Isolation of four forms of acetone-induced cytochrome *P*-450 in chicken liver by h.p.l.c. and their enzymic characterization

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The purpose of this study was to purify and characterize the forms of cytochrome *P*-450 induced in chicken liver by acetone or ethanol. Using high performance liquid ion-exchange chromatography, we were able to isolate at least four different forms of cytochrome *P*-450 which were induced by acetone in chicken liver. All four forms of cytochrome *P*-450 proved to be distinct proteins, as indicated by their *N*-terminal amino acid sequences and their reconstituted catalytic activities. Two of these forms, also induced by glutethimide in chicken embryo liver, appeared to be cytochromes *P*450IIH1 and *P*450IIH2. Both of these cytochromes *P*-450 have identical catalytic activities towards benzphetamine demethylation. However, they differ in their abilities to hydroxylate *p*-nitrophenol and to convert acetaminophen into a metabolite that forms a covalent adduct with glutathione at the 3-position. Another form of cytochrome *P*-450 induced by acetone is highly active in the hydroxylation of *p*-nitrophenol and in the conversion of acetaminophen to a reactive metabolite, similar to reactions catalysed by mammalian cytochrome *P*450IIE. Yet the *N*-terminal amino acid sequence of this form has only 30–33% similarity with cytochrome *P*450IIE purified from rat, rabbit and human livers. A fourth form of cytochrome *P*-450 was identified whose *N*-terminal amino acid sequence and enzymic activities do not correspond to any mammalian cytochromes *P*-450 reported to be induced by acetone or ethanol.

INTRODUCTION

Cytochromes *P*-450 comprise a multigene family of haemo-proteins involved in the oxidative metabolism of numerous endogenous and exogenous chemicals. In most organs, these oxidative reactions convert lipophilic substrates into more water-soluble products to facilitate their excretion. However, certain substrates such as acetaminophen, bromobenzene, carbon tetrachloride and most potential carcinogens are converted into reactive metabolites by this oxidative process, depending on the specific reaction and the form of cytochrome *P*-450 involved (for reviews, see Guengerich, 1988; Gonzalez, 1989).

In mammals, three forms of cytochrome *P*-450 have been purified which are induced by ethanol. These are cytochromes *P*450IIB1 and IIB2, two forms which are also induced by phenobarbital and glutethimide, and cytochrome *P*450IIE (Song *et al.*, 1986; Johansson *et al.*, 1988), which catalyses the bio-activation of dimethylnitrosamine (Tu & Yang, 1985; Thomas *et al.*, 1987) and acetaminophen (Morgan *et al.*, 1983; Raucy *et al.*, 1989). *P*450IIE may have a role in the increased incidence of cancer (for review, see Lieber *et al.*, 1986) and acetaminophen-associated hepatotoxicity (for review, see Seeff *et al.*, 1986) observed in alcoholic patients.

Cytochrome *P*450IIE can also be induced in mammals by acetone, fasting, experimental diabetes and pyrazole treatment (for review, see Koop & Coon, 1986), treatments which result in elevated levels of blood acetone. These results have led to the hypothesis that acetone is the ultimate inducer of this form of cytochrome *P*-450 (Miller & Yang, 1984). In rats, acetone is also an effective inducer of cytochromes *P*450IIB1 and IIB2 (Song *et al.*, 1986).

In rats (Reinke & Moyer, 1985), rabbits (Koop, 1986) and humans (Raucy *et al.*, 1987), cytochrome *P*450IIE has greater activity towards *p*-nitrophenol hydroxylation compared with

other forms of cytochrome *P*-450 from these species. We reported that ethanol induces *p*-nitrophenol hydroxylase in cultured chick hepatocytes, suggesting the induction of a form of *P*-450 similar to *P*450IIE (J. Sinclair *et al.*, 1989). Using an innovative h.p.l.c. technique (Funae *et al.*, 1985; Funae & Imaoka, 1987), we have purified at least four major forms of cytochrome *P*-450 which are induced by acetone in young chickens, and have distinguished them both by their enzymic properties and by their *N*-terminal amino acid sequences. Two of these forms appear to be identical to cytochromes *P*450IIH1 and *P*450IIH2, two greatly similar forms of cytochrome *P*-450 induced by phenobarbital in chicken embryo liver (Hansen & May, 1989; Gonzalez, 1989) (based on *N*-terminal amino acid sequences), and one appears to be equivalent to *P*450IIE (based on enzymic properties).

