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# ROLE OF A HYDROPHOBIC POLYPEPTIDE IN THE N-TERMINAL REGION OF NADPH-CYTOCHROME P-450 REDUCTASE IN COMPLEX FORMATION WITH P-450LM

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SUMMARY: Detergent-solubilized liver microsomal NADPH-cytochrome P-450 reductase is known to retain the ability to catalyze electron transfer to cytochrome P-450, whereas the trypsin-solubilized reductase does not. In the present study, treatment of the highly purified detergent-solubilized rabbit liver enzyme (m.w. 77,700) with trypsin was shown to yield a small peptide (m.w. 6,100) as well as the large peptide (m.w. 71,200) which retains the flavin prosthetic groups. The small peptide, which is hydrophobic in nature as shown by its amino acid composition and solubility properties, is apparently the moiety in the native reductase involved in binding to cytochrome P-450 and to the microsomal membrane. The C-terminal amino acid sequences of the native reductase and large fragment are identical [-Trp-(Leu, Val)-Asp-Ser-COOH], thereby indicating that the hydrophobic peptide is located in the N-terminal region of the enzyme.

After the liver microsomal hydroxylation system was reconstituted from detergent-solubilized cytochrome P-450 and NADPH-cytochrome P-450 reductase in the presence of phospholipid (1,2), we found that trypsin-solubilized "NADPH-cytochrome c reductase" (3) could not replace the latter enzyme (4,5). More recently, the detergent-solubilized reductase has been obtained in highly purified form from rat (6-9) and rabbit liver microsomes (10), thereby permitting a structural comparison with the corresponding protease-treated preparations.

The present paper describes the isolation and properties of a small hydrophobic peptide derived from the amino-terminal region of the native reductase from rabbit liver microsomes upon treatment with trypsin. The large, soluble peptide obtained in this process has previously been purified and characterized in other laboratories (3,11). The small peptide appears to be essential in the binding of the native reductase to cytochrome P-450 and to the microsomal membrane. Gum and Strobel (12) have recently concluded that a membrane-binding domain is present in the rat liver reductase, but the amino acid composition of the domain, calculated by the difference between the detergent- and steapsin-solubilized reductase preparations, was not especially hydrophobic.

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## MATERIALS AND METHODS

Cytochrome c (horse heart, type III), Dowex-50X12 ($H^+$ form), N-bromosuccinimide, and trypsin (type XI) were obtained from Sigma. Silica gel G plates were supplied by J. T. Baker; urea (ultrapure) was from Schwarz/Mann and SDS from BDH Chemicals. Carboxypeptidase Y free of endoproteolytic activity was obtained from Boehringer-Mannheim Biochemicals. All other materials used have been described previously (9).

Protein concentrations were estimated by the method of Lowry (13) using crystalline bovine serum albumin as the standard. SDS-polyacrylamide gel electrophoresis was carried out according to the procedure of Laemmli (14). Cytochrome c reductase activity was measured at 30° in 0.3 M phosphate buffer, pH 7.7 (9,15), and assays for cytochrome P-450 reductase activity were carried out using 0.2 μM P-450$_{LM_2}$ and varying amounts of reductase, according to a procedure described previously (10).

P-450$_{LM_2}$ (16) and NADPH-cytochrome P-450 reductase (10) were purified from liver microsomes of phenobarbital-induced rabbits. Trypsin-solubilized NADPH-cytochrome c reductase was also isolated from such microsomes, which were washed and treated with trypsin according to the method of Iyanagi and Mason (11), modified as follows. Trypsin (50 mg) was added to a suspension of microsomes (10 g of protein) in 500 ml of buffer solution containing 10% glycerol. At the end of the incubation the mixture was made 0.2 mM in p-toluenesulfonyl fluoride and centrifuged at 105,000 X g for 120 min, and the supernatant fraction containing 90 to 95% of the original cytochrome c reductase activity was removed for further treatment. Additional purification steps were as described by French and Coon (10) for Renex-solubilized microsomes, except that the detergent was omitted and the eluate from column chromatography on ADP-agarose (7) was dialyzed against 10 mM potassium phosphate buffer, pH 7.0. Typical preparations had a specific activity of 76 μmol of cytochrome c reduced per min per mg of protein, with $A_{278/455}$ = 7.1.

