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# ISOLATION AND STRUCTURAL ANALYSIS OF MICROSOMAL MEMBRANE PROTEINS

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## 1. ABSTRACT

Investigation of the endoplasmic reticulum requires a methodological background in the solubilization, purification, and structural analysis of membrane proteins. The experience of one laboratory with microsomal proteins over 30 years is summarized in this review. We focus on the isolation and structure of the major proteins of rabbit liver microsomes. The special pitfalls encountered with fragile protein complexes, hydrophobic peptides, and post-translational modifications are emphasized.

## 2. INTRODUCTION

Microsomes are defined operationally as the particulate fraction obtained from a tissue homogenate by ultracentrifugation after the nuclear and mitochondrial fractions have been removed by low speed centrifugation. Electron microscopy has shown that microsomes are composed primarily of closed sacs of membrane called vesicles. Most of the vesicles are derived from rough and smooth endoplasmic reticulum (ER). Membrane vesicles derived from the Golgi apparatus, peroxisomes, endosomes, the trans Golgi network, and other intermediate compartments comprise a minor component of microsomes. Glycogen is also present, but is readily removed. Thus, in essence, microsomes represent a preparation of intracellular membranes derived primarily from the ER. Microsomes prepared from tissues specialized for protein secretion, e.g. pancreas or hen oviduct, consist primarily of rough ER vesicles and are enriched in proteins engaged in the translocation, folding, and post-translational modification of secretory proteins. Liver microsomes contain rough and smooth ER vesicles in a roughly 2:1 ratio, and, in addition to components of the protein secretory pathway, contain a multitude of proteins involved in lipid/lipoprotein biosynthesis, and drug metabolism. The ER is by far the most abundant membrane in metabolically active cells. Some 2-3 mg of microsomal protein is obtained from liver per gram of wet tissue. As such, microsomes are an ideal preparation in which to study the relationships between enzyme structure, protein-protein and lipid-protein interactions, and the functional properties of membrane bound enzymes. Although many of the most abundant microsomal proteins have been studied extensively, many more remain to be isolated and characterized. The aim of this review is to present some of the insights gained from thirty years of working with microsomal membrane proteins in the hope that they may be of some use to others studying membrane proteins. Space did not permit a comprehensive review of all pertinent research. We apologize to the many investigators whose important contributions could only be covered by reference to reviews and discussions in other papers.

## 3. ISOLATION OF MICROSOMAL PROTEINS

The isolation of a microsomal protein is logically divided into three steps: preparation of microsomes, solubilization of microsomes, and fractionation of solubilized microsomes. Anyone facing the problem of isolating a "new" membrane protein will encounter a multitude of detergents and chromatography methods; some will work and others will be useless. Ultimately, the problem of choosing the "right" detergent and fractionation procedures must be solved experimentally. The following paragraphs detail the procedures we have used to isolate a large spectrum of microsomal proteins for structural and functional analysis. These methods have stood the test of time and are useful for microscale procedures as well as for large scale isolation of microsomal membrane proteins.

### 3.1. Preparations of microsomes

After a tissue has been selected for study, the composition of the homogenizing buffer, the method of homogenization, and the time and force of the low speed centrifugation step are the primary variables in the preparation of microsomes. The homogenizing buffer is usually isotonic and contains buffer (usually 10-100 mM Tris, HEPES, or Triethanolamine, pH 7.5-8.1), a chelating agent, and a reducing agent such as 1 mM dithiothreitol (DTT). Depending on the tissue selected and the protein or activity of interest, it may be beneficial to add magnesium (1-5 mM), and/or protease inhibitor(s) to the homogenizing buffer. The choice of technique for homogenization is determined by the nature and volume of tissue to be processed. Delicate tissues such as brain and liver are readily homogenized with Potter-Elvehjem tissue grinders. Tougher tissues, e.g. hen oviduct and lung, require the high shear produced by mechanical homogenizers (e.g. Polytron) to achieve adequate tissue disruption. A large volume of tissue is most conveniently homogenized in a Warring blender. The force of the low speed centrifugation step in reported procedures for preparing microsomes varies from 10,000 to 18,000 g. We have not studied the importance of this variable systematically, but clearly it would be expected to affect the purity and yield of ER vesicles. Microsomes are pelleted by centrifugation of the post-mitochondrial supernatant at approximately 100,000 g for 60-90 minutes. A particulate fraction of microsomes can also be obtained by calcium precipitation of the post-mitochondrial supernatant (1). Calcium precipitation eliminates the need for ultracentrifugation and is useful for processing a large volume, but may result in the degradation of some proteins by calcium activated protease.

We prepare rabbit liver microsomes by homogenizing minced tissue from two animals (combined liver weight approximately 160 g) in 800 ml 100 mM Tris acetate, pH 7.5, 100 mM KCl, 1 mM EDTA, 0.1 mM DTT in a Warring blender (2). The livers are perfused with buffer prior to homogenization to limit contamination with hemoglobin and serum proteins. It is important not to over-homogenize the suspension so as to avoid the formation of nuclear and mitochondrial fragments. Differential centrifugation steps are then applied in sequence to remove unbroken cells, nuclei, and mitochondria. Sequential centrifugation at 600 g and 10,000 g gives pellets designated the "nuclear" and "mitochondrial" fractions. Centrifugation of the postmitochondrial supernatant at 105,000 g for ninety minutes yields the microsomes in a pelleted form. The isolated microsomal fraction consists of smooth and rough microsomes, the latter having ribosomes attached on their outer surface. A density gradient sedimentation step can be used at this point to separate the rough and smooth microsomes. The microsomal pellets are suspended in homogenizing buffer containing 20% glycerol to yield a volume of microsomal suspension equal to half the original weight of the liver. The microsomal suspension is then aliquoted and stored at -80$^{O}$C in a freezer with no defrost cycle. Storing samples in "frost free" freezers will lead to loss of fragile enzyme activities as well as loss of clarity of the bands upon SDS-PAGE analysis.

How "good" is the preparation? Conventionally, this has been assessed by a series of enzyme assays on the subcellular fractions obtained. For example, glucose-6-phosphatase is considered specific for ER membranes and cytochrome oxidase is considered specific for mitochondria. However, very few laboratories have the expertise to perform these assays reliably. An alternative is to compare the SDS-PAGE profiles of the particulate and cytosolic fractions. SDS-PAGE is a rapid and powerful means for separating and visualizing complex protein mixtures. In order to cover a wide molecular mass range, we run 8% and 12% acrylamide Laemmli gels (3). In our hands, this is simpler and more reproducible than running gradient gels. The subcellular fraction is diluted with 5 volumes of SDS-PAGE sample loading buffer (60 mM Tris-acetate, pH 6.8, 3% SDS, 5% beta-mercaptoethanol and 10% glycerol) so that the protein concentration of the sample is suitable for SDS-PAGE. Inclusion of 0.1 M MgCl in the loading buffer will prevent the high viscosity problems encountered with whole cell and nuclear lysates. Although it is common practice to incubate SDS-PAGE samples at 90$^{O}$C for 1-3 minutes to assure that the proteins are completely denatured, in our experience such heating often leads to the disappearance of some protein bands from membrane preparations. Incubation of the sample with loading buffer at room temperature for 30 minutes is sufficient. Overloading the gel is another potential problem. One or two microliters of sample per well is sufficient for a ten lane 10 x 8 cm, 0.75 mm gel. Coomassie Blue is the simplest and most reliable procedure for visualizing protein bands in gels. If protein bands characteristic of the cytosolic, nuclear or mitochondrial fractions are seen in the microsomal preparation, then the fractionation procedure needs to be altered. The SDS-PAGE profile of each fraction provides a permanent record for the evaluation of the entire subcellular fractionation procedure. Figure 1 shows the SDS-PAGE of subcellular fractions obtained by differential centrifugation of liver homogenates.