EXPERIMENTAL

Treatment of chicken embryos and chickens

White Leghorn chicken embryos were housed in a Petersime incubator, as described (Hamilton *et al.*, 1989). Chicken embryos (16 days old) were either untreated or injected with 6 mg of glutethimide per egg as described previously (P. Sinclair *et al.*, 1989). At 48 h after the administration of glutethimide, the embryos were killed and hepatic microsomes were prepared as described (Sinclair *et al.*, 1979).

Hatched embryos were housed in stainless steel rat cages, two chickens per cage, in a room maintained at 37 °C by the use of heating lamps. Chickens had free access to food (Agway chicken starter, White River Junction, VT, U.S.A.) and water, which was changed twice per day. Chickens (10 days old) were either untreated or exposed to 5% ethanol in their drinking water for 13 days. Chickens of 8 days old were either untreated or treated with 5 ml of acetone/day per kg for 4 days by gastric intubation

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of a 20% aqueous solution of acetone. Chickens were killed by decapitation and livers were homogenized (30%, w/v) in Tris/acetate/KCl buffer (0.1 M-Tris base/0.1 M-KCl/1 mM-EDTA, adjusted to pH 7.4 with acetic acid). After 2 strokes of a Potter-Elvehjem homogenizer, butylated hydroxytoluene, an antioxidant, and phenylmethanesulphonyl fluoride, a proteinase inhibitor, were added to final concentrations of 45 μ M and 115 μ M respectively. Homogenization was continued for an additional nine strokes. Microsomes were prepared as described (Sinclair *et al.*, 1979).

Purification of acetone-induced cytochromes *P*-450

Microsomes from livers of 25–30 chickens were solubilized in 10 mM-sodium phosphate, pH 7.4, 1 mM-EDTA, 0.5 M-NaCl, 20% (v/v) glycerol containing 0.5% (w/v) sodium cholate and 0.2% (v/v) Emulgen 911 (Gibson & Schenkman, 1978). The mixture was stirred gently on ice for 30 min and then centrifuged at 100 000 *g* for 45 min. The supernatant, containing approx. 300 nmol of cytochrome *P*-450, was applied to a column (2.5 cm $\times$ 30 cm) of octyl-Sepharose CL-4B, pre-equilibrated with Buffer A. Three buffers were used in the elution of cytochrome *P*-450 from the octyl-Sepharose column, as follows. Buffer A, 10 mM-sodium phosphate, pH 7.4, 1 mM-EDTA, 500 mM-NaCl, 20% (v/v) glycerol and 0.5% (w/v) sodium cholate; buffer B, 10 mM-sodium phosphate, pH 7.4, 1 mM-EDTA, 400 mM-NaCl, 20% (v/v) glycerol, 0.44% (w/v) sodium cholate and 0.1% (v/v) Emulgen 911; buffer C, 10 mM-sodium phosphate, pH 7.4, 1 mM-EDTA, 20% (v/v) glycerol, 0.2% (w/v) sodium cholate and 2% (v/v) Emulgen 911.

Cytochromes *P*-450 were separated according to a modification of the method of Kling *et al.* (1985), as shown in Fig. 1(a). Absorbance was monitored at 405 nm and the flow rate was 1.2–1.7 ml/min. Cytochromes *P*-450 (Omura & Sato, 1964) and *b*₅ (Omura & Takesue, 1970), NADPH-cytochrome *P*-450 reductase (Vermilion & Coon, 1974) and proteins (BCA Protein Assay; Pierce Chemical Co., Rockford, IL, U.S.A.) were measured for each peak.

