Formation of cytochrome *P*-450 containing haem or cobalt-protoporphyrin in liver homogenates of rats treated with phenobarbital and allylisopropylacetamide

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The potent porphyrogen allylisopropylacetamide and related compounds decrease hepatic concentrations of cytochrome *P*-450. This decrease occurs particularly in phenobarbital-induced cytochrome *P*-450 and is caused by suicidal breakdown of the haem of cytochrome *P*-450. Quantitative rocket immunoelectrophoresis showed that the protein moiety of the major phenobarbital-inducible form of hepatic cytochrome *P*-450 was not diminished up to 1 h, but was markedly decreased (to 43% of that of the phenobarbital-treated control) at 20 h after allylisopropylacetamide treatment. In contrast, the concentration of total cytochrome *P*-450, measured spectrophotometrically, decreased to 30–40% of the control at both 1 and 20 h after allylisopropylacetamide. Cytochrome *P*-450-dependent demethylations of ethylmorphine and benzphetamine decreased to a similar extent. When liver homogenates from rats treated with allylisopropylacetamide 1 h before being killed were incubated with haem, functional holocytochrome *P*-450 could be reconstituted from the apoprotein. Incubation with haem increased spectrophotometrically measurable cytochrome *P*-450 to 69%, ethylmorphine demethylase to 64% and benzphetamine demethylase to 93% of the activities in rats treated with phenobarbital alone. At 20 h after allylisopropylacetamide treatment, however, little or no reconstitution of cytochrome *P*-450 occurred after incubation with haem. When liver homogenates were incubated with cobalt and protoporphyrin, and microsomal proteins were then subjected to polyacrylamide-gel electrophoresis, cobalt-protoporphyrin was found specifically associated with proteins of M_r 50 000–53 000. When homogenates from rats given allylisopropylacetamide for 1 h or 20 h were compared, it was found that the extent of this association was higher in livers from the rats containing more apocytochrome *P*-450, suggesting that cobalt-protoporphyrin had associated with the apocytochrome. The data provide insight into the association of haem with the protein moiety of cytochrome *P*-450 and factors affecting breakdown of this protein.

Cytochrome(s) *P*-450 are composed of a protein moiety and a prosthetic haem group. The mechanism by which haem and the protein moiety associate remains imperfectly understood. Correia & Meyer (1975) presented evidence that exogenous haem could be incorporated into apo-cytochrome *P*-450 in liver homogenates from rats pretreated with phenobarbital and CoCl_2 , a metal salt known to decrease cytochrome *P*-450 (for reviews, see

Abbreviation used: AIA, 2-allyl-2-isopropylacetamide.

Maines & Kappas, 1977; Tephly, 1978). However, the degree of restoration by haem of cytochrome *P*-450 and of *P*-450-associated mixed-function oxidase activities was small (only 15–25%). We showed that cobalt-protoporphyrin, which is formed in livers of rats treated with CoCl_2 (Sinclair *et al.*, 1979), associates with apo-cytochrome *P*-450 at the expense of haem (Sinclair *et al.*, 1982). This occupation of the haem site by cobalt-protoporphyrin probably prevented further haem restoration in the study by Correia & Meyer (1975).

AIA is one of several olefins that cause breakdown of haem from cytochrome *P*-450 to green *N*-alkylated protoporphyrin by a process termed 'suicidal inactivation' [for reviews, see De Matteis *et al.* (1980) and Ortiz de Montellano & Correia (1983)]. The major form of cytochrome *P*-450 induced by phenobarbital in rat liver (cytochrome *P*-450 PB-4) is particularly susceptible to suicidal inactivation by AIA (Waxman & Walsh, 1982). Reconstitution of cytochrome *P*-450 with haem has been studied in livers of rats pretreated with phenobarbital and AIA (Bhat *et al.*, 1977; Unseld & De Matteis, 1978; Correia *et al.*, 1979; Farrell *et al.*, 1979; Farrell & Correia, 1980; Correia *et al.*, 1981). Compared with reconstitution produced by administration of haem *in vivo* (Farrell & Correia, 1980), the degree of reconstitution by incubation of haem with liver homogenates *in vitro* has previously been small (Correia & Meyer, 1975; Correia *et al.*, 1981). In addition, no evidence has been presented for reconstitution of mixed-function oxidase activity after incubation with haem *in vitro*.

In the studies reported here, we have quantified, by rocket immunoelectrophoresis, the protein moiety of the major phenobarbital-inducible form of cytochrome *P*-450 at various times after AIA administration, and we have investigated the association of the apo-cytochrome with the protoporphyrin chelates of iron (haem), cobalt and zinc. Our results show that, up to 1 h after AIA administration, the protein moiety was not decreased and could associate with haem *in vitro* to form functional holo-cytochrome *P*-450. However, 20 h after AIA treatment, the protein moiety decreased by 57% and little or no increase in holo-cytochrome occurred after incubation with haem. We have investigated conditions for reconstitution of functional holo-cytochrome *P*-450 *in vitro* and have found that cobalt-protoporphyrin, but not zinc-protoporphyrin, binds tightly to apo-cytochrome *P*-450.

