Differential haemin-mediated restoration of cytochrome *P*-450 *N*-demethylases after inactivation by allylisopropylacetamide

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Administration of allylisopropylacetamide (AIA) to phenobarbital-pretreated rats results in the destruction of several phenobarbital-inducible cytochrome *P*-450 isoenzymes and a correspondingly marked loss of benzphetamine *N*-demethylase and ethylmorphine *N*-demethylase activities. Accordingly, the ion-exchange h.p.l.c. or DEAE-cellulose-chromatographic profile of solubilized microsomal preparations from such rats revealed a marked decrease in the cytochrome *P*-450 content of several eluted fractions compared with that of microsomes from corresponding non-AIA-treated controls. Incubation of liver homogenates from such rats with haemin restores not only cytochrome *P*-450 content from 35 to 62% of original values, but also benzphetamine *N*-demethylase and ethylmorphine *N*-demethylase activities, from 23 to 67%, and from 12 to 36% of original values respectively. Moreover, the chromatographic profiles of microsomes prepared from such homogenates indicated increases of cytochrome *P*-450 content only in some fractions. Reconstitution of mixed-function oxidase activity of cytochrome *P*-450 by addition of NADPH:cytochrome *P*-450 reductase to these fractions indicated that incubation with haemin restored benzphetamine *N*-demethylase activity predominantly, but ethylmorphine *N*-demethylase activity only minimally. After injection of [¹⁴C]AIA, a significant amount of radiolabel was found covalently bound to protein in chromatographic fraction III, and this binding was unaffected by incubation with haemin. Furthermore, the extent of this binding is apparently equimolar to the amount of cytochrome *P*-450 refractory to haemin reconstitution in that particular fraction. Whether such refractoriness reflects structural inactivation of the apo-cytochrome remains to be determined. Nevertheless, the evidence presented very strongly argues for AIA-mediated inactivation of multiple phenobarbital-induced isoenzymes, only a few of which are structurally and functionally repairable by haemin.

The hepatic microsomal haemoprotein cytochrome *P*-450 consists of a family of closely related isoenzymes that catalyse the oxidative metabolism not only of xenobiotics but also of endogenous steroids, prostaglandins and fatty acids (Conney, 1967; Gillette *et al.*, 1972; Lu & West, 1980). Repeated exposure of the animals to many foreign chemicals results in induction of specific cytochrome *P*-450 isoenzymes in the liver and other organs. For instance, PB has been shown to induce several hepatic isoenzymes (Waxman & Walsh,

1982; Bornheim & Franklin, 1982), some of which have been purified to apparent homogeneity (Guengerich, 1978*a,b*; Ryan *et al.*, 1980; Johnson, 1980; Koop *et al.*, 1981; Waxman & Walsh, 1982). Although isolation, purification and characterization of individual cytochrome *P*-450 isoenzymes have permitted elucidation of their structure and function, relatively little is known about the structural assembly of their constitutive moieties. This is in contrast with other haemoproteins, such as the soluble bacterial cytochrome *P*-450_{cam}, horseradish peroxidase and catalase, which can be readily reconstituted to the holoenzyme after structural dissociation into the haem apoprotein moieties (Yu & Gunsalus, 1974; Sano, 1979).

Abbreviations used: PB, phenobarbital; AIA, allylisopropylacetamide.

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Early attempts to reconstitute mammalian cytochrome *P*-450 *in vitro* by addition of exogenous haem to rat liver homogenates that had been relatively enriched in apo-cytochrome *P*-450 (by concomitant treatment of rats with PB and cobalt, an inhibitor of haem synthesis) only had limited success (Correia & Meyer, 1975). More recently, AIA, a suicide substrate of hepatic PB-inducible cytochrome *P*-450 isoenzymes, has been employed to deplete cytochrome *P*-450 haem acutely. Such acute depletion of hepatic cytochrome *P*-450 haem results from cytochrome *P*-450 dependent oxidative metabolism of AIA to a reactive species that irreversibly alkylates the prosthetic haem moiety of the isoenzyme, leaving the apo-cytochrome largely unscathed (De Matteis, 1971; Ortiz de Montellano & Correia, 1983, and references therein). Administration of exogenous haem *in vivo* to PB-treated rats given AIA (Farrell & Correia, 1980) or addition of haem *in vitro* to liver homogenates from such rats (Bhat *et al.*, 1977) has been shown to restore partially both spectrally detectable microsomal cytochrome *P*-450 content and its mixed-function oxidase activity (Farrell *et al.*, 1979; Bonkowsky *et al.*, 1982; Bornheim *et al.*, 1983). We have recently modified this procedure to obtain greater yields of the reconstituted haemoproteins. By using this improved procedure, we have examined the structural and functional reconstitution of AIA-inactivated cytochrome *P*-450 in liver homogenates from PB-treated rats after the addition of haem *in vitro*. We now report that, although AIA destroys several PB-inducible cytochrome *P*-450 isoenzymes, the partial restoration of cytochrome *P*-450 content and function is largely due to differential haemin-mediated reconstitution of specific PB-inducible isoenzymes.

