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Structural and Functional Analysis of NADPH-Cytochrome P-450 Reductase from Human Liver: Complete Sequence of Human Enzyme and NADPH-Binding Sites†

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ABSTRACT: The complete amino acid sequence of human liver NADPH-cytochrome P-450 reductase has been determined by microsequence analysis and mass spectrometry. The total sequence consists of 676 amino acids initiated by an amino-terminal acetyl group. There is no evidence for posttranslational modifications, including Asn-linked glycosylation. The human enzyme exhibits sequence homology in the range of 92–95% with other mammalian enzymes. Sequence differences were mainly confined to several hydrophilic regions in the NH_2-terminal and COOH-terminal domains. Since the human enzyme is immunochemically distinct from the rabbit enzyme despite similar enzymatic properties, it is likely that these variable hydrophilic regions are potential antigenic determinants. The NADPH-depleted enzyme is inactivated by either fluorescein isothiocyanate, a lysine-specific reagent, or 5-(iodoacetamido)fluorescein, a cysteine-specific reagent. In both cases, protection by NADP(H) prevents enzyme inactivation by the reagents. Isolation of fluorescent peptide from 5-(iodoacetamido)fluorescein-inactivated enzyme identified Cys 565 as the specifically NADPH-protected residue.

The flavoprotein NADPH-cytochrome P-450 reductase (EC 1.6.2.4) is an integral component of the cytochrome P-450 xenobiotic metabolizing system (Williams, 1976; Schwab & Johnson, 1987). This enzyme system is found in both hepatic and extrahepatic tissues (McManus et al., 1987a; Hall et al., 1989) and is localized in the endoplasmic reticulum and nuclear membrane of cells (Kasper, 1971). Together with cytochrome P-450, NADPH-cytochrome P-450 reductase is essential for reconstitution of xenobiotic metabolizing activity in purified systems. The reductase functions by catalyzing electron transfer from NADPH to the hemoprotein during catalysis (Lu & West, 1978). In addition to providing reducing equivalents to cytochrome P-450, the reductase is also involved in electron transfer from NADPH to heme oxygenase (Yoshida & Kikuchi, 1978) and cytochrome b_5 (Ilan et al., 1981). The reductase has been purified from a variety of eucaryotic and procaryotic tissues (McManus et al., 1989) including human liver (Guengerich et al., 1981; Abraham et al., 1986; McManus et al., 1987a, 1989) and placenta (Osawa et al., 1981; Muto & Tan, 1986). By contrast with cytochrome P-450, which is comprised of a family of isozymes, only one form of NADPH-cytochrome P-450 reductase has been identified in each species investigated, and in the mouse, this enzyme is located on chromosome 6 (Simmon et al., 1985). Each form of NADPH-cytochrome P-450 reductase isolated has been shown to contain one molecule each of FAD and FMN per polypeptide chain. A cDNA for rat reductase was cloned by Gonzalez and Kasper (1982), while Black and Coon (1982) published protein sequence data on rabbit reductase. Since then, the amino acid sequence of pig liver reductase has been reported from protein sequence analysis (Haniu et al., 1984, 1986; Vogel et al., 1985; Vogel & Lumper 1986), and the cDNA-deduced sequences of the rat enzyme (Porter & Kasper, 1985; Murakami et al., 1986), rabbit enzyme (Katagiri et al., 1986), and yeast enzyme (Yabusaki et al., 1988) have been reported. Partial sequence of trout enzyme has been also reported (Urenjak et al., 1987). The amino acid sequence homology between the pig, rat, and rabbit enzymes is approximately 90%.

Except for two reports giving the amino acid composition (Muto & Tan, 1986; McManus et al., 1989), no structural information on the human NADPH-cytochrome P-450 reductase is available. However, indirect evidence from immunological studies suggest that there are structural differences between human reductase and its animal counterparts (Guengerich et al., 1981; Osawa et al., 1981; McManus et al., 1989). The reductase contains two prosthetic groups, FAD and FMN, and interacts with NADPH, cytochrome P-450, cytochrome b_5, and cytochrome *c*, suggesting the presence of up to six binding domains on the molecule (Porter & Kasper, 1986; Nisimoto, 1986). Since important binding regions on enzymes such as NADPH-cytochrome P-450 reductase exhibit sequence conservation across species, determination of the amino acid sequence of the human reductase is an important first step in understanding its structure–function relationships. We have therefore determined the complete amino acid sequence of the human NADPH-cytochrome P-450 reductase

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and compared it with those known in other species. We have also carried out site-directed alkylation studies to determine the location of sites in the primary sequence responsible for cofactor binding.

