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FUNCTIONAL RECONSTITUTION OF RAT LIVER CYTOCHROME P-450 WITH MESOHEMIN

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SUMMARY: After allylisopropylacetamide-mediated "suicide" destruction of their prosthetic heme moieties, certain rat liver cytochrome P-450 isozymes can be effectively reconstituted by addition of exogenous hemin in vitro. We now report that two of these isozymes will equally accept mesohemin, a 2,4-diethyl heme-analog and result in a "meso-hemoprotein" with altered spectral but not functional characteristics.

In the liver, microsomal hemoproteins collectively termed cytochrome P-450 exist as isozymes which are not only responsible for the oxidative metabolism of a variety of structurally different chemicals but are inducible by them as well. Structural analyses of the individual isozymes following their isolation and purification have shown that the hemoproteins are monomers containing one prosthetic heme per mole of enzyme (1). Aminoacid sequencing and peptide mapping of the various cytochrome P-450 isozymes have indicated that their apoprotein moieties are not homologous (2-6). This feature is believed to contribute to the differential substrate specificity of the various isozymes.

On the other hand, the heme moieties of the cytochrome P-450 isozymes examined apparently are identical and have been identified as iron (III)-protoporphyrin IX (7). In the hemoproteins, the heme iron has been shown to be coordinated to the thiolate of a cysteine residue of the apocytochrome (8-11). Although the apocytochrome P-450 moieties are on the whole heterologous, a tetradecapeptide region containing a cysteine residue is found to be conserved in both rat and rabbit phenobarbital-inducible isozymes, as well as in bacterial cytochrome $P\text{-}450_{CAM}$ (3-5,12). If, as proposed (3-5,12), this region is indeed

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the heme binding site, then the structural specifications for this site appear to be quite rigidly defined.

In contrast, very little is known about the structural specifications of the heme moiety of mammalian cytochrome P-450s. Structure-function relationships of the heme moiety have remained unexplored due to the inability to reconstitute the mammalian holoenzyme (in contrast to bacterial cytochrome P-450_{CAM}) after deliberate dissociation of its heme and apocytochrome moieties. In recent years, this problem has been circumvented by an indirect approach: the existing prosthetic heme moiety of cytochrome P-450 is first destroyed through catalytic turnover of a suicide substrate such as allylisopropylacetamide (AIA) and subsequently replaced with exogenously supplied fresh heme (13-15). Such replacement has been shown to result in both structural and functional reconstitution of the hemoprotein (13-15). We have now improved this procedure so as to obtain greater yields of the reconstituted hemoprotein. That is, we are now capable of restoring approximately 50% of the cytochrome P-450 fraction lost by inactivation, as compared with the previously reported 20% (15). This improvement has permitted us to examine the relative effectiveness of mesohemin, the 2,4-diethyl analog of hemin, as the prosthetic moiety of the cytochrome.

MATERIALS AND METHODS

Materials: AIA was a gift from Dr. W. Scott, Hoffman-La Roche, Inc. (Nutley,NJ). Reduced glutathione and hemin were obtained from Sigma Chemical Co. (St. Louis, MO). Mesohemin was prepared by the catalytic hydrogenation of protoporphyrin-IX dimethyl ester (16) followed by iron insertion and hydrolysis (17). 7-Ethoxyresorufin (ER) and p-chloro-N-methylaniline (PCNMA) were obtained from Pierce Chemical Co., Rockville, IL, and Calbiochem, La Jolla, CA, respectively. Ethylmorphine (EM) was obtained from Merck Chemical Company. Benzphetamine (BZP) was a gift from Dr. J. F. Stiver, Upjohn Company, Kalamazoo, MI.

Hemin refers to iron (III)-protoporphyrin IX chloride, and mesohemin refers to 2,4-diethyldeuterohemin, i.e. the vinyl groups at the 2 and 4 positions of hemin are substituted with ethyl groups in mesohemin.

Experimental Protocol: Male Sprague-Dawley rats (250-300 g) were pretreated daily with sodium phenobarbital (PB, 80 mg/kg, i.p.) for five days. Twenty-four hours after the last injection of phenobarbital, rats were injected with AIA (200 mg/ kg, i.p. in distilled water) 1 hour before killing. Livers were then removed, weighed, perfused with ice-cold saline, and homogenized (50% w/v) in 0.25 M sucrose containing 10 mM glutathione, pH 7.4 at 4° C. Aliquots of liver homogenates were adjusted to 0.1 M with respect to potassium phosphate buffer, pH 7.4 and incubated in the presence or absence of hemin or mesohemin [50 μM,

dissolved in a small volume of 0.1 N NaOH and neutralized with phosphate buffer, pH 7.4] at 37° C for 15 min in a Gyrotory Water Bath Shaker (New Brunswick Scientific). Homogenates were diluted with 3 volumes of ice-cold 1.15% KCl and centrifuged at 9000 x g for 10 min. Supernatants were decanted and sedimented at 105,000 x g for 60 min. Microsomal pellets were resuspended in 1.15% KCl and resedimented at 105,000 x g for 30 min. The washed microsomes were resuspended in 0.1 M phosphate buffer, pH 7.4. Protein was determined by the method of Lowry, et al. (18) and cytochrome P-450 content was determined by the method of Estabrook, et al. (19) using an Aminco DW-2a spectrophotometer. Microsomes were assayed for N-demethylase activity with EM and BZP (final concentration, 2 mM) or PCNMA (final concentration, 1 mM), as substrates, as described previously (14). The amount of formaldehyde generated was quantitated by the method of Nash (20). The rate of ER O-deethylation was measured by the direct fluorometric assay described by Burke and Mayer (21).