### 3.2. Solubilization of microsomal membrane proteins

After microsomes have been prepared, the next step toward isolating a microsomal protein is its solubilization. Solubilizing buffers generally contain ~ 10 mM buffer pH 7.5-8.1 (usually Tris, phosphate, HEPES, or Triethanolamine), 10-20% glycerol to stabilize proteins, 1 mM EDTA to bind metal ions, and 0.1-1 mM dithiothreitol to maintain a reducing environment. The critical "ingredients" in a solubilizing buffer are salt and detergent. Peripheral proteins bound to the cytosolic surface of microsomes by ionic interactions, e.g. ribosomes and the signal recognition particle, are efficiently extracted from rough microsomes without detergent by solutions of high ionic strength (4). Soluble lumenal microsomal proteins are selectively released from microsomal vesicles by nonionic detergents below their critical micelle concentration (5). In fact, if the ionic strength of the solubilizing buffer is low, the capacity of nonionic detergents to solubilize most integral membrane proteins is very limited, even at high detergent concentrations (unpublished observation). Most integral membrane microsomal proteins require either high ionic strength (at least 100 mM KCl) or ionic detergent for efficient solubilization. This creates a dilemma, since both salt and ionic detergent interfere with ion exchange chromatography which is the most powerful general technique available for the fractionation of proteins. There are several approaches to this dilemma. The approach we have used most extensively was developed for the isolation of cytochrome P-450 isozymes (6,7) and epoxide hydrase (8). Microsomes are solubilized with sodium cholate in buffer containing 100 mM Tris buffer and 100 mM KCl. The solubilized preparation is then fractionated by stepwise polyethylene glycol precipitation. Most microsomal proteins are recovered in the 6-12% PEG fraction, and, most importantly, are soluble in nonionic detergent solutions at low ionic strength. As described in the next section, this solubilization procedure has led to the primary

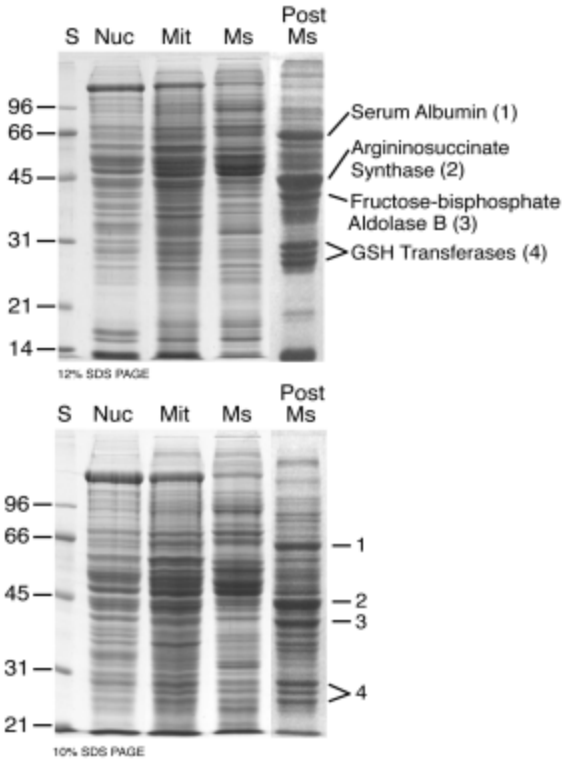


**Figure 1.** SDS-PAGE of subcellular fractions obtained by differential centrifugation of rabbit liver homogenate. Approximately 10 µl aliquot of the subcellular fraction was added to 50-100 µl of gel loading buffer. Ten-microliter aliquots of each sample were subjected to electrophoresis. Gels were stained with Coomassie blue. Molecular mass standards are shown on the left panel of the gel. The identity of some of the predominant cytosolic proteins is indicated in the right panel.

structural characterization of eight microsomal protein families. The 0-6% PEG fraction, which is difficult to solubilize, contains a unique set of proteins including stearyl-CoA desaturase, the oligosaccharyltransferase complex (ribophorin I, ribophorin II, and ost48/50), and the sec61 protein translocation complex. Special solulbilization procedures had to be developed in order to isolate these proteins. The oligosaccharyltransferase complex is solubilized with nonionic detergent in high ionic strength buffer and then transferred to low ionic strength buffer by glycerol gradient centrifugation. Another approach is to wash the microsomes with high salt buffer prior to solubilization. We have found that certain proteins that are not solubilized from native microsomes by nonionic detergent, are solubilized by the same detergent in microsomes that are first washed with high salt buffer (500 mM NaCl, 15 mM EDTA). After a desired microsomal protein or activity is solubilized, it is important to assess its stability. If an enzyme activity is not stable overnight at $4^{o}$, it will be necessary to investigate the effect of essential cofactors, phospholipids, other detergents, and protease inhibitors on the stability of the preparation. For example, oligosaccharyltransferase (OT) can be solubilized with a variety of nonionic detergents, but activity is not stable overnight unless phosphatidylcholine is added. Inclusion of manganese and magnesium, both essential cofactors of OT, in the solubilizing buffer and column buffers also slows the deterioration of OT after solubilization. The detergent used for solubilization also affects the stability of OT. The stability of OT in detergent solution is expressed by digitonin>CHAPS>>Triton X-100. Cytochrome P-450 is another relatively fragile enzyme. It is necessary to include glycerol in all buffers in order to isolate active preparations of cytochrome P-450. We include 20% glycerol in all buffers in order to isolate active preparations of cytochrome P-450. We include 20% glycerol in all buffers used to solubilize and fractionate microsomal proteins. We also include 1 mM EDTA and 0.1 mM DTT. We do not routinely include protease inhibitors in solubilizing/fractionation buffers. Most liver microsomal proteins are remarkably stable even when incubated at $37^{o}$. Exceptions to this rule are stearyl-CoA desaturase (SCD) and heat shock proteins/chaperones GRP94, GRP78(BiP). ER72, and calreticulin. These proteins are rapidly degraded by microsomal protease(s) at $37^{o}$. They are also degraded, albeit more slowly, at $4^{o}$.

