Evidence for Complex Formation between Rabbit Lung Flavin-Containing Monooxygenase and Calreticulin†

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ABSTRACT: Rabbit lung flavin-containing monooxygenase (FMO, EC 1.14.13.8) was denatured, reduced, carboxymethylated, digested with endoproteinase Glu-C or trypsin, and subjected to mass spectrometric analysis. The amino acid sequences of selected peptides were determined by tandem mass spectrometry. Over 90% of rabbit lung FMO was mapped by liquid secondary ion mass spectrometry (LSIMS). The FMO N-terminal amino acid was found to be N-acetylated, and the N-terminal 23 amino acid peptide contained an FAD binding domain consisting of Gly-X-Gly-X-X-Gly. Another peptide was found to contain a $NADP^+$ binding domain consisting of Gly-X-Gly-X-X-Ala. The mapped and/or sequenced peptides were found to be completely consistent with the peptide sequence deduced from the cDNA data and the previously published gas-phase sequencing data. Further mass spectrometry and protein analytical work unambiguously showed that rabbit lung FMO existed in tight association with a calcium-binding protein, calreticulin. Over 68% of rabbit lung calreticulin was mapped by LSIMS. Tandem mass spectrometric and gas-phase sequencing studies provided direct evidence for the identification of the N-terminal and other rabbit lung calreticulin-derived peptide sequences that were identical to other previously reported calreticulins. The complexation of calreticulin to rabbit lung FMO could account for some of the unusual physical properties of this FMO enzyme form.

The mammalian flavin-containing monooxygenase (FMO,[1] EC 1.14.13.8) catalyzes the NADPH-dependent oxygenation of nitrogen-, sulfur-, and phosphorous-containing drugs, chemicals, and pesticides in many tissues. The FMO isolated from hog liver has been the FMO most thoroughly studied (Ziegler, 1988). The only known endogenous substrate of FMO is cysteamine, which is oxidized to the disulfide, cystamine (Ziegler et al., 1983). Ziegler and Poulsen (1977) have postulated that FMO plays a key role in protein disulfide bond biosynthesis by providing a steady-state level of cystamine necessary for mixed disulfide bond formation. Hog liver FMO is an extremely hydrophobic enzyme that is sensitive to inhibition by anionic detergents, is generally stimulated by primary alkylamines, has a pH optimum of 8.5, and is inactivated rapidly above 40 °C in the absence of NADPH (Ziegler, 1980).

Recently, another form of the FMO was isolated from rabbit lung and found to be catalytically and immunologically distinct from the FMO enzyme isolated from either rabbit liver (Tynes et al., 1985; Williams et al., 1985) or hog liver (Williams et al., 1984). That rabbit lung and liver FMOs are different is consistent with the following evidence: (a) marked differences exist between the effects of Hg^{2+} on the activities of FMO from rabbit liver and lung (Devereux et al., 1977); (b) striking differences have been observed in substrate specificities between pulmonary and hepatic FMOs (Nagata et al., 1990; Poulsen et al., 1986; Sabourin et al., 1984; Williams et al., 1984; Ziegler, 1988); (c) in the absence of NADPH, hepatic FMO is markedly more sensitive to thermal inactivation than the pulmonary enzyme (Tynes et al., 1985; Williams et al., 1984, 1985); (d) primary alkylamines are substrates for the pulmonary enzyme (Poulsen et al., 1986); (e) the pulmonary enzyme differs markedly from the porcine hepatic enzyme in exhibiting a broader pH optimum (Tynes et al., 1985; Williams et al., 1985) and rabbit lung FMO is a water-soluble enzyme which is not inactivated by anionic detergents as is the hepatic form of FMO (Ozols, 1990; Ziegler, 1980).

The primary sequences of FMO deduced from cDNA data have been established for hog liver (Gasser et al., 1990) and rabbit liver and rabbit lung (Lawton et al., 1990). The data show that rabbit liver and hog liver FMOs have more than 85% similarity while less than 60% similarity exists between the rabbit liver and lung FMOs. The nature of posttranslational modifications of FMO cannot be determined from the cDNA data. Gas-phase sequencing of peptides from tryptic digests has nevertheless provided some direct amino acid sequence data (Hlavica et al., 1990; Ozols, 1989, 1990). Herein, we describe the mass spectrometric analysis of rabbit lung FMO peptides including posttranslationally modified and unmodified peptides. Over 90% of rabbit lung FMO has been mapped by liquid secondary ion mass spectrometry. Our results indicate that the FMO N-terminal amino acid is acetylated, that the N-terminal region contains an FAD binding domain, and that another peptide region contains a putative $NADP^+$ binding domain. In addition, our results

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[1] Abbreviations: FMO, flavin-containing monooxygenase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reversed-phase high-pressure liquid chromatography; LSIMS, liquid secondary ion mass spectrometry; CID, collision-induced dissociation; DMS, dimethyl suberimidate; DTT, dithiothreitol; HPAE, high-pH anion exchange.

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indicate that highly purified rabbit lung FMO exists as a tightly associated complex with the calcium-binding protein calreticulin.

Experimental Procedures

Chemicals. Chemicals used in this study were of the highest purity available and were purchased from the following companies: Urea, NH_4HCO_3 (Aldrich); dithiothreitol, sodium iodoacetate, sodium dodecyl sulfate, endoproteinase Glu-C (*Staphylococcus aureus* V8 protease, EC 3.4.21.19) (U.S. Biochemicals); trypsin (Sigma); and dimethyl suberimidate (Pierce). All other chemicals and reagents were obtained in the highest purity available from commercial sources.

Instrumental Analysis. Automated Edman degradation (Edman & Begg, 1967) was carried out with a gas-phase sequencer (Applied Biosystems Model 470A) with an on-line Model 120A system and Data Module for identification of the PTH-amino acids at the UCSF Biomolecular Resource Center.

Mass spectrometry was carried out on a Kratos MS-50S double-focusing mass spectrometer equipped with a high field magnet (mass range m/z = 3000 at an acceleration voltage of 8 kV), a Cs^+ liquid secondary ion mass spectrometry source (Aberth et al., 1982; Falick et al., 1986b), and a cooled sample introduction probe (Falick et al., 1986a). Tandem mass spectrometry experiments were performed on a Kratos Analytical Instruments (Manchester, U.K.) Concept IIHH four-sector EBEB tandem mass spectrometer (Walls et al., 1990) fitted with an electrooptical array detector (Cottrell & Evans, 1987), a cesium LSIMS source, and a cooled sample probe. Precursor ions were generated with a 10-kV Cs^+ primary ion beam. The collision energy for collision-induced dissociation was 4 kV. The collision gas (He) was used at a pressure sufficient to suppress the precursor ion beam to about 30% of its initial level. The instrument was controlled, and data were acquired with a DS-90 data system. Data reduction and display were carried out on a Mach 3 data system.

