Cytochrome P-450 Heme Moiety

THE SPECIFIC TARGET IN DRUG-INDUCED HEME ALKYLATION*

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Exogenously administered heme is incorporated into rat hepatic cytochrome P-450 in vivo (Correia, M. A., Farrell, G. C., Schmid, R. S., Ortiz de Montellano, P. R., Yost, G. S., and Mico, B. A. (1979) J. Biol. Chem. 254, 15-17). This was demonstrated in allylisopropylacetamide (AIA)-treated rats by the formation of a radioactively labeled adduct derived from the porphyrin of the administered [3H]heme and AIA. Formation of such adducts requires catalytic participation of cytochrome P-450 in oxidative metabolism of AIA to an active species which subsequently alkylates the prosthetic heme moiety of the cytochrome. These results suggested that the exogenous heme had been incorporated prosthetically into cytochrome P-450 prior to generation of the adduct. However, the possibility remained that a minute portion of the inactivating AIA-species escaped the catalytic site of the generating hemoprotein and alkylated the nonprosthetically bound isotopic heme. To examine this critical possibility, we have employed a chemical derivative of heme which binds to the microsomal membrane. Although this heme derivative is a structurally suitable target for attack by the inactivating drug species, we found that it was unsuitable for incorporation into the prosthetic site of cytochrome P-450. The findings of this study provide irrefutable evidence that the label recovered in drug-porphyrin adducts is derived exclusively from radioactive heme incorporated prosthetically into cytochrome P-450. Drug-porphyrin adducts can therefore be used as reliable probes to follow the transfer of heme from the hepatic "free" heme pool into cytochrome P-450.

Recent findings in our laboratories have demonstrated that heme† administered to intact rats readily gains access to an

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§ Recipient of a C. J. Martin Fellowship of the Australian National Health and Medical Research Council.

¶ Recipient of Grant AM-11275 from the National Institutes of Health.

‖ Recipient of Grant GM-25515 from the National Institutes of Health and an Alfred P. Sloan Fellowship.

* Throughout this report, heme will be used interchangeably with hemin (iron-protoporphyrin IX), irrespective of its oxidation state.

† The abbreviations used are: AIA, allylisopropylacetamide; (C-isopropyl-4-pentenamide); HPLC, high pressure liquid chromatography; TLC, thin layer chromatography.
Because the avid plasma protein binding and potential hydrolysis of diisopropylheme renders experiments in vivo difficult, we had to devise an in vitro system for examining incorporation of labeled exogenous heme into hepatic cytochrome P-450 and subsequent adduct formation. Because technical difficulties were encountered in isolating AIA adducts from in vitro systems, we substituted norethindrone for AIA. To validate this substitution, it was necessary to demonstrate in vivo that following destruction of cytochrome P-450 by norethindrone, isotopic heme is incorporated into the residual apocytochrome. Preliminary experiments (Table II), however, were performed with liver homogenates from AIA-treated rats to ascertain that heme, but not diisopropylheme, can reconstitute cytochrome P-450.

The present finding that only labeled heme but not its labeled diisopropylster results in the appearance of labeled adducts offers unequivocal evidence that adduct formation is restricted to reaction of the activated drug metabolite(s) with heme that has been incorporated prosthethically into holocytochrome P-450.

EXPERIMENTAL PROCEDURES

Materials

Norethindrone acetate, tricaprylin, hemin, NADPH, d(+)-isocitrate, isocitric dehydrogenase (EC 1.1.1.42), subtilisin, rat serum albumin (Fraction V), glutathione, and nicotinamide were obtained from Sigma. All high pressure liquid chromatography solvents were purchased from Burdick and Jackson. ([H]Hemin was prepared by the method of Goldstein et al. (23) and crystallized by the method of Labbe and Nishada (24). The diisopropyl ester of [H]hemin (diisopropylhemin) was chemically prepared by a modification of a previously reported procedure (25) as follows: [H]Hemin was esterified in sulfuric acid/isopropanol (1:20, v/v) at room temperature for 48 h, followed by extraction into chloroform and repeated washing with 1.2 M NaCl. Final purification of the ester was performed by thin-layer chromatography (Analtech: Silica Gel GF, 250 μm; chloroform/acetone/methanol, 20:10:3, v/v) and the product was identified by co-chromatography with a well characterized standard prepared in an identical manner. The authenticity of diisopropylhemin so prepared was established by uv-visible, NMR, and mass spectral analysis following removal of iron. This procedure yielded diisopropylhemin of high radiochemical purity (>99%).

