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STRUCTURAL COMPARISON BETWEEN THE TROUT AND MAMMALIAN HYDROPHILIC DOMAIN OF NADPH–CYTOCHROME P-450 REDUCTASE*

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SUMMARY

The isolation of the protease-solubilized NADPH–cytochrome P-450 reductase from trout liver and its properties are described. The sequence of the "hydrophilic domain" [protease-solubilized NADPH–cytochrome P-450 reductase from trout (residues Lys^{56}–Ser^{678})] is reported. The CNBr fragments of the trout "hydrophilic domain" and their proteolytic subpeptides were sequenced. The CNBr fragments were aligned by homology to the reported sequence of the porcine NADPH–cytochrome P-450 reductase. The structures of the mammalian and the trout NADPH–cytochrome P-450 reductases were compared. Stretches with high exchange rates between the pig and trout reductase were found at the NH_2 and the COOH terminal regions of the hydrophilic domain.

INTRODUCTION

The metabolic functions of the NADPH–cytochrome P-450 reductase has been intensively studied in the last decade, and the catalytic role of this enzyme in the cytochrome P-450-dependent monoxygenase reactions[1,2], the involvement in the NADPH-supported microsomal fatty acid chain elongation[3], the degradation of haeme to biliverdin[4,5] and in the generation of radical metabolites of oxygen[6] or organic molecules[7] have been established.

Recently, the amino acid sequences of the NADPH–cytochrome P-450 reductases from mammalian species have been elucidated[8–10], and the secondary structure of the enzyme was calculated using predictive methods[10,11]. Substantial homology disclosed the evolutionary relationship of the NADPH–cytochrome P-450 reductases to the flavodoxins and the FAD-containing enzymes (glutathione reductase, succinate dehydrogenase and fumarate reductase from *E. coli*, and the ferredoxin $NADP^+$ oxidoreductase)[10,11]. The notion that flavodoxin and ferredoxin $NADP^+$ oxidore-

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ductase are to be considered as "ancestral proteins" will possibly have to be reconsidered, as a soluble cytochrome P-450-dependent monoxygenase has been found in *B. megaterium*, containing the NADPH–cytochrome P-450 reductase and cytochrome P-450 on a single polypeptide chain[12].

The binding sites in the NADPH–cytochrome P-450 reductase for the two flavocoenzyme moieties (FAD and FMN) and the cosubstrate NADPH have been tentatively assigned by the identification of homology areas to the nucleotide-binding regions of other flavin enzymes[10,11]. The location of the two cysteine residues ($Cys^{472}$and Cys^{566}) protected by NADP(H) against attack from thionucleophilic reagents[10,13] is compatible with the results obtained by homology considerations. In addition, classical chemical modification has shown that the integrity of arginine and lysine residues is required for NADPH-binding[14–16]. However, the identification of individual residues awaits detailed studies. Interspecies comparison between the NADPH–cytochrome P-450 reductases of mammalian origin and from organisms at a lower evolutionary level should yield a further insight into the development of the reductase, the structural variation of the nucleotide-binding sites, the hydrophobic membrane segment and the functional role of special amino acid residues or specific sequence segments.

The isolation of the NADPH–cytochrome P-450 reductases from microorganisms[17], plants[18], invertebrates[19] and lower vertebrates[20,21] has been reported. Particular interest was directed to the NADPH–cytochrome P-450 reductases from fish as examples of the reductase from cold-adapted (poikilothermic) species. The catalytic properties, the amino acid composition and the interaction with cytochrome P-450 and lipids in the reconstituted monoxygenase system are well documented for the NADPH–cytochrome P-450 reductase from trout[21]. Differences between the mammalian and the trout enzymes have been detected by immunological methods and amino acid analysis.

In this paper, we report the primary structure of the protease-solubilized NADPH–cytochrome P-450 reductase from trout (*Salmo trutta*).

EXPERIMENTAL

Materials

For the preparation of the NADPH–cytochrome P-450 reductase, the entrails of freshly slaughted rainbow trouts (*Salmo trutta*) were cooled in ice without delay, and the livers were used within 4 h or immediately frozen in liquid nitrogen and stored at −70°C until used.

2′,5′-ADP–Sepharose 4B and agarose–hexane–nicotinamide adenine dinucleotide phosphate AG NADP, Type 4, were obtained from Pharmacia (Uppsala, Sweden). Monobromobimane (Thiolyte MB) was purchased from Calbiochem (Frankfurt, F.R.G.). 2′-AMP, soy bean trypsin inhibitor and PMSF (phenylmethylsulphonyl fluoride) were products of Serva (Heidelberg, F.R.G.). Pepsin A, trypsin and endoproteinase Lys-C from *Lysobacter enzymogenes* were obtained from Boehringer (Mannheim, F.R.G.), pepstatin A from Sigma (Deisenhofen, F.R.G.), haemin from Eastman Organic (Rochester, NY, U.S.A.) and V8 proteinase from Miles Labs. (Elkhart, IN, U.S.A.). All other chemicals were of analytical-reagent grade. The solvents used for the high-performance liquid chromatography (HPLC) of peptides or

the analysis of PTH-amino acids were of HPLC grade. Sequence-grade chemicals were used for solid-phase Edman degradation.

GKC4 [Gynkochrom Hypersil Butyl (C_4) wide-pore column material (5 μm, pore diameter 30 nm)] was purchased from Gynkothek (Germering, F.R.G.), BC18 [Bakerbond Wide-Pore Octadecyl (C_{18}) column material (5 μm, pore diameter 33 nm)] from J. T. Baker (Gross-Gerau, F.R.G.) and SC18 [(Shandon Hypersil (3 μm)] from Shandon (Frankfurt, F.R.G.).

Isolation of the trypsin-solubilized NADPH–cytochrome P-450 reductase from trout liver

Trypsin-solubilized NADPH–cytochrome P-450 reductase was isolated according to the procedures described previously[10,22,23] for the purification of the enzyme from pig liver. To obtain satisfactory yields of the trout reductase, certain carefully defined conditions are required: the proteolytic digestion of the microsomes by trypsin (60 mg per microsomal fraction, isolated from 1 kg of liver) at 4°C was confined to 1 h. After this time, 0.8 *M* calcium chloride solution was added to the digest up to a final concentration of 20 m*M* and the solution was centrifuged (2 h, g_{max} = 10000 *g*). A cold, saturated solution of ammonium sulphate (5.28 *M*) was added slowly to the supernatant up to 45% saturation. The precipitate formed was removed by centrifugation (35 min, g_{max} = 10000 *g*), then the concentration of the ammonium sulphate was raised to 75% saturation in the supernatant. The resulting precipitate was removed by centrifugation, as above. The sediment obtained was redissolved in buffer A (0.05 *M* sodium phosphate–1 m*M* disodium ethylenediaminetetraacetate, pH 7.5). After addition of leupeptin and phenylmethylsulphonyl fluoride to final concentrations of 5 and 0.05 m*M*, respectively, the solution was stirred for 20 min (4°C) and centrifuged for 10 min at g_{max} = 15000 *g*. The supernatant was immediately loaded on a 2′,5′-ADP–Sepharose 4B column (40 cm × 1 cm I.D.), pre-equilibrated with buffer A. The affinity column was protected from particulate material not removed by centrifugation by means of a pre-column, filled with Sepharose 4B (10 cm × 1 cm I.D.). The 2′,5′-ADP–Sepharose 4B column was washed with 50 ml of buffer A–0.5 *M* potassium chloride solution and the reductase was subsequently eluted with 100 ml of 1 m*M* 2′-AMP in buffer A.