Materials and methods

Male Sprague-Dawley rats (250–300g) were pretreated daily with sodium phenobarbital (80mg/kg intraperitoneally) for 5 days. At 24h after the last injection of PB, rats received an injection of AIA (200mg/kg intraperitoneally) administered in distilled water 1h (unless otherwise indicated) before they were killed. Livers were then removed, weighed, perfused with ice-cold 0.96% NaCl, and homogenized (50%, w/v) in 0.25M-sucrose containing 10mM reduced glutathione, pH7.4, at 4°C. Samples of liver homogenates were made 0.1M in potassium phosphate buffer, pH7.4, and incubated in the presence or absence of haemin (50µM, dissolved in a small volume of 0.1M-NaOH and neutralized with phosphate buffer, pH7.4 at 37°C for 15min in a Gyrotory water-bath shaker (New Brunswick Scientific). Homogenates were diluted with 3 vol.

of ice-cold 1.15% KCl and centrifuged at 9000g for 10min. Supernatants were decanted and centrifuged at 105000g for 60min. Microsomal pellets were resuspended in 15ml of 1.15% KCl and resedimented at 105000g for 30min. The washed microsomal preparations were resuspended in 0.1M-phosphate buffer, pH7.4, for the determination of protein concentration by the method of Lowry *et al.* (1951) (human serum albumin was used as standard) and cytochrome *P*-450 content by the method of Estabrook *et al.* (1972). Microsomes were assayed for *N*-demethylase activity with the substrates ethylmorphine and benzphetamine (final concn. 2mM) as described previously (Farrell & Correia, 1980). The amount of formaldehyde generated was quantified by the method of Nash (1953).

Microsomes solubilized with 0.5% (w/v) sodium cholate and 0.2% (v/v) Emulgen 911 (Warner *et al.*, 1978) were subjected either to ion-exchange h.p.l.c. as described by Kotake & Funae (1980) or to DEAE-cellulose (DE-52) column chromatography by the method of Warner *et al.* (1978), modified as previously described (Bornheim & Franklin, 1982). The eluted h.p.l.c. fractions were monitored at 417nm and assayed for cytochrome *P*-450 content. In this h.p.l.c. system, non-prosthetically bound haem is eluted in the void volume. Although tentative, it appears that cytochrome *P*-450 isoenzymes eluted in h.p.l.c. fractions C, D, E and F separate chromatographically as DE-52 fractions II and III. The fractions obtained from DE-52 column chromatography were also assayed for cytochrome *P*-450 content as well as functional activity. For this purpose, fractions were first dialysed for 48h against two changes of 10mM-phosphate buffer, pH7.4, containing 20% (v/v) glycerol. These partially resolved fractions were then concentrated by ultrafiltration over an Amicon PM-30 membrane, and treated with Bio-Beads SM-2 (Holloway, 1973) to lower the Emulgen 911 concentration to less than 0.01% (Garewal, 1973). To determine their relative *N*-demethylase activities, these cytochrome *P*-450 fractions were then reconstituted in the presence of saturating amounts of NADPH (2mM), substrate (ethylmorphine or benzphetamine, 2mM) and partially purified rat liver microsomal NADPH:cytochrome *P*-450 (*c*) reductase. The reductase was prepared by the method of Yasukochi & Masters (1976), as modified by Chung & Chao (1980). The activity of the reductase was 14µmol of cytochrome *c* reduced/min per mg of protein at 37°C.