Protein Studies. Rabbit lung FMO was isolated and purified from pregnant female rabbits by a method previously described (Williams et al., 1985). Rabbit lung FMO had a specific content of 13.4 nmol of flavin/mg of protein. Rabbit lung FMO had characteristically high *N*,*N*-dimethylaniline *N*-oxygenase activity (Ziegler & Petit, 1964) [33.6 nmol/(min·nmol of protein)] and *p*-methoxyphenyl-1,3-dithiolane *S*-oxygenase activity (Cashman & Williams, 1990) [22.3 nmol/(min·nmol of protein)] and was judged to be homogeneous by SDS–PAGE (Laemmli, 1970). The concentration of protein was determined by the method of Lowry et al. (1951).

Rabbit lung FMO (0.6 mg, 10 nmol) was dissolved in 1.0 mL of a denaturing buffer (8 M urea, 0.1 M NH_4HCO_3, 1 mM EDTA, pH 8.2). Disulfide bonds were reduced by adding 0.4 mg of DTT to the sample, which was then purged with argon and incubated for 1 h at 37 °C. Carboxymethylation was carried out by adding 1.6 mg of sodium iodoacetate (about a 3 times molar excess over DTT) followed by an argon purge and incubation at 37 °C for 1 h. The carboxymethylated protein was transferred to the digestion buffer (2 M urea, 50 mM NH_4HCO_3, pH 8.2) with a Centricon 30 filter. The sample was divided into two equal parts (0.4 mL each), and endoproteinase Glu-C (10 μg) was added to one sample and the digestion mixture was incubated at 37 °C for 18 h. To the other sample was added 3 μg of trypsin, and the sample was incubated at 37 °C for 2 h.

Saccharide Analyses. For saccharide analyses, HPAE chromatography of acid hydrolysates of rabbit lung FMO was employed. Rabbit lung FMO (0.6 mg, 10 nmol) was placed in 500 μL of hydrochloric acid (4.0 M) for 4 h at 100 °C. The hydrolysate was lyophilized and dissolved in water (50 μL) and injected onto a Dionex BioLC Model equipped with a Carbopac AS-6 pellicular anion-exchange column, a gradient pump reagent delivery module, and pulsed amperometric detector. The solvent gradient was developed under conditions which readily separate monosaccharides (Hardy et al., 1988).

Peptide and Protein Separation and Analysis. The digested samples were lyophilized to reduce their total volume to about 0.1 mL and directly analyzed by RP-HPLC on a Rainin Model HPX instrument (Emeryville, Ca) controlled by a Macintosh SE computer using a Vydac C-4 column (4.6 mm × 25 cm, Western Analytical). The chromatogram was developed at a flow rate of 1.0 mL/min by a 90-min linear gradient from 0 to 45% of solvent B, where solvent A was 0.1% trifluoroacetic acid in water and solvent B was 0.08% trifluoroacetic acid in CH_3CN (Figure 1a). The absorbance of the HPLC eluate was monitored at 215 nm. The HPLC fractions were individually hand-collected in Eppendorf tubes and lyophilized prior to mass spectrometric analysis (Figure 2). For HPLC of intact protein samples, carboxymethylated FMO (20 μg, approximately 300 pmol) was dissolved in 0.05 mL of denaturing buffer (8 M urea and 0.1M NH_4HCO_3, pH 8.2) and injected onto a Vydac C-4 column. The chromatogram was developed at a flow rate of 1.0 mL/min by a 60-min linear gradient from 0 to 100% of solvent B, where solvents A and B were as above (Figure 1b).

Antisera and Immunoblotting. Antibody to rabbit lung FMO was raised in guinea pigs as described previously (Williams et al., 1984). Antibody to dog pancreas calreticulin was raised in goat and was a generous gift of Professor M. Michalak (University of Alberta, Edmonton, Canada). The guinea pig IgG fraction was isolated from antisera by ammonium sulfate precipitation and DEAE-Sepharose chromatography (Kaminsky et al., 1981). Goat antisera to calreticulin was used as a 1:200 dilution. Rabbit lung microsomes, rabbit lung FMO, and calreticulin were prepared for SDS-PAGE and applied to wells of the stacking gel and subjected to electrophoresis. Immunoblots were performed according to Burnette (1981) as described previously (Williams et al., 1985). SDS–PAGE-separated proteins were transferred electrophoretically to nitrocellulose. Once transferred, the proteins were immunostained with IgG followed by ^{125}I-labeled protein G. Following development of the autoradiograms quantification was performed by laser densitometry. Alternatively, Western blotting was accomplished by detecting immunoreactive bands with horseradish peroxidase secondary antibody, and peroxidase activity was developed with 4-chloronaphthol. The nitrocellulose membranes were scanned for densitometric quantification.

DMS Cross-Linking. Dimethyl suberimidate (10 mg) was dissolved in 1 mL of 0.2 M triethanolamine (pH 8.2)/0.5 M NaCl. A 10-μL aliquot of the cross-linking reagent and a 10-μL sample of rabbit lung FMO protein prepared as described above (30 μg) was combined and the mixture was incubated at room temperature. At various time points a 1-μL aliquot was withdrawn from the reaction, combined with 1 μL of 1 M ammonium acetate, boiled with equal volumes of electrophoresis sample buffer, and analyzed by SDS–PAGE (12% gel).

Results

Peptide Molecular Weight Mapping. Rabbit lung FMO was isolated and purified by standard procedures to apparent homogeneity. The enzyme was judged to be similar to other highly purified rabbit lung FMO preparations as judged by