The active fractions were concentrated by ultrafiltration through an Amicon PM-10 membrane, then passed through a Sephadex G-25 column (90 cm × 1.7 cm I.D.) and finally rechromatographed on a 2′,5′-ADP–Sepharose 4B column, as described. After removal of 2′-AMP by ultrafiltration, leupeptin (enzyme:leupeptin = 50:1, w/w) was added, and the enzyme was stored at −70°C until used.

Chemical and enzymatic cleavage of the protease-solubilized NADPH–cytochrome P-450 reductase and its fragments

The CNBr fragments and subpeptides obtained by digestion with trypsin, pepsin A or V8 proteinase were prepared according to ref. 13. Digestion with the endoproteinase Lys-C was performed at 37°C for 24 h. The proteinase, dissolved in 25 m*M* Tris–HCl–1 m*M* EDTA (pH 7.7), was added in two equal amounts at the start of the reaction and 3 h after to the solution of the peptide in 0.1 *M* ammonium hydrogencarbonate solution (pH 8.0) (peptide:endoproteinase Lys-C = 1 μg:120 μU). The column type and the steepness of the linear gradients of acetonitrile in 0.1%

trifluoroacetic acid used for the reversed-phase high-performance liquid chromatography (HPLC) of peptides are given in the legends to the Figures and Table.

Analytical methods

Amino acid analyses, automated amino acid sequence determination, dansylation, identification of the PTH-amino acids and sodium dodecyl sulphate electrophoresis (SDS-PAGE) were performed as described previously[10,13]. The assay of the cytochrome *c*-reducing activity of the enzyme, the spectroscopic determination of the protein concentration and the flavin content of the purified reductase at A_{273} ($\varepsilon = 1.25 \cdot 10^5$ l mol^{-1} cm^{-1}) and A_{455} ($\varepsilon = 1.85 \cdot 10^4$ l mol^{-1} cm^{-1}), respectively, and the determination of protein were performed as described in ref. 22.

Chemical modification of the protease-solubilized NADPH–cytochrome P-450 reductase from trout

Reductase was modified with monobromobimane under denaturing conditions. Reductase (110 nmol in 0.1 *M* ammonium hydrogencarbonate solution, pH = 8.0) was dried in a Savant Speed-Vac-Concentrator, redissolved in 2 ml of 6 *M* guanidinium chloride–Tris–HCl (pH 8.0), 1.38 μl (22.05 μmol) of 2-mercaptoethanol was added (enzyme:reagent = 1:200, mol/mol) and the mixture was shaken under nitrogen for 3.5 h in the dark. Subsequently, 55.1 μmol (14.9 mg) of monobromobimane (enzyme:reagent = 1:500, mol/mol) were added to block the thiol groups, and the incubation was continued for a further 2 h. The reaction was stopped by addition of 800 μl of formic acid. Excess of reagent was separated from the reductase by filtration through a Sephadex G-50 column (25 cm × 1.8 cm I.D.), using 30% formic acid as equilibrating and eluting solvent.

Peptide nomenclature

CNBr fragments are designated CB... in the text and the legends. The CNBr fragments were numbered according to their corresponding gel permeation chromatography pool (GPC pool) and the acetonitrile concentration at which they are detected during elution from Gynkochrom C4 WP300 columns (GKC4) under the conditions described (Table I). Subfragments of CNBr peptides, obtained by tryptic, peptic, V8 proteinase and endoproteinase Lys-C digestion, are designated CB...T..., CB...P..., CB...V... and CB...EL..., respectively.

RESULTS

Isolation and characterization of the protease-solubilized NADPH–cytochrome P-450 reductase from trout

The protease-solubilized NADPH–cytochrome P-450 reductase from trout was purified to homogeneity by a procedure based on published methods[10,22,23]. However, the high sensitivity of this protein to the attack of proteases was a serious handicap during our efforts to purify and store the trout reductase. The major losses of activity occurring during the affinity chromatography of the enzyme on 2',5'-ADP–Sepharose 4B could be avoided by the addition of leupeptin and PMSF to the ammonium sulphate-precipitated reductase before loading it on the column. The trout reductase was not retained during washing of the affinity column (20 cm × 1

cm I.D.) with buffer A–0.5 *M* potassium chloride solution and, in contrast to mammalian reductase, it was not separated from contaminating protein. Columns with bed heights of 40 cm are required in order to achieve efficient purification on 2',5'-ADP–Sepharose 4B and elution of pure reductase by 2'-AMP. However, the obviously weaker binding of the trout enzyme to the affinity matrix cannot fully be explained by the fact that the K_m value (15 ± 3 m*M*; ionic strength 0.13; 25°C) determined for the protease-solubilized trout enzyme in accordance with preparations from other fish species[24] is double that of reductases from animals and plants (8–10 m*M* under identical conditions)[18,20]. The average yields (8–12 mg/kg liver) were considerably lower than that obtained by using porcine liver as starting material (20–30 mg/kg liver). The average specific activity of preparations purified from trout to apparent homogeneity, as determined by SDS-PAGE (27 ± 3 U/mg protein; 25°C; ionic strength 0.13), was distinctly higher than that of the protease-solubilized reductase from pig liver (18 ± 4 U/mg protein).

The characteristic spectroscopic properties (A_{273}/A_{455} = 7.18 ± 0.19 and A_{455}/A_{380} = 1.13 ± 0.08) determined for the protease-solubilized reductase from trout differ significantly from the values obtained for preparations from mammals only with respect to the A_{273}/A_{455} ratio (pig, 6.8 ± 0.2; rat, 6.7[25]). The slightly higher A_{273}/A_{455} ratio for the trout enzyme could indicate a loss of flavin during the purification procedure. However, attempts to activate the enzyme with FAD or FMN (flavin:reductase = 1:100, pH = 7.5) resulted in only a 10% increase in activity after an incubation period of 60 min. The protease-solubilized NADPH–cytochrome P-450 reductase from trout shows a striking lability to serine and cysteine proteinases.