The extent of AIA binding to microsomal protein was determined 1h after a bolus injection of [¹⁴C]AIA (1mg, 52.5µCi/mg) into the tail vein and AIA (200mg/kg intraperitoneally) into PB-pretreated rats. Microsomes were prepared and

solubilized as described above in the presence of a 2000-fold excess of AIA to displace any non-specifically bound [14 C]AIA. Preliminary experiments, in which equivalent amounts of [14 C]AIA were added to solubilized microsomes in the presence of a 2000-fold excess of AIA, revealed that, on DE-52 chromatography, most (95%) of the radioactivity representing unbound AIA was in the void volume. Fractions eluted from the DE-52 column after the salt gradient (0–0.25M-NaCl) were assayed for cytochrome *P*-450 content as described above. Their radioactivity was determined in Dimilume-30 (Packard Instruments, Downer's Grove, IL, U.S.A.; 10ml) in a liquid-scintillation spectrometer (Beckman LS-7000). The counting efficiency was >95% for all samples monitored. The profile of radioactivity was found to mimic the elution profile of cytochrome *P*-450 isoenzymes.

Covalent binding of [14 C]AIA to protein in the fractions eluted from the DE-52 column was also determined. For this purpose, protein (20mg) was precipitated by the addition of trichloroacetic acid (final concn. 25%, w/v). Samples were kept at 4°C for 30 min before addition of diethyl ether (1 vol. to 3 vol. of sample). To remove the non-specifically bound AIA and its metabolites, as well as the AIA-haem adduct (green pigment) from the protein precipitate, this two-phase system was vortex-mixed for 1 min and, after sedimentation of the pellet, the supernatant was discarded and the pellet washed and resedimented with 3 × 3 ml of diethyl ether/acetone (9:1, v/v). The pellet was dissolved in 1M-NaOH at 60°C and samples were taken for the determination of protein (Lowry *et al.*, 1951) and radioactivity.

Results

Administration of AIA to PB-pretreated rats resulted, within 1 h, in a decrease of about two-thirds in hepatic cytochrome *P*-450 content (Fig. 1a), in confirmation of previous reports (De Matteis, 1970). This loss of cytochrome *P*-450 was associated with decreased mixed-function oxidase activity, as indicated by the considerably decreased *N*-demethylation of benzphetamine and ethylmorphine by liver microsomes of these rats (Figs. 1b and 1c). Such losses were found to be not only time-dependent (Figs. 1b and 1c), but dose-dependent as well (Fig. 2).

Haemin incubation of liver homogenates prepared from PB-pretreated rats killed 1 h after AIA administration resulted in the structural reconstitution of approx. 50% of the inactivated cytochrome *P*-450, as determined spectrophotometrically (Fig. 3a). That is, AIA decreased hepatic cytochrome *P*-450 content from 1.82 to 0.64 nmol/mg of microsomal protein, and haemin incubation restored microsomal cytochrome *P*-450 content to 1.13 nmol/mg. In addition, it appears that haemin incubation restored a constant amount of the original cytochrome *P*-450 content, regardless of the duration of AIA treatment (Fig. 1a) or AIA dosage (Fig. 2). That is, although progressively larger amounts of cytochrome *P*-450 were irreversibly destroyed with time, haemin incubation was able to restore only $32 \pm 4\%$ of the total cytochrome *P*-450 content originally present (Fig. 1a). Such reconstitution represents a marked improvement over our previously reported values for haemin-mediated restoration of only 10% of the original cytochrome *P*-450 content (Farrell &

Fig. 1. Haemin restoration of rat liver microsomal cytochrome *P*-450 and *N*-demethylase activities after time-dependent AIA inactivation

PB-pretreated rats were given AIA (200mg/kg intraperitoneally) and killed at 0, 30, 60, 120, or 180 min thereafter. Liver homogenates were prepared and incubated in the presence (●) or absence (○) of haemin (50 μ M) at 37°C for 15 min. Microsomes were prepared and assayed for cytochrome *P*-450 content (a) and *N*-demethylase activities (b, c). Values (means $\pm$ S.E.M.) are presented as percentages of the original microsomal cytochrome *P*-450 content (1.82 ± 0.09 nmol/mg) and benzphetamine (b) and ethylmorphine (c) *N*-demethylase activities (281 ± 16 and 373 ± 25 nmol of formaldehyde/15 min per mg of microsomal protein respectively) in PB-pretreated control animals.

Correia, 1980; Correia *et al.*, 1981). We attribute this improvement to optimization of haemin concentrations currently utilized in our reconstitution studies.