Experimental Procedures

Materials. TPCK[1]-trypsin (232 units/mg) was purchased from Biomedical Co., and *Staphylococcus aureus* protease (strain V8) was obtained from Pierce Chemical Co. Endoproteinase Asp-N was purchased from Boehringer-Mannheim. DEAE-Sepharose CL-6B and agarose–hexane 2′,5′-diphosphate type 2 were obtained from Pharmacia (Australia), Ultrogel HA was from LKB (Australia), and DE52 was from Whatman Australia. All other chemicals have been described previously (Haniu et al., 1986).

Purification of Human NADPH-Cytochrome P-450 Reductase. Human liver was obtained from two renal transplant donors maintained on life support systems until the kidneys could be removed. Subject 1 was a 62 year old male, and prior to death the patient had received the following drugs: phenytoin, dexamethasone, gentamycin, ranitidine, and cotrimoxazole. The smoking history and alcohol consumption of this subject are unknown. Subject 2 was a 19 year old male who had received dopamine prior to death. This patient was a nonsmoker and was a social alcohol drinker. The use of such tissues in these studies had both local Research Ethics Committee approval and Coroner's permission. The detergent-solubilized human reductase was purified from liver microsomes essentially according to the method of Yasukochi and Masters (1976) as previously described (McManus et al., 1987a,b). The trypsin-solubilized enzyme was purified only from liver microsomes of subject 1, essentially by the method of Iyanagi and Mason (1973) as modified by Haniu et al. (1984). Preparations of the detergent-solubilized reductases used in these studies had specific activities of 51, 39, and 46 units/mg, whereas the trypsin-solubilized enzyme had a specific activity of 44 units/mg. One unit of activity corresponds to the reduction of 1 μmol of cytochrome *c*/min. Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Enzyme Assay. NADPH-cytochrome *c* reductase was measured essentially according to the method of Williams and Kamin (1962) in 0.1 M potassium phosphate buffer, pH 7.7, at 25 °C. The nanomoles of cytochrome *c* reduced per minute was calculated from the initial phase of the reaction by use of an extinction coefficient of 21 000/(M·cm) for the difference in absorbance between oxidized and reduced forms at 550 nm. The reduction of ferricyanide was monitored at 420 nm, and the initial rate of the reaction was determined by use of an extinction coefficient of 1020/(M·cm) (Schellenberg & Hellerman, 1958).

Preparation of Peptides. Purified detergent-solubilized reductase (0.5 mg) was reduced and carboxymethylated with [^{14}C]iodoacetate (50 μCi/mmol) in 0.1 M phosphate buffer, pH 7.7, at 25 °C overnight, followed by a cold chase with iodoacetate (20 mg) in 6 M guanidine hydrochloride for 20 min. The *N*-ethylmaleimide derivative of the protein was also prepared in similar way, from [^{3}H]-*N*-ethylmaleimide (50 μCi/mmol). The reaction mixtures were dialyzed against water for 2 days and finally dialyzed against 0.1 M ammonium bicarbonate, pH 8.0. Trypsin and *S. aureus* protease digestions were performed in the same buffer at 37 °C for 24 h as described previously (Haniu et al., 1986). For endoproteinase Asp-N digestion, the reductase sample was dialyzed against 50 mM sodium phosphate buffer, pH 7.7. The digestion was performed in the same buffer at 37 °C for 20 h with an enzyme to substrate ratio of 1/1000 (w/w) (Drapeau, 1980). Cyanogen bromide digestion was performed in 70% formic acid at 25 °C for 24 h.

Peptide Separation by HPLC. Peptides were separated by HPLC using an Ultrasphere C8 (4.1 × 250 mm), Vydac C4 (4.6 × 250 mm), or C18 (4.6 × 250 mm) column. Chromatographic conditions were similar to those previously reported (Yuan et al., 1982).

Analytical Methods. Amino acid compositional analysis was performed on a Beckman System 6300 amino acid analyzer after hydrolysis in 5.7 N HCl containing 0.2% 2-mercaptoethanol as described (Del Valle & Shively, 1982). An automated gas-phase sequence analysis was performed according to Hawke et al. (1985) and manual analysis according to Haniu and Shively (1988).

Mass Spectrometry. FAB mass spectra were taken with a JEOL HX100HF mass spectrometer at a 5-kV accelerating potential and a nominal resolution setting of 3000 as described (Haniu et al., 1986). Peptide samples (100–400 pmol) in 1–2 μL of 5% acetic acid were added to 1–2 μL of a mixture of DTT/DTE (5/1).