### 3.3. Fractionation of solubilized microsomes

Once a desired microsomal protein/activity is solubilized and stable in low ionic strength buffer with nonionic detergent, a series of ion exchange chromatography and adsorption chromatography steps should lead to substantial purification. The questions arise: a) which column to start with, b) stepwise or gradient elution, c) what to do when the usual fractionation procedures have been employed and the protein still is not homogeneous. In regard to the first two questions, we describe a series of columns and elution procedures that have been applied to the fractionation of liver microsomal proteins since 1982 with extremely reproducible and extensively documented results. In regard to the last question, the chromatographic behavior of microsomal proteins is influenced by protein-protein interactions and may change after partial purification is achieved. It is therefore reasonable to repeat a chromatography step used earlier in the isolation procedure.

Purification of microsomal proteins is frequently monitored by determining the specific activity of column fractions. N-terminal sequence analysis may also be used to monitor the course of the protein purification. During the purification of an enzyme, the number of protein bands will decrease as the specific activity increases. Assignment of enzymatic activity to a particular protein band may be achieved by the integration of specific activity data with the SDS-PAGE profiles of active fractions. SDS-PAGE of partially purified preparations will identify protein bands whose concentration correlates with specific activity. N-terminal sequencing of proteins selected in this way will identify them as known or unknown. Difficulties are encountered when the desired protein has a blocked N-terminus, or SDS-PAGE mobility identical to that of another protein. Another difficult case is when the desired protein has a "high" enzymatic activity, but is present in such small quantities that it is not detected by SDS-PAGE.

Over some 30 years we have isolated and determined the primary structure of several dozen microsomal proteins. These include cytochromes b5 from eight mammalian species (9), NADH-cytochrome b5 reductase (10), stearoyl CoA desaturase (SCD) (11,41), epoxide hydrase (12), four isoforms of cytochrome P-450 (7,12-13), and two isoforms of esterase (14-15). More recently, this list was extended to include the lumenal glucose-6-phosphate dehydrogenase (16), three isoforms of FAD-flavin monooxygenases (17-19), the lumenal 11β-steroid dehydrogenase (20) and the 48/50 kDa subunit of oligosaccharyl transferase (OT) from 3 species (21-23). OT consists of three membranous polypeptides (24) and catalyzes the N-glycosylation of nascent proteins in the

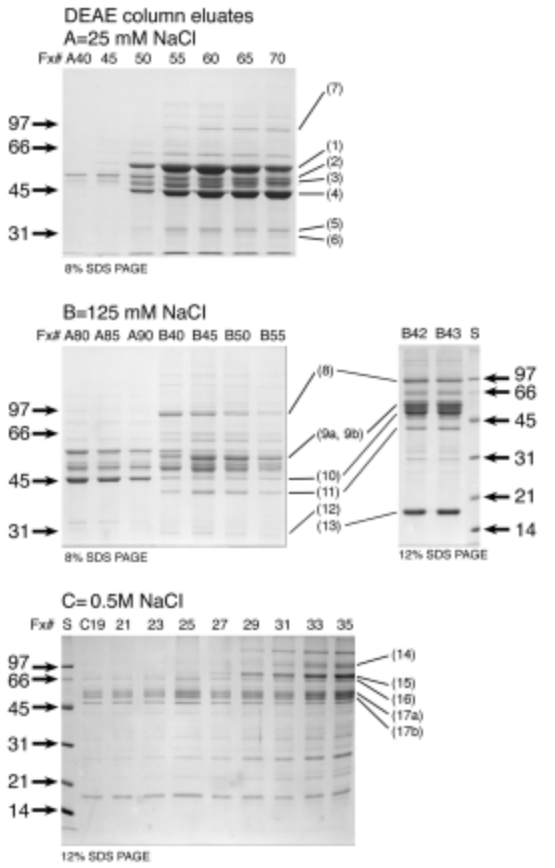


**Figure 2.** SDS-PAGE analysis of the DEAE column eluate. Rabbit liver microsomes were prepared and solubilized as described in the text. The 6-12% polyethylene glycol fraction was solubilized and applied to a 5 x 12 cm DEAE column as described in the text. The column was developed by a stepwise gradient of increasing NaCl concentration as described in the text. The 25, 125 and 500 mM NaCl eluates are designated as Racks A, B, and C, respectively. The number above each lane denotes the rack and fraction number of the sample. Proteins that have been identified are assigned a number to the right of the gel. The N-terminal sequence or a segment of internal sequence is given in table 1 for each identified protein.

endoplasmic reticulum. Except for SCD and OT, which require special methods for solubilization and purification, all the other proteins can be isolated by the following procedure.

### 3.3.1. Fractionation of solubilized microsomes by polyethylene glycol precipitation

We start with microsomes that have been suspended in 20% glycerol, 0.1 M Tris/acetate, pH 8.1, 100 mM KCl, 1 mM EDTA, and 0.1 mM DTT. Glycerol is included to stabilize the proteins. EDTA binds extraneous metal ions, and DTT provides a slightly reduced environment. The microsomes are solubilized by the dropwise addition of 10% sodium cholate to final concentration of 1.7%. To this mixture a 50% solution of polyethylene glycol (PEG) is added dropwise with stirring. The fractions precipitating from 0 to 6% and 6 to 12% PEG are collected by low speed centrifugation. The 6 to 12% PEG fraction is solubilized by the addition of 10% Tergitol NP-10 to a final concentration of 0.5% or 10% Triton X-100 to a final concentration of 2%. The mixture is stirred overnight, since solubilization of this fraction occurs slowly. The 6 to 12% PEG fraction is very sticky and viscous, and is therefore solubilized in the centrifugation bottle in which the precipitate was obtained. Cholate is used as the initial detergent because it solubilizes more proteins than nonionic detergents. PEG precipitation removes a spectrum of insoluble proteins in the 0-6% fraction and permits the exchange of cholate for a nonionic detergent prior to the DEAE-column step. For optimal results, microsomes should be solubilized and fractionated by PEG precipitation immediately after isolation.

### 3.3.2. Isolation of microsomal proteins in the 6-12% PEG fraction

A few days before the isolation of microsomes, a DEAE-cellulose column is poured and equilibrated with 5 mM potassium phosphate, pH 7.4, containing 20% glycerol, 0.5% NP-10, 1 mM EDTA, 0.1 mM DTT (Buffer A). The ion-exchanger (Whatman DE-52) is prepared according to manufacturers directions. The size of the column depends on the quantity of protein to be fractionated. For the quantity of microsomes obtained from two 2 kg. rabbits, we use a 5 x 12 cm column. The clear red solution obtained from solubilization of the 6 to 12% PEG precipitate is applied to the DEAE-cellulose column. The column is developed with the equilibration buffer, and 50 ml fractions are collected until the A415 of the eluate drops to about 0.160. These fractions constitute the void volume of the DEAE column. Adsorbed proteins are then eluted with Buffer A containing 10 mM potassium phosphate followed by a stepwise gradient of increasing concentrations of NaCl. We use 25, 125, and 500 mM NaCl steps and collect 6 ml fractions. The effluent fractions from the 25, 125 and 500 mM NaCl are designated as Racks A, B, and C, respectively. Rack A contains about 100 fractions, Rack B about 70 fractions and Rack C about 80 fractions. Figure 2 and table 1 indicate the proteins present in these fractions. Esterase 1, epoxide hydrase UDP-gal transferase and the NADH-cytochrome b5 reductase elute in Rack A fractions. The predominant proteins of Rack B are cytochrome b5, microsomal paraoxanase, microsomal lipid transfer protein, chaperone proteins PDI, ERp57, and several cyt-P450 isoforms. Rack C contains strongly adsorbed proteins, which include the ER heat shock proteins GRP94, GRP78, GRP72, PDI and calreticulin.