Portions of this work have appeared in abstract form (Bonkovsky *et al.*, 1982a,b).

Methods

Chemicals

Dimethyl sulphoxide, haemin (type III), dithiothreitol, NADPH, isocitrate and isocitrate dehydrogenase were purchased from Sigma, St. Louis, MO, U.S.A. Phenobarbital (Na⁺ form) was purchased from Eli Lilly and Co., Indianapolis, IN, U.S.A. AIA was generously provided by Hoffmann-LaRoche, Nutley, NJ, U.S.A. Protoporphyrin IX, cobalt-protoporphyrin IX and zinc-protoporphyrin IX were obtained from Porphyrin Products, Logan, UT, U.S.A.; lithium dodecyl

sulphate was from BDH Chemicals, Poole, Dorset, U.K.; 3,3',5,5'-tetramethylbenzidine and butylated hydroxytoluene were from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Acrylamide, *NN'*-methylenebisacrylamide and *NNN'*-tetramethylethylenediamine were from Eastman Kodak Co., Rochester, NY, U.S.A. Ethylmorphine and benzphetamine were gifts from Upjohn Pharmaceutical, Kalamazoo, MI, U.S.A. The major phenobarbital-inducible form of hepatic cytochrome *P*-450 was purified from livers of Sprague-Dawley rats (Gasser, 1983). This protein and rabbit polyclonal antibody to the purified cytochrome *P*-450 were generously provided by Dr. R. Gasser and Dr. U.A. Meyer (Department of Pharmacology, University of Basel, Basel, Switzerland).

Treatment of rats

Male Sprague-Dawley rats, weighing 180–250 g and housed as described previously (Bonkovsky *et al.*, 1981), were treated as follows, unless indicated otherwise: phenobarbital (16 mg/ml, dissolved in 0.15 M-NaCl) was administered in two intraperitoneal injections (80 mg/kg body wt.) at 64 h and 40 h before the rats were killed. AIA (15 mg/ml, dissolved in 0.15 M-NaCl) was administered subcutaneously at a dose of 400 mg/kg either 1 h or 20 h before the rats were killed. All animals were starved for 40 h before being killed. Controls in each treatment received appropriate volumes of 0.15 M-NaCl.

Preparation of rat liver homogenates and incubation with metalloprophyrins

Rats were killed by decapitation. Livers were removed, rinsed with and placed in ice-cold 0.15 M-NaCl. Individual livers were then minced and homogenized in 10 ml of 0.25 M-sucrose/0.02 M-Tris/HCl buffer (pH 7.4) with four complete strokes of a Potter-Elvehjem homogenizer. Homogenates were diluted to 20% (w/v) with the sucrose/Tris buffer, and butylated hydroxytoluene (10 mg/ml in ethanol) was added (final concn. 0.002%, w/v). To this homogenate was added dithiothreitol (final concn. 10 mM) and one of the following: (i) haemin (stock solution, 1.31 mg/ml in dimethyl sulphoxide), final concn. 20 μ M; (ii) CoCl₂, final concn. 100 μ M, and protoporphyrin IX (stock solution, 7 mg/ml dissolved in dimethyl sulphoxide), final concn. 125 μ M; or (iii) ZnSO₄, final concn. 100 μ M, and protoporphyrin IX, final concn. 50 μ M. The final concentration of dimethyl sulphoxide in all homogenates was 1% (v/v). Samples were routinely incubated in a shaking water bath for 30 min at 37°C. Before measurement of cytochrome *P*-450 and demethylase activities in homogenates, the samples were further disrupted

with a Brinckmann Polytron apparatus at low speed (15s, 0°C).

We took advantage of endogenous homogenate ferrochelatase activity to form the protoporphyrin chelates of cobalt and zinc, because preliminary studies had shown that commercial cobalt-protoporphyrin incubated with homogenates from rats treated with phenobarbital and AIA (1h before they were killed) did not associate with cytochrome *P*-450. This is probably because the commercial cobalt-protoporphyrin contains liganded pyridine (J. Bommer, personal communication) which blocks insertion into the haem pocket of cytochrome *P*-450.

Preparation of microsomes

Microsomes (microsomal fractions) were usually prepared from rat liver homogenates by the calcium precipitation method, as previously described (Sinclair *et al.*, 1981). In experiments involving zinc-protoporphyrin, however, microsomes were prepared by ultracentrifugation, because the Ca^{2+} -containing buffer interfered with the assay for zinc-protoporphyrin.

