Structural Analysis of NADPH-Cytochrome P-450 Reductase from Porcine Hepatic Microsomes

SEQUENCES OF PROTEOLYTIC FRAGMENTS, CYSTEINE-CONTAINING PEPTIDES, AND A NADPH-PROTECTED CYSTEINE PEPTIDE*

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Detergent-solubilized NADPH-cytochrome P-450 reductase was purified from porcine hepatic microsomes and compared to the rabbit enzyme isolated under identical conditions. The porcine enzyme had an equivalent specific activity toward cytochrome c compared to the rabbit enzyme. When analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the porcine enzyme exhibited a major band at Mr = 80,000 and two additional bands at Mr = 20,000 and 60,000. The 20-kDa fragment was shown to be the COOH-terminal portion of the protein which contains a hydrophobic sequence of 28 residues homologous to the pyrophosphate-binding portion of the FAD-binding protein p-hydroxybenzoate hydroxylase. The 60-kDa fragment corresponded to the NH2-terminal portion of the protein since this peptide and the intact protein have blocked NH2 termini. The trypsin-solubilized porcine enzyme has an NH2-terminal sequence which is homologous to the equivalent trypsin-solubilized enzymes from rat and rabbit (80% sequence homology).

Eight cysteine-containing peptides were isolated from a tryptic digest of the S-carboxymethylated pig enzyme. Significant sequence homology was not found between these peptides and other flavoproteins, except for one peptide (Glu-Val-Gly-Glu-Thr-Leu-Leu-Tyr-Tyr-Gly-Cys-Arg) which exhibited partial homology with the known NADPH-binding site of glutathione reductase. When the NADPH-protected enzyme was first S-alkylated with unlabeled iodoacetate, NADPH depleted, and further alkylated with 14C-labeled iodoacetate, the above radiolabeled peptide was isolated from a tryptic digest. The equivalent peptide was also isolated by a similar procedure from rabbit liver cytochrome P-450 reductase.

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The hepatic NADPH-cytochrome P-450 reductase (EC 1.6.4) participates as an electron carrier in the hydroxylation of various endogenous and foreign compounds (Williams, 1976). The detergent-solubilized enzyme contains one molecule each of FAD and FMN per subunit in every system studied and has a molecular weight of 76,000–79,500 (Iyanagi and Mason, 1973, Enoch and Strittmatter, 1979). These flavins participate in electron transport from NADPH to cytochrome P-450 in a phospholipid environment. In contrast to cytochromes P-450, the reductase does not exhibit substrate specificity, multiplicity of enzyme form, or significant inducibility by drugs. Earlier studies have suggested that cytochrome b5 can be reduced by NADPH-cytochrome P-450 reductase and that reduced cytochrome b5 may provide the second electron in cytochrome P-450-catalyzed reactions (Enoch and Strittmatter, 1979).

Structural studies on rabbit or rat liver reductase have revealed that the detergent-solubilized enzyme contains a hydrophobic domain in the NH2-terminal region (Black and Coon, 1982; Gum and Strobel, 1981). The protease-solubilized enzyme is missing this hydrophobic domain, and it has been suggested that this fragment may be involved in binding the microsomal membrane or cytochrome P-450 (Black and Coon, 1982). The protease-solubilized enzyme retained the ability to transfer electrons to the artificial acceptor, cytochrome c, but not to cytochrome P-450. Nishimoto and Shibata (1982) reported that four sulphydryl groups were modified after treatment with a low concentration of p-hydroxychloromercuribenzoate without loss of enzyme activity. A fifth sulphydryl group was modified at a higher concentration of the reagent, resulting in the complete loss of enzymatic activity. In the presence of NADP+ or 2'-AMP, 1 cysteinyl residue was protected against p-hydroxychloromercuribenzoate titration. This derivative retained enzymatic activity and was completely inactivated by further treatment with sulphydryl reagents. These results suggested that there is an essential sulphydryl group at or near the NADPH-binding site with which FAD or FMN might interact. It was predicted by Nishimoto and Shibata (1982) that the FMN-binding domain would be structurally homologous to that of flavodoxin, a small flavoprotein.

In order to test these possibilities, extensive structural studies are required. Gonzalez and Kasper (1983) have cloned the cDNA complimentary to the mRNA of rat liver NADPH-cytochrome P-450 reductase and determined DNA sequences around the 5' cap site, but were not able to find the TATA or CTCAA box sequences, or a transcription initiation sequence, or a translation initiation codon. We report here protein structural studies of NADPH-cytochrome P-450 reductase isolated from porcine hepatic microsomes. In addition to the native and trypsin-solubilized enzymes, we have characterized a COOH-terminal fragment which has a molecular weight of 20,000. A hydrophobic domain of this fragment was compared with the hydrophobic region of rabbit liver enzyme and the flavin-binding domains in several flavoproteins. We have also characterized the amino acid sequences around each cysteine
residue of the enzyme and identified a unique cysteine residue which is protected by NADPH from alkylation.