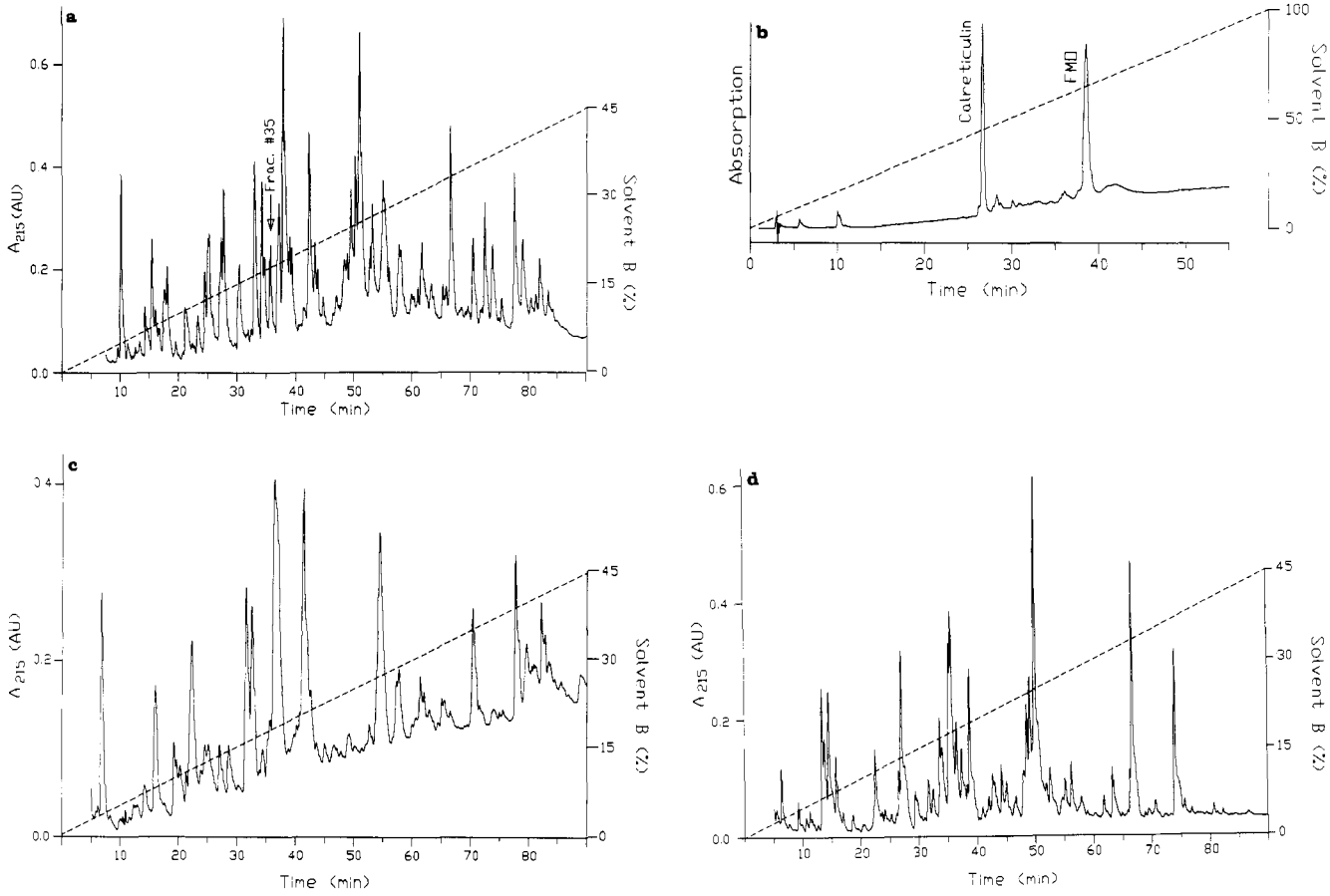

FIGURE 1: (a) RP-HPLC of a tryptic digest of the rabbit lung FMO·calreticulin complex. (b) RP-HPLC separation of carboxymethylated FMO and calreticulin. (c) RP-HPLC of a tryptic digest of pure carboxymethylated rabbit lung FMO. (d) RP-HPLC of a tryptic digest of pure carboxymethylated calreticulin.

the criteria of catalytic activity, immunoreactivity, and SDS–PAGE (Hlavica et al., 1990; Tynes et al., 1985; Williams et al., 1985). Highly purified rabbit lung FMO was subjected to reduction and carboxymethylation. The carboxymethylated protein was digested separately with trypsin or endoproteinase Glu-C. Figure 1a shows the HPLC chromatogram of the tryptic digest mixture of carboxymethylated rabbit lung FMO. Generally, each HPLC fraction contained a number of peptides. For example, fraction 35 of Figure 1a contained 10 peptides as judged by the number of molecular ions in the LSIMS spectra. Figure 2 shows a portion of the LSIMS spectrum resulting from analysis of HPLC fraction 35. It should be noted that even though fraction 35 contained numerous peptides (which precluded direct analysis by gas-phase sequencing methodology), as described below, sequence determination by tandem CID was straightforward and yielded readily identifiable peptide sequences. Approximately 30 of the HPLC fractions were derivatized with hexanol (Falick & Maltby, 1989) to give useful peptide molecular ions. Because the primary sequence of rabbit lung FMO was known from the cDNA data (Lawton et al., 1990), we focused on important peptides determined to be present on the basis of their molecular weights and the known specificities of the proteolytic enzymes used. Figure 3 shows the resulting peptide weight map for rabbit lung FMO. Peaks corresponding to the expected molecular masses of 90% of the expected peptides were found by LSIMS.

Figure 4 shows the CID spectrum of a protonated peptide (MH^+ m/z = 2418.3) from the endo Glu-C digest. The spectrum was consistent with the N-terminal peptide of rabbit lung FMO with the sequence Ac-Ala-Lys-Lys-Val-Ala-Val-

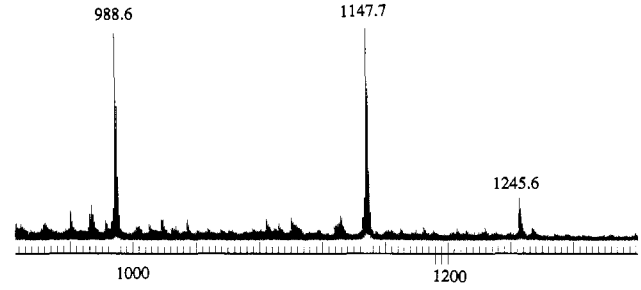

FIGURE 2: A segment of the LSIMS spectrum of HPLC fraction 35 (see Figure 1a). Peaks at m/z = 988.6, 1147.7, and 1245.6 have been identified by tandem mass spectrometry as due to internal peptides of rabbit lung calreticulin. The entire fraction contains at least 10 peptides as observed by LSIMS.

Ile-Gly-Ala-Gly-Val-Ser-Gly-Leu-Ile-Ser-Leu-Cys_c-Cys_c-Val-Asp-Glu. As shown in Figure 3, possible N-glycosylation sites were mapped by LSIMS and/or sequenced by tandem mass spectrometry. The only N-glycosylation consensus sequence Asn_{60}-Thr_{61}-Ser_{62} was determined to be unmodified, suggesting that it was unlikely that the rabbit lung FMO preparation analyzed was N-glycosylated. Exhaustive acid hydrolysis and analyses of hydrolysates by HPAE chromatography (Hardy & Townsend, 1988) also provided no evidence for the presence of significant amounts of saccharides.