Sequencing of the protease-solubilized NADPH–cytochrome P-450 reductase from trout

The complete amino acid sequence of the trypsin-solubilized NADPH–cytochrome P-450 reductase (except *ca.* 30 residues), as determined in this study, is presented in Fig. 1.

The sequence strategy applied (Fig. 2) consisted first in the fragmentation of the S-carboxymethylated trout enzyme by cyanogen bromide. Nineteen CNBr fragments were isolated by gel chromatography on Sephadex G-75 (Fig. 3) and subsequent reversed-phase HPLC of the individual gel chromatography pools (Fig. 4A–D). This number of peptides obtained by CNBr cleavage of the enzyme is consistent with the amount of methionines (18 residues/mol) calculated from the amino acid analyses of the trout enzyme. The CNBr fragments were characterized by their amino acid composition (Table I). The CNBr peptides were aligned by homology comparison with the known structures of mammalian NADPH–cytochrome P-450 reductases (Fig. 1).

Compared with the porcine NADPH–cytochrome P-450 reductase, five methionines are additionally present in the region 119–541 of the trout sequence, and three methionines are replaced by other hydrophobic residues (Met^{253}–Ile^{253}, Met^{263}–Leu^{263}, Met^{346}–Ile^{346}) (Fig. 1). The absence of Met^{346} in the trout reductase results in the formation of a third, large CNBr fragment, His^{302}–Met^{405} (M_r = 11 598), eluted together with the peptides CB1 (Pro^{449}–Met^{511}) and CB2 (Gly^{542}–Met^{636}) in the GPC pool III + IV (Fig. 3). HPLC of these fragments on a Gynkochrom Hypersil Butyl (C_4) wide-pore column with an acetonitrile gradient in 0.1%

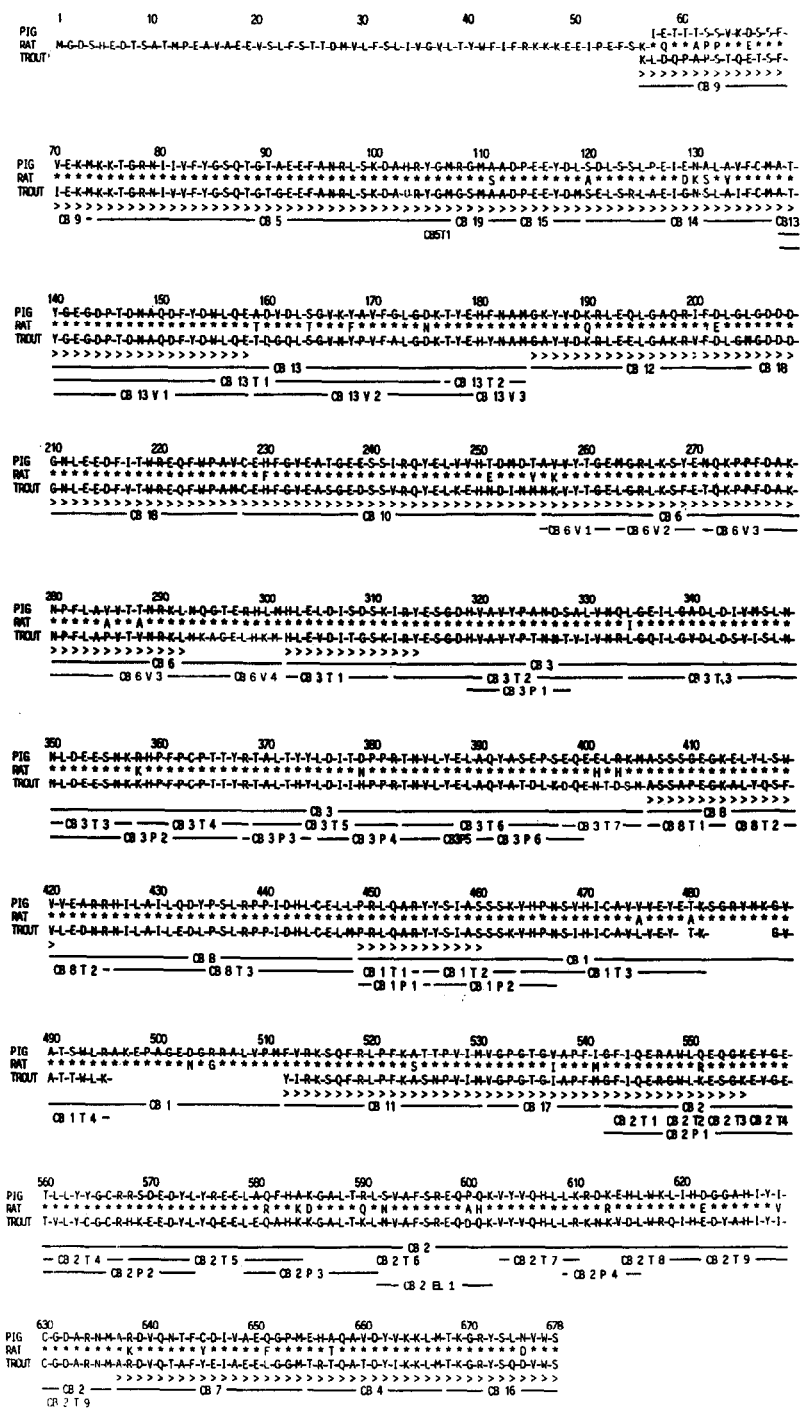

Fig. 1. Primary structures of NADPH–cytochrome P-450 reductases from pig, rat and trout. The strategy for the sequencing of the trout enzyme is shown: > > > indicates identification of PTH derivatives by HPLC, following automated Edman degradation of CNBr peptides; underlined residues (———) indicate identification of PTH derivative following automated Edman degradation of proteolytic subfragments, derived from CNBr peptides.