EXPERIMENTAL PROCEDURES

RESULTS

Preparation of Cytochrome P-450 Reductase—The porcine and rabbit enzymes were purified by a combination of affinity chromatography on 2',5-ADP-Sepharose and hydroxylapatite column chromatography according to the method of Iyanagi et al. (1981). The overall yield of the purified porcine reductase was about 30%. Flavin content was measured from the ratio of absorbance at 278 nm to absorbance at 455 nm. The value obtained, 8.7, was similar to that found by Nishimoto and Shibata (1982). Based on the reduction of cytochrome c, the detergent-solubilized porcine and rabbit liver enzymes had equivalent specific activities. Although the rabbit enzyme showed one major band on analysis by NaDodSO₄-polyacrylamide slab gel electrophoresis, (Fig. 1A), the porcine enzyme showed three major bands (Fig. 1D) corresponding to molecular weights of 80,000 (80K), 60,000 (60K), and 20,000 (20K). Since the A₂₇₈nm/A₄₅₅nm ratio was in agreement with previously reported values (Nishimoto and Shibata, 1982) and the enzyme retained full activity, it was considered likely that the 20- and 60-K fragments of the porcine liver enzyme were derived from the native protein (80K). This possibility was examined further by the isolation and characterization of the fragments. Seven separate preparations of the porcine enzyme showed similar electrophoretic patterns, except that in some cases the 20-K fragment was further split into a doublet at 20 and 19K. Although these fragments were probably derived from proteolytic cleavage of the native enzyme during enzyme purification, both the 20- and 60-K fragments copurified with the native enzyme and retained the ability to bind both FMN and FAD. Treatment with NaDodSO₄ at 100 °C appeared to dissociate the proteolytically cleaved enzyme into the 20-K and 60-K fragments. Addition of the protease inhibitors phenylmethylsulfonyl fluoride, leupeptin, and peptatin A to the microsomal fraction during purification did not prevent the formation of the fragments in the porcine liver preparation. These results suggested that porcine liver microsomes may contain a unique protease which partially cleaves the reductase into two fragments with no effect on its catalytic activity. The enzyme was also prepared by trypsin solubilization from the microsomal preparation (Fig. 1E). The trypsin-solubilized enzyme exhibits bands at 75, 55, and 20K. These results are consistent with the interpretation that the 80-K protein and the 60-K fragment are reduced in molecular weight by 5K by trypsin solubilization and that the 20-K fragment is unaffected.

Separation of Reductase Fragments by HPLC—The separation of the reductase smaller fragments (20K and 60K) from detergent-solubilized enzyme was performed by HPLC on an

Fig. 1. Electrophoretic behavior of porcine and rabbit liver cytochrome P-450 reductases. NaDodSO₄-polyacrylamide gels A through C show increasing loading of the detergent-solubilized enzyme from rabbit liver microsomes (1.4 μg, 3.5 μg, and 10.5 μg, respectively). Gels D and E show the detergent- and trypsin-solubilized porcine enzymes (10 μg each). Determination of molecular weight was according to mobility of standard proteins.

Fig. 2. Purification of proteolytic fragments from porcine cytochrome P-450 reductase. A, HPLC of the detergent-solubilized porcine enzyme. Enzyme solution (0.95 mg, 0.2 ml) containing 10% glycerol and 0.1% Triton N-101 was applied to an alkylphenyl column (300 × 4.1 mm) equilibrated with 0.1% trifluoroacetic acid (pH 2.0). A linear gradient was run from solvent I (0.1% trifluoroacetic acid, pH 2.0) to solvent II (trifluoroacetic acid/H₂O/CH₃CN = 0.1:9:90, v/v/v) over 90 min. Flow rate was 0.9 ml/min. Insets show NaDodSO₄-gel electrophoresis of each peak from HPLC. B, HPLC of the trypsin-solubilized porcine enzyme. Experimental conditions are same as above.

alkylphenyl column (Fig. 2A). The small double peaks eluted early in the chromatogram were identified as dissociable FAD and FMN by inspection of their absorption and fluorescence spectra. The last peak eluted was identified as dissociated detergent. NaDodSO₄-gel electrophoresis of each numbered peak and comparison to standard markers revealed that peaks 1, 2, and 4 corresponded to fragments 20, 80, and 60K, respectively. Peak 3 contained an aggregate of the 60- and 80-

1 Portions of this paper (including "Experimental Procedures," part of "Results," Tables S-I-S-IX, and Figs. S-1 and S-2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, Bethesda, MD 20814. Request Document No. 84M-768, cite the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

The abbreviations used are: NaDodSO₄, sodium dodecyl sulfate; PTH, phenylthiohydantoin; HPLC, high-performance liquid chromatography; ODS, octadecyl silane. PMSF, phenylmethylsulfonyl fluoride; pCMB, p-hydroxychloromercuribenzoate; TPCK, tosylphenylalanyl chloromethyl ketone.
K proteins. These gels demonstrate the high degree of purity of the chromatographed enzyme, since no traces of contaminants were identified.

Trypsin-solubilized enzyme was chromatographed in a similar way as shown in Fig. 2B. Each peak was analyzed by NaDodSO₄-gel electrophoresis. The three major peaks correspond to the 20-, 75-, and 55-K fragments, respectively.

Amino Acid Analysis—Amino acid compositions of the porcine liver microsomes and its fragments were determined after performic acid oxidation. As shown in Table I, the 20-K fragment contained 3 cysteic acid residues, whereas the 60-K fragment had 5 cysteic acid residues. The sum of these values was consistent with that obtained from the native enzyme. Within the experimental limits of amino acid compositional analysis, the sum of the amino acids of the 60- and 20-K fragments is equal to the composition of the 80-K intact protein. Table I also shows the comparison of porcine and rabbit reductase which, in general, are similar except for the number of cysteine residues. Although 6 cysteine residues have been reported for porcine protease-solubilized enzyme (Lumper et al., 1980), 3 cysteine residues were found from our detergent-solubilized enzyme, based on the molecular weight of 80,000.