In the course of analyzing peptides from rabbit lung FMO proteolytic digests, we found and sequenced by tandem mass spectrometry several peptides that bore no relation to the amino acid sequence of any known pulmonary or hepatic FMO. However, computer searches of protein data banks with these unknown sequences found in the rabbit lung FMO di-

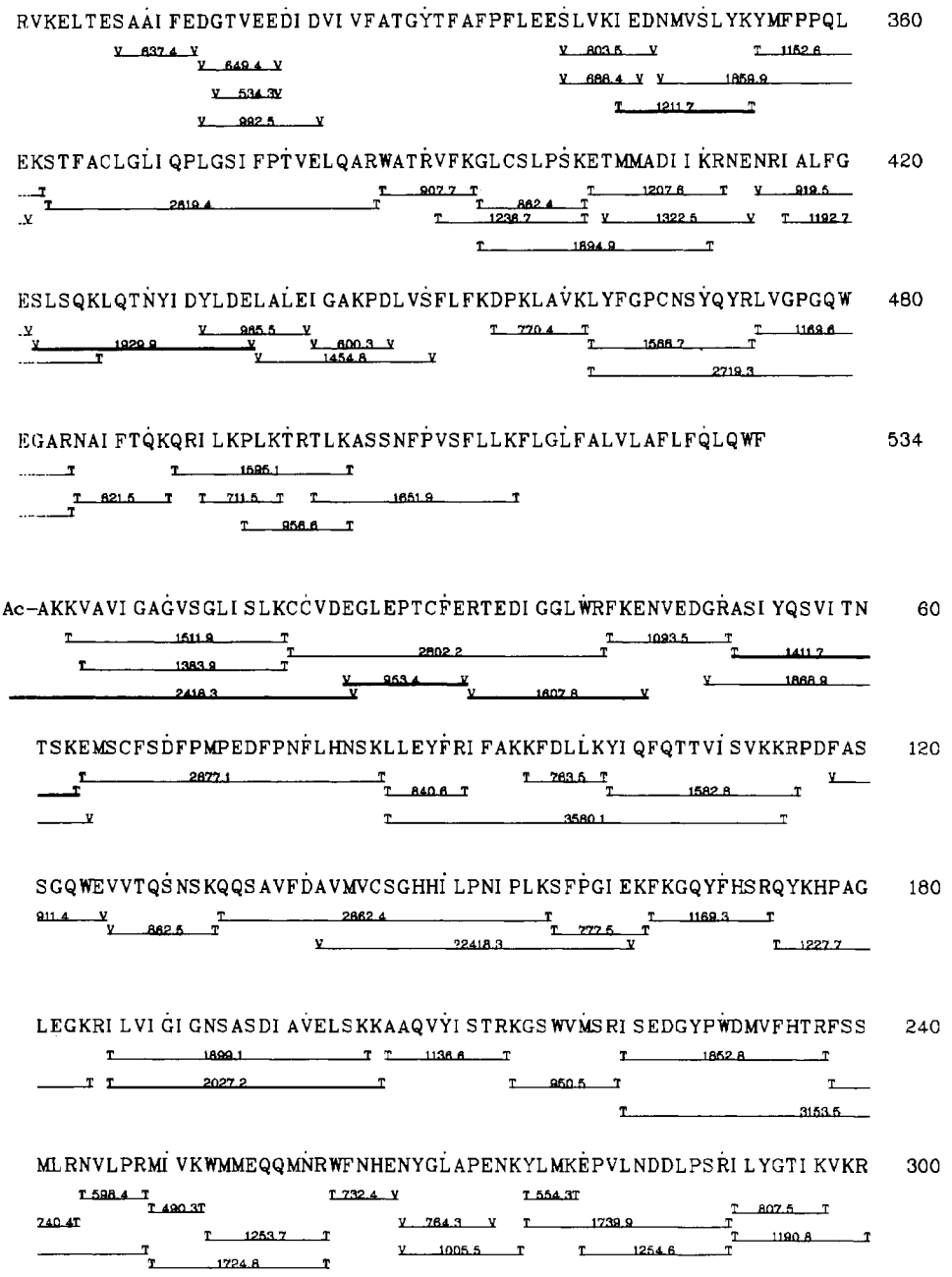

FIGURE 3: Primary sequence of rabbit lung FMO mapped by LSIMS. The peptides heavily underlined were sequenced by tandem MS.

gests provided matches identical to the calcium-binding protein calreticulin (Fliegel et al., 1989). Because calreticulin has been cloned and sequenced and does not contain an N-terminal blocking group, we sequenced the rabbit lung FMO preparation by gas-phase Edman sequencing to confirm the presence of calreticulin. Table I shows the results of the first five cycles of the Edman sequencing of the intact FMO protein sample. The N-terminal sequence obtained (Glu-Pro-Val-Val-Tyr-) was identical to the first five amino acids of calreticulin and was unrelated to the N-terminus of FMO, which is blocked in any case by an acetyl group.

A total of 10 peptides from the digests of the rabbit lung FMO preparation were sequenced by mass spectrometry. Two examples are shown in Figures 5 and 6. In Figure 5 is shown the tandem CID spectrum of a peptide of m/z = 881.5 (MH^+), whose sequence was determined to be consistent with the sequence of the N-terminus of calreticulin (Glu_1-Pro_2-Val_3-Val_4-Tyr_5-Phe_6-Lys_7). Another peptide of m/z = 1147.7 (MH^+) was detected by LSIMS (Figure 2) and was subjected to tandem mass spectrometry. The CID spectrum (Figure 6) shows a complete series of a ions and nearly complete y ion series. This allows the sequence to be read directly from the spectrum (Lys_{126}-Val_{127}-His_{128}-Val_{129}-Ile_{130}-Phe_{131}-Asn_{132}-Tyr_{133}-Lys_{134}) (Figure 6). Although this HPLC fraction (no. 35, Figure 1a) contained nine peptides in addition to the above one, they did not interfere with the sequence determination

Table I: Edman Degradation of the Rabbit Lung Calreticulin Found in the FMO Protein Sample[a]

cycle no.	residue	yield (pmol)
1	Glu	56
2	Pro	33
3	Val	30
4	Val	29
5	Tyr	27

[a] Automated Edman gas-phase sequencing of rabbit lung calreticulin found in the rabbit lung FMO was accomplished as described in the Experimental Procedures section.