Assays

Cytochrome *P*-450. Cytochrome *P*-450 was measured in microsomes by the method of Omura & Sato (1964) and in liver homogenates by a modification of the procedure of Estabrook *et al.*, (1972) with an Aminco DW-2 spectrophotometer. In samples incubated with haem, the presence of dithiothreitol in the incubation decreased or eliminated an absorbance peak at 420nm, without affecting the measurement of cytochrome *P*-450 (Fig. 1).

Demethylases. Reaction mixtures routinely contained 1mM-NADPH, 5mM-isocitrate, 5mM substrate, 1 unit of isocitrate dehydrogenase/ml and 0.3–0.6mg of liver homogenate protein in a total volume of 0.45ml of 0.1M-sodium phosphate buffer (pH 7.4). Duplicate tubes were incubated in the dark in a shaking water bath (120 oscillations/min) for 20min at 37°C. The reaction was terminated by transferring the tubes to an ice/water bath and adding 150 μl of trichloroacetic acid (20%, w/v). The amount of formaldehyde generated was determined (Nash, 1953). Liver homogenates could be stored frozen (–20°C) for at least 3 months with no loss in demethylase activities or concentrations of cytochrome *P*-450. Homogenates rather than microsomes were used for most experiments, to avoid different recoveries of total protein or cytochrome *P*-450 (McLean & Day, 1974). In some experiments, demethylase assays were performed with isolated microsomes. The pattern of results was similar to that obtained with homogenates.

Fig. 1. Effects of haem and dithiothreitol on the cytochrome *P*-450 spectrum of liver homogenate from a rat treated with phenobarbital and AIA

A rat pretreated with phenobarbital was given AIA 1h before being killed. Liver homogenate was prepared and portions incubated with dimethyl sulphoxide alone, or haem (20 μM final concn.) dissolved in dimethyl sulphoxide, each with and without dithiothreitol (DTT; 10mM) (see the Methods section). The homogenate was bubbled with CO and divided between two cuvettes, and the contents of the sample cuvette were reduced by addition of a few crystals of $\text{Na}_2\text{S}_2\text{O}_4$. Difference spectra of cytochrome *P*-450 were performed as previously described (Bonkowsky *et al.*, 1981).

Iron-, cobalt- and zinc-protoporphyrin. Haem and cobalt-protoporphyrin were measured spectrophotometrically in homogenates and microsomes as previously described (Sinclair *et al.*, 1982). Zinc-protoporphyrin was measured fluorimetrically in ethanol extracts of homogenates or microsomal suspensions (Bloomer *et al.*, 1983) as follows: 100 μl of 10% (w/v) homogenate (or 100 μl of microsomal suspension at approx. 5mg of protein/ml) were mixed with 400 μl of ethanol, vortex-mixed and centrifuged (1000g for 10min).

A fluorescence emission spectrum of the supernatant was recorded (excitation, 415 nm; emission scanned from 550 to 650 nm) with a Perkin-Elmer 512 recording spectrofluorimeter equipped with a Hamamatsu R928 red-sensitive photo-tube. When zinc-protoporphyrin was present, a peak was observed at 585 nm, clearly separated from the protoporphyrin peak at 630 nm. The concentration of zinc-protoporphyrin present was measured by the fluorescence at 585 nm, by using zinc-protoporphyrin standards.

In preliminary studies of rats pretreated with phenobarbital, we found that subsequent treatment of rats with AIA 20 h before being killed did not affect the amount of metallo-protoporphyrins formed by liver homogenates incubated with protoporphyrin IX and cobalt or zinc.

Quantification of major phenobarbital-inducible apo-cytochrome P-450

Rocket immunoelectrophoresis was performed as described (Pickett *et al.*, 1980; Gasser *et al.*, 1982) by using microsomes that had been washed with 5 ml of 0.1 M-sodium phosphate (pH 7.4)/10% (w/v) glycerol/0.05% (w/v) NaN₃ and sedimented by centrifugation (105 000 g for 120 min).

Polyacrylamide-gel electrophoresis of microsomal proteins

Microsomal proteins were electrophoresed on polyacrylamide slab gels with lithium dodecyl sulphate instead of the sodium salt to improve retention of haem or cobalt-protoporphyrin with haemoproteins (Sinclair *et al.*, 1982). Samples were applied to three adjacent tracks. After electrophoresis, one track was stained to locate haem and protein (Thomas *et al.*, 1976). The remaining two adjacent tracks were sliced into 0.5 cm × 1 cm × 0.25 cm pieces and were analysed spectrophotometrically for cobalt-protoporphyrin and haem as described previously (Sinclair *et al.*, 1982). Zinc-protoporphyrin was measured in gel slices by homogenizing each slice in 1.5 ml of dimethyl sulphoxide and reading the fluorescence at 590 nm (excitation at 420 nm), by comparing the results with a suitable standard curve. (Peak excitation and emission wavelengths of zinc-protoporphyrin standard dissolved in dimethyl sulphoxide were 420 and 590 nm respectively, and were unaffected by the presence of polyacrylamide.)