Fraction 4 from the octyl-Sepharose column contained most of the cytochrome *P*-450 and was applied on to a TSK DEAE-5-PW preparative h.p.l.c. column (21.5 cm $\times$ 150 mm) pre-equilibrated with buffer D [10 mM-Tris/acetate, pH 7.5, 20% (v/v) glycerol and 0.4% (v/v) Emulgen 911], and chromatographed by a modification of the procedure of Funae and coworkers (Funae *et al.*, 1985; Funae & Imaoka, 1987). Buffer E was buffer D containing 0.8 M-sodium acetate. Cytochrome *P*-450 was eluted in ten separate fractions with a step gradient of 0 to 100% buffer E, as shown in Fig. 1(b). The flow rate was 2 ml/min. The gradient was returned to 100% buffer D linearly over 10 min, then the column was washed with 100% buffer D for 30 min at a flow rate of 2 ml/min before application of the next sample. The major fractions containing cytochrome *P*-450 were dialysed against 5 mM-potassium phosphate (pH 7.4)/20% (v/v) glycerol. Emulgen was then removed from each fraction by chromatography on hydroxyapatite: 5 ml fractions were applied to a hydroxyapatite column (1.5 cm $\times$ 4 cm) previously equilibrated with 5 mM-potassium phosphate (pH 7.7)/0.1 mM-dithiothreitol/0.1 mM-EDTA/20% (v/v) glycerol/0.1% (w/v) sodium cholate. The same buffer was applied until Emulgen was removed, as determined by monitoring the absorbance at 276 nm. Cytochrome *P*-450 was eluted from the column by a buffer identical with the equilibrating buffer, but containing 300 mM-potassium phosphate. The resulting cytochromes *P*-450 were dialysed overnight against 10 mM-sodium phosphate (pH 7.4)/20% (v/v) glycerol to remove the cholate, concentrated by Amicon ultra-filtration (PM 30 filter) and stored at -80°C in 10 mM-sodium phosphate, pH 7.4, containing 20% (v/v) glycerol.

The cycle of cytochrome *P*-450 purification by octyl-Sepharose and preparative h.p.l.c. DEAE-cellulose chromatography was repeated three times with hepatic microsomes from separate batches of acetone-treated chickens. The elution profiles obtained were similar. The results reported in Tables 1 and 2 and Figs. 1–4 show one representative cycle of this purification.

Cytochrome *b*₅, eluting in fraction 9 from the preparative DEAE-cellulose column, was purified to a specific content of 16.8 nmol/mg of protein. NADPH-cytochrome *P*-450 reductase was purified from rat microsomes by the procedure of Yasukochi & Masters (1976) to a specific content of 8 nmol/mg of protein. Cytochrome *b*₅ and cytochrome *P*-450 reductase were each purified to a single protein, as determined by SDS/PAGE (results not shown).

The components of the h.p.l.c. chromatography system consisted of a Milton Roy CM 4000 solvent delivery system, an SM 4000 programmable wavelength detector, a 7125 Rheodyne injector and a Shimadzu C-R6A Chromatopac integrator.

Resolution of microsomal cytochromes *P*-450 by anion-exchange analytical h.p.l.c.

Microsomal cytochrome *P*-450 was resolved by anion-exchange h.p.l.c., using the methodology of Funae and coworkers (Funae *et al.*, 1985; Funae & Imaoka, 1987). Microsomes were solubilized by stirring gently on ice for 30 min in a solution containing 10 mM-sodium phosphate, pH 7.4, 10% (v/v) glycerol, 0.05 mM-EDTA, 0.5% (w/v) sodium cholate and 0.2% (v/v) Emulgen 911, and then centrifuged at 11 000 *g* for 15 min. A portion of the supernatant containing the amounts of protein and *P*-450 indicated in the legend to Fig. 5 was applied directly on to an anion-exchange column (TSK DEAE-5-PW, 7.5 mm $\times$ 75 mm) equilibrated with 10 mM-Tris/acetate, pH 7.5, 20% (v/v) glycerol and 0.4% (v/v) Emulgen 911. The cytochromes *P*-450 were eluted with a linear step gradient of sodium acetate, as described in the legend to Fig. 5, and absorbance was monitored at 405 nm.

Assays

Protein was measured by the BCA Protein Assay (Pierce) using bovine serum albumin as a standard. Cytochrome *P*-450 was measured by the technique of Omura & Sato (1964). NADPH-cytochrome *P*-450 reductase (Vermilion & Coon, 1974) and cytochrome *b*₅ (Omura & Takesue, 1970) were measured as described.

SDS/PAGE and immunoblotting utilized the Bio-Rad Mini Protean II system, as described previously (P. Sinclair *et al.*, 1989).

Microsomal analysis of *p*-nitrophenol hydroxylase activity was carried out as described previously (J. Sinclair *et al.*, 1989). Formation of 3-glutathion-*S*-yl acetaminophen was analysed in a total reaction volume of 0.5 ml containing 0.5 mg of microsomal protein, 10 mM-acetaminophen, 5 mM-GSH and 5 mM-MgCl₂ in 37 mM-Tris/Cl, pH 7.4. The reaction was initiated by the addition of NADPH to 1 mM and the mixture was incubated at 37 $^{\circ}\text{C}$ for 60 min. The reaction was terminated by the addition of HClO₄ to a concentration of 0.15% (w/v) (Tee *et al.*, 1987). After centrifugation, the supernatant was analysed by h.p.l.c., using an isocratic system of 3.5 mM-heptanesulphonic acid, 20% (v/v) methanol and 1% (w/v) acetic acid, at a flow rate of 1.5 ml/min, on a reverse-phase Rainin 5 μ C₁₈ column, 4.6 mm $\times$ 250 mm (Gemborys & Mudge, 1981). 3-Glutathion-*S*-yl acetaminophen, measured by absorbance at 245 nm, was eluted at 14.8–16 min, as confirmed by separate injection of a standard.