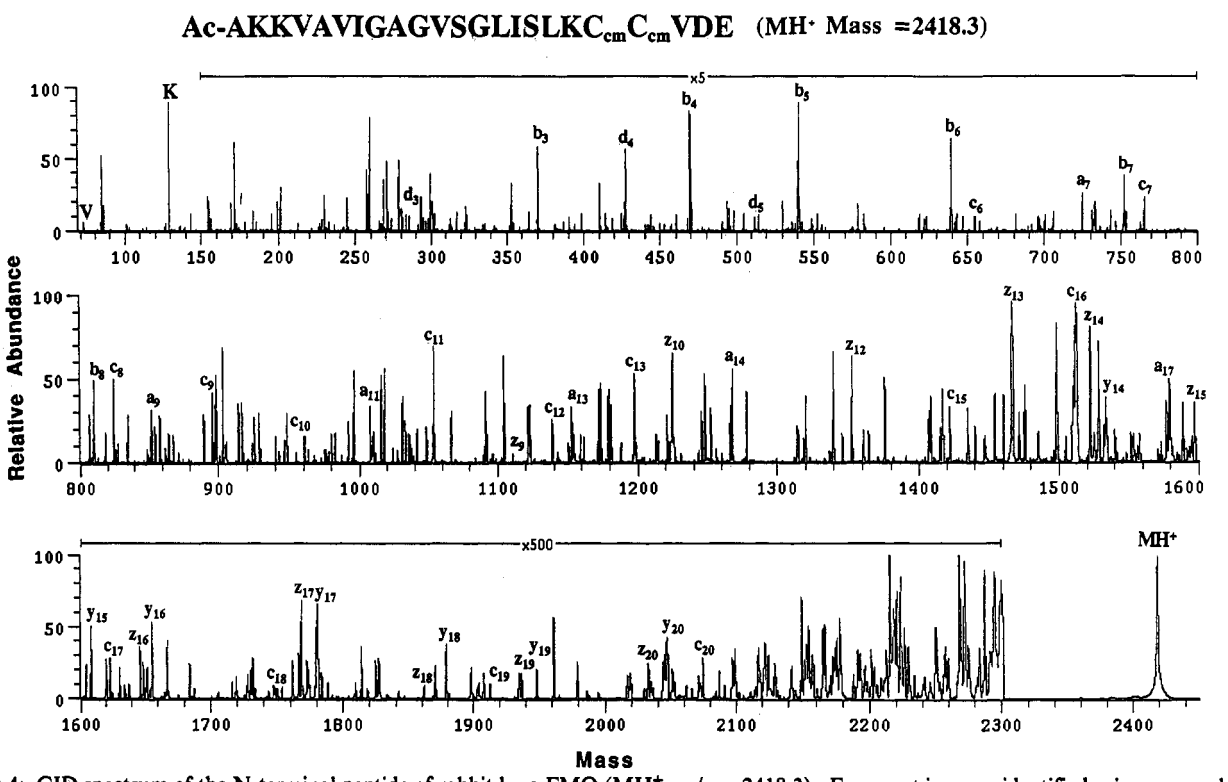

FIGURE 4: CID spectrum of the N-terminal peptide of rabbit lung FMO (MH^+, m/z = 2418.3). Fragment ions are identified using nomenclature described in Johnson et al. (1987). The two cysteine residues were carboxymethylated.

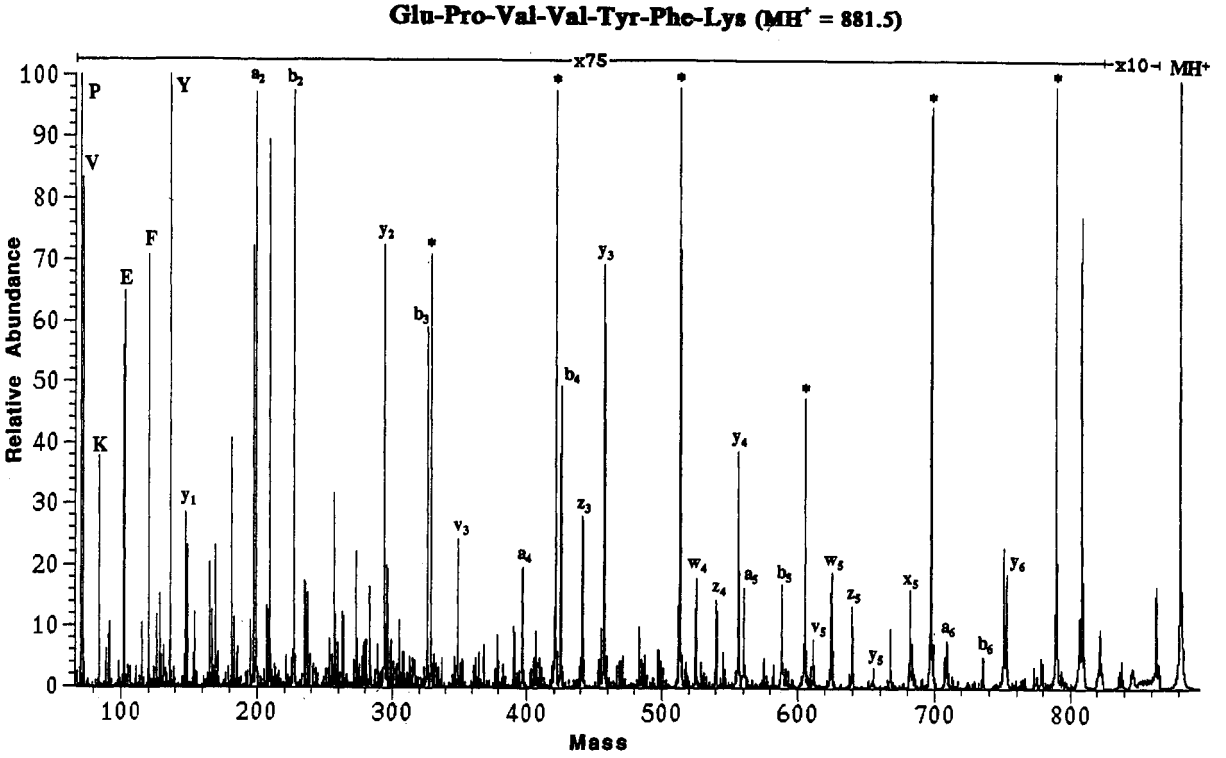

FIGURE 5: CID spectrum of the N-terminal peptide of calreticulin (MH^+, m/z = 881.5). Fragment ions were identified using nomenclature described in Johnson et al. (1987).

by tandem mass spectrometry.

Separation of Rabbit Lung FMO and Rabbit Lung Calreticulin. In agreement with other reports (Hlavica et al., 1990; Tynes et al., 1985; Williams et al., 1985), we found that highly purified rabbit lung FMO appeared as a single band on SDS–PAGE (Figure 8). To confirm that this apparently homogeneous FMO protein contained calreticulin as predicted by N-terminal peptide sequencing and by the mass spectral evidence discussed above, rabbit lung FMO was subjected to RPHPLC. Figure 1b shows the HPLC chromatogram which, in fact, shows two completely separated peaks. Each peak was isolated by preparative HPLC runs and was subjected to proteolytic digestion with trypsin. Panels b and c of Figure 1 show the HPLC chromatograms of the tryptic digests of carboxymethylated rabbit lung FMO and calreticulin, respectively. The disparate HPLC retention times of rabbit lung