Active Site Labeling of the Reductase with FITC and 5-IAF. Inhibition kinetics were studied by use of various concentrations of inhibitors at 25 °C over 90 min. At each time interval, duplicate values were measured with and without inhibitor. Enzyme activity was measured with respect to cytochrome *c* reduction after addition of 10 μL of cytochrome *c* (10 mg/mL) and 10 μL of 10 mM NADPH. For experiments investigating the effect of $NADP^+$ on FITC and 5-IAF inhibitions, enzyme incubation with 0.1 mM $NADP^+$ was carried out at room temperature for 1 h prior to labeling.

Stoichiometry of the Labeled Residues with FITC and 5-IAF. The number of cysteines or lysines that reacted with the fluorescent probes was measured according to the method described by Kirley et al. (1984). The enzyme (1 nmol) was incubated with 0–100 nmol of reagents in 0.1 M phosphate buffer, pH 7.4, for 1 h at room temperature. After gel filtration by Sephadex G-25 column, the fluorescence of the modified enzyme was measured at 495 nm as described previously (Kirley et al., 1984), and the concentration of covalently bound fluorescent probes was calculated [ϵ = 75 000/(M·cm)]. The protein concentration was determined by amino acid analysis. Isolation of fluorescein-labeled peptides was done in the same manner as in the previous peptide separations, except a fluorescent detector (excitation filter at 450 nm and emission filter at 570 nm) was used to minotor the chromatography.

Results

Proteolytic Digestions of Reductase. The tryptic peptide map of the ^{14}C-carboxymethylated protein was obtained by HPLC using a Vydac C18 column. Most of the peptides including the large hydrophobic NH_2-terminal peptide were recovered in suitable yield. Radioactivity measurements revealed that six or seven peptides were labeled with radioactive monoiodoacetate as shown in Figure S1 (supplementary material). *S. aureus* protease digestion was performed on detergent-solubilized reductase, and the peptides were separated on a Vydac C4 column, which permitted elution of the large hydrophobic peptides (Figure S2, supplementary material). Small peptides eluted at the break-through position were pu-

[1] Abbreviations: FITC, fluorescein isothiocyanate; 5-IAF, 5-(iodoacetamido)fluorescein; FAB-MS, fast atom bombardment mass spectrometry; PTH, phenylthiohydantoin; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

Table I: Amino Acid Compositions of Two NH_2-Terminal Peptides

amino acid	Sa-1	T-1
Asp	2.1 (2)[a]	3.1 (3)[a]
Thr	1.7 (2)	4.3 (5)
Ser	3.4 (4)	5.5 (7)
Glu	1.0 (1)	6.4 (6)
Pro		1.1 (1)
Gly	0.8 (1)	2.3 (2)
Ala		2.4 (2)
Val	1.7 (2)	5.4 (6)
Met		1.3 (2)
Ile		2.0 (2)
Leu		6.7 (6)
Tyr		1.2 (1)
Phe		5.0 (5)
Lys		3.2 (4)
His	0.7 (1)	0.8 (1)
Arg		1.1 (1)
Trp		(1)
total	13	55
calcd mol wt	1321[b]	6257[b]
obsd mass unit	1363	6300

[a] Number in parentheses shows residue determined by sequence analysis. [b] Calculated on the basis of the amino acid composition.

Table II: Amino Acid Compositions of Human and Rabbit Liver Microsomal NADPH-Cytochrome P-450 Reductases

	human enzyme		rabbit enzyme
amino acid	HCl hydrolysis[a]	sequence data	sequence data[b]
Asx	66.0	66	65
Thr	33.3	34	34
Ser	44.3	45	40
Glx	77.1	82	85
Pro	26.6	26	28
Gly	48.9	45	46
Ala	50.8	51	62
Val	48.5	49	48
Met	14.7	17	17
Ile	24.5	27	24
Leu	65.3	65	62
Tyr	29.8	31	33
Phe	27.3	28	27
Lys	36.2	36	35
His	17.8	19	18
Arg	38.2	38	38
Cys	*c*	8	7
Trp	*c*	9	9
total		676	678
mol wt (+acetyl)		76 553	76 583

[a] The number of the residues is calculated on the basis of a molecular weight of 77 000. [b] Derived from Katagiri et al. (1987). [c] Not determined.

rified again on Ultrasphere C8 or C18 columns. The endoproteinase Asp-N digest was subjected to HPLC using a Vydac C18 column in order to obtain some overlapping peptides (Figure S3, supplementary material).

