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Complete Amino Acid Sequence of NADPH–Cytochrome P-450 Reductase from Porcine Hepatic Microsomes†

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ABSTRACT: The complete amino acid sequence of porcine hepatic microsomal NADPH–cytochrome P-450 reductase has been determined by microsequence analysis on several sets of proteolytic fragments. Sequence studies were performed initially on a 20-kilodalton (kDa) fragment and then on 80-kDa fragment. The amino-terminal end of the mature protein was blocked with an acetyl group, followed by 676 amino acid residues. It has been revealed that the COOH-terminal 20-kDa fragment has been derived from original enzyme by cleavage at the Asn–Gly (residues 502–503) linkage by an unknown mechanism. An NADPH-protected cysteine residue is located at residue 565, near a region exhibiting high sequence homology with ferredoxin–$NADP^+$ reductase. The FMN and FAD binding regions are possibly located in the amino-terminal region and the middle part of the protein molecule, respectively, as suggested by Porter and Kasper [Porter, T. D., & Kasper, C. B. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 973–977]. When this sequence is compared with that of rat enzyme, 60 amino acid residues are substituted, probably due to species differences. However, total sequence homology between these enzymes is 90%. Hydropathy plot analysis reveals that two regions from residues 27–43 and from residues 523–544 exhibit a high degree of hydrophobicity, suggesting membrane binding or interaction with cytochrome P-450.

Hepatic microsomal NADPH–cytochrome P-450 reductase is a flavoprotein which participates in hydroxylation of various endogenous and foreign compounds (Williams, 1976). This enzyme contains one molecule each of FAD and FMN per subunit in the active form and has a molecular weight of 78 000 (Iyanagi & Mason, 1973; Yasukochi & Masters, 1976). The catalytic function of this enzyme is electron transport from NADPH to cytochrome P-450 or cytochrome b_5 via FAD and FMN in the microsomal systems (French & Coon, 1979; Enoch & Strittmatter, 1979). Furthermore, this enzyme independently donates an electron to anticancer quinone drugs such as adriamycin and produces a free radical form of the anthracycline molecule which causes cell damage by DNA or RNA breakages (Bachur et al., 1978; Berlin & Haseltine, 1981).

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Structural features on this enzyme have been revealed by chemical and biophysical methods. The detergent-solubilized enzyme contains a hydrophobic domain in the NH_2-terminal region, which may be involved in binding the microsomal membrane or cytochrome P-450 (Black & Coon, 1982). Although this enzyme contains an NH_2-terminal blocking group, the protease-solubilized enzyme exhibited a new NH_2-terminus lacking the hydrophobic domain consisting of 48 amino acid residues (Black & Coon, 1982; Haniu et al., 1984). The total amino acid sequence of rat liver enzyme has been recently deduced from its cDNA sequence analysis, and FAD, FMN, and NADPH doamins were proposed according to the sequence homology to the other flavoproteins (Porter & Kasper, 1985). However, the NH_2-terminal residue of the mature protein has not been determined.

Two laboratories have reported a functional sulfhydryl group which was essential for the enzymatic activity, particularly for binding $NADP^+$ (Lumper et al., 1980; Nishimoto & Shibata, 1982). We have studied the amino acid sequence of eight cysteine-containing tryptic peptides from porcine hepatic enzyme, and one out of eight cysteines was able to be protected by NADPH against iodoacetate S-alkylation (Haniu et al., 1984). The COOH-terminal 20-kilodalton (kDa)[1] fragment which contains a functional SH group has been completely sequenced (Haniu et al., 1985). Partial sequences of cyanogen bromide peptides from porcine hepatic NADPH–cytochrome P-450 reductase have recently been reported, and the NADPH-protected cysteine residue has been confirmed (Vogel et al., 1985). We report here the complete amino acid sequence of the detergent-solubilized NADPH–cytochrome P-450 reductase from porcine hepatic microsomes.

Experimental Procedures

Materials. Benzene, acetonitrile, and butyl chloride (HPLC grade) were obtained from J. T. Baker Chemical Co. Trifluoroacetic acid and ethyl acetate were distilled as reported (Shively et al., 1982). Iodoacetic acid was crystallized from petroleum ether. TPCK–trypsin (232 units/mg) and chymotrypsin were purchased from Worthington Biochemical Corp. *Staphylococcus aureus* protease (strain V8) was obtained from Pierce Chemical Co. 4-Sulfophenyl isothiocyanate was obtained from Pierce Chemical Co.