For measurement of dimethylnitrosamine demethylation, microsomes (0.2 mg/0.5 ml of reaction volume) were incubated with increasing concentrations of dimethylnitrosamine in the

following assay buffer: 50 mM-Tris/Cl, pH 7.4, 10 mM-MgCl₂, 20 mM-isocitrate, 0.107 units of isocitrate dehydrogenase and 0.5 mM-NADPH. The reaction mixture was incubated for 30 min at 37 °C and terminated by the addition of trichloroacetic acid to a final concentration of 5% (w/v) formaldehyde was analysed in the supernatant by the Nash reaction (Udenfriend, 1969). The activities are presented using 1 mM-dimethylnitrosamine as substrate, a concentration that was saturating for the low K_m activity.

For the *N*-terminal amino acid sequence analyses, cytochromes P-450 were electrophoresed on SDS/10%-polyacrylamide gels. Proteins were electroblotted on to polyvinylidene difluoride membranes (Immobilon, Millipore), following the procedure described by Matsudaira (1987). Membranes were stained with Amido Black. Bands of interest were excised and submitted to sequence analysis. *N*-Terminal sequences were determined by an Applied Biosystems pulsed liquid-phase protein sequencer, model 477A, in the Protein/Nucleic Acid Shared Facility of the Medical College of Wisconsin. The 50 kDa protein from fraction 8 was alkylated before analysis (Charbonneau, 1989) to identify the amino acid residue at position 18.

Reconstitution of enzyme activities of purified cytochromes P-450

The cytochrome P-450 complex was reconstituted by incubation of dilauryl phosphatidylcholine, cytochrome P-450, NADPH-cytochrome P-450 reductase and cytochrome *b*₅ in a molar ratio of 165:1:1:1 for 60 min at room temperature to form the micelles (Jansson *et al.*, 1985). All enzymic activities employed a NADPH-generating system composed of 10 mM-MgCl₂, 10 mM-glucose 6-phosphate, 0.2 units of glucose-6-phosphate dehydrogenase and 1 mM-NADPH. The *p*-nitrophenol hydroxylase assay (J. Sinclair *et al.*, 1989) contained 100 pmol of cytochrome P-450 per reaction and the reaction mixture was incubated for 60 min at 37 °C. The assay of the benzphetamine demethylase reaction (Sinclair *et al.*, 1982) contained 1 mM-benzphetamine and 50 pmol of cytochrome P-450 per reaction, and the reaction mixture was incubated for 20 min at 37 °C. Formation of the glutathione adduct of acetaminophen was assayed as described earlier, using 100 pmol of reconstituted cytochrome P-450 per assay.

Incubation times and the amounts of cytochrome P-450 used for all assays were determined to be within the linear range of product formation with time and cytochrome P-450 concentration.

Materials

Glutethimide, dimethylnitrosamine, Trizma base, EDTA,

sodium cholate, butylated hydroxytoluene, phenylmethane-sulphonyl fluoride, octyl-Sepharose CL-4B, bovine serum albumin, MgCl₂, isocitric acid, isocitric dehydrogenase, NADPH, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, glutathione, acetaminophen, chymotrypsin, *Staphylococcus aureus* V8 protease and dilauryl phosphatidylcholine were all purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Ethanol, acetone, KCl, acetic acid, sodium phosphate, glycerol, NaCl, sodium acetate, trichloroacetic acid, HClO₄, heptanesulphonic acid and methanol (h.p.l.c. grade) were purchased from Fisher Scientific Co., Fairlawn, NJ, U.S.A. BCA protein kit was purchased from Pierce. Hydroxyapatite (Biogel HTP) was purchased from Bio-Rad, Richmond, CA, U.S.A. Emulgen 911 was a gift from KAO Corporation, Tokyo, Japan. 3-Glutathion-S-yl acetaminophen was kindly provided by Dr. Mark Gemborys, McNeil Laboratories, Fort Washington, PA, U.S.A.

