates at Asn-77 and Asn-281 and a disulfide bond between Cys-115 and Cys-340. This proposal was further confirmed by the observed resistance of the 50-kDa protein toward proteases in intact microsomes. The membrane-traversing segment consists of a stretch of hydrophobic residues between residues 3 and 23. Another hydrophobic segment is found between residues 105 and 125 (Figure 4); however, it is unlikely to traverse the membrane, since the presence of a disulfide bond and the carbohydrate moieties preclude positioning this segment in the membrane. The predicted orientation of the 50-kDa protein is essentially identical to 11β-HSD (*19*). Both proteins have a single membrane-spanning segment at the N-terminus, several high-mannose *N*-glycans, and one disulfide bond all oriented toward the lumen of the membrane.

Recent studies have demonstrated the existence of an additional microsomal 11β-HSD enzyme. This form is found in kidney (*29*) and placenta microsomes (*30*), and is denoted Type 2 11β-HSD. The Type 2 enzyme utilizes NAD^+ instead of $NADP^+$ as the cofactor and catalyzes the dehydrogenase, but not the reductase, reaction. Type 2 dehydrogenase has three hydrophobic segments at the N-terminus (*31*). It has been postulated that the three membrane segments in the Type 2 enzyme function as membrane anchors to the ER (*31*). None of the three segments are related to the membrane-spanning segment of liver microsomal 11β-HSD (Table 3). Studies on the subcellular localization of the Type 2 enzyme have provided evidence that the Type 2 dehydrogenase is bound to the microsomal membrane with a cytosolic orientation (*32*). The lack of similarity between the N-termini of the two steroid dehydrogenases may explain their different topologies.

Table 3: Characteristics of the N-Termini of 11β-HSD Type 2 and Type 1 Enzymes[a]

	1	50
Type 2	MESPWPSGGAW LLVAA RALLQ LLRADLRLGR PLLAALALLA ALDWLCQ	
	1	8
Type 1		A FMKK Y LLP
	51	96
Type 2	R L L P P L A S L A V LAATGWIVLS RLARPQRL PVATRAVLITGCDSGFG	
	9	47
Type 1	. L L GLFLAYY YYS A N E E F. ..... RPEML QGK KV IVTGAKSGIG	

[a] Type 2 is from the cDNA sequence of ovine kidney microsomal (*31*). Type 1 is rabbit liver microsomal (*19*).

Presently, the 11β-HSD and the 50-kDa proteins are the only resident type II proteins that have been identified in the liver ER. Except for the primary structure similarities at their N-termini and the overall topology, these proteins are unrelated in structure and function. The findings of this study imply that a cluster of tyrosyl residues in the uncleaved signal peptide, flanked by positively charged residues at the beginning and negatively charged residues downstream of the hydrophobic domain, may constitute a lumenal orientation signal for a specific set of ER proteins. This proposal should provide the fundamental groundwork for further experiments aimed at elucidation of the molecular basis of protein topology in the lumen of ER, and design approaches for specific targeted delivery of gene products to be anchored to the lumen of the ER.

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