Statistics

Means were compared by analysis of variance procedures and the *F* statistic (Dixon & Massey, 1968).

Results

Electrophoretic analysis of hepatic microsomal proteins after treatment of rats with 2-allyl-2-isopropylacetamide

Previous workers reported that phenobarbital-induced apo-cytochrome *P*-450 is unaffected after treatment of rats *in vivo* with AIA for 1 h, but is decreased after exposure for 5–20 h (Baird *et al.*, 1976; Ivanetich & Bradshaw, 1977; Bhat *et al.*, 1977; Sinclair *et al.*, 1981). Fig. 2 shows the result of a typical experiment in which the system of lithium dodecyl sulphate/polyacrylamide-gel electrophoresis was used to improve the retention of haem with cytochrome *P*-450 (Sinclair *et al.*, 1981). Treatment of rats with AIA for 20 h led to a decrease in microsomal protein(s) of *M*_r 50 000–53 000 (Fig. 2*a*, cf. lanes 3 and 4; see the arrow). The haem associated with the protein(s) (Fig. 2*b*, cf. lanes 3 and 4; see the arrow) was also decreased. (We intentionally overloaded the gel in order to have sufficient haem to detect in the gel samples from rats treated with AIA for 20 h.) In contrast, 1 h after AIA, there was no apparent decrease in microsomal proteins of *M*_r 50 000–53 000 (Fig. 2*a*, cf. lanes 1 and 2). There was a decrease in the amount of haem at the front of the gel after treatment with AIA for 1 h (Fig. 2*b*, cf. lanes 2 and 1); however, there was no decrease in the haem associated with protein(s) of *M*_r 50 000–53 000, suggesting that haem re-associated with apo-cytochrome *P*-450 during electrophoresis. In agreement with this suggestion, in rats exposed to AIA for 20 h, incubation of liver homogenates with haem did not change the electrophoretic pattern of microsomal proteins, or increase haem association with the *M*_r 50 000–53 000 protein(s), but it did increase the amount of haem at the front of the gel (Fig. 2, cf. lanes 3 and 3+H).

Immunological measurement of the protein moiety of the major phenobarbital-induced form of cytochrome P-450

Identification and quantification of the protein moiety of cytochrome *P*-450 necessitate the use of antibody prepared against the purified protein. Accordingly, we performed rocket immunoelectrophoresis of solubilized microsomal proteins, by using a precipitating polyclonal antibody raised in rabbits against the major form of cytochrome *P*-450 induced in rat liver by phenobarbital (Fig. 3).

In this experiment the same amount of spectrophotometrically measured cytochrome *P*-450 (holo-cytochrome *P*-450) was placed into each of the test wells. The results show that samples from rats exposed to AIA for only 1 h had more apo-cytochrome *P*-450 than did rats not exposed to AIA (Fig. 3, cf. wells 8, 10 and 12 with well 6).

(a) Stain for protein

(b) Stain for haem

Fig. 2. Effect of AIA treatment and haem incubations on microsomal haemoproteins of rats pretreated with phenobarbital

Rats were pretreated with phenobarbital. Some were then treated with AIA for 1 or 20h before being killed. Homogenates were prepared and a portion of each was incubated with haem. Microsomes were prepared and solubilized, and their proteins electrophoresed on lithium dodecyl sulphate/polyacrylamide slab gels. The gels were stained and photographed (Sinclair *et al.*, 1982). The arrows indicate M_r 50000. Lanes 1 and 4 are microsomal proteins from rats pretreated with phenobarbital (PB) alone; lane 2, proteins from rats pretreated with phenobarbital and given AIA 1h before being killed; lane 3, proteins from rats pretreated with phenobarbital and given AIA 20h before killing. Lanes designated '+ H' refer to microsomal proteins from homogenates incubated with haem before microsomal isolation. M_r markers (track on the far left in *a*) include: bovine serum albumin (68000), catalase (60000), fumarase (49000), aldolase (40000), lactate dehydrogenase (36000).

