Amino Acid Sequence of an Analogous Peptide from Two Forms of Cytochrome P-450*

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Two cytochrome P-450 preparations, a constitutive isozyme, form 3b, and a phenobarbital-induced isozyme, form 2, were isolated from rabbit liver microsomes and compared by peptide mapping following digestion with trypsin and by partial sequence analysis. The NH₂-terminal sequence of form 3b differed from form 2 in 15 out of 18 amino acids, but both forms have an NH₂-terminal methionine residue followed by an acidic residue. Comparisons of many of the tryptic peptides of the two forms by means of high pressure liquid chromatography, as well as amino acid composition and sequence analysis, indicated that peptides from these forms, with one exception, are different.

A tridecapeptide, differing only in a methionine (form 3b)/isoleucine (form 2) replacement was isolated from both forms. The amino acid sequence of this peptide is as follows: Met-Pro-Tyr-Thr-Asp-Ala-Val-Ile/Met-His-Glu-Ile-Gln-Arg. Taken together, these data indicate that forms 2 and 3 represent dissimilar gene products. The observation that these two cytochromes share an analogous peptide suggests that this tridecapeptide may contribute structural information necessary for common functional properties.

Cytochromes P-450 are a group of membrane-bound oxygenases found in the microsomal fraction of many tissues. These proteins have spectral and biological properties in common and therefore constitute a group of related proteins. Each form studied thus far consists of a single polypeptide chain of M₆, 48,000-60,000 which binds heme and forms a stable CO-Fe(II) complex exhibiting an absorbance maximum at ~450 nm. As an enzyme system, the cytochromes P-450 are responsible for the metabolism of a wide variety of endogenous and exogenous substrates, and the various forms of P-450 show overlapping specificity with regard to substrate metabolism (cf. review in Ref. 1). The existence of spectral and catalytic similarities between the different forms of cytochrome P-450 would be most readily explained by regions of structural homology. However, all structural comparisons to date have suggested a complete lack of homology between the different cytochrome P-450 forms, e.g., they display different peptide maps following limited proteolytic digestion (2-6) as well as distinct NH₂-terminal amino acid sequences (7, 8).

In this communication, we report the isolation and primary structure of an analogous peptide from rabbit microsomal cytochrome P-450 form 2, the principal form of the cytochrome inducible by phenobarbital in the rabbit liver (9, 10) as well as a major constitutive form in pulmonary microsomes (5, 6) and from form 3b, a major constitutive form in rabbit liver (11, 12).

EXPERIMENTAL PROCEDURES

The manner of preparation and characterization of the cytochromes, form 2 (13) and form 3b (11), are given in the references indicated. The cytochromes used in this study had the following specific contents expressed as nanomoles/mg of protein: form 2, 18.7 and 14.3; form 3b, 18.3, 19.3, 18.8, 16.9, 16.3, and 17.4. All preparations exhibited little or no electrophoretic contamination.

Prior to trypsin digestion or NH₂-terminal amino acid sequence analysis, cytochrome P-450 samples were dialyzed against 0.2 M NH₄-acetate, pH 7.4, and lyophilized. Trypsin digestion of cytochromes P-450 was performed as previously described (14). The resulting isolated tryptic peptides were dissolved in 8 M urea, 2 mM NH₄HCO₃ buffer, pH 8.1, and the urea concentration was reduced to 2 M by the addition of water. Trypsin (u-1-tosylamide-2-phenylthiohydantoin ketone-treated, Worthington) was added as a 2 mg/ml solution in 1.2 mM HCl to give a 1:14 molar ratio of trypsin to cytochrome, and digestion was allowed to proceed 12 to 16 h at room temperature.

Reverse phase chromatography was performed using a BONDAPAK C-18 column (0.4 x 30 cm, Waters Associates). Alkyl-phényl columns which were used to identify phenylthiohydantoin derivatives of amino acids were also obtained from Waters Associates. Resolution of peptide mixtures was achieved by gradient elution from the octadecl alkyl silane (C-18) column using 30 mM potassium phosphate, pH 2.2, as the aqueous solvent and acetonitrile (Burick and Jackson) as the organic solvent (15). The additional details concerning the conditions for the separation of the peptides are described in Fig. 1. Peptides isolated in phosphate buffers were desalted on a Bio-Gel P-2 column (0.9 x 12 cm) equilibrated with 88% aqueous acetic acid prior to sequence analysis. In some cases, peptides were purified further by rechromatography on high performance liquid chromatography using 10 mM ammonium acetate, pH 6.0, as the aqueous buffer. Peptides isolated in ammonium acetate buffers were lyophilized and applied to the sequenator cup without desalting. Lyophilized peptides or peptides were transferred to the cup with 0.5 ml of 88% formic acid. The presence of ammonium salts did not interfere with the determination of amino acid composition or sequence analysis at the concentrations used.

