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Liver Microsomes Contain Two Distinct NADPH-Monooxygenases with NH_2-terminal Segments Homologous to the Flavin Containing NADPH Monooxygenase of Pseudomonas fluorescens

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Two NADPH-reductase preparations (FAD-containing monooxygenases) were isolated from rabbit liver microsomes, referred to as form 1 and form 2. Purification was achieved by means of anion-exchange, cation-exchange and hydroxylapatite chromatography in the presence of cholate and Nonidet P-40. Affinity chromatography on 2', 5'-ADP Sepharose was used to increase the purity and to concentrate the enzyme. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis, form 1 exhibited a single band at Mr 58,500 and form 2 at Mr 58,000. The NH_2- terminus of form 1 is blocked, whereas the NH_2-terminus of form 2 is homologous to the NADPH-p-hydroxybenzoate hydrolase from Pseudomonas fluorescens. The latter and the form 2 enzyme share 11 identical residues in the NH_2-terminal segment of 15 residues. Both forms were subjected to tryptic cleavages and peptide mapping. Sequence analysis of the peptides obtained indicated that forms 1 and 2 are similar but not identical proteins. A tryptic peptide, homologous to residues 3 to 32 of form 2 enzyme was isolated from the form 1 protein. This segment has 24 residues that are identical to the form 2 and contains the consensus sequence Gly-X-Gly-X-X-Gly, found in most FAD binding proteins. These results indicate that the NADPH-monooxygenase system consists of at least two distinct proteins representing different gene products.

The FAD-containing monooxygenase is a labile enzyme found in mammalian liver microsomes (1). The monooxygenase from hog liver catalyzes N-oxygenation of a variety of different lipophilic acyclic aliphatic amines (2). Known amine substrates for the monooxygenases include antipsychotic, antihistaminic and narcotic drugs (3). This reductase has been purified from hog (1), rat (4) and mouse (5) liver microsomes. FAD-containing monooxygenase has been also isolated from rabbit lung microsomes (6). The lung reductase resembles the pig liver enzyme with respect to minimum molecular weight on SDS-PAGE, spectral properties, and activity with several substrates including methimazole. The lung enzyme, however, is immunochemically distinct from the liver enzyme (6). To date, only one form of FAD-containing monooxygenase has been identified in mammalian liver microsomes. Although much is known about the substrate specificity and catalytic mechanism of this reductase from hog microsomes, amino acid sequence information of this enzyme has yet to be obtained, and the structural relationship between this

Abbreviations:
SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; DTT, dithiothreitol. KPi, potassium phosphate. Rabbit liver microsomal cytochromes P-450: P-450-2 is phenobarbital-, P-450-4 and -6 are 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin-induced isozymes. P-450-3c is a constitutive form of 502 residues (J.O., manuscript in preparation).

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monooxygenase, cytochrome P-450 monooxygenases, and other microsomal reductases is not known. In a previous study we reported the primary structure of several microsomal cytochrome P-450s (7-9), epoxide hydrolase (10), cytochrome b_5 reductase (11), and stearyl-CoA desaturase (12). Since amino acid sequence information permits a number of generalizations to be made about a protein, and provides an important data base for isolating and cloning the cDNAs of microsomal proteins, I report here the isolation, the nature of NH_2-termini, and amino acid sequence of peptide fragments of two NADPH-monooxygenase forms present in rabbit hepatic microsomes. I find that, in contrast to previous reports, the NADPH-monoxygenase is composed of at least two unique enzymes.

METHODS

Materials - All reagents, detergents (sodium cholate, Tergitol, NP-10, Nonidet P-40), and chromatography media unless stated otherwise were obtained from Sigma. Hydroxyapatite-agarose (HA-Ultragel) was a product of IBF biotechnics. Trypsin-TPCK treated was obtained from Worthington. Solvents for HPLC were from Burdick and Jackson. [^{14}C] iodoacetamide was from New England Nuclear.