Preiliminary Experiments

To validate the use of diisopropylhemin as a suitable heme congenere which is incapable of being incorporated prosthethically into cytochrome P-450, but binds nonspecifically to microsomal membranes, the following preliminary experiments were carried out. Liver microsomes from phenobarbital-treated rats were incubated in vitro with equimolar concentrations of either [H]hemin or [H]diisopropylhemin at 37 °C for 1 h, resedimented, and “washed” (26). Radioactivity recovered in microsomes from preparations incubated with either isotopic heme or diisopropylhemin was found to be 79 and 87%, respectively, indicating comparable microsomal membrane binding of the two heme compounds.

The relative effectiveness of hemin and diisopropylhemin in reconstitution of tryptophan pyrroline in vitro was determined in liver from rats pretreated daily with sodium phenobarbital (80 mg/kg, intraperitoneally) for 5 days. Liver homogenate, supplemented with tryptophan (2.5 mM), was incubated at 37 °C for 1 h with or without either hemin (2 μM) or diisopropylhemin (2 μM) and the kynurenine formed was determined as described previously (26). The results (Table I) demonstrate that, in contrast to heme, diisopropylhemin is not utilized for reconstitution of hepatic tryptophan pyrroline.

The relative effectiveness of hemin and diisopropylhemin in reconstituting microsomal cytochrome P-450 in vitro was estimated in liver from phenobarbital-pretreated rats that had been given AIA (400 mg/kg, subcutaneously) (27). Animals were killed 2 h after AIA administration and liver homogenates were incubated in the presence or absence of either hemin (4 μM) or diisopropylhemin (4 μM) at 37 °C for 30 min (28). Microsomes were obtained and their cytochrome P-450 content was determined spectrophotometrically (28). Whereas, on incubation with heme, a significant rise in hepatic microsomal cytochrome P-450 was obtained (Table II), no reconstitution of the hemoprotein was demonstrable with diisopropylhemin (Table II).

Experimental Protocol

Experiments in Vivo—Male Sprague-Dawley rats (200–300 g) were pretreated daily for 5 days with sodium phenobarbital (80 mg/kg, intraperitoneally) and fasted overnight prior to use. Three identical sets of experiments were carried out, each employing two experimental and two control rats. Eighteen h after the last injection of phenobarbital, two rats were given norethindrone (100 mg/kg, intraperitoneally) in tricaprylin, while control rats received an equal volume of the vehicle. After 50 min, all animals were injected intramuscularly with [H]hemin (80 μg; specific activity, 7.5 x 10^9 dpm/μg), dissolved in 0.1 N NaOH (0.1 ml), neutralized with 0.1 N HCl (0.08 ml), diluted with 0.3 ml of rat serum and 0.1 M potassium phosphate buffer, pH 7.4, to a final volume of 1.0 ml, and filtered through a Millipore filter (0.45 μ). One h later, the animals were killed and the livers were weighed before and after perfusion ex situ with ice-cold 0.15 M KCl. Individual livers were then finely minced and homogenized in 3 volumes of 0.1 M phosphate buffer, pH 7.4, containing glutathione (2 mM) and nicotinamide (10 mM). Microsomes and microsomal carbon monoxide-binding particles (CO-particles, devoid of cytochrome P-450 and hemoprotein) were prepared as described previously (1). Aliquots of the liver homogenate, microsomes, and CO-particles were assayed for total heme, cytochromes P-450 and heme-specific radioactivity (1), as described below. Values were expressed per g of preperfused liver wet weight.