RESULTS

Purification of acetone-induced cytochromes P-450

As shown in Table 1, treatment with acetone for 4 days resulted in increases in microsomal cytochrome P-450, as measured spectrophotometrically. There were also increases in three enzymic activities associated with mammalian P450IIE, i.e. *p*-nitrophenol hydroxylation (Reinke & Moyer, 1985; Koop, 1986; Raucy *et al.*, 1987), acetaminophen activation (Morgan *et al.*, 1983; Raucy *et al.*, 1989) and demethylation of dimethylnitrosamine (Patten *et al.*, 1986; Thomas *et al.*, 1987).

In solubilized microsomes from acetone-treated chickens, compounds absorbing at 405 nm eluted in four fractions from the octyl-Sepharose column (Fig. 1a). Fractions 1–3 contained mainly cytochrome P-450 reductase and cytochrome *b*₅. Fraction 4, which was eluted with the buffer containing 2% Emulgen, contained 76% of the cytochrome P-450 applied to the column (Table 2). Fraction 4 was purified further on a preparative anion-exchange h.p.l.c. system (Fig. 1b). Ten peaks containing cytochrome P-450 were resolved. We determined the antigenic identity of these cytochromes P-450 with a major cytochrome P-450 of 50 kDa purified from glutethimide-treated chicken embryos (P. Sinclair *et al.*, 1989). This antibody detects two proteins of 53 kDa and 50 kDa in hepatic microsomes from glutethimide-treated embryos (results not shown). As can be seen from the immunoblot in Fig. 2, microsomes from acetone-treated chickens contain proteins of 53 kDa and 50 kDa that share epitopes with the glutethimide-induced cytochrome P-450. Fractions 1, 3, 4 and 5 from the DEAE column contained immunoreactive 53 kDa proteins, whereas fractions 5–10 contained immunoreactive 50 kDa proteins.

Table 1. Induction of cytochrome P-450 by acetone

Chickens (8 days old) were either untreated or exposed to acetone for 4 days as described in the Experimental section. A total of 27 livers were pooled from acetone-treated chickens, and four from untreated chickens. Microsomes were prepared and enzyme activities were determined as described in the Experimental section. Each value represents the mean of duplicate measurements with the individual value varying by no more than 10% of the means.

Treatment	Enzyme activity			
	Cytochrome P-450 (pmol/mg of protein)	<i>p</i> -Nitrophenol hydroxylase (nmol of 4-nitrocatechol/min per mg of protein)	Acetaminophen activation (nmol of 3-glutathion-S-yl acetaminophen/min per mg of protein)	Dimethylnitrosamine demethylation (nmol of formaldehyde/min per mg of protein)
None	146	0.25	0.45	0.16
Acetone	387	0.65	0.78	0.56

Fig. 1. Purification of acetone-induced forms of cytochrome *P*-450

Microsomes prepared from 27 chickens were solubilized and applied to an octyl-Sepharose CL-4B column (a), as described in the Experimental section. The buffers described in the Experimental section were applied at the times indicated in the Figure. Fraction 4 was then applied to a preparative h.p.l.c. DEAE-cellulose column and chromatographed as described in the Experimental section. A profile of the gradient is presented as the broken line in (b).

Table 2. Purification of *P*-450 from acetone-treated chickens

Purification step	Fraction no.	Protein (mg)	Cytochrome <i>P</i> -450 (nmol)	Specific content (nmol/mg of protein)	Yield (%)
Microsome		990	278	0.28	(100)
Octyl-Sepharose column	3	23	9.5	0.42	3
	4	118	211	1.78	76
TSK-DEAE-cellulose anion-exchange column	1	26	45.7	1.76	16.4
	2	1.7	1.25	0.7	0.4
	3	7.2	23.7	3.3	8.5
	4	2.6	2.4	0.9	0.9
	5	3.6	7.9	1.2	2.8
	6	1.2	2.3	1.9	0.8
	7	2.3	4.2	1.8	1.5
	8	4.6	18.8	4.1	6.8
	9	4.8	4.2	0.87	1.5
	10	11.2	1.5	0.13	0.5
Hydroxyapatite column	1	12.3	28.4	2.3	10.2
	3	1.44	11.1	7.7	4.0
	5	0.24	2.5	10.5	0.9
	8	0.62	4.7	7.6	1.7

Fractions 1, 3, 5 and 8, containing most of the cytochrome *P*-450 (Table 2), were further purified on hydroxyapatite columns to remove Emulgen 911. The purities of the final preparations were determined by SDS/PAGE (Fig. 3). Purified fraction 1 contained a major protein of 53 kDa and some minor proteins. Purified fraction 3 contained one protein of 53 kDa. Purified fractions 5 and 8 contained major proteins of 50 kDa; fraction 5 also contained small amounts of a 53 kDa protein (Fig. 3). Fractions 5 and 8 often contained another protein of higher molecular mass (100 kDa), which is probably a dimer of the 50 kDa protein, since limited proteolysis of the 50 kDa and 100 kDa proteins with chymotrypsin and *S. aureus* V8 protease gave identical profiles on SDS/polyacrylamide gels (results not shown).