Isolation of the two reductase forms - Liver microsomes were isolated from New Zealand male rabbits as described previously(13). The microsomes (1 to 1.5 g of protein) were suspended in 10 mM KPi, pH 7.4, containing 20% glycerol, 1 mM EDTA, 0.1 mM DTT, (Buffer A), to a concentration of 15 mg of protein/ml. Solubilization was achieved by addition of sodium cholate 1.2 mg/mg protein and the nonionic detergent, Nonidet P-40, 1.3 mg/mg protein. The resulting solution was then passed over a column containing 200 ml of DEAE-cellulose equilibrated with Buffer A containing 0.5% NP-10. Unbound material eluting from the column with the equilibration buffer was dialyzed against 20 volumes of Buffer A containing 5 mM KPi, and 0.2% NP-40 and applied to a column containing 100 ml of CM-Sepharose equilibrated with the same buffer. The column was developed using linear gradient of increasing concentration of KPi in the equilibration buffer (10-300 mM, 250 ml each). Form 2 and form 1 reductase elutes at about 75 mM KPi, and 100 mM KPi, respectively. Reductase containing fractions were dialyzed against 20 volumes of Buffer A, containing 0.1% NP-40 and then applied to a column containing 10 ml of HA-Agarose equilibrated with Buffer A containing 0.1% NP-40. The column was developed with Buffer A containing 125 mM KPi and then with 300 mM KPi. Form 1 is eluted with 125 mM KPi and form 2 with Buffer A containing 300 mM KPi. Fractions containing form 1 reductase were pooled and dialyzed against 100 volumes of 20 mM Tris-acetate, pH 7.4 (room temp.), 0.1% NP-40 containing 20% glycerol, 1 mM EDTA, and 0.1 mM DTT (Buffer B). The dialyzed material was then adsorbed to a column containing 7 ml of packed 2',5' ADP-Sepharose equilibrated with Buffer B. The column was washed with 30 ml of Buffer B, with 40 ml of Buffer B containing 100 mM KCl and with 60 ml of Buffer B containing 100 mM KCl and 0.5 mM NADPH. The latter buffer elutes form 1 reductase in pure form. Reductase, form 2, does not bind to 2',5' ADP-Sepharose equilibrated with Buffer B. When SDS-polyacrylamide gel electrophoresis indicates that the form 2 preparation requires further purification, then dialyzed fractions of form 2 from the HA-Agarose column are applied to a second CM-Sepharose column equilibrated with Buffer A and eluted with a linear gradient of increasing concentrations (20 to 170 mM) of KPi. The yield of homogeneous reductase preparations range from 0.5 to 2 mg and 0.5 to 1 mg/liver of form 1 and 2 respectively.

Measurement of reductase activity - Monooxygenase activity was measured by monitoring methimazole-dependent NADPH oxidation at 340 nm in the presence of glutathione (1,5). NADPH oxidation was measured in a 1 ml reaction mixture containing 0.1 mM NADPH, 1.0 mM methimazole, 0.1 M Tris-acetate, pH 8.4, and 1 mM glutathione. The specific activity of form 1 and 2 preparations was 1.4 and 0.25 μmoles/min/mg, respectively.

SDS-Polyacrylamide Gel Electrophoresis - were run on a gel system (16 x 16 cm, 1.5 mm thick) essentially as described by Laemmli (14). The separating gels contained 7.5% acrylamide, and the stacking gels contained 5% acrylamide.

Protein modifications, chemical and enzymatic cleavage reactions, as well as amino acid analysis were performed as described in reference (13). Sequence analysis of intact proteins and peptides was carried out on an Applied Biosystems Model 470A, gas-phase sequencer, equipped with Model 120A PTH analyzer according to the manufacturers instructions. Peptide mixtures were first separated using a 2.5 x 100 cm column of LH60 Sephadex equilibrated with formic acid/ethanol (3:7) as the solvent, as previously described (17). The peptide mixtures from LH60 columns were further resolved using reverse phase HPLC. The methodology has been detailed in references (9,15). Peptide mixtures were dissolved in 88% formic acid prior to injection. Reverse phase columns used for separations include Vydac C_4 (15 x 0.46 cm), or Waters Associates C_{18} μ-Bondapac (30 x 0.39 cm). The type of aqueous and organic solvents used in these separations is indicated in the legend of each chromatogram.

