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AMINO ACID SEQUENCE OF COOH-TERMINAL 20K Da FRAGMENT FROM PIG LIVER MICROSOMAL NADPH-CYTOCHROME P-450 REDUCTASE

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Summary. We have determined the complete amino acid sequence of a 20K Da COOH-terminal fragment of porcine NADPH-cytochrome P-450 reductase. The 20K Da fragment is probably produced by a proteolytic cleavage of the intact protein in porcine liver microsomes, and since the cleavage does not affect enzymatic activity, the fragment has been studied as a distinct domain. The sequence comprises 175 amino acids including three cysteine residues, one of which has been previously identified as protected by NADPH from S-carboxymethylation. The NADPH-protected cysteine lies in a stretch of 12 residues with partial homology to glutathione reductase, and is adjacent to a hydrophobic region containing a glycine-rich stretch homologous to other FAD-containing proteins. The predicted secondary structure over this entire region is β-sheet/β-turn/-β-sheet/α-helix/β-sheet/β-turn/α-helix corresponding to hydrophobic residues 21-28/glycine-rich residues 29-33/residues 34-38/residues 39-54/residues 56-61/NADPH-protected cysteine residues 62-78/residues 71-82. It is possible that the 20K Da domain provided a significant portion of the sequence responsible for binding FAD and NADPH in the intact enzyme. This data provides a basis for further active site studies.

Introduction. Hepatic microsomal NADPH-cytochrome P-450 reductase is an essential enzyme in the cytochrome P-450 system responsible for the detoxification of drugs, activation of procarcinogens, steroid metabolism, and fatty acid desaturation (1). It is also involved in the free radical formation of anthracycline anti-cancer drugs, which causes cell injury by DNA or RNA breakages (2,3). This enzyme contains one molecule each of NADPH, FAD and FMN

Abbreviations:

FRDA, fumarate reductase; GRase, glutathione reductase; LPDHase, lipoamide dehydrogenase; MRase, mercuric reductase; P-450 Rase; NADPH-cytochrome P-450 reductase; pCMB, p-hydroxychloromercuribenzoate; PHBHase, p-hydroxybenzoate hydroxylase; SDHA, succinate dehydrogenase; TPCK, tosylphenylchloromethyl-ketone.

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and has a molecular weight of approximately 78,000-80,000 (1,4,5). Structural studies (5,6) have revealed that the detergent-solubilized rabbit enzyme contained an NH_2-terminal hydrophobic domain which might span the microsomal membrane or interact with cytochrome P-450 (6). Trypsin-solubilized rabbit enzyme lacks the hydrophobic domain which consists of 48 amino acid residues (6). We have shown that the pig liver enzyme gives two fragments in addition to the original enzyme (5). These fragments include an NH_2-terminal fragment possessing a molecular weight of 60,000 and a COOH-terminal fragment which has a molecular weight of 20,000. The 20K Da fragment contained a hydrophobic region consisting of 22 amino acid residues and a homologous sequence to the pyrophosphate binding region of p-hydroxybenzoate hydroxylase (5,7). Nishimoto and Shibata (8) have shown that one out of six reactive cysteine residues in the rabbit enzyme was protected by NADPH from pCMB titration. In agreement with their finding, we have identified an NADPH-protected cysteine residue which lies within a region possessing homology to glutathione reductase (5). We have now isolated the COOH-terminal 20K Da fragment from the pig liver enzyme and obtained its complete sequence in order to investigate the relationship between structure and function of NADPH-cytochrome P-450 reductase.

Materials and Methods. The NADPH-cytochrome P-450 reductase was isolated and purified from pig liver microsomes according to Iyanagi et al (9). Purification of 20K Da fragment was performed by high pressure liquid chromatography as described previously (5). TPCK-trypsin and chymotrypsin were purchased from Worthington Biochemical Co. Staphylococcus aureus protease (strain V8) was from Pierce Chemical Co. Enzyme digestion was carried out on S-carboxymethylated or on S-carboxymethylated and 4-sulfophenylisothiocyanate-derivatized protein by the method described previously (10). The soluble peptides were resolved by HPLC on an Ultrasphere C-8 column as described (10).

Amino acid compositional analysis was performed as described (11). For NH_2-terminal sequence analysis, peptides were subjected to manual or automated Edman degradation on a modified Beckman 890C sequencer as described (12). PTH-amino acids were identified and quantified by reverse phase HPLC (13).