The amino acid compositions of the peptides and proteins were determined on acid hydrolysates of the samples using an updated single-column Beckman 121 automatic amino acid analyzer. Tyrophan content was estimated fluorometrically as described by Pajot (16), and cysteine residues were quantitated as cysteic acid following performic acid oxidation of the cytochrome. Automated sequence analyses of proteins and peptides were performed on a Beckman 890 C sequenator, using dimethyldiylamine peptide Program No. 102704, or 0.1 M Quadruph Program No. 030176. In the presence of 5 mg of Polybrene which together with 100 mmol of glycol glycyl glycine, was subjected to c prevulence of cationization (17). Conversion of the thiazoline products from the 0.1 M Quadruph program was carried out in-line with a Sequamat P-6 autoconverter using 1.5 N acetylchlordide in methanol (18). Reagents for sequencing were ob-

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FIG. 1. Resolution of tryptic peptides derived from forms 3b (top) and 2 (bottom) using reverse phase chromatography. In each instance, the peptides were eluted with a gradient of 0-70% acetonitrile in 20 mM potassium phosphate, pH 2.2, over a 90-min period. The flow rate was 1 ml/min, and the elution of the peptides was monitored by their absorbance at 214 nm. In the examples shown, approximately 20 nmol of each cytochrome were subjected to digestion with trypsin as described under "Experimental Procedures."

RESULTS AND DISCUSSION

It is now generally accepted that there are multiple forms of microsomal cytochrome P-450. The multiplicity of the purified cytochromes is plainly evidenced by their discrete mobilities on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, specific immunoprecipitation, analysis of their terminal amino acid sequences, and peptide mapping on polyacrylamide gels following limited proteolysis in the presence of sodium dodecyl sulfate (cf. review in Ref. 1).

These contrasting properties of the electrophoretically distinct forms of cytochrome P-450 could conceivably arise as the result of post-translational modifications of a single gene product or through the expression of specific genes coding for the individual cytochromes. Evidence supporting the latter hypothesis is provided by the translation of heterologous liver mRNA in vitro. In experiments conducted in several laboratories (19-22), antibodies to a phenobarbital-inducible form of rat cytochrome P-450 precipitate nascent protein with a similar mobility to the purified cytochrome, indicating that this cytochrome is not derived from a higher molecular weight precursor and that the monospecific antibody recognizes the nascent protein chain. This was corroborated in one case by NH₂-terminal amino acid sequence analysis of the protein synthesized in vitro (19). On the other hand, it has been reported that antibody to a form of rat cytochrome P-450 which is inducible by 3-methylcholanthrene precipitates a higher molecular weight precursor for this cytochrome (20). Thus, it may be somewhat premature to infer from a limited number of studies that post-translational processing does not
Peptide mapping provides additional evidence that the cytochromes represent distinct gene products. The cytochromes are clearly distinguished by one-dimensional maps employing polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate following limited proteolysis (2-6). However, the extent of the differences in amino acid sequence which exist between forms cannot be gauged from peptide mapping studies of this nature. In this report, we describe the application of reverse phase chromatography to the resolution and isolation of tryptic peptides derived from each of two highly purified forms of rabbit microsomal cytochrome P-450, forms 2 and 3b. Comparison of chromatograms of tryptic peptides from both forms showed extensive differences in the retention times of the individual peptides as shown in Fig. 1. Amino acid analysis followed by sequence analysis of all major peaks again indicated a lack of structural similarity. This is illustrated in Table I which displays the first three amino acid residues of the NH2-terminal sequences of several major tryptic peptides isolated from form 3b with peptides of similar mobility from form 2. However, peptides from different retention times, are very similar in structure as indicated in Table II. The yields of these two peptides were both greater than 60%. The amino acid sequences of these peptides are homologous except for a methionyl residue at position 7 of the peptide from form 3b and an isoleucyl residue at that position for form 2. Although this lack of homology may contribute to the differences in retention times observed for the two peptides, the oxidation states of the methionyl residues could also affect the retention times of these peptides. In the example shown in Fig. 1, the NH2-terminal methionine of peptide 19A derived from form 3b occurred as the sulfoxide form. However, this was not observed in all cases examined.