Thus, in rats exposed to AIA for 1h, hepatic microsomes contained similar amounts of the protein moiety of cytochrome *P*-450 to microsomes from rats not exposed to AIA, and only spectrophotometrically measurable cytochrome *P*-450 was decreased because of loss of haem. For rats killed

Fig. 3. Rocket-immunoelectrophoretic assay of the major phenobarbital-inducible form of cytochrome *P*-450

Wells 1-5 (from left to right) contained decreasing amounts of the major phenobarbital-inducible cytochrome *P*-450, purified from rat liver (16.8, 8.4, 4.2, 2.9 and 1.1 pmol/well, respectively). The concentration of the purified cytochrome *P*-450 standard was calculated from its protein concentration and from M_r 50000. Wells 6-13 contained the same amount (7 pmol) of total spectrophotometrically measured cytochrome *P*-450. All rats were pretreated with phenobarbital; additional rat treatments were as follows: wells 6 and 7, none; wells 8 and 9, AIA 30min before killing; wells 10 and 11, AIA 60min before killing; wells 12 and 13, AIA 20h before killing. Wells 7, 9, 11 and 13 contained microsomal proteins prepared from homogenates incubated with haem.

0.5 or 1h after AIA treatment, incubation of liver homogenate with haem decreased the amount of the apo-cytochrome needed to obtain the same amount of holo-cytochrome *P*-450 (Fig. 3, cf. wells 8 and 9 and wells 10 and 11). Similar results were obtained 2h after AIA treatment (results not shown). This haem effect was, however, not seen in homogenates from rats treated with AIA 20h, before being killed (Fig. 3, cf. wells 12 and 13). Overall, these results indicate that in homogenates from rats treated with AIA for 0.5 or 1h, but not for 20h, before being killed, holo-cytochrome *P*-450 was restored by incubation with haem.

Table 1 summarizes results from several rocket-immunoelectrophoresis experiments of the type just described. The data show no significant decrease in the concentration of the protein moiety of the major phenobarbital-inducible apo-cytochrome *P*-450 either 0.5 or 1h after AIA, but a highly significant decrease 20h after AIA. Incubation of homogenates with haem had no effect on the concentration of the protein moiety, but significantly increased ($P < 0.01$) holo-cytochrome

Table 1. *Effect of AIA in vivo and of haem in vitro on microsomal concentrations of cytochrome P-450 determined immunologically or spectrophotometrically*

As described in the Methods section, rats were treated, homogenates of livers were prepared and incubated, microsomes were prepared, and total cytochrome *P*-450 was measured by difference spectroscopy and the immunoreactive major form of cytochrome *P*-450 induced by phenobarbital was measured by rocket immunoelectrophoresis. Results are means $\pm$ S.D. for three animals: ^a*P* < 0.005, ^b*P* < 0.01, compared with phenobarbital alone.

Nature of incubation of homogenates ...	Cytochrome <i>P</i> -450 (nmol/mg of protein)			
	No haem		With haem	
	Immuno-reactive	Spectrally measured	Immuno-reactive	Spectrally measured
Treatment				
Phenobarbital alone	1.29 $\pm$ 0.06	1.75 $\pm$ 0.13	1.08 $\pm$ 0.24	1.77 $\pm$ 0.12
Phenobarbital, then AIA 0.5h before killing	1.11 $\pm$ 0.25	0.84 $\pm$ 0.02 ^b	1.16 $\pm$ 0.11	1.34 $\pm$ 0.14 ^b
Phenobarbital, then AIA 1h before killing	1.07 $\pm$ 0.03	0.62 $\pm$ 0.02 ^b	1.13 $\pm$ 0.16	1.09 $\pm$ 0.11 ^b
Phenobarbital, then AIA 20h before killing	0.56 $\pm$ 0.06 ^a	0.46 $\pm$ 0.04 ^a	0.63 $\pm$ 0.15 ^a	0.48 $\pm$ 0.05 ^a

P-450 concentrations at 0.5 or 1 h, though not at 20h, after AIA treatment. These results establish unequivocally the conclusions suggested by polyacrylamide-gel electrophoresis (Fig. 2; Ivanetich & Bradshaw, 1977; Sinclair *et al.*, 1981) that, although a single injection of AIA rapidly decreases the haem component of cytochrome *P*-450, the protein moiety does not decrease until later.

Haem-dependent reconstitution of functional holo-cytochrome P-450

We studied the effect of varying the haem concentrations on the reconstitution of cytochrome *P*-450. The extent of restoration was nearly maximal with 20 μ M-haem; use of 40 μ M-haem led to little change in the amount of spectrophotometrically measured cytochrome *P*-450, but to a large increase in an absorbance peak at 420 nm. At 37°C, the haem-dependent reconstitution was nearly maximal in 30 min; only slight additional increases were observed up to 250 min. At 0°C, some reconstitution (to 40–50% of phenobarbital-induced values) occurred during this time period. Addition of ATP (1 mM) had no effect. As mentioned above (the Methods section and Fig. 1), addition of dithiothreitol decreased the absorbance peak at 420 nm. Therefore, for haem-dependent reconstitution of cytochrome *P*-450, we routinely incubated homogenates at 37°C for 30 min in the presence of haem (20 μ M) and dithiothreitol (10 mM).