Fig. 2. Haemin restoration of cytochrome *P*-450 after its dose-dependent destruction by AIA

PB-pretreated rats were given 0, 50, 100, or 200 mg of AIA/kg and were killed 30 min thereafter. Liver homogenates were prepared and incubated in the presence (●) or absence (○) of haemin (50 μ M) at 37°C for 15 min. Microsomes were prepared and cytochrome *P*-450 content was determined (See the Materials and methods section). Values represent means $\pm$ S.E.M. from at least three individual animals.

The structural reconstitution of cytochrome *P*-450 by haemin was associated with the restoration of its mixed-function oxidase activity. This was evidenced by the marked restoration of hepatic microsomal benzphetamine *N*-demethylase activity from 23 to 67% of original values and by the restoration, albeit modest, of ethylmorphine *N*-demethylase activity, from 12 to 36% of original values (Fig. 3b). It was unclear whether such differential restoration (67% as against 36% of original values) of these *N*-demethylase activities was due to unequal haemin-dependent reconstitution of specific cytochrome *P*-450 isoenzymes. That is, the isoenzyme(s) responsible for benzphetamine metabolism could have been more effectively reconstituted than those catalysing ethylmorphine *N*-demethylation. To examine this possibility, cytochrome *P*-450 isoenzymes in hepatic microsomes from AIA-treated rats were fractionated before and after haemin reconstitution by both ion-exchange h.p.l.c. and column chromatography.

H.p.l.c. of solubilized microsomes from PB-pretreated rats (control) revealed the presence of several cytochrome *P*-450 isoenzymes, which were eluted in fractions termed A, B, C, D, E and F (Fig. 4). These fractions contained spectrally detectable cytochrome *P*-450, but no spectrally

Fig. 3. Haemin restoration of cytochrome *P*-450 content and microsomal *N*-demethylase activity after AIA-mediated destruction

PB-pretreated rats were given AIA (200 mg/kg intraperitoneally) and were killed 1 h later. Liver homogenates were prepared and incubated with or without haemin (50 μ M) at 37°C for 15 min. Microsomes were prepared and assayed for cytochrome *P*-450 content (a) and *N*-demethylase activities (b). Values represent means $\pm$ S.E.M. for at least three individual animals. Activities are also expressed as a percentage of control values found in PB-pretreated animals. Open bars represent values obtained from control (PB-pretreated) rats; stippled and solid bars represent corresponding values from liver homogenates of AIA-treated rats incubated without or with haemin respectively.

Fig. 4. H.p.l.c. chromatograms of solubilized hepatic microsomes from AIA-treated rats before and after haemin restoration

PB-treated rats were not treated or treated with AIA (200 mg/kg intraperitoneally) for 120 min before being killed. Liver homogenates were prepared and incubated in the presence or absence of haemin (50 μ M) at 37°C for 15 min. Microsomes were prepared, solubilized as described by Kotake & Funae (1980), and 1 mg of microsomal protein was chromatographed. The spectral absorption of the cytochrome haem was monitored at 417 nm. The area bounded by a continuous line represents the chromatogram of liver microsomes from a PB-pretreated rat. The solid area represents the chromatogram of liver microsomes from another PB-pretreated rat given AIA. The stippled areas bounded by a broken line represent the chromatogram of microsomes prepared from this animal after incubation of a sample of its liver homogenate with haemin.

detectable cytochrome *b*₅ or functionally detectable NADPH:cytochrome *c* reductase, confirming previous reports (Kotake & Funae, 1980). AIA treatment of these rats apparently had little effect on the hepatic cytochrome *P*-450 content in fractions A and B, but decreased that of fractions C, D, E and F. Haemin incubation had little effect on cytochrome *P*-450 content of fractions C and D, but markedly increased its content in fractions E

and F. It thus appears that, after AIA-mediated destruction, haemin is capable of preferentially reconstituting specific isoenzymes of cytochrome *P*-450.

Functional characterization of these isoenzyme fractions, however, required larger amounts than those obtainable by h.p.l.c. For this purpose, solubilized microsomes were fractionated by DE-52 ion-exchange chromatography. Cytochrome *P*-450 isoenzymes in solubilized microsomal preparations obtained from PB-pretreated rats were eluted in four (I–IV) fractions (Fig. 5). After AIA treatment of such rats, fractions II and III were found to exhibit markedly decreased cytochrome *P*-450 content. Fractionation of liver microsomes after haemin incubation of homogenates from AIA-treated rats revealed a large increase in cytochrome *P*-450 content of fraction III, but only a small increase in cytochrome *P*-450 content of fraction II. Reconstitution of mixed-function oxidase activity of these two DE-52 fractions by addition of partially purified NADPH:cytochrome *P*-450 reductase and NADPH permitted quantification of their benzphetamine *N*-demethylase and ethylmorphine *N*-demethylase activities. Such reconstitution indicated that fractions II and III from PB-treated rat liver microsomes retain their full complement of microsomal benzphetamine *N*-demethylase activity, but not that of ethylmorphine *N*-demethylase (Table 1). This may partly be due to the removal of cytochrome *b*₅ after fractionation.