Results and Discussion. The 20K Da fragment was purified from native pig enzyme by HPLC using an alkylphenyl reverse phase column. Tryptic digestion was performed on the carboxymethylated fragment and the resulting peptides were

purified by reverse phase HPLC on an Ultrasphere C-8 column. Chymotryptic and S. aureus protease digestions were performed on the 4-sulfophenylisothiocyanate derivative of the 20K Da fragment in order to maintain high solubility in aqueous solvents. Corresponding peptides were resolved by same method as mentioned above. Microsequence analysis on each peptide (0.5 $\sim$ 1 nmole) was performed by automated or manual Edman degradation. Complete amino acid sequence of 20K Da fragment was determined by overlapping each of the peptides as shown in Figure 1. This fragment contained three out of a total of eight

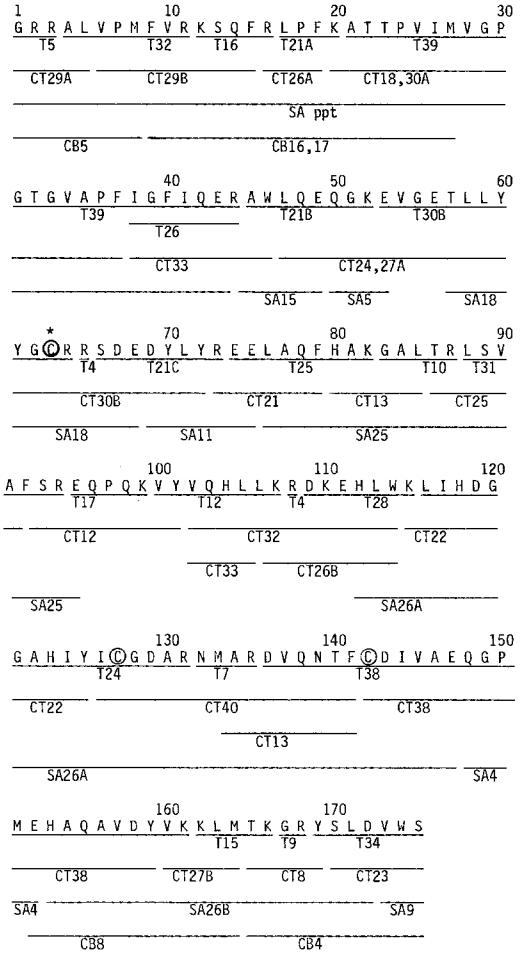

Figure 1. Total sequence of 20K Da fragment of porcine liver NADPH-cytochrome P-450 reductase.

Peptides were generated by tryptic (T), chymotryptic (CT), S. aureus protease (SA), and cyanogen bromide (CB) digestions of 20K Da fragment. The peaks were numbered in increasing order of elution from RP-HPLC and each peptide was sequenced by automated or manual Edman degradation. Cysteine residues are circled and an asterisk shows the essential residue for NADPH protection.

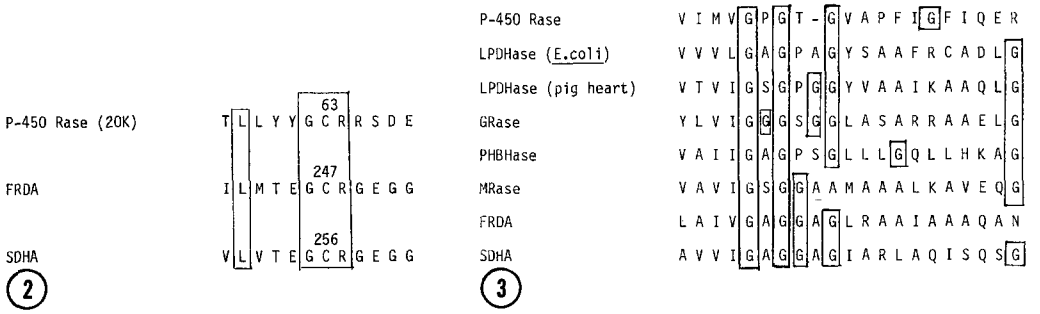

Figure 2. Sequence homology around essential cysteine residues of several flavoproteins.

Cysteine 63 in NADPH-cytochrome P-450 reductase is protected from S-carboxymethylation by NADPH. Cysteine 247 in fumarate reductase (FRDA) and cysteine 256 in succinate dehydrogenase (SDHA) may be in the active sites of these enzymes (14). Homologous residues are boxed.