These conditions were applied to liver homogenates from rats pretreated with phenobarbital and then exposed to AIA for various times up to 20h. Fig. 4 shows that, as early as 15 min after administration of AIA, there was a decrease in both cytochrome *P*-450 and the associated benzphetamine demethylase activity. After a 20h

exposure to AIA the decreases in cytochrome *P*-450 and associated demethylation of either benzphetamine or ethylmorphine were not greater than after a 1h exposure (60–80%; Table 2). Incubation with haem of liver homogenates from rats treated with AIA for 1h resulted in restoration of both spectrophotometrically measured cytochrome *P*-450 and of ethylmorphine demethylase activity, to 60–70% of the values from rats treated with phenobarbital alone. Even as early as 15 min after administration of AIA, haem-dependent restoration of spectrally measured cytochrome *P*-450 was incomplete (Fig. 4). In contrast, at times up to 1h, benzphetamine demethylase activity was almost completely restored. In four separate experiments of 1h exposure to AIA, the average reconstitution ($\pm$ S.D.) by haem was 69 $\pm$ 4% for total spectrophotometrically measured cytochrome *P*-450, 64 $\pm$ 8% for ethylmorphine demethylase activity, and 93 $\pm$ 11% for benzphetamine demethylase activity. These numbers are percentages of values for rats treated with phenobarbital alone.

In contrast, addition of haem to homogenates from rats treated with AIA for 20h resulted in no significant increase in total cytochrome *P*-450 (Table 2). Although there was a 2-fold increase in enzymic activity compared with identical homogenates incubated without haem, activities were restored to only 30–40% of the values with phenobarbital alone.

Incorporation of cobalt-protoporphyrin or zinc-protoporphyrin into apo-cytochrome P-450

Previously we reported that cobalt-protoporphyrin, formed in rats treated with phenobarbital, CoCl₂ and 5-aminolaevulinate, is associated with hepatic microsomal protein(s) of *M_r* 50000–53000, suggesting combination of cobalt-protoporphyrin

Fig. 4. Early time course of AIA-mediated destruction of cytochrome P-450: restoration of cytochrome P-450 and benzphetamine demethylase activity by haem

Rats were pretreated with phenobarbital (PB) as described in the Methods section. As indicated, rats were given AIA 15, 30 or 60 min before being killed, after which liver homogenates were prepared and incubated (37°C, 30 min) either with dimethyl sulphoxide alone or with 20 µM-haem dissolved in dimethyl sulphoxide. Concentrations of total cytochrome P-450 and activities of benzphetamine demethylase were then measured (see the Methods section). Results are means ± S.D.; n = 3–4 for each treatment group.

with apo-cytochrome P-450 (Sinclair *et al.*, 1982). Since we have now demonstrated that apo-cytochrome P-450 can be generated after exposure of phenobarbital-treated rats to AIA for 1 h, we investigated whether cobalt-protoporphyrin could associate with the apoprotein generated in this way. We also tested zinc-protoporphyrin, another metalloporphyrin that can be readily formed in the liver by action of ferrochelatase (Johnson & Jones, 1964; Mazanowska *et al.*, 1966) when large amounts of zinc are available (Bloomer *et al.*, 1983).

Polyacrylamide-gel electrophoresis (Sinclair *et al.*, 1981) was used to investigate whether cobalt- or zinc-protoporphyrin, generated in such homogenates, combined with apocytochrome P-450. Results from a typical experiment are shown in Fig. 5. In microsomes of rats that had been exposed to AIA for only 1 h, 54% of the total cobalt-protoporphyrin recovered from the gel was associated with the microsomal proteins of *M*_r50 000–53 000 (Fig. 5a). In contrast, only 27% of recovered cobalt-protoporphyrin was associated with these proteins in rats that had been exposed to AIA for 20 h (Fig. 5b). Microsomal protein(s) of *M*_r50 000–53 000 from untreated rats or from rats treated with phenobarbital alone also contained only 20–25% of recovered cobalt-protoporphyrin (results not shown).

In contrast with the results for cobalt-protoporphyrin or haem, in similar experiments in which zinc-protoporphyrin was generated, the zinc-protoporphyrin was found only at the gel front. Thus we were unable to demonstrate an association of zinc-protoporphyrin with apo-cytochrome P-450.

Discussion

Previous workers have suggested that exposure of phenobarbital-pretreated rats to AIA for less than 2 h results in a rapid destruction of the haem, but not the protein moiety, of cytochrome P-450

Table 2. Restoration by haem of cytochrome P-450 and cytochrome P-450-catalysed activities

All rats were treated with phenobarbital, and some also received AIA (see the Methods section). Liver homogenates were prepared, incubated without (– haem) or with (+ haem) haem (20 µM) and assayed as described in the Methods section. Results are means ± S.D. for three or four rats.