Purification and Derivatization of Reductase and the 20-kDa Fragment. Detergent-solubilized NADPH–cytochrome P-450 reductase was prepared from pig liver microsomes as reported previously (Iyanagi & Mason, 1973; Haniu et al., 1984). Separation of the 20-kDa fragment from other fragments was performed by HPLC using a μBondapak phenyl column as reported (Haniu et al., 1984). Reductase was solubilized in 2 mL of 6 M guanidine hydrochloride/Tris buffer (pH 8.5) containing 5 mM EDTA and reduced by 10 μL of 2-mercaptoethanol for 2 h. Iodoacetic acid (60 mg dissolved in 1 mL of above buffer) was added to the reaction mixtures and was dialyzed against water after 20 min of reaction. Dialysis was continued for 2 days. The 20-kDa fragment was carboxymethylated with iodoacetic acid in a similar way and further derivatized with S-PITC to increase hydrophilicity (Braunitzer et al., 1971; Kubota & Tsugita, 1980). The protein was treated with 6 mg of S-PITC dissolved in TEA–propanol buffer (TEA/1-propanol/H_2O, 6/44/50 v/v) (pH 9.0) for 12 h at 55 °C. The reaction mixtures were dialyzed against water for 2 days.

Preparation of Peptides. Trypsin and *S. aureus* protease digestions were performed in 0.2 M ammonium bicarbonate buffer (pH 8.0) for 24 h at 37 °C as described previously (Yuan et al., 1983). Chymotrypsin digestion of the S-PITC-labeled 20-kDa fragment was performed in 0.2 M ammonium bicarbonate buffer for 24 h at 37 °C. These digests were directly applied to the HPLC columns without lyophilization.

Peptide Separation by HPLC. Peptides were separated by HPLC using an Ultrasphere C-8 or Synchropak RP C8 column. Chromatographic conditions were similar to those reported (Yuan et al., 1983); peptides were eluted with a linear gradient from buffer A (0.1% TFA, pH 2.0) to buffer B (TFA/H_2O/CH_3CN, 0.1/9.9/90 v/v, pH 2.0). Peptides were detected by the absorbance at 206 nm and manually collected.

Analytical Methods. Amino acid compositions were determined on a Beckman 121MB amino acid analyzer after hydrolysis in 5.7 N HCl containing 0.2% 2-mercaptoethanol at 110 °C for 24 h unless otherwise noted. Automated sequence analyses were performed on a modified Beckman sequencer (Model 890C) in the presence of Polybrene (Pierce Chemical Co.) (Hunkapillar & Hood, 1978; Shively, 1981) or an updated gas phase sequencer, as reported previously from this laboratory (Hawke et al., 1985). Also, manual Edman degradation was performed as follows: peptide samples (0.2–1.0 nmol) were dissolved in 40 μL of TEA–propanol buffer (TEA/1-propanol/H_2O, 6/44/50 v/v, pH 9.0) containing 5 μL of 10% PITC–acetonitrile, and the coupling reaction was performed for 10 min at 55 °C. Acetylated protamine (1–5 μg) was added to the sample instead of Polybrene. The sample was dried under vacuum and washed with 0.5 mL each of benzene and ethyl acetate. The cleavage reaction was carried out at 55 °C for 2 min in 50 μL of HFBA. The sample was dried under vacuum, and the thiazolinone was extracted with 0.5 mL of benzene (HPLC grade) after being warmed at 55 °C for 1 min. The conversion reaction was performed in 25% TFA (100 μL) by heating at 55 °C for 15 min. Phenylthiohydantoin derivatives of amino acids were identified by reverse-phase HPLC using a gradient system (Hawke et al., 1982).

Mass Spectrometry. Mass spectra were taken with a JEOL HX100HF mass spectrometer operating at 5-kV accelerating potenial and a nominal resolution setting of 3000. A neutral xenon atom beam with 3-kV translational energy was used for sample ionization. Peptide samples (50–100 pmol) in 1–2 μL of 5% aqueous acetic acid were added to 1–2 μL of a mixture of dithiothreitol/dithioerythritol (5/1) on a 1.5 × 6 mm stainless-steel sample stage. Repetitive scans over the mass range m/z 30–2500 (cycle time = 45 s) were collected by using a JEOL DA5000 data system. Mass values reported are for the monoisotropic mass of the protonated molecular ion and are accurate to within 0.2 amu.