N-Terminal amino acid sequences of the forms of cytochrome *P*-450 induced by acetone

The *N*-terminal amino acid sequences of the purified proteins are presented in Table 3. Cysteine could not be detected in the *N*-

terminal sequence, since the protein was not alkylated. A drop in stepwise yield is routinely observed after sequencing over unmodified cysteine residues. Therefore an adjacent amino acid would be difficult to detect. The *N*-terminal amino acid sequences of the 50 kDa proteins in fractions 5 and 8 appear to be identical with the sequences derived from the cDNA sequence of two forms of cytochrome *P*-450 induced by phenobarbital in chicken embryos, cytochrome *P*450IIH2 (PCHP7) and cytochrome *P*450IIH1 (PCHP3) respectively (Hansen & May, 1989; Gonzalez, 1989). Purified proteins of 53 kDa from fractions 1, 3 and 4 were distinct from each other and from cytochromes *P*450IIH1 and IIH2.

Reconstitution of enzymic activities

The enzymic activities of reconstituted cytochromes *P*-450 from fractions 1, 3, 5 and 8 were measured, using benzphetamine, *p*-nitrophenol and acetaminophen as substrates (Fig. 4). All enzymic activities were increased when cytochrome *b*₅ was included in the reaction. The greatest increase in activity was

Fig. 2. Immunoblot of fractions isolated from octyl-Sepharose and preparative anion-exchange columns

Cytochrome *P*-450 was purified from the livers of acetone-treated chickens as described in the Experimental section. Fractions 4 from the octyl-Sepharose chromatography and fractions 1–10 from preparative DEAE-cellulose anion-exchange chromatography were subjected to SDS/PAGE and immunoblotted with antibody prepared in rabbits against a cytochrome *P*-450 of 50 kDa purified from glutethimide-treated chicken embryos (P. Sinclair *et al.*, 1989). The details of the immunoblot preparation are as described (P. Sinclair *et al.*, 1989). The following amounts of protein were applied per well: OS₄, octyl-Sepharose (fraction 4), 2 µg; DEAE-cellulose fractions: 1–4, 0.8 µg; 5–8, 0.7 µg; 6, 0.45 µg; 9 and 10, 0.8 µg.

observed with *p*-nitrophenol hydroxylation and acetaminophen activation. All four proteins catalysed the enzymic activities to different extents. Although cytochromes *P*-450 from fractions 5 and 8 had similar benzphetamine demethylase activities, the cytochrome *P*-450 from fraction 5 lacked both acetaminophen-activating and *p*-nitrophenol hydroxylase activity. Cytochrome *P*-450 contained in fraction 1 catalysed both benzphetamine demethylation and *p*-nitrophenol hydroxylation, but was inactive in acetaminophen activation. Cytochrome *P*-450 purified from fraction 3 had the greatest catalytic activity for acetaminophen activation and *p*-nitrophenol hydroxylation, and may therefore be a chicken equivalent of cytochrome *P*450IIE.

Effect of ethanol and glutethimide treatments on h.p.l.c. elution profiles of microsomal cytochromes *P*-450

In Fig. 5 we compare the profiles of cytochrome *P*-450 resolved on analytical anion-exchange h.p.l.c. of solubilized hepatic microsomes from untreated chicken embryos and 12-day-old chickens, glutethimide-treated embryos and ethanol-treated chickens. Fractions 1, 3, 5 and 8 (Fig. 5) corresponded in the elution profile to respective fractions resolved on the preparative anion-exchange h.p.l.c. column (Fig. 1b). Fractions 1, 3, 5 and 8 all contained cytochrome *P*-450, as measured spectrophotometrically. Fraction 9 mainly contained cytochrome *b*₅. Ethanol and glutethimide both induced cytochromes *P*-450 which eluted in

Fig. 3. Purification of acetone-induced cytochromes *P*-450: analysis of purified components by SDS/PAGE

