# Evidence for Functional and Structural Multiplicity of Pregnenolone-16$\alpha$-carbonitrile-Inducible Cytochrome P-450 Isozymes in Rat Liver Microsomes†

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*Received October 3, 1986; Revised Manuscript Received February 24, 1987*

ABSTRACT: Administration of pregnenolone-16$\alpha$-carbonitrile (PCN) to adult female rats caused a 2-fold increase in total liver microsomal cytochrome P-450 along with 5–7-fold increases in four in vitro monooxygenase activities considered diagnostic for the major PCN-inducible cytochrome P-450 isozyme. However, upon administration of chloramphenicol to PCN-treated rats, these monooxygenase activities could be resolved into three groups. Thus, the ability of the microsomes to convert triacetyloleandomycin to a metabolite that forms a spectral complex with the reduced heme iron was decreased by 80% by chloramphenicol, whereas only a 50% decrease was observed in the rate of conversion of (*R*)-warfarin to its 9,10-dehydro metabolite and in the rate of 6$\beta$-hydroxylation of androstenedione. More strikingly, the 10-hydroxylation of (*R*)-warfarin was actually enhanced 2-fold by the chloramphenicol treatment. Fractionation studies were carried out on liver microsomes from PCN-treated adult male rats, and two highly purified cytochromes P-450, referred to as PCNa and PCNb, were recovered. PCNb was found to be identical in the sequence of the first 15 amino acid residues with a PCN-inducible isozyme, the complete amino acid sequence of which has recently been deduced in another laboratory [Gonzalez, F. J., Nebert, D. W., Hardwick, J. P., & Kasper, C. B. (1985) *J. Biol. Chem. 260*, 7435–7441]. The other isozyme, PCNa, differed in amino acid sequence in three of the first 15 positions from PCNb. Upon immunoblot analysis, polyclonal antibodies raised to PCNb also recognized PCNa. Thus, the PCN-inducible family of rat liver cytochrome P-450 comprises at least two separate proteins.

Liver microsomal cytochromes P-450 constitute a superfamily of hemoproteins that play a key role in the metabolism of a wide variety of endogenous and foreign compounds. Many cytochrome P-450 isozymes are inducible and can be classified into families on the basis of a common response to a particular chemical agent such as phenobarbital (PB)[1] or 3-methylcholanthrene (3-MC). Recent evidence from many laboratories has shown that the members of these families also exhibit marked sequence homology with each other at the amino acid and nucleic acid level. In rats, cytochrome P-450b[2] and P-450e are the major PB-inducible isozymes, whereas cytochromes P-450c and P-450d constitute the major 3-MC-inducible forms of the enzyme (Adesnik & Atchison, 1986). A third class of inducing agents, exemplified by the antiglucocorticoid pregnenolone-16$\alpha$-carbonitrile (PCN), stimulate the synthesis of cytochrome P-450 isozymes that are biochemically and immunochemically distinct from the members of the PB- and 3-MC-inducible cytochrome P-450 families and that are alternatively referred to as P-450p (Wrighton et al., 1985a), PB/PCN-E (Guengerich et al., 1982), or PB-2a (Waxman et al., 1985).

Evidence for the possible structural multiplicity of PCN-inducible cytochrome P-450 isozymes in rat liver microsomes is conflicting. On the one hand, no laboratory has isolated more than a single form of the enzyme, and in all cases polyclonal antibodies to this protein have been shown to recognize only a single electrophoretic species upon immunoblot analysis. On the other hand, the major PCN-inducible isozymes isolated in two different laboratories do not have the same amino-terminal sequences (Wrighton et al., 1985a; Gonzalez et al., 1985), and Southern blot analysis of rat liver DNA with a cDNA clone of mRNA encoding for one of these proteins suggests the possibility of three to five homologous genes (Hardwick et al., 1983; Molowa et al., 1986). Conclusive evidence for the functional multiplicity of PCN-inducible cytochromes P-450 has also been difficult to obtain due to their apparent inactivation in many instances during purification (Wrighton et al., 1985a; Guengerich et al., 1982; Waxman et al., 1985). However, on the basis of induction and antibody inhibition studies performed with intact rat liver microsomes,

† This work was supported by U.S. Public Health Service Grants ES 03619 (J.H.) and ES 03516 (L.S.K.). J.H. is the recipient of Research Career Development Award ES00151 from the National Institutes of Health and of a Faculty Development Award in Pharmacology from the Pharmaceutical Manufacturers Association Foundation.

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[1] Abbreviations: PCN, pregnenolone-16$\alpha$-carbonitrile; PB, phenobarbital; 3-MC, 3-methylcholanthrene; TAO, triacetyloleandomycin; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; androstenedione, androst-4-ene-3,17-dione; Tris, tris(hydroxymethyl)aminomethane.

[2] There is no generally accepted nomenclature for rat liver cytochromes P-450 (Waxman, 1986). In this paper, preparations of cytochrome P-450 from different laboratories are referred to according to the nomenclature in use in each respective laboratory. What appears to be the major PCN-inducible isozyme of rat liver cytochrome P-450 has been called P-450p (Wrighton et al., 1985a), PB/PCN-E (Guengerich et al., 1982), or PB-2a (Waxman et al., 1985). We refer to two forms of cytochrome P-450 isolated by us from liver microsomes of PCN-treated rats as PCNa and PCNb and to four forms of cytochrome P-450 isolated from PB-treated rats as PB-B, PB-C, PB-D, and PB-E (Guengerich et al., 1982).

0006-2960/87/0426-3887$01.50/0 

the conversion of (*R*)-warfarin to (*R*)-9,10-dehydrowarfarin and (*R*)-10-hydroxywarfarin (Kaminsky et al., 1984), the 6$\beta$-hydroxylation of androstenedione (Waxman et al., 1985), and the conversion of triacetyloleandomycin (TAO) to a metabolite that forms a complex with reduced cytochrome P-450 (Wrighton et al., 1985a) have all been suggested as marker activities for the major PCN-inducible form of rat liver microsomal cytochrome P-450.

Recently we obtained preliminary evidence for the possible functional multiplicity of PCN-inducible rat liver cytochrome P-450 isozymes in that administration of the irreversible cytochrome P-450 inhibitor chloramphenicol to male rats pretreated with PCN was found to decrease, in subsequently prepared liver microsomes, both the cytochrome P-450 content and the rate of conversion of (*R*)-warfarin to the 9,10-dehydro metabolite without affecting the rate of formation of (*R*)-10-hydroxywarfarin (Halpert et al., 1985a). In this investigation we have extended these studies by examining the effect of chloramphenicol on other monooxygenase activities considered diagnostic for PCN-inducible isozymes. We also report the results of purification studies that indicate the presence in liver microsomes from PCN-induced rats of two chromatographically and electrophoretically distinct yet immunochemically and structurally related cytochrome P-450 isozymes.