For determination of amino acid composition, samples were first dialyzed against 10 mM phosphate buffer, pH 7.0, and brought to dryness under reduced pressure. Hydrolysis for 24, 48, and 72 h at 105° was carried out in constant boiling HCl in evacuated, sealed tubes. Amino acids were quantitated using a modified Beckman 120B amino acid analyzer with the Durrum single column system using Pico system II buffers and DC-1A resin. Half-cystine was determined as cysteic acid after a 48-h hydrolysis in the presence of dimethylsulfoxide (17). Tryptophan was determined by titration of the proteins with N-bromosuccinimide in the presence of 1% SDS (18). The composition of the small tryptic peptide was determined from a 75 h-hydrolysis. Analyses for sequence experiments were carried out on a modified Beckman 120C amino acid analyzer.

For C-terminal sequence analyses, samples were first dialyzed against 0.1 M phosphate buffer, pH 7.0, and concentrated to about 75 μM by ultrafiltration. Urea and SDS were added at final concentrations of 4 M and 0.05 M, respectively, and the solution was incubated at 25° for 10 min. The reaction was then initiated by the addition of carboxypeptidase Y (molar ratio of peptidase to protein, 1:100), and aliquots were withdrawn at various times. Each aliquot was added to a tube containing about 0.4 ml of dry Dowex-50X12, and the mixture was shaken well and transferred to a narrow column having a volume of 1 ml. The column was washed with three 1.0-ml portions of distilled water, and the amino acids were eluted with three 1.0-ml portions of 5 M $NH_3$(aq). The eluates were dried under reduced pressure, and amino acid analyses were carried out. Losses were estimated for each amino acid residue by an external standard run in parallel with experimental samples, and the results presented were corrected accordingly.

## RESULTS AND DISCUSSION

Isolation of Small Tryptic Peptide: The small peptide was prepared by trypsin treatment of the native reductase in the presence of P-450$_{LM_2}$ and dilauroyl-GPC in a molar ratio of 1:1.2:200. In a typical experiment, trypsin was added to the reductase (20 mg protein) in a 10-ml reaction mixture in a 1:100 (w/w) ratio. After incubation of the mixture at 4° for 120 min, 90 ml of chloroform-methanol (1:1) were added, and the mixture was centrifuged to remove the precipitate, which was washed twice with 90 ml of chloroform-methanol. The extracts were combined and brought to dryness under reduced pressure. The residue was dissolved in a minimal volume of chloroform-methanol and applied as a thin band to a 17.5 X 20-cm silica gel G plate, which was developed in chloroform-methanol-water (50:50:5) made 20 mM in HCl. The small tryptic peptide, which was visualized with $I_2$ vapor at an $R_f$ of 0.46, was eluted with choloroform-methanol. The solution was taken to dryness, suspended in aqueous buffer, and gently sonicated before use.

The electrophoretic behavior of the reductase and its tryptic peptides, as shown in Fig. 1, indicates that each polypeptide is apparently homogeneous. The small tryptic peptide binds dye poorly and thus exhibits a lighter band than do the large polypeptides. On the basis of appropriate standards, the molecular weight of the small fragment was estimated as 12,000. This value is about twice that expected by comparison of the native enzyme and large fragment, and is believed to indicate anomalous electrophoretic behavior, as has been reported for other low molecular weight peptides (19).

Amino Acid Analyses: The amino acid composition of the purified proteins is given in Table I. With a few exceptions, the sum of the individual residues in the large and small tryptic fragments agrees well with the values found for