The amino acid compositions of the trypsin-solubilized enzyme and its fragments from porcine liver microsomes (Table SI) revealed that the 75-K fragment has a slight decrease in hydrophobic amino acid content, which might be due to the loss of the NH₂-terminal hydrophobic region as described in the rabbit liver enzyme (Black and Coon, 1982).

### Table I

| Amino acid | Porcine, 20-K fragment | Porcine, 60-K fragment | Porcine, reductase (80K) | Rabbit, reductase (77K)* |
|------------|------------------------|-----------------------|-------------------------|--------------------------|
| Asx        | 12                     | 64                    | 76                      | 64                       |
| Thr        | 8                      | 36                    | 45                      | 33                       |
| Ser        | 7                      | 39                    | 47                      | 41                       |
| Glx        | 25                     | 71                    | 94                      | 82                       |
| Pro        | 8                      | 22                    | 34                      | 29                       |
| Gly        | 15                     | 36                    | 50                      | 44                       |
| Ala        | 15                     | 39                    | 54                      | 56                       |
| Val        | 12                     | 29                    | 42                      | 44                       |
| Met*       | 5                      | 12                    | 16                      | 14                       |
| Ile        | 6                      | 18                    | 22                      | 21                       |
| Leu        | 17                     | 52                    | 70                      | 57                       |
| Tyr        | 6                      | 23                    | 30                      | 28                       |
| Phe        | 6                      | 20                    | 28                      | 25                       |
| Lys        | 10                     | 25                    | 35                      | 30                       |
| His        | 7                      | 13                    | 20                      | 15                       |
| Arg        | 14                     | 25                    | 39                      | 34                       |
| Trp*       | (4)                    | (6)                   | (10)                    | 6                        |
| Cys*       | 3                      | 5                     | 8                       | 10                       |
| Total      | 180                    | 535                   | 720                     | 633                      |

*| Taken from French and Coon (1979).

+a Determined as glutathione sulfone.

* Not accurate due to destruction of Trp on HCl hydrolysis.

* Determined as cysteic acid.

Analysis by NaDodSO₄-polyacrylamide gel electrophoresis (Fig. 1) revealed that 5 kDa of peptide were lost from the native enzyme by trypsin cleavage. Amino acid analysis reveals that the 20-K fragment from trypsin-solubilized enzyme is probably the same fragment as that obtained from detergent-solubilized enzyme, thus supporting the conclusion that the large fragment is from the NH₂-terminal portion of the enzyme, and the small fragment is from the COOH-terminal portion.

NH₂-terminal and COOH-terminal Analyses of Reductase and the Fragments—All three purified fragments (20, 60, and 80K) were subjected to microsequence analysis. Sequence data for the 20-K fragment are summarized in Fig. 3. Approximately 50 residues were identified, of which only 6 were basic amino acid residues. The remaining residues were either hydrophobic or neutral. The sequence from residues 21 to 48 contained a hydrophobic domain, and a number of aromatic amino acids were found from residues 37 to 46. When this sequence was compared with the flavin-binding domain of other flavoproteins, the location of two homologous sequences in p-hydroxybenzoate hydroxylase was noted (Fig. 4). These sequences include the pyrophosphate-binding portion of FAD and the so-called "glycine rich" region. Glycine residues are important in the FAD-binding domain of many flavoproteins (Hofsteenge et al., 1980). Flavin-binding proteins contain the invariant sequence Gly-X-Gly-Y-Z-Gly. The 20-K fragment of NADPH-cytochrome P-450 reductase has a Gly-X-Gly-Y-Gly sequence which is homologous to those of flavodoxin, lactate dehydrogenase, p-hydroxybenzoate hydroxylase, and L-amino acid oxidase (only one of which is shown in Fig. 4).

The NH₂ termini of the 60- and 80-K proteins were blocked as determined by automated Edman degradation. Since the NH₂ terminus of the rabbit enzyme is blocked by an N-acetyl group (Black and Coon, 1982), this finding is not surprising.

The NH₂-terminal sequence of the trypsin-solubilized porcine enzyme (75K) is shown in Fig. 5 and compared to the

FIG. 3. NH₂-terminal sequence of the 20-K fragment. Basic amino acids are denoted by positive charge. Nonpolar and hydrophobic regions are underlined. Arrow shows trypsin cleavage site.
corresponding sequences from the rat and rabbit enzymes. The striking homology among the NH₂-terminal regions of all three enzymes suggests that the NH₂-terminal region is highly conserved, including the sequence which is accessible to trypsin in the native enzyme. Since the 55-K fragment gave an identical NH₂-terminal sequence to that of the 75-K fragment, the 55-K fragment derived from the 75-K fragment by proteolysis, lacking the 20-K COOH-terminal fragment.

COOH-terminal sequence analyses on each fragment showed that both the 20- and 80-K proteins have an unambiguous serine residue by both carboxypeptidase digestion and hydrazinolysis (Table S-IV). However, the COOH terminus of the 60-K fragment was not obtainable by the same methods. Since the 20-K fragment contained the same COOH terminus as the 80-K protein, it is likely that the 20-K fragment is the COOH-terminal fragment of the reductase. A scheme showing

![Diagram of P-450 Reductase](image)

**Fig. 6.** Possible orientation of each fragment in NADPH-cytochrome P-450 reductase from pig liver. Arrows show the site of proteolytic cleavage; X denotes undetermined residue.