The void volume of the DEAE column is applied to a CM-Sepharose column equilibrated with 10 mM potassium phosphate, 20% glycerol, 0.2% Nonidet P-40. The column is eluted by a continuous gradient of increasing phosphate concentration (10 to 300 mM). Proteins retained on the CM column include the flavin monooxygenase form 1 (FMO 1), several cytochrome P450 isoforms, 11beta-hydroxysteroid dehydrogenase, and the cyclosporin binding protein (figure 3A, table 2). When stearoyl CoA desaturase (SCD) induced microsomes are solubilized by this procedure, SCD is found in these fractions.

Proteins not retained on the CM-Sepharose column are applied to a hydroxyapatite (HAP) column (100 ml), equilibrated with 10 mM potassium phosphate, pH 7.4, containing 0.1% N-P 40, 20% glycerol, 1 mM EDTA, and 0.1 mM DTT. The HAP column is initially developed with the equilibration buffer. Two step increases in the phosphate concentration, 125 mM at fraction 10, and 300 mM at fraction 20 yield a spectrum of proteins not present in DEAE or CM-Sepharose column eluates (figure 3B and table 2). The 90-kDa band in the 125 mM phosphate eluate is the lumenal glucose-6-

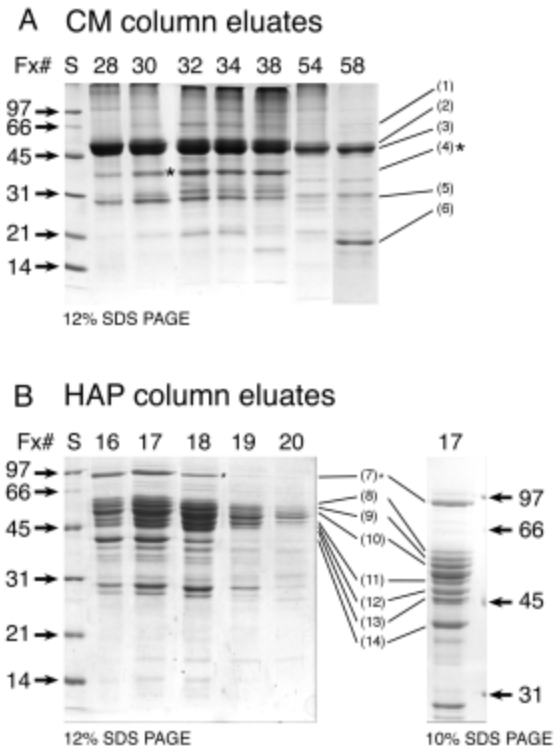


**Figure 3. A**, SDS-PAGE analysis of the CM-Sepharose column eluate. The unretained material from the DEAE column was applied to a 100 ml CM column and developed with a linear gradient of potassium phosphate (10-300 mM) as described in the text. B, SDS-PAGE analysis of the hydroxyapatite (HAP) column eluate. The unretained material from the CM-Sepharose column was applied to a 100 ml HAP column. Fractions 16 to 20 represent the 125 mM potassium phosphate eluate. Proteins that have been identified are assigned a number to the right of the gel. The N-terminal sequence of each identified protein is given in table 2.

**Table 1.** Identification of rabbit liver microsomal (Ms) proteins in DEAE column eluates.

| Band Fig. 2 | Approx k-Da | Sequence | Protein | Position in Sequence |
|---|---|---|---|---|
| 1 | 60 | HPSAPPVVDTVKGKV | Esterase 1 | N-terminal |
| 2 | 52 | GKVLVWPMDFS | UDP-Gal Transferase | N-t |
| 3 | 50 | MDPVVVLVLGLCCLL | Cyt. P-450 | N-t |
| 4 | 47 | MLLELLLASVLGFVI | Epoxide Hydrase (EH) | N-t |
| 5 | 33 | *myGAQLSTLGHVVLTPL | NADH-Cyt. b5 reductase | N-t |
| 6 | 30 | PKSHGLSDEHIFEVIC | EH fragment | res. 163- ? |
| 7 | 100 | MLLELLLASVLGFVI | EH dimer | N-t |
| 8 | 97 | VKLGSTGLSLNNDRL | Ms Lipid Transfer Prot. | N-t |
| 9a | 57 | SDVLELTPDNFE | Erp57 | N-t |
| 9b | 57 | DAPEEEDNVLVLKSS | PDI | N-t |
| 10 | 50 | MDLLIILGICLSCVV | Cyt. P450 | N-t |
| 11 | 43 | LLLTLLGASLAFVGE | Ms paraoxanase | N-t |
| 12 | 31 | LHTKGALPLDTVTFY | 31-KDa (KEEL) Protein | N-t |
| 13 | 17 | *acAAQSDKDVKYYTLEE | Cytochrome b5 | N-t |
| 14 | 94 | KEKQDKIYFM | GRP94 | res. 511- CNBr pep |
| 15 | 78 | EKAVEERIEWLESH | GRP78 | res. 577- CNBr pep |
| 16 | 72 | EPEEFDSDVLREFVT | ERp72 | res. 465- CNBr pep |
| 17 | 57 | DAPEEEDNVLVLKSS | PDI | N-t |
| 18 | 48 | EPVVYFKEQFLDGDG | Calreticulin | N-t |

*ac=acetyl; my=myristoyl

phosphate dehydrogenase. Other proteins eluting in the 125 mM potassium eluate include three flavin-monooxygenases, a variant form of esterase 1, two uncharacterized cytochromes P-450, and esterase 3. Upon elution of the column with 300 mM potassium phosphate the predominant form of FMO2 and a variant form of 11 beta-hydroxysteroid dehydrogenase are eluted. The void volume of the HAP column contains several poorly characterized proteins including a steroid dehydrogenase and a FMO isoform. Pure preparations of the proteins eluted from these three columns can be obtained by subjecting specific enriched fractions to another DEAE-cellulose column equilibrated with 10 mM Tris/acetate pH 8.1, containing 20% glycerol, 2% Triton X-100.