## Materials and Methods

*Materials.* Resolution of racemic warfarin into the optically pure *R* and *S* sodium salts (West et al., 1961) and synthesis of the metabolite standards (Kaminsky et al., 1981) have been described previously. [1,2-$^{14}$C]Chloramphenicol and [4-$^{14}$C]androstenedione were purchased from NEN Research Products (Boston, MA). PCN and TAO were gifts from the Upjohn Co. (Kalamazoo, MI) and Pfizer Inc. (New York, NY), respectively. Unlabeled chloramphenicol, 16$\alpha$-hydroxyandrostenedione, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, $NADP^+$, and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO). Unlabeled androstenedione and 6$\beta$-hydroxyandrostenedione were purchased from Steraloids (Wilton, NH), and 7$\alpha$-hydroxyandrostenedione was a gift from Dr. David Waxman (Harvard Medical School, Boston, MA). Reagents and molecular weight standards for gel electrophoresis were obtained from Bio-Rad (Richmond, CA).

*Assay Procedures.* Cytochrome P-450 and heme contents (Omura & Sato, 1964) and protein (Lowry et al., 1951) were determined by standard methods. Covalent binding of chloramphenicol to microsomal protein (Halpert et al., 1983) and warfarin metabolism (Kaminsky et al., 1981) were monitored as described previously. The formation of a metabolite complex of TAO and reduced cytochrome P-450 was measured spectrally (Wrighton et al., 1985a). The metabolism of androstenedione was assayed essentially as described (Waxman et al., 1983) with the following modifications. Microsomal protein (25 $\mu$g) was incubated with 25 $\mu$M [4-$^{14}$C]-androstenedione and 1 mM NADPH in a final volume of 0.1 mL of 0.05 M HEPES buffer (pH 7.6) containing 15 mM $MgCl_2$ and 0.1 mM EDTA. Incubations were carried out for 5 min at 37 °C and were quenched by the addition of 0.05 mL of tetrahydrofuran. Aliquots of 0.05 mL were spotted on the preadsorbent loading zone of a TLC plate [Baker silica gel, 250 $\mu$m, Si250F (19c)], and the plate was developed twice in chloroform–ethyl acetate (1:2). Metabolites were localized by autoradiography and identified by comparison with unlabeled standards, except for 16$\beta$-hydroxyandrostenedione, for which a radiolabeled standard was generated with the use of a reconstituted system containing the major phenobarbital-inducible isozyme of rat liver cytochrome P-450. The radioactive areas from the plates were scraped into scintillation vials, and the metabolites were quantified by liquid scintillation counting.

*Treatment of Animals and Preparation of Microsomes.* Adult female Sprague-Dawley rats were divided into three groups of three to four animals each. Two groups were administered PCN (100 mg/kg) by gastric intubation once daily for 4 days in 1 mL of 1% Tween 80, and the third group received vehicle only. On the last day of treatment, food was withheld. On the fifth day, one group of PCN-treated animals received chloramphenicol (300 mg/kg) ip in 0.5 mL of propylene glycol, and the other two groups received vehicle only. After 1 h, the animals were killed by cervical dislocation, and liver microsomes were prepared from each individual rat as described previously (Halpert et al., 1983). Pooled liver microsomes from male rats treated with PCN as described above or with PB (0.1% in the drinking water for 5 days) were also prepared and used for isolation of cytochrome P-450 isozymes. Microsomes were stored at −70 °C in 10 mM Tris–acetate buffer (pH 7.4) containing 20% glycerol, 1 mM EDTA, and 0.1 mM phenylmethanesulfonyl fluoride.

*Isolation of Cytochrome P-450 Isozymes from Liver Microsomes of PB-Treated Rats.* Isozymes PB-B, PB-C, and PB-E were isolated essentially according to Guengerich et al. (1982). In brief, liver microsomes from 12 PB-treated adult male Sprague-Dawley rats (Charles River) corresponding to 7000 nmol of cytochrome P-450 were solubilized with 0.6% sodium cholate and chromatographed on a 3.2 × 30 cm column of *n*-octylamino-Sepharose. The cytochrome P-450 peak that eluted with buffer containing 0.06% Lubrol and 0.33% sodium cholate (4000 nmol of cytochrome P-450) was chromatographed on a 5 × 12 cm column of hydroxylapatite (Hypatite C). This column was eluted with 700 mL each of 40, 90, and 180 mM potassium phosphate buffers (pH 7.25) containing 20% glycerol and 0.3% Lubrol. The peak fractions from the 90 mM (1000 nmol of cytochrome P-450) and 180 mM (1500 nmol of cytochrome P-450) washes were pooled separately and chromatographed on a series of Whatman DE-51, DE-52, and DE-53 columns connected in tandem. Cytochrome P-450 PB-B (150 nmol) was recovered in apparently homogeneous form following ion-exchange chromatography of the 180 mM hydroxylapatite peak. Cytochromes P-450 PB-C and PB-E were highly enriched following ion-exchange chromatography of the 180 mM hydroxylapatite peak but required further purification.

The PB-C-containing fractions from the series of Whatman DE columns (300 nmol of cytochrome P-450) were concentrated to 5 mL with an Amicon PM-30 ultrafiltration device and then dialyzed for 24 h at 4 °C against 2 L of 5 mM potassium phosphate buffer (pH 7.7) containing 0.1% Lubrol, 0.2% sodium cholate, 0.1 mM EDTA, and 20% glycerol (5 mM DEAE buffer). The sample was chromatographed at room temperature on a column of DEAE-Sephacel (1.5 × 57 cm) equilibrated with the same buffer. The column was washed at 30 mL/h with 200 mL of 5 mM DEAE buffer, and 4-mL fractions were collected and monitored for absorbance at 417 and 280 nm. Epoxide hydrolase eluted after approximately 100 mL and PB-C after approximately 180 mL of elution with 5 mM DEAE buffer. The column was then washed with 500 mL of the same buffer in which the phosphate concentration had been increased to 10 mM (10 mM DEAE buffer). An additional highly purified cytochrome P-450 (referred to as PB-X) eluted after approximately 150

mL of the 10 mM DEAE buffer. The PB-C-containing fractions (60 nmol) were subjected to a final chromatographic step on a 5-mL column of CM-Sepharose equilibrated with 5 mM potassium phosphate (pH 6.5) containing 20% glycerol and 0.2% Emulgen 911 (Waxman & Walsh, 1983). The column was first washed at room temperature with buffer containing 30 mM KCl, and highly purified PB-C (15 nmol) was eluted with buffer containing 50 mM KCl. The PB-E-containing fractions from the series of Whatman DE columns (150 nmol) were also further purified by chromatography on DEAE-Sephacel. The sample was dialyzed without prior concentration against two 1-L portions of 10 mM DEAE buffer for 24 h at 4 °C and then applied at room temperature to a 1 × 45 cm column of DEAE-Sephacel equilibrated with the same buffer. Immediately after sample application, the column was eluted at 15 mL/h with a 300-mL linear gradient of 0–60 mM sodium chloride in 10 mM DEAE buffer. A single major cytochrome P-450 peak containing highly purified PB-E (50 nmol) eluted at a sodium chloride concentration of 30 mM.