Cysteine-containing Peptides—Eight cysteine-containing peptides were isolated from the tryptic map of reduced and S-alkylated porcine reductase 80-K fragment (Fig. 8). Each of the peptides contained a cysteine residue (Fig. 7). Identifying the NADPH-protected Cysteine Residue—NADPH-cytochrome P-450 reductase was first S-alkylated with unlabeled iodoacetate under native conditions in the presence of an equimolar amount of NADPH. NADPH was removed by gel filtration, and the enzyme was S-alkylated

![Diagram of cysteine-containing tryptic fragments](image)

**Fig. 7.** Sequences of cysteine-containing tryptic fragments. Aromatic residues (tyrosine and tryptophan) are boxed in and hydrophobic or nonpolar regions are indicated by wavy lines. Cysteine residues are shown in boldface.

![Graph](image)

**Fig. 8.** A, NADPH-protected cysteine residue in NADPH-cytochrome P-450 reductase. The protein was S-alkylated with unlabeled iodoacetate in the presence of equimolar NADPH, NADPH depleted, S-alkylated with ¹⁴C-labeled iodoacetate, digested with trypsin, and the resulting peptides separated by reverse-phase HPLC on an Ultrasphere C-8 column. The flow rate was 0.8 ml/min and the gradient from 100% solvent I (0.1% trifluoroacetic acid) to 70% solvent II (trifluoroacetic acid/H₂O/CH₃CN = 0.1:9.9:90, v/v/v) over 90 min. B, purification of NADPH-protected cysteine peptide in rabbit enzyme. Experimental conditions are same as in porcine enzyme.
with $^{14}$C-labeled iodoacetate under native conditions and later with unlabeled iodoacetate under denaturing conditions. The identification of the protected sulfhydryl group was performed by isolating the specifically radiolabeled tryptic peptide by reverse-phase HPLC (Fig. 6A). Only one radiolabeled peptide (T-49) was detected from the NADPH-protected enzyme. The calculated recovery for the $^{14}$C label showed that this radiolabeled peptide contained more than 80% of total original radioactivity. Thus, there is no evidence for additional specifically labeled cysteine residues. A similar experiment was performed on the rabbit enzyme. The tryptic peptide map is shown in Fig. 6. This scheme agrees with data obtained by amino acid compositions, NH$_2$-terminal sequence analysis, and HPLC-terminal analysis of each fragment.

In addition to the 20-K fragment, a smaller fragment (approximately 19K) was detected as a minor component in some cases. This fragment was clearly derived from the native enzyme by proteolytic cleavage. The postulated relationship between these fragments is shown in Fig. 6. This scheme agrees with data obtained by amino acid compositions, NH$_2$-terminal sequence analysis, and COOH-terminal analysis of each fragment.

Although the NH$_2$-terminal sequence of the 20-K fragment was shown to be quite hydrophobic, it was not necessary to isolate this fragment by gel-permeation chromatography on Sephadex LH using organic solvents as used by Black and Coon (1982) for the isolation of the rabbit enzyme NH$_2$-terminal domain. The fragments were easily separated on reverse-phase (alkylphenyl or SynChropak RPP) columns. Under these conditions, a portion of the 60-K fragment still remains noncovalently associated with the protein since these prothetetic groups were completely dissociated under acidic conditions (pH 2.0). This finding is similar to that observed for glutathione reductase, in which FAD was removed by a combination of a high salt concentration and acidic conditions (pH 3.0) (Icen, 1967). The tertiary structure of glutathione reductase as determined by x-ray crystallography indicates that the NH$_2$-terminal region forms a $\beta$-barrel in the secondary structure that interacts with portions of the adenosyl moeity of the FAD (Thieme et al., 1981). Similar observations were made for p-hydroxybenzoate hydroxylase (Hofsteenge et al., 1980) and $\delta$-amino acid oxidase (Ronchi et al., 1982). The invariant Gly-X-Gly-Y-Z-Gly sequence is found in these flavoproteins. The 20-K fragment contains a similar sequence, Gly-X-Gly-Y-Gly. The glycine residues have important structural roles, as in a portion of the nucleotide-binding domain (Hofsteenge et al., 1980) in maintaining the appropriate secondary or tertiary structure for FAD binding. However, it is premature to discuss the interaction of the flavin with the protein solely from this sequence homology. Further studies, including primary and tertiary structural analysis of cytochrome P-450 reductase, are necessary to fully elucidate the interaction.

This report also describes the sequence around each of the 8 cysteine residues in the porcine liver enzyme. A comparison of the cysteine-containing peptides to other types of flavoprotein.