RESULTS

Fig. 1 shows the SDS-PAGE profile of the two purified reductase preparations and compares its mobility to cytochromes P-450 with known amino acid sequence (9). Each reductase appears as a single band. Form 1 enzyme migrates between P-450-6 and P-450-4, and has an apparent molecular weight of 58.5-kDa. Form 2 reductase has an electrophoretic mobility identical to that of P-450-4 (58.0-kDa). Cytochrome P-450-3c (57.3-kDa) moves slightly below form 2 reductase.

Automated Edman degradation of form 1 indicated a blocked NH_2-terminus. In order to obtain sequence information on this protein, form 1 was reduced, alkylated with [^{14}C] iodoacetamide, succinylated and digested with trypsin. The digest was first resolved on column of Sephadex LH60 equilibrated with formic acid/ethanol. Peptide mixtures from this column were further resolved using a reverse phase HPLC. Examples of such resolutions are shown in Fig. 2. CNBr cleavage of form 1, followed by HPLC gave overlap for peptides 1, R-1 and 1, R-2. The results of microsequence analysis of selected peptides is given in Table I.

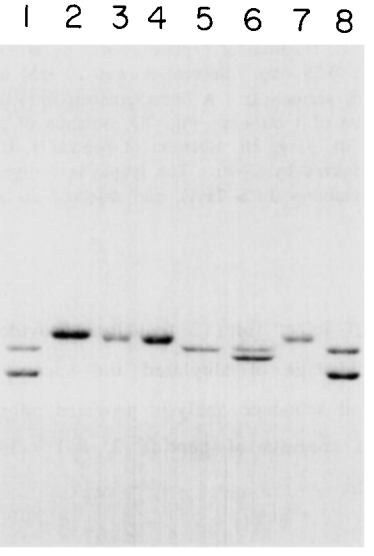

Figure 1: SDS-PAGE analysis of purified NADPH-monooxygenases.
Lane 1 and 8, rabbit liver microsomal cytochromes P-450-2 and P-450-4. Lane 2, cytochrome P-450-6. Lanes 3,4, and 7 form 1 monooxygenase. Lane 5, form 2 monooxygenase. Lane 6, form 2 (top band), and cytochrome P-450-3c (bottom band).

