Amino-Terminal and Carboxy-Terminal Sequence of Hepatic Microsomal Cytochrome P-450d, a Unique Hemoprotein from Rats Treated with Isosafrole†

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ABSTRACT: Cytochrome P-450d is a unique liver microsomal cytochrome P-450 previously purified to electrophoretic homogeneity from rats treated with the (methylenedioxy)phenyl compound isosafrole [Ryan, D. E., Thomas, P. E., & Levin, W. (1980) *J. Biol. Chem. 255*, 7941–7955]. Three approaches have been used to analyze the protein structure of cytochrome P-450d: amino acid composition, automated sequence analysis of the NH_2 terminus, and carboxypeptidase analysis of the COOH terminus. Cytochrome P-450d is composed of 44% hydrophobic amino acid residues, and the NH_2- and COOH-terminal amino acid residues are alanine and lysine, respectively. A comparison of the NH_2- and COOH-terminal amino acid sequences of cytochrome P-450d with those previously reported [Botelho, L. H., Ryan, D. E., & Levin, W. (1979) *J. Biol. Chem. 254*, 5635–5640] for rat liver cytochromes P-450a, P-450b, and P-450c reveals very little homology among these hemoproteins. The COOH-terminal sequence (Tyr-Leu-Arg-Phe-Lys-COOH) of cytochrome P-450d revealed essentially no homology with the COOH termini of the other three cytochromes P-450. The NH_2-terminal sequence of cytochrome P-450d differs markedly from the sequence of the other hemoproteins; there are 4/23 identical residues for cytochromes P-450d and P-450a, 2/21 for cytochromes P-450d and P-450b, and 0/19 for cytochromes P-450d and P-450c. The different amino acid compositions and partial amino acid sequences, with previously reported data on peptide maps of proteolytic digests and cyanogen bromide cleavage products, establish cytochrome P-450d as a fourth isozyme of rat liver cytochrome P-450.

The hemoproteins collectively designated cytochrome P-450 refer to a family of enzymes that function as terminal oxidases in the electron-transport pathway of hepatic microsomes responsible for the metabolism of widely diverse substrates such as steroids, fatty acids, and xenobiotics (Conney, 1967; Lu & Levin, 1974). Four forms of cytochrome P-450 (P-450a, P-450b, P-450c, and P-450d) have been purified from the livers of rats treated with isosafrole [4-propenyl-1,2-(methylenedioxy)benzene] (Ryan et al., 1980). Cytochromes P-450a, P-450b, and P-450c had been previously purified to electrophoretic homogeneity from rats treated with other inducers (Ryan et al., 1979). Cytochrome P-450b is a predominant hemoprotein in phenobarbital-treated rats; cytochrome P-450c is the major form in 3-methylcholanthrene-treated rats; cytochrome P-450a is a minor enzymic form which is modestly induced by both compounds. The three cytochromes P-450 are all inducible in rat liver by Aroclor 1254, a mixture of polychlorinated biphenyls. Cytochromes P-450a, P-450b, and P-450c have been shown to be distinct hemoproteins which differ in minimum molecular weight in sodium dodecyl sulfate ($NaDodSO_4$)[1]–polyacrylamide gel electrophoresis, spectral, catalytic, and immunological properties, peptides generated by proteolytic or chemical digestion, and amino acid compositions as well as NH_2- and COOH-terminal amino acid sequences (Ryan et al., 1979; Botelho et al., 1979; Thomas et al., 1981). These reports were the first to clearly establish that multiple forms of liver microsomal cytochrome P-450 are isozymes.

Cytochrome P-450d is a unique hemoprotein purified to apparent homogeneity as an isosafrole metabolite–cytochrome P-450 complex from hepatic microsomes of rats treated with isosafrole (Ryan et al., 1980; Fisher et al., 1981). This hemoprotein has the same minimum molecular weight as cytochrome P-450b (52 000) in $NaDodSO_4$–polyacrylamide gel electrophoresis and shows spectral and immunological relatedness to cytochrome P-450c but apparently is a distinct enzyme based on substrate specificity and peptide maps of proteolytic and cyanogen bromide digests (Ryan et al., 1980). Specifically, cytochromes P-450d and P-450c exhibit a CO-reduced difference spectral peak at the same wavelength (447 nm) and generate the formation of an isosafrole metabolite–cytochrome P-450 complex with a single absorbance maximum at 455 nm at pH 7.4, and the reduced hemoproteins bind ethyl isocyanide with similar resultant 452:430 ratios at pH 7.4 (1.6–2.0) (Ryan et al., 1980; Levin et al., 1980). Furthermore, cytochromes P-450d and P450c share one or more minor antigenic determinants (Ryan et al., 1980; Reik et al., 1981).