Subsequent experiments revealed that cytochrome P-450 PB-B could be isolated in equal purity and greater yield by replacing the series of Whatman DE columns with a single column of DEAE-Sephacel. The 180 mM peak from the hydroxylapatite column was concentrated to 50 mL by ultrafiltration and then dialyzed for 48 h at 4 °C against four 1-L portions of 5 mM DEAE buffer. The sample was chromatographed at room temperature at 40 mL/h on a 1.5 × 57 cm column of DEAE-Sephacel equilibrated with 5 mM DEAE buffer. The column was washed with 160 mL of 5 mM DEAE buffer, with 200 mL of 10 mM DEAE buffer, and then with a linear gradient of 0–100 mM sodium chloride in a total volume of 1 L of 10 mM buffer. A fraction corresponding to PB-X eluted with the 10 mM wash, and apparently homogeneous PB-B (200 nmol) eluted at 70 mM NaCl. This modified method also allowed the isolation of isozyme PB-D (100 nmol), which eluted in apparently homogeneous form at 80 mM sodium chloride during DEAE-Sephacel chromatography of the 90 mM potassium phosphate peak from hydroxylapatite.

Detergent was removed by chromatography on hydroxylapatite (20 nmol of cytochrome P-450/mL of resin). The samples were dialyzed against two 1-L portions of 10 mM Tris–acetate buffer (pH 7.4) containing 0.1 mM EDTA and 20% glycerol and applied to the hydroxylapatite column equilibrated with the same buffer. After sample application, the column was washed with 10 bed volumes of the buffer containing 0.2% sodium cholate, and the cytochrome P-450 was eluted with 300 mM potassium phosphate buffer (pH 7.25) containing 0.2% cholate, 0.1 mM EDTA, and 20% glycerol. After elution, the cytochrome P-450 was again dialyzed against two 1-L portions of the Tris buffer and then stored at −70 °C.

*Immunochemical Methods.* Antibodies to purified cytochromes P-450 were generated in female New Zealand white rabbits by injecting 100 μg of protein subcutaneously in 1 mL of Freund's complete and 0.5 mL of Freund's incomplete adjuvant. Injections were repeated after 1 and 2 months, and blood was drawn 1 week following the final injection. An IgG-enriched fraction was prepared by passage of sera through a protein A–Sepharose CL-4B column (Pharmacia) as described previously (Haaparanta et al., 1983). The specificity of the antibodies was assessed by immunoblot analysis (Guengerich et al., 1982). Proteins were transferred from the polyacrylamide gel to nitrocellulose with a voltage gradient of 100 mA for 16 h followed by 200 mA for 2 h. The nitrocellulose sheets were incubated with the primary antibody (17 μg/mL in 1% gelatin) for 2 h. Immunoreactive proteins were identified with a horseradish peroxidase Immuno-Blot assay kit (Bio-Rad, Richmond, CA).

Table I: Effect of PCN and Chloramphenicol Administration in Vivo on the Heme and Cytochrome P-450 Content of Liver Microsomes from Adult Female Rats[a]

| | nmol/mg of microsomal protein | | |
|---|---|---|---|
| treatment | heme | cytochrome P-450 | cytochrome P-420 |
| none | 1.74 ± 0.20 | 0.70 ± 0.02 | 0.80 ± 0.27 |
| PCN | 3.02 ± 0.25 | 1.39 ± 0.16 | 0.92 ± 0.12 |
| PCN + chloramphenicol | 3.18 ± 0.15 | 0.95 ± 0.15 | 1.23 ± 0.20 |

[a] Animals were treated and liver microsomes were prepared as described under Materials and Methods. Heme and cytochrome P-450 were determined according to Omura and Sato (1964). Cytochrome P-420 was estimated as described by Guengerich (1982). Results represent the mean ±SD of the values obtained with three to four individual samples per group.

## Results

*Effect of Chloramphenicol Administration on the Cytochrome P-450 Content and Monooxygenase Activity of Liver Microsomes from PCN-Treated Female Rats.* In a previous study (Halpert et al., 1985a), we found that administration of chloramphenicol (300 mg/kg) to PCN-treated adult male Sprague-Dawley rats resulted in a significant decrease in the liver microsomal cytochrome P-450 content. However, the ability of the microsomes to hydroxylate (*R*)-warfarin in the 10-position, a monooxygenase activity considered diagnostic for the major PCN-inducible isozyme of rat liver cytochrome P-450 (Kaminsky et al., 1984), was not affected. In this investigation we extended these findings by examining the effect of chloramphenicol on additional monooxygenase activities induced by PCN. For this purpose we used female rats, our reasoning being that the lower constitutive levels of PCN-inducible cytochromes P-450 in female compared to male rats (Wrighton et al., 1985a,b; Waxman et al., 1985) would allow greater sensitivity in detecting both increases in activity following induction with PCN and decreases in activity following chloramphenicol treatment.