![Diagram of peptide T-49 to a portion of the NADPH-binding region of glutathione reductase](image)

**Fig. 9.** A comparison of peptide T-49 to a portion of the NADPH-binding region of glutathione reductase. The portion of glutathione reductase shown (281–291) is only a small portion of the NADPH-binding site and is believed to be near the adenine moiety in NADPH (Thieme et al., 1981). Cysteine residues are circled, aromatic residues are boxed in, and charged residues are denoted with plus or minus signs. Homologous residues are denoted with vertical lines, and hydrophobic or nonpolar regions are shown by wavy lines. The $\beta$-sheet plots are shown in Fig. S2 (see Miniprint).
teins was made including glutathione reductase, d-amino acid oxidase, p-hydroxybenzoate hydroxylase, and flavodoxins. However, since these flavoproteins are slightly different from NADPH-cytochrome P-450 reductase with respect to the function of sulphydryl groups, it was not anticipated that a high degree of sequence homology would be seen. Nonetheless, we have noted that one peptide (T-49) of NADPH-cytochrome P-450 reductase is homologous to the NADPH-binding region of glutathione reductase (Fig. 9). It is interesting to note that both peptides conserve similar charged groups and hydrophobic sequences, including the location of an aromatic amino acid, which in the case of glutathione reductase is close to the adenine moiety of NADPH (Thieme et al., 1981). One exception to the homologous placement of residues is the location of the cysteines which are inverted within the hydrophobic region. Tryptophan 287 in glutathione reductase was determined to be adjacent to the adenine moiety of NADPH by x-ray crystallographic studies (Thieme et al., 1981), while NADPH-cytochrome P-450 reductase contains tyrosine at the corresponding position. Since the significance of an aromatic residue at this position is not clear and the degree of overall homology is not extensive, we can only hypothesize at this point that the two regions serve similar functions.

It has been shown that a cysteine residue has a functional role in NADPH binding in NADPH-cytochrome P-450 reductase (Nishimoto and Shibata, 1982). However, it is not known whether the cysteine residue at the NADPH-binding region of glutathione reductase is involved in the active site. This homology may suggest that it is, but not in the same way as in NADPH-cytochrome P-450 reductase. The fact that this critical cysteine residue is protected from S-alkylation in the presence of NADPH strongly suggests that it is very close to or actually in the NADPH-binding site. Also, arginine residues are reported to be essential for enzymatic activity (Sugiyama et al., 1983). It is likely that positively charged groups may form stabilizing salt bridges to the acidic pyrophosphate group of NADPH. The conservation of the acidic groups (Asp and Glu) may indicate that these negatively charged residues may interact with the positively charged groups of NADPH. A calculation of secondary structure according to Chou and Fasman (1978) revealed that α-sheet may be predominant in both regions (Fig. S2). Indeed, x-ray crystallographic studies have shown that the NADPH-binding region in glutathione reductase contains α-sheet structure. These findings are consistent with the idea that both active sites contain a similar conformation around the NADPH and strengthen an earlier proposal that NADPH-cytochrome P-450 reductase may contain both a glutathione reductase-like domain and a flavodoxin-like domain (Nishimoto and Shibata, 1982). Although it has been reported that FAD might be located near the active site, this study could not account for the interaction of FAD and NADPH. From our studies, it is hypothesized that the cysteine residue of peptide T-49 is involved in (or adjacent to) NADPH binding but not in FAD binding, since the flavin moiety was still bound even after removal of NADPH by gel filtration. Therefore, [14C]iodoacetate alkylation was performed in the presence of both FAD and FMN.

More extensive active-site and x-ray crystallographic studies are required before the exact nature of the protein-NADPH interactions in NADPH-cytochrome P-450 reductase can be elucidated. But even at this early stage, it is possible to speculate on the nature of these interactions and to design experiments to test them. In this context, we would like to propose at least one possibility which can easily be tested. Is it possible that the sulphydryl group(s) in NADPH-cytochrome P-450 reductase participate in some interaction with cytochrome P-450 in the same manner that glutathione reductase interacts with glutathione? If so, the sulphydryl group(s) play a key role in the redox mechanism of cytochrome P-450.

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**P-450 Reductase from Pig Liver**

**Supplementary Material**

**Structural Analysis of NADPH-Cytochrome P-450 Reductase from Porcine Hepatic Microsomal Protein Fractions.**

**Cysteine Content**

- The enzyme was obtained by dialysis against water.
- The mixture was then diluted with water containing 2 mg/ml of carboxypeptidase A and a mixture of trypsin and cholesterol per liter.
- The active fraction was eluted with buffer A containing 0.2% acetic acid and 0.5 M guanidine hydrochloride.
- The active fraction was then dialyzed against water containing 2 mg/ml of carboxypeptidase A and a mixture of trypsin and cholesterol per liter.
- The active fraction was applied to a Sepharose 4B column and a hydroxylapatite column, respectively.
- The active fraction was applied to a Sepharose 4B column and a hydroxylapatite column, respectively.

**Analytical Methods**

- A 250 M solution was passed through Sephadex G-50 column (5 x 10 cm) to remove the NADPH. The NADPH-free derivative was immediately alkylated with NEM-4-acetic acid (New England Nuclear Co., 20 a) dissolved in 0.5 ml of buffer A at 4°C.

**Protein Analysis**

- A 250 M solution was passed through a DEAE-cellulose column and a hydroxylapatite column, respectively.
- A 250 M solution was passed through a DEAE-cellulose column and a hydroxylapatite column, respectively.

**Tryptophan Content**

- A 250 M solution was passed through a DEAE-cellulose column and a hydroxylapatite column, respectively.

**RESULTS**

- A 250 M solution was passed through a DEAE-cellulose column and a hydroxylapatite column, respectively.