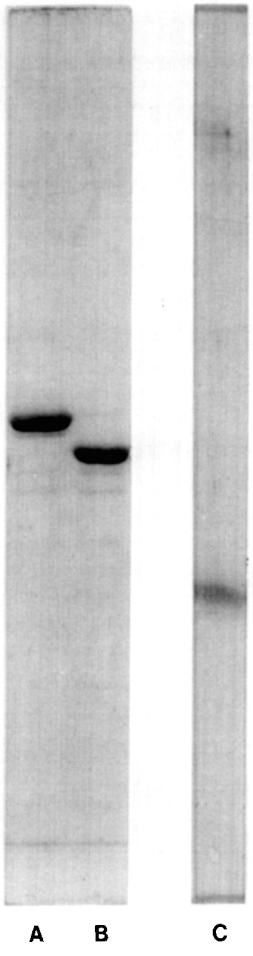


Fig. 1. Electrophoretic behavior of native reductase and tryptic fragments. The proteins (2.0-µg samples) were submitted to SDS-polyacrylamide slab gel electrophoresis, with migration from top to bottom, and fixed and stained as described elsewhere (16). A 7.5% separating gel was used, except in the case of the small peptide, where a 15% gel was used. A, native reductase; B, large tryptic fragment; and C, small tryptic fragment.

the native reductase and, accordingly, the amino acid content of the small fragment determined directly and that calculated by difference are also in good agreement. The disparity seen with several amino acids, particularly lysine, may indicate that several such residues were lost during trypsinolysis. The minimal molecular weight of the small tryptic fragment based on amino acid analysis is 6,100, which is in fairly good agreement with that calculated by difference The values of 77,700 and 71,200 calculated for the native and trypsin-solubilized reductases, respectively, are slightly higher than previously reported for the rabbit liver enzyme (10) and quite close to the values found for the proteins from rat liver (6,20,21). The relative hydrophobicity of these proteins, es-

TABLE I

Amino Acid Composition of Reductase and Tryptic Peptides

| Amino acid residue | Number of residues per molecule | | | |
|---|---|---|---|---|
| | Detergent-solubilized reductase (A) | Trypsin-solubilized reductase (B) | Hydrophobic peptide calculated (A-B) | |
| ASX | 68 | 64 | 4 | 4 |
| THR | 37 | 29 | 3 | 8 |
| SER | 37 | 35 | 4 | 2 |
| GLX | 84 | 80 | 9 | 4 |
| PRO | 31 | 29 | 4 | 2 |
| CYS | 9 | 8 | 0 | 1 |
| GLY | 48 | 47 | 4 | 1 |
| ALA | 62 | 58 | 5 | 4 |
| VAL | 48 | 45 | 5 | 3 |
| MET | 16 | 15 | 1 | 1 |
| ILE | 24 | 21 | 3 | 3 |
| LEU | 66 | 59 | 6 | 7 |
| TYR | 32 | 31 | 1 | 1 |
| PHE | 26 | 21 | 3 | 5 |
| HIS | 21 | 18 | 1 | 3 |
| LYS | 36 | 30 | 1 | 6 |
| ARG | 39 | 37 | 2 | 2 |
| TRP | 7 | 7 | 0[a] | 0 |
| Total | 691 | 634 | 56 | 57 |
| Polypeptide m. w. | 77,700 | 71,200 | 6,100 | 6,500 |

[a]The absence of tryptophan is indicated by the ultraviolet spectrum of this peptide.

timated as an unweighted percent of hydrophobic residues (22), was 31.7, 31.3, and 34.1 for the native enzyme, large fragment, and small fragment, respectively. All of these proteins are hydrophobic as compared to typical proteins, but the small fragment is particularly hydrophobic, as would be expected from its unusual solubility properties.

Inhibition of NADPH Oxidase Activity by Small Peptide: Various amounts of a sonicated aqueous suspension of the small hydrophobic peptide were added to a reconstituted enzyme system containing P-450$_{LM_2}$, phospholipid, and native reductase with benzphetamine as substrate. A marked inhibition of NADPH oxidation was seen at very low concentrations of the small peptide. These data are shown in Fig. 2 as percent inhibition of the oxidase activity, calculated as turnover numbers with respect to the reductase. The $K_I$ calculated from this plot was 55 nM, based on a molecular weight of 6,100 for the small hydrophobic peptide. In

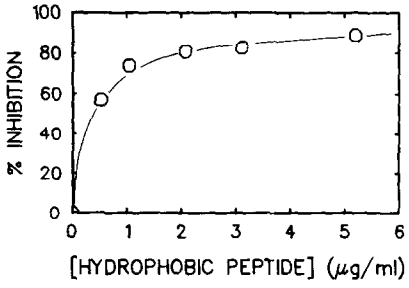


Fig. 2. Inhibition by the small hydrophobic peptide of NADPH oxidation in the reconstituted hydroxylation system. Varying amounts of the small peptide were added to the reconstituted system before the reaction was initiated by NADPH.

other experiments spectral perturbations were seen when the small peptide and P-450$_{LM_2}$ were mixed, thus providing further indication of their interaction (23).