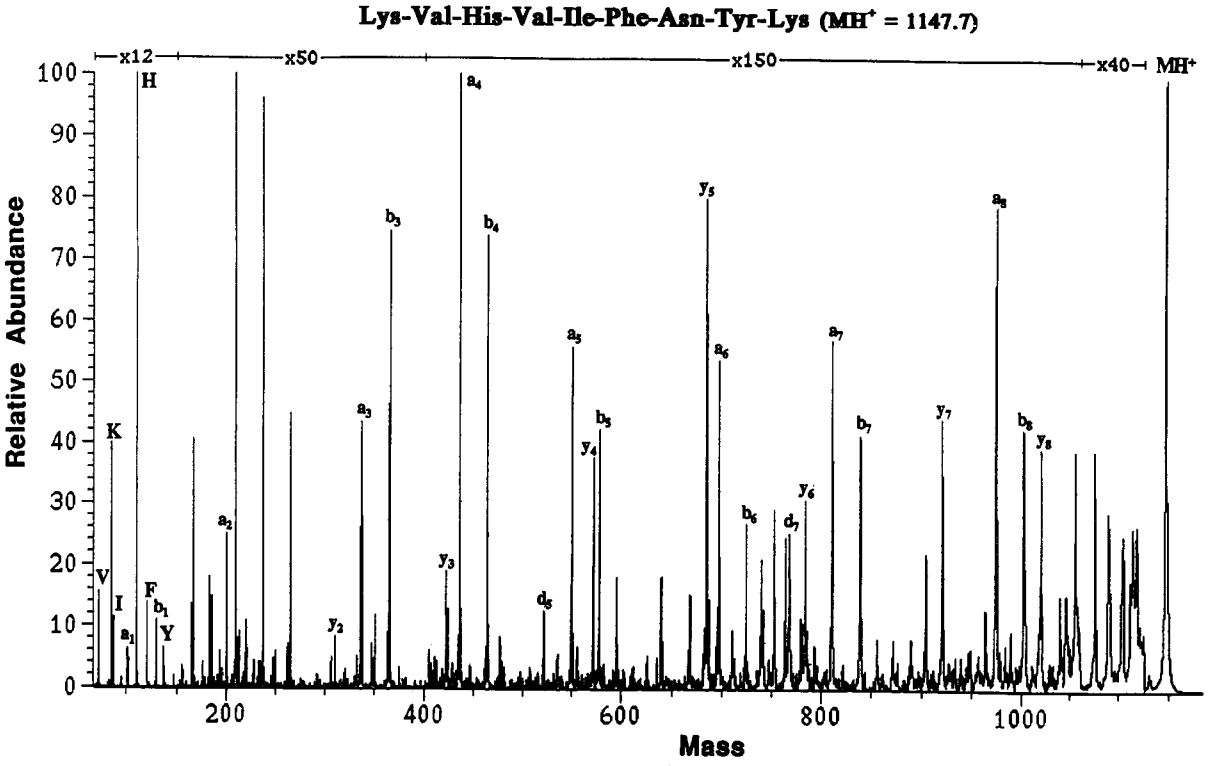

FIGURE 6: CID spectrum of an internal peptide of rabbit lung calreticulin (MH^+ 1147.7 Da). A complete series of a ions and nearly complete b and y series were observed.

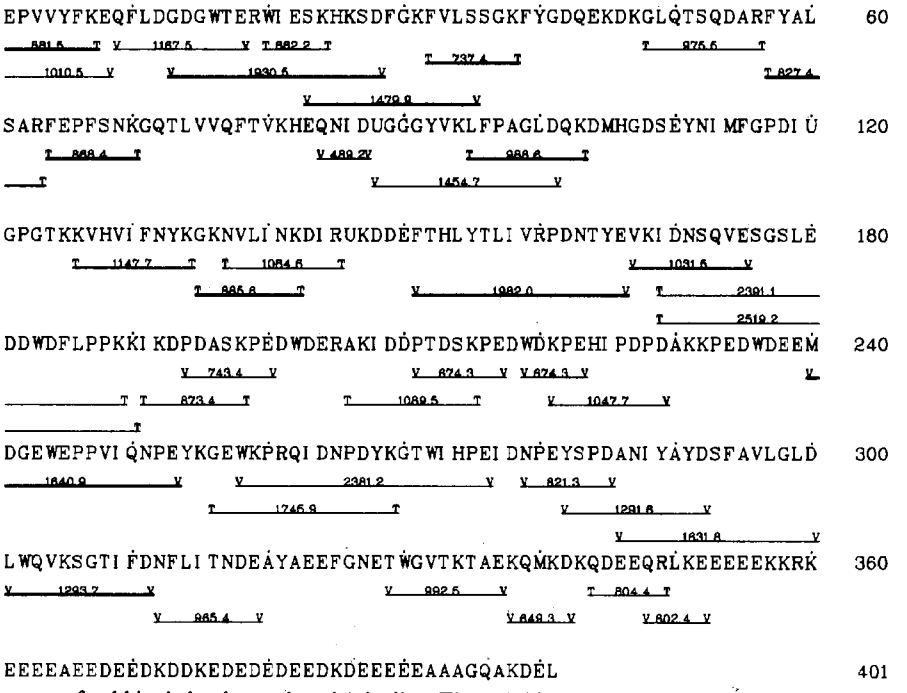

FIGURE 7: Primary sequence of rabbit skeletal muscle calreticulin. The peptides heavily underlined were sequenced by tandem MS.

FMO and calreticulin (Figure 1a,b) and the corresponding peptides (Figure 1c,d) suggest that the proteins have very different properties with regard to hydrophilicity. In agreement with the relative hydrophobicity of the two proteins as determined by HPLC (Figure 1a,b), during the course of reduction and carboxymethylation, lung FMO gradually precipitated from the reaction mixture even in the presence of 8 M urea buffer while calreticulin remained in solution. Once separated by HPLC, the carboxymethylated proteins (run in parallel electrophoresis lanes) separate very slightly from one another by SDS–PAGE. The molecular weights of the tryptic peptides of carboxymethylated calreticulin were determined by LSIMS. Shown in Figure 7 is the primary sequence of calreticulin deduced from cDNA data and a partial (68%) peptide molecular weight map of rabbit lung calreticulin. The partial molecular weight map of rabbit lung calreticulin is in excellent agreement with the deduced primary sequence of skeletal muscle calreticulin from the cDNA data (Fliegel et al., 1989) and also includes the N-terminal peptide determined by Edman sequencing. The results of the HPLC and mass spectrometric experiments suggested that the initial preparation existed approximately as a 1:1 complex of the two proteins. The proteins were not covalently bound to one another because HPLC readily separated the two proteins. The

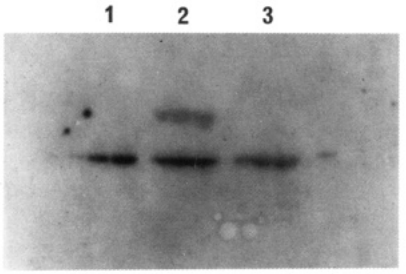

FIGURE 8: Immunoblot quantification of calreticulin in purified FMO (lane 1) and microsomes (lane 2) from rabbit lung. Lane 3 was highly purified dog pancreas calreticulin. The method used is described under Experimental Procedures.

rabbit lung FMO·calreticulin complex was very soluble in water (e.g., ≥100 mg of protein complex/mL of pure water) as noted previously (Williams et al., 1985), but in the absence of calreticulin, lung FMO was quite insoluble in water. In agreement with these observations, other forms of FMO (most notably hepatic FMO) require large amounts of detergent to afford solubility in aqueous solution (Ziegler, 1980).