Results

Proteolytic Digestions of the 20-kDa Fragment and Reductase. The 20-kDa fragment isolated by HPLC using a μBondapak phenyl column was derivatized by carboxymethylation. The tryptic peptide map of the carboxymethylated protein was obtained by using an Ultrasphere C8 column (supplementary material; see paragraph at end of paper regarding supplementary material). Approximately 20 peptides were isolated and further purified by rechromatog-

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; FAB/MS, fast atom bombardment mass spectrometry; FNR, ferredoxin–$NADP^+$ reductase; GR, glutathione reductase; HPLC, high-performance liquid chromatography; HFBA, heptafluorobutyric acid; PTH, phenylthiohydantoin; PITC, phenyl isothiocyanate; pHBH, *p*-hydroxybenzoate hydroxylase; S-PITC, sulfophenyl isothiocyanate; *S. aureus*, *Staphylococcus aureus*; TPCK, tosylphenylalanyl chloromethyl ketone; TFA, trifluoroacetic acid; TEA, triethylamine; kDa, kilodalton(s); Tris, tris(hydroxymethyl)aminomethane.

Table I: Amino Acid Compositions of NH_2-Terminal Peptides

amino acid	SAI	SAIAC3
Asp	3.0 (3)	2.0 (2)
Thr	3.3 (4)	3.3 (4)
Ser	1.7 (2)	1.6 (2)
Glu	1.1 (1)	1.2 (1)
Gly	2.2 (2)	1.2 (1)
Val	1.1 (1)	1.0 (1)
total	13	11
residue no.	1–13	3–13
mol wt, calcd	1283.5	
mass no.	1325.4	

raphy on a different column (i.e., Ultrasphere C18). In a similar way, the peptide maps of chymotryptic and *S. aureus* protease digests were obtained by using an Ultrasphere C8 column (supplementary material). However, in order to complete the structural analysis of the 20-kDa fragment, it was necessary to further derivatize it by 4-sulfophenyl isothiocyanate to increase the hydrophilicity. The tryptic peptide map of the whole enzyme was carried out on the Ultrasphere C8 or the Synchropak C8 column (supplementary material). Since cysteine residues were labeled with iodo[^{14}C]acetic acid, the cysteine-containing peptides were detected by measurement of radioactivity. Hydrophobic peptides were recovered from Synchropak RP C8 (300-μm particle size) in suitable yield. The *S. aureus* peptide mapping of the reductase was also carried out on a Synchropak C8 column (supplementary material). All of the peptide peaks were rechromatographed by a different column prior to amino acid analysis and sequencing. Amino acid compositional study was performed on all of the peptides. However, only compositions of tryptic peptides are presented in the tables (see the supplementary material).

Isolation of the NH_2-Terminal Blocked Peptides. The reductase was digested with *S. aureus* protease, and the digests were initially applied to a column of Dowex 50x8 (H form, 0.5 × 5 cm) equilibrated with 2 N acetic acid. The fraction which was eluted with 2 N acetic acid (4 mL) was directly applied to an Ultrasphere C8 column, followed by a linear gradient elution from buffer A to buffer B. Only one major peak was obtained. To obtain enough material, the *S. aureus* digest was applied to the same column without passing through the ion-exchange column (supplementary material). The first peak, SAI, was collected for further analysis. The amino acid composition of the peptide SAI is shown in Table I, exhibiting a high content of hydroxyamino acids.

Sequence Determination of the NH_2-Blocked Peptide. Peptide SAI was blocked at the NH_2-terminus on Edman degradation. However, after treatment of this peptide with 0.1% TFA at 110 °C for 2 h, a new NH_2 group was detectable (PTH–serine was detected). The acid-treated SAI was purified by HPLC using an Ultrasphere C8 column (supplementary material). Each peak was characterized, and it was found that SAIAC3 had the sequence Ser-Asn-Val-Asp-Thr-Gly-Thr-Thr-Thr-Ser-Glu. Sequence analysis data are summarized in Table II. A comparison of this sequence with the amino acid composition of peptide SAI revealed that two amino acids were lost: Gly and Asx. From the specificity of the acid cleavage reaction, the remaining sequence could be deduced as Gly-Asp. FAB/MS spectral analysis of the intact peptide SAI gave a value for the protonated molecular ion of 1325.4 (monoisotopic mass). The calculated value for the indicated sequence was 1283.5. The 42 mass unit difference between the calculated and observed mass values is consistant with an acetyl blocking group on the NH_2-terminus of the peptide. Thus, the complete sequence of peptide SAI could be determined as CH_3CO-Gly-Asp-Ser-Asn-Val-Asp-Thr-Gly-Thr-Thr-Thr-Ser-Glu.