When expressed on the basis of the specific cytochrome *P*-450 content of these fractions, benzphetamine was found to be *N*-demethylated with a higher specific activity by the isoenzymes present in fraction III than by those in fraction II (Table 1). In contrast, ethylmorphine was metabolized more readily by isoenzymes in fraction II than by those in fraction III (Table 1). Similar reconstitution of mixed-function oxidase activity of fractions II and III from AIA-treated rat liver microsomes revealed that *N*-demethylation of both substrates was markedly decreased in both fractions. After haemin incubation, however, benzphetamine *N*-demethylase activity was significantly restored in fraction III, but not in fraction II, whereas ethylmorphine *N*-demethylase activity was only minimally restored in fraction III and not at all in fraction II (Table 1). Therefore it appears that the cytochrome *P*-450 isoenzyme(s) eluted in fraction III not only exhibits the greatest capacity for benzphetamine *N*-demethylation, but after AIA-induced inactivation is also largely amenable to structural and functional reconstitution with haemin (Table 1). Furthermore, the haemin-mediated increase in activity of benzphetamine *N*-demethylase (472 nmol of formaldehyde/15 min per nmol of

Fig. 5. Effect of AIA-mediated inactivation and haemin restoration on the DE-52 chromatographic elution profile of cytochrome P-450

Microsomes were prepared from animals treated as in Fig. 3, and were solubilized and chromatographed on DE-52 as described in the Materials and methods section (a, typical DE-52 chromatogram). Because AIA treatment and haemin-incubation altered the specific cytochrome P-450 content of the microsomal preparations chromatographed (b), the values have been normalized (as previously detailed; Bornheim & Franklin, 1982) to reflect these differences as follows: (cytochrome P-450 eluted in each fraction as a percentage of total cytochrome P-450 recovered) $\times$ [specific cytochrome P-450 content (nmol/mg of protein) of each microsomal preparation]. Open bars represent values obtained from control (PB-pretreated) rats; stippled and solid bars represent corresponding values from liver homogenates of AIA-treated rats incubated without or with haemin respectively.

cytochrome P-450) in fraction III of AIA-treated rat liver microsomes over corresponding values (245 nmol of formaldehyde/15 min per nmol of cytochrome P-450) from PB-pretreated controls suggests that haemin preferentially restores only the cytochrome P-450 isoenzymes responsible for benzphetamine N-demethylation. In contrast, after AIA-mediated destruction, isoenzymes that were eluted in fractions II and III and largely

responsible for ethylmorphine N-demethylase activity are not similarly reparable by exogenous haemin (Table 1).

It was conceivable that such resistance to structural and functional reconstitution was due to tenacious binding of AIA to a critical site (substrate- or haem-binding site of the apo-cytochrome). In principle, this AIA binding to protein could arise from a reactive metabolite of AIA or residual AIA-porphyrin adduct (green pigment). To determine if AIA bound to microsomal proteins in fractions II and III, [^{14}C]AIA was injected into rats *in vivo* (see the Materials and methods section). DE-52 chromatography of solubilized liver microsomes from these rats revealed that AIA bound to proteins in fractions II and III and could not be displaced by a 2000-fold excess of unlabelled AIA added *in vitro* (Table 2). Fractions II and III, obtained after DE-52 chromatography of solubilized microsomes from haemin-incubated liver homogenates of [^{14}C]AIA-treated rats, exhibited approximately the same extent of ^{14}C binding to proteins (Table 2). When the microsomal proteins were precipitated by trichloroacetic acid and extensively washed with diethyl ether and diethyl ether/acetone, a procedure that is expected to remove not only loosely bound AIA and its metabolites but also the green pigment, a similar pattern of binding was observed, thereby indicating that most of the [^{14}C]AIA eluted in fractions II and III was covalently bound to the microsomal protein. It is intriguing that, on a molar basis, the covalent binding of AIA to proteins in fraction III was equivalent to the cytochrome P-450 content that could not be restored by haemin incubation (Table 2). Thus a significant portion (approx. 60 nmol) of the inactivated cytochrome P-450 in fraction III appears to be refractory to haemin reconstitution. On the other hand, although a similar portion (i.e. 64 nmol) of cytochrome P-450 content in fraction II is refractory to haemin-mediated restoration, the observed covalent binding of AIA is not proportional and by itself may not fully explain such a finding.