C-Terminal Sequence Analysis: The release of C-terminal amino acid residues from the native and trypsin-solubilized reductases by carboxypeptidase Y in the presence of SDS and urea was studied as indicated in Fig. 3. The identity and kinetics of release for each residue were found to be the same for the two polypeptides, with a C-terminal sequence as follows: -Trp-(Leu,Val)-Asp-Ser-COOH.

The results presented indicate that NADPH-cytochrome P-450 reductase is composed of two functional domains, which can be separated by tryptic cleavage. The large fragment is monomeric and more hydrophilic than the native reductase, which exists as an aggregate, and represents the catalytic domain. The small fragment, in contrast, is quite hydrophobic as judged by its solubility behavior and amino acid composition, and apparently plays a role in binding the native enzyme to

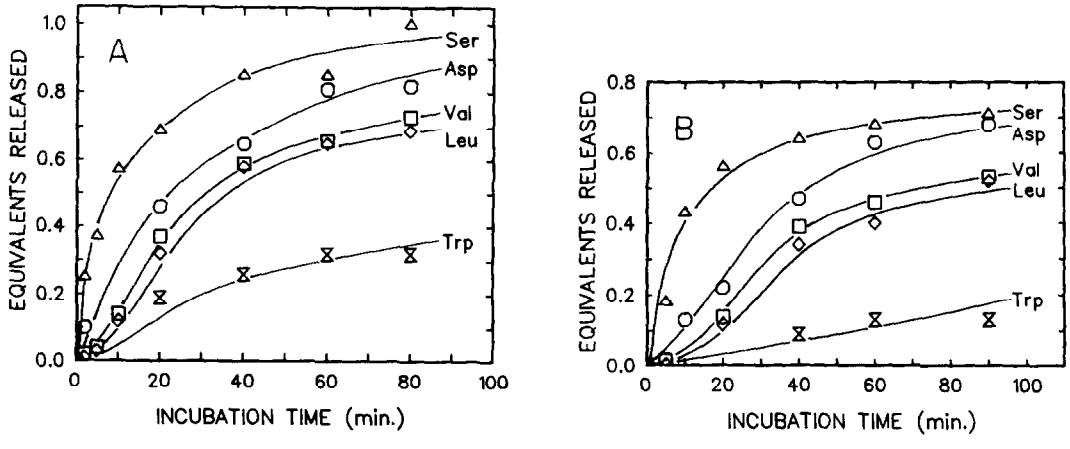


Fig. 3. Determination of C-terminal amino acid sequence of native reductase (A) and trypsin-solubilized reductase (B). Solutions of the native enzyme (74 μM) and trypsin-treated enzyme (78 μM) were treated with carboxypeptidase Y as described in the text.

the microsomal membrane. This function has been shown for the rat liver reductase (12). In addition, the ability of the small peptide to inhibit NADPH oxidation in the reconstituted system and to produce spectral perturbations when added to the cytochrome indicate that it represents the region of the reductase involved in complex formation with cytochrome P-450 (23). Thus, the native reductase appears to be an amphiphilic molecule, similar in this respect to two other microsomal enzymes which have been thoroughly studied, NADH-cytochrome $b_5$ reductase and cytochrome $b_5$ (24,25). However, the hydrophobic domains of the latter two proteins have been shown to function simply as membrane anchors and are not directly involved in protein-protein interactions (26). Another significant difference is that the hydrophobic peptide of cytochrome $b_5$ is located at the C-terminus (27), whereas evidence presented in this paper indicates that the hydrophobic domain of NADPH-cytochrome P-450 reductase is situated in the N-terminal region.

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