**Tryptophan Content**

| Amino acid | % Trp | Value |
|------------|-------|-------|
| Arg        | 11    | 22    |
| Asp        | 19    | 41    |
| Asx        | 12    | 24    |
| Pro        | 10    | 22    |
| Lys        | 15    | 31    |
| Val        | 10    | 20    |
| Thr        | 10    | 20    |
| Leu        | 15    | 31    |
| Ile        | 10    | 20    |
| Ala        | 15    | 31    |

**Acid Analysis**

- A 250 M solution was passed through a DEAE-cellulose column and a hydroxylapatite column, respectively.

**Transthyretin Content**

- A 250 M solution was passed through a DEAE-cellulose column and a hydroxylapatite column, respectively.

**Tryptophan Content**

| Amino acid | % Trp | Value |
|------------|-------|-------|
| Arg        | 11    | 22    |
| Asp        | 19    | 41    |
| Asx        | 12    | 24    |
| Pro        | 10    | 22    |
| Lys        | 15    | 31    |
| Val        | 10    | 20    |
| Thr        | 10    | 20    |
| Leu        | 15    | 31    |
| Ile        | 10    | 20    |
| Ala        | 15    | 31    |

**Acid Analysis**

- A 250 M solution was passed through a DEAE-cellulose column and a hydroxylapatite column, respectively.

**Transthyretin Content**

- A 250 M solution was passed through a DEAE-cellulose column and a hydroxylapatite column, respectively.

**Tryptophan Content**

| Amino acid | % Trp | Value |
|------------|-------|-------|
| Arg        | 11    | 22    |
| Asp        | 19    | 41    |
| Asx        | 12    | 24    |
| Pro        | 10    | 22    |
| Lys        | 15    | 31    |
| Val        | 10    | 20    |
| Thr        | 10    | 20    |
| Leu        | 15    | 31    |
| Ile        | 10    | 20    |
| Ala        | 15    | 31    |
by either standard chromatography steps or affinity chromatography on Figure 52. The secondary structure of the homologous peptides to porcine NADPH-cytochrome P-450 reductase and glutathione reductase are shown in Figure 52.

Regarding the purine and the proteolysis of cytochrome P-450 reductase

The rabbit liver enzyme can be purified to apparent homogeneity by either standard chromatography steps or affinity chromatography on 2.5-Sephadex-Sepharose. In actual fact, if the protein is overloaded on NaDodSO₄-gels traces of proteolytic cleavage products are observable. Figure 1 shown increased loading on a NaDodSO₄-gel of 1.4 ug (A), 3.5 ug (B), and 10.5 ug (C). Clearly, the highest loading traces impurities can be detected. In spite of this, the protein is greater than 99% pure, a purity sufficient for certain enzymatic studies. The point is that the rabbit enzyme does contain traces of proteolytic fragments, at least one of which is similar to major fragments found in the porcine preparations. Previous publications by experts on the rabbit enzyme exhibit NaDodSO₄-gels with a minimum of enzyme loaded (0.5 - 1.5 ug). To our knowledge, no one has ever published an NaDodSO₄-gel of the porcine enzyme even though it can be purified to an equivalent specific activity compared to the rabbit enzyme.

In our report we help to clarify the issue of protein purity of the porcine enzyme. It is fully active on a weight basis but on NaDodSO₄-gels electrophoresis reveals three major bands (lane 5). The A8 band is evidently the native enzyme, and the two other bands are due to proteolysis which cannot be eliminated even with the addition of protease inhibitors during purification. A similar story is evident in the tryptic solubilized enzyme (lane 1). Since the porcine enzyme is rather unusual in yielding these fragments even though it was purified exactly the same way as the rabbit enzyme, we decided to investigate the structure of the fragments in terms of their relationship to the native enzyme.

We are aware of the past history in the literature regarding the controversy over the presence or absence of fragments in the rabbit system. We were the first to point out even in the best preparations of rabbit enzyme, traces of fragments do exist, but this fact can be obscured by underloading NaDodSO₄-gels. In the case of the porcine enzyme, the fragments are clearly derived from the native enzyme as proven in our report. We further suggest that the protease sensitivity of the enzymes varies from one species to another (probably in a sequence specific manner). The fragmentation of the enzyme in no way affects its enzymatic activity, but does allow one to explore the domain structure of the protein. It should be clear that we do not claim that the porcine enzyme exists as fragments in vivo (although it might). We only wish to stress the relationship between the fragments and the native enzyme.

Table SIV

| Fragment         | carboxypeptidase | carboxypeptidase |
|------------------|------------------|------------------|
| 28K Fragment     | (Asp,Val) Ser    | Ser              |
| 28K Fragment     | (Asp,Val) Ser    | Ser              |
| 28K Fragment     | (Asp,Val) Ser    | Ser              |

Approximately 1 mg of each sample was treated with carboxypeptidase A and B (1000 units) in 30 min at pH 7.8 for each sample aliquot was analyzed at T = 0 min, T = 30 min, T = 60 min, T = 60 min, T = 24 h by Beckman 121KB amino acid analyzer.

Hydroxylase catalysis were analyzed by the procedure 121KB derivatization method with HPLC (Jones et al., 1981).

Not determined.