Figure 3. Sequence conservation in a segment of the glycine-rich region of NADPH-cytochrome P-450 reductase and the FAD-binding regions of several flavoproteins.

Glycine residues are boxed. The above sequences were derived from Wood et al. (14) and Rice et al. (15).

cysteine residues of pig liver NADPH-cytochrome P-450 reductase. It is interesting to note that a unique cysteine residue which was protected by NADPH from alkylation or pCMB titration (5,8) was located at residue 63, close to the previously noted hydrophobic and basic amino acid-rich region (5). This cysteine-containing peptide (T-30) is partially homologous to the NADPH-binding region of glutathione reductase (5), and also possesses sequence homology to the active site of fumarate reductase (FRDA) or succinate dehydrogenase (SDHA) (14). These proteins possess a common sequence Gly-Cys-Arg at the active site as shown in Figure 2. The sequence contains a hydrophobic region (residue 21 to 42), which includes a glycine-rich sequence Gly-Pro-Gly-Thr-Gly that has homology to other FAD binding proteins (Fig. 3).

Secondary structure of this 20K Da fragment was predicted according to Chou and Fasman method (16). The results suggest that residues 21-28 are β-sheet, residues 29-33 are β-turn, residues 34-38 are β-sheet, residues 39-54 are α-helix, residues 56-61 are β-sheet, residues 62-68 are β-turn, and residues 71-82 are α-helix. If these structures are in contact in the three dimensional structure then they may provide a significant portion of the FAD

and NADPH-binding sites in the intact protein. Although we have identified a homologous sequence to the pyrophosphate-binding region of p-hydroxybenzoate hydroxylase (residues 1-9) (5), the β α β structure which was found in the nucleotide binding region of dehydrogenases by Rossman et al. (17) was not strongly predicted for this region. This finding may argue against the location of the FAD-binding region in the 20K Da domain, or indicate an alternate secondary structure for FAD binding in NADPH-cytochrome P-450 reductase. Further studies are underway to test each of these hypotheses.

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References

1. Williams, C.H. JR. (1976) in The Enzymes (Boyer, P.D., ed) Vol. 13, pp 89-173. Academic Press, New York.
2. Bachur, N.R., Gordon, S.L., and Gee, M.V. (1978) Cancer Res. 38, 1745-1750.
3. Berlin, V. and Haseltine, W.A. (1981) J. Biol. Chem. 256, 4747-4756.
4. Iyanagi, T. and Mason, H.S. (1973) Biochemistry 12, 2297-2308.
5. Haniu, M., Iyanagi, T., Legesse, K., and Shively, J.E. (1984) J. Biol. Chem. 259, 13703-13711.
6. Black, S.D. and Coon, M.J. (1982) J. Biol. Chem. 257, 5929-5938.
7. Weijer, W.J., Hofsteenge, J., Beintema, J.J., Wierenga, R.K., and Drenth, J. (1983) Eur. J. Biochem. 133, 109-118.
8. Nishimoto, Y. and Shibata, Y. (1982) J. Biol. Chem. 257, 12532-12539.
9. Iyanagi, T., Makino, R., and Anan, K.F. (1981) Biochemistry 20, 1722-1730.
10. Yuan, P.-M., Nakajin, S., Haniu, M., Shinoda, M., Hall, P.F., and Shively, J.E. (1983) Biochemistry 22 143-149.
11. Del Valle, U. and Shively, J.E. (1979) Anal. Biochem. 96, 77-83.
12. Shively, J.E. (1981) Methods Enzymol. 79, 31-48.
13. Hawke, D., Yuan, P.-M., and Shively, J.E. (1982) Anal. Biochem. 120, 302-311.
14. Wood, D., Darlison, M.G., Wilde, R.J., and Guest, J.R. (1984) Biochem. J. 222, 519-534.
15. Rice, D.W., Schulz, G.E., and Guest, J.R. (1984) J. Mol. Biol. 174, 483-496.
16. Chou, P.Y. and Fasman, G.D. (1978) Annu. Rev. Biochem. 47, 251-276.
17. Rossmann, M.G., Liljas, A., Brändén, C.I., and Banaszak, L.J. (1975) in The Enzymes (Boyer, P.D. eds.) Vol 11, pp. 61-102 Academic Press, New York.