Separate but identical experiments were performed to isolate, purify, and quantitate norethindrone-porphyrin adducts. Following liver perfusion, individual livers were finely minced and mixed and an aliquot (1 g) was homogenized in 0.1 M phosphate buffer containing glutathione and nicotinamide, pH 7.4, and used for determinations of cytochrome P-450, total heme, and total and heme-specific radioactivity. The remainder of the minced liver was employed for isolation of norethindrone-porphyrin adducts.

Experiments in Vitro—Five or six rats pretreated with sodium phenobarbital (80 mg/kg, intraperitoneally, daily) for 5 days were fasted overnight and killed 18 h after the last injection. The livers

<table>
<thead>
<tr>
<th>Table I</th>
<th>Relative effectiveness of heme and diisopropylhemin in reconstitution (apoenzyme saturation) of tryptophan pyrroline in rat liver in vitro</th>
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</thead>
<tbody>
<tr>
<td>Addition</td>
<td>Holoenzyme activity</td>
</tr>
<tr>
<td>----------</td>
<td>---------------------</td>
</tr>
<tr>
<td>None</td>
<td>2.24 ± 0.35</td>
</tr>
<tr>
<td>Heme</td>
<td>5.09 ± 0.62</td>
</tr>
<tr>
<td>Diisopropylheme</td>
<td>2.29 ± 0.39</td>
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</table>

<table>
<thead>
<tr>
<th>Table II</th>
<th>Reconstitution of microsomal cytochrome P-450 with heme or diisopropylhemin in AIA-treated rat liver in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Addition</td>
<td>Content</td>
</tr>
<tr>
<td>----------</td>
<td>---------</td>
</tr>
<tr>
<td>None</td>
<td>0.84 ± 0.04</td>
</tr>
<tr>
<td>Heme</td>
<td>1.07 ± 0.08</td>
</tr>
<tr>
<td>Diisopropylheme</td>
<td>0.83 ± 0.08</td>
</tr>
</tbody>
</table>

Norethindrone acetate will hence forward be referred to as noret-indrone.
were perfused in situ with ice-cold 0.15 M KCl, pooled, homogenized in 0.1 M potassium phosphate buffer, pH 7.4, containing 1.0 mM EDTA, following which a "washed" microsomal pellet was obtained as described previously. Batches of microsomes (500 mg of protein) were incubated at 3% C for 1 h in a shaking water bath in a final 100-
ml volume of phosphate/EDTA buffer, pH 7.4, containing norethindrone (62 mM), NADPH (2 mM), dl-isocitrate (2.5 mM), isocitrate dehydrogenase (66 units), and either [3H]hemin or [3H]diisopropyl- 
hecin. [3H]Hemin (95 µg; specific activity, 2.06 × 10^8 dpm/µg) was 
dissolved in 0.1 N NaOH (0.2 ml), neutralized with 0.1 N HCl (0.15 
ml), added to 1% rat serum albumin and phosphate/EDTA buffer, 
pH 7.4, and filtered through a Millipore filter (0.45 µm). An equimolar 
amount of [3H]diisopropylhecin of comparable specific activity was 
dissolved in acetone (0.25 ml) and added to 1% rat serum albumin 
and phosphate/EDTA buffer, pH 7.4, and filtered through a similar 
filter. A similar incubation mixture without norethindrone served 
as the control for non-drug-mediated loss of cytochrome P-450 (8). At 
the conclusion of the incubation, aliquots were removed for measure-
ment of residual cytochrome P-450, and estimation of [3H]hemin or 
[3H]diisopropylhecin in the flask. The remainder of the mixture was 
rapidly chilled in ice and sedimented at 100,000 × g for 1 h at 4 °C. 
The pellets so obtained were used for isolation and purification of 
norethindrone-porphyrin adducts.