PCN caused a 2-fold increase in the cytochrome P-450 content of the female rat liver microsomes, whereas administration of chloramphenicol to the PCN-treated animals caused a 32% decrease in cytochrome P-450 (Table I). This loss of P-450 detectable as the reduced carbon monoxide complex was not accompanied by a loss of heme measured as pyridine hemachromagen but rather by the conversion of a significant fraction of the cytochrome P-450 to P-420. The effect of PCN and of chloramphenicol administration in vivo on the liver microsomal metabolism of (*R*)-warfarin, androstenedione, and TAO in vitro is shown in Table II. The PCN treatment caused approximately 5-fold increases in the rate of conversion of (*R*)-warfarin to (*R*)-9,10-dehydrowarfarin and to (*R*)-10-hydroxywarfarin and in the rate of 6β-hydroxylation of androstenedione. A somewhat greater (7-fold) increase was observed in the ability of the microsomes from the PCN-treated animals to form a metabolite complex with TAO in vitro compared to controls. Although the four monooxygenase activities were therefore affected by the PCN treatment to essentially the same extent, the response to chloramphenicol was very different. Thus, the administration of chloramphenicol to the PCN-treated rats caused an 83% decrease in the extent of TAO metabolite complex formation, a 55% decrease in the rate of 6β-hydroxylation of andros-

Table II: Effect of PCN and Chloramphenicol Administration in Vivo on in Vitro Monooxygenase Activities of Liver Microsomes from Adult Female Rats[a]

| | (*R*)-warfarin (nmol mg$^{-1}$ min$^{-1}$) | | | | | | androstenedione (nmol mg$^{-1}$ min$^{-1}$) | | | | TAO complex (nmol mg$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| treatment | 9,10-dehydro | 4-OH | 6-OH | 7-OH | 8-OH | 10-OH | 7α-OH | 6β-OH | 16β-OH | 16α-OH | |
| none | 0.04 ± 0.00 | 0.09 ± 0.02 | 0.05 ± 0.01 | 0.22 ± 0.05 | 0.04 ± 0.01 | 0.22 ± 0.04 | 0.32 ± 0.02 | 0.20 ± 0.03 | 0.31 ± 0.09 | 0.10 ± 0.02 | 0.11 ± 0.05 |
| PCN | 0.21 ± 0.02 | 0.10 ± 0.03 | 0.14 ± 0.02 | 0.28 ± 0.05 | 0.05 ± 0.01 | 1.26 ± 0.11 | 0.64 ± 0.10[b] | 1.03 ± 0.16 | 0.91 ± 0.16[b] | 0.33 ± 0.02[b] | 0.77 ± 0.07 |
| PCN + chloramphenicol | 0.11 ± 0.00 | 0.12 ± 0.02 | 0.04 ± 0.01 | 0.04 ± 0.01 | 0.00 ± 0.00 | 2.64 ± 0.11 | 0.79 ± 0.25 | 0.46 ± 0.08 | 0.23 ± 0.05 | 0.14 ± 0.01 | 0.13 ± 0.05 |

[a] Treatment of animals, preparation of microsomes, and assays of androstenedione metabolism were performed as described under Materials and Methods. Warfarin metabolism was monitored as described previously (Kaminsky et al., 1981). TAO complex formation was determined from $\Delta A^{457/510}$ following incubation of microsomes (1 mg/mL) with 20 μM TAO and 1 mM NADPH for 30 min at 37 °C, with an extinction coefficient of 68 mM$^{-1}$ cm$^{-1}$ (Wrighton et al., 1985a,b). Results represent the mean ± SD of the values obtained with three to four individual samples per group. [b] Although induced by PCN, these hydroxylase activities are attributable to other isozymes than that responsible for 6β-hydroxylation (Waxman et al., 1985).

tenedione, a 48% decrease in the rate of formation of (*R*)-9,10-dehydrowarfarin, but a 2-fold *increase* in the rate of 10-hydroxylation of (*R*)-warfarin.

*Inactivation of Androstenedione Hydroxylases by Chloramphenicol in Vitro.* Because the stimulation of *R*-10-hydroxylation of warfarin by chloramphenicol was unexpected, in vitro experiments were carried out to corroborate the in vivo findings. Liver microsomes from PCN-treated female rats were incubated for 10 min at 37 °C with and without 100 μM chloramphenicol in the presence and absence of NADPH, and the microsomes were recovered from the incubation mixtures by ultracentrifugation as described previously (Halpert et al., 1985a). The combined treatment with chloramphenicol and NADPH caused a 25% decrease in cytochrome P-450 and in dehydrowarfarin formation, but a 40% increase in *R*-10-hydroxylation compared to controls lacking chloramphenicol, NADPH, or both (data not shown). In order to assess whether the kinetics of inactivation of PCN-inducible cytochromes P-450 were consistent with the same type of suicide inactivation previously documented in the case of the effect of chloramphenicol on the major PB-inducible isozyme of rat liver cytochrome P-450 (Halpert et al., 1985b; Miller & Halpert, 1986), androstenedione metabolism was monitored. As seen in Figure 1, preincubation of liver microsomes from PCN-treated male rats with chloramphenicol in the presence of NADPH caused a time-dependent loss of androstenedione 6β-hydroxylase activity, the pseudo-first-order kinetics of which were consistent with a suicidal mechanism. In contrast, the 7α-hydroxylase activity was refractory to inactivation, in agreement with the results obtained with the liver microsomes from the female rats treated with chloramphenicol in vivo (Table II). Thus, in liver microsomes from PCN-treated rats of both sexes, chloramphenicol inactivates cytochromes P-450 responsible for androstenedione 6β-hydroxylation but not warfarin *R*-10-hydroxylation.

*Isolation of PCN-Inducible Cytochrome P-450 Isozymes.* The results obtained in this investigation on the differential effect of chloramphenicol on PCN-associated monooxygenase activities in female rat liver microsomes were in complete agreement with those obtained previously with male rats (Halpert et al., 1985a) and suggested the possible existence of more than one major form of PCN-inducible cytochrome P-450 in rats of both sexes. We therefore set out to fractionate liver microsomes from PCN-treated rats with the goal of isolating at least two similar and related forms of cytochrome P-450. Since all of our previous purification work had been done with liver microsomes from male rats and since PCN-treated male rats have been reported to contain higher levels of the major form of PCN-inducible cytochrome P-450 than female rats (Waxman et al., 1985), we used pooled liver