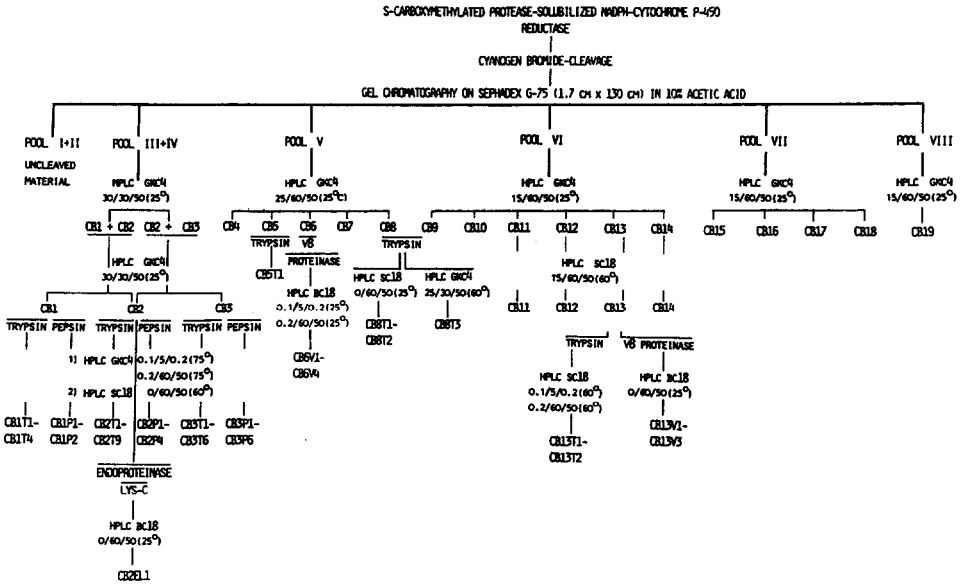

Fig. 2. Purification scheme for peptides from NADPH–cytochrome P-450 reductase (trout). HPLC of peptides was performed by gradient elution from a Gynkochrom Hypersil C4 WP300 (GKC4) column (25 cm × 0.46 cm I.D.) or a Shandon Hypersil C_{18} (SC 18) column (25 cm × 0.46 cm I.D.). The linear gradient is described by the initial concentration (%) of acetonitrile in 0.1% trifluoroacetic acid/time of gradient (min)/final concentration (%) of acetonitrile in 0.1% trifluoroacetic acid. For peptide nomenclature, see text. CB5T1 was isolated from the tryptic digest of CB5 by homoserine lactone coupling to CPG–3-aminopropyl and sequenced by automated Edman degradation.

trifluoroacetic acid resulted in a peak group of the three overlapping large CNBr fragments (Fig. 4). Division of the peak group according to N-terminal analysis and rechromatography under identical conditions was necessary to obtain pure CB1–CB3 in sufficient yield. Use of C_{18} wide-pore (33 nm) material led to a complete separation of the distinctly more retarded CB3 from a poorly resolved peak, containing a mixture of CB1 and CB2. The CNBr peptide, comprising the stretch Gly^{542}–Met^{636} (CB2 from trout), is eluted at higher acetonitrile concentrations than the fragment containing the sequence Pro^{449}–Met^{511}(CB1 from trout) (Table I). The order of elution was the reverse of that of the corresponding peptides from porcine reductase, probably because the trout CB2 is ten residues shorter than the corresponding porcine peptide Val^{531}–Met^{636} (Fig. 1).

Fragments CB1–CB3 were processed by proteolytic degradation to generate peptides suitable for solid-phase sequencing (Fig. 2). The sequence Ser^{595}–Lys^{602} was established from CB2EL1 obtained by digestion of CB2 with endoproteinase Lys-C. CB5 was determined by direct sequencing; the last two amino acids at the COOH-terminal side were confirmed by analysis of CB5T1 (Gly^{106}–Met^{107}). The sequence Asn^{293}–Met^{301} in CB6 (Fig. 1) was determined by Edman degradation of the subpeptides CB6V3 and CB6V4 obtained by cleavage of CB6 with V8 proteinase (Fig. 2). CB8 was sequenced for 15 (Val^{420}) of 43 residues. The structure of CB8 has been completely established by sequencing the three tryptic subfragments isolated.

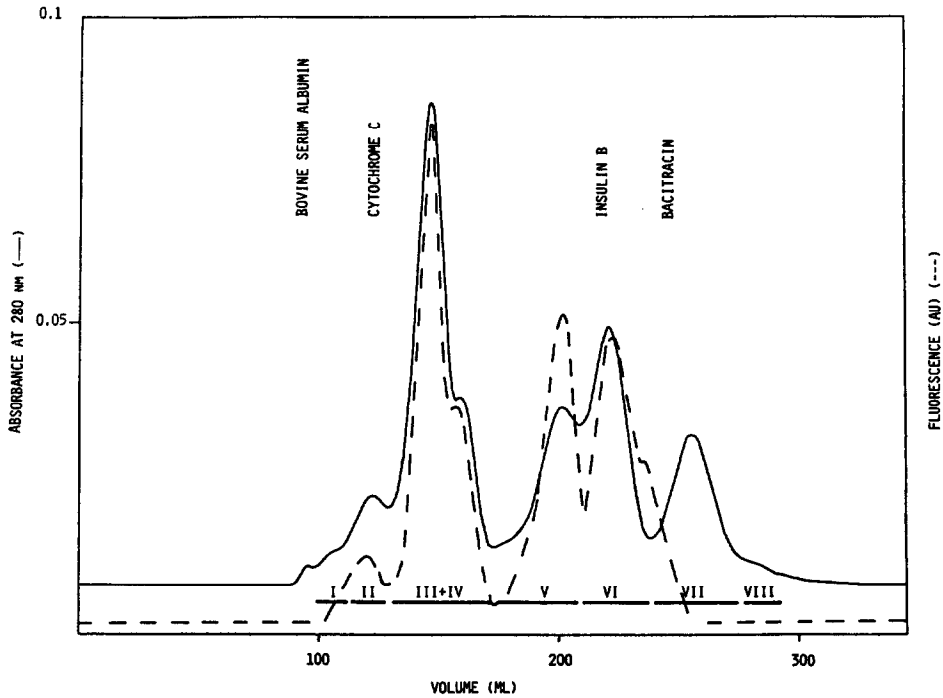

Fig. 3. Gel chromatography of the peptides obtained by CNBr cleavage of the bimane-labelled NADPH–cytochrome P-450 reductase (8 bimane groups per mole of enzyme) on a Sephadex G-75 column (130 cm × 1.7 cm I.D.) with 10% (v/v) acetic acid. Peptides of bimane-labelled NADPH–cytochrome P-450 reductase were prepared from 70 nmol of modified NADPH–cytochrome P-450 reductase. Fractions of 3.5 ml were collected. (———) Absorbance at 280 nm; (– – –) fluorescence (excitation, 395 nm; emission, 475 nm).

Cleavage at the site Arg^{438}–Pro^{439} was obviously in a poor yield (Fig. 1). CNBr fragments CB11–CB14, derived from GPC pool VI, could not be isolated in pure form by HPLC on Gynkochrom Hypersil C_4 WP300 (GKC4). They were therefore rechromatographed on Shandon C_{18} (3 μm) material with acetonitrile gradients in 0.1% trifluoroacetic acid (Fig. 2). CB13 was sequenced by direct Edman degradation up to Glu^{158}. Data from tryptic subpeptides CB13T1–CB13T3 and CB13V2 extended the sequence up to the C-terminal Met^{184}.