To determine whether such refractoriness reflected the disappearance of the apo-(cytochrome P-450) secondary to its haem stripping and to acute AIA-induced haem depletion, animals were given exogenous haem *in vivo*, 30 min before AIA-treatment (Fig. 6). Such pretreatment resulted not only in recovery of increased microsomal cytochrome P-450 content, but also was associated with a much greater restoration of ethylmorphine N-demethylase activity than that observed *in vitro* in haemin-incubated preparations from AIA-treated rats (Fig. 6). However, such haem pretreatment *in vivo* produces no increased restoration of benzphetamine N-demethylase activity over the values

Table 1. *Activity of reconstituted cytochrome P-450 after AIA destruction and haemin restoration*

Microsomes were prepared from animals treated as in Fig. 3, and were chromatographed on DEAE-cellulose (DE-52) as described in the Materials and methods section. Fractions obtained after chromatography were dialysed against 10 mM-phosphate buffer containing 20% (v/v) glycerol, and Bio-Beads SM-2 were used to lower the concentration of Emulgen 911 to <0.01%. Cytochrome *P*-450 (0.2 nmol) in fraction II or III was reconstituted with saturating amounts of NADPH:cytochrome *P*-450 reductase, and *N*-demethylation of benzphetamine and ethylmorphine was determined. Values represent means for two separate experiments and are expressed on the basis of specific cytochrome *P*-450 content, rather than protein, of these preparations.

Activity (nmol of formaldehyde formed/15 min per nmol of cytochrome *P*-450)

Treatment	Benzphetamine <i>N</i> -demethylase			Ethylmorphine <i>N</i> -demethylase		
	Microsomes	Fraction II	Fraction III	Microsomes	Fraction II	Fraction III
PB	167	107	245	225	117	59
AIA	105	26	106	80	16	25
AIA + haemin	186	18	472	139	14	36

Table 2. *Cytochrome P-450 content and [¹⁴C]AIA binding to microsomal proteins after DE-52 chromatography*

Liver homogenates from PB-pretreated rats treated with [¹⁴C]AIA (200 mg/kg) for 1 h were incubated in the presence or absence of haemin (50 μM). Microsomes (200 mg) were prepared, solubilized, and chromatographed on DEAE-cellulose (DE-52) as described in the Materials and methods section. Radioactivity was determined in the eluted fractions before and after precipitation with trichloroacetic acid and extensive washing of the protein pellet with organic solvents.

	Fraction II	Fraction III
Cytochrome <i>P</i> -450 (nmol)		
No AIA treatment	152	156
AIA treatment	66	34
AIA treatment + haemin incubation	88	98
Not restored	64	58
[¹⁴ C]AIA present (nmol)		
AIA treatment	34	53
AIA treatment + haemin incubation	19	53
[¹⁴ C]AIA covalently bound to protein (nmol)		
AIA treatment	15	54
AIA treatment + haemin incubation	14	56

observed after haemin-mediated reconstitution *in vitro* (Fig. 6).

Discussion

Observations from several laboratories, including our own, indicate that AIA preferentially inactivates hepatic cytochrome *P*-450 isoenzymes induced by PB (Loosemore *et al.*, 1981; Waxman & Walsh, 1982; Ortiz de Montellano & Correia, 1983). Such inactivation has been shown to result from alkylation of the prosthetic haem moiety of cytochrome *P*-450 by a reactive electrophilic metabolite of AIA, possibly an iron-co-ordinated alcohol with an adjacent carbonium ion (Ortiz de Montellano & Mico, 1980). Attack of the electrophilic carbonium ion at the porphyrin-ring nitrogen atom of the haem moiety is proposed to yield the haem-AIA adduct, whereas its intramolecular

closure is believed to result in the formation of an epoxide (Ortiz de Montellano & Mico, 1980, 1981; Loosemore *et al.*, 1981). The partition ratio of this reaction is such that for every 200 molecules of the epoxide formed, one haem-AIA adduct is generated, thereby sacrificing a cytochrome *P*-450 haem (Loosemore *et al.*, 1981; Ortiz de Montellano & Mico, 1981). Such alkylation of the haem moiety is believed to account largely for the AIA-mediated loss of hepatic cytochrome *P*-450 content, because the apocytochrome can accept a fresh haem moiety to regenerate the cytochrome (Bhat *et al.*, 1977; Farrell & Correia, 1980) and therefore appears to escape relatively unscathed.