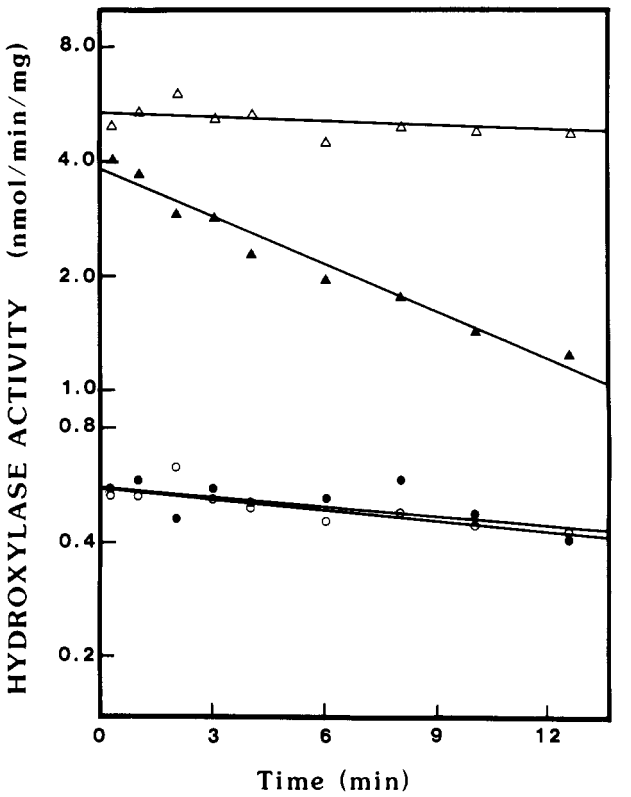


FIGURE 1: Effect of preincubation with chloramphenicol on the androstenedione hydroxylase activity of liver microsomes from PCN-treated male rats. Microsomes were incubated with 250 μM chloramphenicol at 37 °C for 2 min. Reactions were started by the addition of NADPH and were allowed to proceed for the times indicated, at which point 80-μL aliquots were taken and added to 20 μL of [$^{14}$C]androstenedione in buffer. The reactions were allowed to proceed for an additional 1.5 min and were quenched with 50 μL of tetrahydrofuran. The concentrations of the various components of the incubation mixture *after* addition of the androstenedione were 0.25 mg/mL microsomal protein, 200 μM chloramphenicol, 25 μM androstenedione, 1 mM NADPH, 50 mM HEPES buffer (pH 7.6), 15 mM $MgCl_2$, and 0.1 mM EDTA. Identification and quantitation of androstenedione metabolites was performed as described under Materials and Methods. (△) 6β-Hydroxylase activity after preincubation with NADPH alone; (▲) 6β-hydroxylase activity after preincubation with NADPH plus chloramphenicol; (○) 7α-hydroxylase activity after preincubation with NADPH alone; (●) 7α-hydroxylase activity after preincubation with NADPH plus chloramphenicol.

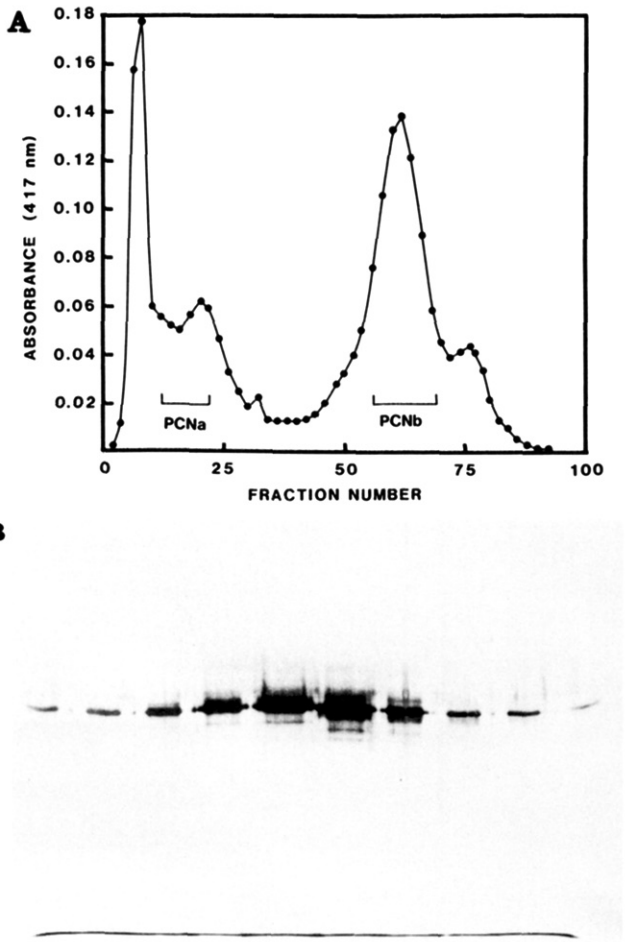


FIGURE 2: (A) Purification of cytochromes P-450 from liver microsomes of PCN-treated male rats. Liver microsomes from nine rats were solubilized and chromatographed on *n*-octylamino-Sepharose and hydroxylapatite (Shimada & Guengerich, 1985). The peak fractions that eluted from hydroxylapatite with 180 mM phosphate buffer were pooled and dialyzed for 24 h at 4 °C against two 1-L portions of 10 mM potassium phosphate buffer (pH 7.7) containing 0.1% Lubrol, 0.2% sodium cholate, 0.1 mM EDTA, and 20% glycerol. The sample was then applied to a column of DEAE-Sephacel (1 × 45 cm) equilibrated at room temperature with the same buffer. After sample application, the column was washed with 15 mL of the equilibration buffer. During the sample application and wash, a single prefraction was collected, which contained no appreciable amounts of cytochrome. At fraction 1, a 300-mL linear gradient of 0–60 mM NaCl in buffer was applied at a flow rate of 15 mL/h. Fractions of 3 mL were collected and monitored for absorbance at 417 nm as well as by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). Fractions containing a single major protein band were pooled as indicated. Some preparations of PCNa required further chromatography on CM-Sepharose (Waxman & Walsh, 1983) with highly purified PCNa eluting at 80 mM KCl. Detergent was removed as described under Materials and Methods for the cytochromes P-450 isolated from phenobarbital-treated rats. (B) SDS–polyacrylamide gel electrophoresis of cytochromes P-450 PCNa and PCNb. The samples were electrophoresed on a 7.5% gel and stained with silver by a commercial kit (Bio-Rad, Richmond, CA). The identities of the samples from left to right are (lanes 1–5) PCNa (0.05, 0.1, 0.2, 0.5, and 1 μg) and (lanes 6–10) PCNb (1, 0.5, 0.2, 0.1, and 0.05 μg). Under the staining conditions used, the limit of detection was 1–2 ng.

microsomes from PCN-treated male rats as the starting material for those studies. The microsomes were solubilized with sodium cholate and chromatographed on a column of *n*-octylamino-Sepharose, followed by a hydroxylapatite column (Shimada & Guengerich, 1985). The material that eluted with 180 mM phosphate was further fractionated on DEAE-Sephacel, which yielded four major cytochrome P-450 containing peaks (Figure 2). Two of these, referred to a PCNa and