### 3.3.3. Purification of esterase 2, stearyl Co-A desaturase, and oligosaccharyl transferase

The 12% PEG supernatant contains esterase 2 and calreticulin in addition to some ten unidentified protein bands. Partial removal of PEG from the supernatant can be accomplished by extensive dialysis. The dialyzed preparation is then subjected to HAP column chromatography. Most of the PEG elutes in the void volume of the column. The isolation of esterase 2 from the 12% PEG fraction has been described in detail (18). The isolation of stearoyl CoA desaturase (SCD) utilizes successive detergent extraction of microsomes with increasing concentrations of deoxycholate to remove other microsomal proteins. SCD is solubilized by extraction with Triton X-100-calcium-deoxycholate (1). A DEAE-column chromatography step is used to remove the final impurities. Oligosaccharyl transferase is solubilized from salt washed microsomes with 2.5% digitonin in buffer containing 500 mM NaCl. The solubilized OT complex is transferred to low ionic strength buffer and substantially purified by glycerol gradient centrifugation (21,24). Further purification is achieved by a series of alternating ion exchange chromatography steps and gel filtration steps. While gel filtration generally has limited usefulness in the isolation of microsomal proteins, it is useful for OT because the enzyme behaves as a 200 kDa protein complex, and there are few microsomal proteins in that molecular weight range. There is synergy between gel filtration and ion exchange chromatography when they are alternated, because one method prepares the sample for the other. Gel filtration requires a highly concentrated sample, which is produced by stepwise elution from ion exchange columns. Ion exchange requires the sample to be in low ionic strength buffer, which is readily achieved by gel filtration.

## 4. STRUCTURE ANALYSIS OF MICROSOMAL PROTEINS

The analytical procedure that provides the most information about a protein from the least amount of material is N-terminal amino acid sequence analysis. The amino acid sequence of ten or so N-terminal residues provides enough information to search data banks and determine whether a protein has been previously cloned or sequenced. However, the N-terminal amino acid sequence of a protein is frequently not enough information to identify it or exclude it as a member of a protein family. For example, the structural relatedness of different cytochrome P450 isozymes is not apparent from a comparison of their N-terminal sequences and only became apparent when internal peptides were isolated and sequenced (25). Conversely functionally unrelated proteins may bear homology in their N-terminal segments because they contain uncleaved signal peptides.

The N-terminus of most ER proteins is blocked as a result of posttranslational modification, precluding N-

**Table 2.** Identification of rabbit liver microsomal (Ms) proteins in CM and HAP column elu

| Band Fig. 3 | Approx k-Da | Sequence | Protein | Position in Sequence |
|---|---|---|---|---|
| 1 | 60 | *acAKRVAIVGAGVSGLA | Flavin-monooxygenase1(FMO) | N-terminal |
| 2 | 54 | MDLIFSLETWVLLAA | CYP2C3 | N-t |
| 3 | 52 | MEFSLLLLLAFLAGL | CYP2B4 | N-t |
| 4 | 38 | AFMKKYLLPLLGLFL | 11-beta-Hydroxysteroid Dehydrogenase | N-t |
| 5 | 31 | MDALVHLLQLLVLLL | Class 1 MHC like protein | N-t |
| 6 | 19 | DEKKKGPKVTVK | Cyclosporin-binding prot | N-t |
| 7 | 96 | *pyQELQGHVSVILLGAT | G-6-P-Dehydrogenase | N-t |
| 8 | 60 | HPSAPPVVDTVKGKV | Esterase 1 | N-t |
| 9 | 59 | GKRVAVIGAGASGLA | FMO 3 | N-t |
| 10 | 58 | GKKVAIIGAGISGLA | FMO 2 | N-t |
| 11 | 56 | MDLFIVLVICLSCL | Cyt. P450x | N-t |
| 12 | 48 | GVKTVLLLIVGVLGA | Esterase 3 | N-t |
| 13 | 47 | APVEGKKAKRGILER | 47 KDa protein | N-t |
| 14 | 40 | *acAHRFPALTPEQKKEL | Ms Fructose Aldolase | N-t |

*ac=acetyl; py=pyroglutamyl

terminal sequence analysis. Sequence information in such cases can be obtained only by fragmentation of the protein, followed by isolation and sequence analysis of the peptides. This approach requires a larger quantity of material than that needed for N-terminal sequence analysis. In addition to obtaining amino acid sequence information on proteins that have a blocked N-terminus, internal amino acid sequence analysis is used to 1) identify the nature and the site of posttranslational modifications, 2) identify the site of a group specific reagent, or affinity label, and 3) determine the complete amino acid sequence of a protein.

Methods for determining N-terminal and internal amino acid sequences of membrane proteins are described in sections 4.1 and 4.2. The structure analysis of post-translational modifications is described in section 4.3.

### 4.1. N-terminal amino acid sequence analysis

Proteins can be sequenced directly or from transblots. In either case, the N-terminal sequence of a protein is determined by automated Edman degradation. The chemistry of Edman degradation, which has been reviewed in detail elsewhere (26), requires the protein sample to be free of detergents and non-volatile buffers and stabilizers. Direct sequence analysis requires that a protein be purified to homogeneity or near homogeneity or at least that the protein of interest is the predominant species in the preparation with an open N-terminus. For direct sequencing the procedure we use to remove detergents and salts is precipitation with trichloroacetic acid (TCA) followed by washing the precipitated protein several times with 20 vol. of cold acetone containing 0.2% (v/v) HCl (27). The washing step is essential to completely remove TCA and other salts. The precipitated protein is then transferred to the automated sequencer cartridge by using a small amount of 88% formic acid. If a protein preparation is not sufficiently purified for direct sequencing, the simplest way to obtain N-terminal sequence information is to resolve the protein mixture by SDS-PAGE, and transfer the separated proteins electrophoretically to a polyvinylidene difluoride (PVDF) membrane. The protein bands trapped on the membrane surface are visualized with a charged dye such as Coomassie Blue. The areas of membrane containing the band of interest are excised, and the membrane strips, usually 8 pieces from a transblotted ten lane minigel (7 x 10 cm), containing 0.5 to 2 g of protein per band are placed directly into the sequencer cartridge. PVDF membrane, unlike nitrocellulose or nylon, withstands the harsh chemistry of Edman degradation (28). Prior to loading the membrane strips into the sequencer cartridge, they are rinsed with methanol and then with water to remove dye and any residual detergent or salt. Proteins are not removed from the membrane by this treatment.

The number of residues identified by N-terminal sequencing depends on the efficiency of the sequencing reaction, which is measured by the repetitive yield (RY). The most important factors affecting the RY are: a) the amount of protein loaded, b) the overall hydrophobicity of the protein, c) the length of the protein, and d) the overall function of the sequencer and the skills of the operator. Hydrophobic proteins are less efficiently transblotted than polar proteins, and are extracted from the sequencer cartridge by the organic solvents used in sequencing reaction. Sequencing proteins above 70-kDa may not provide clear N-terminal sequence data because of high background. Internal peptide bonds are broken during Edman degradation, and since large proteins have many more potential internal cleavage sites, the background rises rapidly as residues are released from newly generated N-termini. After unfolding and denaturation, the peptide bonds of large proteins may be more fragile than those in peptides or small proteins. Despite these limitations, sequencing a transblotted protein band can usually identify 10 to 15 N-terminal residues. The first residue is often the most difficult to identify, since there is no previous cycle for comparison, and because impurities in transblotted samples give false signals for glycine, serine and threonine.