This report provides structural information on cytochrome P-450d including amino acid composition, automated sequence analysis of the first 30 NH_2-terminal amino acids, and carboxypeptidase analysis of the COOH-terminal amino acid sequence. When the results for cytochrome P-450d were compared to previous data for cytochromes P-450a, P-450b, and P-450c (Botelho et al., 1979), the comparison illustrated that the four hemoproteins have different primary structures and are thus isozymic to each other.

Experimental Procedures

Chemicals. Carboxypeptidases A and B were obtained from Worthington Biochemical Corp.; 4-SPITC, 3-SPITC, *N*-methylmorpholine, and authentic PTH-amino acid derivatives were purchased from Pierce Chemical Co.

Purification of Cytochrome P-450d. Liver microsomal cytochrome P-450d was purified to apparent homogeneity from

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[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; 4-SPITC, 4-sulfophenyl isothiocyanate; 3-SPITC, 3-sulfophenyl isothiocyanate; PITC, phenyl isothiocyanate; ATZ, anilinothiazolinone; PTH, phenylthiohydantoin.

0006-2960/82/0421-1152$01.25/0

rats treated with isosafrole as described (Ryan et al., 1980). The purified protein had a specific content of 15.6 nmol of heme per mg of protein when protein was estimated by the method of Lowry et al. (1951) and 19.5 nmol of heme per mg of protein determined by amino acid composition.

Amino Acid Analysis. Amino acid analyses were performed as described for cytochromes P-450a, P-450b, and P-450c (Botelho et al., 1979). Carboxymethylcysteine values were obtained after reduction and alkylation in 6 M guanidine hydrochloride according to Hirs (1967). The procedure included two incubations with iodoacetic acid, one with ^{14}C-labeled and one with unlabeled iodoacetic acid. Amino acid analyses were performed on a two-column Beckman 121 MB analyzer (Del Valle & Shively, 1979) and on a single-column Dionex D-400 analyzer.

COOH-Terminal Analysis. Carboxypeptidases A and B digestion of cytochrome P-450d was performed at 37 °C for 240 min in 0.2 M *N*-methylmorpholine–acetate buffer (pH 8.5)–0.5% $NaDodSO_4$. The incubation contained, at a molar ratio of 25:1, cytochrome P-450d and a mixture of carboxypeptidases A and B (1:1). The reactions were terminated by immediate freezing of the samples in a dry ice–acetone bath. The samples were diluted (1:1 v/v) with 0.2 M sodium citrate buffer (pH 2.2), and free amino acids were analyzed on a Dionex D-400 analyzer. Norleucine was added to the sample prior to digestion as an internal standard.

NH_2-Terminal Analysis. Amino acid sequencing was performed on an updated, modified Beckman 890C sequencer. The modifications, which are similar to those described by Wittman-Liebold (1973) and Hunkapiller & Hood (1978), include reagent–solvent purification, improved vacuum and reagent–solvent systems, retention of samples in the spinning cup with 6 mg of precycled polybrene, and automated conversion of the ATZ- to PTH-amino acid derivatives (Wittman-Liebold et al., 1976). The reduced and alkylated sample (1.5 nmol) was treated with 4-SPITC in the spinning cup as follows. The sample was applied to the cup in aqueous 0.1% $NaDodSO_4$ (1 mL), dried, and dissolved in 0.33 M Quadrol. 4-SPITC in 0.5 M *N*-methylmorpholine buffer (pH 9.5) (300 nmol) was added and allowed to react with the sample for 30 min. The sample was automatically dried and extracted with solvents, recoupled with PITC, and then subjected to regular automated Edman degradation cycles. PTH derivatives were identified by high-pressure liquid chromatography on Ultrasphere (Altex) ODS columns by using a Waters Associates chromatograph. Peaks were integrated, and gradient elution was controlled by a Spectra-Physics 4000 integrator system.

Results and Discussion

The amino acid composition of cytochrome P-450d is shown in Table I. The molecular weight of the protein (52 720) is in good agreement with the value (Ryan et al., 1980) obtained by $NaDodSO_4$–polyacrylamide gel electrophoresis (52 000). Compositions of cytochromes P-450a, P-450b, and P-450c were previously reported (Botelho et al., 1979). Cytochrome P-450d contains 44% hydrophobic amino acids (Pro, Ala, Val, Met, Ile, Leu, and Phe) as do the other three hemoproteins. Comparisons of the amino acid compositions of cytochromes P-450a, P-450b, P-450c, and P-450d reveal significant differences in several amino acids among the four proteins, but cytochrome P-450d appears, however, to be most closely related to cytochrome P-450c.