To locate cysteinyl peptides and the PTH derivative of S-bimanylcysteine by fluorescence, NADPH–cytochrome P-450 reductase labelled by monobromobimane after denaturation of the protein with 6 *M* guanidinium chloride was subjected to CNBr cleavage and subfragmentation of the CNBr peptides by proteases. During solid-phase sequencing, it was observed that the coupling of S-bimanylated peptides, containing cysteine residues near the COOH-terminus [*e.g.*, CB2T4 and CB2T9 (Fig. 1)] to 3-aminopropyl-glass by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide proceeds in very low yield (< 10%) under the conditions described by Laursen[26].

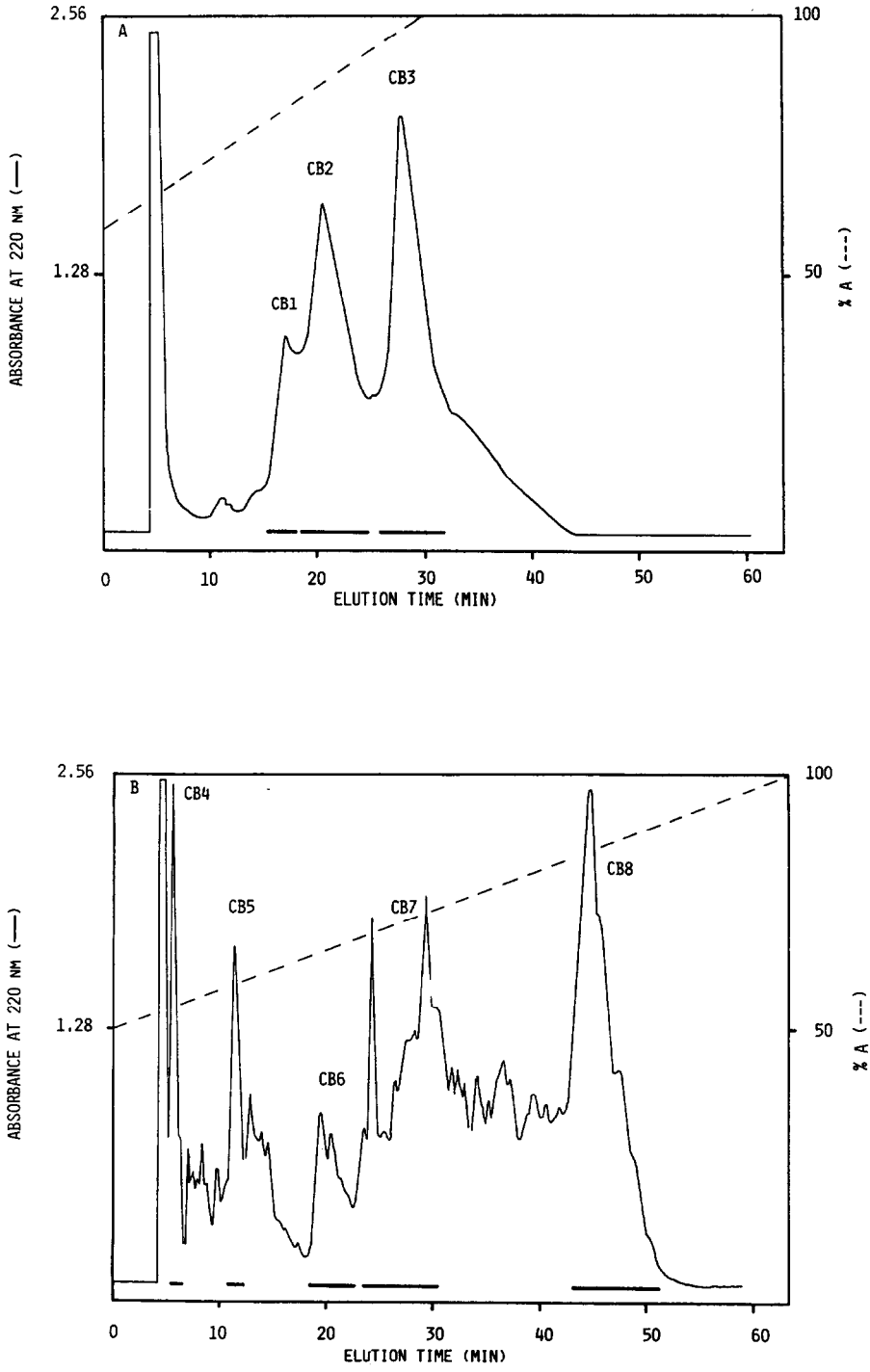

Fig. 4.

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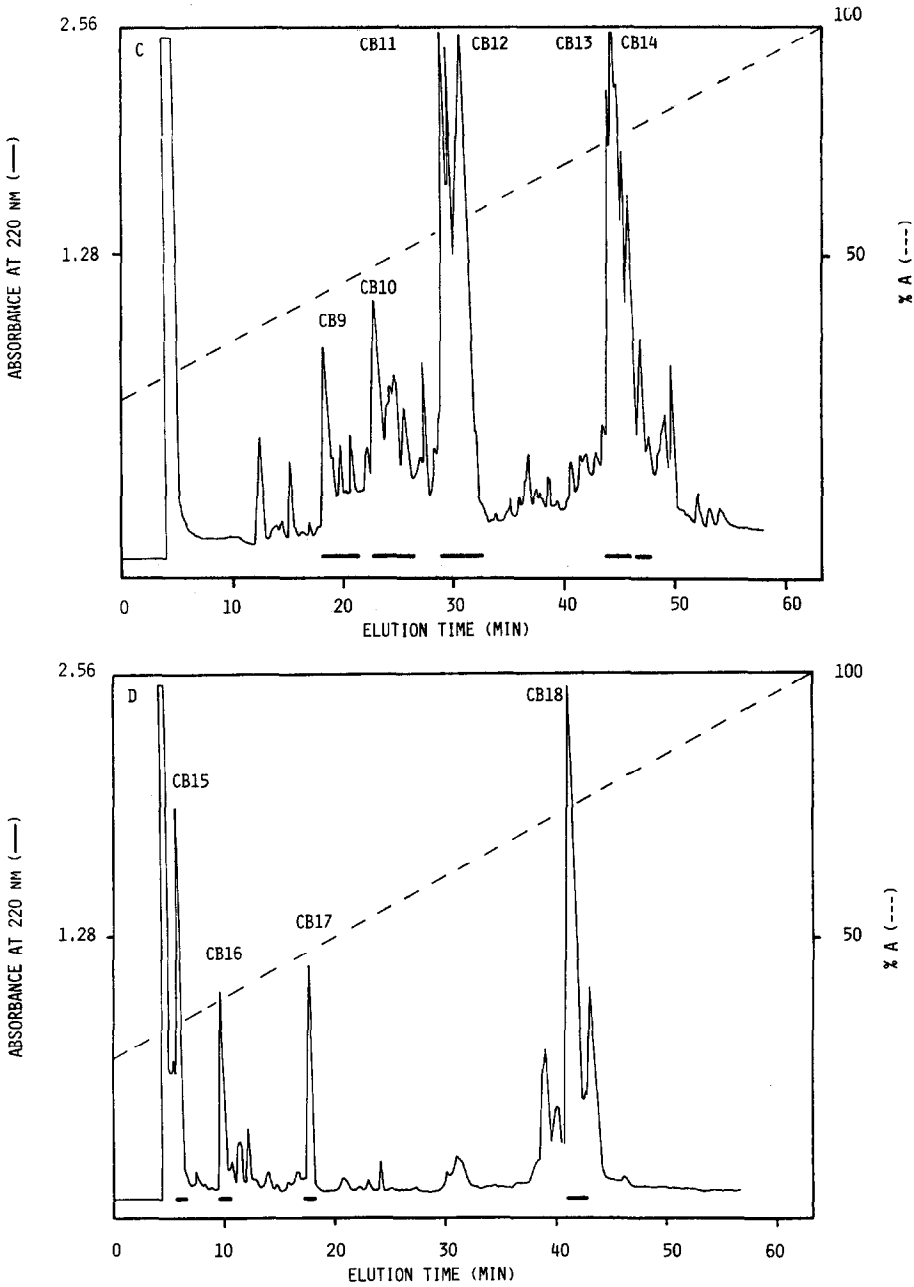