### 4.2. Internal amino acid sequence analysis

Because detergents obscure the elution of peptides from HPLC columns, and are incompatible with automated Edman degradation, they must be removed prior to protein fragmentation. If the protein has been purified to homogeneity, one may precipitate the protein with TCA and wash the protein precipitate with cold HCl/acetone as described in section 4.1. The protein is then dissolved in 88% formic acid or 8 M urea depending on the type of cleavage reaction planned. In an alternative procedure, used to prepare milligram quantities of cytochrome P-450 for digestion, solid guanidine hydrochloride (GNCl) is added to the sample to bring the GNCl concentration to 6 M, cysteine residues are carboxymethylated, lysine residues are succinylated, and the preparation is dialyzed (29). Cytochrome P-450 preparations treated by this procedure precipitate upon dialysis and can be collected relatively free of detergent by centrifugation. Assuming the protein has not been purified to homogeneity, it is possible to obtain a sufficient quantity of protein for digestion and peptide isolation from a preparative SDS-PAGE gel (14 x 16 cm, 1.5 mm). The appropriate band is cut out from the gel, and the protein is eluted from the gel in solution by electroelution, using commercially available elution devices. After elution, the protein is precipitated by TCA to remove SDS and the salts employed in the elution step. The precipitates are then washed with cold acid/acetone as described above to remove TCA. The electroelution procedure provides a 10 to 20 fold greater quantity of protein than electroblotting. Although some laboratories are able to elute sufficient protein from PVDF membranes for fragmentation and peptide isolation, direct electroelution of proteins from SDS-PAGE gels has been a superior procedure in our laboratory. Internal peptides are isolated from chemical or enzymatic digests of the electroeluted protein by gel filtration and HPLC as described in section 4.2.2. Electroeluted protein preparations (ELP) have certain advantages over native protein solutions. ELP are free from detergents and other materials that may interfere

with cleavage and the subsequent peptide isolation methodology. ELP are already denatured which is essential for effective enzymatic cleavage of membrane proteins. One limitation of the current electroelution methodology is that the recovery of high molecular weight proteins (> 150,000-kDa) is poor.

In our experience, most peptides obtained from membrane proteins sequence far better than the parent proteins. Membrane proteins usually contain polar domains as well as membrane spanning segments, and frequently polar domains predominate. Peptides with hydrophobic C-termini usually do not sequence well after the polar residues are removed. This was first illustrated during sequence analysis of the C-terminal membranous segment of cytochrome b5 (30). If the entire peptide is hydrophobic then sequencing will terminate after few cycles, no matter how much material is applied to the sequencer. For example, the C-terminal 17 residues of the flavin monooxygenases (FMO's) are devoid of polar amino acids. Sequence analysis of C-terminal peptides of the FMO's could be achieved only by covalently linking the peptide to a solid support via the C-terminal carboxyl group and then applying some 40 cycles of solid-phase sequencing methodology (19). On the other hand, if a polar segment follows the hydrophobic segment of a peptide, the peptide may sequence down to the last residue. The presence of even a single polar residue at the very C-terminus of a peptide may prevent wash out during liquid/gas phase automated Edman degradation.

#### 4.2.1. Preparation of chemical and enzymatic digests

Of the many methods available for cleavage of proteins, cyanogen bromide (CNBr) is perhaps the best method for initial cleavage of membrane proteins. CNBr cleaves proteins at the C-terminus of methionyl residues. Since the average number of methionyl residues in proteins is low, CNBr usually generates a few large fragments. The reaction is performed in 70% formic acid, which is an excellent solvent for membrane proteins. With few exceptions, cleavage is quantitative in 10-12 hours. Some Met-Thr bonds are not cleaved, and oxidation of methiones prevents cleavage. CNBr and formic acid are volatile and can be readily removed. For initial enzymatic cleavage, the endoproteases Lys-C, Glu-C and trypsin are the most useful. These commercially available proteases selectively cleave proteins at the C-terminus of lysyl residues, glutamyl residues, and both lysyl and arginyl residues, respectively. Native proteins are poor substrates for proteases. Therefore, proteins have to be denatured prior to enzymatic cleavage. This is accomplished by dissolving or suspending the protein in 8 M urea. The urea concentration is then reduced to 2 to 3 M immediately before the protease is added. Many of the other proteases that are available, e.g. chymotrypsin and pepsin, do not give complete cleavage of proteins, or yield too many fragments. Digests containing too many peptides or partially cleaved peptide fragments are difficult to resolve. After obtaining N-terminal sequence data on the peptides obtained from the primary cleavage, large peptide fragments are subcleaved into smaller fragments and the isolation and sequencing steps are repeated. In addition to the primary cleavage reactions described above, a variety of other methods are suitable for subcleavage of peptides. Chemically, peptides can be fragmented at tryptophanyl residues by the use of excess of CNBr (31). Asp-Pro bonds can be cleaved by 88% formic acid at $37^{o}$. For enzymatic cleavage, Asp-N protease, which cleaves at the N-terminus of Asp residues, endoprotease Arg-C, chymotrypsin, and other proteases may be used. For the subcleavage of very hydrophobic peptides, the protease of choice is pepsin. Membranous peptides can be solubilized in a small volume of neat formic acid, which can then be diluted to a concentration compatible with pepsin activity. Because formic acid is volatile, these digests can be readily concentrated and subjected to HPLC. The ability to concentrate the digest is essential, since the sample size for most HPLC systems is limited. Injection of samples with a high salt concentration frequently results in the formation of precipitates in the micro-bore tubing of the HPLC plumbing.

#### 4.2.2. Isolation and structure analysis of peptides

The complex peptide mixtures that result from chemical or enzymatic cleavage of large microsomal proteins can be resolved by High Pressure Liquid Chromatography (HPLC) using micro-bore steel columns containing alkylated silica (32). The latter material is noncompressable as compared with conventional column material, and withstands high flow rates and pressures, parameters that are key determinants of effective chromatographic separation of peptides. A peptide digest is applied to an alkylated silica column and developed first with an aqueous solvent of low ionic strength and then with increasing concentrations of an organic solvent such as acetonitrile. The effluent is monitored at 214- and 280 mu with an in line UV detector. In such systems, polar peptides elute first, followed by large peptides, and finally hydrophobic peptides. The plumbing of HPLC is constructed of micro-bore stainless steel tubing, and the flowcells of the detector have volumes of 1-2 µl to limit sample dilution. Sophisticated solvent delivery systems ensure reproducible elution profiles. HPLC effectively resolves peptides in membrane protein digests ranging from a few residues to fifty residues, on a nanogram scale. Because the salts and solvents employed in the HPLC systems are volatile, peptides obtained by HPLC can be sequenced directly without further desalting steps. Dual wavelength monitoring of the effluent fractions gives the shape of the peptide peak and information regarding the amino acid composition of the peptide. Asymmetric peaks suggest the presence of several peptides. UV absorbance at 280 nm indicates the presence of aromatic amino acid residues. The effectiveness of HPLC resolution is dramatically increased by gel filtration of the digest (29,33). A column of Sephadex LH-60 equilibrated with 88% formic acid/ethanol (3:7 (v/v)) will resolve peptides up to 140 residues in length according to size. The organic solvent used as mobile phase in the LH-60 gel filtration step essentially eliminates peptide-peptide interactions. The gel filtration step can be carried out on samples containing as little as 15 µg of protein. Detergents on this column behave as monomers rather than high molecular weight micelles. HPLC of individual gel filtration fractions yields many more peptides in pure form as compared to results obtained by direct injection of the digest for HPLC. This is very important because, in most laboratories, automated Edman degradation is the only rapid method for determining the purity of a peptide. Trying to determine whether a peptide is sufficiently pure for sequencing by repeating HPLC under different solvent or gradient conditions is time consuming and frequently associated with severe loss of material.