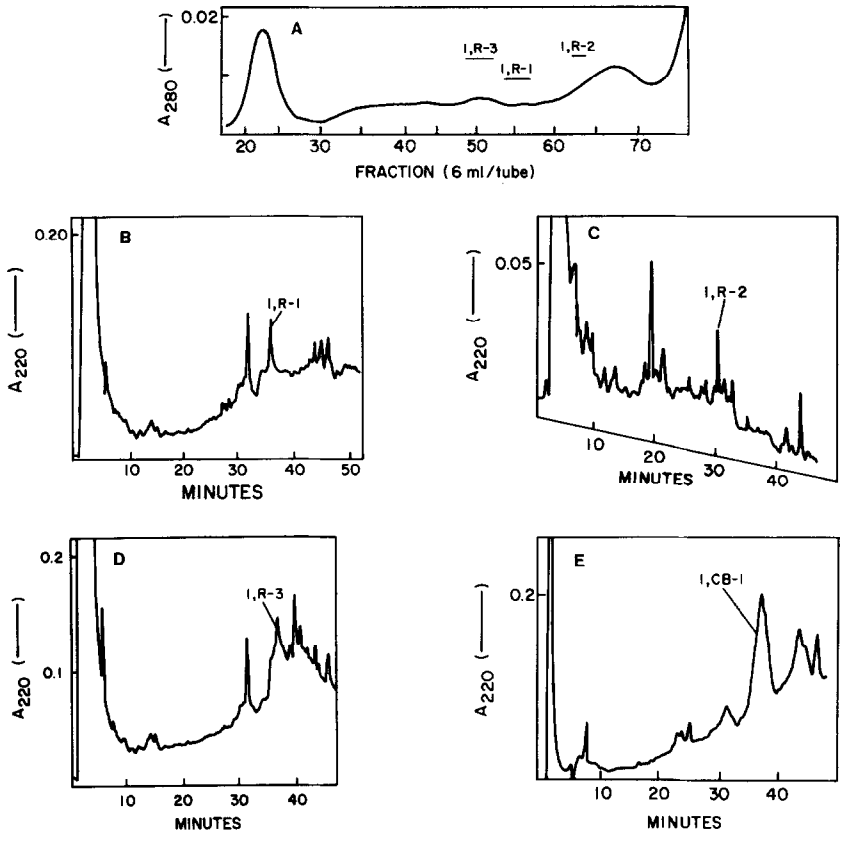

Figure 2A: Gel filtration of tryptic digest of alkylated, succinylated form 1 monooxygenase. Approximately 8 nmol of digested protein applied to a column of LH60 Sephadex (2.5 x 100 cm) equilibrated with formic acid/ethanol (3:7). Six ml fractions were collected at a flow rate of 10 ml/hour. The solid bar indicates the distribution of a particular peptide in the collected fractions. Fig. 2B, Isolation of peptide 1, R-1. Fractions indicated in Fig. 2A were resolved on a Vydac C_4 column (15 x 0.46 cm). Solvent A was aqueous 0.1% TFA and Solvent B was 0.1% TFA in 75% acetonitrile. A linear gradient from 0% to 100% of Solvent B in 70 min was performed at a flow rate of 1.0 ml/min. Fig. 2C, Isolation of peptide 1, R-2 by HPLC of LH60 column fractions on a C_{18} μBondapak column (30 x 0.39 cm). Solvent A was 20 mM ammonium acetate, pH 5.8, and Solvent B was Solvent A in 75% acetonitrile. A linear gradient from 0% to 100% of Solvent B in 70 min was performed at a flow rate of 1 ml/min. Fig. 2D, isolation of peptide 1, R-3. The HPLC was performed as described in Fig. 2B. Fig. 2E, isolation of peptide 1, CB-1 from CNBr digest of form 1. Two nmol of form 1 was cleaved by CNBr. The lyophylized digest was extracted with 100 μl of Solvent B (75% acetonitrile containing 0.1% TFA), and resolved on a Vydac C_4 column (15 x 0.46 cm) as described in Fig. 2B.

Microsequence analysis of intact form 2 reductase provided the sequence of the first 21 residues (Table II). Tryptic cleavage of alkylated and succinylated form 2, followed by gel filtration of the digest, HPLC and sequence analysis provided additional structural data on form 2 reductase (Table II). Sequence analysis of peptide 2, R-1 confirmed and extended the NH_2-terminal sequence to residue 32.

DISCUSSION

Comparison of the NH_2-terminal sequence of form 2 reductase with other flavoproteins shows that it is homologous to the NH_2-terminus of p-hydroxybenzoate hydroxylase from Pseudomonas

Table I

Summary of Sequence Studies on Form 1 Peptides

Form 1, R-1			Form 1, R-2			Form 1, CB-1			Form 2, R-3		
Cycle #	AA	Yield (pmol)	Cycle #	AA	Yield (pmol)	Cycle #	AA	Yield (pmol)	Cycle #	AA	Yield (pmol)
1	Val	109	1	Val	140	1	Val	138	1	Asn	27
2	Ala	70	2	Phe	122	2	Phe	132	2	Ser	16
3	Ile	53	3	Asp	92	3	Thr	58	3	Leu	28
4	Val	56	4	Ser	36	4	Thr	40	4	Pro	22
5	Gly	47	5	Gly	67	5	Arg	39	5	Thr	8
6	Ala	53	6	Tyr	68	6	Phe	53	6	Pro	16
7	Gly	35	7	Pro	64	7	Gln	24	7	Ile	13
8	Val	49	8	Trp	49	8	Asn	27	8	Val	12
9	Ser	18	9	Asp	62	9	Phe	28	9	Ser	5
10	Gly	31	10	Met	44	10	Ile	21	10	Thr	4
11	Leu	50	11	Val	53	11	Arg	15	11	Leu	14
12	Ala	45	12	Phe	39	12	Asn	16	12	Val	10
13	Ser	10	13	Thr	20	13	Ser	4	13	Ala	8
14	Ile	30				14	Leu	10	14	Lys	6
15	Lys	29							15	Lys	7
16	Ser	8							16	Met	4
17	Cys	22							17	Asn	4
18	Leu	25							18	Ser	2
19	Glu	20							19	Trp	1
20	Glu	22							20	Phe	3
21	Gly	17									
22	Leu	15									
23	Lys	11									
24	Pro	9									
25	Thr	3									
26	Cys	4									
27	Phe	6									
28	Glu	4									
29	Arg	2									