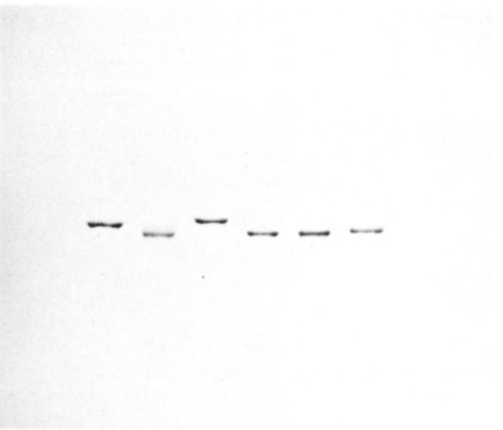

FIGURE 3: SDS–polyacrylamide gel electrophoresis of cytochrome P-450 isozymes purified from liver microsomes of phenobarbital- or PCN-treated adult male rats. The samples (1 μg) were electrophoresed on a 7.5% gel (Laemmli, 1970) and stained with Coomassie Blue. From left to right the identities of the samples along with their apparent molecular weights are (lane 1) PB-B (50 500), (lane 2) PB-C (47 000), (lane 3) PB-D (51 500), (lane 4) PB-E (47 500), (lane 5) PCNb (47 500), and (lane 6) PCNa (48 000). The standards used for the molecular weight determination were phosphorylase *a* (92 500), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400).

PCNb, were highly purified as judged by SDS–polyacrylamide gel electrophoresis (Figure 2). Shown in Figure 3 are four cytochrome P-450 isozymes isolated from liver microsomes of PB-treated adult male Sprague-Dawley rats. One of these, PB-E, exhibited the same chromatographic behavior and electrophoretic mobility as PCNb.

Preparations of isozymes PB-B, PB-C, and PB-D ranged in specific content from 13 to 16 nmol of cytochrome P-450/mg of protein on the basis of reduced carbon monoxide *difference* spectra (Omura & Sato, 1964). The specific contents of PB-E, PCNa, and PCNb determined by this method were only 6–8 nmol of P-450/mg of protein. However, *absolute* reduced carbon monoxide spectra of the latter isozymes revealed that approximately 50% of the cytochrome failed to reduce with dithionite under the conditions of the assay. When the specific contents of PB-E, PCNa, and PCNb were determined from the absorbance at 417 nm of the oxidized protein with a nominal extinction coefficient of 106 $mM^{-1}$ $cm^{-1}$ (Wolf et al., 1980), specific contents of 12–14 nmol of cytochrome P-450/mg of protein were obtained. In contrast, the specific contents of PB-B, PB-C, and PB-D, which appeared to reduce fully upon addition of dithionite, were the same whether calculated from the reduced carbon monoxide difference spectra or from the absolute oxidized spectra. These results indicate that poor reducibility, rather than low heme content, is responsible for the apparent low specific contents of PCNa, PCNb, and PB-E determined by the method of Omura and Sato. In this context, it is of interest that unusual behavior of cytochrome P-450p during the assay of Omura and Sato has been reported (Wrighton et al., 1985a).

The amino-terminal sequences of PCNa, PCNb, and PB-E were determined and are shown in Figure 4, where they are also compared to the amino-terminal sequence of a major PCN-inducible cytochrome P-450, the complete amino acid sequence of which was recently reported (Gonzalez et al., 1985). The latter protein is identical with PCNb in the first 15 residues, whereas PCNa differs from PCNb in three of these positions. However, it should be noted that PCNa and PCNb exhibit far greater amino-terminal homology with each other than they do with any of the other purified rat liver cytochromes P-450, the amino-terminal sequences of which

| | 5 | | | | | 10 | | | | | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| P-450PCN | Met-Asp- | Leu- | Leu- | Ser- | Ala- | Leu- | Thr- | Leu- | Glu- | Thr- | Trp- | Val- | Leu- | Leu |
| PCNb | Met-Asp- | Leu- | Leu- | Ser- | Ala- | Leu- | Thr- | Leu- | Glu- | Thr- | Trp- | Val- | Leu- | Leu |
| PCNa | Met-Asp- | Leu- | Ala* | Ser- | Ala- | Leu- | Val* | Leu- | Glu- | Tyr* | Trp- | Val- | Leu- | Leu |
| PB-E | Met-Asp- | Leu- | Leu- | Ser- | Ala- | Leu- | Thr- | Leu- | Glu | | | | | |

FIGURE 4: Amino-terminal acid sequences of PCN-inducible cytochromes P-450. Purified cytochromes PCNa, PCNb, and PB-E (2–4 nmol) were dialyzed against four 1-L portions of 5% acetic acid for 48 h and then concentrated to 100 μL with a gentle stream of nitrogen. Sequence analysis was performed by Edman degradation with a Beckman 890m sequencer (fully automated for coupling, cleavage, and conversion of protein to stable PTH-amino acid residues) and the manufacturer's program [Protein/Peptide, Micro/Macro Sequencing—dated 5/28/85 (Q/P)]. Determination of PTH-amino acids was done by HPLC (Beckman System, Model 334) with a 5-μm ODS/C18 column (Burdick & Jackson). Elution of the PTH-amino acids was done isocratically with a mobile phase of 64.6% 17 mM sodium acetate in 10% acetonitrile and with 35.4% acetonitrile with 10% tetrahydrofuran at a flow of 1.7 mL/min. The column temperature was 41 °C, and the PTH-amino acids were identified by their retention times with PTH-amino acid standards obtained from Pierce (Rockford, IL) and quantified from their absorbance at 269 nm. The sequence of P-450PCN was determined by Edman degradation and was also deduced from the nucleotide sequence of a full-length cDNA clone (Gonzalez et al., 1985). Residues in PCNa marked with an asterisk differ from the corresponding residues in the other proteins.

have been reported (Black & Coon, 1986). It should also be noted that PB-E was identical in the sequence of its first 10 residues to PCNb, which together with the chromatographic and electrophoretic data confirms the probable identity of these two proteins.

*Monooxygenase Activities of the Purified Cytochrome P-450 Isozymes.* (*R*)-Warfarin and androstenedione hydroxylase assays were performed on PCNa and PCNb and on the octylamino-Sepharose fraction and the intact microsomes from which the purified proteins were derived. Only 25% of the ability to form (*R*)-9,10-dehydrowarfarin and 6β-hydroxyandrostenedione and 60% of the ability to form (*R*)-10-hydroxywarfarin were recovered in the octylamino-Sepharose fraction compared to the microsomes. Neither of the purified proteins was capable of catalyzing appreciable formation of 9,10-dehydrowarfarin or 6β-hydroxyandrostenedione, and only PCNa exhibited appreciable *R*-10-hydroxylase activity (0.18 nmol $min^{-1}$ $nmol^{-1}$). However, PCNa had only 30% of the activity of the octylamino-Sepharose fraction. These findings are consistent with the apparent inactivation of PCN-inducible rat liver cytochrome P-450 isozymes upon purification (Guengerich et al., 1982; Wrighton et al., 1985a). In contrast, the warfarin *R*-7-hydroxylase activity of purified PB-C was 5-fold higher than that of the microsomes (data not shown).