Figure 1 shows the results of limited carboxypeptidases A and B digestion of cytochrome P-450d. The sequence Tyr-Leu-Arg-Phe-Lys-COOH shows virtually no homology with the COOH-terminal amino acid sequences of the other rat liver

Table I: Amino Acid Composition of Purified Cytochrome P-450d[a]

amino acid	no. of residues/ molecule
Asx	44
Thr	24
Ser	31
Glx	49
Pro	29
Gly	37
Ala	26
Val	32
Met	6
Ile	25
Leu	49
Tyr	10
Phe	31
Lys	32
His	13
Arg	21
Trp[b]	4
Cys[c]	5
polypeptide mol wt	52720

[a] The amino acid composition was obtained after hydrolysis of the proteins in 5.7 N HCl for 24, 48, 72, and 96 h in sealed evacuated tubes at 100 °C (Botelho et al., 1979). Values for serine and threonine were derived by extrapolation to zero time. [b] Tryptophan values were determined by hydrolysis with 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole at 118 °C for 21 h (Liu, 1972). Values were corrected with lysozyme as standard. [c] Cysteine values were obtained by carboxymethylation with iodoacetic acid as described under Experimental Procedures.

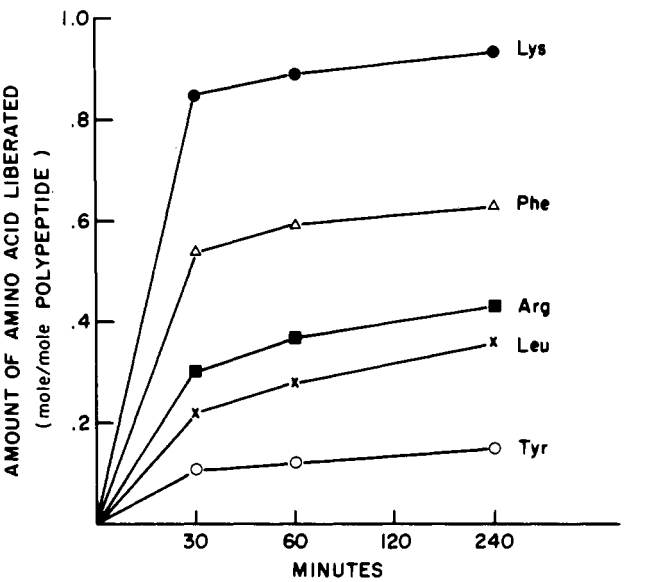

FIGURE 1: Carboxypeptidase digestion of cytochrome P-450d. At a molar ratio of 25:1, cytochrome P-450d and a mixture of carboxypeptidases A and B (1:1) were incubated at 37 °C for 240 min. The released amino acids were determined on a Dionex D-400 analyzer with norleucine as an internal standard.

hemoproteins (Botelho et al., 1979) with the exception of the fourth amino acid (Leu) in cytochromes P-450a and P-450d. The attempted digestion of cytochrome P-450d with carboxypeptidase Y (200:1 molar ratio) for 135 min at 37 °C failed to release any amino acids. Cytochrome P-450c, unlike cytochromes P-450a and P-450b, has been previously reported to be resistant to digestion with carboxypeptidase Y (Botelho et al., 1979).

The results of NH_2-terminal sequence analysis of 1.5 nmol of 4-SPITC-treated cytochrome P-450d are shown in Figure 2. The yield of the single NH_2-terminal amino acid (Ala)