Fig. 4. HPLC of the peptide pools obtained by gel chromatography of the CNBr peptides on Sephadex G-75. The eluate from Sephadex G-75 was divided into pools (III + IV)–VIII (Fig. 3). The CNBr peptides were fractionated by HPLC on a Gynkochrom Hypersil C4 wide-pore column (25 cm × 0.46 cm I.D.) with acetonitrile gradients in 0.1% (v/v) trifluoroacetic acid. Solvent A, 50% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid; solvent B, 0.1% (v/v) trifluoroacetic acid. (A) HPLC of pool III + IV; (B) HPLC of pool V; (C) HPLC of pool VI; (D) HPLC of pool VII.

DISCUSSION

The species specificity of the NADPH–cytochrome P-450 reductase has been questioned during reconstitution experiments of the hepatic mixed-function oxidase system with components of different origin. Williams *et al.*[21] showed that the NADPH–cytochrome P-450 reductase "is apparently not species-specific with respect to enzymatic activity, that is, reductase from any source appears to be effective in reducing cytochrome P-450 from any other source". The structural comparison between the "hydrophilic domains" (protease-solubilized NADPH–cytochrome P-450 reductase) from pig, rat and trout confirms that the NADPH–cytochrome P-450 reductase is, at least in its catalytically active part, a highly conserved protein. The overall homology between the trout and pig protease-solubilized enzymes (79%) is unexpectedly high. This simplified view must be refined by inspection of the regions with defined function in the structural organization of the enzyme. In the intact membrane protein NADPH–cytochrome P-450 reductase the NH_2-terminal sequence Lys^{56}–Ile^{70} of the "hydrophilic domain" is connected with the membrane segment (residues 1–55). The level of identical residues within the sequence Lys^{56}–Ile^{70} is 33% between the trout and pig reductase. The dislocation of the proline residues in the connecting peptide should not orient the peptide chain differently in each protein. The polar character of this region with predicted high flexibility[10] is not changed by the replacements. The degree of structural identity in the sequence 56–70 of mammalian reductases (pig, rat, rabbit[27]) is distinctly higher than that between porcine and trout reductase. A similar accumulation of interchanges is observed in the COOH-terminal region between residues 642 and 663 (12 replacements per 22 residues between trout and pig reductase).

The structural organization of the NADPH–cytochrome P-450 reductase is apparently the result of a gene fusion. This hypothesis is based on the strong homology between the NADPH–cytochrome P-450 reductase and the two ancestral proteins, flavodoxin and ferredoxin–$NADP^+$ oxidoreductase[10,11]. According to the model proposed by Porter and Kasper[11], the FMN-binding region (Val^{82}–Cys^{218}) of the NADPH–cytochrome P-450 reductase homologous to flavodoxins borders on the sequence His^{230}–Leu^{265} at residue 228, showing no homology to other flavoproteins. This region is intercalated between the FMN-binding site and the sequence Leu^{292}–Asn^{326}, which is proposed to be identical with the FAD–pyrophosphate binding site and shows structural relationship to the NH_2-terminal part of the ferredoxin–$NADP^+$ oxidoreductase. The stretch Leu^{292}–Asn^{326} is separated by the region Leu^{330}–Glu^{445} from the sequence Glu^{446}–Ser^{678} with strong homology to the ferredoxin–$NADP^+$ oxidoreductase (Glu^{45}–Tyr^{294})[10,11]. FAD–pyrophosphate-(Leu^{292}–Asn^{326}, exchanges per 35 residues: rat/pig 0; trout/pig, 9) and NADPH-binding region (Gly^{483}–Gly^{554}, exchanges per 72 residues: rat/pig, 6; trout/pig, $\geqslant 11$) are highly conserved.

The exchanges do not influence the degree of homology to the ferredoxin–$NADP^+$ oxidoreductase. The sequence Ala^{295}–Lys^{300} is homologous to the consensus sequence "Gly–X–Gly–X–X–Gly" for the pyrophosphate-binding segment in the glutathione reductase (Gly^{27}–Gly^{32}). None of the characteristic glycine residues of this consensus sequence is conserved in the trout reductase. The high rate of exchange between mammalian and trout reductases does not change the polar character of the

TABLE I

CHARACTERIZATION OF THE CNBr FRAGMENTS OBTAINED FROM THE CATALYTIC DOMAIN OF THE NADPH–CYTOCHROME P-450 REDUCTASE AFTER ELUTION FROM A REVERSED-PHASE HYPERSIL C_4 WIDE-PORE COLUMN

Peptides were characterized as follows: (1) by the elution volume (GPC pool) from a Sephadex G-75 column (130 cm × 1.7 cm I.D.) and (2) by the conditions used for isolation on an GKC-4 column (25 cm × 0.46 cm I.D.) (HPLC GKC-4). Acetonitrile (%) denotes the gradient concentration in the mixing chamber of the gradient former at the time of peptide elution. Amino acids were determined as *o*-phthaldialdehyde derivatives. The amino acid compositions are given in residues per mole of peptide. Numbers in parentheses give the integral number of residues, determined from the composition or obtained by sequence analyses. The amino acid contents were calculated without corrections for destruction. The N-terminal amino acid was determined by the dansyl chloride method. Cysteine was determined as S-carboxymethyl- or as S-bimanyl-cysteine, methionine as the sum of homoserine lactone and homoserine, tryptophan by peptide fluorescence (excitation, 295 nm; emission, 354 nm), and proline qualitatively by dansylation of the peptide hydrolysate and thin-layer chromatography.