Isolation of NH_2-Terminal-Blocked Peptide. Two different methods were used to isolate the NH_2-terminal-blocked peptide from human NADPH-cytochrome P-450 reductase. The human reductase was digested with *S. aureus* protease, and the digests were applied to Dowex 50-X8 (H form) equilibrated with 0.2 N acetic acid as described previously (Haniu et al., 1986). The first fraction (0.2 N acetic acid fraction) was applied to an Ultrasphere C18 column. One major peak was obtained. Alternatively, *S. aureus* digests of pig enzyme and human enzyme were compared on HPLC maps from a Vydac C18 column. The peak corresponding to the NH_2-blocked peptide of pig reductase was collected and analyzed by mass spectrometry and amino acid analysis. The amino acid compositions of the NH_2-terminal peptides from tryptic or *S. aureus* digests of human reductase are shown in Table I.

Sequence Determination of the NH_2-Terminal-Blocked Peptides. Peptide Asp-N-1 derived from the NH_2-terminal portion revealed the sequence D-T-S-S-T-V-S-E-A-V-A-E-E-S (Table SI, supplementary material), corresponding to residues 6–20. The remaining portion (residues 1–5) of the NH_2-terminal sequence was determined by analysis of peptide Sa-1. Since the α-NH_2 group of this peptide was blocked, presumably with an *N*-acetyl group, the peptide was hydrolyzed with 1% TFA at 110 °C for 12 h. After acid cleavage, a portion of the peptide mixture was directly applied to the gas-phase sequencer. The remaining peptide mixture was purified on a Vydac C18 column. The purified peptides revealed sequences of G-D, S-H-V, and T-S-S-T-V. Mass spectral analysis of the intact peptide gave a value of 1363 for the protonated molecular ion (Table SI, supplementary material). The 42 mass unit difference between the observed value and that calculated from the amino acid composition is consistent with an acetylated amino terminus. Thus, the peptide was assigned as the structure Ac-G-D-S-H-V-D-T-S-S-T-V-S-E, consistent with other NADPH-cytochrome P-450 reductases.

Analysis of Tryptic Peptides. All of the tryptic peptides from the detergent-solubilized enzyme were applied onto the gas-phase sequencer after rechromatography by HPLC as necessary. Sequence determinations were confirmed by mass spectrometry and summarized in Table SII (supplementary material). These data also confirm that there is no evidence that human liver NADPH-cytochrome P-450 reductase has posttranslational modifications such as Asn-linked glycosylation or methylation of lysine residues. During sequence analysis of peptide T-39, a polymorphism was observed. Amino acid compositions of these peptides are shown in Table SIII (supplementary material) and the sequence results in Table SIV (supplementary material).

Sequence Studies of S. aureus Protease Peptides. Peptides from the *S. aureus* digestion were separated on a Vydac C4 column. The highly hydrophobic NH_2-terminal peptide eluted as the last peak in the chromatogram (Figure SII, supplementary material). Table SIII in the supplementary material shows the sequence data for the various peptides with the majority confirmed by mass spectrometry. In the cases of several peptides which were not completely sequenced, mass spectral analyses served to confirm the remainder of the sequence. As in the tryptic digests, we found a polymorphism in the peptide sequence; Sa-25 gave the two sequences R-A-W-L-*Q*-Q-Q-G-K and R-A-W-L-*R*-Q-Q-G-K (Table SV, supplementary material).

Total Sequence of Human NADPH-Cytochrome P-450 Reductase. Although the proper order for most of the peptides could be established by overlapping regions with the *S. aureus* peptide fragments, additional peptides derived from endoproteinase Asp-N or cyanogen bromide cleavages were needed. The remaining alignments were resolved by sequence homology with other mammalian reductases. The strategy for determining the total sequence of the human reductase is presented in Figure S4 (supplementary material). The amino acid composition derived from sequence analysis is compared with those from 6 N HCl hydrolysis data in Table III, together with those of rabbit enzyme. These results are consistent with the reported values except for serine and glycine (McManus et al., 1989).

Sequence Comparison of the Human Reductase with Other Reductases. Figure 1 shows a sequence comparison of the