The partial loss of hepatic cytochrome *P*-450 content and its *N*-demethylase activity in PB-pretreated rats given AIA is rationalized on the basis that liver microsomes from such rats contain several PB-inducible cytochrome *P*-450

Fig. 6. Relative efficacy of haemin pretreatment *in vivo* versus haemin incubation *in vitro* on structural and functional restoration of AIA-inactivated cytochrome P-450 isoenzymes

PB-pretreated rats were given haemin (5 mg/rat) via the tail vein 30 min before AIA (200 mg/kg intraperitoneally). Animals were killed 1 h later, liver microsomes were prepared and assayed for cytochrome P-450 content and N-demethylase activity (solid bars), as described in the Materials and methods section. In parallel, liver homogenates from another group of PB-pretreated rats, which were treated for 1 h with AIA alone, were incubated with (cross-hatched bars) and without (stippled bars) haemin exactly as described in Fig. 1. Microsomes were prepared and assayed for structural and functional restoration of cytochrome P-450 (see the Materials and methods section). Original (100%) values for cytochrome P-450 content, benzphetamine N-demethylase and ethylmorphine N-demethylase activities in PB-pretreated rats not given AIA were 1.76 ± 0.09 nmol/mg of protein, 325 ± 32 nmol of formaldehyde formed/15 min per mg of protein and 438 ± 43 nmol of formaldehyde formed/15 min per mg of protein respectively. Values (means $\pm$ S.E.M.) indicated are expressed as percentages of the original values obtained from three individual animals.

isoenzymes, only one of which metabolizes AIA and is thereby suicidally inactivated (Waxman & Walsh, 1982). Evidence for this was presented by Waxman & Walsh (1982), who purified two PB-inducible cytochrome P-450 isoenzymes, one of which was 10-fold more susceptible to AIA-mediated inactivation than the other. The findings of the present study, however, suggest that AIA

inactivates more than one PB-inducible isoenzyme *in vivo*.

The possibility that AIA inactivates multiple PB-inducible cytochrome P-450 isoenzymes is supported by the large decreases in cytochrome P-450 content in selective fractions resolved by DE-52 chromatography or h.p.l.c. of liver microsomes from AIA-treated rats and by the differential restoration of cytochrome P-450 content and specific N-demethylase activity in such fractions after haemin incubation (Figs. 4 and 5; Table 1).

The failure of haemin to restore ethylmorphine N-demethylase activity appreciably after reconstitution of mixed-function oxidase activity with NADPH:cytochrome P-450 reductase cannot be ascribed solely to the absence of cytochrome b_5 from the reconstituted system. This is because, even in liver microsomes, ethylmorphine N-demethylase activity was not appreciably restored after haemin incubation, even though these preparations retain their full complement of cytochrome b_5 after AIA treatment (De Matteis, 1973; Wada *et al.*, 1968). In addition, failure to restore microsomal ethylmorphine N-demethylase activity appreciably after haemin incubation does not appear to reflect competitive substrate inhibition by any residual AIA, since a 10-fold increase in ethylmorphine concentrations in the assay system failed to increase ethylmorphine N-demethylation correspondingly. The above findings thus indicate that haemin differentially restores ethylmorphine N-demethylase and benzphetamine N-demethylase after their inactivation by AIA.

Similar inferences may also be drawn from the report that, although AIA apparently inactivates several cytochrome P-450 isoenzymes (including cytochrome P-450₂), only some isoenzymes are restored by haem administration (Liem *et al.*, 1983). Furthermore, because the loss of cytochrome P-450₂ was monitored immunochemically, such findings also attest to the rapid disappearance of its apoprotein moiety. Such a disappearance might partly account for the failure of haemin to fully restore hepatic cytochrome P-450 content after its AIA-induced loss.

Additionally, the resistance of some cytochrome P-450 isoenzymes to haemin-mediated repair raises the suspicion that their inactivation by AIA may result from mechanisms other than the well-recognized suicidal alkylation of the prosthetic haem. Accordingly, only those cytochrome P-450 isoenzymes that undergo haem alkylation without inactivation or loss of apo-(cytochrome P-450) would be available for haemin-mediated structural and functional repair (i.e. restorable ethylmorphine N-demethylases and benzphetamine N-demethylases). On the other hand, isoenzymes that either incur irretrievable loss of the haem-stripped

apoprotein and/or whose apo-cytochrome moiety is structurally damaged would be refractory to haemin reconstitution.