	Fragment																	
	CB1		*CB2*		*CB3*		*CB4*		*CB5*		*CB6*		*CB7*		*CB8*		*CB9*	
N-Terminus	P		G		H		T		K		N		A		A		K	
GPC pool	III+IV		III+IV		III+IV		V		V		V		V		V		VI	
Aceto-nitrile (%)	41.2		43.5		48.7		27.3		29.8		34.9		36.2		43.7		26.5	
CYS (CM)	1.4	(1)	3.3	(3)	1.1	(1)	–	(–)	–	(–)	–	(–)	–	(–)	1.4	(1)	–	(–)
ASP	4.6	(5)	8.2	(8)	15.0	(17)	1.3	(1)	3.0	(3)	5.4	(5)	1.4	(1)	5.4	(5)	1.1	(1)
THR	4.5	(4)	3.3	(2)	8.6	(10)	2.8	(3)	2.5	(3)	3.8	(3)	1.0	(1)	–	(–)	1.8	(2)
SER	5.8	(6)	2.4	(2)	4.9	(6)	–	(–)	2.2	(2)	1.3	(1)	–	(–)	3.5	(4)	1.6	(2)
GLU	3.4	(3)	18.0	(18)	11.6	(9)	1.2	(1)	3.4	(3)	5.6	(4)	4.7	(4)	5.4	(5)	4.4	(4)
GLY	3.6	(4)	7.3	(7)	4.2	(4)	–	(–)	5.5	(5)	3.4	(3)	1.6	(2)	1.6	(1)	–	(–)
ALA	5.7	(6)	5.5	(5)	5.2	(4)	0.9	(1)	2.2	(2)	2.5	(3)	3.0	(3)	3.8	(4)	1.0	(1)
VAL	5.7	(6)	6.8	(6)	8.1	(8)	–	(–)	1.7	(2)	3.2	(3)	1.2	(1)	1.1	(1)	–	(–)
MET	0.9	(1)	1.2	(1)	1.1	(1)	0.9	(1)	1.0	(1)	1.0	(1)	1.1	(1)	1.2	(1)	1.1	(1)
ILE	2.7	(3)	3.9	(4)	5.6	(7)	0.7	(1)	1.0	(1)	–	( )	0.8	(1)	2.4	(1)	0.9	(1)
LEU	6.2	(6)	9.0	(9)	11.1	(11)	1.0	(1)	1.4	(1)	5.0	(5)	1.3	(1)	7.2	(8)	1.1	(1)
TYR	3.3	(3)	7.1	(6)	5.7	(6)	1.0	(1)	1.8	(2)	1.2	(1)	0.9	(1)	1.3	(1)	–	(–)
PHE	–	(–)	2.0	(2)	1.0	(1)	–	(–)	2.2	(2)	3.0	(3)	0.9	(1)	1.2	(1)	1.0	(1)
HIS	2.5	(2)	4.9	(5)	4.9	(5)	–	(–)	0.8	(1)	0.8	(1)	–	(–)	1.3	(1)	–	(–)
LYS	6.0	(6)	7.5	(9)	5.7	(4)	1.9	(2)	2.9	(3)	5.6	(7)	–	(–)	1.4	(1)	2.2	(2)
ARG	3.0	(3)	5.9	(6)	4.4	(4)	0.9	(1)	2.7	(3)	2.8	(4)	1.2	(1)	2.0	(2)	–	(–)
TRY	+	(2)	+	(2)	–	(–)	–	(–)	–	(–)	–	(–)	–	(–)	–	(–)	–	(–)
PRO	+	( )	–	(–)	+	(6)	–	(–)	–	(–)	+	(4)	–	(–)	+	(4)	+	(2)
Total		( )		(95)		(104)		(13)		(34)		(46)		(18)		(43)		(18)

sequence between residues 295–300. The segments intercalated between regions of supposed nucleotide-binding function in the reductase vary distinctly at their borders [Ala^{235}–Gln^{272} (13 exchanges per 38 residues (trout/pig), Ala^{321}–Ile^{346} (10 exchanges per 26 residues (trout/pig) and Ala^{390}–Pro^{435} (20 exchanges per 45 residues (trout/pig)]. In a similar manner, the sequence Lys^{555}–Ala^{625}, immediately adjacent to the NADPH–binding region, shows large differences between the enzymes of trout and mammals (exchanges per 71 residues: rat/pig, 9; trout/pig, 17).

According to the model proposed, the NADPH–protectable cysteines, Cys^{472} and Cys^{566}, are positioned outside the NADPH–binding region (Gly^{483}–Gly^{554}).

CB10		*CB11*		*CB12*		*CB13*		*CB14*		*CB15*		*CB16*		*CB17*		*CB18*		*CB19*	
C		Y		G		A		S		A		T		V		G		G	
VI		VI		VI		VI		VI		VII		VII		VII		VII		VIII	
28.2		31.8		32.5		40.1		41.1		18.4		22.2		25.2		38.9		3.6	
0.8	(1)	–	(–)	–	(–)	–	(–)	1.0	(1)	–	(–)	–	(–)	–	(–)	–	(–)	–	(–)
4.4	(4)	1.7	(1)	2.6	(2)	9.5	(9)	1.2	(1)	2.1	(2)	1.1	(1)	–	(–)	5.3	(5)	–	(–)
–	(–)	–	(–)	–	(–)	4.0	(4)	–	(–)	–	(–)	1.1	(1)	1.4	(1)	1.0	(1)	–	(–)
3.1	(3)	1.6	(2)	–	(–)	1.3	(1)	2.6	(3)	–	(–)	2.0	(2)	–	(–)	–	(–)	1.0	(1)
6.4	(6)	1.6	(1)	2.7	(2)	5.5	(6)	2.8	(2)	2.2	(2)	1.6	(1)	–	(–)	4.1	(4)	–	(–)
2.5	(2)	–	(–)	3.5	(3)	5.0	(5)	1.5	(1)	–	(–)	1.2	(1)	3.3	(3)	2.1	(2)	1.0	(1)
1.4	(1)	1.4	(1)	2.4	(2)	4.3	(4)	2.4	(2)	1.9	(2)	–	(–)	1.1	(1)	1.6	(1)	–	(–)
1.6	(2)	1.0	(1)	2.1	(2)	2.0	(2)	–	(–)	–	(–)	0.8	(1)	1.0	(1)	1.0	(1)	–	(–)
1.0	(1)	1.0	(1)	1.0	(1)	0.9	(1)	1.1	(1)	0.8	(1)	–	(–)	0.9	(1)	1.1	(1)	1.1	(1)
1.1	(1)	1.5	(2)	–	(–)	–	(–)	1.5	(2)	–	(–)	–	(–)	1.2	(1)	–	(–)	–	(–)
1.2	(1)	1.8	(1)	3.3	(3)	3.0	(3)	3.3	(3)	–	(–)	–	(–)	–	(–)	1.4	(1)	–	(–)
1.1	(1)	1.0	(1)	0.9	(1)	4.0	(5)	–	(–)	1.0	(1)	0.9	(1)	–	(–)	–	(–)	–	(–)
1.0	(1)	2.0	(2)	1.3	(1)	2.0	(2)	1.0	(1)	–	(–)	–	(–)	1.0	(1)	2.1	(2)	–	(–)
2.0	(2)	–	(–)	–	(–)	1.3	(1)	–	(–)	–	(–)	–	(–)	–	(–)	–	(–)	–	(–)
1.6	(1)	2.4	(2)	2.1	(2)	0.8	(1)	–	(–)	–	(–)	1.5	(1)	–	(–)	–	(–)	–	(–)
1.2	(1)	2.0	(2)	2.2	(2)	–	(–)	1.1	(1)	–	(–)	0.8	(1)	–	(–)	1.3	(1)	–	(–)
–	(–)	–	(–)	–	(–)	+	(1)	–	(–)	–	(–)	+	(1)	–	(–)	+	(2)	–	(–)
–	(–)	+	(2)	–	(–)	+	(2)	–	(–)	+	(1)	–	(–)	+	(2)	+	(1)	–	(–)
	(28)		(19)		(21)		(47)		(18)		(9)		(11)		(11)		(22)		(3)