Table II: Sequence Analysis of Peptide SAIAC3

residue	amino acid	PTH (pmol)
1	Ser	23
2	Asn	88
3	Val	75
4	Asp	69
5	Thr	11
6	Gly	41
7	Thr	12
8	Thr	10
9	Thr	12
10	Ser	4
11	Glu	5

Table III: Amino Acid Compositions of Porcine Liver NADPH–Cytochrome P-450 Reductase

amino acid	HCl hydrolysis[a]	sequence data
Asp	71	56
Asn		11
Thr	42	40
Ser	44	44
Glu	88	56
Gln		26
Pro	31	28
Gly	47	45
Ala	50	49
Val	39	50
Met	15	17
Ile	21	26
Leu	66	60
Tyr	28	32
Phe	26	27
Lys	35	36
His	19	18
Arg	39	39
Trp	9	9
Cys	8	8
total	678	677
mol wt (protein)		76600
mol wt (+FAD, FMN)		77908

[a] Data were taken from Haniu et al. (1984), corrected on the basis of total residues.

Sequence Analyses of the 20-kDa Fragment. Determination of the sequence of the 20-kDa fragment was performed by both automated and manual Edman degradations. Since preliminary results were reported previously (Haniu et al., 1985), only quantitative results are presented in the supplementary material.

Sequence Analyses of Whole Enzyme. Sequence analysis data of each peptide are summarized in the supplementary material tables. Each tryptic peptide is overlapped with the *S. aureus* peptides. The complete amino acid sequence of the detergent-solubilized NADPH–cytochrome P-450 reductase from porcine hepatic microsomes is shown in Figure 1. The amino acid composition of the total sequence is presented in Table III, compared with previously reported data exhibiting nearly consistent values with the sequence data.

Discussion

NADPH–cytochrome P-450 reductase has a unique function in the microsomal electron-transport systems for cytochrome P-450 monooxygenase and cytochrome b_5. Since this enzyme consists of four functional domains (NADPH, FMN, FAD, and membrane-anchoring or P-450 binding domains), an understanding of the relationships between structure and function requires the determination of the total structure and