Isolation and Purification of Norethindrone-Porphyrin Adducts

Crude Adducts—Livers from rats treated with norethindrone in vivo or hepatic microsomal pellets obtained from co-incubations of 
norethindrone with either [3H]hemin or [3H]diisopropylhecin in vitro were methylated in cold sulfuric acid/methanol (1:20, v/v; 250 
ml/liver or pellet) at 4 °C in the dark for at least 17 h. After filtration 
to remove denatured protein, water (approximately 125 ml/liver or 
pellet) was added and the crude methylated adducts were extracted 
with a similar volume of dichloromethane (2.5 ml). The organic 
extracts were combined, washed four times with an equal volume of water, 
twice with dichloromethane (v/v) and filtered through a similar 
Millipore filter. The remainder of the mixture was 
rapidly chilled in ice and sedimented at 100,000 × g for 1 h at 4 °C. 
The pellets so obtained were used for isolation and purification of 
norethindrone-porphyrin adducts.

**TABLE I**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Cytochrome P-450</th>
<th>Heme</th>
<th>Radioactivity</th>
<th>Total</th>
<th>Heme</th>
<th>Non-heme</th>
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<tbody>
<tr>
<td>Homogenate, norethindrone</td>
<td>75.1</td>
<td>191.4</td>
<td>90,400</td>
<td>56,800</td>
<td>23,600</td>
<td></td>
</tr>
<tr>
<td>Homogenate, control</td>
<td>120.7</td>
<td>226.4</td>
<td>106,700</td>
<td>89,100</td>
<td>17,600</td>
<td></td>
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<tr>
<td>Microsomes, norethindrone</td>
<td>49.4</td>
<td>89.8</td>
<td>37,300</td>
<td>21,200</td>
<td>16,100</td>
<td></td>
</tr>
<tr>
<td>Microsomes, control</td>
<td>68.3</td>
<td>98.4</td>
<td>42,300</td>
<td>29,700</td>
<td>12,600</td>
<td></td>
</tr>
<tr>
<td>CO-particles, norethindrone</td>
<td>23.7</td>
<td>25.4</td>
<td>29,500</td>
<td>12,800</td>
<td>16,700</td>
<td></td>
</tr>
<tr>
<td>CO-particles, control</td>
<td>30.2</td>
<td>33.6</td>
<td>34,200</td>
<td>20,200</td>
<td>14,000</td>
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**TABLE IV**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Loss of cytochrome P-450 (%)</th>
<th>Loss of [3H]heme (%)</th>
<th>Crude Zn complex (TLC)</th>
<th>Purified Zn complex (HPLC)</th>
<th>Purified [3H]porphyrin adducts (%)</th>
<th>Recovery (%)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>225</td>
<td>5.2</td>
<td>86.4</td>
<td>133</td>
<td>2.8</td>
<td>53</td>
</tr>
<tr>
<td>2</td>
<td>128</td>
<td>1.4</td>
<td>35.4</td>
<td>82.5</td>
<td>0.9</td>
<td>63</td>
</tr>
</tbody>
</table>

* Determined spectrophotometrically using a millimolar extinction coefficient of 125 at 432 nm.
* Corrected for 14% recovery of the adducts based on control experiments using defined amounts of authentic zinc complex (see text).
* Recovery of NE-[3H]porphyrin adducts.

twice with dichloromethane (100 ml; 50 ml). The organic extracts 
were combined, washed four times with an equal volume of water, 
and dried over anhydrous sodium sulfate. Zinc acetate 
(0.5 ml) was added and the crude zinc complex (see text).

**FIG. 1.** HPLC analyses of norethindrone-porphyrin adducts obtained from norethindrone-treated rat liver. A Whatman Partisil 10-PAC column was used with hexane/tetrahydrofuran (1:1, v/v) and a 30-min linear gradient (0-100%) of methanol (2 ml/min). Top Panel, HPLC analysis of the crude zinc complex obtained after TLC separation. The curve delineates the elution profile of the purified zinc complex as monitored by absorbance at 432 nm; the effluent fractions eluting between 15-20 min and 50-66% methanol gradient (arrows) were assayed for radioactivity. Bottom Panel, HPLC analysis of the free bases obtained by demetallation of the purified zinc complex of the adducts. The curve illustrates the elution profile (determined by absorbance at 417 nm) of the zinc-free bases. The methanol gradient was started at 200 min and extended for 20 
min. Aliquots of the effluent, eluting at intervals indicated by the arrows, were monitored for radioactivity. Note the difference in the time scale in the two panels.
Drug-induced Alkylation of Cytochrome P-450 Heme