fluorescens (16). In the segment residues 1 to 33 of the Pseudomonas reductase some 40% residues are identical to the NH_2-terminus of form 2 enzyme (Fig. 3). p-Hydroxybenzoate hydroxylase is a substrate inducible, soluble, flavin monooxygenase catalyzing the incorporation of one atom of molecular oxygen in the substrate p-hydroxybenzoate with reduction by NADPH of the other oxygen atom (17). The enzyme forms dimers with two identical subunits, and the monomer has a molecular weight of 43,000. The three-dimensional structure of p-hydroxybenzoate hydrolase, with bound substrate, has been determined at a resolution of 0.25 nm (16). Peptide 1, R-1 of form 1 reductase shows a striking degree of identity with the NH_2-terminus of form 2. Some 80% of the residues of peptide 1, R-1 are identical to the NH_2-terminus of form 2 reductase (Fig. 3). Interestingly, the NH_2-terminus of these three proteins contains the consensus sequence Gly-X-Gly-X-X-Gly found in the FAD binding proteins (18). In the p-hydroxybenzoate hydroxylase this segment maintains the appropriate secondary or tertiary structure necessary for FAD binding (16). High degree of homology between forms 1 and 2 is also seen in the segment comprised by peptides [1,R-2]-[1,CB-1]-[1,R-3] and form 2 peptides [2,R-2]-[2,R-3] (Fig. 3). In this 42 residue segment 60% of the residues are identical. No clear similarity, however, is evident when this segment is compared to the sequence of p-hydroxybenzoate hydroxylase.

Table II

Summary of Sequence Studies on Form 2 Peptide

Form 2			Form 2, R-1			Form 2, R-2			Form 2, R-3		
Cycle #	AA	Yield (pmol)	Cycle #	AA	Yield (pmol)	Cycle #	AA	Yield (pmol)	Cycle #	AA	Yield (pmol)
1	Gly	109	1	Ser	46	1	Val	77	1	Phe	123
2	Lys	138	2	Cys	28	2	Trp	22	2	Gln	96
3	Lys	102	3	Leu	34	3	Asp	38	3	Thr	34
4	Val	77	4	Glu	31	4	Asp	43	4	Phe	101
5	Ala	83	5	Glu	38	5	Gly	34	5	Leu	110
6	Ile	73	6	Gly	16	6	Tyr	30	6	Lys	85
7	Ile	81	7	Leu	20	7	Pro	24	7	Asn	62
8	Gly	60	8	Glu	19	8	Trp	9	8	Asn	70
9	Ala	88	9	Pro	15	9	Asp	19	9	Leu	80
10	Gly	61	10	Thr	7	10	Met	8	10	Pro	62
11	Ile	70	11	Cys	10	11	Leu	10	11	Thr	30
12	Ser	21	12	Phe	8	12	Tyr	11	12	Ala	42
13	Gly	55	13	Glu	7	13	Val	7	13	Ile	34
14	Leu	59	14	Met	4	14	Thr	4	14	Ser	9
15	Ala	45				15	Arg	4	15	Asp	29
16	Ser	10							16	Met	8
17	Ile	43							17	Trp	10
18	Arg	31							18	Tyr	10
19	Ser	8							19	Val	10
20	Cys	17							20	Lys	9
21	Leu	19							21	Gln	8
									22	Met	4
									23	Asn	8
									24	Ala	8
									25	Trp	2
									26	Phe	6