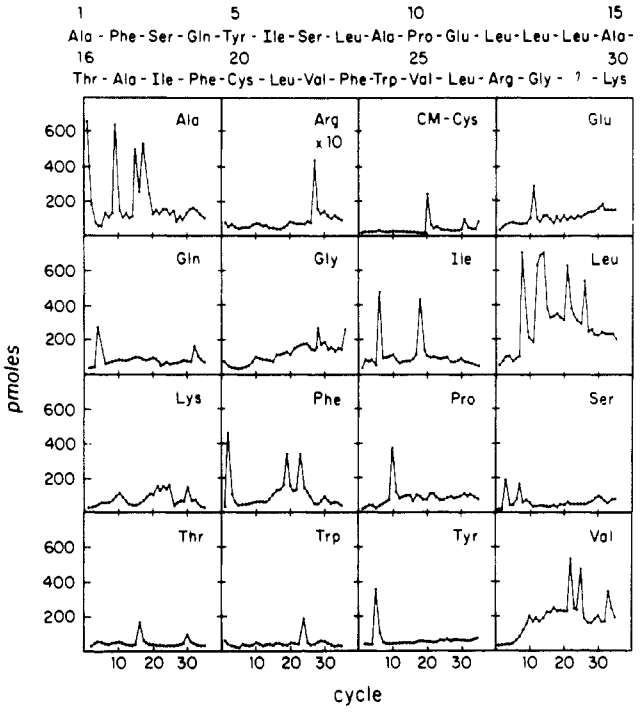

FIGURE 2: NH_2-terminal amino acid sequence of cytochrome P-450d on a modified Beckman 890C sequencer. The hemoprotein (1.5 nmol) was reduced, alkylated, applied to the spinning cup, and treated with 4-SPITC as detailed under Experimental Procedures. Cytochrome P-450d was then subjected to automated Edman degradation. The PTH derivatives were identified by high-pressure liquid chromatography on an Ultrasphere (Altex) ODS column.

was 43%. Although SPITC reduces the NH_2-terminal yield somewhat, this treatment increases the yield at subsequent cycles by preventing sample washout, which is a common problem with the sequence analysis of microsomal cytochromes P-450. The NH_2-terminal amino acid sequence results demonstrate that cytochrome P-450d is composed of a single, unblocked polypeptide chain. Although a single amino acid sequence was obtained through 35 cycles of Edman degradation, an increasing background of hydrophobic amino acids prevented positive identification of PTH-amino acids beyond cycle 30. The cysteine at cycle 20 was further confirmed as the ^{14}C-labeled carboxymethyl derivative by counting aliquots from each cycle.

Initial attempts to sequence the NH_2 terminus of cytochrome P-450d by the procedure developed for cytochromes P-450a, P-450b, and P-450c (Botelho et al., 1979) were unsuccessful. Dialysis of the protein against 10 mM *N,N*-dimethyl-*N*-allylamine buffer (pH 9.5) at 4 °C resulted in essentially irreversible precipitation of the protein. However, addition of $NaDodSO_4$ (0.5% final concentration) to cytochrome P-450d (25 nmol) before dialysis at room temperature against 200 mM *N*-methylmorpholine (pH 9.5)–0.1 mM dithiothreitol (DTT) eliminated the precipitation problem. When this sample was subsequently treated with 3-SPITC as previously described for cytochromes P-450a, P-450b, and P-450c (Botelho et al., 1979), an NH_2-terminal amino acid sequence through cycle 19 was obtained which was identical with that shown in Figure 2. An additional experiment with a lower concentration of $NaDodSO_4$ (0.05% final concentration) before dialysis of the protein (20 nmol) against pH 9.5 buffer and subsequent treatment of the protein with 3-SPITC also yielded the amino acid sequence shown in Figure 2 through 19 cycles. None of the three NH_2-terminal sequence determinations revealed any detectable secondary amino acid sequence, demonstrating the high degree of purity of cytochrome P-450d.

As shown in Figure 3, cytochromes P-450d and P-450c have totally distinct NH_2- and COOH-terminal amino acid sequences. Interestingly, these two hemoproteins have certain similar spectral properties (Ryan et al., 1980; Levin et al., 1980), exhibit a weak immunochemical relatedness (Ryan et al., 1980; Reik et al., 1981), and are resistant to carboxypeptidase Y digestion. Theoretically, these isozymes could contain certain similar internal amino acid sequences resulting in a region which protects the COOH terminus from carboxypeptidase Y but not A and/or B digestion. Similarly, internal regions of these cytochromes may have homologous tertiary structure resulting in certain common antigenic sites. However, as illustrated in Figure 3, cytochromes P-450d and P-450c are distinct hemoproteins with different primary structures.