Table SIV

| Amino Acid Compositions of Cysteine-Containing Peptides | Residues/Molecule |
|--------------------------------------------------------|------------------|
| T-21                                                   | 1.04 (1)         |
| T-23                                                   | 0.30 (1)         |
| T-26                                                   | 0.81 (1)         |
| T-29                                                   | 0.32 (1)         |
| T-34                                                   | 0.54 (1)         |
| T-35                                                   | 0.49 (1)         |
| T-42                                                   | 0.31 (1)         |
| T-52                                                   | 0.51 (1)         |

Approximately 1 mg of each sample was treated with carboxypeptidase A and B (1000 units) in 30 min at pH 7.8 for each sample aliquot was analyzed at T = 0 min, T = 30 min, T = 60 min, T = 60 min, T = 24 h by Beckman 121KB amino acid analyzer.

Hydroxylase catalysis were analyzed by the procedure 121KB derivatization method with HPLC (Jones et al., 1981).

Not determined.

Table SIII

| NH-Terminal Sequence Analysis of Trypsin-Solubilized Enzyme (20K)² | Cycle residue yield (mol) cycle residue yield (mol) cycle residue yield (mol) cycle residue yield (mol) |
|-------------------------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|
| Ile 169                                                             | 18 Lys 30                                       | 18 Lys 30                                       | 18 Lys 30                                       |
| Glu 19                                                              | 19 Thr 13                                       | 19 Thr 13                                       | 19 Thr 13                                       |
| Thr 15                                                              | 15 Ser 24                                       | 15 Ser 24                                       | 15 Ser 24                                       |
| Ser 19                                                              | 19 Asp 24                                       | 19 Asp 24                                       | 19 Asp 24                                       |
| Asp 26                                                              | 26 Arg 15                                       | 26 Arg 15                                       | 26 Arg 15                                       |
| Arg 13                                                              | 13 Lys 30                                       | 13 Lys 30                                       | 13 Lys 30                                       |
| Lys 68                                                              | 68 Lys 30                                       | 68 Lys 30                                       | 68 Lys 30                                       |
| Arg 65                                                              | 65 Arg 15                                       | 65 Arg 15                                       | 65 Arg 15                                       |
| Glu 14                                                              | 14 Glu 19                                       | 14 Glu 19                                       | 14 Glu 19                                       |
| Cys (5)                                                             | 0.21 (1)                                        | 0.21 (1)                                        | 0.21 (1)                                        | 0.21 (1)                                        |

Table SII

| Acid Conversions and Analyses                                 | Cysteine-Containing Peptides |
|---------------------------------------------------------------|------------------------------|
| Ala 0.95 (2)                                                  | 0.15 (1)                     |
| Val 0.72 (1)                                                  | 0.77 (1)                     |
| Leu 0.72 (1)                                                  | 0.78 (1)                     |
| Ile 0.64 (1)                                                  | 0.75 (1)                     |
| Ser 0.70 (1)                                                  | 0.71 (1)                     |
| Asp 1.01 (1)                                                  | 1.01 (1)                     |
| Cys 0.13 (1)                                                  | 0.13 (1)                     |
| Gly 1.15 (1)                                                  | 1.2 (1)                      |
| Val 1.15 (1)                                                  | 1.18 (1)                     |
| Ala 1.54 (2)                                                  | 1.55 (1)                     |
| Ala 1.57 (2)                                                  | 1.59 (1)                     |
| Leu 1.87 (1)                                                  | 1.91 (1)                     |
| Phe 2.31 (2)                                                  | 2.31 (1)                     |
| Tyr 2.59 (4)                                                  | 2.6 (1)                      |
| His 0.77 (1)                                                  | 0.78 (1)                     |
| Arg 0.64 (1)                                                  | 0.65 (1)                     |
| Lys 0.60 (1)                                                  | 0.61 (1)                     |
| 5.26 (1)                                                      | 5.26 (1)                     |

Table SII

| NH-Terminal Sequence Analysis of Trypsin-Solubilized Enzyme (20K)² | Cycle residue yield (mol) cycle residue yield (mol) cycle residue yield (mol) cycle residue yield (mol) |
|-------------------------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|
| Ile 169                                                             | 18 Lys 30                                       | 18 Lys 30                                       | 18 Lys 30                                       |
| Glu 19                                                              | 19 Thr 13                                       | 19 Thr 13                                       | 19 Thr 13                                       |
| Thr 15                                                              | 15 Ser 24                                       | 15 Ser 24                                       | 15 Ser 24                                       |
| Ser 19                                                              | 19 Asp 24                                       | 19 Asp 24                                       | 19 Asp 24                                       |
| Asp 26                                                              | 26 Arg 15                                       | 26 Arg 15                                       | 26 Arg 15                                       |
| Arg 13                                                              | 13 Lys 30                                       | 13 Lys 30                                       | 13 Lys 30                                       |
| Lys 68                                                              | 68 Lys 30                                       | 68 Lys 30                                       | 68 Lys 30                                       |
| Arg 65                                                              | 65 Arg 15                                       | 65 Arg 15                                       | 65 Arg 15                                       |
| Glu 14                                                              | 14 Glu 19                                       | 14 Glu 19                                       | 14 Glu 19                                       |
| Cys (5)                                                             | 0.21 (1)                                        | 0.21 (1)                                        | 0.21 (1)                                        | 0.21 (1)                                        |

References in parentheses were obtained by sequence analysis. Sequence results are not complete, results tentative.
### Table SIV