A

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                                        10
p-HbH     Met-Lys-Thr-Gln-Val-Ala-Ile-Ile-Gly-Ala-Gly-Pro-Ser-Gly-Leu-Leu-Leu-

Form 2    Gly-Lys-----Lys-Val-Ala-Ile-Ile-Gly-Ala-Gly-Ile-Ser-Gly-Leu-Ala-Ser-

Form 1, R-1          Arg-Val-Ala-Ile-Val-Gly-Ala-Gly-Val-Ser-Gly-Leu-Ala-Ser-

                20                                  30
p-HbH     Gly-Gln-Leu-Leu-His-Lys-Ala-Gly-Ile-Asp-Asn-Val-Ile-Leu-Glu-Arg-33

Form 2    Ile-Arg-Ser-Cys-Leu-Glu-Glu-Gly-Leu-Glu-Pro-Thr-Cys-Phe-Glu-Met-32

Form 1, R-1  Ile-Lys-Ser-Cys-Leu-Glu-Glu-Gly-Leu-Lys-Pro-Thr-Cys-Phe-Glu-Arg  X-32
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B

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          1                   10                                  20
Form 1    Arg-Val-Phe-Asp-Ser-Gly-Tyr-Pro-Trp-Asp-Met-Val-Phe-Thr-Thr-Arg-Phe-Gln-Asn-Phe-Ile-Arg-

Form 2    Arg-Val-Trp-Asp-Asp-Gly-Tyr-Pro-Trp-Asp-Met-Leu-Tyr-Val-Thr-Arg-Phe-Gln-Thr-Phe-Leu-Lys-

                    30                                  40
Form 1    Asn-Ser-Leu-Pro-Thr-Pro-Ile-Val-Ser-Thr-Leu-Val-Ala-Lys-Lys-Met-Asn-Ser-Trp-Phe-

Form 2    Asn-Asn-Leu-Pro-Thr-Ala-Ile-Ser-Asp-Met-Trp-Tyr-Val-Lys-Gln-Met-Asn-Ala-Trp-Phe-
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Figure 3A: Comparison of the NH_2-terminal sequences of FAD-containing monooxygenase from Pseudomonas fluorescens (pHbH), rabbit liver microsomal form 2 monooxygenase and peptide 1, R-1 from form 1.
The amino acid sequence of pHbH is from Hofsteenge et al. Eur. J. Biochem. (1983) 133, 91-108.
Fig. 3B. Comparison amino acid sequences of fragment comprised by peptides (1, R-2)-(1, CB-1)-(1, R-3) of form 1 and (2, R-2)-(2, R-3) of form 2 monooxygenase.

The previously isolated FAD-containing monooxygenases from pig and rat liver microsomes are thought to be a single species with respect to minimum molecular weight on SDS-PAGE (1,4). Sabourin et al. (5), however, purified monooxygenase from mouse liver microsomes containing two equally staining protein bands with apparent minimum molecular weight of 56-kDa and 54-kDa, respectively. Whether the 54-kDa band was a contaminant, degradation product, or related protein could not be ascertained from their study. Dannan and Guengrich (19), after separating microsomal proteins by SDS-PAGE and using an antibody to pig FAD-containing monooxygenase as a probe, found one immunoreactive protein band (56.5-kDa) in pig, rat and rabbit liver microsomes. Furthermore, Dannan and Guengrich (19) reported that the rabbit liver monooxygenases migrated along with the cytochrome P-450-4, which corresponds to the mobility of our preparation form 2.

The data presented in the present communication reports the isolation of two structurally related NADPH-monooxygenases from rabbit liver microsomes, and identifies the NH_2-terminal sequences of these two reductases. The determination of complete amino acid sequences of these enzymes will allow for a detailed analysis of the relationship between these and the previously isolated FAD-containing monooxygenases.

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