Period of AIA exposure	Cytochrome P-450 (nmol/mg of protein)		Demethylase activities (nmol of formaldehyde/20 min per mg of protein)			
			Ethylmorphine		Benzphetamine	
	– Haem	+ Haem	– Haem	+ Haem	– Haem	+ Haem
None	0.57 ± 0.04	0.60 ± 0.06	230 ± 20	240 ± 40	150 ± 10	160 ± 7
1 h	0.22 ± 0.05	0.40 ± 0.08	60 ± 20	140 ± 10	50 ± 20	140 ± 10
20 h	0.14 ± 0.02	0.18 ± 0.03	40 ± 10	90 ± 20	40 ± 10	70 ± 10

Fig. 5. Association of cobalt-protoporphyrin with microsomal proteins from rats treated with AIA

Rats pretreated with phenobarbital were given AIA 1 h (a) or 20 h (b) before being killed. As described in the Methods, section, liver homogenates were incubated with CoCl_2 and protoporphyrin; microsomes were isolated and solubilized, and proteins electrophoresed in a lithium dodecyl sulphate/polyacrylamide gel. Gels were stained for haem and protein; separate unstained tracks were sliced and analysed for cobalt-protoporphyrin. The solid arrow indicates M_r 50 000, and the broken arrow indicates the protein and dye front. The gel photographs are mounted such that migration (towards the anode) was from left to right.

(Bhat *et al.*, 1977; Ivanetich & Bradshaw, 1977). A decrease in the protein moiety appeared to occur only at 5 h (Baird *et al.*, 1976) or 12 h (Bhat *et al.*, 1977) after AIA. These interpretations were based on amounts of microsomal proteins, detected on polyacrylamide gels, of M_r values similar to those reported for purified cytochrome(s) *P*-450. In confirmation, we found that microsomal protein(s) of M_r 50 000–53 000 were decreased at 20 h, but not at 1 h, after injection of AIA (Fig. 2). In addition, by use of an antibody to the major phenobarbital-inducible form of cytochrome *P*-450, we demonstrated unequivocally that, in livers from phenobarbital-pretreated rats exposed to AIA, the protein moiety was decreased at 20 h, but not at 1 h, after exposure to AIA, even though the holo-cytochrome was decreased to a similar extent at both times (Table 1 and Fig. 3).

Our results on restoration by haem of spectrophotometrically measurable holocytochrome *P*-450 and of *P*-450-dependent demethylations (Tables 1 and 2, Fig. 4) further support the conclusion that, in the rat, more apoprotein was present at 1 h than at 20 h after treatment with AIA. The extent of reconstitution of total spectrophotometrically measured cytochrome *P*-450 *in vitro* at early times after AIA administration *in vivo* was 3-fold greater than that observed previously (Correia *et al.*, 1981). The greater reconstitution obtained here may be due to the 5-fold higher concentrations of haem in our incubations (20 μM rather than 4 μM). The large extent of reconstitution with exogenous haem suggests there is not an obligatory role for endogenously synthesized haem, complexes of mitochondria with rough endoplasmic reticulum (Meier *et al.*, 1978, 1981; Pickett *et al.*, 1981) or intact endoplasmic reticulum to generate holocytochrome *P*-450.

Under optimal conditions of haem reconstitution, activity of benzphetamine demethylase could be increased significantly more (93%) than that of ethylmorphine demethylase (64%) (Table 2, Fig. 4). Perhaps there are differences in the forms of phenobarbital-inducible cytochrome *P*-450 that catalyse demethylation of ethylmorphine and benzphetamine. The form(s) mainly responsible for demethylation of benzphetamine may be more reconstitutable by incubation with haem *in vitro*. Differences in susceptibility to attack by AIA and in mixed-function oxidase activities by various phenobarbital-inducible forms of cytochrome *P*-450 have been reported (Waxman & Walsh, 1982).

Although the extent of haem-dependent reconstitution of spectrophotometrically measured cytochrome *P*-450 was substantially above those previously reported (Correia & Meyer, 1975; Correia *et al.*, 1981), the reconstitution was still incomplete. We do not know why only partial reconstitution was achieved. Possible explanations include the following: (1) *N*-alkyl-porphyrin, formed by AIA and cytochrome *P*-450 haem, may remain bound to the apoprotein, maintaining the native protein structure; added free haem may not be able to replace the *N*-alkyl-porphyrin completely; (2) *N*-alkyl-porphyrin may be released completely, followed by a conformational change around the binding site that inhibits complete reconstitution; (3) Some form of the microsomal vesicles may be inaccessible to added haem; (4) some species of rat apo-cytochrome *P*-450, other than the major phenobarbital-inducible form, may be broken down very rapidly after AIA attack. This possibility seems unlikely, because incomplete reconstitution occurred as early as 15 min after AIA treatment (Fig. 4). It is not yet possible to decide which if any of these explanations is correct.