RESULTS AND DISCUSSION

Treatment of PB-pretreated rats with AIA, as expected, resulted in destruction of over 60% of hepatic cytochrome P-450 content. In parallel, its dependent mixed function oxidase activity, assessed by monitoring O-deethylation of ER and N-demethylation of PCNMA, EM and BZP was also reduced to 45, 47, 13 and 29% of original values, respectively (Fig. 1, Table 1). In addition,

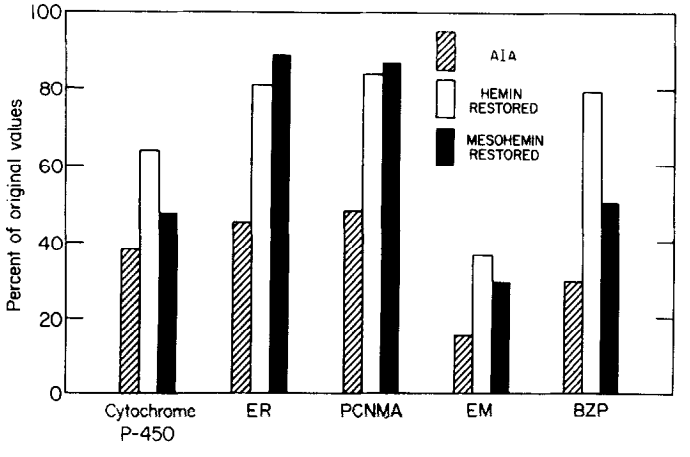

Figure 1. Restoration of Microsomal Cytochrome P-450 Content and Dealkylase Activity after AIA-mediated Destruction.

PB-pretreated rats were given AIA (200 mg/kg, i.p.) for 1 hour. Liver homogenates were prepared and incubated in the presence or absence of hemin or mesohemin (50 μM) at 37°C for 15 min. Microsomes were prepared and assayed for cytochrome P-450 content and dealkylase activity. Values are presented as a percentage of the original microsomal cytochrome P-450 content (1.75 ± 0.15 nmol/mg) and original N-dealkylase activities (123 ± 5 pmol/mg/min, 83 ± 11 nmol/mg/15 min, 381 ± 36 nmol/mg/15 min, and 292 ± 17 nmol/mg/15 min for 7-ER, PCNMA, EM, and BZP, respectively) in corresponding non-AIA-treated (PB-pretreated) controls. On the bases of similar molar extinction coefficients (E_M) for hemin and mesohemin solutions, their corresponding pyridine-hemochromogens, and hemin- and mesohemin-reconstituted cytochrome b_5, E_M for mesohemin-reconstituted cytochrome P-450s was also assumed to be the same as that of the native cytochrome P-450.

TABLE 1 Restoration of Rat Liver Microsomal Cytochrome P-450 Content and Dealkylase Activity after AIA-Mediated Destruction

Treatment	Incubation	Cytochrome P-450 (nmol/mg protein)	DEALKYLASE ACTIVITY			
			ER (pmol/mg/min)	PCNMA	EM	BZP
			(nmol/mg protein/15 min)			
AIA	----	0.66 ± 0.02	55 ± 7	40 ± 3	57 ± 9	84 ± 10
AIA	Mesohemin	0.83 ± 0.04	109 ± 12	72 ± 5	111 ± 9	146 ± 4
AIA	Hemin	1.12 ± 0.04	100 ± 7	70 ± 6	139 ± 4	232 ± 6
None	----	1.75 ± 0.15	123 ± 5	83 ± 11	381 ± 36	292 ± 17

Values determined as described in Figure 1
Values represent the mean ± S.E. from at least 3 individual PB-pretreated animals

when liver homogenates from such AIA treated rats were incubated with hemin, the spectrally detectable hepatic cytochrome P-450 content was restored to 64% of original values observed before AIA treatment (Fig. 1, Table 1). Following AIA treatment, the maximum of the CO-reduced cytochrome P-450 absorption spectrum was shifted from 450 to 452 nm, which, however, on hemin reconstitution was reverted towards 450 nm (Fig. 2A,B). On the other hand, when aliquots of liver homogenates from the same AIA-treated rats were incubated with mesohemin, the spectrally detectable cytochrome P-450 was restored to 47% of original values. However, such reconstitution resulted in a distinctly altered absorption spectrum with a maximum at 446 nm (Fig. 2C). Addition of mesohemin to liver microsomes from PB-pretreated rats not given AIA, failed to shift the absorption spectrum of cytochrome P-450 from its characteristic 450 nm maximum. Similar results were obtained when mesohemin was added to AIA-treated rat liver microsomes. This suggests that the shift to 446 nm observed in microsomes from AIA-treated rat liver homogenates incubated with mesohemin was due to incorporation of mesohemin into the apocytochrome, rather than merely to its nonspecific binding to the microsomal membrane. Moreover, the absorption spectrum of the cytochrome P-450 fraction reconstituted with mesohemin could be visualized by subtracting the spectral contribution of the residual cytochrome P-450, (i.e. the fraction which is resistant to AIA-mediated destruction). Accordingly,