Cross-Linking and Western Blot Analysis. The relative intensity of immunochemically detected rabbit lung FMO and calreticulin was determined by Western blotting using antibody to rabbit lung FMO and dog pancreas calreticulin, respectively. As shown in Figure 8, the antibody to calreticulin cross-reacted quite well with proteins of approximately the same molecular weight as the calreticulin standard in both the apparently pure rabbit lung FMO preparation and rabbit lung microsomes (lanes 1 and 2). Highly purified dog pancreas calreticulin (lane 3) reacts quite well with goat antiserum to calreticulin. In a separate experiment (data not shown), overexposure of the immunoblot revealed very low cross-reaction between an antibody to rabbit lung FMO and highly purified calreticulin. In the same blot, both microsomes and FMO from rabbit lungs were intensely stained by a guinea pig antibody to rabbit lung FMO. As noted above, rabbit lung FMO is extremely hydrophobic and calreticulin is quite hydrophilic. Nevertheless, SDS–PAGE and Western blot analysis suggested that both proteins showed similar apparent molecular weights and affinities, even though their actual molecular weights differ by at least M_r = 10000. Treatment of the rabbit lung FMO·calreticulin complex with DMS for 0.5–1 min led to a cross-linked protein complex (data not shown). Gel electrophoresis showed the emergence of a prominent band at approximately M_r = 100000 that was not present in the absence of the cross-linking reagent. The M_r = 100000 band appeared on gels with the concomitant disappearance of the FMO and calreticulin bands. Incubation of the complex with DMS for 1–2 h resulted in the attenuation of the M_r = 100000 band and the emergence of high molecular weight bands with the concomitant loss of the parent complex band (data not shown). We conclude that longer incubation of the complex with the cross-linking agent caused the initially formed covalently attached dimers to oligomerize upon further cross-linking to high molecular weight aggregates.

Discussion

Rabbit Lung FMO. FMO isolated from rabbit lung is catalytically and immunochemically distinct from the FMO isolated from rabbit liver (Tynes et al., 1985; Williams et al., 1985). Rabbit lung FMO also differs markedly from rabbit liver and other hepatic FMOs. It is unlikely, however, that differences in the primary sequence of these two forms of FMO are solely responsible for the remarkable differences in physical, catalytic, and immunochemical properties.

Over 90% of the rabbit lung FMO sequence was mapped by LSIMS and tandem MS. The N-terminus of rabbit lung FMO was found to be blocked with an acetyl group, which is identical to the case for hog liver FMO (Guan et al., 1990). Rabbit lung FMO contains the N-glycosylation tripeptide consensus sequence -Asn_{60}-Thr_{61}-Ser_{62}- which was also mapped by tandem MS. We found no evidence for glycosylation of rabbit lung FMO as observed by mass spectrometry and HPAE chromatography after acid hydrolysis of FMO.

Rabbit Lung Calreticulin. During the work required to construct the peptide map for rabbit lung FMO, a number of peptides were found that did not match peptides predicted to be present from computer analysis of the cDNA sequence. Several of the peptides were sequenced by tandem mass spectrometry. The peptide sequences determined by tandem mass spectrometry precisely matched peptide sequences deduced from the cDNA data for rabbit skeletal muscle calreticulin, a ubiquitous calcium-binding protein recently cloned and sequenced (Fliegel et al., 1989). Calreticulin is an acidic protein involved in the storage of calcium in the endoplasmic reticulum. The enzyme is markedly zonal in character with an approximately neutral (net charge) N-terminus forming a globular domain attached to a tail-like proline-rich internal zone and an acidic C-terminus (Smith & Koch, 1989). The zonal character may enable the protein to perform multiple functions in addition to its role as a calcium-binding structure. Over 68% of the calreticulin which apparently forms a 1:1 complex with rabbit lung FMO was mapped by LSIMS, and several regions were sequenced by tandem mass spectrometry (Figure 7). The highly conserved endoplasmic reticulum retention signal sequence -Lys-Asp-Glu-Leu at the C-terminus is part of the calreticulin peptide sequence deduced from the cDNA. The C-terminus sequence -Lys-Asp-Glu-Leu (KDEL) is present in several resident luminal endoplasmic reticulum proteins (i.e., disulfide isomerase, prolylhydroxylase, and grp 78 (BiP)) (Munro & Pelham, 1987). The KDEL sequence is apparently required in the mechanism to recognize resident luminal proteins and distinguish them from other proteins which are not targeted for retention in the endoplasmic reticulum. Rabbit lung calreticulin may act as a chaperon protein to help target or translocate rabbit lung FMO to the endoplasmic reticulum and modulate enzyme activity.

Both rabbit lung FMO and calreticulin exhibit anomalous molecular weights when analyzed by SDS–PAGE. The molecular weight for calreticulin deduced from its cDNA sequence is approximately M_r = 45000, but molecular weights calculated from SDS–PAGE are M_r = 55000–60000 (Fliegel et al., 1989). Conversely, the molecular weight of FMO calculated from SDS–PAGE (M_r = 55000–60000) is significantly lower than that deduced from cDNA sequencing. The anomalous behavior of these two proteins presumably results from unusually poor binding of SDS by the hydrophilic, negatively charged calreticulin and enhanced binding of SDS by the positively charged, hydrophobic FMO. The two proteins are not resolved on standard SDS–PAGE gels, which explains why this protein was not detected in preparations of rabbit lung FMO judged to be homogeneous by SDS–PAGE.

Our studies of the rabbit lung FMO show that the enzyme is obtained in highly purified form as a complex with calreticulin. In addition, we have presented Western blot data that shows that rabbit lung FMO is tightly associated with calreticulin in microsomes.