Preparation of the various fractions is described in the Experimental section. Electrophoresis was performed as described and proteins were stained with Coomassie Blue (P. Sinclair *et al.*, 1989). The amounts of cytochrome *P*-450 applied per well were 7 pmol for OS₄ (octyl-Sepharose fraction 4) and for fractions 1, 3, 4, and 8 from the DEAE-cellulose column, repurified on hydroxyapatite, and 3.5 pmol for fraction 5. The markers are (lane M, from top to bottom): bovine serum albumin, 68 kDa, catalase, 57 kDa; glutamic dehydrogenase, 53 kDa; fumarase, 49 kDa; aldolase, 40 kDa; lactate dehydrogenase, 36 kDa.

peaks 5 and 8 (Figs. 5c and 5d). Ethanol, but not glutethimide, also induced the cytochrome *P*-450 induced by acetone which was eluted in fraction 3.

DISCUSSION

In this paper we used a combination of low-pressure and h.p.l.c. chromatography to identify and isolate several forms of hepatic cytochrome *P*-450 induced by acetone in chicken liver. Three forms of cytochrome *P*-450 were not only highly resolved by the h.p.l.c. technique used in this study, but the enzymic activities could be reconstituted after the removal of Emulgen.

Two forms of acetone-induced cytochrome *P*-450 isolated from the preparative DEAE-cellulose column from fractions 5 and 8 are also induced by glutethimide in chicken embryos (Fig. 5). These forms can also be purified to homogeneity from livers of glutethimide-treated embryos by h.p.l.c. methodology identical with that used in this study (results not shown). These data, along with the *N*-terminal amino acid sequences (Table 3), suggest that the 50 kDa proteins from fractions 5 and 8 may be

Table 3. Amino acid sequences of the *N*-termini of acetone-induced cytochromes *P*-450

The *N*-terminal sequences of the cytochromes purified by DEAE-cellulose anion-exchange (fraction 4) and hydroxyapatite chromatography (DEAE fractions 1, 3, 5 and 8) were performed as described in the Experimental section.

Frac- tion no.	Mole- cular mass (kDa)	Amino acid residues																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	53	Met-Leu-Leu-Xaa-Gly-Ala-Ala-Ser-Val-Val-Leu-Leu-Val-Xaa-Val-Ala-Leu-Leu-Asp-Val																							
3	53	Met-Glu-Val-Thr-Ala-Ala-Leu-Leu-Leu-Phe-Leu-Gly-Xaa-Ser-Leu-Val-Val-Leu-Leu-Ala-Val-Arg-Gly-Arg																							
4	53	Met-Glu-Val-Thr-Ala-Ala-Leu-Leu-Leu-Phe-Leu-Gly-Xaa-Xaa-Leu-Val-Val-Leu-Leu-Ala-Xaa-Leu-Gly-Ala																							
5	50	Met-Asp-Phe-Leu-Gly-Leu-Pro-Thr-Ile-Leu-Leu-Leu-Val-Xaa-Ile-Ser-Xaa-Phe-Leu-Ile-Ala																							
8	50	Met-Asp-Phe-Leu-Gly-Leu-Pro-Thr-Ile-Leu-Leu-Leu-Val-Xaa-Ile-Ser-Xaa-Leu-Leu-Xaa-Ala

Fig. 4. Reconstituted enzymic activities of acetone-induced cytochromes *P*-450

(a) Benzphetamine demethylase; (b) *p*-nitrophenol hydroxylase, (c) acetaminophen activation. The forms of cytochrome *P*-450 purified from DEAE-cellulose fractions 1, 3, 5 and 8 were reconstituted as described in the Experimental section. Fractions 5 and 8 were isolated from one purification and 1 and 3 from separate purifications. Reactions in the absence (□) and presence (▨) of cytochrome b_5 are shown. Each value represents the mean and range of duplicate determinations.

identical to cytochromes *P*450IIH2 and IIH1 respectively (Hansen & May, 1989).

Although May and coworkers isolated and sequenced the cDNAs for cytochromes *P*450IIH1 and IIH2, only the holoprotein of cytochrome *P*450IIH1 was isolated (Hobbs *et al.*, 1986). We may have now isolated both proteins, and have shown that they can be distinguished enzymically (Fig. 4), even though the difference in their amino acid sequences is only 8% (Hansen & May, 1989). Two major forms of cytochrome *P*-450 induced by phenobarbital in rats, i.e. cytochromes *P*450IIB1 and IIB2, that show only a 3% difference in their amino acid sequences, can also be distinguished enzymically (Wolf *et al.*, 1988).