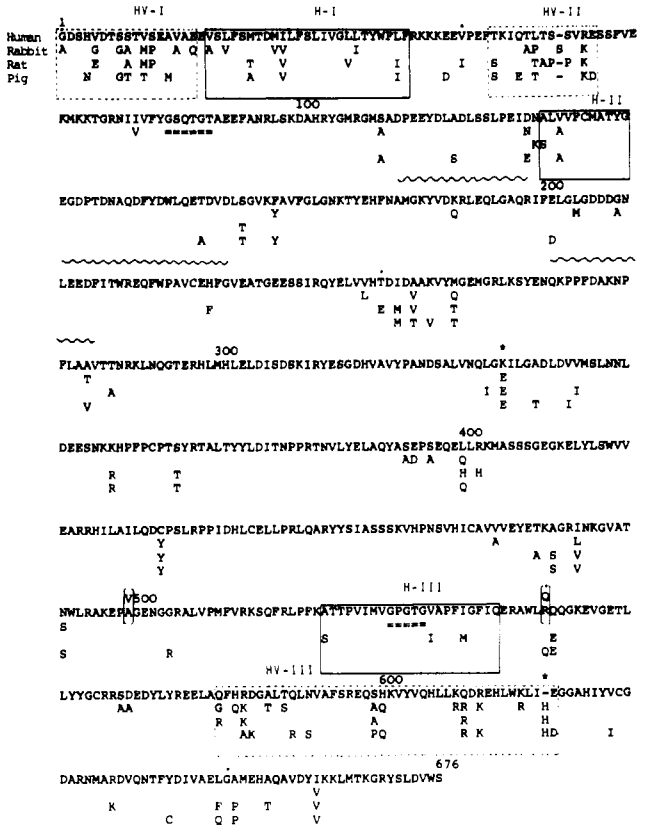

FIGURE 1: Sequence comparison of four mammalian NADPH-cytochrome P-450 reductases. Boxes show hydrophobic (solid line) or hydrophilic and variable (dashed line) regions. The double underlines denote regions which probably interact with the phosphate or pyrophosphate moiety of FMN or NADPH. Asterisks show the sites altering the charge properties and wavy lines show highly acidic regions which function in protein–protein interaction. The amino terminus of the reductase is acetylated. The sequences of rabbit enzyme, rat enzyme, and pig enzyme are derived from Katagiri et al.. (1986), Porter and Kasper (1985), and Haniu et al. (1986).

human enzyme with other mammalian reductases, including the rabbit (Katagiri et al., 1986), rat (Porter & Kasper, 1985), and pig enzymes (Haniu et al., 1986; Vogel & Lumper, 1986). Three hydrophobic regions (H-I, H-II, and H-III) were commonly observed: particularly at both the NH_2-terminal and COOH-terminal domains. Two of the three regions have a length of 22–25 amino acid residues. However, only the NH_2-terminal hydrophobic region (H-I) is predicted to be a

Table III: Sequence Variations among Four Enzymes[a]

	human	pig	rat
human			
pig	56		
rat	50	58	
rabbit	53	68	70

[a] Deletions at residues 62 and 620 have been made to obtain better homology, as shown in Figure 1. The numbers denote the substituted residues.

transmembrane segment (Black & Coon, 1982). The COOH-terminal hydrophobic region (H-III) is in the NADPH-binding domain (Haniu et al., 1984; Porter & Kasper, 1984). An H-II region containing a cysteine residue is proposed as in FMN binding (Porter & Kasper, 1986). Hydrophilic, variable regions were observed in the extreme NH_2-terminal (HV-I and HV-II) and COOH-terminal portions (HV-III) of the sequence. Since these regions are considerably variable between the human enzyme and the rabbit enzyme, they are likely candidates for antibody recognition sites. Other candidates are the single amino acid differences (Lys 335; Cys 433; a deletion at residue 620). A Kyte and Doolittle (1982) hydrophobicity plot (Figure 3) indicates that the protein is highly polar overall except for the NH_2-terminal region and the COOH-terminal NADPH domain. Hydrophobicity plots and sequence variation among species may reveal possible antigenic determinants (Hopp & Woods, 1981), including the HV-I, HV-II, and HV-III regions. Overall sequence differences among four mammalian enzymes are shown in Table III, indicating 92–95% homology.

FITC Inhibition and Labeling at NADPH Site. Since FITC has been used to label the active site of nucleotide-binding enzymes such as Ca^{2+}- and K^+-ATPases (Kirley et al., 1984), we attempted to use this reagent for active site labeling of a human reductase. The NADPH-depleted enzyme was treated with 0–0.5 mM FITC at room temperature. Figure 3A shows the FITC inhibition pattern, indicating pseudo-first-order kinetics. From a double-reciprocal plot of $1/k_{obs}$ against $1/[FITC]$, the inhibition parameters $K_i = 0.25$ mM and $k_2 = 0.021$/min were obtained according to the method described previously (Palczewski et al., 1986). Since the inhibition was prohibited by DTT titration (data not shown), FITC probably reacted with cysteine residues, but not with an ϵ-amino group. A protective effect of $NADP^+$ on FITC inhibition was significant as shown in Figure 3B. These results suggest that FITC initially competes with $NADP^+$ at the site where