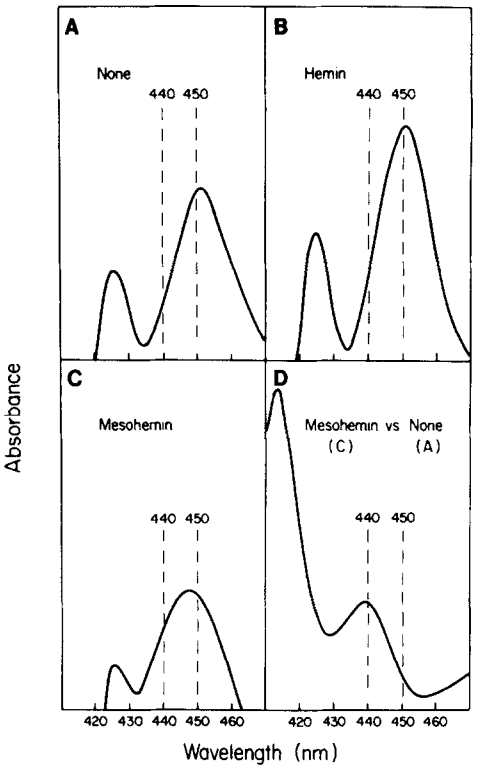

Figure 2. CO-reduced Cytochrome P-450 Absorption Spectra of Hepatic Microsomes Before and After Reconstitution with Hemin or Mesohemin.

Rat liver microsomes were prepared as described in Figure 1. Cytochrome P-450 was determined by the method of Estabrook, et al. (9) in which both sample and reference cuvettes were bubbled with CO, the baseline recorded, and then sodium dithionite added to the sample cuvette. Spectrum 2A was obtained with liver microsomes from AIA-treated rats. Spectra 2B, and C were determined after incubation of liver homogenates from such rats with hemin or mesohemin, respectively. Spectrum 2D represents the absorption difference between the sample cuvette in 2C and the sample cuvette in 2A.

optical subtraction of the CO-reduced cytochrome P-450 spectrum of liver microsomes of an AIA-treated rat (Fig. 2A), from that of corresponding microsomes after mesohemin reconstitution (Fig. 2C), results in a CO-reduced difference spectrum with an absorption maximum at 440 nm (Fig. 2D). This latter absorption spectrum appears to be unique for the microsomal isozyme(s) reconstituted with mesohemin as the prosthetic moiety. It is relevant to note that incorporation of mesohemin into apocytochrome b_5 also shifts the Soret absorption maxima of the oxidized and reduced forms of the hemoprotein from 413 to 406 nm and 423 to 411 nm, respectively (22).

As previously reported (14,15,23-25), the hemin mediated increase in cytochrome P-450 content is associated with a corresponding increase in its

mixed function oxidase activity (Fig. 1). That is, N-demethylation of EM, BZP, and PCNMA was restored to 36, 79, and 84% of original values respectively, whereas ER O-deethylation was likewise increased to 81% after hemin-mediated reconstitution of cytochrome P-450 in liver homogenates from AIA-treated rats (Fig. 1, Table 1). On the other hand, after similar reconstitution of cytochrome P-450 with mesohemin, N-demethylation of EM, BZP, and PCNMA and O-deethylation of ER were increased to 29, 50, 87, and 89% of original (PB-induced) values (Fig. 1, Table 1). These findings thus indicate that mesohemin is just as effective as hemin in fully reconstituting PCNMA and ER dealkylases. It is noteworthy that these two particular isozymes not only are largely inducible by polycyclic aromatic hydrocarbons but that such cytochrome P-450 induction is associated with a characteristic shift of the maximum to 448 nm in the "CO-reduced" absorption spectrum. It is intriguing that the structural reconstitution of these isozymes with mesohemin also results in a similar spectral shift. In contrast, neither hemin nor mesohemin appear to significantly restore EM N-demethylase activity. Whether this refractoriness reflects loss (rapid degradation) of the apocytochrome or irreversible structural damage to the apoprotein inflicted by a reactive metabolite of AIA, remains to be determined. In addition, hemin successfully reconstitutes BZP N-demethylase whereas mesohemin appears to result in only partial restoration. Such partial restoration may be due to a slower rate of incorporation of mesohemin into the apocytochrome as compared to that of hemin, or there may be more than one isozyme responsible for BZP N-demethylation, only one of which is capable of accepting mesohemin as its prosthetic moiety.

Collectively, our findings indicate that some cytochrome P-450 isozymes can very effectively function with mesoheme as the prosthetic moiety and thus appear to be quite promiscuous in their prosthetic acceptance.

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