In contrast with our results with rats, in phenobarbital-treated rabbits the protein moiety of the major phenobarbital-inducible form of cytochrome *P*-450 was decreased as early as 1 h after AIA treatment (Liem *et al.*, 1983). This suggests a difference between rats and rabbits in the rate at which the apo-cytochrome is broken down after attack on the haem moiety by AIA. Since, in the study of Liem *et al.* (1983), the amounts of apoprotein were determined only by immunoprecipitation, an alternative possibility is that 1 h exposure to AIA only decreased the immunoreactivity of the rabbit protein moiety by conformational changes rather than by total breakdown.

At 20 h after exposure to AIA, there was little or no haem reconstitution as determined both spectrophotometrically and enzymically (Tables 1 and 2). In agreement with these data, both the immunological method (Fig. 3) and the electrophoretic analysis (Fig. 2) indicated a loss of the protein moiety of cytochrome *P*-450 at this time. The protein loss is not directly caused by formation of *N*-alkyl-porphyrins, since such derivatives are detected as early as 1.0–1.5 h after exposure to AIA (De Matteis, 1971; Bonkowsky *et al.*, 1980), when immunologically the apoprotein is unchanged (Fig. 3, Table 1). Rather, the loss of protein requires subsequent events.

Apo-cytochrome *P*-450, totally free of metalloporphyrin or *N*-alkylated porphyrin, may be more susceptible than the holocytochrome to proteolytic degradation. This conclusion is consistent with our current findings and with the following observations: (1) formation of cobalt-protoporphyrin led to an increase in the concentration of microsomal proteins of M_r 50 000–53 000 (J.F. Sinclair, P. R. Sinclair & H. L. Bonkowsky, unpublished work); and (2) the 'green pigments' formed by AIA attack on cytochrome *P*-450 haem undergo metabolism (Bonkowsky *et al.*, 1980). Such metabolism is perhaps associated with removal of *N*-alkylporphyrin from the haem pocket of cytochrome *P*-450 and an increase in the rate of apoprotein breakdown. In contrast, when cobalt-protoporphyrin replaces haem in the pocket, the rate of apoprotein breakdown may decrease because of the greater stability of cobalt-protoporphyrin than of haem (De Matteis & Gibbs, 1977).

We previously reported that, in rats treated with phenobarbital, CoCl_2 and 5-aminolaevulinic acid *in vivo*, cobalt-protoporphyrin could be detected in association with hepatic microsomal protein(s) of M_r 50 000–53 000 (Sinclair *et al.*, 1982), providing evidence for the incorporation of this metalloporphyrin into apo-cytochrome *P*-450. In the current experiments, cobalt-protoporphyrin generated *in vitro* by liver homogenates was found to associate with microsomal proteins of M_r 50 000–53 000, particularly at 1 h after AIA treatment, when apo-

cytochrome *P*-450 was present (Fig. 5a). In contrast, there was much less association 20 h after AIA treatment, when apo-cytochrome *P*-450 was decreased (Fig. 5b). Thus, the current results strengthen the conclusion that cobalt-protoporphyrin, like haem, can be incorporated into mammalian apo-cytochrome *P*-450. Such incorporation has also been reported for bacterial apo-cytochrome *P*-450 (Wagner *et al.*, 1981).

In contrast with cobalt-protoporphyrin, zinc-protoporphyrin was not incorporated into apo-cytochrome *P*-450. Perhaps the zinc chelate, unlike the iron or cobalt chelate, does not fit into the haem pocket of the cytochrome, or perhaps the zinc chelate is not bound as tightly and is entirely lost during gel electrophoresis.

In conclusion, our results demonstrate substantial reconstitution by haem of functional hepatic cytochrome *P*-450 in rats treated with the porphyrinogen AIA. The reaction with AIA and subsequent reconstitution with haem indicate that the most of the cytochrome *P*-450 haem pockets are superficially located on the endoplasmic reticulum and can readily and reversibly bind exogenous haem. The results strengthen the rationale for administering haem to patients suffering from acute porphyric syndromes or from poisoning by chemicals that, like AIA, cause accelerated destruction of the haem of hepatic cytochrome *P*-450 (Bonkowsky, 1982).

Note added in proof (Received 4 July 1984)

L. Bornheim (personal communication) recently found that, in phenobarbital-treated rats given AIA, injection of haem 30 min before AIA prevented most of the loss of activity of ethylmorphine demethylase or benzphetamine demethylase otherwise observed 1 h after AIA.

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