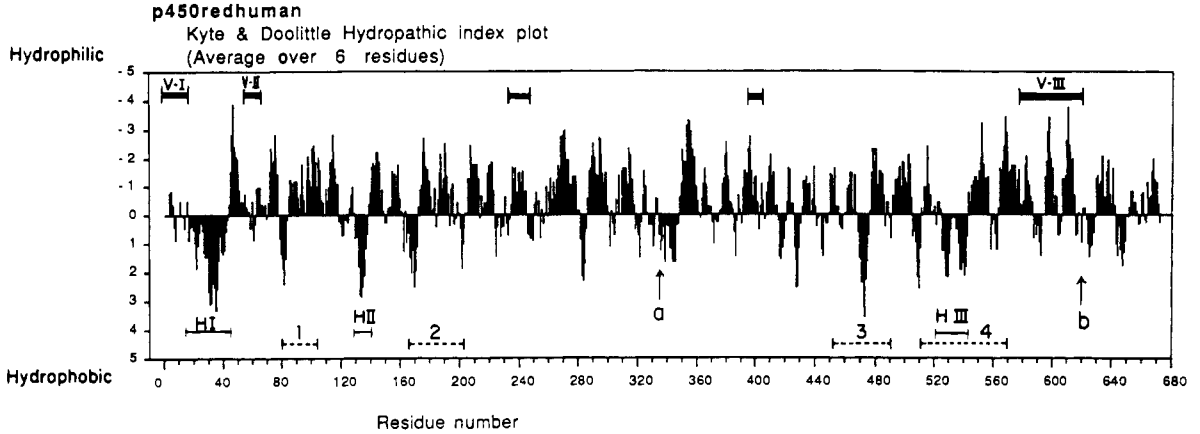

FIGURE 2: Hydropathy plot of human reductase by Kyte and Doolittle and search for antigenic determinants. The hydropathy plot was calculated according to Kyte and Doolittle (1982) with a window of six residues. Three hydrophobic regions (H-I, H-II, and H-III) are indicated, and three hydrophilic–variable regions (V-I, V-II, and V-III) are proposed as potential antibody recognition sites. Regions involved in binding of NADPH (1 and 4), FMN (2), and FAD (3) are proposed. Charge differences are indicated as a or b with arrows.

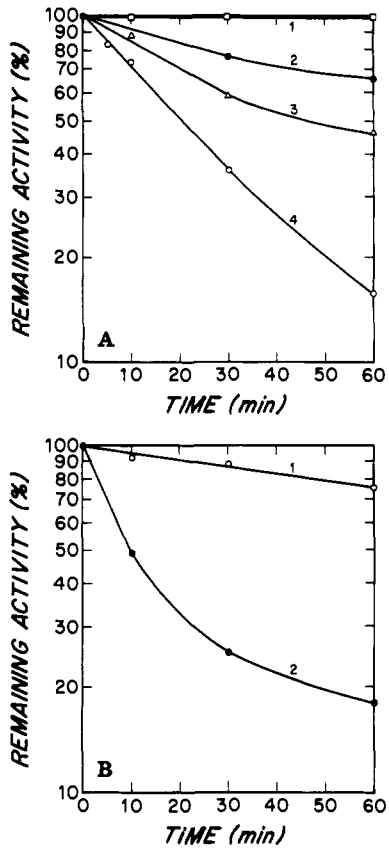

FIGURE 3: (A) FITC inhibition of the human reductase. An NADPH-depleted human reductase was incubated with different concentrations of FITC: (1) control; (2) 0.20 mM; (3) 0.40 mM; (4) 0.58 mM, respectively. (B) Protective effect of $NADP^+$ on FITC inhibition. The NADPH-depleted enzyme was treated with 0.50 mM FITC in the (1) presence of (2) absence of 0.1 mM $NADP^+$.