The markedly different physicochemical properties of these two proteins would argue against the likelihood that they would copurify. The purification procedure involves hydrophobic

Table II: Amino Acid Composition of FMO and Calreticulin

AA	rabbit lung			
	Hlavica et al.[a]	Williams[b]	Lawton et al.[c] (deduced)	calreticulin[d] (deduced)
Ala	4.8	6.2	5.8	4.5
Arg	5.2	5.0	4.5	2.2
Asn and Asp	9.6	13.1	6.9	16.9
Cys	0.8	0.9	1.5	0.7
Gln and Glu	13.5	12.0	10.7	18.0
Gly	9.2	6.2	5.8	6.2
His	1.9	1.4	1.3	1.7
Ile	4.0	4.2	6.2	4.7
Leu	8.5	10.6	10.1	4.5
Lys	6.3	6.7	7.9	10.7
Met	2.1	2.7	3.0	1.0
Phe	4.8	7.8	7.3	4.5
Pro	7.1	6.3	4.9	6.0
Ser	7.3	7.9	7.7	4.0
Thr	5.4	3.9	4.1	3.7
Trp		0.8	1.7	2.7
Tyr	2.3	2.9	3.4	3.5
Val	7.2	6.6	6.5	4.2
total AA			535	401

[a] Amino acid composition reported by Hlavica et al. (1990). [b] Amino acid composition reported by Williams (1990). [c] Amino acid composition deduced from the cDNA data of Lawton et al. (1990). [d] Amino acid composition deduced from the cDNA data of Fliegel et al. (1989).

(*n*-octylamino-Sepharose), anionic (DEAE-Sepharose), and absorptive (hydroxylapatite) chromatography (Williams et al., 1985). The hydrophilic nature of calreticulin and its acidic nature (p*I* 4.1) suggest that calreticulin should behave quite differently from the hydrophobic, basic (p*I* 9.5) rabbit lung FMO, especially during the first two chromatographic steps. In fact, calreticulin isolated from rabbit liver requires NaCl concentrations greater than 0.25 M to elute from DEAE-cellulose (Treves et al., 1990), whereas rabbit lung FMO elutes in the void volume (Williams et al., 1985).

It is possible that selective binding of calreticulin to FMO stabilizes the pulmonary enzyme to thermal denaturation and obviates the requirement for high concentrations of detergents normally required for hepatic FMO activity. It is unlikely that the complexation with calreticulin alters the kinetic or substrate stereoselectivity properties of FMO because numerous laboratories have obtained similar substrate turnover results with various rabbit lung FMO preparations (Hlavica et al., 1990; Tynes et al., 1985; Williams et al., 1985). However, it is quite possible that laboratories investigating rabbit lung FMO are using preparations containing substantial quantities of calreticulin. Table II lists the amino acid compositions deduced from the cDNA data for rabbit lung FMO and rabbit smooth muscle calreticulin. Several significant differences between the reported amino acid composition of purified rabbit lung FMO preparations and the deduced amino acid composition of rabbit lung FMO are apparent. For example, the reported amino acid composition of Asp and Asn was significantly greater than the deduced amino acid composition on the basis of the cDNA data (Table II) (Hlavica et al., 1990; Williams, 1991). The contribution of Asp and Asn from calreticulin could account for the difference. Similarly, the reported amino acid composition of Gln, Glu, and Pro for rabbit lung FMO is substantially greater than the composition predicted to be present based on the basis of the cDNA data. Calreticulin possesses a large percentage of Gln, Glu, and Pro, and this probably accounts for the amino acid composition differences noted (see Table II) (Hlavica et al., 1990; Williams, 1990).

In our studies of the peptides of rabbit lung FMO, the use of tandem mass spectrometry and protein analytical methods for peptide sequence determination had certain important advantages. Peptide sequencing by tandem mass spectrometry does not require complete separation of peptides and is capable of providing sequence information of N-blocked or otherwise modified peptides. Tandem mass spectrometry allowed the rapid determination of the N-terminal acetyl group and confirmed the presence of FAD and $NADP^+$ binding domains in rabbit lung FMO. Finally, tandem mass spectrometry allowed rapid identification of peptides leading to the discovery of the presence of calreticulin. Specific binding of calreticulin to rabbit lung FMO may serve some important physiological function, and we are currently examining this possibility in in vitro experiments.

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Primary Structure of the Assimilatory-Type Sulfite Reductase from *Desulfovibrio vulgaris* (Hildenborough): Cloning and Nucleotide Sequence of the Reductase Gene[†,‡]

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ABSTRACT: The nucleotide sequence encoding the structural gene (651 bp) and flanking regions for the assimilatory-type sulfite reductase from the sulfate-reducing bacterium *Desulfovibrio vulgaris* (Hildenborough) was determined after cloning a 1.4 kb *Hin*dIII/*Sal*I genomic fragment possessing the gene into Bluescript pBS(+)KS. The primary structure of the protein was deduced, and the molecular mass of the apoprotein was estimated as 24 kDa. The amino acid sequence of the polypeptide shows some similarities at putative $[Fe_4S_4]$ cluster binding sites in comparison with the heme protein subunit of the larger *Escherichia coli* and *Salmonella typhimurium* sulfite reductases and spinach nitrite reductase. This is the first reported sequence of a member of a new class of low molecular weight assimilatory sulfite-reducing enzymes recently identified in a number of anaerobic bacteria [Moura, I., Lina, A. R., Moura, J. J. G., Xavier, A. V., Fauque, G., Peck, H. D., & Le Gall, J. (1986) *Biochem. Biophys. Res. Commun. 141*, 1032-1041].

Biological redox chemistry is a diverse field that encompasses both electron transfer proteins and oxidoreductase enzymes (Cowan et al., 1989; Barber, 1984; Witt et al., 1986; Papa, 1983; Hatefi et al., 1985; Mortenson & Thorneley, 1979). Typically, the latter class possesses redox cofactors that serve to bind substrate and facilitate the formation and cleavage of chemical bonds. Important examples are found in the biological sulfur cycle. For instance, many bacteria possess assimilatory enzymes that reduce oxyanions of sulfur to the oxidation level of sulfide, which is the redox state of sulfur in a large number of cellular compounds (Le Gall & Postgate, 1973; Peck & Le Gall, 1982; Siegel, 1975; Vega & Kamin, 1977; Lancaster et al., 1979; Huynh et al., 1984; Murphy et al., 1974). Bacteria that use sulfate as the terminal electron acceptor in anaerobic respiration (dissimilatory reduction) also possess a sulfate (SO_4^{2-}) reducing system[1] and sulfite (SO_3^{2-}) reductases (Le Gall & Postgate, 1973; Peck & Le Gall, 1982; Siegel, 1975).

Our laboratory is engaged in the elucidation of the molecular details of enzymatic sulfite reduction as carried out by the assimilatory-type sulfite reductase from *Desulfovibrio vulgaris* (Hildenborough). The six-electron reduction of SO_3^{2-} to S^{2-} is catalyzed by a single enzyme possessing a siroheme chromophore that is essential for sulfite binding and reduction (Young & Siegel, 1988). On the basis of spectroscopic evi-

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[1] The enzyme ATP sulfurylase activates SO_4^{2-} by formation of the adenosine phosphosulfate (APS) adduct. Subsequent reduction of APS to SO_3^{2-} and AMP is catalyzed by APS reductase (Postgate, 1984).

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