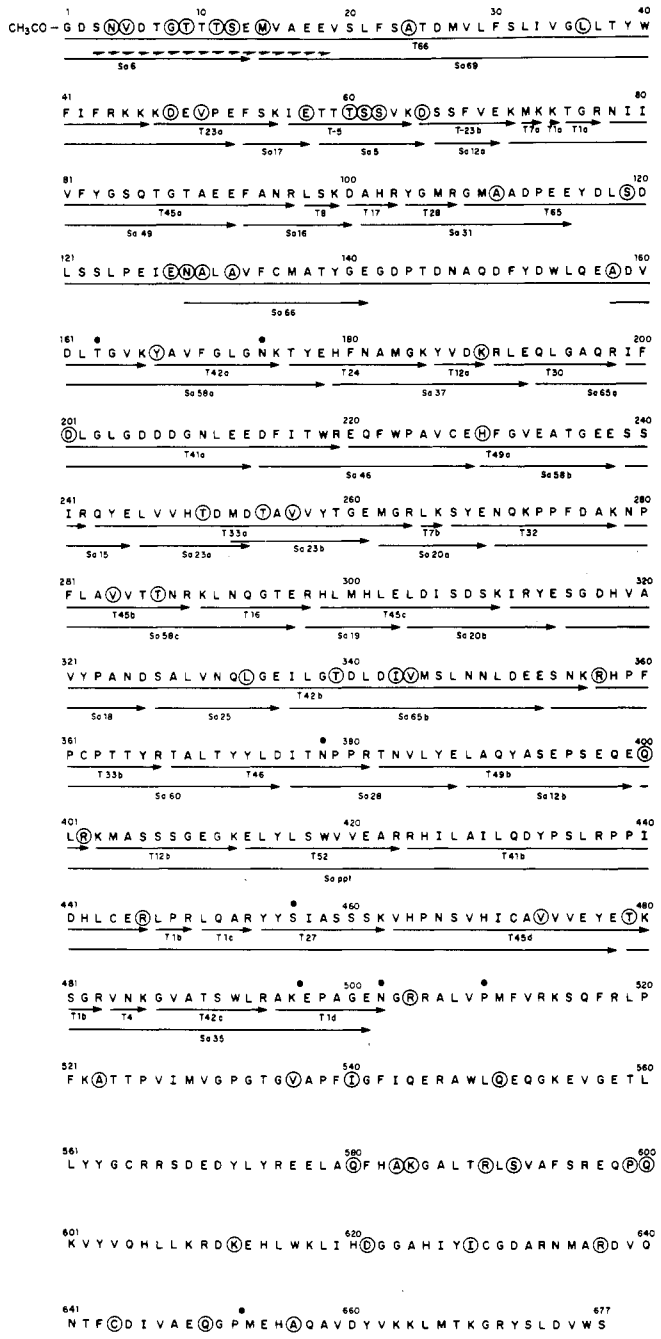

FIGURE 1: Total sequence of porcine hepatic NADPH–cytochrome P-450 reductase. Peptides generated by tryptic and *S. aureus* protease digestions of reductase are represented. The numbers show the order of elution from reverse-phase HPLC, and each peptide was sequenced by an automated gas phase sequencer, a spinning cup sequencer, or the manual method as shown by solid lines with arrows. Residues in circles represent the occurrence of substitution between the porcine sequence and the rat sequence. The closed circles show the differences from the previously reported partial sequence (Vogel et al., 1985). Since the COOH-terminal 20-kDa fragment of the reductase has already been published (Haniu et al., 1985), the sequencing strategy for the COOH-terminal region is omitted.

the identification of some functional amino acid residues (Lumper et al., 1980; Nishimoto & Shibata, 1982; Haniu et al., 1984; Porter & Kasper, 1985). The sequence study of the 20-kDa fragment revealed that it contains a cysteine residue essential for NADPH binding or catalytic function (Haniu et al., 1984). Figure 2 shows comparison of the nucleotide binding region of ferredoxin–$NADP^+$ reductase (Karplus et al., 1984) with the possible NADPH binding region of the cytochrome P-450 reductase. An NADPH-protected cysteine

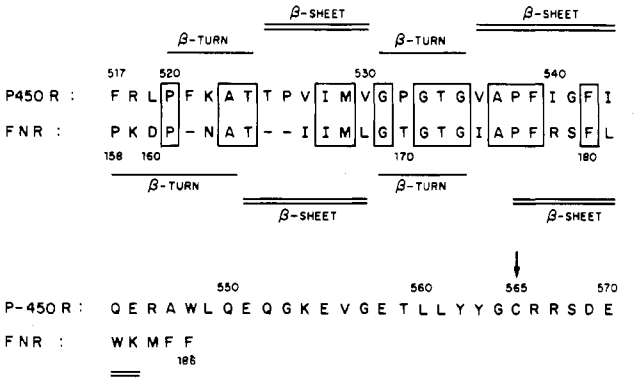

FIGURE 2: Sequence homology at nucleotide binding regions of NADPH–cytochrome P-450 reductase and ferredoxin–$NADP^+$ reductase. Identical residues are boxed. The predicted secondary structure was calculated by the Chou–Fasman method. The triple glycine sequence strongly suggests a β-turn structure for binding NADPH. Cysteine-565 is involved in protection of NADPH from alkylating reagents (Haniu et al., 1984; Vogel et al., 1985). Sequence data for spinach ferredoxin–$NADP^+$ reductase were derived from Karplus et al. (1984).