Some very hydrophobic peptides precipitate in the LH-60 column mobile phase and are lost on the column. To prevent this, protein digests are allowed to stand in the LH-60 column solvent overnight. Any precipitated material is removed from the sample by centrifugation prior to loading on the gel filtration column and examined for peptide material. The observation that some peptides precipitate in the LH-60 column solvent was made during sequence analysis of FMO form 3 (19). Methods used to isolate the hydrophobic C-terminal peptides of FMO1 and FMO2 failed to identify the C-terminal peptide of FMO3.

The C-terminus of FMO3 was ultimately isolated by differential organic solvent extraction. The CNBr digest of FMO3 was extracted first with 0.2% aqueous trifluoroacetic acid, followed by formic acid/ethanol (30/70), and finally with 75% formic acid. The first two solvents extracted the polar and the ordinary hydrophobic peptides of the digest. The C-terminal peptide was recovered in the 75% formic acid extract. It was then purified by passage through a short LH-60 column equilibrated with 75% formic acid. Solid-phase sequencing identified the amino acid order as S-S-N-S-V-T-M-M-T-M-G-K-F-M-L-A-I-A-V-V-Y-FG. This sequence differs from the C-termini of FMO1 and 2 by the absence of polar residues at positions 6 and 10. These substitutions drastically altered the solubility of the form 3 peptide relative to its counterparts in form1 and form2 FMO's.

Recently, a methodology has been introduced that compliments gel filtration and HPLC. Matrix assisted laser deadsorption ionization time-of-flight mass spectroscopy (MALDI/TOF) provides information on the number of peptide components present in a particular peak, as well as their exact molecular size (34,35). MALDI works by bombarding samples embedded on an organic matrix layer with an ultraviolet laser pulse, which vaporizes the sample and brings it into the gas phase. The time-of-flight mass analyzer, which accompanies MALDI, resolves matrix-peptide complexes up to 20-kDa. MALDI/TOF when properly applied permits the evaluation of complex peptide mixtures and often provides a semiquantitative overview. The exact masses of the peptide fragments permit the confirmation of deduced primary structures and/or the identification of possible post-translational modifications.

### 4.3. Structural analysis of post-translational modifications

Membrane proteins may have chemical groups incorporated that have important effects on their biological properties. The predominant modifications found in microsomal proteins include N-terminal modification, glycosylation, and disulfide bond formation. Modifications characteristic of plasma membrane proteins including prenylation, attachment of saturated fatty acids via thio ester linkage, and attachment of phosphatidylinositol at the C-terminus have not been found in microsomal proteins. Only one example of N-terminal fatty acylation has been identified. Post-translational modifications are frequently discovered during peptide isolation and sequence analysis. Anomalous behavior of a peptide on HPLC is an indication that the peptide may be modified. Failure to identify a phenylthiohydantoin (PTH) residue at one cycle during automated Edman degradation is another sign of a modified amino acid. These clues must be followed up with different methodologies. It should be noted that in addition to genuine post-translational modifications, blocked peptides arise from cyclization of N-glutaminyl residues at the N-termini of peptides released by proteases or chemical methods.

#### 4.3.1. Identification of N-terminal blocking groups

Acetyl groups and pyrrolidone rings (pyroglutamic acid) are the most common blocking groups at the N-termini of microsomal membrane proteins. The N-terminus of NADH-cytochrome b5 reductase is myristoylated (36). Thus far b5 reductase is the only microsomal protein covalently modified by a fatty acid. The identification and removal of acyl groups requires the isolation of the corresponding peptide. The behavior of the N-acylated peptide on HPLC provides a good indication whether the blocking group is an acetyl or a fatty acyl residue. N-acetylation of peptides does not significantly alter the elution pattern of the peptide. In contrast, fatty acid acylated peptides display an unusually long retention time on HPLC, and are isolated in poor yield or may be irreversibly bound to the reverse-phase columns.

After isolation, the N-acylated peptide is subjected to partial acid hydrolysis. The digest is characterized by HPLC and the N-acylamino acid identified by mass-spectroscopy. The experiments that led to the identification of myristate as the blocking group at the N-terminus of NADH-cytochrome b5 reductase are illustrative (36). Initially, the results of N terminal sequence analysis of cytochrome b5 reductase were difficult to interpret. After few cycles of automated Edman degradation, several residues appeared at each cycle suggesting that the preparation contained several proteins even though it appeared pure by SDS-PAGE. We now know that b5 reductase is a fragile protein; internal fragmentation occurs when the intact reductase is subjected to automated Edman degradation. Gel filtration and HPLC of a CNBr digest identified a 20 to 30 residue very hydrophobic peptide with a blocked N-terminus. This blocked peptide was refractory to further enzymatic digestion. The presence of a tryptophanyl residue was inferred from the peptides 280 mu absorbance, and specific cleavage at this residue was performed. A short polar peptide was recovered from this digest, but the remainder of the peptide could not be recovered. The removal of few polar residues from the C-terminus of the CNBr peptide had generated a peptide that was irreversibly bound to reverse phase HPLC columns. Subcleavage of the N-terminal CNBr peptide of b5 reductase was accomplished with pepsin in formic acid. HPLC of the pepsin digest yielded several fragments including peptide material eluting at the end of the HPLC elution gradient. Amino acid analysis of one late peak indicated a tripeptide with a blocked N-terminus. These results suggested that the blocking group was responsible for the hydrophobicity of the N-terminal peptide. The structure of the blocking group was determined as myristic acid by combined gas-chromatography/mass spectroscopy (36). N-acetyl groups can be removed by partial acid hydrolysis (17). The acetylated peptide from FMO1 was unblocked by heating for 15 min with 1.2 M HCl at 107$^{o}$.