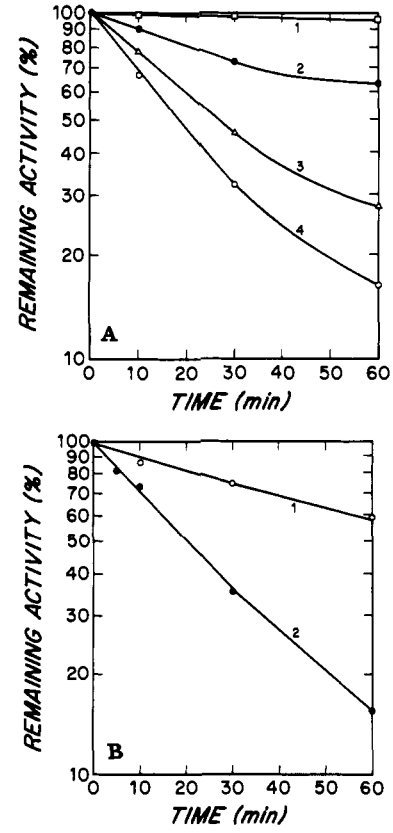

FIGURE 4: (A) Inhibitory effect of 5-IAF on human P-450 reductase. The NADPH-depleted enzyme was incubated with the following concentrations of 5-IAF: (1) control; (2) 10 μM; (3) 30 μM; (4) 50 μM. The enzyme assay was performed without removal of the reagent. (B) $NADP^+$ protection against 5-IAF inhibition. The enzyme was treated with the 5-IAF modification (50 μM) in the (1) presence or (2) absence of 0.1 mM $NADP^+$.

NADPH binds. Although we attempted to isolate the fluorescent peptides by HPLC, the labeled peptides could not be obtained by regular HPLC conditions probably due to the dissociation of the modifying group in the TFA–acetonitrile solvent system. This indirectly suggests that FITC reacts with sulfhydryl groups of the reductase.

Inhibition of the Reductase by 5-IAF and Identification of the Essential Cysteine Residue. Although kinetics for 5-IAF inhibition of the human reductase were similar with those from FITC inhibition (Figure 4A), the concentration of the inhibitor required was 10-fold less (K_i = 15.8 μM, k_2 = 0.02/min). Because of the specificity and stability of the adduct formed with 5-IAF, a cysteine residue at the active site may be labeled. The protective effect of $NADP^+$ (0.1 mM) on 5-IAF inhibition (Figure 4B) was similar to that observed for FITC inhibition, suggesting that this reagent also selectively labels the site which binds NADPH. Stoichiometry of 5-IAF labeling of the NADPH-depleted human reductase indicates 2.7 residues of covalently labeled fluorescein per molecule of enzyme, whereas 2.1 residues of labeled probe were detected for the NADPH-bound enzyme. Identification of the 5-IAF-labeled site was performed as described previously (Bishop et al., 1988). The native enzyme was initially treated with *N*-ethylmaleimide (0.1 mM) in the presence of $NADP^+$ (0.1 mM) at pH 7.4 for 12 h at 4 °C. The modified enzyme was passed through a Sephadex G-25 column and treated with 5-IAF as in the inhibition studies. Isolation of the fluorescent peptides was accomplished by HPLC separation after tryptic digestion of the modified enzyme. Figure 5 shows the tryptic peptide map, as monitored by UV absorbance at 220 nm and fluorescent intensity. Peak 1 did not show any peptide sequence by microsequence analysis and is likely a reagent background peak. The sequence analysis of peak 3 gave the sequence E-V-G-E-T-L-L-Y-Y-G-C-R (Table SVII, supplementary material). Mass spectral analysis indicated that the peptide contain a covalently bound acetamidofluorescein moiety (mass unit of the protonated ion was 1793). Peak 2 was a minor component and did not yield a significant sequence result. These results were consistent with our previous report (Haniu et al., 1984) and an other report (Vogel et al., 1986), indicating the involvement of Cys 565 in the NADPH-binding site.

Discussion

The human NADPH-cytochrome P-450 reductase consists of 676 amino acid residues and has a calculated molecular weight of 76 451, not including cofactors. A comparison of human, rabbit, rat, and pig enzyme sequences suggests that the NADPH-cytochrome P-450 reductase gene may contain at least two introns at residues 62–63 and at residues 619–620, since these sites contain deletions or additions across the four enzymes (Figure 1). Gonzalez and Kasper (1982) have reported that the rat NADPH-cytochrome P-450 reductase gene may contain seven introns.

Immunological studies have shown that a precipitin line could not be detected when the rabbit enzyme was tested against antibody to the human enzyme by the double-diffusion method (McManus et al., 1989). However, the human enzyme slightly cross-reacted with an antibody raised against the rat enzyme (Guengerich et al., 1981). These results suggested that the rat enzyme was more closely related to the human enzyme than to the rabbit enzyme. Although the sequences