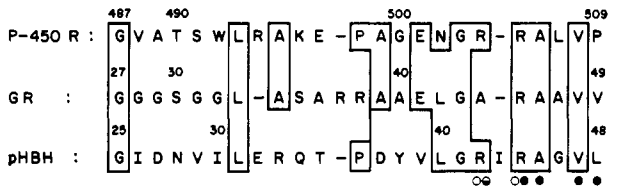

FIGURE 3: Sequence homology between NADPH–cytochrome P-450 reductase and glutathione reductase at proposed FAD binding regions. Residues 487–509 of P-450 reductase and FAD binding regions of glutathione reductase (GR) and *p*-hydroxybenzoate hydroxylase (pHBH) are compared. Circles, dark circles, and the half-dark circle show pyrophosphate, isoalloxazine, and ribosyl binding sites in *p*-hydroxybenzoate hydroxylase, respectively. Data for glutathione reductase and *p*-hydroxybenzoate hydroxylase are taken from Rice et al. (1984) and Weijer et al. (1983).

residue in the cytochrome P-450 reductase probably interacts with the nicotinamide moiety of NADPH and participates in the oxidation–reduction mechanism, as observed in glyceraldehyde-3-phosphate dehydrogenase (Moras et al., 1975). Porter and Kasper (1986) proposed the regions from residues 483–519 and 520–554 for NADPH binding sites in the rat enzyme. It was reported that residues 77–228 homologous to FMN binding regions of several flavodoxins conserved tyrosine (residue 177) which bound the alloxazine ring of FMN (Porter & Kasper, 1986). Tryptophan residues are proposed for FMN binding sites from the photooxidation study (Nishimoto & Shibata, 1982), whereas there is no evidence for tyrosine residues involved in the FMN interaction. The locations of the FAD binding sites are still speculative. Three regions are proposed for an FAD domain: residues 292–331, 452–477, and 503–537 (Porter & Kasper, 1985, 1986; Haniu et al., 1984). However, this is not surprising because multiple binding sites such as the isoalloxazine, pyrophosphate, ribose, and nicotinamide moieties are expected. Porter and Kasper (1985) proposed that histidine-469 was the FAD binding site. Our cysteine modification study in the presence or absence of FAD suggests that cysteine-471 was uniquely protected by this nucleotide moiety.[2] To obtain a better identification of the FAD binding sites, further biochemical experiments are necessary.

The amino-terminal residue of NADPH–cytochrome P-450 reductase was determined to be acetyl-Gly from this study.

[2] M. Haniu, M. Shields, T. Iyanagi, and J. E. Shively, unpublished results.

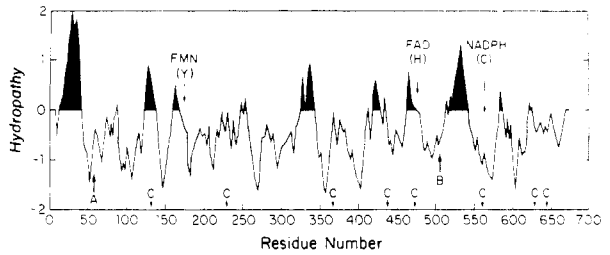

FIGURE 4: Hydropathy plot of porcine hepatic NADPH–cytochrome P-450 reductase and several functional domains. The hydropathy plot was calculated according to Kyte and Doolittle (1982) using a window of 12 residues by the computer program PRINAS (Protein Research Foundation, Osaka, Japan). Significant hydrophobic domains were observed at two regions: residues 27–43 (NH_2-terminal region) and residues 523–544 (near NADPH binding region). Vertical arrows (↑) represent cleavage sites by either trypsin (A) or an unknown type of mechanism (B). Cysteine residues are shown by C. Proposed FMN binding tyrosine and FAD binding histidine are shown according to Porter and Kasper (1985).

Although Black and Coon (1982) have reported the hydrophobic sequence near the NH_2-terminus and identification of an NH_2 blocking group, the NH_2-terminus of the mature protein has not been determined. The cDNA sequence study of the rat enzyme could not predict the correct NH_2-terminus of the mature protein (Porter & Kasper, 1985). The NH_2-terminal sequence of the porcine enzyme is homologous to the deduced rat sequence from cDNA except for a methionine residue which might be removed during the biosynthesis.

Unique sequences of triple hydroxyamino acids are observed in this reductase: Thr-Thr-Thr (residues 9–11 and 58–60), Ser-Ser-Ser (residues 406–408 and 459–461), and Ser-X-Thr-Y-Thr (residues 85–89). These residues may have a role in FMN phosphate binding (Porter & Kasper, 1986). Several acidic and basic regions are observed: Asp-Asp-Asp (residues 206–208), Glu-Glu-Asp (residues 212–214), Asp-Glu-Glu (residues 351–353), Asp-Glu-Asp (residues 569–571), Arg-Lys-Lys-Lys (residues 43–46), and Lys-Met-Lys-Lys (residues 71–74). Regions containing multiply charged residues are possible candidates for the protein–protein interaction with cytochromes b_5, c, or P-450 (Daily & Strittmatter, 1980).