*Immunochemical Studies.* In order to further probe the structural relatedness between PCNa and PCNb, polyclonal antibodies to the latter protein were raised in rabbits. As seen in Figure 5, upon immunoblot analysis these antibodies recognized PCNa as well as PCNb. Moreover, a mixture of PCNa and PCNb could not be resolved by the immunoblotting, which explains why the antibodies appeared to recognize only a single electrophoretic species in intact liver microsomes from PCN-treated rats. In addition to PCNa and PCNb, the antibodies reacted with a P-450 fraction termed PB-X, isolated from liver microsomes of PB-treated rats and which exhibited the same chromatographic and electrophoretic behavior as PCNa. The antibodies also recognized PB-E (data not shown).

The antibodies were also used with intact microsomes to probe the role of PCNa and PCNb in chloramphenicol-mediated cytochrome P-450 destruction. This was not possible to accomplish with a reconstituted system due to the poor ability of the purified cytochromes to metabolize chloramphenicol. Preincubation of liver microsomes from PCN-treated male rats with 6 mg of anti-PCNb IgG/nmol of cytochrome P-450 was sufficient to inhibit more than 80% of the androstenedione 6β-hydroxylase activity. This same concentration of anti-PCNb IgG inhibited 30% of the covalent binding of chloramphenicol to the protein in such microsomes. More important, however, the anti-PCNb IgG inhibited 60% of the in vitro loss of cytochrome P-450 caused by incubation of liver microsomes from PCN-treated male rats with chloramphenicol in the presence of NADPH (data not shown).

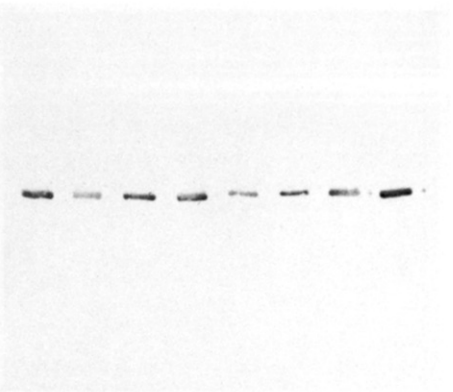

FIGURE 5: Immunoblots of liver microsomes from PCN-treated male rats and of purified cytochromes P-450. Protein samples were resolved by electrophoresis in a 7.5% SDS–polyacrylamide gel and transferred electrophoretically to nitrocellulose. The blot was developed with anti-PCNb IgG and visualized by peroxidase staining. One microgram of microsomal protein or 0.1 μg of purified cytochrome P-450 was applied to the gel. From left to right the identity of the samples is (lane 1) male PCN-microsomes, (lane 2) PCNa, (lane 3) PCNb, (lane 4) mixture of PCNa and PCNb (0.05 μg each), (lane 5) PB-X isolated during the purification of PB-C (see Materials and Methods), (lane 6) PB-X isolated by DEAE-Sephacel chromatography of a 180 mM phosphate–hydroxylapatite fraction (see Materials and Methods), (lane 7) PCNa from liver microsomes of PCN-treated female rats, and (lane 8) male PCN microsomes.

An alternative approach was also employed, whereby the antibodies were used in conjunction with chromatography to identify the cytochrome P-450 isozymes that had been converted to cytochrome P-420 in liver microsomes from the PCN-treated female rats administered chloramphenicol in vivo. This experiment made use of the observation that the chromatography of solubilized microsomes on octylamino-Sepharose removed almost all of the cytochrome P-420 from the P-450 fraction. Therefore, equal amounts on a milligram of protein basis of liver microsomes from PCN-treated (control group) and PCN plus chloramphenicol treated rats were solubilized and chromatographed on octylamino-Sepharose columns. Whereas the microsomes from the two groups contained equal amounts of protein reactive with the antibody to PCNb, only half as much immunoreactive protein and total cytochrome P-450 was recovered in the octylamino-Sepharose fraction from the chloramphenicol-treated rats compared to the control group (data not shown). These findings, together with the inhibition by anti-PCNb of the chloramphenicol-mediated loss of cytochrome P-450 in vitro, indicate that proteins immunochemically related to PCNb are major targets for chloramphenicol in liver microsomes from PCN-treated rats.

## Discussion

Although the possible multiplicity of the major PCN-inducible isozyme of rat liver cytochrome P-450 has been suggested previously (Wrighton et al., 1985b; Hardwick et al., 1983), this paper describes to our knowledge the first isolation in a single laboratory of two chromatographically and elec-

trophoretically distinct yet immunochemically similar forms of cytochrome P-450 from liver microsomes of PCN-treated rats. These two forms, referred to as PCNa and PCNb, differ in 3 of the first 15 amino-terminal acid residues. On the basis of the amino-terminal sequences, PCNb seems to correspond to P-450PCN described by Gonzalez et al. (1985).[3] PCNb also appears to correspond to an isozyme termed PB/PCN-E (Guengerich et al., 1982), since the chromatographic and electrophoretic behavior as well as the amino-terminal sequence of PCNb are identical with those of a cytochrome P-450 isozyme isolated by us from liver microsomes of PB-treated rats using a minor modification of the method for the isolation of PB/PCN-E. In contrast, the relationship between PCNb and P-450p (Wrighton et al., 1985a) is unclear, since the reported amino-terminal sequence of the latter protein is quite different from that of PCNb. Likewise, the relationship between the isozymes isolated in our laboratory and isozyme PB-2a (Waxman et al., 1985) is unknown in the absence of amino-terminal sequence data on the latter protein.

In agreement with reports from several laboratories (Guengerich et al., 1982; Wrighton et al., 1985a; Waxman et al., 1985), we observed an apparent inactivation of isozymes PCNa and PCNb upon purification, as evidenced by the poor recoveries of several microsomal monooxygenase activities generally attributed to the major PCN-inducible cytochrome P-450 isozyme. This precluded the reliable assessment of any functional differences between the two proteins in a reconstituted system. Moreover, the immunological cross-reactivity between PCNa and PCNb precluded the use of antibody inhibition studies with intact microsomes as a means of distinguishing between the two proteins. However, results obtained with the mechanism-based inactivator chloramphenicol provided strong evidence for the existence in rat liver microsomes of at least two functionally distinct PCN-inducible cytochrome P-450 isozymes. Thus, although PCN-treatment of female rats caused 5–7-fold increases in four monooxygenase activities considered diagnostic for the major PCN-inducible cytochrome P-450 isozyme, these activities were not affected in the same manner by chloramphenicol. The major finding in this regard was the 80% decrease in TAO complex formation in liver microsomes from PCN-treated female rats following chloramphenicol treatment compared to the 2-fold increase in the ability of such microsomes to hydroxylate (*R*)-warfarin in the 10-position.