Enzymatic removal of N-acetyl groups is difficult. The acylamino acid releasing enzyme will remove this block only from short peptides and its substrate specificity is confined to N-Gly, -Ala, and -Ser (37). In contrast, pyroglutamate aminopeptidase will remove the N-terminal pyroglutamyl residue from intact proteins or peptides. If N-terminal pyroglutamate is suspected, then microquantities of the protein are incubated with pyroglutamate aminopeptidase and the digest subjected to SDS-PAGE. Sequence analysis of the transblotted band effectively identifies the amino acid sequence of the residues following the blocking group. We used this enzyme to free the N-terminus of the 60-kDa esterase form 2, and the 90-kDa glucose-6-phosphate dehydrogenase (15,16).

The function of blocking groups is unknown. Studies with non-myristoylated forms of b5 reductase suggest that the myristoyl group not only facilitates binding of reductase to the ER membrane, but also provides a specific orientation in the membrane (42). The tilting of reductase into an oblique orientation is apparently necessary for optimal electron transfer to cytochrome b5.

#### 4.3.2. Glycosylation

The covalent attachment of carbohydrate to the amide nitrogen of asparaginyl residues (N-linked glycosylation) is a major biosynthetic function of the ER

(38). Microsomal proteins with lumenal domains often contain N-glycan. O-linked glycosylation occurs in the Golgi, and O-linked carbohydrates are not found in microsomal proteins.

N-glycosylation is initiated in the ER by oligosaccharyltransferase (OT), a heterotrimeric ER protein complex, which catalyzes the transfer of a preformed 14 residue oligosaccharide from dolichol pyrophosphate to asparaginyl (Asn) residues of nacent polypeptides. The Asn must be in an Asn-X-Ser/Thr triplet where X is any amino acid except proline. The initial N-glycosylation step is followed by the rapid removal of three glucose residues from the N-glycan giving the "high mannose" derivative. Further modification of the oligosaccharide chain occurs as the glycoprotein is transported through the Golgi apparatus, resulting in the formation of complex and hybrid N-glycans. Upon sequence analysis of a peptide containing an N-glycan, a "blank" residue will be observed at the cycle corresponding to Asn, followed by PTH-Ser or PTH-Thr at cycle N + 2. The PTH derivative of asparaginyl-linked glycans is poorly soluble in the solvent system used to extract and deliver the sequencer product to the PTH analysis system and, therefore, a blank is observed at the N-glycan site. The presence of an N-glycan is confirmed by deglycosylation of the parent protein by specific N-glycosidases (39). The deglycosylation reaction can be conveniently performed on protein preparations obtained by electroelution. Upon SDS-PAGE, the deglycosylated protein characteristically exhibits faster migration than the parent protein. This increased migration can even be observed in high molecular weight (~ 100-kDa) N-glycosylated proteins having as few as 1 or 2 N-glycans. Because of the specific orientation of OT, N-glycosylation occurs only in the ER lumen. Therefore, identification of an N-glycan provides valuable information about the membrane orientation of a microsomal protein. N-glycosylation of cytosolic proteins or microsomal proteins with a cytosolic orientation has not been described.

Deglycosylation with specific enzymes that distinguish between high mannose and complex or hybrid type N-glycans provides information regarding the subcellular trafficking of a microsomal protein. The presence of complex or hybrid type N-glycans indicates exposure to Golgi enzymes. The specific deglycosidases available commercially are usually free from proteases.

### 4.3.3. Disulfide bonds

Translocation of proteins across the ER membrane into the lumen is initiated by recognition of N-terminal signal peptides. Signal peptide cleavage and N-glycosylation occur during translocation, a unique time when the polypeptide is in an unfolded state. Protein folding and disulfide (S-S) bond formation occur co-translationally and post-translationally in the lumen of ER. Resident proteins of the ER lumen presumably catalyze these processes. One resident protein thought to be involved in the formation of S-S bonds is protein disulfide isomerase (PDI). The ER chaperone proteins calreticulin, GRP94, ER 72 and BiP are believed to be involved in the processes of protein folding and assembly. The specific functions of ER lumenal proteins in protein folding, disulfide bond formation, and assembly of membrane protein complexes have not been elucidated. Most cysteine thiols are fairly unreactive at a physiological pH, but under the influence of chaperones their pKa's can drop to below pH 5 as compared to a pKa of 8.7 for free cysteine. Once formed, disulfide bonds are stable even in a mildly reducing environment. Although PDI has been implicated in the formation of S-S bonds, the extent to which PDI is involved is still a matter of debate. Indirect evidence implies that the lumen of the ER is more oxidative than cytosol. The ratio of oxidized to reduced glutathione (1:3) in the ER lumen is close to the ratio that is optimal for reoxidation of fully reduced and unfolded proteins in vitro. How the lumen maintains the proper oxidative environment is not known. Lumenal glucose-6-phosphate dehydrogenase may play a role in regulating oxidative processes. Disulfide bonds are found in resident proteins of the ER lumen and in segments of membrane proteins that are oriented toward the lumen of ER. Disulfide bonds have not been found in membrane spanning peptides or in segments of microsomal proteins with a cytosolic orientation. Thus, identification of disulfide bond(s) in a membrane protein provides structural information that has to be accounted for in the assignment of a specific topology. The determination of free cysteinyl residues and the assignment of disulfide bonds in membrane proteins can be readily performed on a micro scale (40).

## 5. PERSPECTIVE

Much has been accomplished, and yet many aspects of ER structure and function are unresolved. What has been done? The primary structure and topology of most major liver microsomal proteins has been defined. The major proteins with a cytosolic orientation are the drug metabolizing enzymes and the electron transport system that desaturates stearyl Co-A thereby regulating membrane fluidity. The predominant proteins with a lumenal localization are glucose 6 phosphate dehydrogenase, the esterases, heat shock proteins, protein disulfide isomerase, and calreticulin. Proteins engaged in the translocation and post-translational modification of secretory proteins, e.g. the Sec61p complex, signal peptidase and oligosaccharyl transferase, while critically important from a functional point of view, are relatively minor ER components by quantitative standards. What challenges remain? Currently, the major lumenal proteins of the ER are thought to function as chaperones during the folding and assembly of membrane proteins and secretory proteins. But there are too many chaperone "hands" reaching for nascent polypeptides as they emerge from the translocon and enter the ER lumen. A major challenge for the future will be to provide a more exact description of the activities of the ER lumen. Another field of research that will undoubtedly attract considerable attention in the future is the ER protein degradation system. ER membrane proteins with short half lives, e.g. stearyl Co-A desaturase and HMG Co-A reductase, are selectively degraded in the ER. Proteins that fail to fold correctly or assemble into requisite complexes are also degraded in the ER. In short, protein degradation in the ER serves at least two functions: metabolic control and quality control. The proteosome, a large cytosolic protein complex, has recently emerged as a "universal" protein degradation machine, and has been implicated in the degradation of misfolded ER proteins. If this view is correct, the mechanism(s) by which misfolded proteins are recognized, selected, and transported back across the membrane into the cytosol for degradation remain to be determined. And how are the transmembrane segments degraded? Do proteases operate in the hydrophobic interior of the membrane? Or, are nonpolar peptides chaperoned into the proteosome? Is the role of selective ER proteases limited to metabolic control? The answers to these and other questions in the field of ER protein degradation appear to be on the horizon.

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