Conceivably, such structural damage to the apo-cytochrome could result from its alkylation by a reactive AIA metabolite. One prospective candidate is the 4,5-epoxide, whose formation *in vivo* has been deduced from isolation of the AIA glycol and AIA lactone from the urine of AIA-treated rats (Doedens, 1971; Smith, 1976). The epoxide has in fact been postulated to account for the covalent binding of [¹⁴C]AIA to microsomal proteins and DNA (Loosemore *et al.*, 1981). In principle, it could also attack the nucleophilic thiol groups of the apo-(cytochrome *P*-450) and result in its irreversible inactivation.

Another prospective alkylating metabolite of AIA is the reactive cation radical intermediate, which has been proposed either to alkylate the haem moiety or to rearrange to form the epoxide (Ortiz de Montellano & Mico, 1980; Loosemore *et al.*, 1981; Ortiz de Montellano & Correia, 1983). Significant apoprotein alkylation by this route might occur if a sufficient nucleophilic amino acid residue of apo-(cytochrome *P*-450) is intercalated in this catalytic path. Such an event is consistent with the concept that the unique spatial orientation of each apoprotein moiety around its own prosthetic haem (i.e. the haem environment) critically determines the regiospecific alkylation of each cytochrome *P*-450 haem moiety (Kunze *et al.*, 1983; De Matteis *et al.*, 1983). Accordingly, for any given suicide substrate, the regiospecificity of haem alkylation is found to vary with individual cytochrome *P*-450 isoenzymes (De Matteis *et al.*, 1983), even though their haem moieties are structurally identical. Thus, given the particular haem environment of each cytochrome *P*-450 isoenzyme, it is quite conceivable that some isoenzymes might sustain haem alkylation, whereas others (whose protein moieties protect otherwise-susceptible haem target sites) might incur alkylation of their apoprotein moiety and be irreparably damaged.

It is not immediately clear whether such apoprotein alkylation accounts for our finding that haemin fails to restore fully certain cytochrome *P*-450 isoenzymes inactivated by AIA. However, consistent with such a possibility, we have found that the [¹⁴C]AIA covalently bound to proteins in fraction III is approximately equimolar to the amount of cytochrome *P*-450 destroyed by AIA but resistant to haemin-mediated reconstitution (Table 2). Although it is tempting to speculate that such stoichiometry solely reflects AIA-mediated inactivation of apo-(cytochrome *P*-450), it must be noted that these fractions also contain microsomal proteins other than cytochrome *P*-450. In contrast, the extent of covalent binding of AIA to protein in

fraction II is insufficient to account for most of its non-reconstitutable cytochrome *P*-450 content. This discrepancy might be due to tenacious binding of the green pigment, which prevents incorporation of fresh haem into some apo-cytochromes, or to rapid loss of certain apo-cytochromes after their AIA-mediated alkylation and/or acute AIA-induced haem depletion. Consistent with the latter possibility, we have found that concurrent haem administration *in vivo* to AIA-treated rats largely, but not completely, reverses the loss of hepatic ethylmorphine *N*-demethylase activity, otherwise observed after AIA-induced haem depletion (Fig. 6).

In summary, our findings indicate that AIA inactivates multiple hepatic cytochrome *P*-450 isoenzymes in PB-pretreated rats, and that haemin incubation of liver homogenates from such AIA-treated rats is found to restore differentially cytochrome *P*-450 dependent *N*-demethylation of benzphetamine and ethylmorphine, consistent with other reports that such reactions might be catalysed by different cytochrome *P*-450 isoenzymes (Guengerich *et al.*, 1982). The failure of haemin to restore fully benzphetamine *N*-demethylase activity (fraction III) might be due to partial inactivation of the apo-(cytochrome *P*-450) moiety by AIA-mediated alkylation. The failure to restore ethylmorphine *N*-demethylase activity, on the other hand, might be due not only to alkylation of haem and the apo-cytochrome, but also to rapid degradation of the irreparably damaged cytochrome.

Note added in proof

Since submission of this manuscript, Bonkowsky *et al.* (1984) have found haemin to restore differentially benzphetamine and ethylmorphine *N*-demethylases.

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