In addition to causing a loss of certain monooxygenase activities, chloramphenicol also mediated the conversion to cytochrome P-420 of a significant fraction of the cytochrome P-450 in liver microsomes from PCN-treated rats. The in vitro loss of cytochrome P-450 caused by chloramphenicol could be inhibited by antibodies to PCNb. Further evidence that cytochromes P-450 immunochemically related to PCNb are inactivated by chloramphenicol was obtained by chromatographing solubilized liver microsomes from PCN-treated and PCN plus chloramphenicol treated female rats on octylamino-Sepharose. Chloramphenicol had no effect on the amount of immunoreactive protein in the intact microsomes, which is consistent with the unaltered levels of heme and of total cytochrome P-450 plus cytochrome P-420. However, after removal of the cytochrome P-420 on the octylamino-Sepharose column, only half as much protein that reacted with anti-PCNb was recovered from the microsomes from chloramphenicol-treated compared to the control rats.

A final question concerns the extent to which multiplicity may exist among members of the PCN family of cytochrome P-450 in other species. In this regard it is of interest that rabbit liver microsomes have been reported to contain at least two inducible proteins immunochemically related to rat liver P-450p (Wrighton et al., 1985b). Furthermore, in light of our results that PCNa and PCNb cannot be resolved by immunoblotting, the fact that antibodies to P-450p recognize only a single electrophoretic entity in liver microsomes from other species including humans (Watkins et al., 1985) does not necessarily preclude the possibility of more than one related isozyme. In fact, very recent results suggest that not only rats but also humans may contain three to five homologous genes in the PCN-P-450 family (Molowa et al., 1986). If more than one of these human genes code for active proteins, then the functional multiplicity we have demonstrated in the rat may also occur in humans and could be of pharmacological and toxicological interest.

## ACKNOWLEDGMENTS

We thank Wallace Clark for the sequence analysis and Jeffrey Stevens for the experiments involving in vitro inactivation of androstenedione hydroxylases by chloramphenicol. We also thank David Duignan for his help in preparing and characterizing the antibodies to PCNb. We also gratefully acknowledge the expert assistance of Deborah Dunbar with the warfarin hydroxylase assays and of Leslie Auerbach in the preparation of the manuscript.

**Registry No.** Pregnenolone-16$\alpha$-carbonitrile, 1434-54-4; cytochrome P-450, 9035-51-2; heme, 14875-96-8; monooxgenase, 9038-14-6; (*R*)-warfarin, 5543-58-8; androstenedione, 63-05-8; triacetyloleandomycin, 2751-09-9; 9,10-dehydrowarfarin, 67588-18-5; 10-hydroxy-(*R*)-warfarin, 63740-79-4; 6$\beta$-hydroxyandrostenedione, 63-00-3; androstenedione 6$\beta$-hydroxylase, 9075-83-6; phenobarbital, 50-06-6; (*R*)-warfarin 10-hydroxylase, 104520-86-7.

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[3] During the preparation of this paper, the complete nucleotide sequence was reported (Gonzalez et al., 1986) of a cDNA clone homologous to the previously characterized P-450PCN cDNA (Gonzalez et al., 1985). The putative protein products of the genes corresponding to these two cDNA clones both possess identical amino-terminal acid sequences to our PCNb for the first 15 residues.

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# Intrinsic Fluorescence of Elongation Factor Tu in Its Complexes with GDP and Elongation Factor Ts†

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*Received October 8, 1986; Revised Manuscript Received February 11, 1987*

ABSTRACT: The intrinsic fluorescence properties of elongation factor Tu (EF-Tu) in its complexes with GDP and elongation factor Ts (EF-Ts) have been investigated. The emission spectra for both complexes are dominated by the tyrosine contribution upon excitation at 280 nm whereas excitation at 300 nm leads to exclusive emission from the single tryptophan residue (Trp-184) of EF-Tu. The fluorescence lifetime of this tryptophan residue in both complexes was investigated by using a multifrequency phase fluorometer which achieves a broad range of modulation frequencies utilizing the harmonic content of a mode-locked laser. These results indicated a heterogeneous emission with major components near 4.8 ns for both complexes. Quenching experiments on both complexes indicated limited accessibility of the tryptophan residue to acrylamide and virtually no accessibility to iodide ion. The quenching patterns exhibited by EF-Tu·GDP and EF-Tu·EF-Ts were, however, different; both quenchers were more efficient at quenching the emission from the EF-Tu·EF-Ts complex. Steady-state and dynamic polarization measurements revealed limited local mobility for the tryptophan in the EF-Tu·GDP complex whereas formation of the EF-Tu·EF-Ts complex led to a dramatic increase in this local mobility.

Elongation factor Tu (EF-Tu)[1] mediates the binding of aminoacyl-tRNA to the ribosome of prokaryotic systems and is thus an essential component of the elongation cycle of protein biosynthesis [see Miller and Weissbach (1977), Weissbach (1980), and Bosch et al. (1983) for reviews]. EF-Tu interacts with many components of the protein biosynthesis system at various stages of the cycle; these components include guanosine nucleotides, elongation factor Ts, aminoacyl-tRNA, and ribosomes. The antibiotics kirromycin and aurodox also bind tightly to EF-Tu (Parmeggiani & Swart, 1985). A body of evidence suggests that the binding of the different ligands

† This work was supported by the Medical Research Council, U.K. (J.F.E.), by U.S. Public Health Service Grant GM 29603 (J.F.E.), and by National Science Foundation Grants PCM-8403107 (E.G.), PCM-8402663 (D.M.J.), and INT-8408263 (E.G. and D.M.J.). A preliminary report of this work has been presented at the Feb 1986 meeting of the Biophysical Society.
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[1] Abbreviations: EF-Tu, elongation factor Tu; EF-Ts, elongation factor Ts; ANS, anilinonaphthalenesulfonate; DTT, dithiothreitol; FWHM, full width at half-maximum; CD, circular dichroism; ORD, optical rotatory dispersion; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/87/0426-3894$01.50/0 