**Sequence Analysis of Peptides**

| Cycle | 2-56 (2.0 µg) | 2-56 (1.9 µg) | 2-56 (1.8 µg) | 2-56 (1.7 µg) | 2-56 (1.6 µg) | 2-56 (1.5 µg) | 2-56 (1.4 µg) |
|-------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| 1     | His (3,326)  | Leu (2,574)  | Thr (2,098)   | Ala (1,669)   | Asp (996)     | Glu (867)     | Gly (607)     |
| 2     | Glu (1,669)  | Ala (1,669)   | Asp (996)     | Glu (867)     | Gly (607)     | Asp (996)     | Glu (867)     |
| 3     | Gln (1,669)  | Ala (1,669)   | Asp (996)     | Glu (867)     | Gly (607)     | Asp (996)     | Glu (867)     |
| 4     | Gln (1,669)  | Ala (1,669)   | Asp (996)     | Glu (867)     | Gly (607)     | Asp (996)     | Glu (867)     |
| 5     | Cys (1,669)  | Ala (1,669)   | Asp (996)     | Glu (867)     | Gly (607)     | Asp (996)     | Glu (867)     |
| 6     | Cys (1,669)  | Ala (1,669)   | Asp (996)     | Glu (867)     | Gly (607)     | Asp (996)     | Glu (867)     |
| 7     | Thr (1,669)  | Ala (1,669)   | Asp (996)     | Glu (867)     | Gly (607)     | Asp (996)     | Glu (867)     |
| 8     | Ser (1,669)  | Ala (1,669)   | Asp (996)     | Glu (867)     | Gly (607)     | Asp (996)     | Glu (867)     |

**Table SIVI**

**Cysteine-Terminal Sequence Analyses of Cysteine-Containing Peptides**

| Peptide | Sequence |
|---------|----------|
| 1       | Val-Asp-Arg     |
| 2       | Val-Asp-Arg     |
| 3       | Val-Asp-Arg     |
| 4       | Val-Asp-Arg     |
| 5       | Val-Asp-Arg     |
| 6       | Val-Asp-Arg     |
| 7       | Val-Asp-Arg     |

Table SIVIII

**Amino Acid Compositions of NADPH-Protected Peptide**

| Amino Acid | Cysteinyl-Cysteine | Cysteine-Containing Peptides |
|------------|--------------------|------------------------------|
| Val        | 2.43               | 1.02 (1)                     |
| Glu        | 0.89               | 0.12 (1)                     |
| Cys        | 0.18               | 0.02 (1)                     |
| Tyr        | 0.04               | 0.19 (1)                     |
| Thr        | 0.11               | 0.02 (1)                     |
| Cys        | 0.53               | 0.12 (1)                     |

| Total      | 32                | 4                             |

Results in parentheses were determined by sequence analysis.

### Table SIVII

**Sequence Analyses of NADPH-Binding Peptides**

| Cycle | 1-15 (2.0 µg) | 1-15 (1.9 µg) | 1-15 (1.8 µg) | 1-15 (1.7 µg) | 1-15 (1.6 µg) | 1-15 (1.5 µg) |
|-------|---------------|---------------|---------------|---------------|---------------|---------------|
| 1     | Val (1,034)  | Gly (733)     | Val (1,034)   | Gly (733)     | Val (1,034)   | Gly (733)     |
| 2     | Gly (733)    | Val (1,034)   | Gly (733)     | Val (1,034)   | Gly (733)     | Val (1,034)   |
| 3     | Cys (411)    | Ser (242)     | Cys (411)     | Ser (242)     | Cys (411)     | Ser (242)     |
| 4     | Gly (402)    | Ser (242)     | Gly (402)     | Ser (242)     | Gly (402)     | Ser (242)     |
| 5     | Ser (242)    | Gly (402)     | Ser (242)     | Gly (402)     | Ser (242)     | Gly (402)     |
| 6     | Ser (242)    | Gly (402)     | Ser (242)     | Gly (402)     | Ser (242)     | Gly (402)     |
| 7     | Ser (242)    | Gly (402)     | Ser (242)     | Gly (402)     | Ser (242)     | Gly (402)     |
| 8     | Ser (242)    | Gly (402)     | Ser (242)     | Gly (402)     | Ser (242)     | Gly (402)     |
| 9     | Ser (242)    | Gly (402)     | Ser (242)     | Gly (402)     | Ser (242)     | Gly (402)     |
| 10    | Ser (242)    | Gly (402)     | Ser (242)     | Gly (402)     | Ser (242)     | Gly (402)     |
| 11    | Ser (242)    | Gly (402)     | Ser (242)     | Gly (402)     | Ser (242)     | Gly (402)     |
| 12    | Ser (242)    | Gly (402)     | Ser (242)     | Gly (402)     | Ser (242)     | Gly (402)     |

### Figure 1

**HPLC tryptic map of detergent-solubilized P401 fragment protein reductase.** The tryptic digest contained 0.2 µg of P401 (9.5 µg of P401) and was applied to a SpinCupel RPC-E-8 column (250 x 4.6 mm, 10 µL steel) and eluted with solvent I (0.15 TFA) to solvent II (1% TFA/50% CH2Cl2) over 90 min. Cysteine-containing peptides were detected by measurement of TCEP radioactivity in 20 µL each from each fraction.

### Figure 2

**Secondary structure of homologous peptides in P401 and P408.** A: C-terminal peptides (4) and glutathione reductase (4). Calculations were performed according to Chou and Fasman (1978) for 8 and by X-ray crystallography for 8B.