This could be considered as evidence that the protection of these residues against the attack of –SH reagents is a consequence of a conformational change induced by the binding of the cosubstrate. On the other hand, it has been shown, that the modification of Cys^{472} and Cys^{566} does prevent the binding of NADPH only if these residues are blocked by bulky or charged groups[28]. This implies a steric or electrostatic hindrance of the cosubstrate binding by the blocking groups. In the trout reductase, there is an additional cysteine at position 564. The reactivity of this cysteine in the absence and presence of NADPH has not yet been studied. The glycine-rich region Val^{528}–Arg^{547} in the NADPH–cytochrome P-450 reductases conserved among the

ferredoxin–$NADP^+$ oxidoreductase and the NADH–cytochrome b_5 reductase[10,27] is related to a pyrophosphate binding unit of dinucleotide-binding proteins. The functional role of the stretch Val^{528}–Arg^{547} has been discussed controversially[10,11,27]. The spatial relationship between the glycine-rich sequence and the NADPH-protectable Cys^{566} suggests the participation of the stretch Val^{528}–Arg^{547} in the NADPH–pyrophosphate binding.

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REFERENCES

1 H. W. Strobel, A. Y. H. Lu, J. Heidema and M. J. Coon, *J. Biol. Chem.*, 245 (1970) 4851.
2 B. S. S. Masters, in W. B. Jakoby (Editor), *Enzymatic Basis of Detoxication*, Vol. 1, Academic Press, Orlando, 1980, pp. 183–199.
3 Z. Ilan, R. Ilan and D. L. Cinti, *J. Biol. Chem.*, 256 (1981) 10066.
4 B. A. Schacter, E. B. Nelson, H. S. Marver and B. S. S. Masters, *J. Biol. Chem.*, 247 (1972) 3601.
5 T. Yoshinaga, Sh. Sassa and A. Kappas, *J. Biol. Chem.*, 257 (1982) 7786.
6 N. R. Bachur, S. L. Gordon, M. V. Gee and H. Kon, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 954.
7 S. N. J. Moreno, J. Schreiber and R. P. Mason, *J. Biol. Chem.*, 261 (1986) 7811.
8 S. D. Black and M. J. Coon, *J. Biol. Chem.*, 257 (1982) 5929.
9 T. D. Porter and C. B. Kasper, *Proc. Natl. Acad. Sci. U.S.A.*, 82 (1985) 973.
10 F. Vogel and L. Lumper, *Biochem. J.*, 236 (1986) 871.
11 T. D. Porter and C. B. Kasper, *Biochemistry*, 25 (1986) 1682.
12 L. O. Narhi and A. J. Fulco, *J. Biol. Chem.*, 261 (1986) 7160.
13 F. Vogel, C. Kaiser, I. Witt and L. Lumper, *Biol. Chem. Hoppe-Seyler*, 366 (1985) 577.
14 R. E. Ebel, in M. J. Coon, A. H. Conney, R. W. Estabrook, H. W. Gelboin, J. R. Gilette and P. J. O'Brien (Editors), *Microsomes, Drug Oxidation and Chemical Carcinogenesis*, Vol. 1, Academic Press, New York, 1980, p. 315.
15 H. Inano and B. Tamaoki, *Eur. J. Biochem.*, 155 (1986) 485.
16 H. Inano and B. Tamaoki, *J. Enzyme Inhibition*, 1 (1986) 47.
17 S. Kubota, Y. Yoshida, H. Kumaoka and A. Furumichi, *J. Biochem. (Tokyo)*, 81 (1977) 197.
18 I. Benveniste, B. Gabriac and F. Durst, *Biochem. J.*, 235 (1986) 365.
19 D. R. Vincent and L. C. Terriere, *Insect Biochem.*, 15 (1985) 299.
20 R. J. Pohl, C. J. Serabjit-Singh, S. R. Slaughter, P. W. Albre, J. R. Fouts and R. M. Philpot, *Chem.-Biol. Interact.*, 45 (1983) 283.
21 D. E. Williams, R. R. Becker, D. W. Potter, F. P. Guengerich and D. R. Buhler, *Arch. Biochem. Biophys.*, 225 (1983) 55.
22 T. Omura and S. Takesue, *J. Biochem (Tokyo)*, 67 (1969) 249.
23 Y. Yasukochi and B. S. S. Masters, *J. Biol. Chem.*, 251 (1976) 5337.
24 A. V. Klotz, J. J. Stegeman and C. Walsh, *Arch. Biochem. Biophys.*, 226 (1983) 578.
25 T. C. Pederson, J. A. Buege and S. D. Aust, *J. Biol. Chem.*, 248 (1973) 7134.
26 R. A. Laursen, *Methods Enzymol.*, 47 (1977) 277.
27 M. Katagari, H. Murakami, Y. Yabusaki, T. Sugiyama, M. Okamoto, T. Yamano and H. Ohkawa, *J. Biochem. (Tokyo)*, 100 (1986) 945.
28 L. Lumper, F. Busch, S. Dzelic, J. Henning and T. Lazar, *Int. J. Pept. Protein Res.*, 16 (1980) 83.