A comparison of the porcine sequence to the rat sequence reveals 90% sequence homology: 60 amino acid differences per 677 total residues were found. The NH_2-terminal region (residues 1–13) ajacent to the proposed membrane binding domain is quite variable and is not likely involved in membrane or cytochrome P-450 binding. On the other hand, the proposed functional NADPH (residues 483–554), FAD (residues 292–331 and 452–477), and FMN (residues 77–100 and 133–188) domains are invariable. Minimum base changes of codens for most of the substitutions are single base changes: eight residues require double base changes, and one residue (Gln-Phe at residue 650) requires a triple base change (supplementary material).

A comparison of our sequence with reported partial sequences of porcine hepatic reductase (Vogel et al., 1985) reveals eight positions with amino acid differences. There are two positions with insertions (between residues 496–497 and 652–653) and one deletion (residue 509). Other differences are Asn instead of Asp at residues 174, 378, and 502, Thr instead of Ser at residue 163, and Ser instead of Gln at residue 456.

A hydropathy profile calculated according to Kyte and Doolittle (1982) is shown in Figure 4. Apparently, two regions (residues 27–43 and 523–544) exhibit significant hydrophobicity, suggesting membrane, cytochrome b_5, or cytochrome P-450 binding domains.

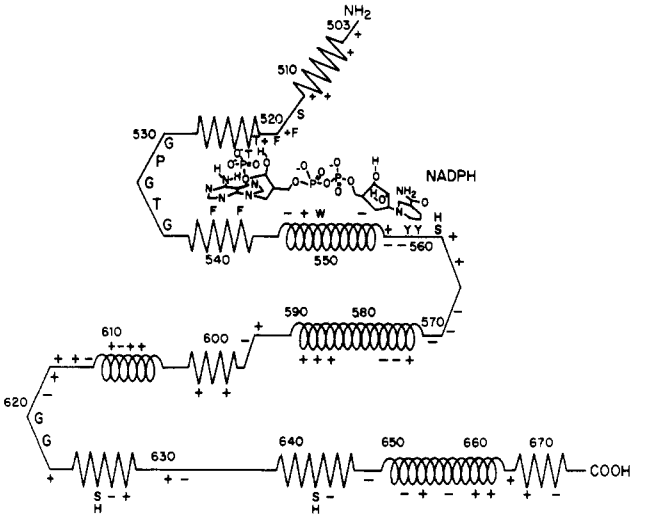

FIGURE 5: Predicted secondary structure of the reductase COOH-terminal domain calculated by Chou–Fasman and Robson methods. Common structures from both methods are taken in this figure. In this figure, α-helical regions are denoted by coils, β-sheets by zig-zag lines, and random coils by straight lines. NADPH is schematically drawn and is located in the homologous region to the nucleotide binding region of ferredoxin–$NADP^+$ reductase.