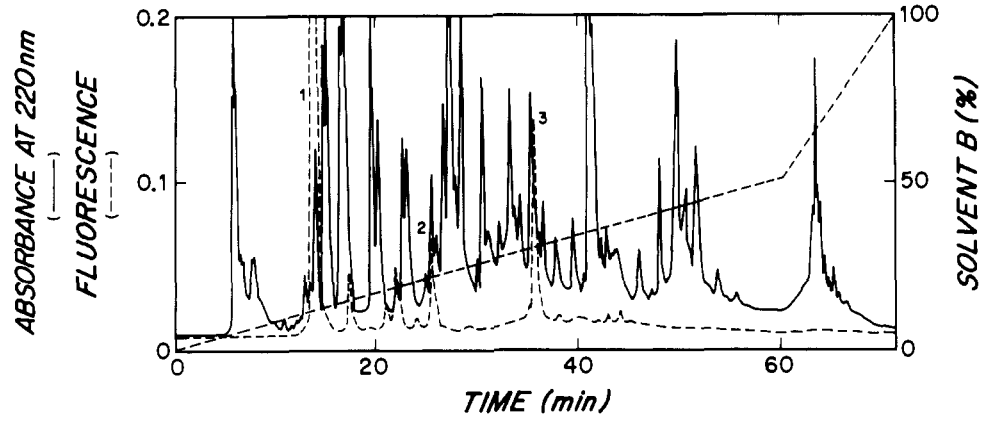

FIGURE 5: Isolation of acetamidofluorescein-labeled peptides. The enzyme was initially treated with *N*-ethylmaleimide (0.1 mM) in the presence of $NADP^+$ (0.1 mM). After gel filtration to remove $NADP^+$ and excess reagents, the sample was treated with 5-IAF (20 μM) at room temperature for 12 h. The modified sample was digested with trypsin (2% w/w), and the peptides were separated on a Vydac C4 column (4.6 × 250 mm). Dotted line shows fluorescence intensity from measurement at 570 nm of emission filter and at 450 nm of excitation filter.

around the proposed functional domains were basically unchanged between the human and rabbit enzymes (Figure 1), some immunochemical properties are distinct from each other, probably due to variability in the hydrophilic regions. These include the NH_2-terminal variable regions (HV-I and HV-II) and the COOH-terminal variable region (HV-III). It is not clear whether the HV-I region penetrates the membrane to the lumen side or not. If this region is located on the cytosolic side, it may be one of the antigenic determinants. However, since the trypsin-solubilized enzyme also recognizes the antibody raised against the human reductase,[2] the HV-II or HV-III region will be suggested as a possible antibody recognition site. Guengerich et al. (1981) have reported that immunological differences between the detergent-solubilized enzyme and the protease-solubilized enzyme may be due to either the presence of antigenic sites in the small NH_2-terminal hydrophobic peptide or changes in the antigenicity of the remaining portion of the enzyme that occur after the release of the small hydrophobic peptide.

Our previous results suggested that Cys 565 might be involved in NADPH binding in the pig enzyme (Haniu et al., 1985). This region contains a Gly-rich hydrophobic sequence similar to those commonly observed in the nucleotide binding region of other reductases. Yabusaki et al. (1988a) have reported that yeast enzyme does not contain a cysteine residue corresponding to cysteine 565 of the pig enzyme. They have emphasized another conservative cysteine residue (Cys 628) participating in enzyme activity. However, since the yeast enzyme exhibits low sequence homology to the mammalian reductases (30%), some functional site may be slightly changed in the yeast enzyme. This paper confirms that IAF reacts with the essential Cys 565, which is probably involved in NADPH binding. Our recent study[3] using 2,4,6-trinitrobenzenesulfonate (TNBS) indicates that one lysine residue in the NH_2-terminal region is specifically labeled with this reagent and the inhibition was protected by $NADP^+$. The essential lysine may interact with the 2′-phosphate (Inano & Tamaoki, 1985). Therefore, these results suggest that both NH_2-terminal and COOH-terminal regions of NADPH-cytochrome P-450 reductase are accessible to each other during the catalytic reaction of this enzyme.

[2] F. J. Gonzalez, personal communication.

[3] M. Haniu, T. Iyanagi, and J. E. Shively, unpublished results.

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SUPPLEMENTARY MATERIAL AVAILABLE

Four figures showing tryptic peptide map of human NADPH-cytochrome P-450 reductase (Figure S1), *S. aureus* protease peptide map of human reductase (Figure S2), HPLC peptide map of endoproteinase Asp-N digest of the reductase (Figure S3), and total sequence of human NADPH-cytochrome P-450 reductase (Figure S4) and seven tables listing sequence analysis of NH_2-terminal peptide Asp-N-1 (Table SI), sequences of tryptic peptides and the mass units observed (Table SII), amino acid compositions of peptides T-39 (Table SIII), sequence analysis of peptides with microheterogeneity (Table SIV), sequences and mass spectral analyses of Sa peptides (Table SV), sequence analyses of Sa peptides with microheterogeneity (Table SVI), and sequence analysis of the major fluorescent peptide (AF-5) (Table SVII) (15 pages). Ordering information is given on any current masthead page.

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