Incorporation of [3H]heme or [3H]diisopropylheme into norethindrone (NE)-porphyrin adducts in vitro

Microsomes obtained from liver of phenobarbital-pretreated rats were incubated in the presence of norethindrone and either [3H]heme or [3H]diisopropylheme. Norethindrone-porphyrin adducts isolated from these incubation mixtures were purified and quantitated as detailed in the text. Experiments were carried out in duplicate with essentially similar results. Values obtained in one of the two experiments are recorded below.

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Final concentration (μM)</th>
<th>Radioactivity (nmol)</th>
<th>Cytochrome P-450 content (nmol)</th>
<th>Cytochrome P-450 de-</th>
<th>Crude Heme</th>
<th>Zn complex</th>
<th>Sequential purification of NE-porphyrin adducts</th>
<th>Zn-free base</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Radioactivity (μM)</td>
<td>Amount (μmol)</td>
</tr>
<tr>
<td>Heme</td>
<td>1.5</td>
<td>19,400,000</td>
<td>1,530</td>
<td>530</td>
<td>182.0</td>
<td></td>
<td>76.8</td>
<td>50.4</td>
</tr>
<tr>
<td>Diisopropylheme</td>
<td>1.5</td>
<td>19,400,000</td>
<td>1,530</td>
<td>480</td>
<td>95.0</td>
<td></td>
<td>65.1</td>
<td>None</td>
</tr>
</tbody>
</table>

*Crude adducts obtained by TLC separation were subjected to repeated HPLC (I, II, III). The metal of the purified zinc complex (HPLC III) was removed and the free base was separated by HPLC IV.

Analytical Determinations

Cytochromes P-450 and b, were determined by the methods of Estabrook et al. (29) and Omura and Sato (30), respectively. Heme was measured by the pyridine-hemochromogen method (31). Total radioactivity and “heme-specific” radioactivity of heme isolated and crystallized by the method of Labbe and Nishida (34) were quantitated in a liquid scintillation spectrometer as described (1).

RESULTS AND DISCUSSION

Norethindrone administration to phenobarbital-pretreated rats reduced the cytochrome P-450 and heme content of liver homogenate, microsomes, and CO-particles (Table III). This reduction was similar to, although somewhat smaller than, that previously reported for AIA-treatment (1). Total and heme-specific radioactivity of these cellular fractions also was decreased (Table III), whereas their non-heme radioactivity was increased, reflecting formation of labeled norethindrone-porphyrin adducts (Table III). These adducts were isolated, purified, characterized and quantitated (Fig. 1, Table IV). Approximately 60% of the total [3H]heme lost from the liver after norethindrole treatment was recovered in the form of labeled adducts (zinc complex) (Table IV).

When liver microsomes containing [3H]heme were incubated with norethindrole, the isolated norethindrone-porphyrin adducts were highly labeled (Table V). By contrast, the adducts recovered after incubation with [3H]diisopropylheme and norethindrole failed to exhibit detectable radioactivity (Table V). Since [3H]heme, but not [3H]diisopropylheme, is incorporated into cytochrome P-450 (Table III), this difference in labeling would be expected only if the alkylating attack by norethindrole metabolite(s) were restricted to the heme moiety incorporated prosthetically into cytochrome P-450. The present findings indeed indicate that no adduct formation occurred with [3H]diisopropylheme that was merely bound to the microsomal membrane without being incorporated prosthetically into holocytochrome P-450 (Table V). It is therefore apparent that the previously reported loss of added [3H]heme (32) on incubation of liver microsomes with AIA can be ascribed entirely to adduct formation with [3H]heme that had been incorporated into the holocytochrome.

REFERENCES

Drug-induced Alkylation of Cytochrome P-450 Heme