The 53 kDa protein present in DEAE-cellulose fraction 1 is

Fig. 5. Resolution of chick hepatic cytochromes *P*-450 by analytical anion-exchange h.p.l.c.

The treatments and solubilization of the microsomes are presented in the Experimental section. A portion of the supernatant containing the amounts of cytochrome *P*-450 and protein injected are listed below. The gradient, whose profile is shown by the broken line in panel (c), consisted of two buffers: buffer D [10 mM-Tris/acetate (pH 7.5)/20% (v/v) glycerol/0.4% (v/v) Emulgen 911]; and buffer E (buffer D containing 0.8 mM-sodium acetate). (a) Untreated embryos: 1 mg of protein, 300 pmol of cytochrome *P*-450; (b) untreated chickens: 1.2 mg of protein, 220 pmol of cytochrome *P*-450; (c) glutethimide-treated embryos: 0.5 mg of protein, 650 pmol of *P*-450; (d) ethanol-treated chickens: 1.2 mg of protein, 600 pmol of *P*-450.

different from the other three forms of cytochrome *P*-450 isolated, as shown by its enzymic activity and by the amino acid sequence of its *N*-terminus. This protein is highly hydrophobic and may prove similar to the novel form of cytochrome *P*-450 isolated recently by Juvonen and coworkers from pyrazole-treated mice (Honkakoski *et al.*, 1988). However, this 53 kDa protein may prove identical to the cytochrome *P*-450 of 56 kDa and 54 kDa isolated from phenobarbital-treated roosters (Oron & Bar-Nun, 1984) and chicken embryos (Ades, 1983), and to the glutethimide-induced 53 kDa protein reported by our laboratory (P. Sinclair *et al.*, 1989).

The form of cytochrome *P*-450 isolated as a 53 kDa protein from DEAE-cellulose fraction 3 is enzymically similar to mammalian cytochrome *P*450IIE, since it is highly active in the hydroxylation of *p*-nitrophenol and in the conversion of acetaminophen to a metabolite that binds to glutathione. Despite the similarity in enzyme activities of chicken cytochrome *P*-450 isolated from DEAE-cellulose fraction 3 and of mammalian cytochrome *P*450IIE, the *N*-terminal regions of fraction 3 (Table 3) had only 30–33% similarity to mammalian cytochrome *P*450IIE isolated from rat, human (Song *et al.*, 1986) or rabbit (Khani *et al.*, 1987) liver. Other related forms of cytochrome *P*-

450 isolated from different species have also been shown to have similar catalytic activities but different *N*-terminal sequences. For example, the *N*-terminal sequence of human cytochrome *P*450IA2 has only 30% similarity with that of mammalian cytochrome *P*450IA2, yet the catalytic activities are almost identical (Raucy *et al.*, 1989).

Another form of 53 kDa cytochrome *P*-450, present in fraction 4, was isolated from acetone-treated chickens and has an *N*-terminal amino acid sequence at least 80% similar to that of the protein in fraction 3. In addition, antibodies prepared against the 53 kDa protein in fraction 3 also react with the 53 kDa protein in fraction 4, but not with the other proteins isolated (results not shown). This protein may prove similar to rabbit *P*450IIE2, an ethanol-induced cytochrome *P*-450 that is 97% identical to rabbit cytochrome *P*450IIE1 (Khani *et al.*, 1988).

The pattern of acetone-mediated induction of cytochrome(s) *P*-450 in chickens appears similar to that observed in rats (Song *et al.*, 1986), namely that two forms are induced which are also the major phenobarbital-induced forms, and a separate form is induced which seems enzymically equivalent to mammalian *P*450IIE. An intriguing question arises as to why these responses are conserved when the actual amino acid sequences of cytochrome *P*-450 are not highly conserved between chickens and mammals. The results suggest that the induction processes for these separate forms of cytochrome *P*-450 are conserved in these different species.

In summary, we have isolated four distinct forms of cytochrome *P*-450 induced by acetone in chickens. One form does not correspond to any mammalian cytochrome *P*-450 reported to be induced by acetone or ethanol. Two appear to be identical to major forms also induced by phenobarbital, and one appears to be equivalent to mammalian cytochrome *P*450IIE.

Note added in proof (received 14 March 1990)

Gupta *et al.* (1990) have recently purified two forms of cytochrome *P*-450 from phenobarbital-treated hens that have the same *N*-terminal sequences as the cytochromes *P*-450 from fractions 1 and 3 in this study.

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