The NH_2-terminal sequence of cytochrome P-450d is extremely hydrophobic, being comprised of 72% hydrophobic amino acids through residue 26. This hydrophobicity is similar to results previously reported for cytochromes P-450a, P-450b, and P-450c where 63–76% of the first 19–23 amino acids were determined to be hydrophobic (Botelho et al., 1979). Strikingly, however, there is very little homology among the four hemoproteins in the NH_2-terminal region. There are only 4/23 identical residues for cytochromes P-450d and P-450a, 2/21 for cytochromes P-450d and P-450b, and 0/19 for cytochromes P-450d and P-450c. Compared to rabbit liver microsomal cytochrome $P\text{-}450_{LM2}$ (Haugen et al., 1977), residues 3–4 and 9–10 are identical with 2–3 and 8–9 of rat liver cytochrome P-450d. The Cys at residue 20 for cytochrome P-450d (Figure 2) is the first reported Cys in the NH_2-terminal sequence of a microsomal cytochrome P-450.

NH_2-TERMINAL AMINO ACID SEQUENCE

Cytochrome	1 5 10 15
P450d	Ala-Phe-Ser-Gln-Tyr-Ile-Ser-Leu-Ala-Pro-Glu-Leu-Leu-Leu-Ala-Thr-Ala-Ile-Phe-
P450c	Ile-Thr-Val-Tyr-Gly-Phe-Pro-Ala-Phe-Glu-Thr-Ala-Ser-Glu-Leu-Leu-Leu-Leu-Val-

COOH-TERMINAL AMINO ACID SEQUENCE

P450d	-Tyr -Leu-Arg-Phe-Lys-COOH
P450c	-(Val-Phe-Lys)-Arg-Ala-His-Leu-COOH

FIGURE 3: Comparison of the NH_2- and COOH-terminal amino acid sequences of cytochromes P-450c (Botelho et al., 1979) and P-450d from rat hepatic microsomes. The amino acid sequence shown in parentheses is tentative.

Although there is little NH_2-terminal homology among the four rat liver cytochromes P-450, they are very similar in having extremely hydrophobic NH_2-terminal amino acid sequences. Possibly a much more hydrophilic sequence lies just beyond the hydrophobic NH_2-terminal amino acid sequence. Rat liver cytochromes P-450b (Botelho et al., 1979) and P-450d and rabbit liver cytochrome P-450_{LM2} (Haugen et al., 1977) have the sequence Arg-Gly (not in registry) which may mark the beginning of a hydrophilic region in these sequences. A greater amino acid sequence homology could exist among the various isozymes of cytochrome P-450 beyond the hydrophobic NH_2-terminal sequence. The length of the hydrophobic stretch may be variable among the isozymes, and it may be more informative to align the amino acid sequences when sequence data are available for the interior regions of the proteins. Nevertheless, the present structural studies in conjunction with biochemical and immunological comparisons of rat liver microsomal cytochromes P-450a, P-450b, P-450c, and P-450d (Ryan et al., 1980) definitively establish cytochrome P-450d as an isozyme (Kenney, 1974) rather than a posttranslationally modified form of cytochrome P-450a, P-450b, or P-450c.

Acknowledgments

We thank D. Hawke for operation of the microsequencer and Ann Marie Williams for her assistance in the preparation of the manuscript.

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Purification and Properties of Type 1 Topoisomerase from Chicken Erythrocytes: Mechanism of Eukaryotic Topoisomerase Action†

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ABSTRACT: A simple method for the purification of the major topoisomerase (topoisomerase 1) from chicken erythrocytes is described. Because of the generally repressed state of the chromatin from these nuclei, the heterogeneity of the nonhistone proteins is reduced, and it is possible to purify this enzyme from a nuclear extract by a single chromatographic step. The chicken erythrocyte topoisomerase appears to be similar to previously described eukaryotic type I topoisomerases with respect to its physical and enzymological properties. The pattern of intermediate products generated during the action of chicken erythrocyte topoisomerase on a supercoiled closed circular DNA substrate has been examined quantitatively and has been shown to be consistent with a mechanism in which the enzyme closes its substrate DNA molecule after the removal of each superhelical turn and in which dissociation of the enzyme substrate complex may, but does not necessarily, occur after each cycle of the reaction.

DNA topoisomerases comprise a ubiquitous and varied class of enzymes that change the linking number of closed circular DNA molecules through the production of transient breaks in the phosphodiester backbones of the molecules. Although these enzymes were originally sought in order to overcome the perceived problems of unwinding DNA at a sufficient rate ahead of a replication fork, they are now believed to participate in several other cellular processes involving DNA. These include facilitation and possible control of RNA transcription (Falco et al., 1978; Yang et al., 1979), participation in both site-specific (Kikuchi & Nash, 1979) and nonspecific (Witkin, 1976) recombination events, and disentanglement of catenated DNA circles (Liu et al., 1979; Kreuzer & Cozzarelli, 1980;

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0006-2960/82/0421-1155$01.25/0