The NH2-terminal amino acid sequences of forms 2 and 3b as obtained by automated sequence analysis of the intact cytochrome are also compared in Table II. There was no significant homology between the NH2 terminus of form 3b and the NH2-terminal sequence reported by Haugen et al. for form 2 (8) or by Botelho et al. (7) for three forms of rat cytochrome P-450. The one common feature is that both forms 2 and 3b have methionyl residues at the NH2 terminus of the molecule followed by an acidic residue and a segment of hydrophobic residues.

Interestingly, some variation in the end-group was noted with form 3b in as much as one preparation completely lacked the NH2-terminal methionine. This preparation was isolated from microsomes of a pool of three rabbits in the usual manner and yielded. The lack of NH2-terminal methionyl in this preparation was unexpected. Methionine serves as the end-group for forms 2 as well as for rat cytochrome P-450 form a (7). It has been pointed out that there is significant homology between NH2-terminal sequences of rat form b and rabbit form 2 provided that the NH2 terminus of the rat form b is aligned with the second residue of rabbit form 2 (7). Both of these cytochromes are induced by phenobarbital in the livers of their respective species and share similar enzymatic properties. In light of the apparent lability of the NH2-terminal methionine of form 3b, a similar loss of methionine may have occurred with rat form b.

The amino acid composition of form 3b and three analyses reported for form 2 are shown in Table III. The composition of form 3b is similar to but not identical with that reported for form 2 by Haugen and Coon (24), Imai et al. (25), and Wolf et al. (5). Differences among the analyses for the two forms occur in several amino acids, most notably lysine, argi...
nine, and cysteine. However, on the whole the compositions are rather similar. Analysis of amino sugars following mild hydrolysis indicated less than 0.1 residue/mol of form 3b. In contrast, form 2 is reported to contain 1 residue of glucosamine/mol of protein (24).

It is surprising that more similarities in sequence were not observed for forms 2 and 3b, since these heme proteins share several spectral and enzymatic properties, and all interact with the same flavoprotein reductase during catalysis (1). These particular properties may ultimately depend on the conformation of the protein chain, the location of only a few critical amino acids, or the conservative substitution of other amino acids. This will be more evident when the primary structures of these proteins have been determined. Such studies are currently being pursued in our laboratories for form 3b and in the laboratory of Dr. M. J. Coon in Ann Arbor for form 2. Thus, additional comparisons between forms 2 and 3b can be expected to contribute to our future understanding of the microsomal cytochrome P-450 proteins.

The peptide mapping procedure described in this report provides further evidence that the multiple forms of cytochrome P-450 represent distinct gene products. The analysis of tryptic digests using reverse phase chromatography and comparisons of the amino acid sequences of selected peptides suggests that these two cytochromes differ extensively in primary structure. Of the peptides compared to date, representing about 70% of the structure (data not shown), only one analogous peptide has been detected as reported here.

The observation of a limited region of marked structural homology in two cytochrome P-450 forms which are by other criteria structurally dissimilar, suggests that the homologous segment contains structural information necessary for common functional properties. The presence of a histidyl residue in this unique peptide is significant, since it has been suggested that histidine is one of the axial heme ligands in cytochrome P-450 (26). Thus, the analogous peptide may be involved in the heme binding segment of cytochrome P-450. Of course, much more evidence will be required to prove this hypothesis, since the two proteins have 11–12 histidyl residues. Dus et al. (27) have reported the isolation of heme peptide from CNBr digests of several forms of cytochrome P-450. Although the compositions of these peptides have been determined, their larger size precludes a meaningful comparison with the two analogous peptides described here.

Nevertheless, at a recent meeting in Tokyo, Fuji Ji-Kuriyama et al. (29) reported the amino acid sequence determined from the cloned complementary DNA of the phenobarbital-inducible cytochrome P-450 mRNA from rat liver. A portion of this sequence is homologous to the tridecapeptide described here for the phenobarbital-inducible rabbit cytochrome P-450 form 2. Presumably, this peptide is conserved between rat and rabbit species as well as between forms 2 and 3 in the rabbit liver.

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