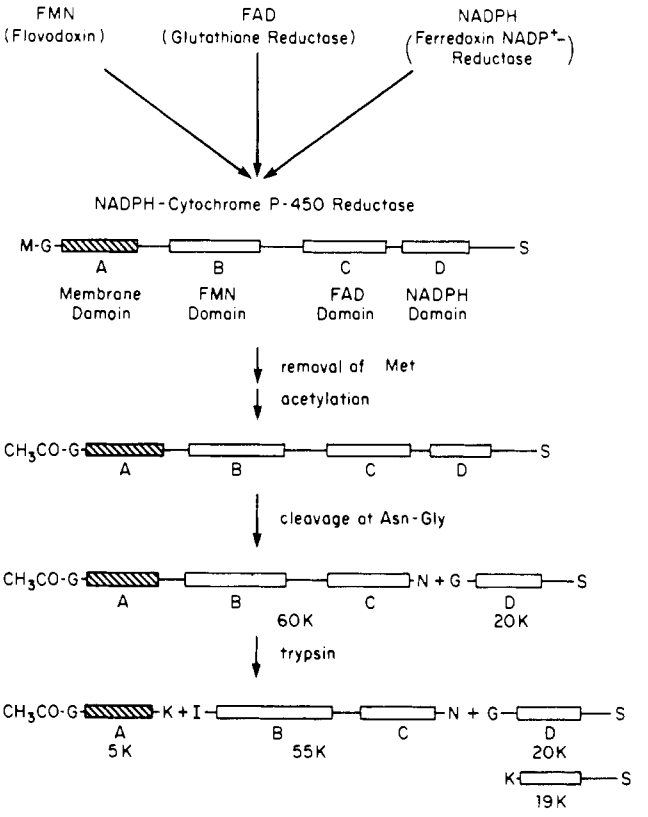

FIGURE 6: Schematical presentation on biosynthesis and degradation of porcine NADPH–cytochrome P-450 reductase. On the basis of the sequence homology with other flavoproteins, the cytochrome P-450 reductase gene may have evolved from genes encoding a flavodoxin domain (FMN), a glutathione reductase or p-hydroxybenzoate hydroxylase domain (FAD), and a ferredoxin–$NADP^+$ reductase domain (NADPH). The protein is synthesized as with the initiation methionine followed by 677 amino acid residues. Removal of methionine is followed by acetylation of the cytoplasmic side of the membrane. Porcine reductase is accessible to a protease or some unknown reaction which generates a 20-kDa fragment. The hydrophobic NH_2-terminal domain is easily cleaved with trypsin, subtilisin, or *S. aureus* protease (Black & Coon, 1982).

Secondary structure prediction is made only on the COOH-terminal 20-kDa fragment (residues 503–677) using

the Chou and Fasman (1978) and Robson (1974) methods as modified by Nishikawa (1983) (Figure 5). Although this portion is proposed as the NADPH binding domain, it is interesting to note that it does not retain a significant $\beta\alpha\beta$ structure which has been commonly observed in nucleotide binding regions of NADH or NADPH enzymes (Moras et al., 1975).

The mechanism of the formation of the 20-kDa fragment from the intact reductase is not understood. The Asn–Gly peptide bond at residues 502–503 was clearly cleaved during preparation of enzyme although no reagents were used which are known to accelerate Asn–Gly cleavages. Other investigators also reported similar degradation of the enzyme from pig liver or pig testes microsomes (Yasukochi et al., 1980; Kuwada et al., 1985). Interestingly, this cleavage was not observed in rat or rabbit enzyme preparations. The porcine enzyme contains the sequence Gly-Glu-Asn-Gly-*Arg*-Arg (residues 500–505) instead of Gly-Glu-Asn-Gly-*Gly*-Arg found in the rat sequence. The amino acid substitution of Arg for Gly (residue 504) may influence this cleavage.

Evolutionary aspects of this enzyme are interesting: the whole molecule contains four domains, three of which are structurally related to flavodoxin (FMN), glutathione reductase or *p*-hydroxybenzoate hydroxylase (FAD), and ferredoxin–$NADP^+$ reductase (NADPH). Figure 6 summarizes the possible construction of the reductase from the evolutionary point of view, biosynthesis, and proteolytic fragmentation.

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Supplementary Material Available

HPLC tryptic peptide map of the 20-kDa fragment of reductase (Figure 1), HPLC chymotryptic peptide map of the 20-kDa fragment (Figure 2), HPLC *S. aureus* protease map of the 20-kDa fragment (Figure 3), HPLC tryptic peptide map of the reductase (Figure 4), HPLC *S. aureus* peptide map of the reductase (Figure 5), isolation of the NH_2-terminal peptide by HPLC (Figure 6), HPLC map of acid cleavage products of peptide SAI (Figure 7), amino acid compositions of tryptic peptides from the 20-kDa fragment [peptide numbers (20T4, 20T5, etc.) are derived from Figure 1] (Table I), amino acid compositions of tryptic peptides from the reductase (peptide numbers are derived from Figure 4) (Table II), sequence analyses of peptides (Table III), sequence differences between porcine enzyme and rat enzyme (Table IV), and sequence differences from reported sequence (Table V) (36 pages). Ordering information is given on any current masthead page.

Registry No. NADPH–cytochrome P-450 reductase (pig liver microsome reduced), 104760-96-5; NADPH–cytochrome P-450 reductase, 9039-06-9.

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